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Characterization of the Molecular Mechanisms Underlying Retinoic Acid Induced Growth Inhibition in MCF-7 Human Breast Cancer Cells

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University of Ottawa

Ottawa, ON, Canada

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ABSTRACT

Retinoids are known to strongly inhibit the growth of estrogen (E2) dependent human breast cancer cells. This growth inhibition was found to be associated with an accumulation of cells in G1 phase of the cell cycle. Since the molecular mechanisms underlying these antiproliferative effects were unknown, we investigated the effects of retinoic acid (RA) on cell cycle progression and the expression and activity of cell cycle regulatory genes in the E2 dependent breast carcinoma cell line, MCF-7. We have found that RA induced accumulation of cells in G1 was correlated with decreased phosphorylation of the retinoblastoma protein (pRb). It also resulted in a down regulation of cyclin dependent kinase 2 (cdk2) and cyclin D1 gene expression and a profound down regulation of cdk2 activity. In contrast, no changes were seen in cdk4 expression or activity. We have further demonstrated that RA did not cause a change in the overall levels of expression of the known cyclin-cdk inhibitors p21 and p27. As well, no change in the activity of the cdk activating kinase, CAK, was detected.

These results suggest that the growth inhibition of MCF-7 cells is associated with a down regulation of cdk2 activity. While this reduced activity occurred in the absence of RA-mediated increases in the levels of the cdk inhibitors p21 and p27, an increased association between p27 and cdk2 was observed in RA treated lysates. Furthermore, assays of cdk2 in pooled lysates taken from RA treated and control cells showed that RA treated cells contain a p27-like transferable cdk2 inhibitory activity. Pretreatment of cells with antisense but not mismatch p27 oligonucleotides attenuated the inhibitory effects of RA on cdk2 associated activity.

These findings demonstrate that RA is capable of regulating cdk2 activity through a mechanism involving p27.

RA also caused an increase in p53, we therefore investigated the role of p53 in growth arrest induced by exposure to RA. MCF-7/E6 cells expressing the human papillomavirus HPV16 E6 protein contained very low levels of p53 and exhibited partial abrogation of both cdk2 inactivation and G1 arrest in response to RA. To further elucidate the role of p53 in RA mediated inhibition of cdk2, MCF-7 cells were transfected with various p53 mutant expression constructs. However, none of the stable clones generated were impaired in their ability to down regulate cdk2 or undergo G1 arrest in response to RA. These results suggest that no functional domain of p53 alone contributes to the RA induced regulation of cdk2, but rather the cooperation of several domains of p53 may be required.

We also observed that RA was able to profoundly down regulate cdk2 gene expression. However, following exposure to RA, no decrease in cdk2 gene transcription was observed in MCF-7 cells which had been stably transfected with a cdk2 promoter firefly luciferase reporter gene construct. These results were confirmed by nuclear run off assays which demonstrated that RA did not cause a decrease in the rate of cdk2 gene expression. Instead we found that RA decreased cdk2 expression by triggering posttranscriptional destabilization of the mRNA.

Taken together, these findings suggest that RA inhibits cell cycle progression in MCF-7 cells through the inactivation of cdk2. Several targets have been identified that participate in the RA mediated down regulation of cdk2 activity.

ACKNOWLEDGEMENTS

Many people have contributed to the experiments and ideas expressed in this thesis. I would first like to thank Dr. Christine Pratt for providing me with the opportunity to pursue research in an exciting and growing field. As well, I would like to thank Drs. Albert, Robertson,Trifaro and Tuana for their support and advice whenever it was sought. To the members of the lab, past and present, your assistance and friendship was greatly appreciated: NK, HM, KW, DN, DD, CC and MM – thanks for everything. I also thank the friends I have made along the way: NW and JW, RG, SC, JJL, MS, SR, TG and JP - for all your support. I acknowldedge furthermore the Natural Science and Engineering Research Council of Canada and the Ontario Ministry of Education and Training for helping fund my tenure here.

I dedicate this thesis to my family. To my parents for their unconditional support during this endeavour but especially to Mike for his endless supply of patience.

TABLE OF CONTENTS

LIST OF TABLES and FIGURES

LIST OF ABBREVIATIONS

Amp	ampicillin
ATP	adenosine triphosphate
bp	base pair
CAK	cdk activating kinase
CAT	chloramphenicol acetyltransferase
cdk	cyclin dependent kinase
cDNA	complementary DNA
CMV	cytomegalovirus
DEPC	diethylpyrocarbonate
dCTP	deoxycytidine triphosphate
DMEM	Dubelcco's minimal essential media
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E2	estrogen
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetracetic acid
EGTA	ethyleneglycol-bis(β-aminoethylether)-N,N'-tetracetic acid
ER	estrogen receptor
FBS	fetal bovine serum
G418	geneticin
GAPD	glyceraldehyde phosphate dehydrogenase
GST	glutathione-S-transferase

HEPES	4-(2-hydroxylethyl)-1-piperazine ethanesulfonic acid
HPV	human papillomavirus
IgG	immunoglobulin
kDa	kilodalton
MOPS	3-[N-morpholino]propanesulfonic acid
NaCl	sodium chloride
NaOH	sodium hydroxide
P_i	orthophosphate
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
RA	retinoic acid
RAR	retinoic acid receptor
RARE	retinoic acid response element
RNA	ribonucleic acid
RT	reverse transcriptase
RXR	retinoid X receptor
SDS	sodium dodecyl sulfate
TBS	Tris buffered saline
TGFβ	transforming growth factor beta

INTRODUCTION

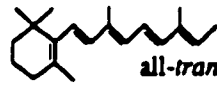
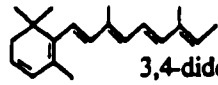
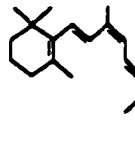
Retinoids

Retinoids are a group of natural and synthetic compounds structurally related to vitamin A (retinol), that function as signaling molecules. Increasing evidence indicates that retinoids play essential roles in vision, growth, and the maintenance of epithelial differentiation, particularly during embryogenesis (reviewed by Gudas, 1994). Both excess and deficiency of vitamin A can cause dramatic changes in cellular differentiation, cell proliferation and development. All *trans* retinoic acid (RA), one endogenous oxidative metabolite of vitamin A, is a known morphogen, regulating the pattern formation of some organs in the developing embryo (Thaller and Eichele, 1987). Administration of exogenous RA during early organogenesis resulted in neural tube defects and malformations in the cardiovascular system, whereas slightly later exposures resulted in limb deformities, genitourinary tract and craniofacial abnormalities (Lammer et al., 1988). In the adult, RA promotes the normal differentiation of many cell types, including the epithelial cells of the skin (Asselineau et al., 1989) and cells of the hematopoietic system (Kizaki et al., 1993).

Retinoids are an essential requirement for the diet of all vertebrate animals. They can be derived from dietary retinol or through the conversion of β-carotene to retinol (Gudas et al., 1994). Retinol is stored in the liver and can be metabolized to various biologically active retinaldehyde and retinoic acid forms in many cell types (Figure 1). To meet cellular needs, retinol is delivered to target tissues through the circulation as retinol bound to retinol binding protein (RBP) (D'Onofrio et al., 1985). The mechanisms involved in the uptake of retinol by cells from RBP are not

Figure 1. Structures of known naturally occurring retinoids that activate nuclear retinoid receptors

Comparison of the retinoid receptor selectivity of biologically active retinoids (Leid et al., 1993).

RETINOID	RECEPTOR ACTIVITY	
	RAR	RXR
 COOH (T-RA) all-*trans* retinoic acid	+	-
 COOH (T-ddRA) 3,4-didehydroretinoic acid	+	-
 9-*cis* retinoic acid (9C-RA)	+	+

clear, although it has been suggested that a specific RBP receptor may be involved. Within target tissues retinol is taken up by cells from the circulation and bound by cytosolic carrier proteins termed cellular retinol binding proteins (CRBPs) (Colantuoni et al., 1985). Once it has been delivered to the cell, it can either be oxidized to retinaldehyde and retinoic acid or esterified for storage.

Retinoic acid is the biologically active form which is needed for mediating and maintaining cellular differentiation. Both all *trans*- and its stereoisomer, 9-*cis*-retinoic acid occur endogenously in mammals. Cellular retinoic acid binding proteins (CRABPs) I and II bind all *trans*-RA (Giguere et al, 1990), but not 9-cis-RA (Allenby et al., 1992), with high affinity to modulate the effects of RA within cells. Although little is known about the functions of CRABPs, it has been suggested that these proteins may be involved in the storage, transport or metabolism of intracellular retinoids. It is thought that CRABP I and II may function in modulating the concentration of free RA available to bind to nuclear retinoid receptors by sequestration.

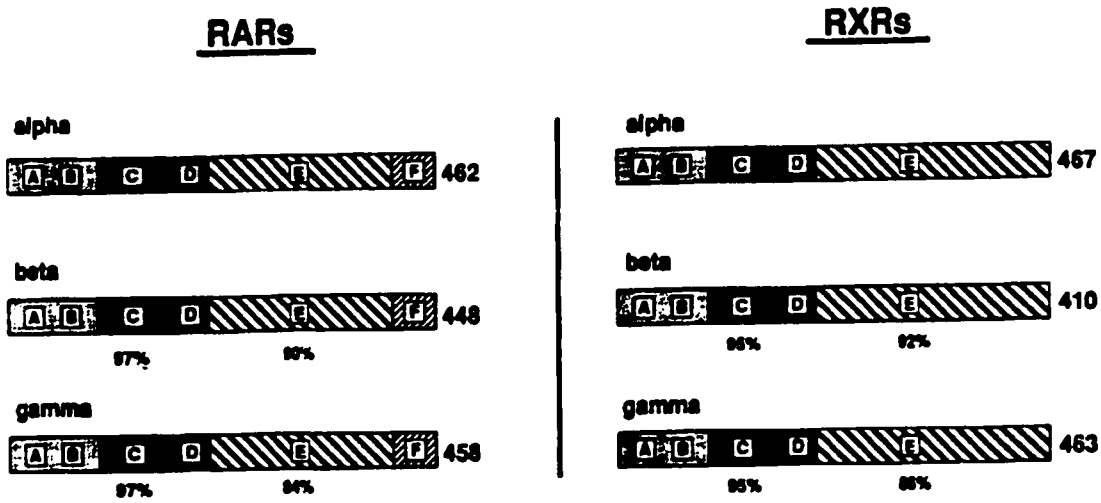
Molecular characterization of retinoid receptors

Many actions of retinoids are mediated through high affinity nuclear retinoic acid receptors (RARs) and retinoid X receptors (RXRs) (Figure 2A) (reviewed by Gudas et al., 1994; Pemrick et al., 1994). These receptors belong to the superfamily of ligand inducible transcriptional regulatory factors which include the steroid, vitamin D3 and thyroid hormone receptors, in addition to a large number of orphan receptors whose ligands have not yet been identified. These nuclear receptors mediate gene

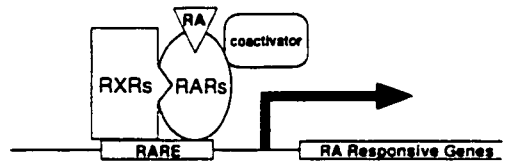
Figure 2. Characterization of nuclear retinoid receptors

A. Schematic illustration of the two families of nuclear retinoic acid receptors, each consisting of three receptor subtypes, α, β, and γ. Comparison of the percentage amino acid identity between RAR and RXR subtypes. Five structural domains (A-E) have been identified based on homology among themselves and with other members of the nuclear receptor superfamily. RARs contain a sixth domain (F) whose function is unknown. **B.** Schematic representation of receptor action. The RARs and RXRs form heterodimers to bind with high affinity and specificity to its cognate response element (RARE). Ligand binding and interactions with other factors influence transcriptional activation by the receptor complex (Pemrick et al., 1994).

A


RARs
RXRs
alpha
462
beta
448
97%
90%
gamma
458
97%
84%
alpha
467
beta
410
86%
92%
gamma
463
85%
88%

B


RA
coactivator
RXRs
RARs
RARE
RA Responsive Genes

transcription through a variety of mechanisms. They can bind to specific DNA consensus sequences termed hormone response elements (HREs), which are located in the regulatory regions of genes, to activate transcription in a ligand dependent manner (Figure 2B). Within this superfamily of receptors there exists two subfamilies that are functionally distinct. The type I class includes the classical steroid receptors, the glucocorticoid, progesterone and estrogen receptors, while the type II class is comprised of receptors for retinoic acid, vitamin D and thyroid hormone. In general, type I receptors require ligand induced homodimerization to bind their respective response elements. In contrast, type II receptors require an auxiliary factor, RXR, for high affinity binding although homodimer binding and subsequent gene activation has also been found. Type II receptors have also been known to bind their response elements in the absence of ligand. The response elements recognized by these two subfamilies also differs. The consensus DNA sequence motif to which the retinoid, vitamin D3 and estrogen receptors bind contains the half site GGTCA in the form of a palindrome (type I receptors) or as a direct repeat (DR) (type II receptors) with varying numbers of spacer nucleotides (Nair et al., 1991; Umesonoet al., 1991).

Characterization of structural features have identified several domains which are responsible for the biological activity of the receptors. A conserved DNA binding domain (domain C) contains two zinc finger motifs that consist of several tandemly repeated cysteine residues coordinated by zinc atoms. This region was found to be required for the interaction of the receptors with their respective response elements. The two Zn finger modules are joined primarily through interaction between the amphipathic helices formed by amino acids at the base of the two fingers. The amino

acids in the stem of the first Zn finger form a α-helix and make base contacts in the major groove of the DNA. This α-helix coincides with the P-box which has been implicated in the determination of specificity of binding site recognition. The ligand binding domain (domain E) is located at the carboxy terminus of the receptor. A ligand dependent transactivation function (AF-2) and a dimerization region are also localized within domain E. The AF-2 is a highly conserved region that was first characterized in the estrogen receptor where it was found to modulate ligand dependent transcriptional activation. It was demonstrated that upon deletion of amino acids 538 to 552 of the mouse ER, transcriptional activation was completely blocked but no effect on DNA or ligand binding was observed (Daneilian et al., 1992). The amino terminal region of nuclear receptors (domains A/B) also contain an isoform specific transactivation domain termed AF-1. In contrast to the AF-2, this region is not well conserved and does not require ligand binding for its function.

The family of RARs is composed of three subtypes, RARα, β and γ. The three subtypes show strong homology in their DNA binding and ligand binding domains (Figure 2A). Numerous isoforms of each subtype also exist as a result of alternative splicing (Leroy et al., 1991), as well as, alternative promoter usage (Kastner et al., 1990). The RXRs, which have been shown to participate with RARs in mediating retinoid responses, also have three subtypes. Most cell types express more than one RAR and RXR receptor. The generation of non functional receptors of RAR subtypes through the use of homologous recombination has suggested that RAR functional redundancy exists among different RARs (Li et al., 1993). However, other studies have indicated that the various receptor subtypes possess distinct functions and may indeed

modulate the activity of distinct genes (Boylan et al., 1995). Evidence also suggests a unique role for each of the receptor subtypes: (1) receptor selectivity towards specific transactivating response elements has been demonstrated (La Vista-Picard et al., 1996; Thacher et al., 1999); and (2) specific cell types have become refractory to the antiproliferative and differentiating effects of RA with the loss of one receptor subtype, despite the presence of other RAR subtypes (Sheikh et al., 1993; Boylan et al., 1995).

Signal transduction through retinoid receptors

Since their initial discovery, retinoic acid receptors have greatly advanced our understanding of RA signaling mechanisms (reviewed by Pfahl, 1993). As previously mentioned, RARs require heterodimerization with an RXR partner for DNA binding and transcriptional activation. Only heterodimers bind effectively to the retinoic acid response element (RARE) to activate gene transcription in the presence of ligand. However, DNA binding of heterodimers can also occur independent of ligand binding, such that the receptors can have dual functions, serving as activators in the presence of ligand while they can be repressors in the absence of ligand. Members of the RAR family are activated by both all-trans (t-RA) and 9-cis (9c-RA) (Allenby et al., 1994), whereas the RXR family are activated exclusively by 9-cis RA (Levin et al., 1992; Heyman et al., 1992). The ability of receptors to function as activators or repressors of gene transcription is mediated through their interactions with additional cofactors. For example, the steroid receptor coactivator-1 (SRC-1) and CREB binding protein (CBP) have been identified as essential components of a coactivator complex that enhances transcriptional activation by RAR-RXR heterodimers (Onate et al., 1995; Kamei et al.,

1996; Westin et al., 1998). Similarly, corepressors, such as silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) and nuclear receptor corepressor (N-CoR) have been identified which mediate the silencing of target gene transcription by unliganded receptors (Seol et al., 1996; Baniahmad et al., 1998).

Besides the classical way of action via DNA binding, the retinoid receptors can also negatively regulate gene expression through interference with other signaling pathways. For example, they have been shown to inhibit estrogen mediated transcriptional activation in hormone dependent breast cancer cells (discussed below). Another important mechanism for inhibition of gene transcription involves interaction of RARs with the cellular oncogenes c-jun and c-fos, which are constituents of the transcription factor AP-1 (reviewed by Pfahl, 1993). AP-1 binds to specific DNA sites and activates gene transcription from adjacent promoters (Halazonetis et al., 1988). Upon ligand activation, RAR and RXR have been shown to repress c-jun/c-fos binding to the AP-1 consensus sequence and subsequent gene activation (Lee et al., 1998; Lee et al., 1999; Zhou et al., 1999). Since c-jun and c-fos have been shown to mediate the signals of peptide hormones, oncogenes and tumour promoters, the ability of liganded RARs to interfere with AP-1 components likely represents a major pathway by which retinoids exert their antiproliferative effects. In addition, negative regulation of RAR-mediated gene transcription has been shown to occur by the competitive binding of the orphan receptor COUP and the thyroid hormone receptor to RAREs (Butler and Parker, 1995; Barettino et al., 1993).

Breast cancer

Breast cancer is the most common malignancy among women in North America, and in much of Europe, Latin America and Australasia (Harrell, 1999). There is substantial evidence demonstrating that hormones play a major role in the etiology of breast cancer. The most common risk factors associated with breast cancer are familial association (Plu-Bureau and Thalabard, 1998) and the levels of exogenous and endogenous estrogen and progesterone (Soderqvist, 1998), although diet and environment have also been implicated (Snyderwine, 1998; Mannisto et al., 1999). Primary breast carcinomas arise from the epithelial cells of the lobular epithelium. They can present as either non invasive lesions, either intraductal or intralobular carcinomas, or as invasive cancers. Tumours that are invasive can spread rapidly by infiltrating through tissue spaces and can invade both lymphatic and blood vessels. They can produce a high incidence of distant metastases, mostly in the lungs, soft tissues, liver, bones and adrenals. Many human breast cancers are dependent on estrogen for growth (Lippman et al., 1988). In post menopausal women, approximately 65% of breast tumours express the ER and 50% of this number initially exhibit a hormone responsive phenotype (Clarke et al., 1994) and it has been suggested that over time many breast tumours evolve into an estrogen independent phenotype. This hormone independent phenotype is often associated with a more malignant and aggressive stage of the disease which also restricts the curative potential of current hormone therapies.

Many experimental models have been developed for the study of breast cancer. Among these are ER negative, hormone independent cell lines that exhibit a down regulation of hormone receptors (eg. MDA-MB-231). In contrast, the MCF-7, T-47D

and ZR-75-1 cell lines represent hormone dependent, ER positive human breast carcinomas (Brunner et al., 1990). Breast cancer established cell lines provide a readily available source of proliferating cells with infinite growth potential. In many cases, these lines retain in vivo properties allowing for physiological studies to be carried out. For example, the characterization of estrogen receptors was made possible because some breast cancers retained estrogen receptors (Green et al., 1986; Greene et al., 1986). Studies using these cell lines also have clinical relevance by supporting the detailed characterization of potential estrogen receptor inhibitors such as tamoxifen (Coradini et al., 1994). The antiestrogen, tamoxifen is frequently used in the treatment of breast cancer. It has the advantage of a low acute toxicity and high rate of compliance. However, the success of treatment is often limited due to the development of an antiestrogen resistant phenotype.

MCF-7 cells were initially derived from malignant effusions of a patient with metastatic mammary adenocarcinoma (Soule et al., 1973). They have been used to study the mechanisms controlling proliferation of hormone dependent breast cancer cells. However, in addition to the expression of the ER, MCF-7 cells also express high levels of the retinoid receptor subtypes RARα and γ (Roman et al., 1992). Thus they represent an excellent model system to investigate the antiproliferative effects of retinoids on hormone dependent breast carcinomas.

Retinoids and Breast Cancer

Retinoids are the most studied class of agents in chemoprevention. Since cancer is often associated with abnormal growth and loss of differentiation, retinoids

have been considered as chemopreventive agents because of their role in regulating cellular proliferation and differentiation (Lippman et al., 1987). The chemopreventive properties of retinoids were first observed in experimental models which demonstrated their efficacy in the prevention of breast, prostate, skin and urinary bladder cancers. Since then, clinical trials have observed significant retinoid chemopreventive activity in head and neck, lung, skin, cervical and breast carcinogenesis. All-*trans* retinoic acid, 13-*cis* retinoic acid and the synthetic retinoid fenretinide, N-(4-hydroxyphenyl)retinamide (4-HPR) have all been used successfully in the treatment of these diseases (Lippman et al., 1994; Weiss et al., 1998; Culine et al., 1999; Copper et al., 1999). In breast cancer trials, fenretinide was found to have little toxicity (impaired night vision) while being effective at reducing the incidence of contralateral breast cancer in women already treated for breast cancer (DePalo et al., 1997). Furthermore, the combination of fenretinide and the antiestrogen tamoxifen was found to be more effective than either drug alone in inhibiting the induction of mammary tumours in carcinogen treated rats (Hong and Lotan, 1993). This has led to the initiation of a Phase III trial of fenretininde plus tamoxifen for advanced breast cancer patients (Moon and Constantinou, 1997).

There is also evidence that retinoids can significantly inhibit the growth of human breast cancer cells. However, their efficacy is limited to hormone dependent breast cancer cells, while they are ineffective at inhibiting the growth of ER negative cells (Sheikh et al., 1993). It has been previously demonstrated that retinoids can mediate their antiproliferative effects by antagonizing the estrogen stimulation of MCF-7 cell growth (Fontana et al., 1990). Retinoic acid has also been shown to inhibit the

expression of estrogen induced genes such as pS2 and TGFα (Fontana et al., 1992), suggesting a role for RA in the inhibition of estrogen receptor transactivation. Transient transfection experiments were used to confirm the ability of RA to antagonize the estrogen induction of a vit-tk-CAT reporter gene (Demirpence et al., 1994). The reporter construct contains the estrogen response element from the vitellogenin A2 gene (vit) linked to the herpes simplex virus promoter for thymidine kinase (tk). These upstream sequences are responsible for driving the expression of the reporter gene, chloramphenicol acetyltransferase (CAT).

The mechanisms by which retinoids inhibit breast cancer cell growth and estrogen induced gene expression are believed to be dependent on RARα mediated signaling pathways (Sheikh et al., 1994). The estrogen independent cell line MDA-MB-231 is not responsive to RA and was found to contain low levels of RARα. However, upon transfection of RARα, these cells were shown to acquire sensitivity to growth inhibition by retinoids. Other evidence indicates that RARβ and γ are not involved in growth inhibition. For example, RARγ is expressed at high levels in breast cancer cells irrespective of ER status, however, RA is unable to inhibit the growth of ER negative cells (van der Leede et al., 1995). In addition, MCF-7 cells do not express detectable levels of RARβ (Sutherland et al., 1992), yet are efficiently growth inhibited. The involvement of RARα in mediating growth inhibition in MCF-7 cells was further demonstrated by the ability of a dominant negative form of the retinoic acid receptor, RARα', to alleviate growth arrest (Pratt et al., 1996). Furthermore, growth inhibition was associated with transcriptional interference of estrogen transactivation which was mediated through the RARα AF-2 domain. Recent evidence also demonstrates the

involvement of the RARα in the inhibition of MCF-7 cell growth through transcriptional interference by repressing gene activation from the transcription factor AP-1 (Jun/Fos) which participates in mediating cell proliferation signals. This inhibition of AP-1 activity is distinct and separable from its transactivating function suggesting that RARα is capable of utilizing several mechanisms to induce growth inhibition (Schule et al., 1991; Nagpal et al., 1994).

Regulation of mammalian cell cycle progression

Past studies have shown that RA inhibition of breast cancer cells is associated with accumulation of cells in G1 phase of the cell cycle (Marth et al., 1985). All eukaryotic cells possess similar mechanisms to regulate the progression of the cell cycle. It is driven by the formation and activation of protein complexes which occurs at specific stages of the cell cycle. Cyclins and cyclin dependent kinases (cdks) have been identified as the key components of these protein complexes (reviewed by Grana and Reddy, 1995). Mammalian cells contain at least 8 classes of cyclins (A to H) which reach maximum abundance at different points in the cell cycle. Similarly, at least 7 cdks (cdc2 and cdk2 to 7) have also been identified. Cyclins act as positive regulatory subunits which bind cdks to initiate phosphorylation cascades which are responsible for progression through the cell cycle. Different cyclin:cdk complexes are assembled and activated at specific points of the cell cycle. The first cyclin:cdk holoenzyme known to be activated after mammalian cells are released from quiescence is composed of a D-type cyclin in association with cdk4, cdk6 or both depending on the cell type (Matsushime et al., 1992; Kato et al., 1993; Meyerson and Harlow, 1994; Lucas et al.,

1995; Sherr, 1995) . Later in G1, cyclin E associates with cdk2 to promote S phase progression (Tsai et al., 1993). Cyclin E levels oscillate during cell cycle, being synthesized thoughout G0 to S phase in many cell types, with maximal levels being detected near the G1/S boundary. However, as cells enter S phase cyclin E is degraded, allowing cdk2 to associate with cyclin A (Koff et al., 1992; Lees et al., 1992). Entry into mitosis is triggered by the maturation promoting factor (MPF), which is composed of cdc2 and cyclin B (Labbe et al., 1989). Finally, cyclin B destruction is required for exit from mitosis.

In addition to association with a cyclin partner, concomitant phosphorylation/dephosphorylation of specific residues on the cdk catalytic complex are required to become an active holoezyme. Activating phosphorylations are catalyzed by the cdk activating kinase CAK (Solomon et al., 1992). CAK is itself a cdk complex composed of a catalytic subunit, cdk7/MO15 (Fesquet et al., 1993; Poon et al., 1993; Solomon et al., 1993), and a regulatory subunit, cyclin H (Fisher et al., 1994). In contrast, the phosphatase cdc25A is responsible for the removal of inhibitory phosphates (Hoffman et al., 1994). Recently it has been shown that a family of cyclin dependent kinase inhibitors (ckis) also play a major role in the control of cell cycle progression. The first cki identified, p21waf1, was cloned as a wild type p53 inducible gene (el-Deiry et al., 1993). Since then it has been shown to participate in the suppression of cell cycle progression by forming quaternary complexes with a cyclin:cdk pair and the proliferating cell nuclear antigen, PCNA. It has two distinct roles, one as a cki, the other as an inhibitor of PCNA (Harper et al., 1993; Gu et al., 1993). p27kip1 is another cki that shares partial identity with p21 and was first noticed as a cyclin E:cdk2

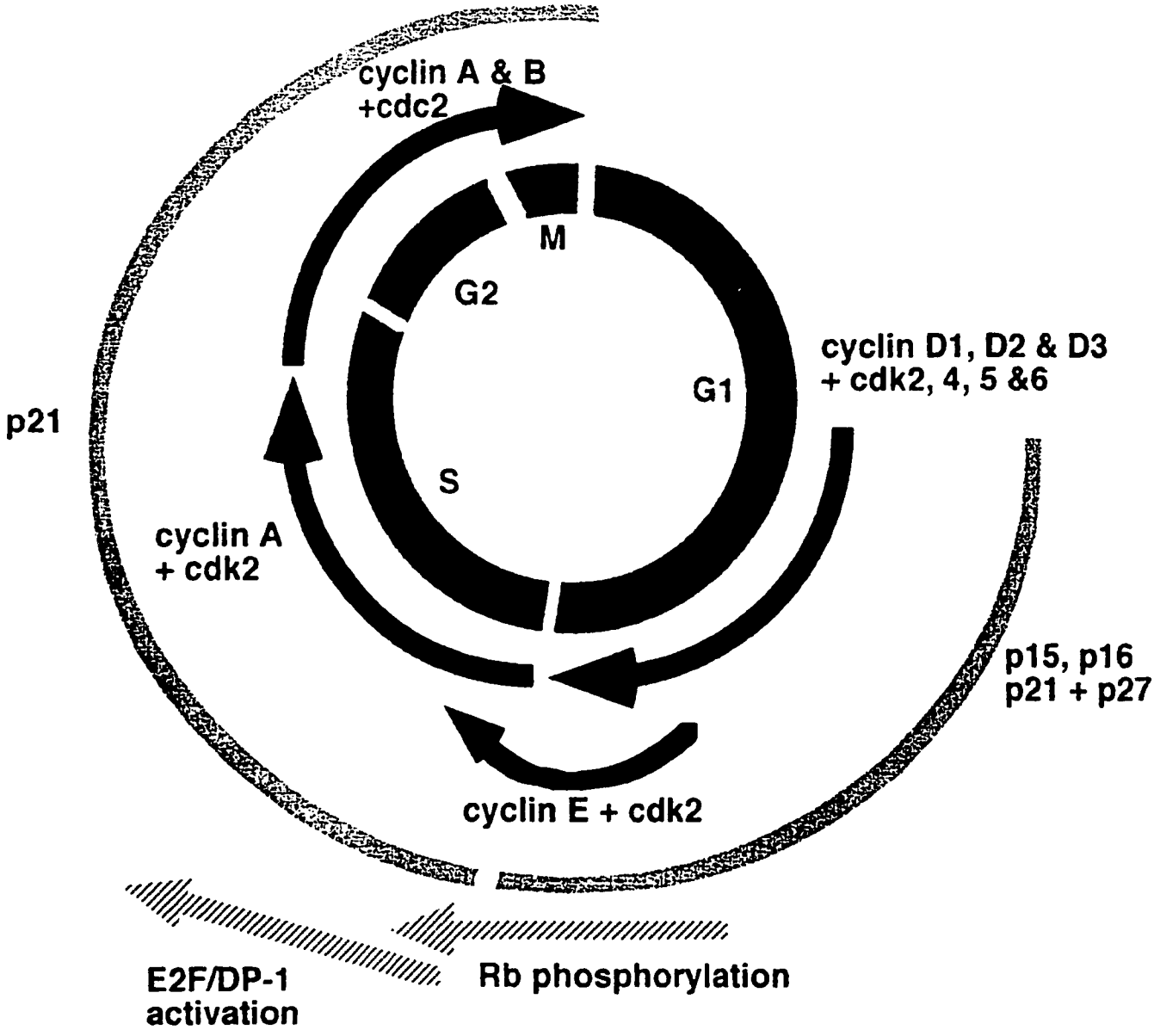
inhibitor present in TGFβ arrested or contact inhibited Mv1Lu mink lung epithelial cells (Polyak et al., 1994). Another member of this family of ckis, p57kip2, has recently been cloned, however, its expression in terminally differentiated cells suggests an involvement for this cki in the cell cycle exit of specific cell types (Matsouka et al., 1995). p15, p16, p18 and p19 are members of the INK4 family of ckis which are specific for cdk4/cdk6 complexes (Serrano et al., 1993; Hannon et al., 1994; Guan et al., 1994; Hirai et al., 1995; Chan et al., 1995). The activation of ckis has been implicated in growth suppression in response to a variety of stimuli.

