MECHANISM OF INHIBITION OF MCF-7 CELL PROLIFERATION BY ALKYLLYSOPHOSPHOLIPIDS (ALPs) : EFFECT OF ALPs ON INSULIN SIGNALLING PATHWAYS

BY

CHRISTINA RICHARD

A Thesis
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Mechanism of Inhibition of MCF-7 Cell Proliferation by Alkyllysophospholipids (ALPs): Effect of ALPs on Insulin Signalling Pathways

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Christina Richard

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of MASTER OF SCIENCE

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Dedication

To my parents for everything they've given and done for me

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ABBREVIATIONS

Ab	antibody
AEBSF	aminoethylbenzenesulfonyl fluoride
ALPs	alkyllysophospholipids
ATCC	American Type Culture Collection
ATP	adenosine 5' triphosphate
BM41.440	1-S-hexadecylthio-2-methoxymethyl-*sn*-glycero-3-phosphocholine (ilmofosine)
BSA	bovine serum albumin
cAMP	cyclic adenosine 3',5'-monophosphate
Ci	Curie
CR 1, 2 or 3	conserved region 1, 2 or 3
CRD	cysteine-rich domain
cys	cysteine
C-terminus	carboxyl terminus or COOH-terminus
DDW	distilled deionized water
DMEM	Dulbecco's modified Eagle medium
DMEM/BSA	DMEM containing 0.5 mg / ml bovine serum albumin
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylene diaminetetraacetic acid
EGF	epidermal growth factor

EGFr	epidermal growth factor receptor
EGTA	ethyleneglycol-bis-(β-amino-ethyl ether)N,N'-tetraacetic acid
ERK1 or 2	extracellular signal-regulated protein kinase 1 or 2
ET-18-OCH_3	1-*O*-octadecyl-2-*O*-methylglycerophosphocholine (edelfosine)
FBS	fetal bovine serum
FGF	fibroblast growth factor
GAP	GTPase-activating protein
GDP	guanosine 5'-diphosphate
Grb2	growth factor receptor-bound protein 2
GSK	glycogen synthase kinase-3
GTP	guanosine 5'-triphosphate
HBSS	Hank's balanced saline solution
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
hsp	heat shock protein
IGF-1	insulin-like growth factor 1
IH1/IH2	IRS-homology 1 (or 2)
IP3	inositol triphosphate
IPTG	isopropyl β-*D*-thiogalactopyranoside
IR	insulin receptor
IRS	insulin receptor substrate

kDa	kilodaltons
KGF	kerotinocyte growth factor
KSR	kinase suppressor of Ras
LB	Luria broth base
LPA	lysophosphatidic acid
LPAF	lysoplatelet-activating factor
LPC	lysophosphatidylcholine
LPE	lysophosphatidylethanolamine
LPI	lysophosphatidylinositol
MAPK	mitogen-activated protein kinase
MBP	myelin basic protein
MEK	mitogen-activated protein kinase/extracellular signal regulated protein kinase kinase
MOPS	(3-[N-Morpholino] propane sulfonic acid
NP-40	nonidet p-40
N-terminus	amino- or NH_2-terminus
PA	phosphatidic acid
PBS	phosphate buffered saline solution
PBST	PBS with Tween-20
PC	phosphatidylcholine
PDGF	platelet-derived growth factor
PDK1/PDK2	phosphoinositide-dependent protein kinase 1 or 2

PH	Pleckstrin homology
PI	phosphatidylinositol
PI3K	phosphoinositide 3-kinase
PIK	phosphoinositide kinase
PIP	phosphatidylinositol (3) phosphate
PIP2	phosphatidylinositol (3,4) bisphosphate
PIP_3	phosphatidylinositol (3,4,5) triphosphate
PI-PLC	phosphoinositide-specific phospholipase C
PKB	protein kinase B
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethylsulfonyl fluoride
PTB	phosphotyrosine binding
PtdIns	phosphatidylinositol
RBD	Ras-binding domain
Shc	Src homology 2/α-collagen-related
SHPTP2	SH2-containing protein-tyrosine phosphatase
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Ser (S)	serine
SH2 or 3	Src homology 2 or 3

Sos	Son of Sevenless
SRI 62-834	2-{hydroxy[tetrehydro-2-(octadecyloxy)methylfuran-2-1-methoxyl]phosphinyloxy}-*N,N,N*-trimethylethaniminium-hydroxide
TBS	tris-buffered saline solution
TBST	TBS with tween-20
TCA	trichloroacetic acid
TDB-PC	ET-16-phosphono-TDB or 2'-(trimethylammonio)-ethyl-4-(hexadecyloxy)-3-methoxy-1-butenephosphonate
Thr (T)	threonine
TLC	thin-layer chromatography
Tris-HCl	Tris[hydroxymethyl]aminomethane hydrochloride
Tyr (Y)	tyrosine

ABSTRACT

Alkyllysophospholipids (ALPs) are ether analogs of lysophosphatidylcholine that display antitumour properties *in vitro* and *in vivo*. The mechanism of its antiproliferative actions remains unknown. Previous studies have shown that 1-*O*-octadecyl-2-*O*-methylglycerophosphocholine (ET-18-OCH_3), the prototype ALP, inhibited MCF-7 cell proliferation by interfering in the activation of Raf-1 by perturbing the association of Raf-1 with the cell membrane. This consequently led to inhibition of mitogen-activated protein kinase (MAPK) and ultimately resulted in inhibition of cell growth. The objective of this study was to investigate whether the inhibition of Raf-1 activation by ET-18-OCH_3 was sufficient to completely inhibit the growth of MCF-7 cells.

We examined the effects of ET-18-OCH_3 on the multiple diverging signalling pathways activated following insulin stimulation. The cells treated with ET-18-OCH_3 showed decreased MAPK activity and decreased Raf-1 association with the membrane, verifying previous results obtained subsequent to epidermal growth factor stimulation. Further examination showed that ET-18-OCH_3 decreased the binding of a GST-Ras-GTPγS fusion protein with Raf-1 providing evidence that ET-18-OCH_3 may inhibit the association of Raf-1 with Ras in intact cells.

ET-18-OCH_3 did not interfere with the tyrosine phosphorylation of insulin-receptor-substrate-1 (IRS-1) nor did it affect the association between IRS-1 and phosphoinositide 3-kinase (PI3K). ET-18-OCH_3 directly inhibited the activity of

PI3K *in vitro* and also inhibited the phosphorylation and activation of protein kinase B (PKB) and p70 S6 kinase, downstream targets of PI3K.

In order to determine whether these inhibitions are causal to the decrease in cell growth, we examined the effect of two enantiomeric ALPs, (*R*)- and (*S*)-2'-(trimethylammonio)-ethyl-4-(hexadecyloxy)-3-methoxy-1-butenephosphonate (TDB-PC), with a differential effect on cell proliferation and MAPK activation, on the activation of PKB and p70 S6K. (*S*)-TDB-PC was previously demonstrated to inhibit MCF-7 cell proliferation to a much greater extent than (*R*)-TDB-PC. Phosphorylation and activation of PKB and p70 S6 kinase were inhibited when MCF-7 cells were treated with either enantiomer, however, the degree of inhibition was much greater with (*S*)-TDB-PC, the compound that inhibited cell proliferation. When the cells were treated with (*S*)-TDB-PC, there was complete inhibition of both PKB and p70 S6 kinase activity while there was only partial inhibition with (*R*)-TDB-PC suggesting that the inhibition of these molecules may have some relevance to inhibition of cell growth.

Taken together, our studies show that ET-18-OCH_3 inhibits the association of Raf-1 with the membrane by inhibiting the association of Raf-1 with Ras. In addition to the inhibition of Raf-1 association with the membrane, the inhibition of the PI3K pathway by ET-18-OCH_3 appears to also contribute to the inhibition of cell proliferation.

1 BACKGROUND AND LITERATURE REVIEW

1.1 CANCER

The malignant transformation of a normal cell into a cancer cell requires the activation of multiple oncogenes and the loss of tumour-supressor genes. Oncogenes code for proteins that are components of growth factor-activated intracellular signalling pathways (Powis, 1991). When an oncogene is overexpressed or constitutively active through mutation, the cell receives a continuous signal to grow. Likewise, loss of function mutations in tumour-supressor genes relieve cells of inhibitions which normally control their numbers. In essence, cancer cells defy the normal controls of cell division, and they may invade and colonize territories normally reserved for other cells. If proliferation of a cell is out of control, it will give rise to a tumour, and when the cells invade surrounding tissue, the cells become malignant (Fialkow, 1976). The more widely a cancer metastasizes, the harder it becomes to eradicate. The need to stop the growth and spreading of cancer cells is obvious. Cancer cells can be removed surgically or destroyed with toxic chemicals or radiation. But, many human cancers are refractory to chemotherapy. The problem with finding a cure for cancer is that treatments that kill cancer cells are often toxic to normal cells as well. Many of the conventional anti-cancer drugs prevent synthesis of DNA or interfere in DNA function. They are, therefore, non-selective in their molecular action and do not discriminate in their binding between tumour and normal DNA. Hence, there is a

necessity to develop new anti-cancer drugs that would kill and eliminate the cancer cells without affecting the normal cells.

1.2 : ALKYLLYSOPHOSPHOLIPIDS

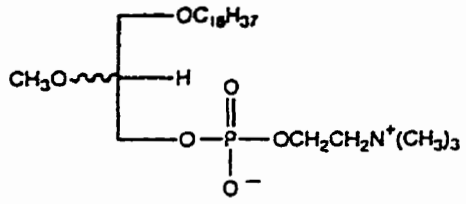
1.2.1 DEVELOPMENT OF ALPs

Alkyllysophospholipids (ALPs) are ether analogs of lysophosphatidylcholine (LPC) and were developed to investigate the involvement of LPC in macrophage activation. During phagocytosis, phospholipase A_2 is activated which leads to an over-production of LPC which accumulates in the cell membrane of the macrophage (Munder *et al.*, 1966). LPC was difficult to study as it has a short half-life *in vitro* and *in vivo.* It could be attacked by both acyltransferases and lysophospholipases. The addition of a fatty acid in the presence of acyltransferase would convert LPC to phosphatidylcholine. Likewise, lysophospholipase will hydrolyze the ester function in position 1 to form glycerophosphocholine. To circumvent the instability of LPC, LPC analogs were synthesized that would have a longer half-life *in vivo.* The ester function at position 1 in the glycerol backbone was replaced with long chain ethers and the hydroxyl function at position 2 was replaced with a short chain ether (Munder and Westphal, 1990). By replacing positions 1 and 2 of LPC, the resulting molecules were no longer susceptible to metabolism. When these ether analogs of LPC were tested, many of them were found to have strong antitumour activities (Munder *et al.*, 1977; Tarnowski *et al.*, 1978). With the ether linkage in

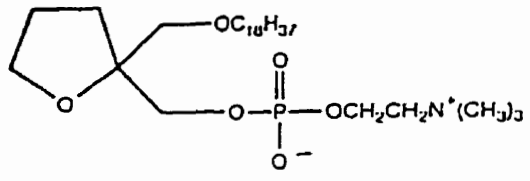
position 1 and a metabolically stable group in the 2 position, these compounds had anti-proliferative effect on various cancer cells and were cytotoxic at micromolar concentrations. However, with an ester analog at position 1, these analogs were inefficient within the same dose range (Andreesen *et al.*, 1978). Hence, the birth of a new class of antitumour compounds was achieved, and these new molecules were termed alkyllysophospholipids or ALPs.

1-*O*-octadecyl-2-*O*-methylglycerophosphocholine (ET-18-OCH_3), in which the ester function at position 1 was replaced with an 18-carbon chain ether and the hydroxyl group at position 2 was replaced with a methoxy group, turned out to be the most active compound and became the prototype of the series. Since the initial development of these antitumour compounds, work has been done to make structurally related compounds with possible antineoplastic activity, and many related compounds have been synthesized. Many of these compounds have been successful in inhibiting the proliferation of cancer cell lines. These drugs include BM 41.440 (or ilmofosine), a thioester phosphopholipid (Herrmann *et al.*, 1987; Girgert *et al.*, 1995) ; SRI 62-834, a cyclic ether analog of ET-18-OCH_3 in which the oxygen atom at position 2 of the glycerol backbone has been incorporated into a five-membered heterocycle (Houlihan *et al.*, 1987); and hexadecylphosphocholine (miltefosine) which lacks the glycerol backbone (Danhauser-Riedl *et al.*, 1990; Verweij *et al.*, 1992). **Figure 1** shows the structures of these different ALPs.

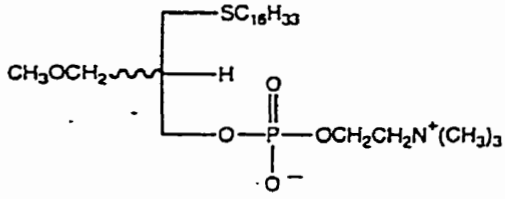
These compounds have direct effects on neoplastic cells; they are cytotoxic, anti-invasive and can induce cell differentiation. Many conventional anticancer



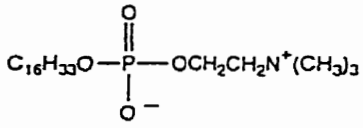
ET18-OCH3 (edelfosine)



SRI 62-834



BM 41.440 (ilmofosine)



HePC (miltefosine)

Figure 1. Structure of the different ALPs

The structure of the different ALPs are shown. ET-18-OCH_3 (edelfosine), the prototype of the series; SRI 62-834, a cyclic ether analog; BM 41.440 (ilmofosine), a thioester phospholipid; HePC (miltefosine), which lacks the glycerol backbone.

drugs act mainly by preventing the synthesis of DNA or by interfering with DNA function. In contrast, ALPs exert their anticancer activity without interaction with DNA (Berdel, 1991; Lohmeyer and Bittman, 1994). These compounds are selective in the sense that normal cell lines are relatively unaffected under conditions that are toxic to cancer cells (Berdel, 1991). Because of their selectivity, these ether lipids are considered to be a promising group of anti-cancer drugs and are being used in clinical trials. ET-18-OCH_3 has been tested for use in purging residual malignant cells from marrows prior to autologous bone marrow transplantation (Berdel *et al.*, 1992; Vogler and Berdel, 1993).

1.2.2 MECHANISM OF ACTION OF ALPs

Although ALPs have been shown to inhibit cell proliferation, the mechanism by which they accomplish this is not yet known. There have been a number of different hypotheses proposed to account for their growth inhibitory actions and their selectivity. It is well known that ALPs are incorporated into the plasma membrane and this is thought to be where they exert their growth inhibitory effects (Scholar, 1986; Berdel *et al.*, 1983). But, their cellular distribution may be broader as the ALPs have been shown to inhibit phosphocholine cytidylyltransferase which is an intracellular enzyme (Muschiol *et al.*, 1987; Houlihan *et al.*, 1987). Hence the effect of ALPs on the cell may be more widespread affecting both plasma membrane and intracellular processes.

Many enzymes with lipid binding sites are inhibited by ALPs and it was

thought that the inhibition of lipid metabolism may be a key component in their antiproliferative effects. ALPs have been shown to interfere with phosphatidylcholine metabolism in a number of different cell lines (Weider *et al.*, 1995; Zhou and Arthur, 1995; Unger *et al.*, 1987), but the contribution of inhibition of phosphatidylcholine biosynthesis to the antiproliferative activity of ALPs has been questioned. It was found that neither overexpression of CTP: phosphocholine cytidylyltransferase nor lysophosphatidylcholine supplementation allowed Hela cells to proliferate in the presence of ET-18-OCH_3 (Baburina and Jackowski, 1998). This indicated that the cytostatic property of ET-18-OCH_3 was independent of its effect on membrane phospholipid biosynthesis. In addition to cytidylyltransferase, a number of enzymes have been shown to be inhibited by ALPs including acyltransferases (Herrmann *et al.*, 1986), diacylglycerol kinase (Salari *et al.*, 1992), phosphatidylinositol 3-kinase activity (Berggren *et al.*, 1993), and phosphatidylinositol specific phospholipase C (PI-PLC) (Powis *et al.*, 1992).

PI3K and PI-PLC are key enzymes that generate second messengers thought to participate in mitogenic signalling; the inhibition of either of these enzymes could result in inhibition of cell growth. ALPs have been shown to be potent inhibitors of PI-PLC, but some ALPs inhibit IP_3 formation while having little effect on cell growth and some ALPs which inhibit cell growth have little effect on IP_3 production (Junghahn *et al.*, 1995). Therefore the exact contribution of inhibition of PI-PLC to decreased cell growth is not known. The role of PI3K in cell growth has also been questioned. Although the activity of PI3K has been shown

to be inhibited by ALPs, whether this results in growth inhibition is not clear.

These ether lipids also cause an increase in intracellular calcium levels but studies show that this increase is likely not the key mechanism by which these drugs lead to growth inhibition (Seewald *et al.*, 1990; Brinkmeier *et al.*, 1996).

Since ALPs have antiproliferative activity, it suggests that ALPs may inhibit signalling molecules that are necessary for cell growth. Many studies have examined the effect of ALPs in signal transduction pathways. The initial step in signal transduction pathways is the binding of ligand to its receptor. Inhibition of this binding could potentially result in inhibition of cell growth. The effects of ALPs on different cell surface receptors have been examined. Upon examination of the epidermal growth factor receptor (EGFr), ET-18-OCH_3 was found to inhibit the internalization of the receptor in MCF-7 cells although it had no effect on ligand binding (Zhou *et al.*, 1996). On the other hand, this inhibition of receptor internalization occurred after 30 minutes with only a 15% inhibition between drug-treated and control cells (Zhou *et al.*, 1996) suggesting that this is unlikely to be the mechanism by which ALPs inhibit cell growth. Also, the kinetics of activation of the EGFr in control and drug-treated cells were found to be the same (Zhou *et al.*, 1996). ET-18-OCH_3 also had no effect on platelet-derived growth factor binding to its receptor (Seewald *et al.*, 1990). Inhibition in receptor number and receptor internalization of the granulocyte-macrophage stimulating factor receptor was observed (Shoji *et al.*, 1994) but because this receptor is not expressed in all cells, signalling via this receptor cannot be a universal mechanism for the action of ALPs.

ET-18-OCH_3 was also found to cause a decrease in estrogen receptor number (Shoji *et al.*, 1994) as well as in progesterone receptors and transforming growth-factor levels (Kosano *et al.*, 1990). It is not known whether these decreases caused by the ALPs are sufficient to affect cell growth. ET-18-OCH_3 also caused an increase in transferrin receptor number and in the affinity of transferrin for its receptor, but because this was observed to a similar extent in sensitive and insensitive cell lines (Kosano *et al.*, 1990; Shoji *et al.*, 1991), it is unlikely to serve as the mechanism of inhibition.

The ALPs also have been shown to affect protein kinase C (PKC) activity. Protein kinase C are a family of ubiquitously distributed Ser/Thr protein kinases that participate in a wide variety of cellular functions. PKC has been shown to have important roles in transducing mitogenic signals. Some conflicting reports show that ALPs inhibit the kinase (Daniel *et al.*, 1987) while others demonstrate that PKC is elevated (Heesbeen *et al.*, 1991). This controversy may be moot as the inhibition of PKC in intact cells does not always correlate with the cytotoxicity of these drugs (Salari *et al.*, 1992). Also, the effect of ALPs on PKC in intact cells has been difficult to attribute. Many of the studies are based on the assumption that translocation of PKC is synonymous with activation which has been shown not to be the case. To get around this problem, the phosphorylation of endogenous proteins by PKC in MCF-7 cells was examined in the presence and absence of ET-18-OCH_3 (Zhou *et al.*, 1997). This study showed that the drug had no effect on PKC translocation but phosphorylation of specific proteins by PKC was inhibited by

the drug. Whether the inhibition of phosphorylation of these proteins correlates with cell growth is not known. It is also possible that the effect of the drugs may be different in different cell types thereby contributing to some of the opposing effects of the drug on PKC.

The mitogen-activated protein kinase cascade is an important signal transduction pathway in the cell and its activation is necessary for cell growth. If the ALPs affect molecules in this signal transduction pathway, this could likely contribute to inhibition of cell growth. An investigation to determine whether ALPs interfere in the MAPK pathway was undertaken (Zhou *et al.*, 1996) and it was found that ET-18-OCH_3 inhibits MAPK activity. The drug had no effect on Ras activation and it did not directly affect Raf-1 kinase activity but it did interfere with the association of Raf-1 with the membrane.

Another question which remains unanswered is why ALPs inhibit the growth of various cancer cells without affecting normal cells. Reasons for this selectivity have been attributed to their rate of uptake (Bazill *et al.*, 1990), their different affinities for acyltransferases (Herrmann and Neumann, 1986), and the rate at which they are metabolized (Bishop *et al.*, 1992). However, there are reports which argue against the above parameters being responsible for their selective effects (Lu and Arthur, 1992a; Lu and Arthur, 1992b; Zhou *et al.*, 1992; Lu *et al.*, 1993).

Although ALPs have been shown to affect a number of different processes in the cell, it was not known whether these effects cause the inhibition of cell proliferation or whether they occur simply because of decreased cell growth. In the

process of trying to address this problem, two additional drugs were developed by Dr. Robert Bittman. These compounds are enantiomeric analogs of ET-18-OCH_3 which have different effects on the proliferation of a number of cancer cells (Samadder, 1998). (*R*)-ET-16-phosphono-TDB has no effect on cell growth, MAPK activity or Raf-1 activation, while the *S* enantiomer, (*S*)-ET-16-phosphono-TDB has a significant effect on cell growth (Samadder, 1998). These compounds should prove useful in determining whether inhibition of molecules in the cell are related to the inhibition of cell growth or if they are merely a nonspecific effect of the ALPs.

1.3 : MAP KINASE SIGNALLING PATHWAYS

The mitogen-activated protein kinase (MAPK) cascade is a signal transduction cascade found in a wide range of organisms from yeast to mammals. This cascade is widely conserved and several MAPK cascades are co-expressed in the same cell (Neiman, 1993). MAPK activation can be initiated by many types of cell surface receptors including receptor tyrosine kinases and receptors with seven transmembrane helices (heterotrimeric G proteins). The activation of MAP kinases results in such cellular processes as proliferation, differentiation and development (**Figure 2**).

1.3.1 MAP KINASE ACTIVATION BY RECEPTOR TYROSINE KINASES

The traditional MAPK pathway is activated by a number of mitogens such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and nerve

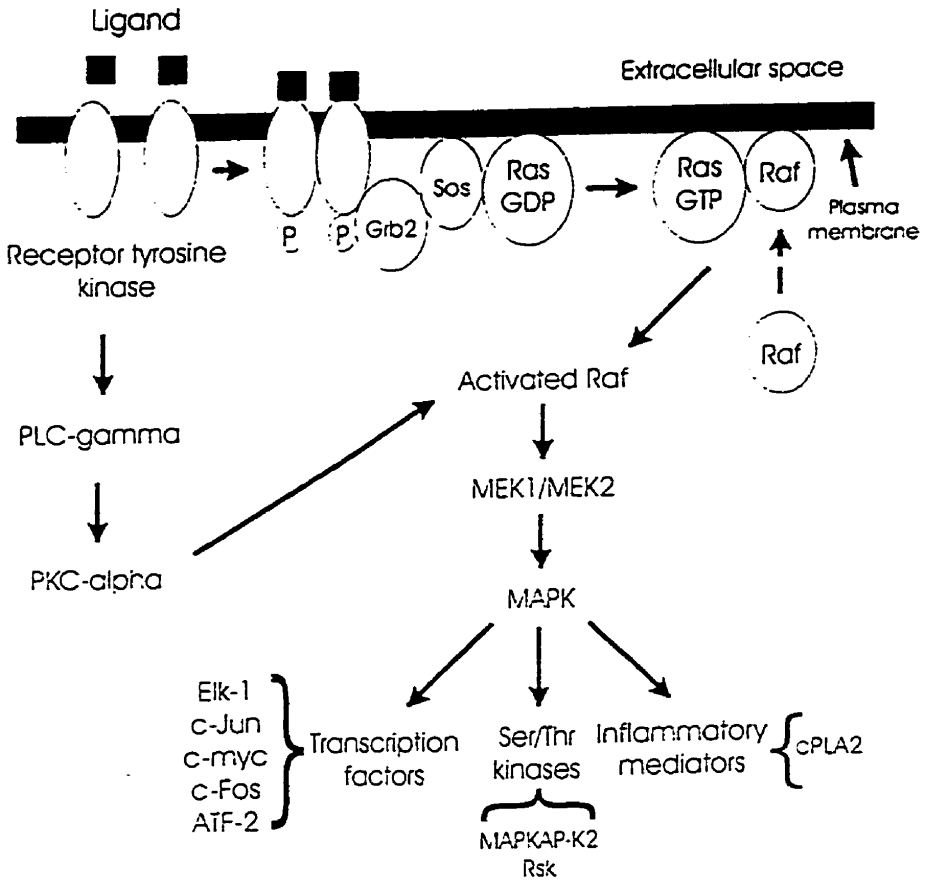


FIGURE 2. MAPK Pathway Ligand binds to its receptor resulting in receptor dimerization and autophosphorylation of tyrosine residues in the cytoplasmic domain. Grb2 binds to specific phosphotyrosine residues on the receptor via its SH2 domain. Grb2 also binds to Sos via its SH3 domain thus bringing Sos to the plasma membrane. Sos catalyzes the release of GDP on Ras and the subsequent binding of GTP thus activating Ras. Active Ras then binds to Raf-1 recruiting it to the plasma membrane where it becomes activated. Activated Raf-1 phosphorylates and activates the dual specificity kinase MEK which in turn phosphorylates and activates MAPK. Activated MAPK phosphorylates a number of different cellular substrates including transcription factors and other ser/thr kinases (from Ashagbley, 1997)

growth factor (NGF). Binding of these mitogens to their respective receptors results in receptor dimerization and induces autophosphorylation of these receptors on tyrosine residues by activation of intrinsic catalytic kinase domains (Ullrich and Schlessinger, 1990). Once the receptor is phosphorylated on multiple tyrosine residues, probably in a trans-phosphorylation reaction with the dimers (Honeggar *et al.*, 1990), it becomes activated and is then able to bind to the src homology 2 (SH2) domains present in many cytoplasmic proteins. These domains are a stretch of approximately 100 amino acids and are called SH2 domains by virtue of their considerable sequence similarity to the non-catalytic region of the src family of protein tyrosine kinases (Pawson and Gish, 1992). One of the proteins that binds to the activated EGF receptor is Grb2 (Egan *et al.*, 1993) which has a high affinity for phosphorylated Try1068 of the EGF receptor. Grb2 is a 23-25 kDa adaptor protein which contains one SH2 domain flanked by two SH3 domains (Matuoka *et al.*, 1992). Grb2 is able to bind to the proline rich domain of the Ras guanine nucleotide exchange factor Sos (named from the Drosophila son of sevenless gene) via its SH3domain. Therefore, when the receptor is activated and Grb2 is bound, Sos is brought in contact with the plasma membrane allowing it access to Ras, its substrate.

