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UNIQUE LOCALIZATION OF PROTEIN KINASE C ISOZYMES IN T51B RAT LIVER CELLS AND THEIR ROLE IN PROLIFERATION AND APOPTOSIS

by

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Thesis submitted to the School of Graduate Studies and Research
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degree of Doctor of Philosophy

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ABSTRACT

The regulation of cell proliferation and apoptosis in T51B rat liver cells has been investigated. These cells have proven to be an excellent *in vitro* model for cellular homeostasis since growth stimulation by re-addition of serum or epidermal growth factor (EGF) induces an initial burst of cell division followed by apoptosis, as determined by flow cytometry, Hoechst staining and TUNEL assay. Furthermore, flow cytometric analyses indicate that as the cells approach confluence, the percentage of apoptotic cells increases with a corresponding decrease in the percentage of the G_2 cell population. I hypothesize that part of the G_2 cell population is eventually eliminated by apoptosis. This could be either directly or after the completion of the cell cycle.

The role of PKC isozymes in the regulation of cellular homoestasis in T51B cells has also been studied. Immunofluorescence and immunoblot analyses demonstrate the presence of PKC-α, -β_I, -δ, -ϵ and -ζ in the cytosol, the plasma membrane and the nucleus. Interestingly, PKC-β_I appears as a doublet in the membrane fractions which is unique for these type of cells. Furthermore, treatment of serum-stimulated cells with the phorbol ester, TPA, down regulates membrane-associated PKC-α, -δ, -ϵ but without effect on PKC-ζ. Moreover, TPA causes an increase in the membrane and the nuclear association of PKC-β_I in confluent serum-stimulated and serum-deprived cells. However, in proliferating cells (one day after plating), TPA causes a re-distribution of nuclear PKC-β_I between the nucleus, the cytoplasm and the plasma membrane. On the other hand, EGF is without effect on PKC-α, -δ, -ϵ and -ζ but induces an increase in the translocation of PKC-β_I to the plasma membrane. These results suggest a possible involvement of PKC-β_I in the mitogenic effect of EGF and TPA.

Immunofluorescence analyses indicate a differential change in the subcellular localization of PKC isozymes as the cells approach confluence. PKC-β_I is totally associated with the nucleus and the nuclear membrane after one day of plating (at which time most of the cells are likely in the G_1 phase or at the G_1/S boundary) and moves to the cytoplasm and the plasma membrane as the cells become confluent. Interestingly, re-addition of serum to quiescent serum-deprived cells results in a substantial increase in the nuclear level of PKC-β_I, suggesting a possible involvement of nuclear PKC-β_I in the proliferative signal. Moreover, in dividing T51B cells, the level of PKC-β_I is much higher than that of non-dividing cells, indicating a possible role of this isozyme in the cell division. On the other hand, no significant changes in the localization of PKC-α, -δ and -ϵ are noted. However, in the case of PKC-ζ, the membrane-associated level increases as the cells reach confluence. [^{32}P] labeling experiments show that TPA treatment or serum re-addition to serum deprived cells results in an increase in the phosphorylation of two nuclear proteins with a molecular weight of approximately 21 and 31 kD. PKC-β_I may be implicated in the observed effect.

Dual immunofluorescence and Hoechst staining data clearly demonstrate a differential localization of PKC isozymes during apoptosis of T51B cells. While PKC-β_I and -α are not detectable in apoptotic cells, the level of PKC-δ substantially increases with no change in the level of PKC-ϵ and -ζ. Furthermore, the PKC inhibitor bisindolylmaleimide (Bis) enhances apoptosis while TPA reduces the number of cells undergoing apoptosis. I hypothesize that differential localization and translocation of PKC isoforms play a role in cellular homeostasis of T51B cells.

DEDICATION

To my Husband, my Children and my Parents

who through their support, encouragement,

and love, have made this work possible.

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LIST OF ABBREVIATIONS

3Y1 cells	rat embryonic fibroblasts
4αPDD	4-α-phorbol-12-13-didecanoate
A431	an epidermal carcinoma cell line
AA	arachidonic acid
Ap-1	a transcriptional factor
aPKC	atypical PKC
ATP	adenosine-5′-triphosphate
BCS	bovine calf serum
Bis	bisindolylmaleimide
BME	eagle's basal medium
c-fos	cellular fos (a nuclear proto-oncogene)
c-myc	cellular myc (a nuclear proto-oncogene)
Ca^{2+}	calcium
$CaCl_2$	calcium chloride
cAMP	cyclic adenosine monophosphate
cdc2	key fission yeast-cell cycle control gene
cDNA	complimentary deoxyribonucleic acid
CHO cells	Chinese hamster ovary cells
COS-7	African green monkey kidney cell, SV40 transformed
cPKC	conventional PKC
CTLL-2 cells	IL-2-dependent mouse T cells
DAG	diacylglycerol
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dPPA	12-deoxyphorbol-13-phenylacetate-20-acetate
dUTP	deoxyuridyltriphosphate
ECM	extracellular matrix
EDTA	ethylenediaminetetra-acetic acid
EGF	epidermal growth factor
EGF-R	epidermal growth factor-receptor
EGTA	ethyleneglycol-bis-(β-aminoethyl ether)N,N, N,N′,tetraacetic acid
FITC	fluorescein isothiocyanate
G protein	GTP-binding regulatory protein
GAP	GTP-ase activating protein
GDP	guanosine diphosphate
GH_4C_1 cells	pituitary gland cells
Gi	inhibitory G protein
gjc	gap-junctional communication
GTP	guanosine triphosphate
H-7	1,5-isoquinolinylsulfonyl-2-methylpiperzine
H33258	Hoechst fluorochrome 33258
HA 1004	N-(2-guanidinoethyl)-5-isoquinoline sulfonamide

HL-60 cells ..... human promelocytic leukemia cells
HT29 cells ..... human colonic carcinoma cells
I-R ..... insulin-receptor
IGF ..... Insulin-like growth factor
IGF1 ..... insulin-like growth factor 1
IGF1-R ..... insulin-like growth factor 1 receptor
IgM ..... immunoglobulin M
IIC9 cells ..... a subclone of Chinese hamster embryo fibroblasts
IL-2 ..... interleukin-2
IL-3 ..... interleukin-3
IL-4 ..... interleukin-4
IP_3 ..... inositol-1,4,5-trisphosphate
K562 cells ..... human erythroleukemia cells
KCl ..... potassium chloride
kD ..... kilodalton
MDBK cells ..... Madin Darby bovine kidney celld
MDCK ..... Madin Darby canine kidney cells
$MgCl_2$ ..... magnesium chloride
MNNG ..... N-methyl-N'-nitrosoguanidine
MPF ..... maturation promoting factor
mRNA ..... messenger ribonucleic acid
$NaHCO_3$ ..... sodium bicarbonate
NaOH ..... sodium hydroxide
NF-kB ..... a nuclear transcriptional factor
NGF ..... nerve growth factor
NIH 3T3 cells ..... mouse fibroblast cells
nPKC ..... novel PKC
PA ..... phosphatidic acid
PAGE ..... polyacrylamide gel
PBS ..... phosphate-buffered saline
PC ..... phosphatidylcholine
PC12 cells ..... rat pheochromocytoma cells
PDBu ..... phorbol dibutyrate
PDGF ..... platelet drive growth factor
PDGF-R ..... platelet-derived growth factor receptor
PI ..... phosphatidylinositol
PIP_2 ..... phosphatidylinositol-4,5-bisphosphate
PKA ..... cAMP-dependent protein kinase
PKC ..... protein kinase C
PKM ..... a catalytic fragment of PKC
PLC ..... phospholipase C
PLD ..... phospholipase D
PMSF ..... phenylmethylsulfonylfluoride
PNF ..... post nuclear fraction
PS ..... phosphatidylserine
RNA ..... ribonucleic acid

RNase ..... ribonuclease
SDS ..... sodium dodecyl sulfate
Ser ..... serine
SH ..... Src homology
SH2 ..... Src homology 2
SH3 ..... Src homology 3
Sos ..... guanine nucleotide releasing factor
T51B cells ..... rat liver epithelial cells
TdT ..... deoxylnucleotidyl transferase
TGFα ..... transforming growth factor α
TGFβ_1 ..... transforming growth factor β_1
TNF ..... tumor necrosis factor
TPA 12-O-tetradecanoyl-phorbol-13-acetate
TUNEL ..... TdT-mediated dUTP nick end labeling
U937 ..... human promonocytic leukemia cells
WB cells ..... non-transformed rat liver epithelial cells

CHAPTER ONE

Characterization of Protein Kinase C Isozymes in T51B cells and Study of The Effects of TPA and EGF on Subcellular Localization of these Isozymes

1.1 Introduction

Protein kinase C (PKC) was first discovered in 1977 as an undefined protein kinase which was activated by limited proteolysis with the neutral protease calpain (Nishizuka, 1988). Its ability to be activated by tumor-promoting phorbol esters and by diacylglycerol (DAG), produced through receptor-stimulated phospholipid metabolism, has led to the realization that this enzyme plays a major role in signal transduction events (Cook and Wakelam; 1992; Nishizuka, 1988). Now it is well known that PKC exists as a family of related genes which code for a number of highly homologous isozymes (Parker et al., 1989). There is considerable interest in the possibility that individual isoforms may have distinct functional roles in the cell (Nishizuka, 1988). Biochemical and immunocytochemical studies with subspecies - specific antibodies suggest that PKC subspecies may be differently located in particular cell types and at limited intracellular locations. Many of the cell types so far studied express more than one subspecies in variable ratios. The fact that multiple PKC members are expressed in a tissue-specific manner suggests fuctional divergence among PKC family members.

1.1.1 Structure of Protein Kinase C Isozymes

Protein kinase C (PKC) is not a single molecular entity but instead consists of a family of closely related isozymes. Molecular cloning analyses have revealed the existence of twelve different PKC isozymes indicated by Greek symbols, α, β_I, β_{II}, γ, δ, ϵ, ζ, η, θ, τ,

λ and μ. Many non-mammalian PKCs have also been identified (Dekker and Parker, 1994). Partial genomic analyses have suggested that β_I-PKC and β_{II}-PKC derive from a single RNA transcript by alternative splicing of the most C-terminal exon of the β-gene producing two polypeptides (approx. 77 kD) which only differ in the sequence of their 50 amino acids at the C-terminal (Parker et al., 1986; Coussens et al., 1986). Similarly, there is evidence for the alternative splicing of the ϵ-PKC gene since a cDNA clone encoding a truncated version of the PKC enzyme (-ϵ-PKC) lacking 240 N-terminal amino acids present in the ϵ isozyme, has been isolated from rat brain (Ono et al., 1988). Shorter transcripts that differ from ϵ-PKC at the 5-end have also been reported in rat lung and brain (Schaap et al., 1989). The PKC family can be divided into two main groups: the Ca^{2+}-dependent and Ca^{2+}-independent PKCs. The primary structure of the isozymes of PKC (Diagram One) shows conserved structural motifs with a high degree of sequence homology. The group α-, β_I-, β_{II}- and γ-isozymes have five variable (V_1-V_5) and four constant (C_1-C_4) regions. A variable region (V_3), the so called hinge region, contains a protease-sensitive cleavage site and links the amino terminal regulatory domain and the carboxy terminal catalytic domain (Haller et al., 1994a). The C_1 region possesses two "zinc finger" cysteine-rich regions and is believed to be the phospholipid, DAG/phorbol ester binding region (Ono et al., 1989), while the C_2 region is the calcium binding region (Nishizuka, 1989). The activity of these PKC isozymes is strongly dependent on calcium, phospholipids and DAG (Nishizuka, 1988). As such they have been designated as conventional PKCs (cPKCs). Subsequently, PKC-δ, -ϵ , -η and -θ are designated novel PKCs (nPKCs) since they lack the conserved C_2 region. These isozymes depend on DAG for activation and require phospholipids as cofactors but are Ca-independent (Haller et al., 1994a). PKC-ζ constitutes a different branch of the PKC family,

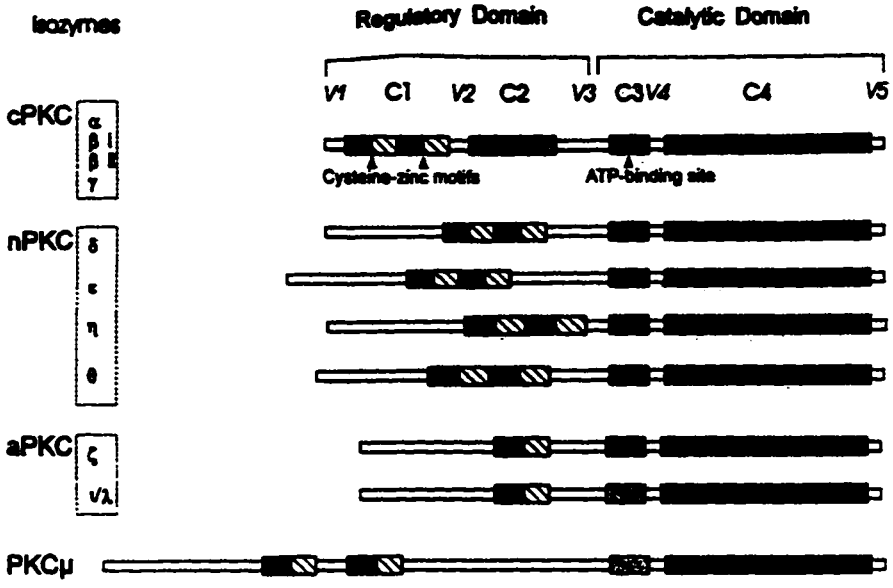
the so called atypical PKCs (aPKCs). This PKC isozyme lacks the conserved C_2 region and contains only one "zinc finger" cysteine-rich region in its C_1 region (Bell and Burns, 1991). PKC-τ, -λ and -μ have only recently been cloned and have not been sequenced so far. However, it appears that PKC-μ is a member of the nPKC subfamily while PKC-τ and -λ are related to PKC-ζ (Dekker and Parker, 1994).

1.1.2 Activation of Protein Kinase C Isozymes

The finding that PKC represents a phospholipid dependent protein kinase has drawn attention to the cellular glycerolipids, sphingolipids, phospholipids and their metabolic breakdown products (Berridge, 1987; Berridge, 1989) and the enzymes involved in lipid metabolism, i.e. phospholipases and phosphatidylinositol kinases (Meldrum et al., 1991). For cPKCs, a model of activation has been proposed by Hug and Sarre, 1993 which includes: (i) the generation of DAG and inositol-1,4,5-trisphosphate (IP_3) from the plasma membrane-associated phosphatidylinositol-4,5-bisphosphate (PIP_2) by the action of phospholipase C (PLC); (ii) the release of Ca^{2+} from intracellular storage sites stimulated by IP_3; (iii) the binding of Ca^{2+} to the C_2 region of PKC and subsequent translocation of the enzyme to the plasma membrane; (iv) where it is activated, via its C_1 region, by DAG and phosphatidylserine (PS), the latter being constitutively present in the membrane. In this model, phorbol ester would mimic the action of DAG and by its persistence in the cellular membrane, leads to a long term activation of PKC (Gschwendt et al., 1991). Activation of the cPKCs is thought to require DAG as an activator and PS as a cofactor of activation, the presence of both leading to a reduction in the Ca^{2+} requirement of PKC to the micromolar range (Lee and Bell, 1991). For activation of cPKCs with phorbol esters, the presence of Ca^{2+} is not required but it lowers the concentration of phorbol esters necessary to obtain full

DIAGRAM ONE

Domain Structure of PKC Family



activity (Ryves et al., 1991). Members of the nPKC group do not require Ca^{2+} for activation, but require either DAG/PS or TPA/PS. PKC-ζ exhibits a low but constitutive activator-independent kinase activity (Liyanage et al., 1992). However, Nakanishi and Exton, 1992 have reported a marked stimulation of PKC-ζ activity (purified from bovine kidney) by PS or unsaturated fatty acids, e.g. arachidonic acid.

Recently, it has been shown that other components of glycerolipid metabolism can be activators of PKC at least *in vitro* (Lee and Bell, 1991). Cardiolipid is able to activate PKC-α, -β_I (Kochs et al., 1993) and -ϵ (Saido et al., 1992). Arachidonic acid (AA) and lipoxin A are capable of activating PKC-β_{II}, -γ (Shearman et al., 1989) and -ϵ (Ogita et al., 1992) in a Ca^{2+} independent manner, while PKC-β_I and -δ do not respond to these activators (Shearman et al., 1989; Burns et al., 1990). Surprisingly, PIP_2 can substitute for DAG as an activator of PKC-α, -β_I and -γ but not of -β_{II}, and phosphatidylinositol (PI) can replace PS as a cofactor of activation, at least for PKC-α and -β_I (Lee and Bell, 1991; Kochs et al., 1993). These findings together may indicate that some if not all PKC isozymes may be activated by different second messengers in a distinct manner. Thus, the signal-induced production of a distinct second messenger or activator may actually decide whether a PKC isozyme becomes activated or not.

1.1.3 Expression of PKC Isozymes

The tissue distribution of PKC isozymes has been determined mostly by northern blot analyses and by western blotting using isozyme-specific antibodies. The tissue distribution of the cPKCs has been studied extensively. PKC-γ appears to be expressed solely in the brain and spinal cord (Saito et al., 1988; Kikkawa et al., 1989). PKC-β is found to be the major isozyme in the liver (Rogue et al., 1990). The tissue distribution of PKC-β_I and -β_{II}

shows that there is much more -β_{II} than -β_{I} in the brain and many other tissues such as liver, kidney, spleen, lung, heart and testis (Kikkawa et al., 1989). Immunofluorscence analyses have revealed a distinct pattern of cellular localization for β_{I} and β_{II} subspecies. For example, in rat cerebellar cortex, β_{I} is present mainly in the granular layer, whereas β_{II} is found primarily in the molecular layer, apparently in the presynaptic nerve endings (Nishizuka, 1988). Immuno-electron microscopy has shown that in some cells such as neuronal cells, the β_{II} subspecies is associated with Golgi apparatus (Nishizuka, 1988). PKC-α is distributed in most tissues and cell types (Kosada et al., 1988).

For the tissue distribution of the nPKCs and aPKCs, northern blot analysis has shown that PKC-δ is present in brain and lung; PKC-ϵ is expressed mainly in brain and PKC-ζ is found in brain, lung and kidney (Ono et al., 1988). Studies using western blot analysis has indicated that PKC-δ is present in brain, lung, heart, spleen, liver, testes and ovary (Wetsel et al., 1992). PKC-ϵ is found in brain and kidney, while PKC-ζ is found in most tissues but particularly in brain, lung, liver and kidney (Wetsel et al., 1992). PKC-η is strongly expressed in skin and lung, and only slightly in brain and spleen (Osada et al., 1990; Bacher et al., 1991). PKC-θ is predominantly expressed in skeletal muscle, and clearly to a lower extent, in lung, spleen, skin and brain (Osada et al., 1992).

1.1.4 Regulation of Protein Kinase C by Phorbol Esters

Several phorbol esters such as 12-O-tetradecanoyl phorbol-13-acetate (TPA or PMA), are well known as potent tumor promoters, and several kinetic studies with various cell types suggest that the primary site of action is the cell surface membrane (Nishizuka, 1984). Many studies have indicated that PKC is a target for phorbol esters, that the tumor promoters directly activate this enzyme both *in vitro* and *in vivo*, and that there is an approximate

correlation between the ability of individual phorbol esters to promote tumors and to activate the enzyme (Castagna et al., 1982; Yamanishi et al., 1983). Kinetic analysis has indicated that TPA, which has a diacylglycerol-like structure, is able to substitute for DAG at extremely low concentrations (Nishizuka, 1984). Like DAG, TPA dramatically increases the affinity of the enzyme for Ca^{2+} to the 10^{-7} M range, resulting in its full activation without detectable cellular mobilization of Ca^{2+} (Yamanishi, 1983). TPA activates the enzyme presumably by some modification of its phospholipid microenvironment (Nishizuka, 1984).

Recently, the distribution of PKC isozymes within resting (unstimulated) cells of various origins has been determined. Upon treatment of these cells with certain stimuli such as phorbol esters a re-distribution of PKC from a cytosolic location in resting cells to a membrane-associated site during stimulation is observed (Kraft and Anderson, 1983). Prolonged treatment (24 h) of intact cells with TPA results in down regulation and proteolytic degradation of PKC. The proteases *in vivo* are thought to be Ca^{2+}-dependent neutral proteases I and II (calpains) which are active in the micromolar and millimolar concentration range of Ca^{2+} respectively (Kishimoto et al., 1989). Calpains cleave PKC in the V_3 hinge region and thus produce two distinct fragments, a protein comprising the regulatory domain and a protein known as PKM containing the kinase domain which is catalytically active in the absence of any activators (Kishimoto et al., 1989).

The distribution, translocation and down regulation of PKC could be visualized by analysis of the so-called particulate (i.e. membrane) and soluble (i.e. cytosolic) fractions of the cells, by means of PKC activity measurement or immunoblotting. For cPKC isozymes, PKC-α seems to be located in the cytosol and is translocated to cellular membranes and down-regulated upon prolonged exposure of many cell types to TPA (Hocevar and Fields,

1991; Huwiler et al., 1991; Borner et al., 1992; Strulovici et al., 1991; Crabos et al., 1991; Standaert et al., 1993). In other cell systems, such as MDBK cells, TPA down-regulates PKC-α in both cytosolic and membrane fractions (Simboli-Campbell et al., 1992). In rat hepatocytes, a short treatment (15 min) with TPA results in a marked loss in total PKC-α activity (Robles-Flores et al., 1991). Similar results have been reported in rat adipocytes where a short exposure to TPA causes a preferential loss of PKC-α activity (Cooper et al., 1989). In GH_4C_1 pituitary gland cells, PKC-α is translocated to the particulate fraction, but not down-regulated upon treatment with thyrotropin-releasing hormone or TPA (Akita et al., 1990; Kiley et al., 1990). In NIH 3T3 cells, PKC-α has been found to translocate to the nucleus upon TPA treatment (Leach et al., 1989).

Protein kinase C-β also appears to be a cytosolic isozyme in some unstimulated cells which is sensitive to down-regulation by TPA (Huang et al., 1989; Crabos et al., 1991; Cook et al., 1992; Duyster et al., 1992; Akita et al., 1990; Kiley et. al, 1990; Strulovici et al., 1991). It should be noted, however, that observations like these should not be generalized since, recently, Van Den Berghe et al., 1992 have reported that PKC-β_I is not down-regulated by TPA treatment in a human colonic cell line. Similarly, in BC3H-1 myocytes, chronic TPA treatment has no effect on PKC-β levels (Standaert et al., 1993). In rat hepatocytes, PKC-β activity is not affected when the cells are treated with TPA for short periods of time (15 min) (Robles-Flores et al., 1991) and in regenerating rat liver PKC-β has been found to be insensitive to TPA (Houweling et al., 1989). Others have reported that a type II (PKC-β) activity persists after TPA treatment (Cooper et al., 1989). In HL-60 cells, TPA treatment leads to only partial translocation of PKC-β_{II} to the plasma membrane fraction, while

bryostatin-1 (a non tumor promoting agent and an activator of PKC) treatment induces a translocation of PKC-β_{II} to the nuclear fraction (Hocevar and Fields, 1991).

Depending on the cell type, PKC-δ seems to be differentially distributed within the cell. While it is located in the cytosol of human platelets (Grabarek et al., 1992), the majority of PKC-δ is found to be associated with the particulate fraction in rat 6 fibroblasts (Borner et al., 1992) and in renal mesangial cells (Huwiler et al., 1992) where the isozyme can be completely down-regulated by TPA treatment. In Jurkat T lymphoma cells a large fraction of PKC-δ exists in the membrane fraction. In these cells the translocation of PKC-δ is not apparent after 1 h treatment with TPA, but the total PKC-δ protein is reduced within 4 to 6 h of treatment (Tsutsumi et al., 1993).

In some cell lines PKC-ϵ is found to be mostly cytosolic such as in GH_4C_1 pituitary gland cells where it is down-regulated by prolonged treatment with TPA (Akita et al., 1990; Kiley et al., 1990). However, in other cells including rat 6 fibroblasts and renal mesangial cells, PKC-ϵ is associated with the membrane and also down-regulated by TPA treatment (Huwiler et al., 1991; Borner et al., 1992). In leukemia cell line U937 as well as in thymocytes, PKC-ϵ is membrane-associated to a certain extent but is resistant to down-regulation by prolonged TPA treatment (Strulovici et al., 1991; Ways et al., 1992b). In Jurkat T lymphoma cells, PKC-ϵ is associated with the cytosolic and the membrane fractions. After 10 min of TPA addition, the isozyme translocates rapidly to the plasma membrane. However, prolonged TPA treatment results in PKC-ϵ down-regulation (Tsutsumi et al., 1993).

In several cell lines investigated, PKC-ζ has been found to be localized within both cytoplasmic and membrane components (Olivier et al., 1992; Borner et al., 1992; Huwiler

et al., 1992; Gschwendt et al., 1991; Wooten et al., 1994). Additionally, PKC-ζ has been observed around and within the nucleus (Hagiwara et al., 1990; Khalil et al., 1992). With respect to the presence of one zinc finger cysteine-rich motif in the C_1 region of PKC-ζ, initial transfection experiments using COS cells (Ono et al., 1989) as well as the use of recombinant baculo virus expressed PKC-ζ have documented that this isozyme is incapable of binding phorbol dibutyrate PDBu (McGlynn et al., 1992). The authors have suggested that the two zinc fingers are required for phorbol ester binding. The activity of PKC-ζ purified from bovine kidney has also been shown to be independent of phorbol esters (Nikanishi and Exton, 1992). While the enzyme clearly does not display any requirement for phorbol ester-induced activation *in vitro*, discrepancies regarding the effects of TPA *in vivo* have been reported. Several studies have demonstrated that PKC-ζ is not translocated or down-regulated in response to TPA (Nakanishi et al., 1993; Olivier et al., 1992; Ways et al., 1992a; Huwiler et al., 1992; Gschwendt et al., 1992). However, Borner et al., 1992 have indicated that in R6 fibroblast cells PKC-ζ is translocated from the cytosol to the membrane in response to TPA treatment. Similarly, in human platelets, TPA treatment results in PKC-ζ translocation, although with less magnitude compared to two other cPKCs (Crabos et al., 1992). Recently, Wooten et al., 1994 have reported that TPA induces translocation of PKC-ζ from cytosolic to membrane fractions in a manner similar to that of other PKC isozymes expressed in PC12 cells.

In several human tumor cell lines, PKC-η has been reported to be present specifically in the cell nucleus; TPA treatment does not lead to down-regulation (Greif et al., 1992). When PKC-θ is expressed in COS-7 cells, the majority of the protein is found in the

particulate fraction (Osada et al., 1992). These results together indicate that PKC isozymes respond differently to phorbol esters depending on the cell type.

1.1.5 Epidermal Growth Factor (EGF) and PKC

Many growth factors and growth-modulating compounds activate protein kinases that mediate signal transduction and regulation of cell growth. One of the best characterized growth factors is epidermal growth factor (EGF). EGF exerts its effects in target cells by binding to and activating the EGF-receptor. Following activation of the tyrosine kinase domain, the receptor phosphorylates a number of proteins including the receptor itself. Ultimately, these events lead to enhanced DNA synthesis and cell division in most cells (Carpenter and Cohen, 1979; Carpenter, 1987; Schlessinger, 1988).

1.1.5a Epidermal Growth Factor (EGF)

Epidermal growth factor is a low molecular weight polypeptide of 6,000 Da (depending on the species). It consists of 53 amino acid residues, and lacks lysine, phenylalanine and alanine. The molecule has six cysteine residues that interact with each other to form three intramolecular disulfide bonds. These disulfide bonds are requisite for the biological activity of the molecule (Savage et al., 1973). EGF has been shown to be expressed in submaxillary glands, kidney and detectable in human urine and plasma. Low levels of EGF have also been reported in the oral cavity, mammary glands , duodenum, pituitary glands, spleen, brain and ovary (Carpenter and Cohen, 1990).

The biological activity of EGF can be divided into early and delayed effects. Stimulation of DNA synthesis in cell cultures as measured by an increase in thymidine incorporation, is a delayed effect (Richman et al., 1976). DNA synthesis begins several hours (6-15 h) after exposure to EGF and reaches a peak at 24 h (Carpenter and Cohen, 1979;

Hanguel-Demouzon et al., 1992). EGF apparently has tumor-promoting activity in some cell lines (Rose et al., 1976; Shoyab et al., 1979) since the addition of EGF to culture medium overcomes contact inhibition. Short term effects of EGF are measurable within seconds or minutes and include activation of calcium influx and Na^+/H^+ exchange (Macara, 1986) combined with an increase in pH (Moolenar, 1986). Short term effects also include activation of phospholipid metabolism and stimulation of the expression of a set of cellular genes called "immediate" early genes (will be discussed later in Chapter Two, section 2.1.3). These genes include nuclear proto-oncogenes c-fos and c-myc which are associated with stimulation of cell growth by hormones and serum (Hauguel-Demouzon et al., 1992; Muller et al., 1984).

1.1.5b The EGF Receptor (EGF-R)

The epidermal growth factor receptor belongs to a family of receptors which includes the avian erythroblastosis virus v-erbB, c-erbB2 and c-erbB3 (Merlino, 1990). All these proteins possess tyrosine kinase activity. The EGF-R is also related to other tyrosine kinases including colony-stimulating factor receptor, platelet-derived growth factor receptor (PDGF-R), insulin receptor (I-R) and insulin-like growth factor 1 receptor (IGF1-R) (Carpenter, 1987). The mature receptor is composed of 1,186 amino acid residues that are preceded at the NH_2-terminal end by a peptide of 24 hydrophobic amino acids. This peptide is cleaved after insertion of the nascent receptor into the membrane of endoplasmic reticulum. The receptor is glycosylated and transported through the Golgi apparatus to the plasma membrane. The mature receptor is composed of three major structural elements. The first is an extracellular EGF-binding domain composed of 621 amino acid residues, anchored to the plasma membrane by a single transmembrane region of 23 hydrophobic amino acids.

The transmembrane region is followed by a sequence of mostly basic residues; a feature common to many membrane proteins. The cytoplasmic domain of EGF receptor is composed of 542 amino acids (Schessinger, 1986). It contains a region of approximately 300 amino acid residues that is homologous to the catalytic domain of the protein tyrosine kinase encoded by the src gene family of oncogenes (Hunter and Cooper, 1985; Ullrich and Schessinger, 1990). Like the other protein tyrosine kinases, the catalytic domain of EGF receptor kinase contains a lysine (lys721) residue that is located in the carboxy-terminal side of a consensus sequence, Gly-X-Gly-Xphe-Gly-X-Val. The lysine residue, together with the consensus sequence form part of the ATP-binding site (Schessinger, 1988). Replacement of the consensus lysine residue of the ATP-binding site in the EGF-receptor completely abolishes its kinase activity *in vitro* and *in vivo* (Kashles et al., 1988). There are four autophosphorylation sites clustered at the carboxy-terminal tail that include Tyr 1068, Tyr 1148, Tyr 1173 and Tyr 1086 (White, 1991) and appear to modulate the interaction of EGF receptors with substrates and other proteins (Bertics et al., 1988). The autophosphorylation sites of EGF receptor compete with exogenous substrates for the substrate binding site of the tyrosine kinase domain. The EGF receptor is, with the exception of hemopoietic cells, detectable in a large variety of cell types or tissues. All available evidence indicates that EGF receptor mediates the biological signals not only of EGF, but also of at least two EGF-like growth factors, transforming growth factor α (TGFα) and the vaccinia virus growth factor (VGF). The three ligands are approximately 22% identical in amino acid sequence, but bind to the EGF receptor with nearly identical affinities and produce the same responses in target cells. The three ligands are, however, the products of distinct genes (Carpenter and Cohen, 1979).

1.1.5c Internalization of EGF-R

Like many other serum proteins, EGF is internalized by receptor-mediated endocytosis. EGF receptors are randomly dispersed on the surface. The occupied EGF receptors cluster in coated pits and, after internalization, both EGF and its receptor are degraded by lysosomal enzymes. Hence, EGF appears to facilitate the entry of its receptor into coated pits (Schlessinger, 1986).

1.1.5d Regulation of EGF-R by PKC

Phosphorylation of EGF receptor is believed to be a primary mechanism for the regulation of its kinase activity. The data shows that treatment of cells with TPA, in most cell types, abolishes the high affinity EGF-R and reduces its protein tyrosine kinase activity (Friedman et al., 1984). Modulation of EGF receptor activity *in vitro* with purified PKC has also been reported (Fearn et al., 1985). The natural activator of PKC, DAG, has also been shown to produce similar results (McCaffrey et al., 1984). In many cases, TPA blocks the early responses of cells to EGF. However, the attenuation of EGF receptor function is somewhat difficult to reconcile with the reported synergism between EGF and TPA in the induction of DNA synthesis (Dicker et al., 1980), a late event in the mitogenic process. The effects of PKC on receptor tyrosine kinase activity are widely believed to be linked to the phosphorylation of threonine 654 (Coutaway et al., 1990). Other major phosphorylation sites on the receptor are threonine 669 and serines 1046/1047, all of which are phosphorylated *in vivo* in response to phorbol esters (Heisermann et al., 1988). Substitution of an alanine residue at the threonine 654 site prevents down-regulation of EGF-stimulated tyrosine kinase activity (Countaway et al., 1990) and EGF-stimulated PLC-γ activity by phorbol esters (Decker et al., 1990). PKC activators have been shown to inhibit PT hydrolysis induced by

EGF treatment WB cells (non-transformed rat liver epithelial cells) (Huckle et al., 1990). Thus, there is a strong association between PKC activation and EGF-R transmodulation which may provide a negative feed back mechanism to control receptor activity (Whiteley and Glaser, 1986).

1.1.5e Regulation of PKC by EGF

The binding of EGF to its cell surface receptor activates its tyrosine kinase activity which results in the phosphorylation of several substrates, including the EGF receptor itself. There are various substrates that are phosphorylated by EGF-R. The best characterized one is phospholipase γ_1 (PLC-γ1). This enzyme is activated by receptor-tyrosine kinases which results in the hydrolysis of inositol phospholipids to produce inositol phosphate and DAG needed for PKC activation (Rhee and Choi, 1992). PLC-γ1 is a unique PLC enzyme in that it contains sequences shared with several cytoplasmic tyrosine kinases (Pawson, 1988). These regions are referred to as the Src homology regions SH2 and SH3, and have many regulatory roles. SH2 and SH3 domains recognize a phosphotyrosine residue within a context of a short amino acid sequence. SH3 domains recognize amino acid sequence containing proline and hydrophobic residues. SH2 and SH3 domains are found in a variety of proteins involved in the control of cellular signaling and architecture (Schessinger, 1994). The EGF-R which has intrinsic tyrosine kinase activity dimerizes on ligand binding, phosphorylates the dimerized receptor protein itself, and thereby binds the SH2 domain of PLC-γ1. Cytosolic PLC-γ1 is then translocated to the membrane to hydrolyze inositol phospholipids (Nishizuka, 1995). Some studies have indicated that the association between PLC-γ and activated EGF-R is essential for tyrosine phosphorylation of PLC-γ and that tyrosine phosphorylation activates PLC-γ leading to phospholipid hydrolysis (Rotin et al.,

1992a,b) However, in other cells, phosphorylation of PLC-γ has no effect on PLC activity as measured using PIP_2 as a substrate (Kim et al., 1990).

Recent evidence has shown that many agonists, including EGF, also stimulate DAG production through the hydrolysis of phosphatidylcholine (PC) and that phospholipase D (PLD) is the major enzyme involved (Exton, 1990). PLD initially produces phosphatidic acid (PA) which can be rapidly converted by PA-phosphohydrolase to DAG (Exton, 1994). Most agonists induce a biphasic production of DAG, with PIP_2-hydrolyzing PLC being responsible for the initial rapid increase, and PC-hydrolyzing PLD for the second sustained increase (Exton, 1994). There have been contradictory results concerning the effect of EGF on phospholipid hydrolysis and activation of PKC. In hepatocytes and A431 cells, EGF induces PIP_2 hydrolysis which results in an increase in IP_3 content and a rapid increase in DAG levels which is accompanied by activation of PKC with its translocation to the membrane (Cerpovicz et al., 1992; Disatnik et al., 1994a). In Swiss 3T3 fibroblasts, EGF has been reported to activate PLD and to stimulate DAG formation from PIP_2. In these cells, EGF induces a biphasic translocation of PKC-δ and -ϵ to the membrane. However, EGF has no effect on PKC-α at any time (Yeo and Exton, 1995). Reynolds et al., 1993 have observed that in dermal fibroblasts, DAG formed through EGF-induced PC hydrolysis is capable of activating PKC; however, in keratinocytes, EGF induces a modest activation of PKC in the absence of any detectable PC hydrolysis or DAG elevation. EGF also increases ^{32}P incorporation into PC concomitantly with PKC activation in colon carcinoma cell line, HT29 (Balogh et al., 1993). Similar results have been shown in other cell systems (Wright et al., 1990; Cook and Wakelam, 1992). However, EGF can also exert its effects without detectable PI hydrolysis (Taylor et al., 1985; L'Allemain et al., 1986).

Many studies have indicated that the mitogenic action of EGF in the induction of cell proliferation is accompanied by activation of PKC. For example, in mammary epithelial cells, EGF treatment enhances cell proliferation which is accompanied by PKC activation and translocation of PKC-α to a membrane fraction (Sylvester et al., 1994). On the other hand, other studies have shown that EGF enhances the growth of 3T3 L1 cells and induces an inactive pool of membrane-associated PKC activity within 10 min of EGF treatment. (Chakravarthy et al., 1994). Stimulation of quiescent 3Y1 cells by serum or EGF results in DNA synthesis but has no effect on activation, translocation or down-regulation of PKC species. However, these stimuli cause an upward shift in the electrophoretic mobility of PKC-δ (Ohno et al., 1994). In addition, there is an increase in the phosphorylation levels of PKC-δ and -ϵ, but only a slight increase for PKC-α (Ohno et al., 1994). EGF has also been observed to increase both PKC activity and the growth rate of glioma cells (Couldwell et al., 1992). However, when the cells are treated with staurosporine (a non specific protein kinase inhibitor that inhibits PKA, PKC, Ca^{2+}-dependent calmodulin kinases, tyrosine kinases, etc.), the growth rate declines to the level of staurosporine treated controls (Couldwell et al., 1992). Thus, PKC may be a common signal transduction system induced by this mitogen. Sharma et al., 1994 have observed an increase in DNA synthesis of serum starved quiescent T51B rat liver cells in response to EGF or serum. In these cells, EGF causes a rapid, transient increase in the activity of membrane-associated PKC followed by a longer sustained increase. However, TPA is without effect on DNA synthesis alone, but when added along with EGF or serum, it causes a significant enhancement of DNA synthesis. A similar potentiation of TPA's effect on DNA synthesis in response to EGF has been reported in primary cultures of rat heptatocytes (Hirata and Fujiwara, 1990).

1.1.6 The T51B Cell Line as a Model System

The T51B rat liver cells are a non-neoplastic epithelial cells. This cell line was established from the liver of a normal adult fisher rat (Swierenga et al., 1978). The cells were derived from the bile duct epithelium (Marceau et al., 1986). Their proliferative response is under stringent control by growth factor, calcium, cAMP and phospholipid signaling pathway. DNA synthesis in the proliferatively quiescent T51B cells is controlled by Ca^{2+} concentration in the medium. Lowering the Ca^{2+} concentration from 1.8 mM to 0.02 - 0.1 mM arrests the growth of approximately 90% of the cells at G_0/G_1 and G_1/S transitions. Adding back Ca^{2+} to the medium to the normal level removes the block and allows the cells to re-enter the cell cycle (Boynton et al., 1985a). In contrast, the tumorigenic T51B clones (produced by treating T51B cells with carcinogens such as N-methyl-N′ nitrosoguanidine, MNNG) continue to proliferate even under Ca^{2+}-deprived conditions (Boynton et al., 1984). It has been found that the amount of EDTA-extractable PKC activity increases transiently during these two Ca^{2+}-dependent $G_0 \rightarrow G_1$ and $G_1 \rightarrow S$ transitions. Moreover TPA, that stimulates PKC in an extract from these cells in the absence of Ca^{2+} enables these cells to transit G_1 in Ca^{2+}-deficient medium. Cyclic AMP elevating agents and DAG are also able to release the low Ca^{2+}-mediated G_1/S block (Boynton et al., 1985a,b). Other agents such as dexamethasone, 1,2-dihydroxyl vitamin D_3 and saccharin also trigger the $G_1 \rightarrow S$ transition in Ca^{2+}-arrested late G_1 T51B cells and like TPA, stimulate PKC activity in cell free preparation (Kleine et al., 1986; cited in Boynton et al., 1985c). PKC, therefore may be one of the normally Ca^{2+}-dependent pre-replicative control mechanisms in T51B cells. The mitogenic effects of EGF on T51B cells are dependent on the extracellular Ca^{2+} in the medium but are independent of freshly added BCS to conditioned low-serum medium (Hill

et al., 1988). Furthermore, addition of EGF or serum to quiescent serum-deprived T51B cells initiates DNA synthesis. In these cells, EGF causes a rapid transient increase in the activity of membrane-associated PKC followed by a longer sustained increase (Sharma et al., 1994). Thus, PKC seems to be involved in the mitogenic action of EGF in T51B cells. Interestingly, when T51B cells are grown in 1 nM EGF, there is an initial period of hyperplasia, lasting about 3 days which is followed by an increase in cell death (apoptosis). However, the cell density returns to the confluent level 5 days after EGF addition (Brabyn et al., 1994). Thus, T51B cells are a very good *in vitro* model for liver homeostasis which facilitates the study of early events in cell proliferation and apoptosis in response to serum or EGF, and the roles of PKC isozymes expressed in T51B cells in these two phenomena. It is also of interest to investigate the effects of TPA and EGF treatment on the subcellular localization of PKC isozymes expressed in these cells and to see how these isozymes respond to these agents which will give a better understanding of the involvement of PKC isozymes in the regulation of cell proliferation and/or apoptosis.

1.2 Objectives

The overall objective of the studies described in this chapter were to investigate which protein kinase C (PKC) isozymes are expressed in T51B cells, their subcellular localization and the effects of TPA and EGF on subcellular localization of PKC isozymes. The specific objectives were:

1.2.1 To establish the profile of PKC isozymes expressed in T51B cells and their subcellular localization by immunoblot and immunofluorescence analyses of intact cells.

1.2.2 To determine the effect of TPA treatment on subcellular localization of PKC isozymes by immunoblot analyses and immunofluorescence of intact T51B cells.

1.2.3 To determine the effect of EGF on subcellular localization of PKC isozymes by immunoblot analyses and immunofluorescence of intact T51B cells.

1.3 Methods

1.3.1 Cell Culture

T51B rat liver epithelial cells were cultured in a medium consisting of 90% (v/v) BME (basal medium eagle) (Life Technologies Inc., Burlington, ON, Canada) and bovine calf serum (BCS) (Life Technologies, Inc.) containing 0.1 g/L gentamycin sulfate (Sigma Chemical Co. Ltd., St. Louis, USA). The cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Prior to experiments, the cells were detached by brief exposure to 0.05% (w/v) trypsin- 1 mM EDTA in phosphate buffered saline (PBS) (all reagents were obtained from Sigma Chem. Co.). The cells were plated at a density of 125 x 10^2 cells/cm^2 in BME medium containing 10% BCS and incubated at 37°C for 4-5 days after which they reached confluence. For experiments to examine the effect of TPA, confluent cells were treated for 24 h with 100 nM TPA (in ethanol/DMSO 50/50; (v/v) to a final concentration of 0.05%) (Life Technologies Inc.). For EGF or okadaic acid experiments confluent cells were serum-deprived by fluid changing to BME containing 0.2% BCS for 48 h followed by treatment with 1 nM EGF in PBS (Life Technologies Inc.) or 1 μM okodaic acid in PBS (Sigma Chem. Co.). Alternatively, for immunofluorescence studies, the cells were plated at a density of 125 x 10^2 cells/cm^2 and gown on glass coverslips. The vehicles for the agents to be added were PBS for EGF or ethanol/DMSO for TPA. No significant changes in the morphology or in the viability were observed due to vehicles alone.

1.3.2 Subcellular Fractions

T51B cells were washed twice with cold PBS, scraped, pelleted and Lysed in hypotonic buffer (1 mM $NaHCO_3$, 5 mM $MgCl_2$, 100 μM PMSF and 20 μg/ml leupeptin)

and buffered with 50 mM Tris-HCl and 0.5 mM EGTA, pH 7.5. The total cell homogenate was centrifuged (500 x g, 5 min 4°C) to obtain a crude nuclear pellet and a post-nuclear fraction (PNF). The PNF was centrifuged (100,000 x g, 60 min, 4°C) to give cytosolic supernatant and a crude plasma membrane/microsomal pellet that was sonicated briefly in 50 mM Tris-HCl, pH 7.5, containing 0.5 mM EDTA, 0.5 mM EGTA, 1% β-mercaptoethanol, 1 mM PMSF, and 20 μg/ml leupeptin. Protein content was determined by the Bradford method (Bradford, 1976).

1.3.3 Immunoblotting of Protein Kinase C Isozymes

Sodium dodecyl sulfate (SDS) in an anionic detergent that very effectively disrupts the quaternary structure of most multimeric proteins. Many SDS molecules bind tightly to the subunits thereby obscuring the original charge on the protein. Thus, during electrophoresis, all SDS-protein complexes migrate towards the positive pole with about the same charge/mass ratio. If electrophoresis is carried out on polyacrylamide gels, the mobility of an SDS protein complex will depend almost exclusively on its size. Thus, a standard curve can be prepared using proteins with known molecular weights. The molecular weight of an unknown can then be easily determined.

Samples were solubilized in SDS sample buffer (0.01% bromophenol blue, 5% (v/v) glycerol, 1% (w/v) SDS and 1.25% (v/v) β-mercaptoethanol). 50 μg of protein/lane was electrophoresed on 10% SDS-PAGE (Laemmli, 1970). Gels were equilibrated for 30 min in 192 mM glycine, 25 mM Tris, 20% methanol, pH 8.3, and electrophoretically transferred to nitrocellulose membranes, and blocked for 2 h at room temperature with blocking buffer (10 mM Tris-HCl, 150 mM NaCl, 0.05% (w/v) skim milk, pH 7.3). The blots were then incubated for 2 h at room temperature with PKC isozyme-specific primary antibodies (Life

Technologies, Inc.), diluted to 2.5 µg/ml with blocking buffer. Non-specific binding was assessed in parallel samples by incubating without primary antibody or in the presence of primary antibody plus peptide antigen (1 µg/ml). Antibody binding was detected using goat anti-rabbit IgG alkaline phosphatase conjugated secondary antibody (Life Technologies, Inc.) diluted 1:3500 with blocking buffer. The reaction product was visualized by incubating the blots with 5-Bromo-4-Chloro-3-Indolylphosphate-P-toluidine salt (BCIP) and Nitroblue Tetrazolium Chloride (NBT) (Life Technologies, Inc.). Briefly, 44 µl of NBT solution was added to 10.0 ml of substrate buffer (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$) and was gently mixed by inverting the tube. 33 µl of BICP solution was then added and followed by gentle mixing. The blots were then incubated in this substrate solution at room temperature for 10-15 min in the dark. The blots were then washed with distilled water and air dried and stored in the dark.

1.3.4 Immunofluorescence of Protein Kinase C Isozymes

T51B cells were plated at a density of 125 x 10^2 cells/cm^2 and grown on glass coverslips. The coverslips were washed twice with cold PBS, fixed for 20 min at -20°C in ethanol: acetone (50:50; v/v), and then blocked overnight at 4°C in PBS/0.5% (w/v) skim milk. After washing with PBS, the coverslips were incubated with 10 µg/ml PKC isozyme-specific antibodies (Life Technologies Inc.) overnight at 4°C in a humid box. After washing with PBS, the coverslips were then incubated with FITC-secondary antibody (Jackson Immuno Research Laboratories Inc.) for 60 min. They were then rinsed three times with PBS and mounted with glycerol containing 0.1% (w/v) p-phenylenediamine and viewed with an Olympus fluorescence microscope and photographed with Ilford-400 black and white film or Kodak ektachrome-400 colour slide film. Non-specific binding was assessed in parallel

experiments by incubating without primary antibody or in the presence of antigenic peptide (1 μg/ml).

1.4 Results

1.4.1 Expression of PKC Isozymes in T51B Cells and Antibody Specificity

1.4.1a Immunoblot Analysis of PKC Isozymes

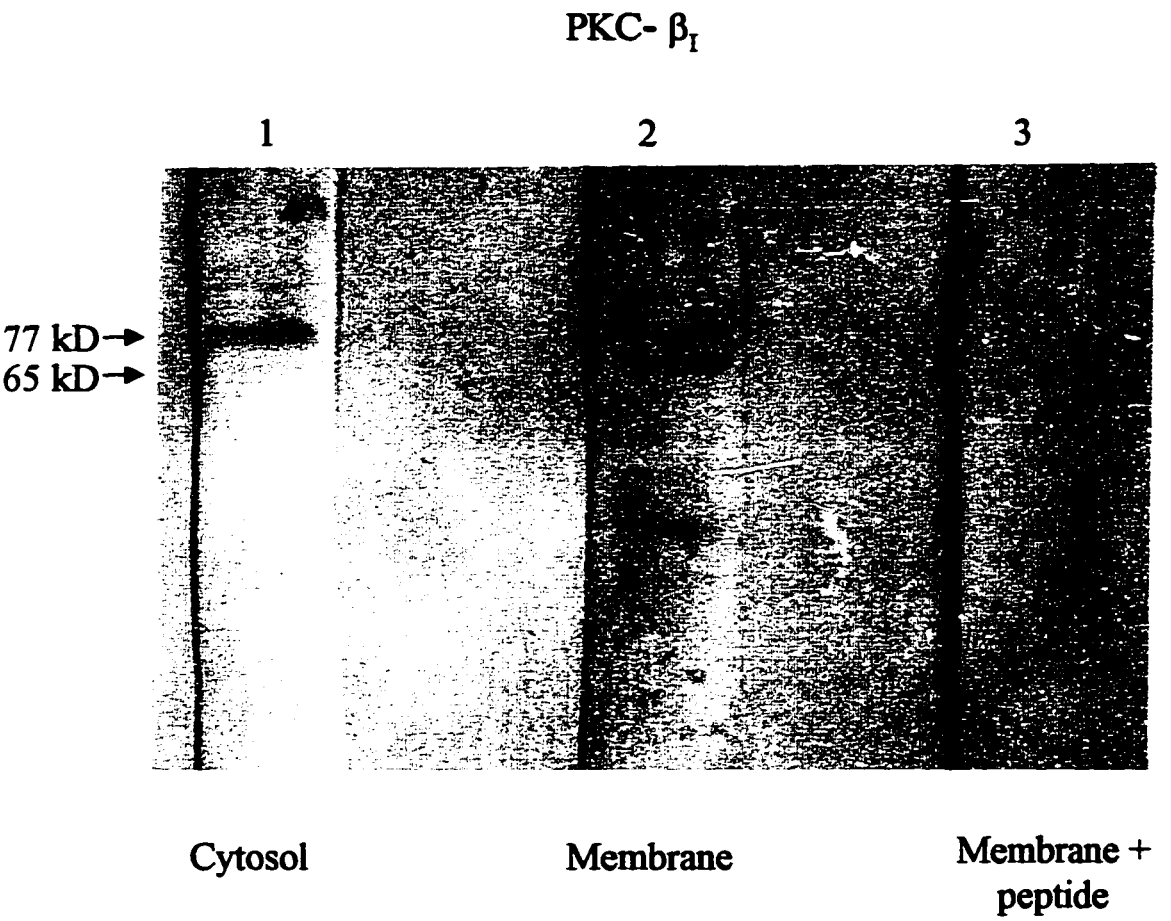
To determine which PKC isozyme is expressed in T51B cells, cytosolic or membrane fractions are immunoblotted with isozyme specific antibodies. As indicated in Fig. 1.1 antibody directed against PKC-β_I recognizes a single band in the cytosolic fraction (Fig. 1.1, lane 1) while in the plasma membrane PKC-β_I appears as two bands (doublet) of approximately 77 and 65 kD respectively (Fig. 1.1, Lane 2). Both these bands are competed out by the PKC-β_I antigenic peptide (Fig. 1.1, Lane 3). On the other hand, when the cytosolic or the membrane fraction is immunoblotted with PKC-β_{II} specific antibody, no immunoreactivity is detected (data not shown). The doublet for PKC-β_I is unique for T51B cells since in most other cells, PKC-β appears as a single band. To investigate the possibility that these two bands may result from differential phosphorylation, T51B cells are treated with 1 μM okadaic acid, (an inhibitor of protein phosphatases, PP1 and PP2A) (Haystead et al., 1985) for 15, 30 min or 1 h. The immunoblot of plasma membrane fraction derived from cells treated with okadaic acid for the indicated times is shown in Fig 1.2a. These results indicate a time-dependent increase in the level of PKC-β_I doublet which reaches a maximum at 1 h (Lane 4) as compared to control (Lane 1). In order to confirm these observations, it is necessary to measure the intensities of the two bands of PKC-β_I in response to okadaic acid treatment with time. Therefore, the three immunoblots are scanned by an HP Laser Scanner and the images then are analyzed by using Sigma Scan Image Software. These data are shown in Table 1.2. These results indicate an increase in the intensities of the two bands of PKC-β_I after 30 min and 1 h of okadaic acid treatment. Such

FIGURE 1.1

Expression of PKC-β_I in T51B Cells and Antibody Specificity

Confluent T51B cells were homogenized and fractionated into cytosol, lane (1), and plasma membrane, lane (2) as described in 1.3.2. Samples were solubilized in sample buffer and electrophoresed on 10% SDS-PAGE, transferred to a nitrocellulose membrane and immunoblotted with PKC-β_I antibody. Antigen-antibody complexes were detected with alkaline phosphatase-conjugated secondary antibody as described in 1.3.3. The specificity of antibody in the presence of PKC-β_I antigenic peptide is shown in lane (3). The molecular weight is expressed as kilodalton (kD). Data is representative of three experiments which gave identical results.

Figure 1.1



increase is significant since the P values are 0.05 and 0.01 respectively. Thus, okadaic acid treatment leads to an increase in the levels of the two subspecies of PKC-β_I phosphoproteins and differential phosphorylation of the two subspecies is not observed.

When cytosolic or plasma membrane fractions are immunoblotted with PKC-α specific antibody, the antibody recognizes a single band of approximately 80 kD in both the cytosolic and the membrane fractions (Fig. 1.3, Lanes 1,2). However, higher level of PKC-α protein is detected in the membrane fraction as compared to the cytosol. The antibody is competed out by the inclusion of PKC-α specific peptide antigen (Fig. 1.3, Lane 3). PKC-δ antibody detects two bands of approximately 78 and 76 kD in both the cytosolic and the membrane fractions (Fig. 1.4, Lanes 1,2). The antigenic peptide is able to compete with the lower band only and fails to displace the upper band (Fig. 1.4, Lane 3) which suggests that the upper band may represent non-specific binding of the antibody. The specific antibody for PKC-ϵ recognizes a single band of approximately 85 kD in both the cytosolic and the membrane fractions (Fig. 1.5, Lane 1,2) which is abolished by the addition of the antigenic peptide (Fig. 1.5, Lane 3). PKC-ζ antibody detects a major single band of approximately 80 kD in both the cytosol and the membranes of T51B cells (Fig. 1.6, Lane 1,2). These results also show that the level of PKC-ζ protein is high in both the cytosol and the membrane fractions. This band is also competed out by PKC-ζ antigenic peptide (Fig. 1.6, Lane 3).

1.4.1b Immunofluorescence of PKC Isozymes

Immunofluorescence localization indicates that PKC-β_I is associated with the plasma membrane with more fluorescence in certain areas along the plasma membrane where, it appears as punctate fluorescence along the cell boundaries between touching cells (Fig. 1.7a). The cytosol exhibits low PKC-β_I immunofluorescence. The most intriguing result, however,

FIGURE 1.2

Effect of Okadaic Acid on PKC-β_I in T51B Cells

Quiescent serum-deprived T51B cells were treated with PBS vehicle, lane (1) or 1 μM okadaic acid for 15 min, lane (2), 30 min, lane (3) or 1 h, lane (4) is shown in (a). Cells were homogenized and fractionated into cytosol and membrane fraction as described in 1.3.2. Plasma membrane samples were solubilized in sample buffer and electrophoresed on 10% SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with PKC-β_I specific antibody. Antigenic antibody complexes were detected with alkaline phosphatase-conjugated secondary antibody as described in 1.3.3. Data is representative of three experiments which gave identical results.

Figure 1.2a

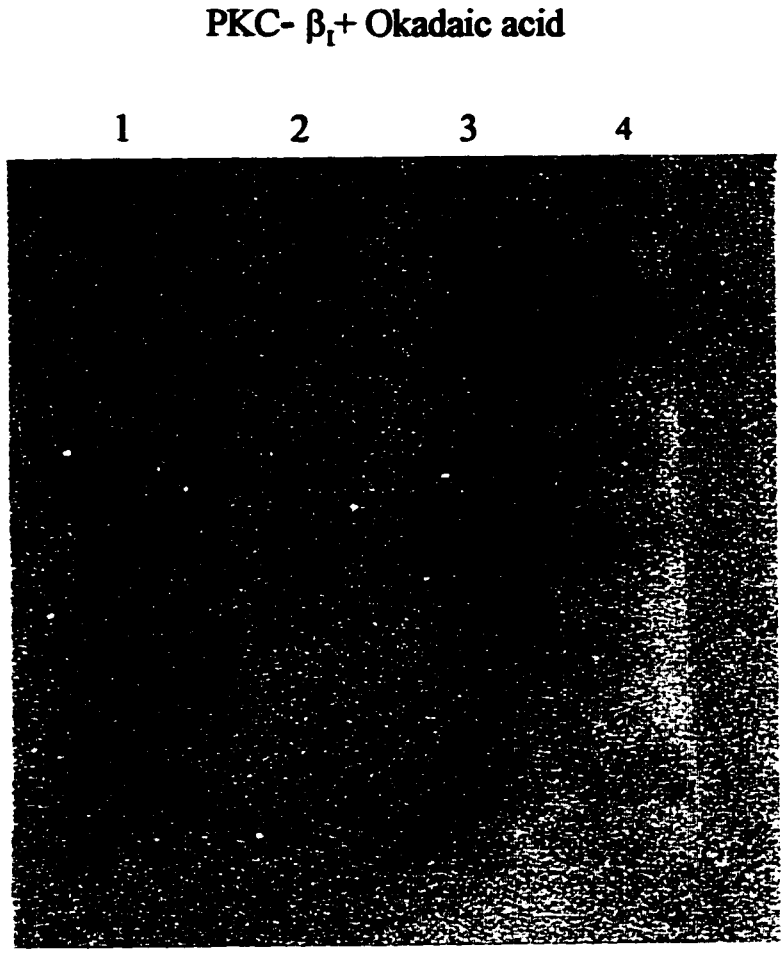


TABLE 1.2

Effect of Okadaic Acid on the Plasma Membrane Level of PKC-β_1 in T51B Cells

The immunoblots of plasma membrane fractions derived from controls and okadaic acid treated cells at various times were prepared as described in Figure 1.2. The blots were scanned with an HP Laser Scanner and the images were then analyzed by using Sigma Scan Image Software. Data are expressed as pixel intensities. Statistical analysis was performed by paired student's t-test, comparing the mean ± SE of three experiments of controls and okadaic acid treated cells. A level of significance of $P < 0.05$ is indicated as * and of $P < 0.01$ is indicated as **.

TABLE 1.2

Effect of Okadaic Acid on the Plasma Membrane Level of PKC-β_I in T51B Cells

	Mean ± SE			
	Control	**15 min**	**30 min**	**1 h**
PKC-β_I (77 kD)	74.2 ± 2.1	81.0 ± 8.7	87.3 ± 2.7*	101.0 ± 4.9**
PKC-β_I (65 kD)	63.5 ± 6.6	70.0 ± 7.4	81.0 ± 5.8*	101.0 ± 5.0**

SE: Standard error

FIGURE 1.3

Expression of PKC-α in T51B Cells and Antibody Specificity

Confluent T51B cells were homogenized and fractionated into cytosol, lane (1) and membrane, lane (2), as described in 1.3.2. Samples were solubilized in sample buffer and electrophoresed on 10% SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with PKC-α specific antibody. Antigen-antibody complexes were detected with alkaline phosphatase-conjugated secondary antibody as described in 1.3.3. The specificity of antibody in the presence of PKC-α antigenic peptide is shown in lane (3). The molecular weight is expressed as kilodalton (kD). Data is representative of three experiments which gave identical results.

Figure 1.3

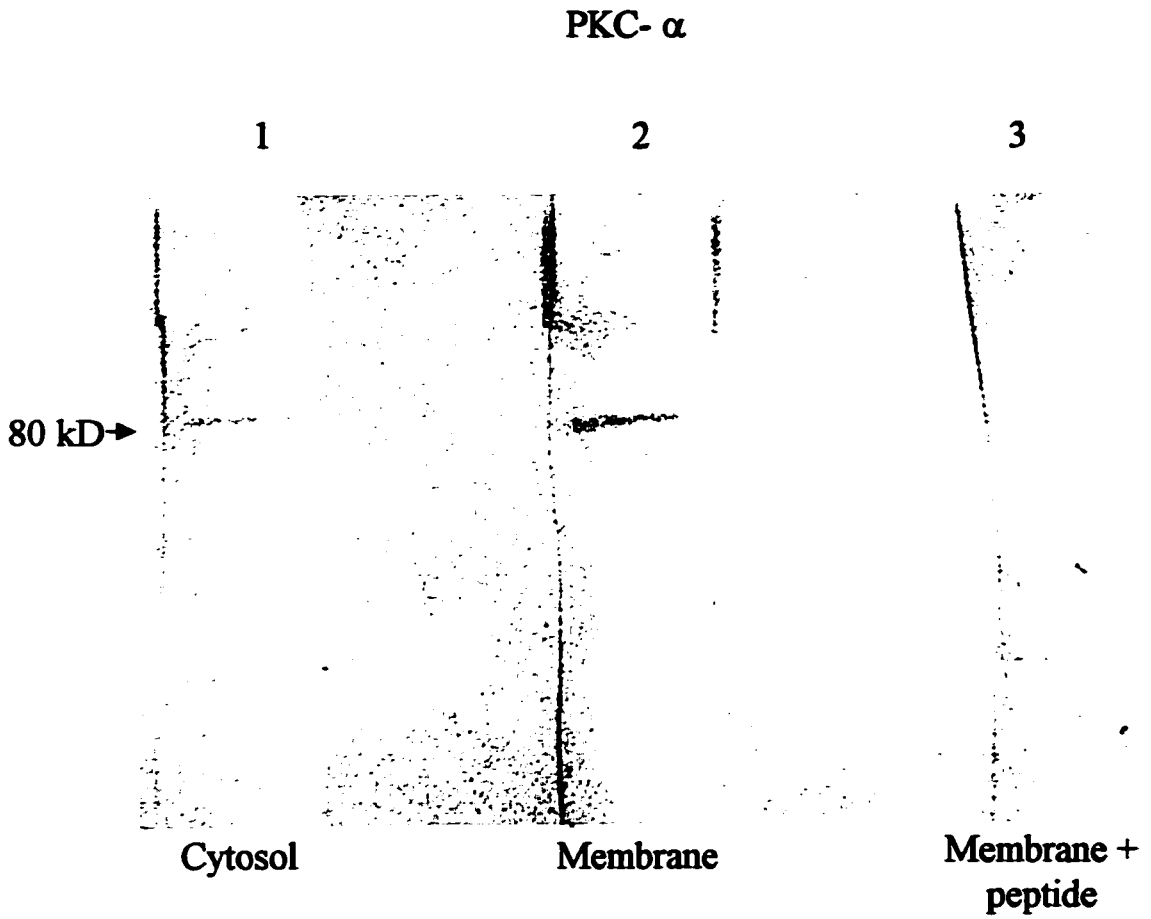


FIGURE 1.4

Expression of PKC-δ in T51B Cells and Antibody Specificity

Confluent T51B cells were homogenized and fractionated into cytosol, lane (1) and membrane, lane (2), as described in 1.3.2. Samples were solubilized in sample buffer and electrophoresed on 10% SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with PKC-δ specific antibody. Antigen-antibody complexes were detected with alkaline phosphatase-conjugated secondary antibody as described in 1.3.3. The specificity of the antibody in the presence of PKC-δ antigenic peptide is shown in lane (3). The molecular weight is expressed as kilodalton (kD). Data is representative of three experiments which gave identical results.

Figure 1.4

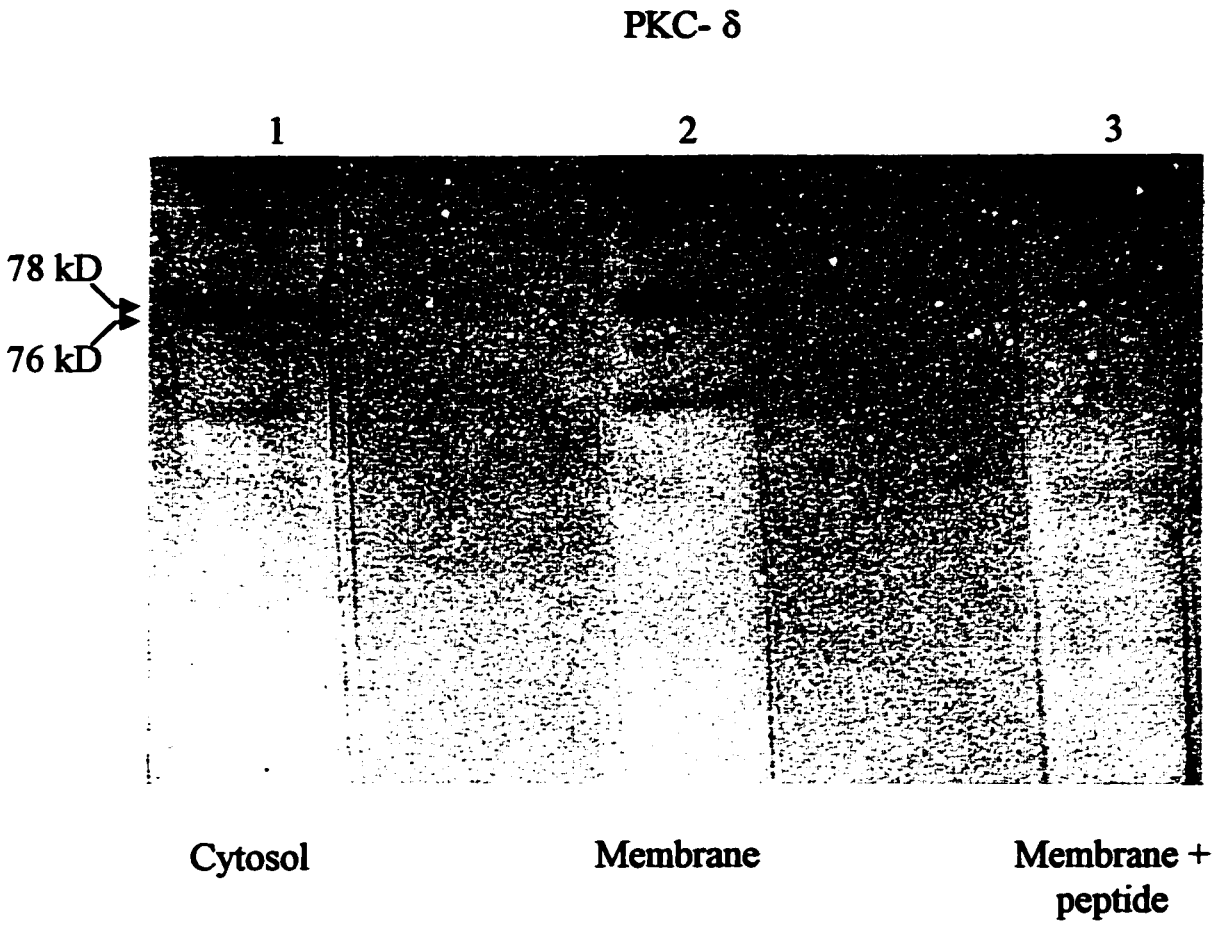


FIGURE 1.5

Expression of PKC-ϵ in T51B Cells and Antibody Specificity

T51B cells were homogenized and fractionated into cytosol, lane (1) and membrane, lane (2), as described in 1.3.2. Samples were homogenized in sample buffer and electrophoresed on 10% SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with PKC-ϵ specific antibody. Antigen-antibody complexes were detected with alkaline phosphatase-conjugated secondary antibody as described in 1.3.3. The specificity of the antibody in the presence of PKC-ϵ antigenic peptide is shown in lane (3). The molecular weight is expressed as kilodalton (kD). Data is representative of three experiments which gave identical results.

Figure 1.5

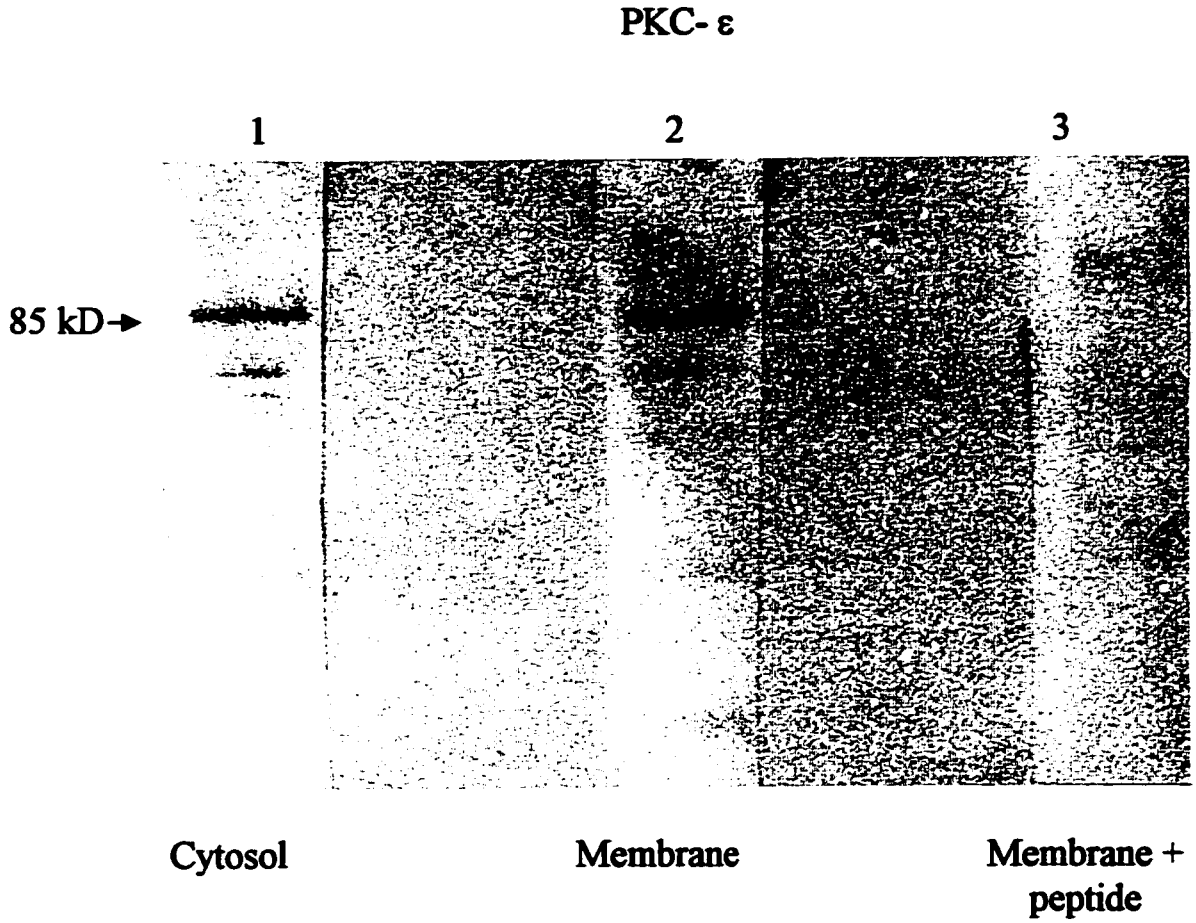
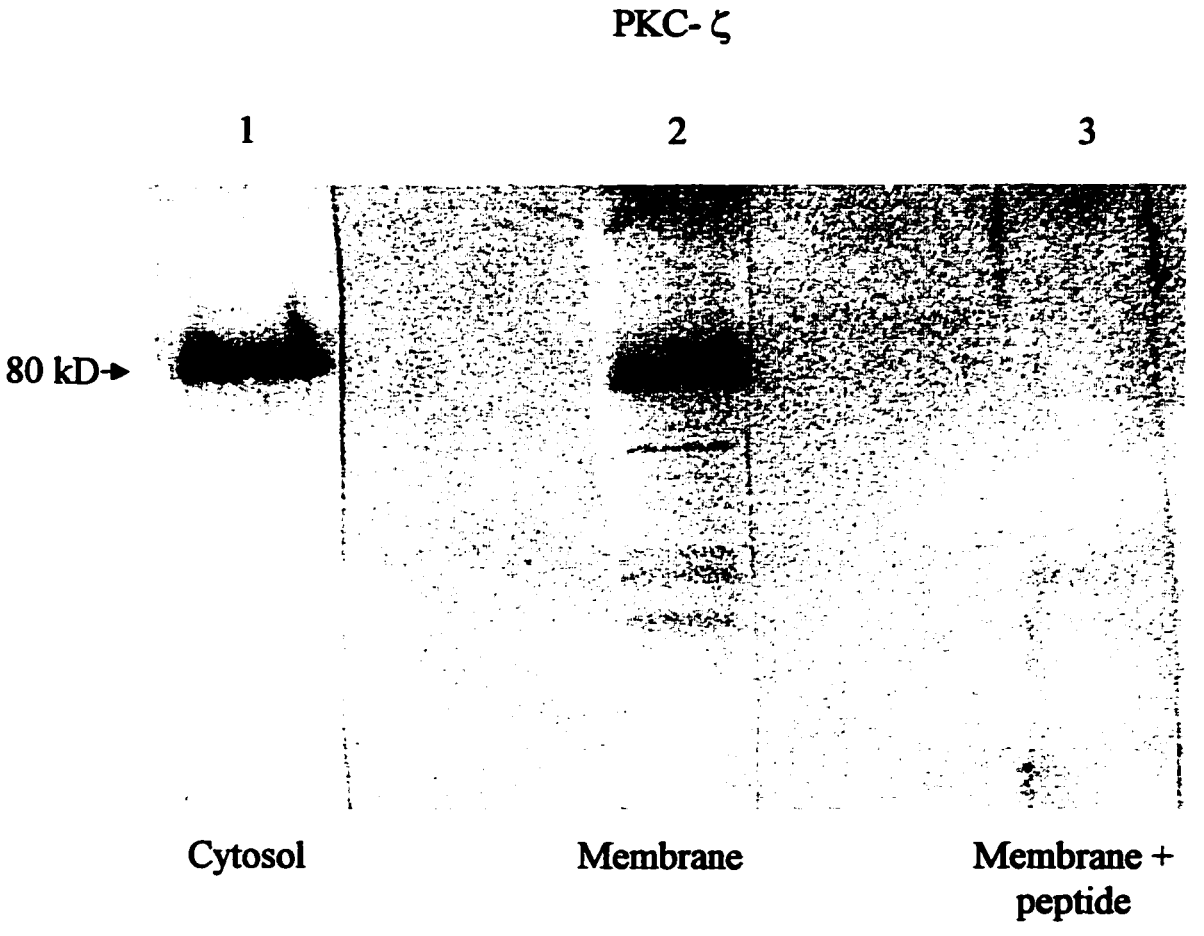


FIGURE 1.6

Expression of PKC-ζ in T51B Cells and Antibody Specificity

T51B cells were homogenized and fractionated into cytosol, lane (1) and membrane, lane (2), as described in 1.3.2. Samples were homogenized in sample buffer and electrophoresed on 10% SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with PKC-ζ specific antibody. Antigen-antibody complexes were detected with alkaline phosphatase-conjugated secondary antibody as described in 1.3.3. The specificity of the antibody in the presence of PKC-ζ antigenic peptide is shown in lane (3). The molecular weight is expressed as kilodalton (kD). Data is representative of three experiments which gave identical results.

