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Regulation of the Tyrosine Phophatase SHP-1 expression by C-jun-N-terminal Kinase and RFX-1 and AP-4 Transcription Factors in Insulin-like Growth Factor-1 (IGF-1) Stimulated Breast Adenocarcinoma MCF-7 Cells

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by

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Abstract

This thesis is devoted to reveal the negative regulators in IGF-1 (Insulin like growth factor 1) stimulated growth of a human breast adenocarcinoma cell line. It is a well-established fact that increased circulating levels of IGF-1 correlate with increased risk of breast cancer. IGF-1 activation of its receptor, IGF-1R, is implicated in the progression of breast cancer, where IGF-1 stimulation leads to proliferative and anti–apoptotic responses by stimulating MAPK Erk and PI3K, respectively. In this study, IGF-1 stimulated MCF-7 cells proliferated more in the absence of MAPK JNK, implicating the involvement of MAPK JNK in the negative regulation of IGF-1 stimulated cell growth.

To understand the anti- proliferative action of MAPK JNK, involvement of the tyrosine phosphatase SHP-1 was examined. SHP-1 is a well studied negative regulator of mitogenic cascades, activated by a number of different ligands in tumors of epithelial origin including breast cancers. Analysis of SHP-1 transcripts in a panel of normal and tumor samples from humans exhibited dramatically increased levels of SHP-1 in cancers of epithelial origin. SHP-1 expression correlates with JNK activation and cell proliferation in IGF-1 stimulated cells. Interfering with either JNK activation or SHP-1 expression resulted in increased MCF-7 cell proliferation. Furthermore, JNK inhibition abrogated SHP-1 expression in epithelial tumors. This discrepancy suggests that SHP-1 expression is not constant but is altered in tumors and prompts us to examine the regulation of SHP-1 expression in breast cancer cells.

SHP-1 gene regulation was examined by isolating the non-hematopoietic cell-specific SHP-1 P-1 promoter from genomic DNA, and a series of 5' deletion mutants were generated. The full length SHP-1 P-1 promoter along with its deletion mutants were fused into the pGL3 basic vector, encoding a luciferase reporter gene, and the ability of P-1 and P-1 fragments to drive the expression of the luciferase reporter gene in MCF-7 breast carcinoma cells was examined. These experiments identified two critical regions in the SHP-1 P-1 promoter, necessary for the low or the high- expression of SHP-1 in MCF–7 cells. Two of the mutant P-1 constructs, P-1/pGL3 and P1D2/pGL3 were analyzed for their capacity to drive luciferase expression following treatment with the JNK inhibitor SP600125. Subjecting these two deletion mutants to the inhibitor

abrogated SHP-1 expression, substantiating the positive role of JNK on SHP-1 expression.

In this research study, we show for the first time that IGF-1 stimulation of breast cancer cells induces SHP-1-expression by activating JNK, which in turn, activates RFX-1 and AP-4 transcription factors to allow them to bind to the high-expression region of the SHP-1 P-1 promoter in breast adenocarcinoma MCF-7 cells.

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The thesis presented here would not have been possible without the assistance of many people. So, here, I would like to thank as many people as I can who have helped in a way or other to make this thesis a successful one.

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I am gratified to my parents, brothers, cousins, and husband. Because my father applied for Canadian immigration, I have been able to immigrate here and pursue higher education at such a prestigious university. He has advised me in every aspects of my life. During the time of my pregnancy, my mother sacrificed her valuable time to aid me so I can finish this thesis on time. I have enjoyed fascinating conversations regarding my research with my elder brother and husband. I am also thankful to two of my younger brothers, Shahrooz and Shahnoor for their caring nature and with whom I enjoy gossiping over many issues. I also want to express my adoration to my baby daughter whose presence provides me with mental strength. Finally, I am thankful to all of my

cousins whom I greatly miss and whose encouragement to me to reach this level cannot be forgotten.

Recently, I have learned one thing from my father and that is, whatever research work I undertake must follow the theme of the word, "SMART." The word, "SMART" is expressed as follows: specific, measurable, achievable, realistic, and tangible. I do believe that the material contained in this thesis follows strictly the true meaning of the given word.

May God aid us so we can make a contribution for the betterment of the human society!

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Key words

AP-4

Breast cancer

Chemiluminescent EMSA kit

C-Jun N-terminal protein kinase (JNK)

ERK

IGF-1R

Insulin like growth factor-1(IGF-1)

MAPK

MCF-7

P38

PD98059

PGL3 basic vector

PI-3K

Proliferation

RFX-1

SB203509

Serine/threonine kinases

SHP-1

SHP-1 P-1 promoter

SHP-2

Signal transduction

SP600125

STAT 5

Transcription factor

ZR-75-1

ABBREVIATIONS

μg – Micro gram

μl – Microlitre

Abl –Abelson tyrosine kinase

AP-1– Activating protein-1

AP-4 –Activator protein 4

ATF-2 – Activating transcription factor-2

Bad – Bcl2-antagonist of cell death

BCR – B cell receptor

BRCA– Breast carcinoma gene

cAMP – Cyclic adenosine monophosphate

CDKN2A – Cyclin dependent kinase N2A

CELP –Converting enzyme-like proteases

c-erbB2 –Erythroblastic leukemia viral oncogene homolog 2

CPL A2 – cytoplasmic phospholipase A2

cPTK – Cytoplasmic protein tyrosine kinase

cPTP – Cytoplasmic protein tyrosine phosphatase

Crk – CT-10 related kinase

CSF1R –colony stimulating factor 1 receptor

CSK – Carboxy terminal Src kinase

DDT–Dichloro-diphenyl-trichloro-ethane

DEP-1 –Density enhanced phosphatase-1

DMSO – Dimethyl sulfoxide

DN SEK– Dominant negative SEK

DNA – Deoxy ribonucleic acid

DSP – Dual specificity phosphatase

DTT– Dithiotreitol

EDTA – Ethylene diamine tetra acetic acid

EGF – Epidermal growth factor

EGF-R – Epidermal growth factor receptor

ELISA – Enzyme linked immuno-sorbant assay

Eph-A7 – Epinephrin A7

EpoR – Erythropoetin R

ER – Estrogen receptor

ERE – Estrogen response element

ERK – Extracellular-signal regulated kinase

FAK– Focal adhesion kinase

Fer –fps/fes related tyrosine kinase

FGF – Fibroblast growth factor

FGFR4 – Fibroblast growth factor receptor

FRK – Fyn related kinase

FSH – Follicle stimulating hormone

GDP – Guanosine diphosphate

GH – Growth hormone

gm – gram

Grb2– Growth factor receptor-bound protein 2

GTP – Guanosine tri phosphate

Hepes –4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

Her2 – Human EGF receptor 2

HGFR –Hepatocyte growth factor receptor

hr – Hour

hr^{-} – Hormone receptor negative

hr^{++} – Hormone receptor positive

HRP– Horseradish peroxide

IFN$\alpha\beta$R– Interferon $\alpha\beta$ receptor

IGF-1 – Insulin like growth factor-1

IGF-1R – Insulin like growth factor-1 receptor

IGFBP – IGF binding protein

IL-1 – Interleukin-1

IP – Inositol Phosphatase

IPTG – isopropyl-beta-D-thiogalactopyranoside

IRS-1 – Insulin receptor substrate-1

JAK – Janus kinase

JNK – C- Jun N-terminal kinase

Lt – Litre

LAR – Leukocyte common antigen-related protein

MAPK – Mitogen activated protein kinase

MEK1– MAPK activating kinase

min – minute

MKK3 – MAP Kinase Kinase Kinase

ml –Millilitre

mM– Milli Mole

MMP-2 –Matrix metalloprotease 2

mV– millivolt

NOEY2 – GTP-binding RAS-like 3,

PAGE – Poly acrylamide gel electrophoresis

PCB–polychlorinated biphenyls

PCD – Programmed cell death

PCR – Polymerase chain reaction

PDGF – Platelet derived growth factor

PDGFR–platelet derived growth factor receptor

PI3K–Phosphotidyl inositol-3-kinase

PLC – Phospholipase C

PMSF – Phenyl methyl sulfoxide

PTEN – Phosphatase and tensin homolog

PTH – Parathyroid hormone

PTK – Protein tyrosine kinase

PTP – Protein tyrosine phosphatase

PTP1B – Protein tyrosine phosphatase-1B

RFX-1 – Regulatory factor X

RPTP – Receptor protein tyrosine phosphatase

RPTPß – Receptor protein tyrosine phosphataseß

Rsk – pp90 ribosomal S6 kinase

RTK – Receptor protein tyrosine kinase

SCID – Severe combined immunodeficiency

SDS– Sodium dodecyl sulphate

Sec – Second

SEK – Stress kinase activating kinase

Shc– SH2 domain containing protein

SHP-1 – SH2 domain containing phosphatase-1

SHP2 – SH2 domain containing phosphatase-2

Src –Sarcoma

sst2R – Somatostatin receptor 2

TBE – Tris- Boric acid– EDTA

TCR– T cell receptor

TGF-β – Transforming growth factor- β

TH – Thyroid hormone

T_m – Melting température

TNF-α – Tumor necrosis factor α

V– Volt

VEGF –Vascular endothelial growth factor

ZO-1 – Zona occludens-1 protein

Chapter 1: INTRODUCTION

In ancient times, cancer was described as a malignant growth which is endured with great pain and ulceration and is so called since large veins surrounding it were compared by the ancients to the claws of a crab (http://www.medterms.com/script/main). The term is now meant as a growth composed of aggregations of cells proliferating in an uncontrolled manner, either without support or embedded in the meshes of a trabecular framework. Cancer cells have a de-differentiated cellular structure, which makes them resemble fetal tissue. The de-differentiation of the cancer occurs from a maturation arrest, which is known as anaplasia. In order for a cancer to be produced, there has to be a way for these abnormally differentiated cells to remain and expand in the body (Basic Pathology, Walter and Israel).

❖ BREAST CANCER

No cancer is feared more by women than carcinoma of the breast, which is one of the leading causes of cancer death. This cancer in the human body depends on the circadian rhythm {defined as biological processes occurring at 24-hour intervals. www.wordreference.com/definition)} of hormone secretion. Breast cancer is one of the potentially life threatening malignancies which develop in one or both of the breasts. Each year, thousands of individuals are diagnosed with breast cancer. In the year 2003 alone, an estimated 267,000 women were diagnosed with breast cancer and 39,800 died of this disease in the United States (American cancer society, Breast cancer facts and figures pages 2–4). It is also recognized as one of the leading causes of death of Canadian women. For example, in 2004, an estimated

21,200 women were diagnosed with breast cancer and it has been estimated that 5,200 will die of breast cancer (Canadian cancer society, Breast cancer statistics).

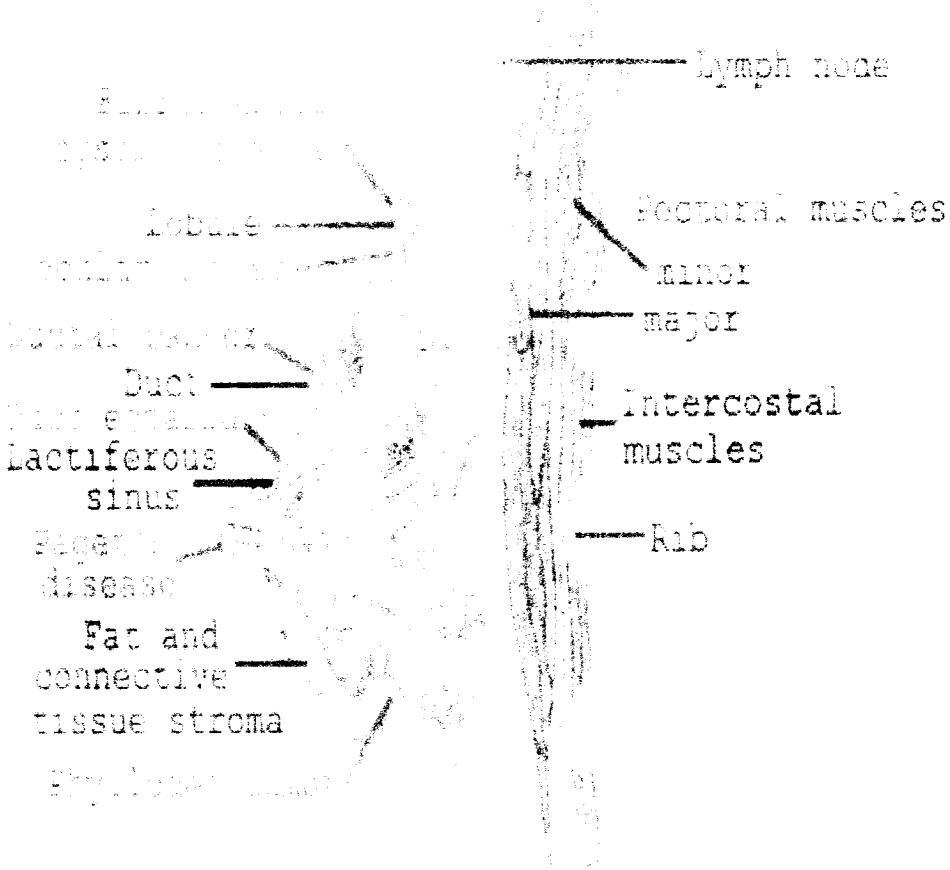
The female breast, which consists mostly of fibrous fatty connective tissues, is divided into 20 sections known as lobes (figure 1.1). From a histological point of view, these lobes are further divided into lobules that contain the milk secreting glands. Produced milk is carried by lobular ducts into a lobular sinus, which then releases milk from the nipple. Breast tumors are heterogeneous with respect to biological and clinical behavior and develop in the epithelium of terminal mammary ducts or lobuli (Weifeng et al, 1999). These discrepancies in breast tumor morphology range from hyperplasia to atypical hyperplasia or dysphasia, from noninvasive (confined to the site of origin) to primarily invasive carcinoma (spreading beyond the basement membrane). This complexity of breast tumors indicates the need for a better understanding of cellular and molecular changes which underlie the genesis and development of breast adenocarcinoma.

The risk factors associated with breast cancer are multiple and include both environmental and genetic causes. Exposure to different harmful chemicals, obesity, cigarette smoking, and mental health have been associated with the development of breast cancer. The risk of developing breast cancer increases with age. It is very rare before 30 years of age. On the other hand an 85-year-old woman has a one in eight chance of developing breast cancer (Robbins's pathological basis of disease). One of the important hormones in breast cancer etiology and treatment is estrogen. Estrogen acts through its receptor ER (estrogen receptor) which is a transcription factor in the cell nucleus. Breast cancer cells contain receptors or binding sites for estrogen and progesterone hormone.

Figure 1.1: Anatomic sites of breast lesions

This is a figure of female human breast showing the sites of different non-inflammatory lesions in the female breast. These lesions include the duct ectasias; the benign breast diseases like fibroadenoma and cystic swellings, phyllodes tumor; pre-malignant conditions like the Paget's disease of the breast and also the malignant tumors of ductal and lobular carcinomas.

Figure 1.1



http://www.wisc.edu/wolberg/anatomy1.jpg

A tumor with binding sites for these hormones is a hormone receptor positive (hr^{++}) tumor, and if it is devoid of binding sites for these hormones, it is referred to as a hormone receptor negative (hr^{-}) tumor. Hr^{++} tumors grow more slowly than the hr^{-} tumors; affording a higher probability to survive (Pavelic et al, 2001). Furthermore, overexposure to estrogen either through oral contraceptives, or due to first trimester abortions increases the risk of breast cancer. This contrasts with decreased risk of breast cancer associated with estrogen overexposure due to hormone replacement therapy in post menopausal women and pregnancy. The harmful chemicals associated with increased breast cancer occurrence are also estrogen like chemicals in the environment namely, PCB (polychlorinated biphenyls), DDT (dichlorodiphenyltrichloroethane), dieldrin, β–hexachlorocyclohexane, and finally diethylstilbestrol indicated to prevent miscarriage (Pavelic et al, 2001). Among psychiatric conditions, major depression carries a four times higher risk of developing breast cancer than other conditions; however, stress has been essentially ruled out as a cause of breast cancer (Pavelic et al, 2001).

Some of the genetic causes that have been found to be associated with breast cancer include mutations in P53 (associated with Li- Fraumeni syndrome), BRCA1 (breast carcinoma 1), BRCA2 (breast carcinoma 2), CDKN2A (cyclin dependent kinase inhibitor 2A), NOEY2 (GTP-binding RAS-like 3, Inherited from the father), and phosphatase and tensin homolog {(PTEN) mutation associated with Cowden's syndrome} (Pavelic et al, 2001). Discovery of *BRCA1* and *BRCA2* (*BRCA1/2*) susceptibility genes have catalyzed research on the clinical implications of genetic testing for inherited breast cancer. Women carrying mutations in these two genes have a higher risk of developing breast cancer and tend to develop tumors that are hr^{-}. The tumor suppressor P53 is another important factor associated with breast cancer. Mutations in

P53 gene are found frequently, varying from 1-40%, in the breast tumors of patients originated from America, Asia and Japan (Keohavong et al, 2004). The gene for P53 maps to chromosome 17p13, and encodes a 53 kDa phospho-protein that is a cell cycle regulator. Most P53 mutations are missense mutations/insertions. These mutations in the P53 gene occur in the pre-malignant lesions, early in cancer development. Deficiency in P53 protein is associated with elevated levels of the apoptosis inhibitor Bcl2 and a concomitant increase in cell growth (Keohavong et al, 2004).

An additional significant factor in breast cancer development is the enhanced expression and activity of cellular enzymes and circulating growth factors. Among the enzymes so far implicated in breast cancer are the protein tyrosine kinases (PTK) and protein tyrosine phosphatases (PTP). For instance, hyper-activation of the receptor PTK (RTK) Her2 (human EGF receptor 2) plays a central role in the development of breast cancer (Zwick et al, 1999) whereas SH2 domain containing PTP-1 (SHP-1) is over-expressed in breast cancer (Tartaglia et al, 2003). Among circulating growth factors, insulin like growth factor-1 (IGF-1), an important growth factor for fetal and childhood development, is a powerful inducer of cell proliferation and has been associated with increased risk of breast cancer in pre-menopausal women. IGF-1 regulates cell growth through activation of RTK IGF-1 receptor (IGF-1R), which leads to tyrosine phosphorylation of cellular proteins (Weifeng et al, 2000).

❖ TYROSINE PHOSPHORYLATION

A key mechanism for balanced control of eukaryotic cellular responses is the phosphorylation of tyrosine residues in proteins. This process is precisely regulated by two types

of enzymes PTKs and PTPs. In general, PTKs activate the signaling proteins by phosphorylating them at key tyrosine residues, whereas the PTPs dephosphorylate tyrosine residues thereby bringing them to their basal activity (Hunter et al, 1989 and Kozlowski et al, 1993).

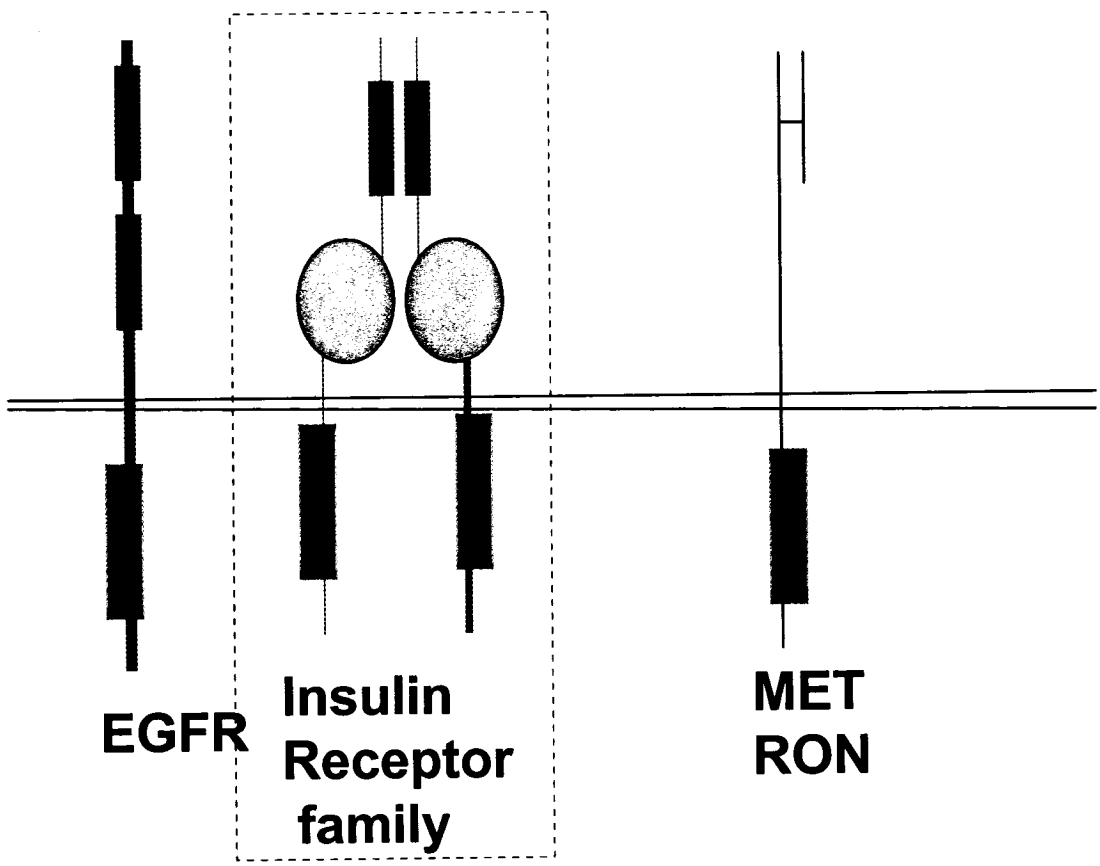
➢ PROTEIN TYROSINE KINASES (figure 1.2)

By phosphorylating proteins at tyrosine residues, PTKs allow protein-protein interactions thereby providing a mechanism for transmission of extracellular signals. PTKs form a large family of kinases found only in metazoans. The human genome contains 90 PTK genes and five presumed PTK pseudogenes. Of the 90 PTKs, 58 are RTKs and 32 are non-receptor or cytoplasmic PTKs (cPTK). The RTKs are further subdivided into 20 subfamilies and the cPTKs into 10 subfamilies based on their domains other then kinase domain (Robinson et al, 2000). So far, the genes for the PTKs can be localized into 19 of the 24 human chromosomes. Frequently, genes encoding RTKs map to adjacent loci. Within a PTK family, the members of a single family exhibit identical exon/intron patterns. This pattern is different from that of other families. Usually, PTKs undergo auto-phosphorylation in contrast to other protein kinases and catalyze the phosphorylation of a few exogenous substrate molecules. Following growth factor stimulation, there is accumulation of phosphotyrosine residues in the stimulated receptor. These phosphotyrosine residues serve as docking sites for the molecules that transmit downstream signals, often including the serine/threonine kinases. Signal amplification results in the high ratio of phosphoserine/ phosphothreonine/phosphotyrosine (3000/300/1) in cells (Craven et al, 2003). All RTKs have an extracellular ligand-binding domain, a single membrane-spanning region, and a cytoplasmic PTK–domain.

Figure 1.2. Receptor protein tyrosine kinase families

This figure shows the family of RTK enzymes. The red box demarcates the family of RTK of interest, the insulin like receptor family, which includes the IGF-1R, IGF-2R and the insulin receptor. Some other members of the RTKs that have been mentioned in this thesis are also included in the figure. These RTKs include the EGF-R family, FGF-R, PDGF-R, CSF-1R, etc.

Figure 1.2



Ligand binding promotes receptor dimerization, consequently stimulating kinase activity and triggering auto-phosphorylation of specific tyrosine residues within the cytoplasmic domain. These phosphorylated residues then serve as docking sites for proteins that are involved in the regulation of intracellular signaling cascades (De Meyts et al, 2004).

RTKs are integral components in the complex signaling network that is necessary for the correct response of a cell to its environment. These trans-membrane RTKs in addition to cPTKs are involved in the regulation of the multicellular aspects of the organism namely, adhesion, proliferation, differentiation, DNA damage repair, hormonal response, and specific alterations in gene expression. Generally, activation of RTKs is tightly controlled, allowing a normal cell to integrate external stimuli with internal signal transduction pathways in a desired and appropriate manner. In contrast, abnormal activation often leads to abnormal cell proliferation and differentiation. In humans, deregulated activities of PTKs have been demonstrated to play a significant role in the development of many types of cancer and diabetes. Historically protein PTKs are defined as the prototypical class of oncogenes implicated in different cancers (Hynes et al, 2000). The activity of PTKs is tightly regulated by dephosphorylation of tyrosine residues by PTPs.

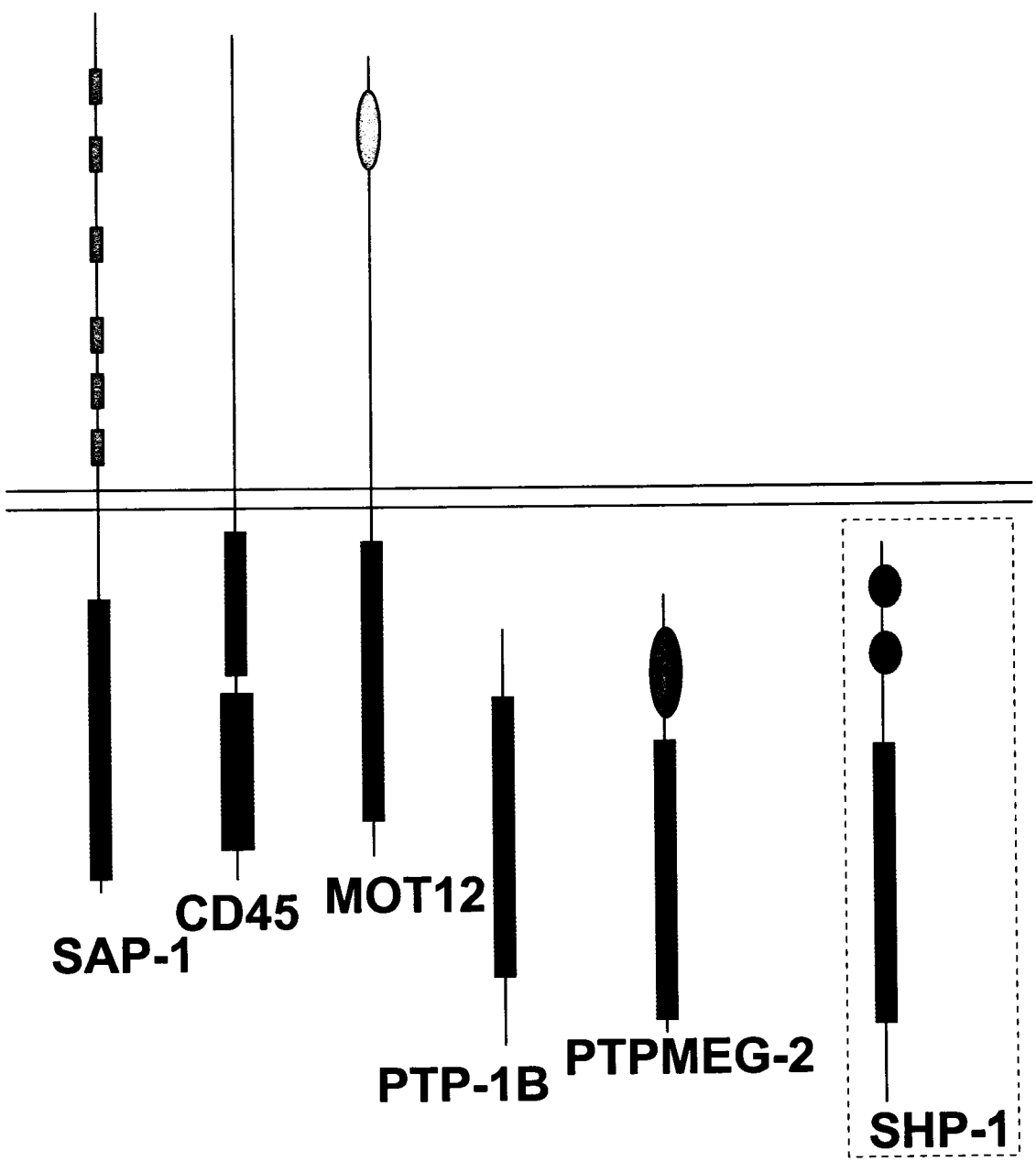
- **PROTEIN TYROSINE PHOSPHATASES (figure 1.3)**

PTPs play specific, active and dominant roles in setting the levels of tyrosine phosphorylation inside the cell and in the regulation of many physiological processes (Alonso et al, 2004).

Figure 1.3. Cytoplasmic protein tyrosine phosphatases

This figure represents the PTPs both transmembrane and cytoplasmic. The protein of interest SHP-1 is included in the SH-2 domain containing group of cPTPs and is demarcated in a red rectangle.

Figure 1.3



PTPs consist of a large family of related enzymes, including two main subgroups, the transmembrane receptor-type (RPTPs) and the non-transmembrane or cytoplasmic PTPs (cPTPs). Although tyrosine phosphorylation is regulated by the equal and balanced action of PTKs and PTPs, proportionately research has been more focused on PTKs. This may in part be due to historical reasons as the first PTP was purified (Charbonneau et al, 1989) and cloned (Guan et al, 1990) ten years after purification of the first PTK (Czernilofsky et al,1980). Recent findings however have led to emerging recognition of PTPs.

The number of genes in the human genome that encode members of the PTP families is higher than anticipated and exceeds the number of genes encoding PTKs. The list of PTPs contains 107 genes, 105 of them having mouse orthologs (Mustelin et al, 1999 and Fischer et al, 1991). All protein PTPs contain a signature motif C $(x)_5R$, and mutations of the critical cysteine residue abolishes phosphatase activity. PTPs can be divided into two major categories within this signature motif, (a) the tyrosine-specific or classical PTPs, typified by the prototypic member PTP1B and, (b) the dual specificity phosphatases (DSPs), which dephosphorylate inositol phospholipids (IP), serine, and threonine residues, in addition to the tyrosine residues (Fischer et al, 1991). Of the 107 PTP genes, 11 are catalytically inactive, 2 encode PTPs that also dephosphorylate mRNA, and 13 encode proteins capable of dephosphorylating IPs. Distribution of human PTP genes is nonrandom, with the largest clusters of loci found on chromosomes 1 and chromosome12. PTP genes have not been found on chromosomes 16, 17, 21, 22, X, and Y, whereas chromosomes 5, 8, and 13 contain PTP pseudogenes only. In general, closely related PTPs (CPTP/PTP1B, PTPD1/PTPD2, and PTPα/PTPε) do not colocalize in the same chromosome; chromosome 12 being the only exception, since it harbors the two SH2 domains

containing PTPs (SHP-1 and SHP-2) and three members of the RPTPß subtype (RPTPß, GLEPP1, and PTPS31) (Andersen et al, 2004).

Compared to the PTKs, many inhibitors of which already are in clinical trials, the PTPs are newcomers in the field of drug development. The effect of disruption of the *PTP1B* gene in mice demonstrated that this PTP acts as a negative regulator of insulin signaling (Elchebly et al, 1999) and ignited the interest of the pharmaceutical industry. Furthermore, the recent discoveries implicating many other PTPs in a variety of human diseases have substantiated the growing interest in PTPs as drug targets. Development of PTP biotherapy for diseases is underway in many biopharmaceutical companies. These include metabolic diseases like type 2 diabetes and obesity (deficiency of PTP1B) (Meyerovich et al, 1989); neurological and muscular diseases like Noonan syndrome (due to SHP-2 deficiency) and myelodysplastic syndrome (SHP-2 deficiency) (Tartaglia et al, 2004); multiple sclerosis (Lynch et al, 2001); autoimmune diseases such a SCID {(Severe combined immuno-deficiency–CD45 missense mutation), (Lynch et al, 2001)}; and most importantly cancer.

At least 30 PTPs, mainly cPTPs have been implicated in cancer. Of these 30 cPTPs, 19 PTP genes map to chromosomal regions frequently deleted in human cancers and 4 to regions frequently amplified in human cancers. In the early days of PTP research, a simplified concept developed suggesting that the main function of this group of enzymes was to act as off-switches to counteract the PTKs, and therefore considered as putative tumor suppressors (Partenan et al, 1996). For example, the gene for PTPγ localized to 3p21 of the short arm of chromosome 3 is often deleted (La Forgia et al, 1991). Based on this observation, it was hypothesized that this enzyme functions as a tumor suppressor whose functional loss underlies the pathogenesis of renal

and lung cancers (Willett et al, 1997 and Daigo et al, 1999). Density enhanced phosphatase-1 (DEP-1) is the first tyrosine-specific PTP that has been assigned a convincing role as suppressor of growth of several human cancers including 19 of 39 human colorectal adenocarcinomas (Palka et al, 2003). DEP-1's role in tumorigenesis is best illustrated by mutations in the DEP-1 gene associated with aberrantly up-regulated expression and signaling of the DEP-1 substrate hepatocyte growth factor receptor (HGFR) Met in several human tumors resulting in enhanced tumor progression (Palka et al, 2003). Another PTP implicated to play a negative role in tumor progression is PTPκ, mapped to a small 140KB region frequently deleted in primary central nervous system lymphomas (Nakamura et al, 2003).

Some PTPs have also been observed to upregulate mitogenic signals in several human carcinomas. For example, over-expression of PTPα has been shown to cause persistent activation of the PTK Src, with concomitant cell transformation, suggesting a positive role of PTPα in tumorigenesis (Tabiti et al, 1995). PTPα has also been shown to act as a negative regulator of cell growth where over-expression of this phosphatase correlated with reduced tumor aggressiveness. For example, PTPα mRNA levels were increased in late-stage colorectal carcinomas (Tabiti et al, 1995) and PTP protein levels were also observed to be higher in about one-third of primary breast carcinomas (Ardini et al, 2000). These studies illustrate that the functional significance of PTPα may depend on the cellular context and type of tumor for control of cell proliferation and cytoskeletal remodeling where it can act in both a positive and a negative role (Ardini et al, 2000).

❖ TYROSINE PHOSPHORYLATION AND BREAST CANCER

➢ TYROSINE KINASES AND BREAST CANCER

Two of the receptor PTKs that are most implicated in breast cancer are EGFR (epidermal growth factor receptor) and IGF-1R. However, the most important intracellular PTKs in breast cancer progression so far implicated is erythroblastic leukemia viral oncogene homolog 2 (c-erbB2) (Zweik et al, 1999). The role of PTKs in breast cancer was first implicated by heregulin (Her), a factor over-expressed in 20% of the breast cancers and a ligand for Her2 PTKs receptor (Olayioye et al, 2003). This led to uncovering the role of other PTKs, both membrane and non receptor types, in the development of breast cancer. The transmembrane PTKs implicated in breast cancer include HER2/neu, IGF-1R, EGFR, fibroblast growth factor receptor 4 (FGFR4), platelet derived growth factor receptor (PDGFR), colony stimulating factor 1 receptor (CSF1R), Met, Tyro-10, Eph-A7, and Tie-1 (Craven et al, 2003). In 1980, Hunter and Sefton demonstrated that the rous sarcoma virus (v-Src) oncogene product was a PTK. Further research demonstrated that v-Src and its cellular homologue c-Src are cPTKs that signal for both proliferation and inhibition of apoptosis in breast cancer. The cPTKs involved in breast cancer growth in addition to Src include Abelson tyrosine kinase (Abl), fps/fes related tyrosine kinase (Fer), carboxy terminal Src kinase (CSK), just another kinase-1 (JAK-1), JAK-2, JAK-3, focal adhesion kinase (FAK) and fyn related kinase (FRK) (Craven et al, 2003).

PTKs have been shown to increase both mitogenic and survival signaling in breast cancer cells (Bosari et al, 1998). Activation of EGF and EGF receptor homologs play important roles in the development and progression of breast cancer (Hynes et al, 2000). The EGF type family of ligands activates EGF type family of receptors. The EGF family of ligands consists of

about a dozen members characterized by an EGF like domain and three disulfide bonded intermolecular loops (Cullen et al, 1989). These peptide ligands are expressed in the extracellular domain of the transmembrane proteins and are generated by regulated proteolysis to yield growth factors that contain 49-85 amino acids (You et al, 1997). The EGFR family consists of four members. For about fifteen years, it has been known that deregulated expression of the EGFR and erbB2 contribute to the development and malignancy of breast cancer (Lippman et al, 1986). In fact, one of the first consistent genetic alterations found in breast tumors was c-*erbB2* gene amplification. The *erbB* family has evolved starting from a combination of a single ligand-receptor combination in C *elegans,* through *Drosophila,* which has one receptor and four ligands, and lastly with vertebrates, in which four *erbB* receptors bind multiple EGF-related ligands. Consequently, in vertebrates numerous *erbB* homodimer and heterodimer combinations are possible, reflecting the greater complexity of receptors and ligands, and suggesting that this complexity has evolved to provide a high degree of signaling diversity, a quick response to a multitude of external cues, necessary for their development. Amplification and over-expression of *neu* proto-oncogene is observed in 20-30% of human breast cancers, and it is inversely correlated with patient survival (Olayioye et al, 2001).

Mutations in EGFR and neu have been linked with enhanced activation of Ras GTPase which in turn activates MAPK (mitogen activated protein kinase) and PTK FAK. FAK mediated signals induce cell-cell adhesion thus increasing cell survival and preventing a specific type of apoptosis called anoikis. Other signaling intermediates that act downstream of PTKs like EGFR involved in breast cancer, include phospholipase C (PLC) PLCγ, Shc, Grb2 and Grb7. These signaling proteins have SH2 phosphotyrosine binding domains, allowing signaling proteins to

bind to specific phosphotyrosine residues in the activated RTKs or other receptor signaling proteins. Many of these signaling intermediates lie downstream of the *erbB* receptors and, not unexpectedly, some (eg PLCγ) show increased activity in tumors that over-express *erbB* RTKs (Hynes et al, 2000). Another RTK, which is a hallmark of breast cancer development, is IGF-1 (Hynes et al, 2000 and Weifeng et al, 1999). Since IGF-1 and IGF-1R play dominant roles in breast cancer development and progression the molecular mechanisms and in particular signal transduction pathways engaged will be discussed in greater detail.