1.3.2 RAS

The ras family of proto-oncogenes encode low molecular weight guanine nucleotide-binding proteins. Activating mutations in Ras result in constitutive

signalling to downstream elements and are found at a high frequency in a wide variety of tumours (Barbacid, 1987). In mammals, three ras genes have been identified and designated as Harvey ras (H-ras), Kirsten-ras (K-ras) and N-ras. These proteins are localized to the membrane and this localization is essential for their biological function. The association to the membrane takes place after farnesylation of a Cys residue at position 186 (Hancock and Marshall, 1993). This process is followed by proteolysis of the last three residues of the molecule (C-AAX) in the carboxyl region conserved in all ras proteins. Also, all ras proteins with the exception of K-ras are palmitoylated in a second Cys residue near Cys186 in the hypervariable domain (Hancock and Marshall, 1993). The Ras proteins are active as signal transducers in the GTP-bound state and are inactive in the GDP-bound state. Ras proteins are capable of a slow exchange of bound nucleotide and free nucleotide but is greatly accelerated by a guanine nucleotide exchange factor (Downward, 1992). Sos catalyzes the release of bound GDP and the subsequent binding of GTP on Ras, hence activating ras (Buday and Downward, 1993). Inactivation of the Ras-GTP complex is stimulated by GTPase-activating proteins (GAPs), which increase the low intrinsic GTPase activity of Ras proteins (Downward, 1992). The activated Ras is able to bind to Raf-1 which is an important step in the activation of Raf-1.

1.3.3 RAF-1

The Raf proteins are a family of cytosolic serine/threonine kinases consisting

of c-Raf-1, A-Raf and B-Raf (Storm *et al.*, 1990) that act downstream of Ras in the MAPK cascade. The Raf family members share a common domain structure (**Figure 3**). The kinase catalytic domain occupies the carboxy-terminal half of the polypeptide and contains the CR3 region while the amino terminal contains the CR1 and CR2 regions. The CR1 and CR2 regions act as a regulatory region as a truncated mutant of Raf-1 not containing the CR1-CR2 region is constitutively active (Cutler *et al.*, 1998). The Raf CR1 domain, encompassing residues 62-194, contains a zinc-finger motif, a Ras-binding domain (RBD) (residues 51-131) and a cysteine-rich domain (CRD). The CR2 is a 14 amino acid sequence that contains many serine and threonine residues that may be phosphorylated.

The activation of Raf-1 is a complicated process involving many molecules that has not been completely elucidated. In unstimulated cells, Raf-1 is found predominantly in the cytosol (Morrison, 1995). In the cytosol, Raf-1 is associated with a number of different proteins including the 14-3-3 proteins, hsp90 and p50 (Koyama *et al.*, 1995; Stancato *et al.*, 1994; Stancato *et al.*, 1993). The 14-3-3 proteins are ubiquitous proteins named according to their electrophoretic mobility. They were originally identified as a series of very abundant 27- to 30-kDa acidic proteins in brain tissue. They are highly conserved and multiple isoforms are present in most cells. The 14-3-3 proteins have been speculated to have a role in the regulation of protein kinases and other signal transduction proteins as they are found to associate with the products of proto-oncogenes and oncogenes such as Raf-1 and Bcr-Abl (Morrison, 1994). Raf-1 contains two phosphorylation sites at

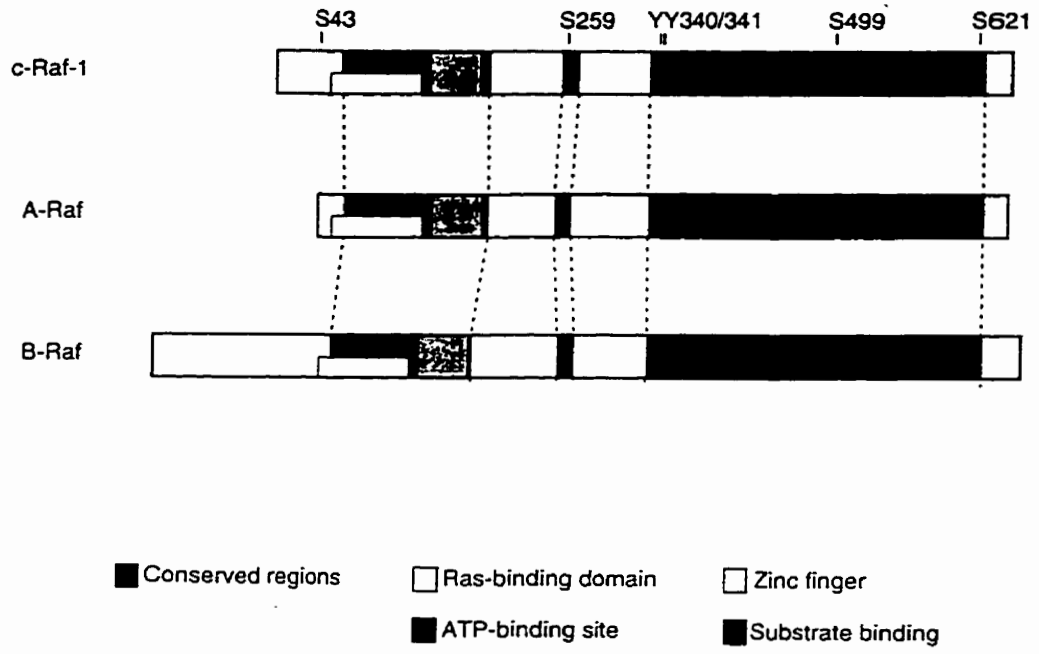


FIGURE 3. Structure of the Raf protein kinases

CR1 (residues 62-196), CR2 (residues 255-268) and CR3 (331-625) represent three conserved regions in the kinase. The phosphorylation sites given for c-Raf-1 are not necessarily present in the other raf kinases. Other functional domains such as the Ras-binding domain, ATP-binding site and substrate binding domain are indicated (from Daum *et al.*, 1994).

Ser259 and Ser621 which match 14-3-3 consensus binding sequence (Muslin *et al.*, 1996). Therefore, the 14-3-3 proteins bind to two sites on Raf-1: one at the amino terminal and one at the carboxyl terminal (Li *et al.*, 1995). The role of these associations is not yet known but have been speculated to keep Raf-1 in a favourable conformation and result in its stabilization.

Ras-GTP binds to Raf-1 and recruits it to the plasma membrane (Marshall, 1995; Avruch *et al.*, 1994). Raf-1 binds to Ras in a GTP-dependent manner through the RBD in the N-terminal region of the CR1 domain of Raf-1. Mutations in the RBD block Ras binding and prevent activation by a number of stimuli (Vojtek *et al.*, 1993; Zhang *et al.*, 1993). This recruitment does not activate Raf-1 (Marais *et al.*, 1995), so at the plasma membrane, additional events are necessary. Artificial targeting of Raf-1 to the membrane *in vivo* by-passed Ras indicating that the main function of Ras was to bring Raf-1 to the membrane for activation (Leevers *et al.*, 1994). Recent results have shown that this is not the case. A mutant form of Ras was able to induce membrane translocation of Raf-1 as effectively as wild-type, but the activity of this mutant to activate Raf-1 was inhibited 70% compared to that of wild-type (Tamada *et al.*, 1997). These results imply that the association of Ras with Raf-1 has another important consequence rather than just the recruitment of Raf-1 with the plasma membrane. Ras also interacts with the C-terminal part of the CR1 region of Raf-1, the CRD. But CRD binding to prenylated ras is not dependent on GTP indicating that the RBD is the primary sensor of ras activity (Hu *et al.*, 1995). 14-3-3 also binds to the cysteine-rich domain in Raf-1 by

a different mechanism, and the availability of this 14-3-3-binding may be activation state dependent (Clark *et al.*, 1997). 14-3-3 zeta is displaced from the amino terminal of Raf-1 upon binding of activated Ras, although it is not displaced from the carboxyl terminal (Rommel *et al.*, 1996). Once at the plasma membrane, overwhelming evidence suggests that Raf-1 becomes phosphorylated on tyrosine and/or serine/threonine residues (Diaz *et al.*, 1997; Jelinek *et al.*, 1996). Phosphorylation on Tyr340, Tyr341, Ser338, and Ser339 have been observed and result in Raf-1 activation. Possible candidate for this kinase include src and PKC (Stokoe and McCormick, 1997; Sozeri *et al.*, 1992). Enzymatic inactivation of Raf-1 by PTP1B, a tyrosine phosphatase, further demonstrate that tyrosine phosphorylation is causally related to Raf-1 activation (Dent *et al.*, 1995). Inactivation of Raf-1 by phosphatases can be inhibited by 14-3-3 and hsp90 by blocking the dephosphorylation, indicating that these molecules may somehow function in the activation of Raf-1 (Dent *et al.*, 1995). Also, phosphorylation of Raf-1 on Thr269 that is mediated by a ceramide-activated protein kinase has been implicated in tumour necrosis factor-induced Raf-1 kinase activation (Yao *et al.*, 1995). Raf phosphorylated at Ser43 by cAMP-dependent protein kinase displays a decreased affinity for Ras *in vitro* (Burgering and Bos, 1995). This demonstrates that phosphorylation of Raf-1 can have both positive and negative effect with regards to its activation.

Interaction between Raf-1 and Ras is transient, and Raf-1 remains attached to the plasma membrane independent of Ras. Recent evidence has shown that

Raf-1 contains binding sites for phosphatidic acid at the carboxyl terminal and phosphatidylserine at the amino terminal (Ghosh *et al.*, 1994; Ghosh *et al.*, 1996). Association with membrane lipids at both the amino and carboxyl terminal may serve to anchor Raf-1 in the membrane. These protein lipid interactions may also result in a conformational switch in Raf-1 from an active to an inactive state and may result in the exposure of the kinase domain. Also, another lipid, ceramide has been shown to bind to and activate Raf-1 (Huwiler *et al.*, 1996). This evidence suggests that lipids may be important in the activation of Raf-1.

Other proteins have also been implicated in the activation of Raf-1. Kinase suppressor of Ras (KSR), a Raf-like serine/threonine kinase, was found to be able to form a complex with 14-3-3 in the cytosol and membrane fractions and with Raf-1 in the membrane fraction suggesting that this protein may also be involved in Raf-1 activation (Xing *et al.*, 1997; Therrien *et al.*, 1996). A new protein in *C. elegans* was recently identified and was called SUR-8 for suppressor of RAS (Sieburth *et al.*, 1998). By the yeast two hybrid assay, SUR-8 was able to bind to Ras and *in vitro*, binds to the effector domain of Ras. The involvement of this protein or the mammalian homologue in Raf-1 activation remains to be determined. All in all, the activation of Raf-1 is complex involving many molecules and phosphorylation events. The activation of Raf-1 with many of the potential key players involved is depicted in **Figure 4**.

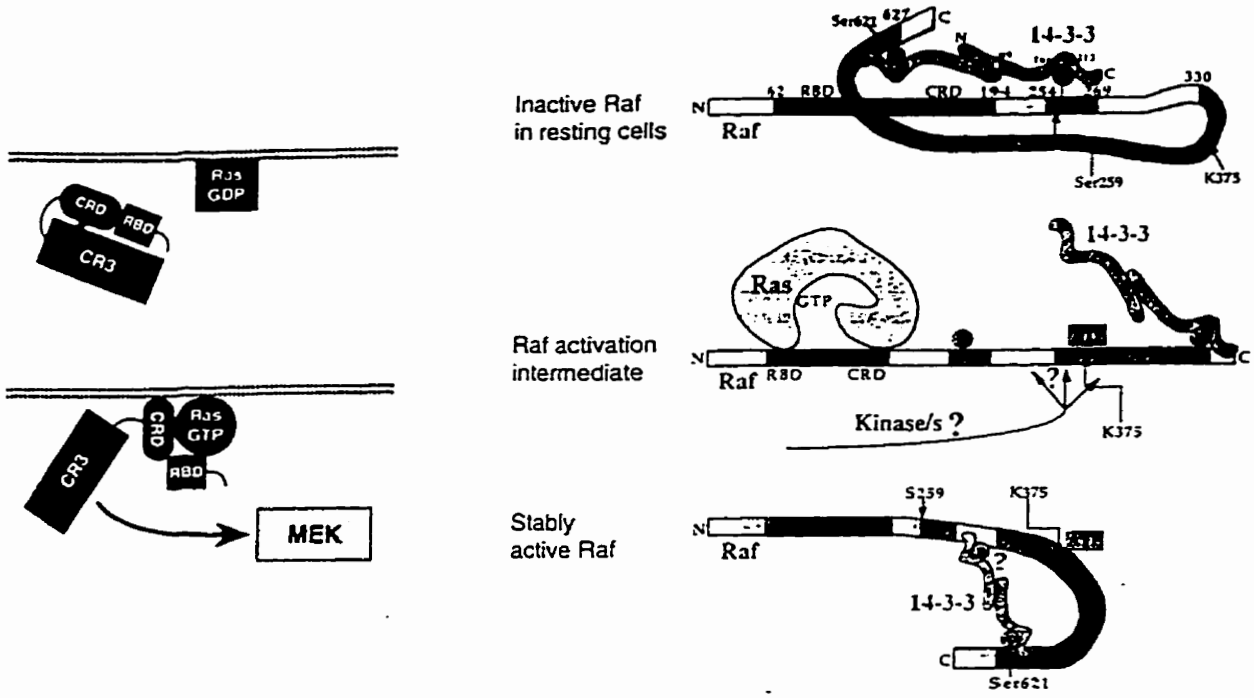


FIGURE 4. Models of Raf-1 activation

A) In quiescent cells, Raf-1 is in an inactive state on the cytosol. The regulatory domain inhibits the activity of the catalytic domain. When Ras is in the GTP-bound state and becomes active, Raf-1 translocates to the plasma membrane where both the Ras-binding domain (RBD) and the cysteine-rich domain (CRD) contact membrane components thereby relieving the repression by the regulatory region (from Cutler *et al*., 1998). B) *Model for the role of 14-3-3 in activation of Raf-1.* Raf is maintained in the cytosol in an inactive state by the binding of a 14-3-3 dimer at two sites on Raf (Ser259, Ser621). Half of the 14-3-3 dimer is displaced from the Ser259 site by Ras-GTP. The 14-3-3 dimer then binds to a newly phosphorylated site on Raf stabilizing an active conformation (from Tzivion *et al.*, 1998)

1.3.4 MAP KINASE

Downstream of Raf-1 are the MEKs (**M**APK / **E**rk **k**inase) or MAPKKs and these constitute an evolutionary family of protein serine/threonine kinases. There are 2 isoforms activated by raf-1, MEK1 and MEK2, and these are the best characterized physiological substrates of Raf-1. The Raf-1 specific phosphorylation sites have been determined for MEK1 and have been found to be Ser218 and Ser222 and either is sufficient for MEK1 activation (Alessi *et al.*, 1994). Other kinases have also been shown to activate and phosphorylate MEK and these are Mos and MEK kinase (Avruch *et al.*, 1994; Blumer and Johnson 1994). The MEK-binding domain on Raf-1 was determined using the yeast two-hybrid method and was found to occur in the CR3 region (Avruch *et al.*, 1994).

The MAPKK phosphorylate and activate the MAPKs. MAPKs are serine/threonine kinases that mediate intracellular phosphorylation events by a variety of external stimuli. Deactivation of MAPK *in vitro* can be achieved by treatment with either protein phosphatase 2A or tyrosine phosphatase CD45 (Anderson *et al.*, 1990) hence it was discovered that activation of MAPK requires the phosphorylation of both threonine and tyrosine residues in the sequence TXY (Payne *et al.*, 1991). Because of this, MEKs are dual specificity kinases that phosphorylate MAPK on these two residues (Ahn *et al.*, 1991). Therefore, inactivation of MAPK can be achieved by three types of phosphatases: the dual-specificity phosphatases, phosphoserine / phosphothreonine phosphatases, and phosphotyrosine phosphatases. The MAPKs are proline-directed protein kinases,

and once activated by MEK are able to phosphorylate their substrates on a PX(T/S)P consensus motif where P is proline and X is any other amino acid.

The first mammalian MAPK family members discovered were the p42 and p44 MAPKs (Ray and Sturgill, 1987). These closely related isoforms are also called ERK2 and ERK1 respectively for extracellular regulated kinases. In ERK2, the residues phosphorylated by MEK1 and MEK2 are Thr183 and Tyr185 (Anderson *et al.*, 1990). In addition to the ERK family of MAP kinases, there have been other groups of the MAPK superfamily discovered including the stress-activated (SAPK)/c-Jun N-terminal kinase (JNK) (Derijard *et al.*, 1994) and the p38 MAPK molecules (Han *et al.*, 1994). Both the JNK and p38 MAPK modules are activated by environmental stress and cytokines. After their activation, MAPKs can phosphorylate a large number of proteins. The substrates of MAPK include other ser/thr protein kinases such as $p90^{rsk}$ and MAPKAP, transcription factors such as c-Jun, c-Myc and ATF-2, and cell surface molecules such as EGFr and other molecules such as cytoplasmic phospholipase A_2 ($cPLA_2$) (Seger and Krebs, 1995).

The MAPK pathways are important pathways in the cell and are implicated in cell proliferation and differentiation.

1.4 : INSULIN SIGNALLING PATHWAY

Insulin is a well studied polypeptide hormone produced by the β cells of the pancreas in response to nutritional stimuli. Insulin activates numerous signals that

regulate a number of important mechanisms including modulation of glucose and amino acid transport, protein synthesis, glucose and lipid metabolism, gene expression and cell growth.

1.4.1 INSULIN RECEPTOR (IR)

All the insulin signal transduction pathways are initiated by the insulin receptor. The IR is present in virtually all vertebrate tissues although the relative number of receptors vary from tissue to tissue. The insulin receptor gene is located on the short arm of chromosome 19 and is approximately 150 kilobase pairs in length containing 22 exons and 21 introns (Yang Feng *et al*, 1985; Muller-Wieland *et al.*, 1989). The IR is a heterotetrameric transmembrane glycoprotein composed of 2 α subunits and 2 β subunits. The α subunits are joined to each other and to the β subunits by disulfide bonds. The α subunit is composed of 723 amino acids and has an approximate molecular mass of 130 kDa as determined by SDS-PAGE. The α subunit is located entirely outside the cell and contains the insulin binding site. **Figure 5** shows the structure of the IR indicating the α and β subunits and the tyrosine phosphorylation sites in the intracellular portion of the β subunit.

The β subunit contains 620 amino acids and has an approximate molecular mass of 95 kDa by SDS-PAGE. Insulin binding to the α subunit modifies the α subunit dimer which mediates trans-autophosphorylation between the β subunits (Lee *et al.*, 1993; Fratalli *et al.*, 1992). The β subunit consists of different regions: the transmembrane spanning region, the tyrosine kinase region, the juxtamembrane

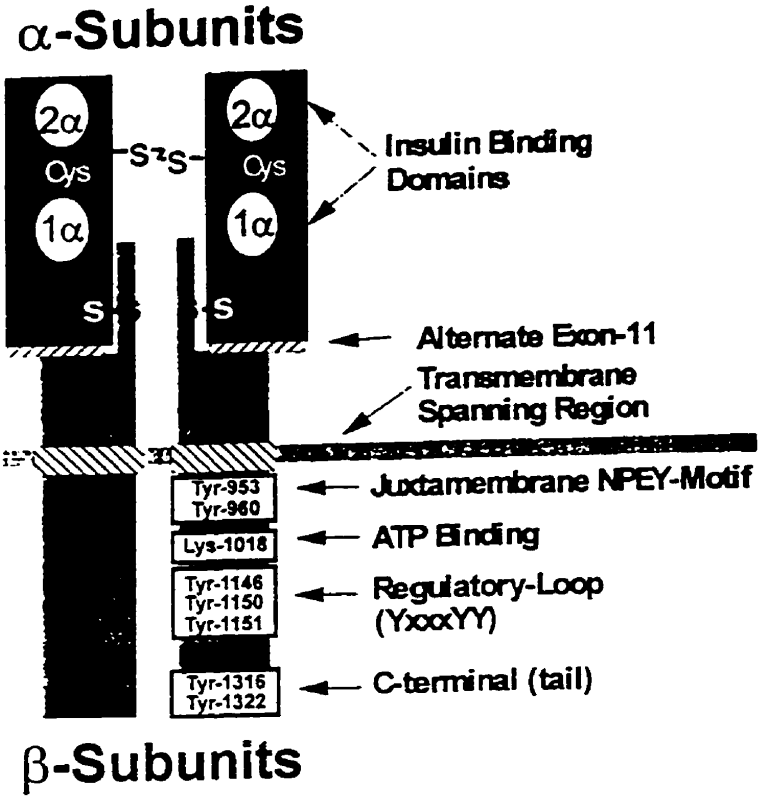


FIGURE 5. The Insulin Receptor

The insulin receptor is a tetramer consisting of 2 α subunits and 2 β subunits. Insulin binds to the extracellular α subunits and results in autophosphorylation of residues in the β subunit. The main phosphorylation sites are shown (from White, 1997).

region, and the COOH-terminal tail.

The external ligand binding domain of the IR is linked to the tyrosine kinase by a single transmembrane segment which stabilizes the insulin-induced conformational change. The binding of insulin activates the tyrosine kinase leading to autophosphorylation of several tyrosine residues in several regions of the intracellular β subunit including Tyr960 in the juxtamembrane region, Tyr1146, Tyr1150, and Tyr1151 in the regulatory loop, and Tyr1316 and Tyr1322 in the COOH-terminus (Feener *et al.*, 1993; White and Kahn, 1994). Using site-directed point mutations in the ATP binding domain which destroy ATP binding demonstrate that the tyrosine kinase activity of the receptor is crucial for insulin action (Chou *et al.*, 1987). Also, naturally occurring mutations of the insulin receptor which inhibit kinase activity are associated with severe insulin resistance (Odawara *et al.*, 1989; Moller *et al.*, 1990). Lys1030, located in the ATP binding site, is a critical residue and mutation of this site completely inactivates the kinase activity (Ebina *et al.*, 1987) Autophosphorylation of all three tyrosine residues in the regulatory loop activates the kinase 10 to 20 fold (White *et al.*, 1988a) and mutation of one, two, or all three sites progressively reduces insulin-stimulated kinase activity (Wilden *et al.*, 1992).

The juxtamembrane region of the β subunit mediates substrate selection presumably by engaging the phosphotyrosine binding domains in the IRS proteins and Shc. This region contains several serine phosphorylation sites and at least one tyrosine autophosphorylation site (Tyr960). Mutation of this site impairs signal

transmission due to the inability to mediate the phosphorylation of IRS-1 and Shc but has no effect on the receptor kinase activity (White *et al.*, 1988b).

The COOH-terminal tail contains two autophosphorylation sites (Tyr1316 and Tyr1322) as well as the serine and threonine phosphorylation sites. Unlike in the EGF and PDGF receptors in which the COOH-terminal region bind to SH2-proteins, the IR COOH-terminal appears to regulate insulin signals rather than to recruit the SH2-proteins to the receptor (Myers *et al.*, 1991; Pang *et al.*, 1994).

The IR is known to phosphorylate several multiple proteins including the two most studied substrates, insulin receptor substrates (IRS proteins) and Shc. Other proteins known to be phosphorylated by the receptor include pp120, an ecto-ATPase which is a liver-specific membrane glycoprotein (Perrotti *et al.*, 1987; Margolis *et al.*, 1990), pp15, an adipose-specific substrate(Hresko *et al.*, 1988), Vav, a 95 kDa proto-oncogene in certain haematopoietic cells (Uddin *et al.*, 1995), pp115, a protein found to associate with SHP2 in adipocytes (Yamauchi *et al.*, 1995a), and pp60, a 60 kDa protein which may be a mini IRS-protein and has been shown to bind p21rasGAP or the PI3K (Hosomi *et al.*, 1994). The function of many of these IR substrates remain unknown

1.4.2 SHC

One known substrate of the insulin receptor are the 46, 52 and 66 kDa proteins called Shc (Src-homology2/α-collagen). The 46 and 52 kDa species are both expressed from the same mRNA transcript by alternate translational initiation

sites resulting in an amino-terminal 59-amino acid truncation of the 46 kDa isoform compared to the 52 kDa isoform. The 66 kDa isoform is likely to result from an alternatively spliced message since there is only one Shc gene and carboxyl-terminal antibodies cross-react with all three molecular weight species (Pronk *et al.*, 1993; Sasaoka *et al.*, 1994a). The three Shc proteins contain a carboxyl-terminal Src homology 2 (SH2) domain, a central glycine/proline-rich region homologous to the α1 chain of collagen, and an amino-terminal region containing a PID (phosphotyrosine-interacting domain) domain (Bork and Margolis, 1995). The Shc proteins are tyrosine-phosphorylated on a single tyrosine residue (317) which serves as a docking site for Grb2 (Skolnik *et al.*, 1993; Sasaoka *et al.*, 1994b). Shc and IRS-1 contain a homologous domain, the SAIN (SHC and IRS-1 NPXY-binding) domain, which interacts with the insulin receptor (Gustafson *et al.*, 1995).

1.4.3 IRS PROTEINS

Although IRS-1 was originally identified as a substrate of the insulin receptor, it is now known to be a substrate for several other receptor systems including those in the IL6 family, the IL2 family and interferons. IRS-1 was initially detected in insulin-stimulated Fao hepatoma cells by immunoprecipitation with antiphosphotyrosine antibodies (White *et al.*, 1985). Because of the way it migrated on SDS-PAGE, it was originally called pp185. The open reading frame of the IRS-1 predicts a protein of 131 kDa, and its slow migration on SDS-PAGE was found to be largely due to the high level of phosphorylation of the protein. IRS-1 mRNA is

detectable in most human tissues by quantitative PCR after reverse transcription of total RNA (Araki *et al.*, 1993). After the identification of IRS-1, another high molecular weight tyrosyl phosphoprotein resembling IRS-1 was observed in myeloid cells. This protein was called 4PS which stands for IL4r Phosphoprotein Substrate since it was initially observed during IL4 stimulation (Wang *et al.*, 1993). Because cloning revealed regions of functional homology between IRS-1 and 4PS, 4PS was renamed IRS-2. There is 43% overall identity between IRS-1 and IRS-2 but regions in the amino-terminus contain higher degrees of identity than the rest of the molecule. These regions have been termed IRS homology-1 (IH1) and IH2 domains.