Figure 1.6



is the association of PKC-β_I with the nucleus. The whole nucleus is stained but there is more fluorescence associated with the nuclear membrane. These results indicate that PKC-β_I is endogenously localized in the nucleus of T51B cells. The antigenic peptide is able to compete with the antibody and the immunofluorescence is observed to be abolished in all areas (Fig. 1.7b). Immunofluorescence data show that PKC-α isozyme is associated with the plasma membrane and also to a lesser extent in the cytosol. The nuclear staining appears to have a punctate fluorescence but, no staining is observed associated with the nuclear membrane (Fig. 1.7c). In the presence of the antigenic peptide, the immunofluorescence is reduced in all the areas with the exception of some faint punctate-like fluorescence associated with the nucleus which could be due to non-specific binding (Fig. 1.7d). The immunofluorescence result indicates that PKC-δ is localized in the cytosol and associated with the plasma membrane. Some fluorescence is also observed associated with the nucleus but not with the nuclear membrane (Fig. 1.7e). In the presence of the antigenic peptide, the immunofluorescence associated with the nucleus, the plasma membrane and the cytosol is completely reduced (Fig. 1.7f). Immunofluroescence data indicate that PKC-ϵ is localized in the cytosol, the plasma membrane and also associated with the nucleus but not with the nuclear membrane (Fig. 1.7g). However, in the presence of the antigenic peptide, no immunofluorescence is detected in the plasma membrane, the cytosol or within the nucleus (Fig. 1.7h) with the exception of some faint dot-like fluorescence observed in some areas which could be non-specific. Strong immunofluorescence of PKC-ζ is observed in both the cytosol and the plasma membrane of the cells with some fluorescence associated with the nucleus which is similar to that observed for PKC-δ and -ϵ (Fig. 1.7i). However, no immunostaining is seen associated with the nuclear membrane. Most of the fluorescence is

abolished in the presence of the antigenic peptide (Fig. 1.7j) with the exception of very faint punctate-like fluorescence observed in some areas which may result from non-specific binding. The specificity of the primary antibodies is also tested by incubating the intact fixed cells with secondary antibody only. As shown in Fig. 1.7k, the cells exhibit no fluorescence in all areas in the presence of the secondary antibody only.

1.4.2 Effect of TPA on Subcellular Localization of PKC Isozymes

1.4.2a Immunoblotting of PKC Isozymes

Previous work in our laboratory with T51B cells and in other laboratories with other cell types indicate that a 24 h treatment with TPA is optimal to demonstrate down regulation of certain PKC isoforms. T51B cells treated with 100 nM TPA for 24 h exhibit an increase in PKC-β_I immunoreactivity in the plasma membrane compared to vehicle control (Fig. 1.8a). This increase is correlated well with a concomitant decrease in the cytosolic fraction in TPA treated cells as compared to vehicle control (Fig. 1.8b). In order to confirm these data three blots of plasma membrane and cytosolic fractions derived from controls and TPA treated cells are scanned and the scanning results are shown in Table 1.8. These results indicate a significant increase in the intensities of the two PKC-β_I bands (77 kD and 65 kD) in the plasma membrane of TPA treated cells with a matching decrease in the intensity of the cytosolic PKC-β_I band (77 kD) as compared to vehicle controls. The intensity level of PKC-β_I (77 kD) band decreased significantly from 65.3 in control to 37.1 in TPA treated cells. Such a decrease corresponds well with a matching increase in the intensity of PKC-β_I (77 kD) in the plasma membrane of TPA treated cells.

Treatment of T51B cells with TPA for 24 h results in a complete reduction of the immunoreactivity of PKC-α in both the membrane and the cytosolic fractions (Figs. 1.9.a

FIGURE 1.7

Immunofluorescence of PKC-β_I, α, δ, ϵ and ζ Isozymes in T51B Cells and Antibody Specificity

T51B cells were plated at a density of 125 x 10^2 cells/cm^2 on glass coverslips in medium containing 10% serum. Confluent cells were fixed and incubated with PKC-β_I (a), PKC-α (c), PKC-δ (e), PKC-ϵ (g) or PKC-ζ (i) antibody. Immunofluorescence in the presence of antigenic peptide for the above PKC isozymes is shown as follows: PKC-β_I (b), PKC-α (d), PKC-δ (f), PKC-ϵ (h) and PKC-ζ (j). Antigen-antibody complexes were detected with a fluorescein-conjugated secondary antibody as described in 1.3.4. Non-specific binding in the presence of secondary antibody is shown in (k). Magnification: Figure 1.7a-k is x1000. Immunostaining was performed four times with similar results.

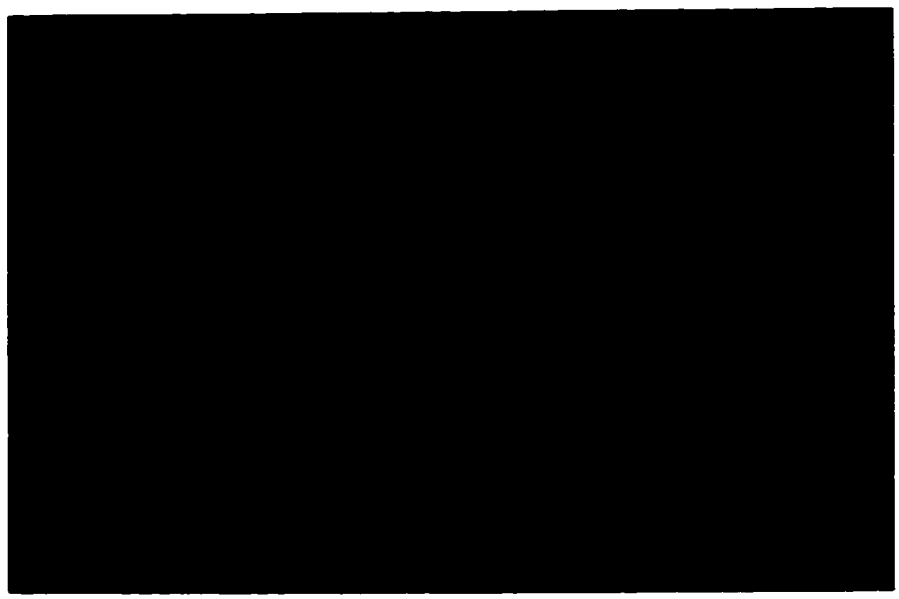
Figure 1.7

(a)

PKC- β_I (control)

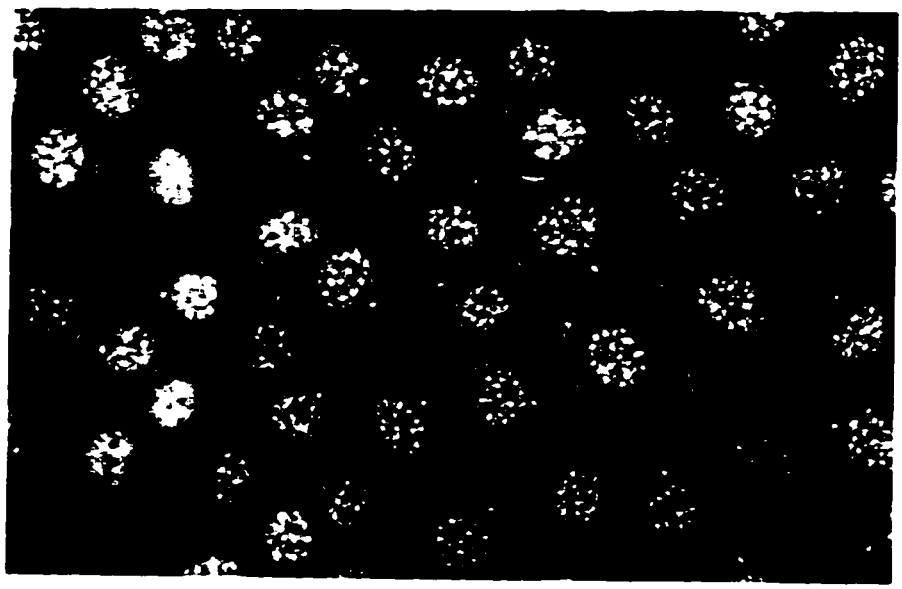
(b)

PKC- β_I + peptide



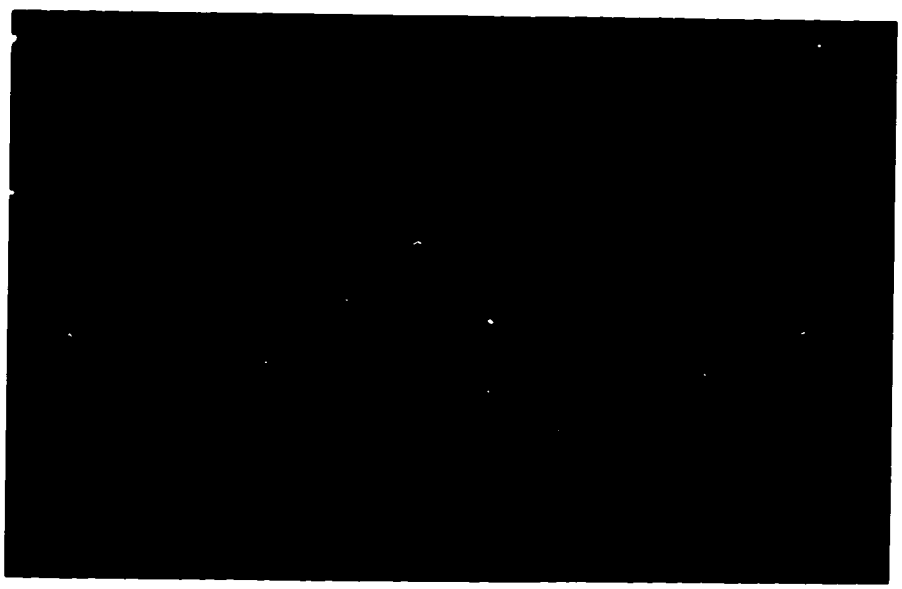
(c)

PKC- α (control)



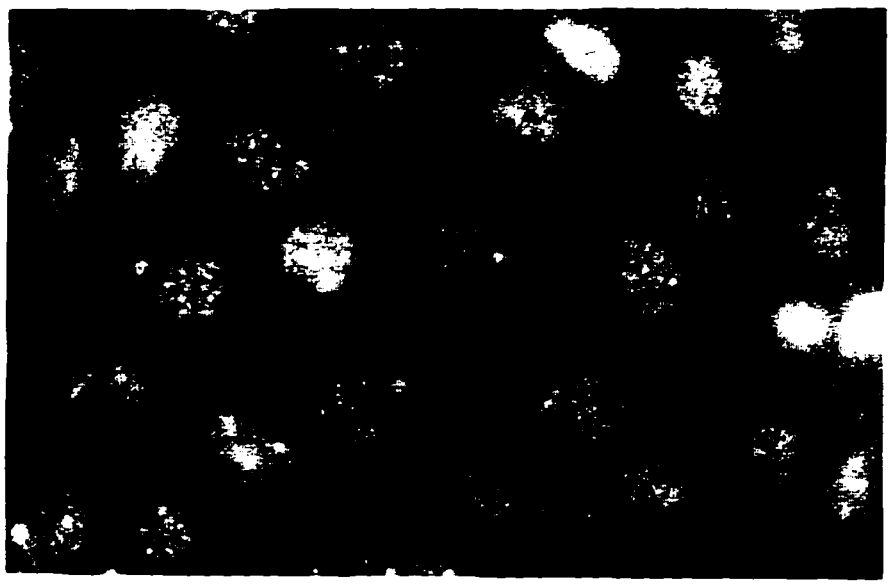
(d)

PKC- α + peptide



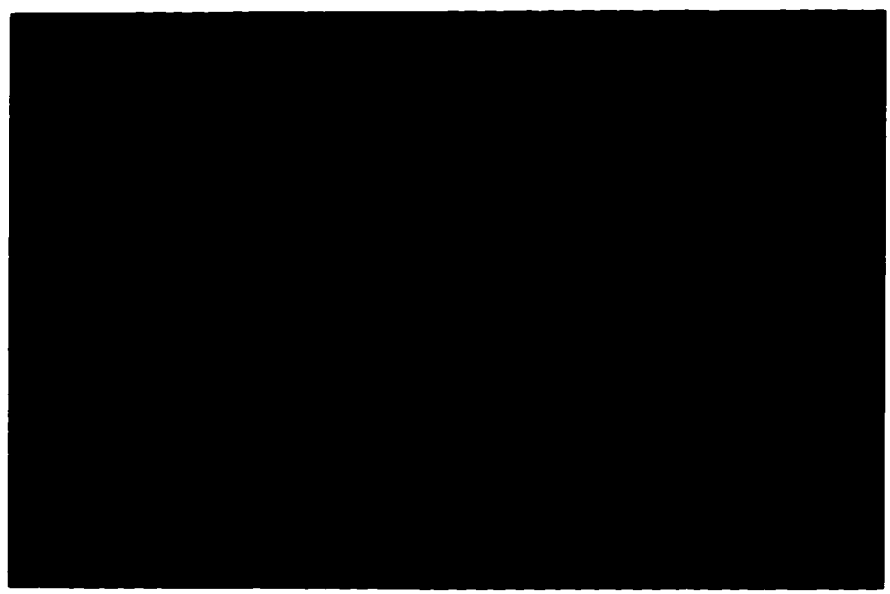
(e)

PKC- δ (control)



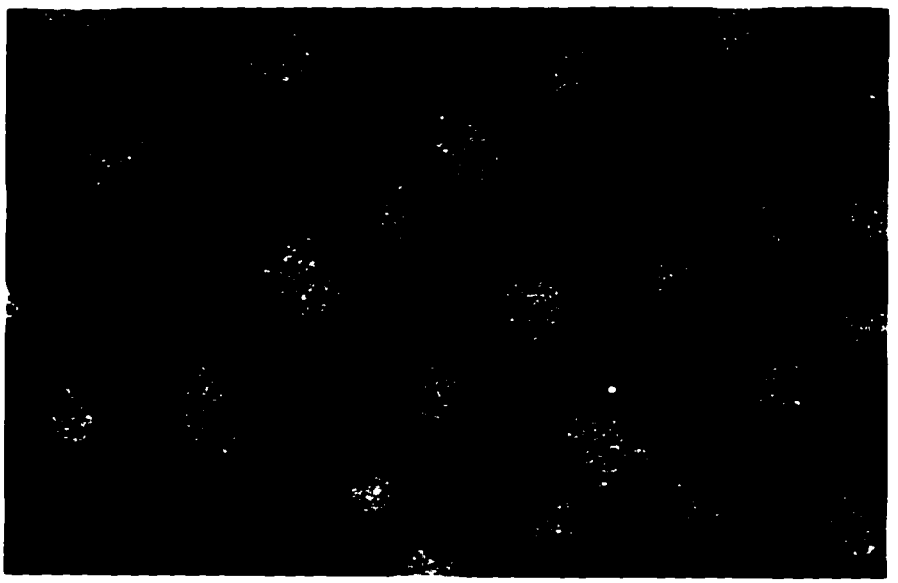
(f)

PKC- δ + peptide



(g)

PKC- ε (control)

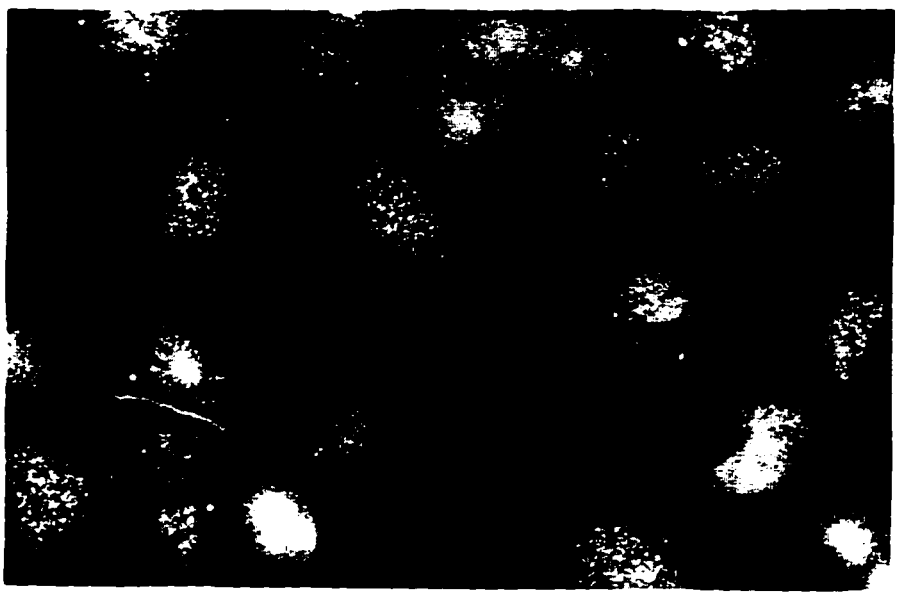


(h)

PKC- ε + peptide

(i)

PKC- ζ (control)

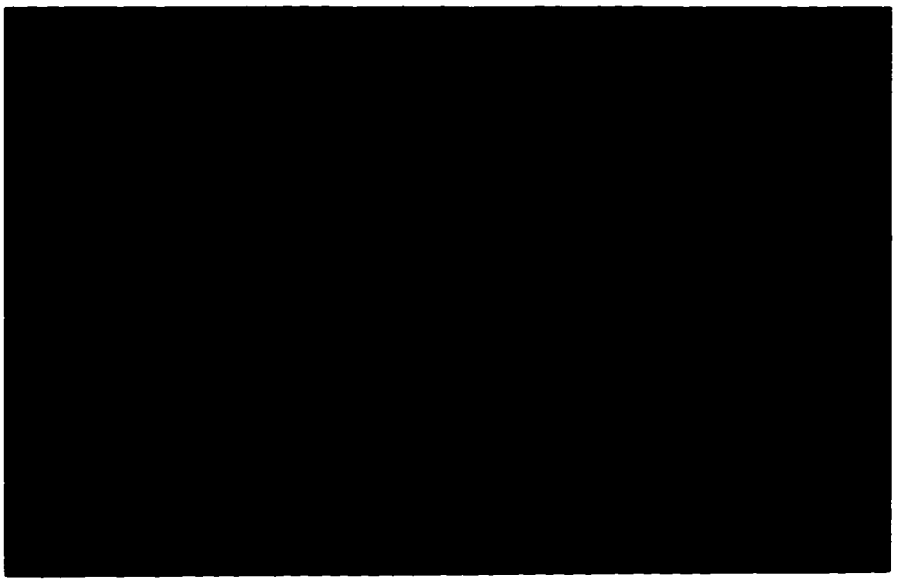


(j)

PKC- ζ + peptide

(k)

Secondary antibody only



and 1.9b). Scanning results of three immunoblots of cytosolic and plasma membrane fractions derived from controls and TPA treated cells are shown in Table 1.9 These results indicate that TPA treatment for 24 h results in a complete reduction of the intensity level of PKC-α band in both the cytosolic and the membrane fractions. The immunoreactivity of PKC-ϵ is also observed to be completely reduced in TPA treated cells in both the plasma membrane and the cytosolic fractions as compared to vehicle controls (Figs. 1.10a and 1.10b). Scanning results of three immunoblots also show that the intensity of PKC-ϵ band in the cytosolic and plasma membrane fractions are completely abolished upon TPA treatment (Table 1.10). Immunoblotting data indicate that PKC-δ appears as a doublet in control cells, however, when the cells are treated for 24 h with TPA, the lower band completely disappears while no effect on the immunoreactivity of the upper band in both cytosolic and membrane fractions is observed (Figs. 1.11a and 1.11b). Scanning results of three cytosolic and plasma membrane immunoblots also indicate that PKC-δ appears as two bands (78 kD and 76 kD) in control cells. Upon treatment with TPA the intensity of 76 kD band is completely apolished without any change in the intensity of the 78 kD band (Table 1.11). On the other hand, 24 h treatment with TPA has no effect on the immunoreactivity of PKC-ζ in either the plasma membrane (Fig. 1.12a) or the cytosolic fraction (Fig. 1.12b). When three blots of cytosolic and plasma membrane fractions are scanned, there is no change in the intensity of PKC-ζ band in TPA treated cells compared to vehicle control (Table 1.12).

1.4.2b Effect of TPA on Immunofluorescence of PKC Isozymes

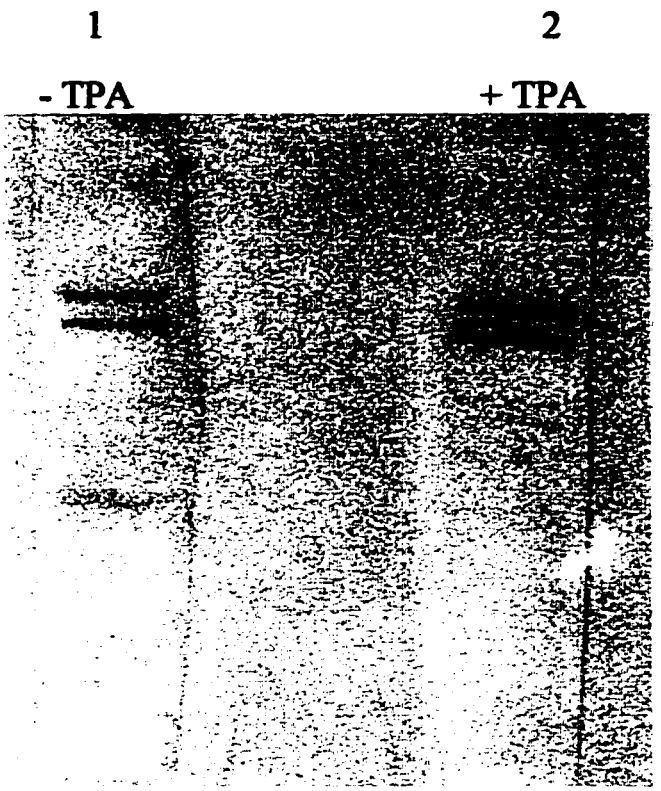
To study the effect of TPA on the intracellular localization of PKC isozymes, the cells are treated with TPA, fixed, prepared for immunofluorescence analysis, and photographed with Kodak ektachrome 400 colour slide film. Consistent with

FIGURE 1.8

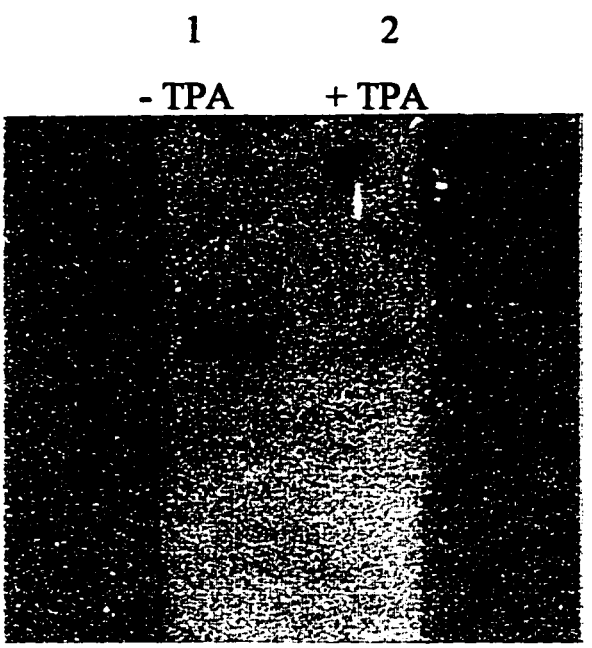
Effect of TPA on the Subcellular Distribution of PKC-β_I in T51B Cells

Confluent T51B cells were treated with ethanol/DMSO vehicle, lane (1) or 100 nM TPA, lane (2) for 24 h. Cells were homogenized and fractionated into plasma membrane (a) and cytosol (b) as described in 1.3.2. Samples were solubilized in sample buffer and electrophoresed on 10% SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with PKC-β_I antibody. Antigen-antibody complexes were detected with alkaline phosphatase-conjugated secondary antibody as described in 1.3.3. Data is representative of three experiments which gave identical results.

Figure 1.8a, b



(a) PKC- β_I (membrane)



(b) PKC- β_I (cytosol)

TABLE 1.8

Effect of TPA on the Cytosolic and the Plasma Membrane Distribution of PKC-β_I in T51B Cells

The immunoblots of cytosolic and plasma membrane fractions derived from controls and TPA treated cells were prepared as described in figure 1.8. The blots were scanned with an HP Laser Scanner and the images were then analyzed by using Sigma Scan Image Software. Data are expressed as pixel intensities. Statistical analysis was performed by paired student's t-test, comparing the mean ± SE of three experiments of controls and TPA treated cells. A level of significance of $P < 0.05$ is indicated as * and of $P < 0.01$ is indicated as **

TABLE 1.8

Effect of TPA on the Cytosolic and the Plasma Membrane Distribution of PKC-β_I in T51B Cells

	Mean ± SE			
	Cytosolic Fractions		**Plasma Membrane Fractions**	
	Control	**+ TPA (24 h)**	**Control**	**+ TPA (24 h)**
PKC-β_I (77 kD)	65.3 ± 3.0	37.1 ± 1.2**	87.0 ± 5.1	113.0 ± 2.0*
PKC- β_I (65 kD)			86.0 ± 4.7	112.0 ± 1.4*

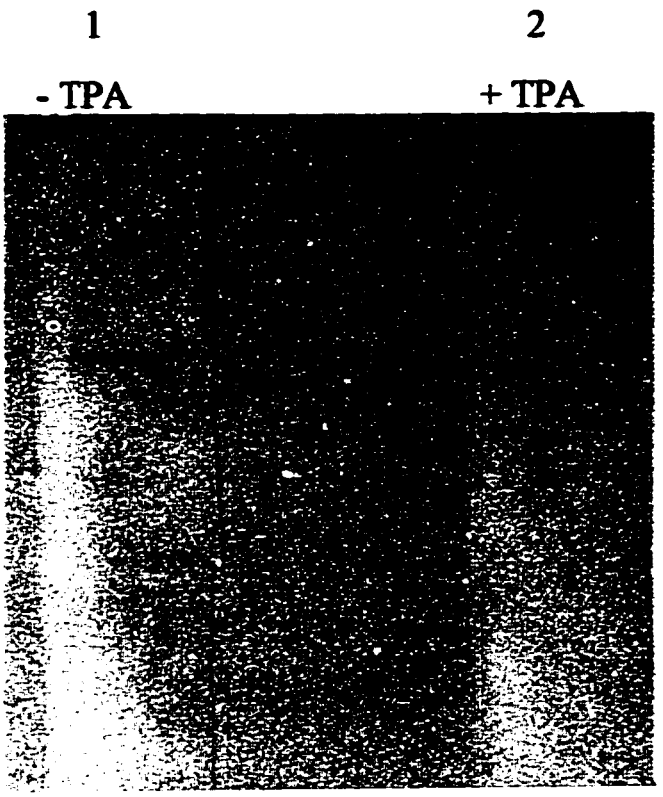
SE: Standard error

FIGURE 1.9

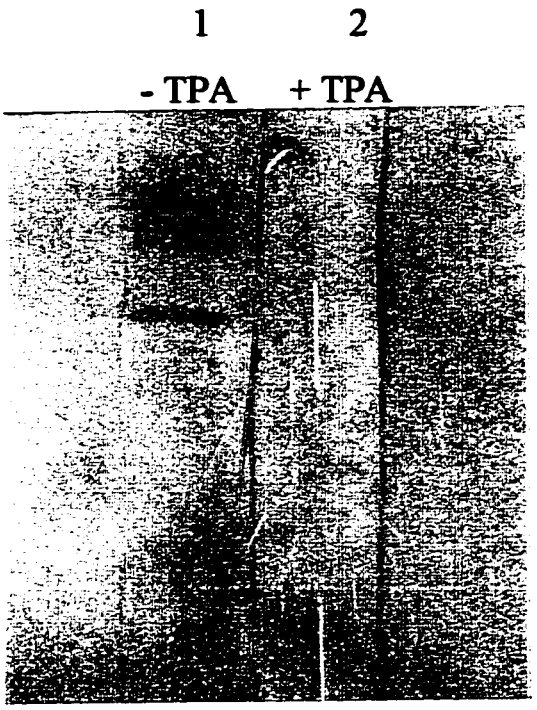
Effect of TPA on the Subcellular Distribution of PKC-α in T51B Cells

Confluent T51B cells were treated with ethanol/DMSO vehicle, lane (1) or 100 nM TPA, lane (2) for 24 h. Cells were homogenized and fractionated into plasma membrane (a) and cytosol (b) as described in 1.3.2. Samples were solubilized in sample buffer and electrophoresed on 10% SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with PKC-α antibody. Antigen-antibody complexes were detected with alkaline phosphatase-conjugated secondary antibody as described in 1.3.3. Data is representative of three experiments which gave identical results.

Figure 1.9a, b



(a) PKC- α (membrane)



(b) PKC- α (cytosol)

TABLE 1.9

Effect of TPA on the Cytosolic and the Plasma Membrane Distribution of PKC-α in T51B Cells

The immunoblots of cytosolic and plasma membrane fractions derived from controls and TPA treated cells were prepared as described in figure 1.9. The blots were scanned with an HP Laser Scanner and the images were then analyzed by using Sigma Scan Image Software. Data are expressed as pixel intensities. Statistical analysis was performed by paired student's t-test, comparing the mean ± SE of three experiments of controls and TPA treated cells.

TABLE 1.9

Effect of TPA on the Cytosolic and the Plasma Membrane Distribution of PKC-α in T51B Cells

	Mean ± SE	
	Cytosolic Fractions	**Plasma Membrane Fractions**
Control	50.3 ± 3.8	61.1 ± 1.5
TPA (24 h)	0.0	0.0

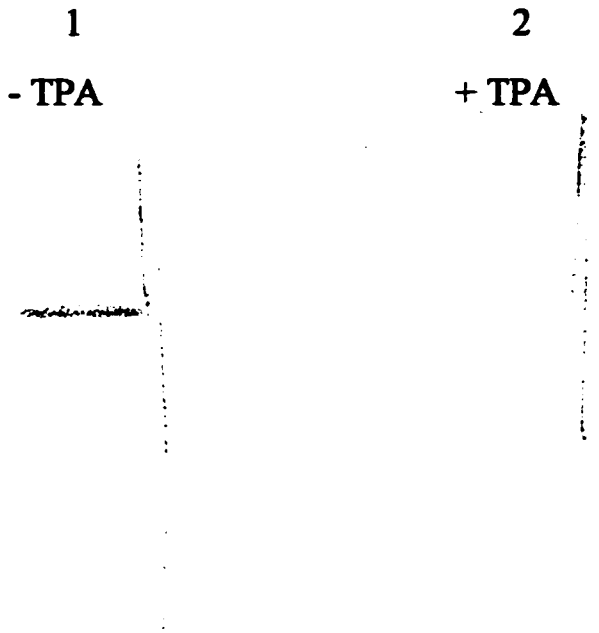
SE: Standard error

FIGURE 1.10

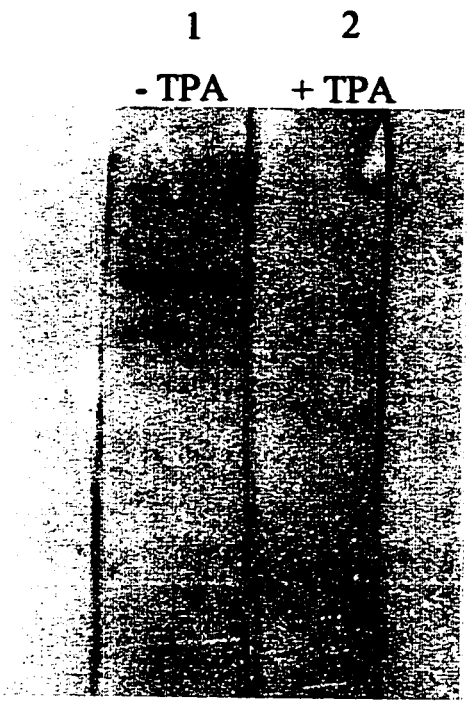
Effect of TPA on the Subcellular Distribution of PKC-ϵ in T51B Cells

Confluent T51B cells were treated with ethanol/DMSO vehicle, lane (1) or 100 nM TPA, lane (2) for 24 h. Cells were homogenized and fractionated into plasma membrane (a) and cytosol (b) as described in 1.3.2. Samples were solubilized in sample buffer and electrophoresed on 10% SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with PKC-ϵ antibody. Antigen-antibody complexes were detected with alkaline phosphatase-conjugated secondary antibody as described in 1.3.3. Data is representative of three experiments which gave identical results.

Figure 1.10a, b



(a) PKC- ε (membrane)



(b) PKC- ε (cytosol)

TABLE 1.10

Effect of TPA on the Cytosolic and the Plasma Membrane Distribution of PKC-ϵ in T51B Cells

The immunoblots of cytosolic and plasma membrane fractions derived from controls and TPA treated cells were prepared as described in figure 1.10. The blots were scanned with an HP Laser Scanner and the images were then analyzed by using Sigma Scan Image Software. Data are expressed as pixel intensities. Statistical analysis was performed by paired student's t-test, comparing the mean ± SE of three experiments of controls and TPA treated cells.

TABLE 1.10

Effect of TPA on the Cytosolic and the Plasma Membrane Distribution of PKC-ϵ in T51B Cells

	Mean ± SE	
	Cytosolic Fractions	**Plasma Membrane Fractions**
Control	71.0 ± 3.2	76.3 ± 4.1
+ TPA (24 h)	0.0	0.0

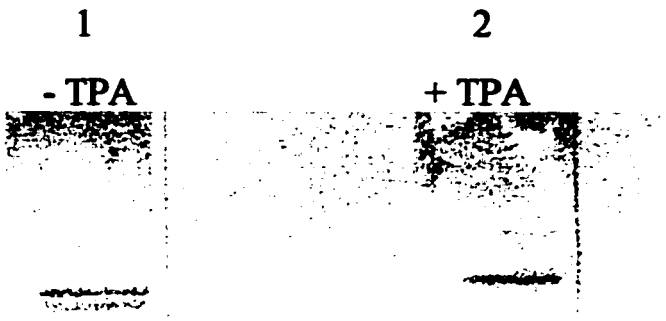
SE: Standard error

FIGURE 1.11

Effect of TPA on the Subcellular Distribution of PKC-δ in T51B Cells

Confluent T51B cells were treated with ethanol/DMSO vehicle, lane (1) or 100 nM TPA, lane (2) for 24 h. Cells were homogenized and fractionated into plasma membrane (a) and cytosol (b) as described in 1.3.2. Samples were solubilized in sample buffer and electrophoresed on 10% SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with PKC-δ antibody. Antigen-antibody complexes were detected with alkaline phosphatase-conjugated secondary antibody as described in 1.3.3. Data is representative of three experiments which gave identical results.

Figure 1.11a, b



(a) PKC- δ (membrane)

(b) PKC- δ (cytosol)

TABLE 1.11

Effect of TPA on the Cytosolic and the Plasma Membrane Distribution of PKC-δ in T51B Cells

The immunoblots of cytosolic and plasma membrane fractions derived from controls and TPA treated cells were prepared as described in figure 1.11. The blots were scanned with an HP Laser Scanner and the images were then analyzed by using Sigma Scan Image Software. Data are expressed as pixel intensities. Statistical analysis was performed by paired student's t-test, comparing the mean ± SE of three experiments of controls and TPA treated cells.

TABLE 1.11

Effect of TPA on the Cytosolic and the Plasma Membrane Distribution of PKC-δ in T51B Cells

	Mean ± SE			
	Cytosolic Fractions		**Plasma Membrane Fractions**	
	Control	**+ TPA (24 h)**	**Control**	**+ TPA (24 h)**
PKC-δ (78 kD)	115.7 ± 2.0	111.0 ± 4.7	84.0 ± 2.7	83.7 ± 1.2
PKC- δ (76 kD)	93.7 ± 2.0	0.0	79.0 ± 3.5	0.0

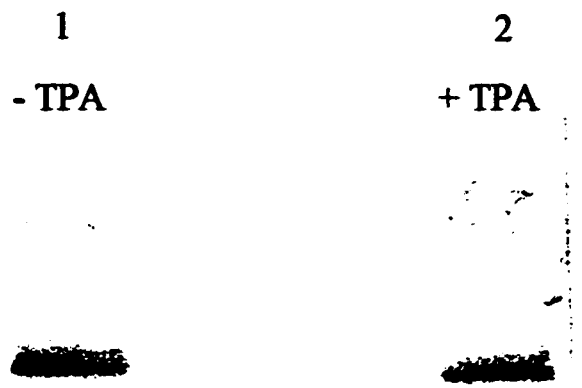
SE: Standard error

FIGURE 1.12

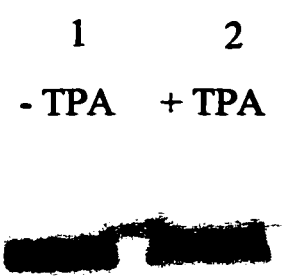
Effect of TPA on the Subcellular Distribution of PKC-ζ in T51B Cells

Confluent T51B cells were treated with ethanol/DMSO vehicle, lane (1) or 100 nM TPA, lane (2) for 24 h. Cells were homogenized and fractionated into plasma membrane (a) and cytosol (b) as described in 1.3.2. Samples were solubilized in sample buffer and electrophoresed on 10% SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with PKC-ζ antibody. Antigen-antibody complexes were detected with alkaline phosphatase-conjugated secondary antibody as described in 1.3.3. Data is representative of three experiments which gave identical results.

Figure 1.12a, b



(a) PKC- ζ (membrane)



(b) PKC- ζ (cytosol)

TABLE 1.12

Effect of TPA on the Cytosolic and the Plasma Membrane Distribution of PKC-ζ in T51B Cells

The immunoblots of cytosolic and plasma membrane fractions derived from controls and TPA treated cells were prepared as described in figure 1.12. The blots were scanned with HP Laser Scanner and the images were then analyzed by using Sigma Scan Image Software. Data are expressed as pixel intensities. Statistical analysis was performed by paired student's t-test, comparing the mean ± SE of three experiments of controls and TPA treated cells.

TABLE 1.12

Effect of TPA on the Cytosolic and the Plasma Membrane Distribution of PKC-ζ in T51B Cells

	Mean ± SE	
	Cytosolic Fractions	**Plasma Membrane Fractions**
Control	113.5 ± 4.3	114.4 ± 5.0
+ TPA (24 h)	111.8 ± 4.4	112.1 ± 4.1

SE: Standard error

immunoblotting results, T51B cells treated with TPA for 24 h exhibit an increase in the immunofluorescence of PKC-β_I associated with the plasma membrane. (Fig. 1.13b) as compared to vehicle control (Fig. 1.13a). This result is consistant with the immunoblot analysis (Fig. 1.8 and Table 1.8). However, the effect of TPA on the immunofluorescence of the cytosolic and the nuclear PKC-β_I is not clear. The immunofluorescence of PKC-α completely disappears in the plasma membrane of TPA treated cells (Fig. 1.13d) compared to vehicle control (Fig. 1.13c) which is consistent with the immunoblot analysis (Fig. 1.9 and Table 1.9). However, the fluorescence associated with the nucleus is not changed by TPA treatment. Similarly, TPA treatment results in a complete reduction of the immunofluorescence of PKC-δ and PKC-ϵ in the plasma membrane (Figs. 1.13f and 1.13h) compared to vehicle controls (Figs. 1.13e and 1.13g) which is consistent with the immunoblot analysis (Figs. 1.10-1.11 and Tables 1.10-1.11). However, TPA treatment has no effect on the immunofluorescence associated with the nucleus for both PKC-δ and PKC-ϵ. Immunofluorescence results indicate that TPA treatment has no effect on the immunofluorescence of PKC-ζ (Fig. 1.13j) in all cellular localization compared to vehicle control (Fig. 1.13i). These findings are consistent with the immunoblotting results which demonstrate that TPA treatment has no effect on PKC-ζ subcellular localization or down-regulation (Fig. 1.12 and Table 1.12).

1.4.3 Effect of EGF Treatment on Subcellular Localization of PKC Isozymes

Previous reports from our laboratory have demonstrated that EGF has mitogenic effects on T51B cells where, it increases DNA synthesis with a rapid increase in membrane-associated PKC activity (Sharma et al., 1994). Therefore, it is of interest to examine the effect of this mitogen on the subcellular localization of PKC isozymes expressed in T51B

FIGURE 1.13

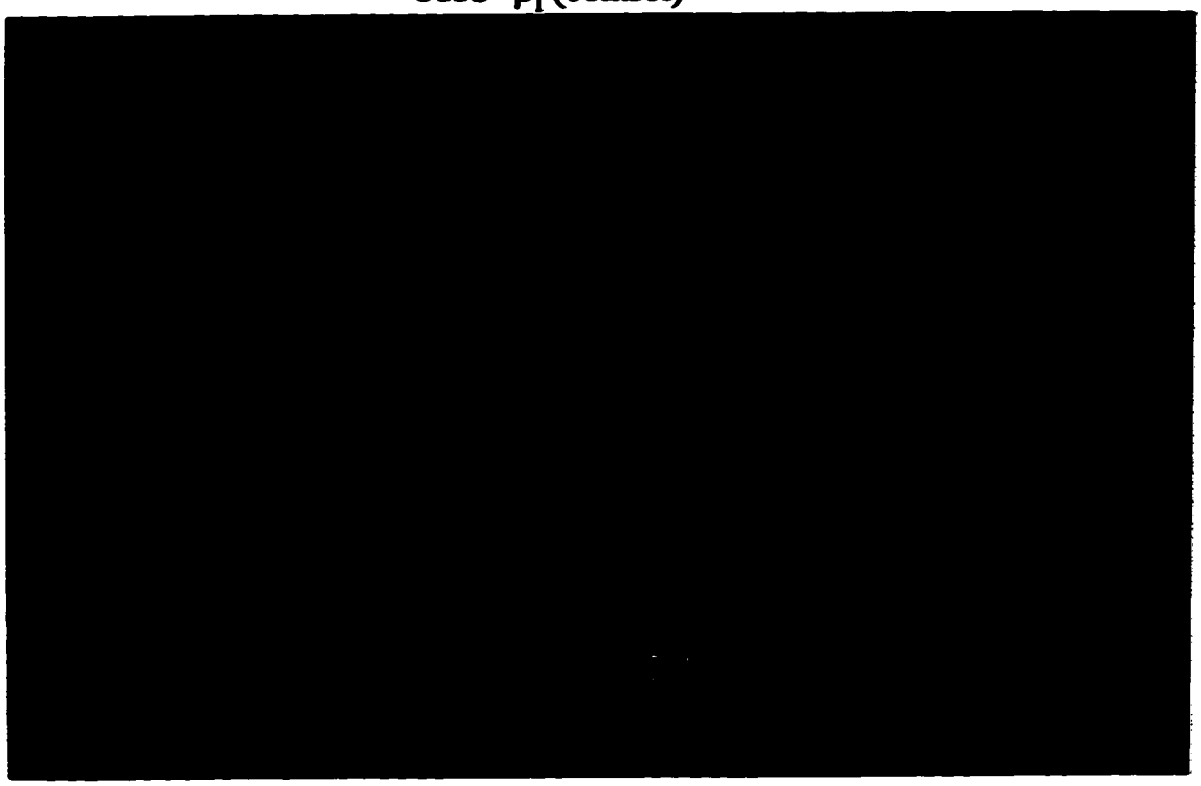
Effect of TPA on PKC-β_I, α, δ, ϵ and ζ Immunofluorescence

T51B cells were plated at a density of 125 x 10^2 cells/cm^2 on glass coverslips in medium containing 10% serum. Confluent cells were then treated with ethanol/DMSO vehicle or 100 nM TPA for 24 h. Cells were then fixed and incubated with PKC-specific antibodies. Immunofluorescence staining for vehicle control are: PKC-β_I (a), PKC-α (c), PKC-δ (e), PKC-ϵ (g) and PKC-ζ (i). Immunofluorescence for TPA treated cells are: PKC-β_I (b), PKC-α (d), PKC-δ (f), PKC-ϵ (h) and PKC-ζ (j). Antigen-antibody complexes were detected with fluorescein-conjugated secondary antibody as described in 1.3.4. Magnification: Figure 1.13a-j is x1000. Immunostaining was performed four times with similar results.

Figure 1.13

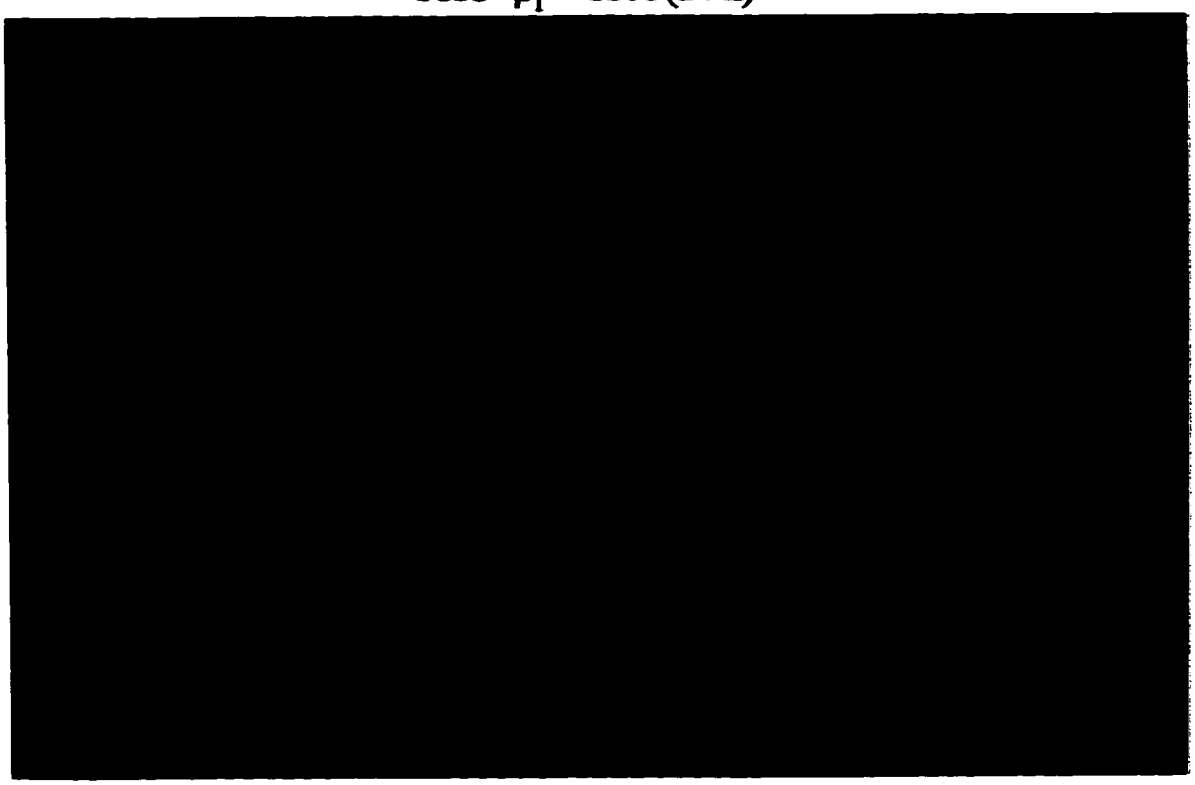
PKC- β_I (control)

(a)



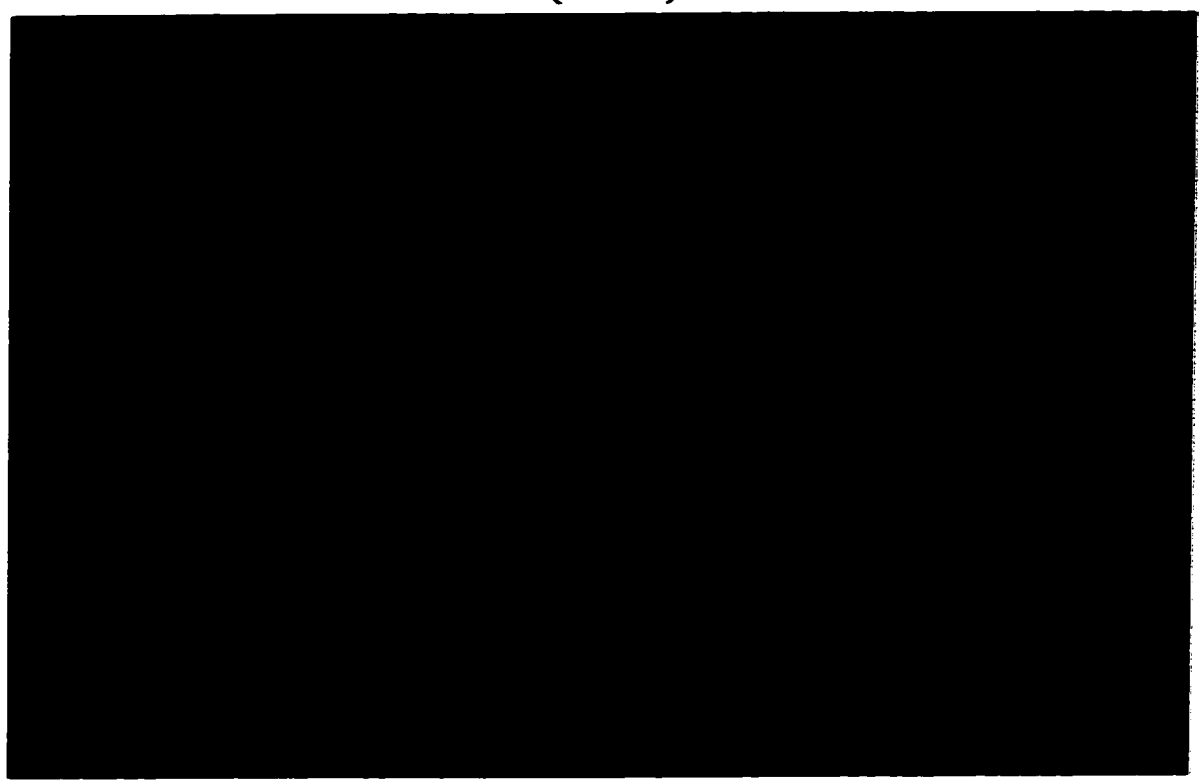
PKC- β_I + TPA (24 h)

(b)



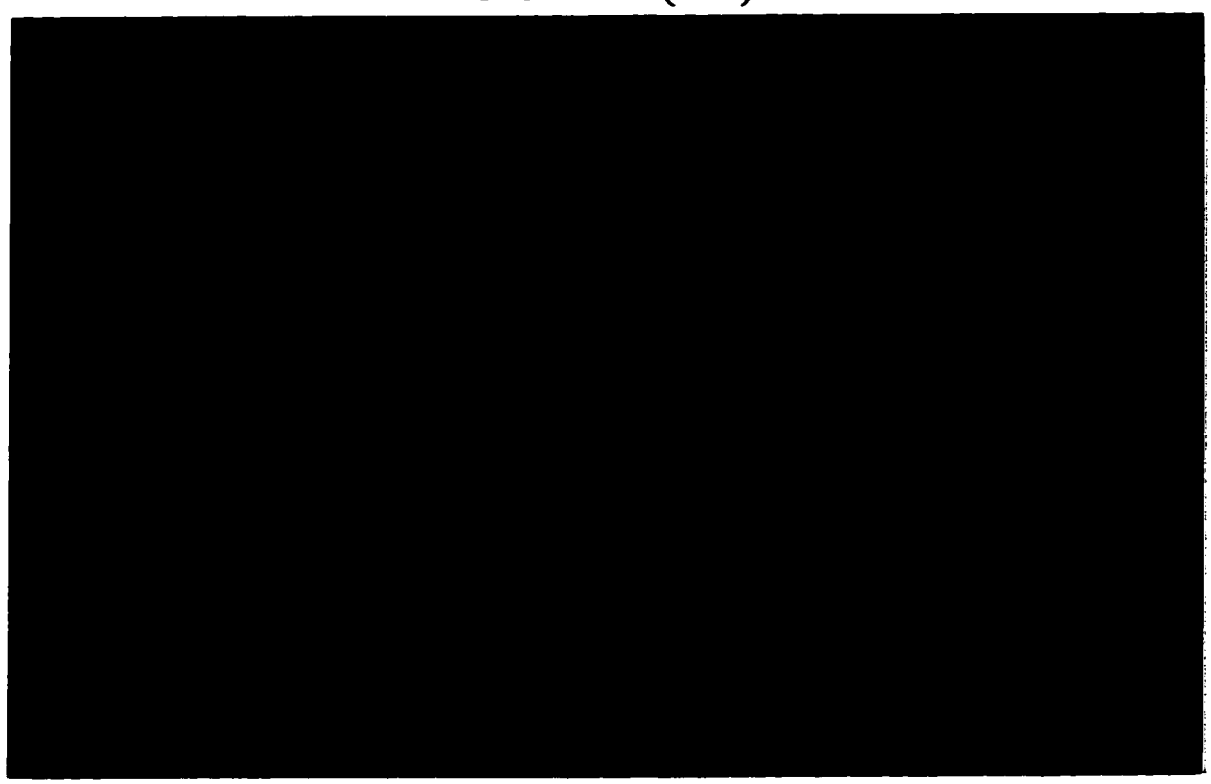
PKC- α (control)

(c)



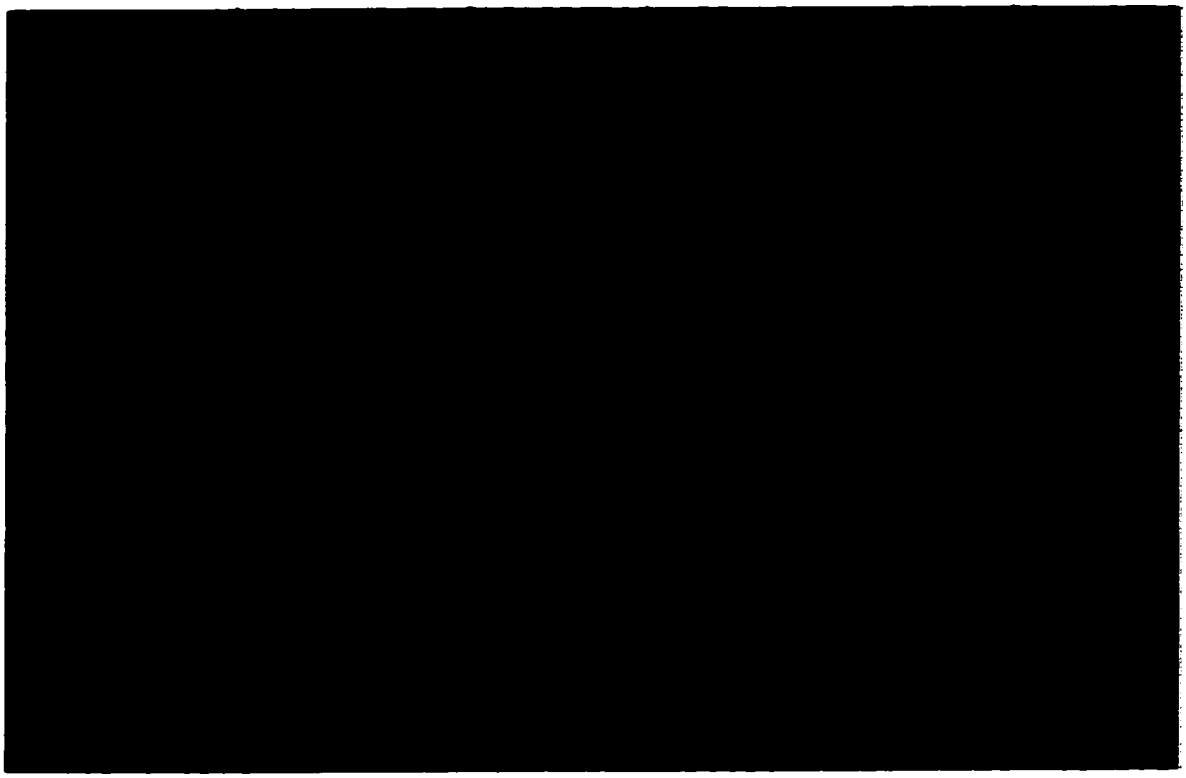
PKC- α + TPA (24 h)

(d)



PKC- δ (control)

(e)

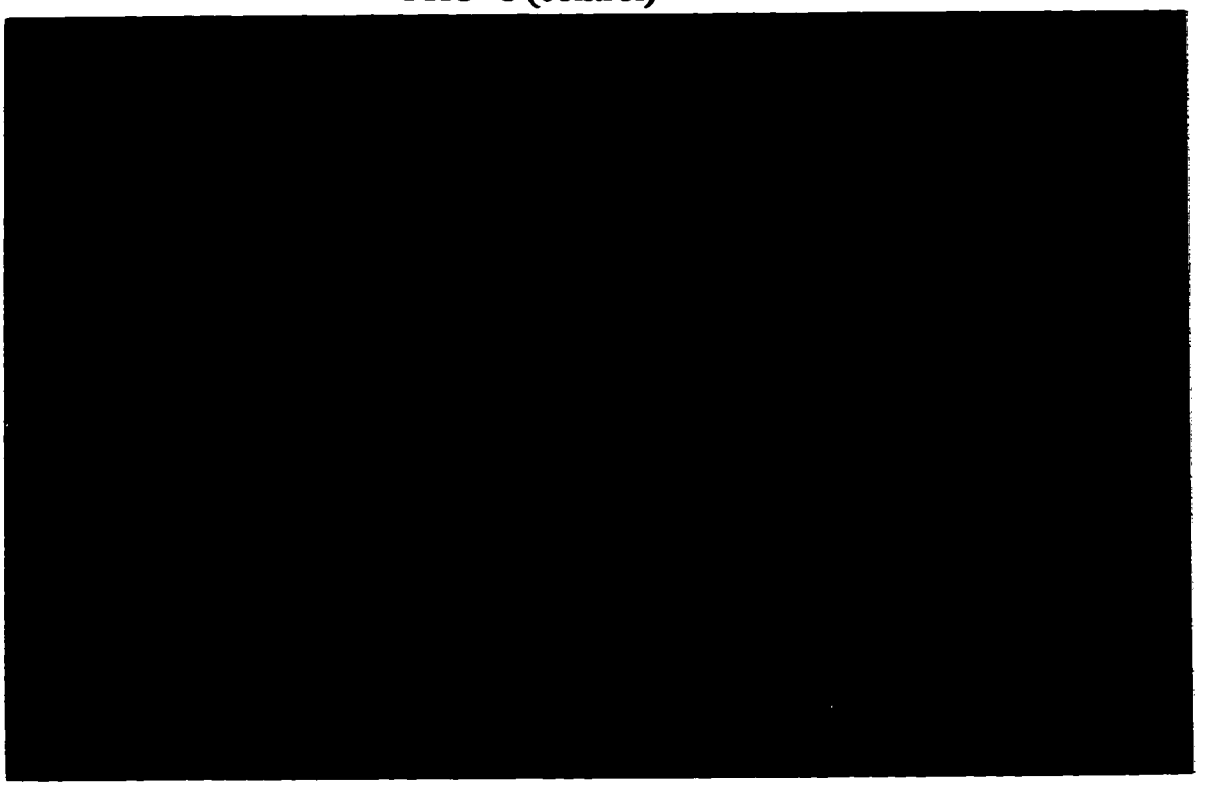


PKC- δ + TPA (24 h)

(f)

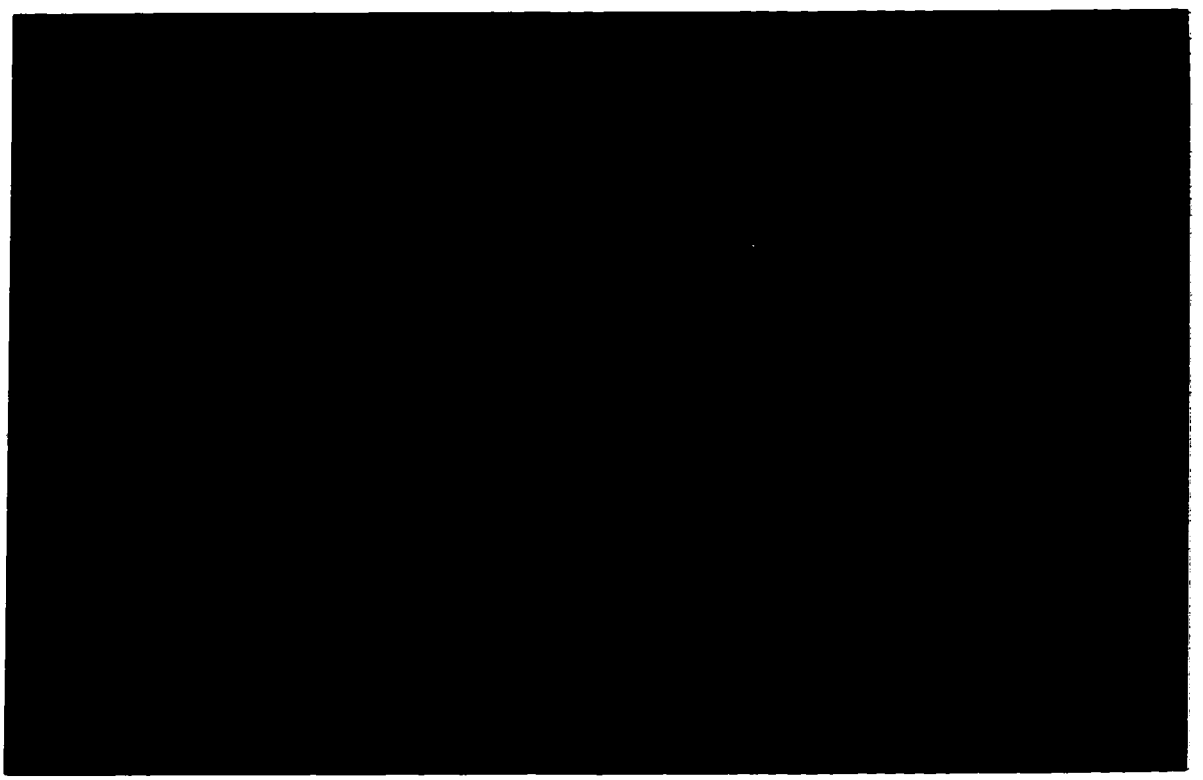
PKC- ε (control)

(g)



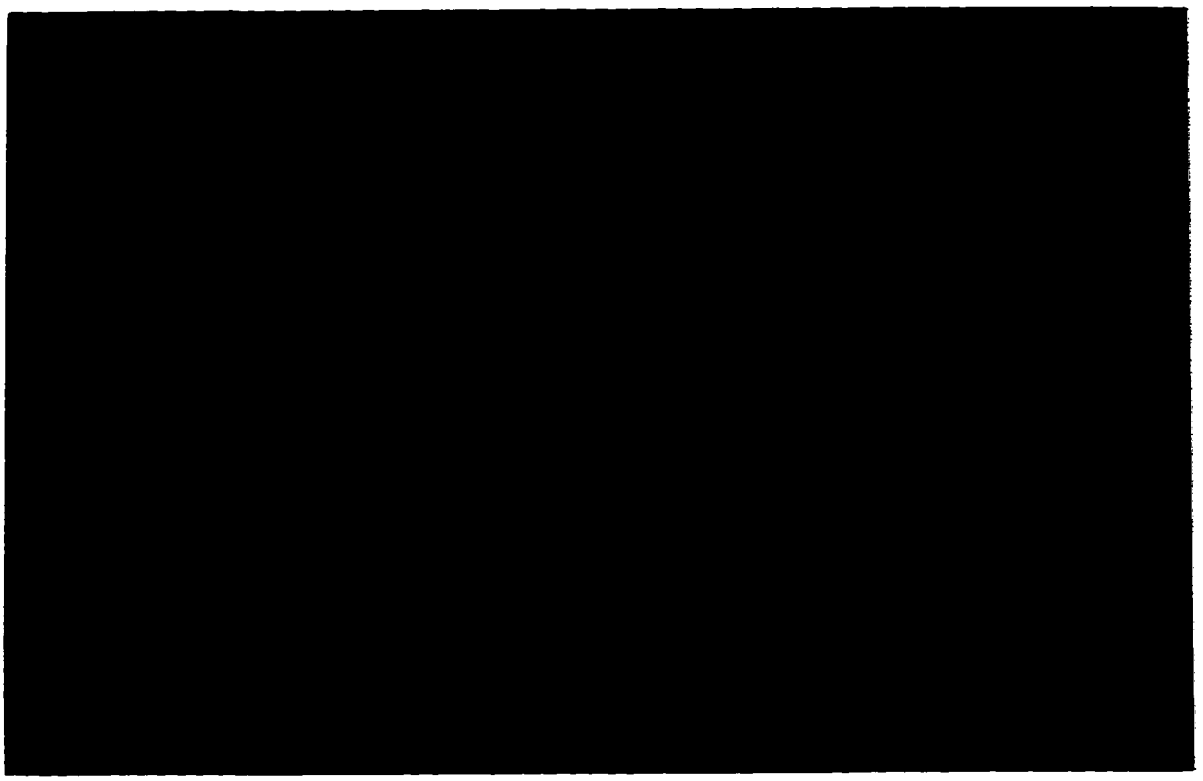
PKC- ε + TPA (24 h)

(h)



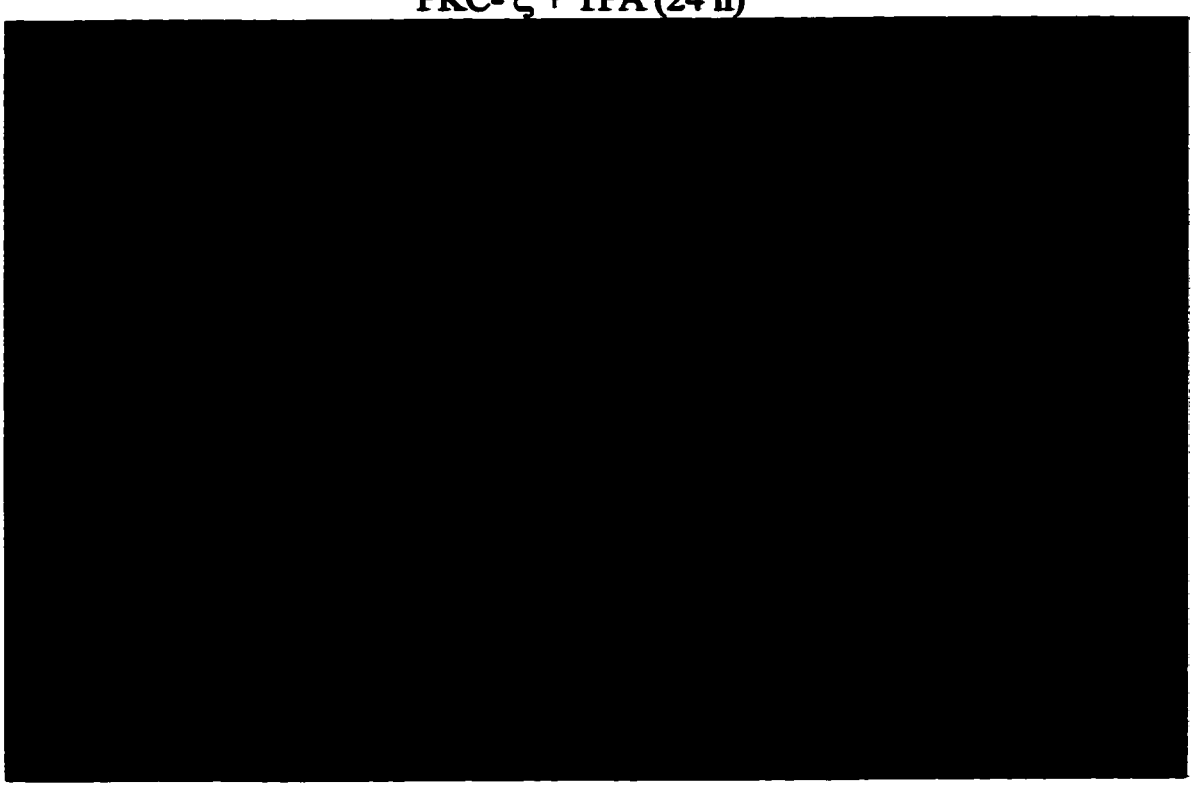
PKC- ζ (control)

(i)



PKC- ζ + TPA (24 h)

(j)



cells both by immunoblotting and immunofluorescence analyses. When quiescent T51B cells are incubated with 1 nM EGF for various times, no effect is observed on the subcellular localization of PKC-α, -δ, -ϵ or -ζ both by immunoblotting and immunofluorescence analyses (data not shown). However, EGF treatment produces a time-dependent increase in the level of PKC-β_I associated with the plasma membrane (Fig. 1.14a, Lanes 1-4), which plateaus at 8 h. In order to confirm these observations three plasma membrane immunoblots derived from controls and EGF treated are scanned and the scanning results are shown in Table 1.14. These data demonstrate a significant increase in the intensity of the two bands of PKC-β_I after 8 and 24 h of EGF treatment. The present results are in agreement with the findings obtained by Sharma et al., 1994 who have observed an increase in PKC activity at the same time exposure (8 and 24 h) after EGF addition. Immunofluorescence results indicate that EGF treated cells exhibit an increase in PKC-β_I immunofluorescence associated with the plasma membrane at 8 and 24 h after EGF exposure (Figs. 1.15c and 1.15d) compared to vehicle control (Fig. 1.15a). However, 4 h exposure to EGF have no obvious effect on the immunofluorescence of PKC-β_I (Fig. 1.15b). These findings are consistent with the immunoblotting results.

FIGURE 1.14

Effect of EGF on Subcellular Localization of PKC-β_I in T51B Cells

Quiescent serum-deprived T51B cells were treated with PBS vehicle, lane (1) or 1 nM EGF for 4 h, lane (2), 8 h, lane (3) or 24 h, lane (4) is shown in (a). Cells were homogenized and fractionated as described in 1.3.2. Plasma membrane samples were solubilized in sample buffers and electrophoresed on 10% SDS-PAGE, transferred to nitrocellulose membrane and blotted with PKC-β_I antibody. Antigen-antibody complexes were detected with alkaline phosphatase-conjugated secondary antibody as described in 1.3.3. Data is representative of three experiments which gave identical results.

Figure 1.14a

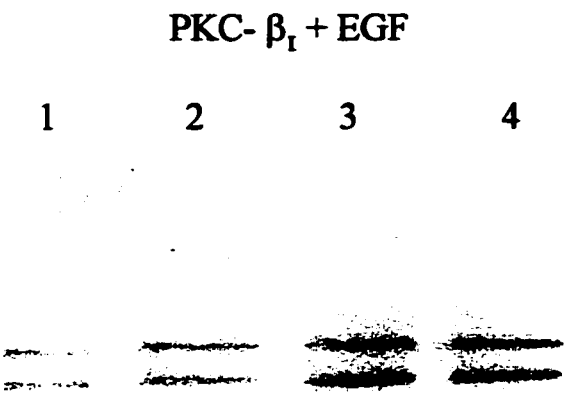


TABLE 1.14

Effect of EGF on the Plasma Membrane Level of PKC-β_t in T51B Cells

The immunoblots of plasma membrane fractions derived from controls and EGF treated cells at varies times were prepared as described in Figure 1.4. The blots were scanned with an HP Laser Scanner and the images were then analyzed by using Sigma Scan Image Software. Data are expressed as pixel intensities. Statistical analysis was performed by paired student's t-test, comparing the mean ± SE of three experiments of controls and EGF treated cells. A level of significance of $P < 0.05$ is indicated as * and of $P < 0.01$ is indicated as **.