IGF-1/IGF-1R signaling (figure 1.4):

IGF-1 is a 7 kDa polypeptide with a high degree of homology to IGF-2. It is synthesized predominantly by hepatocytes in response to growth hormone (GH) where it acts in an endocrine manner. IGF-1 is also synthesized from cells of epiphyseal cartilage (Andriamanalijaona et al, 2003), pituitary gland, breast stromal cells (Yee et al, 1989) and tumors like colon carcinoma, liposarcoma (Tricoli et al, 1986), ovarian tumors (Nakatani et al, 1991) and primary human lung tumors (Minuto et al, 1986) where it acts in an autocrine, paracrine or endocrine manner. The gene coding for IGF-1 is located on chromosome 12q22 and is transcribed from two promoters into a variety of mRNAs, which are translated into four precursor proteins and one single 70 amino acid molecule IGF-1 (De Meytes et al, 2004). There are considerable inter-individual variations in the levels of IGF-1 in humans, and because of this variability the normal range of circulating IGF-1 is difficult to define; however, the levels of this growth factor seem to be constant within an individual. It increases until puberty and declines thereafter. Nutrition status also affects serum IGF-1 levels. IGF-1 level decreases with diseases like Kwashiorkor, Cachexia, anorexia nervosa, celiac disease and inflammatory bowel disease (Caroll et al, 1998). At the

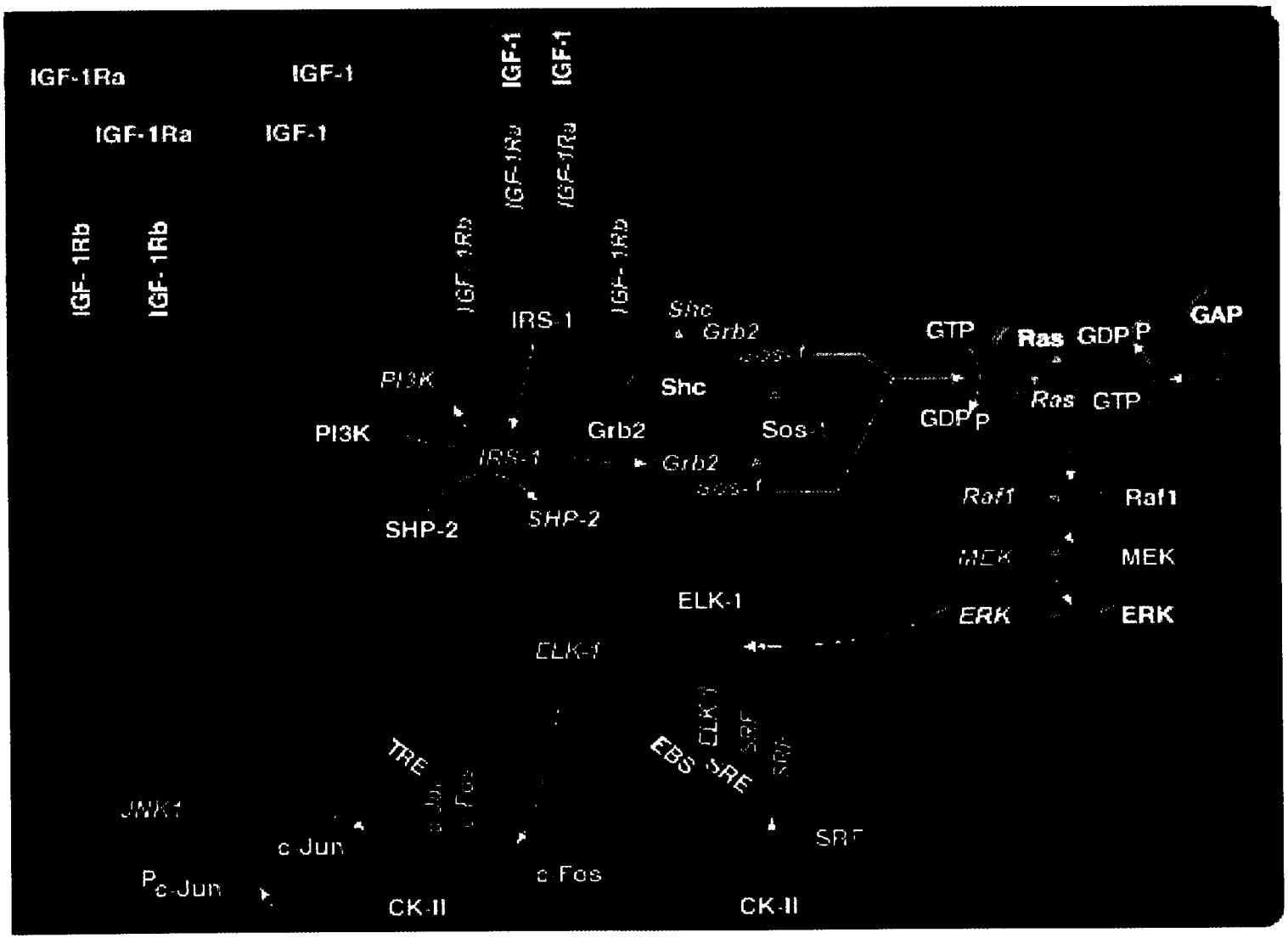
same time short term over-feeding moderately increases IGF-1. Observations demonstrate higher levels of IGF-1 in carbohydrate eaters than in meat eaters. This growth factor is essential for antenatal and postnatal skeletal growth, mediating the effects of growth hormone from two weeks after birth when the pituitary axis matures (Caroll et al, 1998). Transgenic knock-out mice lacking liver IGF-1 have now been generated by specific deletion of the hepatic *igf1* gene by *cre*-mediated recombination. These liver specific knock-out mice have 75% lower circulating IGF-1 levels but are normal, viable, and fertile, give birth to normal litters and have normal tissue expression of IGF-1 (Stergios et al, 2002 and Andrea et al, 2002). This questions the somatomedin hypothesis, stating that somatomedin or IGF-1 is secreted solely from hepatocytes in response to growth hormone, and supports the autocrine or paracrine roles of IGF-1 secreted from cells other than hepatocytes. Although this is a favorable explanation, it is also possible that in this artificial system some hepatocytes are still able to secrete IGF-1 due to incomplete *cre*-mediated excision. This therefore will contribute to circulating IGF-1 levels.

IGF-1 also appears to be a required factor for normal mammary gland development having the capacity to modulate mammary gland morphology at different stages of life including adolescence. IGF-1 is essential for breast stromal cell development in humans during puberty. Additionally, it acts in synergy with estrogen to mediate glandular mammary development (Hadsell et al, 1996).

Figure 1.4. IGF-1 and IGF-1R signaling

This is a key figure demonstrating the signaling pathways that are activated upon IGF-1 stimulation of tyrosine kinase IGF-1R. The figure shows activation of transcription factors in response to MAPK ERK stimulation by IGF-1. As demonstrated in the figure one of the transcription factors activated by ERK is AP-1 which is also activated by JNK-1 another member of the MAPK family.

Figure 1.4



http://kugi.kribb.re.kr/KU/Pathways/BioCarta/igf1Pathway/igf1Pathway.gif

- *IGFBPs:* IGF-1 is distinct from other growth factors due to the way it is carried in the blood and tissues. It is bound to a set of binding proteins IGFBPs which act both as binding proteins and modulators of IGF-1 bioavailability (Nakatani et al, 1991). Although there are six IGFBPs, each of which binds with high affinity to IGFs (Heffner et al, 1998), more than 75% of IGF-1 in circulation is bound to IGFBP-3 (Clemmons et al, 1998). The IGFBPs from 1 to 5 have a similar gene organization, dictating structural similarities, which result in increased affinity for IGF-1 compared to IGFBP-6.Tissue expression of IGFBP is specific for each member. IGFBP-1 is expressed mainly in liver and endometrium. IGFBP-2 is expressed in fetal blood, ovaries, and prostate, and IGFBP-3 is expressed in hepatic tissue (Clemmons et al, 1998). IGFBPs have 16-18 conserved cysteine residues in the amino and carboxy terminal regions. IGFBPs by binding to IGFs serve as their circulating reservoirs, thereby prolonging their half-lives and regulating access of IGF-1 ligands to their receptors (Clemmons et al, 1998).

Interaction of IGFBP with certain proteases is important for release of IGF from IGFBP to its receptor (Clemmons et al, 1998). IGFBP can have both stimulatory and inhibitory actions depending on concentration and proteolytic fragmentation. In addition to the IGF-1 dependent function these BPs have IGF independent actions. IGFBP-1 interacts with integrin to mediate motility (Clemmons et al, 1998) and with other cellular proteins to modulate cell growth. IGFBP-3 and IGFBP-5 have been found in the nucleus of lung cancer cells and can translocate from the extracellular compartment to the nucleus of rapidly dividing breast cancer cell lines (Yee et al, 1989).

The regulation of IGFBP is both ligand and cell specific. Vitamin D, retinoic acid, pituitary hormones {GH, FSH (follicle stimulating hormone)}, thyroid hormones (TH), parathyroid hormone (PTH), insulin, growth factors {PDGF, EGF, fibroblast growth factor (FGF), and transforming growth factorβ (TGF-β)} cytokines{Interleukin-1 (IL-1), tumor necrosis factor α (TNF-α)} and tumor suppressor genes P53 play either stimulatory or inhibitory roles in IGFBP regulation (Defontaine 1995 and Clemmons et al, 1998).

IGF-1R: The mitogenic activity of IGF-1 is mediated by the receptor PTKs, IGF-1R. The IGF-1R belongs to a super family of receptors that also includes the insulin receptor. There are two types of IGFR; IGF-1R and IGF-2R. IGF-1R is a hetero-tetrameric RTKs receptor and binds to both IGF-1 and IGF-2. It is expressed in almost every cell of the body and plays a role in organ development, and absence of this receptor results in generalized organ hypoplasia (Esposito et al, 1996 and De Meyts et al, 2004). It is composed of two extracellular subunits with ligand binding sites and two intracellular subunits having PTK activity bound together by disulfide bonds. Ligand binding induces receptor autophosphorylation, increased RTK activity and tyrosine phosphorylation of a number of substrate molecules. These substrate molecules include the members of the insulin receptor substrate-1 (IRS-1) such as CT-10 related kinase (Crk), SH2 domain containing protein (Shc) and growth factor receptor-bound protein 2 (Grb2). These substrates, in turn, phosphorylate and activate MAPK and phosphoinositol-3-kinase (PI-3K). Among the MAPK, extracellular-signal regulated (ERK) MAPK mediates the proliferative signals. At the same time, PI-3K activates Akt (Manning et al, 2002), which sends the anti-apoptotic and survival signals induced by IGF-1R (Clemmons et al, 1998 and Alexia et al, 2004 and Andrea et al, 2002).

Over-expression of IGF-1R confers resistance to apoptosis under a wide variety of conditions including growth factor withdrawal, serum deprivation, incubation with TNF-α, activation of IL-1 converting enzyme-like proteases (CELP), and UVβ irradiation. At the same time, tumor cells can be driven into apoptosis by using IGF-1R blocking strategies including transfection of antisense oligonucleotides, subjecting cells to dominant negative mutants of IGF-1R and triple helix formation (De Meyts et al, 2004). All these strategies are also associated with decrease in tumorigenesis and metastasis. This anti-apoptotic role of IGF-1R depends on its ability to inhibit IL-1 CELP and to increase the activity and expression of negative death regulator Bcl–XL. IGF-1R promotes cell adhesion, invasion and metastasis possibly by MR 72000 type IV collagen, matrix metalloprotease 2 (MMP-2), interacting with integrin receptors and finally up-regulating adhesion of invading cells to laminin which is a component of the cellular basement membrane (Alexia et al, 2004).

- *IGF-1 and breast cancer:* There is abundant evidence from both in vivo experiments and tissue culture experiments that many adenocarcinomas including breast and prostate are regulated by IGF-1. The first reports implicating IGF-1 in breast cancer were published more than 30 years ago (Lippman et al, 1986). In breast cancer, IGF-1 is expressed mainly in the stromal cells and not in the overlying skin as demonstrated by in situ hybridization (Cullen et al, 1989), where it is localized to stromal fibroblasts and surrounds normal breast epithelium. IGF-1 from paracrine and/or autocrine sources serve as the predominant mitogen in IGF-1 signaling in breast cancer (Lippman et al, 1986). For instance, in transgenic mice over-expressing IGF-1 or Des (1–3) IGF-1, the growth factor induces ductal hypertrophy in lactating mice and prevents post-lactational mammary gland involution (Hadsell et al, 1996). In transgenic

mice, which over-express the Des (1–3) IGF-1 along with mutant P53, the incidence of mammary tumors is 2-3 fold higher than that with only mutant P53 gene (Hadsell et al, 1996). Hepatic IGF-1, which is the endocrine source of IGF-1, also plays a role in breast cancer as suggested by higher circulating IGF-1 levels in breast cancer patients compared to normal controls (Cullen et al, 1989). An increase in IGF-1 levels is also associated with increased risk of breast cancer in pre-menopausal women. There is also evidence that hr^{++} breast tumors are responsive to IGF-1 and that the antiestrogenic drug tamoxifen decreases mean serum IGF-1 levels, decreasing proliferation and metastasis of breast cancer. However, some breast cancer cell lines have also been shown to produce IGF-1; where IGF-1 acts in both paracrine and autocrine fashions in addition to estrogen to increase cell proliferation (Stergios et al, 2002 and Lippman et al, 1986).

The predominant signaling molecule activated by IGF-I in ER^{++} breast cancer cells (MCF-7 and T-47-D) was found to be IRS-1 (Lippman et al, 1986), which further activates other downstream signaling pathways such as the PI-3K and the MAPK cascade with eventual activation of transcription factors which play a role in tumor invasion, metastasis and protection from apoptosis. IGF-1 also regulates the expression of numerous genes implicated in breast tumorigenesis. Some of these include c-myc, CDK, Zona occludens-1 protein (ZO-1), VEGF (vascular endothelial growth factor) and cathepsin D that are activated by PI-3K or ERK MAPK (Peruzzi et al, 1999).

- *Downregulation of IGF-1 signaling in breast cancer:* Recently, IGF-1 and IGF-1R have been selected as therapeutic targets for breast cancer treatment. It was, therefore, of interest to examine the intrinsic signaling molecules that play a negative role in the IGF-1

signaling cascade. Some of the molecules implicated in this negative feedback loop are PTP SHP-2 (Shi et al, 1998), cAMP (cyclic adenosine monophosphate; Lowe et al, 1997), and JNK (c-jun-N-terminal kinase; Satoshi et al, 2000). It has been observed that IGF-1 stimulation of breast cancer cell lines activates two members of the MAPK family namely, JNK1 and JNK 2 by 3.3 and 3.5 fold, respectively. IGF-1 activation of JNK1 and JNK2 is independent of the activation of other MAPK namely, ERK and P38 (Satoshi et al, 2000). IGF-1R induced mitogenic signals are inactivated by increased levels of cAMP (Lowe et al, 1997); the increase in cAMP fails to inhibit the ERK MAPK mediated signaling, thus suggesting an alternative pathway by which cAMP down regulates IGF-1 induced signals probably through JNK.

- *Mitogen activated protein kinases in IGF-1 signaling:* The MAPK are a family of serine/threonine protein kinases and are so far the best characterized proteins that mediate signal transduction pathways from the cell surface to the nucleus (Boulton et al, 1990). The controlled regulation of MAPK cascades is involved in cell proliferation and differentiation, whereas an unregulated activation can result in oncogenesis. One principal MAPK pathway involves the ERK MAPKs, ERK1 and ERK2 (Pavlovic-Surjansev et al, 1992). Upon IGF-IR autophosphorylation, the protein Shc is recruited and tyrosine phosphorylated and activated. Activated Shc then binds to Grb2 in an IRS-1 independent manner (Giorgetti et al, 1994). Grb2, in turn, localizes a guanosine nucleotide exchange factor, Sos, to the plasma membrane. Sos, activates Ras in exchange of guanosine-di-phosphate (GDP) for guanosine tri-phosphate (GTP) (Tanaka et al, 1996 and Chesnel et al, 1997). Ras GTP binds and activates Raf which, in turn, phosphorylates dual specificity kinase MAPK activating kinase (MEK1) on Ser ^{218} and Ser ^{222}. MEK1 binds ERK, phosphorylates either a threonine^{183} or a tyrosine^{185} residue and then dissociates (Weyman et al, 1998). The mono-phosphorylated ERK 1/2 then rebinds to an active

MEK1 for dual phosphorylation. The targets of activated ERK are pp90 ribosomal S6 kinase (Rsk), cytoplasmic phospholipase A2 (CPL A2) and transcription factor Elk-1 (Pavlovic-Surjansev et al, 1992). This pathway of IGF-IR signaling is closely associated with cell differentiation and migration, but in some cases also can regulate the machinery of apoptosis for example, anoikis in fibroblasts where it prevents programmed cell death (PCD) by Bcl2-antagonist of cell death (Bad), although the precise mechanisms involved in this process are unknown (Paugazhenthi et al, 1999).

Distinct from the ERKs, c-Jun N-terminal protein kinase (JNK) and P38 MAP kinases are also activated by IGF-1R and control PCD. Activation of P38 has been shown to prevent induction of PCD following DNA damage through a mechanism that possibly involves regulation of the cell cycle (Weitsman et al, 2004). It shares a 50% homology with ERK. Following its activation by MKK3 P38 gets translocated to the nucleus and phosphorylates activated transcription factor-2 (ATF-2) (Raingeaud et al, 1996).

MAPK JNK is usually activated in response to cellular stress namely in response to cytokines or environmental stress like UV (Hibi et al, 1993), ionizing radiation, heat shock, free radicals (Gupta et al, 1995), etc. JNKs are encoded by one gene whose message is alternatively spliced to form the three JNK isoforms JNK1, JNK2, and JNK3 (Sluss et al, 1994 and Gupta et al, 1996). The two MAPKK proteins that act upstream of JNK are MKK7, primarily activated by cytokines (TNFα, IL-1), and MKK4, primarily activated by environmental stress. Cytokines/environmental stresses activate Rac and Cdc2, two small GTP binding proteins of the Rho/Ras family. In a signaling cascade involving MEKK-1, MKK4/JNKK (SEK)/JNK1-3, each of the serine/threonine kinases is activated sequentially by phosphorylation (Gupta et al, 1996).

Phosphorylated JNK enters the nucleus, and preferentially phosphorylates the transcription factor c-jun at Ser63 and Ser73 (Hibi et al, 1993), inducing formation of homodimers or heterodimers (e.g., c-jun with c-fos forming AP-1) , to modulate the transcription of a number of target genes namely P53, Bcl-2/Bcl-XL, Fas-ligand, tau and caspase-1 (Karin et al, 1997). Activated JNK upon its translocation to the nucleus also phosphorylates several other transcription factors involved in PCD, including ATF-2 and Elk-1. JNK1 and JNK2 are ubiquitously expressed; whereas JNK3 is expressed only in brain tissue (Gupta et al, 1996). Similar to ERK, the sites of activation phosphorylation are conserved in JNK; these sites are located within distinct dual specificity phosphorylation motifs (TPY for JNK and TEY for ERK). JNK-mediated effects can be pro-apoptotic, proliferative, or anti-proliferative (Wilson et al, 1996). Therapeutic inhibition of JNK provides clinical benefit in diseases as diverse as arthritis, inflammatory bowel disease, stroke, Parkinsonism, ischaemic injury and myocardial infarction where its effect is pro-apoptotic. Antisense to JNK 2 blocks the growth factor induced proliferation of lung cancer cells suggesting the importance of JNK in cell proliferation (Heush et al, 1998). In this research attempt, it has been demonstrated that in IGF-1 stimulated cells the role of JNK is anti-proliferative.

The molecular mechanism by which JNK activation induces anti-proliferative signals in IGF-1 stimulated breast cancer cells is not known. Frequently, signal transduction pathways downstream of RTKs like IGF-1R are negatively regulated by PTPs (Mooney et al, 1992). In general, lack of PTPs expression or decreased PTP activity has been associated with enhanced cell proliferation and development of tumors in breast cancer (Andersen et al, 2004).

➢ ROLE OF PROTEIN TYROSINE PHOSPHATASES IN BREAST CANCER :

In breast cancer studies, a comparison of human malignant mammary tumors with normal mammary tissues revealed a large increase in total PTP activity (Partenan et al, 1996). In tumors PTPs play both positive and negative roles in breast cancer progression. For example, the inhibition of growth factor action by anti-estrogens is accompanied by an increase of PTP activity. Vanadate, a specific inhibitor of PTP, abrogated this anti-estrogen effect, thus emphasizing the major role of PTP in negative regulation of growth factor signals (Partenan et al, 1996). Some PTPs implicated in breast cancer are PTP γ, LAR (leukocyte common antigen-related protein), PTP1B, and finally the protein of interest SHP-1 (Wu et al, 2001).

PTPγ has been implicated as a potential tumor suppressor gene in kidney and lung adenocarcinomas. PTPγ mRNA is detected in primary cells isolated from mammoplasty and breast cancer patients and human breast cancer cell lines where the mRNA levels are lower in human breast cancer cells than in normal human breast cells. Also, this lower expression of PTPγ in human breast cancer cells compared to the normal breast tissue is due to higher ERα in human breast cancer tissues (Liu et al, 2002). Furthermore, ERβ significantly inhibits PTPγ expression in ER^{++} human breast cancer cells where its expression is inhibited by ERβ in a dose-dependent manner. After treatment with 20nM ERβ for 24hr, PTP gamma expression was suppressed significantly in primary breast cancer cell isolates, as well as in the ER^{++} MCF-7 cell line but expression did not change in similarly treated ER^{-} MDA-MB-231 cells. Furthermore, sensitivity

to ERβ-induced suppression can be restored (94% inhibition) by transfecting MDA-MB-231 cells with an ER expression plasmid (Zheng et al, 2000).

LAR PTP also acts as a tumor suppressor in human breast carcinoma cells that over express c-erbB2 protein tyrosine kinase (Zhai et al, 1995). In contrast it is also associated with the metastatic potential of rat breast tumors and early LAR expression is associated with poor prognosis (Zhai et al, 1995).

- **Protein tyrosine phosphatase SHP-1 (figure 1.5):**

The SHP-1 gene in humans is located on the short arm of chromosome 12 (12p13) along with the other SH2 domain containing related PTP SHP-2. The SHP-1 gene consists of 17 exons and translates into a 68 KDa cytosolic SH-2 domain containing PTP (figure F), which is expressed predominantly in hematopoietic and to a lesser extent in epithelial and neuronal cells (Kozlowski et al, 1998; Tsui et al, 1993). Two isoforms of SHP-1 protein are generated as a result of differential splicing of the SHP-1 message (figure E1). Both isoforms of SHP-1 proteins are synthesized using identically restricted exons with two different initiation codons in exon 1 and 2 (figure F2). Two different and mutually exclusive tissue-specific promoters regulate expression of the two isoforms of the SHP-1 protein. Promoter 1 (P-1), which is located approximately 7 kb upstream from promoter 2 (P-2), is active in cells of non-hematopoietic origin like epithelial and neuronal cells; whereas P-2 is active exclusively in cells of hematopoietic lineage (Banville et al, 1995) (figure 1.6). SHP-1 expression levels driven by P-1 in epithelial cells attain only 20-30% of SHP-1 level produced from the P-2 in hematopoietic cells (Banville et al, 1995).

Figure1.5:

1. Two isoforms of SHP-1

This is a figure showing the structure of the SHP-1 protein. The SHP-1 protein has two SH-2 domains and a single carboxy-terminal tyrosine phosphatase (PTP) domain. There are two isoforms of SHP-1. One is the 68kDa protein and another one is a 71 kDa protein. In humans the 68kDa protein is more abundant than the 71kDa isoform which contains a 117bp insert in the c-terminal SH-2 domain due to differential splicing of the SHP- 1 mRNA.

2. Mode of action of SHP-1 protein

This figure shows how SHP-1 dephosphorylates the phospho-tyrosine residues of the proteins. SHP-1 is grabbing the phosphotyrosine residue with its SH-2 domains which then causes a conformational change in the PTP domain bringing the PTP domain close to the phosphotyrosine residue and thereby dephosphorylating the protein. The arrow is pointing to the phospho-tyrosine peptide.

Figure 1.5 (1)

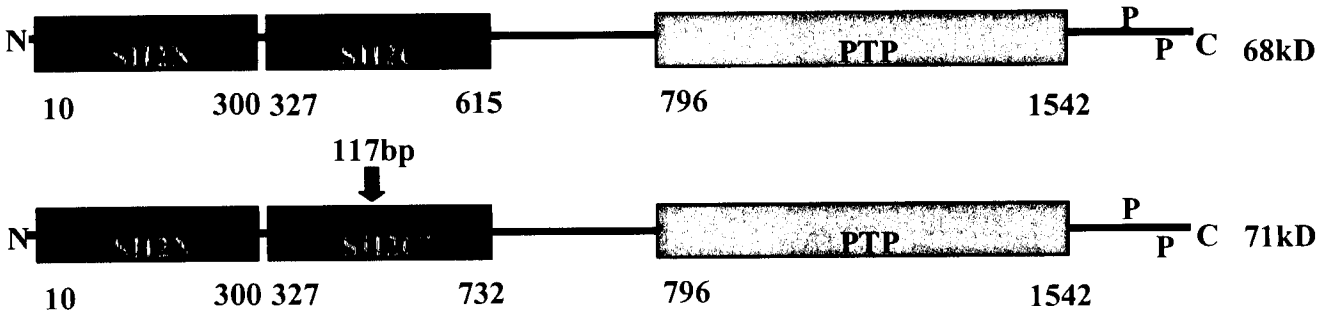


Figure 1.5(2)

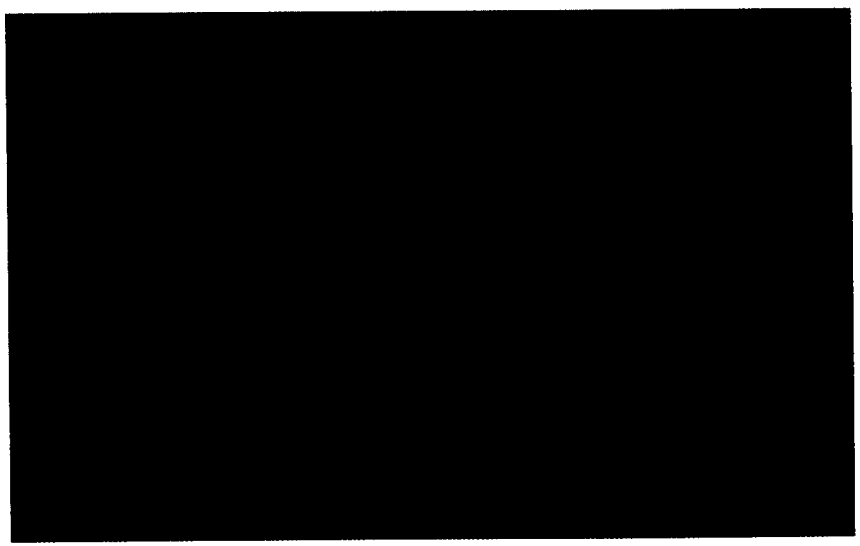
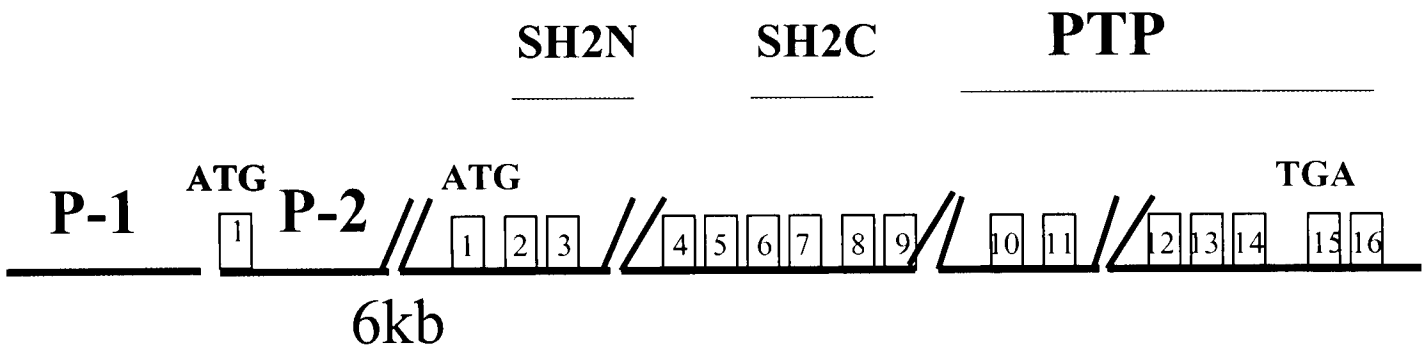


Figure 1.6: Genomic structure of the SHP-1 gene

This is a figure of the SHP-1 genomic DNA showing its 16 exons. The first three exons convert into the N terminal SH-2 domain, exon 4-9 translate into the C-terminal SH-2 domain whereas the exon 10-16 translate into the PTP domain. There are two promoters P-1 and P-2 and two alternate 5' exon 1 as depicted in the figure.

Figure 1.6


SH2N
SH2C
PTP
P-1
ATG
1
P-2
ATG
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
TGA
6kb

This low expression level of SHP-1 in normal epithelial cells contrasts with dramatically up-regulated expression of this phosphatase in a number of human epithelial cancers (Kozlowski et al, 1998).

Both SHP-1 and SHP-2 have two amino-terminal **SH2** domains, a catalytic PTP domain and a carboxyl-terminal regulatory domain. Despite sharing significant sequence identity however, these two phosphatases are distinct and have opposite biological roles. For instance, PTK signaling is usually inhibited by SHP-1 whereas it is enhanced by SHP-2 (Feng et al, 1993).

The function of SHP-1 has been intensively studied in hematopoietic cells where its activity has been associated predominantly with the termination of signals activated by cytokines like growth factors and antigen receptors (Kozlowski et al, 1996). The SH-2 domains of SHP-1 direct this PTP to tyrosine phophorylated cytokine receptors or auto-phosphorylated PTKs, thereby dephosphorylating the tyrosine residues. The RTKs dephosphorylated by SHP-1 include PDGF and EGF, whereas the cPTKs dephosphorylated by SHP-1include Fyn (Lorenz et al, 1996),Lck (Lorenz et al, 1996), Lyn (Borkdorff et al, 1999), ZAP-70 (Borkdorff et al, 1999), vav (Kozlowski et al, 1996), Grb2 (Kozlowski et al, 1996) and Sos-1. Furthermore, activated cytokine receptor such as EpoR (Klingmuller et al,1995), IFNαβR (You et al, 1997) and antigen receptors like TCR (Perez-Viller et al, 1997) and BCR (Nishida et al, 1999) are also SHP-1 targets. SHP-1 mediated tyrosine dephosphorylation is implicated in the signaling of cellular apoptosis. For instance, lymphoid cell apoptosis requires SHP-1 and loss of SHP-1 expression in the motheaten mice is reported to abrogate Fas-mediated lymphocyte apoptosis (Su et al, 1995).

- *SHP-1 and breast cancer*: It has recently been proposed that SHP-1 functions as a tumor suppressor in hematopoietic cells because its expression was diminished or abolished in

most leukaemia and lymphoma cells (Oka et al, 2001). However, very little is known about the role of SHP-1 in non-hematopoietic cells. In particular, its role in IGF-1 stimulated proliferative pathways in breast cancer cells, where SHP-1 expression is up-regulated, is not known.

SHP-1 mRNA levels in 18 human breast cancer cell lines (8 ER^{++} and 10 ER^{-}) and in 72 human primary breast tumors were examined by northern immunoblotting. This experiment revealed over-expression of SHP-1 mRNA in all of the ER^{++} breast tumor cell lines (two to three-fold over-expression in MCF-7 and ZR-75-1), in immortalized primary mammary epithelial cells (HMEC184), in four ER^{-} breast tumor cell lines (two to three-fold over-expression in BT20, MDA-MB-361 and MDA-MB-134) and lastly in 58% of the primary tumors (2–12-fold) (Wu et al, 2003) compared to normal breast cells. Investigations in somatostatin-induced apoptosis further implicated a role for SHP-1 in breast cancer etiology and progression. Indeed, several cellular models, including MCF-7 and T-47D human breast cancer cell lines, demonstrated an association between PTP activity and the sst2R (somatostatin receptor 2) (Lopez et al, 1997). Furthermore, the somatostatin analog SMS 201-995 induced, in both a time-dependent and a dose-dependent manner, relocation of SHP-1 to the membrane of MCF-7 cells. Subsequent co-immunoprecipitation studies demonstrated a direct interaction between sst2R and SHP-1. Furthermore, over-expression of SHP-1 increased the anti-proliferative activity of somatostatin in CHO cells (Lopez et al, 1997). In MCF-7 and T-47D cells, SHP-1 is not involved in apoptosis associated cellular acidification triggered by somastotatin but rather involved in other apoptotic events that follow 6.5 pH acidification as evidenced by SHP-1 translocation to the membrane (Lopez et al, 1997). Within this apoptotic concept SHP-1 may terminate RTK induced proliferative pathways. These anti-proliferative functions of SHP-1 implicate SHP-1 in human breast carcinoma progression.

HYPOTHESIS

A. SHP-1 expression is regulated by the JNK MAP kinases in IGF-1 stimulated adenocarcinoma cells.

B. SHP-1 is the mediator of JNK induced anti-proliferative signals in breast adenocarcinoma cells.

RATIONALE AND OBJECTIVE

It is well established that enhanced circulating levels of IGF-1 correlate with increased risk of breast cancer. IGF-1 stimulation leads to proliferative and anti–apoptotic responses in cancerous cells. IGF-1 is known to mediate its biological effects by activating the mitogen-activated protein kinase (MAPK) pathway. The MAPKs are comprised of three different family members, extracellular-signal regulated kinase (ERK), c-jun-N-terminal kinase (JNK) and p38. To understand the distinctive roles these kinases play in breast cancer cell responses, we have used specific inhibitors to MAPK family members. In contrast to ERK inhibitor PD98059, inhibition of JNK activity by SP600125 led to markedly enhanced cell proliferation of IGF-stimulated MCF-7 breast cancer cells suggesting that JNK activation induces anti-proliferative pathways in these cells.

The molecular mechanism by which JNK activation induces negative anti-proliferative signals in IGF-stimulated breast cancer cells is not known. We previously investigated the role of the tyrosine–phosphatase SHP-1 in hematopietic cells and demonstrated the negative impact of SHP-1 expression on cell proliferation (Kozlowski et al,1993). SHP-1 expression is significantly upregulated by several fold in breast tumors. We, therefore, hypothesized that inhibitory activity

of JNK in IGF-1 stimulated breast adenocarcinoma cell proliferation is accomplished by SHP-1. To understand the mechanism involving the signal transduction pathways achieved by IGF-1 in breast cancer cells the following objectives have been set.

A. Determine the effect of JNK activation and SHP-1 expression on adenocarcinoma cell line proliferation.
B. Analyze the SHP-1 tissue specific promoter P-1 by making deletions in the region of SHP-1 /P1 between nucleotides 987 to -567 and fusing them with pGL3 basic vector.
C. Determine the affect of IGF-1 on SHP-1 expression and JNK activation and to correlate the level of JNK activation with SHP-1 expression

- Examine the expression of P-1 promoter deletion/luciferase constructs in MCF-7 cells with the aim to identify the critical regions necessary for P-1 activity.
- Demonstrate how JNK is involved in the regulation of SHP-1 expression in serum/ IGF-1 stimulated MCF-7 cells.

Chapter 2: MATERIALS AND METHODS

❖ CELL CULTURE :

MCF-7 (HTB 22) a breast adenocarcinoma cell line; A431 (CRL-1555) an epidermoid carcinoma cell line and ZR-75-1 (CRL-1500) a breast cancer cell line were purchased from ATCC (Manassas, VA). Cells were grown in OPTI-MEM media (Canadian Life Technologies, Burlington, ON), supplemented with 10% fetal bovine serum (Cansera, Rexdale, ON) and 100U/ml penicillin-streptomycin (Canadian Life Technologies). Media was changed every two days. These cells were trypsinized, passaged every week and let to recover for two passages following removal from liquid nitrogen storage. Cells used for experiments had been passaged less than 15 times.

❖ ANTIBODIES:

Antibodies to serine/threonine kinases namely JNK1, P38, p-JNK (Mamay et al, 2003), p-P38, to PTKs c-Src, and to transcription factors p-c-Jun, AP-4 and RFX-1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The antibodies to phosphorylated proteins were used to determine the activity of the enzyme whereas the antibodies to the unphosphorylated proteins were used for ensuring equal protein loading. The unphosphorylated P38 and JNK have been shown to be stably expressed in these cell lines and have been routinely used in our laboratory to ensure equal protein loading. To determine expression of SHP-1 and SHP-2 proteins in breast cancer cells mouse monoclonal antibodies to SHP-1, and rabbit polyclonal antibody to SHP-2 were obtained from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal antibody to transcription factor AP-1 (PC06L) was obtained from Cederlane (Hornby, ON). It is an antibody against amino acid 209- 225 in the DNA binding domain in the c-terminus

of v-jun (derived from the avian sarcoma virus 17). This AP-1 antibody was used for gel supershift experiments to determine the role of transcription factor AP-1 on SHP-1 expression in MCF-7 cells by assessing its binding to oligonucleotides derived from the P-1 promoter sequence. For gel supershift assay, 1-2μg of the antibody was used whereas for western immunoblotting 0.1μg of the antibody diluted in BSA (Bovine serum albumin)/TBST (Tris 3gm, NaCl 8gm, KCl 0.2gm, and Tween 20 2ml in 1lt of deionised distilled water).

❖ **INHIBITORS:**

PD98059, (Calbiochem, San Diego, CA), an inhibitor of MAP/ERK kinase-1 kinase, selectively blocks the activity of ERK MAPK and has no effect on the activity of other serine/ threonine protein kinases including Raf-1, P38, MAPK JNKs, PKC or PKA (Dudley et al, 1995). It was reconstituted in DMSO and the toxicity of the inhibitor on MCF-7 breast cancer cells was determined by cell counting. To test the toxic dose the cells were plated in 96 well plates and allowed to recover overnight. The cells were then treated in triplicate with 1, 5, 10, 25, 50 and 100μM concentration of the inhibitor. Cells were counted 48 and 72hr following trypan blue staining. The toxic dose appeared to be 25μM concentration. 1, 5, 10 and 25μM concentration diluted in the media were added to the cells. Since this inhibitor is light sensitive it was kept in a dark box and used within one month of reconstitution.

SP600125 (Calbiochem), is an ATP competitive JNK specific inhibitor with >300 fold selectivity for JNK compared to related MAPKs including ERK-1 and P38 as well as PKA and IkB kinase (Dudley et al, 1995 and Benett et al, 2001). This inhibitor was reconstituted and further diluted in DMSO. The toxic dose of SP600125 for MCF-7 cells was determined by cell counting, as described above and established as 50μM. Cells were treated with 0.2, 0.5, 0.7, 1, 5, 10, 15, and 25μM concentration of the inhibitor. In addition, 0.1% of DMSO for each 1μM

concentration of the inhibitor was added to the cells treated with the above mentioned concentration of inhibitor. This inhibitor was light sensitive and thus was stored in the dark and used within two months of reconstitution.

SU6656 (Calbiochem) is a Src family PTK inhibitor inhibits Src (IC_{50} 280nM) and closely related PTKs including Fyn, Lyn, Lck and PDGFR (Blake et al, 2000 and Bowman et al, 2001). This inhibitor was reconstituted in DMSO and its toxic dose for MCF-7 cells assessed by cell counting was established to be 30μM. SU6656 was further diluted in the media and used at 5, 10and 25μM concentration.

SB203509 (Calbiochem) is a specific inhibitor of P38 MAPK (Jeanmarie 1998). This inhibitor was reconstituted in DMSO, and toxic dose of the inhibitor for MCF-7 cells was determined by cell counting. This inhibitor appeared to be relatively non-toxic to the cells and used at 5, 10, 15 and at 25μM concentration.

All the inhibitors were stored at –20°C.

❖ **NORTHERN BLOTTING:**

A blot containing normal and human epithelial tumor samples was purchased from Canadian Life Technologies. Membranes were first pre-hybridized in 10ml of QuickHyb (Qiagen) solution at 65°C for 20min. The blot was then transferred to 800μl of hybridization solution containing α-P^{32} ATP labelled probes encoding either human SHP-1 or 18S cDNAs. Hybridization was conducted for 1hr at 65°C after which time the blot was extensively washed in 2xSSC (1X SSC = 150mM NaCl and 15mM Na- Citrate), 0.1% SDS followed by a wash in 0.1xSSC, 0.1% SDS at 65°C. The membrane was then exposed to X-ray film (Kodak, VWR, Missisauga, ON).

❖ PROLIFERATION ASSAY:

Rates of cell proliferation were assayed using *The cell titre 96® Non- radioactive Cell Proliferation MTT assay kit* (Promega, Madison, WI) according to the manufacturer's manual. Briefly, after testing viability of the cell lines, by cell counting using trypan blue stain, $5x10^3$ cells were plated in 96 well flat bottom plates and allowed to attach for 24hr. On day 1, 3, 4, 7,10 and 13, 15µl of the MTT dye was added to each well followed by solubilization solution after 4hr. The plates were thereafter incubated in a 37°C incubator overnight after which the conversion of tetrazonium salts to formazan was measured colorimetrically using an ELISA microplate reader (Dynex Laboratories, Chantilly, VA) at 570nm.

Examining the effect of JNK on cell increase in cell proliferations: $5x10^3$ cells, of the three cell lines, were plated in 100µl of media and left to recover overnight. These cells were either left untreated or treated with different concentration of JNK inhibitor II SP600125 (Calbiochem) for 4hr followed by stimulation with IGF-1 (1-5ng/ml). Final volume was adjusted to 100µl. Cell increase in cell proliferations were measured three days after exposure to the JNK inhibitor II. The experiments were conducted in triplicate.

Effect of SHP-1 expression on cell proliferation: $5x10^3$ MCF-7 cells plated in 96 well plates in 100µl of media were allowed to recover overnight. The antisense SHP-1 oligonucleotide was designed using the SHP-1 DNA sequence (ncbi.nlm.nih.gov) and synthesized by the University of Calgary DNA laboratory. The oligonucleotide was purified by HPLC and had phosphorothioate modifications at all the cytosine residues to minimize digestion of the oligonucleotide by DNA nucleases. It was dissolved in de-ionized distilled water in 20µM concentration and transfected using the oligofectamine reagent (Canadian Life Technologies)

according to the manufacturer's manual. For this, 0.6µl of the oligofectamine stock was dissolved in 3µl of media and in parallel 4µl of antisense oligonucleotide was mixed with 16µl of media and both tubes were incubated for 5 min at room temperature. The oligofectamine solution was then mixed with the oligonucleotide cDNAs solution and allowed to incubate for 15min at room temperature to allow formation of the oligonucleotide–liposome complexes, which were then added to wells. The media was changed 6hr after adding the oligonucleotide–liposome complexes and were then stimulated with IGF-1 6hr post transfection. Cells were transfected thereafter every 72hr for 10 days. To optimize the cell loss due to transfection procedure cells were transfected with antisense to CD-4. Cell viability was monitored by tryphan blue exclusion assay. Proliferation was measured using MTT assay in a time–course manner.