The IRS-proteins contain many tyrosine phosphorylation motifs. IRS-1 contains 21 potential tyrosine phosphorylation sites (Sun *et al.*, 1991). Fourteen of the 21 potential tyrosine phosphorylation sites of IRS-1 are conserved in IRS-2, four sites contain alternative surrounding sequences, 3 sites are novel to IRS-1 and 4 are novel to IRS-2. During insulin stimulation, at least 8 of the sites in IRS-1 undergo phosphorylation by the activated IR including residues 608, 628, 939, and 987 which are all in YMXM motifs. Other residues phosphorylated include Tyr 460, 895, 1172 and 1222 (Sun *et al.*, 1993). In addition to the tyrosine phosphorylation sites, the IRS-proteins also contain over 30 potential serine/threonine phosphorylation sites (**Figure 6**). Prior to insulin stimulation, IRS-1 is strongly serine phosphorylated and it is thought that serine and threonine phosphorylation of IRS-1 appears to inhibit its tyrosine phosphorylation during insulin stimulation

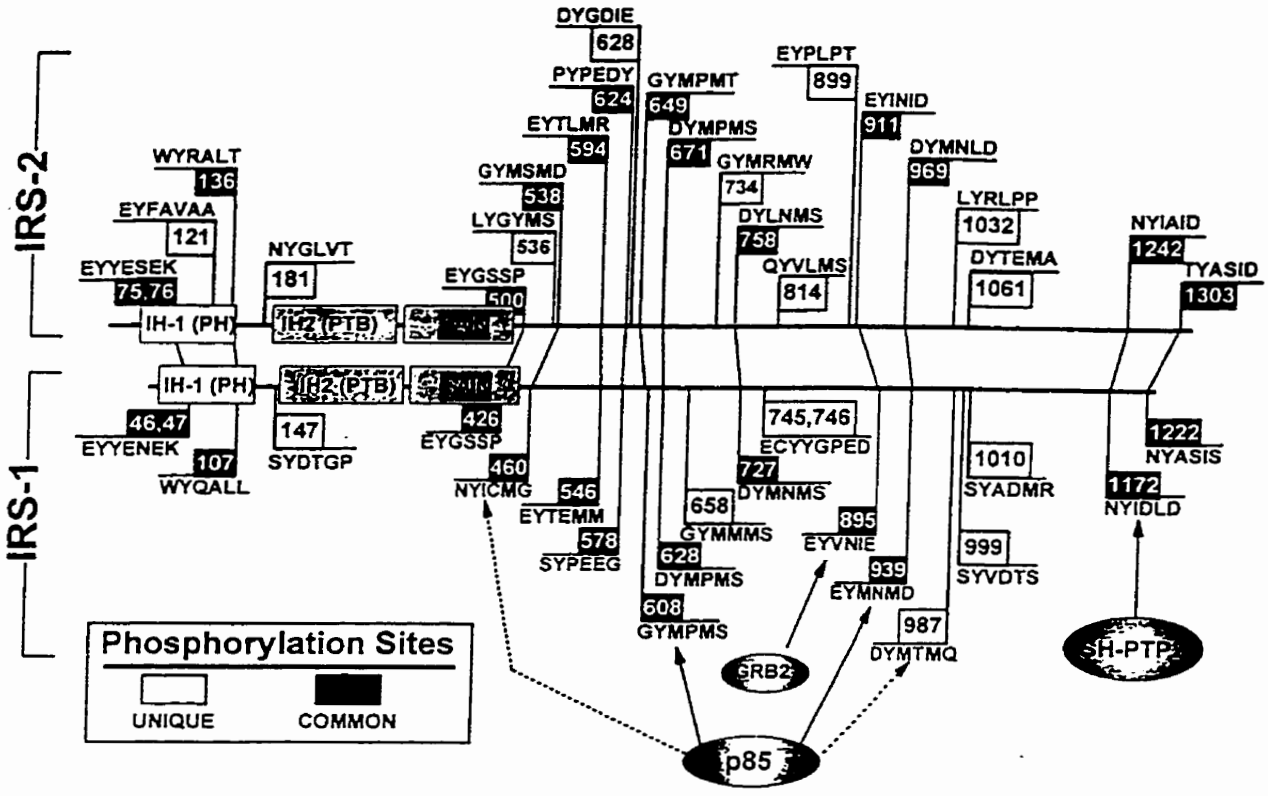


FIGURE 6. Insulin-receptor-substrates

Putative tyrosine phosphorylation sites in the IRS-1 and IRS-2 molecules are shown. The unique phosphorylation sites between the two substrates are indicated by the open boxes. The conserved modules ($IH1^{PH}$ and $IH2^{PTB}$) are shown. IRS-2 is about 100 residues longer than IRS-1 (from White, 1997).

(Jullien *et al.*, 1993).

As mentioned earlier, the IRS-proteins contain two regions of high similarity termed IH1 and IH2 at the amino-terminal end. IH1 has similarity to pleckstrin homology domains and IH2 has homology to PTB domains in various proteins ($IH1^{PH}$ and $IH2^{PTB}$). The IH2PTB domain binds to the phosphorylated NPXY-motif in the insulin and IL4 receptors and specifically the amino-terminal portion of IRS-1 was shown to mediate an interaction with Tyr960 of the insulin receptor (Craparo *et al.*, 1995; Gustafson *et al.*, 1995). More recently, two additional members to the IRS family, IRS-3 and IRS-4 have been characterized (Lavan *et al.*, 1997a; Lavan *et al.*, 1997b). These two new family members, like IRS-1 and IRS-2, also consist of an amino-terminal PH domain followed by a PTB domain and a carboxy-terminal domain containing multiple sites for tyrosine phosphorylation. Presumably, the PH and PTB domains of IRS-3 and IRS-4 also function in the presentation of the sites for tyrosine phosphorylation to the kinase domain of the receptor like in IRS-1 and IRS-2. The expression of IRS-3 and IRS-4 is largely restricted to adipocytes and pituitary-thyroid respectively (Lavan *et al.*, 1997a; Lavan *et al.*, 1997b).

Once the IRS-1 protein has been phosphorylated by the insulin receptor, it is then able to bind to SH2-proteins. The binding of proteins to IRS-1 serve several purposes. Firstly, enzymes associated with IRS-1 through their SH2 domain may be activated. Secondly, binding of proteins to IRS-1 may serve to position them near other signalling molecules to generate their signal, and thirdly, the IRS/SH2-protein complex is mobile (unlike the IR). SH2-containing molecules

known to bind to IRS-1 include SHPTP2, Grb2, Nck and PI3K. The SH2-containing proteins for the different IRS family members vary : IRS-2 associates with PI3K and Grb2 but not with SHPTP2 (Sun *et al.*, 1995; Tobe *et al.*, 1995), IRS-3 associates with PI3K and SHPTP2 (Ross *et al.*, 1998), and IRS-4 binds to PI3K and Grb2 (Fantin *et al.*, 1998).

1.4.4 SHPTP2

SHPTP2 (also called PTP1D, SHPTP3, PTP2C, PTPL1, Syp or SHP2) is a 65 kDa nontransmembrane SH2-containing tyrosine phosphatase that is expressed ubiquitously in mammalian cells and tissues (Freeman *et al.*, 1992). Several growth factor receptors also bind to the SH2 domain in SHPTP2 including the EGFr and the PDGFr (Lechleider *et al.*, 1993). In the PDGF signalling system, the phosphorylated SHPTP2 serves as an adaptor protein between the activated PDGFr and the Grb2-Sos complex (Li N *et al.*, 1993). The precise role of SHPTP2 in insulin signalling remains unclear but it is thought to be essential for the insulin pathway. SHPTP2 contains two amino-terminal SH2 domains and binds to IRS-1 at Tyr1172 (Kuhne *et al.*, 1993) and a carboxyl terminal catalytic domain. Inactive SHPTP2 inhibits insulin-stimulated MAP kinase activity and c-fos transcription (Yamauchi *et al.*, 1995b; Noguchi *et al.*, 1994). This dominant negative effect of inactive SHPTP2 is partially reversed by co-expression of v-ras or Grb2, indicating that SHPTP2 may act upstream of Ras (Noguchi *et al.*, 1994). Recently, it was found that SHPTP2 associates with Tyr1146 in the regulatory loop of the β-subunit

of the insulin receptor (Kharitonenkov *et al.*, 1995). This interaction may act to form a more stable link between IRS-1 and the receptor than can normally be obtained with the $IH1^{PH}$ or $IH2^{PTB}$ domain alone. The binding of SHPTP2 to IRS-1 provides a potential mechanism to its activation. SHPTP2 appears to be the mammalian homolog of a *Drosophila* protein termed corkscrew that is downstream of the receptor Tyr kinase *torso* and is involved in regulating the *Drosophila* homolog of Raf-1 (Perkins *et al.*, 1992). It may have a similar role in mammalian cells and tissues.

1.4.5 GRB2

Grb2, a small cytoplasmic protein, can bind to both IRS-1 and Shc. Grb2 is an adaptor molecule that links SOS, a guanine nucleotide exchange factor for $p21^{ras}$, to proteins such as the EGFr, IRS-1 and Shc (Simon *et al.*, 1993). Grb2 binds to IRS-1 at Tyr895 after insulin stimulation (Baltensperger *et al.*, 1993). An alternate pathway leading to ras activation upon insulin stimulation involves the binding of Grb2 to Shc as mentioned earlier. The relative contribution of Shc and IRS-proteins to the MAPK cascade are likely to be cell and tissue specific and may be influenced by the relative concentrations of these substrates.

1.4.6 NCK

Nck is a 47 kDa adaptor protein composed of three SH3 domains and one SH2 domain. Nck binds to Tyr-phosphorylated PDGF and EGF receptors and has

been shown to bind to IRS-1 (Li CH *et al.*, 1993). Overexpression of Nck in NIH 3T3 fibroblasts results in cellular transformation suggesting a role in regulation of cell proliferation (Chou *et al.*, 1992). Since Nck appears to have no enzymatic activity, it may serve as an adaptor protein that links upstream Tyr kinases and IRS-1 to downstream pathways.

1.4.7 PI3K

The first SH2-protein found to bind to IRS-1 and the best studied protein is phosphoinositide 3-kinase (PI3K). PI3Ks are a family of enzymes that catalyze the phosphorylation of inositol phospholipids in the D-3 position of their inositol ring (Stephens *et al.*, 1993). The PI3K enzyme consists of a regulatory (adaptor) subunit and a catalytic subunit. There are 3 main classes of PI3K based on their different subunits and their substrate specificity (**Figure 7**). There are currently 7 forms of the catalytic subunit and 5 forms of the adaptor subunit. Class I enzymes can use PtdIns, PtdIns(4)P and PtdIns(4,5)P_2 as substrates but seem to preferentially phosphorylate PtdIns(4,5)P_2 (Irvine, 1992). The class I enzymes can be further divided into 2 subclasses: class IA and class IB. The class 1B PI3K are activated by binding of βγ subunits of heterotrimeric G-proteins to specific sites on both the adaptor and the catalytic subunit (Stephens *et al.*, 1997). Only the class IA molecules can be activated following insulin stimulation and the rest of this section will concentrate on their activation in response to insulin. The class IA molecules consist of a 110 kDa catalytic subunit and an 85 kDa (usually) adaptor

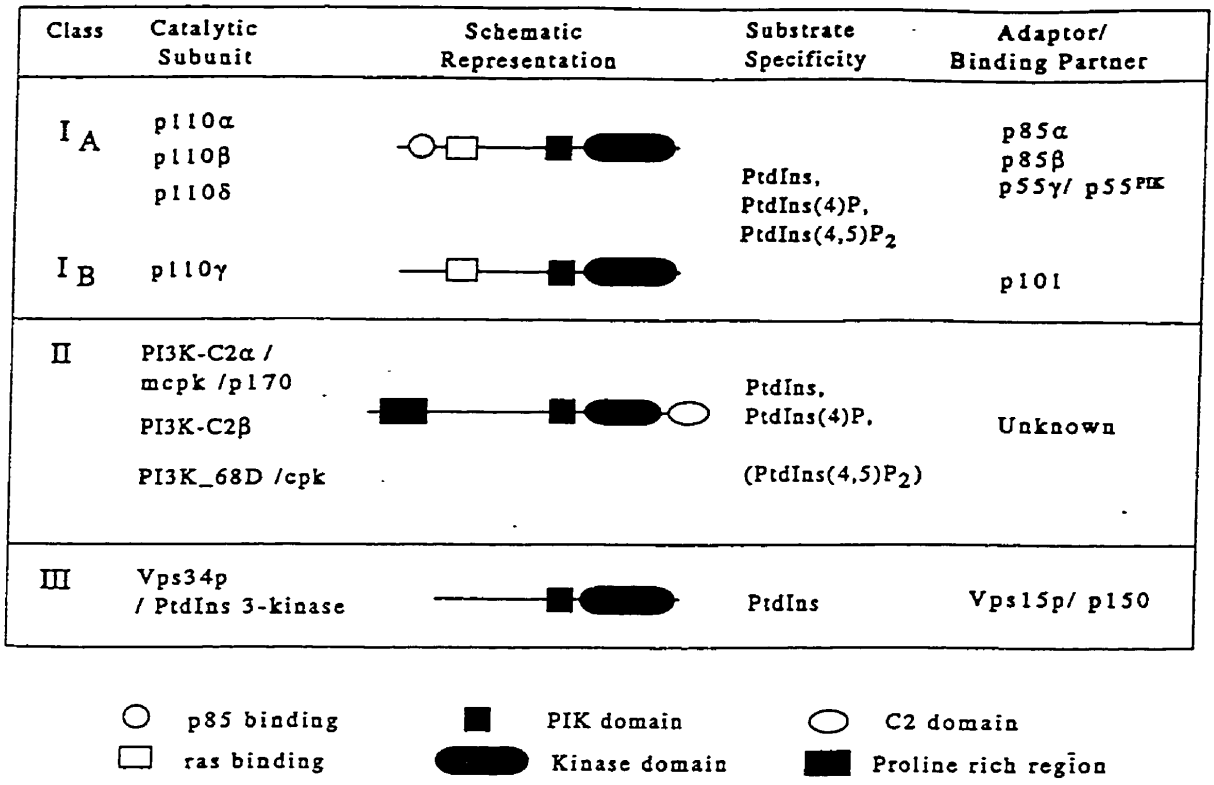


FIGURE 7. Different PI3K Subclasses

The three different PI3K family members are depicted. Different functional domains such as the kinase domain, PIK domain and the Ras-binding domain are shown (from Domin and Waterfield, 1997).

protein. The catalytic domain contains a domain which binds to p85, a region for ras association, a PI kinase (PIK) domain and a C-terminal catalytic domain. The role of the PIK domain is currently unclear but it is conserved in all the PI3 kinases and is thought to perhaps function in substrate presentation (Flanagan *et al.*, 1993). The p85 adaptor protein (p85α and p85β) contain 2 SH2 domains separated by an inter SH2 domain that binds the catalytic subunit (Dhand *et al.*, 1994). They also contain an N-terminal SH3 domain, a breakpoint cluster region homology (BH) domain which has homology with the GAP (GTPase activating protein) domain of the BCR gene product (Musacchio *et al.*, 1996), as well as two proline-rich regions which are located on either side of the BH domain (Gout *et al.*, 1992). The *in vivo* roles of the SH3 domain, the proline-rich domain and the BH domains are not known. Since SH3 domains are known to interact with specific proline-rich sequences and the proline-rich sequences in p85 match the consensus sequence for this interaction (Yu *et al.*, 1994), there may be some intramolecular interactions between these regions. Also, the p85 SH3 domain and the p85 proline-rich domain have been shown to interact *in vitro* with other cellular proteins: the SH3 domain can interact with $p125^{FAK}$ (Guinebault *et al.*, 1995) and the proto-oncogene *cbl* (Hunter *et al.*, 1997) and the proline-rich domain can interact with Grb2 (Wang *et al.*, 1995) and other Src family of tyrosine kinases (Yuan *et al.*, 1997) thus allowing for the formation of terniary signalling complexes. The BH domain is likely not a functional Rac GAP although it is still able to bind to GTP-loaded forms of the small GTPases Rac (Bokoch *et al.*, 1996) and it is thought that these interactions may

regulate the activity of the PI3K catalytic subunit. In addition to the p85 adaptor subunit, there is also a smaller adaptor protein identified ($p55^{PIK}$) which is not generated by the alternate splicing of the p85α gene (Antonetti *et al.*, 1996). The reason for the number of variants of both the catalytic and the adaptor subunits are not known.

The SH2-domains of PI 3-kinase bind preferentially to YXXM-motifs and more specifically to YMXM motifs. IRS-1 contains at least 4 sites that interact with the SH2 domains of p85: Y_{608}MPM>Y_{939}MNM>Y_{987}MTM and Y_{460}ICM (Sun *et al.*, 1993). The activity of PI3K by binding to IRS-1 is brought about by two mechanisms. Firstly, the interaction of the SH2 domains with these specific phosphotyrosine motifs can increase the catalytic activity of the p110 subunit (Giorgetti *et al.*, 1993). Secondly, the binding of PI3K to IRS-1 brings the enzyme close to the membrane allowing access to its substrate (Kelly and Ruderman, 1993). It is also possible that activation of Ras could contribute to increases in cellular PI3K activity (Rodriguez-Viciana *et al.*, 1996) as PI3K contains a binding site for Ras. The binding of p110 to Ras may serve to localize PI3K to the membrane as ras is a membrane-bound protein, or ras may be an effector of PI3K (Yamauchi *et al.*, 1993).

The exact role of PI3K in the cell remains questionable but the most obvious way that PI3K functions is through the transduction of signals via their lipid products. PIP_3 is the major product of PI3K and the rise in PIP_3 levels correlate with the activation of downstream responses and occurs before the activation of

molecules thought to lie downstream of PI3K (Cross *et al.*, 1997). PIP_3 has been shown to directly modulate downstream events (Jiang *et al.*, 1998) and this is thought to occur in one of two ways. One is by directly affecting the properties of cellular membranes as it has been proposed that localized production of phosphoinositide products could cause membrane curvature and possibly contributing to vesicle budding (Schu *et al.*, 1993). The other possibility is that PIP_3 may interact directly with molecules and help to relay downstream signals. They may bind and alter the catalytic activity of targets or promote some sort of conformational change which may expose regulatory sites or the interactions may regulate the co-localization with other proteins to form complexes at the membrane (Shepherd *et al.*, 1998). One structural feature shared by many of the PIP_3-binding proteins is the presence of a PH domain which may mediate the interaction between them and PIP_3.

PI3K has been implicated in a variety of events in the cell including cell growth and proliferation, differentiation, apoptosis, vesicle traffic and glucose-transporter translocation, glycogen synthesis, lipid metabolism, protein synthesis, and cytoskeletal rearrangements (**Figure 8**). The role of PI3K in many of these events has not been elucidated and the involvement of PI3K has largely been identified using specific inhibitors to the kinase and dominant negative forms of the kinase. There have also been studies to determine the cellular targets of PI3K: downstream molecules that have been identified include protein kinase B, glycogen synthase kinase, p70 ribosomal S6 protein kinase, protein kinase C, Jun kinase and

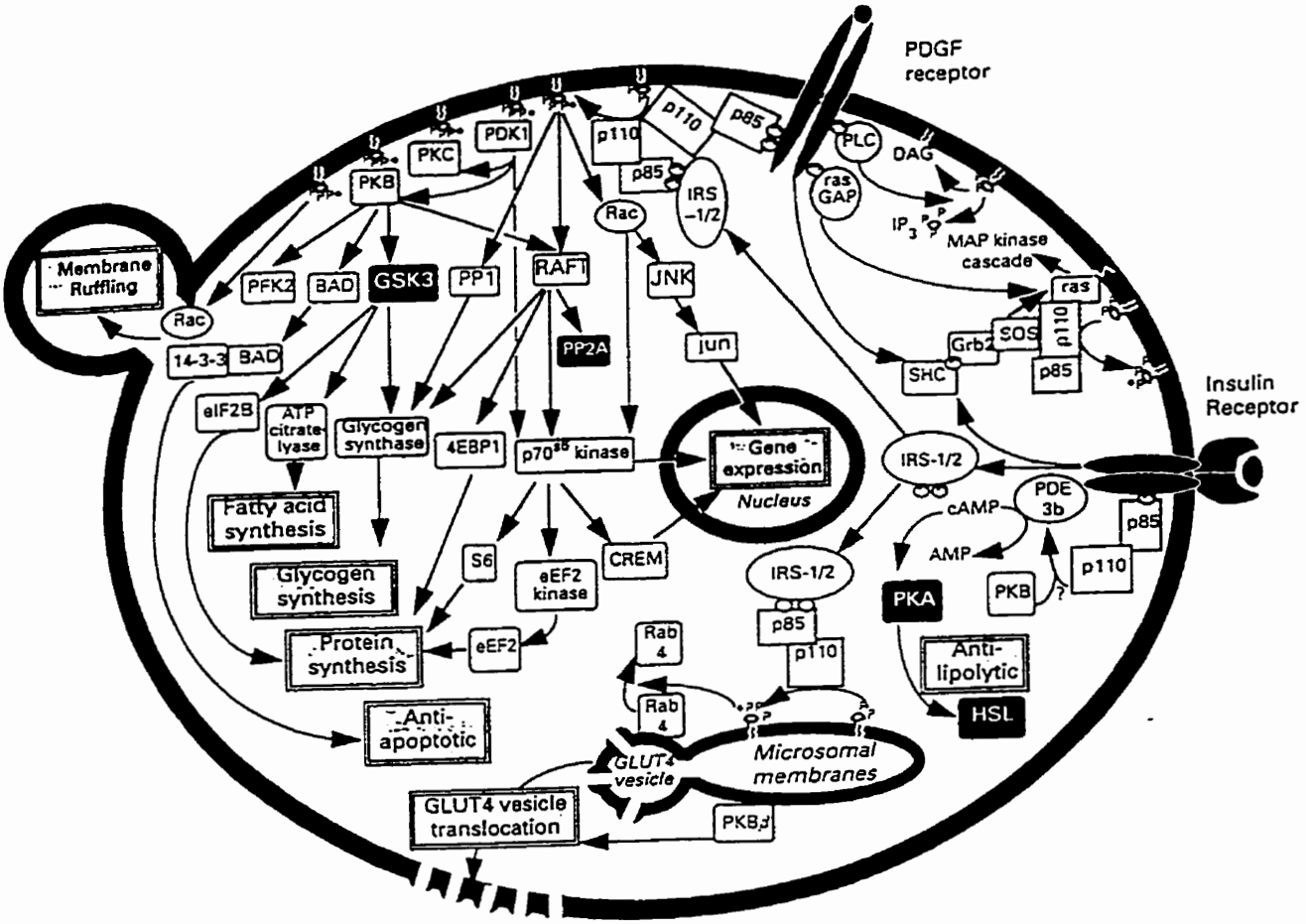


FIGURE 8. Different PI3K Pathways

PI3K is an important signalling molecule in the cell. The activation of this enzyme is responsible for many cellular actions including gene expression, fatty acid synthesis and protein synthesis. The endpoint responses are indicated by the double lined rectangles (from Shepherd *et al.*, 1998).

serine phosphatases (Shepherd *et al.*, 1998).

1.4.8 PROTEIN KINASE B

Protein kinase B (PKB) or Akt is a 60 kDa serine/threonine kinase and is the cellular homologue of the oncogene v-akt of the acutely transforming retrovirus AKT8 found in a rodent T-cell lymphocyte (Bellacosa *et al.*, 1991). PKB was identified as a result of its homology with both protein kinase C and protein kinase A and was originally called RAC (related to the A and C kinases). PKB is activated upon ligand stimulation of receptor tyrosine kinases (Bellacosa *et al.*, 1991). The Akt protein contains an N-terminal PH domain in addition to the protein kinase domain. The PH domain makes up the major part of the amino terminal regulatory domain (amino acids 1-147) and has been referred to as the Akt homology domain. Mammalian genomes contain three genes encoding PKBs (PKBα, PKBβ and PKBγ). PKBβ and PKBγ are approximately 82% identical with the α isoform although PKBγ lacks 23 amino acids at the C-terminus compared with the others (Konishi et al., 1995). PKBα and PKBβ are widely expressed while the expression of the γ-isoform is more restricted (Bellacosa *et al.*, 1993) but all tissues contain at least one form of PKB (**Figure 9**).

Expression of receptor mutants that do not bind p85 and using inhibitors of PI3K block ligand induced PKB activation indicating an involvement of PI3K (Burgering and Coffer, 1995; Franke *et al.*, 1995). Also, expression of constitutively active PI3K results in the activation of PKB (Klippel *et al.*, 1996). Further

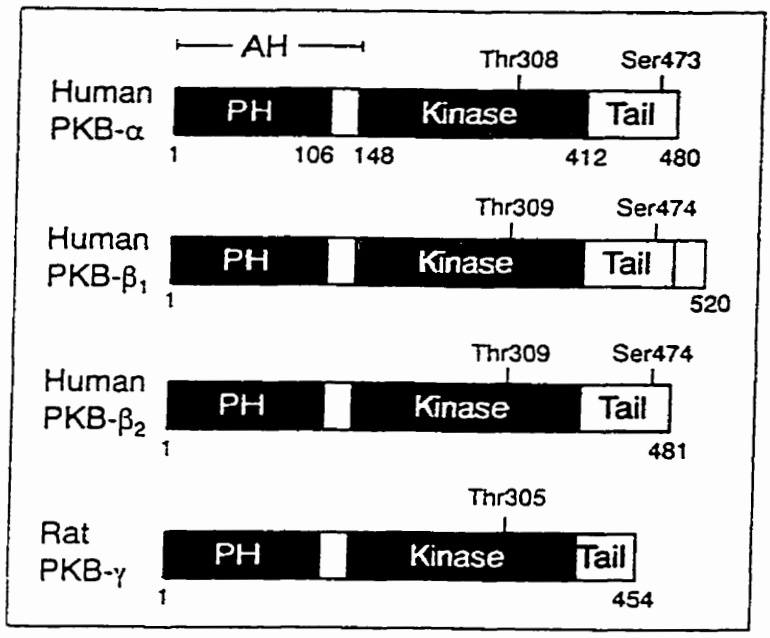


FIGURE 9. Different PKB Isotypes

The different PKB isotypes are indicated. They all contain an amino terminal PH domain as a main portion of the regulatory domain (Akt homology, AH domain). PKB-β_1 and -β_2 are alternative spliced forms derived from the same gene. The insulin-induced phosphorylation sites are indicated (from Marte and Downward, 1997).

implicating a role of PI3K in the activation of PKB, the activation of PKB closely correlates to the increases in cellular PIP_3 levels (Cross *et al.*, 1997). It was initially suggested that PIP_2 and PIP_3 directly bound to PKB and resulted in its activation (Klippel *et al.*, 1996; Franke *et al.*, 1997), but reports have shown that they can not directly activate the kinase (Stokoe *et al.*, 1997; Klippel *et al.*, 1997).