Table 1.14

Effect of EGF on the Plasma Membrane Level of PKC-β_I in T51B Cells

	Mean ± SE			
	Control	**4 h**	**8 h**	**24 h**
PKC-β_I (77 kD)	90.7 ± 3.3	107.0 ± 5.7	127.7 ± 2.5*	137.0 ± 2.0**
PKC-β_I (65 kD)	103.3 ± 3.6	105.3 ± 4.4	134.7 ± 3.2*	138.0 ± 2.7**

SE: Standard error

FIGURE 1.15

Effect of EGF on PKC-β_I Immunofluorescence in T51B Cells

T51B cells were plated at a density of 125 x 10^2 cells/cm^2 and grown on glass coverslips in medium containing 10% serum. Confluent cells were serum-deprived by fluid change to medium containing 0.2% serum for 48 h. The cells were then treated with PBS vehicle (a) or 1 nM EGF for 4 h (b), 8 h (c) or 24 h (d). They were then fixed and incubated with anti-PKC-β_I antibody. Antibody-antigen complexes were detected with fluorescein-conjugated secondary antibody as described in 1.3.4. Magnification: Figure 1.15a-d is x1000. Immunostaining was performed four times with similar results.

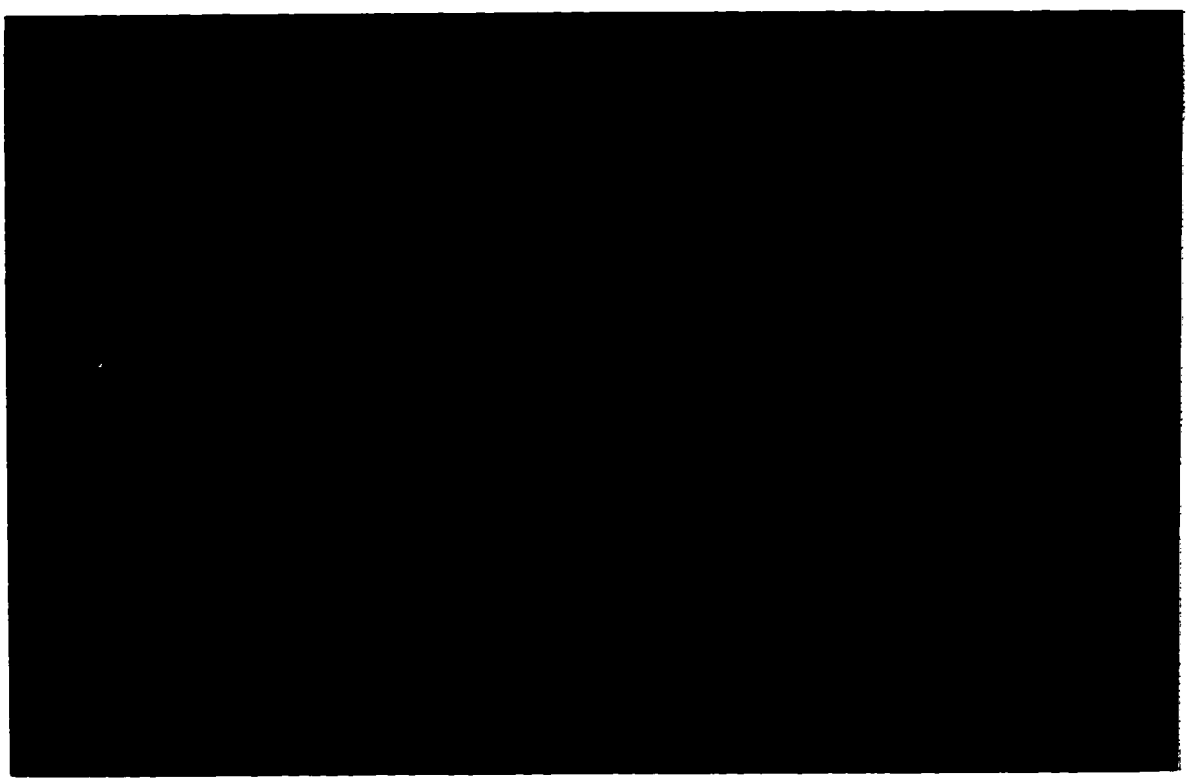
Figure 1.15

Control (0.2 % serum)

(a)

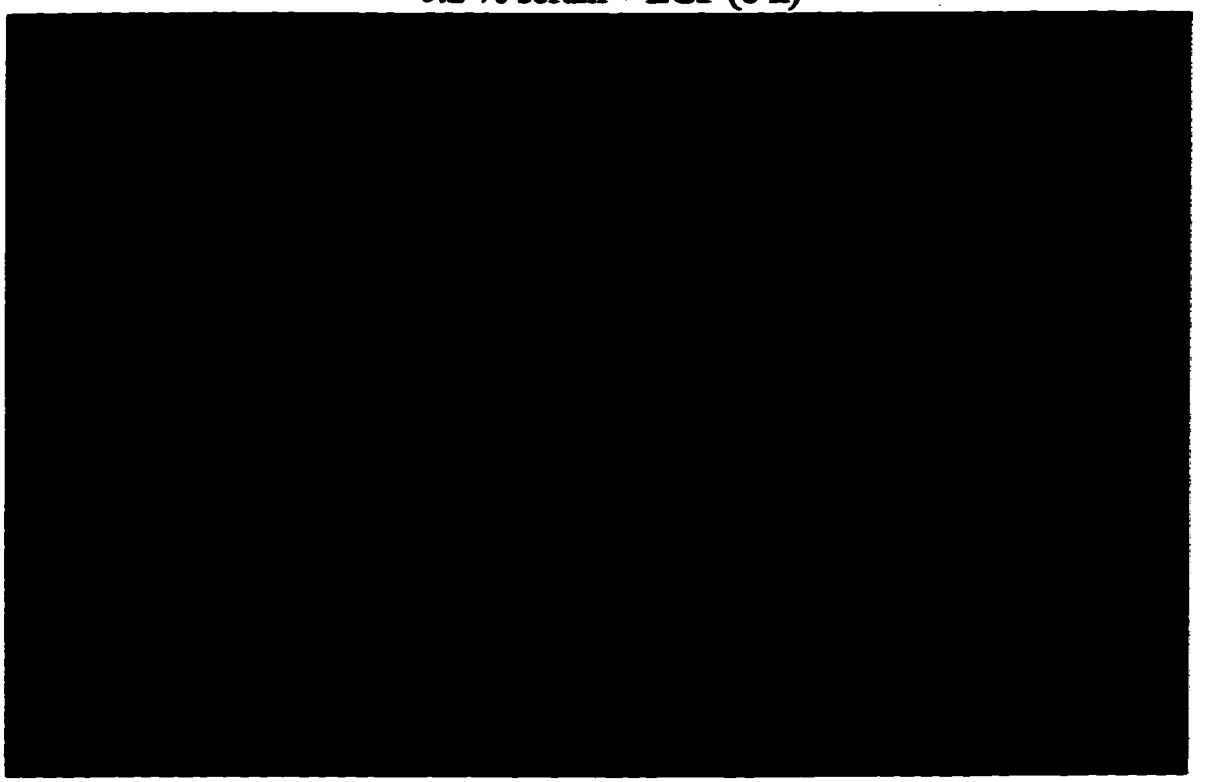
0.2 % serum + EGF (4 h)

(b)



0.2 % serum + EGF (8 h)

(c)



0.2 % serum + EGF (24 h)

(d)

1.5 Discussion

1.5.1 Expression of PKC Isozymes in T51B Cells and Antibody Specificity

To identify the subcellular localization of specific PKC isozymes and their selective modulation by TPA and EGF, isozyme-specific antibodies are used for both immunoblotting and immunofluorescence. Both these techniques indicate that T51B cells express five PKC isozymes, -β_I, -α, -δ, -ϵ and -ζ in both of the cytosolic and membrane fractions. However, in primary cultured hepatocytes, PKC-β_{II}, -α, -ϵ, -γ and -ζ have been detected (Tang et al., 1993). Other studies have indicated the presence of PKC-α, -δ and -ζ in whole liver extract and no immunoreactivity could be observed for PKC-β_I, -β_{II}, -γ or -ϵ (Wetsel et al., 1992). The differences between these results could be attributed to differences in experimental conditions, sources of antibodies used, their specificity and also different cell types.

Intriguingly, however, analysis of the membrane fraction with antibody specific for PKC-β_I leads to the detection of an additional immunoreactive species which migrates with the apparently lower molecular size of approximately 65 kD, compared to that of the 77 kD species found in both membrane and cytosol fractions (Fig. 1.1a). Both of these species, which are detected with anti-PKC-β_I antibody, appear to be authentic PKC-β_I entities in that their detection is specifically abolished when the PKC-β_I antigenic peptide is included in the immunoblotting assay. The 77 kD species is the same size as that reported by others (Huang et al., 1987; Wetsel et al., 1992). In addition, this size is consistent with the cDNA cloning and sequencing data (Knopf et al., 1986). The second subspecies of PKC-β_I which has a molecular size of 65 kD is unique for these cells since in other cell types PKC-β_I is found as a single 77 kD band. However, Wetsel et al., 1992 have observed dark immunostaining with PKC-β_I antiserum at 39 kD in a testis extract. The authors have suggested that the 39 kD

could represent a proteolytic fragment of PKC-β_I. The 65 kD subspecies of PKC-β_I has a higher molecular size than that detected by Wetsel et al. and thus, does not represent the proteolytic fragment detected by Wetsel et al nor the proteolytic fragment of the 36 kD reported by Azzi et al., 1992. There are at least three possibilities to explain the presence of two PKC-β_I subspecies. The first is that PKC-β_I and other isoforms are subject to post-translational modifications such as phosphorylation which could lead to marked alterations in their apparent molecular size upon SDS-PAGE as reported by Pears et al., 1992. Such modifications could explain the apparent presence of multiple forms of PKC-β_I seen here and by others for other PKC isoforms (Tang et al., 1993; Pears et al., 1992). The second possibility is that the two PKC-β_I subspecies may have resulted from differential phosphorylation. This possibility is investigated by incubating the cells for various times with okadaic acid, a protein phosphatase inhibitor. Immunoblotting results indicate a time dependent increase in the level of both PKC-β_I subspecies which is highest at 1 h after the addition of okadaic acid (Fig. 1.2 and Table 1.2). Thus, these results demonstrate that these two subspecies of PKC-β_I are not the result of differential phosphorylation since there is an increase in the levels of the two subspecies of PKC-β_I phosphoproteins in response to okadaic acid. The third possibility is that the two PKC-β_I subspecies may be a degradation product of PKC-β_I which is produced during the preparation of the plasma membrane fraction. However, this is less likely since during the preparation of cysolic and membrane fractions, I included protease inhibitors such as PMSF and leupeptin in the lysis and homogenization buffer. More studies are required in the future to address these possibilities. Such experiments include solubilization of membrane fractions in buffer containing 1% Triton X-100 followed by purification on DEAE-Sephacel columns which give semipurified

PKC (Simboli-Camphell et al., 1994). The significance of these findings is presently unknown. However, one could speculate that the two PKC-β_I subspecies may play different roles in phosphorylating different substrate proteins associated with the plasma membrane. The presence of the 65 kD PKC-β_I subspecies in the plasma membrane and not in the cytosol may suggest that the two PKC-β_I subspecies in the plasma membrane could represent an "active" and an "inactive" pool of PKC-β_I which is detected by immunoblot analysis and results in the observed two subspecies of PKC-β_I. The technique used in the present study for the preparation of cytosolic and membrane fractions is similar to the direct PKC assay (Chakravarthy et al., 1989) which measures PKC activity directly in its native membrane-associated state, without prior detergent solubilization. Indeed, several studies using the direct PKC assay have shown that PKC exists on the membranes of a variety of cells and tissues in both an "active" and an "inactive" state, and that the activation of this pool of inactive membrane PKC may be an important step in many PKC-mediated cellular events (Durkin et al., 1992; Chakravarthy et al., 1992; Lu et al., 1994). However, these studies have not shown if the two pools of PKC represent one or several PKC isozymes. Other reports have not prepared native membrane-associated PKC as I have done. They may have lost the 65 kD subspecies of PKC-β_I during purification of the enzyme (Simboli-Camphell et al., 1994). Whether the two subspecies of PKC-β_I in plasma membrane represent two pools of PKC is yet to be determined in future studies.

Immunofluorescence studies reveal that PKC-β_I is associated with the plasma membrane and the cytoplasm (Fig. 1.7a). However, in the presence of the antigenic peptide, no immunofluorescence is detected (Fig. 1.7b). These findings are consistent with the immunoblotting results which confirm the specificity of the antibody. The most intriguing

results, however, is the association of PKC-β_I with the nucleus, specifically with the nuclear membrane. Such finding is unique for T51B cells since in most cell lines studied so far, no such strong association with the nucleus has been reported. PKC-β_{II} has been found to be translocated to the nucleus only after stimulation of cells with bryostatin-1 or TPA (Hocevar and Fields, 1991; Haller et al., 1994b). The present results are in agreement with other studies which have illustrated the presence of PKC-β in rat liver nuclei (Rogue et al., 1990; Masmoudi et al., 1989). However, these reports have not specified which species of PKC-β is detected in rat liver nuclei. Therefore, this data is the first demonstration that PKC-β_I is localized in the nuclei of T51B cells. This finding leads to the notion that PKC-β_I may be responsible for some of the nuclear functions of PKC. Other studies in rat liver have shown that treatment of isolated nuclei with prolactin or TPA results in a rapid and dramatic (200-fold) increase in PKC activity (Russell, 1989). How hormones such as prolactin can bring about an activation of PKC in isolated nuclei remains unclear. The localization in the nuclei of a particular isozyme of PKC raises questions concerning its role. The nuclear function of PKC will be discussed later.

Immunoblotting results show that PKC-α is expressed as a single protein of approximately 80 kD in both the cytosolic and the membrane fractions of T51B cells. However, more PKC-α is associated with the plasma membrane compared to the cytosol (Fig. 1.3). These results are in agreement with previous Western blot studies (Huang et al., 1987). In addition, 80 kD protein is similar to that predicted from the cDNA sequence (Coussens et al., 1986; Parker et al., 1986; Ono et al., 1988). The present finding is in line with former studies which have indicated the localization of PKC-α in both the cytosol and the plasma membrane of MDBK kidney cells and other cell types (Simboli-Campbell et al.,

1994; Kiley et al., 1990). The immunofluorescence data is consistent with immunoblotting results for the presence of PKC-α in both the cytosol and the plasma membranes of T51B cells. In addition, some immunostaining is also observed within the nucleus which is not abolished completely in the presence of antigenic peptide (Fig. 1.7c,d). Such results could be due to nonspecific binding of the anti-PKC-α antibody to proteins associated with the nucleus.

Both immunoblotting and immunofluorescence results demonstrate that the Ca^{2+}-independent PKC-δ and -ϵ are localized in the cytosol and the membranes of T51B cells. The results indicate that PKC-δ appears as a doublet in both the cytosolic and membrane fractions with molecular masses of approximately 76 and 78 kD (Fig. 1.4). This data is in agreement with previous studies which have shown that PKC-δ isolated from rat brain and artificially expressed in COS-7 cells appears as a doublet of 76 and 78 kD (Ogita et al., 1992). However, the specificity of PKC-δ antibody has not been tested by the inclusion of specific antigenic peptide for PKC-δ. Wetsel et al., 1992 have also detected PKC-δ as an 80-84 kD doublet in brain, lung, spleen, ovary and kidney. However, in heart, liver and testes, it appears as a single band of 80 kD. However, the present data indicate that in the presence of PKC-δ antigenic peptide the 76 kD band is completely abolished while the 78 kD band is not affected. These results raise the possibility that the 78 kD band could represent non-specific binding of the antibody. PKC-ϵ is also localized in both the cytosolic and the membrane fractions where it is found as a band of approximately 85 kD (Fig. 1.5). This is of a similar size to that noted by Schaap et al., 1989 for the brain enzyme expressed in COS-7 cells and reported by Wetsel et al., 1992 for PKC-ϵ detected in rat liver extract. In addition, this size is consistent with cDNA cloning and sequencing data (Ono et al., 1987).

Immunofluorescence analyses also demonstrate the localization of PKC-δ and -ϵ in the plasma membrane and the cytoplasm. Anti-PKC-δ and -ϵ antisera also give some fluorescence within the nuclei of T51B cells which is found to be specific as tested by the inclusion of the antigenic peptides for -δ and -ϵ isoforms (Fig. 1.7e-h). These findings are in agreement with previous immunoblotting studies which have shown both cytosolic and membrane localization of PKC-δ and -ϵ in many types of cells (Leach et al., 1992; Strulovici et al., 1991; Ohno et al., 1994). Furthermore, previous immunofluorescence studies have also demonstrated the localization of PKC-δ and -ϵ in the cytoplasm and the membranes of stratified squamous epithelial cells with faint staining associated with the nuclei (Knox et al., 1993). However, in their study they have not tested the specificity of their antibodies.

The immunoblot result demonstrates the presence of high levels of PKC-ζ in both the cytosol and the membrane fractions of T51B cells. The antibody detects a dense single band of approximately 80 kD (Fig. 1.6). Its size compares with a value reported for PKC-ζ detected by immunoblotting technique in a variety of cell lines and tissues (Ways et al., 1992a; Wetsel et al., 1992; Tang et al., 1993). This value, however, is greater than that predicted (67 kD) from the cDNA sequence (Ono et al., 1987). This result is consistent with previous immunoblot studies which have indicated the localization of PKC-ζ in both the cytosol and the membranes of many cell types (Leach et al., 1992; Simboli-Campbell et al., 1994; Standaert et al., 1993). Immunofluorescence data also support the presence of high level of PKC-ζ in T51B cells where dense staining is observed all over the cells. The immunostaining is almost abolished in the presence of the antigenic peptide of PKC-ζ (Fig. 1.7j,k). These results are consistent with previous immunofluorescence studies which have indicated the localization of PKC-ζ in the cytoplasm, the membrane and the nucleus of

squamous epithelium cells (Knox et al., 1993). Moreover, Hagiwara et al., 1990 have demonstrated the specific expression of PKC-ζ in the nuclei of rabbit nerve cells. In addition, Khalil et al., 1992 have indicated that during both Ca^{2+}-independent and Ca^{2+}-dependent phases of smooth muscle contraction, perinuclear-concentrated PKC-ζ is translocated to the intra-nuclear compartment upon activation.

To my knowledge the present data is the first to demonstrate the expression and the subcellular localization of PKC-β_I, -α, -δ, -ϵ and -ζ in T51B cells by both immunoblotting and immunofluorescence techniques. The presence of multiple isozymes of PKC in the same cell type suggests that individual isozymes may mediate distinct cellular functions.

The membrane association of PKC-β_I, -α, -δ, -ϵ and -ζ isozymes in T51B cells suggests a possible role for these isozymes in phosphorylating substrate proteins associated with the plasma membrane. However, little data exist to directly compare isozyme substrate specificity. Several lines of evidence suggest that PKC isozymes can directly interact with target proteins in the cytoskeleton (Jaken et al., 1989; Kiley et al., 1992). Many cytoskeleton or cytoskeleton-associated proteins including vinculin (Werth et al., 1984), talin (Litchfield and Ball, 1986), integrin (Burn et al., 1988), caldesmon (Litchfield and Ball, 1987), annexin II (Gould et al., 1986), glycoprotein 85 (Kalomiris et al., 1989), adducin (Kaiser et al., 1989) and myristoylated alanine-rich C-kinase substrate or p80 (Rosen et al., 1990) are good PKC substrates. Vinculin, talin and integrin play a functional role in the attachment of microfilament bundles to the plasma membrane (Burridge et al., 1987). Immunofluorescence studies have shown that PKC-α staining resembles that of the focal contact protein vinculin and co-localized with talin (another focal contact protein) (Jaken et al., 1989). Adducin has been observed to be associated with sites of cell-cell contacts in cultured epithelial cells

(Kaiser et al., 1989). Furthermore, overlay approaches have demonstrated direct interaction of PKC with cytoskeleton proteins (Haytt et al., 1990; Wolf and Sahyoun, 1986). Hence, PKC isozymes may affect cytoskeleton reorganization by phosphorylating proteins that regulate cytoskeleton integrity. Future studies should be directed to identify possible membrane-associated proteins as potential substrates for PKC isozymes.

The cytoplasmic association of PKC-β_I, -α, -δ, -ϵ and -ζ isozymes in T51B cells may suggest a potential role for these isozymes in phosphorylating cytoplasmic proteins such as intermediate filament proteins. In the cell, these proteins form a complex network that radiates from the nuclear surface to the cell membrane (Jones and Goldman, 1985). An important post-translational modification of intermediate filament proteins is phosphorylation and modulation of their level of phosphorylation which is probably an important regulatory mechanism for their function (Celis et al., 1983; Evans, 1988). Cytokeratins form the intermediate filaments of epithelial cells and constitute two types; type I, CK18 (acidic) and type II, CK8 (neutral-basic) (Moll et al., 1982). Previous studies have demonstrated that both types of cytokeratins, are *in vitro* (Yano et al., 1991) and *in vivo* (Cardin et al., 1992) substrates for PKC. However, in cultured rat liver hepatocytes the CK8 (55 kD) is more phosphorylated than CK18 in response to TPA treatment (Cardin et al., 1992). Since T51B cells have been found to express cytokeratin CK8 and vimentin (another type of intermediate filament) which often are expressed by cells in culture (Marceau et al., 1986), I would speculate that these proteins are potential substrates for cytosolic PKC isozymes expressed in these cells. Hence, future research should be directed to study these possibilities.

With respect to how PKC regulates nuclear events, there are two general mechanisms through which PKC-mediated signals might be received and interpreted in the nucleus (Olson et al., 1993). First, PKC can activate "downstream" protein kinase cascades leading to the nucleus where they modulate programs of gene expression. In this context, PKC-β_I, -α, -ϵ, -δ and -ζ expressed in T51B cells may have differential roles in transducing the signals to the nucleus. The second, PKC itself may reside or translocate to the nucleus where it directly phosphorylates nuclear regulatory proteins. Since PKC-β_I, -α, -δ, -ϵ and -ζ are associated with nuclei of T51B cells, I would speculate that these isozymes may phosphorylate nuclear regulatory proteins. With respect to DNA replication, PKC has been implicated in the activation of DNA polymerase α (Krauss et al., 1987). In addition, histone H1 and histone H3 have been found to be phosphorylated by PKC *in vitro* and *in vivo* (Martelli et al., 1989; Mahadevan., 1988). These proteins are associated with DNA and are thought to play a regulatory role in cell cycle events such as mitosis and chromatin condensation. Other potential nuclear targets for these isozymes may include topoisomerase II and DNA topoisomerase 1. These are chromatin-bound enzymes found to be phosphorylated by PKC *in vitro* and *in vivo* (Sahyoun et al., 1986; Rottmann et al., 1987; Samuels and Shimizu, 1992; Pommier et al., 1990). Immunofluorescence studies have also demonstrated the association of PKC-β_I with the nuclear membrane. Potential protein targets in the nuclear membrane may include lamin A, B, and C since these polypeptides have been shown to be phosphorylated by PKC *in vitro* and *in vivo* (Hornbeck et al., 1988; Fields et al., 1989; Tsuda and Alexander, 1990; Goss et al., 1994). Phosphorylation of lamins regulates disassembly of the nuclear envelope during mitosis (Laskey and Leno, 1990). Another potential substrate is the 106 kD nuclear pore complex protein. Phosphorylation of this

protein by PKC has been found to decrease the nuclear envelope NTP-ase activity required for mRNA export from the nucleus (Schröder et al., 1988). However, at this point it is not clear whether PKC-β_I is associated with the inner side or the outer side of the nuclear membrane. Confocal microscopic studies are required to answer these questions. Moreover, future research should be directed to identify potential nuclear proteins as substrates for nuclear PKC-β_I, -α, -δ, -ϵ and -ζ isozymes expressed in T51B cells.

1.5.2 Effect of TPA on Subcellular Localization of PKC Isozymes

Treatment of T51B cells with 100 nM TPA for 24 h increases PKC-β_I immunoreactivity in the plasma membrane with a subsequent decrease in the cytosolic fraction (Fig. 1.8a,b and Table 1.8). This data correlates well with the immunofluorescence analysis (Fig. 1.13a,b). This is the first demonstration that TPA treatment increases the level of PKC-β_I in the plasma membrane of cultured cells. This effect of TPA has not been demonstrated in other cell types. In contrast, several other studies have reported either a down-regulation of PKC-β protein level by TPA (Huang et al., 1989; Crabos et al., 1991; Cook et al., 1992; Duyster et al., 1992; Akita et al., 1990; Kiley et al., 1990; Strulovici et al., 1991) or no effect of the TPA on PKC-β (activity or protein level). For example, in BC3H-1 myocytes and a human colonic cell line, chronic treatment with TPA has no effect on the level of PKC-β (Van Den Berghe et al., 1992; Standaert et al., 1993). In regenerating rat liver and pituitary gland cells, a type II (β-sequence) activity persists after TPA treatment (Cooper et al., 1989; Houweling et al., 1989). On the other hand, TPA treatment leads to partial translocation of PKC-β_{II} to the plasma membrane of HL-60 cells while bryostatin-1 (another PKC activator) induces complete translocation of PKC-β_{II} to the nuclear membrane (Hocevar and Fields, 1991). Other reports, however, have indicated that prolonged TPA

treatment (24 h) increases the translocation of PKC-β to the nuclear membrane of MDBK kidney epithelial cells (Simboli-Campbell et al., 1994). However, the authors have not indicated which subspecies of PKC-β is translocated. The differences between these results could be due to differences in the sensitivity of PKC to TPA treatment in different cell types and also differences in experimental conditions or antibodies used. Other factors may also contribute to whether PKC responds or binds to phorbol esters. It has been reported that PDBu binding to PKC varies greatly with coexistence of substrate proteins, in fact, membrane-associated PKC-binding proteins have been reported which may serve to anchor or compartmentalize PKC isozymes to different intracellular membrane (Wolf and Baggiolini, 1990; Mochly-Rosen et al., 1991).

Treatment of T51B cells with TPA for 24 h results in down-regulation of PKC-α, -δ and -ϵ but has no effect on PKC-ζ. Immunoblot analyses show that PKC-α, -δ and -ϵ immunoreactivity is completely abolished in both the cytosolic and the membrane fractions in the presence of TPA (Figs. 1.9-1.11 and Tables 1.9-1.11). Immunofluorescence data indicate that TPA treatment abolishes the immunostaining of anti-PKC-α, -δ and -ϵ antibodies in the cytoplasm and the plasma membrane but have no effect on the fluorescence associated with the nucleus (Fig. 1.13c-h). The lack of effect of TPA on the nuclear PKC-α, -δ and -ϵ is not clear. The site of the nuclear localization of these isoforms is not known and hence, TPA might not affect these isoforms if they reside inside the nucleus. Confocal microscopic studies are required to study these possibilities. The present results are in agreement with previous reports which have also demonstrated down-regulation of these PKC isozymes by TPA in different cell types (Hocevar and Fields, 1991; Huwiler et al., 1991; Borner et al., 1992, Strulovici et al., 1991; Crabos et al., 1991; Standaert et al., 1993,

Huwiler et al., 1992; Tsutsumi et al., 1993; Akita et al., 1990; Kiley et al., 1990). In contrast, other studies have shown that membrane-associated PKC-α and -ϵ are resistant to down-regulation by prolonged TPA treatment (Akita et al., 1990; Kiley et al., 1990; Ways et al., 1992a; Strulovici et al., 1991). On the other hand, in NIH 3T3 cells, PKC-α has been reported to translocate to the nucleus upon TPA treatment (Leach et al., 1989). However, the authors have not tested the specificity of their antibody by the inclusion of specific antigenic peptide.

Both immunoblot and immunofluorescence analyses demonstrate that 24 h exposure to TPA has no effect on the level or localization of PKC-ζ (Fig. 1.12a,b, Table 1.12 and Fig. 1.13j,i). These results are consistent with other reports which have demonstrated that PKC-ζ is not translocated or down-regulated in response to TPA (Nakanishi et al., 1993; Olivier et al., 1992; Ways et al., 1992a; Huwiler et al., 1992; Gschwendt et al., 1992). In contrast, TPA treatment in other cells leads to translocation of PKC-ζ from the cytosol to the membrane fraction (Borner et al., 1992; Crabos et al., 1992; Wooten et al., 1994). The discrepancies between these results could be due to differences in cell types, their sensitivity to TPA treatment and experimental conditions. The insensitivity of PKC-ζ to TPA observed in this study is generally believed to be due to the existence of only one cysteine-rich, zinc finger in its C_1 domain (Ono et al., 1989; Bell and Burn, 1991).

The present data clearly demonstrate that TPA treatment has different effects on PKC-β_I, -α, -δ, -ϵ and -ζ expressed in T51B cells. While prolonged TPA treatment increases membrane association of PKC-β_I, it down-regulates both cytosolic and membrane-associated PKC-α, -δ and -ϵ but has no effect on PKC-ζ. The down regulation of PKC-α, -δ, and -ϵ isozymes by TPA may result in reduced transcriptional activation through both a TPA

response element (TRE) or a serum response element (SRE). Many of TPA-responsive genes such as collagenase (Angel et al., 1987), metallothionein IIA (Hata et al., 1993) and c-Jun (Angel et al., 1988) share a common cis-acting element, TRE, which binds Jun/Fos heterodimer protein (Cantley et al., 1991). Another kind of TPA-responsive genes, including c-fos gene, contains the cis-acting element SRE which has been shown to bind the serum response factor SRF (Contley et al., 1991). Previous studies have demonstrated that overexpression of a truncated constitutively active form of PKC-α is involved in a signaling pathway toward the transcriptional activation of TRE (Hata et al., 1989). Moreover, overexpression of cPKC-α and -β_{II} or nPKC-ϵ results in the enhancement of transcriptional activation through both TRE and SRE (Hata et al., 1993). Hence, one could speculate that down-regulation of PKC-α, -δ and/or -ϵ isozymes may reduce the transcriptional activation through TRE and/or SRE. Further experiments are required to address these possibilities. Also future work should be directed to study the effect of short time incubation with TPA on subcellular localization of these PKC isozymes.

One could speculate that PKC-β_I may mediate some of the TPA effects observed in T51B cells. TPA has been shown to decrease cytosolic PKC activity temporarily in T51B cells (Asheim et al., 1989). Earlier experiments in Dr. Kleine's laboratory have shown that, as early as 3-5 min, TPA treatment increases particulate PKC activity in T51B cells. Furthermore, Sharma et al., 1994 have demonstrated that long term TPA treatment of serum-deprived T51B cells down-regulates particulate PKC activity. However, in these studies the response of specific PKC isozymes to TPA has not been determined. The present results indicate that, in fact, TPA increases the membrane association of PKC-β_I after 24 h of treatment and thus, it does not down-regulate this isozyme. Previous studies have also

demonstrated that the amount of EDTA-extractable PKC activity increases transiently during the two Ca^{2+}-dependent $G_0 \rightarrow G_1$ and $G_1 \rightarrow S$ transitions of serum-stimulated T51B cells in confluent cultures (Boynton et al., 1985a,b). It has also been found that TPA that stimulates PKC in an extract from these cells in the absence of Ca^{2+} enables these cells to transit G_1 in Ca^{2+}-deficient medium (Boynton et al., 1985a,b). Other agents such as saccharin and dexamethasone also trigger $G_1 \rightarrow S$ transition in Ca^{2+}-arrested late G_1 T51B cells and like TPA, stimulate PKC activity in cell free preparation (Kleine et al., 1986). The authors have shown that these agents do not stimulate PKC activity by attaching to the enzyme's DAG/TPA- binding site. They have suggested that the physiological functions of these agents may stimulate PKC by increasing the enzyme's affinity for signal tranducers such as Ca^{2+} and DAG. Hence, one could speculate that PKC-β_1 may be, in fact, the isozyme that is involved in the proliferative control mechanisms in T51B cells. However, one cannot rule out the involvement of other PKC isozymes especially PKC-ζ in the mitogenic signaling pathway since this isozyme is not down-regulated by prolonged TPA treatment. This notion is supported by studies that have linked PKC-ζ with growth control. For example, transformation of mouse keratinocytes results in a decrease in the abundance of PKC-ζ transcript (Dlugosz et al., 1992). Both biochemical and genetic studies strongly suggest that PKC-ζ may play a critical role in cell cycle control of yeast (Ogita et al., 1990; Levin et al., 1990). Moreover, a kinase defective mutant of PKC-ζ has been shown to inhibit DNA synthesis in mouse fibroblast (Berra et al., 1993). The authors have also demonstrated that micro-injection of the pseudo substrate inhibitory peptide of PKC-ζ dramatically impairs the re-initiation of DNA synthesis in response to serum in quiescent fibroblasts (Berra et al., 1993), indicating that PKC-ζ is involved in mitogenic signaling pathway.

1.5.3 Effects of EGF on Subcellular Localization of PKC Isozymes

Immunoblotting results demonstrate that treatment of serum-starved T51B cells with EGF increases the labeling with anti-PKC-β_I antibody on the plasma membrane in a time-dependent manner (Fig. 1.14 and Table 1.14). In addition, the immunofluorescence of PKC-β_I associated with the plasma membrane increases with time in response to EGF treatment (Fig. 1.15a-d). These findings are in agreement with previous reports from our laboratories which have indicated an increase in the membrane-associated PKC activity in response to EGF treatment at the same time exposure tested in my study (Sharma et al., 1994). However, I did not observe any changes with regard to other PKC isozymes in response to EGF. In contrast, in other cell systems such as Swiss 3T3 fibroblasts, EGF induces a biphasic translocation of PKC-δ and -ϵ to the membrane with no effect on -α isozyme at any time (Yeo and Exton, 1995). In mammary epithelial cells, EGF treatment enhances cell proliferation that is accompanied by PKC activation and a translocation of PKC-α to the membrane fraction (Sylvester et al., 1994). Furthermore, stimulation of quiescent 3Y1 cells by serum or EGF results in DNA synthesis but has no effect on the activation of PKC isozymes such as translocation or down-regulation. However, these stimuli increase the phosphorylation levels of PKC-δ and -ϵ, with a slight increase in the phosphorylation level of PKC-α (Ohno et al., 1994). The discrepancies between these results and the present data may be due to differences in the cell types, the doses of EGF applied and the experimental conditions. Earlier reports from Dr. Franks' laboratory have demonstrated that EGF induces DNA synthesis in serum-deprived T51B cells which is associated with a sustained increase in the membrane-associated PKC activity up to 24 h. Moreover, TPA alone is without effect on DNA synthesis but when added along with EGF or serum it causes a significant

enhancement of DNA synthesis (Sharma et al., 1994). Thus, PKC-β_I may be a common signal transduction system induced by these mitogens. Because PKC-β_I is the only isozyme that is up-regulated by EGF and TPA, and since PKC is implicated in the mitogenic effect of EGF and TPA in T51B cells, these observations strongly suggest the involvement of PKC-β_I in the mitogenic action of EGF and TPA in these cells. Nevertheless, other PKC isozymes may also be involved in the mitogenic effects of EGF. Further studies are required to test these possibilities. For example, measurements of specific activities of individual PKC isoforms in response to EGF; inhibition of the synthesis of specific PKC isozymes by the use of specific antisense oligodeoxynucleotides, peptide inhibitors, and intracellular delivery of isozyme-specific antibodies. These studies will allow for depletion and inactivation of individual isoforms and thus, will provide a better understanding of the functions of individual PKC isozymes in the mitogenic signaling pathway.

1.6 Conclusions

The overall conclusion of this chapter is that TPA and EGF have differential effects on the protein level and the localization of PKC isozymes in T51B cells.

The specific conclusions are:

1.6.1 T51B cells express PKC-β_I, -α, -δ -ϵ and -ζ which have different subcellular localization.

1.6.2 The membrane-associated PKC-β_I appears as a doublet which is unique and novel for T51B cells.

1.6.3 A 24 h treatment with TPA increases the membrane-associated level of PKC-β_I. On the other hand, it down-regulates the cytosolic and the membrane-associated PKC-α, -δ and -ϵ and yet, does not affect PKC-ζ in T51B cells.

1.6.4 A 24 h treatment with EGF increases the membrane-associated level of PKC-β_I and yet, does not affect PKC-α, -δ, -ϵ or -ζ in T51B cells.

CHAPTER TWO

Establishment of Cell Cycle Profile During the Growth and Death of T51B Cells

2.1 Introduction

Dividing cells go through a series of metabolic changes which constitute the cell cycle. The balance between cell proliferation and cell death in an organism can be controlled by regulating the rates of proliferation, differentiation or death of constituent cells. If one of the sister cells is destined not to divide again, it leaves the cell cycle and becomes a non-dividing cell that may eventually die or persist for the entire life of the organism without dividing and remain in a quiescent state (G_0) (Pardee, 1989). In practice, it is often difficult to differentiate non-proliferating cells from cells with a very long cycle. For instance, there are cells with a long cell cycle which are still capable of DNA synthesis and which under appropriate conditions may shorten the length of the cycle. A remarkable example of these kinds of cells are the cells of the liver which can be considered as a quiescent tissue with a low thymidine index (Braun et al., 1988). However, in response to partial hepatectomy, the tissue can markedly increase its capacity for DNA synthesis and proliferate rapidly. Kidney cells, basal cells of epidermis, smooth muscle cells of arteries and arterioles, skeletal muscle cells are also known to respond to an appropriate stimulus with an increase in the number of cells synthesizing DNA and a shortening of cycle time. Other cells, like keratinizing epithelial cells of the upper layer of epidermis, various nerve cells are considered as cells that have left the cycle. These cells are non-dividing cells which may eventually die or persist for the entire life of the organism without dividing as terminally differentiated cells. Other

organs that rapidly sustain cell losses such as the hematopoietic system and gastrointestinal tract have a large number of cells that are continuously cycling (Baserga. 1965, Zajicek et al., 1985). In higher organisms, homeostatic control of cell number is thought to be the result of the dynamic balance between cell proliferation and cell death. In most tissues of higher organisms, cell death can be classified into one of three different categories: necrosis, which occurs as a result of massive tissue damage; terminal differentiation of specialized tissues such as skin, intestine, and red blood cells; and apoptosis (programmed cell death), which is a process of active cellular self destruction (Bursch et al., 1990a).

2.1.1 Cell Proliferation

Proliferation is defined as the increase in the cell number resulting from completion of cell cycle, as contrasted to hypertrophy, which is the increase in the cell mass. A cell activated to undergo cell division performs a series of orderly, integrated biochemical events culminating in the formation of two daughter cells. The cell cycle is subdivided into four distinct phases: (a) a posmitotic phase called G_1, during which the cell synthesizes ribonucleic acid (RNA) and proteins; (b) an S phase, during which the amount of deoxyribonucleic acid (DNA) is duplicated while protein and RNA synthesis continue; (c) a postsynthetic phase, called G_2, characterized by no net synthesis of DNA and continued RNA and protein synthesis as in postmitotic phase; and (d) mitosis in which there is a segregation of the constituents of one cell into two daughter cells. In this phase there is no DNA synthesis and protein synthesis is reduced to a minimum. The key events of mitosis are: (i) prophase - the condensation of the duplicated chromosomes from a dispersed and metabolically active state to a compacted condition, suitable for transport; (ii) prometaphase - the positioning of the condensed chromosome, first by orientation so one copy of each

chromosome addresses one end of the cell and then by the motion of each chromosome to the cell's midplane to form the "metaphase plate"; (iii) anaphase - the separation of each chromosome into two identical parts, followed by their movement toward the opposite ends of the cell; (v) telophase - the reformation of nuclei and the decondensation of the chromosomes to re-establish the interphase condition (McIntoch and Koonce, 1989). Studies on vertebrate tissue culture cells have demonstrated that the nuclear lamina undergoes major architectural changes during cell division (Gerace et al., 1978). This structure is a protein meshwork closely associated with nucleoplasmic surface at the nuclear periphery (Gerace and Burke, 1988). It has been suggested that the nuclear lamina serves as a major chromatin anchoring site during interphase (Gerace and Blobel, 1980). When the nuclear envelope is disassembled during mitotic prophase, the lamins, major constituents of the nuclear lamina, are depolymerized and become dispersed throughout the cytoplasm as monomers. Subsequently, the lamins reassemble at the surfaces of the daughter cell chromosomes during telophase, when the nuclear envelope is reconstructed (Gerace et al., 1978; Gerace and Burke, 1988).

There is considerable variation in the time spent in G_0, G_1, S and G_2. Neurons and muscle cells may spend a life time in G_0. G_1 phase may be as short as two hours or last for days. S phase in human cells is known to vary from 11 to 34 hours; G_2 phase occupies from 3 to 16 hours. Mitosis continues for about 30 minutes to 1 hour (Alberts et al., 1989). The control of cell proliferation involves both regulatory events initiated at the plasma membrane that control the re-entry into the cell cycle and biochemical changes that regulate and direct the cell division and progress through the cell cycle. The regulatory events are initiated by growth factors, such as EGF which transduces the signal through binding to its plasma

membrane receptor and thereby activating its tyrosine kinase activity. Subsequent phosphorylation of other proteins on tyrosines, serines and threonine may further transmit the signal to the nucleus. These signal transduction events in response to EGF will be discussed later.

2.1.2 Cell Death (Apoptosis)

Cell death is a fundamental phenomenon of biological organisms. During evolution, the death program has been modified in a cell- and tissue-specific manner to accomplish a number of physiological functions. These include elimination of cells that (a) have no function; (b) are generated in excess; (c) develop improperly; (d) have already completed their life span (terminal differentiation); or (e) are harmful; and (f) production of dead cells for specific functions (e.g. cornification and lens cells). During metamorphosis, cell death is readily apparent in both insects and amphibian (Fesus, 1993). In mammals, cell death is conspicuous from the very beginning of development. Cells of the endometrium die at the site of implantation of the blastocyst, and in the developing nervous system, neurons that fail to make the correct connections die (Vaux, 1993). Cell death also maintains homeostasis in developing and adult organisms. In the immune system, B and T lymphocytes are removed when they fail to correctly rearrange their antigen receptors, when they are self-reactive, or if they fail to see foreign antigen (Vaux, 1993). Other organs such as rat liver and kidney, when exposed to mitogenic stimuli, proliferate very rapidly and there is an initial burst of hyperplasia and an increase in organ weight. Once the stimuli is removed there is an increase in apoptosis and over time the organ is restored to its normal mass (Bursch et al., 1984).

The term "apoptosis" is often used to describe the physiological death of mammalian cells. When individual cells die in a healthy organ, their death is not accompanied by

changes that are characteristic of pathological cell death. Apoptosis is associated with death of isolated cells, rather than contiguous patches or areas of tissue. Affected cells shrink in volume, lose contact with their neighbours and lose specialized surface elements such as microvilli and cell-cell junctions (Wyllie, 1993). Time lapse studies have shown that these changes occur rapidly and are accompanied by extraordinary surface convolutions and then the explosion of the cell into a series of membrane-bound, condensed apoptotic bodies. The endo-plasmic reticulum dilates and a series of crater-like cavities appear where the dilated cisternae fuse with the cell surface. Otherwise, cytoplasmic organelles are largely intact. Initially, and in contrast to cells dying by other means (e.g. necrosis), mitochondria are normal in structure (Cohen, 1994). The most outstanding internal structural changes, however, occur in the nucleus. Chromatin condenses into dense granular caps under the nuclear membrane. Adjacent to these, nuclear pores are absent. The nucleolus dissociates to leave a shower of osmiophilic particles near the centre of the nucleus (Wyllie, 1993). Apoptotic cells do not induce an inflammatory reaction but they are targets of immediate phagocytosis, either by macrophages already present nearby, or by other adjacent viable cells. Within these, the compacted organelles and condensed chromatin of the apoptotic cells may be visible for a few hours but eventually reduce to large nondescript lysosomal residual bodies (Cohen, 1994). How the phagocytes recognize that a nearby cell is undergoing apoptosis is not yet fully understood. There appears to be more than one mechanism, depending on the origins of the apoptotic cells and of the phagocytes (Savill, et al., 1993). Some cells, when apoptotic bind as-yet-uncharacterized extracellular factors that are then engaged by the vitronectin receptors on certain macrophages. The vitronectin receptor of human macrophages is responsible for binding apoptotic human neutrophils (Savill et al.,

1990). Others alter their surface phospholipids; phosphatidylserine, normally confined to the inner leaflet of the plasma membrane, "flips out" to the outer leaflet, where it is recognized by a receptor present on macrophages and probably other cell types. The outcome of this recognition is the quiet disposal of the apoptotic cells without disturbing the neighbors (Cohen, 1994). Apoptotic cells express a new transglutaminase activity that cross links cytoplasmic proteins (Fesus et al., 1991; Piacentini et al., 1991). Some of the shrinkage and membrane blebbings may be attributable to activation of this enzyme. While the role of transglutaminase in apoptosis has not been firmly established, it is likely that the cross-linking of proteins stabilizes the apoptotic bodies and limits the leakage of intracellular constituents into the extracellular space (Fesus et al., 1991). Necrosis, on the other hand, affects many adjoining cells. In necrosis, the primary lesion appears to be in the mitochondria, which become unable to supply sufficient energy in the form of adenosine triphosphate. Cells lose the ability to regulate their osmotic balance, thus they swell and burst. An inflammatory response is called up by the release of intracellular contents, and the damage is eventually resolved by repair or scar formation. DNA degradation, if it occurs, is a late event (Cohen, 1994).

The effector mechanisms of apoptosis are still not completely understood. The nuclear changes are caused by activation of an endogenous Ca^{2+} and Mg^{2+}-dependent endonuclease (Wyllie, 1980). This enzyme cleaves chromatin between nucleosomes, reducing the DNA of apoptotic cells to a series of fragments having integer size multiples of 180-200 base pairs, and thus producing a characteristic "ladder" on agarose gel electrophoresis. Recently cysteine proteases have been implicated in the induction of apoptosis . In mammals, six different homologs of C. elegans cell death protein Ced-3 have

been identified (interleukin 1B converting enzyme (ICE), Nedd2, CPP32, $ICE_{rel\ II}$/ TX/Ich-2, $ICE_{rel\ III}$ and mch2) (cited in Hale et al., 1996, Vaux and Strasser, 1996). Over expression of these proteases has been shown to cause apoptosis in various cells which can be inhibted by interfering with protease function (Vaux and Strasser, 1996). These precursors can be found in most nucleated mammalian cells, but in order to become active and cause apoptosis, they must be cleaved at aspartate residues and assembled into heterotetramers. Recent work has shown that a protease which can cleave poly(ADP-ribose) polymerase (PARP) plays an important role in apoptosis. PARP is involved in DNA repair, in the supervision of genome structure and integrity in stressed cells (Nicholson et al., 1995). The Ca^{+2} dependent-endonuclease implicated in the internucleosomal DNA cleavage is negatively regulated by poly(ADP-ribos)ylation; therefore the loss of PARP function could result in the activation of the nuclease in dying cells (Vermes and Haanen, 1994). Nicholson et al., 1995 have demonstrated that the active form of CPP32 is able to cleave PARP and probably other substrates whose proteolysis calminates in apoptosis.

However, several types of cells undergo apoptosis without DNA fragmentation (Schwartz et al., 1993) and in other instances of apoptosis the activation of an endonuclease has been shown to be neither a trigger nor a necessary or defining component of the early phase of death (Zakeri et al., 1993). It has been suggested that, in addition to increased intracellular Ca^{2+} levels, lamin phosphorylation and solubilization, and breakdown of the nuclear envelope, are early events in apoptosis, allowing the rapid access of activated endoplasmic reticulum enzymes to the nucleus. (Fesus, 1993). The cellular triggers of apoptosis have proved elusive. Shortly before onset of the chromatin changes, at least in some cell types, free cytosolic Ca^{2+} rises to sustained, but moderate levels of around 800 nM

(McConkey et al., 1989a). This appears to be important for the rest of the process, as blockade of Ca^{2+} movement by pharmacological means can inhibit apoptosis (Wyllie, 1993). Binding of ligands, such as transforming growth factor β_1 ($TGF\beta_1$) to its cell surface receptor initiates apoptosis in appropriate cell types (Fesus et al., 1991). In several circumstances, blockade of protein and RNA synthesis inhibits apoptosis, but cyclohexamide does not always block and indeed may initiate apoptosis in some cell types (Wyllie et al., 1993).

Regulatory genes influence cellular susceptibility to enter apoptosis. Some of these have been identified and are already familiar as oncogenes and oncosuppressor genes. At present, there is convincing data relating to c-myc, c-fos, bcl-2, P53 and ras. Expression of c-myc is of particular interest, as it seems to determine whether continuous proliferation or apoptosis occurs depending on the availability of critical growth factors (Evan et al., 1992). The c-myc gene product, when over-expressed in cells deprived of growth factors (Evan et al., 1992) or treated with heat (Bissonnette et al., 1992) induces apoptosis and inhibition of c-myc expression with antisense oligodeoxynucleotides prevents activation-induced death of T cell hybridomas (Shi et al., 1992). There is evidence that T cell activation-induced transcriptional factor AP-1 (homo-or heterodimer of members of the Jun and fos family) is involved in apoptosis (Colotta et al., 1992). It has been reported that cells which overexpress a mutated ras oncogene (Wyllie et al., 1987) fail to undergo apoptosis in response to a wide variety of stimuli. Overexpression of the wild-type P53 anti-oncogene product could induce apoptosis in cultured cells (Yonish-Rouach et al., 1991). Wild type P53 is known to cause temporary G_1 arrest in some cell types (Kastan et al., 1991) and it is thus possible that this growth arrest co-operates with endogenous c-myc expression to initiate apoptosis. The expression of some genes increases during apoptosis. These include the gene for TRPM-2,

or clusterin which may be involved in secretion of a number of mRNAs that were isolated from apoptotic thymocytes; their role in regulation of apoptosis is not yet clear (Leger et al., 1987). It has been suggested that TRPM-2 may be necessary for maintaining the integrity of the cell membrane and for preventing complement fixation during the formation of apoptotic bodies (Wong et al., 1993; Guenette and Tenniswood, 1994). While TRPM-2 may be a good marker for apoptosis in the regressing rat prostate it is not so useful in T51B cells. Work on an earlier cell death model developed with these cells using the calcium ionophore A23187, have shown that while TRPM-2 expression does not increase during apoptosis, the gene is also expressed constitutively and its expression increases during cell proliferation (Brabyn and Kleine, 1990).

In the last few years, the function of the bcl-2 proto-oncogene has been linked to negative regulation of apoptosis. Initially, it has been shown that after introducing bcl-2 into cells and then cutting off the cytokine supply, the cells have survived even though they are supposed to die (Nunez et al., 1990). Subsequently, transgenic mice have been made bearing the bcl-2 oncogene in B cells and show extended survival of these cells (Strasser et al., 1991a). However, bcl-2 cannot inhibit all the pathways of induction of apoptosis; cytotoxic T-cell killing is not inhibited by bcl-2 (Vaux et al., 1993) and thymocytes over-expressing bcl-2 are still negatively selected, though less rapidly (Strasser et al., 1991b). There are a number of additional biological and biochemical observations related to prolonged survival of cells. The absence of bcl-2 from tissues (e.g. liver) as well as other cell types (e.g. endothelial and smooth muscle cells) with regular cell turnover (Hockenbery et al., 1991), and the regulated occurrence of bcl-2-independent forms of apoptosis (i.e. which occur in the presence of even over-expressed bcl-2 protein) suggest the existence of other inhibitor

elements (Fesus, 1993). Apoptosis has been prevented by down-regulation, or in other types of cells, by activation of PKC (Jin et al., 1992; Rajotte et al., 1992). It is inhibited by L-acetylcarnitine (Galli and Fratelli, 1993), by tumor promoters (Bursch et al., 1992), and after induction of sustained elevation of cytoplasmic calcium (Franklin and Johnson, 1992). The biochemical target(s) of these effectors and their relationship to bcl-2 expression or function is presently not known.

2.1.3 Epidermal Growth Factor (EGF), Cell Proliferation and Apoptosis

The progression of non-neoplastic cells through their growth-division cycle is regulated by fluctuations in the metabolic generations and intracellular levels of second messengers in response to growth factors such as EGF. The tyrosine kinase activity of growth factor receptors is critical for the signal transduction pathways required for mitogens, transformation and cell differentiation. Signaling pathways initiated by tyrosine phosphorylation lead to various nuclear events. Ultimately these events lead to enhanced DNA synthesis and cell division in most cells (Carpenter and Cohen, 1979; Carpenter, 1987; Schlessinger, 1988) and elicit other different biological responses.

Binding of growth factors to their receptors such as EGF receptors is followed rapidly by internalization and degradation of the receptor-ligand complex (Schlessinger, 1988). Before this occurs, however, the intracellular stimulus generated by the EGF receptors is recognized and processed by signal transduction systems, with which a number of defined changes in cytoplasmic biochemistry are associated. These include cytoplasmic alkalinization via activation of the Na^+/H^+ antiport and increases in ion flux across the plasma membrane, increases in intracellular level of the second messenger cAMP, and the hydrolysis of phosphoinositides to produce two second messengers, IP_3 and DAG which are

instrumental in the release of calcium ions from intracellular stores and the activation of PKC respectively (Nishizuka, 1995). The most immediate event following EGF stimulation is transcriptional activation of early response genes. Among these are at least two proto-oncogenes c-fos and c-myc. C-fos, which itself encodes a transcription factor (Curran and Franza, 1988), is activated almost immediately (Greenberg and Ziff, 1984), while c-myc, the product of which is required for progression to DNA replication (Heikkela et al., 1987), is transcribed somewhat later (Greenberg and Ziff, 1984). Several groups have shown that upon stimulation of cells with EGF, both PLC-γ and Ras-GTPase activating protein (Ras-GAP) become associated with and are tyrosine phosphorylated by the EGF receptor (Schlessinger and Ullrich, 1992; Schlessinger, 1994). Subsequent studies have demonstrated that only the tyrosine-phosphorylated forms of the growth factor receptor bind PLC-γ and GAP which contain conserved noncatalytic domains termed Src homology (SH) domains SH2 and SH3 (Schlessinger and Ullrich, 1992). Both genetic studies in C elegans and Drosophila, and biochemical studies in mammalian cells, have indicated that the adaptor protein, Grb2 which contains one SH2 flanked by two SH3 domains links receptor tyrosine kinases to the ras signaling pathway (Pawson and Schlessinger, 1993; Lowenstein et al., 1992; Schlessinger, 1993). It has been shown that the SH3 domain of Grb2 binds to short proline-rich regions in the carboxy-terminal tail of a protein known as ras guanine nucleotide releasing factor Sos. In response to EGF receptor activation and autophosphorylation, the Grb2/Sos complex binds to EGF receptor, recruiting Sos to the plasma membrane where the ras protein is located. Since changes in the guanine nucleotide releasing activity of Sos are not detected in response to EGF stimulation, it has been proposed that the interaction of the Grb2/Sos complex with the EGF receptor functions to increase the local concentration of Sos

in the plasma membrane. This would lead to the exchange of GDP for GTP on ras, and subsequent activation of a kinase cascade including Raf, mitogen-activated protein kinase and many other responses which are regulated by these kinases in the cytoplasm and the nucleus. This results in the phosphorylation of transcription factors, such as components of AP-1, and ultimately to changes in gene expression (Schlessinger, 1994).

In many tissues and cell lines, EGF has been shown to enhance DNA synthesis, increase cell survival and ultimately prevent apoptosis. For example, in mammary epithelial cells (Sylvester et al., 1994), dermal fibroblasts and keratinocytes (Reynolds, 1993), glioma cells (Couldwell et al., 1992), BalLb/c/3T3 fibroblasts (Besterman et al., 1986) and in Chinese hamster embryo fibroblasts (11C9 cells) (Wright et al., 1988), EGF has been reported to have a potent mitogenic effect which leads to DNA synthesis and cell division. In other cells such as the human breast cancer cell line MDA-231, EGF has been shown to protect these cells from death induced by anti-cancer drugs adriamycin and actinomycin D (Geier et al., 1994a,b) or by the protein synthesis inhibitor cyclohexamide (Geier et al., 1993). In PC12 cells, both EGF and nerve growth factor (NGF) rescue these cells from apoptosis induced by etoposide, an inhibitor of topoisomerase II (Nakajima et al., 1994). In neuronal cells, EGF increases cell survival and prevents cell death during anoxia or nitric oxide exposure (Maiese et al., 1993). Other studies have indicated that deprivation of serum or EGF triggers apoptosis in a variety of cell types which could be inhibited upon re-addition of EGF (Tilly et al., 1992; Luciano et al., 1994; Dremier et al., 1994). In contrast, in other cells that overexpress EGF receptors such as MB-468 human breast cancer cells, EGF treatment leads to inhibition of cell proliferation and DNA fragmentation (Armstrong et al., 1994). In other tissues and organs such as liver, EGF has been shown to induce an initial

rapid burst of hyperplasia and an increase in organ weight. When EGF is removed there is an increase in apoptosis and over time, the organ is restored to its original size (Bursch et al., 1984; Columbano et al., 1985). Similar observations have been reported in neonatal rat liver hepatocytes and stromal cells where EGF induces first hyperplasia, followed by involution characterized by a high incidence of apoptosis (Armato et al., 1986). Recently, Brabyn et al., (1994) have demonstrated that when T51B rat liver cells are grown in the presence of 1 nM EGF, the cells undergo a period of hyperplasia which is then followed by a decrease in cell numbers due to an increase in apoptosis. It is clear from the above studies that EGF has different effects depending on tissue or cell type. In this chapter I studied the cell cycle kinetics of T51B cells in both serum and EGF treated cultures using flow cytometric techniques combined with Hoechst staining and TUNEL assay to determine the percentage of apoptotic cells as the cells progress through the cycle.

2.2 Objectives

The overall objective of this chapter was to establish the cell cycle kinetics of T51B cells in the presence of 10% serum or EGF. The specific objectives were:

2.2.1 To establish the cell cycle kinetics of T51B cells at various times after plating in medium containing 10% serum by flow cytometry.

2.2.2 To determine the effect of EGF addition at various times on the cell cycle kinetics of T51B cells by flow cytometry.

2.2.3 To examine cell death (apoptosis) in serum or EGF treated T51B cells during the cell cycle progression by Hoechst staining of condensed nuclei.

2.2.4 To determine DNA fragmentation in apoptotic cells using an *in situ* apoptosis detection method (TUNEL).

2.3 Methods

2.3.1 Cell Culture

T51B rat liver cells were cultured in BME medium (basal medium eagle) (Life Technologies, Inc., Burlington, ON, Canada) supplemented with 10% (v/v) bovine calf serum (BCS) (Life Technologies) and 0.1 g/l gentamycin sulfate (Sigma Chemical Co. Ltd., St. Louis, USA). The cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Prior to experiments cells were detached by brief exposure to 0.05% (w/v) trypsin- 1 mM EDTA in phosphate buffered saline (PBS) (all reagents were obtained from Sigma Chemical Co. Ltd.). For the majority of experiments the cells were plated in culture dishes at a density of approximately 125 x 10^2 cells/cm^2 and grown for 24 h before addition of EGF dissolved in PBS, to a final concentration of 1 nM. Control culture were treated with PBS vehicle. All assays were performed at least in duplicate.

2.3.2 Flow Cytometry

DNA analysis by flow cytometry is based on the ability to stain the cellular DNA with a fluorescent DNA binding dye in a stoichiometric manner (the amount of the bound dye is directly proportional to the amount of DNA within the cell. Thus, the machine can readily quantify the proportion of cells in G_0/G_1, S and G_2/M phase. Since apoptotic cells typically decrease in size, in part because of nuclear condensation with an apparent decrease in DNA content per cell, the flow cytometer aught to be able to detect apoptotic cells as smaller, less fluorescent entities. Therefore, the appearance of cells with low DNA stainability, lower than that of G_1 cells (sub G_1 peak, A_0 cells) has been considered as a marker of cell death by apoptosis (Nicoletti et al., 1991; Drzynkiewicz et al., 1992). Cells were detached by brief exposure to 0.05% trypsin, 1 mM EDTA in PBS (PBS-E buffer). The cells were washed

with PBS, centrifuged at 500 x g for 5 min. They were then resuspended in 1 ml of PBS-E buffer. A single cell suspension was obtained by passing the suspension through a 21 gauge needle two times and then counted. An aliquot containing 1 x 10^6 cells/ml were permeabilized with an equal volume of permeabilization buffer (supplied by Coulter) and mixed with 1 ml picogreen (5 µg/ml; Molecular Probes, Inc.) and analyzed in a Coulter Epics X L Flow Cytometer equipped with a 480 nm argon Laser. The fluorescence emission intensity of picogreen-stained cells was detected at 520 nm.. The voltage coming from photo multiplier tube (PMT) was adjusted to 580 volts so that the G_1 population was detected in channel 400. Noise discrimination was applied to eliminate the unwanted pulses usually from the debris. Cell cycle analysis was performed using Multicycle AV software which is designed to determine the percentage of cells at different phases of the cell cycle. The number of cells undergoing apoptosis can also be analyzed by this means. Because the analysis performed by Multicycle AV considers apoptotic population distinct from cycling cells, the totals obtained are greater than 100%.

2.3.3 Hoechst Staining

Cells were grown on coverslips, were washed twice with PBS and fixed by addition of 4% paraformaldehyde (BDH Chemicals Ltd.) (w/v) in PBS for 30 min at room temperature. The coverslips were then washed twice with PBS and followed by incubation with 1 µg/ml Hoechst dye, fluorochrome 33258 (Sigma Chemical Co. Ltd.) in PBS for 20 min. The coverslips were then washed with PBS, rinsed with distilled water, air dried, mounted and viewed under an Olympus fluorescence microscope and photographed with Kodak ektachrome 400.

2.3.4 Nuclear DNA Fragmentation Using *In Situ* Cell Death Detection Kit (TUNEL Assay) Combined with Hoechst Staining "Dual Staining"

The *in situ* cell death detection kit (Boehringer Mannheim) was used to detect and quantitate apoptotic bodies. The TUNEL (TdT-mediated dUTP nick end labeling) reaction preferentially labels DNA strand breaks generated during apoptosis. In this kit, DNA of fixed cells was labeled by the addition of fluorescein dUTP at strand breaks by terminal deoxynucleotidyl transferase (TdT). This allowed the direct detection of DNA fragmentation by fluorescence microscopy. Briefly, cells were grown on coverslips, and washed twice with PBS, fixed with 4% paraformaldehyde (freshly prepared) in PBS for 30 min at room temperature. The coverslips were then washed twice with PBS and incubated in permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate) for 2 min on ice (4°C). The coverslips were then washed twice with PBS and 50 µl TUNEL reaction mixture was added to the coverslips, which were then incubated in a humidified chamber for 60 min at 37°C in the dark. For negative controls coverslips were incubated with 50 µl label solution which contained the nucleotide mixture in reaction buffer without TdT instead of TUNEL reaction mixture (TdT + nucleotide mixture). The coverslips were then washed twice with PBS and incubated with 1 µg/ml Hoechst dye in PBS for 20 min. The coverslips were then washed twice with PBS and mounted with glycerol containing 0.1% (w/v) p-phenylenediamine in PBS. They were then viewed under an Olympus fluorescence microscope and photographed with Kodak ektachrome 400 colour slide film.

2.4 Results

2.4.1 Cell Cycle Kinetics of T51B Cells in the Presence of 10% Serum by Flow Cytometry

Cell cycle analysis is performed on T51B cells at various times after plating in medium containing 10% serum using flow cytometry. The data are then analyzed by Multicycle AV software. In sparse cultures, most of the cells are still in the G_1 phase with a very low population of cells in the S or the G_2 phase. However, 7.7% of cells are undergoing apoptosis (Fig. 2.1 and Table 2.1). Within 2 days, approximately 34% of the cells progress into the S phase and 10% are in the G_2 phase, however, there is no change in the apoptotic population. The increase in the percentage of cells in the S phase corresponds well with a decrease in the percentage of cells in the G_1 phase. By day 3, the cells are still proliferating with 32% of the cells in S phase but significant numbers of cells are now accumulating in the G_2 phase (17.4%) (Fig. 2.3 and Table 2.1). By day 5, the cells reach confluence, however, their rate of proliferation is still quite high (26% of the cells are in S phase). In addition, the G_2 population decreases while the apoptotic population starts to increase (Fig. 2.4 and Table 2.1). By day 6, the cells are confluent as examined by phase contrast microscopy. Nevertheless, 30% of the cells are still proliferating but a significantly greater population of apoptotic cells accumulate (18.1%) with a corresponding decrease in the G_2 cell population (Fig. 2.5 and Table 2.1). DNA synthesis does not cease until day 8 by which time the apoptotic population reaches 21.4% (Fig. 2.6 and Table 2.1). It is obvious that as cells reach confluence there is an increase in the percentage of apoptotic cells with a corresponding decrease in the percentage of the G_2 cell population.

FIGURES 2.1 - 2.6

Flow Cytometric Analysis of T51B Cells at Various Times after Plating in Medium Containing 10% Serum

T51B cells were plated at a density of 125 x 10^2 cells/cm^2 in medium containing 10% serum. At various time intervals the cells were detached by brief exposure to 0.05% trypsin in PBS. The cells were washed with PBS, centrifuged, resuspended in PBS and counted. An aliquot containing 1 x 10^6 cells/ml was used for flow cytometry as described in section 2.3.2. Cells were analyzed in a Coulter Epics XL Flow Cytometer equipped with a 488 nm argon laser.