❖ **CELL LYSIS AND WESTERN IMMUNOBLOTTING:**

IGF-1 or serum induced phosphorylation of JNK or ERK and the expression of SHP-1 or SHP-2 were determined by western blot analysis using antibodies specific to MAPKs, SHP-1 or SHP-2.

To examine the effect of inhibitors SP600125 on JNK and PD98059 on ERK activity and on the expression of SHP-1, the serum-starved cells were pre-incubated with different concentration of specific inhibitors for 2hr, followed by stimulation with 10ng/ml of IGF-1. To determine the effect of transcription factors AP-4 and RFX-1 on SHP-1, serum starved MCF-7 cells were transfected with either RFX-1 or AP-4 antisense oligonucleotides using oligofectamine reagent followed by determination of SHP-1 expression over a 96 hr period. The media was changed every 6hr post transfection. These cells were stimulated with IGF-1 for 24 hr i.e.IGF-1 was added to these cells 24 hr before they were collected for western immunoblotting.

To prepare cell lysate, cells were trypsinized and cell pellets were resuspended in lysis buffer (50mM HEPES pH7.5, 150mM NaCl, 10% glycerol, 1% triton X-100,1.5mM $MgCl_2$, 100mM NaF, 1mM EGTA pH7.7) supplemented with 0.25μg/ml of aprotinin (Sigma), 0.25μg/ml of leupeptin (Sigma), 1mM Na3VO4 (Sigma) followed by incubation on ice for 45min. Cell lysate was cleared by centrifugation at 14,000*g* for 20min. The protein concentration of the clear lysate was determined using the BIO-RAD protein determination assay (BIO-RAD, Mississauga, ON). 120μg of total cell protein was resolved on a 10% SDS–PAGE gel at 107V. The proteins were transferred to Immobilon-P transfer membranes (Millipore, Mississauga, ON) using the BIO-RAD transfer apparatus. For this, the transfer was allowed to continue at 100mV for 1hr at 10°C (~400mAmps). Following the transfer, the membrane was blocked with 5% BSA in TBST and incubated for 1hr with gentle shaking. The membrane was then probed with the appropriate primary antibodies in 5% BSA/TBST for 1hr to overnight at 4°C. The primary antibody was removed and the membrane was washed three times for 10min with TBST buffer. Following the washes the membrane was incubated with the secondary antibodies conjugated to HRP (horse radish peroxidase) in the dark for 1hr at room temperature. Blotted proteins were detected using western blotting luminol reagent (Santa Cruz Biotechnology, Santa Cruz, CA) where the membrane was submerged in the mixture of 1.5 ml of each solution A and B of the luminol reagent for 2min, followed by exposure to X-ray films (KODAK) and pictures were taken following exposure for 10-50sec depending on the intensity of the blot.

Membranes were stripped to reprobe with different antibodies. The antibody was stripped off the membrane by incubating it for 45min at 50°C in the western blotting stripping buffer (62.5mM Tris-HCl pH6.7, 100mM mercaptoethanol). Stripped membranes were reprobed with a second antibody as outlined above. A membrane was usually stripped three times.

Erase it Background eliminator kit (MJX BioLynx, Mississauga, ON) was used to eliminate heavy background following the manufacturer's manual.

❖ IMMUNOPRECIPITATION AND MEASURING SHP-1 ACTIVITY:

The effect of JNK phosphorylation on SHP-1 activity was measured by PTP assay kit 1 (Upstate biotechnology). For this, SHP-1 was immunoprecipitated from 1.2mg of IGF-1 stimulated total cell lysate untreated or treated with JNK inhibitor SP600125. For immunoprecipitation, lysate was first pre-cleared with 50μg of Protein A sepharose beads for 45min at 4°C in a nutator shaker (Fisher Scientific, Ottawa, ON). Then 1μg of rabbit polyclonal α-SHP-1 antibody custom made in the laboratory and 50μg of protein A sepharose beads were added to the pre-cleared cell lysate. This mixture was inverted for 2hr at 4°C and thereafter centrifuged at 14000*g* for 20min. Collected beads thus obtained were washed 3 times with lysis buffer supplemented with protease inhibitors, twice with lysis buffer without protease inhibitors and twice with pNPP tyrosine assay buffer (25mM HEPES, pH 7.2; 50mM NaCl, 5mM DTT, 2.5mM EDTA) and centrifuged at 14000*g*.

The immunoprecipitate was finally resuspended in 200μl of the pNPP tyrosine assay buffer. 75μl from this resuspended immunoprecipitate was incubated with 25μl of 1mM tyrosine phospho-peptide from the PTP assay kit, for 25min and added in duplicates to the 96 well plates supplied by the kit. To stop the reaction 100μl of the malachite green solution was added to each well and colour development was allowed to form for 15min. Phosphatase activity was measured using a 96 well microtiter plate reader at 630nm. The highest absorbance reading was taken as 100% and all other values were calculated as a percentage (%) ratio of the highest value. The plot was constructed by comparing % absorbence to the dose of the JNK inhibitor II SP600125.

❖ CLONING OF THE P-1 PROMOTER AND PRODUCING LUCIFERASE REPORTER GENE PLASMIDS:

Cloning the full-length P-1 promoter in pGL3 basic vector:

PCR amplification of the human the SHP-1 P-1 promoter, -987 to +1, was performed using the Advantage-HF PCR kit (Clontech, Palo Alto, CA) with specific 5' and 3' P-1 promoter primers encoding Xho1 or HindIII restriction sites, respectively. The primers were synthesized by University Core DNA services, University of Calgary (Calgary, AB). The amplification consisted of denaturation at 94°C for 2min, 30 cycles of denaturation at 94°C for 20sec, annealing at 58°C for 30sec, extension at 72°C for 4min and finally elongation at 72°C for 7min. DNA amplicons were resolved on a 1% agarose gel and the correct fragment was gel extracted and sequenced. It was then adenylated on the 3′ end of both strands of the amplicons. 100-500ng of the amplified promoter fragments were then ligated to 1µl of TA cloning PCR 2.1 vector using 1µl of T4 DNA ligase in a 20µl reaction at 16°C using a TA cloning kit (Canadian Life Technologies). These TA clones were used to transform *E. coli* XL-1 blue competent cells and plated on 2YT+Ampicillin plates containing 1.6mg of X-gal and 40µl of 100mM IPTG. The plates were incubated overnight at 37°C and for 2hr at 4°C for development of blue colour. The white colonies (clones containing the insert) were cultured in 2YT+Ampicillin broth and the plasmid DNA was extracted by *Gene–elute miniprep kit* (Sigma). The TA clone was screened for the presence of the P-1 promoter insert. The clone containing the P-1 insert, the P-1–TA clone, was sequentially cut with Xho1 and HindIII, the fragment extracted from the gel using the Qiagen gel extraction kit and ligated into the multiple cloning site of pGL3 basic luciferase reporter vector cut with HindIII and XhoI enzymes using the T4 DNA ligase. XL-1 blue cells were then transformed with the P-1/pGL3 clone and the transformed cells were plated on 2YT+

Ampicillin agar. The clones on the agar were grown in 2YT+ Ampicillin broth and the plasmids were extracted by *Gene–elute plasmid miniprep kit* (Sigma). The plasmid DNA was then digested with HindIII and XhoI enzymes to detect the presence of the insert. Clones containing the insert were grown in 250ml of 2YT broth+Ampicillin and maximum preparation of DNA from colonies was done by using *Endo-free Plasmid maxi-prep kit* (Qiagen) according to the manufacturer's manual.

Generating the P-1deletion mutants (figure 2.1):

The deletion fragments were generated by PCR amplification of the P-1/pGL3 basic construct. This was done using different 5' primers and the same 3' primer. The 5' primers were designed every 100-200bp away from the P-1 promoter 5' end (Table-1). The PCR product was verified by gel electrophoresis, sequenced, and purified by PCR purification kit. Cloning was carried out as discussed above for generating the P-1/pGL3 construct.

Generating P1D2 site directed mutants

The mutant constructs were produced using the Quick Change II XL site directed mutagenesis kit (Cat #200521, Stratagene, La Jolla, CA). For this, three pairs of mutagenic oligonucleotides primers were synthesized by University Core DNA services, University of Calgary (Calgary, AB). Each primer pair represented both the strands of the same sequence containing the mutation. These primers were also later used for gel super-shift assays.

The *Tm* of the primers were determined by the following equation :

Tm = 81.5 +0.41 (%GC)–675/N– %mismatch. (Here N= number of nucleotides)

To increase the T_m to around 78°C an additional 5mM MgSO4 was added to the reaction mixture before PCR and the concentration of primers was adjusted to 0.5μM as per manufacturer's suggestions.

Figure 2.1: P-1 and deletion constructs generated by PCR

A. Promoter and its 5' deletion mutants were synthesized by HF-PCR (high fidelity-polymerase chain reaction) kit using different 5' primers and one 3' primer and cloned into the pGL3 basic luciferase reporter vector. The mutants were mapped every 50-200bp.
B. An agarose gel electrophoresis of the PCR samples of P-1 and its initial 5' deletion mutants. The mutants were synthesized by HF-PCR using different 5' primers and one 3' primer from the genomic DNA of the P-1 promoter and mapped every 200bps.
C. An agarose gel electrophoresis of the PCR samples of P-1 and its new 5' deletion mutants. The mutants were synthesized by HF-PCR using different 5' primers and one 3' primer from the P-1 promoter cut with HindIII and Xho-1 from the P-1/pGl3 construct. These new mutants were mapped every 50-100bp between P-1 and P1D4.

Figure 2.1

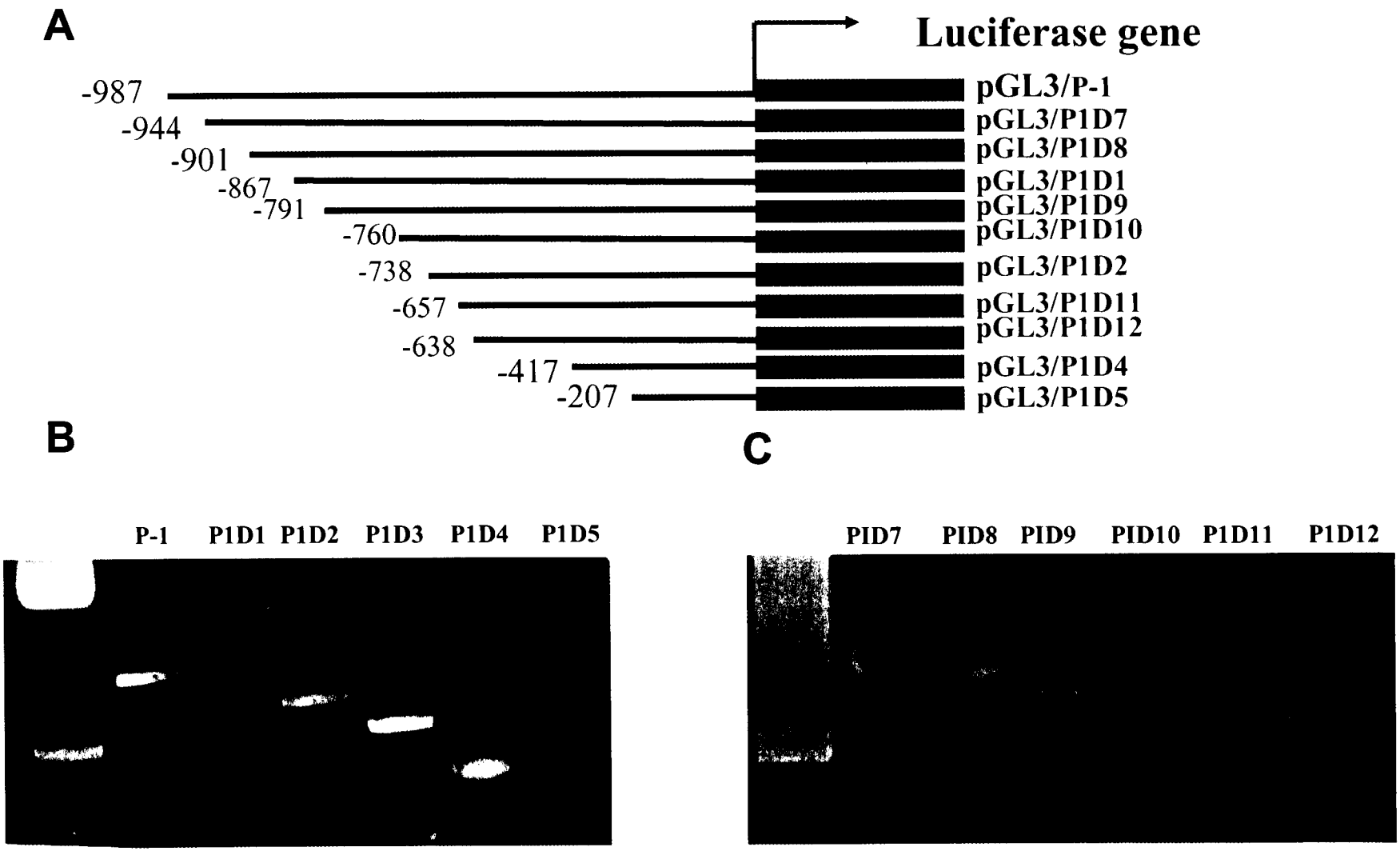

A
Luciferase gene
-987
-944
-901
-867
-791
-760
-738
-657
-638
-417
-207
pGL3/P-1
pGL3/P1D7
pGL3/P1D8
pGL3/P1D1
pGL3/P1D9
pGL3/P1D10
pGL3/P1D2
pGL3/P1D11
pGL3/P1D12
pGL3/P1D4
pGL3/P1D5
B
P-1
P1D1
P1D2
P1D3
P1D4
P1D5
C
PID7
PID8
PID9
PID10
P1D11
P1D12

Figure 2.2: The P-1 promoter sequence with the position of its deletion primers from 5' end.

This is a sequence of the SHP-1 P-1 promoter, showing the location of the deletion primers and their sequence from the 5' end. This sequence codes the P-1 promoter for bp 1-987. The primers for the initial P-1 fragments are highlighted in green and for the new deletions are highlighted in pink. The name of the primer is written in blue in the superscript. The arrows represent the direction of DNA synthesis. The arrows in the figure show the direction of polymerase synthesizing primer extension.

The cycling parameters included 95°C for 1min followed by 18 cycles of 95°C for 50sec, 60°C for 50sec, 66°C for 5min and a final extension time of 7min at 66°C. The reaction was then cooled to 4°C. The reaction mixture was subjected to Dpn-1 digestion to get rid of the parental methylated or hemimethylated DNA for 2hr. Generated mutated plasmids were used to transform XL-1 blue cells using the manufacturer's manual and plated on 2YT + Ampicillin agar. The colonies were screened for the presence of mutated plasmid. A Dpn-1 digested P1D2 was taken as a negative control whereas the P1D2 +/– mutation not subjected to Dpn-1 digestion was taken as a positive control. All DNA sequencing was performed by the Centre for Biologics Research, Health Canada.

- **TRANSIENT TRANSFECTION OF PLASMIDS AND EXPRESSION OF THE LUCIFERASE REPORTER GENE (FIGURE 2.3):**

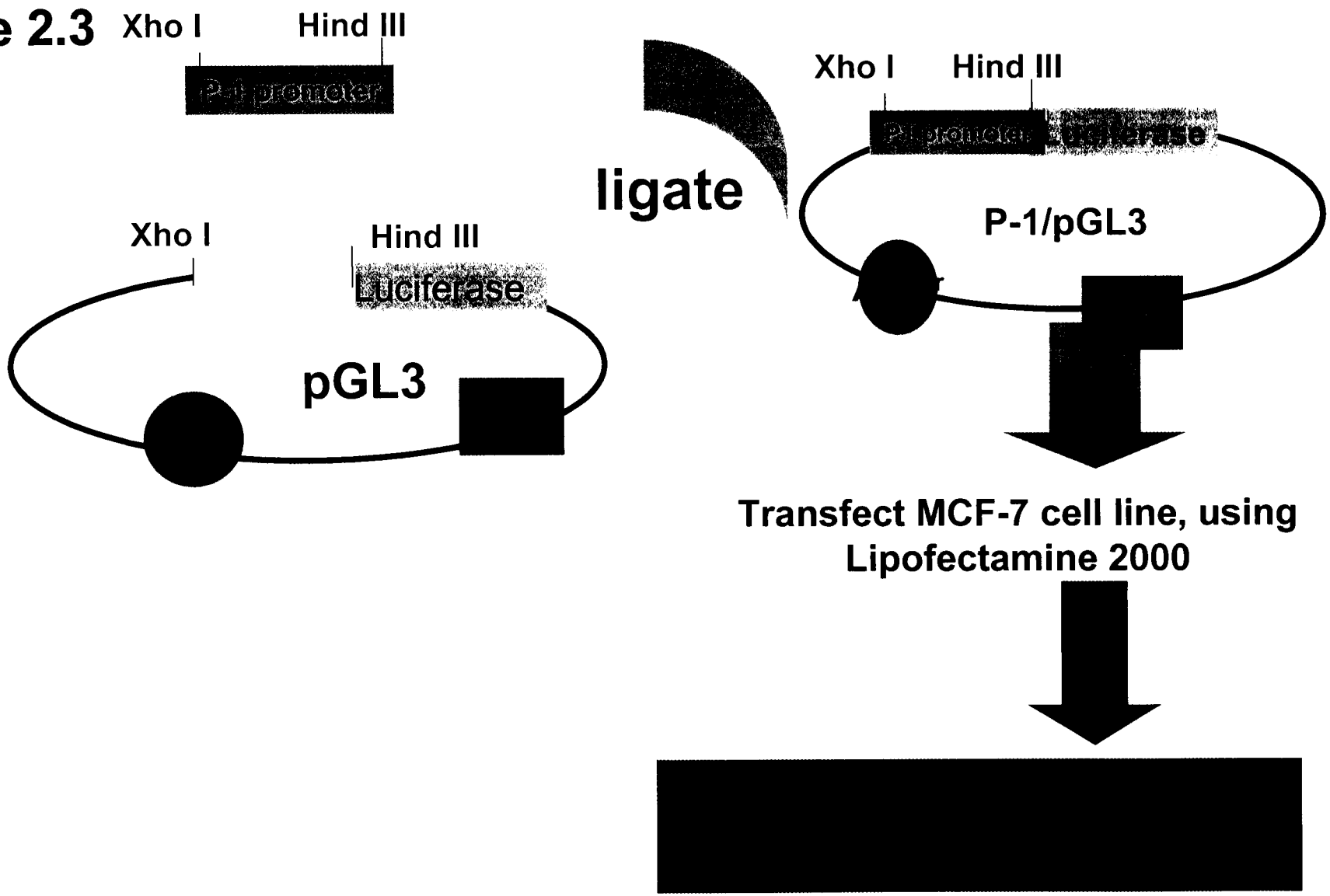
Transient transfection of MCF-7 cells was performed using *Lipofectamine 2000* reagent (Canadian Life Technologies) according to the manufacturer's manual.

The purity of plasmid as determined by the absence of protein or RNA contamination was determined by establishing a ratio OD at 260/280. 260/280 ratio between 1.7–1.9 ensured good transfection efficiency. When the ratio was more than 1.9, indicating RNA contamination, RNAase A was added to the plasmid DNA. A 260/280 ratio lower than 1.7 implicated presence of contaminating proteins and required DNA precipitation. Briefly, to the DNA one volume of phenol/chloroform/isoamyl-alchohol at a ratio of 25:24:1 was added and this reaction mixture centrifuged at the 14000*g* for 2min. This spinning separated the reaction mixture into two layers.

Figure 2.3. Summary of the transfection experiment using P-1/pGL3 and deletion constructs

This figure shows the strategy adopted to measure the activity of the P-1 promoter and its deletion mutants and point mutants. The mutants were fused with the pGL3 basic vector between its HindIII and Xho-1 sites and the P-1/pGL-3 and its mutant plasmids were then transfected into MCF-7 cells using Lipofectamine 2000 reagent. transfectants were analyzed for Luciferase activity 24 hr post transfection.

Figure 2.3


Xho I
Hind III
P-1 promoter
Xho I
Hind III
Luciferase
pGL3
ligate
Xho I
Hind III
P-1/pGL3
Transfect MCF-7 cell line, using
Lipofectamine 2000

The top aqueous layer containing the DNA was transferred to a new eppendorf tube and 10% of ammonium acetate (1M ammonium acetate pH 7.5) and two volumes of 95% ethanol were added. This mixture was chilled in –80°C for 2hr to overnight followed by centrifugation for 15min at 14000*g*. The supernatant was discarded and 100µl of 70% ethanol was added to each eppendorf tube, incubated for 15min at –80°C, spun for another 15 min at 14000*g*. The supernatent was discarded, the pellet air dried and dissolved in 100µl of 10mM Tris-HCl, pH 8.

The DNSEK/ pcDNA3 plasmid (generous gift provided by Dr J.R. Woodgett; Princess Margaret Hospital, Toronto, ON) was transfected into MCF-7 cells. Cells grown to 95–100% confluence were serum starved overnight. 12µg of DNA was incubated with 36µl of Lipofectamine 2000 reagent for 20min to allow the formation of DNA–liposome complexes. These complexes were then added to MCF-7 cells. Cells were stimulated with IGF-1 24hr post-transfection and collected 1hr following stimulation to determine the effect of IGF-1. Some cells were collected 24hr after IGF-1 stimulation (48hrs post transfection). These transfectents were then lysed using reporter lysis buffer and subjected to analysis by western immunoblotting.

Luciferase activity of P-1/pGL3 or its mutant constructs (deletion and point mutated) was assessed in 24 well plates. For this purpose, the cells were trypsinized and resuspended in media without antibiotics and 2% serum and approximately $3–5x10^5$ cells were plated in each well. Cells were allowed to recover and grown overnight to more than 95% confluence. 2µg of each of P-1/pGL3 constructs and 3µg of pSV-β-galactosidase control vector was mixed with 3µg of Lipofectamine 2000 reagent and incubated in room temperature for 20min to allow the formation of DNA–liposome complexes. These complexes were added to MCF-7 cells. Cells were trypsinized for 10min and collected in eppendorfs tubes 24hr post transfection. The cell pellets were frozen at –80°C and luciferase and β-galactosidase assays were done 24hr post transfection

using the luciferase assay system (Promega Corporation) and the β-galactosidase enzyme assay system (Promega Corporation) respectively according to the manufacturer's suggestions.

To evaluate the effect of inhibitors on luciferase activity driven by the promoter, cells were treated with the inhibitors 5hr before each transfection.

To examine the effect of AP-4 and RFX-1 on luciferase expression, serum starved cells were counted, plated in 6 well plates, allowed to grow to 95% confluence, and transfection was performed with 150nM of antisense to AP-4 and RFX-1 using oligofectamine. The media was changed 6hr and 24hr post antisense transfection. 24hr after transfection with the antisense oligonucleotides, these antisense treated cells were transfected with the P-1/ P1D2 luciferase constructs using Lipofectamine 2000 reagent (figure 2.4).

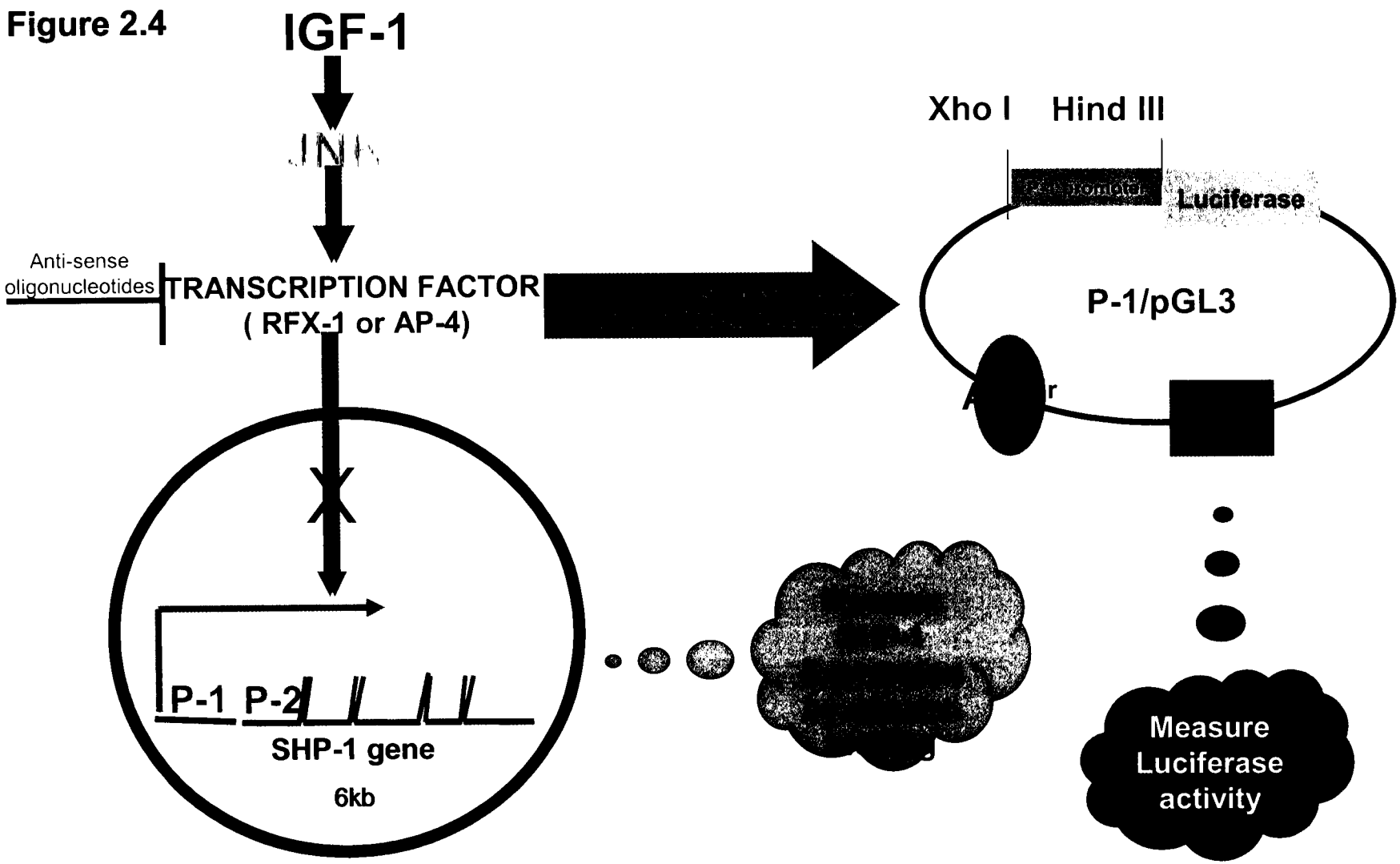
❖ **PREPARATION OF NUCLEAR EXTRACTS:**

MCF-7 cells ($3x10^6$) were trypsinized, collected and resuspended in 1ml of cold PBS. Cell signalling inhibitors were added to the cell suspensions in a dose dependent manner and incubated for 1hr at 37°C. Cells were subjected to stimulation by IGF-1 for 30min, 1hr and 4hr following inhibitor treatment. The cells were centrifuged and the supernatant discarded. Cells were then washed in cold working buffer A (10mM HEPES, 10mM KCl, 1.5mM $MgCl_2$, 0.5mM, 0.5mM PMSF), re-suspended in 70µl of working buffer A supplemented with 0.1% NP- 40 and kept on ice for 10min. These cells were centrifuged at 20,000*g* for 10min and the supernatant consisting of the cytoplasmic protein was removed and saved. The pellet was suspended in 50µl of working buffer B (20mM HEPES, 420mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 25% glycerol, 0.5mM DTT, 0.5mM PMSF, 0.5mM spermidine, 0.15mM spermine, 5µg/ml aprotinin, 5µg/ml leupeptin and 5µg/ml of pepstatin) and kept on ice for 15min followed by centrifugation again at 4°C at 20,000*g* for 10min.

Figure 2.4. Using antisense technology to examine the effect of AP-4 and RFX-1 on SHP-1

This diagram shows two other methods employed in examining the effect of the transcription factors AP-4 and RFX-1 on the SHP-1 P-1 promoter. Here antisense technology was used on both luciferase assay and western immunoblotting. Initially cells transfected with antisense oligonucleotides were further transfected with the P1D2/pGL3 and luciferase assay was measured 24hrs post transfection of P1D2/pGL-3. Alternatively the antisense technology was employed in examining the effect of AP-4 and RFX-1 on transcription of intrinsic SHP-1. For this the cells transfected with antisense oligonucleotides to AP-4 and RFX-1 were lysed and 100μg of total cellular protein was subjected to analysis by western immunoblotting.

Figure 2.4


IGF-1
Anti-sense
oligonucleotides
TRANSCRIPTION FACTOR
(RFX-1 or AP-4)
P-1 P-2
SHP-1 gene
6kb
Xho I
Hind III
Luciferase
P-1/pGL3
Measure
Luciferase
activity

The supernatant consisting of nuclear proteins was stored at -80°C in an equal volume of buffer C (20mM HEPES, 50mM KCl, 0.2mM EDTA, 20% glycerol, 0.5mM DTT, 0.5mM PMSF).

❖ ELECTROPHORETIC MOBILITY SHIFT ASSAYS (EMSA):

The SHP-1 P-1 promoter was subjected to analysis by the Genomatrix MatInspector software, which analyzed the promoter for potential transcription factor binding sites and also provided information as to the expression of these transcription factors in different human cells. 20-50bp oligonucleotide pairs (Table-2) were derived from the P-1 promoter sequence based on the presence of consensus sequences for key transcription factors. Individual complementary strands were synthesized by the University Core DNA services, University of Calgary (Calgary, AB), and were purified by HPLC/gel electrophoresis. Primers were first reconstituted in annealing buffer (10mM Tris-HCl pH7.5–8, 50mM NaCl, 1mM EDTA) and biotin labelled at their 3' end using the *Biotin 3' end labelling kit* (Pierce Biotechnology, Rockford, IL). These biotinylated oligonucleotides were annealed with their complementary strands. For this, the two strands were mixed, heated to 95°C, and cooled at the rate of 0.1°C/sec until 10°C.

Electrophoretic mobility shift assay (EMSA) was conducted using *Lightshift chemiluminescent EMSA kit* (Pierce). Briefly, approximately 23fMs of biotin labelled DNA probes were mixed with 5μg of nuclear proteins for 20min at room temperature. The reaction mixture consisted of 1-5μg of sonicated salmon sperm DNA (Sigma Aldrich), Poly (dA).Poly (dT) (Amersham Biosciences, Montreal, QU), or poly (dA-dC).poly (dG-dT) (Amersham Biosciences) or a mixture of these poly-nucleotides depending on the GC content of the labelled DNA probe. The cold competitor was a 200X excess of the unlabelled DNA probe. The DNA–protein complexes were resolved on a 5% TBE (IX TBE buffer is 10.8gm of tris base, 5.5gm

boric acid and 0.93gm of Na4EDTA) polyacrylamide gel, transferred to nylon membrane, and DNA was cross-linked by a Stratagene cross-linker. The membrane was then blocked in blocking buffer for 15min, blocking buffer supplemented with *Streptavidin-HRP*, for 15min in the dark, washed 4 times for 5min with washing buffer, once for 5min with equilibration buffer and finally once for 5min with the working solution. The kit supplied the buffers and solutions. The blots were detected by exposing the membrane to an X-ray film (Kodak) for 5min to overnight. In case of high background the film was washed using the *Erase it background elimination kit (*Pierce).

Alternatively, the oligonucleotide probes were annealed as described above, 5' end labelled with γP32 ATP using T4-polynucleotide kinase. 200ng of the probe was incubated with 5μg of nuclear extract and poly (dI)poly (dC) and run on a 5%Tris-Glycine native gel. The gel was dried and exposed overnight to an X-ray film (Kodak) at –80°C.

For gel super-shift assay the reaction mixture consisted of 10mM Tris–HCl pH 7.5, 50mM NaCl, 1mM DTT, 1mM EDTA, 5% glycerol, 100mM KCl, 1μg of poly (dG-dT), 1μg of salmon sperm DNA. It was mixed with the supershift antibody for 2hr at 4°C before addition of labelled DNA oligonucleotide probes.

Chapter 3: RESULTS

3.1 INHIBITION OF JNK INCREASED PROLIFERATION OF A SERUM/ IGF-1 STIMULATED MCF-7 BREAST ADENOCARCINOMA CELL LINE IN A DOSE DEPENDENT MANNER

3.1.1 IGF-1 increases proliferation of breast cancer cells

IGF-1 is a multifunctional peptide that modulates cell proliferation, differentiation and apoptosis, factors that are important in tumorigenesis. It has been well established that IGF-1 increases proliferation of breast cancer cells. We therefore first confirmed this effect of IGF-1 on breast cancer cell proliferation employing the human breast cancer cell line MCF-7 as our model system. To accomplish this, serum-starved MCF-7 cells were stimulated with 5ng/ml of IGF-1 and allowed to grow for 4 days. The MCF-7 cells when stimulated with 5ng/ml of IGF-1 proliferated more than their unstimulated counterparts (figure 3.1).

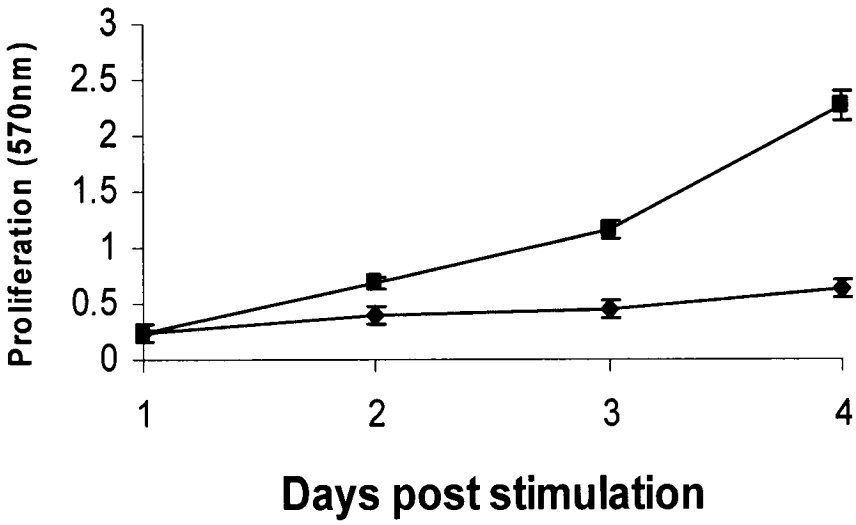
3.1.2 Effect of inhibition of JNK activation on MCF-7 cell proliferation

IGF-1 stimulates three different MAPKs which play a major role in IGF-1 and serum mediated induction of mitogenic signals (Stergios et al, 2002). To understand the distinctive roles of MAPK in IGF-1 stimulated breast cancer cell proliferation specific inhibitors to MAPK family members were employed. Of the MAPKs, the stress activated MAPK JNK has been documented to be activated in adenocarcinomas, and associated with an anti-mitogenic response (Lin et al, 2002 and Wilson et al, 1996).

FIGURE 3.1: IGF-1 induces cell proliferation

The rate of proliferation of MCF-7, a breast adenocarcinoma cell line, left untreated or treated with 5ng/ml of IGF-1 in a time course manner. 5000 cells/well were plated in 96 well flat bottom plates and proliferation was measured using the cell titre 96 well cell proliferation kits at 570nm with a ELISA microtitre plate reader. This is an average of four experiments done in duplicate.

3.1



t-Test: Two-Sample

	-IGF-1	*+IGF-1*
Mean	0.15275	1.1612
Variance	0.002751	0.120178
Observations	4	4
Hypothesized Mean Difference	0	
df	4	
P(T<=t) one-tail	0.001519	
t Critical one-tail	2.131846	
P(T<=t) two-tail	0.003037	
t Critical two-tail	2.776451	

To investigate the role of JNK in MCF-7 cells, the JNK specific inhibitor SP600125 was utilized. SP600125, an anthra-pyrazoline derivative and competitive JNK inhibitor, inhibits JNK dependent phosphorylation of c-jun by binding to the activation pocket of JNK (Dudley et al, 1995). To determine the activity of SP600125, serum stimulated MCF-7 cells were subjected to 5, 10 and 25μM of SP600125 overnight. Cells were lysed, and the activity of JNK was assayed by western immunoblotting using anti-pJNK (αpJNK) antibody and protein lysate (figure 3.2A). Results show that the level of JNK2 phosphorylation was higher than that of JNK1 in MCF-7 cells that were not treated with the inhibitor and taken as positive control (lane-1 panel A figure 3.2A). JNK2 phosphorylation decreased in cells exposed to 5 and 10μM concentration of SP600125 (panel A lane-2 and lane-3 figure 3.2A) in a dose dependent manner and was abolished at a 25μM concentration of SP600125. On the other hand, the JNK1 phosphorylation increased in a dose dependent manner in 0, 5 and 10μM concentration of SP600125. This increase in JNK1 activity most likely compensated for loss of JNK2. However at higher concentration of SP600125 namely at 15μM (data not shown) and 25μM concentration JNK1 phosphorylation (lane-4, panel A, figure 3.2A) was also abolished. Equal loading of protein was confirmed when the same blot was re-probed with α–JNK1 antibody (panel B figure 3.2A).

3.1.3 Stimulation of JNK activation in serum starved MCF-7 cells by IGF-1

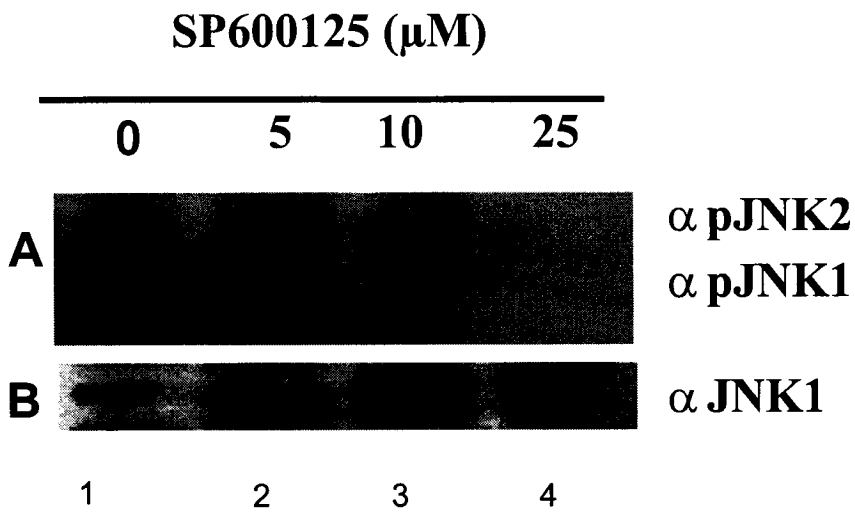
Stimulation of MCF-7 cells with 5ng/ml of IGF-1 increases JNK phosphorylation (lane-2 figure 3.2B panel A). This JNK activation was lowered when the same cells were treated with SP600125 (lane-4 figure 3.2B, panel A). Unstimulated cells treated with the inhibitor had even lower levels of JNK activation (lane-3 figure 3.2B, panel A).