PKB is phosphorylated on two residues, Thr308 and Ser473, and phosphorylation of these residues results in its activation (Alessi *et al.*, 1996). Mutagenesis of each residue to alanine revealed that both are required for full activation (Alessi *et al.*, 1996). The phosphorylation sites are conserved in PKBβ (Thr309, Ser474) and because PKBγ contains a C-terminal truncation, it lacks a Ser473 homologue but contains Thr305 which is analogous to Thr308 in PKBα (Walker *et al.*, 1998). The kinase responsible for phosphorylating Thr308 has been identified and termed PDK1 for 3-phosphoinositide-dependent protein kinase-1 (Stephens *et al.*, 1998). PDK1 is ubiquitously expressed and has a PH domain C-terminal to its kinase domain which binds PIP3 and it was thought that binding of PIP_3 to this PH domain activated PDK1 (Stokoe *et al.*, 1997). But, it has been found that PIP_3 binding to PDK1 does not greatly increase the activity of PDK1 and that PDK1, in fact, is constitutively active (Pullen *et al.*, 1998). The most accepted mechanism of activation of PKB involving PIP_3 is that PIP_3 binding to PKB induces some sort of conformational change which makes Thr308 accessible for phosphorylation by PDK1. Also, PIP_3 may serve to bring PDK1 and PKB together at the membrane to ensure efficient phosphorylation.

The kinase responsible for phosphorylating Ser473 is distinct from PDK1 and is tentatively named PDK2 (Alessi *et al.*, 1997). PDK2 also appears to be under the control of PI3K as phosphorylation of Ser473 in response to insulin is sensitive to wortmannin, a PI3K inhibitor (Alessi *et al.*, 1996). *In vitro*, the site can be phosphorylated by mitogen-activated protein kinase-activated protein (MAPKAP) kinase-2, an enzyme that is regulated by the p38 MAP kinase pathway, but it is unlikely to be the physiological kinase that phosphorylates Ser473 since agents that induce MAPKAP kinase-2 activity have no effect on PKB activation (Alessi *et al.*, 1996) (**Figure 10**).

Glycogen synthase kinase 3 (GSK3) lies downstream of PKB and appears to act as a negative inhibitor of both transcription and translational factors such as c-jun and eIF-2B in addition to metabolic enzymes such as glycogen synthase (Welsh *et al.*, 1997). Insulin stimulation leads to inhibition of GSK3 isoforms by inducing the phosphorylation of GSK3α on Ser21 and GSK3β on Ser9. Inhibition of this phosphorylation is observed when inhibitors of PI3K are used indicating that PI3K is required for this to occur (Welsh *et al.*, 1994). Both of these residues are substrates of PKB *in vitro* (Cross *et al.*, 1995) suggesting that PKB is responsible for the *in vivo* phosphorylation (Cross *et al.*, 1997). This suggests that activation of PKB, indirectly by PI3K, results in the phosphorylation and inhibition of GSK3 which activates a number of key regulatory proteins.

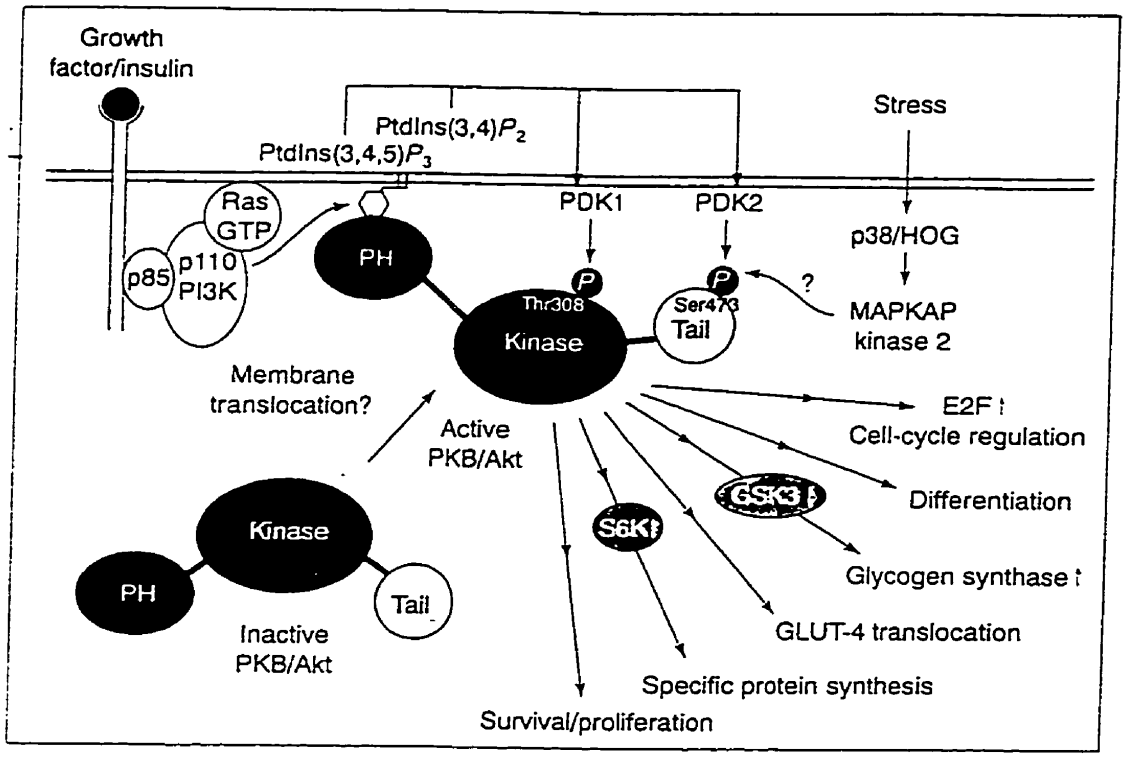


FIGURE 10. Activation of PKB

Upon ligand binding, PI3K becomes activated and produces PI (3,4,5)P_3 and PI(3,4)P_2. The PH domain of PKB binds to the phosphoinositides recruiting PKB to the plasma membrane. Full activation of PKB requires phosphorylation of Thr308 by PDK1 and Ser473 by PDK2. Once activated, PKB phosphorylates its substrates leading to cellular events such as survival and glycogen synthesis (from Marte and Downward, 1997).

1.4.9 p70 S6 RIBOSOMAL PROTEIN KINASE

p70 S6 kinase is activated by most growth factors and cytokines and is responsible for phosphorylating the 40S ribosomal protein S6. Inhibition of this kinase results in inhibition of the cell to progress through the G1 phase of the cell cycle (Lane *et al.*, 1993; Chung *et al.*, 1992). This protein also plays a critical role in regulating the translation of a class of mRNA transcripts which contain an oligopyrimidine tract at their translation start site (Jefferies *et al.*, 1997) indicating the importance of this enzyme in cellular processes. There are two isoforms of this protein that result from alternatively spliced messenger RNAs from the same gene. These two isoforms are identical except one form contains a 23 amino acid extension at its amino-terminal that encodes a nuclear localization sequence and hence is located primarily in the nucleus (Coffer and Woodgett, 1994). The longer isoform is termed p85 S6 kinase and the shorter, mainly cytosolic isoform is termed p70 S6 kinase.

The regulation of the activity of p70 S6K is very complicated and many of the kinases responsible have yet to be identified. There is evidence for the involvement of protein kinase C (PKC) in p70 S6 kinase activation. Treatment of cells with a phorbol ester (such as PMA) which are tumour promoters that directly activate cPKC by mimicking diacylglycerol result in a rapid activation of p70 S6 kinase and leads to S6 phosphorylation (Blenis *et al* ., 1984). Also inhibition of cPKCs by downregulation with prolonged treatment with PMA result in the inhibition of p70 S6 kinase (Blenis and Erikson, 1986).

As mentioned earlier, PI3K has also been shown to have a role in p70 S6 kinase activation. The activation of p70 S6 kinase is increased by constitutively active PI3K (Klippel *et al.*, 1996) and activation of p70 S6K is blocked by dominant negative forms of PI3K as well as inhibitors (wortmannin and LY294002) of PI3K (Weng *et al.*, 1995). The activation of p70 S6 kinase by PI3K appears to occur separately from activation of p70 S6 kinase by PKC. PMA activates p70 S6 kinase in a wortmannin insensitive manner but which can be inhibited by down regulation (Chung *et al.*, 1994). Also, activation of p70 S6 kinase by insulin is completely inhibited by wortmannin and insensitive to downregulation consistent with the ability of insulin to activate PI3K but not cPKC (Chung *et al.*, 1994).

Protein kinase B has been speculated to act upstream of p70 S6 kinase. Since co-transfection of activated PKB increases p70 activity (Burgering and Coffer, 1995), PKB was thought to mediate PI3K-dependent activation of p70 S6 kinase. But the exact role of PKB in p70 S6K activation has yet to be elucidated as a kinase inactive PKB mutant failed to block p70 S6K activation (Burgering and Coffer, 1995).

The inhibition of p70 S6K by rapamycin indicates that other molecules are also involved in this pathway as rapamycin has no effect on the activation of PI3K or PKB but yet inhibits the activation of p70 by both the PKC and the PI3K pathway (Chung *et al.*, 1994). Rapamycin binds to an intracellular receptor, FKBP (FK506-binding protein where FK506 is an immunosuppressant), and the rapamycin:FKBP complex exerts its antiproliferative effects by targeting other intracellular proteins

(Schreiber, 1991). The target of this complex was identified in yeasts and called TOR for target of rapamycin (Cafferkey *et al.*, 1993) and the mammalian homologue was later identified and called a number of different names including RAPT1, FRAP, RAFT1, SEP and mTOR (Brown *et al.*, 1994). This protein appears to be the rapamycin sensitive p70 S6K upstream regulator.

The regulation of p70 S6 kinase has been difficult to elucidate primarily due to the complexity of the process. There are a number of different phosphorylation sites in the enzyme which further complicates the activation process. p70 S6 kinase is predominantly phosphorylated on serine and threonine residues and at least seven sites have been identified to be necessary for activation (**Figure 11**). There are four phosphorylation sites (Ser411, Ser418, Thr421, Ser424) located in the C-terminal region of this enzyme. These residues all contain a proline in the +1 position and reside within the putative autoinhibitory domain (Ferrari *et al.*, 1992). These residues are phosphorylated in response to mitogens but the kinase responsible for the phosphorylation of these residues in unknown. Because of the proline in the +1 position, these residues are possible targets of proline directed kinases. Although Cdc2 and MAP kinases have been shown to phosphorylate these sites *in vitro*, there is no evidence that this occurs *in vivo* (Mukhopadhyay *et al.*, 1992). These four residues are hypophosphorylated in quiescent cells and become hyperphosphorylated upon serum activation (Han *et al.*, 1995). When these four residues are mutated to Asp or Glu (to mimic phosphoserine or phosphothreonine respectively) the basal kinase activity is increased (Ferrari *et al.*,

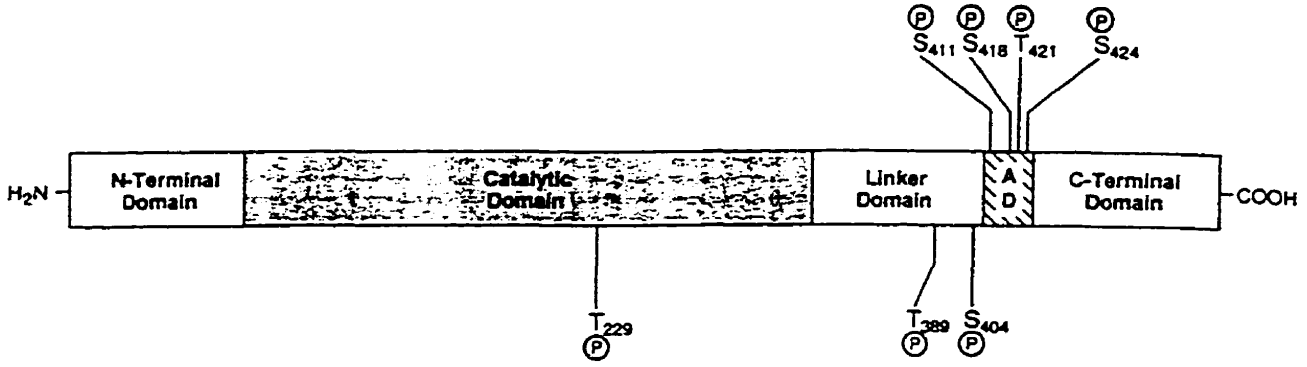


FIGURE 11. Structure of p70 S6K

Multiple phosphorylation sites are required for the activation of p70 S6K. Ser411, Ser418, Thr421 and Ser424 are in the autoinhibitory domain (AD). Thr229 is in the catalytic domain while Thr389 and Ser404 are in the linker domain (from Han *et al.*, 1995).

1993) and mutation to alanine suppresses the kinase (Han *et al.*, 1995) indicating the importance of these residues to activation. Full activation of the kinase occurs with phosphorylation of Thr229 in the T-loop or catalytic domain, as well as phosphorylation of Thr389 and Ser404 in the catalytic domain. Mutation of Thr229 to an alanine renders the kinase inactive while a change to serine restores activity (Weng *et al.*, 1995). Thr389 phosphorylation is important and phosphorylation of this residue results in full activation of the kinase. The rapamycin and wortmannin sensitive sites in p70 S6K are Thr229, Thr389, Ser404, and Ser411 although the principal target is Thr389 (Pearson *et al.*, 1995). The effects of these drugs can be suppressed by a T389E change while a T389A fully activates p70 S6K. There are indications that phosphorylation of Thr389 regulates phosphorylation on Thr229.

The model of p70 S6 kinase is thought to proceed by binding of an effector molecule at the N-terminus which contains a stretch of acidic amino acids that is hypothesized to be involved in substrate recognition. It is speculated that the C-terminus acts as a pseudosubstrate/autoinhibitory region by occluding the catalytic site (Price *et al.*, 1991). It is thought that p70 S6K is maintained in an inactive conformation by the intramolecular interaction of the C-terminal pseudosubstrate domain with the catalytic domain. The binding of an effector molecule at the N-terminus allows segregation from the C-terminus. Hyperphosphorylation of the four C-terminal sites, induced by mitogens, may release the pseudosubstrate domain from the catalytic site. This allows a mitogen-regulated kinase to phosphorylate

Thr389 resulting in the phosphorylation of the T-loop site (Thr229) leading to full activation of the kinase. Additional phosphorylation sites on p70 S6 kinase have also been identified including Ser371 (Moser *et al.*, 1997). The contribution of this site to p70 S6 kinase activation is not known.

Recently PDK1 was identified as the *in vivo* kinase responsible for mediating Thr229 phosphorylation in the catalytic domain of p70 S6 kinase (Pullen *et al.*, 1998). These studies showed that PDK1 is constitutively active and dependent on prior phosphorylation of Thr389 to provide access to Thr229. The kinase responsible for phosphorylating Thr389 has yet to be identified. The proposed model for p70 S6 kinase activation and the key players involved are depicted in **Figure 12**.

The insulin pathway (**Figure 13**) is a complicated network of interacting proteins. There are many redundancies of the signalling molecules in this pathway. For example, Grb2 can bind to both Shc and IRS-1 and lead to MAPK activation. The activation of this signalling complex therefore is very important in the cell and is responsible for initiating many diverse cellular responses.

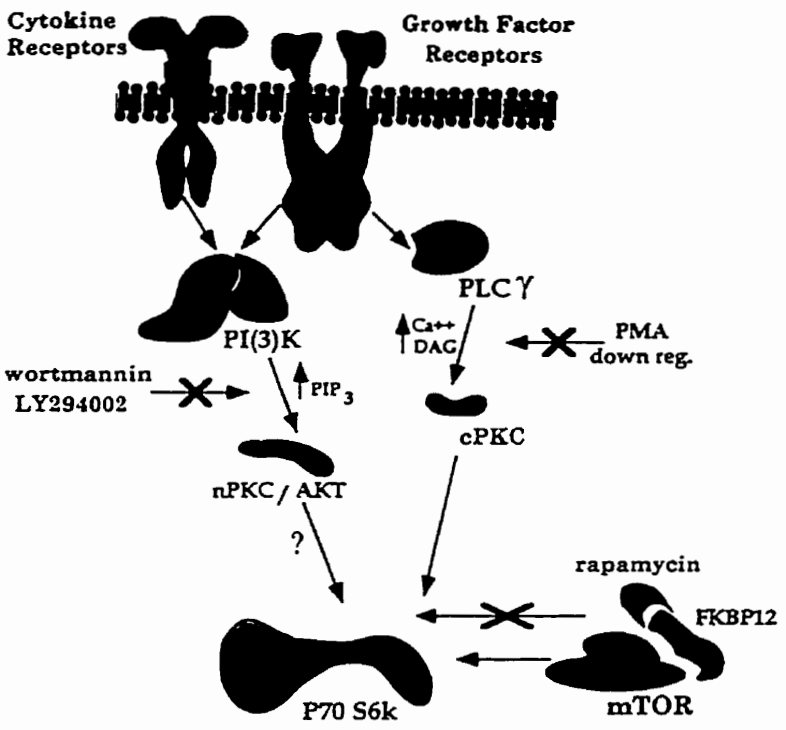


FIGURE 12. Activation of p70 S6K

There are multiple signals that culminate in the activation of p70 S6K. Activation of p70 S6K can be inhibited by wortmannin and LY294002, PI3K inhibitors, as well as rapamycin (from Grammar *et al.*, 1996)

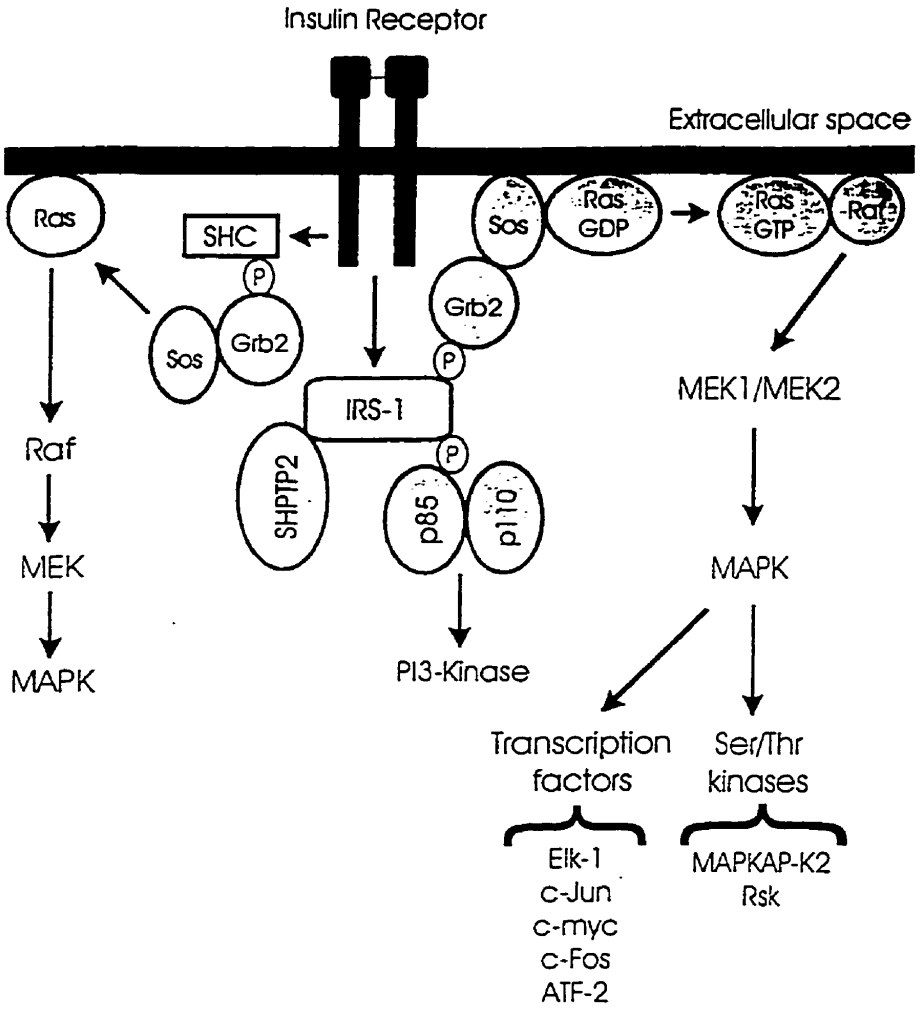


FIGURE 13. Insulin Pathway

Insulin is able to activate multiple signal transduction pathways in the cell. Once insulin binds to its receptor, substrates of the insulin receptor are phosphorylated including Shc and IRS-1. The phosphorylation of Shc leads to the binding of Grb2 and the activation of MAPK. A number of cytosolic proteins containing SH2 domains are able to bind to phosphorylated IRS-1 including Grb2, PI3K, and SHPTP2 (from Ashagbley, 1997).

1.5 RESEARCH AIMS

The overall objective of this research is to investigate how ET-18-OCH_3 inhibits the proliferation of cancer cells. From previous results, it is known that in EGF-stimulated cells, ET-18-OCH_3 inhibits MAPK activity by inhibiting the association of Raf-1 with the membrane. Because of the numerous signal transduction pathways in the cell, it was unclear whether inhibition of Raf-1 kinase activation by ET-18-OCH_3 was sufficient to cause the observed decrease in cell growth or whether inhibition of additional signalling pathways by ET-18-OCH_3 also contributed to the overall inhibition of cell proliferation. In order to attempt to answer these questions, we decided to look at the insulin signal transduction pathways in MCF-7 cells. We wanted to examine the effect of ET-18-OCH_3 on many of the proteins activated following insulin stimulation, and to determine whether any inhibitions we observed could contribute to a decrease in cell growth. To aid in this quest, we used the (*R*) and (*S*)-TDB-PC compounds to further ascertain whether the inhibitions we observed correlate to a decrease in cell proliferation.

In addition to looking at other molecules in the cell, we also wanted to further investigate the mechanism of inhibition of Raf-1 activation by ET-18-OCH_3.

HYPOTHESIS

We hypothesized that because of the number of signal transduction pathways in the cell that are involved in regulating proliferation, ET-18-OCH_3 may

inhibit other molecules in addition to Raf-1 which could contribute to the overall inhibition of cell proliferation that is observed when ET-18-OCH_3 is added to MCF-7 cells.

2 MATERIALS

2.1 Cell Lines, Media, and Other Tissue Culture Materials

MCF-7 (human breast adenocarcinoma) cells were grown from frozen stocks originally obtained from American Type Culture Collection (ATCC) (Rockville, Maryland)

Fetal bovine serum (FBS) was from Hyclone Lab (Logan, Utah).

Bovine serum albumin and trypsin/EDTA were purchased from Sigma Chemical Co. (St. Louis, Missouri).

Penicillin, streptomycin, fungizone, and DMEM were obtained from Gibco BRL (Burlington, Ontario).

2.2 Antibodies

Rabbit polyclonal anti-phospho-MAPK, rabbit polyclonal anti-phospho-PKB, rabbit polyclonal anti-phospho-p70 S6K, rabbit polyclonal anti-PKB antibodies and chemiluminescent detection kit were from New England Biolabs (Mississauga, Ontario).

Rabbit polyclonal anti-IRS-1, mouse monoclonal anti-phosphotyrosine , rabbit polyclonal anti-Raf-1, rabbit polyclonal anti-p70 S6K, rabbit polyclonal anti-ERK1, rabbit polyclonal anti-ERK2, protein A-agarose and protein G plus-agarose were from Santa Cruz Biotechnology, Inc. (La Jolla, California).

Sheep polyclonal anti-PKB, sheep polyclonal anti-p70 S6K, rabbit polyclonal

PI 3-Kinase, and Crosstide were from Upstate Biotechnology Incorporated (Lake Placid, New York).

Mouse monoclonal anti-Raf-1 was from Transduction Labs (Lexington, Kentucky).

Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (H+L) and HRP-goat anti-mouse IgG (H+L), 0.2 μM nitrocellulose membrane were obtained from Bio-Rad Laboratories (Mississauga, Ontario).

2.3 Radiochemicals and Related Reagents

[^{3}H]Thymidine and adenosine 5'-[γ-^{32}P] triphosphate ([γ-^{32}P]ATP) and Ecolite scintillation fluid were from ICN Biomedicals (Montreal, Quebec).

2.4 Chemicals and Reagents

ET-18-OCH_3 was a product of MedMark Pharmaceuticals (Gruenwald, Germany).

(*R*) and (*S*)-ET-16 phosphono-TDB were synthesized and generously provided by Dr. R. Bittman (Queens College, City University of New York).

Iletin regular insulin (beef and pork) was from Eli Lilly and Company (Toronto, Ontario).

Wortmannin, LY294002, microcystin, ATP, Calmidazolium, Tris-HCl, β-glycerophosphate, aprotinin, leupeptin, AEBSF, benzamidine, EDTA, EGTA, sodium orthovanadate, sodium fluoride, sodium pyrophosphate, sodium chloride,

bovine serum albumin, lysozyme, epidermal growth factor (EGF), nonidet P-40 (NP-40), Triton X-100, PMSF, MOPS, morpholine-ethanesulfonic acid, potassium chloride, potassium phosphate (KH_2PO_4), magnesium chloride, potassium oxalate, lithium chloride, Brij 35 solution, fibroblast growth factor (FGF), trichloroacetic acid (TCA) and GSH-agarose were from Sigma Chemical Co. (St. Louis, Missouri).