Figure 2.1

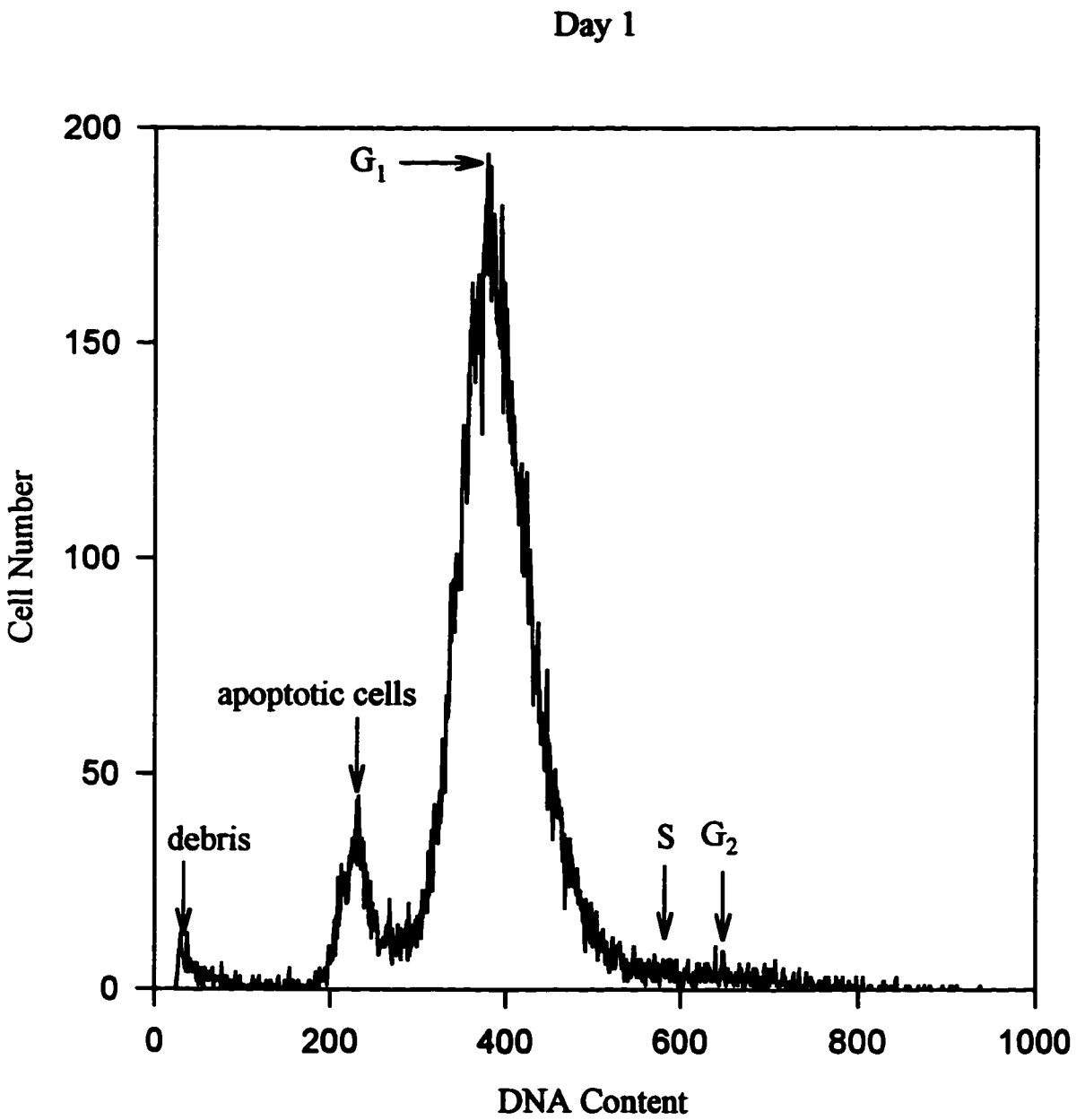

Day 1
200
150
100
50
0
Cell Number
G1
apoptotic cells
debris
S
G2
0
200
400
600
800
1000
DNA Content

Figure 2.2

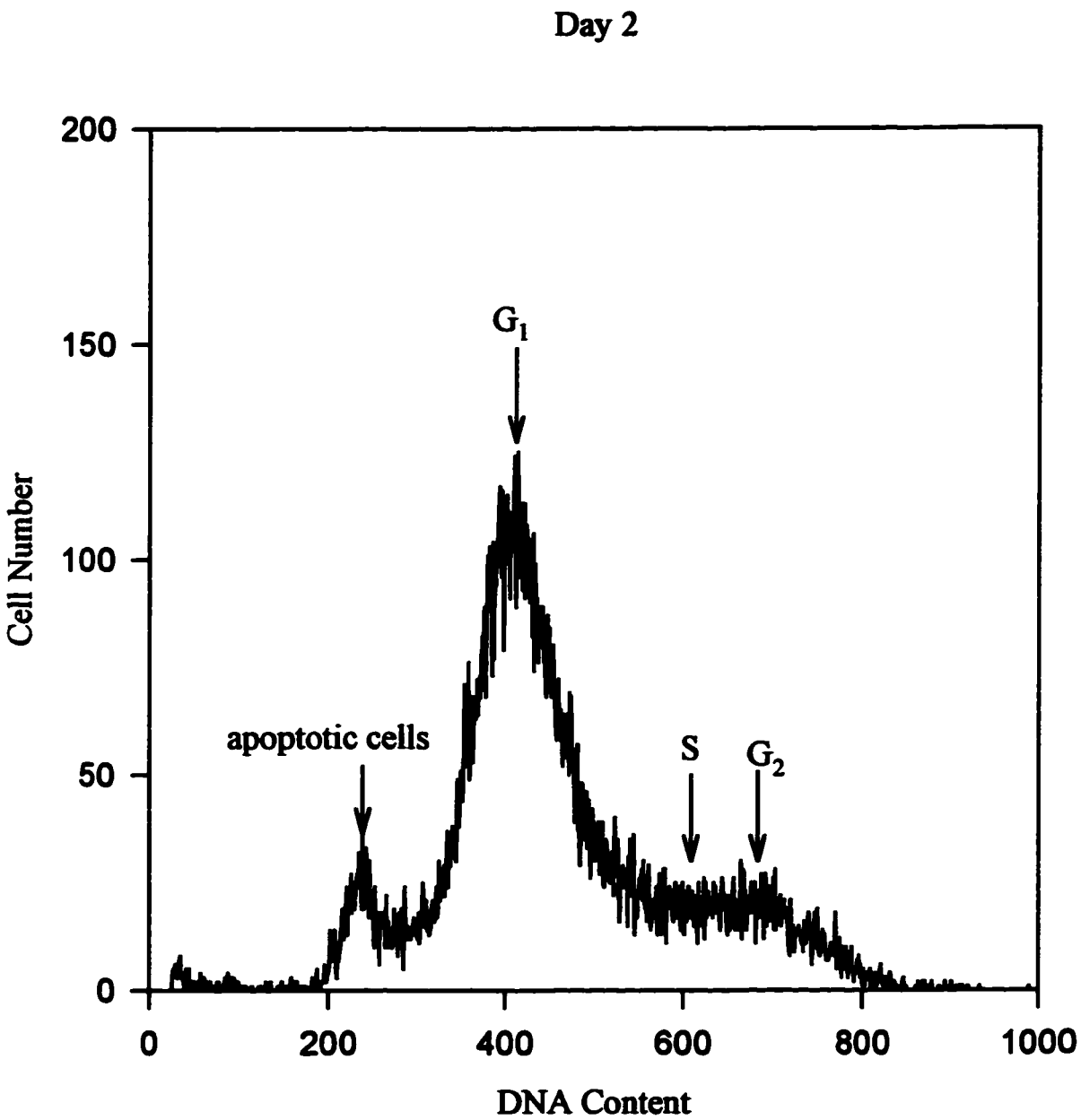

Day 2
200
150
100
50
0
Cell Number
apoptotic cells
G1
S
G2
0
200
400
600
800
1000
DNA Content

Figure 2.3

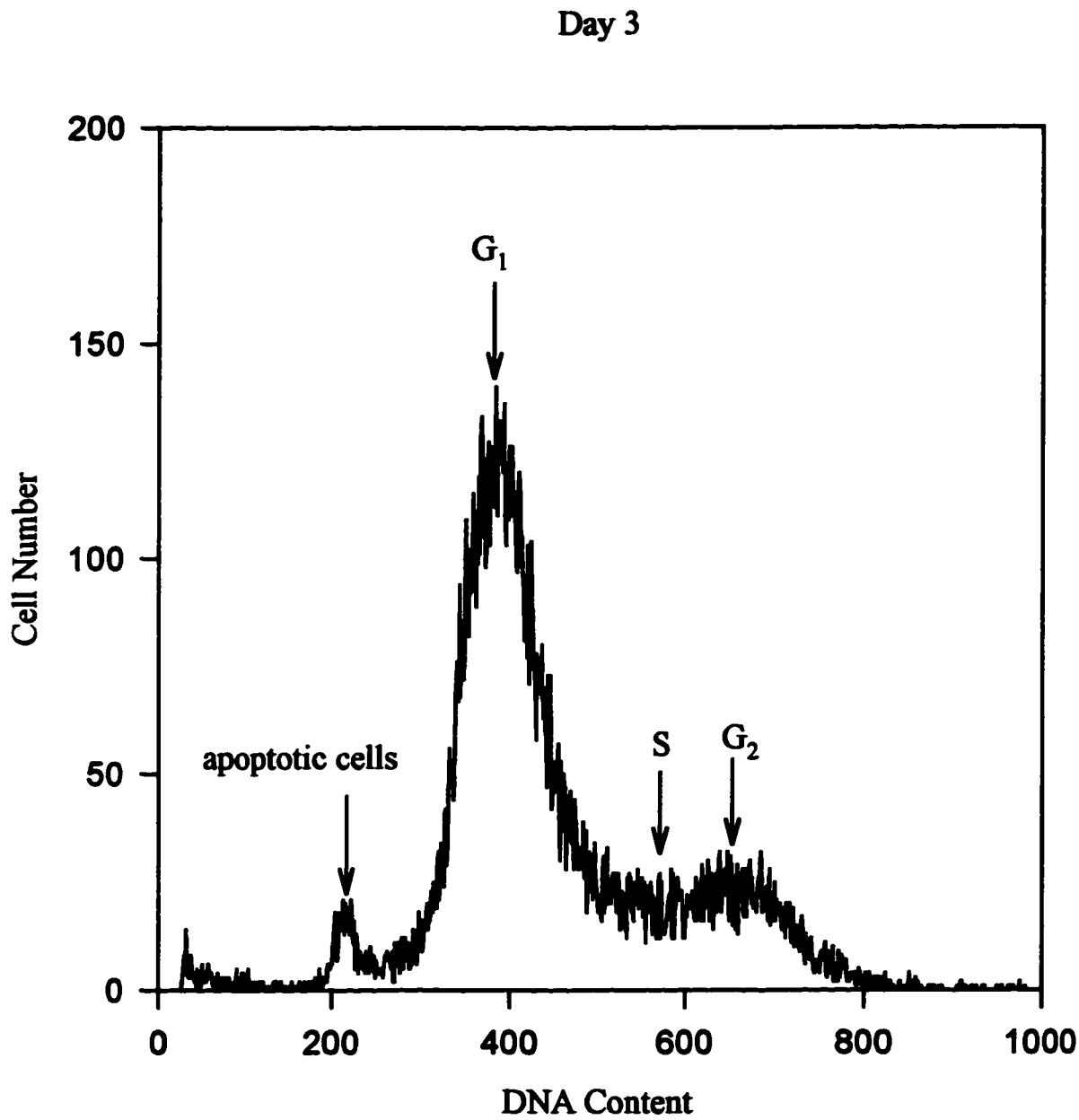

Day 3
200
150
100
50
0
Cell Number
G_1
apoptotic cells
S
G_2
0
200
400
600
800
1000
DNA Content

Figure 2.4

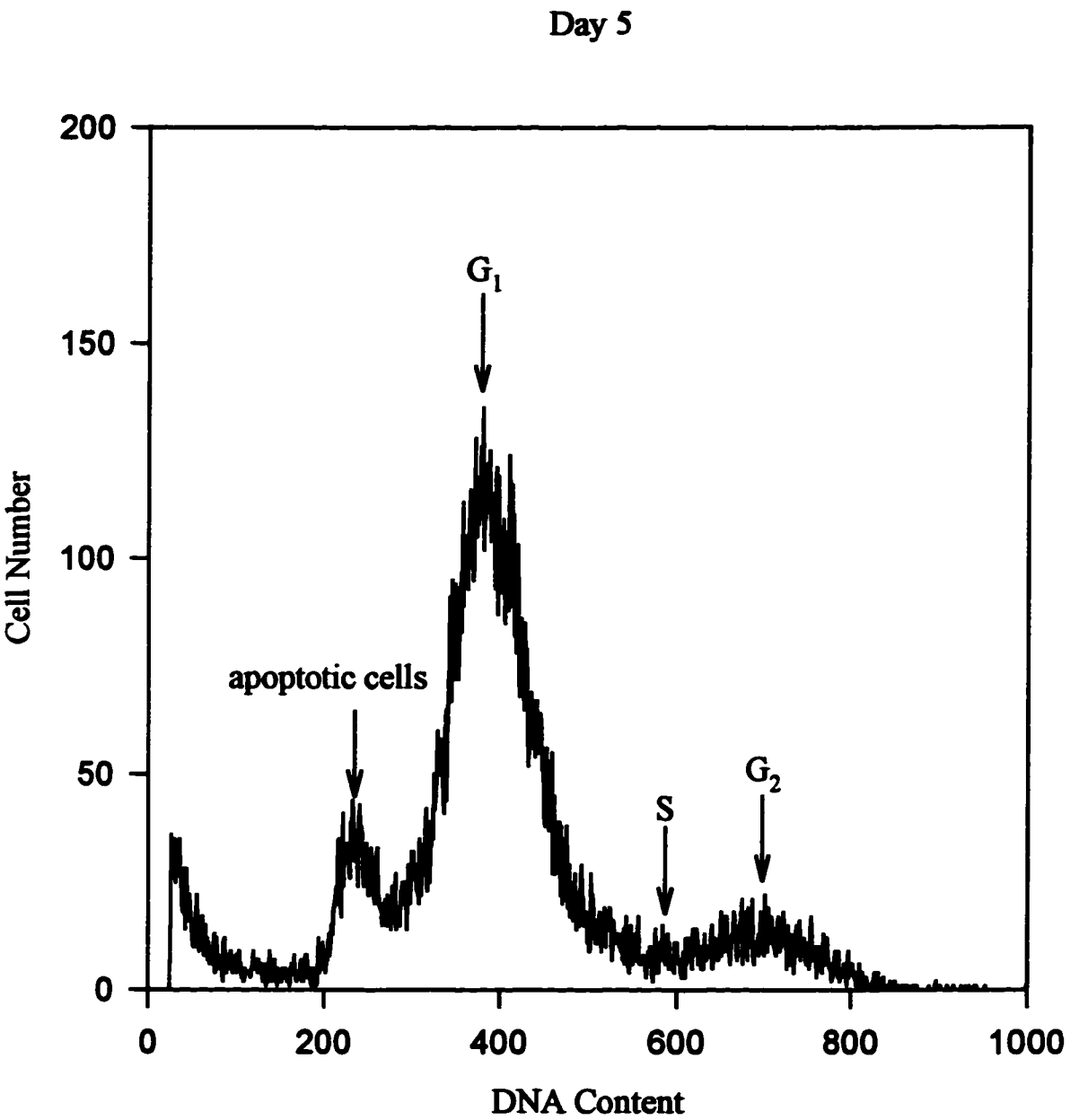

Day 5
200
150
100
50
0
Cell Number
apoptotic cells
G1
S
G2
0
200
400
600
800
1000
DNA Content

Figure 2.5

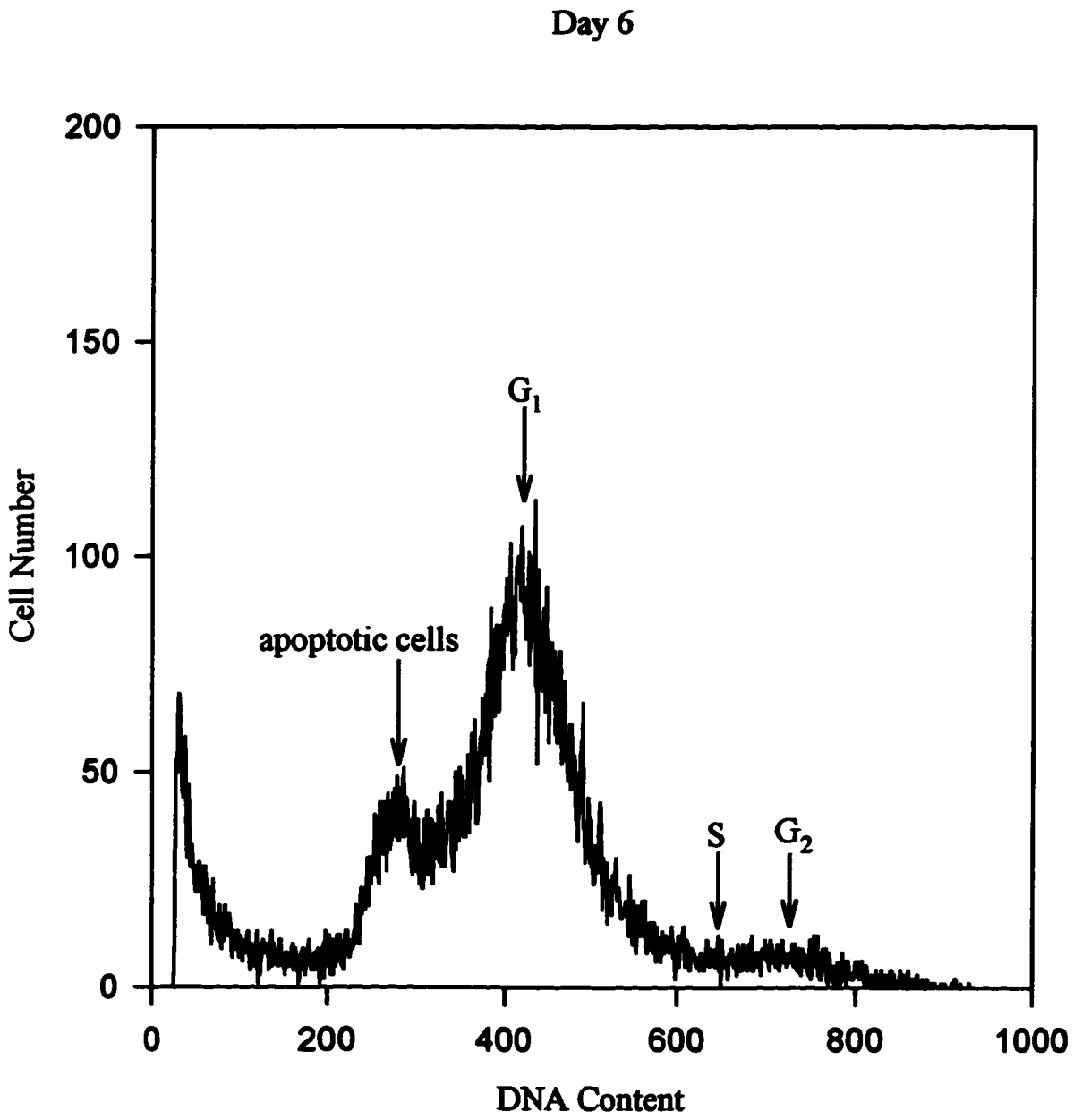

Day 6
200
150
100
50
0
Cell Number
apoptotic cells
G1
S
G2
0
200
400
600
800
1000
DNA Content

Figure 2.6

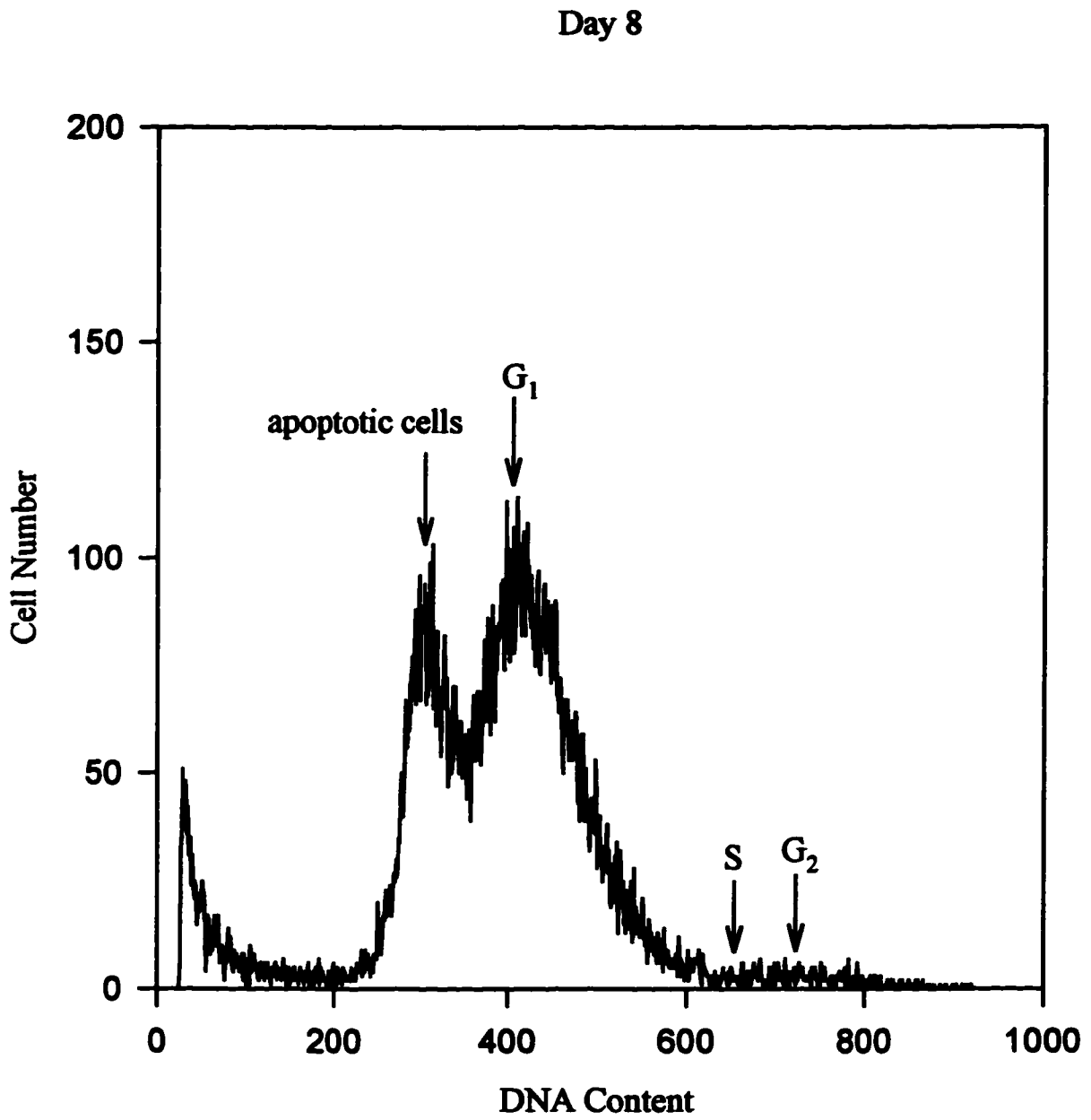

Day 8
200
150
100
50
0
Cell Number
apoptotic cells
G1
S
G2
0
200
400
600
800
1000
DNA Content

TABLE 2.1

Cell Cycle Analysis of T51B Cells at Various Times After Plating in Medium Containing 10% Serum

T51B cells were plated at a density of 125 x 10^2 cells/cm^2 in medium containing 10% serum. At various time intervals the cells were detached by brief exposure to 0.05% trypsin in PBS. They were then washed with PBS, centrifuged, resuspended in PBS and counted. An aliquot containing 1 x 10^6 cells/ml was used for flow cytometry as described in section 2.3.2. Cell cycle analysis were performed using Multicycle AV software.

TABLE 2.1

Cell Cycle Analysis of T51B Cells at Various Times after Plating in Medium Containing 10% Serum

Time	G_1		S	G_2		Apoptotic	
	mean ± SD	%	%	mean ± SD	%	mean ± SD	%
Day 1	95.7 ± 9.5	79.1	18.1	166.5 ± 15.4	2.8	58.3 ± 4.9	7.7
Day 2	101.0 ± 11.9	55.7	34.3	175.8 ± 15.1	10.0	61.0 ± 6.2	7.1
Day 3	95.5 ± 9.5	50.4	32.2	166.3 ± 15.4	17.4	56.6 ± 5.5	3.3
Day 5	95.3 ± 10.8	57.7	26.0	165.9 ± 22.7	13.1	60.4 ± 6.4	11.0
Day 6	104.3 ± 11.5	62.0	30.0	181.6 ± 16.1	7.6	75.3 ± 5.8	18.1
Day 8	103.7 ± 15.3	90.0	9.1	180.5 ± 31.0	1.0	71.2 ± 10.7	21.4

SD: Standard deviation

2.4.2 Effect of EGF Treatment on Cell Cycle kinetics of T51B Cells

To examine the effect of EGF on cell cycle kinetics T51B cells are plated and grown in medium containing 10% serum for one day before addition of EGF. One day after EGF treatment, 37% of the cells progress to the S phase and 17% are in the G_2 phase. However, there is no change in the apoptotic population (Fig. 2.7 and Table 2.2). By day 2, the cells are still proliferating but a significant number of cells are now accumulating in the G_2 phase with a slight decrease in the percentage of apoptotic cells (Fig. 2.8 and Table 2.2). By day 4, EGF treated cells are confluent, however, their rate of proliferation is still quite high with 21% of cells in the S phase. On the other hand, the number of cells in the G_2 phase starts to decrease with a slight increase in the population of apoptotic cells (Fig. 2.9 and Table 2.2). By day 5, the G_2 cell population decreases while the apoptotic cell population continues to increase. Nevertheless, a considerable number of cells are still proliferating (Fig. 2.10 and Table 2.2). After 7 days of EGF addition, DNA synthesis ceases while the apoptotic cell population increases to 18% (Fig. 2.11 and Table 2.2). However, the onset of apoptosis is delayed in EGF treated cells. For example the percentages of apoptotic cells in serum stimulated cells after 5, 6, and 8 days of plating are 11.0, 18.1 and 21.4 % while in EGF treated cells are 4.4, 7 and 18.0 % respectively (Tables 2.1 and 2.2). Hence, the cells in the presence of EGF may servive for longer time before they undergo apoptosis. These results are in agreement with previous studies which have indicated that the addition of EGF to proliferating T51B cells results in a period of hyperplasia followed by apoptosis (Brabyn et al., 1994).

FIGURES 2.7 - 2.11

Flow Cytometric Analysis of T51B Cells at Various Times after Addition of EGF

T51B cells were plated at a density of 125 x 10^2 cells/cm^2 in medium containing 10% serum and grown for one day before addition of 1 nM EGF. Control cultures were treated with PBS vehicle. The cells were then detached and prepared for flow cytometry at various time intervals after addition of EGF to cell cultures as described in section 2.3.2. Cells were analyzed in a Coulter Epics XL Flow Cytometer equipped with a 488 nm argon laser.

Figure 2.7

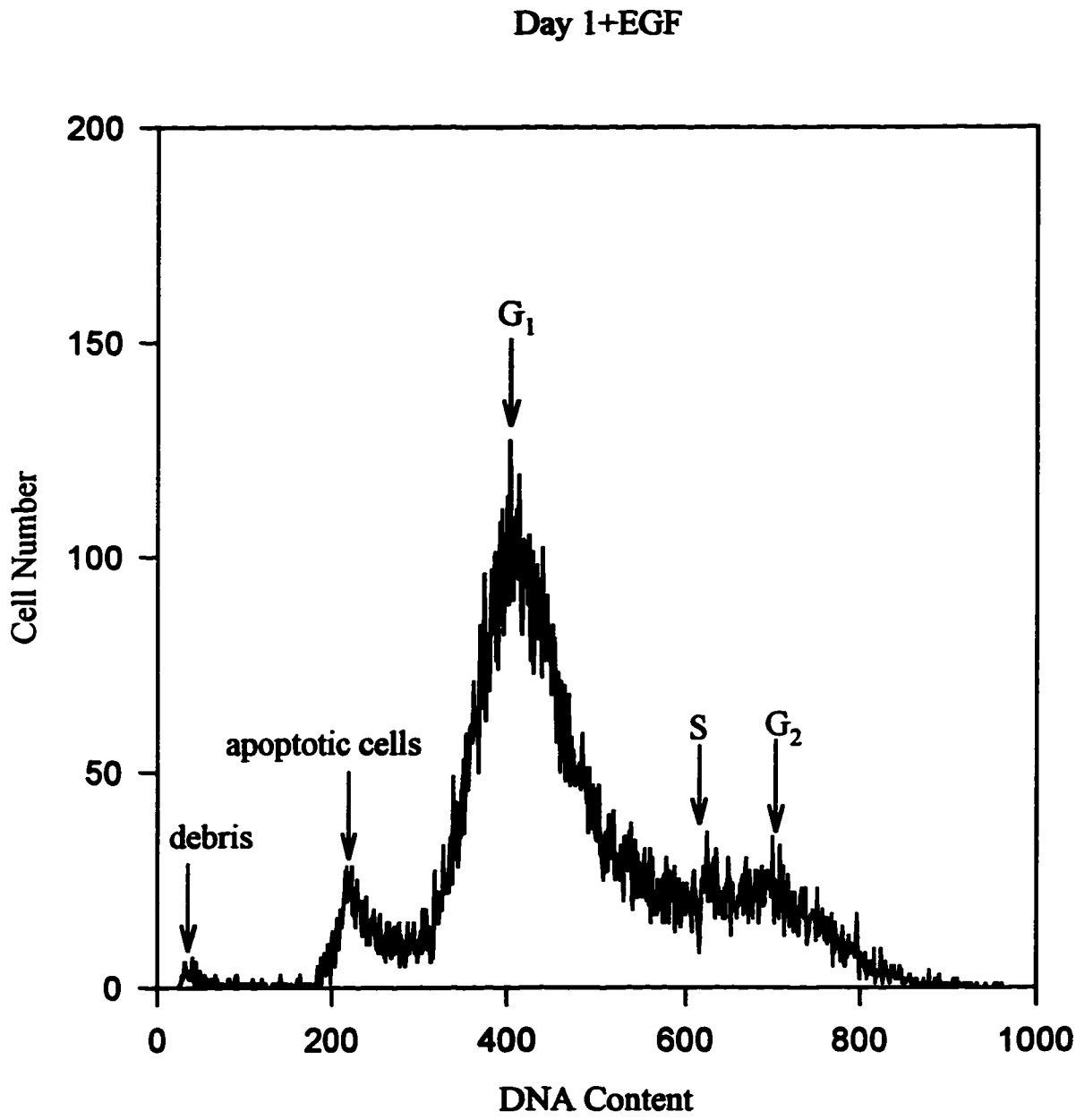

Day 1+EGF
200
150
100
50
0
Cell Number
G_1
apoptotic cells
debris
S
G_2
0
200
400
600
800
1000
DNA Content

Figure 2.8

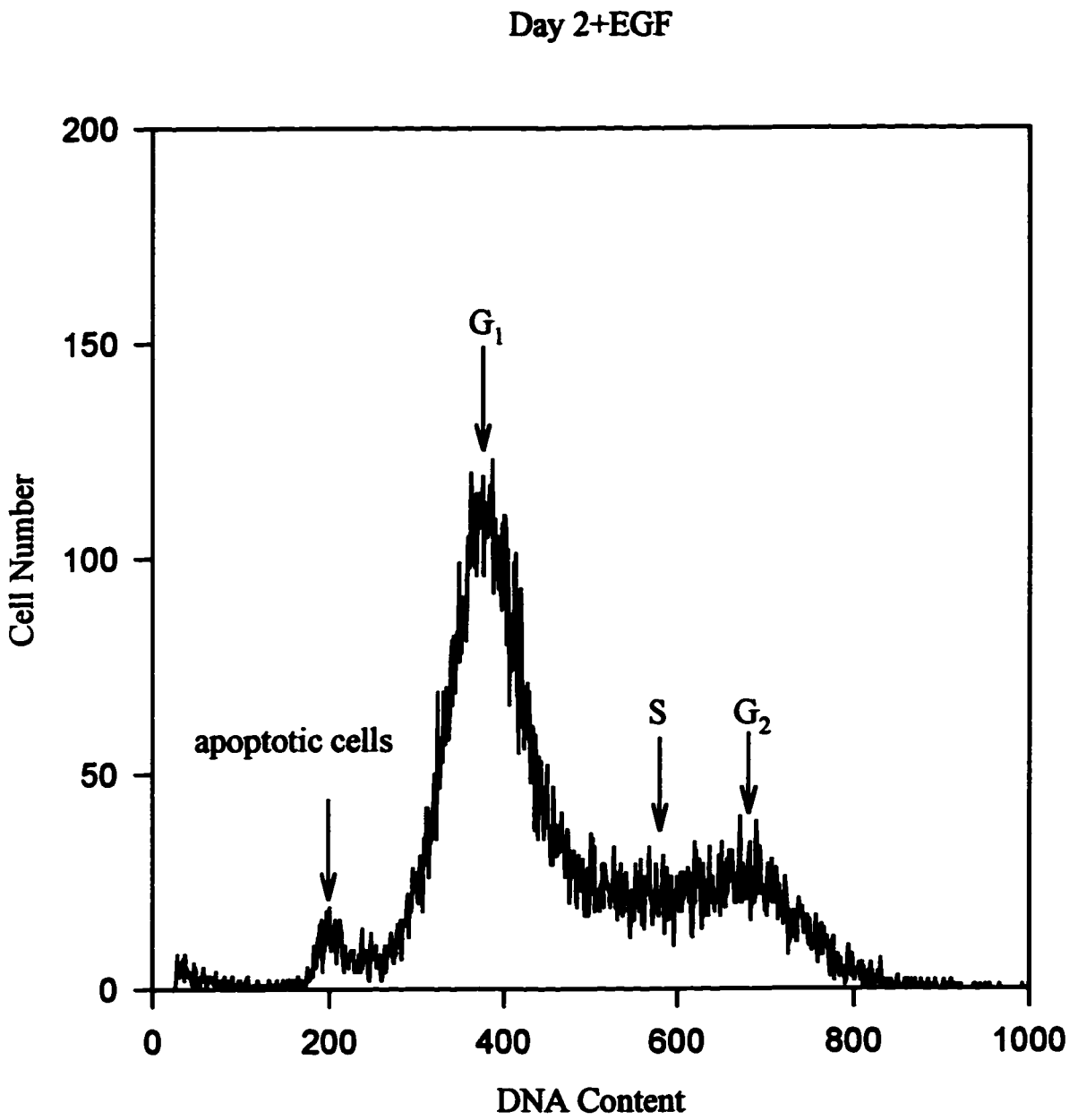

Day 2+EGF
200
150
100
50
0
Cell Number
G1
S
G2
apoptotic cells
0
200
400
600
800
1000
DNA Content

Figure 2.9

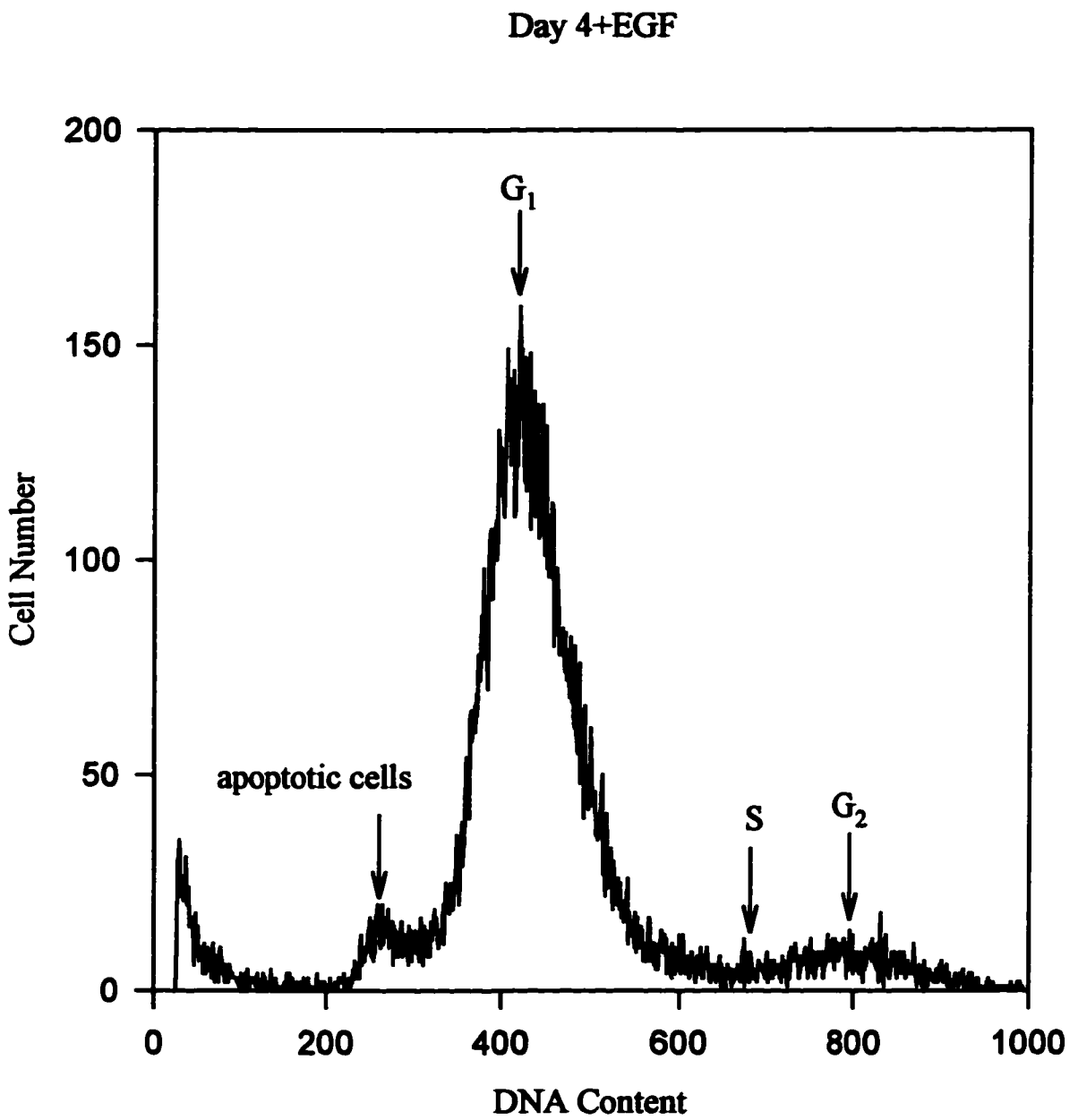

Day 4+EGF
200
150
100
50
0
Cell Number
apoptotic cells
G1
S
G2
0
200
400
600
800
1000
DNA Content

Figure 2.10

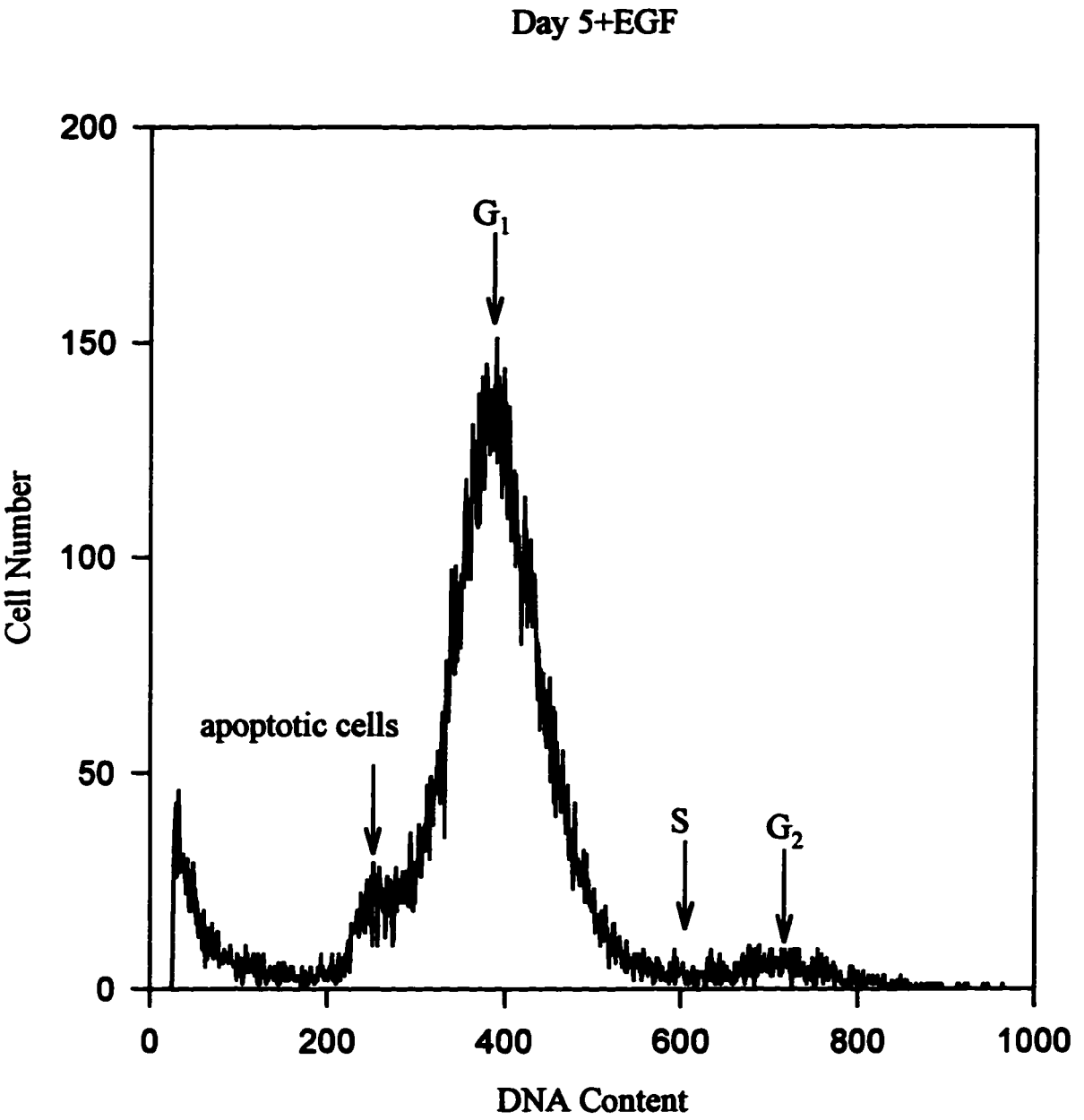

Day 5+EGF
200
150
100
50
0
Cell Number
G1
apoptotic cells
S
G2
0
200
400
600
800
1000
DNA Content

Figure 2.11

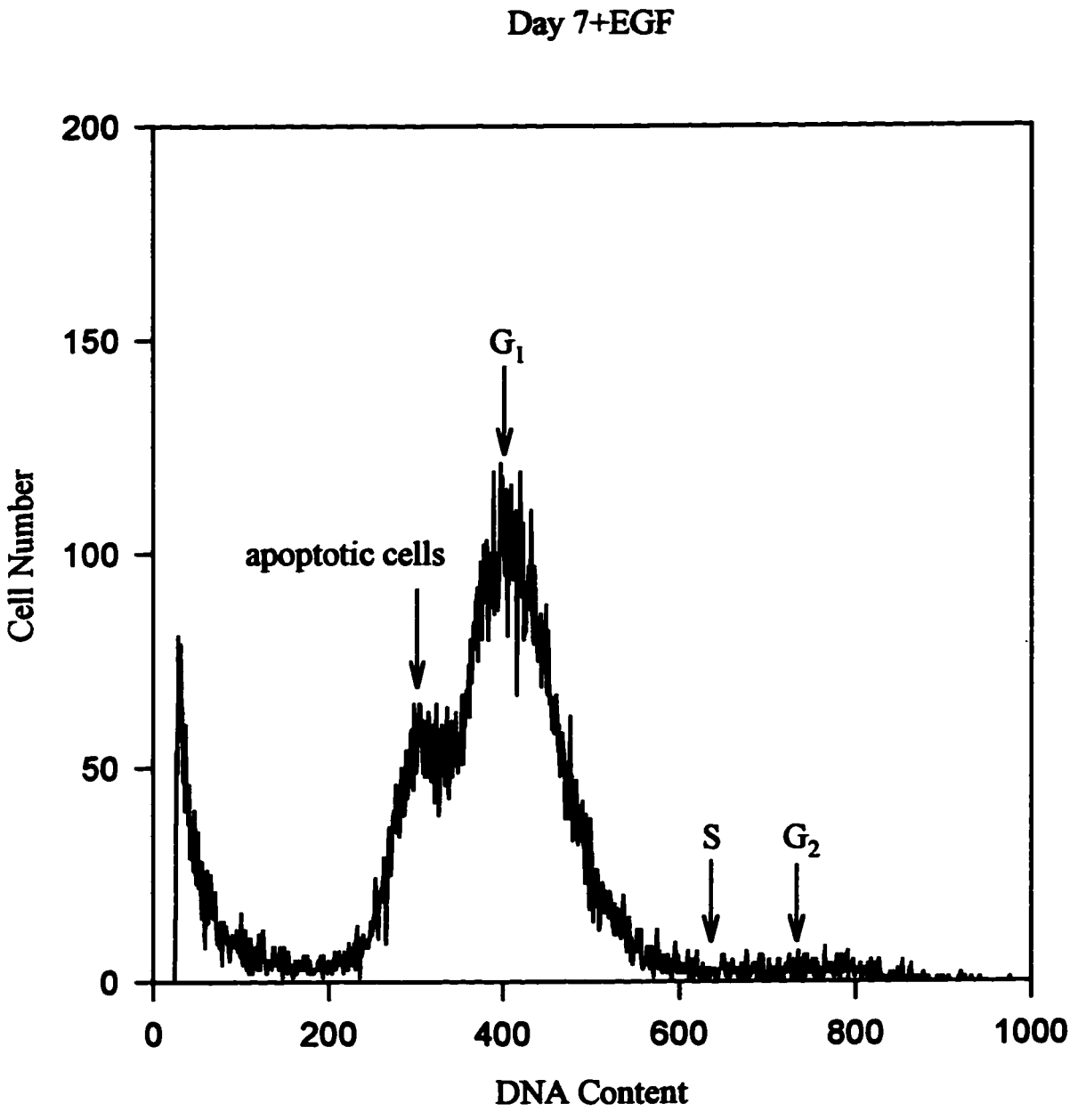

Day 7+EGF
200
150
100
50
0
Cell Number
apoptotic cells
G_1
S
G_2
0
200
400
600
800
1000
DNA Content

TABLE 2.2

Cell Cycle Analysis of T51B Cells at Various Times after Addition of EGF

T51B cells were plated at a density of 125 x 10^2 cells/cm^2 in medium containing 10% serum and grown for one day before addition of 1 nM EGF. Control cultures were treated with PBS vehicle. The cells were then detached and prepared for flow cytometry at various time intervals after addition of EGF to cell cultures as described in section 2.3.2. Cell cycle analysis were performed using Multicycle AV software.

TABLE 2.2

Cell Cycle Analysis of T51B Cells at Various Times after Addition of EGF

Days after EGF Addition	G_1		S	G_2		Apoptotic	
	mean ± SD	%	%	mean ± SD	%	mean ± SD	%
Day 1 (control)	95.7 ± 9.5	79.1	18.1	166.5 ± 15.4	2.8	58.3 ± 4.9	7.7
Day 2 +EGF (1 day)	100.5 ± 11.5	45.7	37.4	175.0 ± 17.5	17.3	58.9 ± 6.3	6.0
Day 3 + EGF (2 days)	93.2 ± 11.0	46.5	26.5	162.2 ± 21.0	27.6	53.4 ± 5.7	3.2
Day 5 + EGF (4 days)	105.9 ± 11.1	63.9	21.1	184.3 ± 35.5	15.0	68.0 ± 6.5	4.4
Day 6 + EGF (5 days)	96.6 ± 11.7	71.2	21.5	168.2 ± 22.1	7.30	64.3 ± 7.6	6.8
Day 8 + EGF (7 days)	102.2 ± 13.5	86.1	8.9	177.3 ± 24.8	5.00	74.2 ± 8.6	18.0

SD: Standard deviation

2.4.3 Nuclear Fragmentation of T51B Cells in the Presence of Serum or EGF

2.4.3a Hoechst Staining of T51B Cells in the Presence of 10% Serum

Condensation of chromatin and nuclear fragmentation reflect stages of apoptosis (Bursch et al., 1990). Staining with Hoechst dye provides a good method to distinguish between normal and apoptotic cells that have fragmented nuclei. Cells grown on coverslips in medium containing 10% serum at various times after plating are used for both flow cytometric analysis and Hoechst staining. Phase contrast and fluorescence microscopy show that by day 1 the cells are sparse and the Hoechst-stained nuclei appear intact with pale fluorescence staining (Fig. 2.12a,b). Within three days, the cells are actively proliferating and numerous mitoses could be seen. However, early stages of apoptosis could not be detected by Hoechst staining, though a few cells with blebbing are observed (Fig. 2.13a,b). By day 5, the cells reach confluence and the number of cells with condensed nuclei which have a bright Hoechst fluorescence staining increases significantly (Fig. 2.14a,b). After 8 days, the cells are confluent and no mitosis could be seen but there is a dramatic increase in the number of cells with condensed nuclei that are brightly stained with Hoechst dye (Fig. 2.15a,b). Hence, Hoechst staining confirms the flow cytometric observations which indicate an increase in the number of apoptotic cells as the cells reach confluence (section 2.4.1).

2.4.3b Hoechst Staining of EGF Treated T51B Cells

Phase contrast and fluorescence microscopy reveal that after one day of EGF addition, there is an increase in the number of cells and numerous number of mitotic cells could be seen (Fig. 2.16a,b). By day 2, few cells with condensed nuclei are observed, nevertheless, the cells are still actively proliferating (Fig. 2.17a,b). Within 4 days, the cells are still proliferating, though at a slower rate, but the number of cells with condensed nuclei

FIGURES 2.12 - 2.15

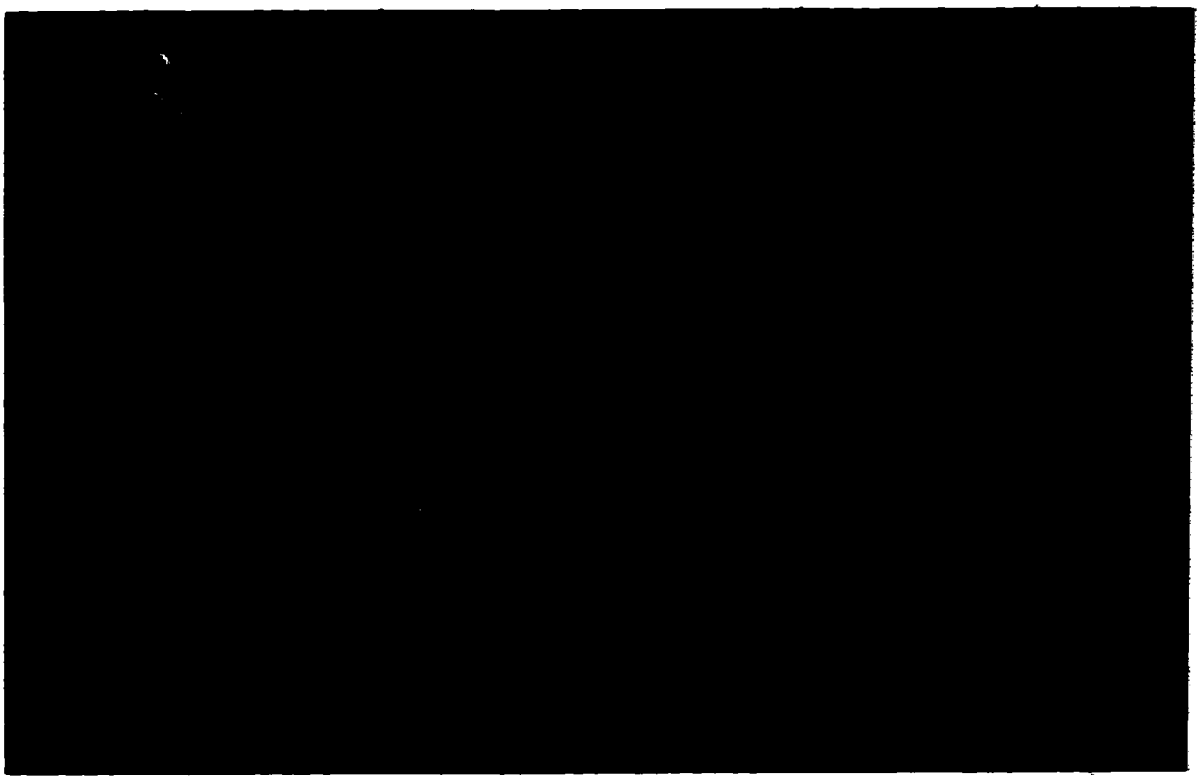
Nuclear Fragmentation in T51B Cells at Various Times after Plating in Medium Containing 10% Serum Using Hoechst Staining

T51B cells were plated at a density of 125 x 10^2 cells/cm^2 in medium containing 10% serum. At various time intervals the cells were washed with PBS and fixed with 4% paraformaldehyde and stained with Hoechst dye 33258 as described in section 2.3.3. The cells were viewed using an Olympus fluorescence microscope and photographed with Kodak ektachrome 400. The figures show (a) phase contrast photographs of T51B cells; (b) fluorescence photographs of Hoechst staining where fragmented nuclei have bright blue Hoechst staining (arrowed) and normal nuclei have pale Hoechst staining. Magnification: Day 1 - Day 8 is x400. Experiments were performed three times with similar results.

Figure 2.12

Day 1 (Control)

(a)



(b)

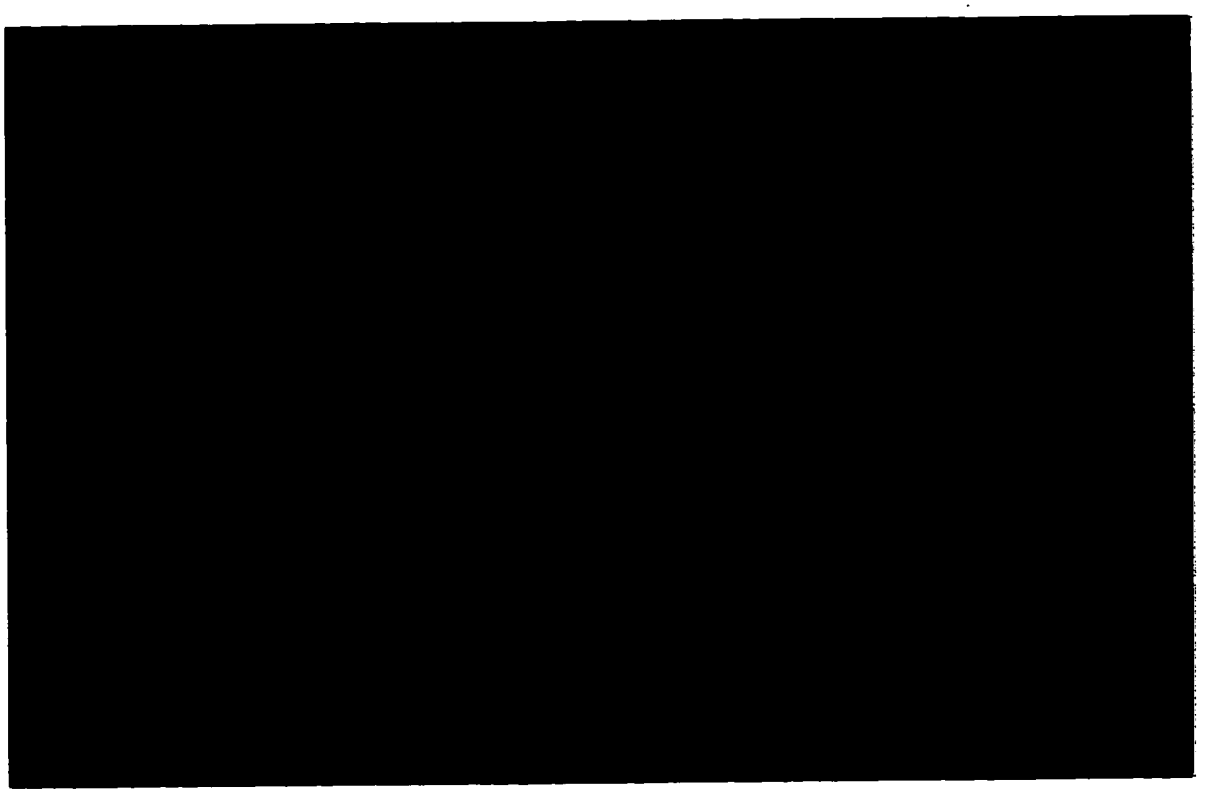
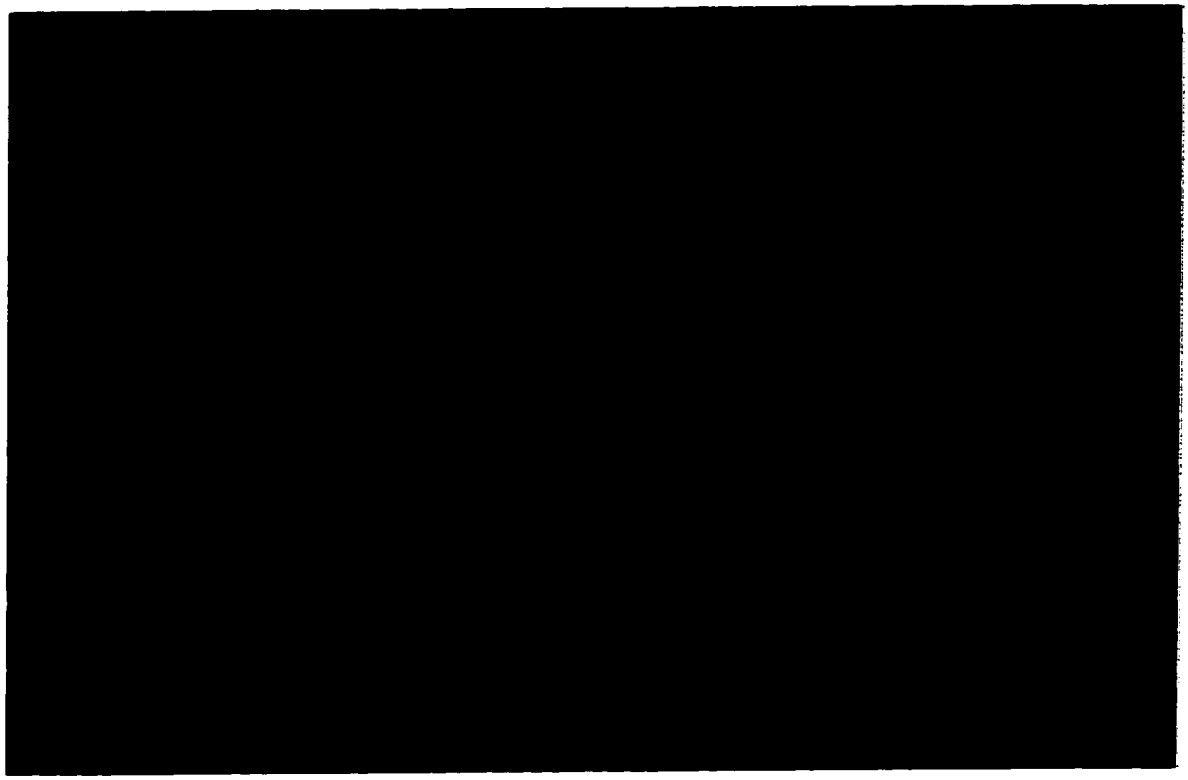


Figure 2.13

Day 3 (Control)

(a)



(b)

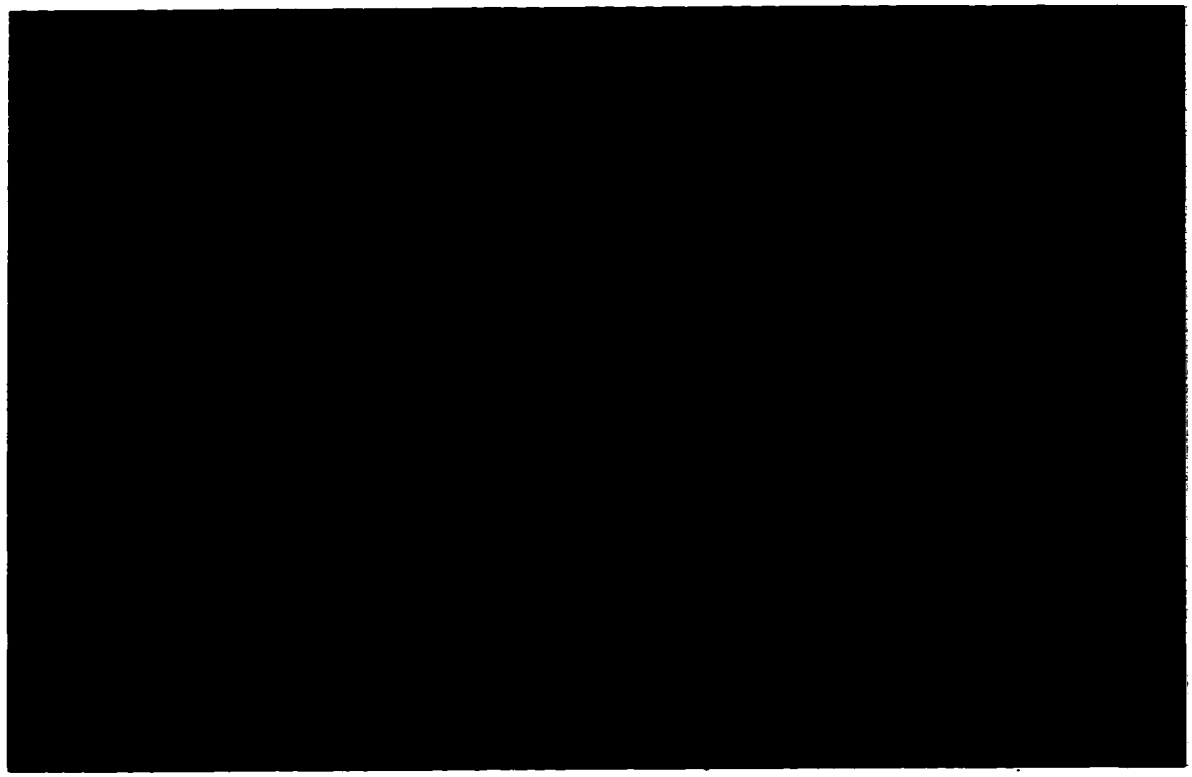
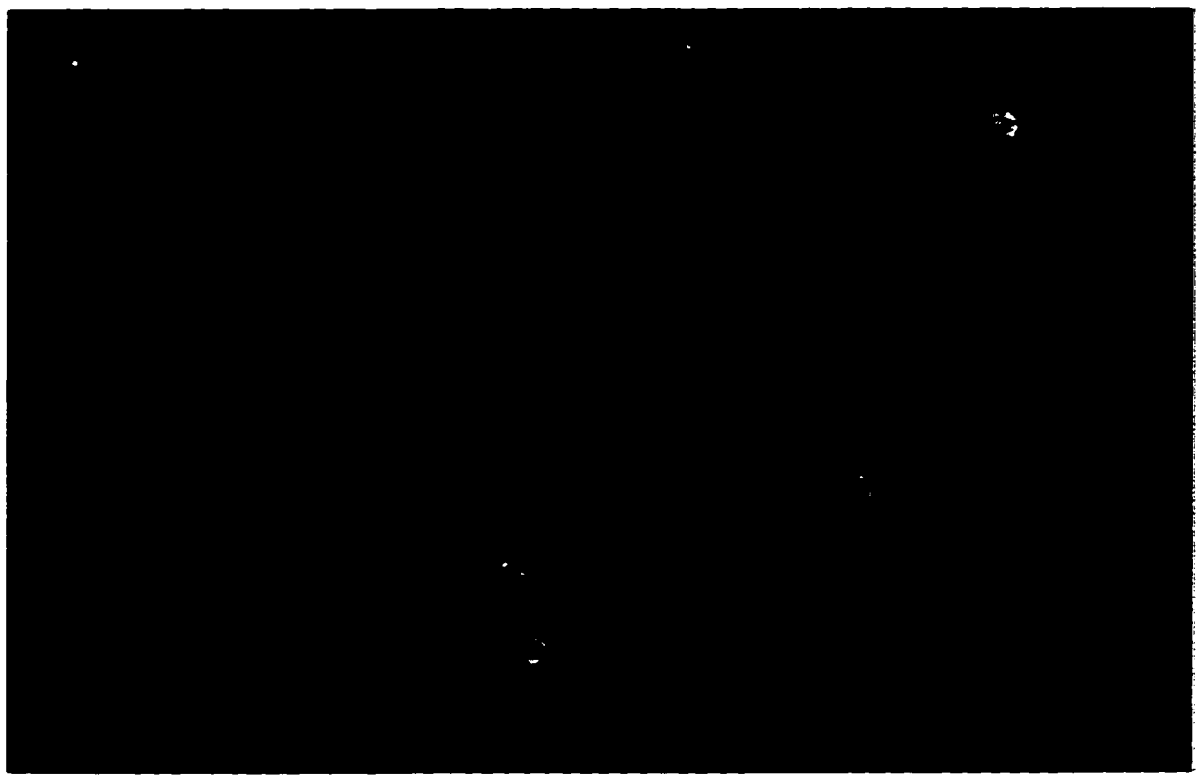


Figure 2.14

Day 5 (Control)

(a)



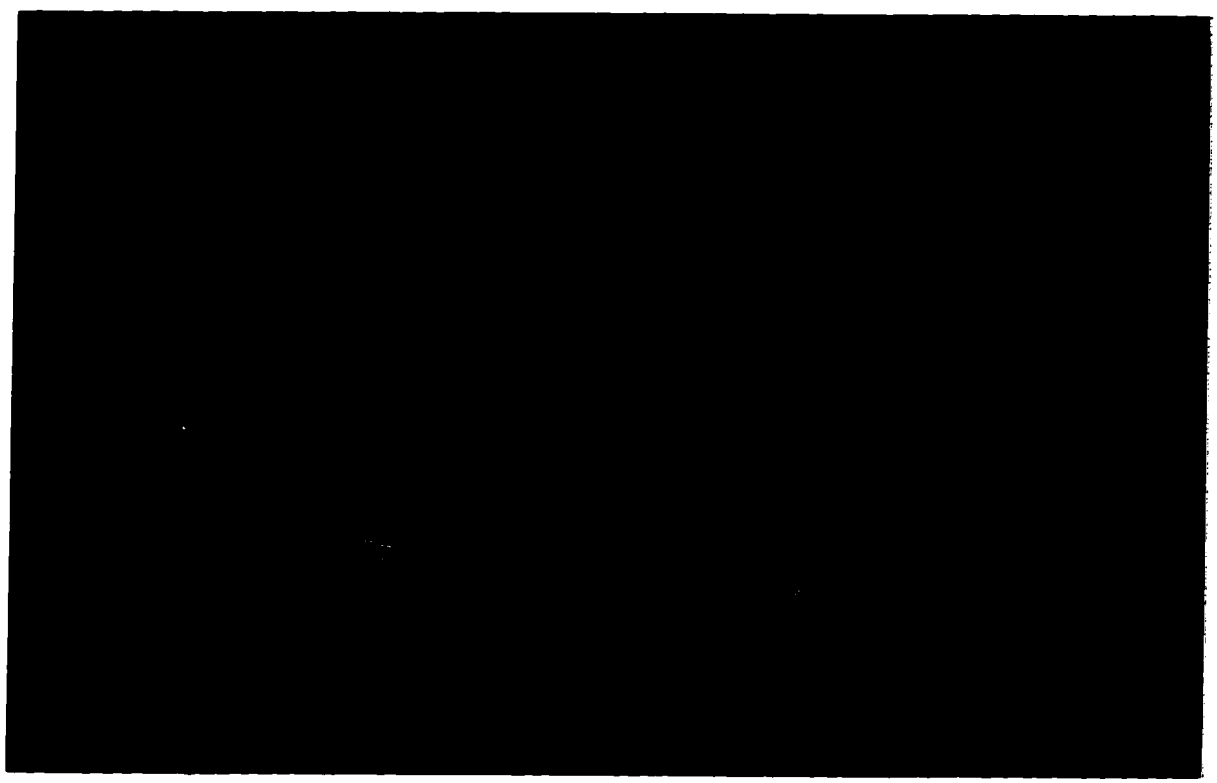
(b)

Figure 2.15

Day 8 (Control)

(a)

(b)



starts to increase (Fig. 2.18a,b). By day 5, the cells reach confluence and more cells with condensed nuclei are detected (Fig. 2.19a,b). After 8 days in the presence of EGF, the cells are confluent by which time there is a further increase in the number of brightly stained condensed nuclei (Fig. 2.20a,b).

2.4.3c DNA Fragmentation Using TUNEL Assay

The *in situ* cell death detection kit is designed to detect and quantitate apoptotic cell death at the single cell level in cells and tissues. This can be done by enzymatic *in situ* labeling of apoptosis induced DNA strand breaks. The tailing reaction using terminal deoxynucleotidyl transferase (TdT) is described as TUNEL (TdT-mediated dUTP nick end labeling) (Gavrieli et al., 1992). Fluorescence microscopy shows that one day after plating in medium containing 10% serum, T51B cells are sparse and no cells with condensed nuclei are detected by Hoechst dye or TUNEL assay (Fig. 2.21a,b) using dual Hoechst and TUNEL assay. There is a difference between Hoechst staining and TUNEL assay. In the case of Hoechst staining, all the nuclei are stained weakly with the dye (Fig. 2.21a) while in the case of TUNEL staining the intact nuclei are not stained and no fluorescence could be seen (Fig. 2.21b). These data indicate that TUNEL assay very precisely labels apoptosis-induced DNA strand breaks only (fragmented DNA). By day 3, (Fig. 2.22a,b) the cells are still proliferating and Hoechst staining shows the presence of weakly stained intact nuclei with some cells in different stages of mitosis. However, no brightly stained condensed nuclei are observed (Fig. 2.22a). On the other hand, TUNEL staining of the same field cells shows the presence of some cells with fragmented DNA which have a bright green fluorescein staining (Fig. 2.22b). However, no intact nuclei are stained in TUNEL assay compared to Hoechst method. Therefore, TUNEL assay is a much more sensitive method to detect early stages of apoptosis

FIGURES 2.16 - 2.20

Nuclear Fragmentation in T51B Cells at Various Times after Addition of EGF using Hoechst Staining

T51B cells were plated at a density of 125 x 10^2 cells/cm^2 and grown on glass coverslips in medium containing 10% serum. At various times after addition of 1 nM EGF cells were washed with PBS and fixed with 4% paraformaldehyde. Cells then washed and stained with Hoechst dye 33258 as described in section 2.3.3. The cells were viewed using an Olympus fluorescence microscope and photographed with Kodak ektachrome 400. The figures show (a) phase contrast photographs of T51B cells; (b) fluorescence photographs of Hoechst staining where fragmented nuclei have bright blue Hoechst staining (arrowed) and normal nuclei have pale Hoechst staining. Magnification: Day 1 - Day 7 is x400. Experiments were performed three times with similar results.