A key function of the cyclin/cdk holoenzyme complexes is the phosphorylation of the retinoblastoma protein (pRb) (Figure 3) (reviewed by Bartek et al., 1997). pRb is an archetypal tumour suppressor and cell cycle regulator whose participation in controlling progression through late G1 allows cells to enter S phase and complete the cell cycle. It has no apparent catalytic function of its own, but instead exerts its biological effects by binding to other proteins including transcription factors of the E2F/DP family (Chellappan et al., 1991; Weintraub et al., 1992; Shirodkar et al., 1992; Hiebert et al., 1992). The activity of E2F/DP is modulated by binding to hypophosphorylated forms of pRb, thus preventing transcriptional activation of proliferation associated genes such as cdc2, DNA polymerase α, dihydrofolate reductase and thymidylate kinase. The activity of pRb as a repressor of G1 progression is regulated by cycles of phosphorylation and dephosphorylation on a series of its serine and threonine residues. Phosphorylation of pRb, and subsequent passage through the G1 restriction point is controlled primarily by cdk4 and cdk2 associated with the D-type cyclins (Ewen et al., 1992; Matsushime et al.,

1992; Matsushime et al., 1994; Kato et al., 1994) and cdk2 associated with cyclin E (Fang and Newport, 1991; Tsai et al., 1992).

Figure 3. Schematic representation of the cell cycle

The specific cyclin/CDK complexes that function at different stages of the cell cycle are shown along with the various inhibitory proteins that control their catalytic activity. Active cyclin/CDK complexes are responsible for the cell cycle dependent phosphorylation of pRb and the interaction of pRb with members of the E2F/DP family of transcription factors.


cyclin A & B
+cdc2
M
G2
G1
S
cyclin D1, D2 & D3
+ cdk2, 4, 5 &6
p21
cyclin A
+ cdk2
p15, p16
p21 + p27
cyclin E + cdk2
E2F/DP-1
activation
Rb phosphorylation

STATEMENT OF THE PROBLEM

As discussed above, retinoids have been shown to strongly inhibit the growth of the estrogen (E2) dependent breast cancer cell lines MCF-7, T-47D and ZR-75-1. Several mechanisms have been identified which play a role in this growth inhibition, including inhibition of estrogen induced transcription and inhibition of AP-1 activity. However, retinoids are also believed to act through induction of genes that inhibit growth and the repression of genes that promote growth. Since the antiproliferative effects of retinoids on human breast cancer cells are known to occur by cell cycle specific actions during G1 phase, we hypothesized that the effects of RA on the proliferation of ER positive breast cancer cells could also be the result of the modulation of cell cycle regulatory and regulated genes. Since the molecular mechanisms underlying the growth inhibition associated with RA are not well defined, the present study was undertaken to investigate the effects of RA on the expression of cell cycle genes and the activities of cell cycle proteins and to examine their contributions to the underlying growth arrest.

MATERIALS AND METHODS

Cell culture

MCF-7 ER+ breast cancer cells (American Type Culture Collection) were maintained in Dulbecco's minimal essential media (DMEM) (Gibco BRL) containing phenol red and supplemented with 1% non-essential amino acids, 5% heat inactivated fetal bovine serum (FBS) (Gibco BRL), 0.3% glucose and 2 mg/mL gentamicin sulfate. Incubations were at 37°C in a 5% CO_2 containing atmosphere. In some experiments cells were synchronized by contact inhibition. Following release from contact inhibition by trypsinization, cells were plated in the presence or absence of 1μM all *trans* RA (Sigma) for the indicated times. For experiments involving exponentially growing cells, cells were grown to 50-80% confluence and treated for the indicated times with a final concentration of 1 μM RA. For longer treatments, medium was changed every 48 hours and fresh drug added as required.

Flow Cytometry

Samples to be analyzed for DNA content were harvested by trypsinization and collected by centrifugation. Cells were fixed by the drop wise addition of 70% ethanol followed by a minimum 1 hour incubation at 4°C. The resuspended pellets were washed twice with 1X phosphate buffered saline (137 mM sodium chloride (NaCl), 27 mM potassium chloride, 10 mM di-sodium hydrogen orthophosphate, 1.76 mM potassium dihydrogen orthophosphate) (PBS) prior to staining with 32μM propidium iodide (45 minutes at room temperature). Propidium iodide was used in conjunction with 500μg mL-1 RNase A to remove interference from any double stranded RNA. Cells were analyzed by flow cytometry using a Coulter Epics V flow cytometer which

detected the red fluorescence emitted by propidium iodide upon laser excitation at 488 nanometers. Cell cycle phase distributions were estimated by computer fit using the program Multicycle AV software (Phoenix Flow Systems).

Plasmid Isolation

Plasmids were routinely purified from overnight bacterial cultures grown in Luria-Bertani (LB) broth containing 50 µg/mL ampicillin (Amp) (Sambrook et al., 1989). The host strain DH5α was used for plasmid propagation. Plasmid DNA was isolated by the alkaline lysis method (Birnboim and Doly, 1979) in the presence of RNase A (500 µg/mL) prior to purification on a mega prep column (Qiagen) according to the manufacturer's protocol.

Isolation of cDNA for cdk7 by RT-PCR

cDNA was synthesized from 1 µg of total RNA isolated from asynchronously growing MCF-7 cells. Random hexamers were used to prime the template in a reaction catalyzed by reverse transcriptase (RT) (Gibco BRL). The cdk7 coding sequence was amplified by polymerase chain reaction (PCR) using cdk7 specific forward (5'-GGTACCGGATGGCTCTGGACGTG-3') and reverse (5'-GGATCCTCAGTAGTAAAATGTTG-3') primers (University of Ottawa Biotechnology Research Institute) and Taq DNA polymerase (Gibco BRL). Cycling conditions were 94°C for 3 minutes, 30 cycles of 94°C/50 seconds, 60°C/2 minutes and 72°C/2 minutes, followed by termination of the reaction by incubation at 72°C/5 minutes (Innis et al., 1990). PCR products were resolved by agarose gel electrophoresis, excised and purified using a gel extraction kit (Qiagen) according to supplier's directions. Using primer generated restriction enzyme sites (Kpn I/Bam HI), the purified PCR products

were subcloned into the pCMX expression vector and sequenced. Clones whose sequence had been confirmed were used to generate cDNA probes for Northern blot analysis (see below).

Preparation of plasmid DNA for sequencing

Positive clones which had been identified by restriction enzyme digestion analysis were verified by sequencing. Qiagen purified mini preps (5 µg) were denatured with .1 volume of .2 M sodium hydroxide (NaOH) for 10 minutes at room temperature. The denatured plasmid DNA was neutralized with .1 volumes of 3 M sodium acetate (pH 5.2) and precipitated with 2 volumes of ethanol. The DNA was pelleted by centrifugation and resuspended in TE (10 mM Tris-HCl pH8.0, 1 mM EDTA (ethylenediaminetetracetic acid)). The denatured template DNA was then used for dideoxy chain terminator sequencing (Sanger et al., 1977) using Sequenase DNA polymerase (Amersham).

RNA isolation and Northern analysis

Total RNA was isolated using TRIzol reagent (Gibco BRL) following the manufacturer's supplied protocol and resuspended in diethylpyrocarbonate (DEPC) treated water. Northern analysis was performed with 20 µg of total RNA per lane. Sample RNA was characterized by gel electrophoresis through a 1% agarose gel containing 2% formaldehyde and 1X MOPS buffer (20mM 3-[N-morpholino]propanesulfonic acid (MOPS), 5mM sodium acetate, 1mM EDTA, pH 7.0). These denaturing conditions were chosen to allow good size separation and resolution of single stranded RNA. The RNA was subsequently transferred by capillary electrophoresis to nylon membranes (Amersham) using 10X SSC (1.5M sodium

chloride, .15M sodium citrate, pH 7.0). The membranes were briefly rinsed with DEPC treated water before RNA was immobilized by UV cross-linking (1200 mJ for 30 seconds). The membranes were pre-hybridized for a minimum of 4 hours at 42°C in hybridization solution (50% deionized formamide, 5X SSPE (0.75M NaCl, 50mM sodium orthophosphate, 5mM EDTA, pH 7.0), 5X Denhardts (0.5% polyvinylpyrrolidone, 0.5% bovine serum albumin, 0.5% Ficoll 400), 0.1% sodium dodecyl sulfate (SDS) and 250 µg/mL denatured, sonicated herring sperm DNA) prior to addition of radiolabeled probes. Blots were incubated overnight at 42°C with various random primed cDNA probes labeled with [α-^{32}P]dCTP (DuPONT NEN) to a specific activity of at least 1 x 10^9 cpm/µg of DNA using a multiprime labeling kit (Amersham). Following hybridization, blots were washed twice at low stringency (2X SSC, 0.1% SDS; room temperature, 20 minutes) and once at high stringency (0.1X SSC, 0.1% SDS; 65°C, 5-30 minutes). Membranes were exposed to Kodak Biomax MR X-ray film for anywhere from 2 hours to several days at –70°C, depending on the intensity of signal detected by a geiger counter. Accuracy of RNA loading was estimated using a 600 bp probe from the glyceraldehyde phosphate dehydrogenase (GAPD) gene whose mRNA levels do not change during RA treatment of MCF-7 cells.

Plasmids

Human cyclin and cyclin dependent kinase cDNAs (cyclins A, E, D1, D2, D3, cdk2 and cdk4) were provided by Paul Hamel (University of Toronto). The cDNA for cdk7 was generated by PCR. The cDNA for the human papillomavirus (HPV) 16 E6 oncoprotein was provided by Peter Howley (Harvard Medical School). The p53 mutant constructs (173L, 22/23 and δ291) were a generous gift from Karen Vousden (Ludwig

Institute for Cancer Research). The murine p53 mutant construct δPP was a generous gift from Daitoku Sakamuro (Wistar Institute). GAPD was obtained from Balwant Tuana (University of Ottawa). A cdk2 expression vector was generated by ligating a 1.2 kilobase (kb), blunt ended (Eco RI/Hind III) fragment, corresponding to the cDNA for cdk2, to a dephosphorylated and blunted, Hind III digested pCMX expression vector (Invitrogen). This ligation ensured that the cDNA for cdk2 was placed down stream of the constitutive cytomegalovirus (CMV) promoter. Ligation reactions were used to transform DH5α competent bacteria, and plated on LB/Amp agar overnight for colony formation.

Screening for recombinants

Colonies were picked with sterile toothpicks and grown as overnight cultures in LB containing ampicillin (4 mL). Plasmid DNA was isolated from clones using the alkaline lysis method (Birnboim and Doly, 1979) in the presence of RNase A (500 μg/mL) and purified on mini prep columns (Qiagen) according to the manufacturer's instructions. Purified plasmids were analyzed by restriction enzyme digestion and size fractionated on an agarose gel. Plasmids containing the cDNA for cdk2 inserted in the proper orientation were identified and prepared for double stranded DNA sequencing (as described previously) .

Western blot analysis and immunoprecipitation

Protein extracts were prepared from cultures washed twice with PBS and incubated with 1 mL per 10^7 cells of lysis buffer (20 mM Tris-HCl pH7.5, 250 mM NaCl, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride (PMSF),10 mM sodium fluoride (NaF), 1 mM sodium orthovanadate) for 30 minutes on ice (Foster and Wimalasena,

1996). Insoluble material was removed by centrifugation at 12000 rpm for 20 minutes at 4°C. Protein concentrations were determined using the BIO-RAD DC protein assay. Typically, 5-50 µg of protein were denatured in sample buffer (63 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol and .25% bromophenol blue) andresolved on 7.5% or 10% SDS polyacrylamide gels by electrophoresis (SDS-PAGE) (Laemmli, 1970) and transferred to PVDF polyscreen membranes (DuPONT NEN). The blots were blocked with 5% Carnation skim milk powder dissolved in Tris buffered saline (20 mM Tris-HCl pH7.6, 137 mM NaCl) containing .1% Tween-20 (TBS-T). The filters were incubated for 1 hour at room temperature with the appropriate primary antibodies diluted to a final concentration of 1:1000 in blocking solution. The blots were rinsed two times 5 minutes with TBS-T prior to incubation with horseradish peroxidase conjugated goat anti-mouse or anti-rabbit IgG diluted 1:5000 in TBS-T for 30 minutes at room temperature, rinsed four times 5 minutes with TBS-T and detected using the enhanced chemiluminescence (ECL) system (DuPONT NEN) according to manufacturer's protocol.

For immunoprecipitations followed by Western blotting, cell lysates were pre-cleared by incubation with 50 µL of protein A - Sepharose for 30 minutes at 4°C. The supernatants were then precipitated overnight at 4°C with protein A-Sepharose beads pre-coated with 10 µL of anti-cdk2 (M-2) (Santa Cruz). Immunoprecipitated proteins were washed four times with 500 µL of lysis buffer. Immune complexes were resuspended in sample buffer, separated by SDS-PAGE, transferred to PVDF membrane (DuPONT NEN) and analyzed by immunoblotting with the desired antibodies.

Antibodies

The following primary antibodies: anti-human pRb (Pharmingen), p27 (Transduction Laboratories), cyclin D1 (HD11), cyclin E (HE12), cyclin D3 (C-16), cdk2 (M-2), cdk4 (H-22), cdc2 (17), cdk7 (C-19), p21 (187), E2F-1 (KH95), p53 (Bp53-12) (all from Santa Cruz Biotechnology) and anti-mouse p53 (Ab-1) (Calbiochem) were used for western blot analysis. Secondary goat anti-rabbit and goat anti-mouse IgG horseradish peroxidase conjugated antibodies were purchased from Transduction Laboratories. A rabbit antibody against α actin (Sigma) was used as an internal control for protein loading.

Metabolic labeling

For $^{32}P_i$ labeling, synchronized cells were released from contact inhibition and were plated in the presence or absence of 1μM RA. At 68 hours following release from contact inhibition, cells were washed twice with PBS, pre-incubated for 30 minute with 10mL of DMEM without phosphate, and then incubated for 4 h in 3mL of phosphate free medium supplemented with 1mCi of $^{32}P_i$ (Amersham). Cells were washed twice with PBS and then lysed as previously described. Insoluble material was removed by centrifugation at 12000rpm for 20 minutes at 4°C. Equivalent amounts of cell lysate were pre-cleared by incubation with 50 μL of protein A - Sepharose for 30 minute at 4°C. The supernatants were then precipitated overnight at 4°C with protein A-Sepharose beads pre-coated with 10 μL of anti-cdk2 antibody. Immunoprecipitated proteins were washed four times with 500 μL of lysis buffer. To visualize radioactivity incorporated into cdk2 immunoprecipitates, samples were denatured in SDS sample

buffer and phosphorylated proteins were visualized by autoradiography of the dried gels.

***In vitro* kinase assays**

For cdk2 and CAK activity assays, 400 μg of cell lysates (prepared as above) were pre-cleared by incubation with 50 μL of protein A - Sepharose (BIO-RAD) for 30 minute at 4°C on a rotating platform. The supernatants were then precipitated for 4 hours to overnight at 4°C with protein A-Sepharose beads pre-coated with 10 μL of the appropriate antibody. Immunoprecipitated proteins were washed four times with 500 μL of lysis buffer. For cdk2 kinase activity, beads were resuspended in 30 μL of 40 mM Tris-HCl pH7.5, 10 mM magnesium chloride ($MgCl_2$), 5 μM adenosine triphosphate (ATP), .5mM dithiothreitol (DTT), .5mM ethyleneglycol-bis(β-aminoethylether)-N,N'-tetracetic acid (EGTA), 400 μg/mL histone H1 (Sigma), 50 μCi [γ-^{32}P]ATP (Amersham) and incubated at room temperature for 20 minutes (Foster and Wimalasena, 1996). For CAK kinase activity, beads were resuspended in 30 μL of 50 mM 4-(2-hydroxylethyl)-1-piperazine ethanesulfonic acid (HEPES) pH7.5, 10 mM $MgCl_2$, 4 mM ATP, 1mM DTT, 400μg/mL glutathione-S-transferase-cdk2 fusion protein (GST-cdk2) and 10 μCi [γ-^{32}P]ATP (Amersham) and incubated for 30 minutes at 30°C (Tassan et al., 1994). For cdk4 activity assay, protein was extracted using the following lysis buffer (50mM HEPES (pH7.5), 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT, .1% Tween 20 containing 10% glycerol, .1 mM PMSF, 10 mM β-glycerophosphate, 1 mM sodium fluoride (NaF), .1 mM sodium orthovanadate, and 3 μg/mL aprotinin) and sonicated at 4°C (Matsushime et al., 1994). Lysates were clarified by centrifugation at

12000rpm for 20 minutes at 4°C. Supernatants were pre-cleared by incubation with 50 µL of protein A - Sepharose for 30 minutes at 4°C on a rotating platform. The supernatants were then precipitated for 2 hours at 4°C with protein A-Sepharose beads pre-coated with 10 µL of the anti-cdk4 antibody. Immunoprecipitated proteins were washed four times with 500 µL of cdk4 lysis buffer and twice with 50mM HEPES (pH7.5) containing 1mM DTT. The beads were resuspended in 30 µL of 50mM HEPES (pH7.5), 1mM NaF, 2.5mM EGTA, 1mM DTT, 10mM β-glycerophosphate, .1mM sodium orthovanadate, 10mM $MgCl_2$, 20 µM ATP, 400µg/mL glutathione-S-transferase-pRb fusion protein (GST-pRb), 10 µCi [γ-^{32}P]ATP (Amersham) and incubated for 30 minute at 30°C. Following incubation the samples were denatured in sample buffer and analyzed by SDS-PAGE. Phosphorylated proteins were visualized by autoradiography of the dried gels.

GST fusion protein expression and purification

GST-cdk2 in pGEX-2T (Tsai et al., 1991) was a generous gift from Tim Hunt (Imperial Cancer Research Fund). GST-pRb (379-928) in pGEX-2T (Ewen et al., 1993) was a generous gift from Paul Hamel (University of Toronto). After transformation of E.coli strain BL21(DE3), 50 mL overnight cultures were grown to saturation, diluted 1:10 in LB and incubated at 37°C for an additional 3 hours. Production of the recombinant GST fusion protein was then induced by the addition of 100 µM isopropylthioglycoside (IPTG) at 30°C for 2 hours. Cells were recovered by centrifugation and lysed in buffer A (500 mM NaCl, 1% Triton-X 100, 50 mM HEPES (pH 7.9), 2 mM EDTA, .1 mM PMSF and 10% glycerol) (5 mL). The lysate was sonicated and clarified by centrifugation.

The supernatant was incubated for 1 hour on a rotating platform at 4°C with 500 µL of glutathione sepharose 4B (Pharmacia Biotech) that had been equilibrated in buffer A containing 1 mM DTT. Beads were washed 4 times with buffer A , and GST fusion proteins were released by incubation in 5 mM glutathione (Sigma) in 50 mM Tris-HCl pH 8.0 for 15 minutes at 4°C. The eluted GST fusion proteins were analyzed by SDS-PAGE and their concentration and purity was estimated by Coomassie blue staining of eluted proteins in comparison to protein standards of known concentration.

Cdk2 inhibitor assay

For inhibition of cdk2 activity, equal amounts of protein extracts isolated from exponentially growing MCF-7 cells were mixed with lysates isolated from cells cultured in the presence of 1 µM RA for 96 hours and brought to 1 mM DTT (Koff et al., 1993; Slingerland et al., 1994). After a 30 minute incubation at 37 °C, cdk2 associated kinase activity was assessed as previously described. Control immunoprecipitates from exponentially growing MCF-7 or RA treated MCF-7 were subjected to the experimental conditions and assayed for histone H1 activity in parallel.

Association of cdk2 with p27

A fixed amount of recombinant GST-cdk2 fusion protein was added to 400 µg of lysate isolated from exponentially proliferating MCF-7 cells cultured in the presence or absence of 1 µM RA for 96 hours, as previously described. After a 1 hour incubation at 4 °C to allow association, the resulting complexes were immunoprecipitated by incubation with equilibrated glutathione sepharose 4B beads (Pharmacia Biotech), as previously described. Samples of immunoprecipitated proteins were resuspended in SDS-PAGE sample buffer and analyzed by electrophoresis followed by transfer to nylon

membranes. Specific proteins were visualized by ECL as previously described.

Treatment with antisense p27 oligonucleotides

An antisense (AS) p27 oligonucleotide (GS3162) and the corresponding mismatch (MSM) sequence oligonucleotide (GS3436) were generous gifts from Mike Flanagan (Gilead Sciences). The sequences of the antisense and mismatch C-5-propyne-modified oligonucleotides used were GS3162, 5'-GCGUCUGCUCCACAG-3' and GS3436, 5'-GCAUCCCCUGUGCAG-3' (Flanagan et al., 1996). For maximum and uniform delivery, all oligonucleotide treatments were performed on monolayer cultures of MCF-7 cells at 50% confluence. For transfection, C-5-propyne-modified oligonucleotides (20X) were mixed with the liposome GS2888 cytofectin (20X) (Gilead Sciences). After a 10-15 minute incubation at room temperature, the oligo:cytofectin complexes were diluted to 1X concentration and overlaid onto cells. Final concentrations used were 30 nM for oligonucleotides and 2.5 ug/mL for GS2888. After a 5 hour incubation with the oligonucleotide mixture, the medium was aspirated and fresh medium with or without 1 μM RA was added. Cells were harvested for p27 immunoblotting (as previously described) and cdk2 associated kinase activity (as previously described).

Generation of stable clones in MCF-7 cells

To generate stable cell lines, MCF-7 cells were trypsinized and plated at a confluence of 30-50%. The following day cells were co-transfected with the desired expression plasmid and a selectable expression construct using the $CaPO_4$/DNA precipitate method (Chen and Okayama, 1987). After 2-3 weeks of selection in the appropriate antibiotic (2 μg/mL puromycin (Sigma) or 100 μg/mL G418 (Gibco BRL)),

colonies were isolated and expanded prior to assaying for expression of the transfected gene by northern blot analysis.

Stable expression of RARα' in MCF-7 cells

To generate stable clones, MCF-7 cells were co-transfected with the CMV-RARα' expression plasmid (Pratt et al., 1990) and CMV-puromycin. The resultant RARα' clones were assayed for growth inhibition, inhibition of ligand induced transactivation function, cdk2 protein expression (as described previously) and cdk2 associated kinase activity (as described previously) in response to RA. To assess the number of viable cells, triplicate cultures of MCF-7 cells or MCF-7 (RARα') cells were plated at a density of 25 x 10^4 cells/mL in 60 mm dishes and cultured in the presence or absence of 1 μM RA. Fresh medium and drug were added every 48 hours as required. Cells were harvested by trypsinization and a hemocytomer was used for the enumeration of viable cells, as assessed by their ability to exclude trypan blue.

Chloramphenicol acetyltransferase (CAT) assays

The C-terminal truncated mutant RARα' has been previously shown to be a dominant repressor of RA induced transcription from RA response elements (Pratt et al., 1990). For determination of ligand induced transcription, MCF-7 or MCF-7(RARα') cells were plated at 30-50% confluence. The following day, transient transfections were performed by the calcium phosphate method (Chen and Okayama, 1987) using 7.5 μg CMV-β-gal and 7.5 μg pTRE(MMTV)-CAT. All transfections were performed in duplicate and repeated at least three times. The transfected cells were cultured in the presence or absence of 1 μM RA for 48 hours and harvested in 0.25M Tris-HCl pH 7.8. Extracts were lysed by three consecutive freeze-thaw cycles and endogenous

deacetylases were heat inactivated by incubation at 65°C for 10 minutes. Lysates were clarified by centrifugation and the supernatants stored at –80°C until assayed. Chloramphenicol acetyltransferase (CAT) and β-galactosidase (β-gal) activities were determined as described previously (Gorman et al., 1982; Rosenthal, 1987). Results presented have been normalized to β-gal activity to control for transfection efficiency.

Generation of p53 deficient MCF-7 cells

To generate p53 deficient clones, MCF-7 cells were co-transfected with the expression plasmid pBR1322, encoding HPV16-E6, and CMV-puromycin. The E6 expressing clones were further screened by western blot analysis to confirm p53 expression was deficient. The resultant clones were assayed for growth inhibition (as described previously), cdk2 protein expression (as described previously) and cdk2 associated kinase activity (as described previously) in response to RA.

Stable expression of p53 mutant proteins in MCF-7 cells

MCF-7 cells were co-transfected with an expression plasmid encoding one of the p53 mutant proteins (173L, 22/23, δ291, δPP) (Marston et al., 1994), and CMV-puromycin. The expression of mutant p53 was confirmed by northern (where possible) and western blot analysis. The resultant clones were assayed for growth inhibition (as described previously), cdk2 protein expression (as described previously) and cdk2 associated kinase activity (as described previously) in response to RA.

Stable integration of cdk2 promoter driven reporter construct

DSC37, which carries a 2.4 kb 5' proximal region of the cdk2 gene fused to a promoterless luciferase reporter gene (Shiffman et al., 1996), was a generous gift from Dov Shiffman (CV Therapeutics). The plasmid pGL2-Basic (Promega) was used to

generate the luciferase reporter construct DSC37. Stable cell lines were generated by co-transfecting MCF-7 cells with either DSC37 or pGL2-Basic and CMV-puromycin as described previously. Integration of the reporter constructs was detected by Southern blot analysis. Briefly, genomic DNA was purified from clones as described previously (Sambrook et al., 1989). DNA (10 ug) was characterized following restriction enzyme digestion (Eco RV/Sac I) on a 0.8% agarose gel. After size fractionation, DNA was alkaline denatured, neutralized, transferred to a nylon support (Amersham) and cross-linked by UV irradiation. The blots were pre-hybridized as described above for Northern analysis. A 700 base pair (bp) fragment corresponding to the coding region for the luciferase gene was radiolabeled with [α-^{32}P]dCTP (DuPONT NEN) to a specific activity of at least 1 x 10^9 cpm/μg of DNA using a multiprime labeling kit (Amersham). Following overnight hybridization at 42°C, blots were washed twice at low stringency (2X SSC, 0.1% SDS; room temperature, 20 minutes) and once at high stringency (0.1X SSC, 0.1% SDS; 65°C, 5-30 minutes). Membranes were exposed to Kodak Biomax MR X-ray film for several days at –70°C for detection.

Luciferase activity assay

For luciferase reporter activity, cultures of exponentially growing MCF-7 (pGL2-Basic) or MCF-7 (DSC37) cells were treated with 1 μM RA for the indicated times. Cell lysates were prepared by washing monolayers twice with PBS followed by incubation of the cells in reporter lysis buffer (RLB) (Promega) for 20 minutes on ice. Insoluble material was removed by centrifugation at 12000 rpm for 2 minutes at 4°C. Protein concentrations were determined using the BIO-RAD DC protein assay. Luciferase activity was measured in equal amounts of extracts using a luciferase assay system

(Promega) as described by the supplied protocol. RA induced changes in luciferase reporter activity were quantified using a luminometer.

Nuclear run-on transcription assays

Intact nuclei were isolated from MCF-7 cells treated with 1μM RA for 24 hours or untreated. The nuclei were isolated by incubating cells in buffer A (15 mM NaCl, 60 mM potassium chloride, 15 mM HEPES pH 7.5, 2 mM EDTA pH 8.0, 0.5 mM EGTA pH 8.0, 0.15 mM spermine, 0.5 mM spermidine, 14 mM β-mercaptoethanol, 0.3M sucrose and 0.5% NP-40) for 10 minutes on ice. Following lysis, nuclei were collected by centrifugation at 1800rpm for 5 minutes and resuspended in buffer E (20 mM Tris-HCl pH 7.9, 75 mM NaCl, 0.5 mM EDTA pH 8.0, 0.85 mM DTT, 1 mM PMSF and 50% glycerol). The transcription assay was carried out by incubating 5-10 x 10^6 nuclei in a reaction buffer containing 0.3 M ammonium sulfate, 100 mM Tris-HCl pH 7.9, 4 mM MgCl2, 4 mM manganese chloride, 200 mM NaCl, 0.4 mM EDTA, 0.1 mM PMSF, 0.1 mM DTT, 10 mM creatine phosphate, 2 units RNase inhibitor, 29% glycerol, 1 mM each of ATP, CTP and GTP, and 150μCi-[α-^{32}P]UTP (3000Ci/nmol, Amersham) for 30 minutes at 26-28°C (Celano et al., 1989). The reaction was terminated by digestion with DNase I for 15 minutes at 37°C and the ^{32}P labeled nascent RNA was isolated using TRIzol reagent (Gibco BRL) according to the manufacturer's direction. RNA was resuspended in 20 mM HEPES pH 7.5 and 5 mM EDTA prior to alkaline denaturation with 0.2M NaOH (15 minutes on ice). The reaction was neutralized with 0.25M HEPES pH 5.5, and RNA precipitated. The cDNAs for cdk2 and GAPD were heat (65°C) and alkaline denatured (0.3 M NaOH) for 30 minutes, followed by neutralization with 1 M ammonium acetate pH 7.0. The denatured cDNA inserts were slot blotted (Schleicher

and Schuell Minifold II) onto nylon membranes (Amersham) and immobilized by UV crosslinking (1200 mJ for 30 seconds). Pre-hybridization was performed as described above for Northern blot analysis. Equal counts of newly elongated nuclear RNA were hybridized to the blots of denatured cDNA for 48 hours at 42°C. The membranes were washed twice at low stringency (2X SSC, .1% SDS; room temperature, 20 minutes), treated with 10 µg/mL RNase A for 5-30 minutes at room temperature, followed by an additional low stringency wash. Membranes were exposed to Kodak Biomax MR X-ray film for several days at –70°C for signal detection.

Cdk2 mRNA stability

Exponentially growing MCF-7 cells cultured in the presence or absence of 1 µM RA for 24 hours were treated with 10 µg/mL of actinomycin D (Sigma) for the indicated times. Total RNA was isolated using TRIzol reagent (Gibco BRL), following the manufacturer's supplied protocol, and resuspended in diethylpyrocarbonate (DEPC) treated water. Northern analysis was performed as previously described. The blots were probed for cdk2, GAPD and p21 mRNAs successively.