FIGURE 3.2: IGF-1 induces JNK activation

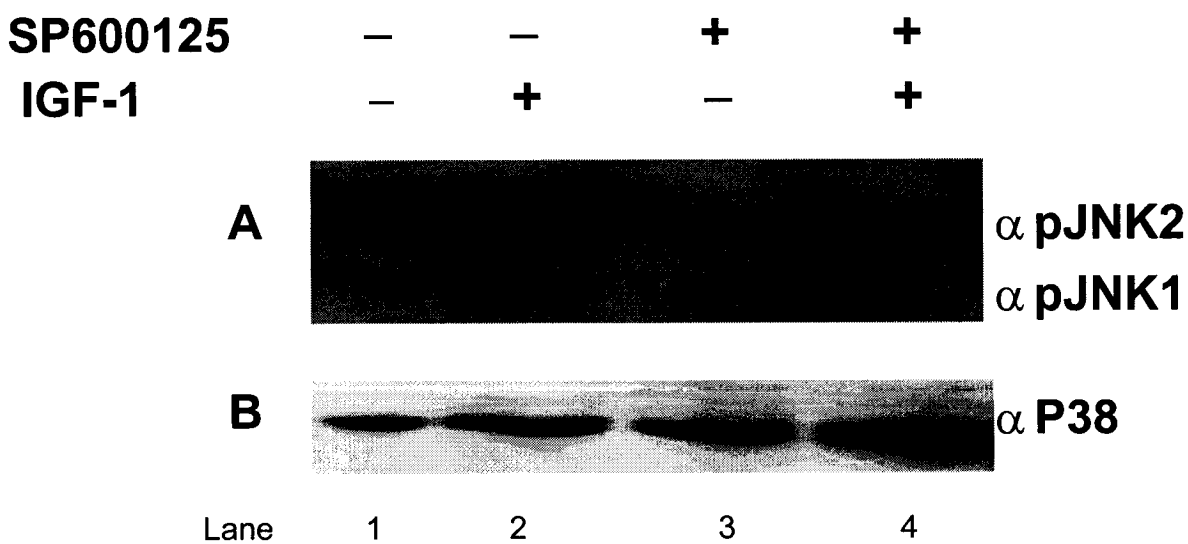
A. 120μg of total cellular protein lysate from MCF-7 breast cancer cells was analyzed by western immunoblotting for the effect of JNK inhibitor SP600125 on JNK1 and JNK2 phosphorylation. Lane-1 represents lysate from serum stimulated MCF- 7 cells. Lane-2, lane-3 and lane- 4 represent protein lysates from MCF-7 cells treated with 5μM, 10μM and 25μM concentration of SP600125 for 24hr, respectively. In (A) the membrane was blotted with pan αpJNK antibody and in (B) the same membrane was blotted with αJNK antibody to confirm equal loading of proteins in all lanes. This experiment was repeated three times.

B. IGF-1 induces MAPK JNK phosphorylation in MCF–7 breast adenocarcinoma cell lines, pre-treated with 25μM concentration of SP600125 for 2hr prior to IGF-1 stimulation (5ng/ml) overnight. Total protein extracts (120μg) were subjected to SDS-PAGE followed by western blot analysis. Lane-1 represents lysate from unstimulated MCF-7 cells, lane-2 lysate from cells stimulated with 5ng/ml of IGF-1, lane-3 lysate from unstimulated MCF-7 cells subjected to 25μM of JNK inhibitor SP600125 and lane- 4 lysate from MCF-7 cells treated with 25μM of SP600125 in addition to IGF-1. In **A** the membrane was blotted with antibody to pJNK whereas in **B** the same membrane was blotted with antibody to P38 to confirm equal loading.This experiment was repeated three times with similar results.

3.2A


SP600125 (μM)
0
5
10
25
A
α pJNK2
α pJNK1
B
α JNK1
Lane
1
2
3
4

3.2B


SP600125
IGF-1
–
–
+
+
–
+
–
+
A
α pJNK2
α pJNK1
B
α P38
Lane
1
2
3
4

Cells not subjected to either SP600125 or IGF-1 (lane-1 figure 3.2B, panel A) were taken as a control. The same gel was re-probed with αP38 antibody to confirm equal loading of proteins in the well (panel B figure 3.2B). P38 protein level is not affected by IGF-1 stimulation and therefore is routinely used in the lab to ensure equal protein loading.

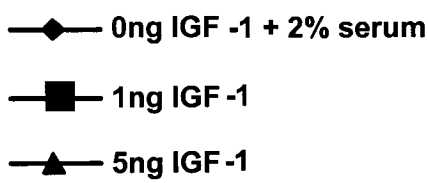
3.1.4 JNK inhibition increased proliferation of IGF-1 stimulated MCF-7 cells

Most breast cancer cells, including MCF-7, express high levels of IGF-1R. IGF-1 stimulation induces cell proliferation and activates JNK. To associate the role of JNK with IGF-1 induced proliferative signals, proliferation in serum starved MCF-7 cells pre-treated with JNK inhibitor SP600125 for 24hr was assessed. In parallel, the level of pJNK in cells treated with the same doses of SP600125 was determined. Results showed that the rate of MCF-7 cell proliferation was highly sensitive to the JNK inhibitor SP600125 where 15μM concentration of SP600125 increased the proliferation by 60% (figure 3.3). Comparison of the rate of MCF-7 proliferation in IGF-1 stimulated cells, pre-treated with different concentration of the JNK inhibitor SP600125 was also performed. IGF-1 stimulation increased the cell proliferation (point-1), and pre-treatment of cells with SP600125 exhibited even higher proliferation following stimulation with either 1 or 5ng/ml of IGF-1 (points 3μM – 30μM). Also, the rate of cell proliferation could be inversely correlated with the level of phosphorylated JNK (inset figure 3.3). This unexpected rise in cell proliferation following SP600125 treatment raised the possibility that IGF-1 activated JNK induces a protein with anti-proliferative effects. In an effort to reveal the nature of the JNK induced protein with anti-proliferative function, expression of PTP SHP-1, a known negative regulator of cell growth was analyzed.

FIGURE 3.3: Inhibition of JNK increases proliferation of serum/IGF-1 stimulated MCF-7 breast adenocarcinoma cell line in a dose dependent manner

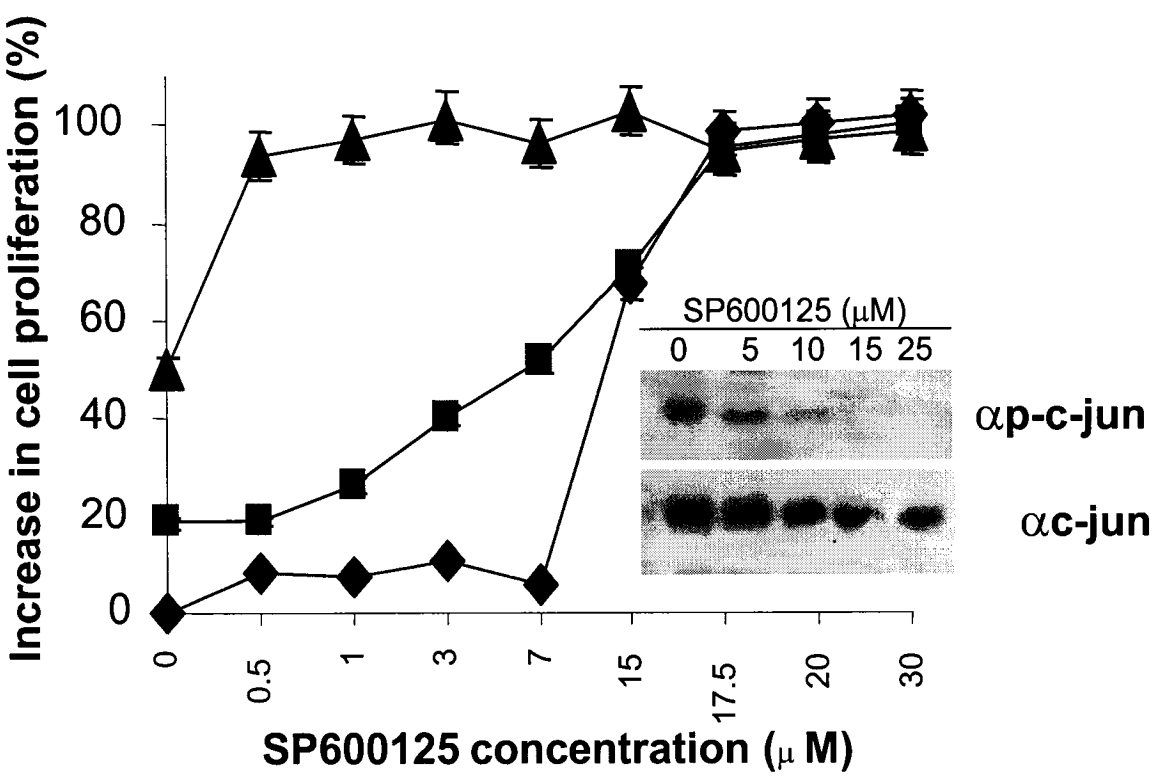
MCF-7 cells were seeded at $7x10^3$ cells/well. Cells were allowed to recover for 24hr. After 24hr cells were treated with 0μM to 30μM of JNK inhibitor SP600125 for 4hr prior to stimulation by 1ng and 5ng of IGF-1. 48hr following IGF-1 stimulation the proliferation was measured employing a non-radioactive cell proliferation kit that uses the MTT dye to measure the conversion of tetrazonium salts to formazan crystals. The formation of formazan crystals in the cell were measured at 570nm using the cell titre microplate reader. The curves represent the percentage of increase in the rate of proliferation, where the highest reading i.e. cells treated with 5ng/ml of IGF-1 and 30mM of SP600125 was taken as 100%. The rest of the readings are the average of increase in cell proliferation compared to the 100%. This graph depicts the results of four experiments done in triplicate.
Rate of proliferation (Y) = average of increase cell number on the day of experiment / cell growth in 0μM on the day of culture (X).

The value of Y for cells treated with 30μM SP600125 +5ng of IGF-1=100% =Y(100)
Reading = {Y /Y(100)} X100%



Inset: Western blot analysis on 120μg of protein lysate from MCF-7 breast cancer cells showing the effect of JNK inhibitor SP600125 on phosphorylation of the JNK target c-jun. Lane-1 represents lysate from IGF-1 stimulated MCF- 7 cells; lane-2, lane-3, lane-4, and lane-5 MCF-7 cells treated with 5μM, 10μM, 15μM and 25μM concentration of SP600125 for 24hr respectively. In the upper panel the membrane was blotted with αp-c-jun antibody and in the lower panel the same membrane was stripped and blotted again with α c-jun antibody to confirm equal loading of proteins in all lanes. The blot was reproduced four additional times in parallel with the proliferation experiment.

3.3


Increase in cell proliferation (%)
100
80
60
40
20
0
0
0.5
1
3
7
15
17.5
20
30
SP600125 concentration (μ M)
SP600125 (μM)
0 5 10 15 25
αp-c-jun
αc-jun

3.2 ENHANCED EXPRESSION OF SHP-1 IN EPITHELIAL TUMORS

It is well established that SHP-1 is expressed at high levels in hematopoietic cells and to a much lower level in non-hematopoietic cells including epithelial and neuronal cells (Lorenz et al, 1996). However, the expression levels of SHP-1 are altered in a number of human tumors suggesting SHP-1's role in tumor growth, an observation consistent with a potential function of SHP-1 as a tumor suppressor. For example, SHP-1 expression is markedly diminished in hematopoietic tumors (Oka et al, 2001). In contrast, SHP-1 expression is up regulated in some epithelial tumors such as breast and ovarian cancers (Wu et al, 2003). To confirm these observations and to get an insight into the involvement of SHP-1 in epithelial tumors, the expression of SHP-1 transcripts in a panel of normal and epithelial tumor samples of breast, uterine, fallopian and ovarian origin was analyzed (figure 3.4). Results verified earlier observations and further demonstrated that the levels of SHP-1 are highly up regulated in adenocarcinoma tumor samples when compared to normal tissues. These observations suggested that SHP-1 expression was modulated in cancer cells, and up-regulated in adenocarcinomas.

3.3 DEPENDENCE OF SHP-1 EXPRESSION ON JNK ACTIVATION

3.3.1 JNK inhibitor SP600125 decreased SHP-1 expression in a dose dependent manner

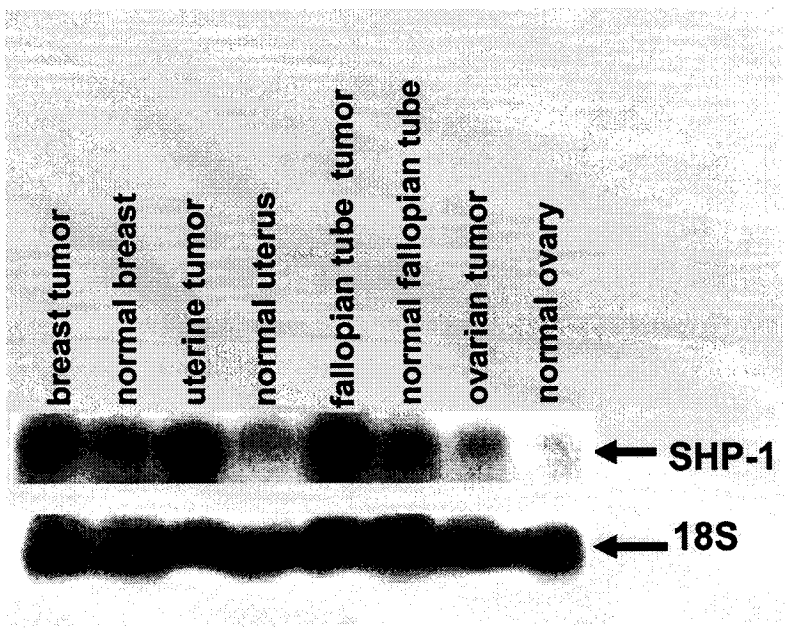
SHP-1 has been implicated in the termination of proliferative signals. Analysis of breast tumor samples suggested that in cancer, the stress-activated kinase JNK has an anti-proliferative (Mamay et al, 2003) effect attributed to the activation of transcription factors that regulate expression of proteins involved in anti-proliferative responses (Lin et al, 2002).

FIGURE 3.4: Epithelial tumors express enhanced level of SHP-1 transcripts.

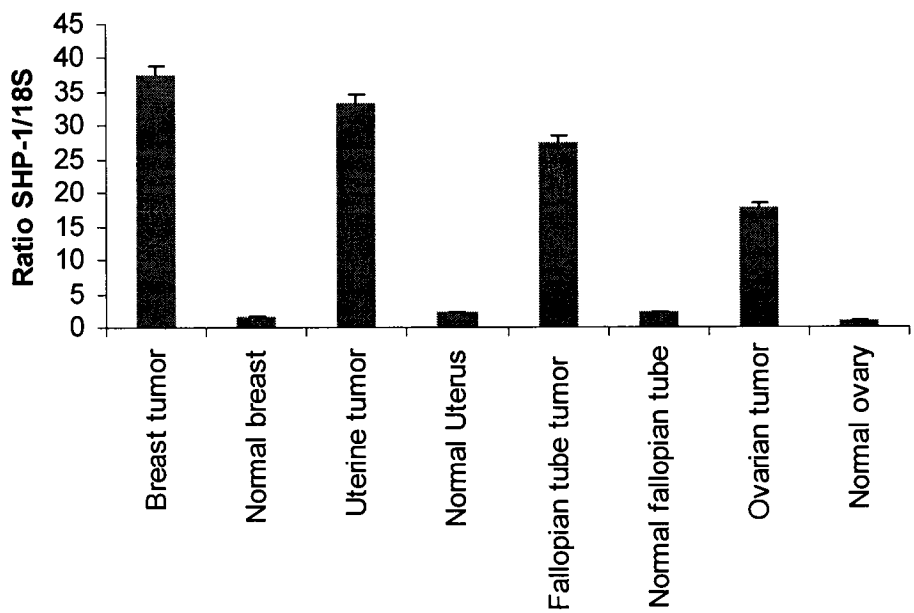
A. Northern blot analysis of the expression of SHP-1 transcripts in normal and tumor samples from human subjects. In the upper blot the membrane was probed with the αP^{32}ATP labelled SHP-1 cDNA to compare SHP-1 expression in these epithelial cells and in the lower blot the same membrane was probed with αP^{32}ATP labelled 18S cDNA to ensure equal RNA loading in all wells. As observed the tumor samples exhibit higher levels of SHP-1 compared to their normal counterparts.

B. This is the densitometry of figure 3.4A. The values are the ratio of SHP-1 mRNA to that of 18S and error bars is a standard deviation in the mean of three densitometry readings of the same blot.

3.4A


breast tumor
normal breast
uterine tumor
normal uterus
fallopian tube tumor
normal fallopian tube
ovarian tumor
normal ovary
SHP-1
18S

3.4B


Ratio SHP-1/18S
45
40
35
30
25
20
15
10
5
0
Breast tumor
Normal breast
Uterine tumor
Normal Uterus
Fallopian tube tumor
Normal fallopian tube
Ovarian tumor
Normal ovary

It was therefore hypothesized that anti-proliferative effect of JNK was mediated by modulating SHP-1 gene expression in breast cancer cells. To test this hypothesis, SHP-1 expression was analyzed in IGF-1 stimulated cells treated overnight with different doses of SP600125. Western blot analyses exhibited a dose dependent inhibition of SHP-1 expression by SP600125 (figure 3.5, lane-2 – lane-6, upper panel). The level of SHP-1 protein was reduced to almost half in the presence of 10μM concentration of SP600125 (figure 3.5, lane-4, upper panel), and there was almost no protein at 25μM concentration of SP600125 (figure 3.5, lane-6, upper panel). The same blot was re-probed with αp-c-jun antibody, a downstream target of JNK to correlate JNK activity with the level of SHP-1 expression in cells treated with different concentration of SP600125. The level of p-c-jun correlated with the level of SHP-1 protein. The level of p-c-jun was reduced by 50% in the presence of 5μM concentration (figure 3.5, lane-3, middle panel) of SP600125, whereas a 25μM concentration of SP600125 completely inhibited c-jun phosphorylation (figure 3.5, lane-6, middle panel). We ensured equal protein loading by probing the same membrane with αc-jun antibody (figure 3.5, bottom panel).

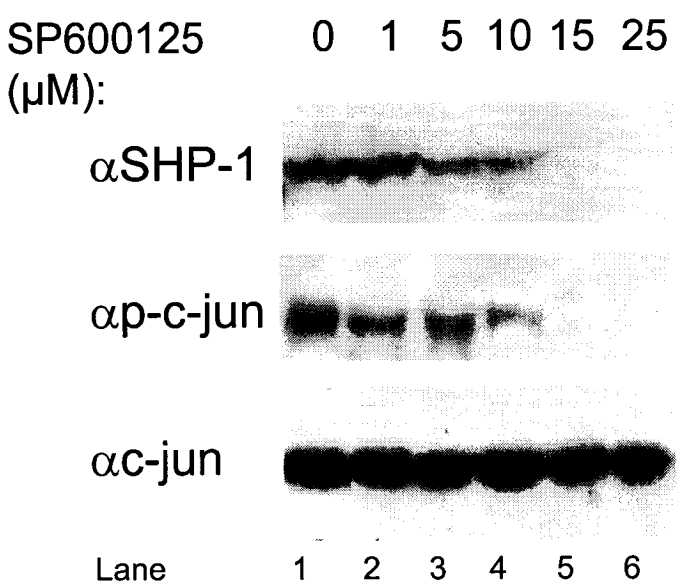
3.3.2 Dependence of SHP-1 activity on JNK phosphorylation in IGF-1 stimulated MCF-7 cells

Previous results correlated decreased SHP-1 expression with increasing doses of the JNK inhibitor SP600125, in IGF-1 stimulated cells. To extend this observation the effect of JNK activation on SHP-1 function was measured. For this, SHP-1 immunoprecipitated from IGF-1 stimulated cells, pre-treated with JNK inhibitor SP600125, was subjected to phosphatase assay using the PTPase assay kit-1 (Upstate Biotechnology).

FIGURE 3.5: JNK inhibitor SP600125 decreases SHP-1 expression in a dose dependent manner.

Serum starved MCF-7 cells stimulated with 5ng/ml of IGF-1 were exposed to the JNK inhibitor SP600125 in the mentioned doses overnight (lane-2 – lane-6) 2hr after IGF-1 stimulation. The effect of JNK inhibition on SHP-1 expression was determined by western immunoblotting using 120μg of total cell lysate. In the upper panel the membrane was incubated with 1μg of polyclonal αSHP-1 antibody custom made in the laboratory. In the middle panel the same blot was probed with αp-c-jun antibody to ensure the integrity of the inhibitor which prevents the activation of c-jun by JNK. In the bottom panel the membrane was blotted with αc-jun antibody to confirm equal loading of protein in all lanes. This experiment is a representative of three experiments with equivalent results.

3.5



The kit uses malachite green stain to colorimetrically determine the amount of free phosphate released by SHP-1 PTP from a tyrosine phospho-peptide. IGF-1 stimulation increased SHP-1 activity by 2.5 fold in IGF-1 stimulated cells as measured by the amount of free phosphate ion (bar-1 versus bar-7, figure 3.6). This increase in SHP-1 activity was reduced in the presence of increasing doses of SP600125 and was almost completely depleted in presence of 25μM of SP600125 (bar-2 to bar-6, figure 3.6) . Here experiments demonstrated that inhibition of JNK in IGF-1 stimulated cells reduced SHP-1 phosphatase activity.

3.3.3 JNK inhibitor SP600125 inhibits SHP-1 expression in a time course manner in IGF-1 stimulated MCF-7 cells

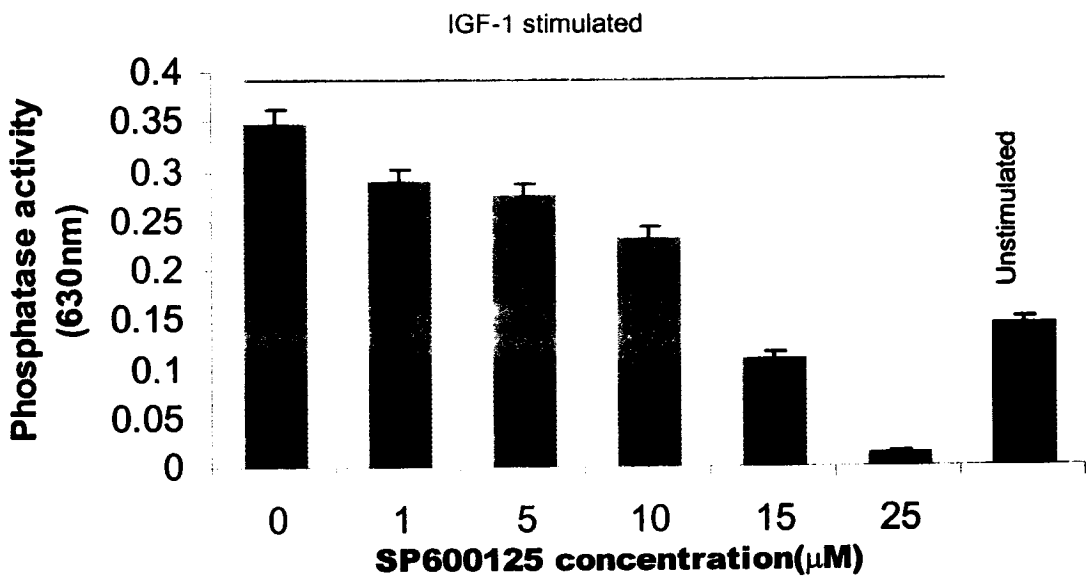
To further demonstrate the dependence of SHP-1 expression on JNK activity, SHP-1 expression in MCF-7 cells treated with 15μM of SP600125 was followed over a 48hr period. Cells pre-treated for 4hr to 48hr with SP600125 were stimulated for 6hr with 5ng/ml of IGF-1. Analysis of SHP-1 expression and JNK activation was conducted by western immunoblotting. IGF-1 stimulation increased SHP-1 expression and JNK activation (lane-2 versus lane-1, figure 3.7). SHP-1 expression and JNK activation were both reduced in a time course manner in the presence of JNK inhibitor SP600125 (lane-4 to lane-8, figure 3.7). The membrane was also blotted with αJNK2 antibody, which confirms equal loading of proteins in all lanes (bottom panel, figure 3.7).

FIGURE 3.6: SHP-1 activity is dependent on JNK phosphorylation in IGF-1 stimulated MCF-7 cells.

A. The phosphatase activity of the SHP-1 protein was colorimetrically measured by malachite green. Serum starved MCF-7 cells stimulated with 5ng/ml of IGF-1 were exposed to 0μM to 25μMs of the JNK inhibitor SP600125 overnight and lysed. 600μg of total cell lysate was immunoprecipitated using 10μg of polyclonal αSHP-1 antibody custom made in our laboratory. The activity of this phosphatase was measured using the PTP assay kit-1. The reading was done at 630nm. The readings were plotted against the SP600125 concentration. Unstimulated cells were taken as negative control. The reading is the average of two separate experiments in duplicate and the error bars represent the standard deviation of readings derived from reading individual wells using the same conditions.

B. Plate showing the phosphatase assay. The top wells A1-A9 and continued B1 through B6 are different dilution of standard provided in the kit. Well C1 has the IGF-1 stimulated cells. Well C2 is the unstimulated control. Well C3 to well C7 is IGF-1 stimulated cells treated with 1μM to 25μM concentration of SP600125. C8 is a negative control.

3.6A


IGF-1 stimulated
Phosphatase activity (630nm)
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Unstimulated
0
1
5
10
15
25
SP600125 concentration(μM)

3.6B

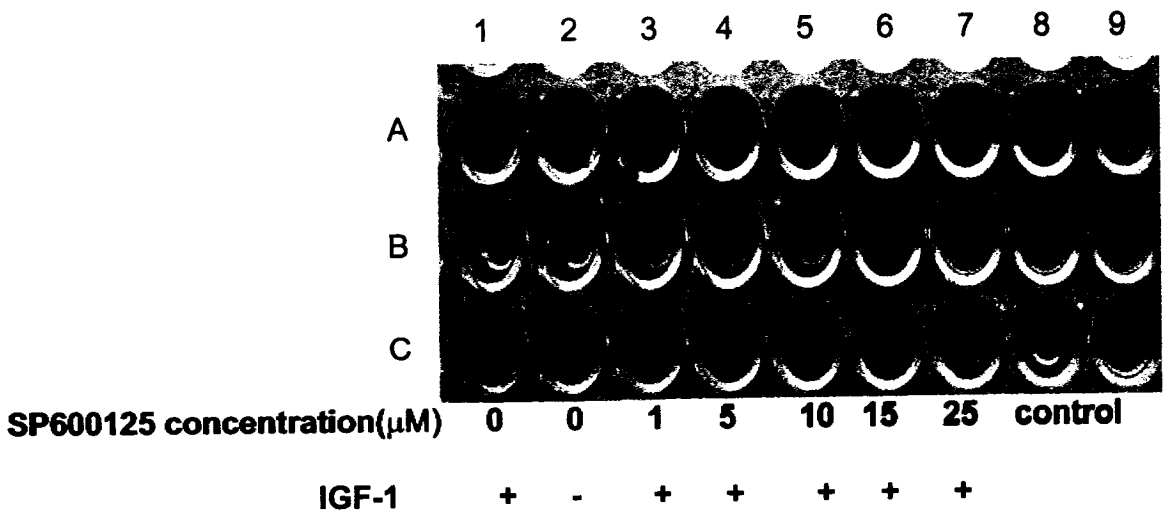
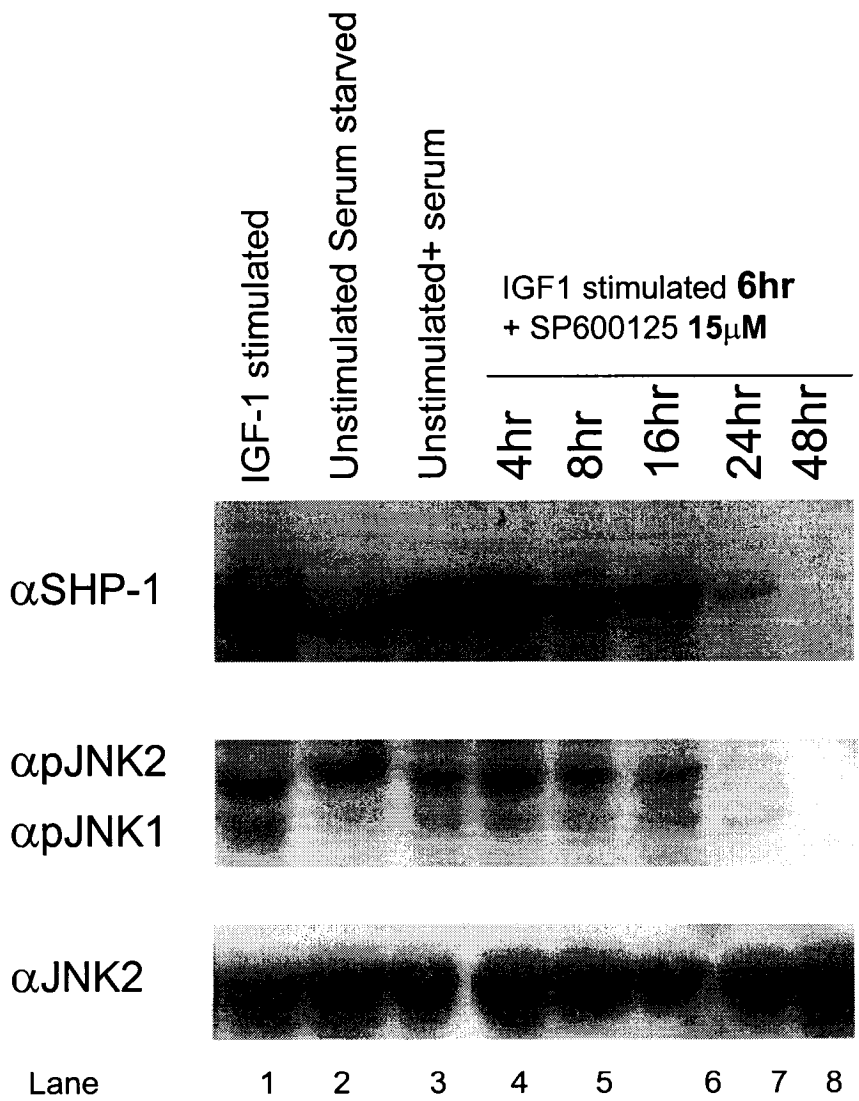

1 2 3 4 5 6 7 8 9
A
B
C
SP600125 concentration(μM) 0 0 1 5 10 15 25 control
IGF-1 + - + + + + +

FIGURE 3.7: JNK inhibitor SP600125 inhibits SHP-1 expression in a time course manner in IGF-1 stimulated MCF-7 cells.

Serum starved MCF-7 cells were exposed to 15μM concentration of JNK inhibitor SP600125 in a time course manner from 4hr to 48hr (lane-4 to lane-8). The cells were stimulated with IGF-1 for 6hr before collecting Unstimulated cells +/– serum (lane-3/lane-2) were taken as negative controls and IGF-1 stimulated serum starved cells (lane-1) were taken as positive controls. A western blot was performed on 120μg of total cell lysate, the membrane probed with αSHP-1 antibody to see the effect of the inhibitor on SHP-1 expression (upper panel) and reblotted with αpJNK antibody (middle panel) to see the effectiveness of the inhibitor on JNK activity. This blot was probed with antibody to JNK2 to confirm equal loading of proteins (lower panel).

3.7


IGF-1 stimulated
Unstimulated Serum starved
Unstimulated+ serum
IGF1 stimulated 6hr
+ SP600125 15μM
4hr
8hr
16hr
24hr
48hr
αSHP-1
αpJNK2
αpJNK1
αJNK2
Lane
1
2
3
4
5
6
7
8

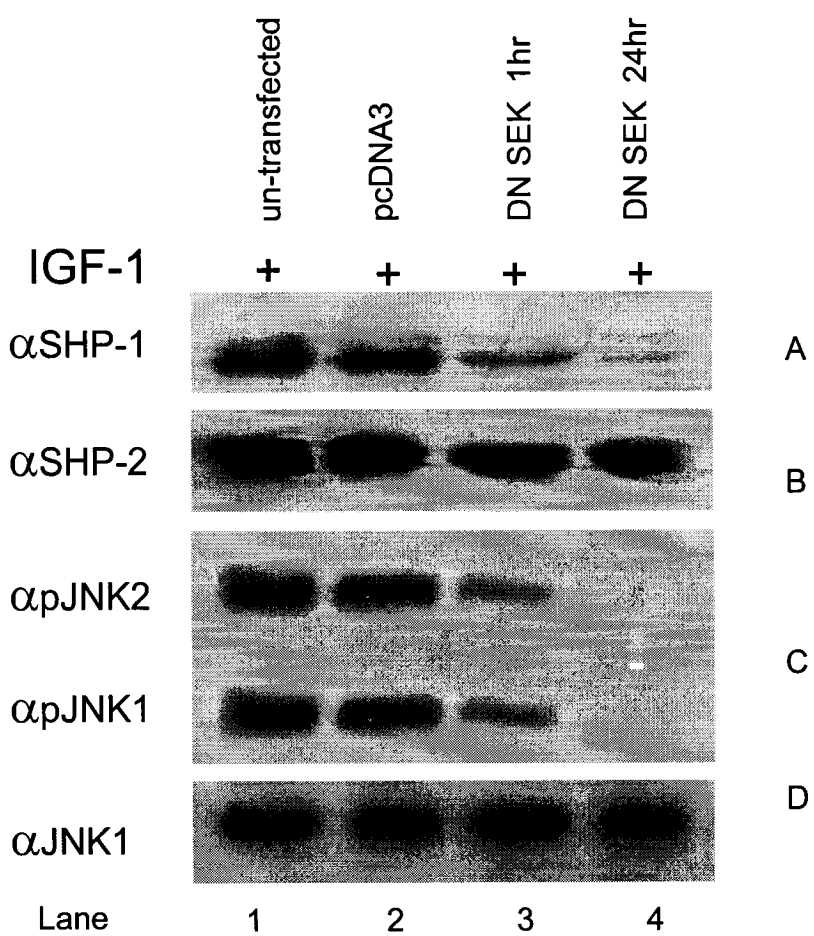
3.3.4 Inhibition of SHP-1 expression by dominant negative SEK (DN SEK)

To further determine the role of MAPK JNK in IGF-1 induced SHP-1 expression, MCF-7 cells were transiently transfected either with a DN SEK or with a control plasmid pcDNA3. Activation of SEK is responsible for the induction of the JNK signaling cascade, and the presence of DN SEK effectively blocks JNK activation (Yao et al, 1997). MCF-7 cells transfected for 24hr with either DN SEK or pcDNA3 were stimulated with IGF-1 and analyzed for JNK phosphorylation and SHP-1 expression by western immunoblotting. IGF-1 stimulation of MCF-7 cells for 1hr and 24hr post transfection with the DN SEK led to reduced JNK phosphorylation compared to cells transfected with pcDNA3 (panel C figure 3.8). IGF-1 stimulation of MCF-7 cells transfected with DN SEK also showed significantly lower expression of SHP-1 compared with that of the IGF-1 stimulated cells transfected with pcDNA3 in a time course manner as shown in panel A of figure 3.8. SHP-1 belongs to a family of two SH-2 domain containing intracellular PTPs with the only other member of this group being SHP-2 or Syp (Feng et al, 1993). SHP-2 was shown to function downstream of IGF-1R and was required for the insulin dependent activation of JNK (Shi et al, 1997 and Tanaka et al, 1996). To test whether JNK inhibition abrogated SHP-2 expression, the same membrane was re-blotted with αSHP–2 antibodies. Data showed that JNK inhibition has no effect on SHP-2 expression, suggesting that MAPK JNK does not play a role in SHP-2 expression in IGF-1 stimulated cells (panel B, figure 3.8). Equal protein loading was further confirmed by blotting the membrane with αJNK1 antibody. The results provide evidence for the regulation of SHP-1 expression by the MAPK JNK in IGF-1 stimulated MCF-7 cells. Since SHP-1 has predominantly a negative effect on cell growth, it was of interest to assess if SHP-1 played a role in IGF-1 induced cell proliferation.

FIGURE 3.8: Dominant negative SEK (DN SEK) plasmid in MCF-7 cells inhibits SHP-1 expression.

This is a western blot of MCF-7 cells stimulated with IGF-1. Cells were transfected with the DN SEK in the eukaryotic expression vector pcDNA3 using Lipofectamine2000 (lane-3 and lane-4). Untransfected cells (lane-1) and cells transfected with the empty pcDNA3 vector (lane-2) were taken as controls to check the effectiveness of the DN SEK construct. Except for lysate in lane–3 which was collected from cells stimulated for 1hr, lysates in lane-1, lane-2 and lane-4 were prepared from cells stimulated with IGF-1 for 24hr. Lystes were collected from cells 24hr post transfection (lane-3). To determine the effect of DN SEK/pcDNA3 plasmid on SHP-1 expression lysate from cells were collected 48hr post transfection (lane-4) and membrane containing 120μg total cellular protein was probed with αSHP-1 antibody (A). The same membrane was re-probed with αpJNK antibody to confirm the effect of the construct in inhibiting JNK phosphorylation (C). Membrane was further re-probed with αJNK1 antibody to check for equal loading in each lane (D) and with αSHP-2 antibody to check the specificity of DN SEK/pcDNA3 (B) and JNK on SHP-1 and not SHP-2 expression.

3.8


un-transfected
pcDNA3
DN SEK 1hr
DN SEK 24hr
IGF-1
+
+
+
+
αSHP-1
αSHP-2
αpJNK2
αpJNK1
αJNK1
A
B
C
D
Lane
1
2
3
4

To address the role of SHP-1 in IGF-1 stimulated cell proliferation, SHP-1 expression was inhibited by transfecting MCF-7 with 170nM of antisense SHP-1 oligonucleotides. The presumed absence of SHP-1 expression resulted in enhanced MCF-7 cell proliferation (figure 3.9). The proliferation of cells transfected with antisense SHP-1 oligonucleotides was higher following stimulation with 10ng/ml concentration of IGF-1 as shown in figure 3.9. These results suggest that SHP-1 expression has a negative impact on IGF-1 induced proliferative signals.

In summary, results above indicate that IGF-1 signaling activates a negative feedback involving JNK and SHP-1 to modulate excessive breast cancer cell proliferation.

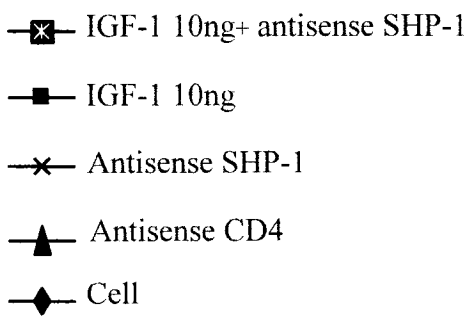
3.4 ASSESSING THE ROLE OF IGF-1 STIMULATED JNK IN EPITHELIAL CANCER CELL PROLIFERATION

3.4.1 Correlating the proliferation of epithelial cancer cells with SHP-1 expression and JNK activation

It was observed that in MCF-7 breast adenocarcinoma cells, an increase in JNK activation decreased IGF-1 stimulated cell proliferation. Furthermore, in IGF-1 stimulated MCF-7 cells proliferation is modulated by JNK by inducing SHP–1 expression. It was therefore of interest to confirm this observation by examining JNK phosphorylation and SHP-1 expression in other cell lines in addition to MCF-7 cells. For this, we compared the proliferation of breast adenocarcinoma ZR-75-1 with that of the pancreatic cell line A431 and further correlated growth of these cells with SHP-1 expression and JNK activation.

FIGURE 3.9: SHP-1 mediates the anti-proliferative role of JNK in IGF-1 stimulated MCF-7 cells.

MCF-7 cells were seeded at $5x10^3$ cells/well in 7 plates. They were allowed to recover for 24hr. Cells were then transfected with 170nM of antisense to SHP–1 using oligofectamine reagent in triplicate. 5hr post transfection media was changed and cells were exposed to 2% serum or 10ng/ml of IGF-1. Plates were incubated in a 37°C CO2 incubator for up to 7 days. Every day a plate was subjected to assay by the cell titre 96 well non-radioactive cell proliferation kit. 15µl of the MTT dye from the 96 well non-radioactive cell proliferation kit was added to all wells. The formation of the formasan crystals was stopped after 4hr by adding the stop solution from the kit. Cell proliferation was measured using the ELISA plate readerat at 570nm



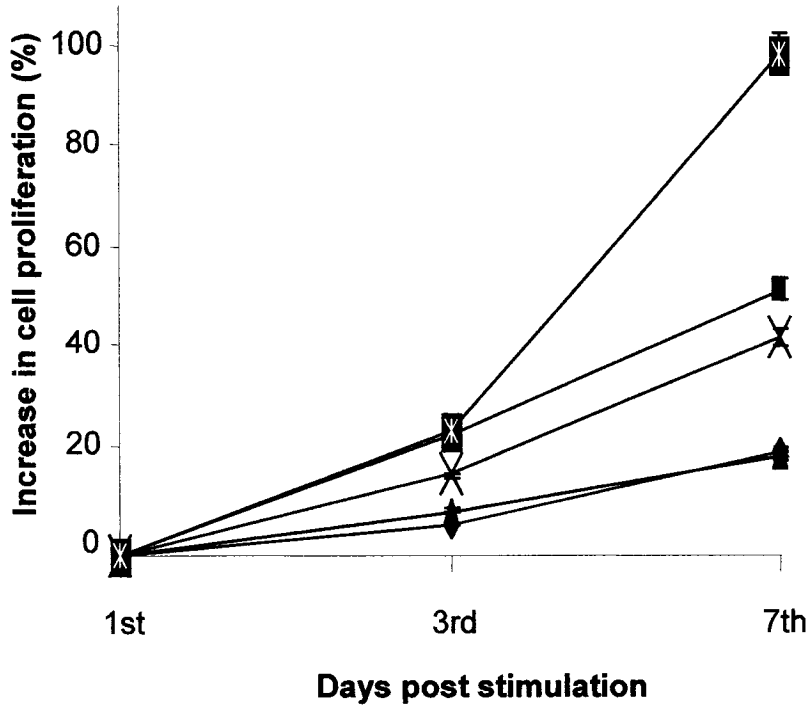
increase in cell proliferation (Y) = (Reading on day X– reading on day 0 for untreated cells)/reading on day0 for untreated cells.