Figure 2.16

Day 1 + EGF (2 days)

(a)

(b)

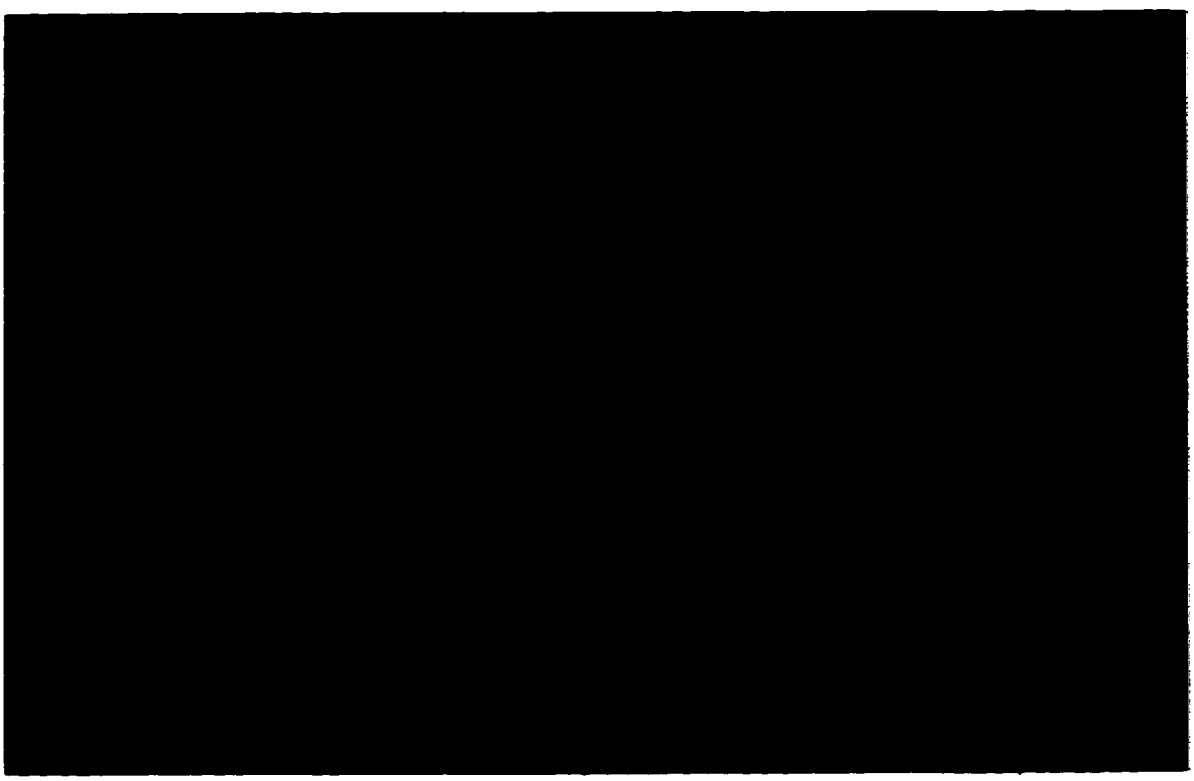
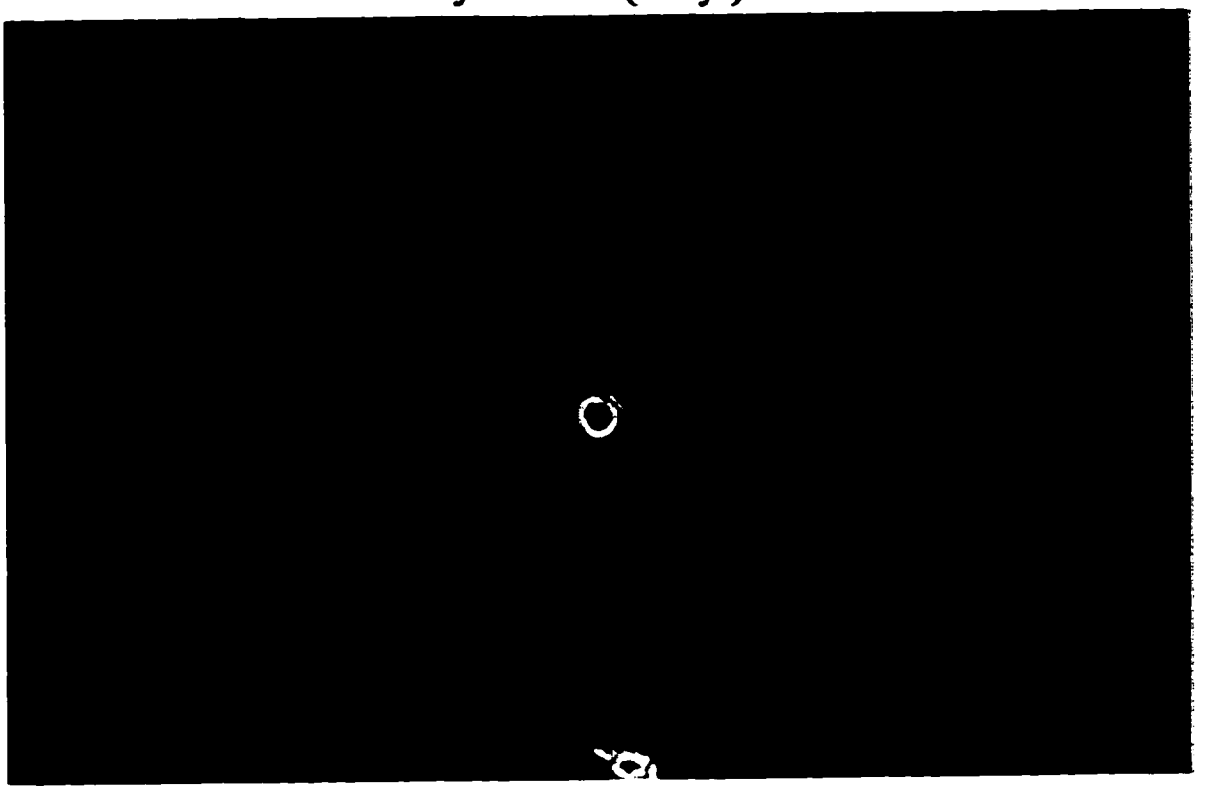


Figure 2.17

Day 2 + EGF (3 days)

(a)



(b)

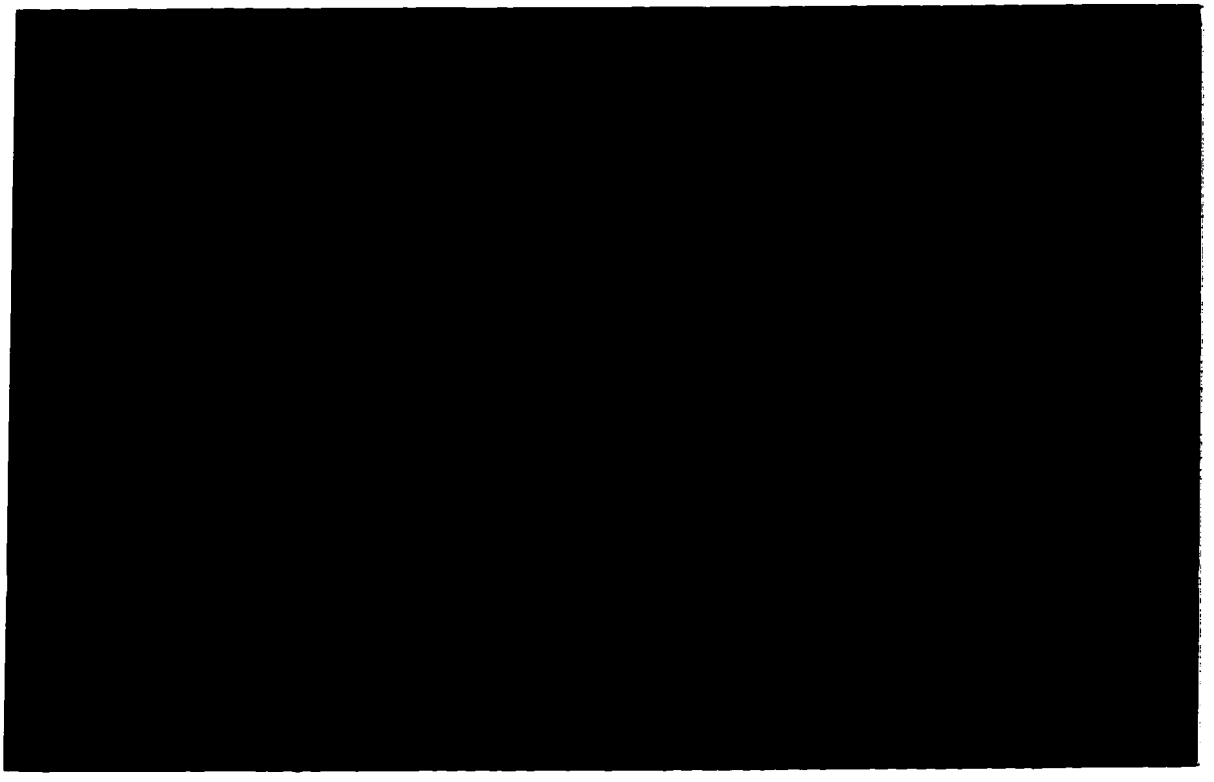


Figure 2.18

Day 4 + EGF (5 days)

(a)

(b)

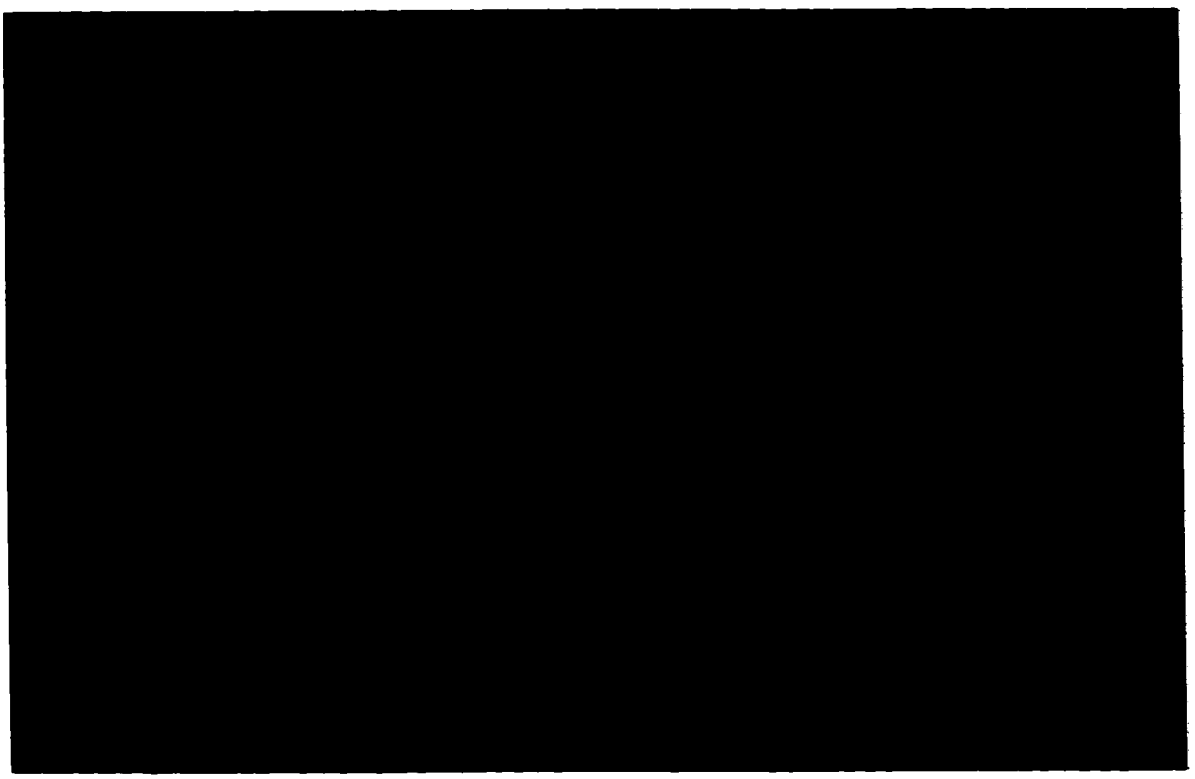
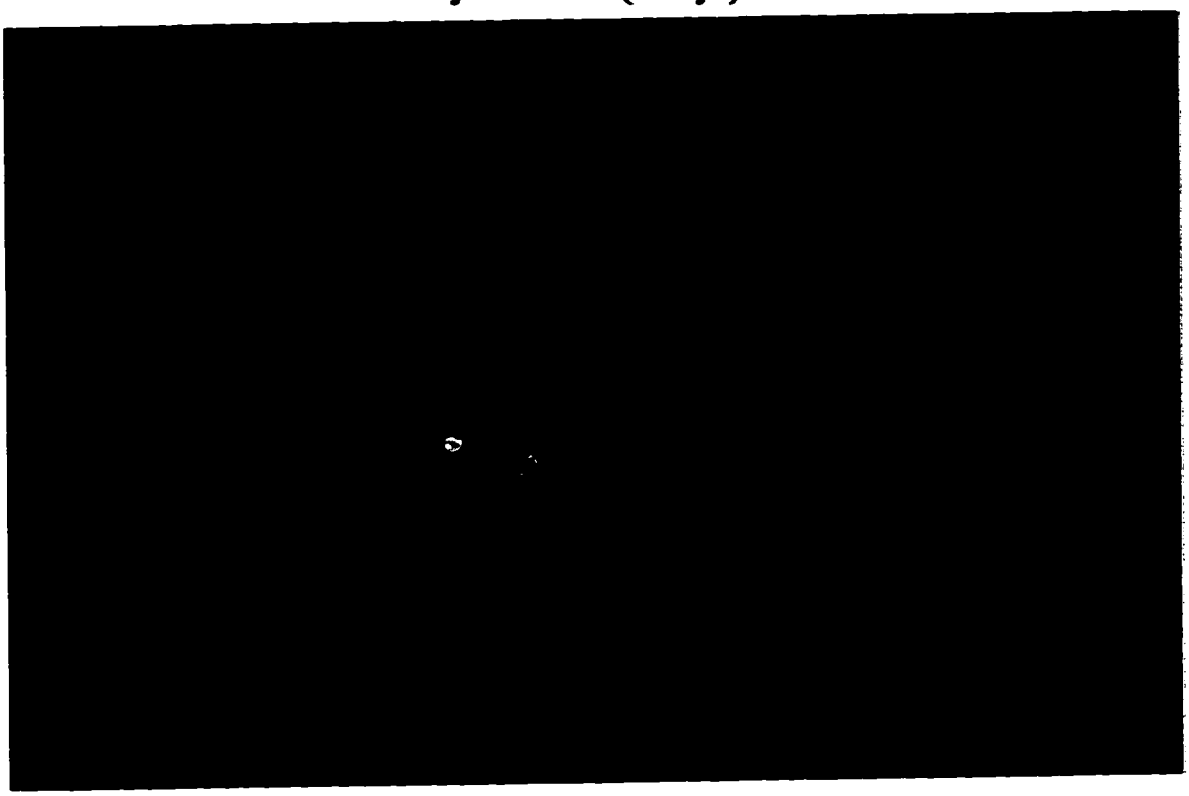


Figure 2.19

Day 5 + EGF (6 days)

(a)



(b)

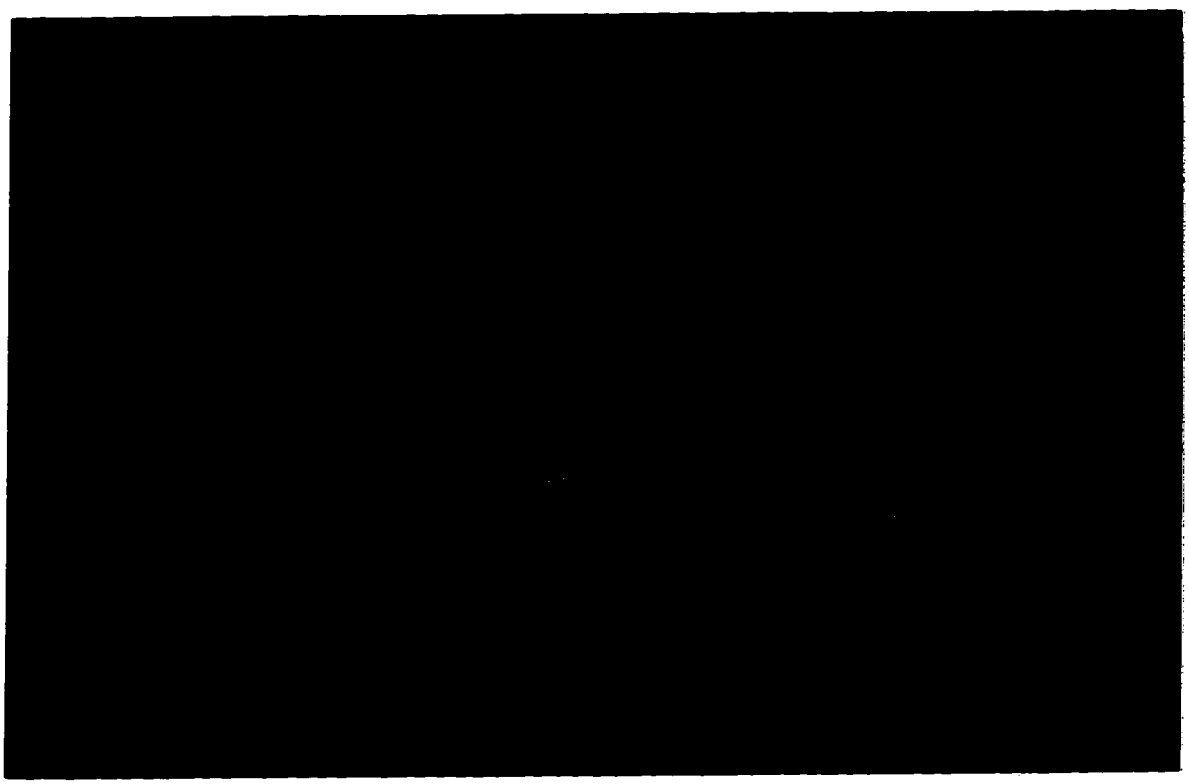
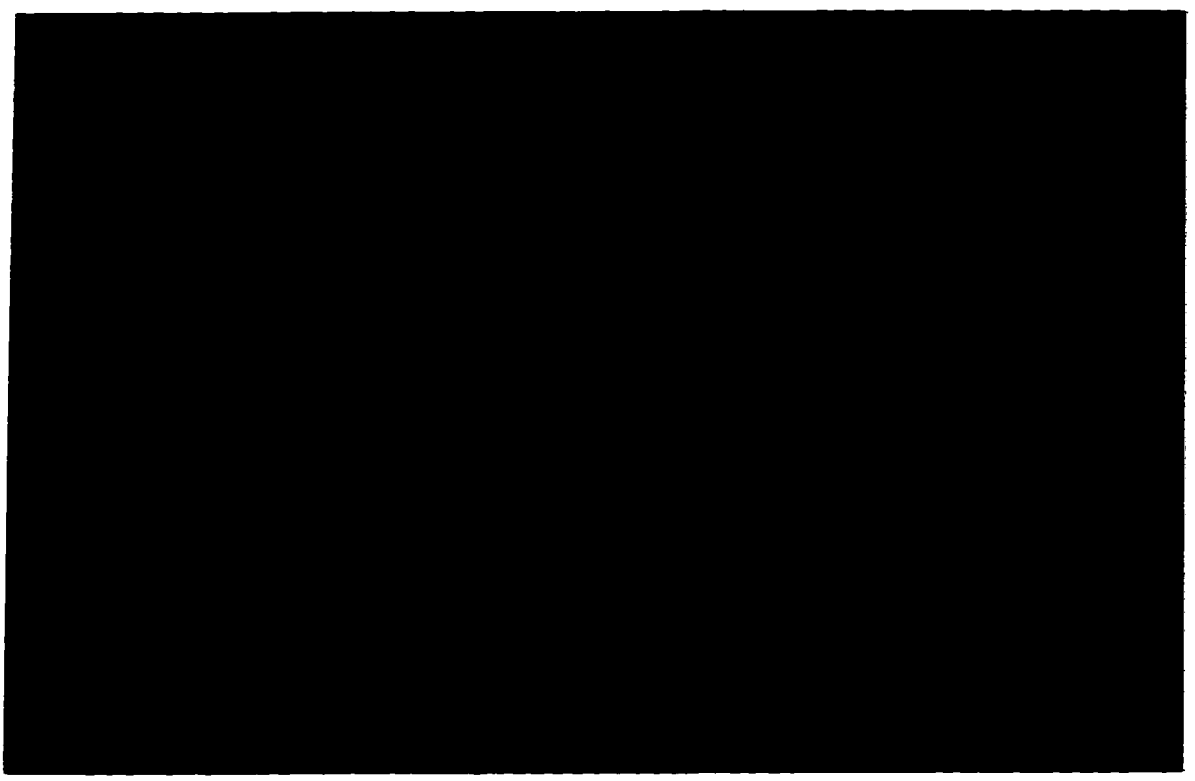


Figure 2.20

Day 7 + EGF (8 days)

(a)

(b)



which the Hoechst staining fails to detect. By day 5, the cells reach confluence and Hoechst staining reveals an increase in the number of cells with brightly stained condensed nuclei (Fig.. 2.23a). However, more cells with fragmented nuclei are detected with TUNEL assay (Fig. 2.23b) compared to that detected with Hoechst dye (Fig. 2.23a). After 8 days, the cells are confluent and Hoechst staining indicates a further increase in the number of apoptotic cells with condensed nuclei (Fig. 2.24a). Again, higher number of cells with fragmented nuclei are detected with TUNEL assay (Fig. 2.24b) compared to Hoechst staining (Fig. 2.24a). Table 2.3 shows the percentage of apoptotic cells at day 4 and day 8 after plating in the presence of 10% serum as determined by TUNEL, Hoechst or flow cytometry. These data indicate that at day 4 the percentage of cells with fragmented nuclei detected by TUNEL assay and flow cytometry is approximately 6% and 8% respectively compared to 2.8% detected by Hoechst dye. After 8 days of plating, the cells are confluent, and at this time the percentage of apoptotic cells increases which reaches about 16% and 21% as determined by TUNEL assay and flow cytometry respectively compared to approximately 8% detected by Hoechst staining. Hence, TUNEL assay is more sensitive than Hoechst staining in detecting cells with fragmented nuclei at different stages of apoptosis. The percentage of cells detected with TUNEL assay is comparable to that obtained by flow cytometric analysis at both day 4 and day 8 after plating. Hence, flow cytometric method is as sensitive as TUNEL assay in detecting apoptosis and is better than Hoechst staining method. In order to test the specificity of TUNEL assay, two controls are included in these experiments. In one type of controls, T51B cells are fixed, permeabilized and incubated with 50 µl/coverslip label solution which contains the nucleotide mixture only without TdT enzyme instead of TUNEL reaction mixture. Phase contrast and fluorescence microscopic data for T51B cells (negative

FIGURES 2.21 - 2.25

Nuclear Fragmentation in T51B Cells at Various Times after Plating in Medium Containing 10% Serum using Dual Hoechst Staining and TUNEL Assay

T51B cells were plated at a density of 125 x 10^2 cells/cm^2 and grown on glass coverslips in medium containing 10% serum. At various time intervals the cells were washed with PBS and fixed with 4% paraformaldehyde. Cells then washed, permeabilized and incubated with TUNEL reaction mixture and then stained with Hoechst dye as described in section 2.3.4. The cells were then viewed using an Olympus fluorescence microscope and photographed with Kodak ektachrome 400. Figures 2.21-2.24 show (a) Hoechst staining of T51B cells where fragmented nuclei have bright blue Hoechst staining (arrowed); (b) TUNEL assay where fragmented nuclei have bright green fluorscein staining (arrowed). The specificity of TUNEL assay was performed by incubating cells with label solution without TUNEL reaction mixture is shown in Figure 2.25 where (a) phase contrast photographs of T51B cells; (b) Hoechst staining; (c) TUNEL assay. Magnification: Figures 2.21 - 2.25a-c is x400. Experiments were performed three times with similar results.

Figure 2.21

Day 1 (Control)

(a)

(b)

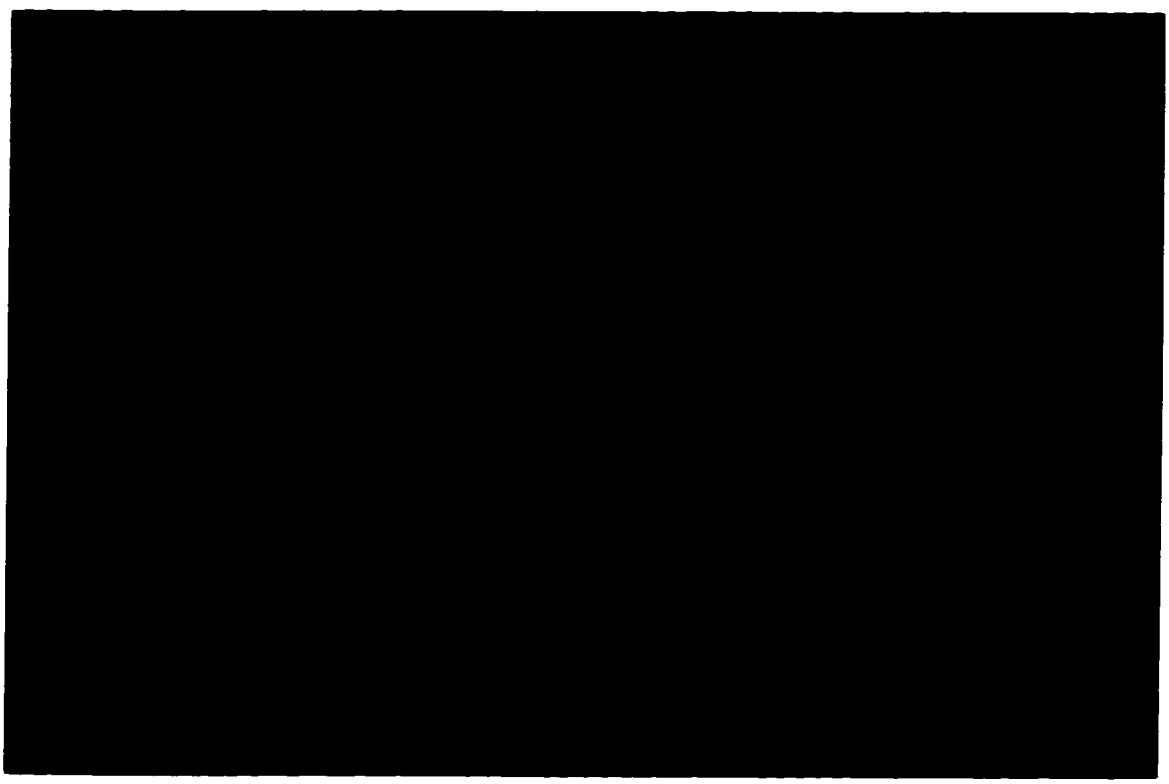


Figure 2.22

Day 3 (Control)

(a)

(b)

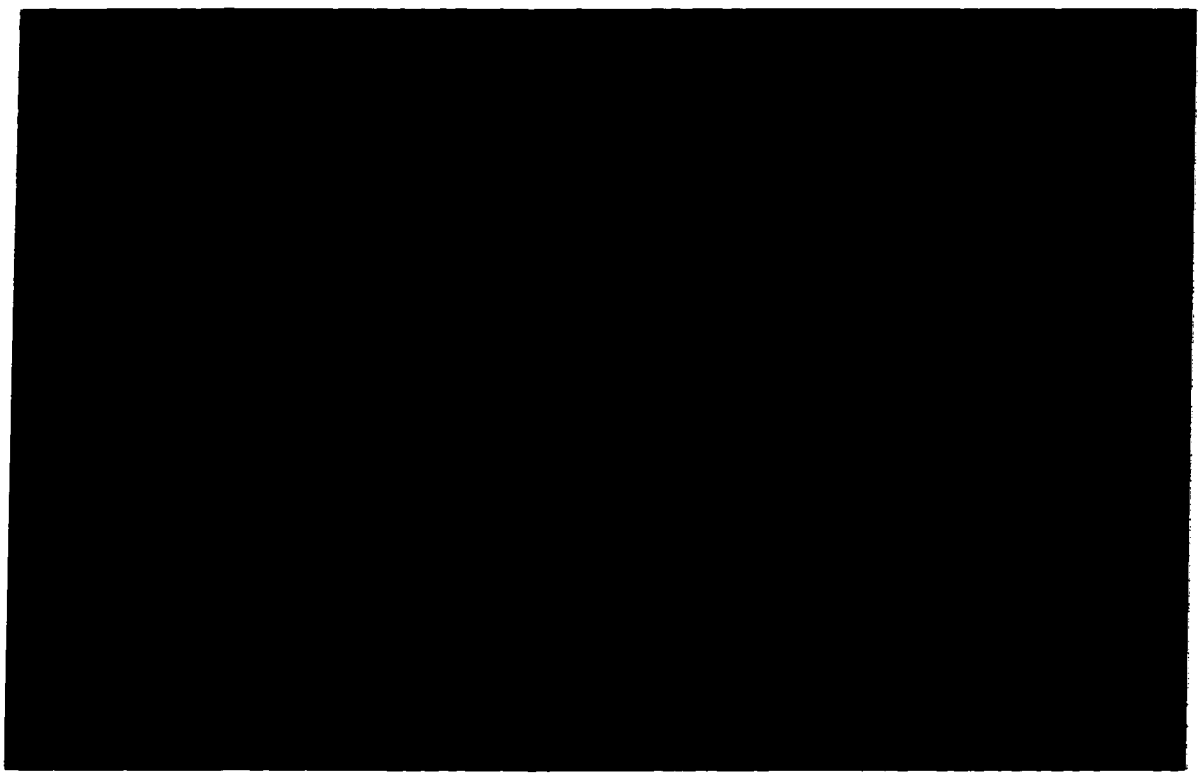
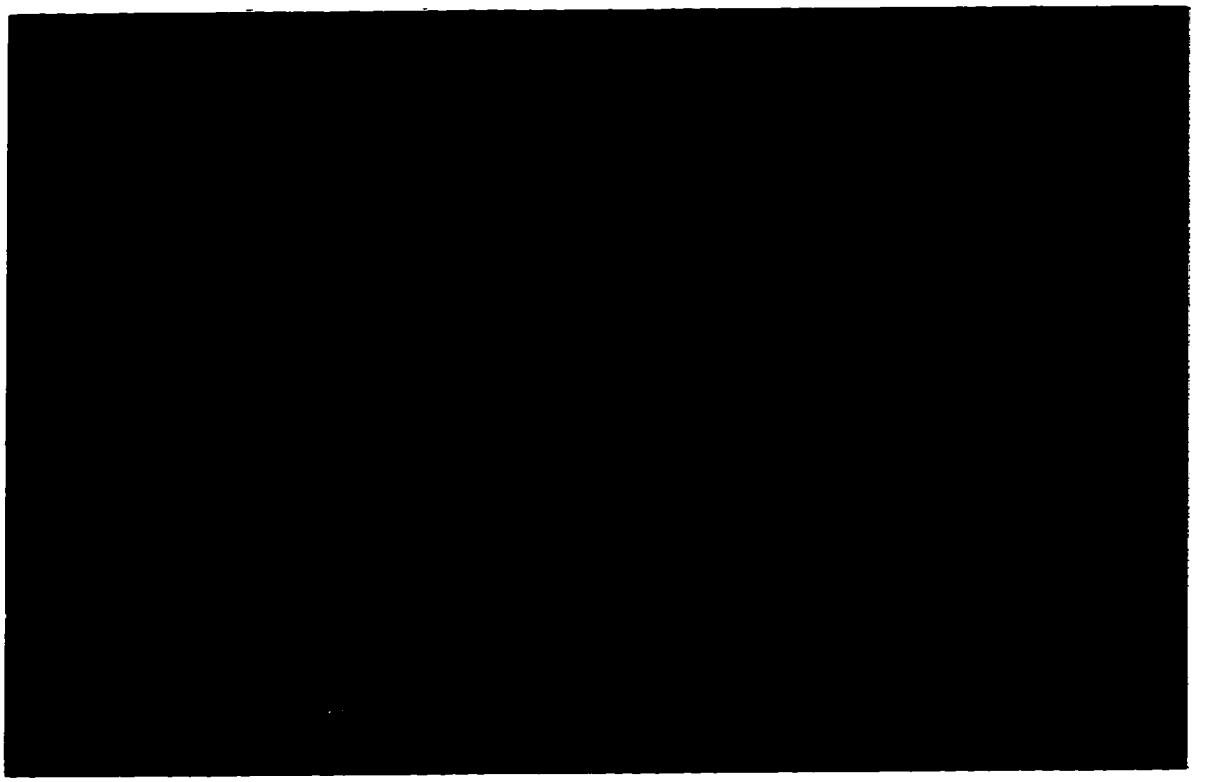


Figure 2.23

Day 5 (Control)

(a)



(b)

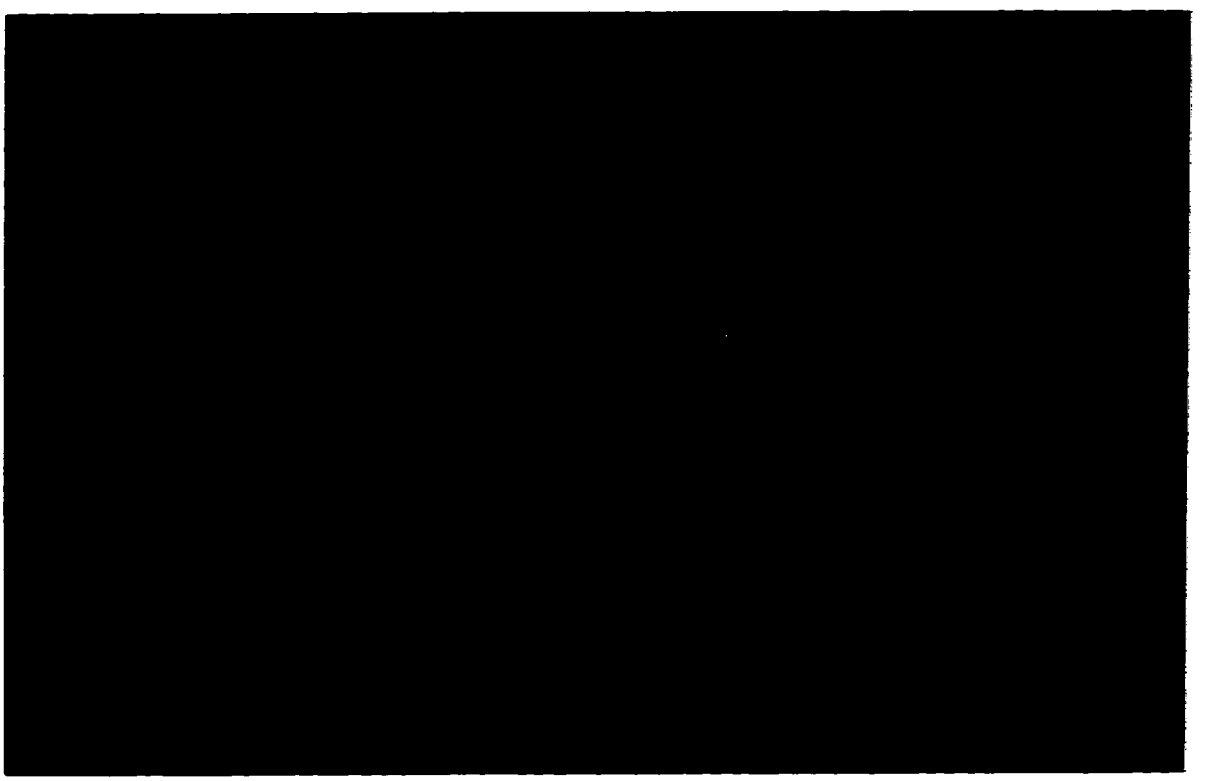


Figure 2.24

Day 8 (Control)

(a)

(b)

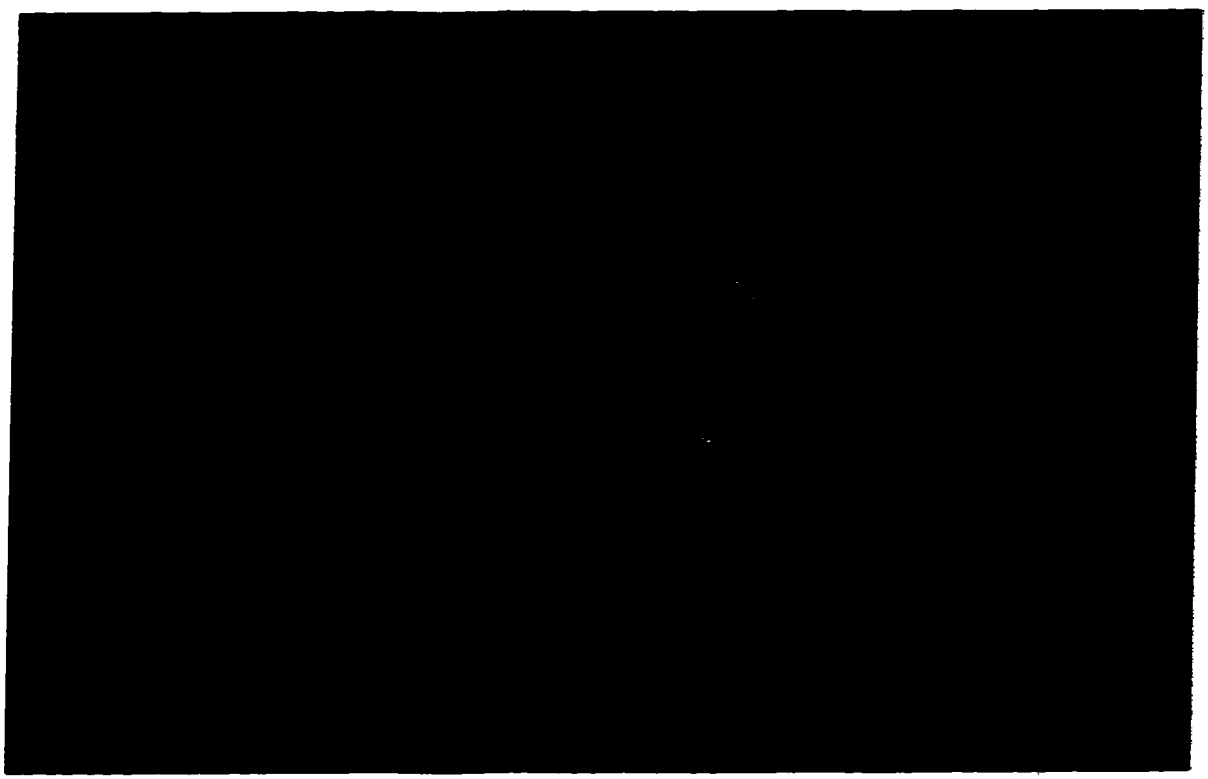
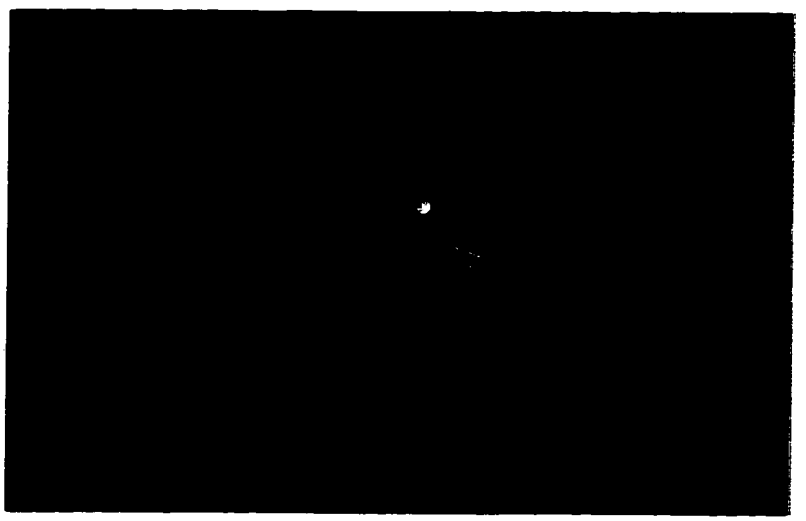


Figure 2.25

Day 4 (T51B cells, negative control)

(a)

(b)



(c)

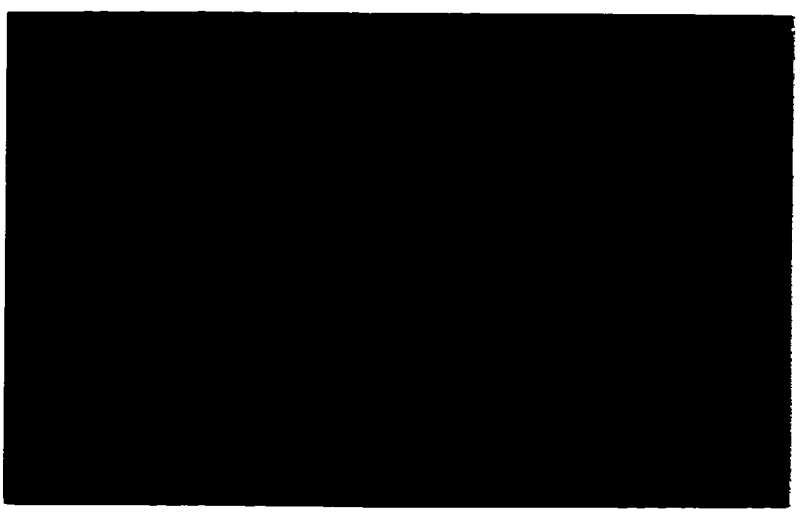


TABLE 2.3

Percentage of Apoptotic Cells at Day 4 and Day 8 after Plating in Medium Containing 10% Serum as Determined by TUNEL Assay, Hoechst Staining, or Flow Cytometry

T51B cells were plated at a density of 125 x 10^2 cells/cm^2. After 4 and 8 days of plating in medium containing 10% serum, the cells were washed with PBS and fixed with 4% paraformaldehyde. They were then washed and prepared for flow cytometry, or dual Hoechst staining and TUNEL assay as described in sections 2.3.2 and 2.3.4 respectively. The cells were then viewed under an Olympus fluorescence microscope. Three fields per dish were counted and the average number of nuclei per field was calculated. Statistical analysis was performed by analysis of variance test (ANOVA). A level of significance of $P < 0.05$ is indicated as * and of $P < 0.01$ is indicated as **.

TABLE 2.3

Percentage of Apoptotic Cells at Day 4 and Day 8 after Plating in Medium Containing 10% Serum as Determined by TUNEL Assay, Hoechst Staining and Flow Cytometry

Method	Day 4		Day 8	
	Mean ± SD	Apop. %	Mean ± SD	Apop. %
TUNEL	156.6 ± 32.0	5.8*	130.3 ± 10.0	16.3**
Hoechst dye	156.6 ± 32.0	2.8*	130.0 ± 10.0	7.8**
Flow-cytometry	61.0 ± 8.6	7.9*	71.2 ± 10.8	21.4**

SD: Standard deviation

control) after 4 days of plating in 10% serum are shown in Figure 2.25a-c. These results show that the fragmented nuclei are easily detected by Hoechst staining (Fig. 2.25b) whereas the label solution (without terminal transferase) fails to detect any fragmented nuclei (Fig. 2.25c). These results clearly indicate the high specificity of TUNEL assay. I also used MDBK cells as negative controls since these are a kidney epithelial cells that do not undergo apoptosis (Walker et al., 1991). Figure 2.26a-c represents phase contrast and fluorescence microscopic photographs of these cells after 4 days of plating in medium containing 10% serum. When the cells are fixed and stained with Hoechst dye, the nuclei appear intact with pale Hoechst fluorescence staining and no cells with brightly stained condensed nuclei could be seen (Fig. 2.26b). TUNEL staining of the same field of cells also shows no cells with fragmented nuclei (Fig. 2.26c) which demonstrates that MDBK cells do not undergo apoptosis under normal growth conditions. These results confirm the specificity of the TUNEL assay in detecting cells with fragmented DNA only.

FIGURE 2.26

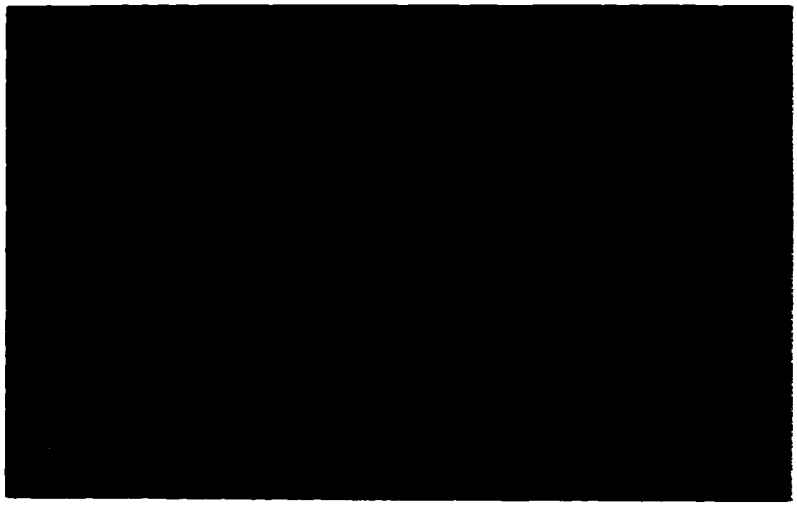
Nuclear Fragmentation in MDBK Cells after 4 Days of Plating in Medium Containing 10% Serum using Dual Hoechst and TUNEL Staining

MDBK cells were plated at a density of 125 x 10^2 cells/cm^2 and grown on glass coverslips. After 4 days of plating in medium containing 10% serum, the cells were washed with PBS and fixed with 4% paraformaldehyde. They were then washed and prepared for dual Hoechst staining and TUNEL assay as described in sections 2.3.4. The cells were then viewed using an Olympus fluorescence microscope and photographed with Kodak ektachrome 400. The figure shows (a) phase contrast of MDBK cells, (b) Hoechst staining and (c) TUNEL assay. Magnification: Figure 2.26a-c is x400. Experiments were performed three times with similar results.

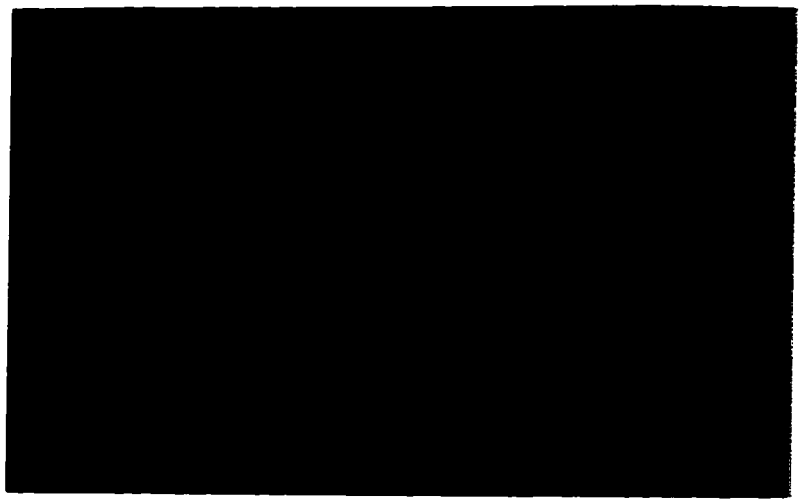
Figure 2.26

Day 4 (MDBK cells, negative control)

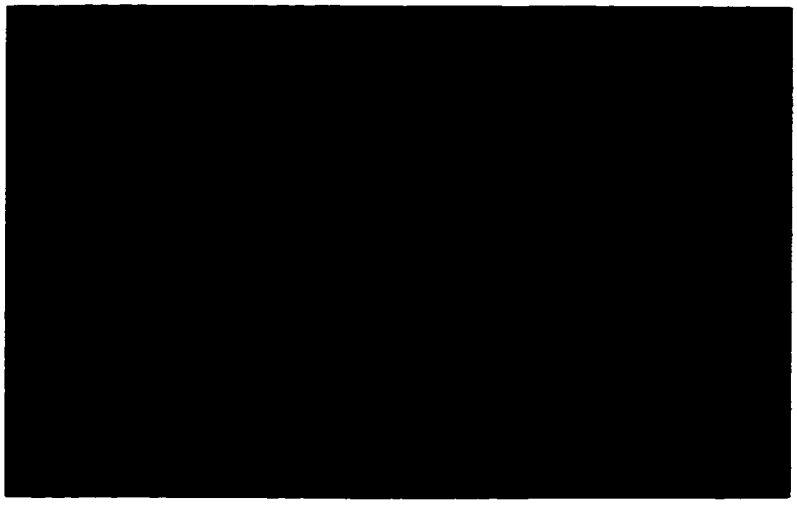
(a)



(b)



(c)



2.5 Discussion

2.5.1 Cell Cycle Kinetics of T51B Cells in the Presence of 10% Serum or EGF

Flow cytometric analysis of cells is widely utilized for evaluating cell death, determining ploidy in tumor samples and measuring cell cycle parameters of cultured cells. This technique appears to be a good choice for the accurate quantification of apoptosis and a methodology has been described to distinguish apoptotic and non-apoptotic cells by means of DNA staining (Nicoletti et al., 1991). All DNA specific dyes allow the identification of apoptosis, irrespective of the chemical interaction between the dye and DNA (Telford et al., 1992). Apoptosis is discerned in flow cytometry by a reduction in cell size and an apparent decrease in DNA content per cell. Flow cytometry has been used to detect apoptosis in thymocytes (Nicoletti et al., 1991; Szondy, 1994), lymphocytes (Illera et al., 1993; Delia et. al., 1995); monocytes (Munn et al., 1995), and various tumor cell lines such as HL-60 cells (Delia et al., 1995; Zamai et al., 1993), promonocytic cell line U937 (Belloc et al., 1994) and HeLa-S3 tumor cell line (Sherwood et al., 1994). These studies have utilized the reduction in cell size and in DNA content of apoptotic cells as criteria to detect the apoptotic cell population. Moreover, most of these cells grow in suspension and they undergo apoptosis in a large percentage upon stimulation with various drugs and the apoptotic population can be easily detected by flow cytometry. A method developd in Dr. Frank's laboratory enabled me to determine the DNA content in each phase of the cell cycle as well as the apoptotic cell population by flow cytometry. These data show that as the cells reach confluence there is an increase in the number of apoptotic cells which reaches about 21% after 8 days of plating, a time point at which the cells are fully confluent. However, even when cells are confluent, a significant population is still proliferating (9% in the S phase at day 8, Figure 2.1-2.6 and

Table 2.1). These results are consistent with Brabyn et al., 1994 who have also observed that when T51B cells reach confluence there is still DNA synthesis in these cultures, yet the cell numbers do not change. It is possible that in T51B cells, growth equilibrium is maintained by a process in which cell division is counter balanced by apoptosis. Hence, cellular homeostasis, i.e. the establishment of confluence in T51B cells appears to involve two processes: (a) growth arrest that occurs presumably as a result of contact inhibition; and (b) removal of "surplus" cells by apoptosis. These results suggest that rat liver T51B cells represent a good *in vitro* model of liver homeostasis because these cells behave similarly to the *in vivo* system. When rat liver is exposed to a mitogenic stimulus such as EGF, there is an initial burst of hyperplasia and an increase in organ weight. Once the stimulus is removed, there is an increase in apoptosis and over time the organ is restored to its original size (Bursch et al., 1984; Columbano et al., 1985). It has also been shown that when primary culture hepatocytes are exposed to mitogenic stimulus they go through the hyperplasia/cell death cycle seen in the *in vivo* systems (Armato et al., 1986). Furthermore, Brabyn et al., 1994 have observed apoptosis in proliferating T51B cells grown in 10% serum. While T51B cells are not hepatocytes, it has been found that during liver regeneration and presumably regression all cell types in the liver are affected (Callea et al. 1991). Since T51B cells have been derived from the bile duct epithelium (Marceau et al., 1986), it seems that these cells can also be induced to undergo the growth pattern observed in the liver *in vivo* and thus, these cells are good *in vitro* model of both liver hyperplasia/regression and liver homeostasis.

The flow cytometric data (Table 2.1) also indicate that as T51B cells progress through the cycle they start to accumulate in G_2 phase. However, once the cells reach confluence the G_2 population starts to decrease with a corresponding increase in the apoptotic cell

population. It is observed that by day 8, the percentage of cells in the G_2 phase declines to 1% while that of the apoptotic cell population reaches to 21%. These results may suggest that the cells enter apoptosis at the G_2/M transition or after the completion of mitosis.

Until recently, it has been assumed that most cells enter apoptosis in the G_1 phase of the cell cycle. However, recent work on different systems shows that cells can enter apoptosis from any point in the cell cycle. Many studies have demonstrated that cells may enter apoptosis at the G_2/M transition. For example, Kruman et al., 1991 have shown that murine T-thymoma BW5147 cells enter apoptosis at the G_2/M transition upon dexamethasone treatment and γ-irradiation. Sorenson et al., 1990 have demonstrated that the anti-cancer drug cisplatin induces apoptosis in L1210/0 cells which has been observed to occur at the G_2/M transition. The same group of investigators (Barry et al., 1990) have tested the effect of cisplatin on Chinese hamster ovary cell line, CHO/AA8 and CHO/UV41. They have indicated that these cells progress to and arrest in the G_2 phase of the cell cycle before dying. In addition, DNA double-strand breaks have been detected following G_2 arrest and prior to loss of membrane integrity. It has been found that synchronized CHO/UV41 cells are damaged by cisplatin in the early S phase. These damaged cells progress to and arrest in the G_2 phase (Demarcq et al., 1994). Upon subsequent dephosphorylation of $P34^{cdc2}$, the cells undergo an aberrant mitosis and subsequently die via apoptosis (Demarcq et al., 1994). Other workers (Willingham and Bhalla, 1994) have shown that treatment of human carcinoma cell lines KB and OVCAR-3 with the anti-cancer drug Taxol (an agent that interferes with the formation of mitotic spindle) causes mitotic arrest and apoptosis after a prolonged incubation period. They have suggested that apoptosis may occur at the G_2/M transition. Furthermore, Davidoff and Mendelow, 1992 have reported that the cytokinetic

effects induced by puromycin include a G_2 arrest, a metaphase-mitotic arrest and apoptosis in leukemia cells, suggesting that these cells enter apoptosis at the G_2/M transition. On the other hand, Darzynkiewicz et al., 1992 have indicated that human leukemic HL-60 cells treated with DNA topoisomerase II inhibitors enter apoptosis in the S phase, the same drugs trigger apoptosis in rat thymocytes when the cells are in the G_0 phase. They have suggested that apoptosis could be selective to different cells at different stages of the cell cycle. Berges et al., 1993 have shown that androgen ablation induces apoptosis in prostatic glandular cells, where more than 95% die in the G_0 phase. El Alaoui et al., 1992 have indicated that metastatic hamster fibrosacroma cells (Met B) enter apoptosis in mid-S-phase under normal growth conditions. Other reports have shown that T cell hybridoma cells exposed to chemotherapeutic agents can enter apoptosis in all phases of cell cycle (Cotter et al., 1992). Sherwood et al., 1994 have demonstrated that treatment of HeLa-S3 tumor cells with colcemid induces apoptosis either in mitosis as a result of terminal mitotic arrest, or in interphase (G_1/S) following aberrant mitosis. Others have found that intestinal crypt cells from irradiated mice enter apoptosis in the G_1 phase (Ijiri and Potten, 1990). Brabyn, 1994 has shown that in T51B cells very few fragmented nuclei have incorporated tritiated thymidine, suggesting that the dying cells could be in G_1 or even G_0 phase. However, in her studies the cells are exposed to tritiated thymidine for 24 h before being fixed and double labeled with tritiated thymidine and Hoechst staining. Thus, it is not clear whether apoptosis is linked to a specific phase of the cell cycle for all the cells. The flow cytometric data in the present study demonstrate an inverse relationship between the percentage of apoptotic cells and the G_2 cell population. As the cells reach confluence the percentage of apoptotic cells increases with a corresponding decrease in the percentage of the G_2 cell population (Table

2.1). These data may suggest that the G_2 cell population are eventually eliminated by apoptosis either directly or after the completion of the cell cycle. It is possible that some of the newly divided cells do not enter the cycle but instead undergo apoptosis by signal(s) that may induce them to do so.

In the second part of the flow cytometric experiments I studied the effect of EGF addition on the cell cycle kinetics of T51B cells. These data demonstrate that the effect of EGF treatment is very similar to serum except that the cells reach a higher density before the induction of apoptosis (Figs. 2.7-2.11 and Table 2.2). Similar results have been obtained by Brabyn et al., 1994 who have indicated that treatment of T51B cells with EGF results in a period of hyperplasia followed by apoptosis. The authors have also shown that EGF-treated cells are more dense and smaller in size than controls. However, they also have detected apoptosis in confluent control cultures. These results parallel *in vivo* systems where rats are given various stimuli that give rise to liver hyperplasia (Bursch et al., 1984; Columbano et al., 1985). After the removal of the hyperplastic stimulus, the livers atrophy through apoptosis back to the original organ weight. Similar effect of EGF has also been described by Armato et al., 1986 in primary cultures of neonatal rat liver cells. However, in other cell types EGF has been found to increase cell survival and ultimately prevents apoptosis. Geier et al., 1994a,b have shown that EGF protects the human breast cancer cell line MDA-231 from apoptosis induced by anti-cancer drugs or cyclohexamide (Geier et al., 1993). Maiese et al., 1993 have reported that EGF rescues hepatoma cells from apoptosis induced by nitric oxide. Others have indicated that EGF protects PC12 cells from cell death induced by etoposide (Nakajima et al., 1994). While EGF has potent growth promoting effects in most cell types (Carpenter and Cohen, 1979), other cells are growth inhibited by EGF. Lagarrigue

et al., 1992 have shown that normal rat liver cells do not increase their growth rate in the presence of 0.7 nM EGF. Other reports have demonstrated that EGF has a growth inhibitory effect on neoplastic cells. For example, high doses of EGF inhibit the growth of A431 epidermoid carcinoma cells (Barnes 1982), human hepatoma Li7A cells (Knowles, 1988), MKN-28 human gastric carcinoma transplanted into nude mice (Murayama et al., 1992) and MDA-MB-486 human breast cancer cells (Filmus et al., 1985). There is generally an inverse relationship between EGF-R number and EGF-induced mitogenesis such that relatively low expression of EGF-R is associated with growth stimulation by EGF, while high EGF-R expression is associated with growth inhibition of most neoplastic cells by EGF (Kawamoto et al., 1984; Filmus et al., 1985). The mechanism of this EGF-induced growth inhibition is not yet clear. Recent studies have shown that EGF not only inhibits growth of MDA-MB-486 cancer cells but also induces apoptosis which is associated with an increase in the expression of c-myc, c-fos, c-jun family members and TGF β1 (Armstrong et al., 1994). Recently, Brabyn and Kleine, 1995 have suggested that the occupancy of the low affinity binding site of the EGF receptor may be responsible for the hyperproliferation seen when T51B cells are grown with high doses of EGF. The authors have hypothesized that apoptosis could be triggered by down-regulation of the receptor in a manner analogous to the removal of a trophic hormone in other systems. In T51B cells, it is the addition of serum or growth factor to the cell culture that results in apoptosis. Most of the other cell systems rely on serum or growth factor removal to induce apoptosis, e.g., EGF removal or serum deprivation of dog thyroid cells (Dremier et al., 1994), granulosa cells (Luciano et al., 1994; Tilly et al., 1992), astrocyte progenitor cells (Yoshida et al., 1993), mature lymphocytes (Lucas et al., 1991), IL-2 withdrawal from CTLL-2 cells (Rodriguez-Tarduchy and Lopez-Rivas, 1989, Lu

et al., 1994), and granulocyte macrophage colony stimulating factor or IL-3 removal from hemopoietic cells (Rajotte et al., 1992).

It is not clear precisely what role EGF is playing in T51B cells. One could speculate that EGF may act as a growth factor for T51B cells, which at a high dose (1 nM) stimulates the cells to grow to double their normal density as observed by Brabyn et al., 1994. However, the cells cannot remain at this density and there is an increase in apoptosis possibly due to the depletion of a serum factor(s) by the treated cells or due to the disruption of cell-cell or cell-extracellular matrix (ECM) interaction. The first possibility is supported by the observation made by Jiang et al., 1993, who have suggested that the spontaneous nucleosomal DNA fragmentation detected in leukemia cell line L1210 grown to high densities is most likely due to the depletion of serum growth factors. Furthermore, Brabyn and Kleine, 1995 suggested that apoptosis of T51B cells could be triggered by down-regulation of EGF-R in a manner analogous to the removal of a trophic hormone in other systems. The second possibility is that EGF may alter the normal contact inhibition. This is supported by previous studies which have indicated that EGF may have a tumor promoting activity since its addition to culture medium overcomes contact inhibition in some cell lines (Rose et al., 1976; Shoyab et al., 1979; Carpenter and Cohen, 1979). Exactly how EGF reduces or removes the normal contact inhibition is not known. Lau et al., 1992 have demonstrated that short-term exposure (30 min) to 1 nM EGF disrupts gap-junctional communication (GJC) in T51B cells. However, the observed disruption does not seem to be long lasting since GJC is re-established within 3 h and thus, it may not affect contact inhibition. Since EGF structural motif occurs in many proteins including some found in ECM (Browne, 1991), it is possible that in T51B cells this growth factor interferes with

normal cell-cell and/or cell-ECM interaction. Once these interactions are removed or reduced, the cells are able to grow over one another. However, the cells cannot remain at high density in the culture dish; therefore, they either float and die via apoptosis or some of the attached cells die by apoptosis. The first possibility is unlikely because: (a) the cells growing in the top layer still maintain strong contacts with cells of the lower layer, since trypsin treatment is required to dislodge them; and (b) apoptosis in the floating T51B cells is very minimal compared to the attached cells as detected by gel electrophoresis of the fragmented DNA (Brabyn et al., 1994). An attractive possibility is that EGF in these cells may disrupt cell-ECM interactions which induce apoptosis in T51B cells to counter balance the increase in cell proliferation induced by this mitogen. This is supported by a number of reports demonstrating that cell-ECM interactions are involved in preventing apoptosis; in fact, disruption of these interactions induces apoptosis in some cells (Meredith et al., 1993; Frisch and Francis, 1994; Montgomery et al., 1994). Future experiments should be directed to explore these possibilities.

2.5.2 DNA Fragmentation

Morphologically, apoptosis is characterized by nuclear and cytoplasmic condensation of single cells (shrinkage) followed by loss of the nuclear membrane, fragmentation of the nuclear chromatin, and subsequent formation of multiple fragments of condensed nuclear material and cytoplasm. These apoptotic bodies are then engulfed by adjacent cells. I applied Hoechst staining method to distinguish between normal and fragmented nuclei. Hoechst staining data demonstrate that there is an increase in the number of condensed nuclei with time as the cells reach confluence in serum containing medium (Figs. 2.12-2.15). A similar effect is obtained with EGF (Figs. 2.16-2.20). However, in the case of EGF, the cells

reach a higher density before the induction of apoptosis. Hence, EGF seems to speed up this process as the cells reach confluence in a shorter time. Thus, Hoechst staining confirms the flow cytometric observations that apoptosis plays an important role in maintaining cellular homeostasis in T51B cells. These results are consistent with previous studies which have used Hoechst staining to distinguish between normal and fragmented nuclei in T51B cells (Brabyn et al., 1994). The authors have observed that fragmented nuclei could be seen both in control cultures and EGF treated cells, and while there is still DNA synthesis in these cultures, yet the cell number does not change. They have also carried out electron microscopic studies to characterize the morphological changes typical of apoptosis. These results together suggest that in T51B cells a growth equilibrium has to be reached, where any cell division is counter balanced by apoptosis.

The earliest biochemical marker taken to be specific for apoptosis is the activation or de novo synthesis of endonuclease(s) (Wyllie, 1980). This enzyme cleaves DNA at linker regions between nucleosomes to form fragments of double stranded DNA. Gel electrophoresis of the fragmented DNA yields the so called "DNA ladders" in some types of cells (Cohen, 1994). However, this technique is not particularly quantitative, so it is difficult to determine the amount of apoptosis occurring in any one sample. Recently, several groups have described the possibility to detect DNA fragmentation *in situ* through end labeling of single- or double-strand DNA breaks using DNA polymerase or terminal deoxyribonucleotidyl transferase (TdT) respectively (Gavrieli et al., 1992; Prabhaker et al., 1995; Portera-Calliau et al., 1995). The latter appears particularly promising because double-strand breaks typically occurs during apoptotic DNA fragmentation. In the present study I carried out direct *in situ* labeling of DNA fragmentation (TUNEL assay) combined with

Hoechst staining (dual staining). Figures 2.21-2.24 clearly demonstrate that the number of condensed nuclei dramatically increases as the cells reach confluence. Interestingly, TUNEL assay is found to be more sensitive than Hoechst staining since at earlier times between 2-3 days, Hoechst staining fails to detect any condensed nuclei while TUNEL assay is able to do so (Fig. 22b,c). By comparing the percentage of cells with condensed nuclei detected by Hoechst staining and TUNEL assay (Table 2.3), it is clear that higher percentage of cells with fragmented nuclei are detected by TUNEL assay than that determined by Hoechst staining method. One explanation for this difference could be attributed to the fact that TUNEL assay is based on end labeling of double-strand DNA breaks that occurs during apoptotic DNA fragmentation which is considered as an earliest biochemical marker for apoptosis (Wyllie, 1980). A combination of TUNEL assay and Hoechst staining does not influence Hoechst staining performance. This has been tested by performing TUNEL assay in the absence of Hoechst staining (data not shown). Hence, TUNEL assay is more efficient and sensitive in detecting early stages of apoptosis. These results are in agreement with Brabyn et al., 1994 who have detected DNA ladders in both T51B control confluent cultures and EGF treated cells. However, they could not determine the amount of apoptosis in a quantitative manner.

In the present study, TUNEL assay enabled me to quantitate apoptotic cell death at single cell level in T51B cells. However, it should be noted at this point that a recent report by Grasl-Kraupp et al., 1995 has indicated that TUNEL assay fails to discriminate among apoptosis, necrosis and autolytic cell death. Apoptosis is a physiological death of mammalian cells. It is distinguishable from necrosis or accidental cell death by unique events including the degradation of chromatin into internucleosomal fragments by the Ca^{2+}-dependent endonuclease and the loss of cellular volume associated with cytoskelctal

breakdown and membrane blebbing. On the other hand, necrosis or accidental cell death, occurs in response to harmful insults such as physical damage, hypoxia, hyperthermia and chemical agents. The earliest morphological changes which occur are swelling of the cytoplasm and the organelles and irregular clumping of chromatin without changes in distribution. Autolysis is induced when tissue sections are kept for more than 3 h at 22°C before fixation which results in cell swelling and the appearance of balloon-like structures. Apoptosis can be discriminated from necrosis and autolysis by the detection of DNA ladders on gel electrophoresis which is not observed in necrosis or autolytic cell death. Necrosis and autolysis yield DNA smears on gel electrophoresis which is probably results from diffuse degradation of DNA (Verms and Hannen, 1994). Therefore, it is always recommended to include negative controls in the TUNEL assay to verify the results. In the present study, two types of negative controls are included in the assay: (1) incubating the cells in the presence of the label solution only which contains the nucleotide mixture without TdT enzyme as recommended by TUNEL manual and; (2) analysing for apoptosis in MDBK kidney epithelial cells by dual Hoechst and TUNEL staining since these cells do not undergo apoptosis (Walker et al., 1991). The results (Figs. 2.25-2.26) show that no apoptotic cells could be detected in these negative controls using dual Hoechst and TUNEL staining. Thus, TUNEL assay under these experimental conditions could be considered as a marker of apoptosis. It has also been established that the form of cell death in T51B cells is via apoptosis as characterized morphologically by electron microscopic studies and the detection of DNA ladders on agarose gel electrophoresis (Brabyn et al., 1994). The percentage of apoptotic cells detected by flow cytometry is higher than that observed by TUNEL and Hoechst staining at both day 4 and day 8 after plating (Table 2.3). Thus, the flow cytometric

method is more sensitive than TUNEL assay in detecting apoptosis and is better than Hoechst staining method. The number of apoptotic cells determined by TUNEL assay and flow cytometry reaches about 16% and 21% respectively after 8 days of plating which is significantly high (Table 2.3). Studies on the duration of the histological stages of apoptosis in liver have suggested that the visible stages could be completed in 3-4 h (Bursch et al., 1990b). Since the overall rate of cellular destruction is very rapid, it is only necessary for 2-3% of the cells to be undergoing apoptosis at any one time to obtain a very substantial regression of the tissue in the order of 25% per day (Bursch et al., 1990a).

2.6 Conclusion

Combining the results from flow cytometric data and fluorescence microscopic studies for both Hoechst staining and TUNEL assay one could conclude the following:

2.6.1 Flow cytometric method and TUNEL assay are more sensitive than Hoechst staining in detecting apoptosis.

2.6.2 There is an increase in apoptosis as cells reach confluence in both serum or EGF-treated T51B cells.

2.6.3 The increase in apoptosis appears to be part of a regulatory mechanism triggered by these cells to counteract the effect of the hyperplasia seen in serum or EGF treated cells.

2.6.4 Hence, apoptosis plays an important role in cellular homeostasis in T51B cells.

CHAPTER THREE

Establishment of Subcellular Localization of Protein Kinase C Isozymes during Cell Cycle Progression of T51B Cells and Effects of TPA and Serum on the Phosphorylation of Nuclear and Membrane Proteins

3.1 Introduction

3.1.1 Protein Kinase C Isozymes and the Cell Cycle

Numerous studies on the regulation of eukaryotic cell cycle have indicated the importance of a variety of protein kinases in controlling the G_0/G_1 (Hunter and Cooper, 1985), G_1/S (Pardee, 1989). and G_2/M transitions (Nurse, 1990) respectively. It has been suggested that PKC is part of the Ca^{2+}-dependent mechanism that controls the proliferation of non-neoplastic cells (Boynton et al., 1985a,b; Boyton et al., 1984). This notion is supported by the fact that several mitogens such as EGF, platelet-derived growth factor (PDGF), bradykinin, serum and vasopressin trigger a cascade of events starting with the activation of membrane phospholipases that catalyze the hydrolysis of phospholipids which releases a Ca^{2+}-transient generator, IP_3 and PKC-stimulating DAG (Berridge, 1989; Nishizuka, 1988; Haller et al., 1994a; Nishizuka, 1995). Evidence has indicated that this enzyme (Isoform not specified) is involved in both the $G_0 \rightarrow G_1$ and $G_1 \rightarrow S$ transitions of the cell cycle (Boynton et al., 1985a). There are two Ca^{2+}-dependent stages in the G_1 phase of serum-stimulated T51B cells in confluent cultures (Boynton et al., 1985a,b). The first of these immediately follows serum addition, lasts 1-2 h and is associated with transition from quiescent G_0 state to the cycling G_1 state, i.e., $G_0 \rightarrow G_1$ transition. The second Ca^{2+}-dependent stage occurs between 8 and 10 h after serum addition and is associated with the $G_1 \rightarrow S$ transition. It has been found that the amount of EDTA-extractable PKC activity increases

transiently during these two Ca^{2+}-dependent $G_0 \rightarrow G_1$ and $G_1 \rightarrow S$ transitions. It has also been observed that a compound such as TPA, that stimulates PKC in an extract from these cells, enables these cells to transit G_1 in Ca^{2+}-deficient medium, while a TPA analogue (4α-phorbol-12-13-didecanoate, 4αPDD) that does not stimulate the enzyme in cell-free preparations does not promote $G_0 \rightarrow G_1$ or $G_1 \rightarrow S$ transit in Ca^{2+}-deficient medium (Boynton et al., 1985a,b). Similarly, TPA by itself has been shown to induce $G_1 \rightarrow S$ transition in Balb/c 3T3 mouse cells (Boynton et al., 1976) that have been arrested in late G_1 by Ca^{2+}-deprivation. Other agents such as dexamethasone, 1,25-dihydroxyvitamin D_3 and saccharin also trigger the $G_1 \rightarrow S$ transition in Ca^{2+}-arrested late G_1 T51B cells and, like TPA, stimulate PKC activity in a cell free preparation (Kleine et al., 1986; Boynton et al., 1985c). These studies together also suggest that a persistence of PKC activity is the basis of the preneoplastic or neoplastic cells' ability to continue proliferating after being transferred to a severely Ca^{2+}-deficient medium. Indeed, non-neoplastic T51B cells have three- to four-fold less EDTA/EGTA extractable PKC activity when grown in Ca^{2+}-deficient medium (0.02 mM) than when they are in medium containing 1.25-1.8 mM Ca^{2+}. However, preneoplastic and neoplastic T51B cells have a normal or even supranormal level of EDTA/EGTA extractable PKC activity when incubated in Ca^{2+}-deficient medium (Boynton et al., 1983). It is interesting to note that treatment of carcinogen (MNNG)-initiated T51B cultures with the tumor promoter saccharin for 21 consecutive days enables a significant number of cells to form colonies in Ca^{2+}-deficient medium. The cells from several of these colonies can be cultured indefinitely in Ca^{2+}-deficient medium where they maintain high levels of EDTA/EGTA extractable PKC activity (Boynton et al., 1985b). Thus, PKC may be one of the normally Ca^{2+}-dependent prereplicative control mechanisms. These reports, however,

have not specified which PKC isozyme is expressed in T51B cells and is involved in G_0/G_1 and G_1/S transitions of the cell cycle.

Recently, PKC inhibitors have been used to investigate the possible role of PKC in the cell cycle. Miyamoto et al., 1993 have studied the effect of H-7 (a non-specific PKC inhibitor) on the proliferation of A65T and TPA-independent revertant A65 IND murine leukemia cells. They have shown that the inhibitor significantly increases the proportion of cells in the G_0/G_1 phase for both cell types, decreases the proportion in the S phase, and does not change the proportion in the G_2/M phase. The authors have suggested that H-7 may block the G_1/S transition by inhibiting PKC. According to their report, PKC-α is the only isozyme expressed in these cells, suggesting a possible role for PKC-α in the G_1/S transition. Abe et al., 1991 have shown that low concentration of staurosporine(a non-selective PKC inhibitor), blocks the early G_1 phase and that high concentration blocks the G_2 phase in rat 3Y1 fibroblasts. They have speculated that the block of the G_2 phase caused by staurosporine may result from the inhibition of $P34^{cdc}$ while the G_1 block may be caused by the inhibition of PKC. It has also been found that sangivamycin (an inhibitor of PKC) can cause growth arrest at the G_1 phase in exponentially growing rat 3Y1 fibroblasts (Usui et al., 1991). These studies, however, have used non-specific PKC inhibitors; therefore, other protein kinases could also have been inhibited which may have resulted in the observed effects.

Molecular genetic approaches have been used to explore the role of the PKC family in cell proliferation by expressing defined enzyme subspecies in mammalian cells. For example, in rat embryo fibroblasts, overexpression of PKC-β_1 has been observed to enhance growth rates and to increase saturation densities as well as anchorage independent growth (Housey et al., 1988; Persons et al., 1988). Such derivatives of rat fibroblasts contain a 20-

30-fold increase in PKC activity. In addition, the cells display alterations in morphology and an increase in the expression of c-myc, ornithine decarboxylase as well as an increase in the susceptibility to transformation by H-ras, myc and fos (Krauss et al., 1989; Hsiao et al., 1989). However, in two rat liver epithelial cell lines, K16 and K22 that stably overexpress PKC-β_I, do not exhibit gross morphological changes, anchorage independent growth or tumorigenicity despite high PKC activity (Hsieh et al., 1989). On the other hand, when PKC-β_I is overexpressed, growth inhibition and tumor suppression in HT29 colon cancer cells have been observed (Choi et al., 1990). The discrepancies in these results could be due to differences in cell types, species and experimental conditions.

When a mutant α- subspecies of PKC, designated UV25 is expressed in NIH 3T3 cells, it appears to cause malignant transformation (Megidish and Mazurek, 1989). However, Borner et al., 1991 have shown that UV25 does not induce such malignant changes in the transfected cells. In several lines of CHO cells that stably overexpress PKC-α, -β_{II}, -ζ and -δ subspecies, TPA treatment has been found to inhibit growth of the cells that express PKC-δ, whereas cells expressing PKC-α, -β_{II} and -ζ are not significantly affected (Watanabe et al., 1992). Moreover, flow cytometric analyses have shown that cell lines overexpressing PKC-δ accumulate in the G_2/M phase in response to TPA. The authors have suggested that PKC-δ may play a role in cell cycle progression (Watanabe et al., 1992). In other studies, probes derived from cDNAs encoding isozymes of rat PKC have been used to screen the genome of the budding yeast S. cerevisiae (Levin et al., 1990). A single gene (PKC-1) has been isolated that encodes a putative protein kinase closely related to α, β and γ subspecies of mammalian PKC. Deletion of PKC-1 has been found to result in recessive lethality and PKC-1-depleted cells are growth arrested. The above study has indicated that PKC-1 is

essential for cell growth and for the G_2 → M transition of the cell division cycle (Levin et al., 1990). Other workers have demonstrated that the specific inhibition of PKC-ζ by its depletion with antisense RNA or oligodeoxynucleotides leads to the blockade of the maturation pathway activated by insulin, ras and PC-PLC in Xenopus Laevis oocytes overexpressing PKC-ζ (Dominguez et al., 1992). In another study, Berra et al., 1993 have indicated that activation of PKC-ζ is not only necessary but also sufficient by itself to activate maturation in oocytes and to produce deregulation of growth control in mouse fibroblasts overexpressing PKC-ζ. Furthermore, by using a dominant kinase defective mutant of PKC-ζ, they have found that this kinase indeed, is required for mitogenic activation in oocytes and fibroblasts.

Several studies have implicated a differential role for PKC isozymes in cellular growth and differentiation. Murray et al., 1993 have indicated that PKC isoforms present in K562 erythroleukemic cells are differentially regulated by PKC-α and -β_{II} expressed in these cells. Increased level of PKC-β_{II} correlates with the cellular proliferative capacity and appears to be necessary for K562 proliferation. In contrast, increased level of PKC-α corresponds to the induction of megakaryocytic differentiation (Murray et al., 1993). An increase in the level of PKC-α , -β and -γ has been shown during the induction of granulocyte differentiation of HL-60 by vitamin A or dimethylsulfoxide (Makowske et al., 1988). Others have indicated that the differentiation of B16 mouse melanoma cells induced by retinoic acid (RA) is preceded by a large increase in PKC-α mRNA and protein level (Gruber et al., 1992). Furthermore, the authors have indicated that overexpression of PKC-α in these cells results in phenotypic changes similar to those induced by RA treatment of control cells, suggesting a key role for PKC-α in RA-induced melanoma differentiation. On

the other hand, hexamethylene bisacetamide-induced differentiation of murine erythroleukemic cells has been shown to result in a decrease in the cellular PKC-β protein level with a corresponding decrease in the kinase activity (Melloni et al., 1989). In the mouse neuroblast cell line, neuro 2a, down-modulation of mRNAs for both PKC-α and PKC-ϵ has been observed in association with *in vitro* differentiation (Wada et al., 1989). These studies together have demonstrated a differential role for PKC isozymes in cell proliferation and/or cell differentiation depending on the cell type. It seems likely that DNA sequences that encode different isozymes of PKC are flanked by different promoter-enhancer sequences that respond to specific transcription factors during the course of differentiation and proliferation.

3.1.2 Nuclear Protein Kinase C and Signal Transduction

Until recently, immunocytochemical analyses have indicated that intracellular localization of PKC appears to vary with cell types, but that the enzyme appears to be absent or poorly represented in the nucleus. During the past few years, researchers from a number of laboratories have demonstrated that PKC is present in the nuclei derived from various sources. It has become evident that PKC has specific functions in the nuclear events activated during signal transduction (Buchner, 1995).

Translocation/activation of PKC isoforms from the cytosol to the nucleus has been observed in a number of cases. In NIH 3T3 and II9C cells, PKC-α translocates to the nucleus upon TPA or α-thrombin treatment (Leach et al., 1992; Eldar et al., 1992). In HL-60 cells, TPA , which induces monocytic-type differentiation, does not induce PKC-α or -β_{II} translocation to the nucleus but rather to the plasma membrane (Hocevar and Fields, 1991). In contrast, bryostatin-1 (a non-tumor promoting agent and a PKC activator) that enhances

proliferation in these cells, induces the translocation of PKC-β_{II} but not the -α isoform to the nucleus (Hocevar and Fields, 1991). In vascular aortic endothelial cells, PKC-α translocates to the nucleus when the cells are stimulated with TPA (Rosales et al., 1992). In Swiss 3T3 cells, treatment with insulin-like growth factor (IGF-1) leads to the translocation of PKC-α to the nucleus (Divecha et al., 1991; Martelli et al., 1991). In cardiac myocytes, PKC-α, -β_{II} and -ζ have been shown to localize in the cytosol of non-stimulated cells and to translocate to the nuclear envelope after stimulation with TPA or norepinephrine (Disatnik et al., 1994b). Moreover, PKC-α and -β (isoform not specified) have been shown to translocate to the nuclei of vascular smooth muscle cells upon stimulation with PDGF and angiotensin II (Haller et al., 1994b).

The nature of the signal directing PKC isoforms to the nucleus is unknown. A bipartite nuclear localization signal (NLS) located in the regulatory domain was suggested by Malviya and Block, 1992. This has not been experimentally confirmed, e.g. by site directed mutagenesis. Several findings argue against the suggested role of the sequence motif in nuclear targetting of PKC. This motif is absent from PKC-α, although the isoform has been found inside the nucleus (Buchner, 1992). This also applies to the nonconventional PKC isoforms, which also do not have the above mentioned sequence motif but, nevertheless, have been found in the nucleus of various tissues and cells (cited in Buchner, 1995). The author also suggested that if PKC isoforms have a NLS, it is likely to be a modified motif located in the hinge region or the catalytic domain. It is also possible that PKC may be directed to the nucleus and to the other intracellular sites by the action of PKC-binding proteins such as annexin I and RACKs (Liu, 1996).

Several studies have reported the localization of PKC isozymes endogenously in the nuclei. Immunohistochemical (Wood et al., 1986) as well as immunochemical (Capitani et al., 1987) studies have indicated the nuclear localization of PKCs. Masmoudi et al., 1989 have demonstrated the presence of PKC in rat liver nuclei. In their report, livers have not been stimulated by any ligand treatment, which reinforces the contention that PKC activity seen in the nuclei is not due to translocation. Using monospecific antibodies, Rogue et al., 1990 have observed that PKC-β is the isozyme predominantly present in rat liver nuclei. In the nuclei of cerebellar cells, PKC-β and γ have been found (Huang, 1988), whereas in the brain as a whole all three types (α, β and γ) are present. However, the above studies have not indicated which subspecies of PKC-β is detected in the nuclei of these cells. Among the calcium-independent isozymes, PKC-ζ, -η and -δ have been shown to be endogenous to the nuclei derived from nerve cells, smooth muscle cells, epidermal A431 cells, and in rat liver during regeneration (reviewed in Buchner, 1995). In cardiac myocytes , PKC-δ and -ϵ have been found to be intra-nuclear in resting cells and, after stimulation with TPA, translocate to the nuclear envelope and to myofibrils, respectively (Disatnik et al., 1994b). Recently, Beckmann et al., 1994 have used confocal laser-scanning microscopy to study the subcellular distribution of PKC isoforms in neuroblastoma x glioma hybrid NG 108-15 cells. They have shown that PKC-δ is localized in the cytoplasm and in the nucleoli, while PKC-ϵ is co-localized with the nuclear-pore complexes at the nuclear envelope.

An important question relates to how the nuclear PKC is activated during signal transduction. Russell, 1989 has suggested that with respect to signal transduction, the nuclear membrane is similar to the plasma membrane in that it has receptors, G-proteins and messenger generating enzymes. GTP binding proteins have been reported to be present in

the nucleus as well as a translocation of the α-subunit of Gi (Takei et al., 1992). An ATP-stimulated calcium transporting system has also been observed in the rat liver nuclei (Nicotera et al., 1989). The phosphatidylinositol (PI) cycle has been suggested to be present in the nucleus of mammalian cells (reviewed in Irvine and Divecha, 1992). For example, Divecha et al., 1991 have shown that when Swiss 3T3 cells are treated with IGF-1, a rapid decrease in the mass of polyphosphoinositol lipids occurs within the nuclei, with a concomitant increase in nuclear DAG and a translocation of PKC to the nuclear region. Both phosphoinositidase PIC-γ (McBride et al., 1991) and -β_1 (Divecha et al., 1993) have been shown to be present in the nucleus. Sources other than phosphatidylinositol hydrolysis as a source of DAG exist (Buchner, 1995), and whether they operate in the nucleus for nuclear DAG generation is not yet known.