Stable overexpression of cdk2 in MCF-7 cells

For constitutive overexpression of cdk2, MCF-7 cells were co-transfected with a plasmid encoding the cdk2 transcript downstream of the CMV promoter (CMV-cdk2) and CMV-neomycin. Individual colonies were isolated and assayed for expression of the transfected gene by northern blot analysis and for protein expression by western blot analysis.

In situ end labeling (ISEL) for the detection of apoptotic nuclei

Asynchronous populations of MCF-7 and MCF-7(CDK2) cells, grown on

coverslips, were cultured in the presence or absence of 1 μM RA for 72 hours. Cells were rinsed once with PBS and fixed in a solution of methanol (MeOH): acetone (1:1) for 10 minutes at room temperature. Cells were air dried and washed for two times 5 minutes with PBS. Cells were labeled for 1 hour at 37°C with biotinylated-16-UTP (Boehringer Mannheim) in a reaction catalyzed by exogenous terminal deoxytransferase (Boehringer Mannheim) (Gorczyca et al., 1992). Cells were washed three times 5 minutes with PBS and stained by incubation with CY2 conjugated avidin in 4X SSC, .1% Triton x-100 and .5% carnation skim milk powder for 45 minutes in the dark. Free 3'-OH ends were visualized by fluorescence microscopy (Zeiss Axiophot microscope).

Densitometry

Quantification of data was done using photo densitometry (MCID Imaging Research Incorporated) of the autoradiogram.

RESULTS

Retinoic acid inhibition of cell cycle progression is associated with inhibition of cdk2 activity

A. Effect of retinoic acid on cell cycle phase distribution

While it has been previously demonstrated that RA inhibits the growth of MCF-7 cells through cell cycle specific actions during G1 (Marth et al., 1985; Fontana et al., 1990), it has also been suggested that there is some clonal variation in responsiveness (Fontana et al., 1992). Initial experiments sought to define the effects of RA on cell cycle progression in our MCF-7 cells. Cells were partially synchronized by contact inhibition to help clarify RA effects on specific phases of the cell cycle. Although loss of contact inhibition is one of the classical hallmarks of transfomation, many tumour cell also appear to maintain a degree of contact inhibition. We found that MCF-7 cells were partially susceptible to contact-dependent growth inhibition and were able to achieve a 70% accumulation of cells in G1 phase (Table 1). Following release from contact inhibition by trypsinization, cells were plated in the presence or absence of 1 μM RA. This concentration of RA has previously been shown to cause accumulation of MCF-7 cells in G1 (Marth et al., 1985; Fontana et al., 1990). Furthermore, it was shown that growth inhibition was not due to nonspecific cytotoxicity as growth resumed after removal of the retinoid. Cell cycle phase distribution was determined at various intervals by flow cytometric analysis (Table 1). Little change was apparent over the first 24 hours of exposure to RA, but the proportion of cells in S phase then fell continuously to reach a minimum of approximately 60% of the control value by 96 hours (Figure 4). These decreases were accompanied by a clear accumulation of cells in

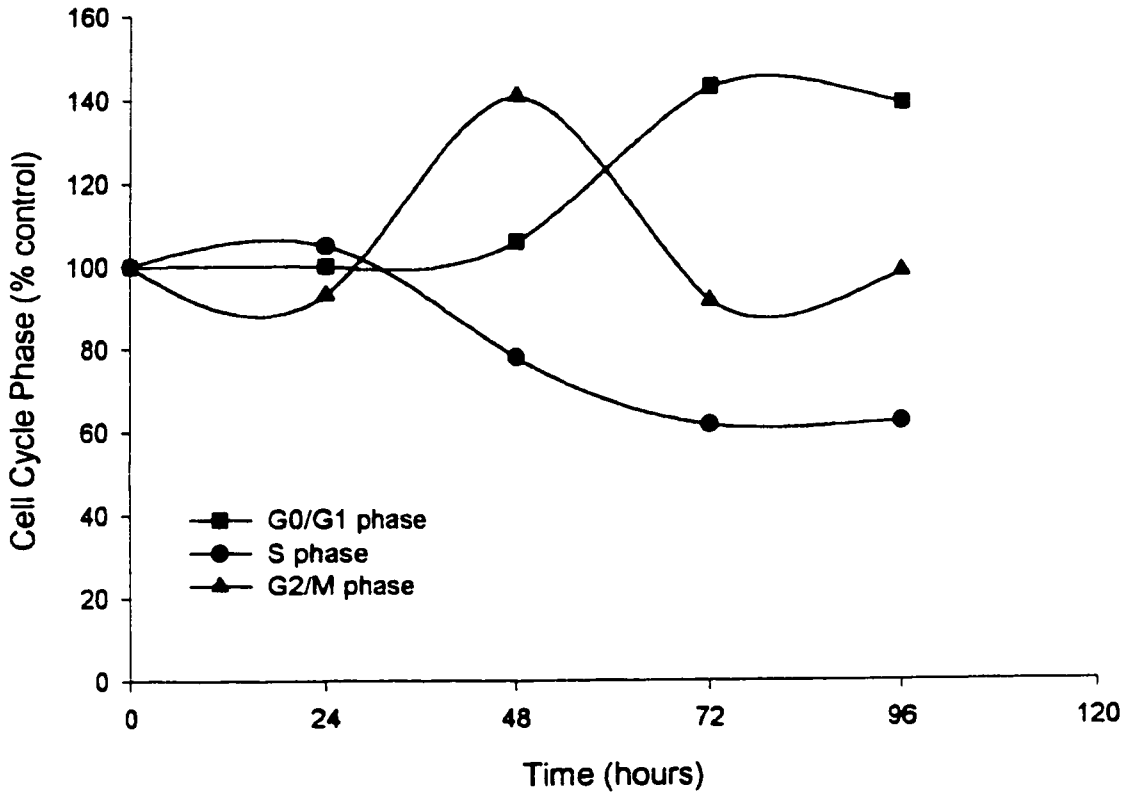
Table 1. Cell Cycle Distribution of Contact Inhibted MCF-7 Cells and Cells Released From Contact Inhibition in the Presence or Absence of RA

	Cell Cycle Distribution (%)		
	G_0/G_1	S	G_2/M
contact inhibited	69.0 ± 4.7	21.3 ± 4.8	9.7 ± 5.1
24 h	61.2 ± 3.6	22.7 ± 4.1	16.2 ± 5.8
24 h +RA	61.3 ± 4.6	23.8 ± 4.6	15.1 ± 4.4
48 h	42.7 ± 4.5	39.5 ± 3.2	16.8 ± 3.8
48 h +RA	45.3 ± 3.2	31.0 ± 2.2	23.7 ± 3.1
72 h	39.8 ± 1.4	37.5 ± 1.9	22.3 ± 1.9
72 h +RA	57.0 ± 5.0	23.1 ± 2.6	20.4 ± 3.1
96 h	40.3 ± 4.8	43.0 ± 3.5	17.4 ± 2.7
96 h +RA	55.9 ± 1.8	26.8 ± 1.3	17.1 ± 3.5

Values represent the mean percentage of cells in the indicated phase from five independent experiments ± SD.

Figure 4. Effect of RA on MCF-7 cell cycle phase distribution

Semi-synchronous populations of MCF-7 cells maintained in DMEM were treated with 1 μM RA or left untreated. At various time intervals, cells were harvested for DNA analysis by flow cytometry as described in "Materials and Methods". RA induced changes in cell cycle phase distribution are expressed relative to values obtained for control cells. Data points represent the mean of at least three individual experiments.


160
140
120
100
80
60
40
20
0
Cell Cycle Phase (% control)
G0/G1 phase
S phase
G2/M phase
0
24
48
72
96
120
Time (hours)

G0/G1. No significant effects were seen on the proportion of cells in G2/M, however at later time points it has been reported that a decrease in the G2/M fraction is also observed (Zhou et al., 1997) .

B. Inhibition of pRb phosphorylation by retinoic acid

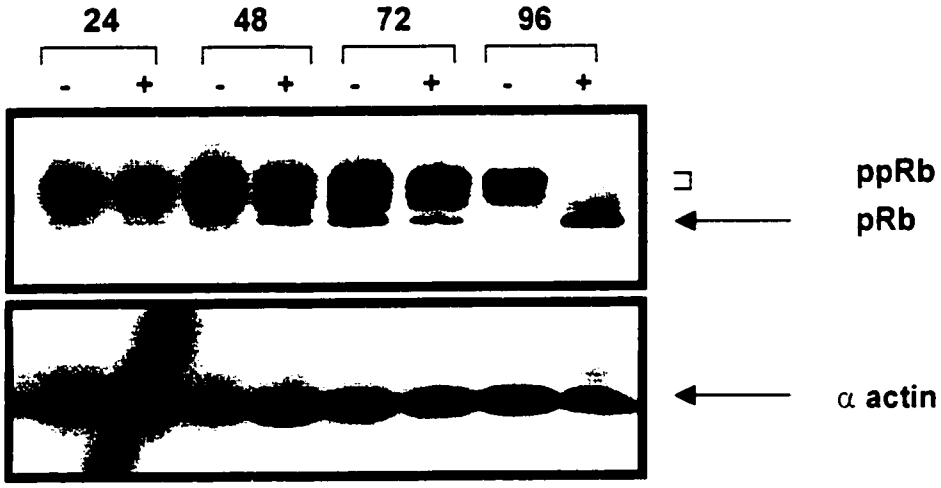
The retinoblastoma protein is a known regulator of cell cycle progression acting in late G1 (reviewed by Bartek et al., 1997). Furthermore, since its activity as a repressor of G1 progression is regulated by phosphorylation mediated by the G1 cyclin:cdk complexes, we examined the effects of RA on pRb phosphorylation (Figure 5). MCF-7cells were semi synchronized by contact inhibition, and released in the presence or absence of 1 μM RA. At 24 hours following release from contact inhibition, no difference in the phosphorylated states of pRb were observed between control and RA treated cells. However, by 48 hours, the appearance of the hypophosphorylated species was detected in RA treated cells. At the same time a decrease in the amount of total pRb was also observed. By 96 hours post RA, additional hypophosphorylation and a decrease in total pRb protein were observed, such that none of the hyperphosphorylated form remained. In contrast, control cells contained only the hyperphosphorylated forms of pRb.

C. Effects of retinoic acid on cyclin and cyclin dependent kinase gene expression

To identify changes in G1 cyclin or cdk expression that might account for the RA induced decreases in pRb phosphorylation and accumulation of cells in G1, Northern

Figure 5. RA decreases pRb protein levels and phosphorylation

Whole cell lysates from semi-synchronous MCF-7 cells treated with 1 μM RA or left untreated were used for western blot analysis of pRb. Immunoreactive bands were developed using chemiluminescence, as described in "Materials and Methods". The multiple hyperphosphorylated forms of Rb are represented by ppRb, while pRb indicates the hypophosphorylated form. Immunoreactivity with an α-actin antibody was used as an internal control for equivalency of protein loading.


24
48
72
96
- + - + - + - +
ppRb
pRb
α actin

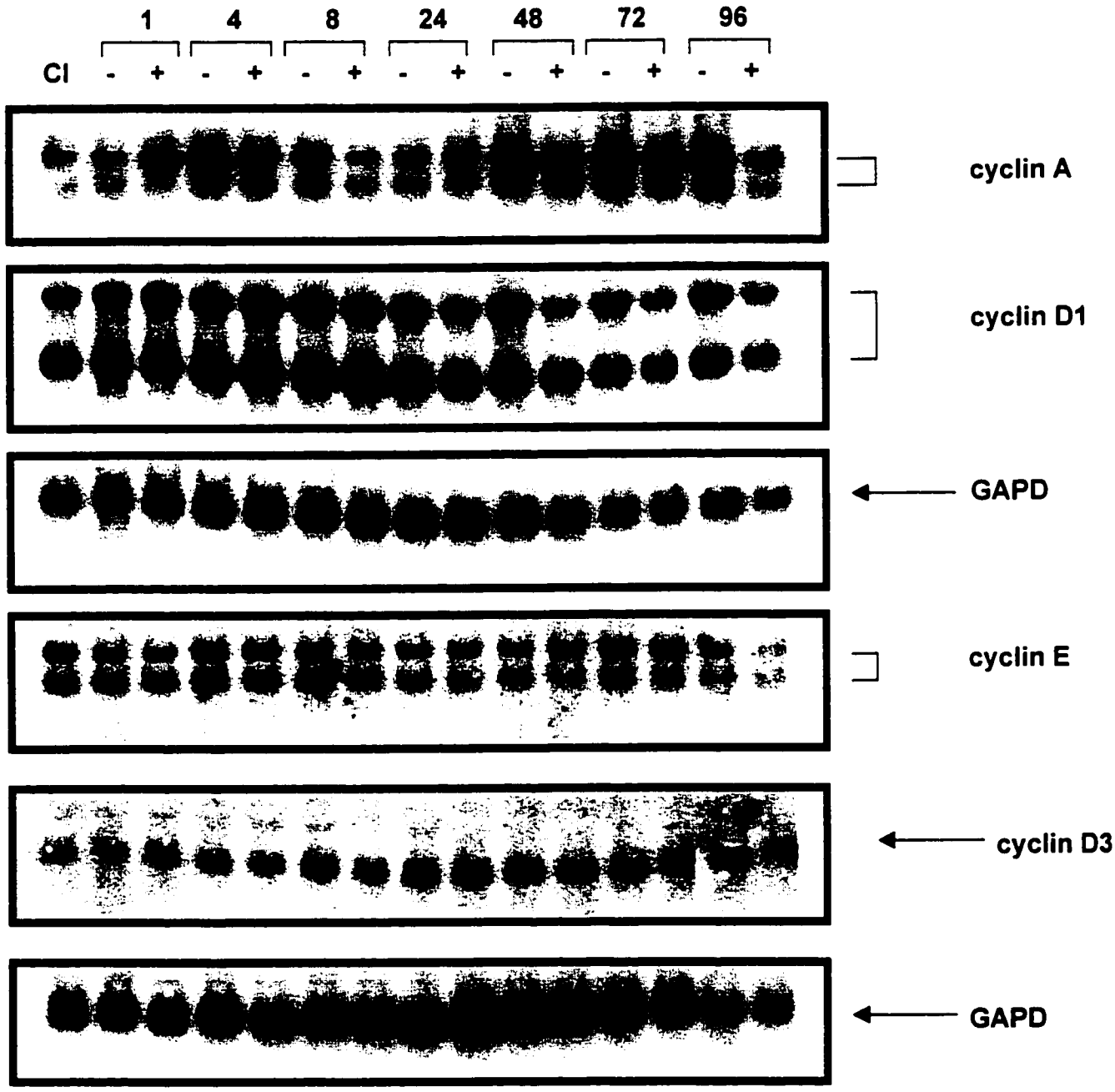
blot analysis was performed on RNA isolated from MCF-7 cells released from contact inhibition in the presence or absence of RA. Immediately following release in the presence of RA, cyclin A mRNA levels were increased, however, by 4 hours they began to decline (Figure 6A). At 24 hours they underwent a second increase before becoming reduced to approximately 50%, relative to control cells, at 96 hours post RA. The early decrease in cyclin A appeared without major effects on cell growth since both control and RA treated cells were equally distributed in the cell cycle by 24 hours after release. The later decline in cyclin A levels can be attributed to the RA mediated decrease in the S phase population. RA treatment also produced a decrease in cyclin D1 mRNA levels, which was detected after 24 hours, and persisted throughout the time course of treatment (Figure 6A). The levels of the remaining cyclins D3 and E were unaffected after treatment with RA (Figure 6A). Cyclin D2 mRNA was present at barely detectable levels and was not further investigated.

MCF-7 cells synchronized by contact inhibition also expressed readily detectable levels of cdk2 and cdk4. Following exposure to RA, the expression of cdk2 was dramatically down regulated by 48 hours compared to untreated control cells, while no change in the abundance of cdk4 was detected (Figure 6B). To ensure that the decrease in cdk2 mRNA was not due to RA preventing exit of cells from G1, Northern blot analysis was performed on RNA isolated from exponentially growing MCF-7 cells cultured in RA for the indicated times. We observed that RA was capable of producing a similar decrease in cdk2 expression under these MCF-7 conditions (Figure 7).

Figure 6. Effect of RA on G1 cyclin and cdk mRNA levels

Semi-synchronous populations of cells were released from contact inhibition (CI) in the presence or absence of 1 μM RA for various times and harvested for RNA, as described in "Materials and Methods". Northern blot analysis was performed by probing replicate filters, containing 20 μg of total RNA per lane, for each mRNA species **(A)** cyclins A, D1, D3, E and **(B)** cdk2 and cdk4. Blots were also hybridized to GAPD as an internal control for RNA loading.

A


1
4
8
24
48
72
96
CI
- + - + - + - + - + - + - +
cyclin A
cyclin D1
GAPD
cyclin E
cyclin D3
GAPD

B

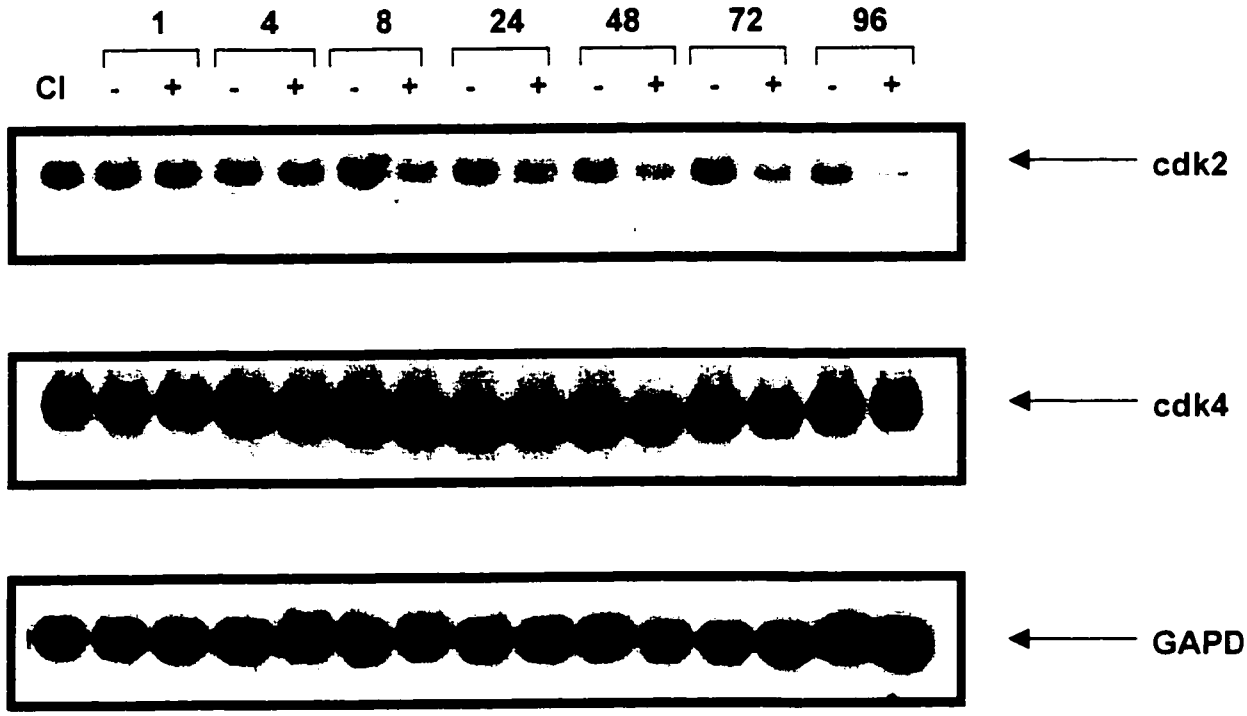
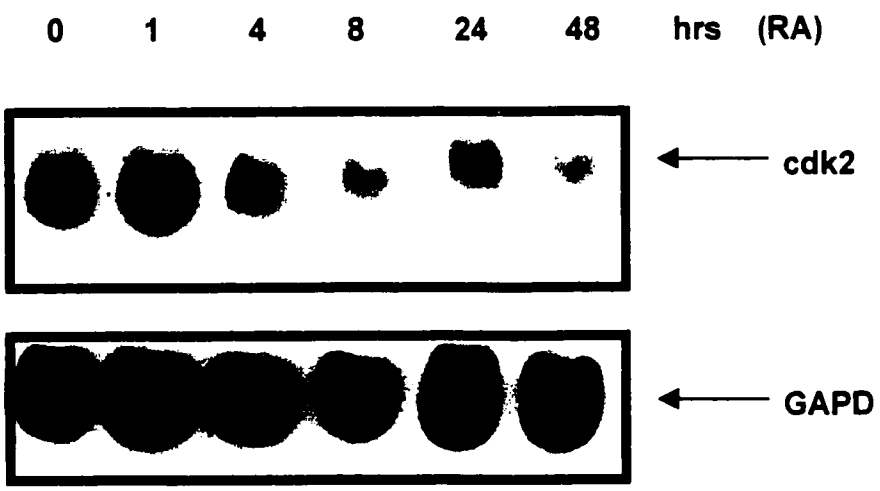


Figure 7. RA decreases cdk2 mRNA levels

Exponentially growing MCF-7 cells were treated with 1 μM RA for the indicated time intervals. Northern blot analysis was performed on 20 μg of total RNA from each time.


0
1
4
8
24
48
hrs
(RA)
cdk2
GAPD

D. RA decreases cyclin D1 and cdk2 protein levels

To determine whether the steady state protein levels were reduced in parallel with mRNA levels, Western blot analysis of cyclin D1 and cdk2 proteins was also performed. Cyclin D1 was readily detectable in contact inhibited cells. Following release from contact inhibition, we observed a decrease in the amount of cyclin D1 protein (p34), and this reduction corresponded closely with the fall in mRNA levels demonstrated in Figure 6A, decreasing by 48 hours post RA (Figure 8A). Treatment with RA also resulted in a 2 fold decrease in cdk2 protein (p33) that was evident at 72 hours and this reduction persisted to the 96 hour time point (Figure 8B). Surprisingly, the change in cdk2 protein levels was minimal compared with the dramatic decrease observed in mRNA levels. In contrast, no effects on cdk4 (p34) or cdc2 (p34) protein levels were observed following exposure to RA (Figure 8B).

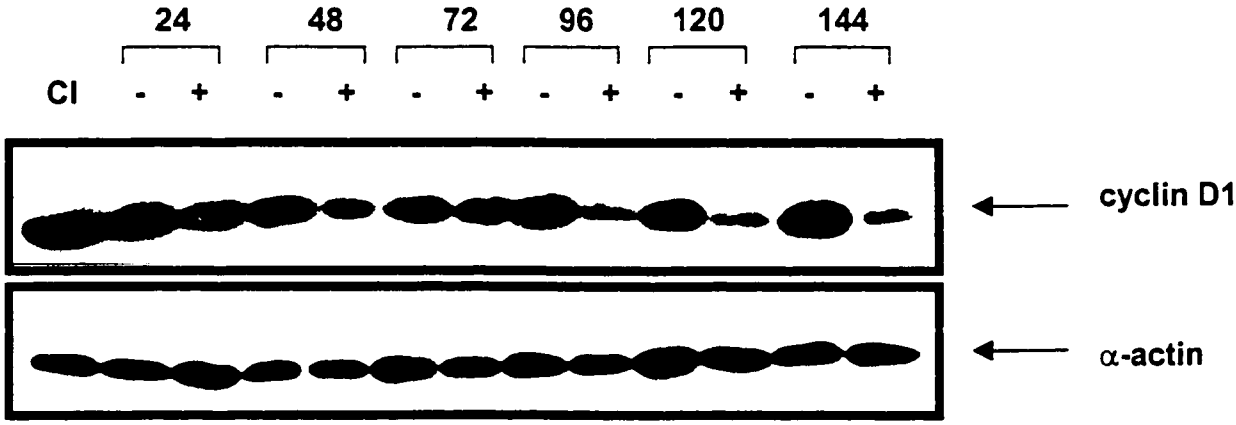
E. RA decreases phosphorylation of cdk2

Since the rapidly migrating form of cdk2 (Thr-160 phosphorylated) could not be resolved by western blot analysis, we wanted to explore the possibility that RA was able to regulate cdk2 phosphorylation. MCF-7 cells released from contact inhibition were cultured in the presence or absence of RA and metabolically labeled with $^{32}P_i$. The resultant phosphate labeled cell lysates were immunoprecipitated with anti-cdk2. We observed a significant reduction in the amount of $^{32}P_i$ incorporated in cdk2 immunoprecipitates from lysates of RA treated cells (Figure 9).

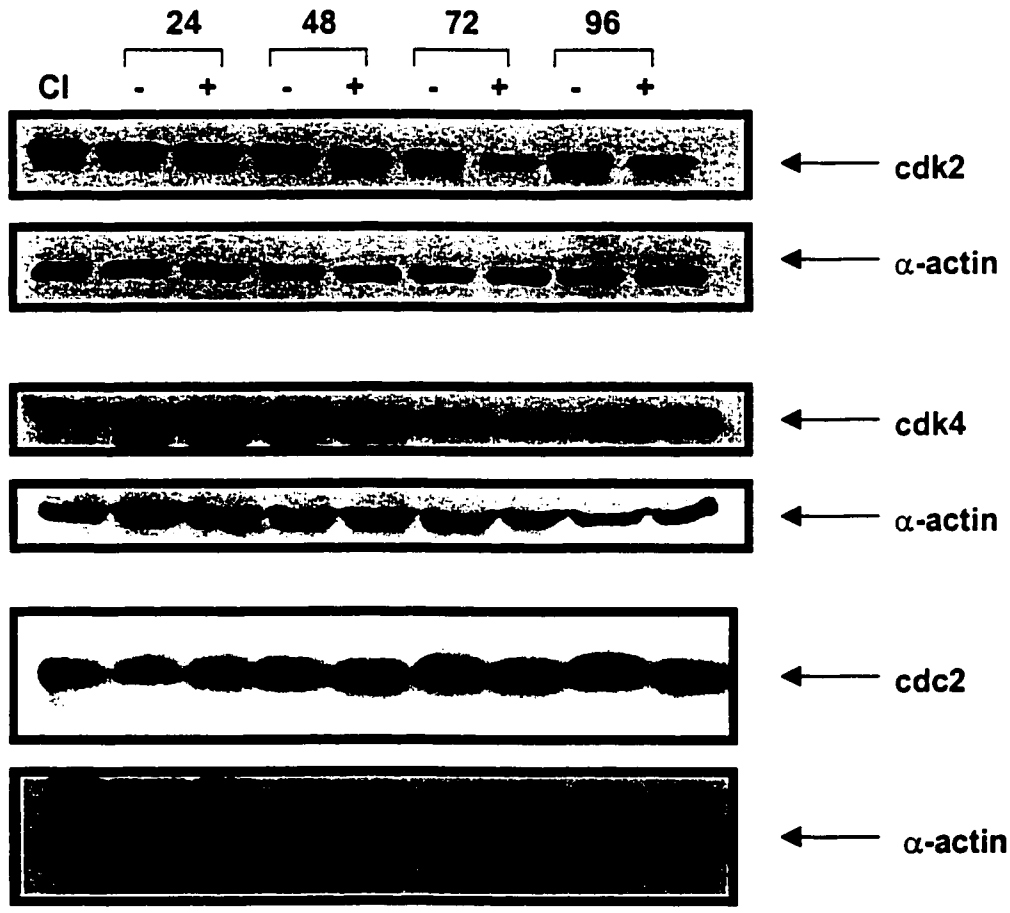
Figure 8. RA decreases cyclin D1 and cdk2 protein levels

Cell extracts from contact inhibited cells (CI) and cells released in the presence or absence of 1 μM RA were resolved on 10% SDS polyacrylamide gels. Western blot analysis was performed by probing with the indicated antibodies and immunoreactive proteins were visualized by chemiluminescence. An α-actin antibody was used as a protein loading control for all blots.

A


24
48
72
96
120
144
CI
- + - + - + - + - + - +
cyclin D1
α-actin

B


24
48
72
96
CI
- + - + - + - +
cdk2
α-actin
cdk4
α-actin
cdc2
α-actin

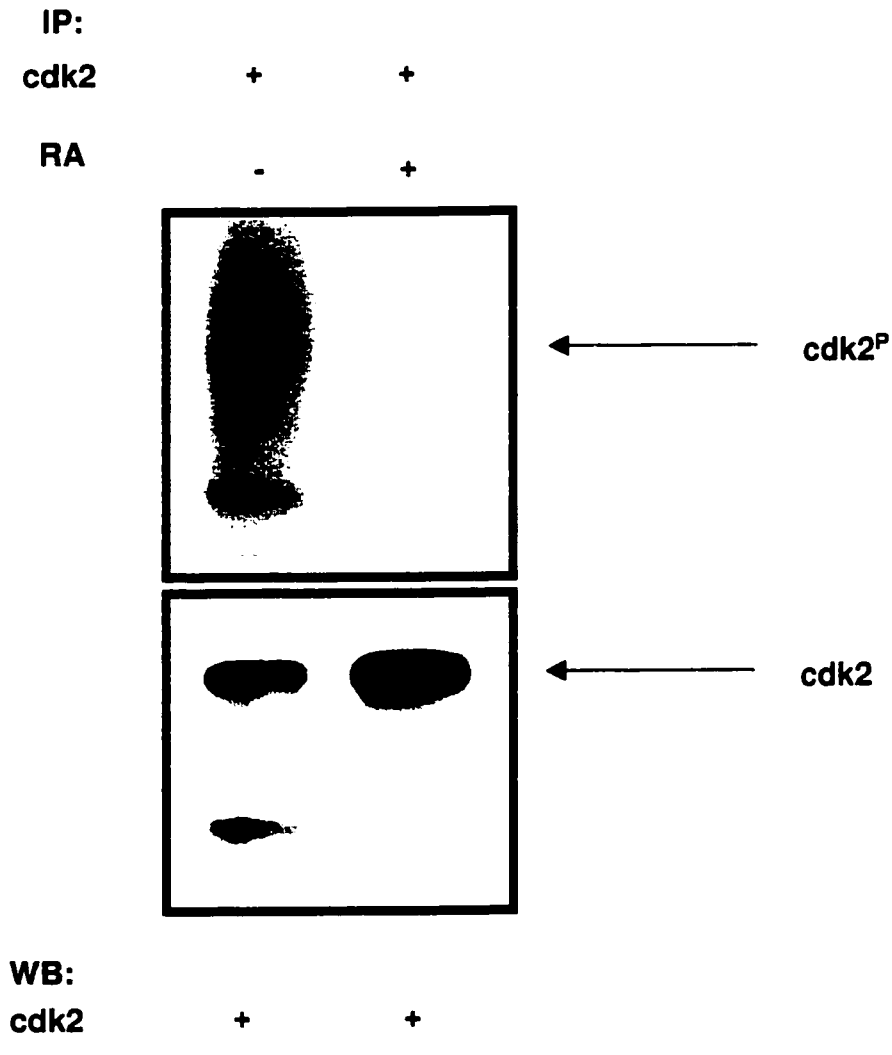
Previous experiments performed in TGFβ arrested myeloid cells also demonstrated a similar decrease in the phosphorylation status of cdk2. Furthermore, the decrease was attributed to the more rapidly migrating form of cdk2 which has a faster rate of turnover than the two inhibitory phosphates (Bang et al., 1996). Western blot analysis with the same anti-cdk2 antibody (shown below) confirmed that there was a marked decrease in phosphorylation of cdk2 following exposure to RA. An additional labeled band at 30kDa coimmunoprecipitated with cdk2 and was also found to cross react with the cdk2 antibody by western blot analysis.