Proliferation of cells transfected with antisense SHP-1 on day 7 was taken as 100%.(X)

Reading = (Y/X) X100%

The graph was plotted with the readings against the days. 2% Serum stimulated cells treated with an irrelevant antisense to CD-4 oligonucleotides, were taken as a control to test specificity of the antisense SHP-1 oligonucleotides. This graph depicts the results of three experiments done in triplicate.

3.9



t-Test: Two-Sample Assuming Unequal Variances

	IGF-1 + antisense SHP-1	*IGF-1*
Mean	1.8235	1.26125
Variance	0.014266	0.093504
Observations	4	4
t Stat	3.425395	
P(T<=t) one-tail	0.013324	
t Critical one-tail	2.131846	
P(T<=t) two-tail	0.026648	
t Critical two-tail	2.776451	

For this purpose, the increase in cell proliferation of ZR-75-1 and A431 cells were followed over a period of 7 days. A431 cells proliferate 3-6 fold higher (figure 3.10A) than ZR-75-1 cells. To correlate these different rates of cell proliferation with expression of SHP-1, total cellular proteins from two cell lines were analyzed by western immunoblotting using αSHP-1 monoclonal antibodies. Results showed that, SHP-1 expression in A431 cells was 10 fold lower than the expression of this phosphatase in ZR-75-1 (upper panel, figure 3.10B). These data suggest a link between cell proliferation and SHP-1 expression, an observation consistent with the negative impact of SHP-1 on mitogenic cascades (figure 3.9). The same blot (middle panel, figure 3.10B) when re-probed with αpJNK antibody demonstrated that the level of JNK phosphorylation correlated with SHP-1 expression in these cell lines. Furthermore, the level of pJNK was higher in ZR-75-1 cells than in A431 cells. The same blot was also re-probed with αJNK1 antibody to ensure equal protein loading (lower panel, figure 3.10B). It should be noted that ZR-75-1 exhibit a two fold increased expression of SHP-1 and activation of JNK compared to that in MCF-7 cells (figure 3.10B). In addition ZR-75-1 cells proliferate at a lower rate compared to MCF-7 cells.

3.4.2 Inhibition of SHP-1 expression and increase of ZR75-1 and A431 proliferation by SP600125

It was shown that MAPK JNK manifests its effect on MCF-7 breast cancer cell proliferation by regulating PTP SHP-1 expression. The results obtained for MCF-7 cells were further emphasized by determining the effect of SP600125 on SHP-1 expression in other cancer cell lines (figure 3.11) and by examining the role of JNK and SHP-1 on proliferation of epithelial cell lines (figure 3.12).

FIGURE 3.10: Proliferation of epithelial cancer cell lines correlates with SHP-1 expression and JNK activation.

A. The rate of proliferation of A431 (an epitheloid carcinoma cell line) and ZR-75-1 (a breast cancer cell line) was determined by cell titre 96 non-radioactive cell proliferation assay kit. Cells were grown in 24 well plates in serum-starved conditions for a period of seven days. Formation of formazan crystals from MTT was measured 24hr after adding the stop solution from the kit at 570nm using the ELISA plate reader. The reading was determined using the formula:

increase in cell proliferation (Y) = (Reading on day X– reading on day 0 for untreated cells)/reading on day0 for untreated cells.

The highest reading indicating maximum cell proliferation was taken as 100%. The graph was plotted with the percentage of readings against the number of days.

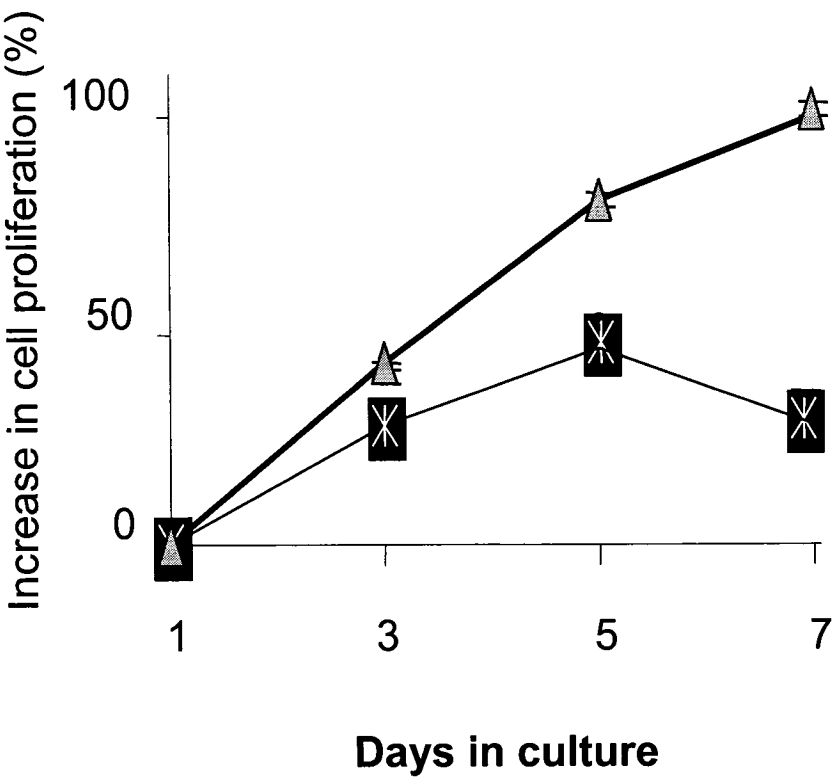
The graph depicts results compiled from three experiments done in triplicates.

B. 10μg of lysate from serum starved A431 and ZR-75-1 cells were run on a 10% SDS-PAGE and then transferred to a PVDF membrane. The membrane was blotted with both αpJNK (middle panel) and αSHP-1 (top panel) to see JNK activation and SHP-1 expression respectively. The membrane was probed with αJNK antibody to confirm equal protein loadings.


A431


ZR-75-1

3.10A


Increase in cell proliferation (%)
100
50
0
1
3
5
7
Days in culture

3.10B

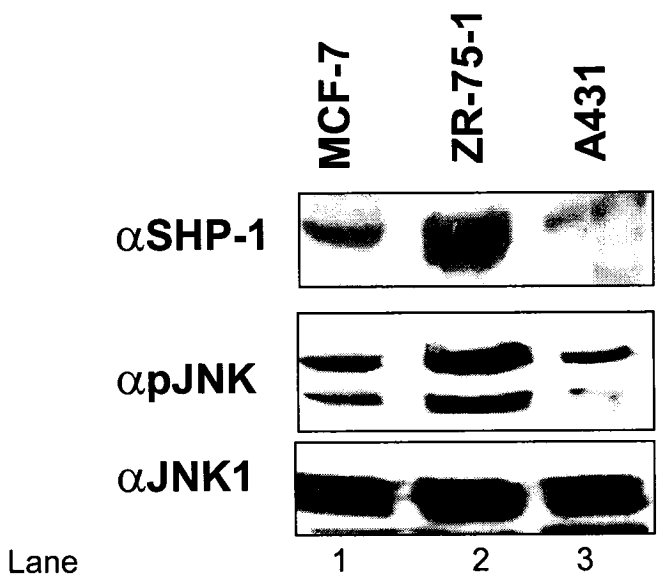
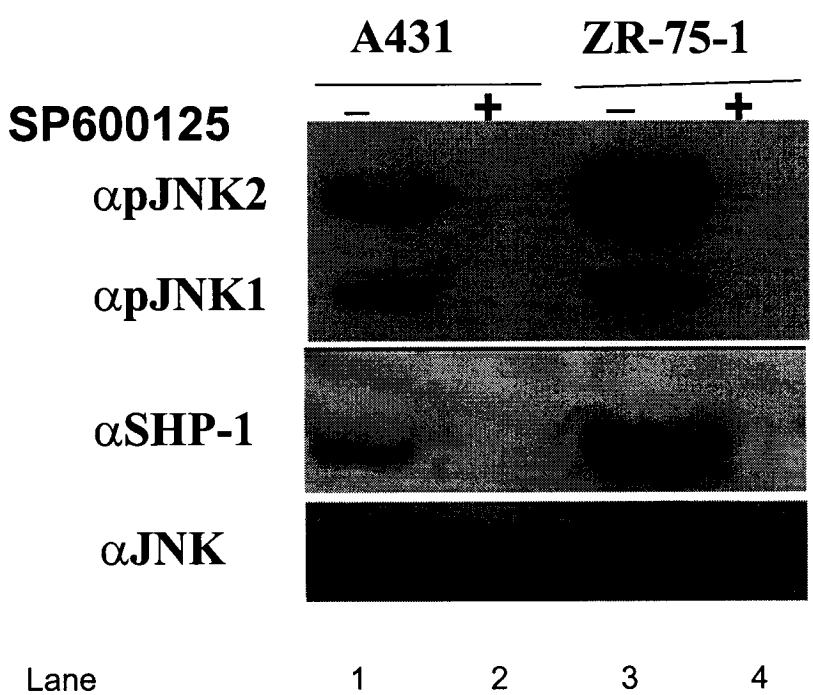

MCF-7
ZR-75-1
A431
αSHP-1
αpJNK
αJNK1
Lane
1
2
3

FIGURE 3.11: SP600125 inhibits SHP-1 expression in ZR-75-1 and A431 epithelial cancer cell lines.

Serum starved A431 and ZR-75-1 cells were treated with 25μM of SP600125 overnight. The cells were lysed and 200μg of protein was run on 10% SDS-PAGE and transferred to a nylon membrane. Lane-2 and lane-4 are from cells treated with SP600125 whereas lane-1 and lane-3 are A431 and ZR-75-1 cells not treated with the inhibitor. The effect of SP600125 on JNK was determined by blotting the membrane with αpJNK antibody (top panel). To see the effect of SP600125 on SHP-1 expression the membrane was blotted with 1μg of polyclonal αSHP-1 antibody custom made in the laboratory (middle panel). The equal JNK expression in all lanes was confirmed by blotting the membrane with antibody to JNK (bottom panel).

3.11



For this purpose, serum starved cells that were both left untreated or treated with 25μM concentration of SP600125 overnight were lysed and 120μg of the lysate were subjected to SDS-PAGE gel followed by analysis by western immunoblotting. Results confirmed that decreased JNK phosphorylation (upper panel, figure 3.11) correlated with diminished SHP-1 expression (middle panel, figure 3.11). Simultaneously, proliferation of ZR-75-1 and A431 cells treated with 0 – 25μM of SP600125 and grown for 9 days and 7 days respectively was determined. There was a several fold increase in cell proliferation in these cell lines, in both dose dependent and time course manner following SP600125 treatment (figure 3.12).

Taken together, these sets of experiments demonstrated that JNK phosphorylation increased SHP-1 expression in IGF-1 or serum stimulated MCF-7 cells and in serum stimulated ZR-75-1 or A431 cells. This increased SHP-1 expression, thereby, led to decreased proliferation of afore-mentioned cell lines.

3.5 ANALYSIS OF P-1 PROMOTER: DISTINCT REGIONS OF THE SHP-1 P-1 PROMOTER ARE DEPENDENT ON JNK ACTIVATION

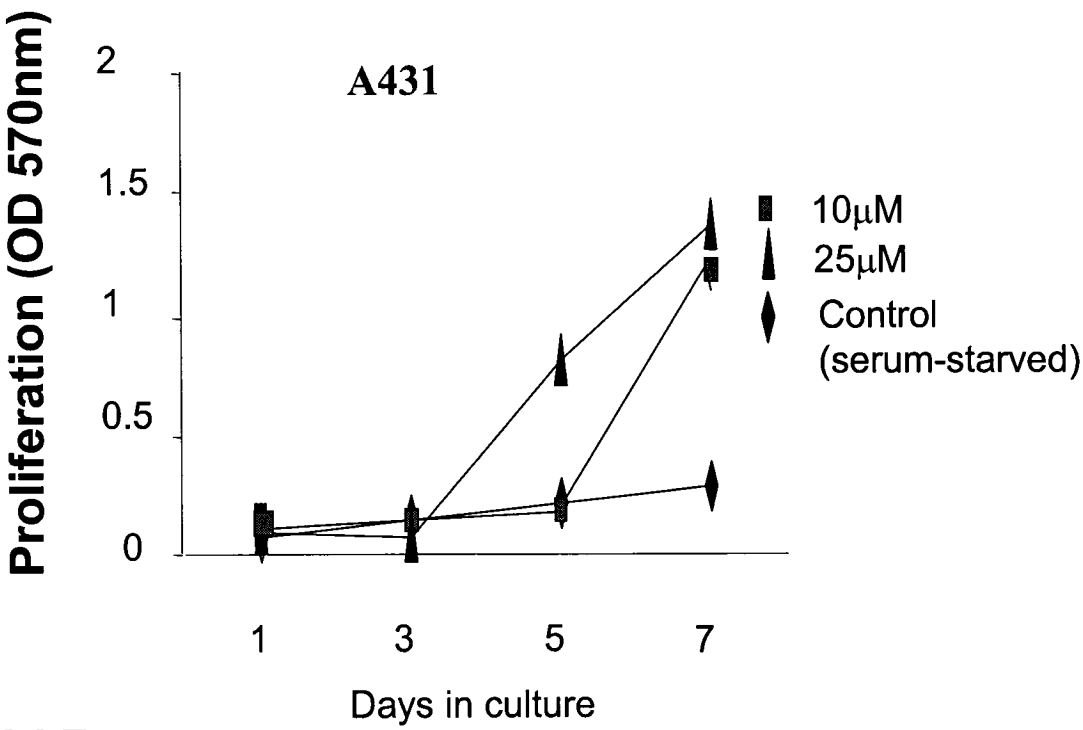
3.5.1 Increase of luciferase reporter gene expression driven by the SHP-1 P-1 promoter and its 5' deletion mutants

Evidence showed that SHP-1 expression was controlled by JNK in IGF-1 stimulated cells. Two tissue specific promoters namely, P-1 promoter and P-2 promoter drive SHP-1 expression. In epithelial cells, including MCF-7, the distal P-1 promoter regulates SHP-1 expression (Banville et al, 1996).

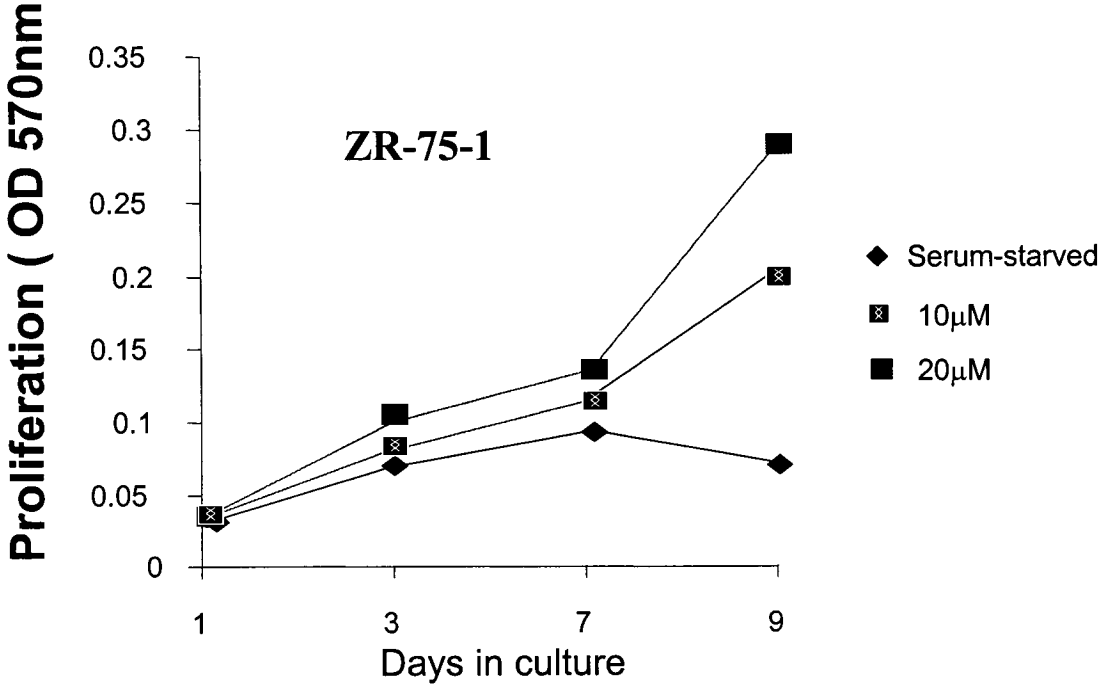
FIGURE 3.12: SP600125 increases proliferation of A431 and ZR-75-1 cells

A431cells (A) and ZR-75-1cells (B) were seeded at $5x10^3$ cells/ well in medium without FBS in 5 plates. They were allowed to recover for 24hr. JNK inhibitor SP600125 was added at a concentration of 10μM and 20μM to ZR-75-1 cells and 10μM and 25μM to A431 cells. Using the cell titre 96 well non-radioactive cell proliferation kit, the proliferation of the A431 cell line was assayed on day 1, day 3, day 5 and day 7 while proliferation of ZR-75-1 cell line was measured on day 1, day 3, day 7, and day 9. Briefly, cells were treated with 15μl of the MTT dye on the day of the assay. The formation of the tetrazonium salts to formazan was stopped after 4hr by adding the stop solution from the kit. Formation of formazan crystals in these cells was measured using the ELISA plate reader at OD 570nm 24hr after adding the stop solution. The reading was plotted against the days to observe increase in cell proliferation. This is a representative of three experiments done in triplicate.

3.12A


A431
Proliferation (OD 570nm)
2
1.5
1
0.5
0
1
3
5
7
Days in culture
10μM
25μM
Control
(serum-starved)

3.12B


ZR-75-1
Proliferation (OD 570nm)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
1
3
7
9
Days in culture
Serum-starved
10μM
20μM

To further investigate the role of JNK in the activation of the SHP-1 gene, a panel of successive 5' deletion mutants was built, spanning every 200bp between –987bp and –207bp of the SHP-1 P-1 promoter sequence. The full-length promoter and the generated deletion mutants were then fused to a luciferase reporter gene in pGL3 basic vector and referred to as P-1/pGL3, P1D1/pGL3, P1D2/pGL3, P1D4/pGL3, and P1D5/pGL3. The capacity of the promoter fragments to drive luciferase expression was assayed in MCF-7 cells. For this, the cells were plated in 24 well plates and transfected with 5μg of β–galactosidase encoding plasmid and 2μg of the pGL3 constructs or the pGL3 control vector, using 2μl of Lipofectamine2000 per construct. Transfected cells were collected 24hr post transfection and were subjected to the luciferase and β-galactosidase assays following the manufacturer's manual. The results were expressed as a ratio of luciferase activity to β-galactosidase activity. It was observed that the luciferase expression driven by the P-1 promoter increased with progressive 5' deletions in the P-1 promoter namely in P1D1 and P1D2, compared to the full-length promoter (figure 3.13). In more extensive deletions (P1D4 and P1D5), the activity of the promoter decreased.

3.5.2 Deletion analysis revealed two regions for high and low level expression of the SHP-1 P-1 promoter in MCF-7 cells

To define the SHP-1 P-1 promoter further, a second set of deletions in the SHP-1 P-1 promoter was made. These deletion mutants spanning every 50-100bp between P-1 and P1D4 (–987 to –417) were generated by PCR. In total, there were six new mutants. Of these six new mutations, there were two new mutations before P1D1 namely, P1D7 (deletion of –987 to –944) and P1D8 (deletion of -987 to -901), two constructs with 5' end located between P1D1 and P1D2 namely, P1D9 (deletion of -987 to -791) and P1D10 (deletion of -987 to -760). Finally, two

mutants after P1D2 were P1D11 and P1D12. These new mutations and the old mutations were also subjected to luciferase assay as described in 3.5.1. This experiment revealed two regions within the SHP-1 P-1 promoter responsible for low and high activity of P-1 in MCF-7. A region between nucleotides –760 and –417 and included in P1D10/pGL3 and P1D4/pGL3 produced progressively higher (3-15 fold higher) luciferase expression compared to the full length P-1 promoter. This region has been referred to as a high-expression region (figure 3.14A and figure14B). A second region of P-1 between nucleotides –987 and –760, and included in P-1, P1D7, P1D8, P1D1 and P1D9; contained genetic elements specifying low levels of SHP-1 in MCF-7 cells. Deletion of this region resulted in markedly increased levels of SHP-1 in MCF-7 cells. This region is referred to as an inhibitory-region (figures 14A and 14B). Since the expression level of SHP-1 was highly elevated in adenocarcinomas, the DNA sequence of the inhibitory-region was determined from genomic DNA of a number of mouse and human breast tumor samples (data not shown). These analyses showed no DNA sequence alterations in these samples. This implied the presence of distinct nuclear factors regulating the activity of the SHP-1 P-1 promoter.

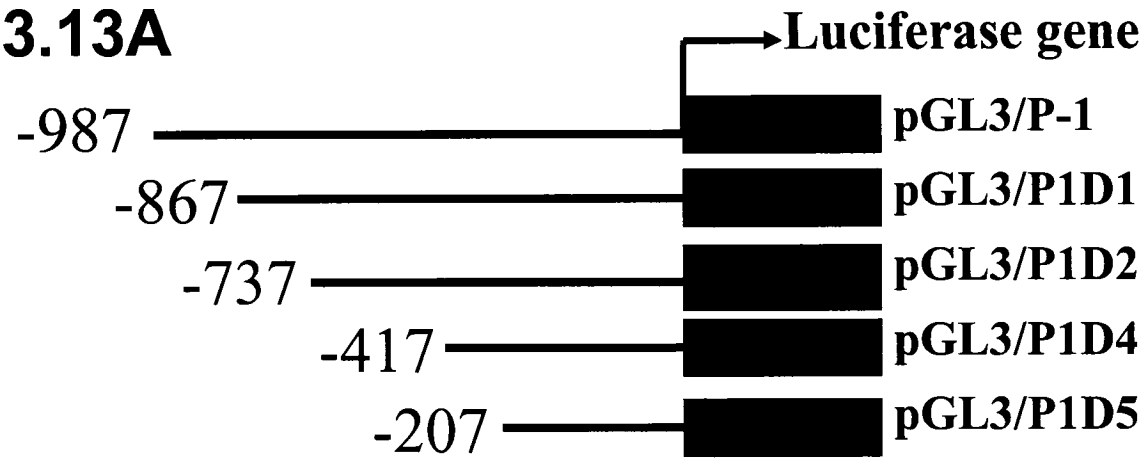
3.5.3 SP600125 inhibits luciferase activity driven by the SHP-1 P-1 and P1D2 promoter in IGF-1 stimulated MCF-7 cells

It was demonstrated that SHP-1 expression was dependent on JNK activation in IGF-1 or serum stimulated MCF-7 cells. The SHP-1 P-1 promoter contains the inhibitory and high-expression regions. Furthermore, SHP-1 protein expression was not constitutive but modulated in different cancers.

FIGURE 3.13: Deletion of 5' DNA sequences from the SHP-1 P-1 promoter increases luciferase gene expression.

SHP-1 promoter and its 5' deletion mutants were synthesized by HF-PCR using different 5' primers and one 3' primer and cloned into the pGL3 basic luciferase reporter vector. The mutants were mapped every 200bp. Expression of the luciferase reporter gene driven by P-1 deletion mutants was measured in MCF-7 cells used as a model system. $3x10^5$ cells were plated in 24 well plates in media with serum but no antibiotics and left overnight to attach and gain 95–100% confluence. 2μg of DNA and 5μg of β–galactosidase vector was incubated with 2μg of Lipofectamine 2000 and transfected into the MCF-7 cells following manufacturer's suggestions. Cells were collected 24hr post transfection and lysed in 50μl of lysis buffer. 20μl lysate was taken for luciferase assay and 20μl was taken for β-galactosidase assay following manufacturer's manual. Dynex luciferase plate reader measured luciferase activity while the ELISA plate reader at OD 405nm measured β-galactosidase activity. The readings are a ratio of the β-galactosidase and luciferase assays and represents an average of two experiments done in duplicate. Cells transfected with the empty vector (basic), with β-galactosidase vector were taken as negative controls. Due to technical difficulties it was impossible to generate a P1D3/pGL3 construct.

3.13A


Luciferase gene
-987
pGL3/P-1
-867
pGL3/P1D1
-737
pGL3/P1D2
-417
pGL3/P1D4
-207
pGL3/P1D5

3.13B

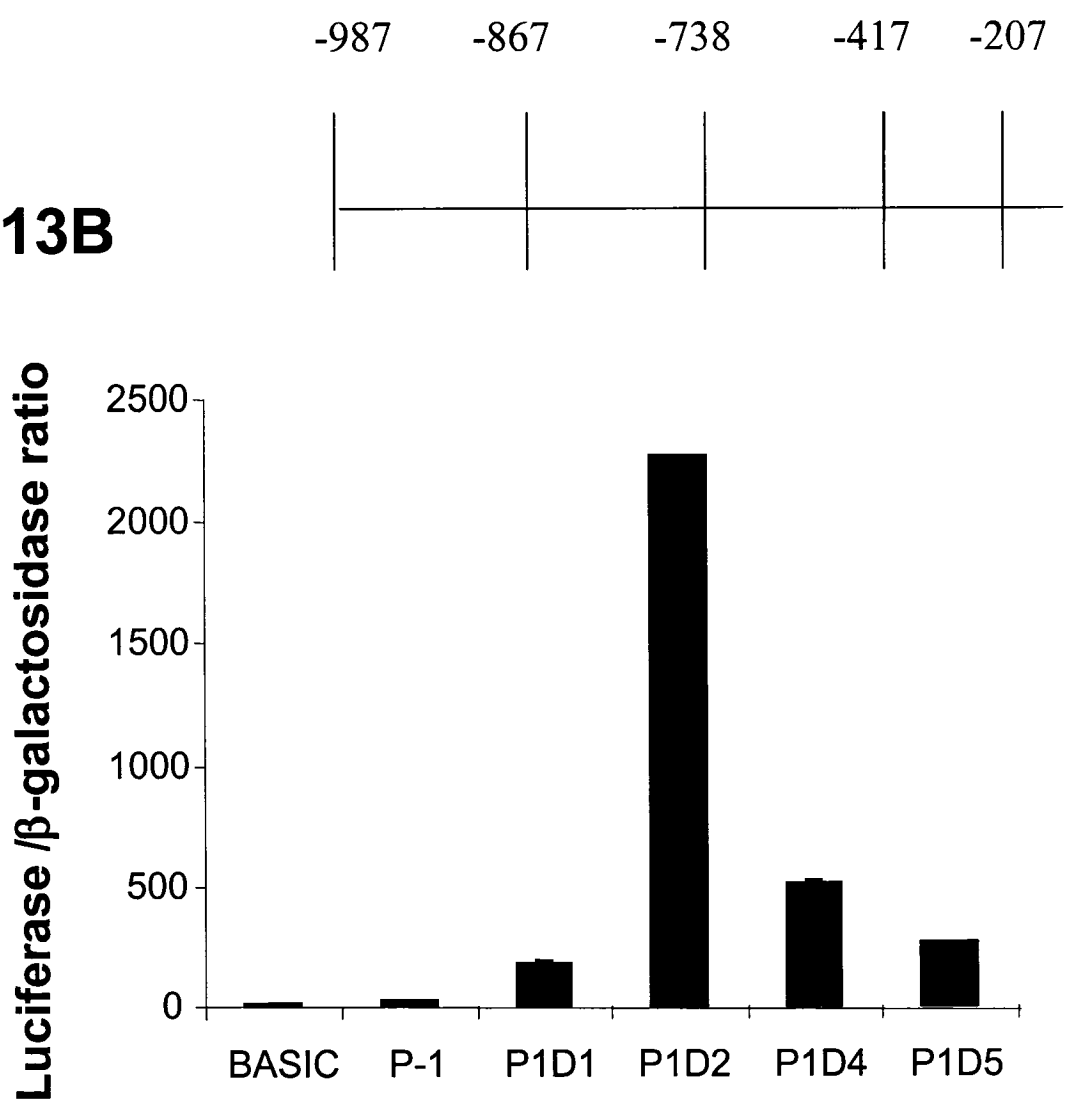
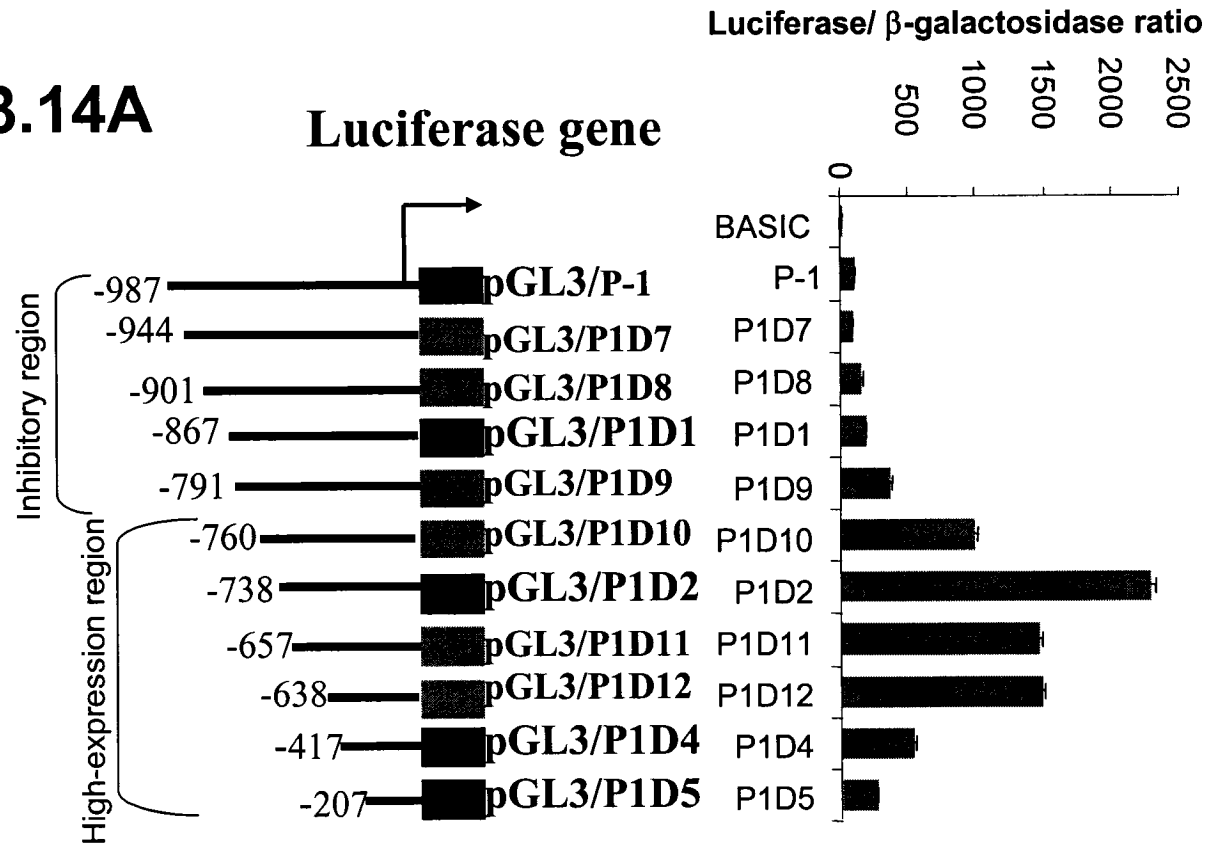

-987
-867
-738
-417
-207
Luciferase /β-galactosidase ratio
2500
2000
1500
1000
500
0
BASIC
P-1
P1D1
P1D2
P1D4
P1D5

FIGURE 3.14: Deletion analysis reveals two regions for high and low level expression of the SHP-1 P-1 promoter in MCF-7 cells.

A. Deletion constructs in the SHP-1 P-1 promoter and included between P-1 and P1D4 pGL3 were made. Each deletion construct was on an average between 50-100bps. There were two deletions before P1D1/pGL3 i.e.P1D7 and P1D8, two deletions before P1D2 i.e. P1D9 and P1D10, and two deletion constructs after P1D2 i.e. P1D11 and P1D12. The TATA box was located between the 386bp-399bp from the 5' end of the promoter. These constructs were transiently co-transfected with β–galactosidase control vector into the MCF-7 cells. Luciferase activity and β–galactosidase activity was measured 24hr post transfection from cell lysate in each condition using the luciferase plate reader and ELISA plate reader at 405nm respectively. The graph represents percentage of ratio of luciferase and the β–galactosidase readings of three experiments done in duplicate.

B. A sequence of the SHP-1 P-1 promoter that shows the two critical regions in the SHP-1 P-1 promoter namely the high-expression region from 230bp to 420 bp and the region (1–230bp) that inhibits this high-expression.

3.14A



3.14B

-987 CCCAGTGTAG ACCAAATGTC ATCATCCAAA CCACACGGGA CAGAGGCCTG(P1D7)
-937 CATGCGTCCT GTTTGGCAAA CAGCTGCCCA GCCAGTGGGG(P1D8) GAGCAGTTCA
-887 TGCTTAAACT ACCACCCCCT CCAGGTGTCT TAGGCACGCT GGTCCTTAGG(P1D1)
-837 AGAAGGGTTG ACCTTCCACT CCCTCTTGCA GGTGTCCTTA AGTTTGCTCG
-787 CTTGGTCAAG(P1D9) TCCTACGAAG CCCAGGATCC

An inhibitory region

-757 TGAGATCGCC(P1D10)AGCCTGTCAG
-737 GCAAGCTGAA GGCGCTGTTT(P1D2)CTGTTGCCCA TGACCCTGCC ACCCCATGGG
-687 C CTGCTGCTG GGCACGGTGG CCGCCTCCTG AGAGTTGGCC(P1D11)CTCCCTTGTG
-637 CCACTGCCAG GGGAGGAAAG GCCTTGAT GT(P1D12)TCCAGACAAT AATAAATGCG
- 587 CCTGTGAC TT AGCCTTGG(P1D3)

A region necessary for high levels of SHP-1 expression in MCF7 cells

-567 TGTCAGTCTC(P1D13) TTGCGGACCT GACAACCCCC

-537 ATCTCTCCTT CCCTGATTCC CTCTCCGTTT CCAGGCCCCA TCCCCCTGAA
-487 CAGCTCCTCC CTATGGTCCT GGCTGGGCCT AACCCTCGCC CAGGGCCTAA
-437 CCCTACCTGA GGCTCCTCCC CTTCCCCCGG GGCAGGTTGA GAGGCTGGAG(P1D4)
-387 TGGGTCCCTC AGCGCCCTGG GTGGGTGGGC CTGCACAGGG GGTACCTCCT
-337 TCTCTGAGGA(P1D14)ACTGGGCTGT TAGGGATTTT CCTTAGGCCC TTTGGTTTCC
-287 GCTACGGAGA GGTTTCCCCC ATTGGTTGCT CTTCCTCAGC CAGGGTTACT
-237 TCCTGGTCTG(P1D5) TTCCCCTACC CAATACCCCG CCGCTCTGTC AGCTTGAGCT
-187 CCAGGTGGAG CTCCAGGTGG CTCCTCCTCT CCCGGGGGAA GGCGGCCCTG
-137 GACCAGCAGG CGGGCCTGCT GTACTCCCGC TTTGGGGCTG CAGGGAAGCT
-87 GGCCGCTGTG GGCGGTCTCG GGCCAGCCCC GCCCCACCTG TCCTTTTCCT
-37 GGAGACTATT AGTCCAGGGT TTGTCCCTGC

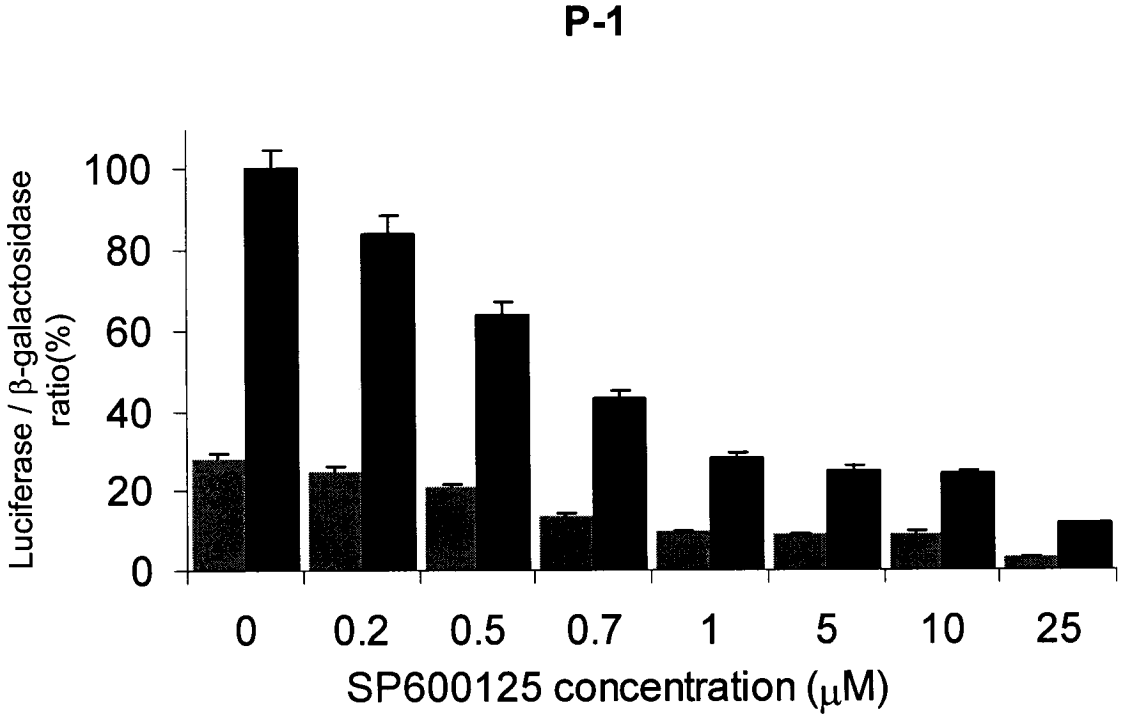
FIGURE 3.15: JNK inhibitor SP600125 inhibits luciferase activity in IGF-1 stimulated MCF-7 cells transfected with P-1/pGL3 and P1D2/pGL3 constructs.

$3x10^5$MCF-7 cells were plated in 24 well plates and left overnight to recover. The media was changed the next day when the cells were exposed to JNK inhibitor SP600125 in 0, 0.7, 1, 5, 10 and 25μM concentration. These cells were co-transfected with P-1/pGL3 construct (3.15A) or P1D2/pGL3 construct (3.15B) together with the β–galactosidase vector. Cells were treated with 5ng/ml of IGF-1 4hr post transfection. The expression of luciferase and β–galactosidase was measured 24hr post transfection using the luciferase plate reader and the ELISA plate reader at 405nm respectively. The graph represents the percentage of the ratio of these two readings in MCF-7 cells +/– IGF-1 and SP600125 and is an average of four separate experiments done in duplicate. Here the highest ratio i.e. that of P-1 and P1D2 was taken as 100%. The error bars are a standard deviation of the mean of reading.