Acrylamide, ammonium persulfate, N, N'-methylene bisacrylamide, sodium dodecyl sulfate (SDS), and N,N,N',N'-tetrymethyl ethylenediamine (TEMED), and 2-mercaptoethanol were obtained from Crescent Chemical Co., Inc (New York).

Hydrogen chloride, 70% perchloric acid ($HClO_4$), phosphoric acid, glycerol, calcium chloride, chloroform ($CHCl_3$), methanol (MeOH), ammonium hydroxide (NH_4OH) were from Fisher Scientific Company (Winnipeg, Manitoba).

HEPES, MBP, LB medium, and glucose were from Gibco BRL (Burlington, Ontario).

KGF, IPTG, GTP-γ-S were from Boehringer Mannheim (Laval, Quebec).

PKI, IGF-1 was from Bachem (Torrance, California).

Phosphatidylinositol, lysophosphatidic acid, lysoplatelet-activating factor, lysophosphatidylethanolamine, lysophosphatidylinositol, phosphatidic acid, phosphatidylcholine, and phosphatidylserine were from Serdary Research Laboratories (St. Catherines, Ontario).

Kodak GBX developer and replenisher, Kodak rapid fixer with hardener and Kodak X-OMAT film was from Picker (Brampton, Ontario).

DTT, sodium hydroxide (NaOH), sodium phosphate ($Na_2PO_4.7H_2O$) were

from Mallinckrodt (Montreal, Quebec).

Coomassie protein assay solution was from Pierce (Rockford, Illinois).

Whatman p81 filter paper was from Mandel Scientific Company (Guelph, Ontario).

Silica gel K6 thin layer chromatography (TLC) plates were purchased from Baxter Canlab Diagnostics Inc. (Winnipeg, Manitoba).

2.5 Equipment

Model ZM Coulter Counter (Coulter Electronics Inc.), Model TMS-F Microscope (Nikon, Japan), L-80 Ultracentrifuge (Beckman), J2-HS Centrifuge (Beckman), LS3801 Liquid Scintillation System (Beckman), U-2000 Double beam Spectrophotometer (Hitachi, Tokyo), Model PDI 325oe High Resolution Color Scanner (Protein + DNA Imageware Systems, Huntington, New York), and Microsonic XL Ultrasonic cell disrupter were utilized for this thesis research.

2.6 Buffers

Distilled deionized water (DDW) (conductance $\leq$-18$\mu\Omega$) from a Milli Q Plus water purification system was used throughout these studies.

Buffer A: 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 0.5 mM sodium orthovanadate, 0.1% 2-mercaptoethanol, 1% Triton X-100, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 10 mM sodium glyerophosphate, 0.1 mM

PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin.

Buffer B: 50 mM Tris-HCl (pH 7.5), 0.03% Brij-35, 0.1 mM EGTA, 0.1% 2-mercaptoethanol.

Buffer C: 100 mM β-glycerophosphate, 2 mM EGTA, 1 mM sodium orthovanadate, 20 mM Tris-HCl (pH 7.5), 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.1 mM AEBSF, 1 mM DTT, 0.1 mM PMSF, 0.2 mM benzamidine.

Buffer D: 50 mM HEPES (pH 7.5), 150 mM sodium chloride, 1% Triton X-100, 1 mM sodium orthovanadate, 100 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, 1 mM DTT, 0.1 mM AEBSF, 20 mM Tris-HCl (pH 7.5), 2 mM EGTA, 0.2 mM benzamidine, 100 mM β-glycerophosphate.

Buffer E: 50 mM HEPES (pH 7.5), 150 mM sodium chloride, 100 mM sodium fluoride, 1% Triton X-100, 0.1% SDS.

Buffer F: 137 mM sodium chloride, 20 mM Tris-HCl (pH 8.0), 1 mM magnesium chloride, 10 % glycerol, 0.4 mM sodium orthovanadate, 1 mM DTT, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM calcium chloride, 100 mM β-glycerophosphate, 0.1 mM AEBSF, 0.2 mM benzamidine, 1% NP-40.

Buffer G: 40 mM Tris-HCl (pH 7.4), 1 % Triton X-100, 1 mM DTT.

Buffer H: 10 mM Tris-HCl (pH 7.4), 0.5 M lithium chloride, 1 mM DTT.

Buffer I: 10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM DTT.

Buffer J: 20mM MOPS (pH 7.2), 25 mM β-glycerophosphate, 5mM EGTA, 1 mM sodium orthovanadate, 1 mM DTT.

Buffer K: 20 mM Tris-HCl (pH 8.0), 100 mM sodium chloride, 1 mM EDTA,

1% Triton X-100, 1 mM PMSF.

Buffer L: 20 mM Tris-HCl (pH. 8.0), 100 mM sodium chloride.

Buffer M: 25 mM Tris, 192 mM glycine, 0.1% SDS

Hanks' balanced saline solution (HBSS): 0.04% potassium chloride, 0.8% sodium chloride, 0.006% KH_2PO_4, 0.009% $Na_2HPO_4.7H_2O$ and 1% glucose (all were w/v).

PBS: 8 g sodium chloride, 0.2 g potassium chloride, 1.15 g $Na_2HPO_4.7H_2O$, 0.2 g KH_2PO_4 per liter.

TBS: 2.42 g Tris-Base, 8 g sodium chloride per liter.

5 X SDS sample buffer: 60 mM Tris-HCl (pH 6.8), 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, 0.1% bromophenol blue.

Exchange Buffer: 50 mM sodium chloride, 25 mM morpholine-ethanesulfonic acid (MES)-NaOH, 2.5 mM EDTA, 0.05% Triton X-100, pH 6.5.

3 EXPERIMENTAL METHODS

3.1 Cell Culture Conditions

Cells were grown in plastic tissue culture plates in DMEM containing 10% FBS supplemented with penicillin (50 U/ml), streptomycin (50ug/ml), and fungizone (0.3 μg/ml). Cells were grown at 37°C in 5%CO_2/ 95% humidified air atmosphere.

Quiescent MCF-7 cells were obtained by seeding cells in 150 mm tissue culture dishes overnight in DMEM supplemented with 10% FBS. After 24 hours, the cells were washed and incubated in DMEM containing 0.5 mg/ml BSA. The cells

were considered quiescent when the 24 hour increase in cell number was less than 10% (usually around 5-8 days).

For monitoring quiescence, MCF-7 cells growing in 6-well plates, growing at the same density as in 150 mm plates, were detached with trypsin and an aliquot was taken for counting on a Model ZM Coulter Counter.

3.2 Protein Determination

The protein concentration of samples was determined with Coomassie Protein Assay Reagent (Pierce). Briefly, 10 μl of sample was added to 990 μl DDW. One ml of Coomassie protein assay reagent was added and the absorbance was measured at 595 nm. A standard curve with BSA was used to extrapolate the protein concentrations in the sample.

3.3 Electrophoresis and Immunoblots

SDS-PAGE separates proteins based primarily on molecular mass. Different percentages of separating gel were used depending on the resolution desired. To vary the percentage, different amounts of acrylamide was used. The separating gel was poured between glass plates on the mini gel apparatus filling about two-thirds of the gel sandwich. Water was added on top of the separating gel to keep the gel surface flat. Once the separating gel polymerized, the stacking gel was added and the combs were inserted. After polymerization of the stacking gel, the samples were loaded into the wells and the gel was run between 100-150 volts in Buffer M.

The gel was then transferred to nitrocellulose for two hours at 100 volts in Buffer M + 20% methanol. The nitrocellulose membrane was then blocked to reduce non-specific binding. For the phospho-antibodies, the membrane was blocked in 5% milk / TBS with 0.1% Tween-20 (0.1% TBST) for one hour. When other antibodies were used, the membrane was blocked in 8% milk / PBS with 0.1% Tween-20 (0.1% PBST) for three hours or overnight. The phospho-antibodies were added overnight to the membrane in 5% BSA / 0.1% TBST while the other antibodies were added in 6.5% milk / 0.05% PBST for three hours or overnight. The membranes were washed (0.1% TBST for the phospho-antibodies and 0.05% PBST for the other antibodies) and the HRP-conjugated secondary antibody was added for one hour (in 5% milk / 0.1% TBST for the phospho-antibodies and in 5% milk / 0.05% PBST for the other antibodies). The membranes were washed and incubated in chemiluminescence solution, exposed to film and developed.

3.4 Quantitation of Band Density

Quantitation of immunoblots was achieved by densitometric analysis with a high resolution colour scanner model PDI 325oe using the ImageMaster scanning program (Pharmacia)

3.5 [^{3}H]Thymidine Incorporation in MCF-7 cells

To determine the mitogenic effect of various growth factors on MCF-7 cells, the cells were plated in 6-well plates and were made quiescent. Various growth

factors [EGF (10 ng/ml), insulin (1μg/ml), IGF-1 (10 ng/ml), serum (10%), FGF (10 ng/ml), KGF (15 ng/ml)] were added for 18 hours in DMEM/BSA. [^{3}H]thymidine (0.5 μCi/well) was then added for an additional 6 hours. The medium was then aspirated and the cells were washed 3 times with HBSS. 1 ml of ice-cold trichloroacetic acid (TCA, 10%) was added to each well and incubated for 15 minutes at 4°C. The TCA was aspirated and each well was washed twice with ice-cold TCA (10%). The wells were then air-dried for 10 minutes followed by solubilization in 1% SDS / 0.3 NaOH. [^{3}H] thymidine was measured by scintillation counting and a blank was subtracted that did not contain any growth factor but remained in DMEM/BSA.

To determine the effect of ET-18-OCH_3 on [^{3}H]thymidine incorporation induced by insulin, the above procedure was followed except that ET-18-OCH_3 (10 μg/ml) was added to the wells for selected periods (0, 1, 2, 3 hrs) prior to the addition of insulin (1 μg/ml) for 18 hours. After the drug was added for the appropriate time, the wells were washed and fresh DMEM/BSA plus insulin was added.

3.6 Phosphorylation of MAPK

Quiescent MCF-7 cells growing in 150 mm dishes were incubated with ET-18-OCH_3 (10 μg/ml) for 3 hours. The cells were then washed and incubated in fresh DMEM/BSA and stimulated with insulin for selected times. After stimulation, the cells were washed three times with ice-cold HBSS and scraped into buffer C.

The lysates were sonicated (3X10 s), centrifuged at 200,000 x g for 30 minutes at 4°C. The supernatants were transferred to eppendorf tubes and stored at -80°C.

Equal protein (65 μg) was loaded on a 10% SDS-polyacrylamide gel. The samples were separated by electrophoresis and then transferred to nitrocellulose membrane and probed with phospho-MAPK antibody (1:1000 dilution). A parallel blot (with 10 μg protein) was probed with ERK1/2 antibody (1:250 dilution). The proteins were detected using HRP-goat anti-rabbit IgG antibody and chemiluminescence was used to detect the bands.

3.7 MAPK Assay

Cells were harvested as described above and the cytosolic fractions were subjected to MAPK assay as described in Ahn *et al.*, (1990). MAPK activity was measured as the phosphorylation of myelin basic protein. The reaction mixture (total volume = 30μl) contained 2 μM protein kinase A inhibitor (PKI) peptide, 10 μM calmidazolium, 8 μg of myelin basic protein, 20 mM $MgCl_2$, 0.1 mg/ml BSA, 40 mM β-glycerophosphate, 0.15 mM Na_3VO_4 and 0.15 mM ATP (3 x 10^6 cpm/nmol). The reaction was initiated by the addition of 1 μg of cytosolic protein and the mixture was incubated at 30°C for 10 minutes. The reaction was terminated by spotting 20 μl of the mixture on P81 paper which was washed several times with 150 mM phosphoric acid. After drying the paper, the amount of bound radioactivity was determined by scintillation counting and the values were corrected for blanks which were without myelin basic protein.

3.8 Translocation of Raf-1 to the Plasma Membrane

Quiescent MCF-7 cells growing in 150 mm plates were preincubated with or without ET-18-OCH_3 (10 μg/ml). The media was then replaced with fresh DMEM/BSA and stimulated with insulin (1 μg/ml) for selected times. The cells were washed three times with ice-cold HBSS and then scraped into buffer C. The lysates were sonicated, centrifuged at 7,000 x g for 15 minutes at 4°C to remove unbroken cells, nuclei and cell debris. The supernatants were then centrifuged at 200,000 x g for 30 minutes at 4°C to separate the cytosol (supernatant) from the membrane (pellet). The pellets were resuspended in buffer C containing 1% Triton X-100 and 0.5% Nonidet P-40, sonicated and re-centrifuged at 200,000 x g for 30 minutes to obtain a solubilized membrane fraction. Equal protein samples from the membrane (25 μg) were loaded on 10% SDS-polyacrylamide gels, the samples were separated by electrophoresis and transferred to nitrocellulose membrane and immunoblotted with anti-Raf-1 antibody (1:100 dilution with the Santa Cruz anti-raf-1 antibody or 1:1000 dilution with the Transduction Lab anti-Raf-1 antibody) and detected by chemiluminescence.

3.9 Ras-Raf-1 binding

Quiescent MCF-7 cells were incubated with or without ET-18-OCH_3 (10 μg/ml) and the cells were washed three times with ice-cold HBSS and scraped into buffer C. The lysates were sonicated, centrifuged at 200,000 x g at 4°C for 30 minutes and stored overnight on ice at 4°C.

Purification of GST-Ras

GST-Ras cloned into a pGEX vector and provided by Dr. Bhullar and the fusion protein was purified as previously described (Jilkina and Bhullar, 1996). The GST-Ras construct was grown up overnight in 5 ml LB medium supplemented with ampicillin (50 μg/ml) at 37°C. The small culture was transferred to a large culture and grown overnight at room temperature. The next morning, IPTG (0.5 mM) was added for 3 hours and the bacteria were pelleted by centrifugation (6,000 rpm, 15 min, 4°C). The pellet was resuspended in buffer L, homogenized and lysozyme (1mg/ml) was added. The bacteria were shaken with the lysozyme for 15 minutes at room temperature and then Buffer K containing 3% Triton X-100 was added to give a final Triton X-100 concentration of 1%. The bacteria were sonicated twice and centrifuged at 16,000 rpm for 45 minutes at 4°C. One ml of GSH-agarose beads, pre-equilibriated in Buffer L was added to the supernatant and the tube was placed on a shaker and mixed for 60 minutes at room temperature. The solution was centrifuged to pellet the beads and the beads were washed three times with Buffer K and two times with Buffer L. The beads were then resuspended in 1:1 Buffer L and were stored at 4°C until used (up to 2 weeks). Ten μl of the bacteria extract was run on an SDS-gel and stained to check the purity of the Ras. On the day the purified Ras construct from the bacteria was used, it was loaded with GTP-γ-S (Manser *et al.*, 1995). The beads were spun down and 500 μl of 2 X Exchange buffer containing 0.5 mM GTP-γ-S was added for 5 minutes at room temperature. The beads were centrifuged (1500 rpm for 50 seconds) and washed once with 100

mM MOPS / 5 mM $MgCl_2$ containing 0.5 mM GTP-γ-S and once in 100 mM MOPS / 5 mM $MgCl_2$. The beads containing the purified Ras loaded with GTP-γ-S were resuspended in 100 mM MOPS / 5 mM $MgCl_2$.

100 μl of the GST-Ras loaded with GTP-γ-S was added to 1 mg of cell lysate and rotated for 90 minutes at 4°C. The samples were centrifuged and washed three times with 100 mM MOPS / 5 mM $MgCl_2$. The beads were dissolved in 75 μl SDS sample buffer, heated for 10 minutes, centrifuged and 20 μl was run on an SDS-polyacrylamide gel. The gel was transferred to nitrocellulose membrane and Western blotted with Raf-1 antibody (1:100 with the Santa Cruz Raf-1 antibody or 1:1000 with the Transduction Lab Raf-1 antibody).

3.9.1 Ras-Raf-1 binding *in vitro*

Quiescent MCF-7 cells were washed and scraped into buffer C, sonicated, and centrifuged. The lysates were stored overnight on ice at 4°C. 1 mg cell lysate was incubated with ET-18-OCH_3 (different concentrations) for 3 hours at room temperature, 37°C and 4°C. Then the lysates were treated with the GST-Ras loaded with GTP-γ-S as described in section 3.9.

3.10 Effect of Lysolipids on Ras-Raf-1 binding

Quiescent MCF-7 cells growing in 150 mm dishes were treated with or without the different lysolipids (20 μM, 3 h), washed three times with ice-cold HBSS and scraped into buffer C. The lysates were sonicated, centrifuged and stored on

ice at 4°C overnight. The lysates were treated with GST-Ras loaded with GTP-γ-S and the Ras-Raf-1 binding was assessed as previously described in Section 3.9.

3.11 · Immunoprecipitation of IRS-1

Quiescent MCF-7 cells were treated with or without ET-18-OCH_3 (10 μg/ml) and stimulated with insulin (1 μg/ml) for selected times. The plates were washed in HBSS and scraped into buffer D. The lysates were sonicated, and centrifuged as described in Section 3.6. The samples (protein concentration = 1 mg) were precleared with Protein A-agarose for 1 hour on a rotator at 4°C. The samples were then incubated with IRS-1 antibody (2 μg/tube) overnight on a rotator at 4°C to immunoprecipitate the IRS-1 protein. The IRS-1 immunoprecipitates were washed three times in buffer E and SDS sample buffer was added. The IRS-1 immunoprecipitates were heated at 100°C for 10 minutes and resolved by SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were either Western blotted with phosphotyrosine antibody (1:250 dilution) to examine the tyrosine phosphorylation of IRS-1 or with PI3K antibody (1:1000 dilution) to examine the association of PI3K with IRS-1.

3.12 PI3K Activity

Quiescent MCF-7 cells were treated with or without ET-18-OCH_3 (10 μg/ml), stimulated with insulin (1 μg/ml), washed in HBSS, and scraped into buffer F. The lysates were harvested as described previously in Section 3.6. Samples (1 mg)

were precleared with Protein A-agarose for 90 minutes on a rotator at 4°C. IRS-1 antibody was then added (2.5 μg/tube) and incubated overnight on a rotator at 4°C. The next morning, Protein A-agarose was added for 2 hours. The IRS-1 immunoprecipitates were washed twice with buffer G, twice with buffer H, and twice with buffer I. The IRS-1 immunoprecipitates were incubated in a reaction mixture and assayed for PI3K activity basically as described in Okada et al., (1994). Each immunoprecipitate was incubated in a reaction mixture (total volume=100 μl) containing 50 mM Tris HCl (pH 7.4), 0.5 mM EGTA, 5 mM $MgCl_2$, 40 μM ATP (20 μCi), 20 μg PI. The kinase reaction was incubated at 30°C for 10 minutes and was stopped by the addition of 20 μl 8% $HClO_4$ and 0.45 ml $CHCl_3$ / MeOH (1:2). The mixture was vortexed and 0.15 ml of $CHCl_3$ and 0.15 ml 8% $HClO_4$ were added. The organic layer was separated and could be kept at -20°C at this point. The samples were spotted on a potassium oxalate-coated TLC plates that had been heated at 110°C for 60 minutes. The TLC plates were run in $CHCl_3$ / MeOH / H_2O / NH_4OH (60 / 45 / 11.3 / 3.2) solvent system and were then air-dried. The plates were exposed to film and the areas of the plates corresponding to PIP were scraped and the amount of radioactivity determined by scintillation counting.

3.13 Phosphorylation of PKB and p70 S6 Kinase

Quiescent MCF-7 cells were pretreated with or without ET-18-OCH_3 (10 μg/ml) and stimulated with insulin (1 μg/ml) for various times. The cells were washed three times in HBSS and scraped into buffer F and harvested as previously

described in section 3.6. SDS-polyacrylamide gels were run using the cell lysates (65 μg) and Western blots were performed using the different phospho-antibodies (1:1000 dilution). The same procedure was followed when looking at the effect of (*R*)-TDB-PC and (*S*)-TDB-PC on the phosphorylation of PKB and p70 S6K except that the MCF-7 cells were treated with or without the ALPS (30 μM, 4 h) prior to stimulation with insulin. The phospho-PKB and phospho-p70 S6K bands were identified by molecular weight, by comparing to the band obtained with the non-phospho antibody and by comparing to a positive control.

3.14 p70 S6 Kinase Activity

Lysates obtained as described in section 3.10 were also used to measure p70 kinase activity.

Protein G was equilibrated with PBS and a 1:1 Protein G / PBS (v/v) solution was prepared. Three μg of p70 antibody was added to 80 μl Protein G / PBS and 400 μl of PBS for 1 hour at 4°C on a rotator. The antibody / protein G complex was centrifuged for 15 seconds at 14,000 rpm in a microfuge and washed twice with buffer A. 100 μl of buffer A was added to the beads and cell lysate (protein concentration = 1 mg) was added and rotated for 2 hours at 4°C. The p70 S6K immunoprecipitates were washed once in buffer A containing 0.5 M NaCl, once in buffer A and once in buffer J. The immunoprecipitates were incubated in a kinase reaction mixture containing buffer J, 2 μM PKI, 10 μM calmidazolium, 30 μM Crosstide (substrate) and 500 μM cold ATP / 75 mM $MgCl_2$ / 10 μCi [γ-^{32}P]ATP

(total reaction volume = 40 μl). The kinase reaction was incubated for 10 minutes at 30°C. At the end of the reaction, 25 μl was spotted on P81 paper and washed with 0.75% phosphoric acid. The amount of bound radioactivity was determined by scintillation counting. A blank was carried through the whole procedure which had protein G but did not contain any antibody.

3.15 Direct Effect of Drugs on p70 Kinase Activity

To examine the direct effect of the ALPs on p70 S6K activity, quiescent MCF-7 cells were stimulated with insulin (1 μg/ml) for 20 minutes and washed and harvested as in section 3.6. p70 S6K was immunoprecipated from the cell lysates as described in section 3.11. The kinase assay was essentially the same as in section 3.11 except that different concentrations of ET-18-OCH_3, (*R*)-TDB-PC and (*S*)-TDB-PC were added to the kinase reaction mixture.

3.16 PKB Kinase Assay

Quiescent MCF-7 cells were treated with or without ET-18-OCH_3 (10 μg/ml) or with or without (*R*)-TDB-PC and (*S*)-TDB-PC (30 μM, 4 h), stimulated with insulin (1 μg/ml) for various times, washed with HBSS and scraped into buffer A containing 1 μM microcystin and harvested as in section 3.6. Protein G was equilibrated with buffer A. 4 μg PKB antibody was added to 80 μl Protein G / buffer A mixture and 450 μl buffer A for 1 hour on a rotator at 4°C. 1 mg cell lysate was added to the antibody / Protein G complex and rotated for 90 minutes at 4°C. The PKB

immunoprecipitates were washed three times in buffer A containing 0.5 M NaCl, twice with buffer B and twice with 100 μl of buffer J. The PKB immunoprecipitates were incubated in the kinase reaction mixture (Cross et al., 1995) containing buffer J, 80 μM PKI, 30 μM Crosstide, ATP (500 μM ATP / 75 mM $MgCl_2$ / 10 μCi [γ-^{32}P]ATP) (total reaction volume = 40 μl). The reaction was incubated at 30°C for 10 minutes. At the end of the reaction, 25 μl was added to P81 paper. The paper was washed in 0.75% phosphoric acid and the amount of bound radioactivity was determined by scintillation counting. The same procedure was followed for the PKB kinase assay with the exogenous drugs except that the quiescent MCF-7 cells were stimulated with insulin (1 μg/ml) for 30 minutes and different concentrations of ET-18-OCH_3, (*R*)-TDB-PC and (*S*)-TDB-PC were added to the kinase reaction mixture prior to the assay for PKB activity.

3.17 Effect of PI3K Inhibitors on Phosphorylation of PKB and p70 S6K

Quiescent MCF-7 cells were treated with different concentrations of LY294002 for 20 minutes or with different concentrations of wortmannin for 60 minutes. The cells were stimulated with insulin (1 μg/ml) for 20 minutes. The cells were scraped into Buffer F and harvested as previously described in section 3.6. SDS-polyacrylamide gels were run with cell lysates (65 μg) and Western blots were performed using either phospho-PKB (Ser 473) or phospho-p70 S6K (Ser 411) antibodies (1:1000 dilution).

4. RESULTS

4.1 Incorporation of [^{3}H]thymidine into MCF-7 cells

Insulin is known to activate a number of different signal transduction pathways in the cell (Myers *et al.*, 1994). Also, insulin and IGF-1 have been suggested to be mitogenic in many cell lines including MCF-7 cells (Hill and Sousa, 1990; Cambrey *et al.*, 1995; Dufourny *et al.*, 1997). Initially, we conducted studies to confirm the mitogenic effects of insulin as well as a number of other growth factors. We examined the ability of these growth factors to stimulate [^{3}H]thymidine incorporation into MCF-7 cells indicating the progression into S-phase. Treatment of the cells with either insulin or IGF-1 caused the greatest amount of [^{3}H]thymidine incorporation (**Figure 14**) verifying their mitogenic ability in these cells. Since we confirmed the mitogenic effect of insulin, we decided to more closely examine the effect of ET-18-OCH_3 on the signalling molecules involved in the insulin pathways.

4.2 Effect of ET-18-OCH_3 on Insulin-stimulated [^{3}H]thymidine Incorporation in MCF-7 cells (contribution by Xiaoli Lu)

After establishing the mitogenic properties of insulin in these cells, the effect of ET-18-OCH_3 on the mitogenic effect were assessed. Preincubation of the MCF-7 cells with ET-18-OCH_3 resulted in a time-dependent decrease in insulin-stimulated [^{3}H]thymidine incorporation (**Figure 15**) verifying that ET-18-OCH_3 was affecting insulin-stimulated growth. As a result, we decided to investigate the effect of ET-18-OCH_3 on different insulin signal transduction pathways in these cells.