The cell nucleus is an essential site for the signal transduction processes. Short-term signals generated at the level of the plasma membrane are propagated towards the nucleus where they elicit long-term adaptation through changes in gene expression. The transcription of AP-1 has been a focus of attention since it has been known that AP-1 controls basal as well as inducible transcription of a number of genes in response to stimulation of cell surface receptors that are linked to the PKC signal transduction pathway (reviewed in Malviya and Block, 1993). Two examples where phosphorylation caused by cytosolic activation of PKC have been shown to regulate nuclear events, are phosphorylation of the transcription factor AP-1 and I-kB (Cantley et al., 1991) by PKC. NF-kB (a transcription factor) moves to the nucleus only when its inhibitor is phosphorylated by cytosolic PKC since in the cytosol, this transcription factor exists as a complex protein with its inhibitor I-kB. When a protein, serum response factor (SRF), is phosphorylated by PKC or other protein kinases such as

MAP kinases, it binds to the serum response element (SRE) in the fos enhancer. This induces the transcription of c-fos. C-fos protein then binds to c-Jun forming Jun/Fos heterodimer protein. The complex so formed binds through a common DNA binding domain to a phorbol ester-response element (TRE), thereby activating transcription of TPA-responsive genes which have a wide spectrum (reviewed in Malviya et al., 1990). Activation of cytosolic PKC also induces dephosphorylation of a critical threonine residue in the c-Jun protein that allows c-Jun to associate with the TRE (Cantley et al., 1991) and thereby activates transcription.

A pertinent question is the importance of PKC being present directly at the level of the nucleus, in contrast to signals transduced by cytoplasmic PKC leading to nuclear events. A role for its nuclear localization is highlighted by the involvement of PKC in the regulation of activity of a number of proteins that are nuclear PKC substrates. Substrates involved in nuclear RNA processing and export are poly(A) polymerase and a 106-kD nuclear pore complex protein (Schröder et al., 1988). Phosphorylation of this latter protein by PKC leads to a decrease in nuclear envelope NTPase activity required for mRNA export from the nucleus (Malviya and Block, 1993). Phosphorylation of lamins regulates disassembly of the nuclear envelope during mitosis (Laskey and Leno, 1990). Although lamins are now known to be PKC substrates *in vitro* and *in vivo* (Hornbeck et al., 1988; Fields et al., 1989; Tsuda and Alexander, 1990), a physiological role of this phosphorylation is unclear. In HL-60 cells, Hocevar et al., 1993 have observed depolymerization and solubilization of nuclear lamins as a consequence of PKC phosphorylation *in vitro*. Recently, Goss et al., 1994 have indicated that PKC-β_{II} translocates to the nucleus during the G_2/M phase concomitant with phosphorylation of lamin B at ser^{405}, thus providing a rationale for PKC as a mitotic lamin

kinase. Histone H1 and histone H3 have been found to be phosphorylated by PKC *in vitro* and *in vivo* (Martelli et al., 1989; Martelli et al., 1990; Mahadevan et al., 1988). Topoisomerase II, a chromatin-bound enzyme, is phosphorylated by PKC *in vivo* as well as *in vitro* with a concomitant rise in enzymatic activity (Sahyoun et al., 1986; Rottmann et al., 1987) and so is the DNA topoisomerase 1 (Samuels and Shimizu, 1992; Pommier et al., 1990). Current knowledge about nuclear events taking place in transcription has indicated that the phosphorylation of nuclear factors (Malviya et al., 1990) is a key regulatory phenomenon mediating inducible gene expression. It is at this stage that nuclear PKC seems to intervene.

Flow cytometric data as well as Hoechst and TUNEL assays in Chapter Two indicate that serum stimulated T51B cells undergo a period of hyperplasia (an increase in cell proliferation) followed by apoptosis as they reach confluence. Thus, the localization of PKC isozymes may change as the cells grow and reach confluence. In this chapter I have an interest in determining the subcellular localization of PKC isozymes in serum stimulated T51B cells in sparse and confluent cultures.

3.2 Objectives

The overall objective of this chapter was to establish the subcellular localization of PKC isozymes in T51B cells as they reach confluence and to study the effects of TPA and serum re-addition on the phosphorylation of nuclear proteins. The specific objectives were:

3.2.1 To determine subcellular localization of PKC isozymes in sparse and confluent T51B cells by immunofluorescence analysis.

3.2.2 To determine subcellular localization of PKC-β_I in dividing cells.

3.2.3 To determine the level of PKC-β_I in the plasma membrane and the nuclear fractions as the cells reach confluence.

3.2.4 To establish the effect of re-addition of 10% serum to quiescent serum-deprived T51B cells on the localization of PKC-β_I by immunofluorescence and immunoblot analysis.

3.2.5 To determine the effect of TPA addition on the localization of PKC-β_I after 1 day of plating by immunofluorescence analysis.

3.2.6 To study the effect of TPA treatment on the localization of PKC-β_I in quiescent serum-deprived T51B cells.

3.2.7 To determine if any nuclear proteins are phosphorylated as a result of TPA treatment or serum re-addition to serum-deprived T51B cells.

3.3 Methods

3.3.1 Cell Culture

T51B cells were cultured in BME medium (basal medium eagle) (Life Technologies, Inc.), supplemented with 10% (v/v) bovine calf serum (BCS) (Life Technologies, Inc.) and 0.1% gentamycine sulfate (Sigma Chemical Co., Ltd.). The cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Prior to experiments, cells were detached by brief exposure to 0.05% (w/v) trypsin- 1 mM EDTA in PBS. The cells were plated at a density of 125 x 10^2 cells/cm^2. For TPA or serum re-addition experiments confluent cells were serum deprived by changing the medium to BME medium containing 0.2% BCS and incubated for 48 h before addition of 100 nM TPA or medium containing 10% serum.

3.3.2 Subcellular Fractions

Cells were washed twice with cold PBS, scraped, pelleted and lysed in hypotonic buffer (1 mM $NaHCO_3$, 5 mM $MgCl_2$, 100 µM PMSF and 20 µg/ml leupeptin) and made to 50 mM Tris-HCl and 0.5 mM EGTA, pH 7.5. The total cell homogenate was centrifuged (500 x g, 5 min, 4°C) to obtain crude nuclear pellet and post-nuclear fraction (PNF). The PNF was centrifuged (100,000 x g, 60 min, 4°C) to give the cytosolic supernatant and a plasma membrane/microsomal pellet that was sonicated briefly in buffer A (50 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 1.0% (v/v) β-mercaptoethanol, 1 mM PMSF and 20 µg/ml leupeptin). The nuclear membrane was prepared according to Simboli-Campbell et al., 1994. Briefly, the nuclear pellet was washed three times with hypotonic lysis buffer, layered over 45% (w/v) sucrose, centrifuged (1660 x g, 30 min, 4°C), resuspended and sonicated in buffer A. Lamin B (a nuclear envelope protein) was used as

a nuclear marker (Franke, 1987). Protein content of subcellular fractions was determined by the Bradford method (Bradford, 1976).

3.3.3 Immunoblotting of PKC-β_I Isozyme

Samples were solubilized in SDS sample buffer (0.01% bromophenol blue, 5% glycerol, 1% SDS and 1.25% β-mercaptoethanol). 10 µg (nuclear membrane) or 50 µg (plasma/microsomal membrane) of protein/lane was electrophoresed on 10% SDS PAGE (Laemmli, 1970). Immunoblotting of PKC-β_I was performed as described in section 1.3.3. For lamin B the primary antibody used was a mouse monoclonal antibody (NCL-LAM-B, Dimension Laboratories Inc., Mississauga, Ont.) diluted 1:200 with blocking solution.

3.3.4 Immunofluorescence of Protein Kinase C Isozymes

Immunofluorescence of PKC isozymes was performed as described in section 1.3.4.

3.3.5 Dual Immunofluorescence and Hoechst Staining of PKC-β_I Isozyme

Cells were plated at a density of 125 x 10^2 cells/cm^2 and grown on glass coverslips in medium containing 10% serum. Coverslips were washed twice with PBS and fixed for 30 min in 3.5% formaldehyde in PBS at room temperature. The coverslips were then rinsed three times in PBS. They were then sequentially incubated at 4°C for 3 min in cold 1:1 (v/v) acetone/water mixture, for 5 min in cold acetone, for 3 min in a 1:1 (v/v) acetone/water mixture, and finally for 3 min in PBS. The coverslips were then rinsed with PBS and blocked overnight at 4°C in 0.5% (w/v) PBS/skim milk. They were then washed with PBS and incubated with 10 µg/ml anti-PKC-β_I primary antibody (rabbit) (Life Technologies Inc.) overnight at 4°C in a humid box. After rinsing with PBS, the coverslips were incubated with FITC-conjugated secondary antibody (goat anti-rabbit, Jackson Immunoresearch Laboratories Inc.) for 60 min. Coverslips were then washed twice with PBS and then incubated in 1

μg/ml Hoechst dye, fluorochrome 33258 (Sigma Chemical Co., Ltd.) in PBS for 20 min. They were then rinsed with PBS and mounted with glycerol containing 0.1% (w/v) p-phenylenediamine in PBS, viewed with an Olympus fluorescence microscope equipped with a special filter cube for dual fluorescence (UV/FITC) and photographed with Kodak ektachrome 400 film.

3.3.6 [^{32}P] Orthophosphate Labeling

Confluent T51B cells were serum deprived by fluid change to BME medium containing 0.2% BCS for 48 h. The cells were then washed two times with phosphate free BME medium and fluid changed to phosphate free BME. 25 μCi/ml ^{32}P orthophosphate was added to each culture dish and incubated for 3 h at 37°C. A single addition of 100 nM TPA was added and the cells were harvested at appropriate times (5, 10, 30 min and 24 h). Alternatively, the cells were fluid changed to medium containing 10% serum and incubated for the same indicated times. The cells were then washed twice with cold PBS, scraped, lysed in hypotonic buffer (1 mM $NaHCO_3$, 5 mM $MgCl_2$ 100 μM PMSF and 20 μg/ml leupeptin) and made to 50 mM Tris-HCl and 0.5 mM EGTA, pH 7.5 The subcellular fractions (nuclear, membrane and cytosol) were prepared as described in section 3.3.2. Nuclear samples were solubilized in SDS sample buffer (0.01% bromophenol blue, 0.05% glycerol, 1% SDS and 1.2% β-mercaptoethanol). 20 μg of protein/lane was electrophoresed on 10% SDS-PAGE (Laemmli, 1970). Gels were stained for 30 min with Coomassie blue solution (0.1% Coomassie R-250 in 40% methanol, 10% acetic acid) (BioRad Laboratories, Hercules, CA, USA) and then destained (40% methanol and 10% acetic acid) for 1-2 h. The destained gels were soaked in 5% (v/v) glycerol solution for 1.5 h. Gels were dried and

placed with an X-ray film (Kodak XAR-5, Rochester, NY) in the dark for 5-6 h at -70°C and autoradiographed.

3.4 Results

3.4.1 Subcellular Localization of Protein Kinase C Isozymes in Sparse and Confluent T51B Cells

3.4.1a Immunofluorescence of PKC Isozymes as the Cells Reach Confluence

Immunofluorescence localization indicate that after one day of plating, PKC-β_I is localized in the nucleus with the majority of the fluorescence associated with the nuclear membrane. No immunofluorescence is observed in the plasma membrane or the cytoplasm (Fig. 3.1b). A phase contrast photograph of the same cells is shown in Figure 3.1a. By day 3, the number of cells increases, though they are not yet confluent as examined under phase contrast microscopy (Fig. 3.2a). Fluorescence microscopy reveals the localization of PKC-β_I in the nucleus and the nuclear membrane but now PKC-β_I appears in the cytoplasm and the plasma membrane where punctate fluorescence could be seen in certain areas along the cell boundaries (Fig. 3.2b). By day 5, the cells reach confluence as examined under phase contrast microscopy (Fig. 3.3a). At this time, the immunofluorescence localization indicates an increase in the immunostaining of PKC-β_I in the plasma membrane, where intense punctate fluorescent staining is seen at cell-cell contacts forming distinct bright lines (Fig. 3.3b). Also there is an increase in the immunofluorescence of PKC-β_I in the cytoplasm. By day 8, the cells are confluent as seen under phase contrast microscopy (Fig. 3.4a). At this time, the immunofluorescence of PKC-β_I seems to decrease in the nucleus compared to day 1 and appears as diffused staining (Fig. 3.4b). In the plasma membrane, the punctate fluorescence staining appears to be intensified in certain areas along the cell boundaries between touching cells. However, no change is observed for the immunofluorescence staining in the cytoplasm. These results indicate that there is a change in the intracellular localization of PKC-β_I as the cells approach confluence which has not been reported in other

cell types. Similar immunofluorescence analyses are performed for PKC-α, -δ, -ϵ and -ζ at various times (1-8 days) after plating in medium containing 10% serum, however, only day 2 and day 5 are shown since no significant changes are observed. After 2 days of plating, PKC-α is observed to be associated with the plasma membrane, the cytoplasm and in the nucleus where the immunostaining appears as punctate fluorescence but no fluorescence is seen associated with the nuclear membrane (Fig. 3.5a). The nuclear association of PKC-α, however, is not highly specific as tested by the inclusion of the antigenic peptide (Chapter One, section 1.4.1b). After 5 days of plating, the cells reach confluence by which time there is only a slight increase in the immunofluorescence of PKC-α especially in the plasma membrane (Fig. 3.5b). Similar results are obtained for PKC-δ and -ϵ, where there is only a little change in the immunofluorescence of these isozymes after 5 days of plating (Fig. 3.6b-3.7b) compared to 2 days (Fig. 3.6a-3.7a). Immunofluorescence results show that after 2 days of plating, high level of PKC-ζ is seen in the nucleus and the cytoplasm with very weak immunofluorescence associated with plasma membrane (Fig. 3.8a). However, the membrane-associated level of PKC-ζ increases by day 5 as the cells approach confluence (Fig. 3.8b). These results indicate that while there is a slight change in the immunofluorescence of PKC-α, -δ and -ϵ, the level of PKC-ζ increases in the plasma membrane as the cells approach confluence. On the other hand, PKC-β_I appears to be associated only with the nuclei and the nuclear membranes in sparse (1-2 days) cultures, and then partially moves to the cytoplasm and the plasma membranes as the cells reach confluence (Figs. 3.1 - 3.4).

Figures 3.1 - 3.4

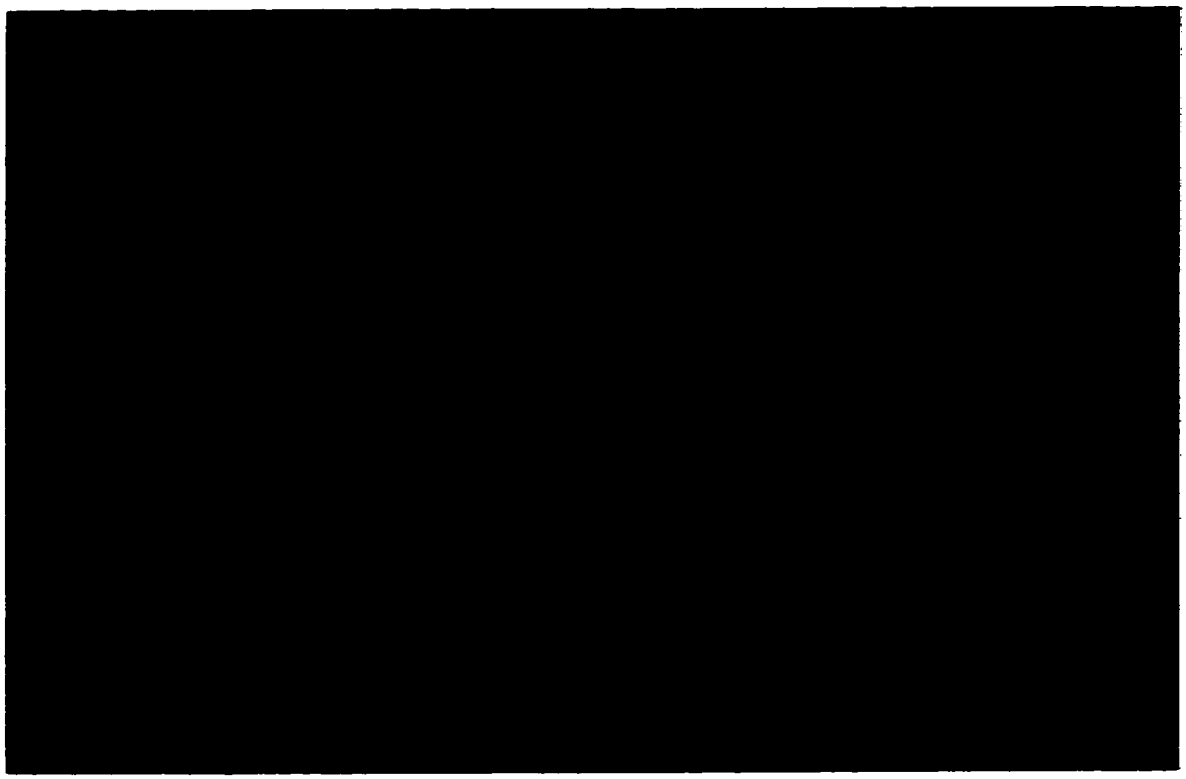
Immunofluorescence of PKC-β_I as T51B Cells Grow and Reach Confluence

T51B cells were plated at a density of 125 x 10^2 cells/cm^2 and grown on glass coverslips in medium containing 10% serum. At various times, the cells were fixed and incubated with PKC-β_I specific antibody. Antigen-antibody complexes were detected with fluorescein-conjugated secondary antibody as described in 1.3.4. Fixed cells at day 1; day 3; day 5; day 8 after plating in 10% serum are shown in Figures 3.1-3.4. In these figures (a) shows phase contrast photographs and (b) shows the corresponding immunofluorescence photographs. Magnification: Figures 3.1-3.4a,b is x1000. Immunostaining was performed three times with similar results.

Figure 3.1

Day 1 (PKC- β_I)

(a)



(b)

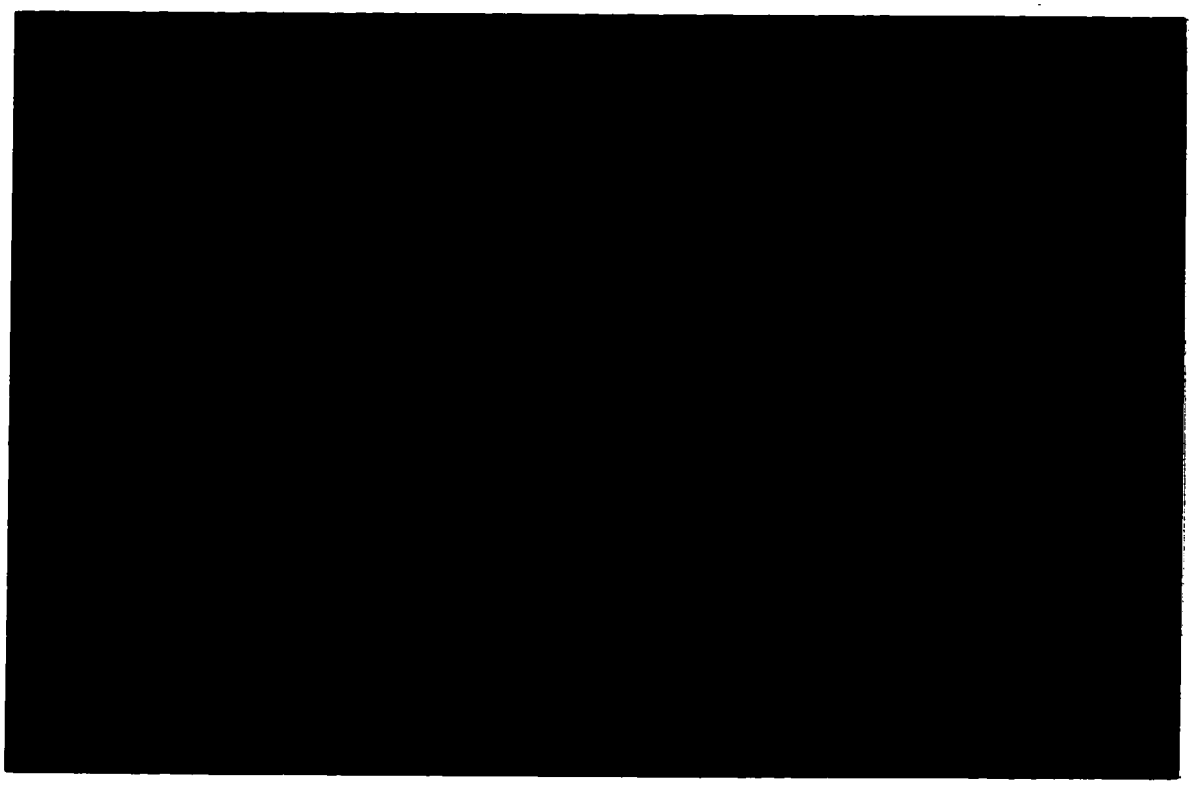
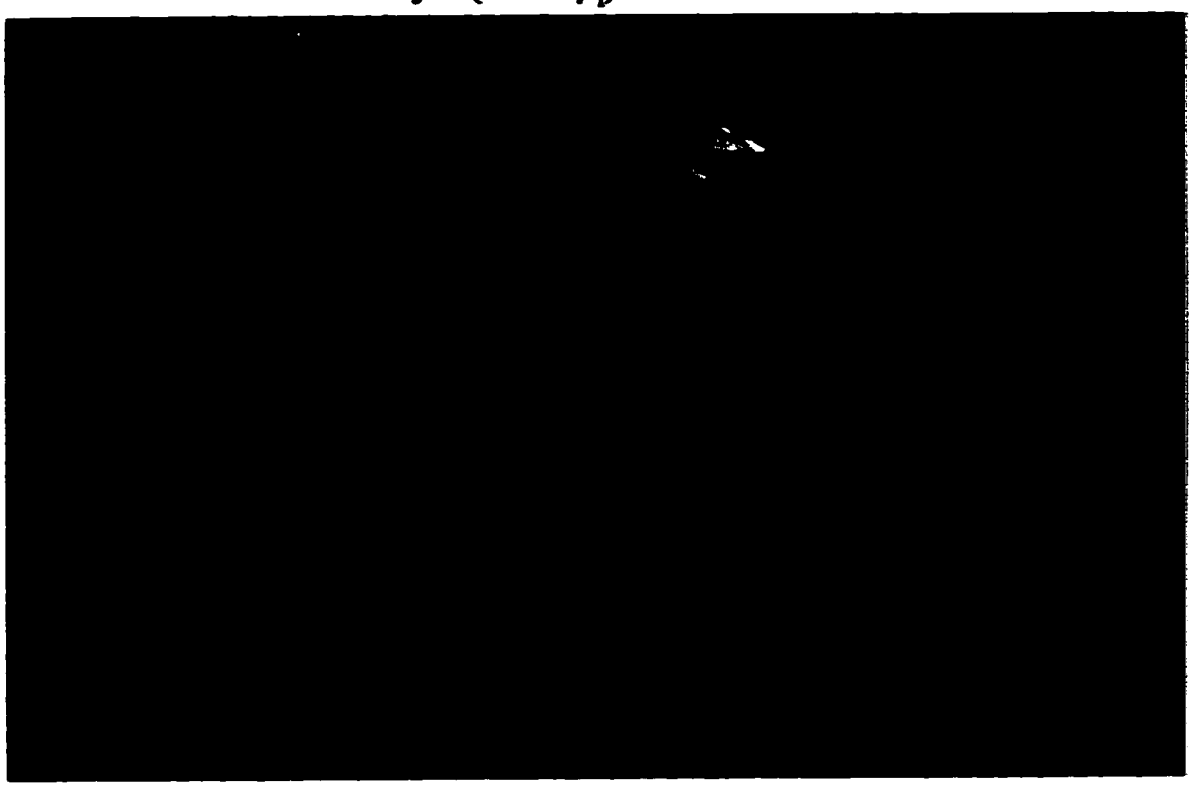


Figure 3.2

Day 3 (PKC- β_I)

(a)

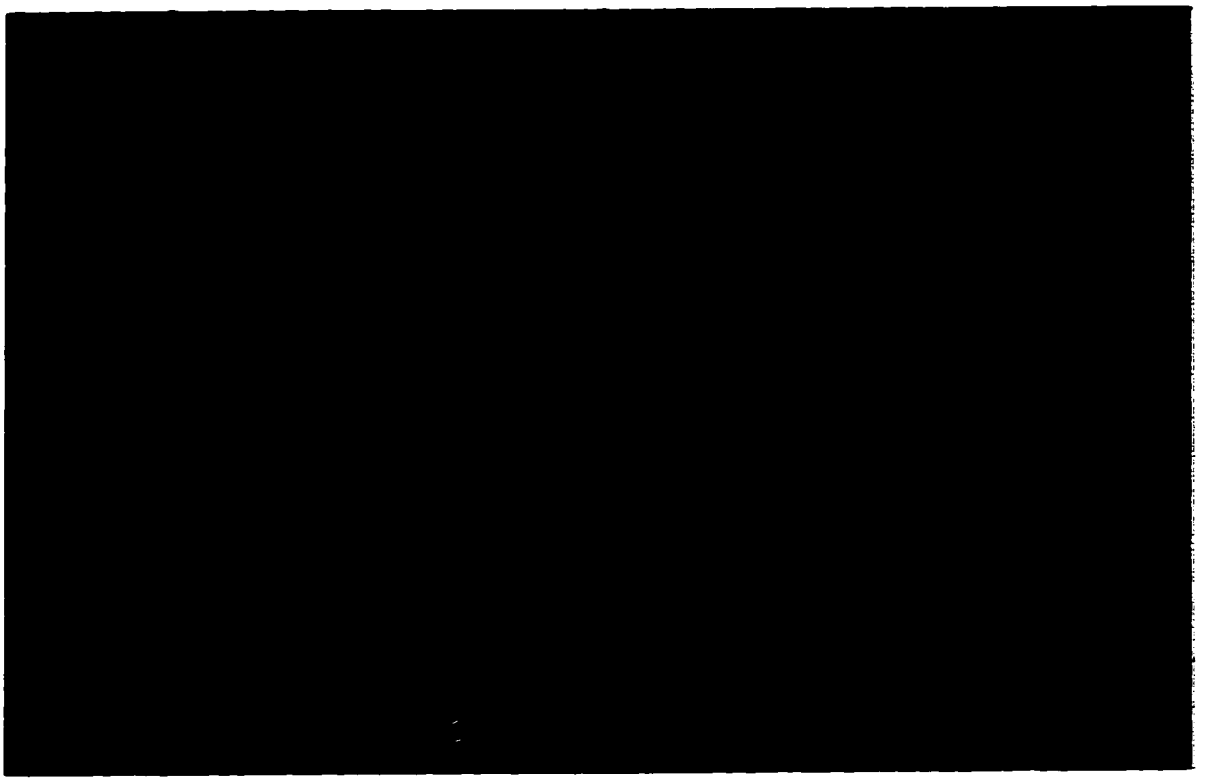


(b)

Figure 3.3

Day 5 (PKC- β_I)

(a)



(b)

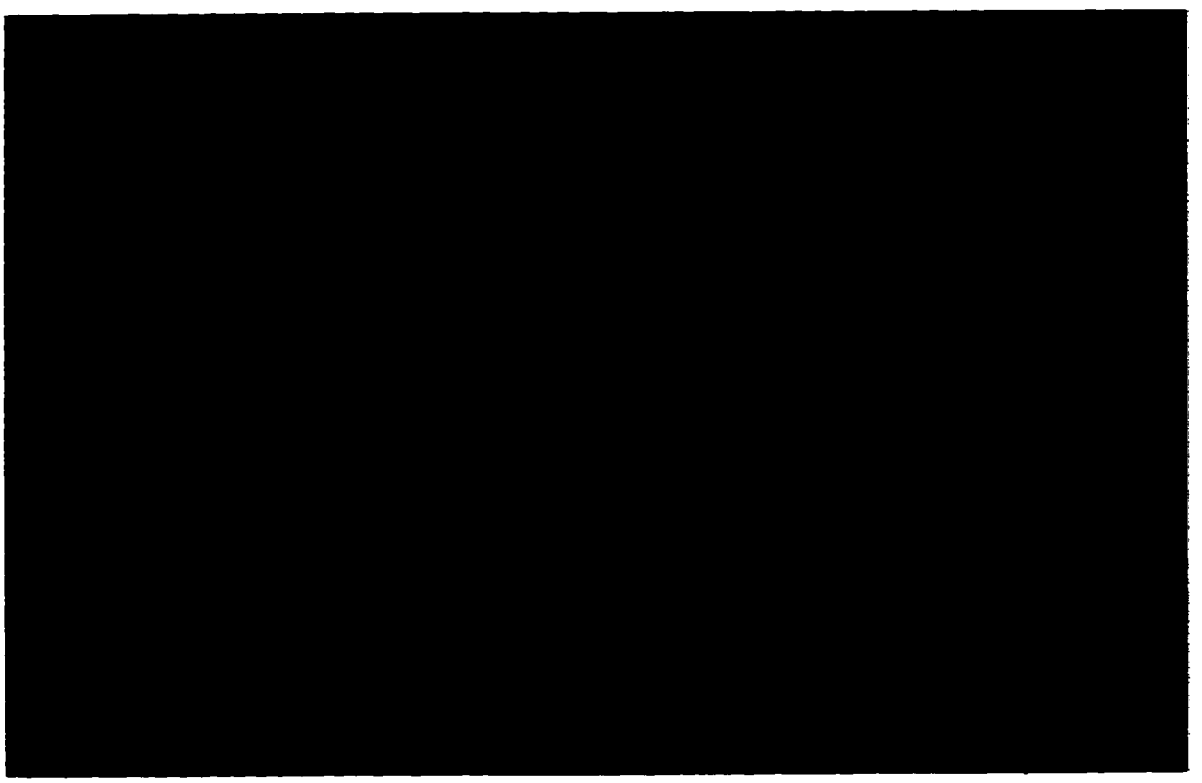
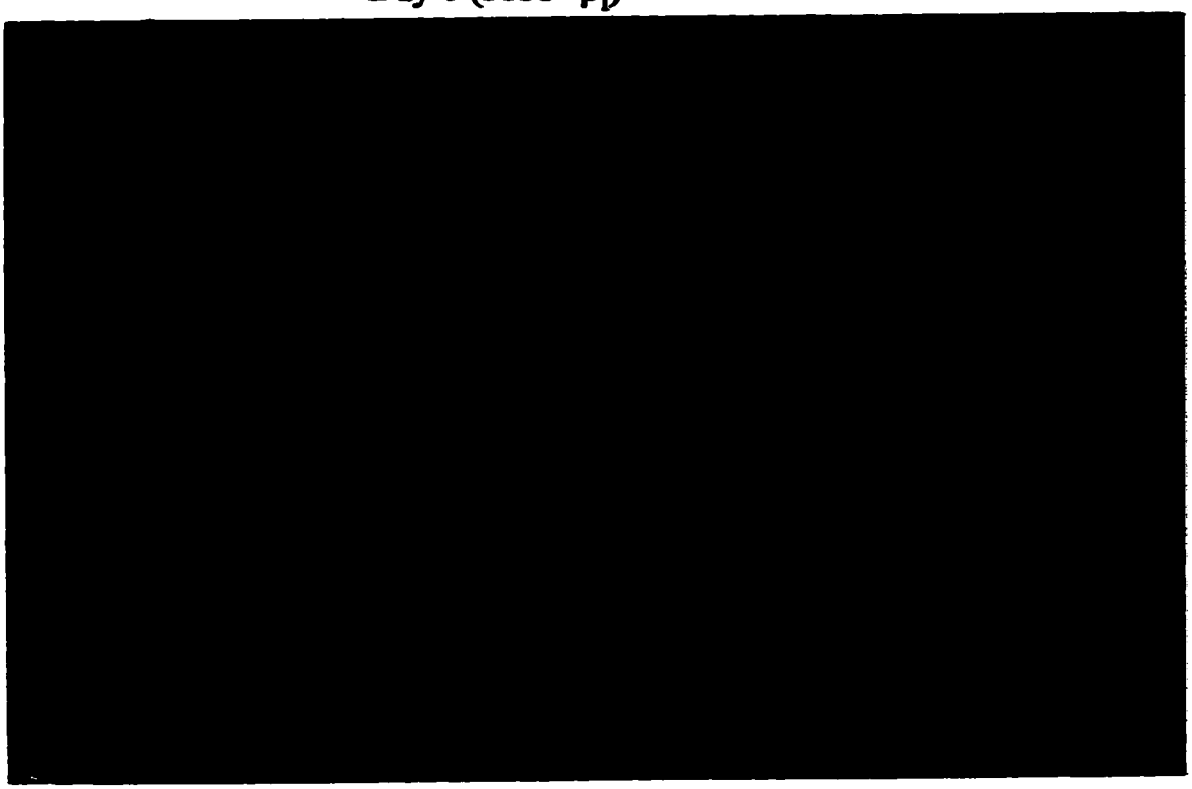


Figure 3.4

Day 8 (PKC- β_I)

(a)



(b)

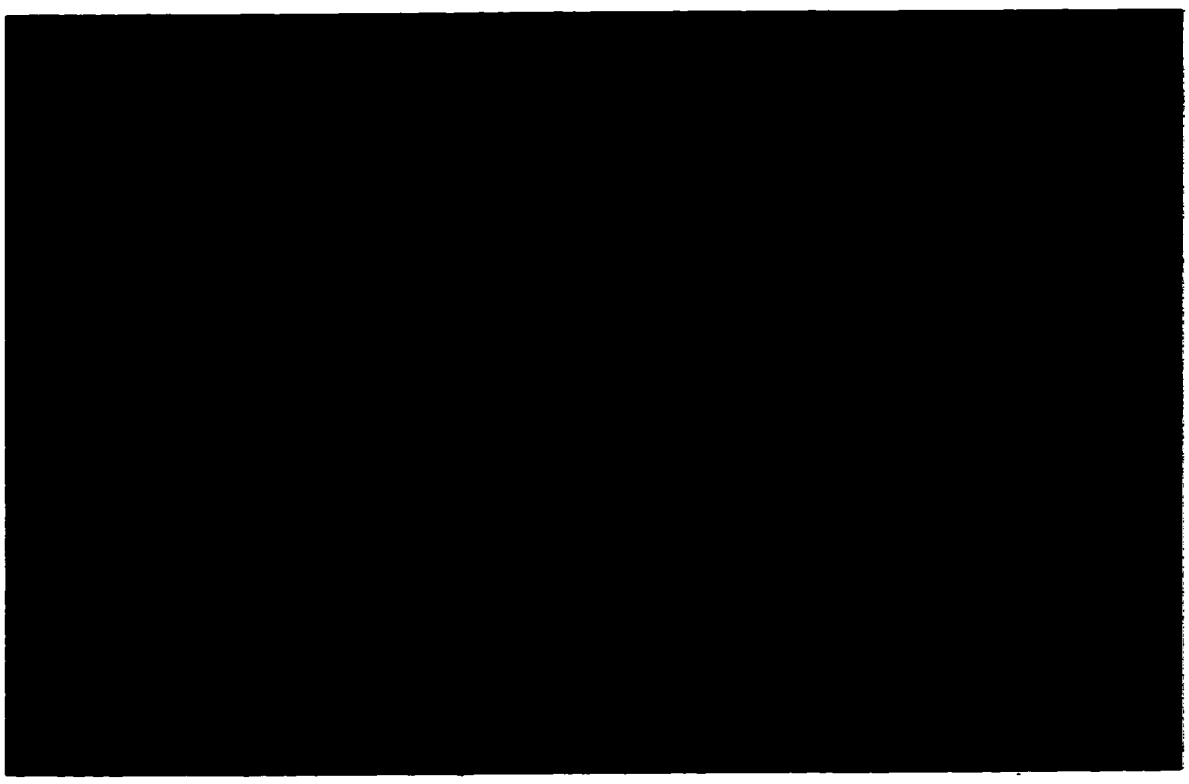


Figure 3.5

Immunofluorescence of PKC-α as T51B Cells Grow and Reach Confluence

T51B cells were plated at a density of 125 x 10^2 cells/cm^2 and grown on glass coverslips in medium containing 10% serum. At various times, the cells were fixed and incubated with PKC-α specific antibody. Antigen-antibody complexes were detected with fluorescein-conjugated secondary antibody as described in 1.3.4. Immunofluorescence photographs of cells at day 2 and day 5 after replating in 10% serum are shown in (a) and (b) respectively. Magnification: Figure 3.5a,b is x1000. Immunostaining was performed three times with similar results.

Figure 3.5

Day 2 (PKC- α)

(a)

Day 5 (PKC- α)

(b)

Figure 3.6

Immunofluorescence of PKC-δ as T51B Cells Grow and Reach Confluence

T51B cells were plated at a density of 125 x 10^2 cells/cm^2 and grown on glass coverslips in medium containing 10% serum. At various times, the cells were fixed and incubated with PKC-δ specific antibody. Antigen-antibody complexes were detected with fluorescein-conjugated secondary antibody as described in 1.3.4. Immunofluorescence photographs of cells at day 2 and day 5 after replating in 10% serum are shown in (a) and (b) respectively. Magnification: Figure 3.6a,b is x1000. Immunostaining was performed three times with similar results.

Figure 3.6

Day 2 (PKC- δ)

(a)

Day 5 (PKC- δ)

(b)

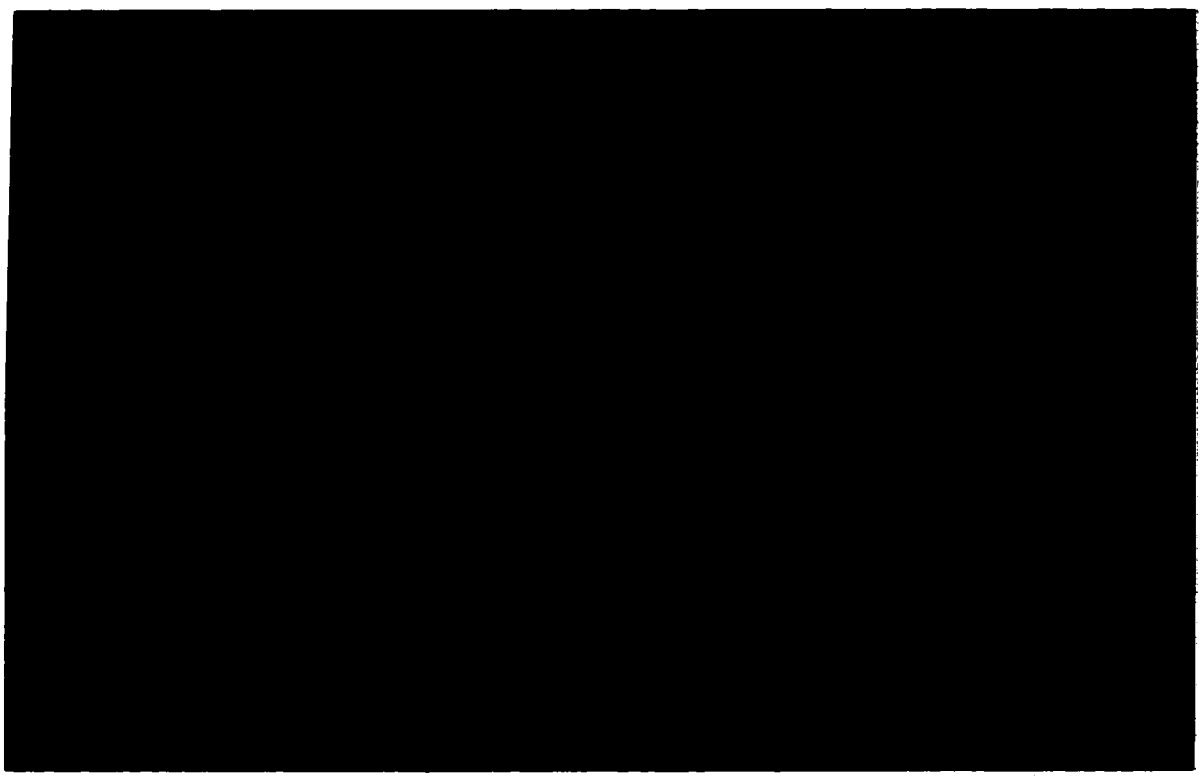


Figure 3.7

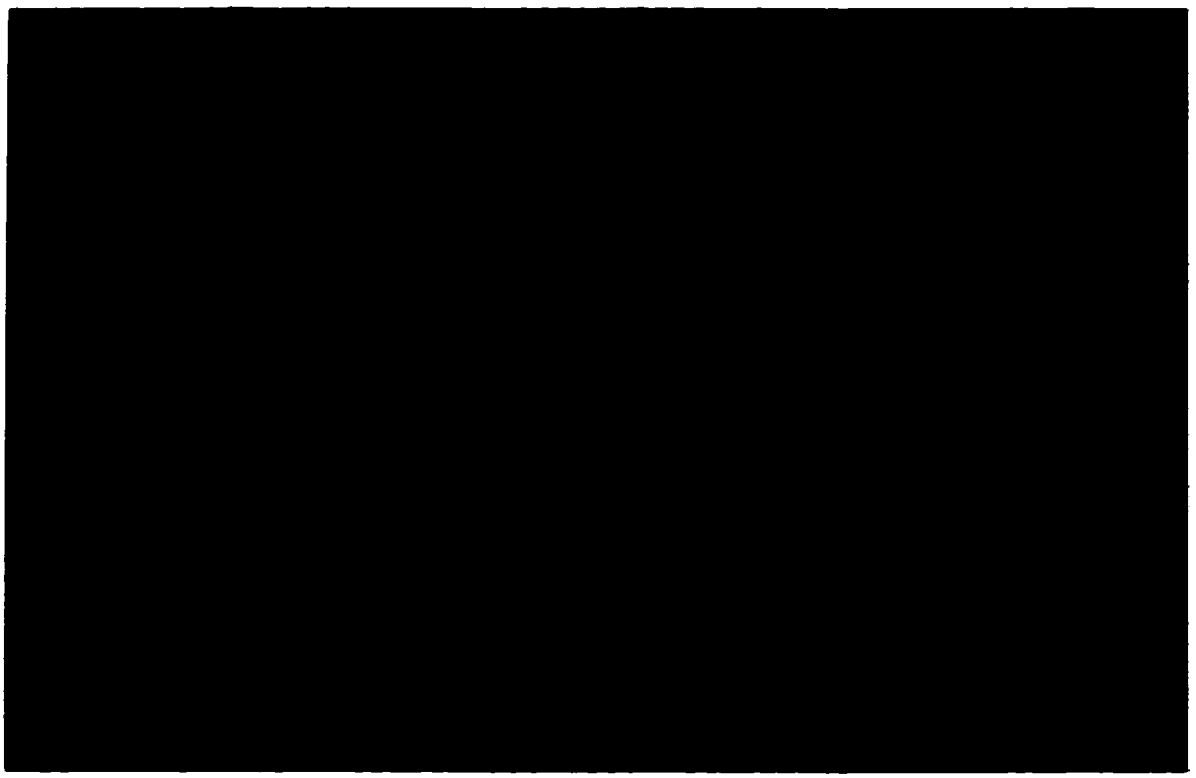
Immunofluorescence of PKC-ϵ as T51B Cells Grow and Reach Confluence

T51B cells were plated at a density of 125 x 10^2 cells/cm^2 and grown on glass coverslips in medium containing 10% serum. At various times. the cells were fixed and incubated with PKC-ϵ specific antibody. Antigen-antibody complexes were detected with fluorescein-conjugated secondary antibody as described in 1.3.4. Immunofluorescence photographs of cells at day 2 and day 5 after replating in 10% serum are shown in (a) and (b) respectively. Magnification: Figure 3.7a,b is x1000. Immunostaining was performed three times with similar results.

Figure 3.7

Day 2 (PKC- ε)

(a)



Day 5 (PKC- ε)

(b)

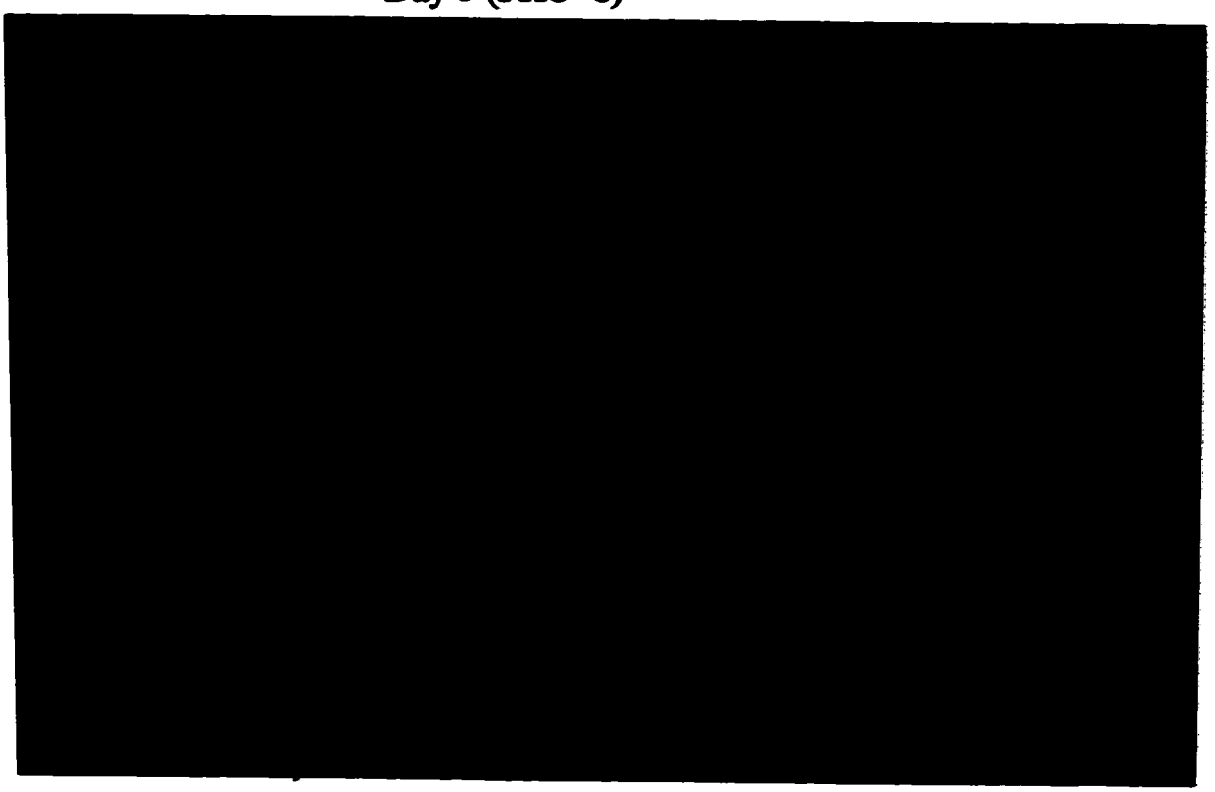


Figure 3.8

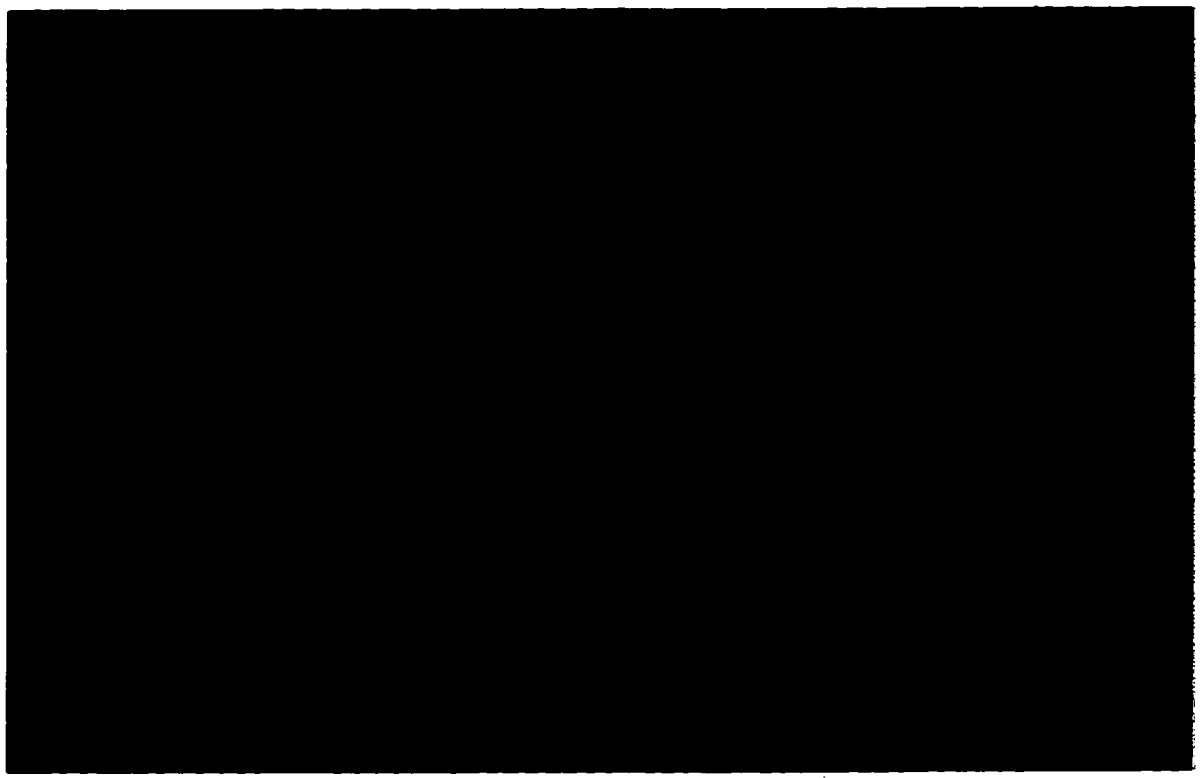
Immunofluorescence of PKC-ζ as T51B Cells Grow and Reach Confluence

T51B cells were plated at a density of 125 x 10^2 cells/cm^2 and grown on glass coverslips in medium containing 10% serum. At various times, the cells were fixed and incubated with PKC-ζ specific antibody. Antigen-antibody complexes were detected with fluorescein-conjugated secondary antibody as described in 1.3.4. Immunofluorescence photographs of cells at day 2 and day 5 after plating in 10% serum are shown in (a) and (b) respectively. Magnification: Figure 3.8a,b is x1000. Immunostaining was performed three times with similar results.

Figure 3.8

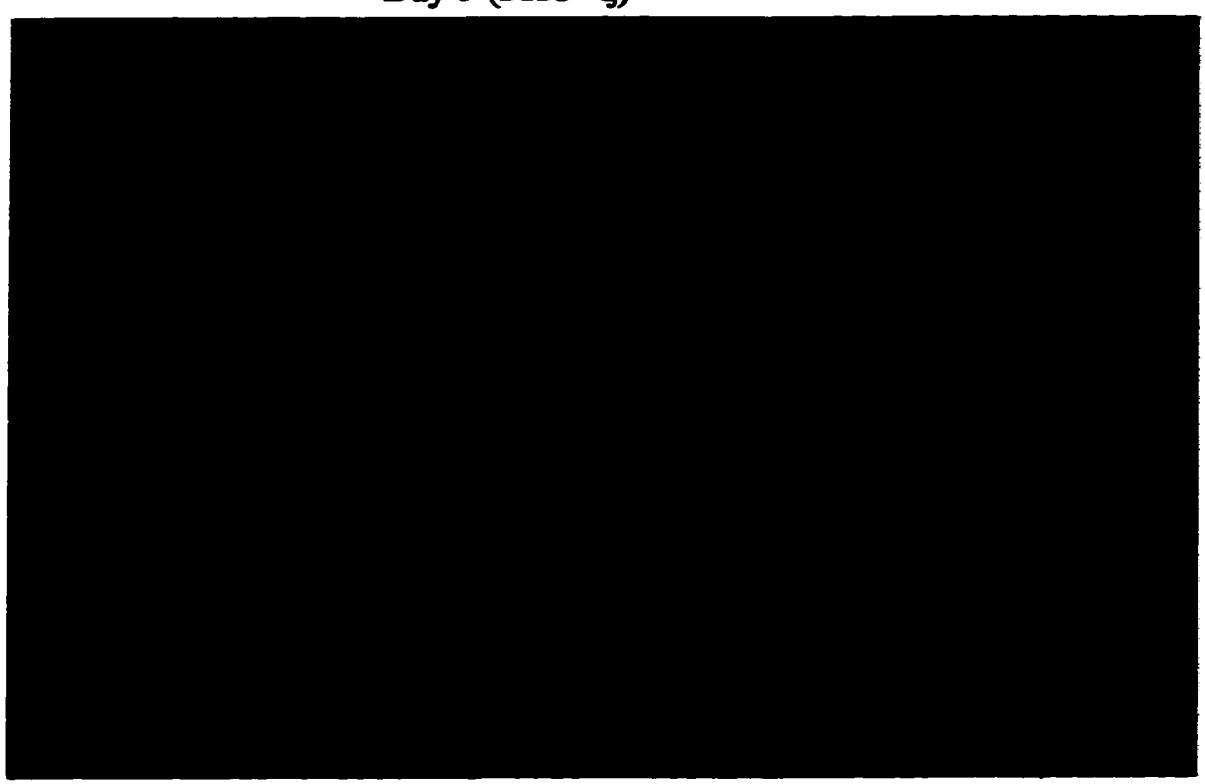
Day 2 (PKC- ζ)

(a)



Day 5 (PKC- ζ)

(b)



3.4.1b Immunofluorescence of PKC-β_I in Dividing Cells

Due to the above results, I was interested in determining the localization of PKC-β_I in dividing cells (during mitosis). Dual immunofluorescence and Hoechst staining assay was performed in order to recognize dividing cells by staining the DNA with the Hoechst dye, fluorochrome 32258. Figure 3.9a shows a phase contrast photograph of a dividing cell in early telophase of mitosis as well as intact non-dividing cells. The immunofluorescence photograph of the same field of cells indicates a strong PKC-β_I immunofluorescence in the dividing cell compared to that of non-dividing cells (Fig. 3.9b). The dual immunofluorescence and Hoechst staining photograph of the same field of cells shows the bright blue fluorescence of Hoechst dye associated with the chromosomes that are in the process of being separated in the early telophase of mitosis toward the opposite ends of the cell (Fig. 3.9c). Some green fluorescence of PKC-β_I is seen associated with the dividing cell (Fig. 3.9c). However, non-dividing intact cells are stained only with Hoechst dye and have blue fluorescence staining. In these experiments, the same observations were noted for all mitotic cells. Figure 3.9c is a representative field of three different experiments. Hence, these results demonstrate for the first time, a high level of PKC-β_I in dividing T51B cells compared to non-dividing cells.

3.4.1c Immunoblotting of PKC-β_I as the Cells Approach Confluence

Immunoblotting of PKC-β_I at various times after plating in medium containing 10% serum (day 1 - day 8) was performed to confirm the immunofluorescence observation seen in 3.4.1a. Lamin B (a nuclear marker envelope protein) was used as a nuclear marker. Anti-lamin B antibody detects a single band of 70 kD in the nuclear fraction only and no immunoreactivity is detected in cytosolic or membrane fractions (Fig. 3.10). When the

Figure 3.9

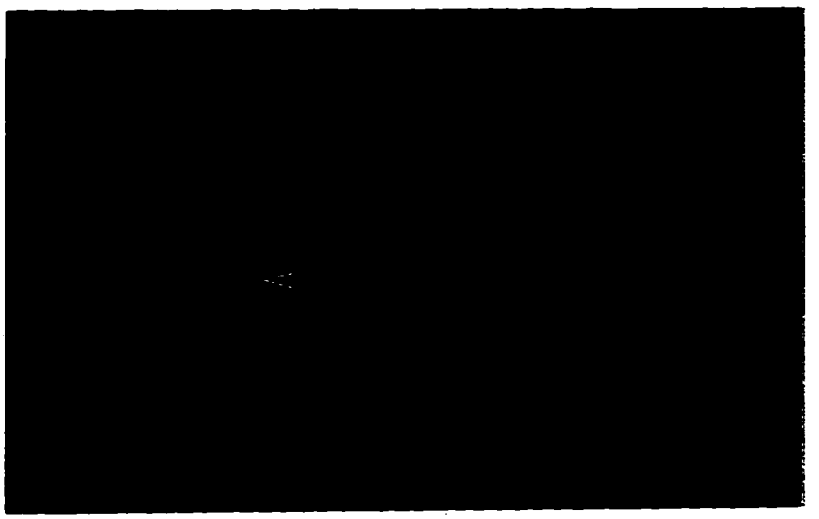
Dual Immunofluorescence and Hoechst Staining of PKC-β_I in Dividing Cells after One Day of Plating in 10% Serum

T51B cells were plated at a density of 125 x 10^2 cells/cm^2 and grown on glass coverslips in medium containing 10% serum. After 1 day of plating the cells were fixed and incubated with PKC-β_I specific antibody followed by incubation with a fluorescein-conjugated secondary antibody as described in 3.3.5. After washing, the coverslips were incubated with Hoechst dye, fluorochrome 33258 as described in 3.3.5 and viewed under an Olympus fluorescence microscope equipped with a special filter cube for dual fluorescence (UV/FITC). In this figure: (a) shows a phase contrast photograph of dividing (arrowed) and non-dividing cells one day after plating; (b) shows the corresponding immunofluorescence photograph of dividing (arrowed) and non-dividing cells; (c) shows a photograph of dual immunofluorescence and Hoechst staining of dividing (arrowed) and non-dividing cells one day after plating. The dividing cells are brightly stained with Hoechst dye while the non-dividing cells have pale staining. PKC-β_I in dividing cells has green fluorescein staining. Magnification: Figure 3.9a-c is x1000. Immunostaining was performed three times with similar results.

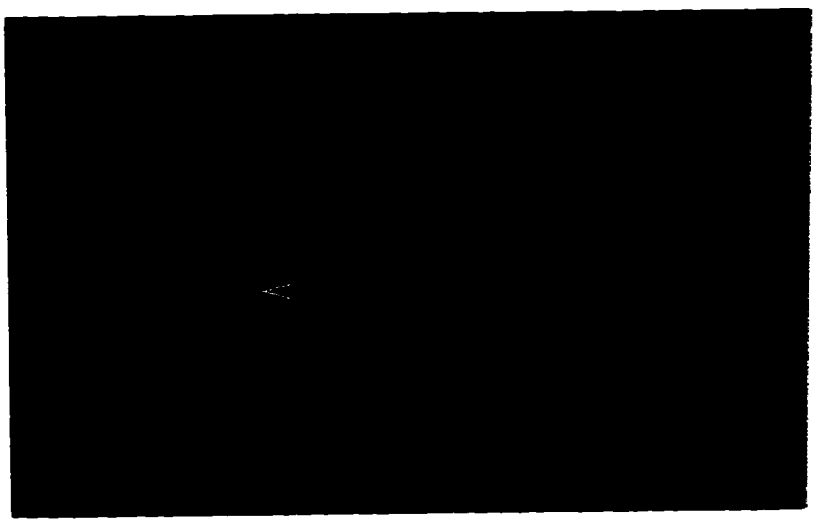
Figure 3.9

PKC- β_I

(a)



(b)



(c)

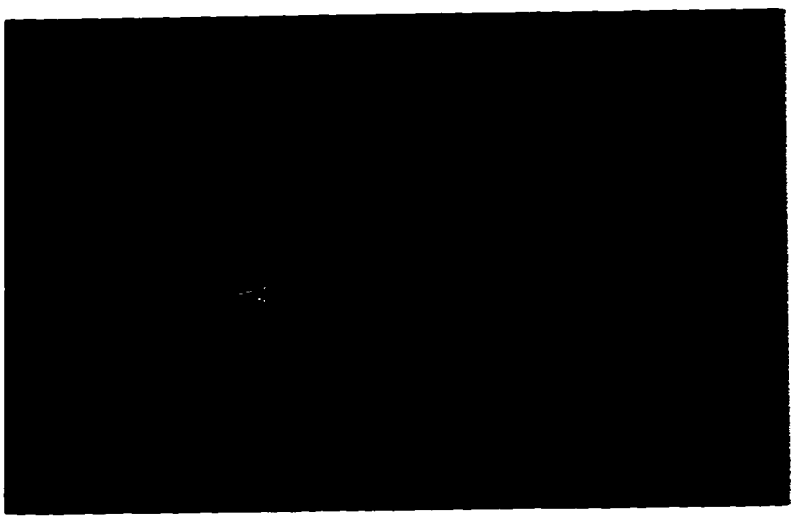
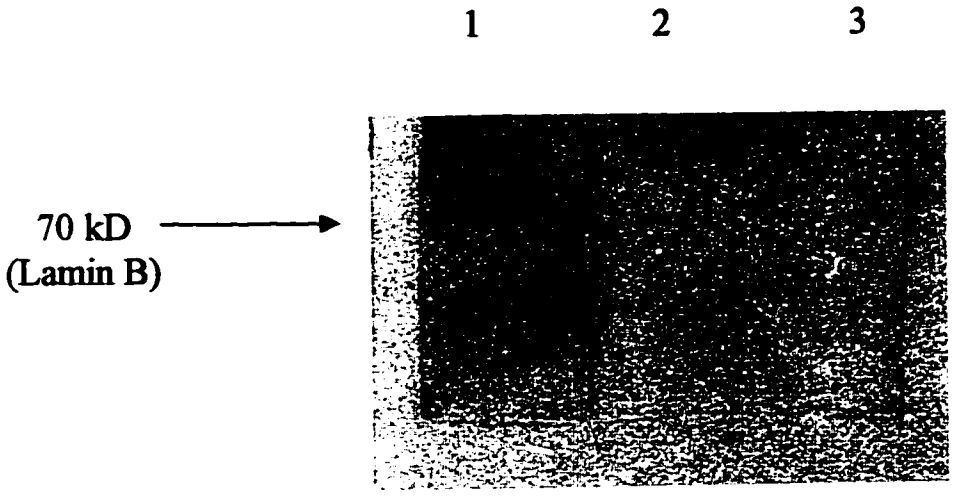


Figure 3.10

Immunoreactivity of Lamin B, a Nuclear Marker in Subcellular Fractions

T51B cells were plated at a density of 125 x 10^2 cells/cm^2 in medium containing 10% serum. Confluent cells were homogenized and fractionated into (1) nuclear; (2) membrane; and (3) cytosol fraction as described in 3.3.2. Samples were solubilized in SDS sample buffer and electrophoresed on 10% SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with anti-lamin B monoclonal specific antibody. Antigen-antibody complexes were detected with alkaline phosphatase-conjugated secondary antibody as described in 1.3.3. Data is representative of three experiments which gave identical results. Molecular weight is expressed as kD (kilodalton).

Figure 3.10



nuclear fractions derived from cells at different days after plating are immunoblotted with anti-PKC-β_I antibody, the antibody recognizes two bands (doublet) of approximately 77 and 67 kD respectively (Fig. 3.11a). The doublet is identical to that seen for PKC-β_I in the plasma membrane of T51B cells (section 1.4.1a). Consistent with the immunofluorescence results, immunoblotting show that between 1 and 2 days of plating, at which time the cells are still sparse, high levels of nuclear PKC-β_I doublet are observed. By day 3, there is a decrease in the level of the nuclear PKC-β_I followed by a slight increase at day 5. Eight days after plating, the level of nuclear PKC-β_I is similar to that observed at day 1, day 2, and day 5. The scanning results of three immunoblots of nuclear fractions at various times are shown in Table 3.11. These data indicate that the intensity of the two PKC-β_I bands (77 kDand 67 kD) do not change significantly between day 1 and day 2. By day 3, the intensity of the two bands decreases and then increases by day 5 and day 8 to reach the same level of day 1 and day 2. Immunoblotting results of plasma membrane fractions derived from cells at different days after plating reveals an increase in the level of PKC-β_I as cells approach confluence (Fig. 3.12a). One day after plating, low levels of PKC-β_I in both bands are detected. By day 2, the level of PKC-β_I starts to increase especially in the lower band which continues to rise through day 3. By day 5, the cells reach confluence and the level of PKC-β_I increases substantially in the two bands of PKC-β_I. However, higher level of PKC-β_I is observed in the lower band than that of the upper band. After 8 days of plating, the level of PKC-β_I starts to decline again, though it is still higher than that detected at day 1. Scanning data of the three immunoblot are shown in Table 3.12. These data clearly demonstrate that there is an increase in the intensity level of the two PKC-β_I bands of the plasma membrane of T51B cells as they reach confluence. Interestingly, there is a difference between the intensity level

Figure 3.11

Immunoreactivity of PKC-β_I in the Nuclear Membrane of T51B Cells at Various Times after Plating in the Presence of 10% Serum

T51B cells were plated at a density of 125 x 10^2 cells/cm^2 in medium containing 10% serum. At various times after plating, cells were homogenized and fractionated into cytosol, membrane, and nuclear fractions as described in 3.3.2. The nuclear membrane samples were solubilized in SDS sample buffer and eletrophoresed on 10% SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with PKC-β_I specific antibody. Antigen-antibody complexes were detected with alkaline phosphatase-conjugated secondary antibody as described in 1.3.3. Immunoblot of nuclear fractions at various times after plating is shown in (a) where: (1) day 1; (2) day 2; (3) day 3; (4) day 5; (5) day 8. Data is representative of three experiment which gave identical results.

Figure 3.11a

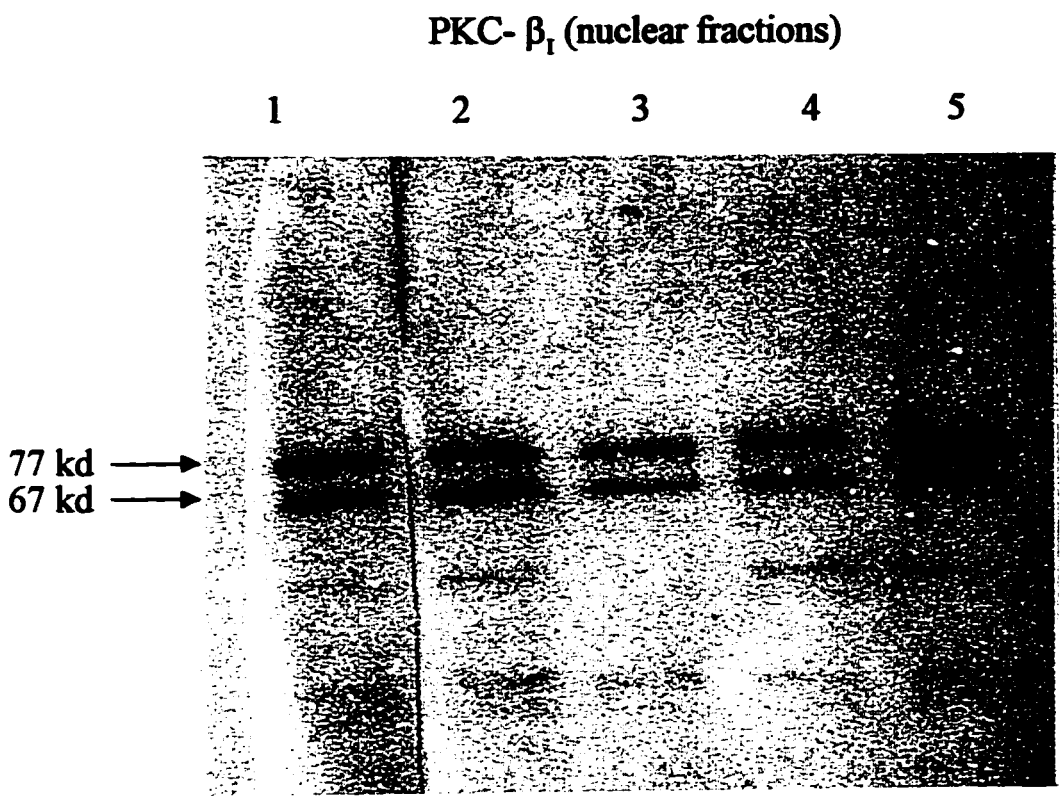


TABLE 3.11

Immunoreactivity of PKC-β_I in the Nuclear Membrane of T51B Cells at Various Times After Plating in the Presence of 10% Serum

The immunoblots of nuclear fractions at various times after plating were prepared as described in Figure 3.11. The blots were scanned with an HP Laser Scanner and the images were then analyzed by using Sigma Scan Image Software. Data are expressed as pixel intensities. Statistical analysis was performed by paired student's t-test, comparing the mean ± SE of three experiments of day 1 - day 8. A level of significance of $P < 0.05$ is indicated as * and of $P < 0.01$ is indicated.

TABLE 3.11

Immunoreactivity of PKC-β_I in the nuclear membrane of T51B Cells at various times after plating in the presence of 10% serum

	Mean ± SE				
	Day 1	**Day 2**	**Day 3**	**Day 5**	**Day 8**
PKC-β_I (77 kD)	111.0 ± 1.2	117.0 ± 1.2	85.0 ± 5.4*	114.0 ± 4.1	110.0 ± 4.0
PKC-β_I (67 kD)	110.0 ± 2.6	118.0 ± 1.5	83.0 ± 6.4*	115.0 ± 4.0	112.0 ± 2.5

SE: Standard error

Figure 3.12

Immunoreactivity of PKC-β_I in the Plasma Membrane of T51B Cells at Various Times after Plating in the Presence of 10% Serum

T51B cells were plated at a density of 125 x 10^2 cells/cm^2 in medium containing 10% serum. At various times after plating, cells were homogenized and fractionated into cytosol, membrane, and nuclear fractions as described in 3.3.2. The plasma/microsomal membrane samples were solubilized in SDS sample buffer and eletrophoresed on 10% SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with PKC-β_I specific antibody. Antigen-antibody complexes were detected with alkaline phosphatase-conjugated secondary antibody as described in 1.3.3. Immunoblot of membrane fractions at various times after plating is shown in (a) where: (1) day 1; (2) day 2; (3) day 3; (4) day 5; (5) day 8. Data is representative of three experiment which gave identical results.

Figure 3.12a

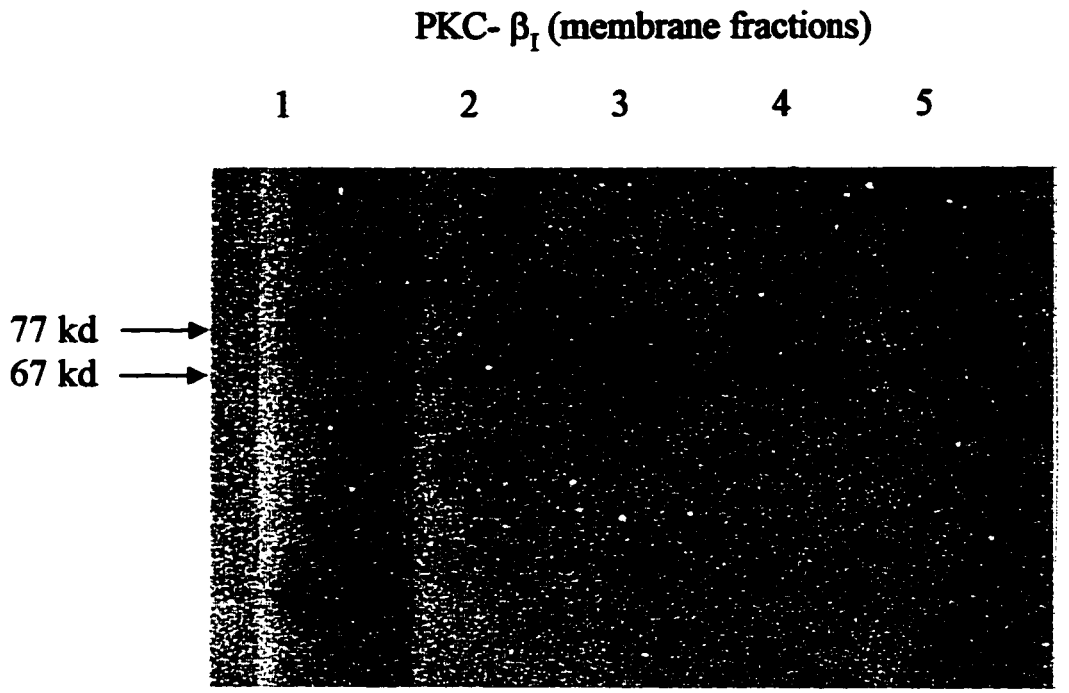


TABLE 3.12

Immunoreactivity of PKC-β_I in the Plasma Membrane of T51B Cells at Various Times After Plating in the Presence of 10% Serum

The immunoblots of plasma membrane fractions at various times after plating were prepared as described in figure 3.12. The blots were scanned with an HP Laser Scanner and the images were then analyzed by using Sigma Scan Image software. Data are expressed as pixel intensities. Statistical analysis was performed by paired student's t-test, comparing the mean ± SE of three experiments of day 1 - day 8. A level of significance of $P < 0.05$ is indicated as * and of $P < 0.01$ is indicated as **.

TABLE 3.12

Immunoreactivity of PKC-β_I in the plasma membrane of T51B cells at various times after plating in the presence of 10% serum

	Mean ± SE				
	Day 1	**Day 2**	**Day 3**	**Day 5**	**Day 8**
PKC-β_I (77 kD)	36.3 ± 2.4	41.3 ± 1.0	45.0 ± 3.8	52.7 ± 2.2*	38.7 ± 1.2
PKC-β_I (67 kD)	41.3 ± 2.0	60.0 ± 2.1*	77.7 ± 3.2**	86.0 ± 3.6**	60.0 ± 3.5**

SE : Standard error

of the two PKC-β_I bands. The intensity of the PKC-β_I band (67 kD) increases as the cells approach confluence between day 2 - day 5 (Table 3.12). However, the intensity level of the 67 kD band declines by day 8 though it is still higher than that detected at day 1.