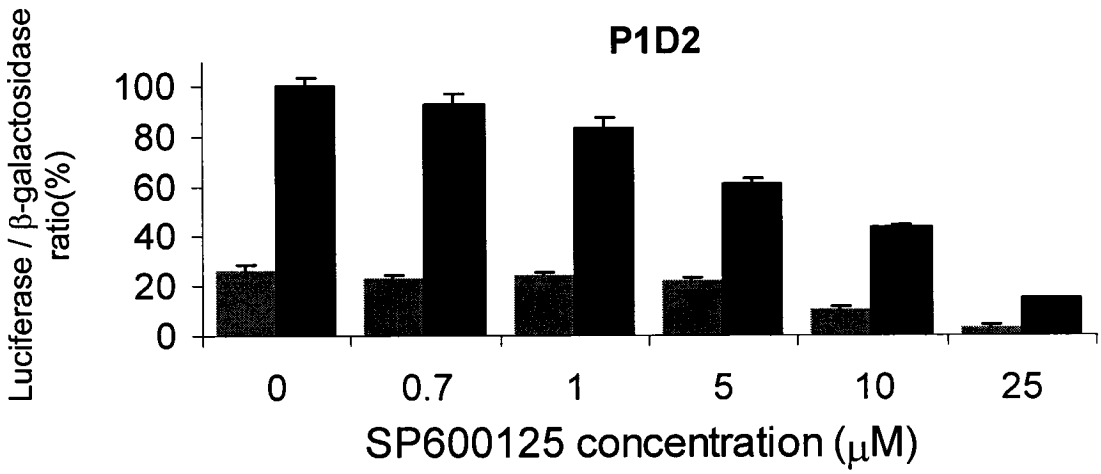

+ IGF-1


–IGF-1 + 10% serum

3.15A


P-1
Luciferase / β-galactosidase ratio(%)
100
80
60
40
20
0
0
0.2
0.5
0.7
1
5
10
25
SP600125 concentration (μM)

3.15B


P1D2
Luciferase / β-galactosidase ratio(%)
100
80
60
40
20
0
0
0.7
1
5
10
25
SP600125 concentration (μM)

Furthermore, since there was no change in nucleotide sequences of the P-1 inhibitory region isolated from a number of breast cancer tumors it was reasonable to suggest that signaling proteins including transcription factors regulate SHP-1 gene transcription through JNK activation in IGF-1 stimulated breast cancer cells. To identify a region of the SHP-1 P-1 promoter which is sensitive to JNK inhibition, the MCF-7 cells were pre-treated in the presence of 2% serum with different concentration of SP600125 ranging from 0μM to 25μM for 5hr. Inhibitor treated cells were transfected with P1D2/pGL3 plasmid (specifies high-expression of luciferase) and with the P-1/pGL3 plasmid (contains the intact P-1 promoter) followed by stimulation with either 5ng of IGF-1 or 10% serum for 6hr. Cells were collected and assayed for luciferase activity 24hr post transfection. These results demonstrated high sensitivity of the SHP-1 P-1 promoter activity towards SP600125. the SHP-1 P-1 promoter exhibited high sensitivity to SP600125 with as low as .2μM decreasing promoter activity. SP600125 inhibited the luciferase expression driven by P-1/pGL3 and P1D2/pGL3 in the MCF-7 cells in a dose dependent manner. 5μM of SP600125 dramatically down regulated the luciferase expression driven by either P-1 (figure 3.15A) or P1D2 (figure 3.15B) in IGF-1 stimulated cells. When cells were treated with 25μM SP600125 luciferase expression from P-1/pGL3 and P1D2/pGL3 constructs was only 10% of cells not treated with SP600125.

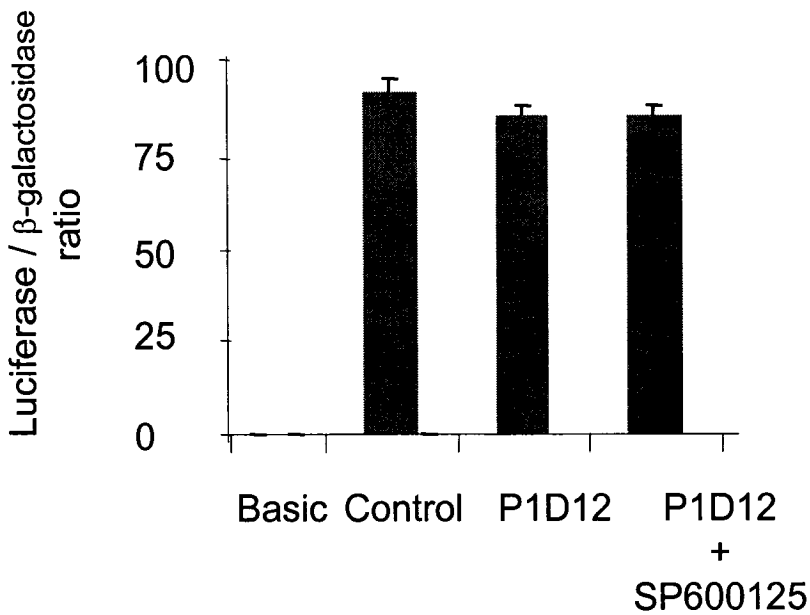
3.5.4 Delineating the JNK sensitive region in the SHP-1 P-1 promoter

JNK inhibitor SP600125 inhibited luciferase activity of P-1/pGL3 and P1D2/pGL3 constructs in IGF-1 stimulated MCF-7 cells. In contrast, SP600125 had no effect on the expression of luciferase protein driven by P1D12 (figure 3.16) thus defining the elements sensitive to JNK inhibition to be located within a 100bp region between nucleotides –738 and –638.

FIGURE 3.16: JNK activated elements in the P-1 promoter lies upstream of P1D12 and TATA box.

Basic consists of co-transfection of the MCF-7 cells with the promoter-less pGL3 basic vector. These three conditions (Cell, Mock and Basic) were taken as negative controls. Cells co-transfected with the pGL3 basic control vector and β–galactosidase vector were taken as positive control (bar *control*). MCF-7 cells transfected with P1D12/pGL3 +/– 25μM SP600125 and with β–galactosidase vector (bar-4/bar-5) were included in this study to demonstrate the JNK sensitive region in the SHP-1 P-1 promoter. The luciferase and the β–galactosidase readings were taken 24hr post transfection. This graph represents an average of four separate experiments done in duplicate and the error bars represent the standard deviation.

3.16


Luciferase / β-galactosidase ratio
100
75
50
25
0
Basic Control
P1D12
P1D12 + SP600125

Furthermore, JNK did not have an effect on the binding of TBP (TATA binding protein) to the TATA box of the promoter. In other words, the JNK sensitive element in the SHP-1 P-1 promoter was located in the high-expression region downstream of the inhibitory-region and upstream of the SHP-1 P-1 promoter TATA box.

Untransfected cells ensured specificity of the reading. Cells transfected with the empty basic vector served as a negative control while the one transfected with the pGL3 basic control plasmid, encoding the CMV promoter served as the positive control. The reading of the negative controls was negligible to the reading of cells transfected with P1D12/pGL3 treated or left untreated with SP600125. The reading from cells transfected with P1D12/pGL3 treated or left untreated with SP600125 was comparable to the pGL3 basic control plasmid. The results suggest that the P1D12/pGL3 construct is not sensitive to JNK (figure 3.16).

3.5.5 Role of other signaling molecules on SHP-1 expression in MCF-7 cells

3.5.5.1 Role of P38 MAPK, ERK MAPK and PI-3K on SHP-1 expression

It has been reported that P38 and ERK MAPKs as well as PI-3K (Dufourny et al, 1997) play a major role in the IGF-1 and other stimuli i.e. cytokine mediated induction of mitogenic signals in breast adenocarcinoma cells (Peruzzi et al, 1999). It was, therefore, of interest to examine a potential role of other signal transduction pathways in the regulation of SHP-1 expression in MCF-7 breast cancer cells. To assess the involvement of PI-3K, P38 and ERK the regulation of P-1/pGL3 in MCF-7 human breast cancer cells was examined using specific kinase inhibitors. For instance, Ly294002 was shown to inhibit PI-3K specifically, whereas SB203509 was shown to inhibit the P38 MAPK, and PD98059 specifically inhibited p42/44 ERK MAPK

signaling (Jeanmarie 1998). To confirm the activity of these inhibitors in our model system, serum starved MCF-7 cells were pre-treated separately with these inhibitors for 4hr followed by serum stimulation overnight and phosphorylation of distinct signaling proteins were visualized by western immunoblotting. Prior treatment of MCF-7 cells with Ly294002, SB203509 and PD98059 resulted in the inhibition of serum-induced phosphorylation of PI-3K (data not shown), P38 (figure 3.19A) and ERK (figure 3.18A), respectively.

To determine the role of these signaling molecules on SHP-1 expression, serum stimulated MCF-7 cells pre-treated with 25μM of either P38 inhibitor SB203509 or the ERK inhibitor PD98059 or PI-3K inhibitor Ly294002 were transfected with P-1/pGL3 (figure 3.17A) or with the P1D2/pGL3 (figure 3.17B) plasmid. Pre-treatment of MCF-7 cells with Ly294002 resulted in a modest insignificant increase in luciferase activity driven by either P-1/pGL3 or P1D2/pGL3. On the other hand, pre-treatment with SB203509 and PD98059 decreased the luciferase activity driven by both P-1/pGL3 and P1D2/pGL3 plasmids (figure 3.17).

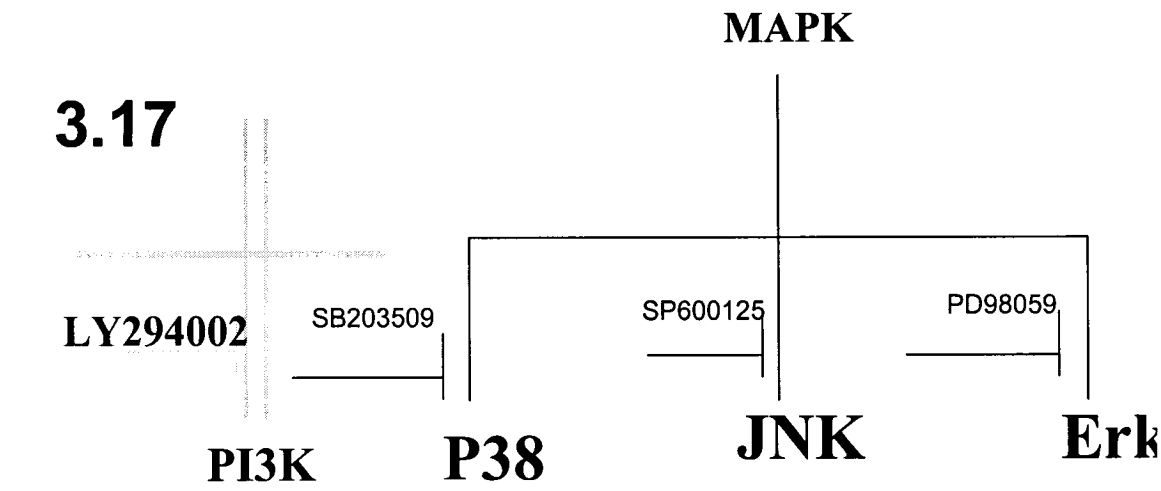
3.5.5.2 Inhibition of ERK activation does not significantly inhibit the luciferase expression driven by P-1/pGL3 and P1D2/pGL3 plasmids

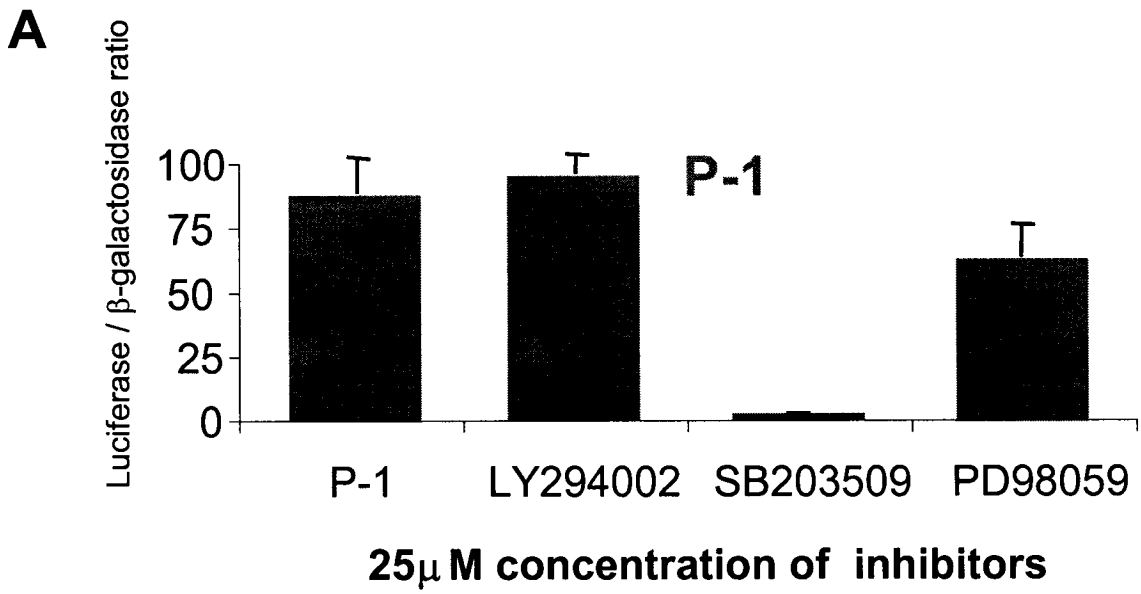
The above observation suggested that ERK and P38 MAPK might act in synergy with MAPK JNK to regulate SHP-1 expression. Alternatively, the high dose of inhibitors used for the assay was toxic to the cells. To better understand the role of ERK in SHP-1 regulation, PD98059 was employed. To assess the effectiveness of PD98059 on phosphorylation of ERK in a dose dependent manner, serum induced MCF-7 cells were subjected to PD98059 inhibitor at 10μM and 25μM concentration for 24hr followed by analysis of ERK phosphorylation by employing monoclonal αpERK antibody with western immunoblotting.

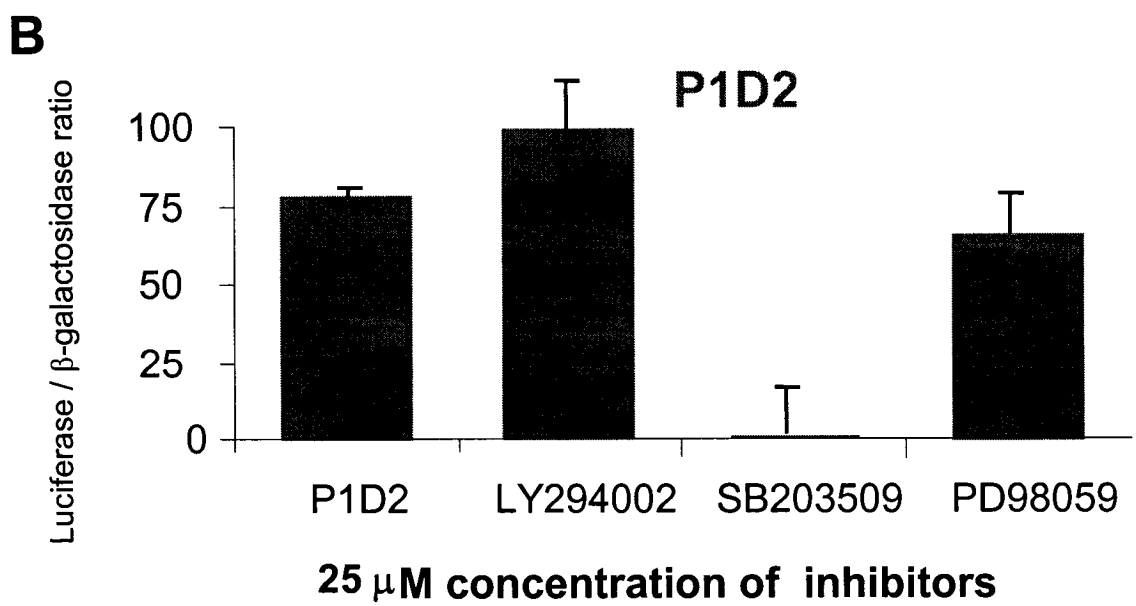
FIGURE 3.17: JNK activates transcription factors binding to the high-expression region in the SHP-1 P-1 promoter in IGF-1 stimulated MCF-7 cells.

Cell signaling inhibitors were checked for toxicity by cell counting. Briefly after checking viability by trypan blue staining cells were plated in 96 well plates with 5000 cells/well. Cells were allowed to recover overnight and thereafter subjected to different doses of the respective inhibitor and incubated at 37C. Cells were counted after 24hr to check viability by trypan blue staining. Then $3x10^5$ MCF-7 cells/well was plated in 24 well plates and left overnight to recover. These cells were subjected to the highest concentration of the inhibitor that was not toxic to the cells. The inhibitors used were PI-3K inhibitor Ly294002 (bar-2), P38 inhibitor SB203509 (bar-3) and ERK inhibitor PD98059 (bar-4). 4hr following inhibitor treatment cells were transfected with β–galactosidase vector and P-1/pGL3 (A) or P1D2/pGL3 (B). Cells were then collected 24hr post transfection, lysed using reporter lysis buffer (provided with the kit) and subjected to luciferase and β–galactosidase enzyme assay kit to determine luciferase and β–galactosidase expression respectively. The readings are the percentage of the ratio of average luciferase and β–galactosidase expression of three experiments.

	P-1	*P1+Ly294002*
Mean	125.0531	152.1871
Observations	3	3
df	4	
t Stat	-6.64645	
P(T<=t) one-tail	0.00133	
t Critical one-tail	2.131846	
P(T<=t) two-tail	0.00266	
t Critical two-tail	2.776451	


MAPK
3.17
LY294002
SB203509
SP600125
PD98059
PI3K
P38
JNK
Erk


A
Luciferase / β-galactosidase ratio
100
75
50
25
0
P-1
P-1
LY294002
SB203509
PD98059
25μM concentration of inhibitors


B
Luciferase / β-galactosidase ratio
P1D2
100
75
50
25
0
P1D2
LY294002
SB203509
PD98059
25 μM concentration of inhibitors

Results showed that PD98059 inhibited the serum stimulated ERK phosphorylation in a dose dependent manner (figure 3.18A).

To understand the role of ERK in SHP-1 expression, serum starved MCF-7 cells were treated with 1, 5, 10 or 25μM concentration of PD98059 for 5hr prior to transfection with P-1/pGL3 or P1D2/pGL3 constructs along with the β–galactosidase encoding plasmid, followed by luciferase and β–galactosidase assays 24hr post transfection. Results show that treatment of MCF-7 cells with increasing concentration of PD98059 inhibited the SHP-1 P-1 promoter activity only partially and at very high concentration namely, at 25μM concentration, (figure 3.18B and figure 3.18C), thus, ruling out ERK as a significant regulator of the SHP-1 P-1 promoter activity.

3.5.5.3 Inhibition of P38 activation inhibits the luciferase expression as driven by the P-1/pGL3 and P1D2/pGL3 at high doses only

Previously, it was demonstrated that SB203509 inhibited the SHP-1 P-1 promoter activity at high doses (25μM) (figure 3.17A and figure 3.17B). To expand this observation the effect of SB203509 on activation of P38 was assessed. For this, serum stimulated MCF-7 cells were pre-treated with 12.5 and 25μM concentration of SB203509 for 24hr followed by analysis of P38 phosphorylation by employing goat polyclonal αpP38 antibodies in western immunoblotting (figure 3.19A). $5x10^5$ cells pre-treated with 5–25μM concentration of SB203509 were transfected with P-1/pGL3 (figure19B) or P1D2/pGL3 (figure 3.19C) construct followed by luciferase assay at 24hr post transfection.P38 activation inhibits the luciferase expression as driven by the P-1/pGL3 and P1D2/pGL3 only at 25μμ concentrations

FIGURE 3.18: Inhibition of ERK activation does not inhibit the luciferase expression derived by the P-1/pGL3 and P1D2/pGL3.

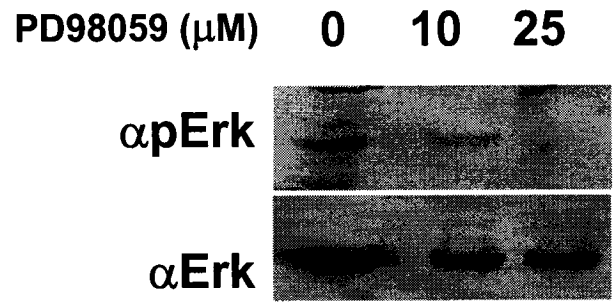
A. For this, $3x10^5$ MCF-7 cells were treated with 10μM (lane2) and 25μM (lane3) of PD98059 overnight. The cell lysate were collected and 120μg of protein was subjected to analysis by western immunoblotting. The membrane was blotted with monoclonal antibody to pERK (upper panel) to ensure the activity of this inhibitor and with antibody to ERK (lower panel) to confirm equal loading in all lanes. This gel represents three other experiments with same results.

B. MCF-7 cells exposed to the mentioned concentration of ERK inhibitor PD98059 were co-transfected with P-1/pGL3 constructs and β–galactosidase encoding plasmids, cells collected 24hr post transfection were subjected to luciferase and β–galactosidase assay as described above. The reading is a percent ratio of three separate experiments done in duplicate.

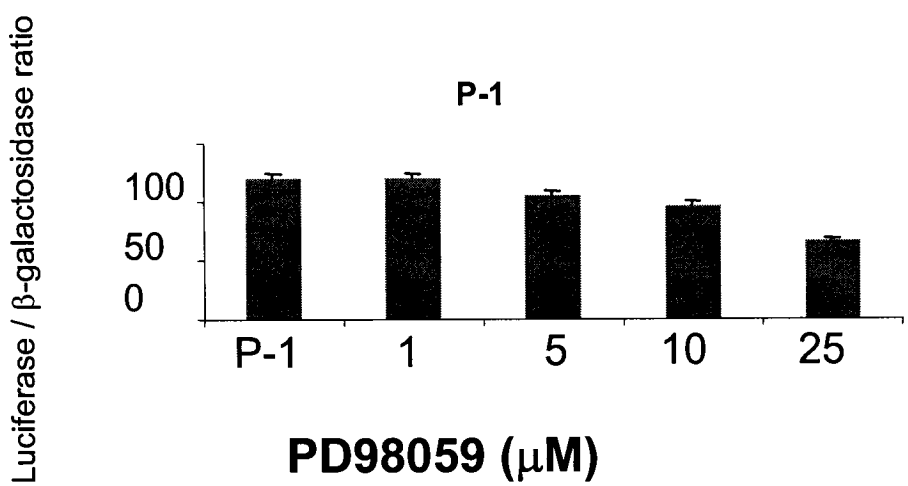
C. MCF-7 cells exposed to the mentioned concentration of ERK inhibitor PD98059 were co-transfected with P1D2/pGL3 constructs and β–galactosidase encoding plasmids, cells collected 24hr post transfection were subjected to luciferase and β–galactosidase assay. The reading is a percent ratio of three separate experiments done in duplicate.

t-Test: Two-Sample Assuming Equal Variances		
	P1D2	*P1D2+PD98059*
Mean	2999.956	1869.848
Variance	351828.9	184526.4
Observations	2	3
Pooled Variance	240293.9	
Hypothesized Mean Difference	0	
df	3	
t Stat	2.525454	
P(T<=t) one-tail	0.042883	
t Critical one-tail	2.353363	
P(T<=t) two-tail	0.085765	
t Critical two-tail	3.182449	

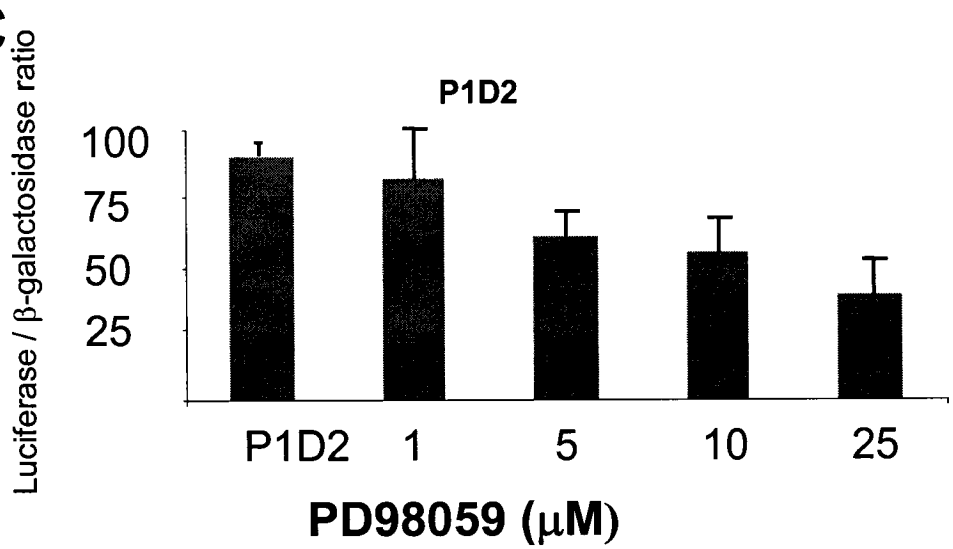
3.18A



3.18B



3.18C



This set of results showed that the SHP-1 P-1 promoter activity was predominantly dependent on JNK activation although other MAPK namely, P38 might at high concentration also play a role in SHP-1 expression in breast adenocarcinoma cells (figure 3.19).

3.6 ANALYSIS OF THE SHP-1 P-1 PROMOTER FOR TRANSCRIPTION FACTOR BINDING SITES

3.6.1 The inhibitory-region of the SHP-1 P-1 promoter

The SHP-1 P-1 promoter was analyzed for the presence of the binding sites for known transcription factors expressed in human breast epithelial cells using the genomatrix MatInspector program. Based on these analyses, three oligonucleotide probes with key transcription factor binding sites derived from the sequence of the inhibitory-region of the SHP-1 P-1 promoter (C3, C4, C5) were designed (figure 3.20A). Binding of C3, C4, C5 oligonucleotide probes to nuclear factors from MCF-7 cells was tested using electrophoretic mobility shift assay (EMSA). P^{32}ATP labeled oligonucleotides were allowed to bind to nuclear factors from serum stimulated MCF-7 cells, and the binding reaction was continued for 20 minutes. The DNA/nuclear factor complex was then resolved on a 5% Tris-glycine gel. Results showed strong binding of nuclear proteins to C5 probes and a lack of binding to C3 and C4 DNA (figure 3.20B).

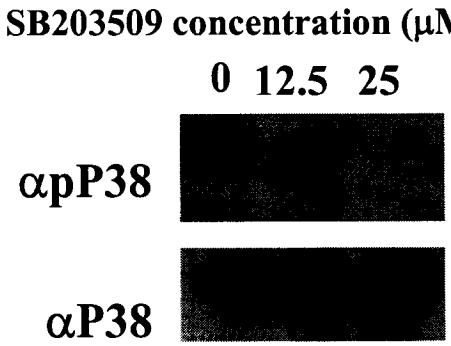
FIGURE 3.19: Abrogation of P38 activity inhibits the luciferase expression derived by the P-1/pGL3 and P1D2/pGL3 at high doses only.

A. The ability of SB203509 to inhibit P38 activity was determined in MCF-7 cells. For this, MCF-7 cells were treated with 12.5μM (lane-2) and 25μM (lane-3) of SB203509 overnight. Untreated MCF-7 cells served as a positive control (lane-1). The cell lysate was collected and 120μg of the lysate was subjected to analysis by western immunoblotting. The membrane was blotted with monoclonal antibody to pP38 (upper panel) to ensure the effectiveness of this inhibitor and with antibody to P38 (lower panel) to confirm equal loading of protein in all lanes.

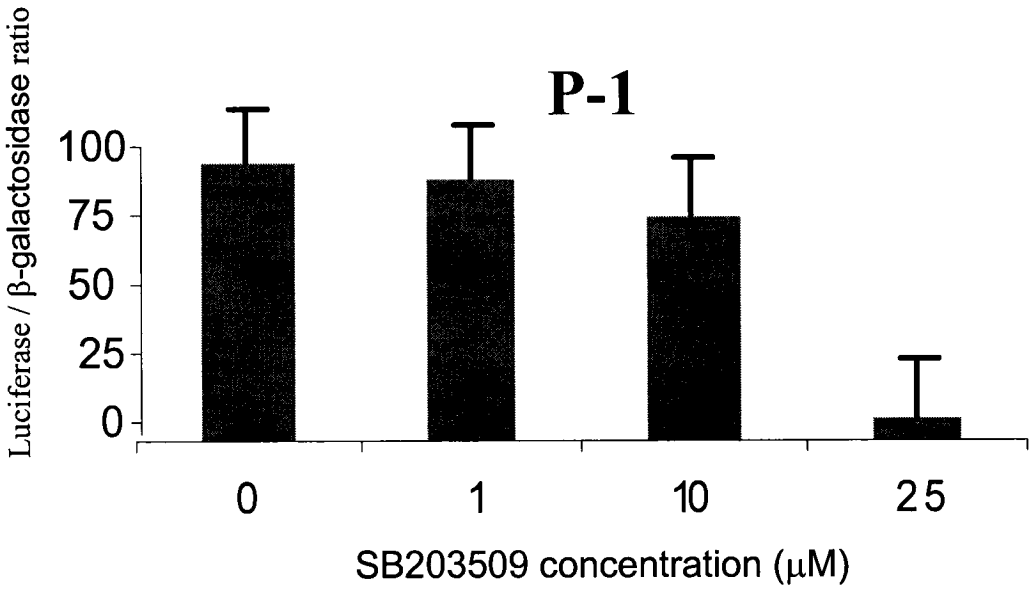
B. MCF-7 cells exposed to the mentioned concentration of SB203509 were co-transfected with P-1/pGL3 construct and with β–galactosidase plasmid. Cells collected 24hr post transfection were subjected to luciferase and β–galactosidase assays as described in Materials and Methods. The reading represents the ratio of luciferase over β-galactosidase activity in one experiment done in triplicate, which represents three other experiments. This experiment was done to determine the role of P38 MAPK in the activation of P-1/pGL3.

C. MCF-7 cells exposed to the mentioned concentration of SB203509 were co-transfected with P1D2/pGL3construct and β–galactosidase vector. Cells collected 24hr post transfection and subjected to luciferase and β–galactosidase assays as described in Materials and Methods. The reading is the ratio of luciferase over β-galactosidase activity in one experiment done in triplicate and represents three other experiments with similar results. This experiment was done to determine the role of P38 MAPK on P1D2/pGL3 activity.

3.19A



3.19B



3.19C

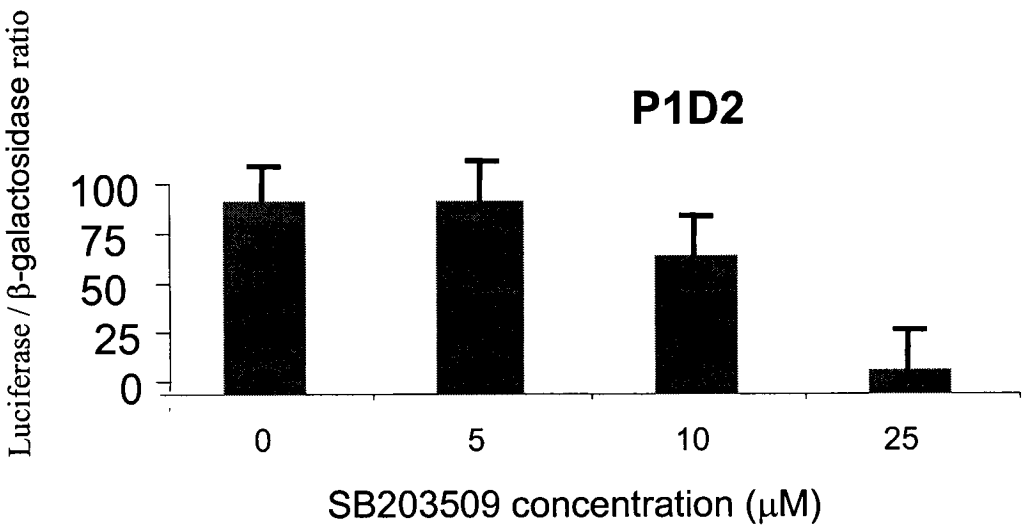
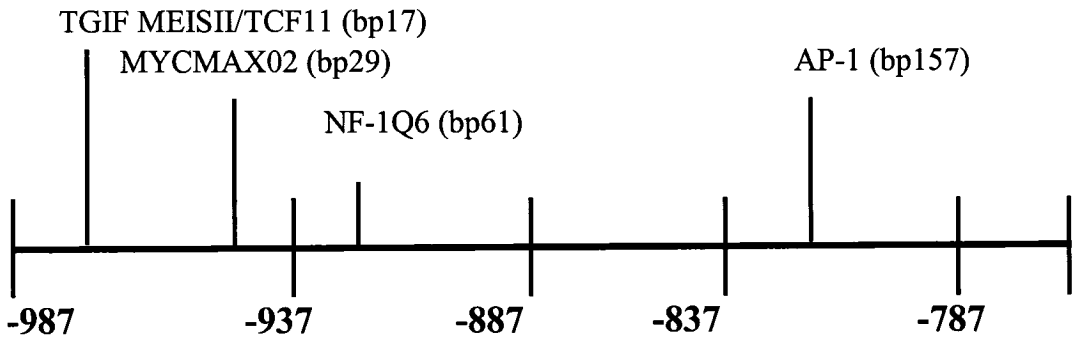


FIGURE 3.20: Nuclear proteins bind to the inhibitory-region of the SHP-1 P-1.

A. The map and sequence of the inhibitory-region showing the putative sites for transcription factor binding and the site of the oligonucleotides derived from the P-1 inhibitory-region respectively.

B. Binding of putative transcription factors in MCF-7 cell nuclei to γ P^{32} ATP labeled C3 (lane-2, lane-3), C4 (lane-4, lane-5), and C5 (lane-6, lane-7) oligonucleotides is determined by EMSA. Here oligonucleotides were radio-labeled, allowed to bind to the nuclear protein from MCF-7 cells in a reaction mixture that contained 5μg of poly-dI–dC, ran at 200V in a native 5% Tris–glycine gel, dried and exposed to X-ray film overnight at -80C. The binding of nuclear factors to commercial DNA containing a consensus NF-κB binding site was taken as positive control (lane-8).

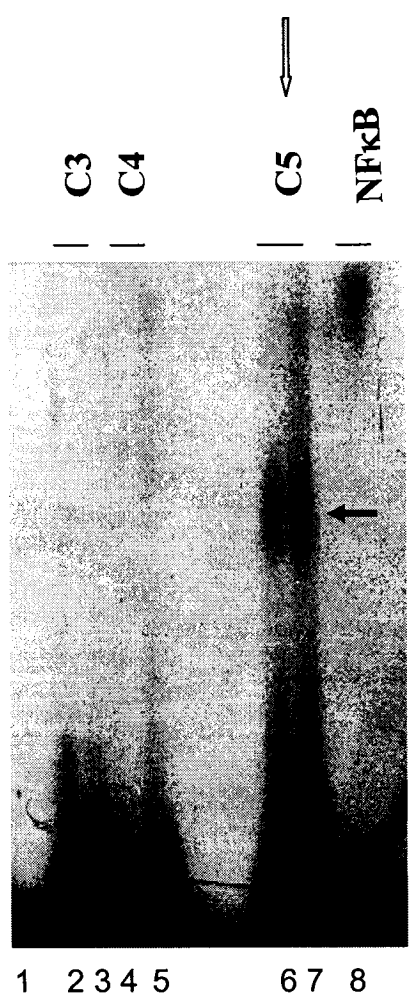
3.20A



The P-1inhibitory region

```
  1  CCCAGTGTAG ACCAAATGTC ATCATCCAAA CCACACGGGA CAGAGGCCTG
 51  CATGCGTCCT GTTTGGCAAA CAGCTGCCCA GCCAGTGGGG GAGCAGTTCA
101  TGCTTAAACT ACCACCCCCT CCAGGTGTCT TAGGCACGCT GGTCCTTAGG
151  AGAA(C3) GG GTTG ACCTTCCACT CCCTCTTGCA GGTGTCCTTA(C4) AGTTTGCTCG
201  CTTGGTCAAG TCCTACGAAG CCCAGGATCC(C5)
```

3.20B



3.6.2 Binding of nuclear proteins to the inhibitory-region of the SHP-1 P-1 is not regulated by JNK.

Since the SHP-1 gene is regulated predominantly by JNK activity, it was of interest to determine whether the transcription factors whose binding sites are encoded by the C5 sequence could also be regulated by JNK. The MCF-7 cells were pre-treated with SP600125 (25μM) and nuclear extract from these cells was subjected to EMSA using C5 DNA probes. Although the result of this experiment demonstrated strong binding of nuclear proteins to the C5 probe, and this interaction was effectively inhibited by cold competition, the binding was not decreased and in fact increased by SP600125 (figure 3.21). This result confirmed our earlier findings suggesting lack of JNK involvement in the regulation of nuclear proteins binding to the inhibitory-region of the SHP-1 P-1 promoter (figure 3.16).

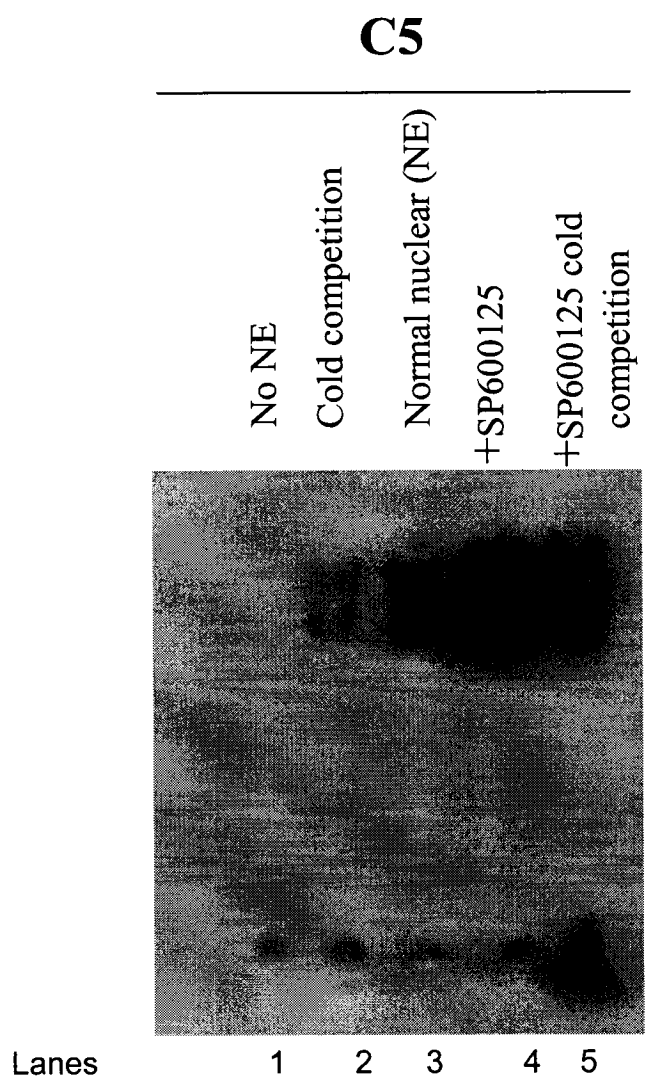
3.6.3 JNK activation regulates transcription factors binding to the high-expression region of the SHP-1 P-1 promoter in MCF-7 cells

To identify the transcription factors regulated by JNK, the sequence of the high-expression region of the SHP-1 P–1 promoter was analyzed using MatInspector software. Several transcription factors including RFX-1, AP-4, AP-1 and NF-κB were found to have binding sites in the high-expression region of the P-1 promoter. 30mer oligonucleotide probes encoding the binding sites for these transcription factors (E1, E2, and E3) were designed. E1 was contained in the region between –739 and –710, which included consensus-binding sites for AP–1 and AP-4 transcription factors.

FIGURE 3.21: Nuclear protein binding to the inhibitory-region of the SHP-1 P-1 is not regulated by JNK.