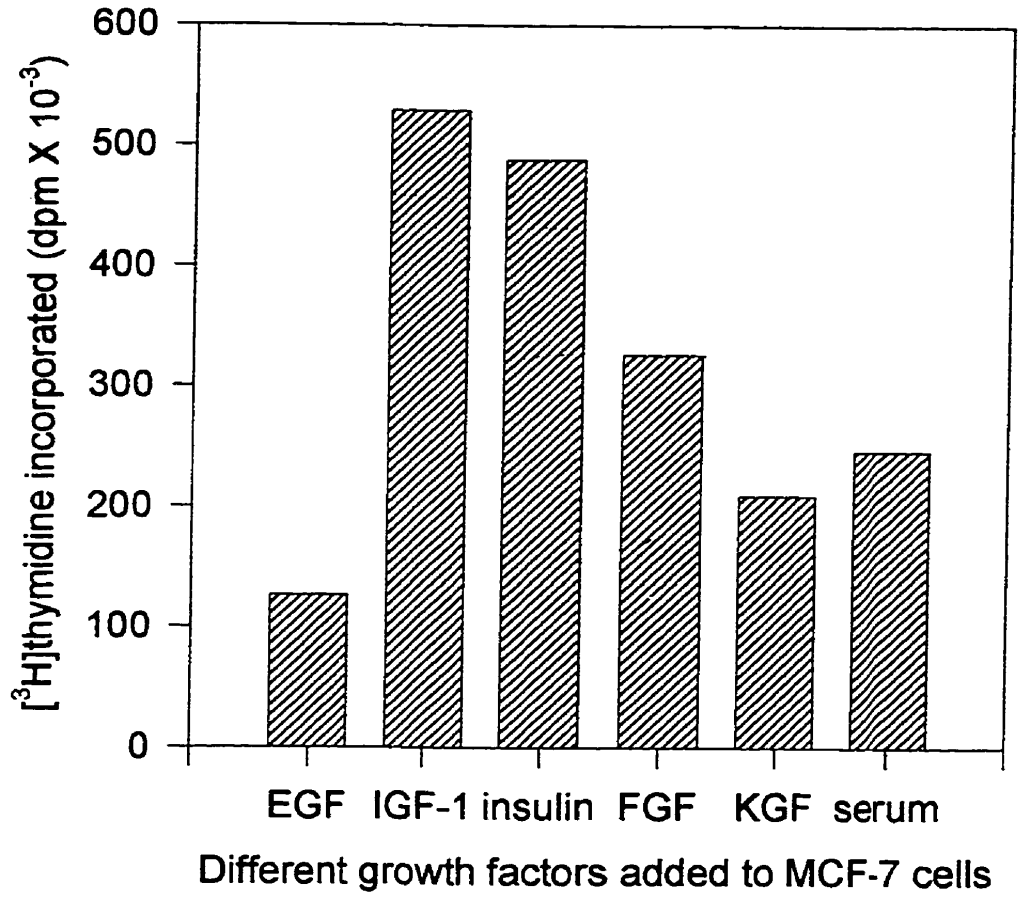


FIGURE 14: Incorporation of [^{3}H]thymidine into MCF-7 Cells

Quiescent MCF-7 cells were incubated with various growth factors (serum 10%; EGF 10ng/ml; insulin 1μg/ml; IGF-1 10ng/ml; FGF 10ng/ml; KGF 15ng/ml) in DMEM/BSA for 18 h followed by the addition of [^{3}H]thymidine for 6 h. Blanks incubated only with DMEM/BSA were used as controls and were subtracted from the wells treated with the growth factors. The amount of [^{3}H]thymidine incorporated was determined (as described in Section 3.5). This experiment is a representative of two separate experiments.

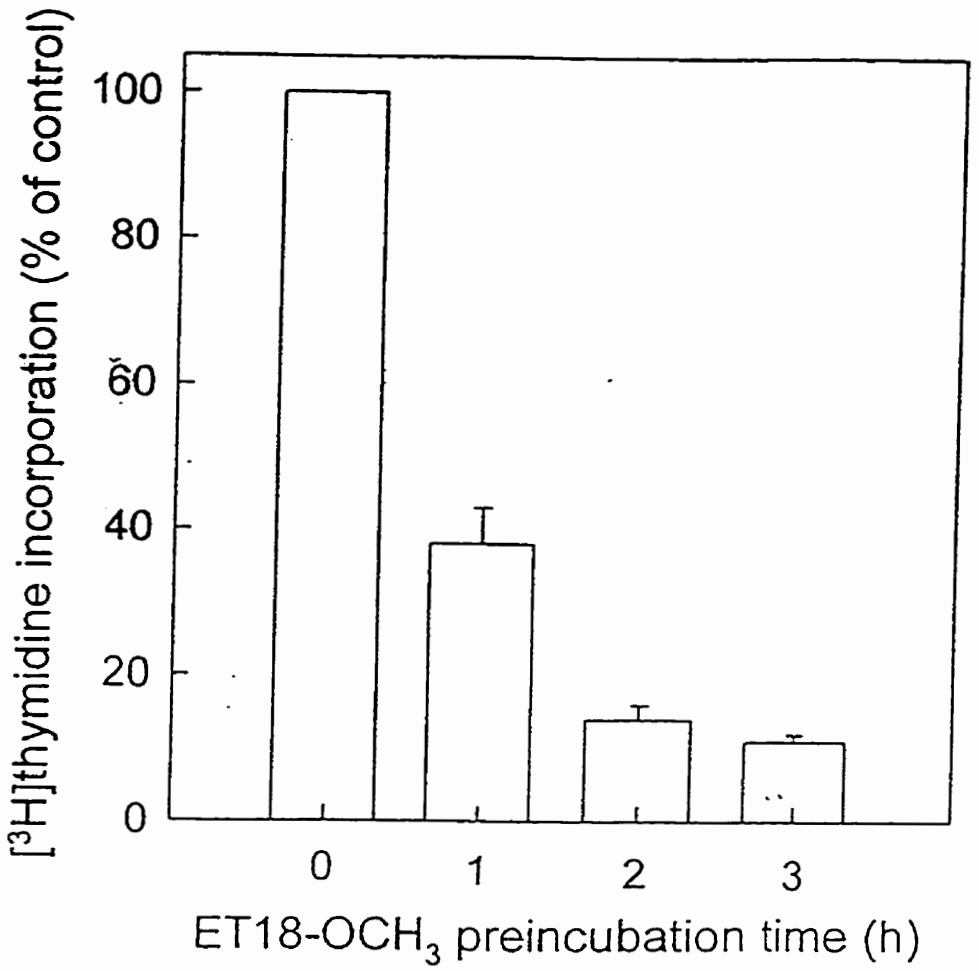


FIGURE 15: Effect of ET-18-OCH_3 on Insulin-stimulated [^{3}H]thymidine Incorporation Quiescent MCF-7 cells were incubated with 10 μg/ml ET-18-OCH_3 for 0, 1, 2, or 3 h. The cells were washed and incubated with DMEM/BSA and stimulated with insulin (1 μg/ml) for 18 h followed by addition of [^{3}H]thymidine for 6 h. The amount of [^{3}H]thymidine incorporated into DNA was determined as described in Section 3.5.

4.3 Effect of ET-18-OCH_3 on Insulin-stimulated MAPK Activation in MCF-7 Cells

The MAPK pathway is an important signal transduction pathway in the cell activated by many types of growth factors and cell surface receptors. The activation of MAPK results in cellular processes such as proliferation and differentiation (Seger and Krebs, 1995). Since the activation of the MAPK cascade is important in controlling cell proliferation, examining the effect of ET-18-OCH_3 on this enzyme can be helpful in determining whether the site of action lies upstream or downstream. It was previously shown that ET-18-OCH_3 inhibited MAPK activation in MCF-7 cells stimulated with EGF and serum (Zhou *et al.*, 1996). To determine whether the drug had the same effect on insulin-stimulated cells, MAPK activity was determined in cells treated with and without ET-18-OCH_3 followed by stimulation with insulin for various times. MAPK activity was determined by both the P81 paper assay using MBP as a substrate and using a phospho-MAPK antibody which specifically recognizes phosphorylated p44 and p42 MAPKs on residues both Thr183 and Tyr185. Insulin stimulated the level of phosphorylated MAPK (**Figure 16a**) and also stimulated the activity of MAPK as determined by the ability of MAPK to phosphorylate MBP (**Figure 16b**). The stimulation of MAPK by insulin was inhibited when the cells were treated with ET-18-OCH_3. The results in **Figure 16b** show that in control cells, MAPK activity peaked at 325 pmol/min/mg protein whereas in cells preincubated with ET-18-OCH_3, the activity was 3 times lower. Analysis of the cell lysates by immunoblotting with the phospho-MAPK Ab revealed

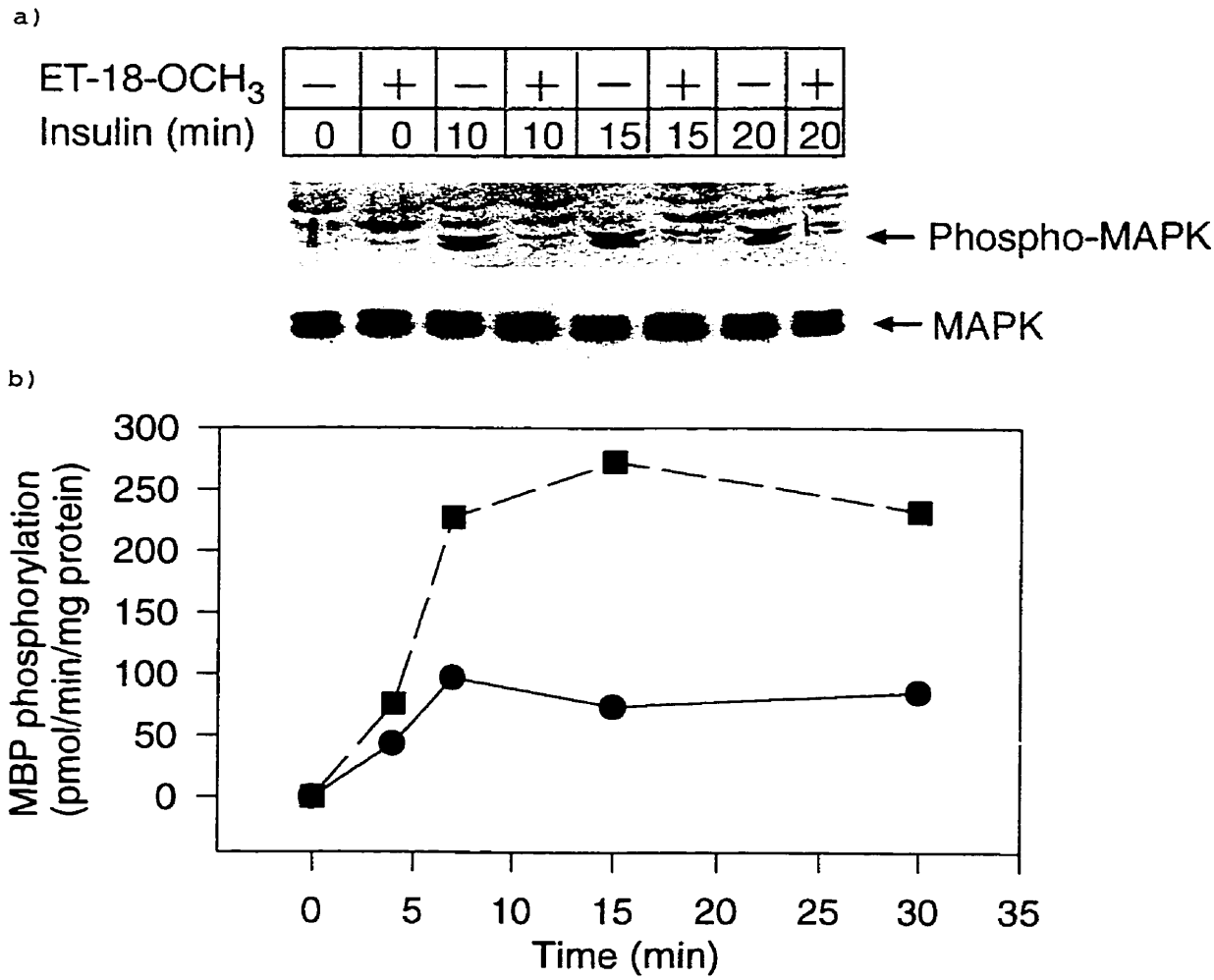


FIGURE 16: Effect of ET-18-OCH_3 on the Activity and Phosphorylation of MAPK a) Quiescent MCF-7 cells in 150mm dishes were incubated with or without ET-18-OCH_3 (10 μg/ml, 3 h) followed by stimulation with insulin (1 μg/ml). The cells were washed and harvested in Buffer C as described in Section 3.6 and the lysates were resolved on 10% SDS gels and subjected to Western blot analysis with either phospho-MAPK Ab or ERK1/ERK2 antibodies. Three independent experiments with different cell preparations showed similar results.
b) Quiescent MCF-7 cells were incubated without (squares) or with ET-18-OCH_3 (circles) (10 μg/ml) for 3 h, washed, and stimulated with insulin (1 μg/ml). MAPK activity in the cytosol fractions was measured as the phosphorylation of MBP as described in Section 3.7. The results are the means of triplicate incubations from a single experiment that is representative of three separate experiments.

a similar inhibitory effect of ET-18-OCH_3 on insulin-stimulated MAPK phosphorylation. The bottom panel in **Figure 16a** demonstrated that there were equal amounts of ERK1 and ERK2 in both the cells treated with and without ET-18-OCH_3. These results suggested that ET-18-OCH_3 inhibited events upstream of MAPK.

4.4 Effect of ET-18-OCH_3 on Tyrosine Phosphorylation of IRS-1

Once insulin binds to its receptor, the receptor becomes phosphorylated and activated. One of the substrates of the insulin receptor is IRS-1. When insulin binds to its receptor, IRS-1 becomes phosphorylated on multiple tyrosine residues leading to its activation (White, 1997). Since we have demonstrated that ET-18-OCH_3 was affecting an event upstream of MAPK, we decided to examine the effect of the ALP on the tyrosine phosphorylation and hence the activation of IRS-1, an initial event in the insulin pathway. IRS-1 was immunoprecipitated from cell lysates treated with and without ET-18-OCH_3, and the level of tyrosine phosphorylation was assessed by immunoblotting with an anti-phosphotyrosine antibody. Prior to stimulation with insulin, there was no phosphorylation of IRS-1, but the phosphorylation was rapidly induced by 2 min upon addition of insulin (**Figure 17**). The level of tyrosine phosphorylation of IRS-1 was similar in the cells treated with and without ET-18-OCH_3 indicating that the ALP has no effect on IRS-1 phosphorylation or activation.

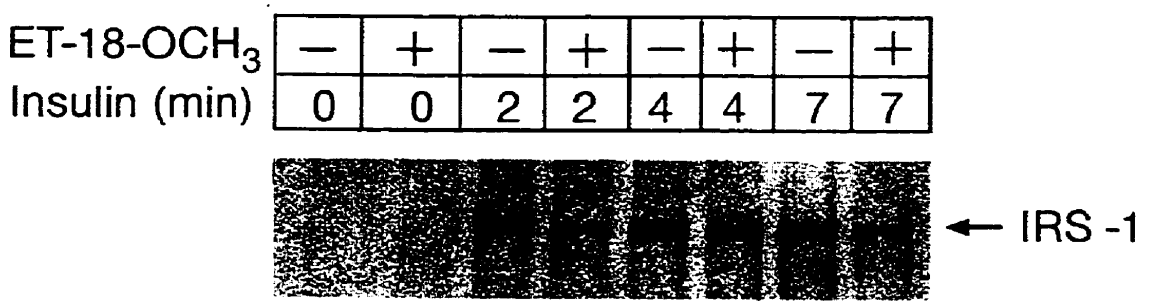


Figure 17: Effect of ET-18-OCH_3 on Tyrosine Phosphorylation of IRS-1

Quiescent MCF-7 cells were incubated with or without ET-18-OCH_3 (10 μg/ml, 3 h) followed by stimulation with insulin (1 μg/ml) for 2, 4, or 7 min, or left untreated as control. The cells were washed, lysed in detergent containing buffer (Buffer D), sonicated and centrifuged. IRS-1 was immunoprecipitated from the supernatant using anti-IRS-1 Ab. The immunoprecipitates were resolved on 12 % SDS PAGE gels, transferred to nitrocellulose membranes, and immunoblotted with anti-phosphotyrosine Ab as described in Section 3.11. Two independent experiments with different cell preparations showed similar results.

4.5 Effect of ET-18-OCH_3 on Raf-1 Association with the Cell Membrane

Since ET-18-OCH_3 had no effect on the activation of IRS-1, we wanted to examine events downstream of IRS-1 phosphorylation. It was previously demonstrated that inhibition of MAPK activation by ET18-OCH_3 was due to perturbation of Raf-1 association

with the membrane in cells stimulated with EGF (Zhou *et al.*, 1996). Since we have shown that ET18-OCH_3 inhibited insulin-stimulated MAPK activity, as it did in EGF-stimulated cells, we next needed to investigate whether the drug inhibited the Raf-1 association with the membrane upon activation with insulin. After insulin stimulation, there are a number of molecules activated that can lead to raf-1 activation. Grb-2, a molecule involved in the activation of raf-1 can bind directly to activated IRS-1 (Skolnik *et al.*, 1993). Grb-2 is also able to bind to Shc, another substrate of the insulin receptor, leading to raf-1 activation (Skolnik *et al.*, 1993; Sasaoka *et al.*, 1994b). To investigate the effect of ET18-OCH_3 on Raf-1, we compared the level of Raf-1 present in the membrane in insulin-stimulated cells treated with or without ET-18-OCH_3. In the absence of stimulation, there was a basal level of Raf-1 already present in the cell membrane (**Figure 18**). The membrane-associated Raf-1 increased in response to insulin stimulation, but the levels present in the membrane were decreased when the cells were pretreated with ET-18-OCH_3. Therefore, ET-18-OCH_3 has the same effect on both insulin and EGF-stimulated MCF-7 cells in inhibiting MAPK activity as a result of decreasing the levels of Raf-1 associated with the membrane.

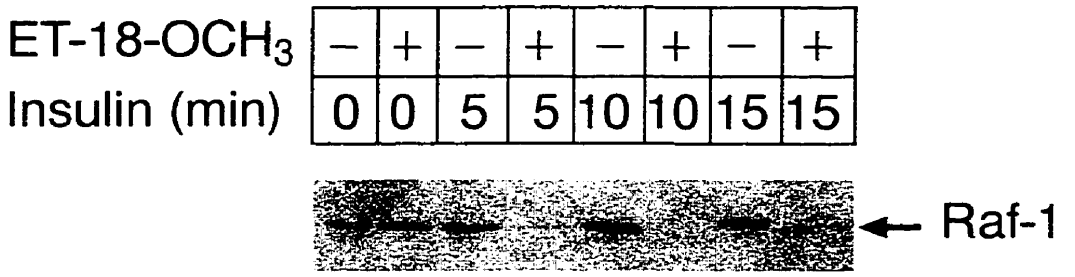


FIGURE 18: Effect of ET-18-OCH_3 on the Association of Raf-1 with MCF-7 Cell Membrane

Quiescent MCF-7 cells were incubated with or without ET-18-OCH_3 (10 μg/ml, 3 h), washed and subsequently stimulated with 1 μg/ml insulin for the times indicated. Membranes were prepared, solubilized, and equal amounts of protein were separated on 10% SDS PAGE gels, transferred to nitrocellulose membranes, and subjected to Western blot analysis with anti-Raf-1 Abs as described in Section 3.8. The results are from a single experiment that is representative of results obtained for 3 different cell preparations.

4.6 Effect of ET-18-OCH_3 on Ras-Raf-1 Interaction

We have demonstrated in section 4.5 above that ET-18-OCH_3 inhibited the Raf-1 association with the membrane in insulin-stimulated MCF-7 cells. Similar effects of the compound on Raf-1 were obtained with EGF-stimulated MCF-7 cells. The mechanism via which ET-18-OCH_3 decreases membrane raf-1 levels is unknown. Since activated p21 ras mediates the translocation of Raf-1 from the cytosol to the membrane in response to cell stimulation (Marais *et al.*, 1995), we investigated whether ET-18-OCH_3 affects the binding of Ras to Raf-1. A GST-Ras construct was purified from bacteria and loaded with GTP-γ-S and was used to examine the binding. The purified protein was added to untreated and ET-18-OCH_3 - treated cell lysates and an immunoblot was performed with anti-Raf-1 antibody as described in section 3.5. Cells pretreated with ET-18-OCH_3 resulted in a 50% inhibition of Raf-1 association with the GST-Ras protein as compared to the control cells (**Figure 19**) indicating that ET-18-CH_3 may be able to inhibit the binding of activated Ras (that has GTP bound) to Raf-1. The same phenomena was also observed with cell lysates in which different concentrations of exogenous ET-18-OCH_3 was added for 3 h at room temperature indicating a direct effect of the drug on the interaction of the GST-Ras protein with Raf-1.

4.7 Effect of Exogenous Lipids on Ras-Raf-1 Binding

We have shown that ET-18-OCH_3 inhibited the ability of Raf-1 to bind to the

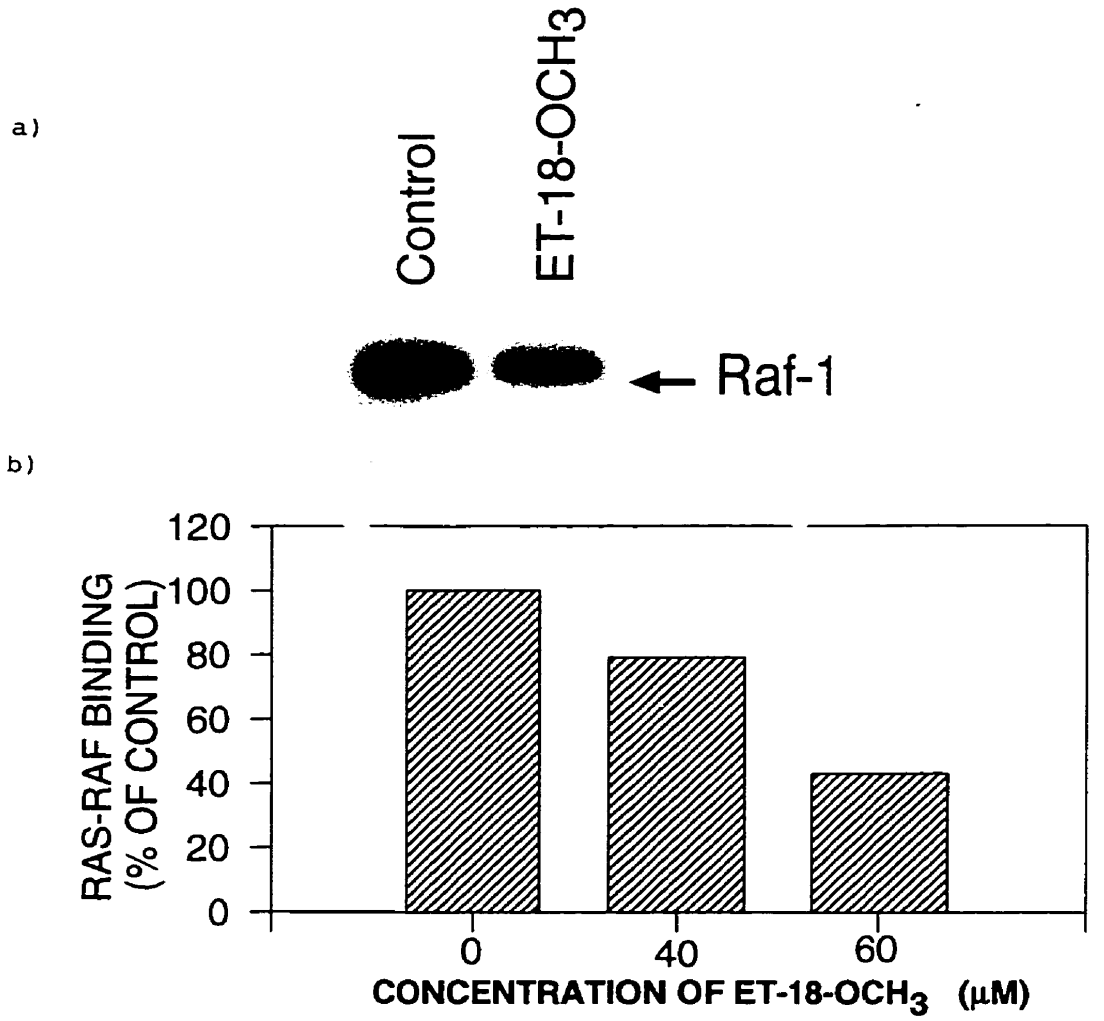


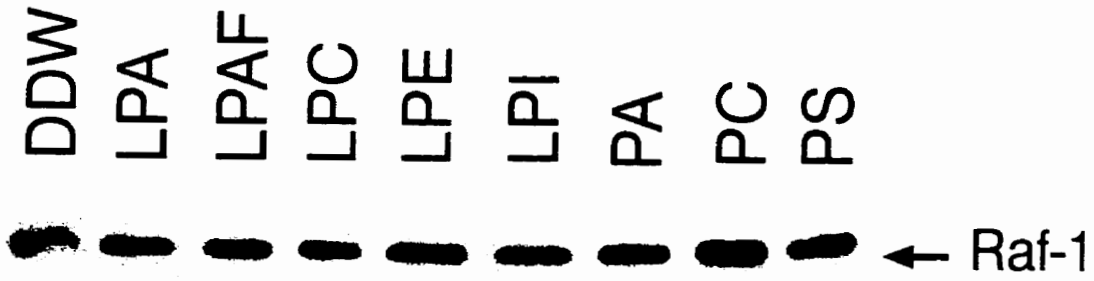
FIGURE 19: Effect of ET-18-OCH_3 on Ras-Raf-1 Binding a) Quiescent MCF-7 cells were incubated with or without ET-18-OCH_3 (10 μg/ml, 3 h) and then harvested. Cytosolic fractions were prepared and were incubated with purified GST-Ras loaded with GTP-γ-S for 90 min at 4°C as described in Section 3.9. The beads were washed, dissolved in SDS sample buffer and resolved in 10% SDS gels. The gels were transferred to nitrocellulose and immunoblotted with Raf-1 Ab. Four independent experiments with different cell preparations showed similar results. b) Quiescent MCF-7 cells were harvested into Buffer C and the cytosolic fractions were isolated by centrifugation. Cytosolic fractions with equal amounts of protein were incubated with ET-18-OCH_3 (0, 40, 60 μm, 3 h) on a rotator at room temperature. The cell lysates were then incubated with purified GST-Ras loaded with GTP-γ-S and Ras-Raf-1 binding was assessed as described above and in Section 3.9. Four independent experiments with different cell preparations showed similar results.