3.4.2 Effect of Re-addition of 10% Serum to Serum-Deprived T51B Cells on the Localization of PKC-β_I

Serum deprivation represents a good method by which one can block the cells in G_0 phase of the cell cycle. It has been shown that when T51B cells are fluid changed to medium containing 0.2% serum for 48 h, the cells become quiescent, are in the G_0 phase and no DNA synthesis is observed until the cells are re-stimulated by addition of medium containing 10% serum or EGF (Sharma et al., 1994). Thus, I have an interest in studying the effect of serum re-addition to quiescent T51B cells on the nuclear localization of PKC-β_I. Immunofluorescence results indicates that the level of PKC-β_I increases in the nucleus and the nuclear membrane after 24 h of serum re-addition to quiescent cells (Fig. 3.13b) as compared to control serum-deprived cells (Fig. 3.13a). Moreover, in the plasma membrane punctate fluorescence staining of PKC-β_I increases slightly along the cell boundaries as compared to serum-deprived cells. The immunoblot analysis indicates that there is a an increase in the level of PKC-β_I in the nuclear fraction after 24 h of serum re-addition to quiescent cells (Fig. 3.14, lane 2) compared to that observed for control serum-deprived cells (Fig. 3.14, lane 1). The scanning results of three immunoblots derived from serum-deprived and serum-stimulated cells are shown in Table 3.14. These results demonstrate an increase in the intensity of the two bands of nuclear PKC-β_I in serum-stimulated cells compared to serum deprived cells. Hence, these results confirm the immunofluorescence observation seen in Figure 3.13a,b.

Figure 3.13

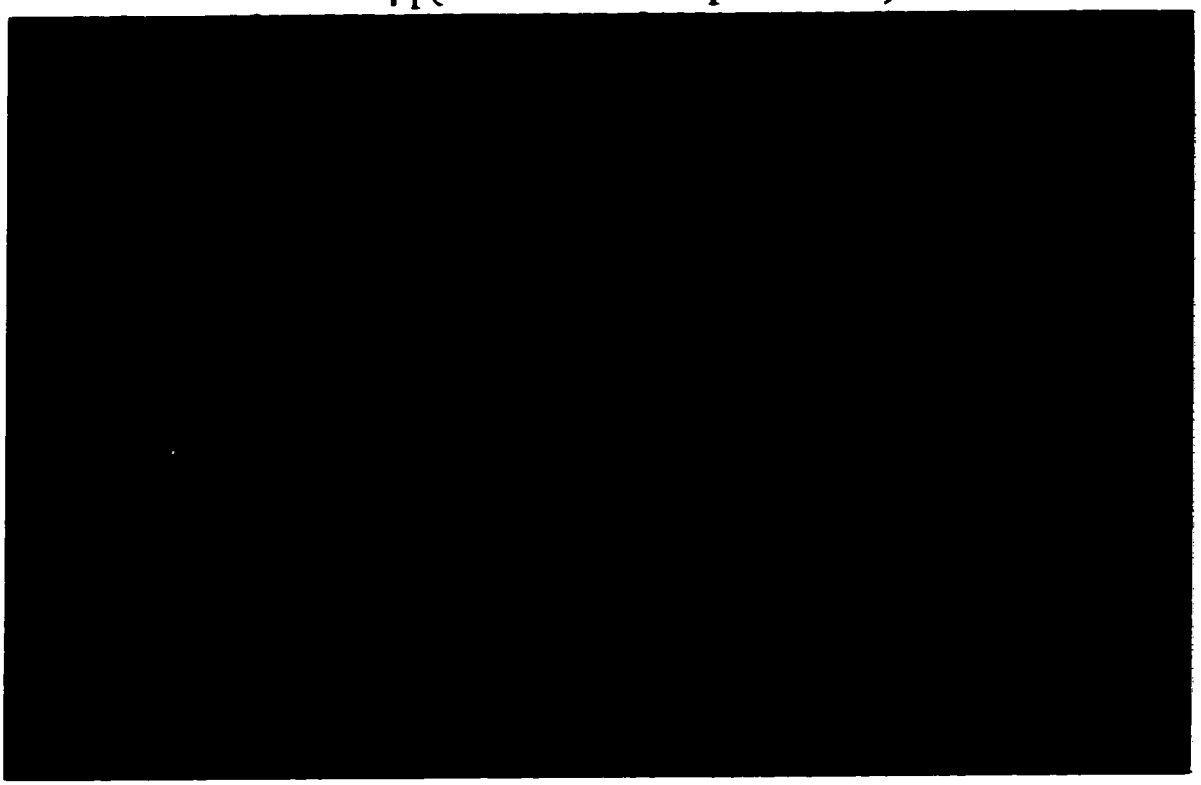
Effects of Serum Re-addition on the Immunofluorescence Localization of PKC-β_I in Quiescent Serum-Deprived T51B Cells

T51B cells were plated at a density of 125 x 10^2 cells/cm^2 and grown on glass coverslips in medium containing 10% serum. Confluent cells were then serum-deprived by fluid change to medium containing 0.2% serum. After 48 h, the cells were fluid changed to medium containing 10% serum for 24 h. Cells were then fixed and incubated with PKC-β_I specific antibody. Antigen-antibody complexes were detected with fluorescein-conjugated secondary antibody as described in 1.3.4. Immunofluorescence photographs of control serum-deprived cells and of cells, 24 h after serum re-addition to quiescent cells are shown in (a) and (b) respectively. Magnification: Figure 3.13a,b is x1000. Immunostaining was performed three times with similar results.

Figure 3.13

PKC- β_I (control serum- deprived cells)

(a)



PKC- β_I (serum- deprived cells + 10% serum, 24 h)

(b)

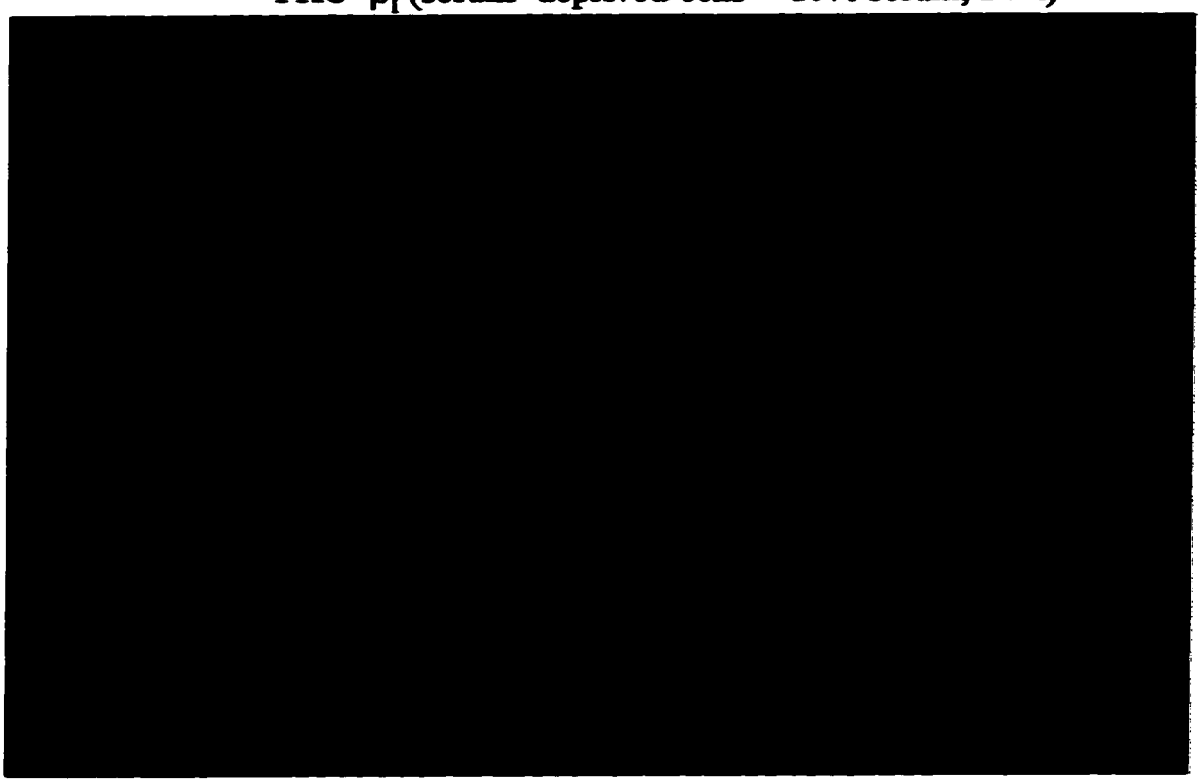


Figure 3.14

Effects of Serum Re-addition on the Immunoreactivity Levels of PKC-β_I in the Nuclei of Quiescent Serum-Deprived T51B Cells

T51B cells were plated at a density of 125 x 10^2 cells/cm^2 in medium containing 10% serum. Confluent cultures were then serum-deprived by fluid change to medium containing 0.2% serum. After 48 h, the cells were fluid changed to medium containing 10% serum for 24 h. Cells were then homogenized and fractionated into cytosol, membrane and nuclear fractions as described in 3.3.2. The nuclear membrane samples were solubilized in SDS sample buffer and electrophoresed on 10% SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with PKC-β_I specific antibody. Antigen-antibody complexes were detected with alkaline phosphatase-conjugated secondary antibody as described in 1.3.3. Immunoblot of nuclear fraction for control serum-deprived cells is shown in (1) and for serum-treated cells is shown in (2). Data is representative of three experiments which gave identical results.

Figure 3.14

PKC- β_I (nuclear fraction in absence or presence of 10% serum)

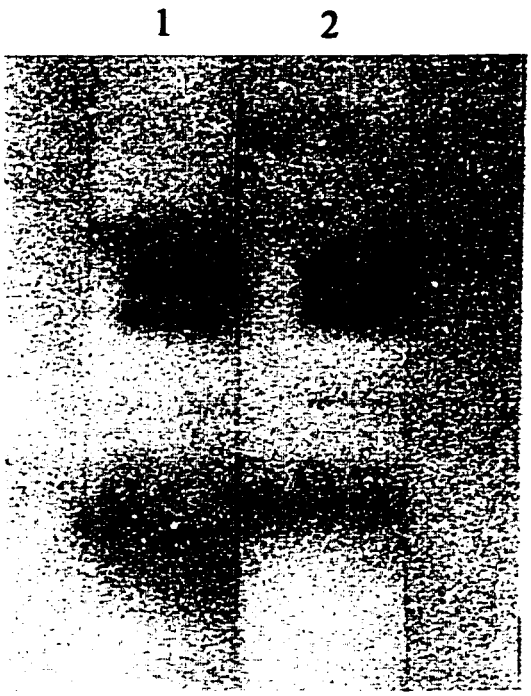


TABLE 3.14

Effect of Serum Re-addition on the Nuclear Levels of PKC-β_I in the Nuclei of Quiescent Serum-Deprived T51B Cells

The immunoblots of nuclear fractions derived from Serum deprived cells and serum stimulated cells were prepared as described in figure 3.14. The blots were scanned with an HP Laser Scanner and the images were then analyzed by using Sigma Scan Image software. Data are expressed as pixel intensities. Statistical analysis was performed by paired student's t-test, comapring the mean ± SE of three experiments of serum-deprived and serum-stimulated cells. A level of significance of $P < 0.05$ is indicated as *.

TABLE 3.14

Effect of Serum Re-addition on the Nuclearlevels of PKC-β_I in the Nuclei of Quiescent Serum-Deprived T51B Cells

	Mean ± SE	
	Serum Deprived Cells	**Serum of Stimulated Cells**
PKC-β_I (77 kD)	84.5 ± 3.6	100.4 ± 3.5*
PKC-β_I (67 kD)	86.6 ± 2.6	105.0 ± 4.1*

SE: Standard error

3.4.3 Effects of TPA Treatment on the Localization of PKC-β_I after 1 Day of Plating

Results from Chapter One (section 1.4.2) have shown an increase in the level of PKC-β_I in the plasma membrane of confluent T51B cells after 24 h treatment with TPA. In the present study I examined the effect of TPA on the localization of PKC-β_I after 1 day of plating. Immunofluorescence localization indicates that after 1 day of plating, PKC-β_I is associated with the nucleus and the nuclear membrane only (Fig. 3.15a). After 5 min of TPA addition, PKC-β_I immunofluorescence starts to appear in the cytoplasm and the plasma membrane (Fig. 3.15b). After 10 min of TPA addition, there is a slight increase in the immunofluorescence of PKC-β_I in the cytoplasm and the plasma membrane as well as in the nucleus especially on the nuclear membrane (Fig. 3.15c) compared to day 1 controls. After 30 min of TPA treatment, the immunofluorescence of PKC-β_I is still observed in the nucleus, the cytoplasm and the plasma membrane, though to a lower extent compared to that observed after 10 min of TPA treatment (Fig. 3.15d). After 24 h of TPA addition, the immunofluorescence of PKC-β_I in the cytoplasm starts to decrease with a slight increase in the immunostaining associated with the plasma membrane. However, no change is observed for the nuclear PKC-β_I immunofluorescence (Fig. 3.15e). These results indicate that TPA treatment after 1 day of plating results in a re-distribution of nuclear PKC-β_I between the nuclear, the cytosolic and the membrane compartments in a time dependent manner.

3.4.4 Effects of TPA Treatment on the Localization of PKC-β_I in Serum-Deprived T51B Cells

Results from Chapter One have indicated that treatment of serum stimulated cells with TPA for 24 h results in an increase in membrane-associated level of PKC-β_I. In the present study, I examined the effect of TPA treatment at various time intervals (5, 10 and 30 min and 24 h) on the localization of PKC-β_I in serum-deprived T51B cells. At 5, 10 and 30

Figure 3.15

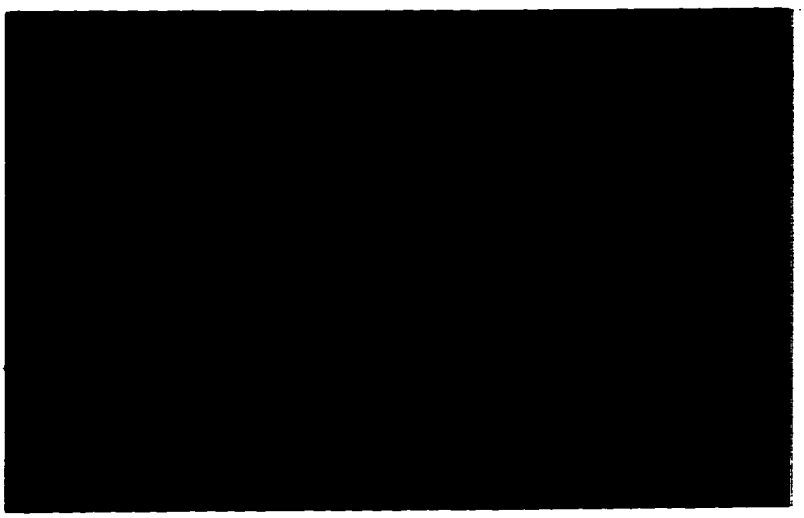
Effects of TPA Treatment at Various Time Points on the Immunofluorescence Localization of PKC-β_I in T51B Cells after One Day of Plating in the presence of 10% serum

T51B cells were plated at a density of 125 x 10^2 cells/cm^2 and grown on glass coverslips in medium containing 10% serum After 24 h, cells were treated with vehicle control (a); or 100 nM TPA for 5 min (b); 10 min (c); 30 min (d); 24 h (e). Cells were then fixed and incubated with PKC-β_I specific antibody. Antigen-antibody complex were detected with fluorescein-conjugated secondary antibody as described in 1.3.4. Magnification: Figure 3.15a-e is x1000. Immunostaining was performed at least two times with similar results.

Figure 3.15

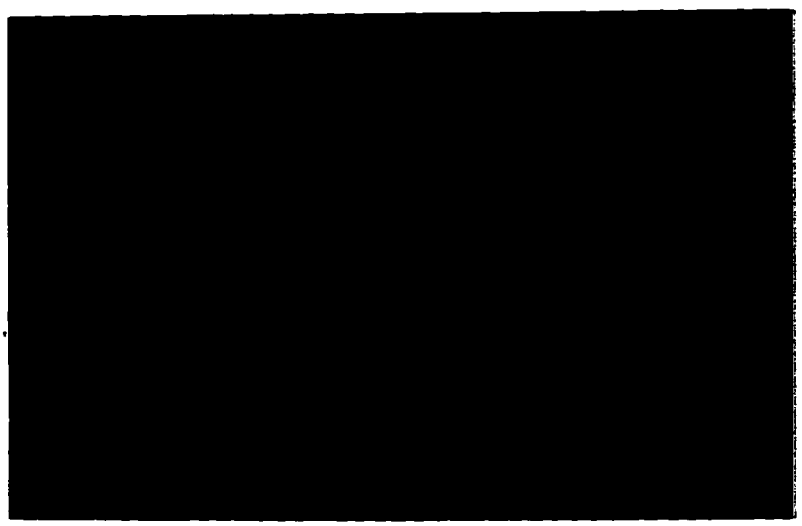
(a)

PKC-β_I
(control, 1 day)



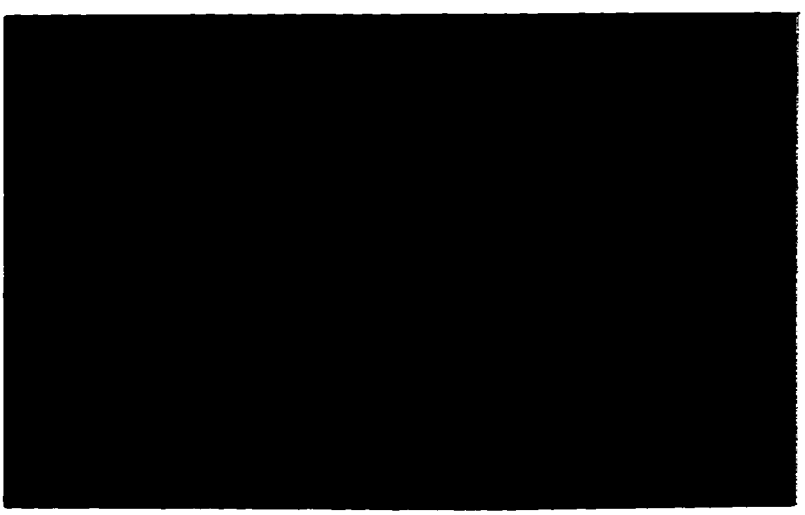
(b)

PKC-β_I+TPA,
5 min



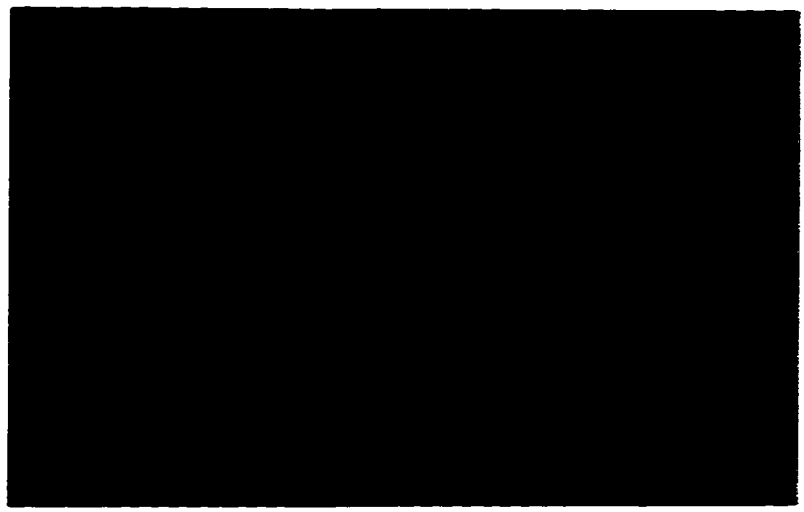
(c)

PKC-β_I+TPA,
10 min



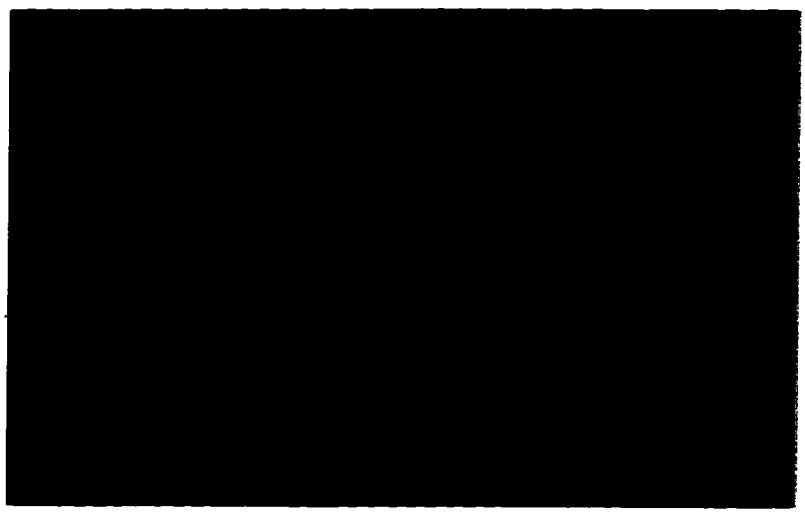
(d)

PKC-β_I +TPA,
30 min



(e)

PKC-β_I +TPA,
24 h



min of TPA addition, the immunofluorescence of PKC-β_I is almost the same (Fig. 3.16b,c,d) as compared to control serum-deprived cells (Fig. 3.16a). After 24 h of TPA treatment, the immunofluorescence of PKC-β_I associated with the nucleus and the plasma membrane slightly increases (Fig. 3.16e). Hence, these results indicate that TPA treatment for 24 h results in a slight increase in the immunofluorescence of PKC-β_I associated with the plasma membranes and the nuclei of T51B cells.

3.4.5 Effects of TPA Treatment on the Phosphorylation of Nuclear Proteins of Serum Deprived T51B Cells

Many studies have indicated that PKC plays a direct role in the phosphorylation of a number of nuclear proteins such as lamin B, histones and many others. In the present work, I studied the effect of TPA (a PKC activator) on the phosphorylation of nuclear proteins *in vitro*. The autoradiogram of labeled nuclear fractions (Fig. 3.17) shows an increase in the phosphorylation of two nuclear proteins after 30 min of TPA addition and this level of phosphorylation is maintained for up to 24 h as compared to controls. The nuclear proteins have a molecular weight of approximately 21 and 31 kD as calculated from a standard curve of SDS-PAGE molecular weight standards.

3.4.6 Effects of Serum Re-addition on the Phosphorylation of Nuclear Proteins of Serum-Deprived T51B Cells

In the present study I examined the effect of serum re-addition on the phosphorylation of nuclear proteins *in vivo*.. The autoradiogram of labeled nuclear fractions (Fig. 3.18) shows an increase in the phosphorylation of two nuclear proteins after 5 min of serum stimulation which decreases within 10 min as compared to controls. The nuclear proteins have a molecular weight of approximately 21 and 31 kD which are the same nuclear proteins observed in the TPA experiment.

Figure 3.16

Effect of TPA at Various Times Points on the Immunofluorescence Localization of PKC-β_I in Quiescent Serum-Deprived T51B Cells

T51B cells were plated at a density of 125 x 10^2 cells/cm^2 and grown on glass coverslips in medium containing 10% serum. Confluent cells were then serum-deprived by fluid change to medium containing 0.2% serum. After 48 h, the cells were treated with vehicle control (a); or 100 nM TPA for 5 min (b); 10 min (c); 30 min (d); 24 h (e). Cells were then fixed and incubated with PKC-β_I specific antibody. Antigen-antibody complexes were detected with fluorescein-conjugated secondary antibody as described in 1.3.4. Magnification: Figure 3.16a-e is x1000. Immunostaining was performed at least two times with similar results.

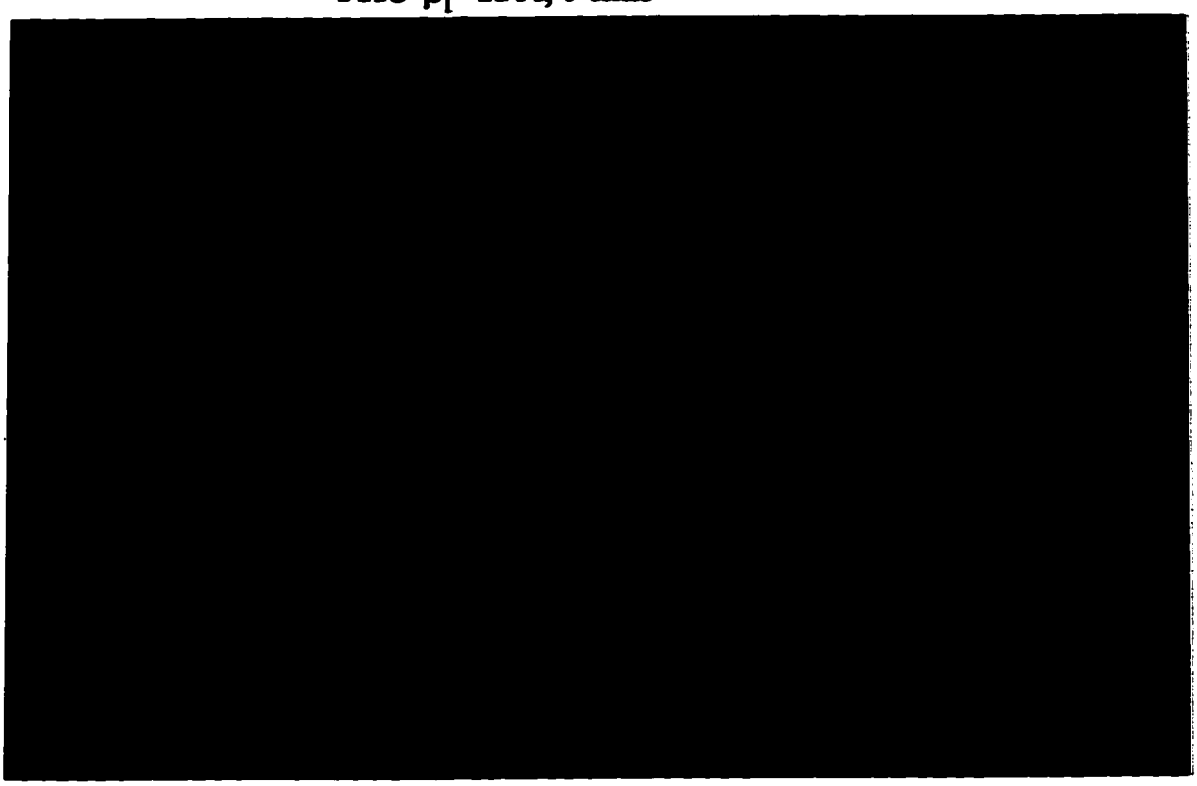
Figure 3.16

PKC-β_I (control serum-deprived cells)

(a)

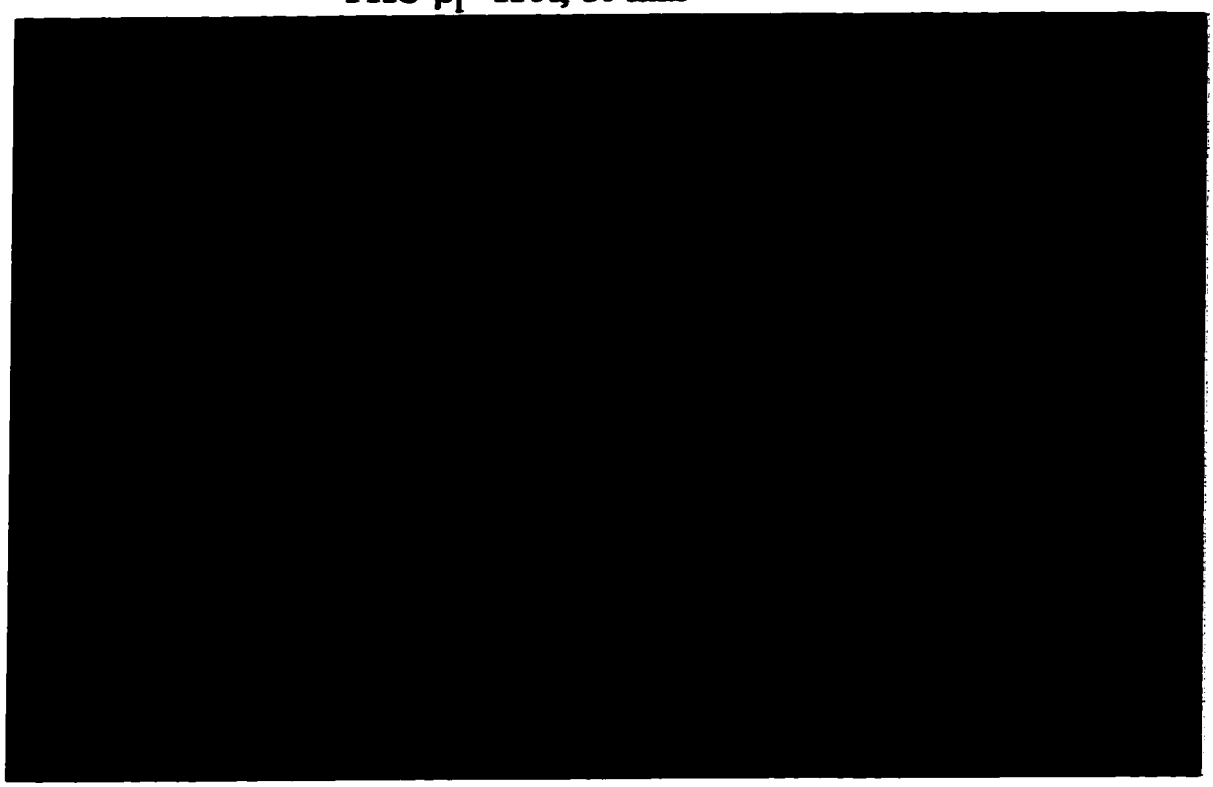
PKC-β_I+TPA, 5 min

(b)



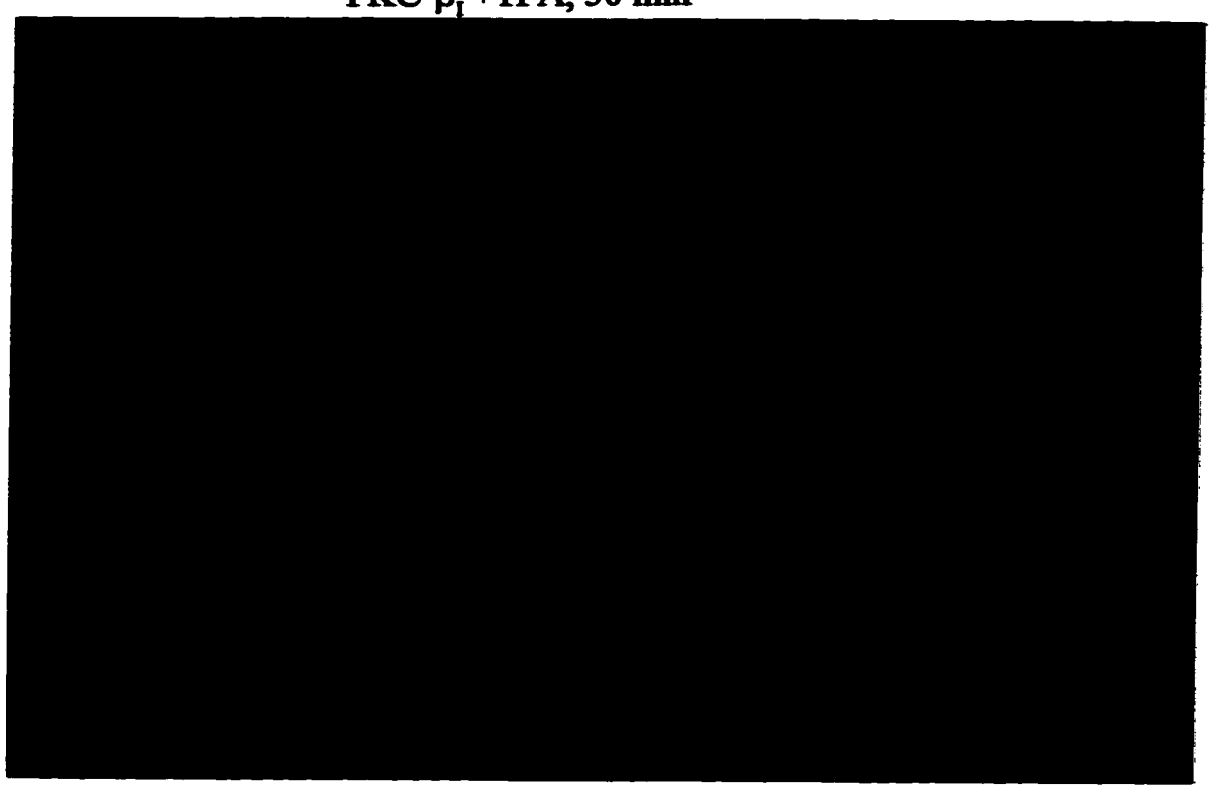
PKC-β_I+TPA, 10 min

(c)



PKC-β_I +TPA, 30 min

(d)



PKC-β_I +TPA, 24 h

(e)

Figure 3.17

Effects of TPA Treatment on the *In vivo* Phosphorylation of Nuclear Proteins of Quiescent Serum-Deprived T51B Cells

T51B cells were plated at a density of 125 x 10^2 cells/cm^2 in medium containing 10% serum. Confluent cells were then serum-deprived for 48 h and then labeled for 3 h with [^{32}P]PO_4 as described in 3.3.6. 100 mM TPA was added to cells for the indicated times. Lane 1, commassie stained lane; Lane 2, no treatment; Lane 3, 5 min TPA; Lane 4, 10 min TPA; Lane 5, 30 min TPA; Lane 6, 24 h TPA. Cells were then homogenized and fractionated into cytosol, membrane and nuclear fractions as described in 3.2.2 and 3.3.6. Nuclear samples were then solubilized in SDS sample buffer. 20 μg of protein/lane was electrophoresed on 10% SDS-PAGE. Gels were stained and autoradiographed as described in 3.3.6. Molecular weight is expressed as kilodalton (kD). The arrows represent the position of the molecular weight (M.W.) standards on the gel. These M.W. standards are as follows: the 93 kD is phosphorylase B, the 66 kD is Bovine Serum Albumin, the 45 kD is Ovalbumin, the 31 kD is Carbonic Anhydrase, the 21 kD is Soybean Trypsin Inhibitor and the 14 kD is Lysozyme.

Figure 3.17

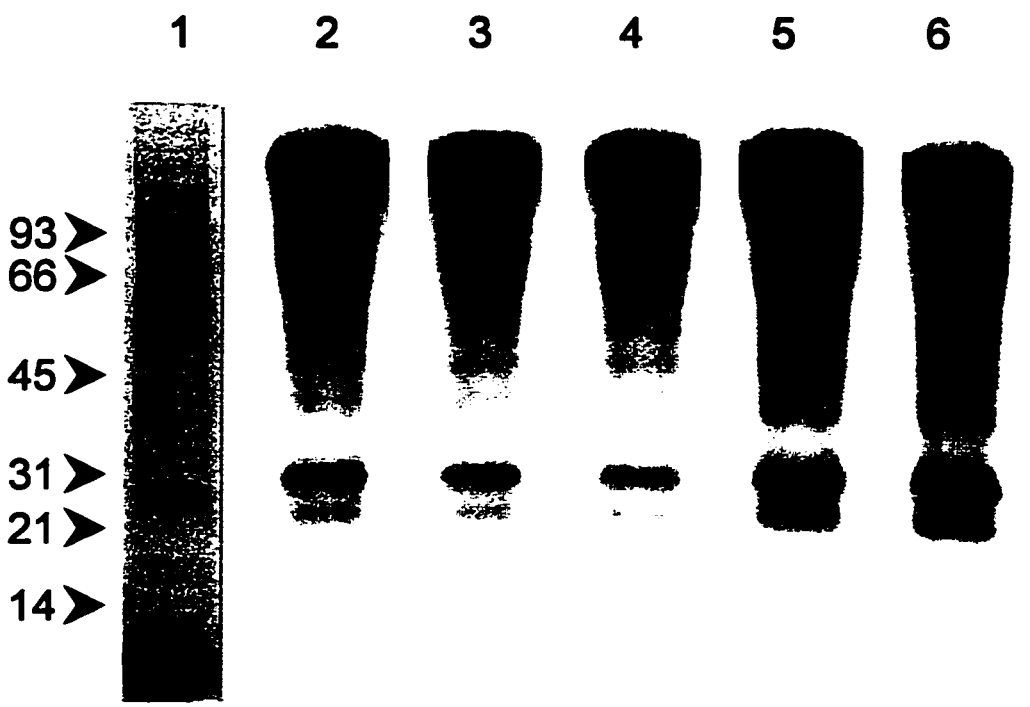
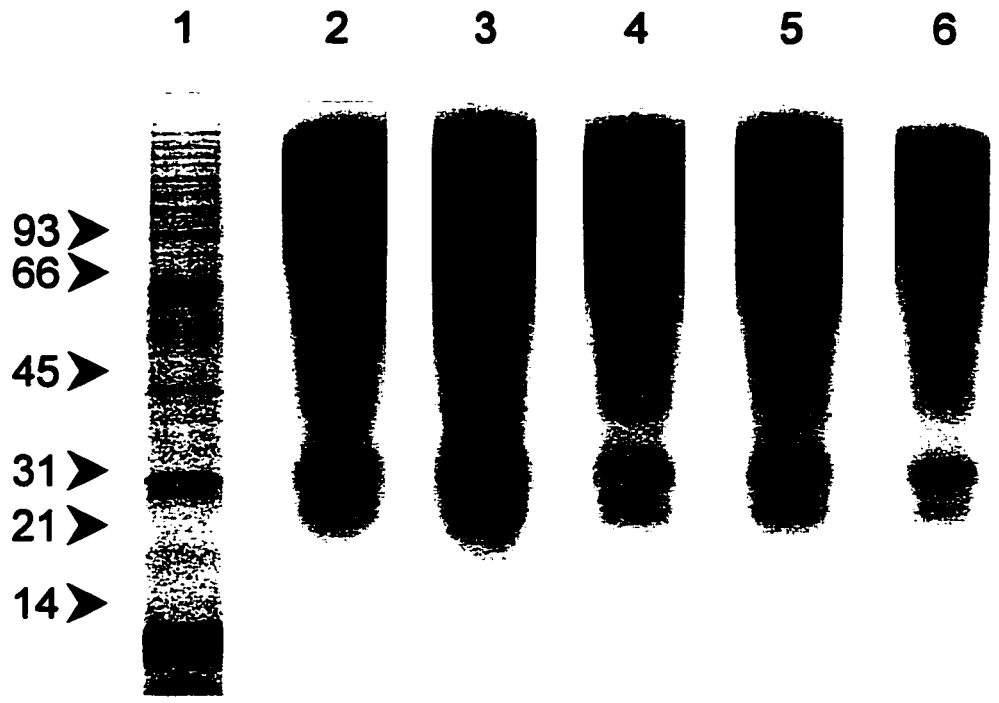


Figure 3.18

Effect of Serum-Re-addition on the *In vivo* Phosphorylation of Nuclear Proteins of Quiescent Serum-Deprived T51B Cells

T51B cells were plated at a density of 125 x 10^2 cells/cm^2 in medium containing 10% serum. Confluent cells were then serum-deprived for 48 h and then labeled for 3 h with [^{32}P]PO_4 as described in 3.3.6. The cells were then fluid changed to a medium containing 10% serum for the indicated times. Lane 1, commassie stained gel; Lane 2, no treatment; Lane 3, 5 min in serum; Lane 4, 10 min in serum; Lane 5, 30 min in serum; Lane 6, 24 h in serum. Cells were then homogenized and fractionated into cytosol, membrane and nuclear fractions as described in 3.2.2 and 3.3.6. Nuclear samples were then solubilized in SDS sample buffer. 20 μg of protein/lane was electrophoresed on 10% SDS-PAGE. Gels were stained and autoradiographed as described in 3.3.6. Molecular weight is expressed as kilodalton (kD). The arrows represent the position of the molecular weight (M.W.) standards on the gel. These M.W. standards are as follows: the 93 kD is phosphorylase B, the 66 kD is Bovine Serum Albumin, the 45 kD is Ovalbumin, the 31 kD is Carbonic Anhydrase, the 21 kD is Soybean Trypsin Inhibitor and the 14 kD is Lysozyme.

Figure 3.18



3.5 Discussion

3.5.1 Subcellular Localization of PKC Isozymes as the Cells Approach Confluence

Immunofluorescence and immunoblot results clearly demonstrate a change in the localization of PKC-β_I as the cells become confluent (Figs. 3.1-3.4). The results also indicate that PKC-β_I is endogenously localized in the nucleus and appears to move to the cytoplasm and the plasma membrane as the cells reach confluence. Immunoblotting results also show a decrease in the level of PKC-β_I in the nuclear fractions derived from these cells at day 3 of plating (Table 3.11) by which time PKC-β_I appears in plasma membrane and slightly in the cytoplasm (Fig. 3.2). However, the nuclear level of the two PKC-β_I bands at day 5 and day 8 is similar to that detcted for day 1 and day 2 (Table 3.11). On the other hand, no significant changes are observed in the localization of PKC-α, -δ, -ϵ but rather a slight increase in the immunofluorescence, especially in the plasma membrane (Figs. 3.5-3.7). However, the immunostaining of PKC-ζ appears to increase in the plasma membrane as the cells grow and become confluent (Fig. 3.8a,b). Hence, these results are the first to demonstrate a differential localization of PKC isozymes as the cells approach confluence. The observed change in the localization of PKC-β_I ,to my knowledge, has not been reported before in any cell system. Immunofluorescence data (Figs. 3.1-3.4) also demonstrate that PKC-β_I is totally associated with the nucleus and the nuclear membrane for the first two days after plating at which time the cells are sparse in culture. As the cells establish contact with each other, as they increase in number, PKC-β_I starts to appear in the cytoplasm and plasma membrane, where punctate fluorescence staining at the cell-cell contacts is observed. Immunoblotting results (Fig. 3.12a) also indicate an increase in the level of PKC-β_I in plasma membrane derived from these cells at various time points after plating. The scanning results

of plasma membrane blots (Table 3.12) demonstrate a differential increase in the intensity level of the two bands of PKC-β_I in plasma membrane as cells reach confluence. These results may suggest a differential role for the two subspecies of PKC-β_I in transducing the signal across the plasma membrane. The present data also may suggest a possible role for PKC-β_I in cell-cell contact formation. Indeed, previous studies in human squamous carcinoma cells have indicated that TPA induces a transient increase in membrane-bound PKC activity in association with cell-cell contact formation (Sheu et al., 1989). Moreover, immunofluorescence studies have shown that TPA, PDBu or 1,2-dioctanoylglycerol (DOG) induces rapid cell-cell contacts and re-distribution of desmoplakins (major components of desmosomes) from the cytoplasm to the desmosomes in the plasma membrane (a major cell-cell adhesion structure) (Sheu et al., 1989). Similar results have also been reported in kidney epithelial cells (Pasdar and Nelson, 1988).

Several studies have demonstrated that PKC can directly interact with target proteins in the cytoskeleton by phosphorylating proteins that regulate cytoskeleton integrity (Kiley et al., 1992). Of particular interest is adducin, a calmodulin-binding protein that promotes binding of spectrin to actin. Both of these proteins are cytoskeleton proteins present in a detergent-stable form and are concentrated at sites of cell-cell contact in cultural epithelial cells (Nelson and Veshnock, 1987). Previous immunofluorescence studies have indicated the presence of high level of adducin at sites of cell-cell contact in epithelial cells such as MDCK (Madin-Darby canine kidney) (Kaiser et al., 1989). Moreover, double labeling experiments have shown that vinculin (component of intracellular junctions) co-localizes with adducin at cell-cell contacts where punctate fluorescence is observed (Kaiser et al., 1989). It has been shown that adducin and vinculin can be phosphorylated by PKC *in vitro*

and *in vivo* (Werth et al., 1984; Bennett et al., 1988). Although functional consequences of phosphorylation are not yet known, it may affect the re-distribution of these proteins at cell-cell contacts. Indeed, TPA treatment of MDCK cells has been shown to result in a re-distribution of adducin at cell-cell contacts (Kaiser et al., 1989). The increase in the immunofluorescence staining of PKC-β_I along the cells boundaries suggest a possible role for PKC-β_I in phosphorylating these proteins which promotes cell-cell contact formation. Since other PKC isozymes (α, δ, ϵ and ζ) are localized in the plasma membrane of T51B cells, they may also contribute in the phosphorylation of these cytoskeleton proteins.

Future studies using monoclonal antibodies for these cytoskeleton proteins will be required to determine the localization of these proteins in T51B cells and whether they are co-localized with specific PKC isozymes expressed in these cells. In addition [^{32}P] prelabeling and immunoprecipitation experiments in response to TPA treatment will be useful in determining potential PKC target proteins that affect the cytoskeleton reorganization.

Immunofluorescence results also indicate that while the membrane-associated level of PKC-β_I and -ζ increases as the cells reach confluence, only a slight increase in the level of membrane-associated PKC-α, -δ, -ϵ is observed. Earlier studies have shown that actively proliferating 10T1/2 cells display PKC phosphorylating activity in the membrane and the cytosolic fractions. However, the activity of PKC is higher in the cytosol of confluent cells than that of the actively proliferating cells (Miloszewska et al., 1991). The authors, however, have not indicated which PKC isozyme is expressed in these cells and is involved in such changes. Recently, Moreton et al., 1995 have shown that the total PKC activity and the protein level of PKC-α and -δ increases as C6 glioma cells grow from low density to the

contact-inhibited quiescent G_0 state in the presence of serum. At the same time, the protein level of PKC-ϵ and -γ decreases while the levels of -β_I, -β_{II}, -δ and -ζ isozymes have not changed. The authors have suggested that the changes in PKC subspecies may regulate the entry of these cells to G_0 or G_1 phase. The differences between my results and the data reported by Moreton et al. could be due to variations in species, cell types and experimental conditions. In addition, the growth pattern of T51B cells appears to be different from that of C6 cells. Flow cytometric data (Chapter Two) indicate that although cells are confluent after 8 days of plating in the presence of 10% serum, some cells (9%) are still proliferating in the S phase. Hence, the establishment of confluence in T51B cells may involve growth arrest as a result of contact inhibition yet, the cells seem to lack the signal that stop them completely from proliferating and become absolutely contact-inhibited as seen in C6 cells. Indeed, Brabyn et al., 1994 have indicated that some DNA synthesis is still observed in confluent serum-stimulated T51B cells. Exactly how the changes in PKC isozymes are triggered as the cells grow and become confluent are not yet known. Increasing the surface contact between the cells as they approach confluence could influence such changes and may contribute in increasing the level of PKC-β_I and -ζ associated with the plasma membranes of T51B cells. Such an increase may be involved in enhancing the cells to reach and establish confluence. Because PKC-β_I is associated only with the nucleus and the nuclear membrane after 1-2 days of plating and moves to the plasma membrane as the cells reach confluence, and since no major changes in the nuclear localization of PKC-α, -δ, -ϵ or -ζ are noted, it is possible that the nuclear PKC-β_I could be involved in the proliferative signal. This may enhance cell proliferation. Once the cells start to approach confluence, the level of PKC-β_I and PKC-ζ increases in the plasma membranes especially at cell-cell contacts.

Perhaps, such an increase could promote the cells to reach confluence and to become growth-arrested by contact inhibition which is not an absolute phenomenon in T51B cells (9% of the cells are still proliferating in confluent cultures) Future studies, including activity measurements of PKC isozymes in T51B cells at various times after plating in the presence of 10% serum, will be helpful in determining the role of specific PKC isozyme in the establishment of confluence in T51B cells.

The nuclear localization of PKC-β_I in T51B cells may be a unique phenomenon for these types of cells because this isozyme is endogenously localized in the nuclei under normal growth conditions without any external stimuli. Other results have indicated that PKC-β (isoform unspecified) is endogenously present in the nuclei of rat liver, (Masmoudi et al., 1989; Rogue et al., 1990). In other cell systems, however, PKC-β translocates to the nucleus upon stimulation with various stimuli. For example, in HL-60 and K562 leukemia cells, PKC-β_{II} has been shown to translocate to the nuclei when these cells are treated with bryostatin-1 (a non-tumor promoting agent and an activator of PKC) (Hocevar and Fields, 1991; Hocevar et al., 1992). Similarly, PKC-β_{II} translocates to the nuclei of cardiac myocytes upon TPA or norepinephrine treatment (Disatnik et al., 1994b). In MDBK kidney epithelial cells, 1,25-dihydroxy vitamin D_3 induces translocation of PKC-β to the nucleus (Simboli-Campbell et al., 1994). Furthermore, in vascular epithelial cells as well as in neutrophils (isoform unspecified), PKC-β translocates to the nucleus upon stimulation with TPA (Rosales et al., 1992; Perletti et al., 1991). In the present study, however, it is the PKC-β_I isoform that is endogenously localized in the nuclei of T51B cells. Such finding is novel for T51B cells since in other cell systems, PKC-β or -β_{II} isoform has been found to be endogenously localized or translocates to the nucleus upon stimulation.

The nuclear localization of PKC-β_I observed in the present study strongly suggests a possible role for PKC-β_I in cell proliferation because this isozyme is observed to be associated only with the nucleus and the nuclear membrane after 1-2 days of plating at which time most of the cells are in the G_1 phase of cell cycle (see Chapter Two). Hence, the nuclear PKC-β_I may be involved in the proliferative signal that promotes the cells to progress in the cycle. Indeed, previous studies have shown that PKC activity increases transiently during the two Ca^{+2}-dependent $G_0 \rightarrow G_1$ and $G_1 \rightarrow S$ transition of cell cycle in T51B cells (Boynton et al., 1985a,b). It has also been demonstrated that tumor promoters such as TPA, that stimulates PKC activity in an extract from T51B cells in the absence of Ca^{2+}, enables these cells to transit G_1 in Ca^{2+}-deficient medium (Boynton et al., 1985a,b; Kleine et al., 1986; Boynton et al., 1985c). In addition, Sharma et al., 1994 have shown that EGF treatment of serum-deprived T51B cells results in an increase in the DNA synthesis which is accompanied by a rapid increase in the membrane-associated PKC activity, suggesting a possible role for PKC in the mitogenic action of EGF. However, these reports have not specified which PKC isozyme is involved in the mitogenic action of EGF, Ca^{2+}-dependent proliferative signals and TPA effects in T51B cells. Hence, it is attractive to speculate that PKC-β_I is the isozyme that is involved in the proliferative signal, because results from Chapter One demonstrate that this isozyme is up-regulated by TPA and EGF treatment. However, PKC-β_I must have other functions in the nucleus since at confluence (day 8) most of PKC-β_I is still observed in the nucleus, yet the cells are mostly quiescent (90%). The involvement of PKC-β_I in cell proliferation has been shown in rat fibroblasts, where overexpression of PKC-β_I enhances growth rates and increases saturation densities (Housey et al., 1988; Persons et al., 1988). However, in other cell systems PKC-β_{II} has been shown to be the isozyme that is involved

in cell proliferation. Furthermore, proliferation of TPA-treated K562 cells is partially inhibited by an antisense PKC-β_{II} oligodeoxynucleotide, suggesting that PKC-β_{II} is required for K562 cell proliferation (Murray et al., 1993). Such differences in the effects of PKC isoforms could be due to differences in cell types.

Immunofluorescence results also demonstrate the association of PKC-α, -δ, -ϵ and -ζ with the nuclei of T51B cells which is found to be not highly specific in the case of PKC-α as examined by the inclusion of specific antigenic peptide (Chapter One). However, the nuclear level of these isozymes is almost the same in sparse and confluent cultures (Figs. 3.5-3.8). Nevertheless, these isozymes may still be involved in the proliferation of T51B cells.

One question is: how is this nuclear PKC activated and how does it regulate nuclear functions? Evidence has been presented that demonstrates the presence of the PI cycle in the nucleus (Irvine and Divecha, 1992). Furthermore, Ca^{2+} ions are known to be present inside the nucleus and to be mobilized by IP_3 for activating nuclear PKC (Martelli et al., 1990). It has been shown that treatment of Swiss 3T3 cells with both IGF-1 and bombesin induces proliferation and translocation of PKC to the nuclei as well as specific generation of DAG in the nuclei (Divecha et al., 1991). Moreover, Banfic et al., 1993 have indicated that after partial hepatectomy, there is a rise in nuclear DAG levels and a translocation of PKC to the nucleus. Kuriki et al., 1992 have also demonstrated that during liver regeneration, there is a transient increase in the nuclear PKC activity in the S phase (20-22 h post-hepatectomy) which is associated with an increase in the activity of DNA polymerase α (a marker for S phase cells; Davis et al., 1976). Thus, the nuclear PI cycle may be important for the regulation of PKC activity within the nucleus during specific points in the cell cycle. Since T51B cells are a rat liver epithelial cell line established from the liver of a normal adult rat,

I would speculate that the PI cycle may exist in the nuclei of these cells and possibly be involved in the activation of nuclear PKC-β_I. However, other sources of DAG other than phosphatidylinositol hydrolysis exist. It has been shown that treatment of IIC9 fibroblasts with α-thrombin increases the level of nuclear DAG via hydrolysis of both phosphoinositides and phosphatidylcholine (PC) parallel to the translocation of PKC-α to the nucleus (Leach et al., 1992; Jarpe et al., 1994). The mechanism of nuclear PC hydrolysis and the location and function of phospholipase D (PLD) in the nucleus has not yet been determined. Since PKC-α, -δ, -ϵ and -ζ are also localized in the nuclei of T51B cells, it is possible that these isozymes may be activated via PC and/or PI hydrolysis in the nucleus. For example, PKC-ζ has been shown to be activated by phosphatidylinositol 3,4,5-trisphosphate (PIP3) (Nakanishi et al., 1993) and arachidonic acid (AA) (Nakanishi and Exton, 1992), a primary product of PC hydrolysis (Exton, 1994). Hence, future studies will be directed to reveal the role of the PI cycle or PC hydrolysis as sources of nuclear DAG in regulating the nuclear PKC-β_I and other nuclear PKC isoforms. Measurements of nuclear PKCs activities would be helpful in determining the possible roles of these isozymes in the nuclear events.

3.5.2 Immunofluorescence Localization of PKC-β_I in Dividing Cells

Dual immunofluorescence and Hoechst staining method is performed to stain the DNA and observe the subcellular localization of PKC-β_I in dividing cells. Immunofluorescence results (Fig. 3.9b) clearly indicate a strong immunostaining of PKC-β_I in the dividing cells compared to non-dividing cells with intact nuclei. Dual Hoechst and immunofluorescence result (Fig. 3.9c) shows that the dividing cell is in the early telophase of mitosis and at this stage a higher level of PKC-β_I is observed in the dividing cell compared to non-dividing cells with intact nuclei. The present data is very interesting because it is the

first to demonstrate an increase in the level of PKC-β_I during mitosis, which is a novel finding. Hence, these results may strongly suggest a possible role for nuclear PKC-β_I in the cell division of T51B cells. The exact role of nuclear PKC-β_I in cell division is not yet known. However, several studies have implicated PKC in the regulation of a number of nuclear proteins that are substrates for PKC and are involved in DNA replication or mitotic processes as discussed in the Introduction, section 3.1.2.

3.5.3 Effects of Serum Re-addition on the Localization of PKC-β_I in T51B Cells

Immunofluorescence results demonstrate an increase in the immunostaining of PKC-β_I in the nuclei of T51B cells and to some extent in the plasma membranes after 24 h of serum re-addition to quiescent serum-deprived cells (Fig. 3.13b) compared to control serum-deprived cells (Fig. 3.13a). The immunoblotting results (Fig. 3.14 and Table 3.14) indicates an increase in the level of PKC-β_I in the nuclear fraction derived from these cells after re-addition of serum compared to control serum-deprived cells. The present data may suggest a possible involvement of nuclear PKC-β_I in the cell cycle progression of T51B cells probably in $G_1 \rightarrow S$ transition. This possibility is supported by: (a) the flow cytometric data (Chapter Two) which indicate that one day after plating most of the cells are in the G_1 phase of the cycle; and (b) the immunofluorescence results which demonstrate that PKC-β_I is associated only with the nucleus and the nuclear membrane one day after plating with the highest level during mitosis (section 3.4.1a). Hence, the increase in the nuclear level of PKC-β_I after serum re-addition may trigger $G_1 \rightarrow S$ transition. Previous studies on T51B cells (Sharma et al., 1994) in our laboratory have shown that serum-deprivation stops the proliferation of T51B cells in the G_0/G_1 transition of their growth division cycle. When these cells are treated with EGF there is an increase in DNA synthesis as the cells progress in the

cycle with a rapid increase in the membrane-associated PKC activity which remains elevated for up to 24 h in the presence of EGF. In their study, however, they have not measured the nuclear PKC activity, nor have they specified which PKC isoform is involved in the EGF effects. Thus, it is attractive to speculate that PKC-β_I may be the isoform that is involved in the cell cycle progression of T51B cells.

Future studies using cell cycle blockers in combination with specific PKC inhibitors including specific PKC-β_I peptide inhibitor as well as the intracellular delivery of PKC-β_I antibody or antisense oligodeoxynucleotides that result in inactivation or depletion of PKC-β_I from the cells would be very useful in revealing the role of PKC-β_I in cell cycle transitions.

3.5.4 Effects of TPA on the Immunofluorescence Localization of PKC-β_I in T51B Cells

Immunofluorescence results demonstrate that treatment of T51B cells with 100 nM TPA after one day of plating resulted in redistribution of nuclear PKC-β_I immunofluorescence between nuclear, cytosolic and plasma membrane compartments which is time-dependent (Fig. 3.15a-e). Interestingly, in control cultures the immunofluorescence of PKC-β_I is only observed in the nucleus (Fig. 3.15a). Within 5 min of TPA addition, PKC-β_I immunostaining appears in the cytoplasm and some areas along the plasma membrane (Fig. 3.15b). The immunofluorescence of PKC-β_I associated with the nuclear, the cytoplasmic and the membrane compartments is observed to be maximum after 10 min of TPA addition (Fig. 3.15c). After 24 h of TPA exposure, the immunostaining of PKC-β_I is still present at the cell-cell contacts forming distinct bright lines (Fig. 3.15e) but a decrease in the immunostaining of PKC-β_I in the cytoplasm is observed. These results suggest that TPA treatment induces rapid re-distribution or movement of PKC-β_I between the nuclear, the cytoplasmic and the plasma membrane compartments in proliferating serum-stimulated

T51B cells. In serum-deprived cells, TPA treatment induces a slight increase in the immunofluorescence of PKC-β_I associated with the nuclei and the plasma membranes after 24 h as compared to serum-deprived cells. No change has been observed at 5, 10 and 30 min of TPA addition (Fig. 3.16a-e). These results are almost similar to that noted for confluent serum-stimulated cells after 24 h of TPA addition (Chapter One). However, the immunofluoresence of nuclear PKC-β_I seems to be slightly higher in serum-deprived than serum-stimulated cells after 24 h of TPA treatment. Such difference could be due to differences in the experimental conditions and in the responsiveness of the cells to TPA in the presence or absence of 10% serum-containing medium. Nevertheless, the present data demonstrate that treatment of serum-deprived cells with TPA up-regulates PKC-β_I in the plasma membrane and the nucleus. Previous work on T51B cells (Sharma et al., 1994) have indicated that treatment of serum-deprived cells for 18 h with TPA decreases membrane-associated PKC activity. However, the authors have not specified which PKC isoform is involved in the TPA effect. Since TPA is observed to down-regulate membrane-associated PKC-α, -δ and -ϵ with no effect on PKC-ζ (Chapter One), the observed effects of TPA on PKC activity in the experiments of Sharma et al could be due to the other PKC isoforms associated with the membrane fractions.

The present results are very interesting and novel because such effects of TPA on PKC-β_I have not been reported before for T51B cells or for other types of cells. Previous studies have shown that treatment of neutrophils with TPA causes a 3.8-fold increase in the endogenous nuclear PKC-β activity (isoform not specified) (Perletti et al., 1991). Furthermore, studies in rat liver have indicated that PKC-β (isoform not specified) endogenous to the nuclei can be activated (Bukley et al., 1988), as evidenced by several

hundred-fold increase in nuclear PKC activity following treatment of isolated nuclei with TPA. In cultures of tumor cell lines, it has been found that treatment with TPA causes translocation of PKC-α to the nuclei of HL-60 (Thomas et al., 1988), NIH 3T3 (Leach et al., 1989) and IIC9 cells (Leach et al., 1992). In other cells, however, treatment with TPA can cause a re-distribution of PKC to the particulate fraction (isoform not specified) (Kraft and Anderson, 1983; Nishizuka, 1988). Such differences could be due to variation in cell types. The present data also demonstrate an increase in the immunofluorescence of PKC-β_I in the cytosol and plasma membrane after TPA addition especially in areas of cell-cell contacts. Hence, one could speculate that PKC-β_I may be involved directly or indirectly in cell-cell contact formation through phosphorylation of specific cytoskeletal proteins such as adducin and vinculin which have been reported to be involved in cell-cell contact formation as discussed earlier. Other possible functions for the increased membrane-associated level PKC-β_I in response to TPA could be the signaling at the level of the plasma membrane. Such effects may be involved in the phosphorylation of membrane and cytosolic proteins which in turn may activate "downstream" protein kinase cascades leading to the nucleus.

Further experiments, including activity measurements of individual PKC isoform in response to TPA in the cytosolic, the membrane and the nuclear fractions of proliferating serum-stimulated or quiescent serum-deprived T51B cells, would be helpful in determining the possible role of PKC isozymes in the signal transduction process.

3.5.5 Effects of TPA and Serum Re-addition on the *In vivo* Phosphorylation of Nuclear Proteins in Serum-Deprived T51B Cells

Autoradiographs of labeled nuclear fractions (Fig. 3.17) demonstrate an increase in the phosphorylation of two nuclear proteins after 30 min of TPA addition and this level of phosphorylation is maintained for up to 24 h as compared to control untreated cultures.

These nuclear proteins have a molecular weight of approximately 21 and 31 kD. These results are consistent with other studies which have indicated an increase in the phosphorylation of nuclear proteins with a molecular weight nearly identical to those observed in my study in response to 1,25-dihydroxy vitamin D_3 (Martell et al., 1992) and a combination of IGF-1 and bombesin (Martelli et al., 1989). Moreover, a low dose and a short exposure to TPA has been found to enhance the activity of a nuclear-associated PKC with an increase in the phosphorylation of nuclear proteins that are almost similar to those observed in my experiments (Thomas et al., 1988). The 31 kD nuclear protein has been identified as histone H1 (Martelli et al., 1989; Martelli et al., 1990), which is a basic protein found to be associated with DNA and is thought to play a regulatory role in cell cycle events such as mitosis and chromatin condensation (Hill et al., 1991). Phosphorylation of histone H1 has been shown to be high in mitosis and low in S phase, suggesting a role of histone H1 in the cell cycle (Matthew and Huebner, 1984). It has been demonstrated that nuclei purified from Swiss 3T3 cells treated with a mitogenic combination of bombesin and IGF-1 contain a PKC activity which selectively phosphorylates *in vitro* two nuclear proteins of approximately 21 and 31 kD (Martelli et al., 1990). The 21 kD nuclear protein has been identified as a high mobility group non-histone DNA binding protein. The same proteins are hyper-phosphorylated *in vivo* within 45 min of exposure of quiescent 3T3 cells to the same drugs (Martelli et al., 1989). Other studies have indicated that exogenous histone H1 is hyperphosphorylated in 22 h regenerating rat liver nuclei (Martelli et al., 1991). The authors have also indicated that the phosphotransferase activity involved in this phenomenon is completely suppressed by staurosporine (a non selective PKC inhibitor) and largely inhibited by an anti-PKC antibody, suggesting the involvement of PKC for the observed effects. It is

worth noting that phosphorylation of histone H1 is associated with cell proliferation in regenerating rat liver, and different histone H1 subspecies are phosphorylated to different extents (Laks et al., 1981). Phosphorylation of nuclear lamins has also been reported to occur during mitosis (Ottaviano and Gerace, 1985) and takes place in quiescent 3T3 cells after only 10 min of insulin treatment (Friedman and Ken, 1988). Recently, Goss et al., 1994 have shown that PKC-β_{II} translocates to the nucleus concomitant with the phosphorylation of lamin B. However, in the present study I could not detect any increase in the phosphorylation of nuclear lamins in response to TPA. Hence, it is possible that lamin B may not be a specific substrate for PKC-β_I. More studies are required to address this possibility.

The above studies together with the present findings strongly suggest a possible involvement of nuclear PKC-β_I in the phosphorylation of the 21 and the 31 kD nuclear proteins because: (a) TPA treatment induces an increase in the phosphorylation of the above nuclear proteins after 30 min that remains elevated for up to 24 h (Fig. 3.18); and (b) it produces a slight increase in the immunofluoresence of nuclear PKC-β_I (Fig. 3.16a-e). However, due to the nuclear association of PKC-α, -δ, -ϵ and -ζ, it is also possible that these isozymes may phosphorylate the same nuclear proteins observed in the present study. This possibility is less likely since TPA treatment has no effect on the nuclear localization of these isozymes (Chapter One). Nevertheless, one should not completely rule out this possibility since the activities of these isozymes were not looked at.

Because of the association of nuclear protein phosphorylation especially histone H1 with the cell cycle (Matthews and Huebner, 1984; Friedman and Ken 1988; Hill et al., 1991), the localization of PKC-β_I in the nuclei of T51B cells with the highest level during mitosis,

it is attractive to speculate that the nuclear PKC-β_I may be involved in the nuclear protein phosphorylation especially the 31 kD protein which is probably histone H1. Future experiments including [^{32}P] prelabeling and immunoprecipitation of histone H1 using anti histone H1 antibody are required to confirm these findings.

The present data also demonstrate an increase in the phosphorylation of two nuclear proteins with a molecular weight of approximately 21 and 31 kD after 5 min of serum re-addition to quiescent T51B cells (Fig. 3.18). This level of phosphorylation decreases within 10 min of serum-stimulation. The pattern of the phosphorylation of nuclear proteins is similar to that noted in TPA treated cells, except that the increase in the phosphorylation of these nuclear proteins is observed to occur after 5 min of serum re-addition which is a rapid effect. The difference in the time of stimulation could be due to the difference between TPA and serum in their mode of stimulation. In the case of serum-stimulation, it has been shown that serum re-addition induces a variety of changes in the cell signaling network including those leading to transcriptional activation of sets of early response genes, subsequently followed by DNA synthesis. However, the processes involved in serum-stimulation are rather complex (Ohno et al., 1994).

In the present study serum-stimulation induces a rapid (5 min) increase in the phosphorylation of two nuclear proteins (31 kD and 21 kD). The mechanism by which serum-stimulation produces this response may be due to the following possibilities: (a) serum-re-addition may induce rapid activation of nuclear PKC-β_I which leads to the observed effect, since serum re-addition to serum-deprived T51B cells increases the nuclear level of PKC-β_I; (b) other nuclear associated PKC such as -α, -δ, -ϵ and/or -ζ may also be activated in response to serum and leads to a rapid increase in the phosphorylation of these nuclear

proteins; (c) the rapid increase in the phosphorylation of the nuclear proteins may be achieved by the serum-stimulated activation of other nuclear enzymes such as histone kinases especially "M phase-specific" histone H1 (Arion et al., 1988); and (d) serum-stimulation may generate signal(s) at the level of the plasma membrane activating tyrosine kinases and/or other kinases including PKCs which may induce a cascade of signals to the cell nucleus activating nuclear PKCs or other nuclear kinases that lead to the observed effects.

Because both TPA treatment and serum re-addition increase the nuclear level of PKC-β_I within 24 h of addition (Figs. 3.13 and 3.16), they induce an increase in the phosphorylation of the same nuclear proteins, and since TPA has no effect on the nuclear level of the other PKC isoforms, it is most likely that nuclear PKC-β_I is the isozyme that is involved in such effects.

3.6 Conclusion

The overall conclusion of this chapter is that there is a change in the localization of PKC-β_I as the cells approach confluence. Furthermore TPA treatment and serum-re-addition to serum-deprived T51B cells results in an increase in the phosphorylation of two nuclear proteins. The specific conclusions are:

3.6.1 Immunofluorescence and immunoblot results demonstrate a change in the localization of PKC-β_I as the cells reach confluence. It is totally localized in the nucleus after 1 day of plating and partially moves to the cytoplasm and plasma membrane as cells approach confluence.

3.6.2 PKC-α, -δ, -ϵ and -ζ are localized in the nucleus, cytoplasm and the plasma membranes. Their immunofluorescence increases slightly in the plasma membranes with a higher level in the case of PKC-ζ as the cells reach confluence.

3.6.3 High level of PKC-β_I is observed in dividing cells (during mitosis) compared to intact non-dividing cells.

3.6.4 Re-addition of 10% serum to serum-deprived T51B cells results in an increase in the immunofluorescence of nuclear PKC-β_I, suggesting a possible involvement of this isozyme in the G_1 - S transition of the cycle.

3.6.5 Treatment of proliferating T51B cells (1 day after plating) with TPA results in a re-distribution of the nuclear PKC-β_I between the nucleus, the cytoplasm and the plasma membrane which is time dependent.

3.6.6 Treatment of serum-deprived cells with TPA induces a slight increase in the level of PKC-β_I associated with the nucleus and the plasma membrane after 24 h.

3.6.7 TPA treatment of serum-deprived T51B cells results in an increase in the phosphorylation of two nuclear proteins (21 and 31 kD) after 30 min and their level of phosphorylation is maintained for up to 24 h.

3.6.8 Re-addition of serum to serum-deprived cells increases the phosphorylation of the same nuclear proteins observed in TPA treated cells after 5 min which declines within 10 min of serum re-addition. PKC-β_I is probably involved in the observed TPA-mediated and serum-stimulated effects on the increased phosphorylation of these proteins.

CHAPTER FOUR

Establishment of Subcellular Localization of Protein Kinase C Isozymes in T51B Cells during Apoptosis and Effects of PKC Inhibitors and TPA on Apoptosis

4.1 Introduction

4.1.1 Regulation of Apoptosis

It is now widely recognized that apoptosis plays a central role in the regulation of a wide variety of tissue processes, both developmental and homeostatic. These include many aspects of immune function, for example the clonal selection of B lymphocytes (Liu et al., 1989), detection of autoreactive thymocytes (Smith et al., 1989), T-cell-mediated cell lysis (Rahelu et al., 1993) and apoptosis of neutrophils to mediate resolution of the acute inflammatory response (Savill et al., 1989). In liver, apoptosis has been found to be responsible for the regression of liver hyperplasia (Columbano et al., 1985) caused by the withdrawal of the stimuli such as EGF, and the regulation of cell turnover in the alteration of liver mass in tumor-bearing rats (Tesstore et al., 1989). Apoptosis is now being viewed as a fundamental process, on a par with cell proliferation and differentiation, that plays a major role in the day-to-day functioning of the organism (e.g., in tissue homeostasis). While apoptosis is an intrinsic property of cells, it can be modulated by extracellular factors, including cytokines (Afford et al., 1992). Our current understanding of the molecular species involved in the apoptotic process and those signaling mechanisms regulating apoptosis is incomplete. In many cell types de novo protein synthesis is not required for apoptosis (Cohen, 1991), and the post-translational modification of existing proteins is therefore likely to be involved in the regulation and initiation of apoptosis (Cohen, 1993). Both protein

kinases (Vintermeyr et al., 1993) and phosphatases (Song and Lavin, 1993) have been implicated in the regulation of apoptosis. Possible intracellular signaling mechanisms in the initiation of apoptosis include influx of calcium (McConkey et al., 1989a), altered expression of oncogenes c-fos and c-myc (Buttyan et al., 1988) and of PKC (Lucas et al., 1991).

PKC is involved in the regulation of many cellular events, including cell proliferation and differentiation (reviewed in Clemens et al., 1992). Both of these processes are central to the maintenance of tissue cell population, size and phenotype, and their dysregulation is a feature of neoplasia. PKC was initially implicated in the regulation of cell proliferation and differentiation following its identification as the cellular receptor for the tumor-promoting phorbol ester TPA. TPA is able to replace diacylglycerol (DAG), the physiological activator of PKC. Unlike DAG, TPA is not metabolized and produces a prolonged activation and ultimate down-regulation of PKC (Nishizuka, 1989). Modulation of apoptosis has now also been recognized as a mechanism involved in tumor promotion (Williams, 1993) and, consequently, PKC has been identified as an agent involved in the regulation of apoptosis.

4.1.2 Protein Kinase C and Apoptosis

The evidence suggesting that PKC plays a role in the regulation of apoptosis has come mainly from studies involving the use of TPA and inhibitors of PKC. Efforts to define a role for PKC in the induction of apoptosis have been complicated by conflicting reports. In rat thymocytes, activation of PKC by TPA blocks apoptosis when these cells are exposed to Ca^{2+} ionophore or glucocorticoid hormone. Also incubation with a non-specific PKC inhibitor H-7 induces apoptosis (McConkey et al., 1989b). Moreover, TPA inhibits Ca^{2+}-dependent DNA fragmentation in isolated thymocyte nuclei. The authors have suggested that PKC activation blocks apoptosis by preventing Ca^{2+}-stimulated endonuclease activation

(McConkey et al., 1989b). A similar effect of TPA in blocking apoptosis has been reported in human thymocytes (McConkey et al., 1989c; Szondy, 1994). In contrast, in mouse thymocytes, apoptosis induced by dexamethasone or by epidophyllotoxin VM-26 (an inhibitor of topoisomerase II) is blocked by H-7 and by sangivamycin (a specific inhibitor of PKC). However, apoptosis is relatively unaffected by HA-1004 which is a more specific inhibitor of PKA (Ye et al., 1993). Similarly, Ojeda et al., 1990 have reported that hydrocortisone-induced apoptosis in mouse thymocytes is blocked by H-7 while HA-1004 fails to do so. They have suggested that PKC activation is involved in hydrocortisone mediated apoptosis. Others have demonstrated that activation of PKC by TPA actually induces apoptosis in mouse thymocytes (Perdandones et al., 1993; Kizaki et al., 1989). It has been shown that in mouse and rat thymocytes, TPA potentiates DNA fragmentation after radiation. However, without radiation, TPA induces DNA fragmentation in mouse but not in rat thymocytes (Shaposhnikova et al., 1994).