This figure shows the effect of JNK inhibitor SP600125 on the binding of nuclear proteins to C5. In lane-1 the reaction mixture contains no nuclear proteins (no NE). In lane-2 C5 DNA was allowed to bind to the nuclear protein from MCF-7 cells in the presence of 200X molar excess of unlabeled C5. In lane-3 the binding of serum starved MCF-7 cell nuclear protein to C5 was allowed to continue for 20min. Lane-4 represents the binding of oligonucleotides to nuclear proteins from cells treated for 1hr with JNK inhibitor SP600125 and lane-5 represents the same binding in presence of 200X molar excess of unlabeled C5 DNA. This figure is a representative of three other experiments with same results

3.21


C5
No NE
Cold competition
Normal nuclear (NE)
+SP600125
+SP600125 cold competition
Lanes
1
2
3
4
5

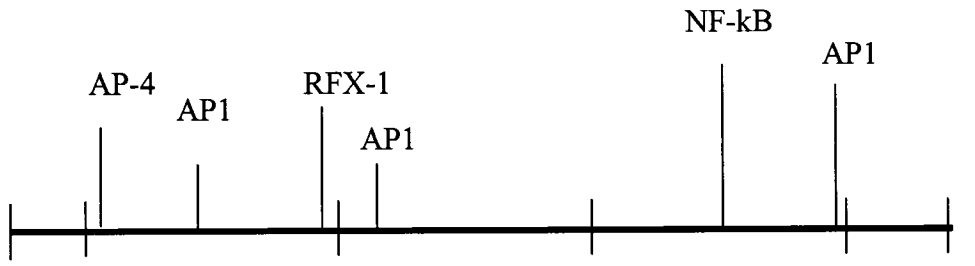
E2 oligonucleotide encoding the −709 to −680 of P-1 sequence, specified consensus binding sequences for AP-1 and RFX-1 transcription factors. E3 oligonucleotide encoding −619 to −590 of P-1 has consensus sequences for NF-κB and AP-1.We used these probes (E1, E2, E3) in EMSAs with nuclear extracts prepared from MCF-7 cells (figure 3.22B). Our results showed the binding of nuclear proteins to all three probes as revealed by a slower migration of DNA-protein complexes in non denaturing polyacrylamide gels (lane-2, lane-7, lane-11; figure 3.22B). The shift in DNA-protein complex was not observed when nuclear extract was excluded from the binding reactions (lane-5 and lane-10 figure 3.22B). To assess the impact of JNK inhibitor SP600125 on binding capacity of nuclear factors to E1, E2 and E3 DNA probes, MCF-7 cells were pre-treated with different doses of the inhibitor prior to the preparation of the nuclear extracts. SP600125 treated nuclear extracts were used for EMSAs with biotin labeled E1, E2 and E3 probes. We demonstrate that the binding of nuclear proteins to E1 (lane-3, lane-4 figure 3.22B) and E2 DNA (lane-8, lane-9, figure 3.22B) was inhibited in a dose dependent manner by SP600125. In contrast, the binding of nuclear proteins to E3 was not sensitive to 25μM of SP600125 (lane-12 figure 3.22B). These results suggest that JNK regulates the transcription factors binding to a distinct sequence of the SHP-1 P-1 high-expression region specified by 60bp of E1 and E2 (−740 to −680).

FIGURE 3.22: JNK activates transcription factors binding to the SHP-1 P-1 promoter high-expression region in MCF-7 cells

A. The map and sequence of the SHP-1 P-1 high-expression region showing the sites for transcription factor binding and the sequence and location of the oligonucleotides derived from this region

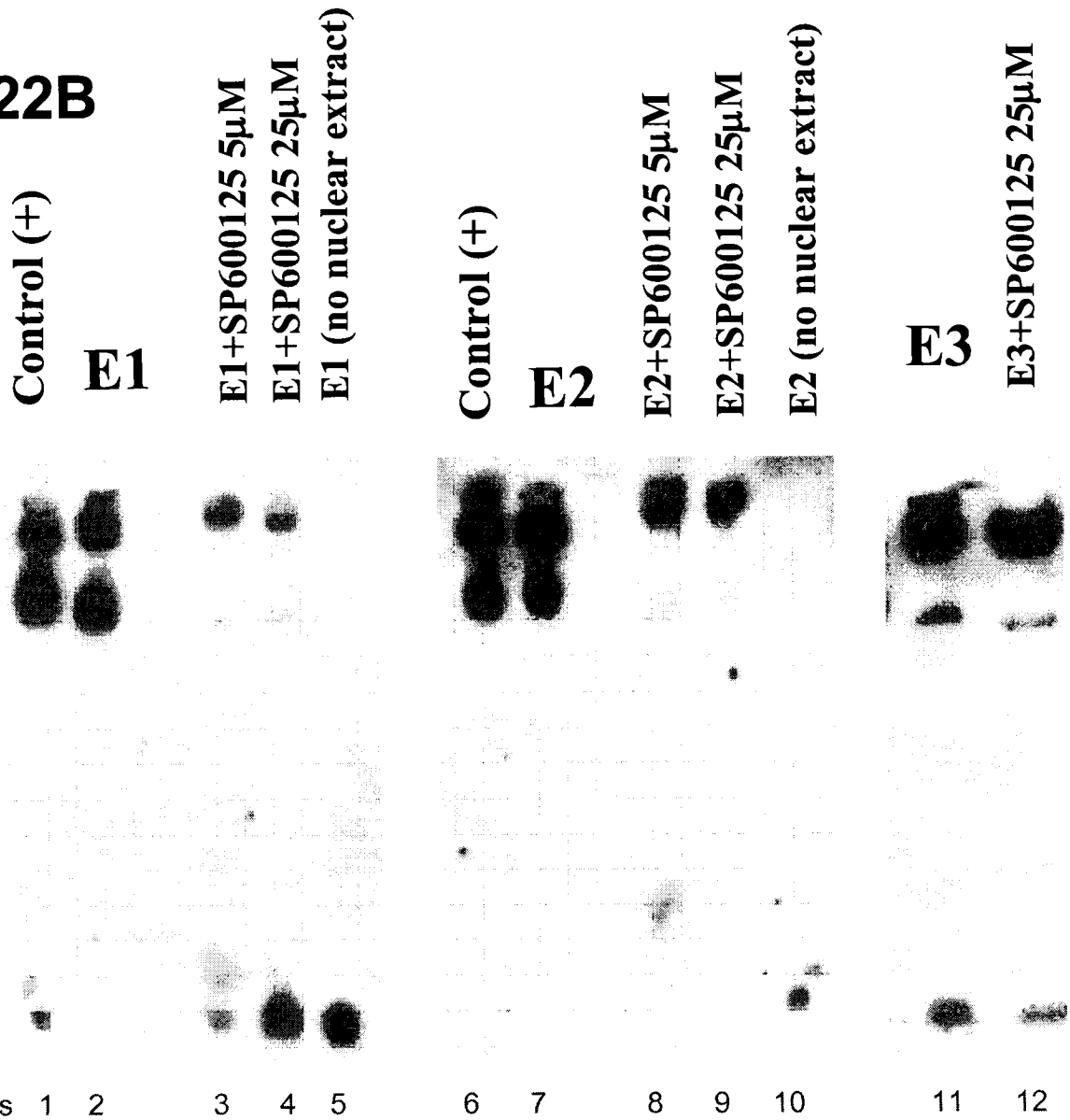
B. The binding of the putative transcription factors in MCF-7 cells to the 3' biotin labeled E1 (lane-2), E2 (lane-7) and E3 (lane-11) oligonucleotide are determined by EMSA using the chemiluminescent EMSA kit. To examine the effect of SP600125 on the binding of these three putative transcription factors, serum starved MCF-7 cells were treated with 5μM (lane-3, lane-8) and 25μM (lane-4, lane-9, lane-12) of SP600125 for 8hr before collecting the nuclear extract. Negative control consisted of E1 (lane-5) and E2 (lane-10) oligonucleotide in a reaction mixture depleted of nuclear extract. In positive control (lane-1, lane-6) consisted of the reaction mixture which was supplied by the 3′ Biotin labeling kit.

3.22A



231 TGAGATCGCC AGCCTGTCAG
251 E1 GCAAGCTGAA GGCGCTGTTT CTGTTGCCCA E2 TGACCCTGCC ACCCCATGGG
301 C CTGCTGCTG GGCACGGTGG CCGCCTCCTG AGAGTTGGCC CTCCCTTGTG
351 CCACTGCCAG GGGAGGAAAG E3 GCCTTGAT GT TCCAGACAAT AATAAATGCG
401 CCTGTGAC TT AGCCTTGG

3.22B



3.7 CHARACTERIZATION AND IDENTIFICATION OF TRANSCRIPTION FACTORS BINDING TO E1 AND E2

3.7.1 Binding of transcription factors to the SHP-1 P-1 promoter was mediated by IGF-1 stimulated JNK

Evidence provided shows that JNK negatively modulates IGF-1 mediated proliferative signals by inducing SHP-1 expression (figure 3.4 to figure 3.6). To get further insight into the involvement of JNK in the regulation of SHP-1 expression, the capacity of IGF-1 stimulated nuclear factors to bind to E1 and E2 oligonucleotides derived from the SHP-1 P-1 promoter was assessed. Data generated demonstrate several fold increased interaction of E1 and E2 with MCF--7 cell nuclear proteins upon IGF-1 stimulation compared to unstimulated MCF-7 cells in a time course manner. This DNA/protein interaction peaked at 1hr and decreased at 4hr (figure 3.23; lane-4 versus lane-7 for E1 and lane-12 versus lane-15 for E2). The specificity of this interaction was demonstrated by cold competition with 500X excess of unlabeled oligonucleotides (figure 3.23 lane-5 and lane-13, E1 and E2, respectively). Furthermore, data showed that the binding of nuclear proteins to E1 and E2 DNA was drastically diminished in cells treated for 1hr with JNK inhibitor SP600125 prior to stimulation with 5ng of IGF-1 for 1hr (figure 3.23 lane-4 versus lane-6 for E1, and lane-12 versus lane-14 for E2). These results demonstrated that JNK regulated the binding of IGF-1 activated transcription factors to E1 and E2 regions of the P-1 promoter in MCF-7 cells.

3.7.2 SHP-1 transcription is regulated by IGF-1 activated AP-4 and by RFX-1

To identify the putative transcription factors regulated by activated JNK in MCF-7 cells, the DNA sequence of the E1 and E2 regions of the SHP-1 P-1 was analyzed using MatInspector software. The E1 oligonucleotide encodes consensus sequences for the transcription factors AP–1 and AP-4. To examine the involvement of AP-1 and AP-4 in JNK activated SHP-1 transcription, five mutagenic primers, encoding altered AP-1 and AP-4 sites namely E1A, E1B, E1C, E1D and E1E, were designated (Table-2). These mutated DNA probes were then used in binding reactions with 10μg of IGF-1 stimulated nuclear proteins from MCF-7 cells. The results of EMSA experiments demonstrated loss of binding of IGF-1 stimulated nuclear factors to E1B, E1D and E1E containing mutated AP-4 consensus sequence as shown in figure 3.24. This was in contrast with efficient binding of nuclear proteins to E1A and E1C, which encoded an intact AP-4 consensus sequence but had a mutagenized AP-1 binding site. Furthermore, the binding of nuclear proteins to these two probes could be decreased by cold competition using unlabeled E1A and E1C probes. To confirm that the putative transcription factor is AP-4, the nuclear extracts from either unstimulated or IGF-1 stimulated MCF-7 cells were incubated with the αAP-4 specific antibody and DNA/AP-4/αAP-4 complex was visualized by EMSA. The antibody-transcription factor DNA complexes were resolved on a 4.5% polyacrylamide gel. The binding of αAP-4 specific antibody to protein DNA complexes is obvious by slower migration of the antibody-protein complex in polyacrylamide gels (figure 3.26 lane-3). This supershift was not observed when αAP-1 antibody was substituted for αAP-4 antibody (figure 3.26, lane-5) suggesting that AP-4 was one of the transcription factors required for SHP-1 gene transcription in MCF–7 cells.

FIGURE 3.23: IGF-1 stimulation induces the binding of transcription factors to the SHP-1 P-1 promoter by activating JNK

The effect of IGF-1 on the binding of transcription factors present in 10μg of crude nuclear extract from MCF-7 cells to E-1 and E-2. Serum starved MCF-7 cells were stimulated with 5ng/ml of IGF-1 for 30min (lane-3, lane-11), 1hr (lane-4, lane-12) and 4hr (lane-7, lane-15). To determine the effect of SP600125 on the binding reaction the cells were pre-treated with SP600125 inhibitor for 1hr before IGF-1 stimulation for 1hr (lane-6, lane-14). To further determine the specificity of IGF-1 stimulation the reaction mixture was supplemented with 500X molar excess of unlabeled E1 and E2 oligonucleotides, in lane-5 and lane-13 respectively, in addition to nuclear extract from cells stimulated with IGF-1 for 1hr. This is a representative of five other independent experiments with a similar outcome.

3.23

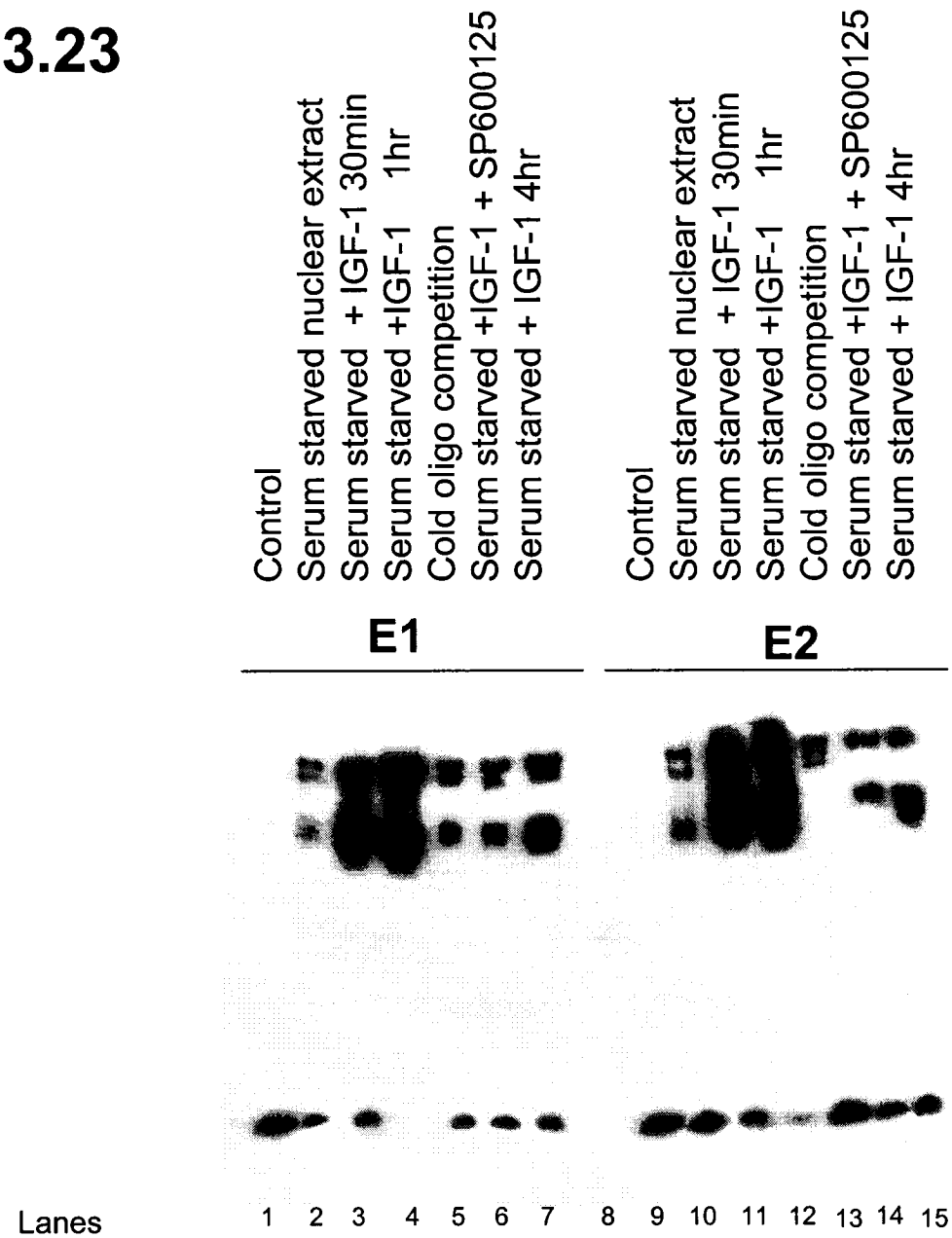

Control
Serum starved nuclear extract
Serum starved + IGF-1 30min
Serum starved +IGF-1 1hr
Cold oligo competition
Serum starved +IGF-1 + SP600125
Serum starved + IGF-1 4hr
Control
Serum starved nuclear extract
Serum starved + IGF-1 30min
Serum starved +IGF-1 1hr
Cold oligo competition
Serum starved +IGF-1 + SP600125
Serum starved + IGF-1 4hr
E1
E2
Lanes
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

FIGURE 3.24: Binding of transcription factors from IGF-1 stimulated MCF-7 cells to E1 can be abolished by a mutation in the AP-4 site.

The binding affinity of the nuclear factors of IGF-1 stimulated cells to the mutagenic primers derived from E1 namely E1A, E1B, E1C, E1D and E1E was determined by EMSA. E1A and E1B have mutations in the AP-1 and AP-4 sites respectively and they are 30bp long DNA fragments. E1C and E1D are 25bp long DNA fragments with mutation in the AP–1 and AP-4 sites respectively. In E1E both AP-4 and AP-1 sites were mutated. To prevent non-specific binding the reaction mixture contained in addition to poly-dI-dC 5μg of sonicated salmon sperm DNA.

a) Labeled oligonucleotides (no nuclear extract)
b) Nuclear extract + labeled oligonucleotide+ unlabeled oligonucleotide (cold competition)
c) Nuclear extract + labeled oligonucleotide

3.24

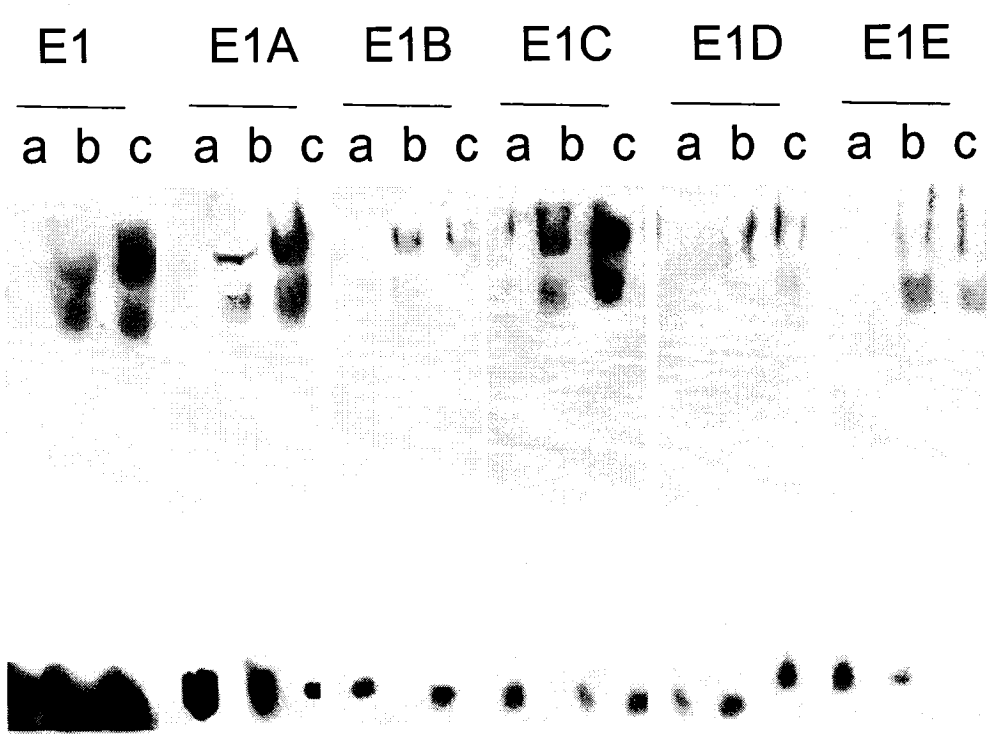
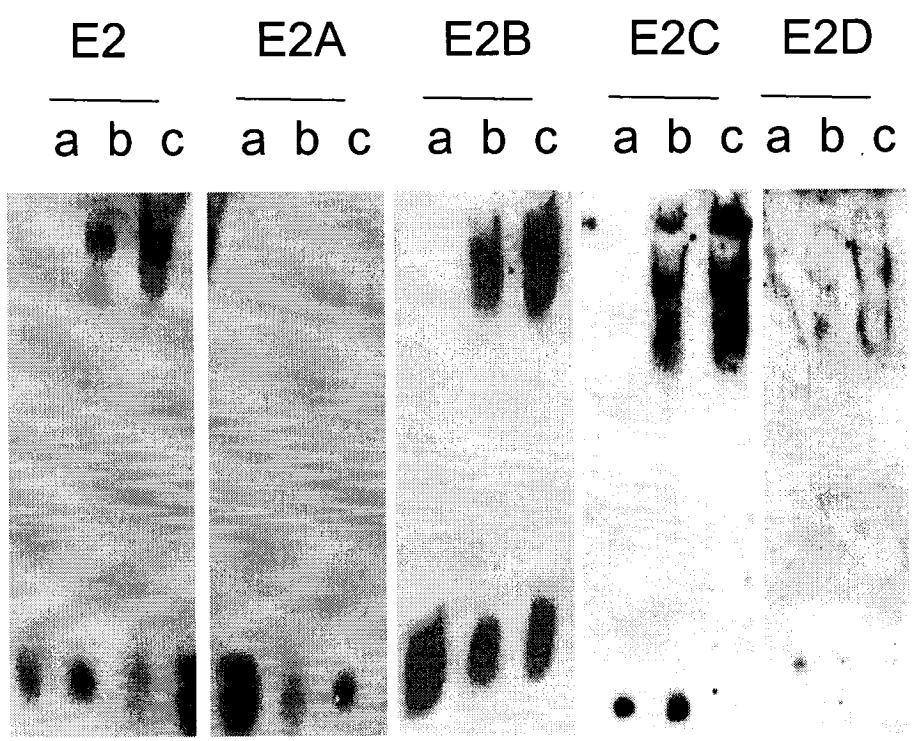


FIGURE 3.25: Binding of transcription factors from IGF-1 stimulated MCF-7 cells to E2 can be abrogated by mutating the RFX-1 binding site.

The binding affinity of the nuclear factors to the mutated DNA derived from E2 namely E2A, E2B, E2C and E2Din IGF–1 stimulated cells was determined by EMSA. In E2A and E2D the RFX-1 binding sequence was mutated and they were 30 and 25bp DNa fragments respectively. In E2B and E2C the AP-1 site was mutated and they were also 30bp and 25bp primers respectively To prevent non-specific binding the reaction mixture contained in addition to poly-dI-dC 5μg of sonicated salmon sperm DNA and 1μg of poly-dG-dT.

a) Labeled oligonucleotide (no nuclear extract)
b) Nuclear extract + labeled oligonucleotide + unlabeled oligonucleotide (cold competition)
c) Nuclear extract + labeled oligonucleotide

3.25



Similar experiments were conducted to identify nuclear factors binding to the E2 region of the SHP-1 P-1. For this, IGF-1 stimulated nuclear proteins were allowed to bind to a panel of five oligonucleotides, namely E2A, E2B, E2C and E2D containing mutated sequences for RFX-1 and AP-1 (table–2). Data showed loss of binding of E2A and E2D which contain a mutated RFX-1 site, to MCF-7 nuclear extracts as shown in figure 3.25. This stood out against the efficient binding of three other DNA probes, E2B and E2C which contained intact sequences for RFX-1 but mutated sequences for AP-1 (figure 3.25). Furthermore, these interactions could be specifically reduced by including the unlabelled oligonucleotides in the reaction mixture (figure 3.25). To confirm RFX-1 binding to the E2 probe, a gel supershift assay was also performed using αRFX-1 specific antibody. Inclusion of αRFX-1 specific antibodies in the binding reactions resulted in a supershift of the IGF-1 stimulated protein-DNA complexes (figure 3.26, lane-11).

3.8 ROLE OF RFX-1 AND AP-4 IN MCF-7 BREAST CANCER CELLS

3.8.1 SHP-1 expression in MCF-7 cells is regulated by RFX-1 and AP-4

RFX-1 and AP-4 bind to the high-expression region of the SHP-1 P-1. Therefore, it was of interest to examine the effect of these two transcription factors on SHP-1 expression. To determine whether IGF-1 induced SHP-1 expression is mediated through RFX-1 and AP-4 activation, MCF-7 cells were transfected using oligofectamine with either the RFX-1 antisense oligonucleotides or the AP-4 antisense oligonucleotides or the control CD-4 antisense oligonucleotides. In parallel, it was also determined whether transfection with RFX-1 antisense oligonucleotide or the AP-4 antisense oligonucleotide affected the expression of RFX-1 or AP-4 in a time course manner (figure 3.28 and figure 3.27 middle panels).

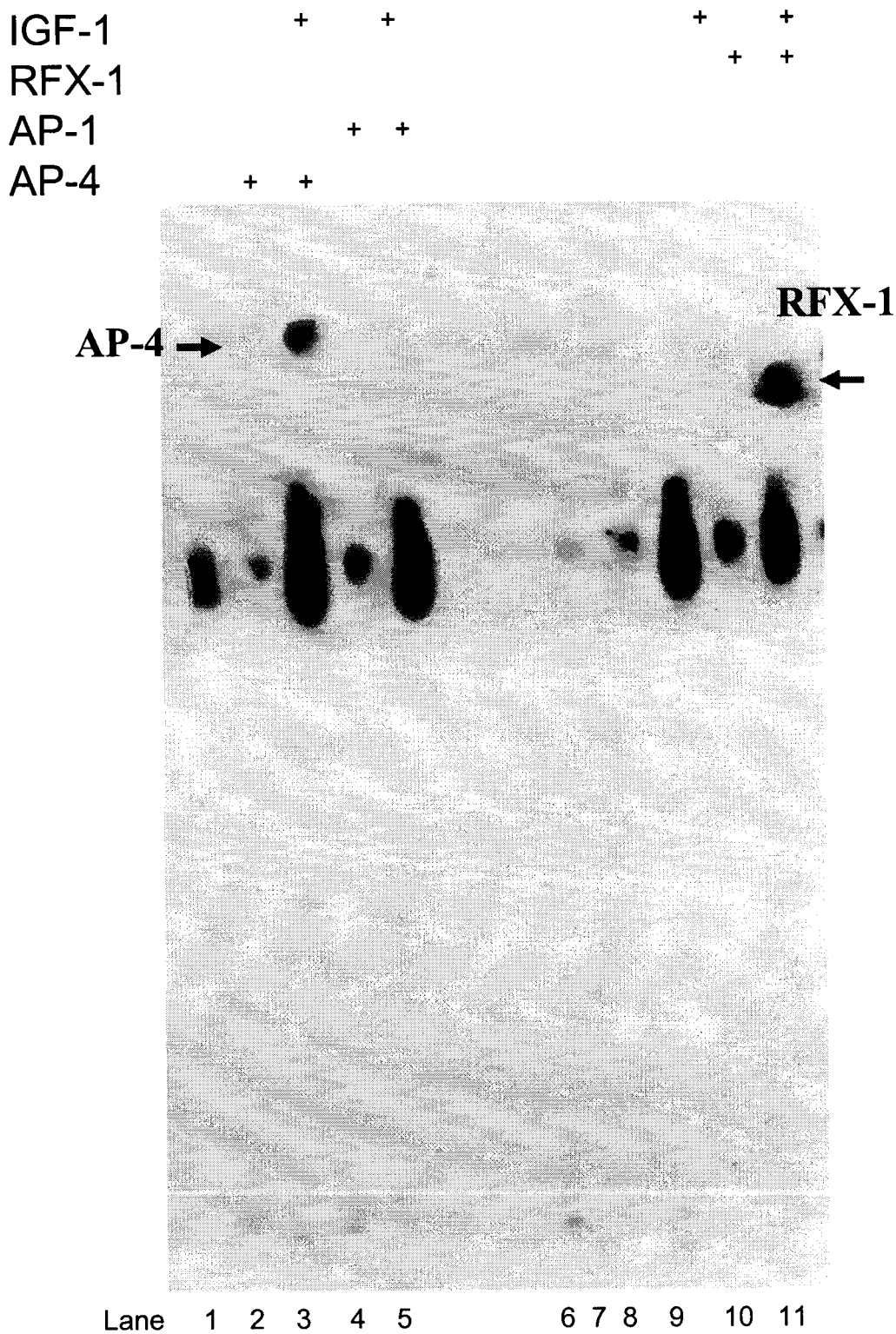
FIGURE 3.26: SHP-1 transcription is regulated by IGF-1 activated AP-4, which binds to the E1 region, and RFX-1which binds to the E2 region.

This is a gel supershift experiment performed using the biotin labeled E1 and E2 using the light shift chemiluminescent EMSA kit.

Lanes

1. 10μg normal nuclear extract + IGF-1 + E1
2. 10μg normal nuclear extract + E1+ 1μg AP-4 antibody
3. 10μg normal nuclear extract + IGF-1 + E1 + 1μgAP-4 antibody
4. 10μg normal nuclear extract + E1+ 2μg AP-1 antibody
5. 10μg normal nuclear extract + IGF-1 + E1 + 2μg AP-1 antibody
6. Labeled E2 oligonucleotide
7. 10μg normal nuclear extract + 200X unlabeled E2 + labeled E2 oligonucleotide
8. 10μg normal nuclear extract + E2
9. 10μg normal nuclear extract + IGF-1+ E2
10. 10μg normal nuclear extract + 1μg RFX-1 antibody
11. 10μg normal nuclear extract + IGF-1 + 1μg RFX-1 antibody

3.26



Cells transfected in a time course manner were stimulated with IGF-1 for 24hr, and crude cell lysate was subjected to analysis by western immunoblotting. Results showed that transfection of MCF-7 cells with antisense oligonucleotides to RFX-1 or antisense to AP–4 inhibited RFX-1 and AP-4 expression respectively in a time dependent manner compared to the cells transfected with the antisense CD-4 control oligonucleotide. The effect of antisense RFX-1 and AP-4 oligonucleotides on RFX-1 and AP-4 protein expression was gradual with limited effect at day 2 and the strongest reduction in protein expression at day 3 post transfection. To check that the effect was not due to cell death, the cell viability was determined by trypan blue staining. To determine whether RFX-1 and AP-4 expression influenced SHP-1 expression, the same membrane was blotted with αSHP-1 antibody. Results showed that SHP-1 expression was decreased in a time dependent manner following transfection with either AP-4 antisense or RFX-1 antisense oligonucleotides. SHP-1 expression paralleled the expression level of these two transcription factors in IGF-1 stimulated MCF-7 cells (figure 3.28 and figure 3.27, upper panel).

3.8.2 RFX-1 and AP-4 regulate SHP-1P-1 promoter activity in MCF-7 cells

To confirm the role of RFX-1 and AP-4 in the regulation of IGF-1 induced SHP-1 expression in MCF-7 breast cancer cells, their effect on luciferase activity driven by either P-1/pGL3 or P1D2/pGL3 plasmids was also investigated. In order to determine the role of these two transcription factors, the cells were transfected with 200μM of either antisense oligonucleotide to RFX-1 or with antisense oligonucleotide to AP-4 for 24hr prior to transfection with either P–1/pGL3 or P1D2/pGL3 constructs followed by the luciferase assay 24hr post transfection of the pGL3 constructs.

FIGURE 3.27: SHP-1 transcription requires AP-4.

MCF-7 cells were serum starved overnight and transfected with 170nM of AP-4 antisense oligonucleotide, which has phospho-thioate modification at all cytosine residues, using oligofectamine reagent. The cells grown for 1 to 3 days (lane-3 to lane-5) were stimulated with 5ng of IGF-1 overnight. Unstimulated cells (lane-1) and IGF-1 stimulated untransfected cells (lane-2) were taken as controls. To ensure the specificity of AP-4 antisense some cells were transfected with antisense to CD-4 and grown for three days (lane-6). 66μg of lysate collected were analyzed by western immunoblotting. The membrane was blotted with 1μg of monoclonal SHP-1 antibody (Upstate Biotechnology) to determine effect of AP-4 on SHP-1 expression (top panel). The same membrane was blotted with 3μg of AP-4 antibody to determine the effectiveness of the antisense (middle panel) and with αP38 antibody to confirm equal protein loading (bottom panel).

3.27

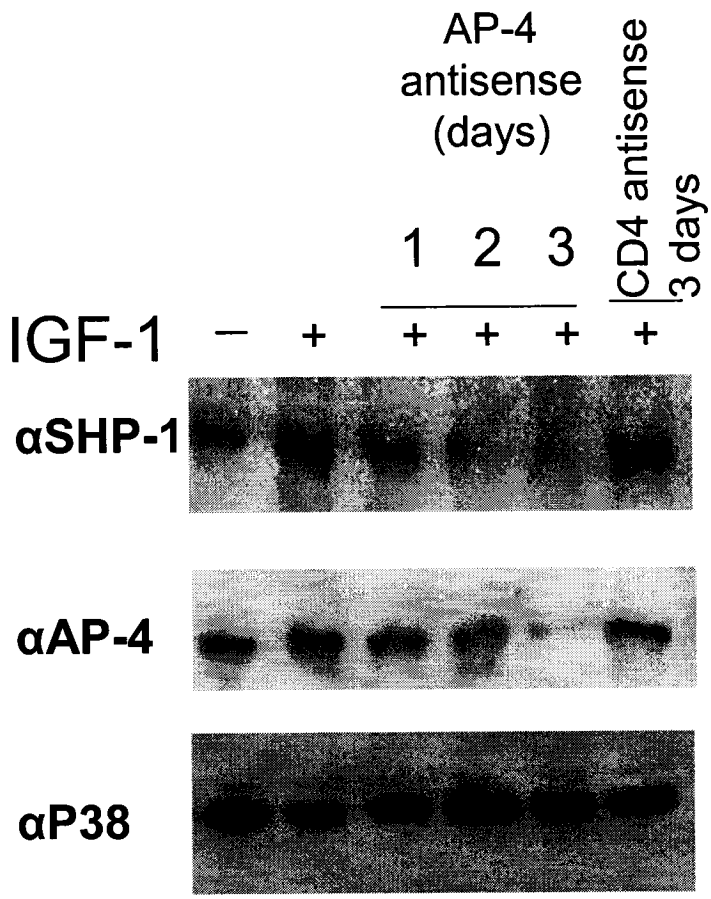

AP-4
antisense
(days)
1 2 3
CD4 antisense
3 days
IGF-1 – + + + + +
αSHP-1
αAP-4
αP38

FIGURE 3.28: SHP-1 transcription requires RFX-1 in addition to AP-4.

MCF-7 cells were serum starved overnight and transfected using oligofectamine with 170nM of RFX-1 antisense oligonucleotide containing phospho-thioate modification at all cytosine residues. Cells were grown for 1– 3 days (lane-3 to lane-5) post transfection and stimulated with 5ng/ml of IGF-1 for 24hr before collecting the pellets. Unstimulated cells (lane-1) and IGF-1 stimulated untransfected cells (lane-2) were taken as controls. To ensure the specificity of RFX-1 antisense some IGF-1 stimulated cells were transfected with antisense CD-4 oligonucleotides and grown for 3 days (lane-6). 66μg of lysate independently collected were analyzed by western immunoblotting. The membrane was blotted with 1μg of monoclonal αSHP-1 antibody (Upstate Biotechnology) to see effect of RFX-1 on SHP-1 expression (top blot). The same membrane was blotted with 1μg of αRFX-1 antibody to determine the effectiveness of the antisense (middle panel) and with antibody to P38 to confirm equal protein loading (bottom blot).

3.28

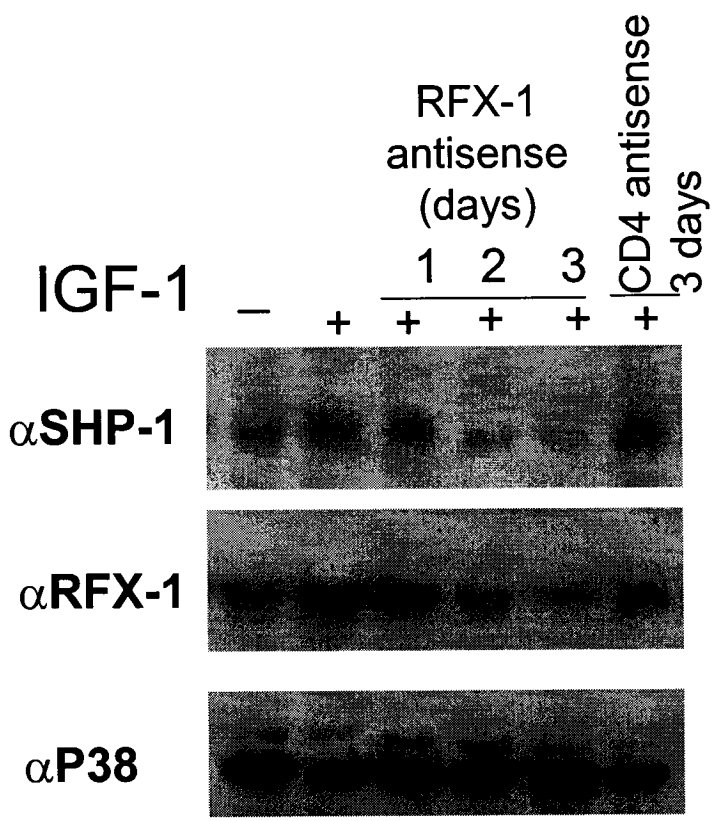
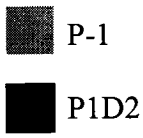

RFX-1
antisense
(days)
1 2 3
CD4 antisense
3 days
IGF-1
– + + + + +
αSHP-1
αRFX-1
αP38

FIGURE 3.29: Transcription factors RFX-1 and AP-4 are required to drive SHP-1 expression by P-1 promoter.

$1x10^5$ MCF-7 cells were plated in serum free medium and kept overnight to recover. Cells were then transfected with 200ng antisense AP-4, antisense RFX-1or antisense CD4 using oligofectamine. The cells were co-transfected with β–galactosidase encoding plasmid and P-1/pGL3 (grey bars) or P1D2/pGL3 constructs (black bars) 24hr after antisense transfections, using Lipofectamine 2000. Untransfected cells (Mock) were taken as negative controls and cells not transfected with the antisense (no antisense) as positive controls. Cells were stimulated with 5ng/ml of IGF-1 6hr after transfecting with the P-1/pGL3 constructs, collected 24hr after transfection with the P-1/pGL3 constructs, and subjected to luciferase and β–galactosidase assays. The toxicity of oligonucleotides to cells was determined by transfecting cells with irrelevant antisense oligonucleotides (antisense CD-4 oligonucleotides) in addition to P-1/pGL3 and P1D2/pGL3. The graph reading is the percentage of ratio of the luciferase and β–galactosidase activity to that of the positive control i.e. cells transfected only with P1D2/pGL3 and not with any antisense oligonucleotides (no antisense). The figure represents the compiled data of three separate experiments done in duplicate.