F. Inhibition of cdk2 activity following exposure to retinoic acid

The decreased phosphorylation of both cdk2 and pRb suggested that cdk2 kinase activity could be inhibited by RA. Therefore, synchronized cultures of MCF-7 cells were released in the presence or absence of RA to determine whether RA mediated G_1 arrest was associated with changes in cdk2 activity. Histone H1 kinase assays were performed on cdk2 immune complexes prepared from cell lysates harvested at various times following exposure to RA. At 24 hours following release, cdk2 activity was reduced in both control and RA treated cells. However, this decrease was only transient, and at 48 hours both control and RA treated cells contained increased cdk2 activity, although this activity was significantly lower (2.5 fold) in RA lysates. By 96 hours post RA cdk2 activity was severely inhibited (10 fold reduction) compared to control cells (Figure 10A), strongly suggesting a role for this kinase as a mediator of RA induced growth inhibition. Densitometric analysis was used to quantify the time dependent inhibition of cdk2 activity upon exposure to RA (relative to control)

Figure 9. Metabolic labeling of cdk2 with $^{32}P_i$

Cell extracts were prepared from MCF-7 cells 72 hours following release from contact inhibition in the presence or absence of 1 μM RA. The cells were metabolically labeled for the last 16 hours with $^{32}P_i$, as described in "Materials and Methods". Radioactivity incorporated into cdk2 present in cdk2 immunoprecipitates is shown in the top panel. In the lower panel the same cdk2 immunoprecipitates were immunoblotted to determine total cdk2 protein.

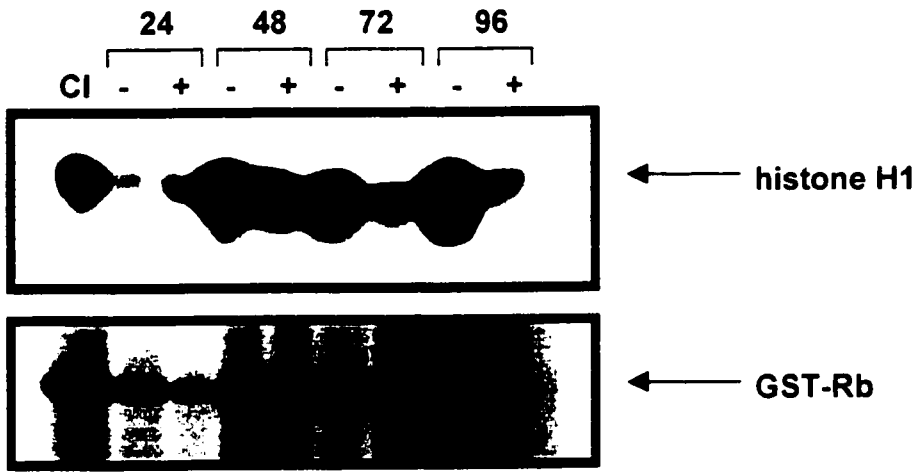

IP:
cdk2 + +
RA - +
cdk2P
cdk2
WB:
cdk2 + +

(Figure 10B). Cdk4 is a major partner of D-type cyclin complexes, therefore, we tested whether the RA induced decrease in cyclin D1 levels could affect cdk4 kinase activity. Using a GST-pRb fusion construct as a substrate (379-928), no change in cdk4 activity was detected in cdk4 immune complexes from untreated or RA treated cells lysates (Figure 10A).

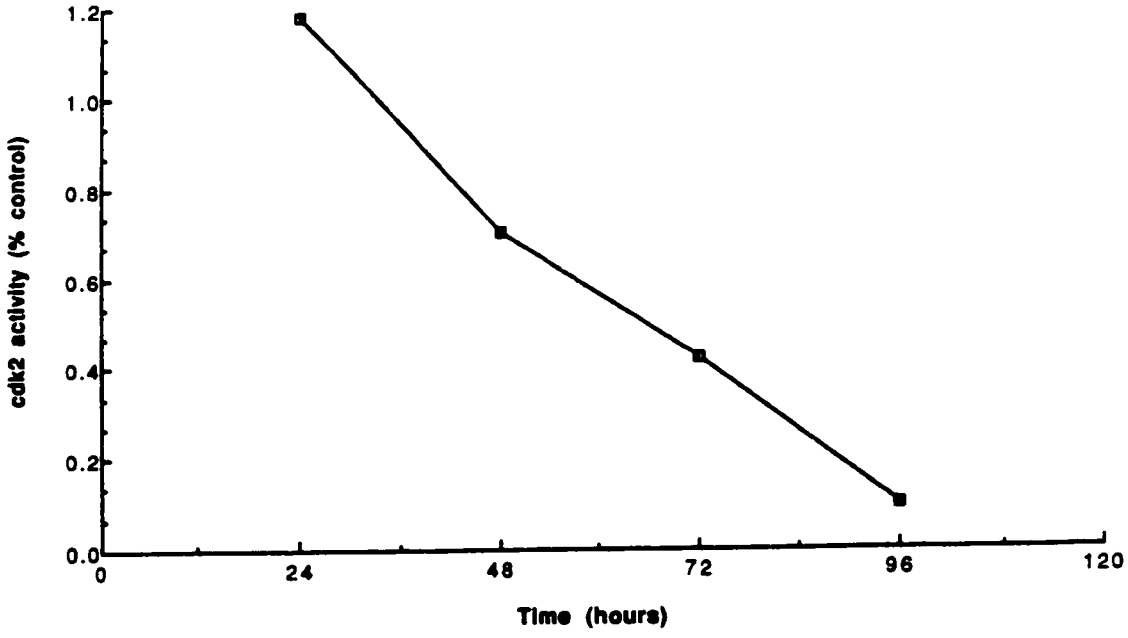
Figure 10. RA inhibits cdk2 activity

A. Cell lysates used for immunoprecipitation were isolated from contact inhibited cells (CI) and from cells following release in the presence or absence of 1 μM RA, as described in "Materials and Methods". The histone H1 kinase activity in anti-cdk2 immunoprecipitates was assayed in extracts containing 400 μg of protein. The radiolabeled products were separated on 10% denaturing gels and detected by autoradiography (exposure time, 2 hours). pRb associated kinase activity in anti-cdk4 immune complexes was assayed in extracts containing 100 μg of protein. The radialabeled GST-pRb fusion protein was resolved on 7.5% denaturing gels and detected by autoradiography (exposure time, 12 hours). **B.** Determination of cdk2 activity in RA treated cells by densitometric scan of histone H1 phosphorylation, activity is expressed relative to untreated control lysates at each time point.

A


24
48
72
96
CI - + - + - + - +
histone H1
GST-Rb

B


cdk2 activity (% control)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
24
48
72
96
120
Time (hours)

Effects of retinoic acid on known regulators of cyclin dependent kinases

A. Retinoic acid has no effect on cdk activating kinase (CAK)

We next examined whether the loss of cdk2 phosphorylation and activity could be attributed to a change in the levels of activity of cdk2 activating kinase (CAK). Northern blot analysis showed no change in the mRNA levels for cdk7, the kinase responsible for the catalytic activity of CAK (Figure 11A). In contrast, western blot analysis of protein levels showed increasing amounts of a faster migrating species following release from contact inhibition, and by 144 hours following exposure to RA, a slight decrease in cdk7 levels was evident (Figure 11B). We also looked at whether the inactivation of cdk2 was due to an inhibition of CAK activity. Using a GST-cdk2 fusion construct as a substrate, no change in CAK activity was detected in cdk7 immune complexes from untreated or RA treated cell lysates until 96 hours post RA when a slight decrease in CAK activity was observed (Figure 11C).

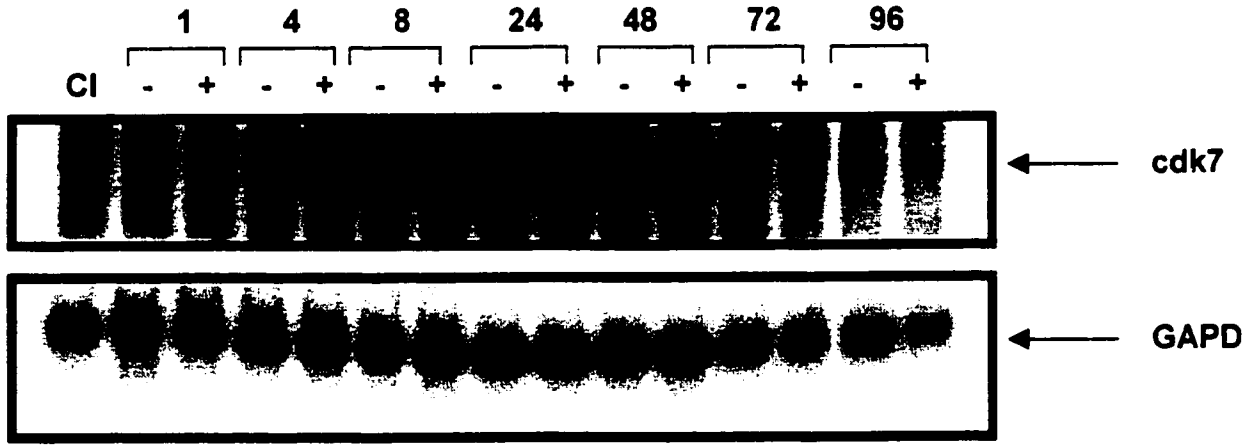
B. Retinoic acid effects on the cyclin dependent kinase inhibitors p21 and p27

Since RA did not appear to regulate cdk7 expression or CAK activity, we wished to determine whether RA inhibition of cdk2 activity was accompanied by an increase in the expression of an inhibitor of cdk2 activity. It has been previously shown that cdk inhibitors play a role in mediating growth arrest induced by a variety of stimuli including TGFβ (Koff et al., 1993; Polyak et al., 1994; Slingerland et al., 1994; Reynisdottir et al., 1995), antiprogestins (Musgrove et al., 1997) and vitamin D3 (Wu et al., 1997). We therefore examined whether RA might regulate the levels of expression of the specific inhibitors of cdk2 activity, p21 and p27. Western blot analysis showed that p21 levels

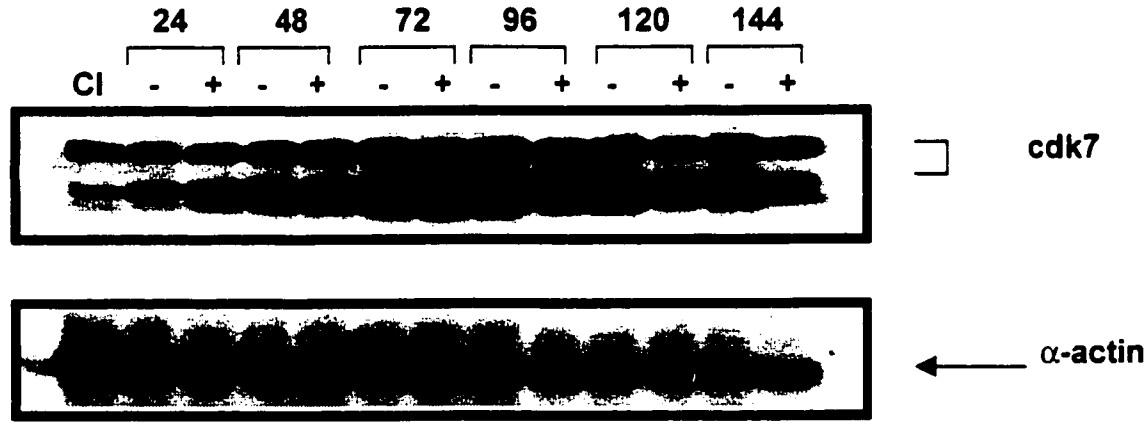
Figure 11. RA has no effect on cdk7 expression or CAK activity

Semi-synchronous populations of cells were released from contact inhibition (CI) in the presence or absence of 1 μM RA for the indicated times and harvested for RNA or protein, as described in "Materials and Methods". **A.** Northern blot analysis was performed on 20 μg of total RNA per lane to assess cdk7 mRNA levels. GAPD hybridization was used as an internal control for RNA loading. **B.** Western blot analysis of cdk7, showed two immunoreactive bands which were present in all samples except in CI cells. Equivalency of loading was assessed by reactivity with an α-actin antibody. **C.** The cdk2 kinase activity in anti-cdk7 immunoprecipitates was assayed in extracts containing 400 μg of protein, as described in "Materials and Methods". The radiolabeled products were separated on 10% denaturing gels and detected by autoradiography (exposure time, 4 days).

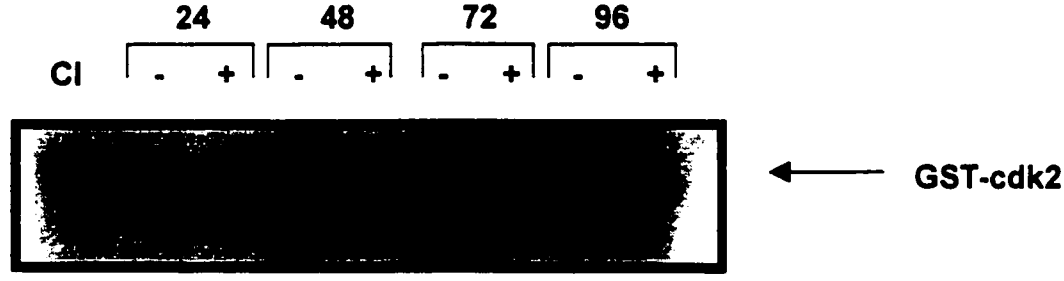
A


1
4
8
24
48
72
96
CI
- + - + - + - + - + - + - +
cdk7
GAPD

B


24
48
72
96
120
144
CI
- + - + - + - + - + - +
cdk7
α-actin

C


24
48
72
96
CI
- + - + - + - +
GST-cdk2

were elevated in contact inhibited cells and decreased upon release in both control and RA treated lysates. We observed that the level of p21 did not change in control cells following release from contact inhibition. However, during the first 48 hours, p21 expression was elevated following exposure to RA, compared to control cells before becoming down regulated at 96 hours post RA (Figure 12). However, these early increases in p21 occurred well before any major effects in cdk2 activity were observed. In contrast, cells cultured in the presence of RA displayed a slight reduction in p27 levels at all times assayed (Figure 12).

C. An inhibitor of cdk2 activity is present in RA treated cells

It has been previously demonstrated that the cdk inhibitory activity induced by TGFβ in mink lung epithelial cells was capable of inhibiting cdk kinase activity in untreated lysates (Slingerland et al., 1994). The absence of substantial changes in the expression and activity of various regulators of cdk2 led us to examine whether RA treated cells contained a soluble inhibitor of cdk2 activity. Lysates were extracted from cells cultured in the presence or absence of RA following the release from contact inhibition. Cdk2 complexes were immunoprecipitated from pooled lysates of control cells or from pooled lysates of control and RA treated cells which had been incubated at 37°C for 30 minutes. In doing so, we hoped to determine whether RA treated cells contained a transferable inhibitor of cdk2 activity. The results of these *in vitro* kinase assays, shown in Figure 13, indicated that RA arrested cells did contain an activity capable of reducing the activity of cdk2 immunoprecipitated from pooled lysates of control and RA treated cells to levels as low as those observed with extracts from RA

Figure 12. Effect of RA on cdk inhibitors

Cell extracts were isolated from contact inhibited cells (CI) and cells released in the presence or absence of 1 μM RA. Western blot analysis was performed by incubation with anti-p21 and anti-p27 antibodies, respectively. Immunoreactive proteins were visualized by chemiluminescence. An α-actin antibody was used to control for equivalency of loading.

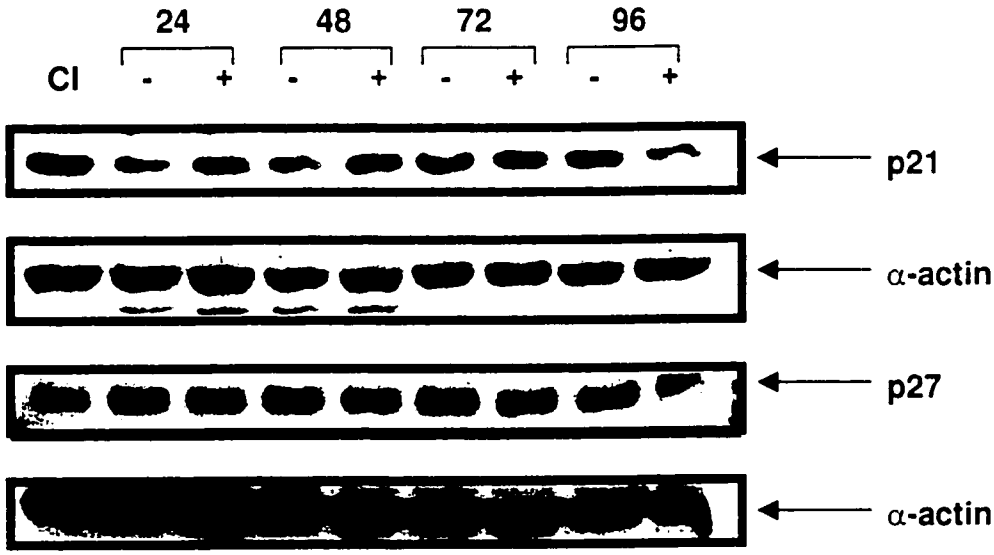
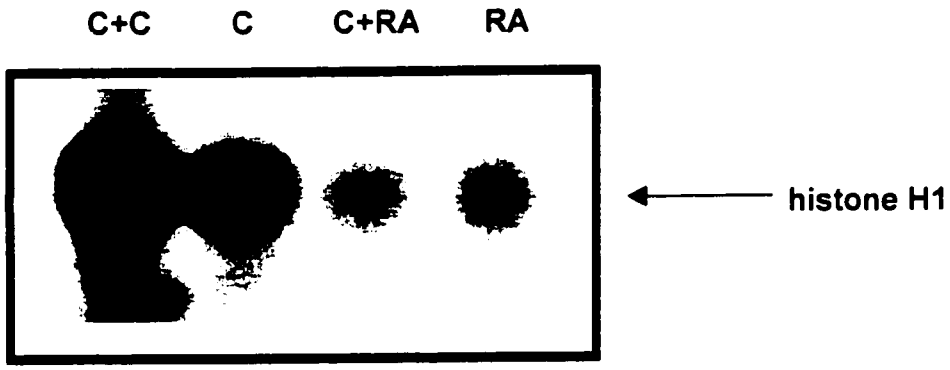

24
48
72
96
CI
- + - + - + - +
p21
α-actin
p27
α-actin

Figure 13. RA treated MCF-7 cells contain a transferable inhibitor of cdk2 activity

Cell extracts were prepared from contact inhibited cells released in the presence (RA) or absence (C) of 1 μM RA for 96 hours. Cdk2 immunoprecipitates were isolated from individual lysates or equivalent amounts of premixed lysates (C+C or C+RA) following a 30 minute incubation at 37°C. Anti-cdk2 immune complexes were assayed for histone H1 kinase activity, as described in "Materials and Methods".


C+C
C
C+RA
RA
histone H1

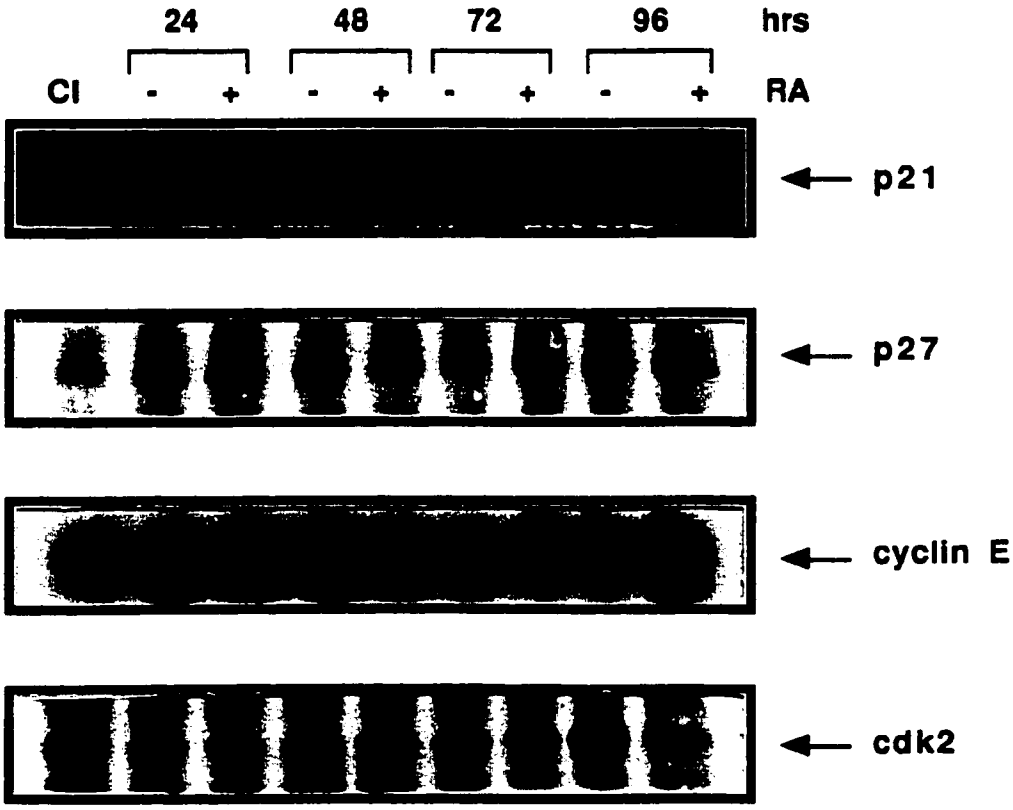
treated cells. These findings are consistent with the presence of a transferable inhibitor of cdk2 activity in RA treated cells.

D. Characterization of cdk2 immune complexes

As shown in Figures 6A and 8A, RA treatment of MCF-7 cells caused a decrease in the expression cyclin D1 suggesting the possible release of inhibitors from cyclin D1:cdk4 complexes, making them available to bind cyclin:cdk2. It has been previously demonstrated that such a model of inhibitor action occurs in other cell types. Treatment of Swiss 3T3 mouse fibroblasts with lovastatin led to the inactivation of cdk2 through the redistribution of p27 between different cyclin:cdk complexes (Poon et al., 1995). Therefore, in the absence of RA mediated increases in the overall levels of the cdk inhibitors p21 and p27, we looked for changes in the abundance of inhibitors associated with cdk2 that might account for the inhibition of cdk2 activity. Cdk2 immunoprecipitates were sequentially immunoblotted for p21, p27, cyclin E and cdk2 (Figure 14). We observed an early increase in the level of associated p21 at 24 hours post RA, however this increase was not sustained and at all later time points the amount of p21 co-immunoprecipitating with cdk2 was reduced compared with control cells. The levels of associated p27 and cyclin E remained relatively constant in control and RA treated cells. Western blot analysis of immunoprecipitated cdk2 was also performed as a control for cdk2 levels in the complexes. The decreased level of cdk2 present at 96 hours likely reflects the overall decrease in cdk2 previously observed. Thus the data revealed that at 96 hours post RA, the level of p27 associated with cdk2 had increased by approximately 2 fold. Considering the substantial inactivation of cyclin:cdk2 complexes occurred at approximately the same time, this suggested a

Figure 14. Characterization of cdk2 immune complexes

Cell extracts were isolated from contact inhibited cells (CI) and cells released in the presence or absence of 1 μM RA. Equal amounts of cell extract (400 μg) were immunoprecipitated with anti-cdk2 antiserum, as described in "Materials and Methods". Cdk2 immunoprecipitates were sequentially immunoblotted for p21, p27, cyclin E and cdk2.


24
48
72
96
hrs
Cl
-
+
-
+
-
+
-
+
RA
p21
p27
cyclin E
cdk2

possible link between these events.

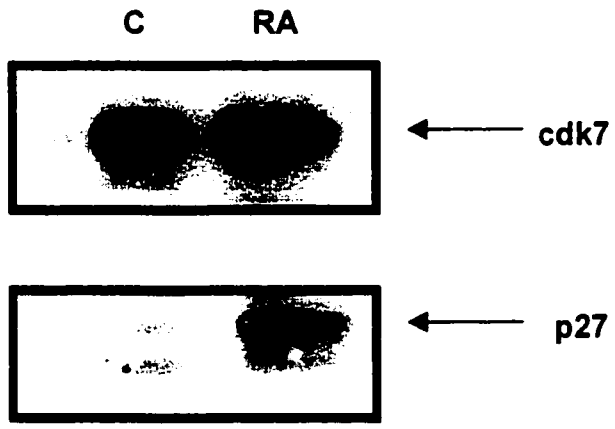
E. Effect of RA on p27 association with cdk2

To further substantiate the increased association of p27 with cdk2 in RA treated cells, we analyzed the level of associated p27 in lysates obtained from contact inhibited cells that had been released in the presence or absence of control and RA for 96 hours. Lysates were incubated with a recombinant GST-cdk2 fusion protein for 1 hour at 4°C to allow association to occur. Western blot analysis revealed that RA treated cells showed an increase in the amount of p27 associated with the GST-cdk2 fusion protein (Figure 15). Immunoblotting to detect associated cdk7 was also performed as a control for the amount of exogenous cdk2 added (Figure 15). However, one limitation of our method for determining the level of associated p27 is that it may not accurately reflect the true nature of the association between p27 and cdk2. Certainly the possibility exists that the association of p27 with recombinant cdk2 may differ from their interactions in vivo.

The presence of a transferable inhibitory activity in RA treated lysates and the increased level of association of p27 with cdk2 suggested that p27 might play a role in RA mediated inhibition of cdk2 activity. To further strengthen the correlation between increased p27 binding to cdk2 and RA mediated inhibition of cdk2 activity, p27 expression was down regulated by the addition of antisense oligonucleotides. MCF-7 cells were treated with 30nM of either antisense or mismatch p27 oligonucleotides prior to being cultured in the presence or absence of RA. These same oligonucleotides have previously been shown to be specific for p27 (Coats et al., 1996). p27 levels were determined by densitometric analysis and normalized to the levels of α-actin

Figure 15. Association of cdk2 with p27

Whole cell lysates isolated from exponentially growing MCF-7 cells treated with 1 μM RA were incubated with a fixed amount of recombinant GST-cdk2 fusion protein, as described in "Materials and Methods". After a one hour incubation at 4°C to allow association, the resulting complexes were immunoprecipitated with an anti-cdk2 antibody and resolved on 10% SDS polyacrylamide gels. The levels of associated p27 and cdk7 were determined by immunoblotting.


C
RA
cdk7
p27

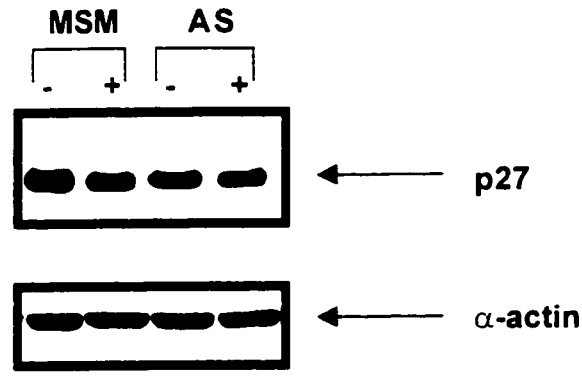
present. Because we had previously observed a decrease in p27 following exposure to RA, quantification of p27 was assessed by comparing the levels of p27 in control cells that had been pretreated with either the mismatch or antisense oligonucleotides. Antisense treatment led to a reduction in the level of p27 protein (2 fold) most notable after 48 hours (Figure 16A), which is consistent with the observations of St. Croix et al., 1996. We also compared the kinase activity of mismatch and p27 pretreated lysates and found that incubation with antisense p27 was able to completely abrogate the RA mediated inhibition of cdk2 activity normally observed but mismatch p27 did not (Figure 16B). Collectively, these results suggest RA is able to cause an increased association of p27 with cdk2 which resulted in the observed inhibition of cdk2 kinase activity.

This increased association may have been due to the reduction in cyclin D1 expression which would release p27 from cyclin D1:cdk4 complexes, allowing it to bind and inhibit cdk2 immune complexes.

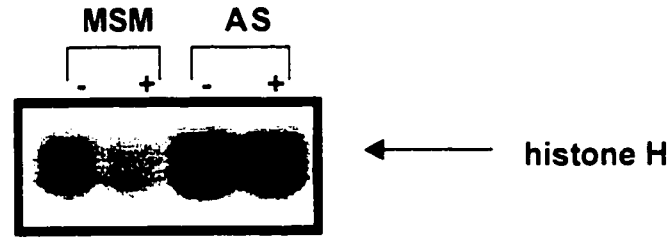
Figure 16. Effect of antisense and mismatch p27 oligonucleotides on p27 expression and cdk2 activity

MCF-7 cells were treated with 30 nM of either antisense or mismatch p27 oligodeoxynucleotides, as described in "Materials and Methods". Following a 6 hour incubation, cell medium was aspirated and replaced with fresh medium with or without 1 μM RA. **A.** Western blot analysis of p27 in cell extracts after mismatch or antisense p27 pretreatment followed by 48 hour of RA exposure. **B.** H1 histone kinase activity of cdk2 immunoprecipitates from lysates after mismatch or antisense p27 pretreatment and 48 hour exposure to RA.

A



B



Role for p53 in RA induced growth arrest

A. Increased expression of p53 following exposure to RA

The involvement of p53 in mediating DNA damage induced G1 arrest or apoptosis observed in certain cell types is well documented (reviewed by Levine, 1997). It has also been previously reported that retinoic acid was capable of inducing stabilization of wild type p53 protein, but not mutant p53, in non-small cell lung carcinoma cells (Maxwell and Mukhopadhyay, 1994). Since MCF-7 cells are known to contain only wild type p53 (O'Connor et al., 1997), we decided to examine the effect of RA on p53 expression. Northern blot analysis of RNA harvested from asynchronous populations of cells treated with RA confirmed that RA did not act at the message level (Figure 17A). In contrast, western blot analysis revealed that exposure to RA caused an accumulation of p53 which persisted throughout the time course of treatment (Figure 17B).