GST-Ras construct both when ET-18-OCH_3 was added to the cells or when exogenous ET-18-OCH_3 was added to the cell lysates. Next we wanted to determine whether this inhibition was specific for ET-18-OCH_3 or whether it was a non-specific inhibition. To test this, we treated MCF-7 cells with 20 μM of different lysolipids including lysophosphatidic acid (LPA), lysoplatelet-activating factor (LPAF), lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), lysophosphatidylinositol (LPI), phosphatidic acid (PA) and phosphatidylserine (PS) for 3 h. These compounds have structures that are similar to ET-18-OCH_3 so we can assess whether the inhibition of Raf-1 association with the GST-Ras protein was specifically due to ET-18-OCH_3. None of the other lysolipids tested resulted in a substantial decrease in Ras-Raf-1 binding and many actually increased the binding such as LPAF, LPE, and PA (**Figure 20**). Only ET-18-OCH_3 caused a decrease providing evidence that the inhibition of Raf-1 association with the ras construct was specific for the ALP.

4.8 Effect of ET-18-OCH_3 on IRS-1 Association with PI3K

We have examined the effect of ET-18-OCH_3 on the MAPK pathway and more specifically on Raf-1 association with the membrane. The purpose of this work was to determine whether ALPs affect other molecules in the cell which could result in decreased cell proliferation. Once IRS-1 is phosphorylated, it serves as a docking molecule and binds to a number of different SH2-domain containing proteins. This leads to activation of other signalling pathways which can culminate

a)



b)

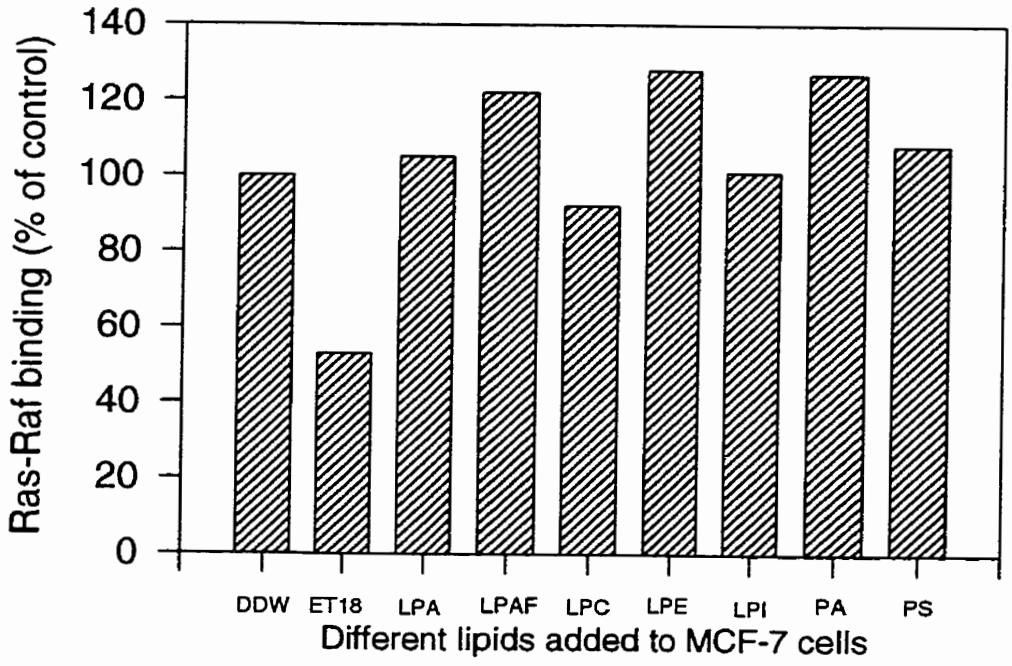


FIGURE 20: Effect of Lipids on Ras-Raf-1 Binding

Quiescent MCF-7 cells were pretreated with different lysolipids (20 μM, 3 h) and then harvested. The amount of Ras-Raf-1 binding was determined as described in Section 3.10. Three independent experiments with different cell preparations showed similar results. ET-18-OCH_3 is abbreviated as ET18.

a) Western blot of the samples with Raf-1 Ab

b) Densitometric scanning of the Western blot represented as a percentage of the control.

in cell proliferation. One of these molecules is the heterodimeric PI3K molecule which phosphorylates the inositol ring of PI, PI 4-P, and PI 4,5-P_2 at the 3 position to produce PI 3-P, PI 3,4-P_2, and PI 3,4,5-P_3 (Whitman *et al.*, 1988). PI3K has been shown to have a number of different functions in the cell including gene expression, protein synthesis, apoptosis and glycogen synthesis (Shepherd *et al.*, 1998). Since the inhibition of the PI3K pathway may result in the inhibition of cell proliferation, it is important to determine whether ET-18-OCH_3 affects this signalling pathway. The association of PI3K with IRS-1 is required for its activation (Backer *et al.*, 1992). Since we have shown in section 4.4 that ET-18-OCH_3 had no effect on IRS-1 phosphorylation, we next investigated whether it had any effect on the association of PI3K with IRS-1. IRS-1 was immunoprecipitated from cell lysates treated with and without ET-18-OCH_3 and the association was assessed by immunoblotting with anti-PI3K antibody. Our results revealed that the association of PI3K paralleled the tyrosine phosphorylation of IRS-1 (**Figure 21**). Prior to stimulation with insulin, when there was no phosphorylation of IRS-1, PI3K was not associated with IRS-1. Stimulation of the cells with insulin led to IRS-1 phosphorylation (**Figure 17**) and association with PI3K (**Figure 21**). Similar levels of PI3K associated with IRS-1 in cells treated with and without ET18-OCH_3. These results suggest that ET-18-OCH_3 did not affect the association of IRS-1 and PI3K.

4.9 Effect of ET-18-OCH_3 on PI3K Activity

The above results demonstrate that ET-18-OCH_3 had no effect on the

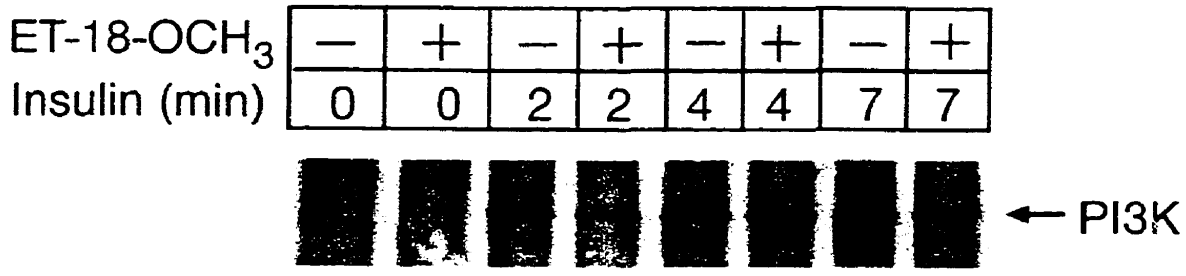


FIGURE 21: Effect of ET-18-OCH_3 on IRS-1 Association with PI3K

Quiescent MCF-7 cells were incubated with or without ET-18-OCH_3 (10 μg/ml, 3 h) followed by stimulation with insulin (1 μg/ml). The cells were harvested and IRS-1 was immunoprecipitated. The immunoprecipitates were resolved on 8% SDS gels, transferred to nitrocellulose and immunoblotted with anti-PI3K Ab. Two independent experiments with different cell preparations showed similar results.

association of PI3K with IRS-1. However, ET-18-OCH_3 could affect PI3K activity subsequent to its association and activation by IRS-1. To investigate this possibility, IRS-1 immunoprecipitates were assayed for PI3K activity by measuring the phosphorylation of phosphatidylinositol to phosphatidylinositol 3-phosphate. The results which are displayed in **Figure 22** show that the kinetics of PI3K activation were similar in the cells treated with or without ET-18-OCH_3. The activity of PI3K peaked between 5 and 15 minutes and subsequently decreased similarly in both groups.

4.10 Direct Effect of ET-18-OCH_3 on PI3K Activity

Although the results in section 4.9 indicate that preincubating cells with ET-18-OCH_3 did not affect PI3K activity immunoprecipitated from cells, it did not preclude a direct effect of the ALPs on the activity of PI3K in cells. It was possible that ET-18-OCH_3 might directly affect PI3K activity but once it was washed off the cells, the activity would appear unaffected. In order to examine whether ET-18-OCH_3 had a direct effect on PI3K activity, IRS-1 immunoprecipitates were assayed for PI3K activity in the presence of ET18-OCH_3 added to the reaction mixture. The amount of PIP produced was assayed in the presence of ET-18-OCH_3 and was found to be decreased by 50% (**Figure 23**) indicating that ET-18-OCH_3 could directly inhibit PI3K activity.

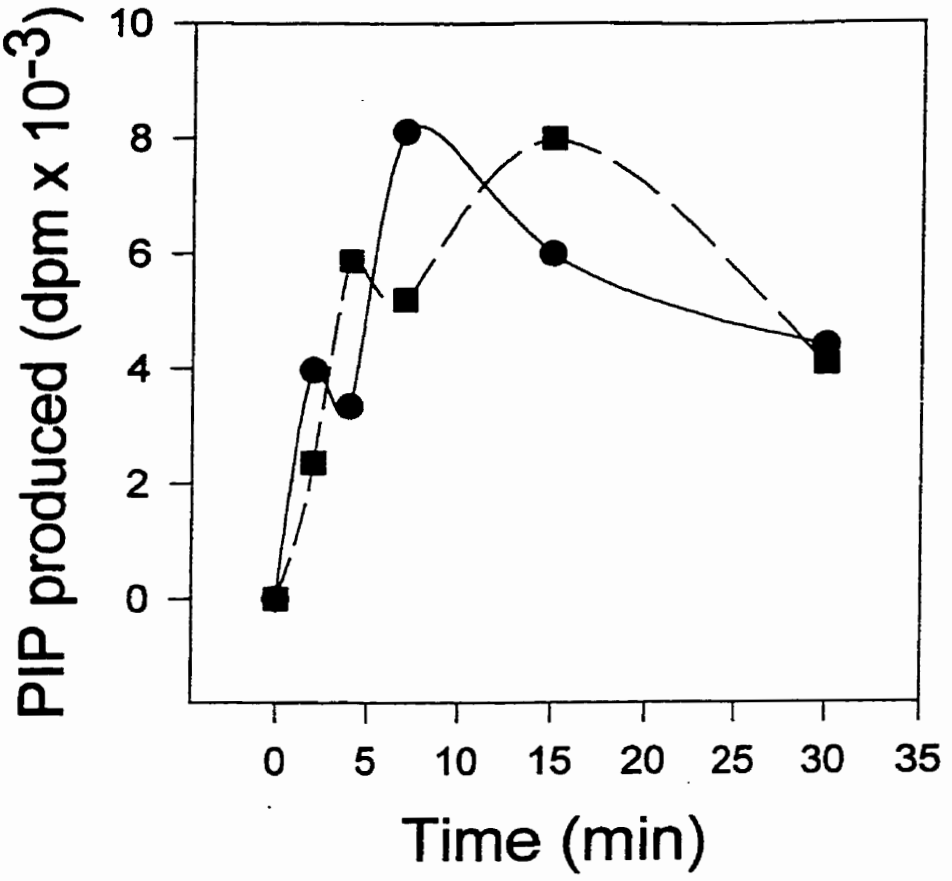


FIGURE 22: Effect of ET-18-OCH_3 on PI3K Activity

Quiescent MCF-7 cells were treated with (circles) or without (squares) ET-18-OCH_3 (10 μg/ml, 3 h). The cells were stimulated with insulin (1 μg/ml) for various times. The cells were harvested in Buffer F and centrifuged. IRS-1 was immunoprecipitated from the cell lysates. The immunoprecipitates were incubated in a reaction mixture containing PI and [γ-^{32}P]ATP as described in Section 3.12. PI3K activity was measured as the phosphorylation of PI to PIP. The PIP fraction was separated from the reaction mixture by TLC and the bands were scraped and counted by scintillation counting. Three independent experiments with different cell preparations showed similar results.

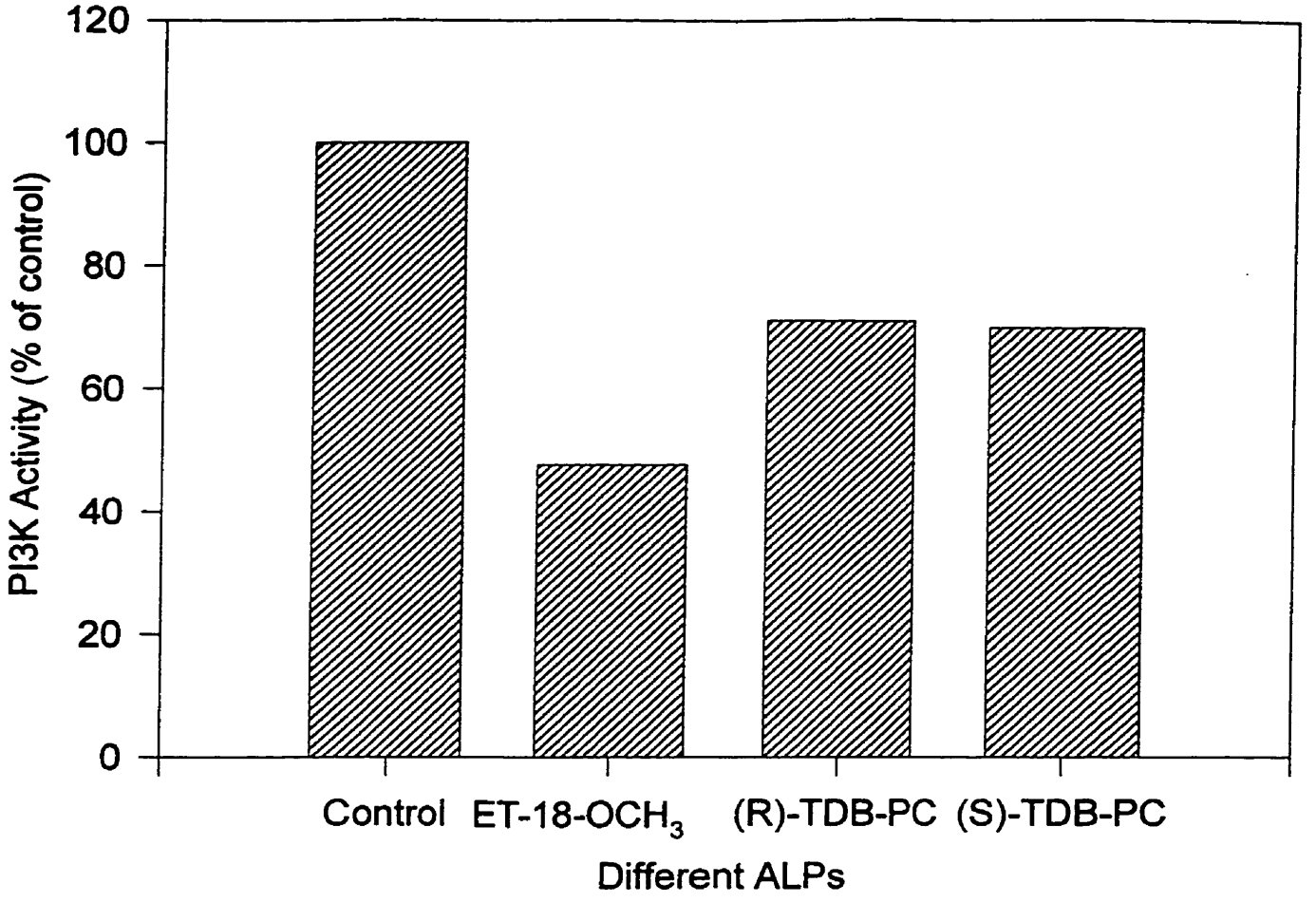


FIGURE 23: Direct Effect of ALPs on PI3K Activity

Quiescent MCF-7 cells were stimulated with insulin (1 μg/ml) for 20 minutes. Cell lysates were prepared and IRS-1 was immunoprecipitated as described in the legend to figure 17. The immunoprecipitates were washed and incubated in a reaction mixture to measure PI3K activity (Section 3.12) which also contained PI, [γ-^{32}P]ATP, and either ET-18-OCH_3 (30 μM), (*R*)-TDB-PC (30 μM), (*S*)-TDB-PC (30 μM), or without any ALP as a control. PI3K activity was measured as the phosphorylation of PI to PIP as described in the legend to Figure 22. These results are representative of two different immunoprecipitates.

4.11 Effect of (*R*)- and (*S*)-TDB-PC on PI3K Activity

The purpose of this project was to identify molecules whose inhibition by ET-18-OCH_3 could account for the decrease in cell proliferation. As mentioned in the introduction, there are two enantiomeric phosphonocholine analogs which have a differential effect on cell proliferation. (*S*)-TDB-PC inhibited cell proliferation to a much greater extent than (*R*)-TDB-PC (Samadder, 1998). The use of these two molecules provides a valuable tool in determining whether the inhibition of a particular molecule is relevant to the inhibition of cell growth. Exogenous (*S*) and (*R*)-TDB-PC were added to the PI3K reaction mixture and the ability of PI3K to produce PIP from PI was assayed. Like ET-18-OCH_3, both of these ALPs inhibited the amount of PIP produced (as compared to the control without any drug added) (**Figure 23**). The inhibition of PI3K due to (*S*) and (*R*)-TDB-PC was not as drastic as with ET18-OCH_3, but the important thing being that they were equivalent. The compound that inhibited cell proliferation and the compound that did not inhibit cell proliferation both inhibited PI3K activity to the same extent indicating that the inhibition of PI3K activity was unlikely to be related to decreased cell proliferation if the inhibition observed *in vitro* also occurred in the cell. However, since our studies have not indicated whether an inhibition of PI3K by ET-18-OCH_3 or (*R*)- and (*S*)-TDB-PC occurred in intact cells, the significance of these results are unclear. We therefore investigated the effect of ET-18-OCH_3 and (*R*)- and (*S*)-TDB-PC on molecules downstream of PI3K.

4.12 Effect of ET-18-OCH_3 on Phosphorylation of PKB

Although preincubation of MCF-7 cells with ET-18-OCH_3 for 3 h had no effect on PI3K activity immunoprecipitated from the cells, ET-18-OCH_3 did directly inhibit PI3K activity when added to the assays. It is therefore possible that ET-18-OCH_3 taken up into the cells could directly inhibit PI3K in the whole cell. This inhibition of PI3K activity should result in inhibition of downstream signalling molecules. Since PKB is activated in a PI3K-dependant manner, we examined the effect of preincubating cells with ET-18-OCH_3 on PKB activation. Activation of PKB requires phosphorylation on two residues, Thr 308 within the P-loop of the protein kinase domain and Ser 473. The availability of antibodies that specifically recognize PKB phosphorylated at either Thr 308 or Ser 473 allowed us to examine PKB phosphorylation by Western blot analysis. Phosphorylation at both of these residues was induced upon insulin stimulation in the control cells (**Figure 24**). Treatment with ET-18-OCH_3 resulted in an inhibition of phosphorylation of both Ser 473 and Thr 308. Panel C is a Western blot with PKB that showed that equal amounts of protein were loaded on the gel in both groups. ET-18-OCH_3 is able to act downstream of PI3K and inhibit the phosphorylation of the two residues of PKB that are necessary for activation of the enzyme.

4.13 Effect of ET-18-OCH_3 on PKB Kinase Activity

We have shown that ET18-OCH_3 inhibited the insulin-induced phosphorylation of both Ser 473 and Thr 308 residues on PKB. We needed to

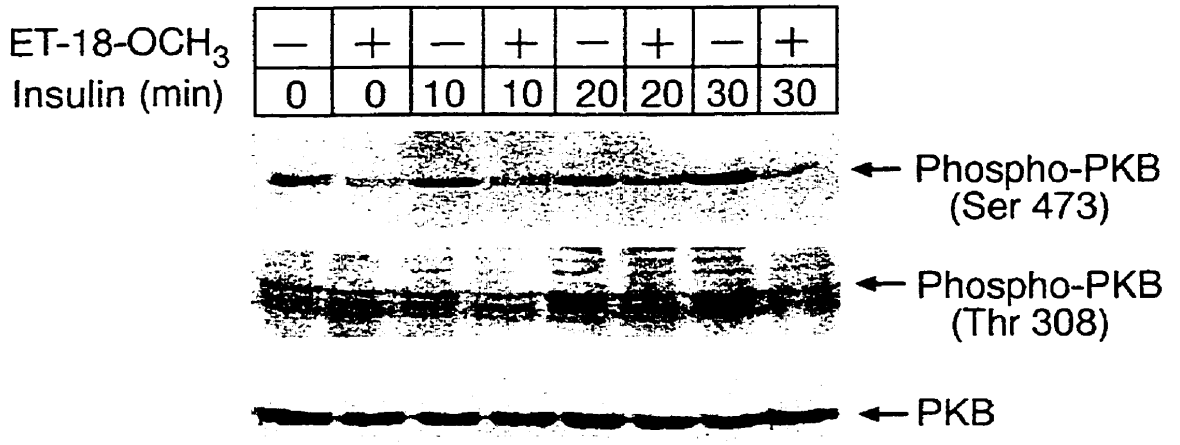


FIGURE 24: Effect of ET-18-OCH_3 on Phosphosphorylation of PKB

Quiescent MCF-7 cells were incubated with or without ET-18-OCH_3 (10 μg/ml, 3 h) followed by stimulation for various times with insulin (1 μg/ml). After harvesting, equal amounts of protein (65 μg) were resolved on 10% SDS gels, transferred to nitrocellulose membranes and probed with either anti-phospho-PKB (Ser473), anti-phospho-PKB (Thr308) or anti-PKB Abs. Three independent experiments with different cell preparations showed similar results.

verify that the inhibition of phosphorylation of both of these residues resulted in an inhibition of PKB kinase activity. PKB in ET-18-OCH_3 and control cells was immunoprecipitated following insulin stimulation of the cells and PKB kinase assays were performed on the PKB immunoprecipitates as described in section 3.13 with Crosstide as the substrate. The PKB kinase activity in the control cells increased with insulin stimulation (**Figure 25**). In the ET-18-OCH_3 treated cells, there was a 5-fold reduction in peak activity (15 minutes) compared to the control cells and an even greater fold inhibition (14-fold) at 30 minutes. These studies clearly showed that preincubating cells with ET-18-OCH_3 resulted in a decrease in PKB activity.

4.14 Effect of (*R*)- and (*S*)-TDB-PC on PKB Phosphorylation and Kinase Activity

To investigate whether inhibition of phosphorylation of PKB by ET-18-OCH_3 could lead to inhibition of cell growth, we investigated the effects of the active and inactive phosphonocholines on PKB activation. Cells were treated with (*R*) or (*S*)-TDB-PC and the cells were subsequently stimulated with insulin. PKB was immunoprecipitated and the phosphorylation of Ser473 was assessed by immunoblotting with the phosphospecific antibody. Both the *R* and *S* enantiomers if TDB-PC inhibited the phosphorylation of PKB on Ser473 although to different extents (**Figure 26**). Cells treated with (*R*)-TDB-PC had decreased phosphorylation relative to the controls but the phosphorylation in cells treated with (*S*)-TDB-PC was

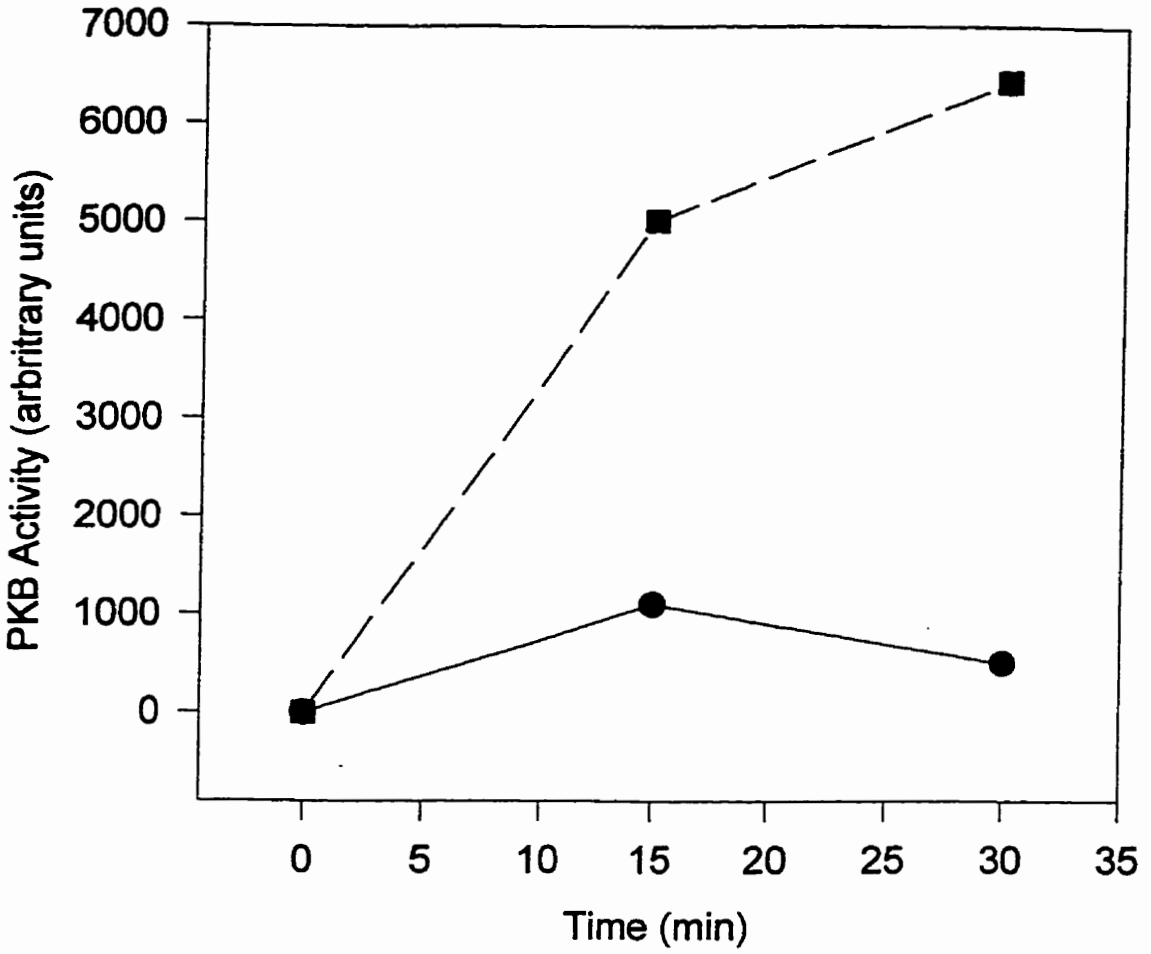


FIGURE 25: Effect of ET-18-OCH_3 on PKB Kinase Activity

Quiescent MCF-7 cells were treated with (circles) or without (squares) ET-18-OCH_3 (10 μg/ml, 3 h) and then stimulated with insulin (1 μg/ml) for various times. The cells were harvested and PKB was immunoprecipitated from the cell lysates (1 mg). The immunoprecipitates were subjected to a kinase assay using Crosstide as substrate as described in Section 3.16. Two independent experiments with different cell preparations showed similar results.