3.29

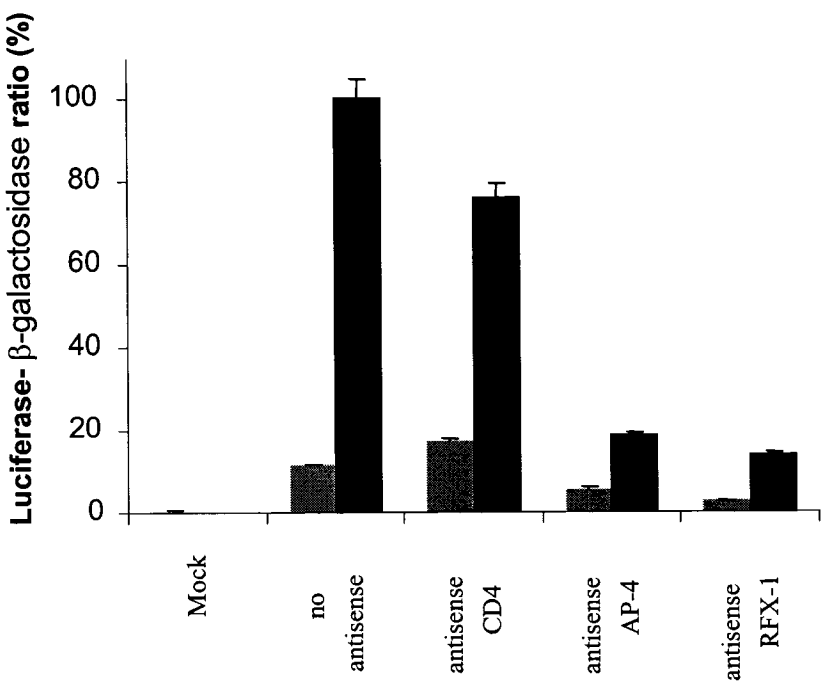
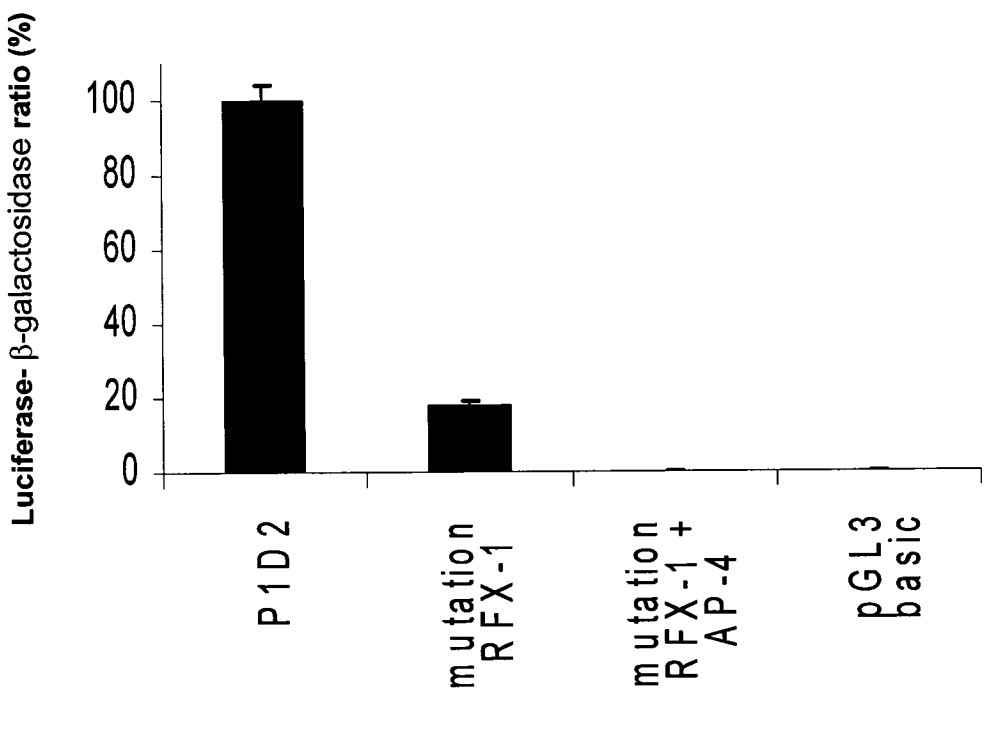

Luciferase- β-galactosidase ratio (%)
100
80
60
40
20
0
Mock
no antisense
antisense CD4
antisense AP-4
antisense RFX-1

FIGURE 3.30: Transcription factor AP-4 and RFX-1 binding to the high-expression region of the P-1 promoter regulates SHP-1 expression.

The P1D2 construct was mutated at the RFX-1 site and in both AP-4 and RFX-1 sites using the Site directed mutagenesis kit and sequenced to confirm the mutation. MCF-7 cells were then transfected with the constructs containing the RFX-1 mutation (bar-2) and with that containing both RFX-1 and AP-4 mutations (bar-3). Cells transfected with promoter-less pGL3 basic vector were taken as a negative control (bar-4) and cells transfected with unmutated P1D2/pGL3 construct as positive control (bar-1).

3.30


Luciferase- β-galactosidase ratio (%)
100
80
60
40
20
0
P1D2
mutation RFX-1
mutation RFX-1 + AP-4
pGL3 basic

Results showed that inhibiting either AP-4 or RFX-1 expression by their respective antisense oligonucleotides decreased P-1/pGl3 and P1D2/pGL3 driven luciferase expression in MCF-7 cells (figure 3.29). In contrast, there was no dramatic change in the luciferase activity driven by P-1/pGL3 and P1D2/pGL3 when the cells were transfected with antisense to CD–4 (figure29).

3.8.3 Interaction of transcription factors AP-4 and RFX-1 with the high-expression region of the P-1 promoter regulate SHP-1 expression

To further establish the role of RFX-1 and AP-4 in the regulation of SHP-1 expression driven by the P-1 promoter in human breast cancer MCF-7 cells, constructs with point mutations in the RFX-1 and/or AP-4 sites in the P1D2/pGL3 construct were generated. These mutant constructs were then transfected into MCF-7 cells along with the β-galactosidase encoding plasmid. The transfectants were collected and were subjected to luciferase and β-galactosidase assays 24hr post transfection. Results show a significant fall in the luciferase expression of P1D2/pGL3/RFX-1 mutants. This down regulation of luciferase expression in P1D2/pGL3 was even more dramatic in cells transfected with P1D2/pGL3 where both RFX-1 and AP-4 sites were mutated as shown in figure 3.30. Technical difficulties prevented generation of a P1D2/pGL3 construct with mutation at the AP-4 site only.

These results demonstrated that RFX-1 and AP-4 control SHP-1 expression by interacting with the high-expression region of the SHP-1 P-1 promoter.

Chapter 4: DISCUSSION

Breast cancer cells expressed enhanced levels of SHP-1 protein. To understand this up-regulated expression of SHP-1 in breast cancer cells the regulation of the SHP-1 gene in response to IGF-1 was analyzed. The results of this study demonstrate that SHP-1 expression correlates with the activity of JNK in IGF-1 stimulated MCF-7 cells and with the rate of cell proliferation. Furthermore, this study shows that the expression of SHP-1 in IGF-1 stimulated breast adenocarcinoma MCF-7 cells is regulated by JNK activity, which, in turn, activates the binding of RFX-1 and AP-4 transcription factors to the high-expression region of the SHP-1 P-1 promoter.

4.1 NEGATIVE FEEDBACK IN IGF-1 SIGNALING

4.1.1 IGF-1 stimulated JNK drives the negative feedback on breast cancer cell proliferation

IGF-1 is a potent mitogen of breast cancer cell growth and the importance of IGF-1 signaling in carcinogenesis is obviously evident from a variety of studies (Esposito et al, 1997). Circulating IGF-1 levels are higher in breast cancer patients. This 7kDa polypeptide growth factor has a high degree of homology to insulin and is an essential factor in modulating normal mammary gland development (Hadsell et al, 1996 and Rosen et al, 1996 and Weifeng et al, 1999). The mitogenic activity of IGF-1 is mediated by the receptor PTK IGF–1R. Many human tumors, including tumors originating from colon (Tricoli et al, 1986), lung (Minuto et al, 1986 and Heush et al, 1986), and breast (Yee and Paik et al, 1989), were shown to over-express IGF-

1R and/or IGF-1. My data confirmed this observation and further demonstrate that binding of IGF-1 to IGF-1R promotes survival and proliferation of the cancer cells as shown in figure 3.1. The importance of IGF-1 in tumorigenesis was also demonstrated by using neutralizing antibodies or antisense strategies aimed at IGF-1R blockade (Caroll et al, 1998; Esposito et al, 1997 and Guvacova et al, 1997). These experimental strategies reversed the tumorigenic phenotype both in vitro and in vivo in various tumor types including melanoma, prostatic carcinoma, colorectal carcinoma and ovarian carcinoma (Alexia et al, 2004). Recently, it has been shown that ER blocker tamoxifen and related drugs, namely droloxifene and roloxefene have caused a moderate suppression of plasma IGF-1, which was further pronounced in combination with somatostatin analogues (Rees et al, 1998; Guvacova et al, 1997 and Buck et al, 2004)

Survival signaling in the IGF-1 stimulated cells is attributed to the activation of the PI-3K/Akt pathway which prevents heterodimer formation of the pro-apoptotic protein BAD, a protein required to counteract anti-apoptotic pathways involving Bcl2 or Bcl-XL. Proliferation in the IGF-1 stimulated cells on the other hand is accomplished by activating the MAPK ERK pathway (Pavlovic-Surjancev, 1992 and Peruzzi et al, 1999). In addition to the ERK pathway and the PI-3K pathway proliferation of breast cancer cells in also dependent on MAPK JNK. The data presented in this study show that IGF-1 stimulation activates the MAPK JNK (figure 3.2B). JNK is a serine-threonine kinase which activates the transcription factor c-jun and a variety of other transcription factors, and thus activates genes regulating both proliferative and apoptotic cellular responses. For instance, in breast tumors JNK prevents tumor development by inhibiting angiogenesis and proliferation and promoting apoptosis. Here, we show that JNK modulated the rate of MCF-7 breast cell proliferation in both untreated and IGF-1 treated cells albeit to

different degrees as shown in figure 3.3. Unstimulated or IGF-1 stimulated MCF-7 cells proliferated more in the presence of the JNK inhibitor SP600125 (figure 3.3). Additionally, the rate of cell proliferation could be correlated with the level of activated JNK in two other adenocarcinoma cell lines namely, ZR-75-1 and with A431 as shown in figure 3.10. Similar to MCF-7 cells, these two cell lines when subjected to JNK inhibition by SP600125 proliferated more as shown in figure 3.11. These findings support our view that proliferation in IGF-1 stimulated MCF-7 breast cancer cells is tightly regulated by MAPK JNK which upon IGF-1 stimulation down-regulates proliferative signals.

4.1.2 Tyrosine phosphatase SHP-1 inhibits proliferation in breast cancer cells

It is well documented that breast adenocarcinoma is not a highly proliferating cancer. This suggests that during tumor growth, there is activation of signaling molecules which are capable of mediating anti-proliferative signals in breast cancer cells. Tyrosine phosphatases, such as SHP-1, may in part fulfill this activity. By examining SHP-1 expression in a panel of normal and tumor tissues, it became evident that SHP-1 might be involved in a negative regulation of cancer cell proliferation. It was observed that expression levels of SHP-1 were much higher in the tumor samples compared to their normal counterparts as shown in figure 3.4. Furthermore, highly invasive tumors like ovarian tumors (figure 3.4) and pancreatic adenocarcinoma (figure 3.10B) had lower SHP-1 expression than the slow growing breast cancer supporting further a role for SHP-1 as a tumor suppressor in cancer cells. This is consistent with the well-documented role of SHP-1 in the negative regulation of mitogenic pathways in hematopoietic cells (Perez-Viller et al, 1999, and Oka et al, 2001). Further evidence was provided by correlating the levels of SHP-1 expression with the rate of tumor cell growth and by showing that tumor cells

expressing higher levels of SHP-1 proliferate less than tumor cell lines which had a diminished level of SHP-1 as shown in figure10A. The negative effect of SHP-1 in breast cancer cell growth was further substantiated by inhibiting SHP-1 expression with SHP-1 specific antisense oligonucleotides. Such treatment increased growth of MCF-7 breast cancer cells (figure 3.9).

Recently, it has been observed that JNK activated by other growth factors like PDGF results in cell increase in cell proliferations much higher compared to IGF-1 induced increase in cell proliferations in the same cells (Mooney et al, 1992 and Cossette et al, 1996). This observation suggests that IGF–1 mediated proliferative signals are modulated by the induction of an additional inhibitory activity. To elaborate on this observation evidence was provided demonstrating SHP-1's role in the negative regulation of IGF-1 induced proliferative signaling. IGF-1 stimulation increases both SHP-1 expression (figure 3.7 and 3.8) and SHP-1 activity (figure 3.6). Furthermore, IGF-1 stimulated cells devoid of SHP-1 proliferate more as demonstrated by inhibiting SHP-1 expression using SHP-1 antisense oligonucleotides (figure 3.9).

4.1.3 JNK mediates its anti-proliferative effect by increasing SHP-1 expression

Results in this thesis implicate JNK and SHP-1 in IGF-1 induced cell growth, where JNK negatively regulates cell proliferation by inducing SHP-1 expression in breast cancer cells. This notion is based on following evidence provided in this study where it is shown that :

1. The level of SHP-1 expression could be correlated with the level of JNK activation and inhibition of either JNK or SHP-1 increased proliferation of IGF-1 stimulated cells.

2. Inhibition of JNK activation in IGF-1 stimulated cells by SP600125 or DN SEK inhibited SHP-1 but not SHP-2 expression (figure 3.5, 7, 8).

3. Inhibition of JNK activation in IGF-1 stimulated cells by SP600125 decreased SHP-1 phosphatase activity in a dose dependent manner (figure 3.6).

These results support our hypothesis and provide strong evidence that SHP-1 expression was dependent on IGF-1 induced JNK activation, and that JNK was mediating its anti-proliferative role in breast cancer cells through SHP-1.

The expression of SHP-1 in normal epithelial tissues is low compared to the expression of this phosphatase in hematopoietic cells. This suggests that in normal epithelial cells SHP-1 expression is tightly controlled by the activity of an upstream signaling molecule. In breast cancer cells, enhanced expression of SHP-1 may be a direct consequence of hyperactivation of a signaling molecule, namely IGF-1 activated JNK. Circulating IGF-1 is higher in individuals with breast cancer, and individuals with higher circulating IGF-1 are also more prone to develop breast cancer (Yee and Favoni et al, 1989). IGF-1 influences breast cancer cell growth in both paracrine and autocrine manners (Lippman et al, 1986 and Yee and Paik et al, 1989). Parallel to increased IGF-1 expression, IGF-1R expression was also 10–12 fold higher in breast adenocarcinomas where over-expression of a functional IGF-1R protected the cells from apoptosis (Peruzzi et al, 1999 and Alexia et al, 2004). This study further demonstrates that

increased IGF-1/IGF-1R activity is responsible for higher SHP-1 expression and JNK activation in breast cancer cells (figure 3.5-9).

In view of the results generated in this study it is of interest to examine how SHP-1 exerts its anti-proliferative affect on MCF-7 cells. One possible mechanism is that SHP-1 may dephosphorylate signaling proteins activated by IGF-1 and also by other ligands such as TNF-α. The interaction of TNF-α with its receptor initiates both apoptoptic and anti-apoptotic signaling pathways (Sluss et al, 1994). The balance between them determines the outcome *i.e.* survival or death. TNF-α engaged anti-apoptotic pathway occurs by activation of P38 MAPK; whereas the apoptotic pathway involves activation of calcitonin which activates TRADD, FADD and caspase 8 (Weitsman et al, 2004).

4.2 CHARACTERIZATION OF THE SHP-1 P-1 PROMOTER

SHP-1 expression levels in epithelial cells are normally very low but become highly up-regulated in breast tumors. It is reasoned that SHP-1 expression levels are altered during breast cancer growth or development. Furthermore, it is shown that SHP-1 expression and activity is regulated by IGF-1 stimulated JNK (figure 3.5 –figures 8). To understand better the regulation of the SHP-1 gene, the SHP-1 epithelial tissue specific P-1 promoter was isolated, and a series of 5' deletion fragments were made and were fused to the luciferase reporter gene in the pGL3 basic vector. Comparing the activity of the promoter fragments to full length P-1 in MCF-7 transfected cells revealed two functional regions in the P-1 promoter specifying the low or the high level of SHP-1 expression. To look for potential mutations in the inhibitory region of the SHP-1 P-1 promoter, which could have resulted in the highly up-regulated SHP-1 expression levels in breast cancer cells, the inhibitory and excitatory regions were isolated from a panel of human breast

cancer cell lines and murine mamary cancer tissue biopsies and their DNA sequences determined. Because DNA sequence analysis did not show any mutations in either the inhibitory or high-expression regions of the SHP-1 P-1 promoter, it was hypothesized that the hyperactivation of certain signaling proteins in breast cancer underlies intrinsic SHP-1 expression in breast cancer cells.

4.2.1 Dependence of luciferase activity of the P-1/pGL3 and P1D2/pGL3 on JNK

The data presenteddemonstrated that SHP-1 expression is regulated by JNK in IGF-1 stimulated MCF-7 cells, and that the enhanced expression of SHP-1 in breast cancer is not a consequence of mutation in the SHP-1 P-1 promoter in breast adenocarcinoma cells (data not shown). To extend this observation and to further understand the regulation of the SHP-1 P-1 promoter, the impact of JNK inhibitor SP600125 on P-1 and P1D2 promoter activity was examined in MCF-7 cells following IGF-1 stimulation. P-1 or P1D2 promoter driven luciferase expression dropped in a dose dependent manner upon inhibition of JNK activation by SP600125 in IGF-1 stimulated cells but not upon inhibition of ERK using a specific inhibitor (PD98059) in serum stimulated cells. This data suggests that signaling molecule(s) activated by JNK but not by ERK distinctively regulated SHP-1 expression.

The P38 inhibitor (SB203590) decreased P-1/pGL3 driven luciferase activity at a very high concentration in serum stimulated cells (figure 3.19B and19C). P38 is activated in response to the Rho family of GTPases by environmental stress or by inflammatory cytokines induced by LPS in a manner not similar to that for JNK (Raingeaud et al, 1996; Buck et al, 2004; Dent et al, 2003). It is also an important enzyme involved in the regulation of inflammatory cytokine

biosynthesis and cell proliferation. Depending on the cellular context, P38 MAPK may enhance or inhibit cell death, and P38 signaling is dependent on the expression of a functional ATM protein (Jeanmarie, 1998). For example, in MCF-7 cells treated with TNF-α, P38 MAPK plays an anti-apoptotic role. The protective effect of P38 MAP kinase upon TNF-α has been attributed to two mechanisms. The first mechanism involves the activation of MAPK-activated protein kinase-2 (PK-2) that phosphorylates heat shock protein 27 (HSP27). HSP27, in turn, inhibits procaspase-3 activation by sequestering both cytochrome-c and procaspase-3. The second mechanism involves the activation of Akt which ultimately leads to the degradation of IκB (Weitsman et al, 2004) with a consequent activation of transcription factor NFκB (nuclear factor of κ light polypeptide gene enhancer in B-cells) and activation of survival signaling by activation of growth promoting genes (Weitsman et al, 2004).

On the other hand, in tamoxifen therapy, P38 MAPK is required for tamoxifen induced growth inhibition (Buck et al, 2004). Therefore, even in the same breast cancer cell line, MCF-7, P38 inhibits proliferation when it is stimulated by tamoxifen; whereas when it is stimulated by TNF-α, P38 increases cell proliferation. It has also been documented that IGF-1 stimulates P38 activation; however, the role of P38 in MCF-7 cells upon IGF-1 stimulation is not well understood. Furthermore not much is known about the role of P38 on IGF-1 stimulated SHP-1 expression. Inhibition of SHP-1 expression by high doses of SB203509 suggests the presence of some cross-talk between JNK and P38 upstream to the SHP-1 gene. For example it is possible that JNK and P38 activate transcription factors whose cooperation drives SHP-1 expression. For instance, one of the downstream cellular targets of P38 is ATF2 which is also a JNK target in breast cancer cells stimulated by TGF-β (Buck et al, 2004). Alternatively, activation of dual specificity kinases that phosphorylate P38 in addition to JNK is required for SHP-1 expression in

breast cancer cells. MKK6, MKK4, MKK3 and MKK3b are the dual specificity kinases that phosphorylate P38 (Raingeaud et al, 1996). Among these, MKK4, a signaling protein upstream of P38 and activated in response to environmental stress, also activates JNK. The precise role of P38 in SHP-1 expression in breast cancer cells awaits further investigation.

4.2.2 Analysis of JNK dependent transcription factors which are essential for the SHP-1 P-1 promoter expression in IGF-1 stimulated MCF-7 cells

A search for transcription factors involved in SHP-1 gene regulation in the IGF–1/JNK pathway identified binding activities interacting with both the inhibitory and high-expression regions of P-1. Although the P-1 inhibitory region contains consensus sites for OCT1 (octamer-binding transcription factor 1), USF2 (upstream transcription factor 2, c-fos interacting), STAT 5 (signal transducer and activator of transcription 5A), CEBP-β {CCAAT/enhancer binding protein (C/EBP)β}, E-47 (transcription factor E2A), AP-1 (activator protein 1) and TCF-II (transcription factor 2), none of these transcription factors' binding activity was modulated by JNK (figure 3.20 and figure 3.21). This suggests the requirement of a cis element within the inhibitory region of the P-1 promoter, whose presence down-regulates the activity of the high-expression level and the transcription of SHP-1 in epithelial cells. Deletion of 227 nucleotides (–987 to –760) specifying the inhibitory region resulted in highly up-regulated levels of luciferase activity in the P1D2 construct, which could be effectively modulated by SP600125. The P-1 high-expression region was confined to 122bp contained between –760bp to –638bp as defined by deletion constructs between P1D2 and P1D12. P1D12 is only 20bp away from the TATA box in the P-1 promoter, and since its activity is not dependent on JNK (figure 3.16) it may only be due to opening of the TATA box. Furthermore, as JNK inhibition is not having much effect on

luciferase activity driven by P1D12, it is concluded that the binding site of the JNK dependent transcription factor must lie upstream of P1D12. The P-1 high-expression region contains consensus sequences for binding of CEBPβ, RFX-1 (regulatory factor binding to X box of MHCII) , Sp-1, Rel NF-κB 65, AP-1, AP-4 (activator protein 4) and TBP (TATA binding protein). The E1 and E2 oligonucleotide probes specifying binding sites for AP-4 and RFX-1 were sensitive to JNK inhibition in IGF-1 stimulated cells as demonstrated by EMSA (figure 3.22). The binding of E1 and E2 oligonucleotide probes to IGF-1 stimulated nuclear factors was dramatically decreased by increasing doses of SP600125 (figure 3.23). These results provided further evidence that the transcription factors binding to the high expression region of P-1 were regulated by JNK in IGF-1 stimulated cells.

4.3 NEED FOR BOTH RFX-1 AND AP-4 FOR SHP-1 EXPRESSION IN MCF-7 CELLS

The oligonucleotide E2 specifies consensus sequences for transcription factors AP-1 and RFX-1. RFX-1 is a member of a unique winged helix family of related transcription factors that form homodimers and heterodimers, and bind to the conserved X box in the MHC class II antigen promoter region (Reith et al, 1994). In mammals, the RFX family is composed of five members, RFX-1, RFX-2, RFX-3, RFX-4 and RFX-5. RFX-1, RFX-2, and RFX-3 are expressed ubiquitously. RFX-4 is expressed specifically in testis, and RFX-5 is identified as being essential for expression of the tissue-specific MHC class II genes (Nakayama et al, 2003). Partially palindromic sequences to which RFX-1, RFX-2 or RFX-3 binds have been found in the enhancers of hepatitis B virus (HBV), polyoma virus, cytomegalovirus (CMV), and Epstein-Barr virus (EBV). The candidate target genes to which RFX-1 binds include genes encoding the

ribosomal rpL30 protein, IL-5R, and proliferating cell nuclear antigen. RFX-1 forms heterodimers with other family members such as RFX-2 and RFX-3 (Reith et al, 1994). Previous observations of the RFX family members demonstrated that interaction of RFX with DNA sequences could be modified by the formation of RFX complexes along with other family members. RFX-1 can act as both a positive and a negative regulatory factor. For example, binding of RFX-1 to genes encoding c-myc, proliferating cell nuclear antigen and collagen II has a negative effect on gene expression. This is in contrast to RFX-1 binding to viral promoters, the genes for ribosomal protein rpL30 or IL-5 where it functions as a positive regulatory factor (Hibi et al, 1993 and Juliger et al, 2003). A similar function for RFX-1 as a positive regulatory factor was observed in the regulation of the SHP-1 P-1 promoter in IGF-1 stimulated MCF-7 cells as demonstrated by antisense technology (figure 3.28 and figure 3.29). Furthermore, a positive role of RFX-1 in the SHP-1 P-1 promoter was substantiated by making point mutations in the RFX-1 binding site in the P1D2 promoter (figure 3.30).

The oligonucleotide E1 has the consensus sequence for binding of the transcription factors AP-4 and AP-1. The role of the AP-4 transcription factor was demonstrated by using antisense technology (figure 3.27 and figure 3.29). As described SHP-1 expression in MCF-7 breast cancer cells was inhibited most strongly by the AP-4 specific antisense oligonucleotide. In addition to RFX-1, point mutation of the AP-4 binding site decreased luciferase expression from the P1D2/pGL3 luciferase construct substantiating the role of AP–4 on SHP-1 expression. AP-4 is a 48kDa protein, which recognizes a conserved symmetric core DNA sequence, CANNTG and belongs to the helix-loop-helix (HLH) family of DNA binding proteins. The members of this group play a role in cellular differentiation and proliferation (Juliger et al, 2003). The HLH motif is required for transcription factor dimerization and DNA binding. In addition to the HLH motif,

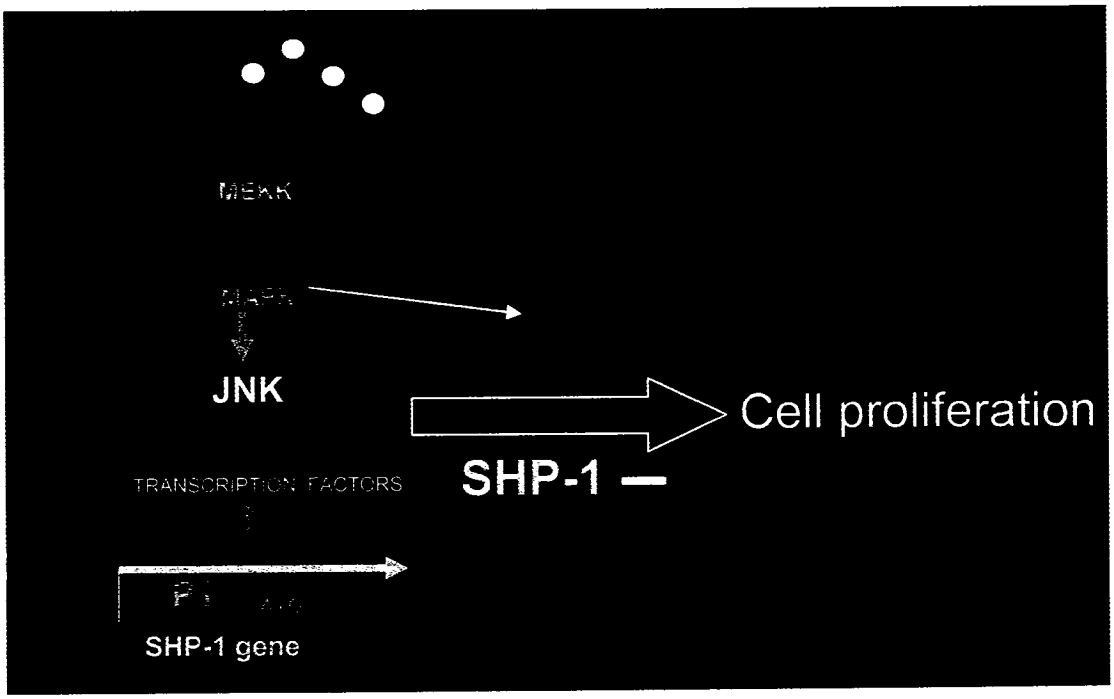
two other distinct leucine repeat elements direct AP-4 dimerization and allow selective complex formation. AP-4 was shown to activate human metallothionein IIA gene transcription and to participate in the regulation of human pro-enkephalin. Some other genes requiring AP-4 include IFN-γ1R (Xiu et al, 2002), FcγRIIβ (Andriamanalijaona et al, 2003) and TGF-1β (Schlechter et al, 1986). AP-4 binding sites often overlap with the recognition sequences of AP-1 transcription factor (Schlechter et al, 1986). This suggests that AP-1 and AP-4 may function in concert to activate transcription of a variety of genes by co-operatively binding to their promoter. For instance, AP-1 can act synergistically with AP-4 to activate SV40 late gene transcription. AP-1 consists of a complex of jun and fos proteins. The oncoprotein c-jun has been shown to be hyper-activated by JNK in a variety of cells including epithelial cells (Juliger et al, 2003). Although the AP-1 binding site is present in the E1 oligonucleotide, derived from the high-expression region of P-1, mutation of this site or the addition of anti-c-jun specific antibody did not diminish E1 binding to IGF-1 stimulated MCF-7 nuclear proteins, suggesting that c-jun did not play a major role in this interaction (figure 3.24 and figure 3.26).

Taken together SHP-1 is an important negative regulator of breast cancer cell growth where SHP-1 level is highly up-regulated in these cells. Results presented in this thesis demonstrate that in IGF-1 stimulated cells the anti-proliferative activity of JNK was correlated with SHP-1 expression. For the first time, in this study it has been shown that IGF-1 stimulation induces SHP-1 expression by activating JNK and P38. JNK in turn activates RFX-1 and AP-4 transcription factors to bind to the high expression region of the SHP-1 P-1 promoter in breast adenocarcinoma MCF-7 cells. It has also been shown that SHP-1 expression is dependent on the binding of RFX-1 and AP-4 transcription factors to the P-1 promoter high- expression region, in IGF-1 stimulated MCF-7 cells.

Chapter 5: **CONCLUSION AND SIGNIFICANCE**

This research was aimed at revealing the negative regulators of IGF-1-induced proliferation of human breast-adenocarcinoma cells. We have defined for the first time the negative feedback mechanism by which IGF-1 exerts both positive and negative effects on the growth of breast cancer cell lines. We demonstrate that enhanced SHP-1 expression resulted in inhibition of IGF-1-induced cell proliferation. Studies designed to understand the molecular mechanism revealed that SHP-1 gene expression was regulated by JNK through activation of RFX-1 and AP-4 transcription factors in IGF-1 stimulated breast cancer cells. Taken together, these results reveal a mechanism by which breast cancer cells modulate excessive growth induced by up-regulated expression of growth factors (IGF-1) as depicted in figure K. To escape from IGF-1 stimulated enhanced proliferation, breast cancer cells activate protective "SOS responses" involving a negative regulator of cell growth, SHP-1, through activation of the stress kinase JNK. This mechanism provides an explanation for several folds enhanced levels of SHP-1 protein in breast adenocarcinoma cells. These findings may be beneficial in designing novel therapeutic interventions, based on signaling proteins identified in this study, for treatment of not only breast cancer but other degenerative disease including ageing.

Figure 5.1 The negative feedback mechanism by which IGF-1 exerts both positive and negative effects on the growth of breast cancer cells.



Chapter 6: REFERENCES

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TABLE 1 : LIST OF PRIMERS

3' PRIMER

AAAAAAAAGCTTTCCTGTTAGTTTGGAGGGA

5' PRIMERS

P-1:	**AAAAAACTCGAGCCCAGTGTAGACCAAATGTC**
P1D1	**AAAAAACTCGAGGCACGCTGGTCCTTAGGAGA**
P1D2:	**AAAAAACTCGAGGGAAGCTGAAGGC GCTGTT**
P1D4:	**AAAAAACTCGAGCAGGTTGAAGAGGCTGGAGTG**
P1D5:	**AAAAAACTCGAGGGTTACTTCCTGGTCTGTTC**
P1D7:	**AAAAAACTCGAGGGCCTGCATGCGTCCTGTTT**
P1D8:	**AAAAAACTCGAGCAGTGGGGGAGCAGTTCATG**
P1D9:	**AAAAAACTCGAGCTCGCTTGGTCAAGTCCTA**
P1D10:	**AAAAAACTCGAGTCCTGAGATCGCGAGCCTGT**
P1D11:	**AAAAAACTCGAGAGAGTTGGCCCTCCCTTGTG**
P1D12:	**AAAAAACTCGAGGCCTTGATTTCCAGACAAT**

TABLE 2: LIST OF OLIGONUCLEOTIDES

E1 Oligonucleotides

E1: **5'TGTC $^{(AP-1)}$AGGCAAGC $^{(AP-4)}$TGAAGGCGCTGTTT**

MUTATION E1A: **5'**AGCC **GCGAGTGCAAGCTGAAGGCGCTGTTT**

MUTATION E1B: 5' **TGTC**TGTGAGGCAA**TACTAAGGATTGCC**

MUTATION E1C: **5'**AGCC**GCGAGGAGCATACTAAGGATTGCCTT**

MUTATION E1D: **5'AGCCT GTCAGGCAAGCTGAAGGCGCTGTT**

MUTATION E1E: 5'AGCC**TGTCAGGCAAGCTGCGATCGCTGTTT**

E2 Oligonucleotides

E2 **5'CTGTTGCCCATGACCCTGCCACCCCATGGG**

MUTATION E2A: 5'CT**ACCTACCATGACCCTGCCACCCATGGG**

MUTATION E2B: 5'CTGTTGCCCA**GTCTGCTACCACCCCATG**GG

MUTATION E2C : 5'CT**ACCTACCAGTCTGCTACCACCCCATGGG**

MUTATION E2D: **5'**CTGTTGCCCATGACCCTGCCACCCCA**CTTT**

- Obtained scholarship from supervisor's grant of Genomics Canada.
- Ranked in the top 1% in the 5 years of medical school.
- High school merit scholarship from **Comilla Board** of Secondary and higher secondary education, Comilla , Bangladesh
- Merit scholarship for outstanding achievement in grade 8 from **Chittagong Division**, Chittagong, Bangladesh

* Presentations*

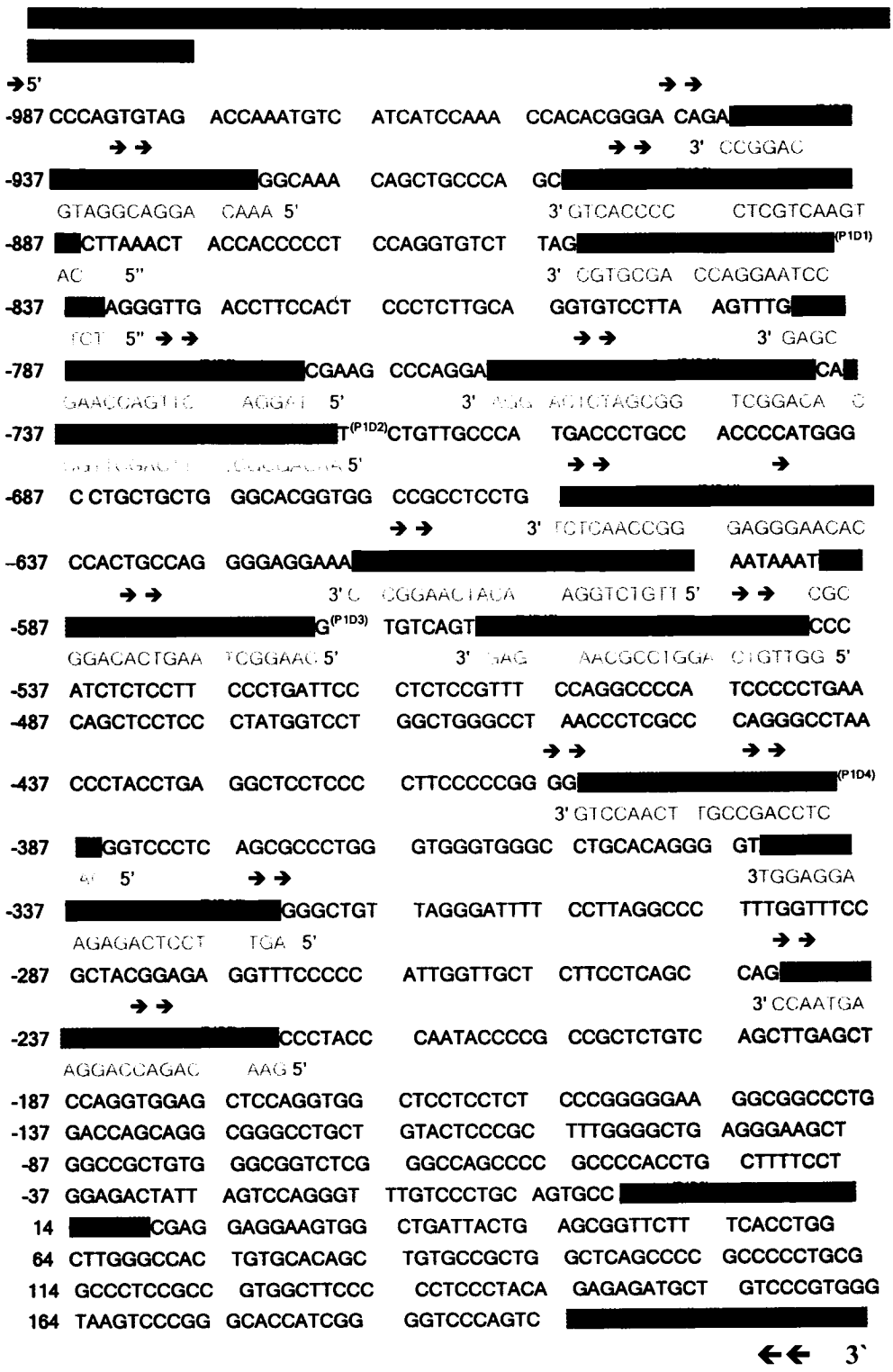
- The role of tyrosine phosphatases in cancer, poster presentation, University of Ottawa, Roger Guidon Hall, Department of Biochemistry, Microbiology and Immunology, April 2002
- Protein Tyrosine Phosphatase SHP-1 in breast cancer, seminar presentation, University of Ottawa Heart Institute, Ottawa, ON, November 2002.
- Protein tyrosine Phosphatase IGF-1 and Breast cancer , Seminar presentation, Health Canada Research Forum, October 2003, Ottawa, ON.
- Regulation of Tyrosine Phosphatase SHP-1 in Breast Cancer is dependent on transcription factors RFX-1 and AP-4, poster presentation, University of Ottawa, Rogers Guidon Hall, Ottawa, ON, April 2004.
- Regulation of Tyrosine Phosphatase SHP-1 in Breast Cancer is dependent on transcription factors RFX-1 and AP-4, seminar and poster presentation, Health Canada Research Forum, October 2004.

Abilities/ Interests

- Performed research in Health Canada on *breast cancer.*
- Performed experiments for making *Rabies vaccines* in NIPSOM, Dhaka Bangladesh.
- Experience in treating patients with *chronic chest infections.*
- Adept in Microsoft word, Microsoft powerpoint and Microsoft excel.
- Adept in using Lotus notes, Corel presentation and Wordperfect software.
- Adept in using Adobe acrobat reader, writer and photoshop.
- Adept in using **Clone-manager** software, **MatInspector** software and **reference manager** software.
- Adept in searching and writing scientific articles for publication.

References

- References available upon prompt request


5'
-987 CCCAGTGTAG ACCAAATGTC ATCATCCAAA CCACACGGGA CAGA
3' CCGGAC
-937 GGCAAA CAGCTGCCCA GC
GTAGGCAGGA CAAA 5'
3' GTCACCCC CTCGTCAAGT
-887 CTTAAACT ACCACCCCCT CCAGGTGTCT TAG (P1D1)
AC 5"
3' CGTGCGA CCAGGAATCC
-837 AGGGTTG ACCTTCCACT CCCTCTTGCA GGTGTCCTTA AGTTTG
TCT 5"
3' GAGC
-787 CGAAG CCCAGGA CA
GAACCAGTTC ACGAT 5'
3' AGG ACTCTAGCGG TCGGACA C
-737 T(P1D2) CTGTTGCCCA TGACCCTGCC ACCCCATGGG
5'
-687 C CTGCTGCTG GGCACGGTGG CCGCCTCCTG
3' TCTCAACCGG GAGGGAACAC
-637 CCACTGCCAG GGGAGGAAA AATAAAT
3' C CGGAACTACA AGGTCTGTT 5' CGC
-587 G(P1D3) TGTCAGT CCC
GGACACTGAA TCGGAAC 5'
3' GAG AACGCCTGGA CTGTTGG 5'
-537 ATCTCTCCTT CCCTGATTCC CTCTCCGTTT CCAGGCCCCA TCCCCCTGAA
-487 CAGCTCCTCC CTATGGTCCT GGCTGGGCCT AACCCTCGCC CAGGGCCTAA
-437 CCCTACCTGA GGCTCCTCCC CTTCCCCCGG GG (P1D4)
3' GTCCAACT TGCCGACCTC
-387 GGTCCCTC AGCGCCCTGG GTGGGTGGGC CTGCACAGGG GT
5'
3TGGAGGA
-337 GGGCTGT TAGGGATTTT CCTTAGGCCC TTTGGTTTCC
AGAGACTCCT TGA 5'
-287 GCTACGGAGA GGTTTCCCCC ATTGGTTGCT CTTCCTCAGC CAG
3' CCAATGA
-237 CCCTACC CAATACCCCG CCGCTCTGTC AGCTTGAGCT
AGGACCAGAC AAG 5'
-187 CCAGGTGGAG CTCCAGGTGG CTCCTCCTCT CCCGGGGGAA GGCGGCCCTG
-137 GACCAGCAGG CGGGCCTGCT GTACTCCCGC TTTGGGGCTG AGGGAAGCT
-87 GGCCGCTGTG GGCGGTCTCG GGCCAGCCCC GCCCCACCTG CTTTTCCT
-37 GGAGACTATT AGTCCAGGGT TTGTCCCTGC AGTGCC
14 CGAG GAGGAAGTGG CTGATTACTG AGCGGTTCTT TCACCTGG
64 CTTGGGCCAC TGTGCACAGC TGTGCCGCTG GCTCAGCCCC GCCCCCTGCG
114 GCCCTCCGCC GTGGCTTCCC CCTCCCTACA GAGAGATGCT GTCCCGTGGG
164 TAAGTCCCGG GCACCATCGG GGTCCCAGTC
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