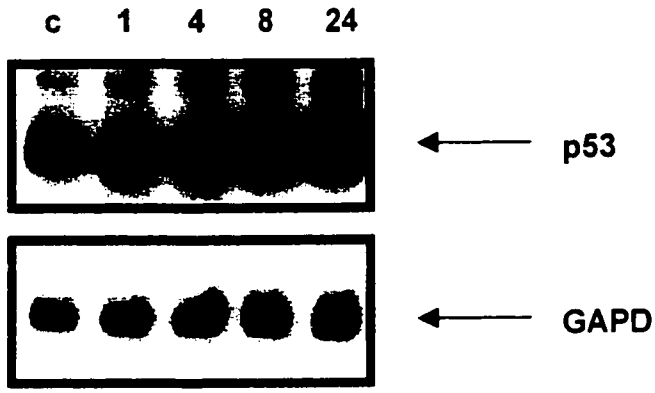
B. Abrogation of p53 function inhibits RA induced down regulation of cdk2 activity and reductions in viable cell numbers

Wild type p53 acts as a transcription factor for genes regulating growth control. The major p53 inducible gene in human cells is WAF-1 (wild type p53 activated fragment 1) which encodes the cdk inhibitor, p21. The elevation of p21 following exposure to DNA damaging agents has been shown to play a major role in the subsequent growth arrest. However, the activity of p53 as a transcription factor can be modulated through its interaction with other proteins. For example, cellular and viral proteins such as mdm2 and E6 can inactivate p53 by direct interaction with different

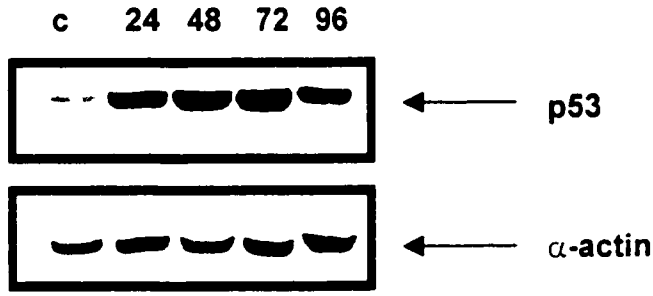
Figure 17. RA increases p53 protein but not mRNA in MCF-7 cells

Exponentially growing MCF-7 cells were treated with 1 μM RA for the indicated time intervals. **(A)** Northern blot analysis was performed on 20 μg of total RNA at various time intervals. **(B)** Western blot analysis was performed by probing with an anti-p53 antibody and immunoreactive protein was visualized by chemiluminescence. An α-actin antibody was used as a protein loading control.

A



B

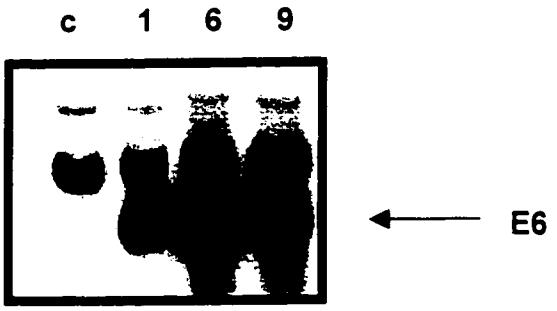


structural domains of p53 (reviewed by Levine, 1997). In contrast, binding of p53 to other proteins such as the c-abl tyrosine kinase leads to enhanced p53 transcriptional activity (Goga et al., 1995). More recently it has been demonstrated that G1 arrest induced by certain genotoxic drugs involves the down regulation of cdk2 activity by a c-abl/p53 dependent mechanism, which occurs independently of p21 (Yuan et al., 1996). To determine whether the RA induced accumulation of p53 was involved in mediating the inhibition of cdk2 activity and growth arrest we had observed, MCF-7 cells were stably transfected with the E6 oncoprotein (Figure 18A). The E6 protein, encoded by the oncogenic human papillomavirus types 16 and 18, has been shown to promote the degradation of p53 through ubiquitin dependent proteolysis (Scheffner et al., 1990). Western blot analysis confirmed that these cells expressed very low levels of p53 (Figure 18B). Subsequent analysis of cdk2 protein levels failed to reveal any effect of p53 abrogation on the RA mediated down regulation of cdk2 (Figure 18C). We also investigated the effect of RA on cdk2 activity in the absence of p53. Histone H1 kinase assays were performed on cdk2 immune complexes prepared from lysates obtained from asynchronous populations of cells treated with RA for 96 hours. Following exposure to RA cdk2 activity did not decrease to the same extent as in parental MCF-7 cells, with one clone retaining 70-80 percent of cdk2 activity compared to untreated control cells (as assessed by densitometry) (Figure 18D). We also observed an increase in the basal activity of cdk2 in E6 expressing clones, suggesting that p53 may also be involved in regulating cdk2 activity in exponentially growing MCF-7 cells.

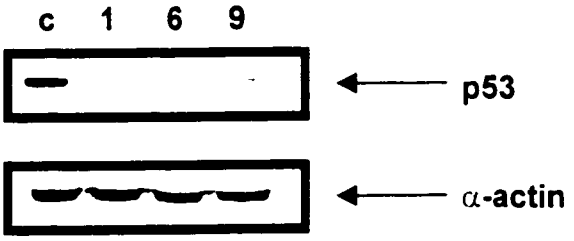
Figure 18. Effect of RA on cdk2 activity in p53 deficient cells

(A) Northern blot analysis of MCF-7 cells stably expressing HPV16-E6. Stable clones were selected by co-transfection with a plasmid expressing the puromycin resistance gene driven by the cytomegalovirus (CMV) promoter. **(B)** Western blot analysis of p53 levels in MCF-7/E6 expressing clones. **(C)** Lysates obtained from exponentially growing MCF-7/E6 expressing clones incubated in the presence of RA for 96 hours were immunoblotted with anti-cdk2 or **(D)** immunoprecipitated with anti-cdk2 and analyzed by histone H1 kinase assay.

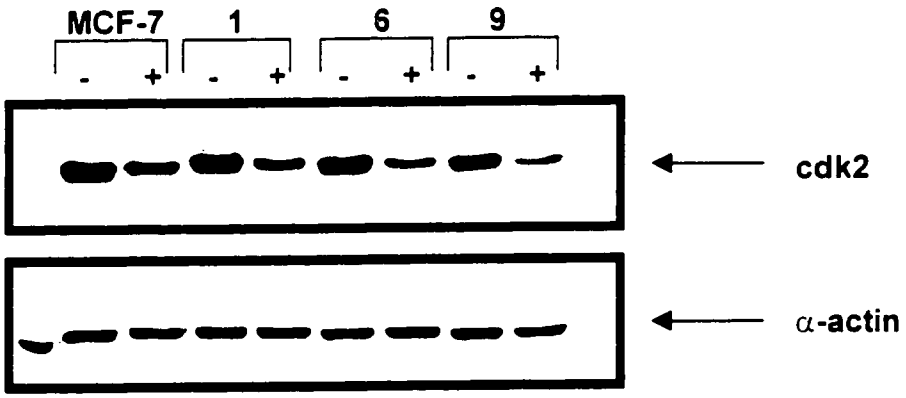
A


c 1 6 9
E6

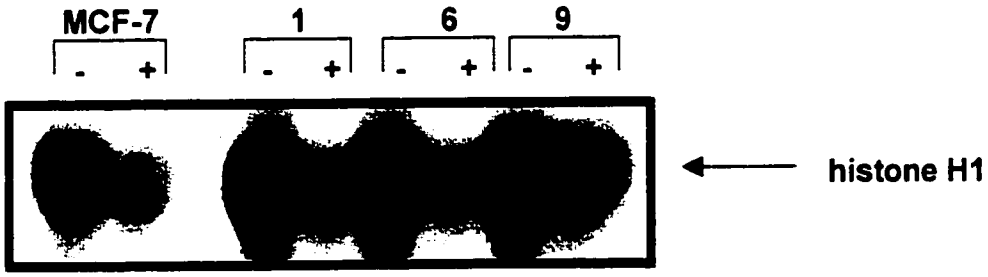
B


c 1 6 9
p53
α-actin

C


MCF-7 1 6 9
- + - + - + - +
cdk2
α-actin

D


MCF-7 1 6 9
- + - + - + - +
histone H1

The involvement of p53 in RA mediated growth arrest was tested by the ability of RA treated MCF-7 and MCF-7/E6 to cause decreases in viable cell numbers, relative to untreated cells. Viable cell numbers, as measured by trypan blue exclusion, demonstrated the ability of p53 deficient cells to abrogate RA mediated effects on the number of viable cells (Figure 19). As previously demonstrated (Pratt et al., 1996), treatment with RA over a 96 hour time period resulted in a 70% reduction in the number of viable cells, relative to untreated controls, in parental MCF-7 cells. In contrast, all clones of MCF-7/E6 exhibited a less than 50% reduction in the number of viable cells. Furthermore, the level of increase in numbers of viable cells observed with each of the E6 expressing clones correlated closely with their ability to abrogate RA induced down regulation of cdk2 activity.

C. Effect of RA on cdk2 activity and viable cell numbers by p53 mutants

Our results obtained using p53 deficient cells suggested that p53 may be involved in regulating the growth response to RA in MCF-7 cells. To further characterize the role of p53, we sought to identify which functional domains of p53 were involved. Previous studies have divided the p53 protein into five functional domains (Figure 20); (i) a transcriptional activation domain that binds to TAF components of TFIID; (ii) a putative proline rich signaling domain; (iii) a sequence specific DNA binding domain; (iv) a tetramerization domain; and (v) a domain that recognizes and binds to damaged DNA non specifically (reviewed by Levine, 1997). A series of p53 mutants (Figure 20) were tested for their ability to suppress RA mediated down regulation of cdk2 activity and growth arrest. Deletion and point mutants specifically designed to target the conserved regions of the p53 protein, including a transcriptionally impaired

Figure 19. Effect of RA on viable cell numbers in p53 deficient MCF-7 cells

Three independent clones of MCF-7 cells stably transfected with HPV16-E6 were incubated in the presence or absence of 1 μM RA. The number of viable cells was assessed by trypan blue exclusion and is expressed relative to values obtained for untreated control cells. Data represents the mean and standard deviation of three independent experiments.

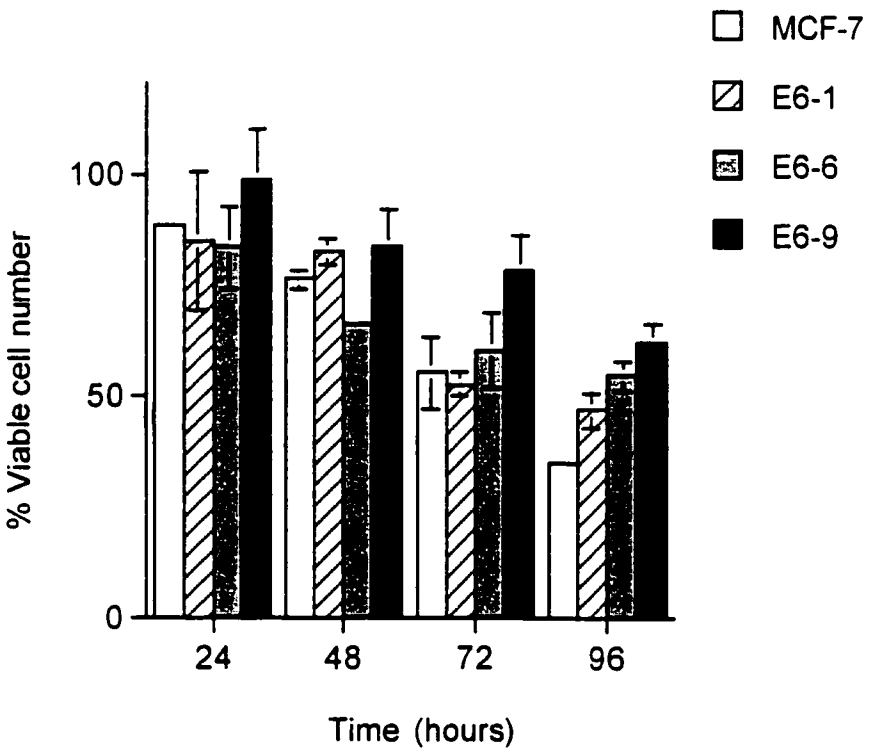
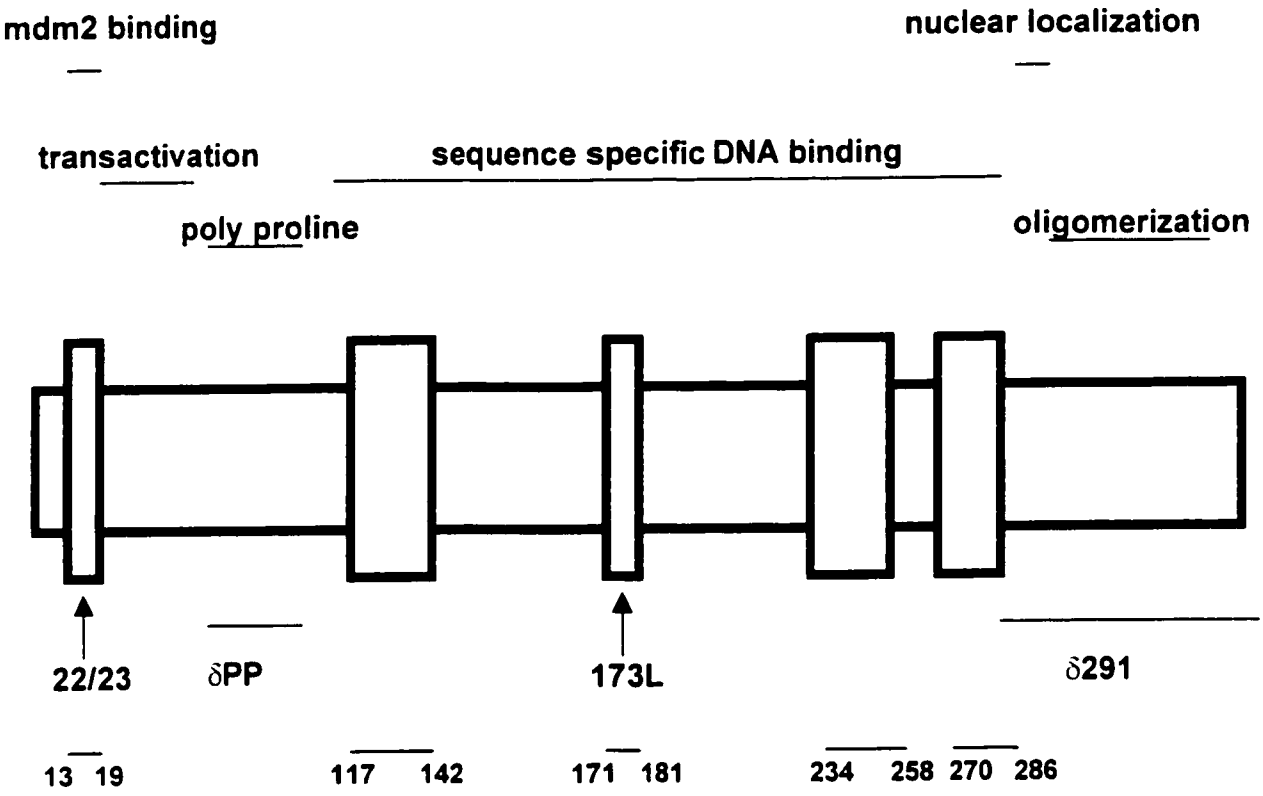

MCF-7
E6-1
E6-6
E6-9
% Viable cell number
100
50
0
24
48
72
96
Time (hours)

Figure 20. Functional domains of the p53 protein

Shown are the mdm-2 binding, transactivation, polyproline, sequence specific DNA binding, nuclear localization and oligomerization domains. The boxed regions represent evolutionarily conserved regions of the protein. The mutants used for our studies are shown below.


mdm2 binding
nuclear localization
transactivation
sequence specific DNA binding
poly proline
oligomerization
22/23
δPP
173L
δ291
13 19
117 142
171 181
234 258
270 286

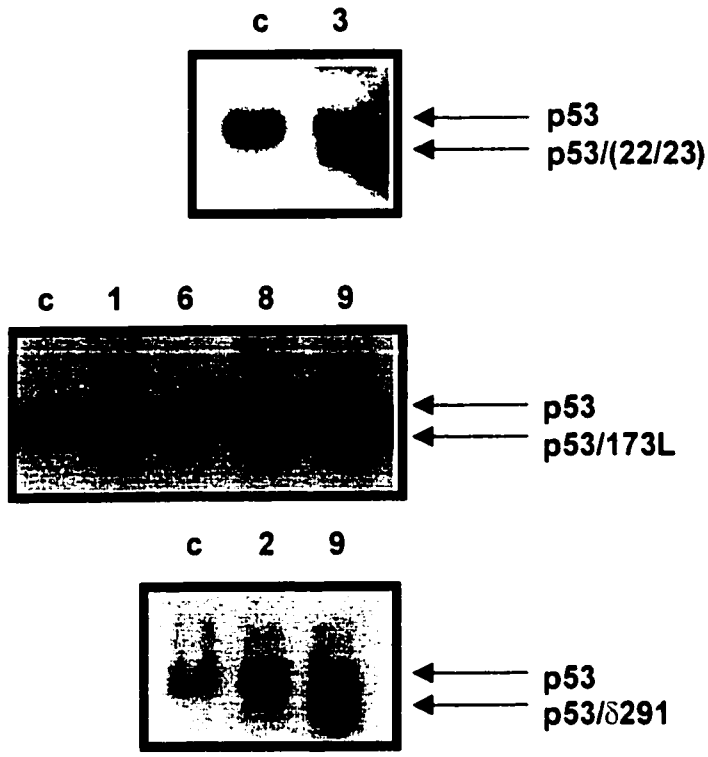
double point mutant (22/23) (Lin et al., 1994), the polyproline region deletion mutant (δPP) (Sakamuro et al., 1997), the tumour derived DNA binding defective mutant (173L) (Crook et al., 1992) and a premature termination mutant that removed the C-terminal 102 amino acids (δ291) (Marston et al., 1994) were analyzed in parallel. MCF-7 cells were co-transfected with each p53 mutant construct and a puromycin resistance marker. Stable cell lines were isolated by selection in puromycin and characterized by Northern blot where possible (Figure 21A). Western blot analysis for the levels of mutant p53 revealed that each of the mutants was efficiently expressed in MCF-7 cells (Figure 21B). The transactivation mutant p53 22/23 and the transcriptionally inactive p53 173L both showed consistent overexpression, as did the C-terminal deletion mutant p53 δ291 and the polyproline deletion mutant p53 δPP. Stable clones were cultured in the presence or absence of RA to assess the effect of each of the p53 mutants on cdk2 expression and activity. Not surprisingly, none of the mutants had any effect on cdk2 expression (Figure 21C), consistent with the inability of E6 expressing cells to prevent the RA mediated down regulation of cdk2. We next looked at the effect of RA on cdk2 activity in the different p53 mutant expressing clones. Histone H1 kinase assays were performed on cdk2 immune complexes prepared from lysates obtained from asynchronous populations of cells treated with RA. Following exposure to RA we see that cdk2 activity was down regulated in the presence of mutant p53 to the same extent as observed in parental MCF-7 cells (Figure 21D).

Interestingly, we also observed an increase in the basal activity of cdk2 in δ291 expressing clones, suggesting that the C-terminus of p53 may be involved in repressing cdk2 activity in exponentially growing MCF-7 cells, however it does not regulate the

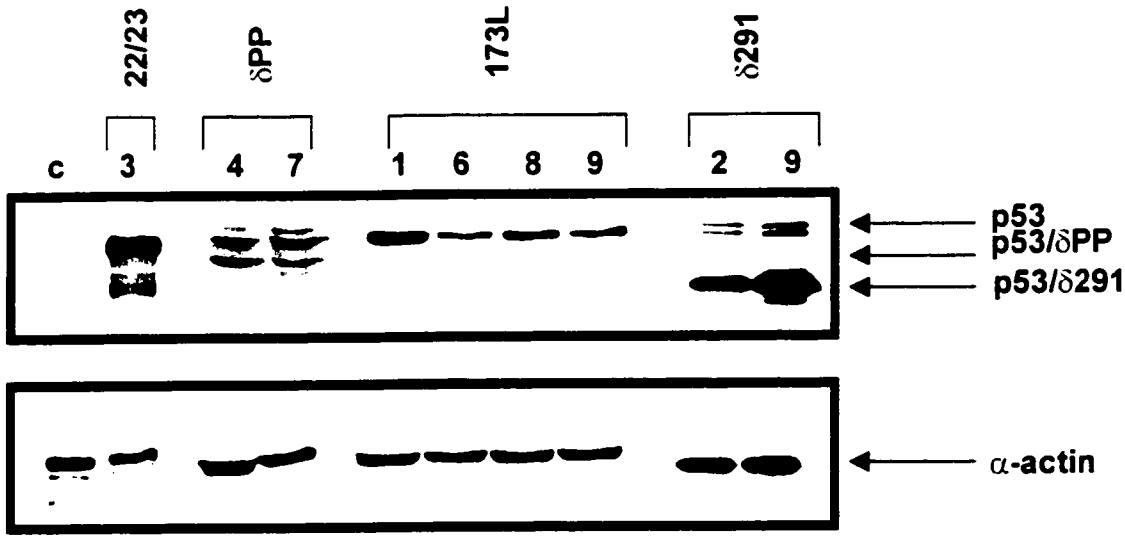
Figure 21. Effect of RA on cdk2 expression and activity in p53 mutant expressing MCF-7 cells

(A) Northern blot analysis of MCF-7 cells stably expressing the following p53 mutant constructs: p53 22/23, p53 δPP, p53 173L or p53 δ291. Stable clones were selected by co-transfection with a plasmid expressing the puromycin resistance gene driven by the cytomegalovirus (CMV) promoter. **(B)** Western blot analysis of p53 levels in p53 mutant expressing MCF-7 clones. **(C)** Lysates obtained from exponentially growing p53 mutant expressing clones incubated in the presence or absence of RA were immunoblotted with anti-cdk2 or **(D)** immunoprecipitated with anti-cdk2 and analyzed by histone H1 kinase assay.

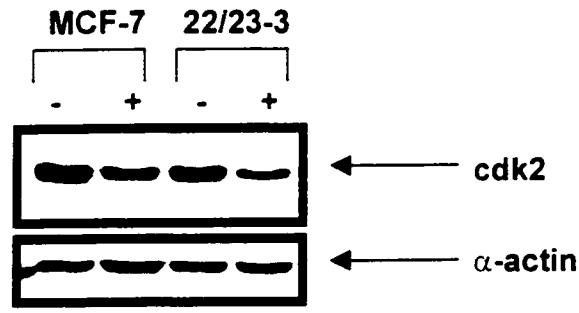
A

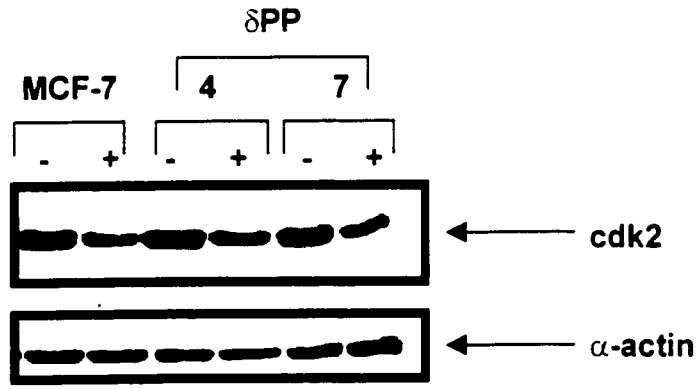

c 3
p53
p53/(22/23)
c 1 6 8 9
p53
p53/173L
c 2 9
p53
p53/δ291

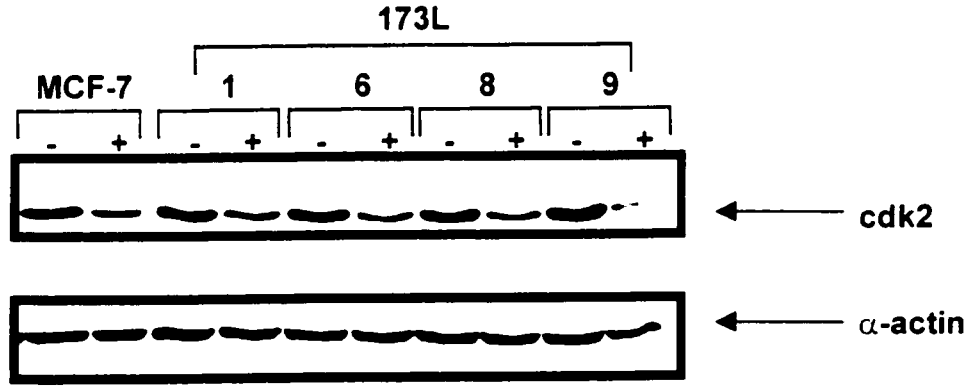
B

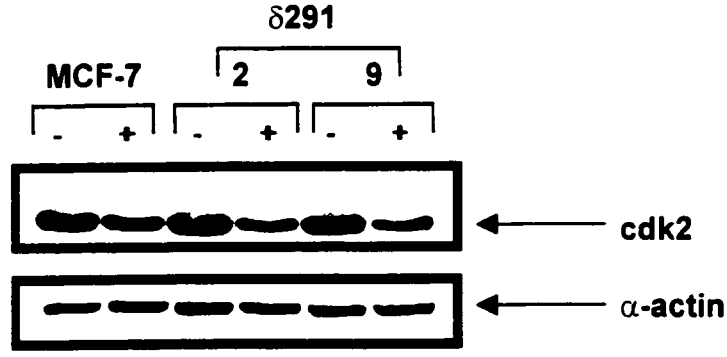

22/23
δPP
173L
δ291
c 3 4 7 1 6 8 9 2 9
p53
p53/δPP
p53/δ291
α-actin

C

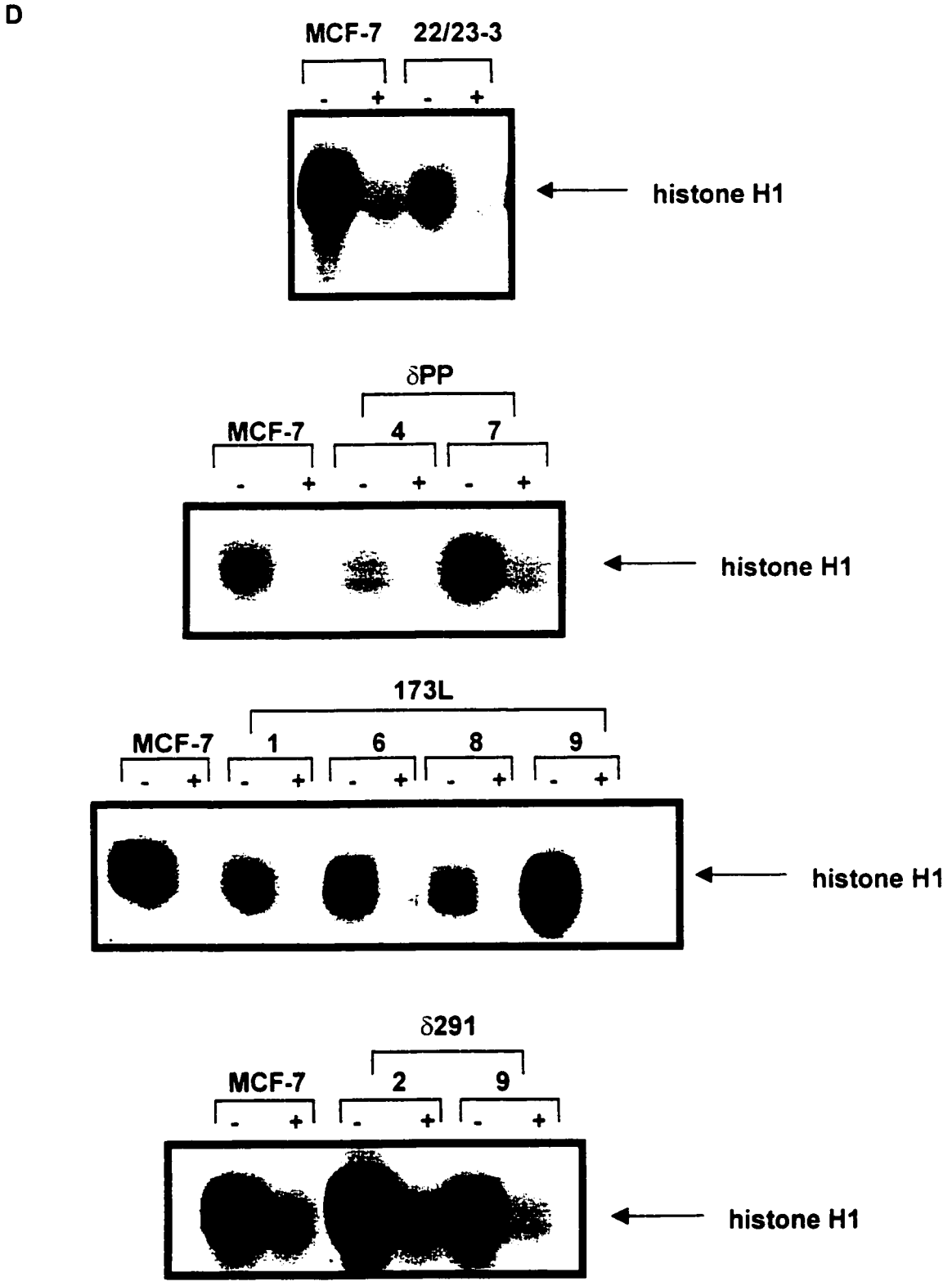

MCF-7
22/23-3
- + - +
cdk2
α-actin


δPP
MCF-7
4
7
- + - + - +
cdk2
α-actin


173L
MCF-7
1
6
8
9
- + - + - + - + - +
cdk2
α-actin


δ291
MCF-7
2
9
- + - + - +
cdk2
α-actin

D

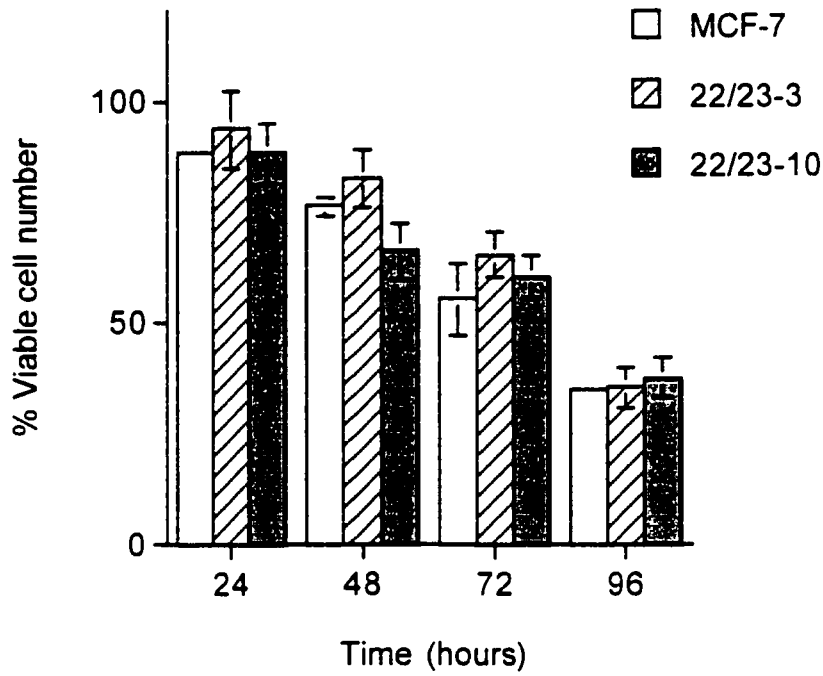

MCF-7
22/23-3
- + - +
histone H1
δPP
MCF-7
4
7
- + - + - +
histone H1
173L
MCF-7
1
6
8
9
- + - + - + - + - +
histone H1
δ291
MCF-7
2
9
- + - + - +
histone H1

RA mediated effects. With a single clone, that expressed the 22/23 mutant p53, we observed a down regulation in the basal activity of cdk2. Surprisingly, the 173L mutant which has been shown to be transdominantly repressing, did not display this characteristic, which may reflect the ability of this mutant to bind cell type specific transcription factors which mediate these effects of p53. Viable cell numbers, as measured by trypan blue exclusion, also demonstrated the inability of any of the p53 mutant expressing cells to abrogate RA mediated reductions in viable cell numbers (Figure 22), thus suggesting a correlation between the inability to prevent RA induced cdk2 inactivation and the inability to prevent RA induced reductions in viable cell numbers. These results were surprising as three of the mutants: 22/23, 173L and δ291 have previously been shown to function as dominant negative inhibitors of wild type p53 transactivation function (Lin et al., 1994; Crook et al., 1992; Marston et al., 1994) which is usually correlated with a loss of growth suppression. The δPP region has also been implicated in mediating growth arrest (Walker and Levine, 1996).