Controversial data regarding PKC and apoptosis have also been reported in cells of myeloid origin. In HL-60 cells, TPA has been found to have little effect on apoptosis (Martin et al., 1994). Others have demonstrated that TPA suppresses apoptosis in HL-60 cells induced by the anti-cancer drug all-trans retinamide (HR) (Delia et al., 1995). In contrast, Macfarlane et al., 1988 have reported that the phorbol ester-sensitive (wild type) S variant of HL-60 cells undergo apoptosis when exposed to TPA. Moreover, the PKC-β selective activator 12-deoxyphorbol 13-phenylacetate-20-acetate (dPPA) induces both growth arrest and cell adherence as well as apoptosis in S cells but not in the phorbol ester-tolerant PET mutant of HL-60 cells (Macfarlane and Manzel, 1994). Likewise, in the myeloid cell line U937, phorbol ester dPPA, has been shown to induce apoptosis more

efficiently than TPA (Pongracz et al., 1994). Interestingly, in control cultures 43% of PKC-β_I is localized in the nuclei of these cells. However, after 1 h of dPPA treatment, PKC-β_I translocates to the cytoplasm and there is no down-regulation (Pongracz et al., 1994). The authors have proposed a possible role of PKC-β_I in the induction of apoptosis. Other studies have shown that acute exposure of HL-60 or U937 leukemia cell lines to sphingomyelinase or synthetic ceramide promotes apoptosis (Jarvis et al., 1994a; Obeid et al., 1993). In contrast, exposure of these cells to PLC or synthetic diglyceride fails to promote apoptosis and abolishes the lethal actions of ceramide (Jarvis et al., 1994a; Obeid et al., 1993). Furthermore, the authors have indicated that ceramide-induced apoptosis is also reduced by acute exposure to the tumor promoters phorbol dibutrate and mezerein as well as to the non-tumor-promoting agent bryostatin-1 (a macrocyclic lactone natural product, and a potential activator of PKC). Conversely, chronic (24 h) pretreatment with these agents fails to block ceramide actions (Jarvis et al., 1994a; Obeid et al., 1993). Moreover, the capacity of diglyceride to prevent very early genomic lesions (generation of DNA fragments) suggests that acute activation of PKC prevents apoptosis at an initial stage (Jarvis et al., 1994b). Similarly, the induction of apoptosis by a potent anti-cancer drug (ara C) is blocked by endogenous and exogenous diglycerides which moderately increase total PKC activity (Jarvis et al., 1994c). Using murine myeloid factor-dependent FDC-PI/ER cells, May et al., 1994 have shown that interleukin-3 (IL-3), erythropoietin or bryostatin-1 suppresses apoptosis and induces a rapid serine phosphorylation of bcl-2α following PKC activation. Furthermore, H-7 or staurosporine blocks not only IL-3 and bryostatin-1-induced hyperphosphorylation of bcl-2α but also their anti-apoptotic effect on growth factor-dependent cells (May et al., 1994). Recently, Jarvis et al., 1994d have studied the effect of several PKC inhibitors on

DNA fragmentation in HL-60 cells. They have indicated that transient exposure to chelerythrine and calphostin C (highly specific inhibitors of PKC) or H-7 and gossypol (non-specific PKC inhibitors) produces concentration dependent increase in DNA fragmentation that leads to apoptosis. The authors have suggested a protective involvement of basal PKC activity in the regulation of apoptosis in myeloid leukemia cells.

The role of PKC in induction of apoptosis has also been investigated in cells of lymphoid origin. For example, small dense splenic T or B lymphocytes undergo spontaneous apoptosis which is blocked by addition of interleukin-4 (IL-4) or TPA but not by the inactive phorbol ester 4α PDD. In contrast, H-7 or staurosporine increases the percentage of cells undergoing apoptosis (Illera et al., 1993; Perandones et al., 1993). Lu et al., 1994 have demonstrated that IL-2 induces a transient activation of an inactive pool of membrane associated PKC activity within 10 min of IL-2 addition. Moreover, the inhibition of PKC activity by a highly specific inhibitor bisinolylmaleimide (Bis) blocks the ability of IL-2 to suppress the onset of apoptosis in IL-2 and serum-deprived IL-2-dependent mouse T cell line (CTLL-2). The authors have suggested that the activation of membrane-associated PKC may be an important step in the mechanism(s) by which the cytokine suppresses cell death in T lymphocytes. Knox et al., 1992 have indicated that staurosporine induces apoptosis in group-I Burkitt's lymphoma cells when used at concentrations relatively selective for PKC activity. Other studies have shown that the induction of apoptosis in murine B cell lymphocytes WEHI-231 by dexamethasone, irradiation or anti-IgM, which increases ceramide production, could be blocked by TPA treatment (Quintans et al., 1994). Moreover, TPA has been shown to inhibit both ceramide production induced by these agents and the lethal effects of exogenously added ceramide (Gottschalk and Quintans et al., 1995). In contrast, Komada

et al, 1994 have demonstrated that in the human B lymphoma cell line MBC-1, cross-linking of surface immunoglubulin (SIgM) by anti-IgM antibodies and activation of PKC by TPA induce apoptosis. Furthermore, the ability of IL-4 to protect these cells from apoptosis suggests that this cytokine inhibits the transduction of the apoptotic signal following activation of PKC (Komada et al, 1994). Likewise, activation of PKC by exposure to TPA, either alone or in conjunction with Ca^{2+} ionophore induces apoptosis in a T cell hybridoma (Mercep et al., 1989).

In other cell systems, the published data regarding the phorbol ester TPA and PKC inhibitors are also conflicting. For example, Mailhos et al., 1994 have indicated that TPA synergizes with retinoic acid (RA) in the induction of apoptosis in the neuronal cell line ND7. On the other hand, chronic down-regulation of PKC dramatically reduces the ability of RA to induce apoptosis. Other studies have shown that the inhibition of PKC activity by H-7 prevents the cell killing and DNA fragmentation induced by etoposide (a topoisomerase II inhibitor) in mouse L929 fibroblasts (Mizumoto et al., 1994). These reports have suggested that PKC activation promotes apoptosis. In contrast, Tomei et al., 1988 have indicated that TPA inhibits apoptosis in cultures of C3H-10T1/2 mouse fibroblasts following either serum removal or exposure to beta or gamma irradiation. Likewise, TPA has been shown to protect the human breast cancer cell line MDA-231 from apoptosis induced by the anti-cancer drug adriamycin (Geier et al., 1994a). Others have shown that the PKC inhibitors staurosporine and tamoxifen induce apoptosis in the malignant glioma cell lines A172 and U87-MG. Moreover, down-regulation of PKC by chronic treatment with TPA also results in apoptosis (Couldwell et al., 1994). Other studies have demonstrated that TPA protects bovine aortic or human vascular endothelial cells from apoptosis induced by serum removal

or irradiation. However, treatment of cells with H-7 prevents the protective effect of TPA. (Araki et al., 1990; Haimovitz-Friedman et al., 1994). Similarly, in rat coronary vascular smooth muscle cells, calphostin C and the calcium channel blocker verapamil induce apoptosis (Leszczynski et al., 1994). Other workers (Sanchez et al., 1992) have shown that apoptosis of freshly isolated rat heptocytes is induced by serum removal or addition of PKC inhibitors polymyxin B or staurosporine. This effect is prevented by TPA treatment, however, after 8 h incubation, TPA fails to counteract this action and itself induces apoptosis. Thus, the above reports are consistent with the antagonistic influence of PKC activation in apoptosis.

Presently, little information is available concerning the specific role of PKC isozymes and their subcellular localization during the apoptotic process. Hence, it is of interest to investigate the possible roles of PKC isozymes and their subcellular localizations during apoptosis of T51B cells and the effects of a PKC inhibitor (bisindolylmaleimide) and a PKC activator (TPA) on this process.

4.2 Objectives

The overall objective of this chapter was to establish the subcellular localization of PKC isozymes in apoptotic cells and the effects of the PKC inhibitor, bisindolylmaleimide and the PKC activator, TPA, on apoptosis of T51B cells. The specific objectives were:

4.2.1 To establish the subcellular localization of PKC isozymes in apoptotic T51B cells by combined immunofluorescence and Hoechst staining methods.

4.2.2 To determine the effect of the PKC inhibitor, Bisindolylmaleimide (GF109203X) on apoptosis of T51B cells by Hoechst staining and TUNEL assay.

4.2.3 To investigate the effect of TPA on apoptosis of T51B cells by Hoechst staining.

4.3 Methods

4.3.1 Cell Culture

T51B cells were cultured in BME medium (Life Technologies, Inc.) supplemented with 10% (v/v) bovine calf serum (BCS) (Life technologies, Inc.) and 0.1% gentamycine sulfate (Sigma Chemical Co. Ltd.). The cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Prior to experiments, cells were detached by brief exposure to 0.05% (w/v) trypsin and 1 mM EDTA in PBS. Cells were plated on glass coverslips in culture dishes at a density of 125 x 10^2 cells/cm^2. For TPA or bisinolylmaleimide experiments, cells were plated on coverslips at the same density and after 24 h., they were treated with 100 nM TPA (in ethanol/DMSO, 50/50 v/v) (Life Technologies, Inc.) or 5 μM bisinolylmaleimide (GF 109203X; Calbiochem. Biochemicals) in DMSO. The vehicles for the agents to be added were DMSO or ethanol/DMSO. No significant changes in the morphology or in the viability of the cells was observed due to vehicles alone.

4.3.2 Hoechst Staining

Hoechst staining was performed as described in Chapter Two, section 2.3.3.

4.3.3 Dual Immunofluorescence and Hoechst Staining

Dual immunofluorescence and Hoechst staining was performed as described in Chapter Three, section 3.3.5

4.3.4 Dual Hoechst Staining and TUNEL Assay

Dual Hoechst staining and TUNEL assay was performed as described in Chapter Two, section 2.3.4.

4.4 Results

4.4.1 Subcellular Localization of PKC Isozymes during Apoptosis of T51B Cells

Dual immunofluorescence and Hoechst staining analysis is performed to determine the localization of individual PKC isozymes in apoptotic cells. In the present study confluent cells are viewed under an Olympus microscope equipped with a special cube for dual fluorescence (UV/FITC). For PKC-β_I, a dual Hoechst and immunofluorescence photograph (Fig. 4.1a) indicates the presence of both intact weakly stained uncondensed nuclei and a brightly stained condensed nucleus which has blue fluorescence staining. It also shows the association of PKC-β_I with the plasma membrane and cytoplasm which has a faint light green fluorescein staining. However, the nuclear staining of PKC-β_I is not observed. The immunofluorescence photograph of the same field of cells (Fig. 4.1b) indicates that PKC-β_I is not detectable in the apoptotic cell with brightly stained condensed nucleus shown in Figure 4.1a. In contrast, in intact viable cells, PKC-β_I immunofluorescence is observed associated with the nucleus, the nuclear and the plasma membranes and weakly with the cytoplasm. For PKC-α, a dual Hoechst and immunofluorescence photograph (Fig. 4.2a) shows the presence of brightly stained fragmented nuclei and weakly stained intact nuclei. It also indicates the presence of a faint green fluorescein staining of PKC-α associated with the plasma membrane and weakly with the cytoplasm. However, nuclear staining of PKC-α is not observed. The immunofluorescence photograph of the same field shows that PKC-α is not seen in the apoptotic cell with brightly stained fragmented nucleus shown in Figure 4.2a. In contrast, in intact viable cells, PKC-α immunofluorescence is observed associated with the nuclei, the plasma membranes and the cytoplasm (Fig. 4.2b). For PKC-δ, a dual Hoechst and immunofluorescence photograph (Fig. 4.3a) indicates the presence of an

Figure 4.1

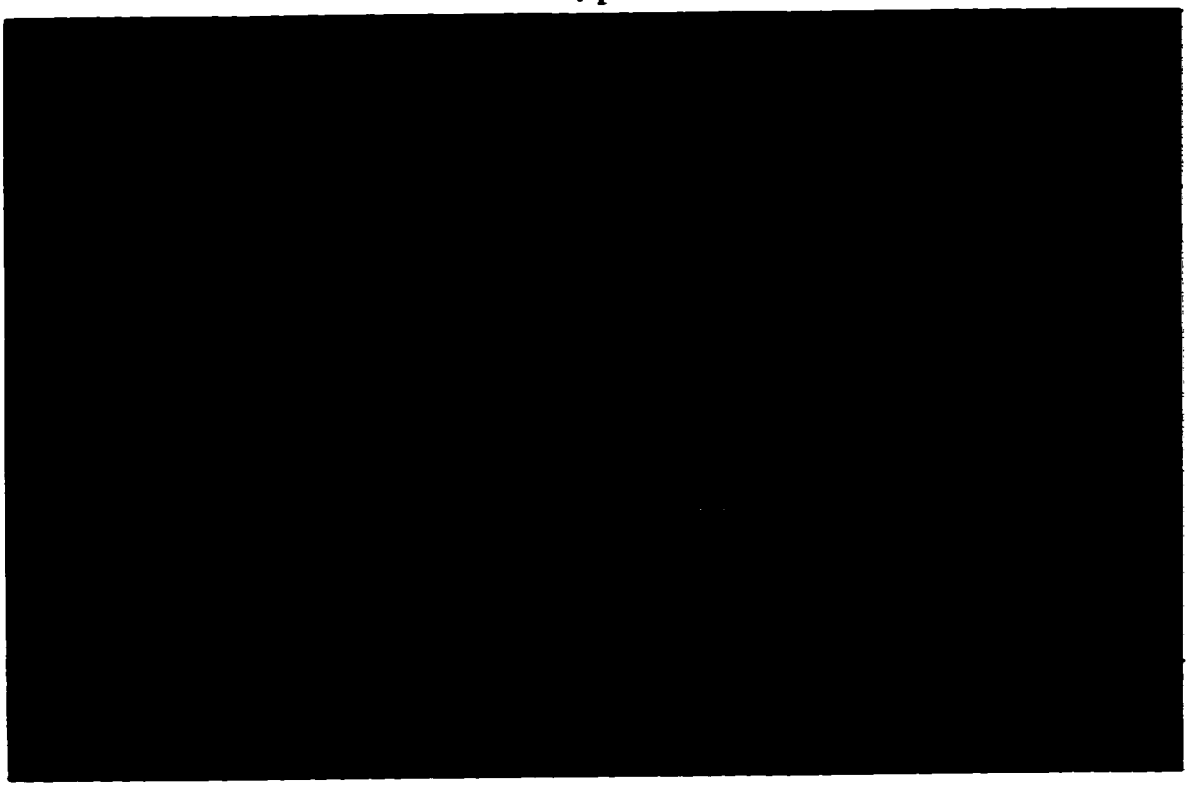
Dual Immunofluorescence and Hoechst Staining of PKC-β_I in T51B Cells

T51B cells were plated at a density of 125 x 10^2 cells/cm^2 and grown on glass coverslips in medium containing 10% serum. After 6 days of plating the cells were washed with PBS and fixed in 3.5% formaldehyde in PBS as described in 3.3.5. After washing with PBS the coverslips were then incubated with PKC-β_I-specific antibody followed by incubation with fluorescein-conjugated (FITC) secondary antibody as described in 4.3.3. After washing with PBS the coverslips were then incubated with Hoechst dye fluorochrome 33258, washed 3 times with PBS and mounted as described in 4.3.3. The figure shows: (a) Hoechst fluorescence photograph in which the apoptotic bodies (arrowed) with condensed nuclei have bright Hoechst staining while the intact nuclei of viable cells have pale staining; (b) Immunofluorescence photograph of the same cells shown in (a). Magnification: Figure 4.1a,b is x1000. Experiments were performed three times with similar results.

Figure 4.1

PKC- β_I

(a)



(b)

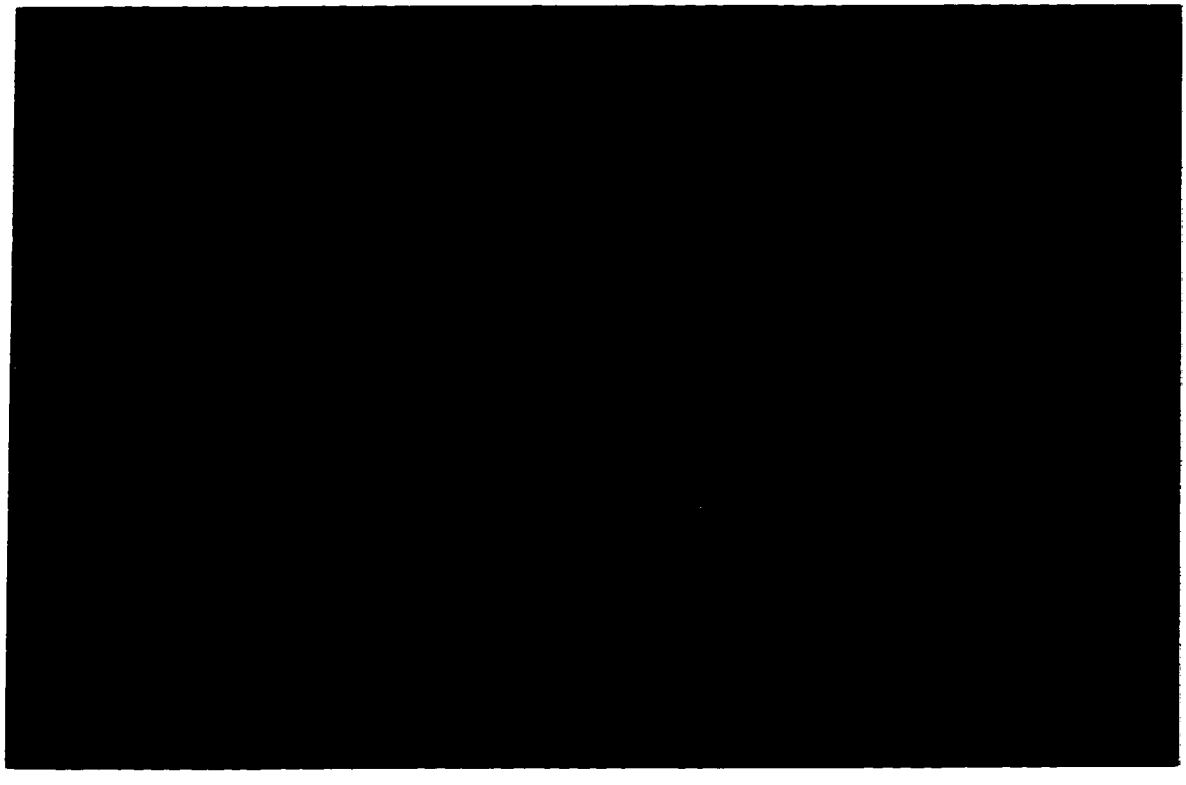


Figure 4.2

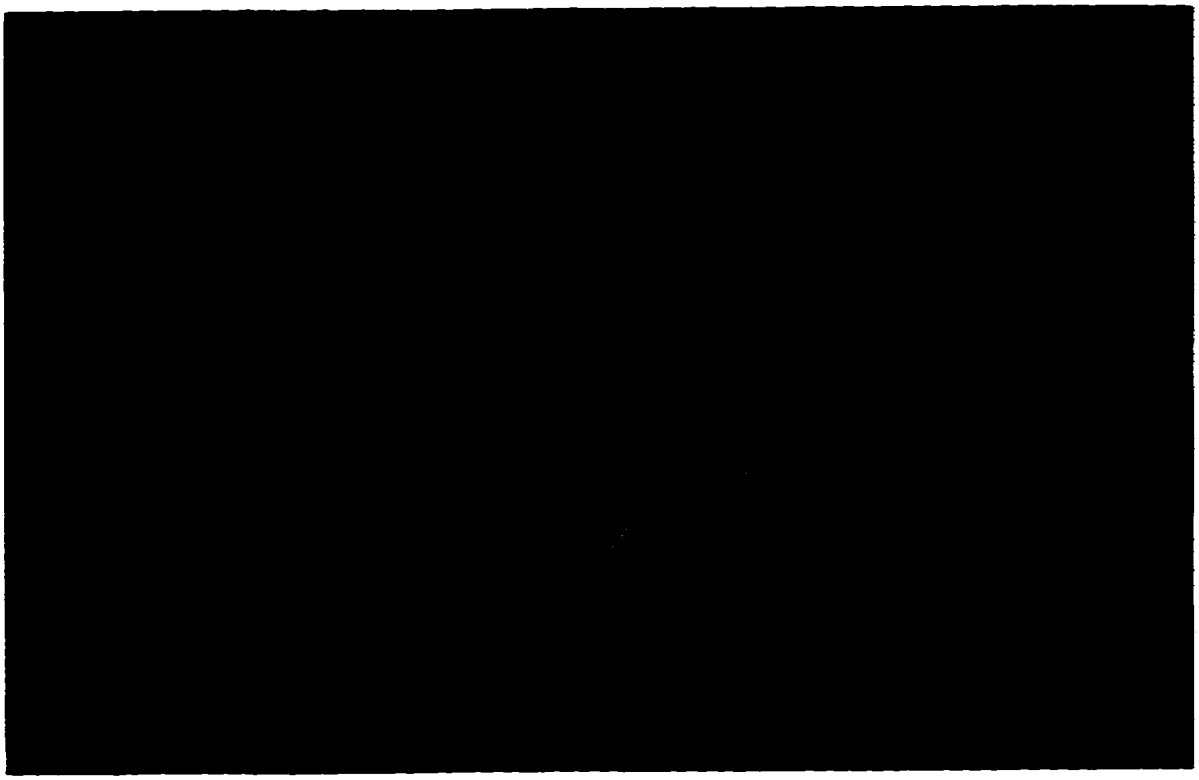
Dual Immunofluorescence and Hoechst Staining of PKC-α in T51B Cells

T51B cells were plated at a density of 125 x 10^2 cells/cm^2 and grown on glass coverslips in medium containing 10% serum. After 6 days of plating the cells were washed with PBS and fixed in 3.5% formaldehyde in PBS as described in 3.3.5. After washing with PBS the coverslips were then incubated with PKC-α-specific antibody followed by incubation with fluorescein-conjugated (FITC) secondary antibody as described in 4.3.3. After washing with PBS the coverslips were then incubated with Hoechst dye fluorochrome 33258, washed 3 times with PBS and mounted as described in 4.3.3. The figure shows: (a) Hoechst fluorescence photograph in which the apoptotic bodies (arrowed) with condensed nuclei have bright Hoechst staining while the intact nuclei of viable cells have pale staining; (b) Immunofluorescence photograph of the same cells shown in (a). Magnification: Figure 4.2a,b is x1000. Experiments were performed three times with similar results.

Figure 4.2

PKC- α

(a)



(b)

Figure 4.3

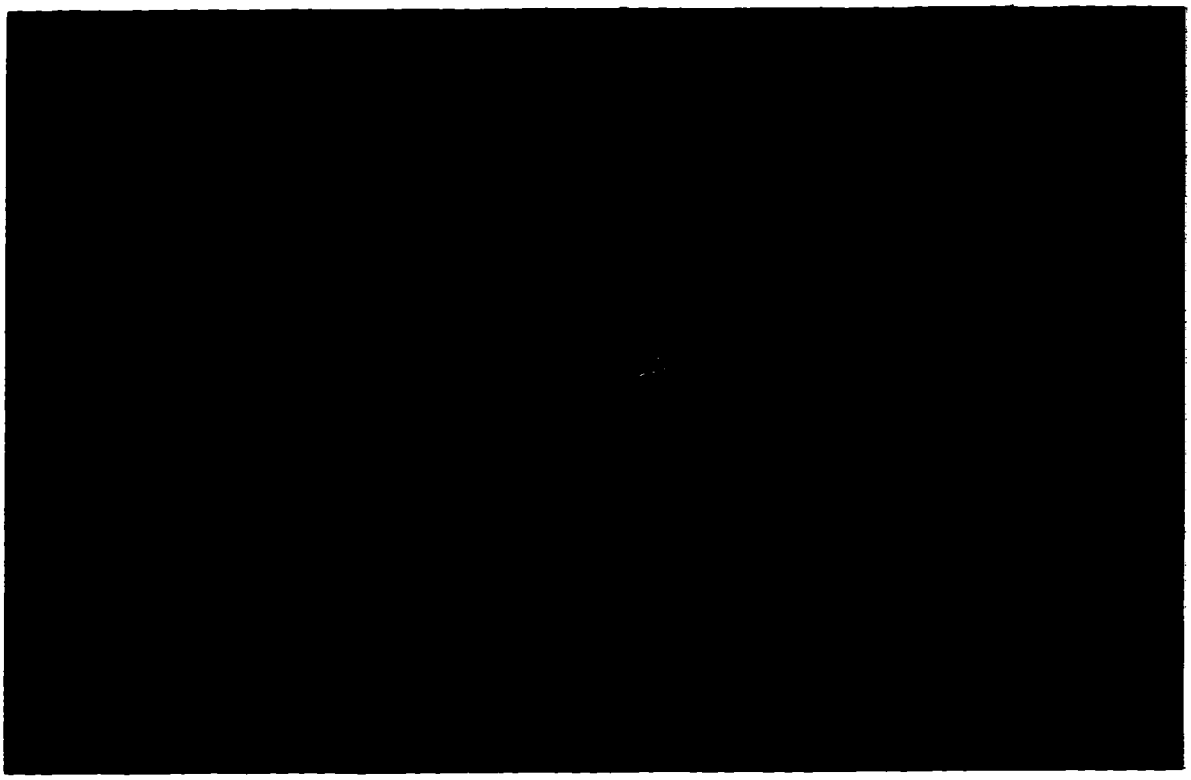
Dual Immunofluorescence and Hoechst Staining of PKC-δ in T51B Cells

T51B cells were plated at a density of 125 x 10^2 cells/cm^2 and grown on glass coverslips in medium containing 10% serum. After 6 days of plating the cells were washed with PBS and fixed in 3.5% formaldehyde in PBS as described in 3.3.5. After washing with PBS the coverslips were then incubated with PKC-δ-specific antibody followed by incubation with fluorescein-conjugated (FITC) secondary antibody as described in 4.3.3. After washing with PBS the coverslips were then incubated with Hoechst dye fluorochrome 33258, washed 3 times with PBS and mounted as described in 4.3.3. The figure shows: (a) Hoechst fluorescence photograph in which the apoptotic bodies (arrowed) with condensed nuclei have bright Hoechst staining while the intact nuclei of viable cells have pale staining; (b) Immunofluorescence photograph of the same cells shown in (a). Magnification: Figure 4.3a,b is x1000. Experiments were performed three times with similar results.

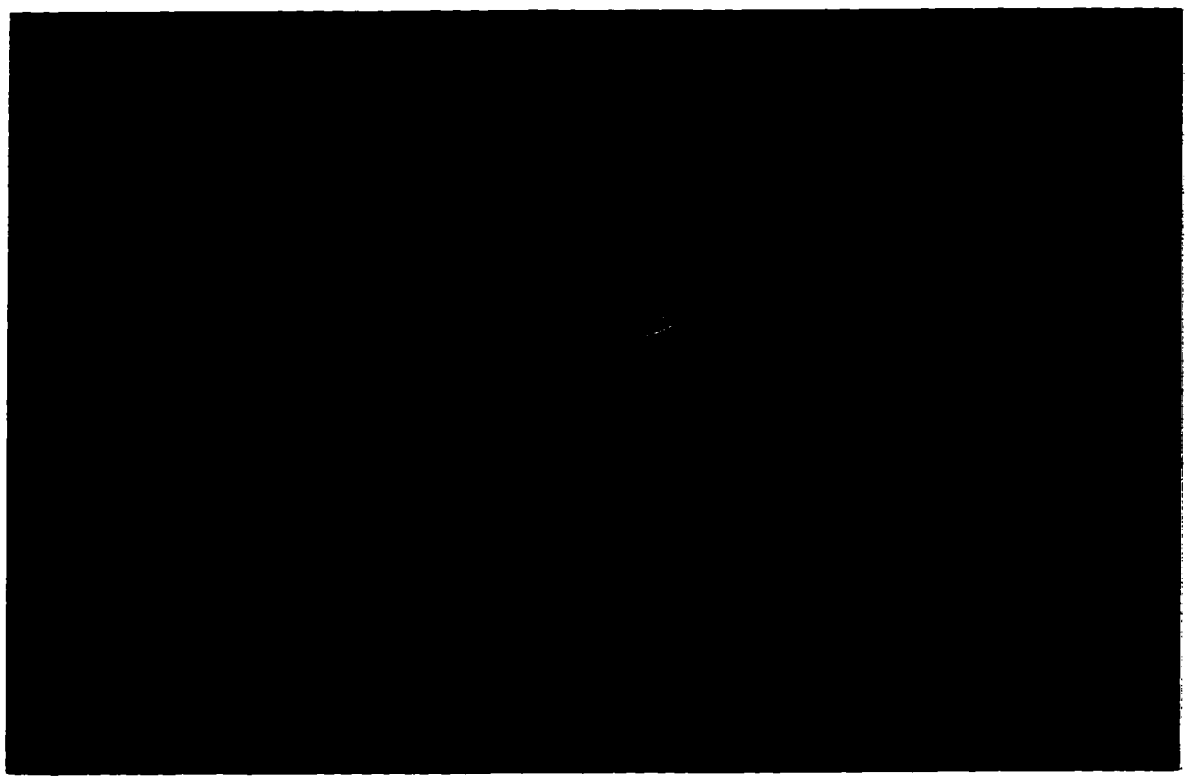
Figure 4.3

PKC- δ

(a)



(b)



apoptotic cell with brightly stained condensed nucleus and weakly stained intact nuclei. It also reveals that the apoptotic cell exhibits increased immunofluorescence of PKC-δ which appears as green fluorescein staining compared to intact nuclei which have Hoechst fluorescence staining only. The immunofluorescence photograph of the same field of cells (Fig. 4.3b) also demonstrates an increase in the immunofluorescence of PKC-δ throughout the apoptotic cell seen in Figure 4.3a compared to intact viable cells. On the other hand, the immunofluorescence of PKC-ϵ in apoptotic cells with brightly stained condensed nuclei, as identified by Hoechst staining (Fig. 4.4a), is almost similar to that observed in intact viable cells (Fig. 4.4b). Similarly, there is no change in the immunofluorescence of PKC-ζ isozyme in an apoptotic cell with brightly stained condensed nucleus as identified by Hoechst staining (Fig. 4.5a) compared to intact viable cells (Fig. 4.5b). Thus, these results indicate a differential localization of PKC-α, β_I and δ isozymes in apoptotic cells compared to intact viable T51B cells.

4.4.2 Effect of PKC Inhibitor Bisindolylmaleimide (Bis) and TPA on Apoptosis of T51B Cells

The possible involvement of PKC in apoptosis is investigated using TPA, an activator of PKC and a specific PKC inhibitor bisinolylmaleimide, GF 109203X (Bis), a staurosporine derivative that is more PKC-specific than its parent compound (Toullec et al., 1991). This compound is a competitive inhibitor of the ATP-binding site of the enzyme, and has been reported to inhibit endogenous PKC activity in the 2.5 - 10.0 μg/ml range (Toullec et al., 1991). Within this concentration, Bis has been found to be highly specific and selective for PKC relative to other cellular kinases (PKA, phosphorylase kinase, myosin light chain kinase and three different tyrosine kinases), and not to directly inhibit DNA replication (Toullec et

Figure 4.4

Dual Immunofluorescence and Hoechst Staining of PKC-ϵ in T51B Cells

T51B cells were plated at a density of 125 x 10^2 cells/cm^2 and grown on glass coverslips in medium containing 10% serum. After 6 days of plating the cells were washed with PBS and fixed in 3.5% formaldehyde in PBS as described in 3.3.5. After washing with PBS the coverslips were then incubated with PKC-ϵ-specific antibody followed by incubation with fluorescein-conjugated (FITC) secondary antibody as described in 4.3.3. After washing with PBS the coverslips were then incubated with Hoechst dye fluorochrome 33258, washed 3 times with PBS and mounted as described in 4.3.3. The figure shows: (a) Hoechst fluorescence photograph in which the apoptotic bodies (arrowed) with condensed nuclei have bright Hoechst staining while the intact nuclei of viable cells have pale staining; (b) Immunofluorescence photograph of the same cells shown in (a). Magnification: Figure 4.4a,b is x1000. Experiments were performed three times with similar results.

Figure 4.4

PKC- ε

(a)

(b)

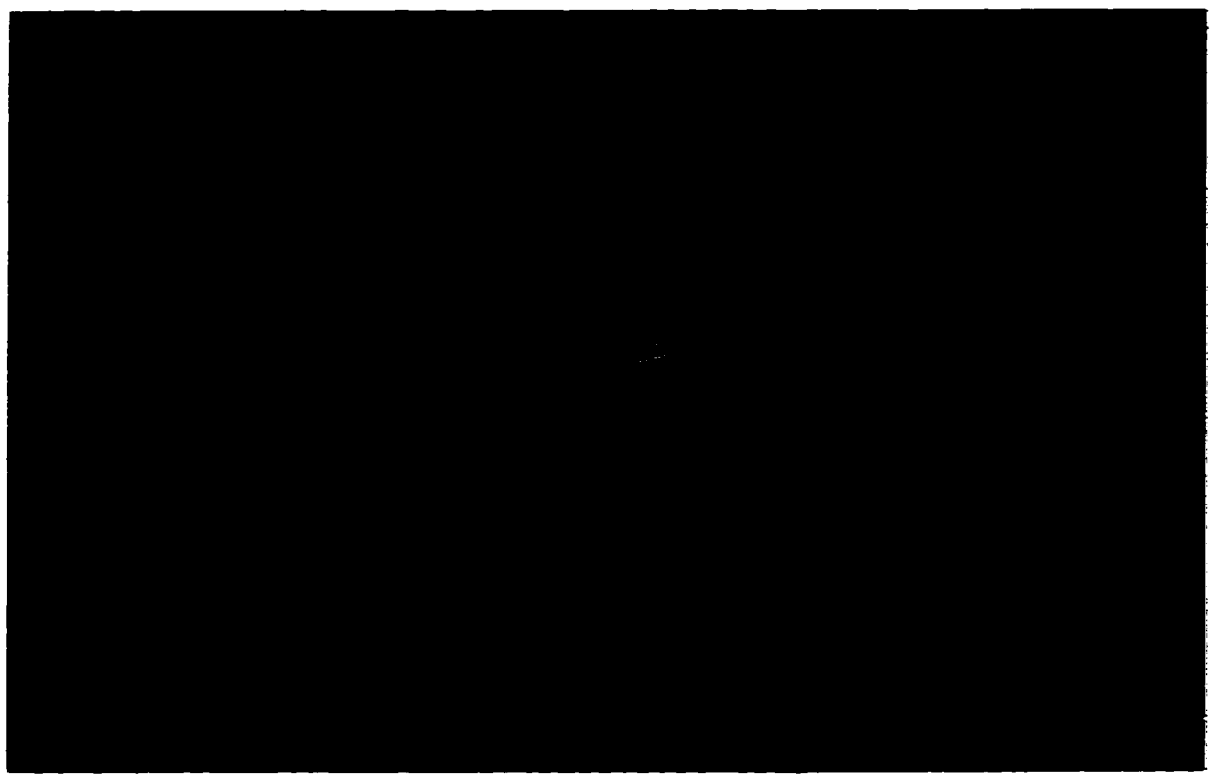


Figure 4.5

Dual Immunofluorescence and Hoechst Staining of PKC-ζ in T51B Cells

T51B cells were plated at a density of 125 x 10^2 cells/cm^2 and grown on glass coverslips in medium containing 10% serum. After 6 days of plating the cells were washed with PBS and fixed in 3.5% formaldehyde in PBS as described in 3.3.5. After washing with PBS the coverslips were then incubated with PKC-ζ-specific antibody followed by incubation with fluorescein-conjugated (FITC) secondary antibody as described in 4.3.3. After washing with PBS the coverslips were then incubated with Hoechst dye fluorochrome 33258, washed 3 times with PBS and mounted as described in 4.3.3. The figure shows: (a) Hoechst fluorescence photograph in which the apoptotic bodies (arrowed) with condensed nuclei have bright Hoechst staining while the intact nuclei of viable cells have pale staining; (b) Immunofluorescence photograph of the same cells shown in (a). Magnification: Figure 4.5a,b is x1000. Experiments were performed three times with similar results.

Figure 4.5

PKC- ζ

(a)

(b)

al., 1991). Preliminary studies indicate that treatment of T51B cells with Bis in the range of 1 to 10 μM increases the number of cells with condensed nuclei at various time intervals. Both 5 and 10 μM Bis are effective in increasing the number of apoptotic cells (data not shown) and therefore 5 μM Bis is used for subsequent experiments. As shown in Table 4.1, Hoechst staining results indicate that when cells are incubated with 5 μM Bis for 2 days, there is about 2% increase in the number of apoptotic cells with condensed nuclei compared to control cultures (day 5). However, when the cells are incubated with Bis for 4 days there is a significant increase in the percentage of apoptotic cells which reaches to about 9% compared to approximately 3% in control cultures (day 5). Thus, PKC inhibition may be linked to the increase in the number of cells undergoing apoptosis. These results are supported by dual Hoechst and TUNEL assay experiments which show a small increase in the number of nuclei with fragmented DNA in Bis-treated cells (Fig. 4.7a,b) compared to control untreated cultures (Fig. 4.6a,b). The effects of Bis on the intracellular localization of PKC isozymes is also investigated by immmunofluorescence analysis. However, no changes in the level or the localization of PKC isozymes is observed (data not shown). On the other hand, when the cells are treated with TPA for 4 days the percentage of apoptotic cells decreased compared to control cultures (Table 4.1). Hence, TPA treatment reduces the number of cells undergoing apoptosis.

Table 4.1

Effect of Bisindolylmaleimide and TPA on Nuclear Fragmentation of T51B Cells

T51B cells were plated at a density of 125 x 10^2 cells/cm^2 and grown on glass coverslips in medium containing 10% serum. After 24 h of plating the cells were treated with 5 μM Bis in DMSO and continued to grow for 2 or 4 days. Alternatively cells were treated with 100 nM TPA after 24 h of plating and continued to grow for 4 days. Control cultures were treated with vehicle only and grown for 5 days. After 5 days in culture the cells were washed with PBS, fixed with 4% paraformaldehyde in PBS and stained with Hoechst dye fluorochrome 33258 for 20 min as described in 4.3.2. The cells were then viewed under an Olympus fluorescence microscope. Three fields per dish were counted and the average number of nuclei per field was calculated. Statistical analysis was performed by student's unpaired t-test. A level of significance of $p < 0.05$ is indicated as * and of $p < 0.01$ is indicated as **. Experiments were performed three times with similar results.

TABLE 4.1

Effects of Bisindolylmaleimide (Bis) and TPA on Nuclear DNA Fragmentation of T51B Cells

Agent	Mean number of cells ± SD	Mean number of apoptotic cells ± SD	Apop. % ± SD
Control (5 days)	151.3 ± 15.6	4.3 ± 0.47	3.0 ± 0.07
+ Bis (2 days)	135.3 ± 10.0	6.7 ± 1.70	5.0 ± 0.90*
+ Bis (4 days)	132.3 ± 4.5	11.7 ± 1.25	7.0 ± 1.02**
+ TPA (4 days)	196.00 ± 30.2	3.8 ± 0.79	1.94 ± 0.4**

SD: Standard Deviation

Figures 4.6 - 4.7

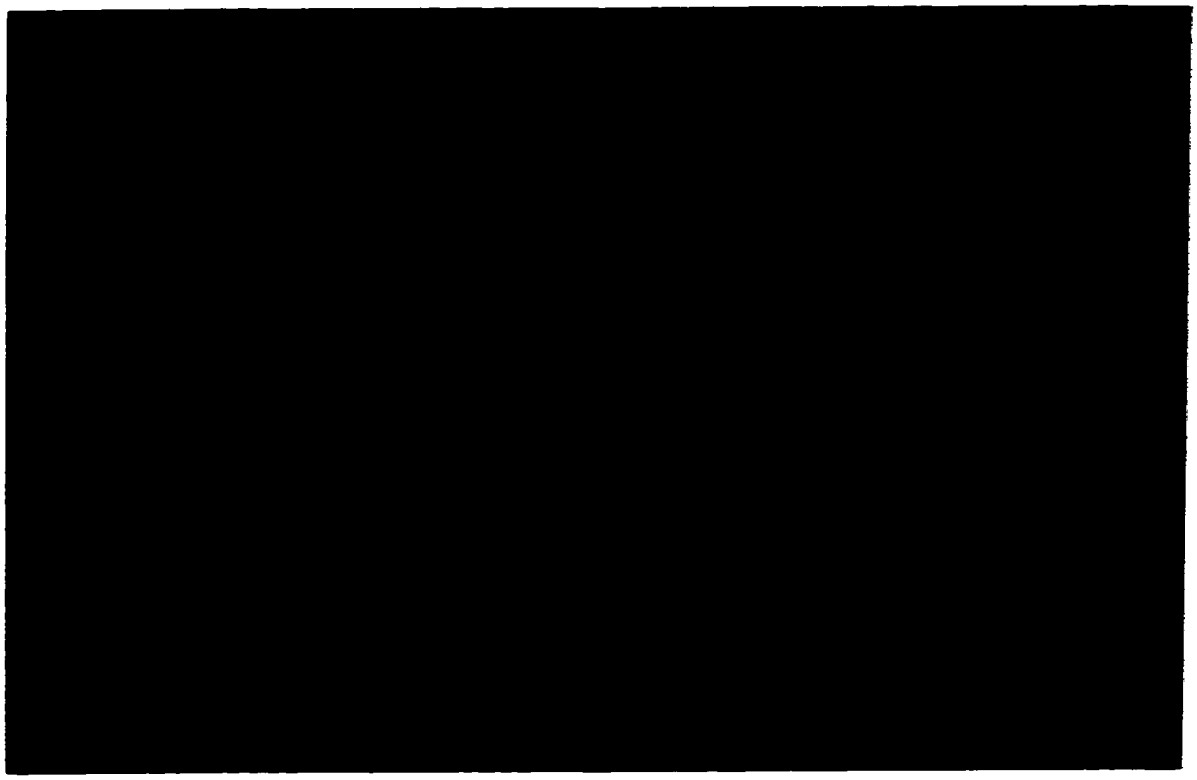
Effect of Bisindolylmaleimide Inhibitor on Nuclear DNA fragmentation of T51B Cells using Dual Hoechst and TUNEL Assay

T51B cells were plated at a density of 125 x 10^2 cells/cm^2 and grown on glass coverslips in medium containing 10% serum. After 24 h of plating the cells were treated with 5 μM Bis in DMSO and incubated for 4 days. Control cultures were treated with vehicle only and grown for 5 days. After 5 days of plating the cells were washed with PBS, fixed with 4% paraformaldehyde, permeabilized and incubated with TUNEL reaction mixture as described in 2.3.4. The coverslips were then stained with Hoechst dye fluorochrome 33258, washed with PBS and mounted as described in 4.3.3. The figures 4.6 (control) and 4.7 (Bis-treated) show: (a) Hoechst fluorescence photograph in which fragmented nuclei have bright Hoechst staining and intact nuclei have pale staining; (b) TUNEL fluorescence photograph in which fragmented nuclei have bright green fluorescein staining. Magnification: Figures 4.6-4.7a,b is x400. Experiments were performed three times with similar results.

Figure 4.6

T51B cells (control 5 days)

(a)

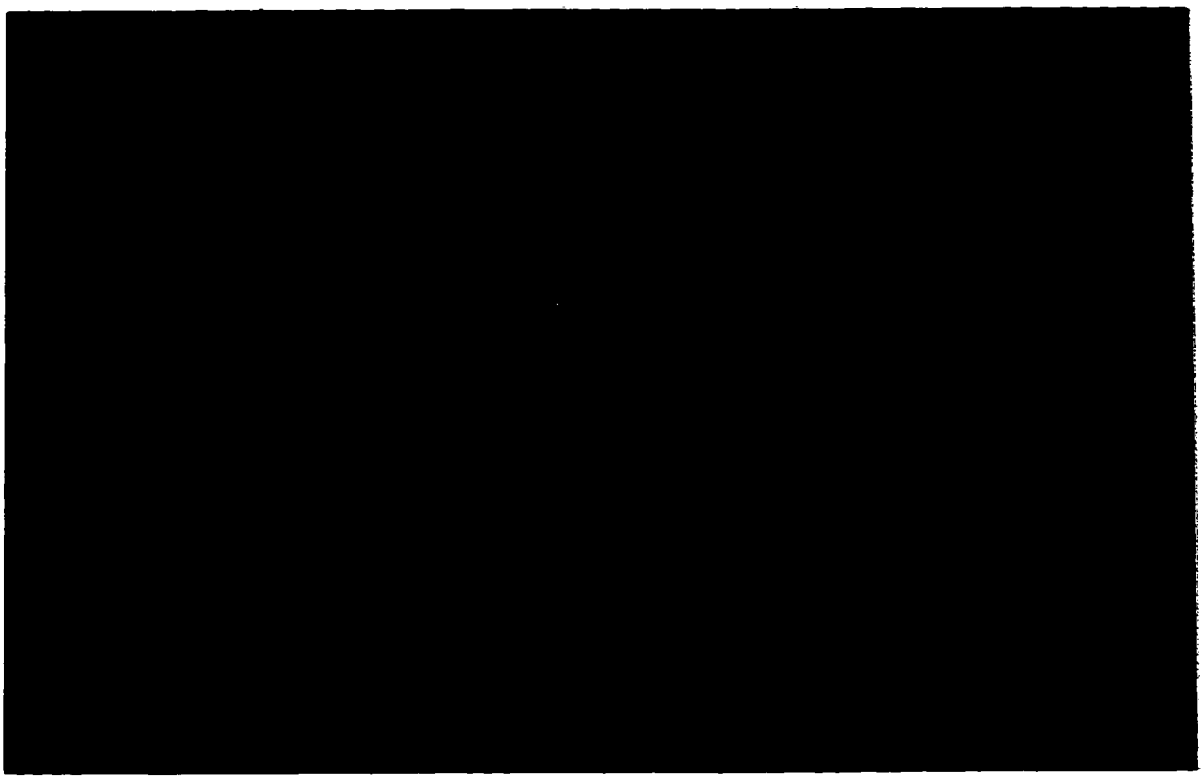


(b)

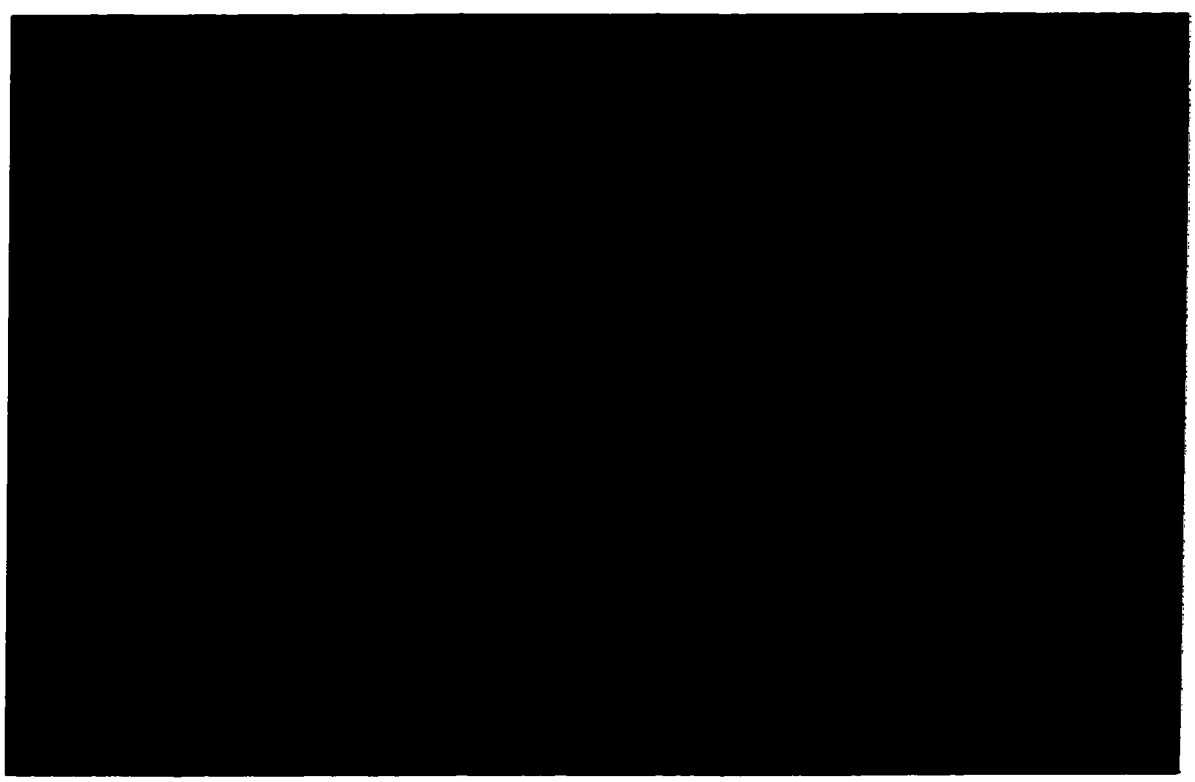
Figure 4.7

T51B cells + 5μM Bis (4 days)

(a)



(b)



4.5 Discussion

4.5.1 Subcellular Localization of PKC Isozymes during Apoptosis

The balance between proliferation and apoptosis is critical to the maintenance of steady-state numbers for cell populations in a variety of normal tissues. Clearly, dysregulation of this mechanism could disrupt homeostasis with the resultant potential overproduction of the affected cells thus predisposing them to the malignant state or tissue dysregulation (Schulte-Hermann et al., 1993). Results from Chapter Two demonstrate that apoptosis plays an important role in cellular homeostasis in T51B cells which is similar to that observed for liver. The intracellular processes which determine whether cells live or die are uncertain. It has become apparent over the last few years that PKC, now known to be a family of enzymes comprising at least twelve members, plays a fundamental role in the regulation of cellular proliferation. More recently, its involvement in the regulation of cell survival has been noted (Kizaki et al., 1989; Knox et al., 1992; McConkey, 1989a-c; Maiese, 1994). The present study is designed to explore the subcellular localization of PKC isozymes during apoptosis of T51B cells and possible involvement of these enzymes in this process.

Dual immunofluorescence and Hoechst staining results clearly demonstrate a differential localization of PKC isozymes during apoptosis. In apoptotic cells while the conventional PKC-β_I and -α are not detectable (Figs. 4.1 and 4.2), the level of the novel PKC-δ increases (Fig. 4.3). On the other hand, no changes in the level or the localization of the novel PKC-ϵ and the atypical PKC-ζ is observed (Figs. 4.4 and 4.5) respectively. Hence, these results are the first to demonstrate a differential localization of multiple PKC isozymes during apoptosis of T51B cells as well as other cell types. The apparent increase in the immunofluorescence level of PKC-δ in apoptotic cells raises the possibility that this isozyme

may be involved in the regulation of the entry of these cells into apoptosis after the completion of mitosis. This notion is supported by the flow cytometric data in Chapter Two (Table 2.1) which indicate that as the cells reach confluence, the percentage of cells with condensed nuclei increases dramatically with a subsequent decrease in the percentage of cells in the G_2 phase. These results may suggest that the G_2 cell population are eventually eliminated by apoptosis either directly or after the completion of the cell cycle. Hence, one could speculate that the increase in the immunofluorescence of PKC-δ may take place in G_2 or at G_2/M transition. The PKC-δ may subsequently promote the entry of these cells into apoptosis. This suggestion is also supported by recent studies which have indicated that the macrolide polyether toxin, bistratene A, which induces apoptosis in a variety of cells (Song et al., 1992), is a selective activator of PKC-δ (Watters et al., 1994). Thus, these data may implicate this isozyme in the regulation of apoptosis. Furthermore, in CHO cells, overexpression of PKC-δ isoform results in an arrest of cell division at the G_2/M phase in response to TPA treatment (Watanabe et al., 1992).

The disappearance of PKC-β_I and -α immunofluorescence in apoptotic cells raises the possibility that these isozymes could be key factors in determining cell survival and may have a protective role in the regulation of apoptosis (Figs. 4.1 and 4.2). One possibility is that PKC-β_I and/or -α may prevent Ca^{2+}-stimulated endonuclease activation directly or indirectly by phosphorylating specific proteins involved in this activation. This possibility is supported by previous studies which have demonstrated that PKC activation by TPA blocks apoptosis by preventing Ca^{2+}-stimulated endonuclease activation (McConkey et al., 1989b,c). A second possibility is that the presence of PKC-β_I and -α in the nuclei of T51B cells may have a direct role in modulating the expression of genes that are involved in the

protection against apoptosis in these cells. In contrast to my studies, previous reports have indicated that selective activation of PKC-β_I by 12-deoxyphorbol-13-O-phenyl-acetate-20-acetate (dPPA) may be involved in apoptosis of U937 leukemia cell line (Pongracz et al., 1994). The authors have observed that dPPA induces apoptosis as determined by DNA fragmentation on gel electrophoresis with translocation of PKC-β_I from the nuclei to the cytoplasm of U937 cells. However, in their studies they have not demonstrate whether the translocation of PKC-β_I occurs only in apoptotic cells since no dual staining to distinguish between apoptotic and viable cells has been performed. In stratified squamous epithelial cells, the immunofluorescence of PKC-α and -β (isoform not specified) has been found to increase in cells undergoing apoptosis (Knox et al., 1993). Nevertheless, the authors have used the co-localization of bcl-2 (an oncogene product which protects from apoptosis) as a marker to differentiate between viable and apoptotic cells. However, this method is indirect and may not be appropriate since the overexpression of bcl-2 protein has been shown to occur in some cells undergoing apoptosis (Fesus, 1993). Thus, the protein expression of bcl-2 is not an accurate biochemical marker to distinguish between viable and apoptotic cells. In my studies however, I used dual Hoechst staining and immunofluorescence which I believe is a direct and very good method to detect apoptotic cells with brightly stained condensed or fragmented nuclei and to determine the localization of specific PKC isozymes in these apoptotic cells. Based on the above results, it is also possible to combine immunofluorescence and TUNEL assay to detect apoptotic cells and to determine the localization of PKC isozymes in apoptotic cells. Such experiments would be helpful in determining whether the change in the localization of specific PKC isoforms takes place at early or late stages of apoptosis.

With respect to the role of PKC-ε and -ζ in apoptosis, previous studies have shown that glucocorticoid-induced apoptosis in immature thymocytes involves selective activation of PKC-ε through de novo synthesis of macromolecules (Iwata et al., 1994). In addition, several studies have demonstrated that ceramide (a selective activator of PKC-ζ) induces apoptosis in a variety of cells such as U937 and HL-60 leukemia cell lines (Jarvis et al., 1994a,b; Obeid et al., 1993), suggesting a role of this isoform in the regulation of apoptosis (Müler et al., 1995). However, in T51B cells, there is no change in the level of the localization of PKC-ε and -ζ during apoptosis (Figs. 4.4 and 4.5). Thus, it is possible that PKC-ε and/or -ζ may play a role in apoptosis of T51B cells. Results from Chapter Three demonstrate an increase in the immunofluorescence of PKC-ζ associated with the plasma membrane after 5 days of plating by which time the cells reach confluence with an increase in the number of cells undergoing apoptosis. Thus, the increase in the membrane-associated level of PKC-ζ may have a role in enhancing the cells to reach confluence and hence, enter apoptosis. The precise role of PKC isozymes in apoptosis of T51B cells is yet to be determined.

4.5.2 Effects of PKC Inhibitor Bisindolylmaleimide (Bis) and TPA on Apoptosis of T51B Cells

One way to define a role of PKC in the regulation of apoptosis is the use of selective PKC inhibitors and TPA (an activator of PKC). The present results demonstrate that treatment of T51B cells with 5 μM Bis increases the percentage of cells with condensed nuclei which is time-dependent. This effect is greatest when the cells are incubated with Bis for 4 days. At this time point there is about 9% increase in the number of cells with condensed nuclei compared to about 3% in control cultures (5 days) (Table 4.1). The effect

of Bis on DNA fragmentation is further confirmed by TUNEL assay which demonstrates an increase in the number of cells with fragmented DNA after 4 days of Bis addition (Fig. 4.7) compared to control cultures (Fig. 4.6). I also examined the effect of Bis on subcellular localization of PKC isozymes to see if this inhibitor down-regulates a specific isozyme. However, no changes were observed and hence, the inhibitor may act by inhibiting PKC activity rather than down-regulation. The Bis inhibitor is chosen in this study because it is among the most selective PKC inhibitors known and it inhibits PKC activity by acting as a competitive inhibitor at the ATP-binding site on PKC (Toullec et al., 1991). However, not all PKC isozymes may be inhibited to the same extent by Bis. The increase in the percentage of apoptotic cells in response to Bis treatment may suggest that the inhibition of one or more PKC isozymes enhances apoptosis. Since the Bis inhibitor has been found to be highly selective and more sensitive for cPKCs than nPKCs and aPKCs (Wilkinson et al., 1993) and because PKC-β_I and -α are not detectable in apoptotic cells, these isoforms are potential candidates as survival agents. However, PKC-α is less likely to be involved because its nuclear association is less specific than that of PKC-β_I. Hence, PKC-β_I is the most likely candidate as a survival agent and its inhibition is permissive for apoptosis. At this point one should not rule out completely the possibility that Bis may also inhibit other PKC isozymes. Thus, the effect of this inhibitor on the activities of PKC isozymes in T51B cells during apoptosis should be investigated in the future. Nevertheless, the present study suggests that PKC inhibition may be directly involved in enhancing apoptosis of T51B cells. These data are consistent with a previous report by Lu et al., 1994 who have indicated that Bis inhibitor in the same concentration range increases apoptosis of IL-2-dependent mouse T cell line (CTLL-2). However, the authors have not indicated which PKC isozyme is inhibited and is

involved in inducing apoptosis. Other PKC inhibitors have also been reported to induce apoptosis in a variety of cell types including hepatocytes which supports the notion that PKC has antagonistic effects on apoptosis (Araki et al., 1990; Sanchez et al., 1992; Knox et al., 1992; Illera et al., 1993; Leszczynski et al., 1994; Jarvis et al., 1994d; May et al., 1994). In contrast to my data and the above studies, other workers have shown that PKC inhibitors block apoptosis induced by various stimuli (Ojeda et al., 1990; Ye et al., 1993; Mizumoto et al., 1994). Such differences could be due to variations in species, cell types, specificity of the inhibitors, and experimental conditions. Although Bis is one of the most selective PKC inhibitors known (Toullec et al., 1991), one cannot completely rule out the possibility that the inhibitor-induced effects on apoptosis arise from inhibition of other uncharacterized protein kinases with a PKC-like function. Thus, more experiments are required to study this possibility.

Previous studies have shown, by using TPA as a selective activator of PKC, that this agent either protects or induces apoptosis as mentioned earlier in the Introduction. In the present study, TPA is used to investigate the possible role of PKC in the regulation of apoptosis. The data indicates that treatment of T51B cells with TPA after 1 day of plating and continued for 4 days reduces the number of cells undergoing apoptosis. There is a significant decrease in the percentage of cells with condensed nuclei in TPA-treated compared to 5 day control cultures (Table 4.1). Thus, TPA treatment appears to reduce the number of cells undergoing apoptosis. The present data is consistent with previous studies which have indicated that TPA prevents or reduces apoptosis by various stimuli in many cell types (McConkey et al., 1989b; Sanchez et al., 1992; Obeid et al., 1993; Jarvis et al., 1994a; Leszczynski et al., 1994; Delia et al., 1995). In contrast to these studies, others have reported

that TPA either induces (Kizakic et al., 1989; Komada et al., 1994; Mailhos et al., 1994) or has little or no effect on apoptosis (Martin et al., 1994). The discrepancies in these studies may be attributed to differences in cell types, species, duration of incubation and concentration of TPA used. In addition, interpreting TPA effects on cellular behavior can be difficult because PKC is not a single entity and consists of a family of at least twelve isozymes. The isozymes show differential requirements for cofactors, are regulated independently and are differentially expressed according to individual cell types and developmental status (Hug and Sarre, 1993). In addition, TPA is able to activate the classical (α, β_I, β_{II} and γ) and the novel (δ, ϵ, η and θ) PKCs, but cannot activate the atypical PKCs (ζ, λ and μ). In T51B cells, TPA is shown in Chapter One and Three to have differential effects on PKC isozymes (β_I, α, δ, ϵ and ζ) expressed in these cells. While TPA down-regulates PKC-α, -δ and -ϵ, it actually increases membrane- and nuclear-associated PKC-β_I but has no effect on PKC-ζ isozyme. Thus, in T51B cells PKC-β_I is most likely to be a candidate as a survival agent and its inhibition results in reduced cell proliferation and is permissive for apoptosis because: (a) it is not detected in apoptotic cells; (b) it is up-regulated by TPA treatment; (c) it is most likely to be inhibited by Bis as this inhibitor has been shown to be much more highly specific for cPKCs than for nPKCs and aPKCs (Wilkinson et al., 1993). Since TPA treatment reduces the number of apoptotic cells and at the same time down-regulates PKC-α, this isozyme is unlikely to be involved as a survival agent because its down-regulation does not induce apoptosis. On the other hand, PKC-δ may be involved in inducing apoptosis because: (a) its level increases in apoptotic cells; (b) it is down-regulated by TPA; (c) TPA treatment reduces the number of cells undergoing apoptosis; (d) it is less likely to be inhibited by Bis since it is less specific for nPKCs; and

(e) treatment with Bis induces apoptosis. Hence, the physiological balance between cell proliferation and apoptosis in T51B cells may be regulated in part by nuclear PKC-β_I and PKC-δ. However, other PKC isozymes such as PKC-ϵ and -ζ may have a role in apoptosis since these isozymes are observed to be present in apoptotic cells with no change in their localization or their level during apoptosis. Because TPA treatment down-regulates PKC-ϵ and also reduces the number of cells undergoing apoptosis, this isozyme could be involved in enhancing apoptosis. PKC-ϵ is unlikely to be inhibited by Bis since it is less sensitive for nPKCs and also treatment with Bis induces apoptosis. The role of PKC-ζ in the regulation of apoptosis is not clear since this isoform is not down-regulated by TPA and is unlikely to be inhibited by Bis because this inhibitor is less sensitive for aPKCs (Wilkinson et al., 1993). However, results from Chapter Two and Three indicate that as the cells approach confluence there is an increase in the percentage of apoptotic cells by which time the level of membrane-associated PKC-ζ increases substantially. Hence, PKC-ζ may be involved in enhancing the entry of cells into apoptosis as they reach confluence. It is also possible that other pathways which are PKC-independent are involved in the regulation of apoptosis in T51B cells. Indeed, measurement of PKC activity of specific isozymes during apoptosis will be helpful in understanding the role of PKC isozymes during apoptosis of T51B cells. It is of interest in the future to study the effects of other PKC activators such as synthetic DAG, bryostatin-1 (a selective activator of PKC-β), ceramides (selective activators of PKC-ζ) and bistratene A (a selective activator of PKC-δ) on apoptosis of T51B cells. The identification of specific substrates for PKC isozymes will be helpful in determining the role of PKC isozymes in the regulation of apoptosis. Very few substrates for specific PKC isozymes have been identified so far, but these do include proteins involved in the modulation of nuclear morphology

(Hocevar et al., 1991), DNA topology (Sahyoun et al., 1986) and cytoskeletal protein interactions (Lord et al., 1994). Although there are potent PKC inhibitors available now, the development of isozyme-specific PKC inhibitors will be extremely useful. Likewise, the inhibition of the synthesis of individual PKC isozymes by the use of antisense oligodeoxynucleotides and the intracellular delivery of PKC isozyme-specific antibodies will allow for the depletion and inactivation respectively of distinct PKC isozymes. These experiments would be helpful in determining the exact role of PKC isozymes in the regulation of apoptosis.

4.6 Conclusion

The overall conclusion of this chapter is that PKC isozymes expressed in T51B cells are differentially localized during apoptosis of these cells. Furthermore, the PKC inhibitor bisindolylmaleimide (Bis) enhances apoptosis while TPA reduces the rate of this process. The specific conclusions are:

1. In apoptotic cells, while PKC-α and -β_I are not detected, the level of PKC-δ increases with no change in the level of PKC-ϵ and -ζ isozymes.

2. Treatment of T51B cells with Bis inhibitor enhances apoptosis.

3. Treatment of T51B cells with TPA reduces the number of cells undergoing apoptosis.

GENERAL CONCLUSION

The major objective of this work was to study the subcellular localization of PKC isozymes and their roles in cell proliferation and cell death (apoptosis) in T51B rat liver epithelial cells. Diagram Two represents a model of these studies.

In Chapter One I have established the profile of PKC isozymes expressed in T51B cells and their subcellular localization in response to the phorbol ester TPA, a potent activator of PKC, and epidermal growth factor (EGF), a mitogenic stimulus for these cells. Immunofluorescence and/or immunoblotting results demonstrate the presence of PKC-α, -β_I, -δ, -ϵ, and -ζ in the cytosol, the plasma membrane and the nucleus. The membrane-associated PKC-β_I appears as a doublet which is unique and novel for T51B cells. A 24 h treatment with TPA increases the membrane and the nuclear association of PKC-β_I, down regulates the cytosolic and the membrane-associated PKC-α, -δ and -ϵ and yet, does not affect PKC-δ. On the other hand, EGF which enhances both DNA synthesis and apoptosis in these cells (Brabyn et al., 1994) is without effect on PKC-α, -δ, -ϵ and -ζ but induces a time-dependent increase in the level of PKC-β_I associated with the plasma membrane. In addition, the immunofluorescence of nuclear PKC-β_I increases slightly in response to EGF. These findings are in agreement with previous reports from our laboratory which have indicated an increase in the membrane-associated PKC activity in response to EGF treatment. (Sharma et al., 1994). Furthermore, TPA alone is without effect on DNA synthesis but when added along with EGF or serum it causes a significant enhancement of DNA synthesis (Sharma et al., 1994).

In Chapter Two I have studied the cell cycle kinetics of T51B cells at various times after plating in medium containing 10% serum alone or in the presence of 1 nM EGF by flow cytometry, Hoechst staining and TUNEL assay. The results indicate an increase in apoptosis as the cells approach confluence in both serum or EGF treated cells. However, in the case of EGF, the cells reach a higher density before the induction of apoptosis. The results also show that as the cells reach confluence, the percentage of apoptotic cells increases with a corresponding decrease in the percentage of the G_2 cell population. The data also demonstrate that even when the cells are confluent, a significant population is still proliferating (9% in the S phase at day 8). Thus, in T51B cultures, growth equilibrium is maintained by a process in which cell division is balanced by apoptosis. Hence, cellular homeostasis in T51B cells appears to involve two processes: (a) growth arrest that occurs presumably as a result of contact inhibition; and (b) the removal of "surplus" cells by apoptosis. The fact that there is a balance between cell proliferation and apoptosis means that T51B cells represent a good *in vitro* model for both liver hyperplasia/regression and liver homeostasis.

In Chapter Three I have demonstrated a differential change in the subcellular localization of PKC isozymes as the cells reach confluence. PKC-β_I is totally associated with the nucleus and the plasma membrane after 1 day of plating, and moves to the cytoplasm as the cells approach confluence. Interestingly, in the plasma membrane, the immunofluorescence of PKC-β_I appears as a punctate fluorescence along the cell boundaries at the cell-cell contacts forming bright lines as the cells become confluent. These results suggest a possible involvement of PKC-β_I in cell-cell contact formation. On the other hand, no significant changes in the localization of PKC-α, -δ, and -ϵ are noted. However, in the

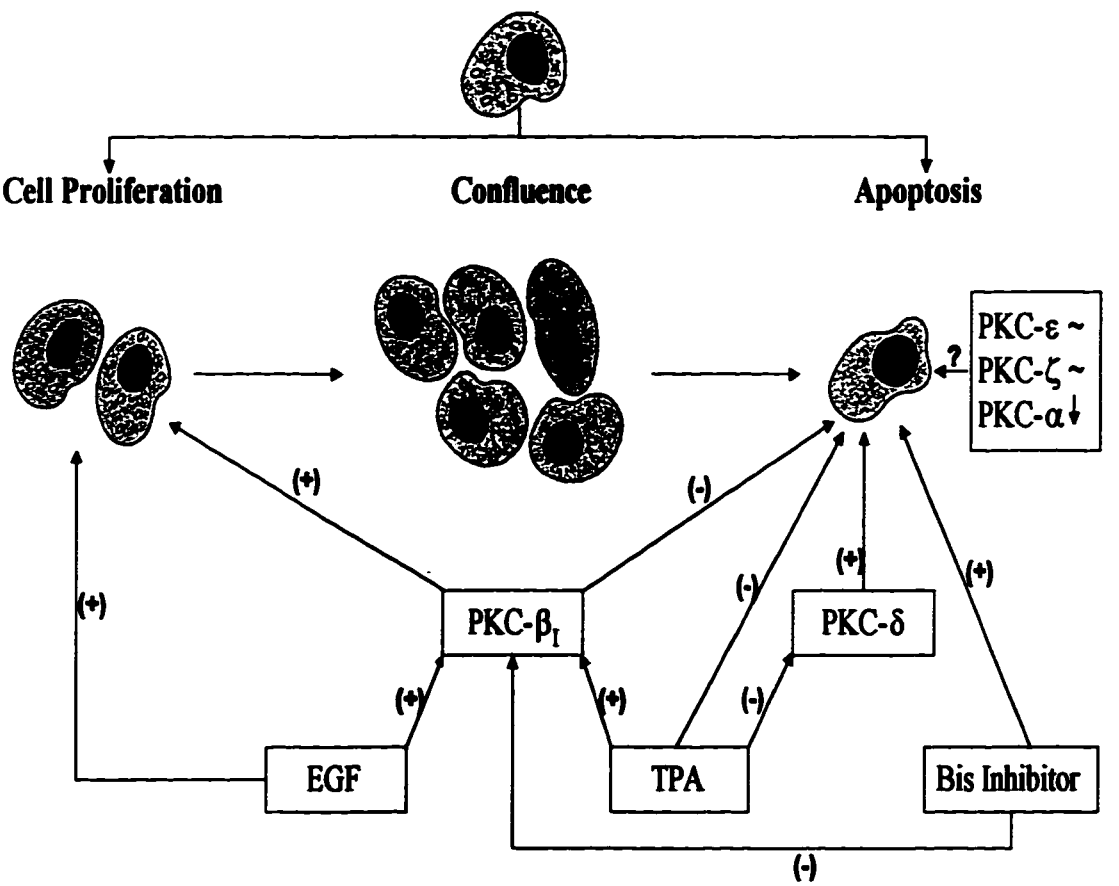
case of PKC-ζ the membrane-associated level increases as the cells reach confluence. The immunfluorescence results also show that in proliferating T51B cells (1 day after plating), TPA induces a re-distribution of the nuclear PKC-β_I between the nucleus, the cytoplasm and the plasma membrane in a time dependent manner. Furthermore, in confluent serum-deprived cells, TPA increases the nuclear- and the membrane-associated level of PKC-β_I which is also observed to be time-dependent. The involvement of nuclear PKC-β_I in cell proliferation has been further investigated. Re-addition of serum to serum-deprived quiescent cells results in a substantial increase in the nuclear level of PKC-β_I. Since the flow cytometric data show that after 1 day of plating most of the cells are in the G_1 phase at which time PKC-β_I is localized only in the nucleus, it is possible that the nuclear PKC-β_I is involved in the proliferative signal probably in the G_1→S transition. Furthermore, in dividing cells the level of PKC-β_I is higher than that of non-dividing cells, suggesting a possible role for this isozyme during mitosis. Prelabeling experiments show that TPA and serum re-addition to serum-deprived cells increase the phosphorylation of two nuclear proteins with a molecular weight of approximately 21 and 31 kD. However, in the case of serum, a more rapid effect is noted compared to that of TPA.

In Chapter Four I have shown a differential localization of PKC isozymes during apoptosis of T51B cells. While PKC-β_I and -α are not detected, the level of PKC-δ substantially increases with no change in the level of PKC-ε and -ζ isozymes. Furthermore, treatment of T51B cells with the specific PKC inhibitor bisinolylmaleimide (Bis) enhances apoptosis, while treatment of cells with TPA (an activator of PKC) reduces the number of cells undergoing apoptosis. These results suggest that PKC-β_I is the most likely candidate as a survival agent and its inhibition results in reduced cell proliferation and is permissive

for apoptosis because: (a) it is not detectable in apoptotic cells; (b) it is upregulated by TPA; (c) it is most likely inhibited by the Bis inhibitor since this inhibitor is more specific for cPKCs than nPKCs and aPKCs (Wilkinson et al., 1993). On the other hand, PKC-δ may be involved in initiating apoptosis because: (a) its level increases in apoptotic cells; (b) it is down-regulated by TPA; (c) TPA treatment reduces the number of cells undergoing apoptosis. However, PKC-ϵ and -ζ may also be involved in apoptosis since these isozymes are detected in apoptotic cells with no change in their localization or their levels. Thus, the physiological balance between cell proliferation and apoptosis may be regulated in part by nuclear-associated PKC-β_I and PKC-δ.

DIAGRAM TWO

A Model System of the Role of PKC Isozymes in Cellular Homeostasis of T51B Cells



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