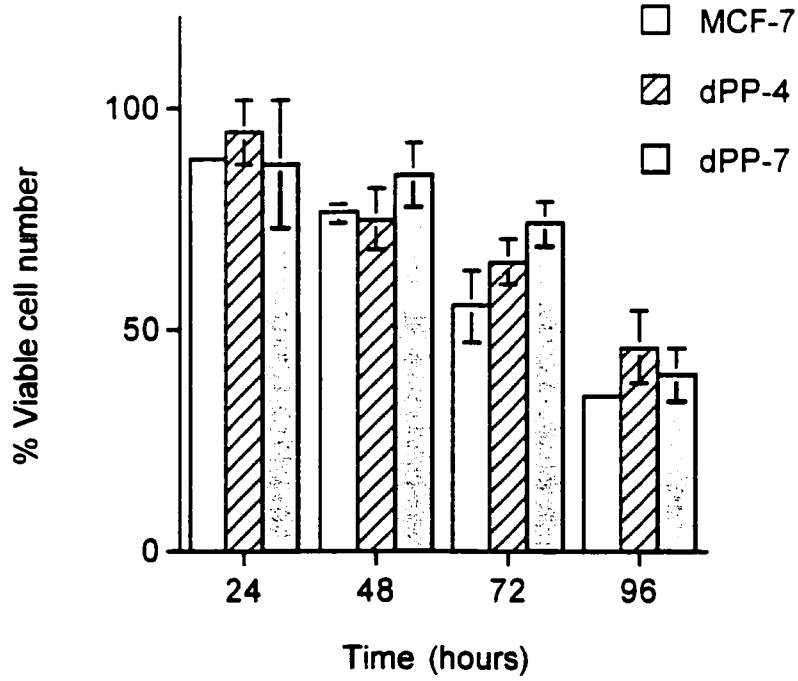
Figure 22. Effect of RA on viable cell numbers in p53 mutant expressing MCF-7 cells

Two or three independent clones of MCF-7 cells stably transfected with the indicated p53 mutant constructs were incubated in the presence or absence of 1 μM RA. Viable cell numbers were assessed by trypan blue exclusion and is expressed relative to values obtained for untreated control cells. Data represents the mean and standard deviation of three independent experiments.

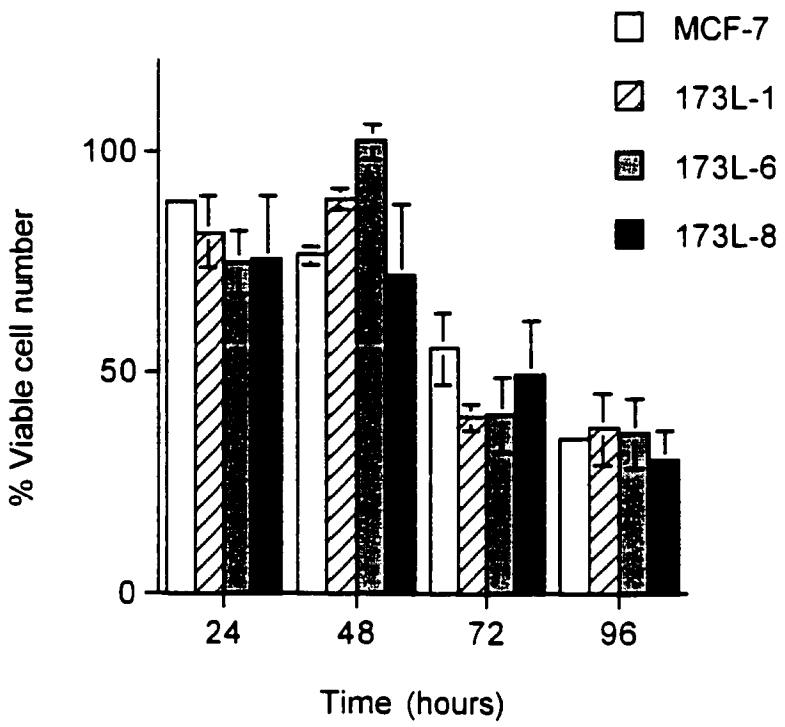
A


MCF-7
22/23-3
22/23-10
100
50
0
% Viable cell number
24
48
72
96
Time (hours)

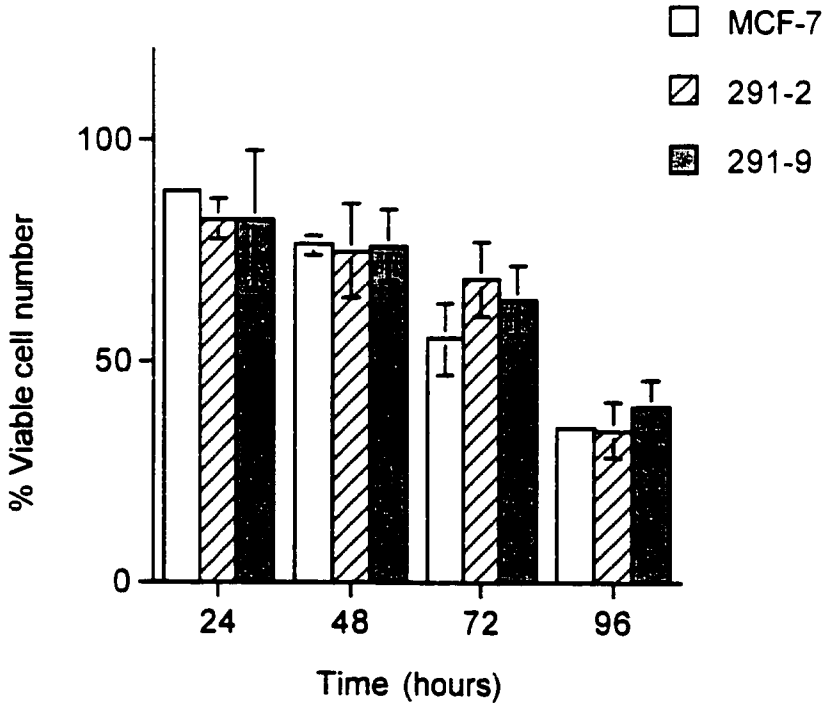
B


MCF-7
dPP-4
dPP-7
100
50
0
% Viable cell number
24
48
72
96
Time (hours)

C


MCF-7
173L-1
173L-6
173L-8
100
50
0
% Viable cell number
24
48
72
96
Time (hours)

D


MCF-7
291-2
291-9
100
50
0
% Viable cell number
24
48
72
96
Time (hours)

Posttranscriptional regulation of cdk2 expression

A. RA effect on cdk2 promoter activity in MCF-7 cells

To further investigate the mechanism by which RA regulates cdk2 gene expression, MCF-7 cells were transiently transfected with a cdk2 luciferase construct containing a 2.4 kb region of the cdk2 promoter (Shiffman et al., 1996). Luciferase activity was corrected for differences in transfection efficiency by co-transfection with a plasmid expressing the β-galactosidase gene driven by the CMV promoter. Unfortunately, the basal cdk2 promoter activity, in the presence of serum, was similar to background activity generated by the vector alone (pGL2-Basic). Therefore, MCF-7 cells were stably transfected with a cdk2 promoter luciferase reporter construct. Stable integration of the reporter construct was determined by Southern blot analysis (Figure 23A). Although exposure of MCF-7 cells to RA results in an approximately 10 fold decrease in cdk2 mRNA, a 1.3 fold increase in cdk2 promoter mediated gene transcription was observed (as detected by luciferase activity) (Figure 23B). These results would suggest that either the required RA responsive elements are not present in the 2.4 kb promoter fragment or that posttranscriptional regulation plays a major role in RA mediated decreases of cdk2 mRNA levels.

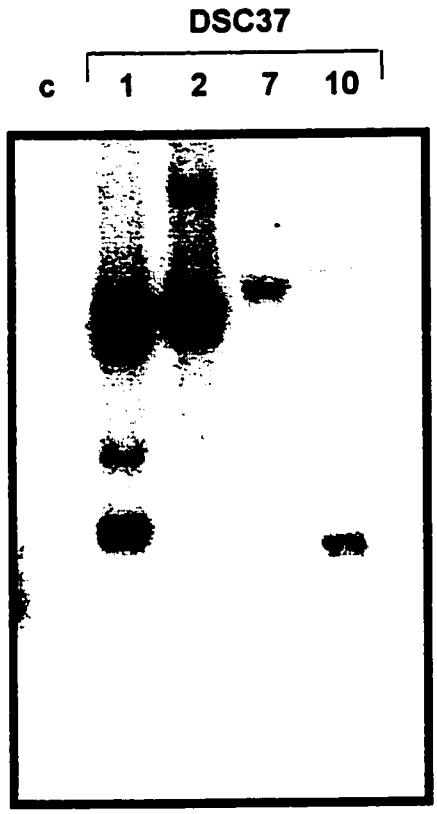
B. Effect of RA on cdk2 regulation at the transcriptional level

To investigate the possibility that the retinoid responsive elements are absent in the 2.4 kb promoter construct, nuclear run on assays were performed to assess the level of cdk2 transcript in extracts isolated from asynchronous populations of MCF-7

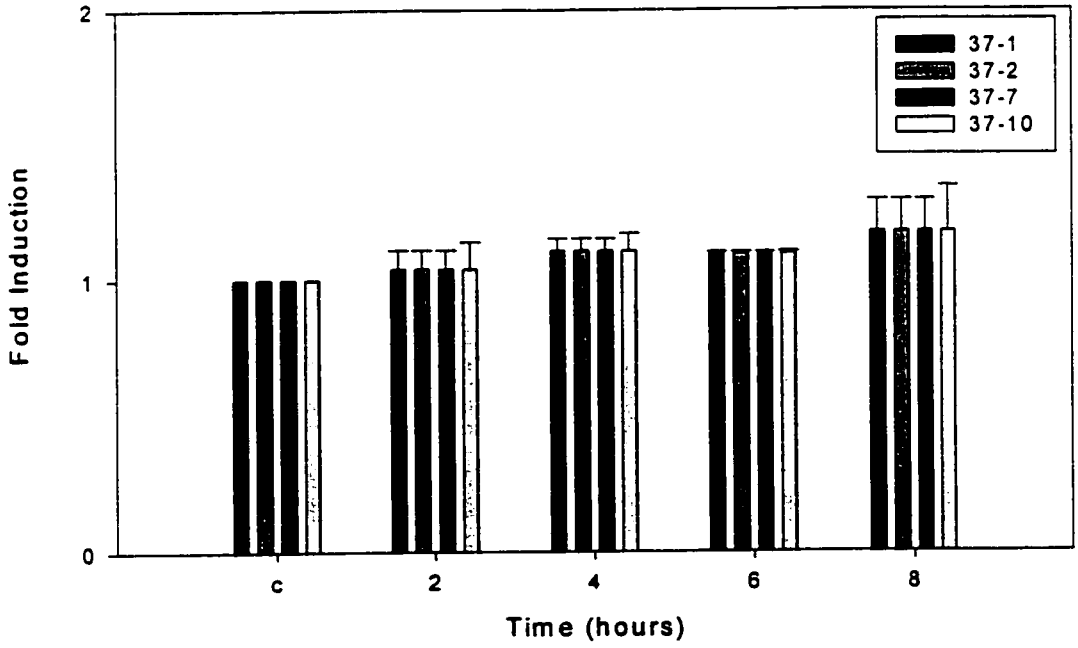
Figure 23. RA effect on cdk2 promoter activity in MCF-7 cells

A. Southern blot analysis of MCF-7 cells transfected with the cdk2 promoter driven luciferase construct, DSC37. Stably transfected cell lines were selected by co-transfection with a plasmid expressing the puromycin resistance gene driven by the cytomegalovirus (CMV) promoter. **B.** The effect of RA on cdk2 promoter activity was examined in MCF-7 cells stably expressing the cdk2 promoter luciferase reporter construct DSC37 as described in "Materials and Methods". Luciferase activities are expressed relative to values obtained for control cells.

A


DSC37
c
1
2
7
10

B


37-1
37-2
37-7
37-10
Fold Induction
0
1
2
c
2
4
6
8
Time (hours)

cells treated with 1 μM RA for 24 hours. Radiolabeled nascent transcripts prepared from intact nuclei were hybridized to cDNA probes for cdk2 and GAPD immobilized on nylon membranes. Exposure to RA did not cause a decrease in the rate of transcription of cdk2, strongly suggesting that there are no RA responsive regulatory elements further upstream in the cdk2 promoter (Figure 24).

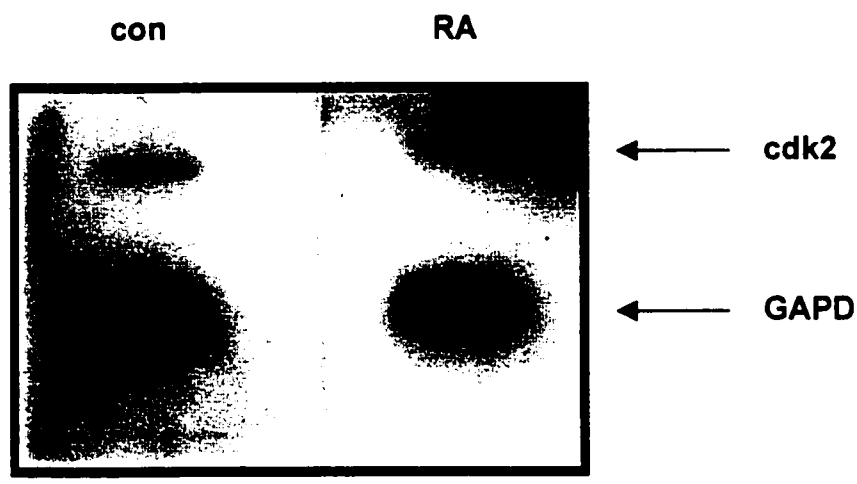
C. RA decreases cdk2 mRNA stability

Profound decreases in cdk2 mRNA levels were noted in the absence of any inhibition of cdk2 gene transcription. We therefore examined whether exposure of MCF-7 cells to RA decreased cdk2 mRNA stability. Cells were incubated in the presence or absence of 1 μM RA for 24 hours, followed by addition of actinomycin D (10 μg/mL). RNA was harvested at various times and Northern blot analysis was performed to determine changes in cdk2 expression (Figure 25A). Exponentially growing cells expressed high levels of cdk2 which remained unchanged after the addition of actinomycin D. In contrast, RA treated cells expressed lower levels of cdk2 mRNA, and the levels decreased at a much faster rate than in untreated cells (Figure 25A). The amount of cdk2 mRNA was determined by normalizing to GAPD expression at the same time point. A semi logarithmic plot of the decay of cdk2 mRNA indicated that exposure of MCF-7 cells to RA caused a significant decrease in mRNA stability (Figure 25B). The half life of cdk2 mRNA expressed in RA treated cells was calculated to be about 6.5 hours; however the half life in untreated cells could not be determined as the cells were severely growth inhibited when exposed to this concentration of actinomycin D for longer than 8 hours.

Figure 24. Nuclear run-on analysis of cdk2 gene expression in RA treated MCF-7 cells

A. Nuclei harvested from exponentially growing MCF-7 cells, or cells treated with 1μM RA for 24 hours, were used to perform the nuclear run-on transcription assay as described in "Materials and Methods". **B.** Densitometric analysis was used to determine the transcriptional rate of cdk2 relative to GAPD in control and RA treated MCF-7 cells.

A



B

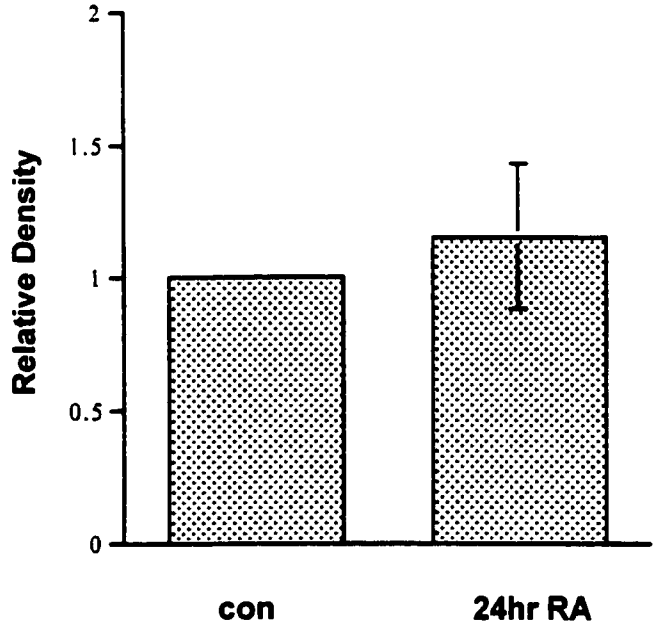
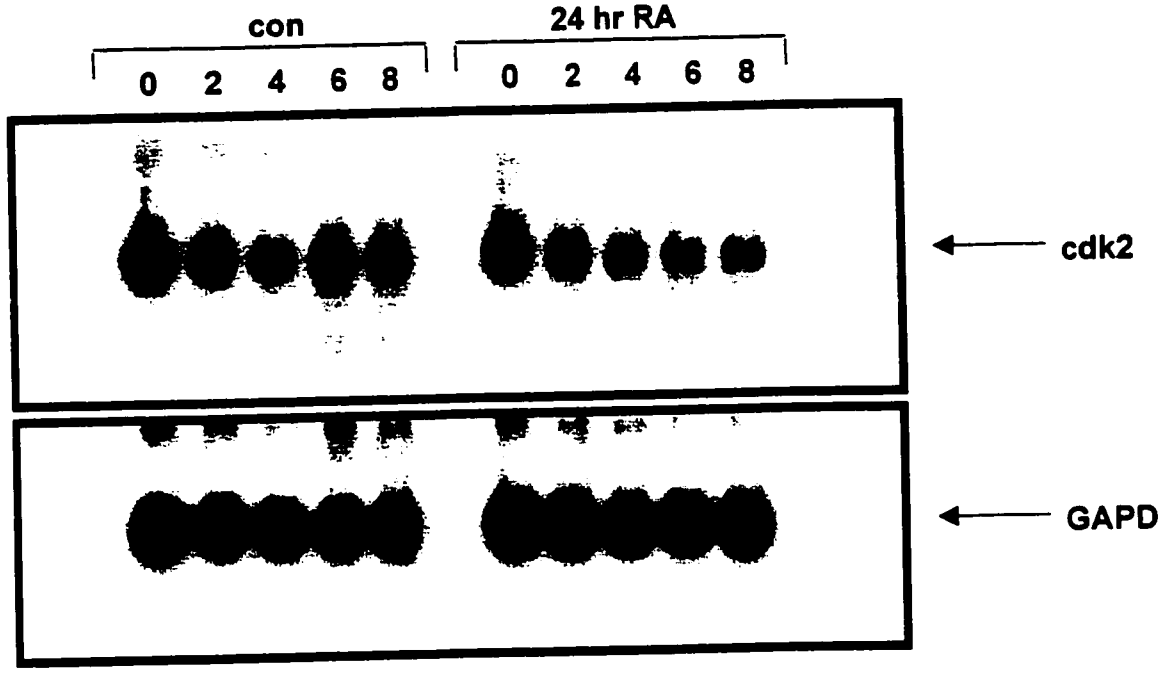


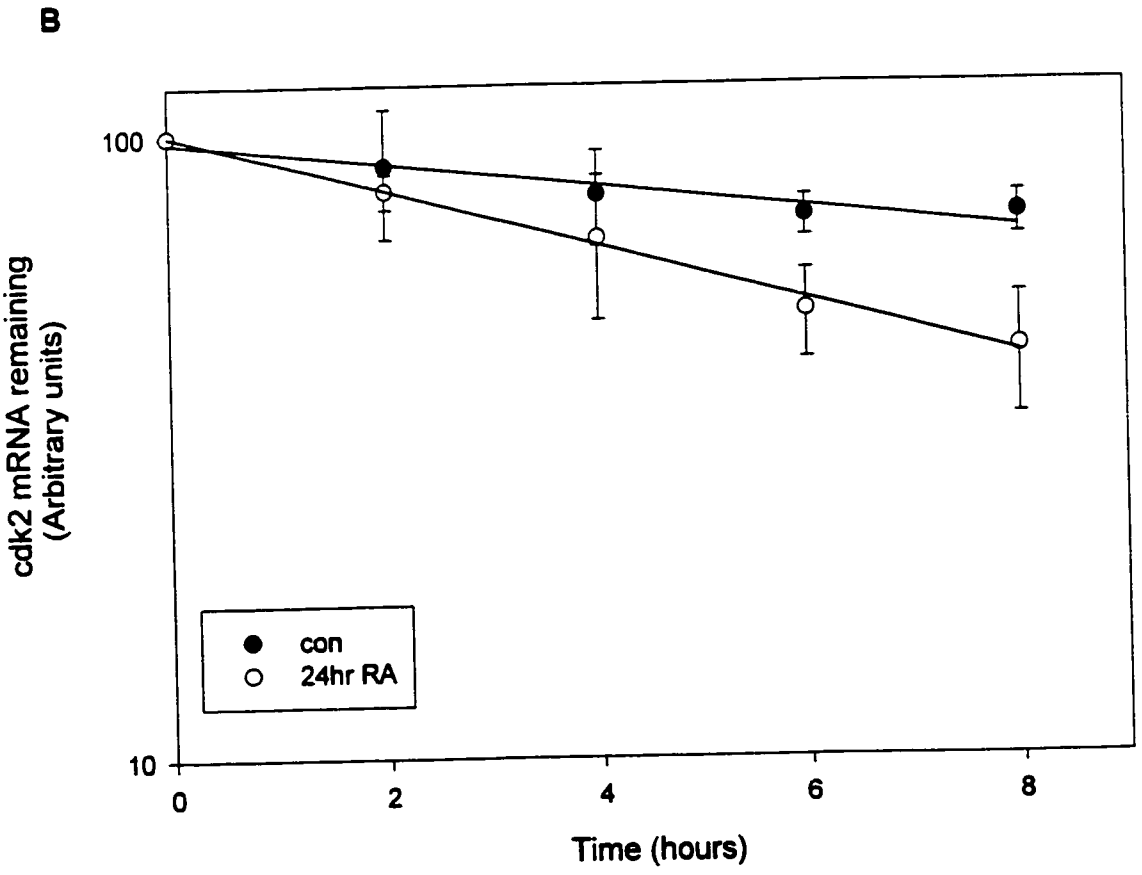
Figure 25. RA induced destabilization of cdk2 mRNA in MCF-7 cells

Exponentially growing MCF-7 cells incubated in the presence or absence of 1 uM RA for 24 hours, were treated with actinomycin D (10 μg/mL), as described in "Materials and Methods". At the indicated time intervals, cells were harvested for RNA and Northern blot analysis performed. **A.** The blots were probed for cdk2 and GAPD mRNA successively. **B.** Semi logarithmic plot of the decay of cdk2 mRNA in the presence or absence of RA. Quantification of signals was performed by densitometric analysis. At each time interval, the cdk2 signals were normalized relative to the respective GAPD signal. Cdk2 mRNA levels at time 0 in either the presence or absence of RA were arbitrarily defined as 1, respectively. Data represent the means from three independent experiments; bars represent standard deviation. **C.** Northern blot analysis was also performed to measure the decay of p21 mRNA.

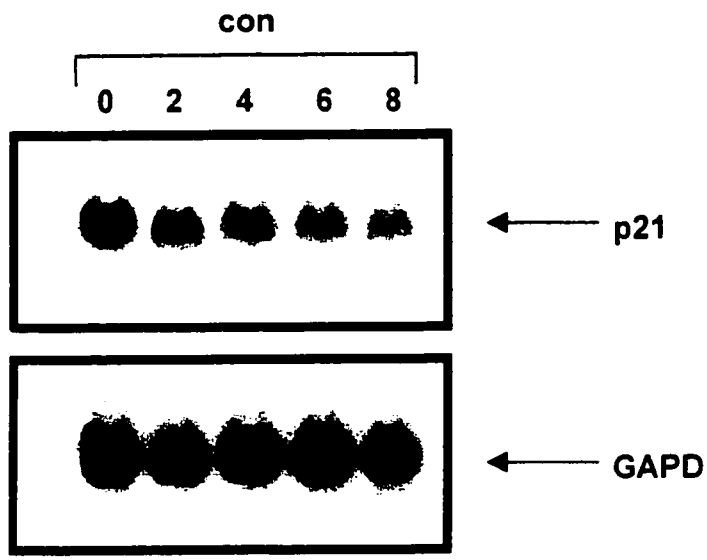
A


con
24 hr RA
0 2 4 6 8
0 2 4 6 8
cdk2
GAPD

B


100
10
cdk2 mRNA remaining
(Arbitrary units)
con
24hr RA
0
2
4
6
8
Time (hours)

C



These results are consistent with the previous demonstration that Rat-1A cells cultured in the presence of serum displayed a marked stabilization of the cdk2 transcript (Oda et al., 1995). Northern blot analysis was also performed to measure the decay of p21 mRNA following exposure to RA as a positive control for treatment with actinomycin D. It has been reported that p21 has a half life of 3 hours in untreated MCF-7 cells (Li et al., 1996), this value is in agreement with our own observations (Figure 25C).

Stable Expression of the RARα' in MCF-7 cells does not prevent RA mediated inactivation of cdk2

A. Effect of RARα' on RA mediated loss of viable cell numbers

It has been previously shown in our lab that RA induced changes in viable cell numbers in MCF-7 cells could be alleviated by the expression of a dominant negative form of the retinoic acid receptor, RARα', which is missing the ligand binding domain due to a C terminal truncation (Pratt et al., 1996). MCF-7 cells co-transfected with a pCDNA3-RARα' construct and a puromycin resistance marker were selected in puromycin. Several high expressing clones were obtained (Figure 26A) as assessed by Northern blot analysis. MCF-7 cells express the RARα as two abundant mRNA species which are also detected. Viable cell counts were then assayed by following treatment of these clones with RA. As previously demonstrated, treatment with RA resulted in a 70% reduction in viable cell counts of parental MCF-7 cells. In contrast, clones expressing RARα' showed a less than 50% reduction in viable cell numbers after being cultured in the presence of RA for 96 hours (Figure 26B).

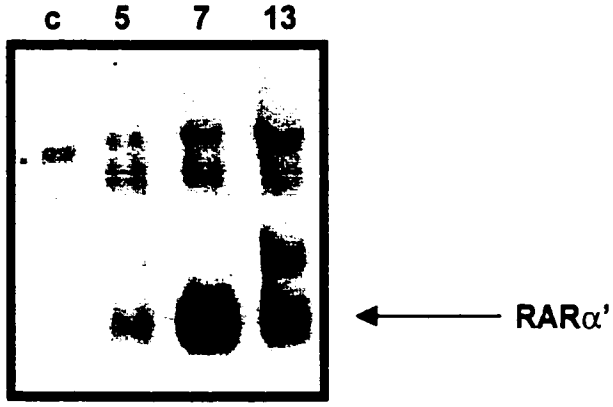
B. Effect of RA on cdk2 expression and activity in RARα' expressing MCF-7 cells

Since we have seen a correlation between RA induced inhibition of cdk2 activity and G1 arrest, we wished to determine whether both of these effects were being mediated through ligand mediated transactivation of the retinoic acid receptor. Stable clones were cultured in the presence or absence of RA to assess the effect of RARα' expression on cdk2 expression and activity. Western blot analysis of lysates obtained from RARα' clones demonstrated RA mediated decreases in cdk2 protein that were

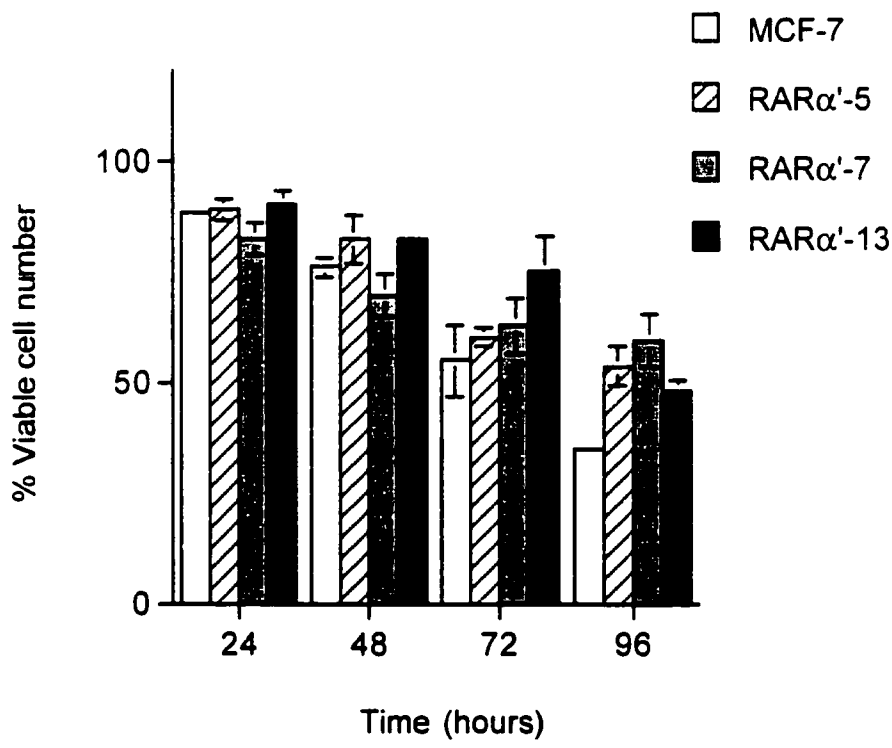
Figure 26. RA mediated decrease in viable cell numbers in MCF-7 cells

(A) Northern blot analysis of RARα' expressing MCF-7 cells using the RARα as a probe. Stable clones were selected by co-transfection with a plasmid expressing the puromycin resistance gene driven by the cytomegalovirus (CMV) promoter. **(B)** Three independent clones of MCF-7 cells stably transfected with RARα' were incubated in the presence or absence of 1 μM RA. Viable cell numbers, as assessed by trypan blue exclusion, are expressed relative to values obtained for untreated control cells. Data represents the mean and standard deviation of three independent experiments.

A


c
5
7
13
RARα'

B

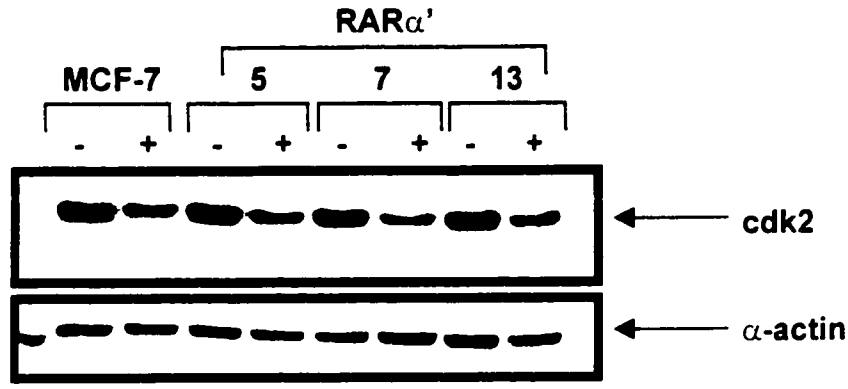

MCF-7
RARα'-5
RARα'-7
RARα'-13
100
50
0
% Viable cell number
24
48
72
96
Time (hours)

similar to those observed with MCF-7 cells (Figure 27A). We also investigated the ability of RARα' expressing clones to regulate cdk2 activity. Histone H1 kinase assays were performed on cdk2 immune complexes prepared from lysates obtained from asynchronous populations of cells cultured in the presence or absence of RA (Figure 27B). Following exposure to RA, we observed that all clones exhibited an inhibition of cdk2 activity that was comparable to what was observed in parental MCF-7 cells, suggesting that while RA induced loss of viable cell numbers is partially mediated through the transactivating domain of RARα, the down regulation of cdk2 expression and activity are not. To confirm that these RARα' expressing clones were capable of repressing RA induced transcription, transient transfections were performed using a thyroid hormone responsive element (TRE)(MMTV)-CAT reporter construct. In the presence of RA, binding of any of the RARs to TRE has been shown to enhance transcription from adjacent promoters. RA dependent transcriptional activity was analyzed by measuring CAT activity following exposure to RA. In parental MCF-7 cells, a 5 fold increase in CAT activity was observed in the presence of RA. In contrast, clones expressing RARα' only showed a 2 fold increase in CAT activity, demonstrating their ability to inhibit RA mediated gene transcription (Figure 27C).

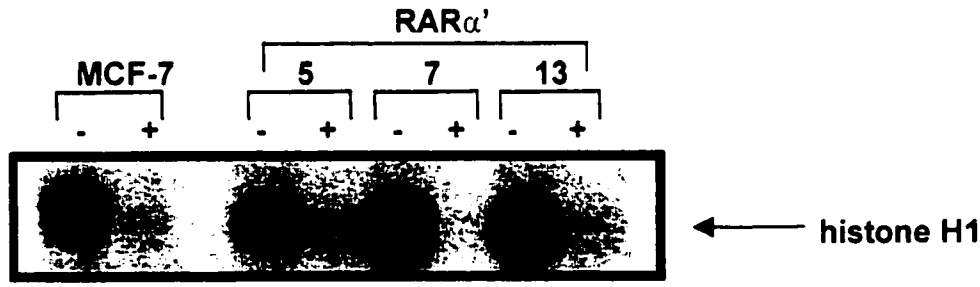
Figure 27. Effect of RA on cdk2 activity in RARα' expressing MCF-7 cells

(A) Lysates obtained from exponentially growing RARα' expressing clones incubated in the presence or absence of RA were immunoblotted with anti-cdk2 or **(B)** immunoprecipitated with anti-cdk2 and analyzed by histone H1 kinase assay. **(C)** MCF-7 cells and MCF-7(RARα') were transiently transfected with a pTRE(MMTV)-CAT reporter construct. Cells were cultured in the presence or absence of 1 μM RA and CAT activity was measured 48 hours post transfection. The CAT activities were normalized to β-galactosidase activities and are expressed relative to untreated controls. Columns represent the mean of three independent experiments; bars represent standard deviation.

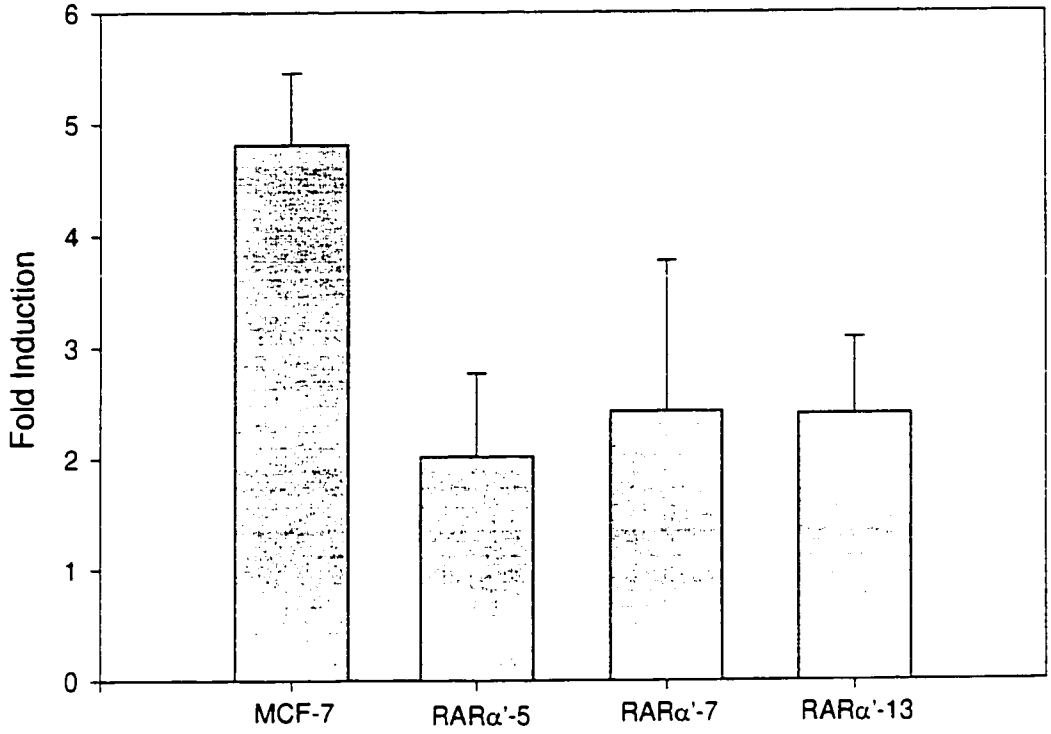
A



B



C



Cdk2 overexpressing MCF-7 cells are sensitized to RA induced growth inhibition and apoptosis

A. MCF-7 cells transfected with cdk2 exhibit greater reductions in viable cell numbers than parental MCF-7 cells following exposure to RA

Due to the profound decreases in cdk2 mRNA and activity observed following exposure to RA, we wanted to determine whether overexpression of cdk2 would be able to alleviate these effects. MCF-7 cells were stably transfected with a construct expressing the cdk2 gene driven by the CMV promoter. Two high expressing clones were obtained as assessed by Northern blot analysis (Figure 28A). Immunoblotting with anti-cdk2 antiserum revealed that the protein was efficiently overexpressed in the presence or absence of RA (Figure 28B). We next assayed the ability of these clones to resist RA mediated reductions in viable cell numbers. Asynchronous populations of MCF-7 cells and MCF-7/cdk2 clones were maintained in DMEM and cultured in the presence or absence of 1μM RA. Cells were harvested at various times by trypsinization and viable cell numbers determined by trypan blue exclusion. The time course of the effects of RA on the proliferation capacities showed that parental MCF-7 cells underwent a 75% decrease in viable cells after 120 hours of RA exposure. In contrast, MCF-7 /cdk2 clones showed even greater decreases in viable cell numbers (80% for cdk2-9 and 84% for cdk2-11), at this time point (Figure 28C).

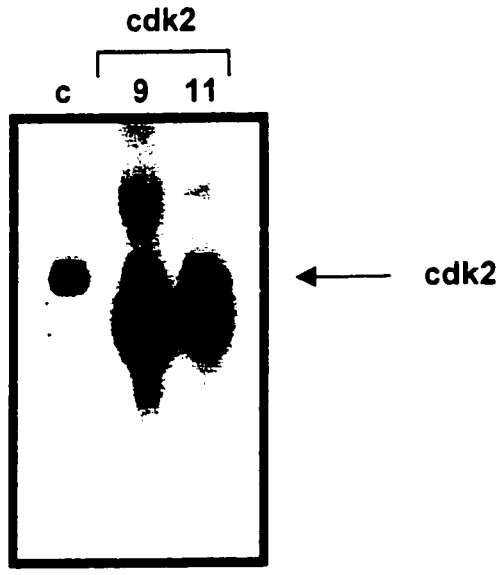
B. Overexpression of cdk2 in MCF-7 cells results in apoptosis following RA treatment

As evidenced in Figure 28C, there was not only a failure to increase the cell population of cdk2 overexpressing clones in the presence of RA, but an actual decline

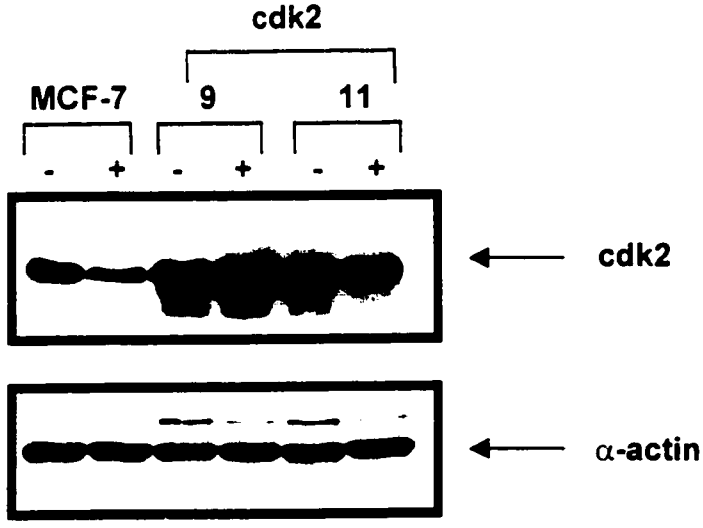
Figure 28. MCF-7 cells transfected with cdk2 are more sensitive to RA mediated reductions in viable cell numbers than parental MCF-7 cells

(A) Northern blot analysis of cdk2 overexpressing MCF-7 cells. Stable clones were selected by co-transfection with a plasmid expressing the neomycin resistance gene driven by the cytomegalovirus (CMV) promoter. **(B)** Lysates were obtained from two independent exponentially growing cdk2 overexpressing clones incubated in the presence or absence of RA and immunoblotted with anti-cdk2 **(C)** Asynchronous populations of MCF-7 cells and cdk2 clones were maintained in DMEM and treated with 1μM RA for various times or untreated. Cells were harvested by trypsinization and viable cell numbers determined by trypan blue exclusion. Viable cell numbers is expressed relative to values obtained for untreated control cells. Data represents the mean and standard deviation of three independent experiments.

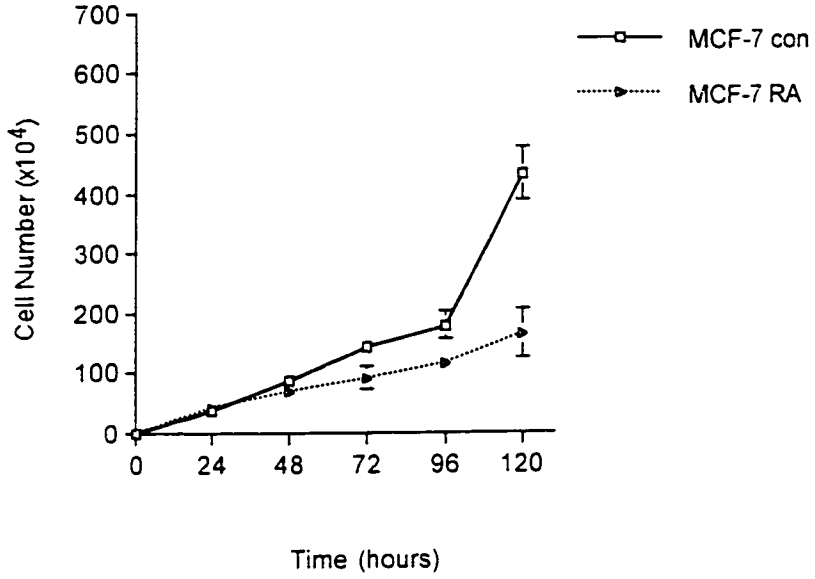
A

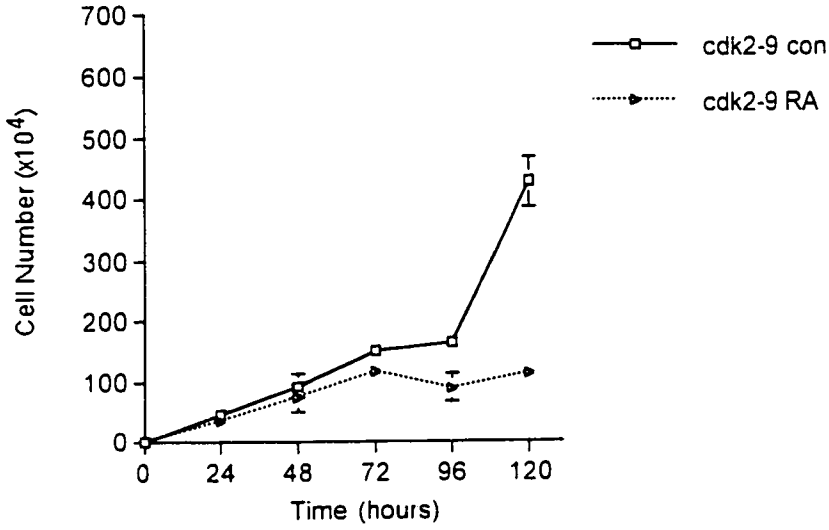


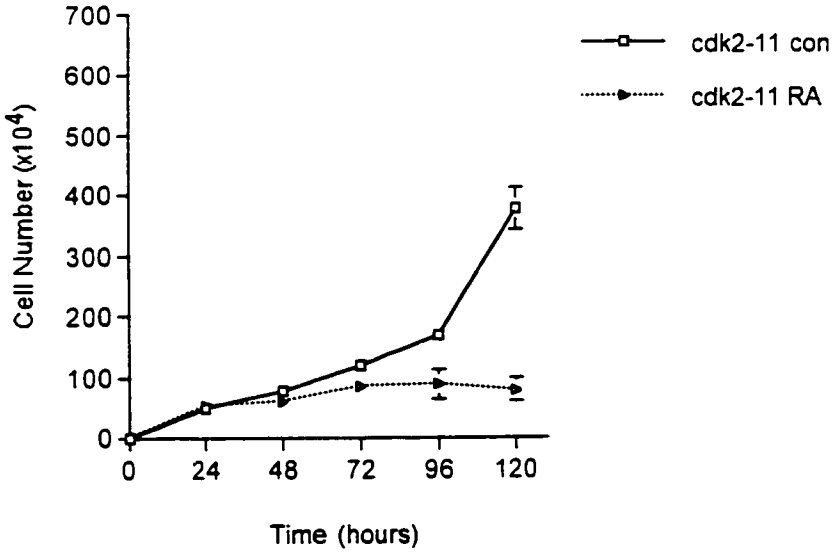
B



C

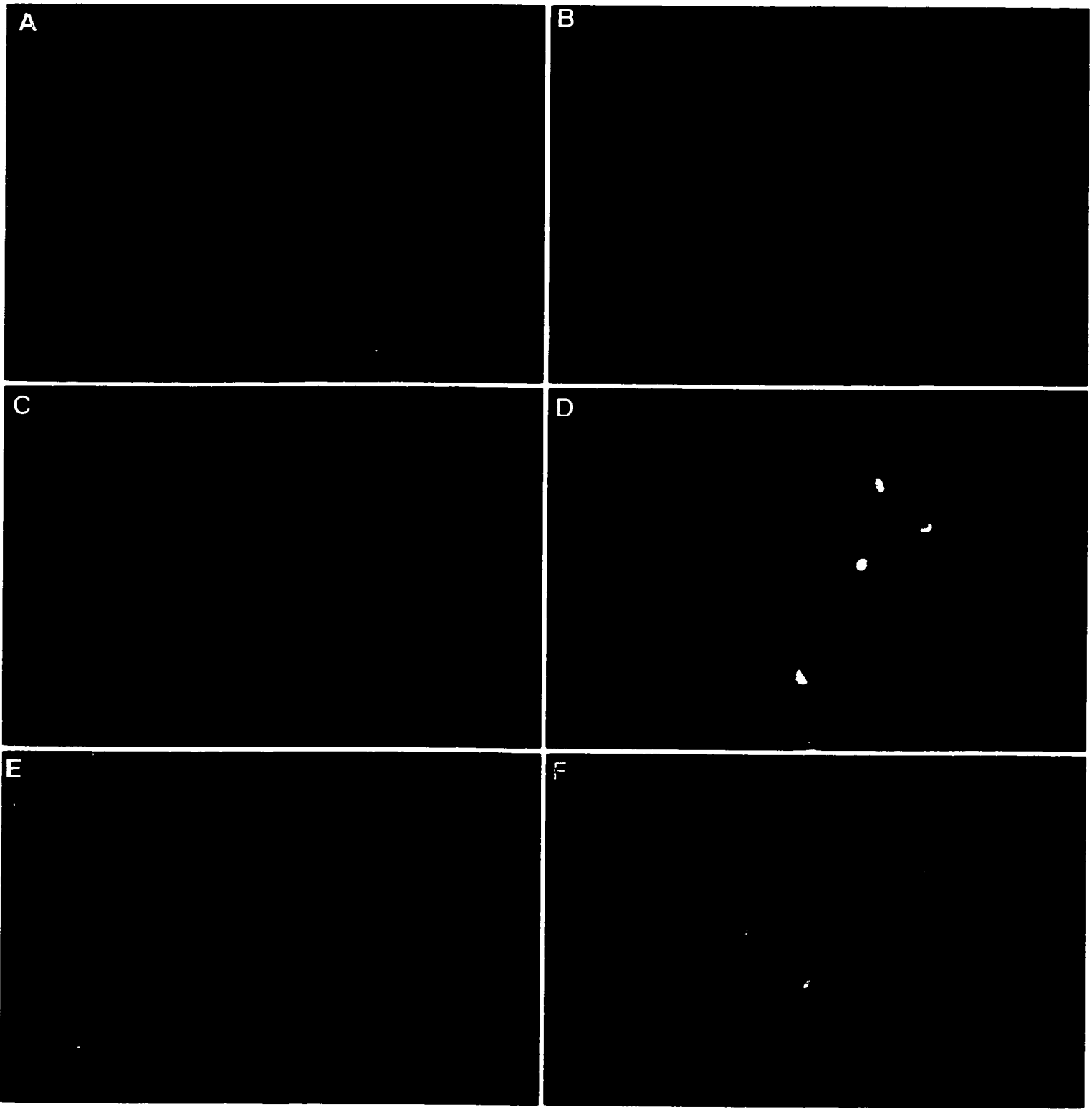

700
600
500
400
300
200
100
0
Cell Number (x10^4)
0
24
48
72
96
120
Time (hours)
MCF-7 con
MCF-7 RA


700
600
500
400
300
200
100
0
Cell Number (x10^4)
0
24
48
72
96
120
Time (hours)
cdk2-9 con
cdk2-9 RA


700
600
500
400
300
200
100
0
Cell Number (x10^4)
0
24
48
72
96
120
Time (hours)
cdk2-11 con
cdk2-11 RA

in cell number for cdk2-11. We therefore examined whether RA was capable of inducing apoptosis in MCF-7/cdk2 cells. Apoptosis is a gene directed process for the removal of unnecessary, aged or damaged cells. It is characterized by several distinct morphological changes including nuclear condensation, cytoplasmic vacuolization, cell shrinkage and membrane blebbing. It has been shown to be activated in response to a wide variety of stimuli including, retinoids, however, the specific signal transduction pathways have not been fully elucidated (reviewed by Kroemer, 1997). To further investigate the possibility that RA induced cell death could be contributing to the observed decreases in cell number, asynchronous populations of MCF-7 cells and cdk2 overexpressing clones were cultured in the presence or absence of 1μM RA for 72 hours and assayed for the onset of morphologic changes indicative of apoptosis. Cells were labeled with biotinylated-16-UTP, in a reaction catalyzed by terminal dideoxytransferase, to detect any free 3'-OH ends which would be generated by internucleosomal cleavage. In untreated and treated parental MCF-7 cells there was no evidence of free 3'-OH ends (Figure 29 A, B). In contrast, MCF-7/cdk2 cells cultured in the presence of RA exhibited signs of apoptosis, as detected by the presence of free 3'OH ends, (Figure 29D, F) which were not observed in untreated control cells (Figure 29C, E). RA treated MCF-7/cdk2 cells also displayed additional morphological changes characteristic of apoptosis including, nuclear condensation and cell shrinkage, relative to untreated controls.

Figure 29. RA causes apoptosis in MCF-7 cells overexpressing cdk2 Asynchronous populations of MCF-7 cells (A and B) and two overexpressing clones, MCF-7/cdk2-9 (C and D) and MCF-7/cdk2-11 (E and F), were cultured in the presence (B, D, and F) or absence (A, C, E) of 1μM RA for 72 hours. Cells were fixed and permeabilized prior to *in situ* end labeling (as described in "Materials and Methods") to visualize fragmented DNA.


A
B
C
D
E
F

DISCUSSION

The growth inhibition of hormone dependent breast cancer cells by retinoids has been well documented, however the molecular basis of inhibition is not well characterized. One way in which retinoids function is through preventing activation of estrogen induced transcription (Pratt et al., 1996). However, this is unlikely to be the sole mechanism by which RA inhibits the growth of breast cancer cells since this interference is incomplete. Furthermore, ER negative cells transfected with the RARα have been shown to acquire sensitivity to RA induced growth inhibition (Sheikh et al., 1994). Therefore, it is likely that RA mediated transcriptional inhibition or activation also contributes to growth inhibition of MCF-7 cells. Several years ago it was shown that treatment of MCF-7 cells with RA was able to inhibit cell cycle progression by blocking transit through G1 (Marth et al., 1985, Musgrove et al., 1993). We undertook this study to investigate whether RA could induce changes in the expression and activity of cell cycle regulatory proteins which would account for the antiproliferative effects associated with RA.

RA regulation of cell cycle progression

We have studied the mechanism by which RA is able to cause MCF-7 human breast carcinoma cells to accumulate in G1 and found that it is able to modulate the expression of G1 cyclins and associated kinase activities at multiple levels. The changes in cell cycle phase distribution observed suggests that the mechanism of action of RA occurs in late G1. The gene product of the retinoblastoma protein, pRb, has been shown to be phosphorylated by activated cdk4 and cdk2 during G1 and it is

growth inhibitory in its underphosphorylated state (reviewed by Weinberg, 1995). The results of this study show that RA prevents phosphorylation of pRb in G1, thereby maintaining it in its growth suppressive state. The accumulation of hypophosphorylated pRb (Figure 5) was coincident with the decrease in S phase fraction (Figure 4). Both the level of hyperphosphorylated pRb and the percentage of cells in G1 phase reached a maximum after 96 hours exposure to RA. Given the known function of pRb in controlling progression through G1 to S phase, this effect likely contributes to the inhibition of entry into S phase that is the ultimate consequence of RA action. It is also probable that RA is modulating the phosphorylation status of additional members of the pRb family, namely p107 and p130 (Schwarz et al., 1993; Xu et al., 1994), as we observed a significant decrease in the percentage of cells in S phase at 72 hours when pRb was still present in its hyperphosphorylated state.

The profound effects of RA on pRb phosphorylation and subsequent decrease in viable cell numbers have previously been demonstrated in another breast cancer cell line, T-47D (Wilcken et al., 1996) suggesting that this is not a cell line specific effect of RA. However, there do appear to be some differences in the mechanism of RA mediated G1 arrest . RA treatment of T-47D cells resulted in an inhibition of cdk4 activity that was not associated with changes in cyclin D1 or cdk4 expression. As well, the kinetics of RA reduction of the S phase population in these cells was faster than what we observed in MCF-7 cells, with decreases in S phase apparent by 24 hours. The reasons for the differences in response to RA between MCF-7 and T-47D are not clear, but likely reflect cell specific responses.

Since pRb is a key substrate for G1 cyclin associated kinases, these data

suggested that decreased pRb phosphorylation might result from decreased cdk activity. Investigation of possible mechanisms underlying the decrease in pRb phosphorylation led us to examine the effect of RA on the abundance of G1 associated cyclins and cdks. The effect of RA on cdk2 mRNA was evident at 8 hours (Figure 6B), however, examination of the level of cdk2 protein by western blot analysis (Figure 8B) demonstrated restriction of cdk2 mRNA had little effect on the accumulation of cdk2 protein. This suggests that control of cdk2 accumulation is likely to have a significant posttranscriptional component. In contrast, RA down regulation of cyclin D1 message (Figure 6A) was reflected by low cyclin D1 protein levels (Figure 8A). Close correspondence between the timing of mRNA and protein is in agreement with the known short half life of cyclin D1 protein in the MCF-7 cell line (Watts et al., 1995). We have found that the onset of RA down regulation of cyclin D1 levels occurred just prior to the time that the proportion of cells in S phase begins to fall (Figure 4). Hence this effect is a potential cause of decreased cell cycle progression and not merely a consequence of these changes. We also observed that cyclin D1 mRNA and protein was readily detectable in contact inhibited cells. Previous studies have shown that the level of cyclin D1 decreases upon serum depletion or at high densities (Won et al., 1992; Sherr et al., 1992). The presence of cyclin D1 in our contacted inhibited cells likely reflects our ability to only partially synchronize cells (70%) in G0/G1 phase of the cell cycle (Table 1).

Although cyclin D1 levels are significantly altered, it remains to be determined whether these are direct transcriptionally mediated effects or require prior activity of other gene products. In particular, the role of c-myc needs to be investigated given its

ability to down regulate cyclin D1 levels (Phillip et al., 1994). However, this effect appears to be cell type specific, as others have reported an up regulation of cyclin D1 after activation of c-myc (Daksis et al., 1994). In MCF-7 cells, RA has been show to increase c-myc levels as early as 30 minutes following treatment, with expression being sustained at 2 to 3 fold above basal levels after 48 hours (Sheikh et al., 1993). Although, with prolonged exposure to RA, the levels of c-myc have been observed to decrease below basal levels (Tsai et al., 1997).

Because the activity of both cyclin D1 and cyclin E associated kinases is required for progress into S phase (Baldin et al., 1993, Ohtsubo et al., 1995), inhibition of either could account for growth inhibition following exposure to RA. However, despite the RA mediated decrease in cyclin D1 expression, no accompanying decrease in cdk4 associated kinase activity was observed (Figure 10). This was an unexpected result due to the dramatic decrease in cyclin D1 levels following exposure to RA. However, the lack of proper controls to measure nonspecific pRb kinase activity limits our ability to say with certainty that cdk4 activity is unaffected by treatment with RA. Although lysates were precleared by incubation with protein A-Sepharose beads, we did not test for kinase activity that could have been precipitated from the cells using either nonimmune serum or control antibodies, which have both been shown to give background kinase activity (Matsushime et al., 1994). Nevertheless, it is also possible that the level of cyclin D1 present is not rate limiting for cdk4 activation. Because cyclin D1 is overexpressed in MCF-7 (Russell et al., 1999), RA mediated decreases in expression may not be sufficient to prevent activation of cdk4. Alternatively, since cyclin D3 was abundant in both RA treated and control cells (Figure 6A), it may reflect

some functional redundancy that would allow cyclin D3 to substitute for cyclin D1 in an active kinase complex (Matsushime et al., 1994).

In contrast, a significant decrease in cdk2 kinase activity was observed (Figure 10), consistent with the inhibition of cyclin E associated cdk2 in preventing S phase progression. Thus the decrease in cdk2 activity observed with RA suggests that it likely contributes to the changes in phosphorylation status of pRb and the concommitant blockade of cell cycle progression. Furthermore, the significant reduction of cdk2 associated activity occurred at 96 hours coincident with pRb being present only in the hypophosphorylated state.

The quantitatively greater decline in cdk2 associated kinase activity relative to the decreases in overall levels of cdk2 suggested additional regulatory factors might be involved. The activity of cyclin dependent kinases is regulated by phosphorylation and dephosphorylation (Gu et al., 1992; Poon et al, 1993). Phosphorylation of residues T14 and Y15 can inhibit its activity, conversely, phosphorylation of T160 is required for activation. The T160 residue is phosphorylated by cdk activating kinase, CAK (Gu et al., 1992; Poon et al., 1993). Metabolic labeling of RA treated cells demonstrated a decrease in the overall phosphorylation status of cdk2. It also appears as though there is an increase in the amount of immunoprecipitable cdk2 in RA treated lysates. This actually reflects the fact that equivalent amounts of cell lysates were not used to immunoprecipitate cdk2 from control and RA treated cells. Due to the high levels of radioactivity present in the extracts, accurate protein concentrations were not obtained and estimates were used instead.

The loss of cdk2 phosphorylation suggests that a critical step in RA induced

growth arrest may be failure to activate cdk2 (Figure 9). Our results show that a decrease in CAK activity did not occur until 96 hours post RA (Figure 11C) at which time the loss of phosphorylation of cdk2 and inhibition of cdk2 activity were already evident. Therefore, the decrease in CAK activity is likely a consequence of RA mediated effects, as it occurs too late to be a causitive effect. However, it is possible that the ability of CAK to phosphorylate cdk2 is blocked in RA treated cells (Kato et al., 1994).

Co-immunoprecipitation experiments have demonstrated that cyclin dependent kinases bind to a number of different proteins in addition to cyclins. For example, PCNA and p21 are both components of cell cycle complexes found in many cell types (Xiong et al., 1992). Proteins that interact with cdk2 either as substrates or regulators are likely to have important roles in cell cycle progression. Recently a novel group of proteins have been identified which inhibit cdks by their association. High levels of the cdk inhibitors, p21 (El-Deiry et al., 1993; Harper et al., 1993; Xiong et al., 1993) and p27 (Polyak et al., 1994; Slingerland et al., 1994) have been known to cause G1 arrest by inhibition of preformed/active complexes. They can also prevent activation of cdk complexes by CAK although they do not inhibit CAK directly (Kato et al., 1994; Aprelikova et al., 1994). An obvious question was whether the antiproliferative response to RA was due to an induction of one of these known inhibitors. We observed an early increase in p21 levels (Figure 12) that may have been due to p53 mediated transactivation, although, there appeared to be no corresponding recruitment of p21 into cyclin-cdk2 complexes (Figure 14). However, overall levels of p21 were decreased by the time RA mediated effects on cdk2 activity were observed.

In contrast, following an initial increase in p27 levels, by 96 hours a slight decrease in the expression of p27 was observed (Figure 12), coincident with an increase in the level of cdk2 associated p27 (Figure 14). One important factor in the formation of complexes between these proteins is the relative affinities of the cdk inhibitors for different cyclin-cdk complexes. Experiments using purified components have indicated that the affinity of p27 for cyclin E-cdk2 is ~ 10 fold higher than its affinity for cyclin D2-cdk4, while in contrast, p21 has a higher affinity for cyclin D2-cdk4 than for cyclin E-cdk2 (Harper et al., 1995). It has been proposed that cyclin D-cdk4 complexes act as high-capacity, low-affinity binding sites for p27, in essence they represent a sink for p27 (Sherr and Roberts, 1995). Therefore, when reductions in the abundance of cyclin-cdk complexes occur, this may lead to a redistribution of cdk inhibitors. Similar models have been proposed to explain the mechanism of action of several growth inhibitors. For example, cdk2 inhibition through the redistribution of p27 to cdk2, has previously been observed in fibroblasts arrested in G1 by lovostatin (Poon et al., 1995), contact inhibition (Zhu et al., 1996) or a calmodulin dependent kinase II inhibitor (Morris et al., 1998). Transforming growth factor-β, which arrests mink lung epithelial cells in mid to late G1, was also found to promote association of p27 with cdk2 (Reynisdottir et al., 1995).

Based on our observations, one possible hypothesis is that the down regulation of cdk2 activity took place as a consequence of decreased cyclin D1 expression induced by RA. The decreased cyclin D1 led to reduced cyclin D1-cdk4 complexes, resulting in the release of associated p27, and a corresponding increase in the amount of p27 bound to cdk2 complexes. Although the levels of p27 were diminished after

exposure to RA, cyclin D1 was reduced even more rapidly. This means that the rate of release of p27 from cdk4 was quicker than the rate of loss of total p27, allowing for a net increase in the p27 available to bind to cdk2. This redistribution of p27 led to the inactivation of cdk2 associated complexes. Another explanation for the increased association of p27 with cdk2 may be a change in the phosphorylation status of p27. It has been previously demonstrated that p27 bound to cyclin E and cyclin A:cdk2 complexes differs from p27 bound to D-type cyclins as it is phosphorylated in at least one additional position (Muller et al., 1997). Therefore, exposure to RA may elicit the phosphorylation of p27, with the differential phosphorylation of p27 resulting in an increased affinity for cdk2.

While it is likely that the composition of cyclin-cdk complexes is largely determined by the relative abundances and affinities of the cyclins, cdks and cdk inhibitors involved, it is also possible that interactions with other intracellular proteins contribute to the availability or association of these molecules. For example, a myc regulated heat labile inhibitor of p21 has been described (Hermeking et al., 1995). Therefore, it is possible that the early RA mediated increases in c-myc levels observed by Sheikh et al., 1993, are responsible for interfering with the inhibitory action of p21, contributing to the inability of p21 to participate in the inactivation of cdk2. Interestingly, prolonged exposure to RA also caused a decrease in p21 levels, however, this was not accompanied by an increase in cdk2 activity. By an entirely different mechanism, in cells which express abundant p27 but little p21, myc induction inhibits the ability of p27 to remain bound to cyclin E-cdk2 (Vlach et al., 1997). The inability of p27 to remain bound to cdk2 was found to be due to its phosphorylation by cdk2, thus targeting p27

for degradation (Muller et al., 1997). Furthermore, degradation of p27 was found to occur only following the induction of c-myc. While this may represent an early response to RA, as previously mentioned, longer treatments with RA resulted in decreased c-myc expression. Interestingly, the investigators also demonstrate that cyclin E:cdk2 complexes can actively phosphorylate p27 even when their kinase activity towards histone H1 is inhibited by more than 95%. This suggests that while phosphorylation of p27 by cyclin E:cdk2 occurs constitutively, phosphorylation of exogenous substrates is blocked due to steric hindrance by p27. Their findings also suggest that while activation of myc induces the degradation of p27, phosphorylation of p27 by cyclin E:cdk2 does not depend on myc function.

We have also demonstrated that RA treated lysates contain a transferable inhibitor of cdk2 activity, similar to p27, which was capable of acting on already active cdk2 complexes present in control extracts. In addition, the inhibitory action of p27 can involve binding to cdk2 complexes, thereby blocking or altering the conformation of the catalytic domain, rendering it inactive. The obstruction of CAK activation of cdk4 by p27 has been reported in cyclic AMP treated macrophages (Kato et al., 1994). Previous studies have also demonstrated the ability of p27 to bind unphosphorylated cdk2 and prevent its activation by CAK, simply by occupying the catalytic cleft (DeBondt et al., 1993).

These earlier studies suggested that p27 might play a critical role in RA mediated cdk2 inactivation. As discussed earlier, CAK activity was not compromised by RA treatment, however it may have been prevented from phosphorylating and activating cdk2 by the presence of increased p27 bound by cdk2 complexes. Therefore,

we performed functional studies with antisense p27 to determine its role in growth inhibition. Antisense reagents have been successfully used to inhibit the synthesis of a wide range of proteins, including those involved in cell cycle progression. Antisense inhibition of cyclin B synthesis was one of the first demonstrations that cyclin synthesis was necessary for entry into mitosis (Minshull et al., 1989). More recently, several investigators have used the same antisense, mismatch and liposomes used in our study to show that p27 is required for restriction point control in fibroblasts (Coats et al., 1996), and to demonstrate its involvement in IL-4 regulation of astrocyte proliferation (Liu et al., 1997). Our results using antisense p27 oligonucleotides provide confirmatory evidence that p27 represents the braking mechanism required for the inhibition of cdk2 activity in MCF-7 cells (Figure 16).

E6 expression can interfere with RA induced inhibition of cdk2 activity

It has been previously reported that retinoic acid was capable of inducing stabilization of wild type p53, but not mutant p53, in non-small cell lung carcinoma cells (Maxwell and Mukhopadhyay, 1994). Furthermore, recent studies have implicated p53 in the cellular response to various genotoxic agents. This p53-dependent G1 arrest was associated with a down regulation of cdk2 activity that was independent of p21 expression (Yuan et al., 1996). While the mechanism of cdk2 inactivation in these studies was not clear, it was found to require the interaction of p53 with the c-abl tyrosine kinase. The abrogation of either p53 function or c-abl kinase function was sufficient to alleviate both genotoxic drug induced cdk2 down regulation and the G1 arrest response, demonstrating that these effects are not exclusively mediated by

activation of p21. Our own results with E6 overexpressing clones suggest that p53 may participate in the RA mediated inactivation of cdk2 (Figure 18) and effects on viable cell numbers (Figure 19). However, this does not preclude the fact that E6 may also be acting to target other cell cycle related proteins for degradation. Cyclins D, E and p27 have been shown to undergo ubiquitin mediated degradation (Russell et al., 1999; Elledge and Harper, 1998; Shirane et al., 1999) and c-myc has been shown be a target of E6 (Gross-Mesilaty et al., 1998).

The increase in p53 may also be responsible for our observed down regulation of pRb (Figure 5), since p53 is known to repress pRb transcription (Demers et al., 1994). These observations may also explain the difference in response to RA between MCF-7 cells (wild type p53) and T-47D cells (mutant p53). Furthermore, it has been demonstrated that the induction of p53 by uv irradiation correlates with parallel decreases in E2F, pRb and cyclin D1 in inducing apoptosis in B16 melanoma (Rieber and Strasberg-Rieber, 1998), which may account for the decrease in cyclin D1 following exposure to RA.

The p53 protein generally acts as a transcription factor by binding to certain genes and regulating their expression. Most notably, expression of the cdk inhibitor p21 has been implicated in p53 growth arrest induced by various stimuli (Dulic et al., 1994). We did not observe a sustained increase in p21 levels even though p53 was still in abundance. However, it has been suggested that the specificity of the p53 dependent response appears to be determined by multiple factors, including the nature of the signal as well as the physiologic status of the cell. For example, depending on the cause of induction, p53 has been shown to selectively transactivate target genes which

are involved in the ultimate fate of the cell (Borner et al., 1997). In addition, it has been previously demonstrated that cell lines in which p53 activation is achieved through the conditional expression of high levels of wild type p53 protein can be blocked in a G1 cell cycle arrest (Martinez et al., 1991). However, these same cells simultaneously expressing high levels of E2F-1 will instead undergo apoptosis (Wu and Levine, 1994). Thus p53 mediated events appear to be both stimulus and cell type specific.

Recent evidence has provided another function for p53 in protein-protein signaling. A proline-rich motif has recently been identified as a fifth functional domain for p53 and was found to be involved in signaling for both apoptosis and growth suppression (Walker and Levine, 1996). It has been postulated that this domain functions by binding an SH3 domain of another protein. The possibility of a regulated interaction between p53 and SH3 domain containing proteins is one way to communicate with signal transduction pathways. It has been shown that p53 binds to and acts on several cellular proteins. Among these, the c-abl protein has several noteworthy properties. It contains an SH3 domain, and has been shown to bind to p53 enhancing its transcriptional activity. Interestingly, one functional consequence of the overexpression of c-abl in normal cells has been the inhibition of cell cycle progression that is dependent on wild type p53 (Goga et al., 1995).

Previous studies have demonstrated a correlation between the ability of p53 mutants to suppress growth with their ability to transactivate transcription specifically (Crooks et al., 1994). However, there is evidence that p53 can activate several target promoters, and different sequence specific DNA binding motifs have been identified (Zambetti and Levine, 1993). A correlation between sequence specific transcriptional

activation and growth suppression has also been noted through the use of a series of C-terminal deletion mutants (Pietenpol et al., 1994). Transcriptional repression has also been shown to be cell type specific. Thus, the p53 protein may use multiple mechanisms to inhibit cell proliferation, and the degree to which transcriptional regulation and direct signaling contribute to signal transmission will vary depending on the particular conditions.

Analysis of a series of p53 mutants was performed to identify if any region(s) of p53 might participate in the transmission of RA mediated antiproliferative signals. Our data showed that none of the clones assayed were able to prevent RA mediated inactivation of cdk2 (Figure 21) or to resist RA induced reductions in viable cell numbers (Figure 22). However, the small number of clones tested make it difficult to interpret our results. It is possible that the lack of effect with the mutants may simply reflect clonal variation resulting from positional effects of the transfections. This possibility can not be ruled out as no vector alone clones were isolated to test for changes in RA induced effects on viable cell numbers and cdk2 activity. Alternatively, although our results with the E6 overexpressing clones did suggest that RA mediated increases in p53 may be participating in the inactivation of cdk2 (Figure 18) and effects on viable cell numbers (Figure 19), it is possible that E6 may also be acting to target other cell cycle related proteins which are responsible for the RA mediated inactivation of cdk2 and G1 arrest. For example, p27 has been shown to undergo ubiquitin mediated degradation (Shirane et al., 1999) and we have previously seen that antisense mediated reductions in p27 were sufficient to abrogate RA induced inhibition of cdk2 activity (Figure 16).

As previously mentioned, cdk2 inactivation and G1 arrest in response to genotoxic stress has been demonstrated to be dependent on p53 (Yuan et al., 1996). Therefore, it is also possible that either none of the p53 mutants have targeted the region necessary to mediate inhibition of cdk2 activity, or that the inactivation of any one of these functional domains of p53 may be insufficient to abrogate RA mediated inhibition of cdk2. Thus the transmission of antiproliferative signals would likely involve the participation of several p53 functions including, transactivation, protein-protein interactions and perhaps sequence specific DNA binding region, with the mutation of no single domain being sufficient to prevent wild type function. Finally, it may be that the level of mutant p53 expression was not sufficient to inhibit wild type function. In fact, continued passage of tumour cell lines that express either the 22/23 or δPP mutants has revealed that expression of these proteins is selected against over time (Walker and Levine, 1996).

Posttranscriptional regulation of cdk2 expression

Retinoid receptors have been thought to act primarily through the direct modulation of gene transcription, but additional evidence has emerged that ligands for these receptors are capable of influencing steady-state mRNA levels through posttranscriptional mechanisms, mainly through mRNA stabilization. For example, retinoic acid has been shown to stabilize the c-fos mRNA (Busam et al., 1993). However, it has also been reported that retinoic acid is capable of destabilizing mRNAs for adipsin (Antras et al., 1991), tyrosine aminotransferase (Pan et al., 1992), myeloblastin (Labbaye et al., 1993) and a binding protein for fibroblast growth factors (Liaudet-Coopman and Wellstein, 1996).

Although previous studies report that γ-irradiation led to a decrease in cdk2 protein levels (Walker et al., 1995), our data demonstrate that exposure to RA results in a significant decrease in cdk2 mRNA. This is the first known observation of a growth inhibitory factor capable of decreasing cdk2 transcript levels. This decrease was not accompanied by any change in the rate of transcription of the cdk2 gene as indicated by a cdk2 promoter-luciferase reporter gene construct (Figure 23), as well as, nuclear run on experiments (Figure 24). Instead, the modulation of cdk2 expression at a posttranscriptional level was demonstrated by a decrease in message stability following exposure to RA (Figure 25). While exposure to RA did cause a destabilization of cdk2 mRNA, the half life of cdk2 mRNA was still considerable (6.5 hours). We were not able to determine the half life of cdk2 mRNA in normally cycling cells because blockade of transcription by exposure to this concentration of actinomycin D for longer than 8 hours led to severe cytotoxicity. This result is consistent with the observation of Oda et al., 1995, who found that the half life of cdk2 mRNA in untreated Rat-1A cells was indeterminate for similar reasons.

Multiple mechanisms have been implicated in the regulation of message stability involving either the 3' or 5' untranslated region (UTR) or the coding region itself. The selectivity of mRNA decay is determined by the presence of certain cis-elements, referred to as destabilizing elements, which are recognized by specific trans acting factors which function on the elements (Schiavi et al., 1992; Sachs, 1993). A specific sequence promoting mRNA decay has been identified in the 3' coding region of a variety of mRNAs. This AUUUA motif is a highly conserved sequence that is often repeated 3 or more times in the 3'UTRs of short lived transcripts (Akashi et al., 1994). It

is unlikely that the destabilization of cdk2 involves its 3' UTR since there are no known destabilizing elements in human cdk2 mRNA (Ninomiya-Tsuji et al., 1991). Instead, cdk2 mRNA has been shown to decay more rapidly in quiescent Rat-1A cells, but is transiently stabilized in proliferating cells, suggesting the presence of an alternative regulatory mechanism for the degradation of cdk2 mRNA which is active in quiescent cells and which is suppressed at the G1/S transition (Oda et al., 1995). Therefore, it is possible that exposure to RA interferes with the serum induced suppression of this regulatory mechanism for degradation of cdk2. Identification of novel destabilizing elements or factors that interact with cdk2 mRNA would shed light on the mechanism by which RA triggers destabilization of the transcript.

Nevertheless, based on the results of the northern blot analysis, the increased decay rates for cdk2 mRNA do not seem sufficient to account for the significant decrease in cdk2 expression. It may be that the cdk2 message is regulated in a complex manner by RA, with additional posttranscriptional events, such as processing of pre-mature transcripts and transport of mature mRNAs into cytoplasm being involved.

RARα' is insufficient to prevent RA mediated inactivation of cdk2

The mutant protein, RARα', is truncated in its C-terminus thereby deleting the ligand binding domain and the AF-2 domain. It has previously been shown to act as a dominant repressor of RA induced transcription from a thyroid responsive element driven (TRE)-CAT reporter construct (Pratt et al., 1990). In the presence of RA, binding of any of the RARs to the TRE enhances transcription from adjacent promoters. The

mutant receptor has also been shown to repress RA inhibition of estrogen induced gene transcription. This inhibition was found to be ligand dependent and mediated through the AF-2 domain, possibly through the sequestering of common transcriptional accessory factors (Pratt et al., 1996). These observations led us to investigate the possible role of these two mechanisms in mediating RA induced inhibition of cdk2 expression and activity. MCF-7 cells transfected with RARα' did show a partial alleviation of RA mediated reductions in viable cell numbers (Figure 26) consistent with the observations of Pratt et al., 1996, although, they were not able to prevent the down regulation of cdk2 expression and activity (Figure 27). These results suggest that the effects of RA on cdk2 could not be prevented by either the dominant repression of RA induced transcription, or the inhibition of estrogen induced gene transcription. The inability of RARα' to completely prevent RA mediated decreases in viable cell numbers suggests that several distinct mechanisms, including the inhibition of cdk2 activity contribute to the growth arrest observed following exposure to RA.

Although it is possible that the level of RARα' expression might not have been sufficient, this is not the first example of RA mediated responses being detected in the presence of high levels of RARα'. The rapid decline in abundance of small RNA polymerase III transcripts (Bladon et al., 1990) and an induction of RARβ mRNA (Pratt et al., 1990), two early RA induced events, have been shown to occur in its presence. Analysis of the human RARβ promoter indicated an RA-responsive element whose sequence is distinct from the TRE but which could mediate RA-induced transcriptional activation in the presence of either RARα or RARβ (deThe et al., 1990), suggesting that RARβ expression may be mediated by receptors other than RARα. Alternatively, RA

induced activation of RARβ may occur as a result of the inability of RARα' to completely repress this element. The induction of RARβ observed in the presence of RARα' was weak (Pratt et al., 1990), suggesting that RARα' may not be efficiently titrating co-activators (squelching) that are required for transcriptional activation. Therefore, it is possible that RA mediated regulation of cdk2 may be occurring through similar mechanisms which cannot be efficiently repressed by the RARα'. In support of this hypothesis, recent evidence suggests the inhibition of MCF-7 cell growth by retinoids does not require transcriptional activation by the RARα on a RAR response element (Dawson et al., 1995). Instead it appears that the growth inhibitory pathway may proceed through transcriptional interference by repressing gene activation from the transcription factor AP-1 (Jun/Fos) which participates in mediating cell proliferation signals. Several mechanisms have been proposed for the antagonism of AP-1 function. It has been recently demonstrated that nuclear receptors prevent c-Jun phosphorylation by interfering with the Jun amino terminal kinase (JNK) signaling pathway (Caelles et al., 1997). Phosphorylation of c-jun is required to activate AP-1 dependent gene expression. It has also been suggested that competition for the common transcriptional co-activator CBP is responsible for disrupting AP-1 activity (Horvai et al., 1997). Similar to the inhibition of gene transcription, the inhibition of AP-1 activity is also dependent on ligand binding (Schule et al., 1991; Nagpal et al., 1994). Therefore, it is possible that the levels of RARα' achieved were not sufficient to inhibit both RA mediated transactivation and inhibition of AP-1 activity. Alternatively, RARα' may not be efficiently titrating common accessory factors that are required for repressing transcriptional activation. In fact, CBP has been shown to integrate both

positive and negative effects on gene expression by serving as an essential co-activator of nuclear receptors (Horvai et al., 1997). Interestingly, the cyclin D1 promoter has been reported to contain a functional AP-1 consensus site (Herber et al., 1994), therefore RA induced transcriptional interference of AP-1 activity is another possible mechanism by which cyclin D1 levels are reduced. Alternatively, it has been previously demonstrated that estrogen also affects the expression of cyclin D1 (Foster and Wimalasena, 1996; Planas-Silva and Weingerg, 1997). And given that RA is capable of inhibiting ER activated transcription, it is possible that reduced levels of cyclin D1 may be partially attributable to RA blocking ER mediated transactivation.

RA induced apoptosis occurs in MCF-7 cells overexpressing cdk2

It appears that the critical regulation by RA occurs at the level of modification and activation of cdk2. The importance of cdk2 in the G1/S transition was previously demonstrated by van den Heuvel and Harlow in 1993, when they showed that expression of a dominant negative form of cdk2 (but not cdk4,5,6 or cdc2) could block cells in G1. It has also been reported that human fibroblasts (HDF2) overexpressing cdk2 were impaired in their ability to down regulate cdk2 kinase activity in response to DNA damage and therefore were not arrested in G2 (Walker et al., 1995). The fact that RA mediated cdk2 inactivation occurred concomitantly with G1 arrest led us to use overproduction of cdk2 to confirm its involvement. By overexpressing cdk2 in MCF-7 cells, we hoped to ascertain whether preventing cdk2 down regulation would also abrogate RA mediated G1 arrest. Our results demonstrate that the exogenous introduction of cdk2 did not prevent RA mediated decreases in viable cell numbers in

these cells. Instead we found that exposure to RA caused an even more profound decrease in the number of viable MCF7/cdk2 cells (Figure 28C), and that this decrease in viable cell number was associated with the induction of programmed cell death (Figure 29).

The hypothesis that apoptosis can occur as the result of unscheduled and unsynchronized induction of cell cycle mediators is supported by recent evidence that has implicated the activation of cdk2 in apoptosis induced by a variety of stimuli. In apoptotic cells, the cleavage of specific regulators of cdk2, including the inhibitory kinase wee1 (Zhou et al., 1998) and the cdk inhibitors p21 and p27 (Levkau et al., 1998), was shown to lead to the rapid induction of cdk2 activity. The precise involvement of cdk2 activation in mediating apoptosis is not clear, although it is believed to be an early step responsible for the activation of as yet unknown downstream targets. In MCF-7/cdk2 cells, it is likely that upon exposure to RA, the misregulated expression of cdk2 leads to an imbalanced cell cycle progression resulting in apoptosis. In contrast, our previous studies with MCF-7/E6 demonstrated that suppression of cdk2 activity is critical in RA mediated growth inhibition. Taken together, these data demonstrate the importance of tightly regulating the levels of cdk2.

Summary

In summary, this study advances our understanding of the molecular basis for retinoid mediated inhibition of human breast cancer cells. Our data demonstrates the first evidence of an antiproliferative agent capable of inactivating cdk2 by decreasing cdk2 levels. It represents the identification of a novel mechanism by which RA is able to inhibit the activity of cdk2, and thereby cell cycle progression. In contrast, numerous other studies involving growth inhibitory factors have cited elevations in, or redistribution of, the cdk inhibitory proteins p21 and/or p27 as the sole mechanism for inhibition of cdk activity.

We have identified multiple targets of RA whose actions converge at the regulation of cyclin E-cdk2 complexes. A model of the proposed pathway(s) leading to cell cycle arrest is presented (Figure 30). RA caused a significant down regulation in cdk2 mRNA levels through a posttranscriptional event. However, the mechanism by which RA destabilizes the cdk2 message is unclear. Experiments to identify novel motifs or factors which may be involved in enhancement of message instability will help delineate the mechanism of destabilization.

Our functional studies with antisense p27 oligonucleotides also demonstrated the requirement of p27 for RA mediated cdk2 inactivation, however, the events leading to the increased association of this functional inhibitor with cdk2 remain unclear. The increased association of p27 with cdk2 may be a result of a change in the phosphorylation status of p27 or an alteration in the cellular levels of cyclin:cdk complexes (Muller et al., 1997). Previous studies have demonstrated that integrin linked kinase induction led to an increase in cdk2 activity by altering the inhibitory potential of

p27 (Radeva et al., 1997). This altered inhibitory potential was associated with an altered electrophoretic mobility for p27. While the functional consequence of the alteration is a decreased inhibitory activity, the nature of the alteration is unknown, though clearly it could result from an altered phosphorylation status. Therefore, it is possible that additional modifications to p27 may also result in an increased inhibitory activity. Alternatively, an as yet unknown factor might modify the ability of p27 to bind cdk2 and promote concomitant inhibition of catalytic activity. Experiments aimed at identifying whether different residues on p27 are phosphorylated in the presence or absence of RA would help address these hypotheses.

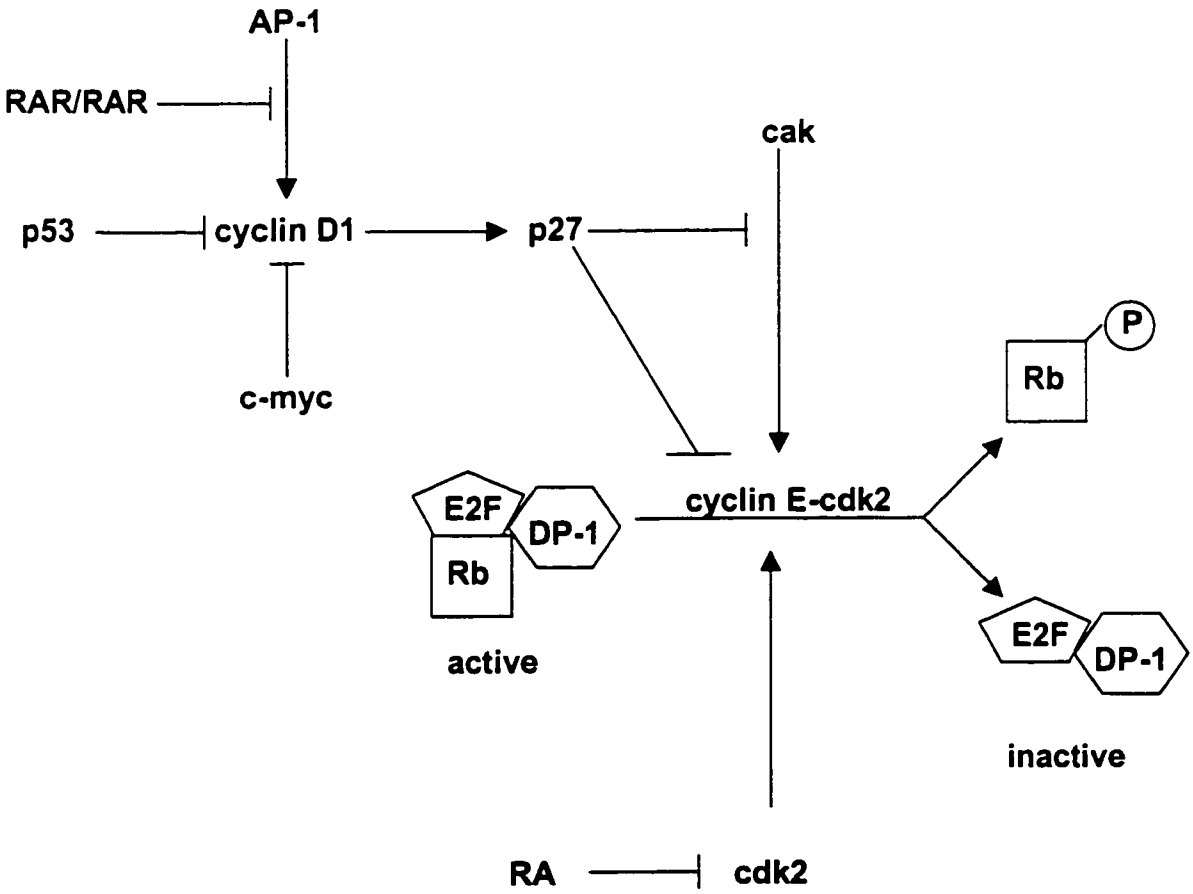
We have also observed an RA mediated down regulation in both cyclin D1 mRNA and protein levels which may begin a cascade effect leading to the inactivation of cdk2. Previous reports have demonstrated that decreases in cyclin D1 expression can cause a redistribution of p27 from cyclin D1:cdk4 complexes to cyclin E:cdk2, leading to the inactivation of cdk2 (Poon et al., 1995; Zhu et al., 1996; Morris et al., 1998). Based on our own observations we have proposed several potential upstream targets for the down regulation of cyclin D1, although their contribution is purely speculative. Promoter-reporter assays can be performed using deletion mutants of the cyclin D1 promoter to test the individual activities of the p53 (Rieber and Strasberg-Rieber, 1998), c-myc (Phillip et al., 1994) and AP-1 responsive elements (Herber et al., 1994) in the presence or absence of RA.

By identifying the upstream targets which participate in mediating retinoid action we can further define the signal transduction pathway involved in RA induced growth inhibition of human breast cancer cells. This information will also validate the rationale

for use of retinoids in the prevention and treatment of breast cancer.

Figure 30. Proposed model for RA induced association of p27 with cdk2

Tumour suppressors p53 and pRb may act in the same pathway to arrest the cell cycle. Several upstream signals have been implicated in the inactivation of cdk2. Exposure to RA was found to decrease cdk2 mRNA levels through destabilization of the message. In addition, RA mediated down regulation of cyclin D1 levels was observed. Several potential regulators of cyclin D1 expression have been proposed. The loss of cyclin D1:cdk4 complexes leads to a redistribution of p27 to cyclin E:cdk2 thus inhibiting the phosphorylation of pRb. The increased association of p27 with cdk2 may also be a result of a change in the phosphorylation status of p27 giving it a higher affinity for cdk2. Hypophosphorylated pRb sequesters the transcription factor complex E2F/DP-1, thereby preventing transcription of components required for cell cycle progression. Inactivation of cyclin E:cdk2 can also occur through prevention of CAK mediated phosphorylation and the down regulation of cdk2 levels.


AP-1
RAR/RAR
p53
cyclin D1
c-myc
p27
cak
P
Rb
E2F
DP-1
Rb
active
cyclin E-cdk2
E2F
DP-1
inactive
RA
cdk2

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