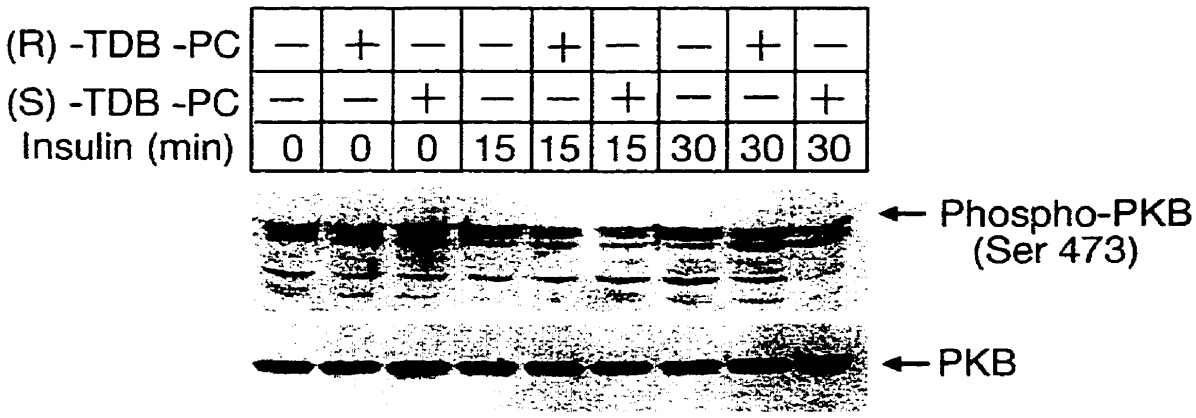


FIGURE 26: Effect of (*R*)- and (*S*)-TDB-PC on PKB Phosphorylation

Quiescent MCF-7 cells were treated with either (*R*)-TDB-PC, (*S*)-TDB-PC (30 μM, 4 h) or left untreated and stimulated with insulin (1 μg/ml) for different times. The cells were harvested and the lysates (65 μg) were resolved on 10% SDS gels, transferred to nitrocellulose membranes and immunoblotted with either anti-phospho PKB (Ser473) or anti-PKB Abs. Three independent experiments with different cell preparations showed similar results.

completely abolished. This compound, which completely inhibited cell proliferation, completely inhibited the phosphorylation of PKB on Ser473. The bottom panel demonstrated that in cells treated with (*S*)-TDB-PC, the levels of PKB were similar to that of the control and (*R*)-TDB-PC - treated cells.

To investigate whether the inhibition of PKB phosphorylation correlated with an inhibition in PKB activity, the cells treated with (*R*) and (*S*)-TDB-PC were harvested and the lysates were subjected to a PKB kinase assay using Crosstide as the substrate. In cells treated with (*S*)-TDB-PC a much greater decrease in kinase activity was observed in PKB immunoprecipitates than in similar immunoprecipitates from the cells treated with (*R*)-TDB-PC (**Figure 27**). The PKB kinase activity increased with the duration of insulin stimulation up to 30 minutes. After 30 minute insulin stimulation, the PKB kinase activity in the control cells was 3.5-fold greater than those treated with (*R*)-TDB-PC and almost 40-fold greater than those treated with (*S*)-TDB-PC. The PKB kinase activity in (*R*)-TDB-PC was almost 11-fold greater than the PKB kinase activity in (*S*)-TDB-PC. Therefore, like the phosphorylation of PKB, the kinase activity in (*R*) and (*S*)-TDB-PC treated cells was inhibited but there was a much greater inhibition with (*S*)-TDB-PC.

4.15 Direct Effect of ALPs on PKB Kinase Activity

We have shown that ET-18-OCH_3, (*R*)-TDB-PC, and (*S*)-TDB-PC inhibit PKB phosphorylation and kinase activity, but it is not known whether these drugs directly

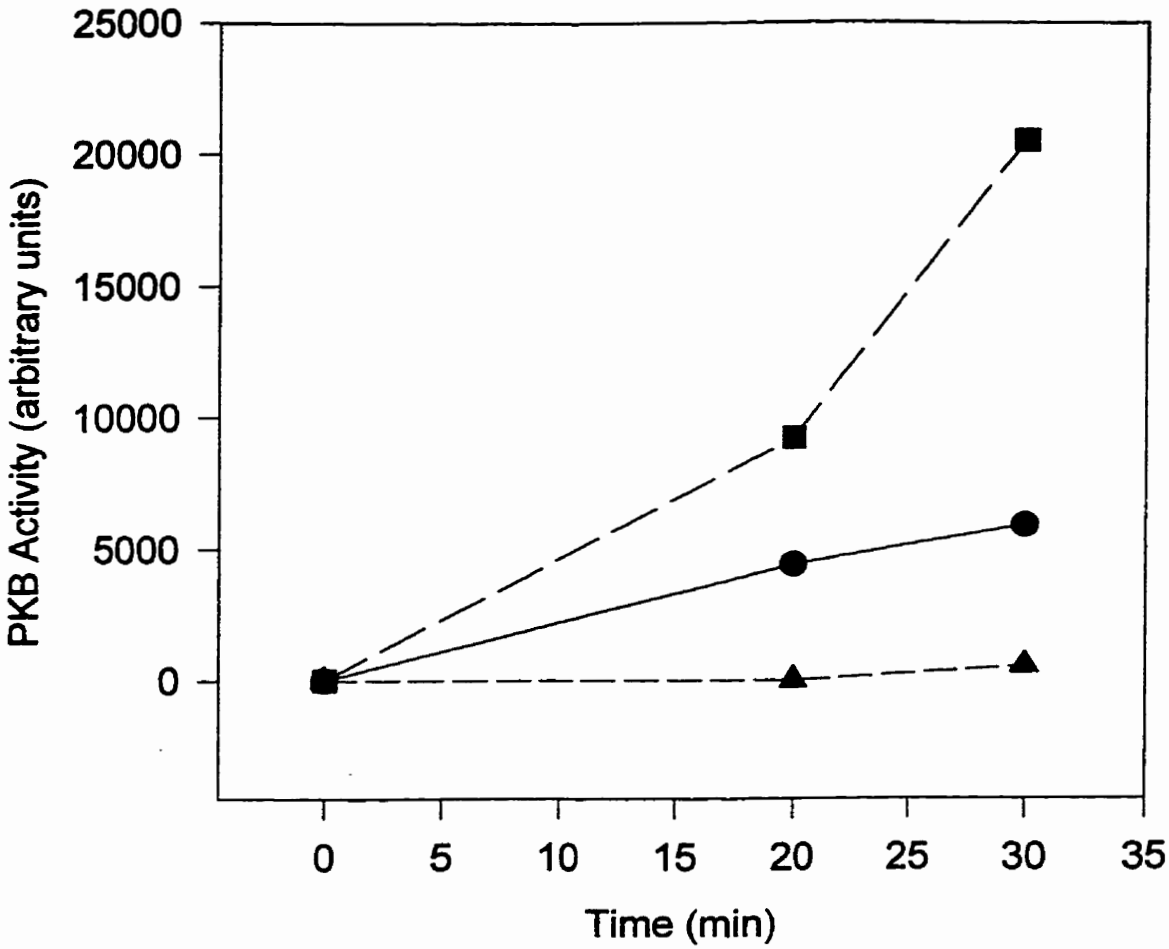


FIGURE 27: The Effect of (*R*)- and (*S*)-TDB-PC on PKB Kinase Activity

Quiescent MCF-7 cells were incubated with either (*R*)-TDB-PC (circles), (*S*)-TDB-PC (triangles) (30 μM, 4 h) or left untreated (squares) followed by stimulation with insulin (1 μg/ml). The cells were harvested and PKB was immunoprecipitated from the lysates (1 mg) and used in PKB kinase assays with Crosstide as substrate using the procedure described in Section 3.16. Two separate experiments with different cell preparations showed similar results.

inhibit PKB kinase activity. Kinase assays were performed with the addition of different concentrations of ET-18-OCH_3, (*R*)-TDB-PC and (*S*)-TDB-PC to determine whether they had a direct effect on PKB activity. When 15 and 30 μM of the drugs were added, there was no inhibition of PKB kinase activity (**Figure 28**). The results show that the ALPs do not directly inhibit PKB kinase activity.

4.16 Effect of ET-18-OCH_3 on p70 S6K Phosphorylation

p70 S6K is a Ser/Thr protein kinase which phosphorylates the 40S ribosomal protein S6. The inhibition of this kinase severely compromises the ability of the cell to progress through the G1 phase of the cell cycle (Jefferies *et al.*, 1997). Since p70 S6K is thought to lie downstream of PI3K, we investigated the effect of ET-18-OCH_3 on its activation. p70 S6K is activated by phosphorylation of a number of residues (S411, S418, T421, S424, T389, S404 and T229) and full kinase activation is accompanied by phosphorylation of all these sites. Antibodies that specifically recognize p70 S6K phosphorylated at Ser411 and another that recognizes p70 S6K phosphorylated on Thr421/Ser424 are commercially available. These reagents permit the analysis of the phosphorylation of p70 S6K by Western blot analysis. To investigate the effect of ET-18-OCH_3 on the phosphorylation of p70 S6K, cells were treated with or without ET-18-OCH_3. Following insulin stimulation, the cells were harvested and the cell lysates were run on a gel and immunoblotted with the phospho-antibodies. The phosphorylation of Ser411 or Thr421/Ser424 occurred

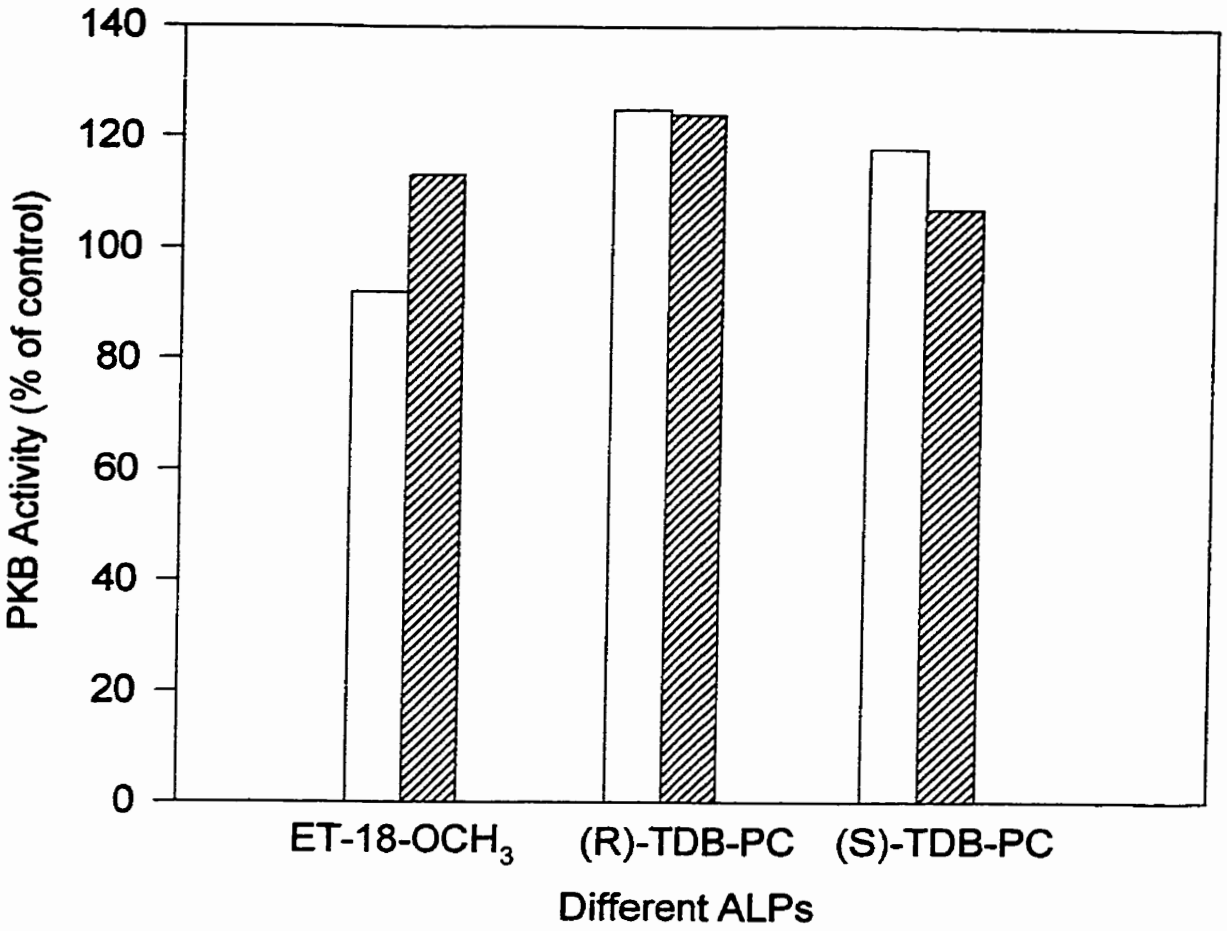


FIGURE 28: Direct Effect of ALPs on PKB Kinase Activity

Quiescent MCF-7 cells were stimulated with insulin (1 μg/ml) for 30 minutes and harvested. PKB was immunoprecipitated from the lysates (1 mg) with anti-PKB Ab as described in Section 3.16. The immunoprecipitates were incubated in a reaction mixture containing either 0, 15 (open bars) or 30 μM (hatched bars) of ET-18-OCH_3, (*R*)-TDB-PC or (*S*)-TDB-PC. PKB kinase activity was measured as the phosphorylation of Crosstide and was expressed as a percentage of the controls without any drugs. Two separate assays with separate immunoprecipitations showed similar results.

upon insulin stimulation and peaked after 30 minutes (**Figure 29**). In cells treated with ET-18-OCH_3, an inhibition of phosphorylation of p70 S6K at both Ser411 and Thr421/Ser424 was observed. The bottom panel demonstrated that both groups of cells contained equivalent amounts of the p70 S6K protein.

4.17 Effect of ET-18-OCH_3 on p70 S6 Kinase Activity

Although the above results revealed that ET-18-OCH_3 inhibited the insulin-stimulated phosphorylation of p70 S6 kinase on residues Ser411, Thr421, and Ser424, the effect of this on the activity of the kinase is not known as numerous sites are phosphorylated on p70 S6K in response to insulin stimulation which could all be essential for catalytic activity. In the absence of specific antibodies to the other phosphorylated sites, determination of catalytic activity was the most direct means of assessing whether the inhibition of phosphorylation at Ser411 and Thr421/Ser424 correlated with inhibition of kinase activity. p70 S6 kinase assays were performed on p70 S6K immunoprecipitates from cells treated with and without ET-18-OCH_3. In the control cells, the p70 S6 kinase activity increased with increasing stimulation time (**Figure 30**). In the cells treated with ET-18-OCH_3, there was a slight increase in p70 S6 kinase activity upon insulin stimulation but the activity was 5-fold less than that observed in the control cells. ET-18-OCH_3 not only inhibited the phosphorylation of p70 S6K at Ser411, Thr421 and Ser424, but it also inhibited the kinase activity.

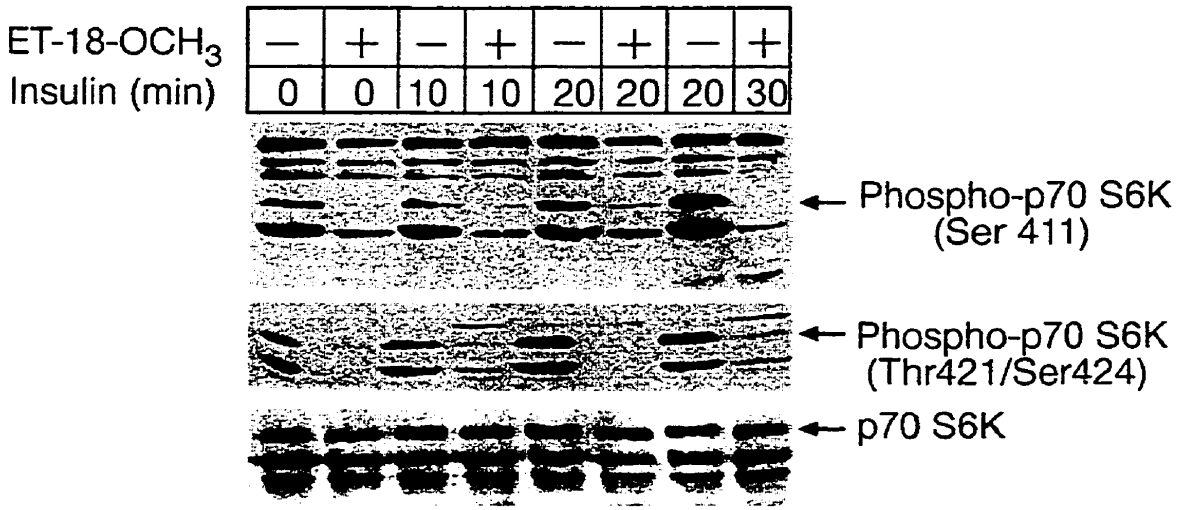


FIGURE 29: Effect of ET-18-OCH_3 on p70 S6K Phosphorylation

Quiescent MCF-7 cells were incubated with or without ET-18-OCH_3 (10 μg/ml) and then stimulated with insulin (1μg/ml) for various times. The cells were harvested and the lysates (65 μg) were resolved on 10% SDS gels and transferred to nitrocellulose membranes. Immunoblots were performed with either a) anti-phospho-p70 S6K (Ser411), b) anti-phospho-p70 S6K (Thr421/Ser424), or c) anti-p70 S6K Abs. Three independent experiments with different cell preparations showed similar results.

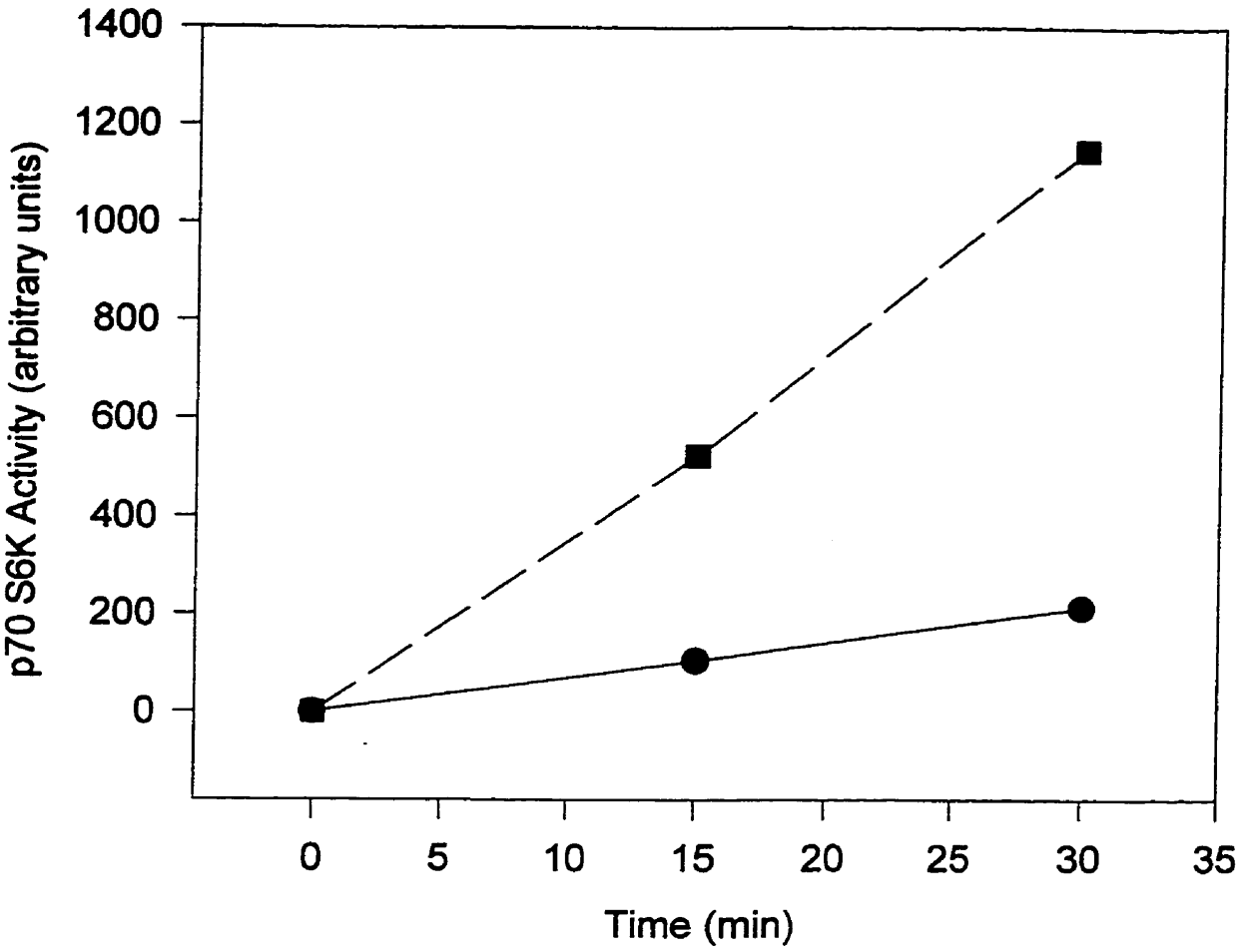


FIGURE 30: Effect of ET-18-OCH_3 on p70 S6 Kinase Activity

Quiescent MCF-7 cells were treated with (circles) or without (squares) ET-18-OCH_3 (10 μg/ml, 3 h) and stimulated with insulin (1 μg/ml). The cells were harvested and p70 S6K was immunoprecipitated from the cell lysates (1 mg). The immunoprecipitates were assayed for p70 S6 kinase activity as the phosphorylation of Crosstide according to the procedures described in Section 3.14). Three independent experiments with different cell preparations showed similar results.

4.18 Effect of (*R*)- and (*S*)-TDB-PC on p70 S6K Phosphorylation and Kinase Activity

The results in the previous section showed that ET-18-OCH_3 inhibited p70 S6K phosphorylation and kinase activity. To investigate whether this inhibition could be relevant in inhibition of cell growth, the effect of the active and inactive enantiomeric double bond phosphonocholines on p70 S6K phosphorylation and activity were examined. Cells were treated with (*R*) and (*S*)-TDB-PC, stimulated with insulin and cell lysates were prepared as described in section 3.10. The lysates were subjected to Western blot analysis with the anti-phospho-p70 antibodies to assess the phosphorylation state of p70 S6K. The phosphorylation of p70 S6K at residue 411 was only slightly inhibited with the (*R*)-TDB-PC relative to the controls while a much greater inhibition was observed with (*S*)-TDB-PC, the compound that inhibits cell growth (**Figure 31**).

To examine whether this decrease in phosphorylation resulted in decreased kinase activity of p70 S6K, p70 S6K was immunoprecipitated from the cells treated with the ALPs and assayed for kinase activity. In the cells treated with (*R*)-TDB-PC, there was an increase in p70 kinase activity although at the peak of 20 minute stimulation, the activity was inhibited about 2.5-fold compared to the control cells without any drug (**Figure 32**). In the cells treated with (*S*)-TDB-PC, there was only basal kinase activity which did not significantly increase upon insulin stimulation. Like the PKB kinase activity, the p70 S6 kinase activity was also moderately inhibited by (*R*)-TDB-PC but was almost completely abolished by (*S*)-TDB-PC.

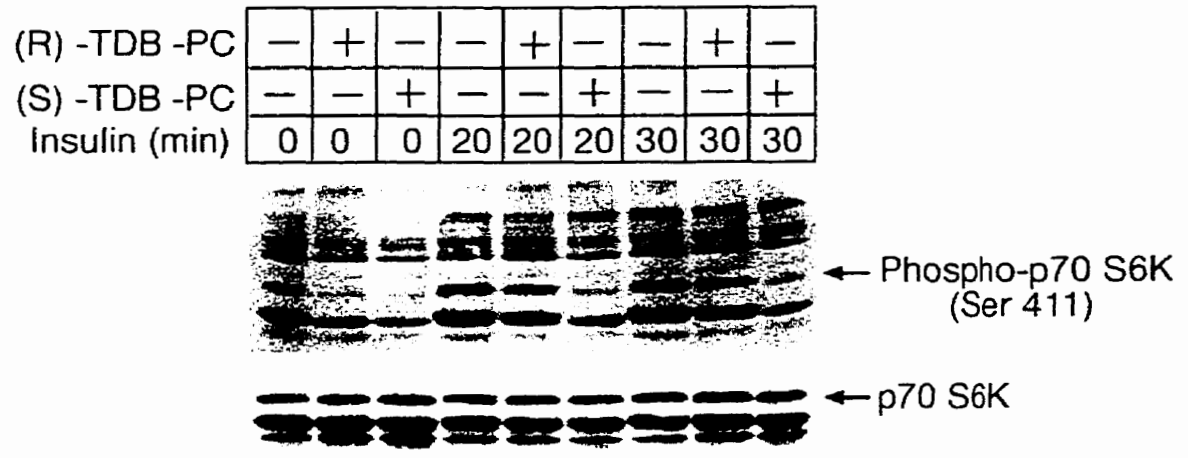


FIGURE 31: Effect of (*R*)- and (*S*)-TDB-PC on p70 S6K Phosphorylation

Quiescent MCF-7 cells were incubated with either (*R*)-TDB-PC, (*S*)-TDB-PC (30 μM, 4 h) or left untreated followed by insulin stimulation (1 μg/ml) for various times. The cells were harvested and equal amounts of lysate protein (65 μg) were resolved on 10% SDS gels and transferred to nitrocellulose membranes. Immunoblots were performed with either a) anti-phospho-p70 S6K (Ser411) or b) anti-p70 S6K Abs.

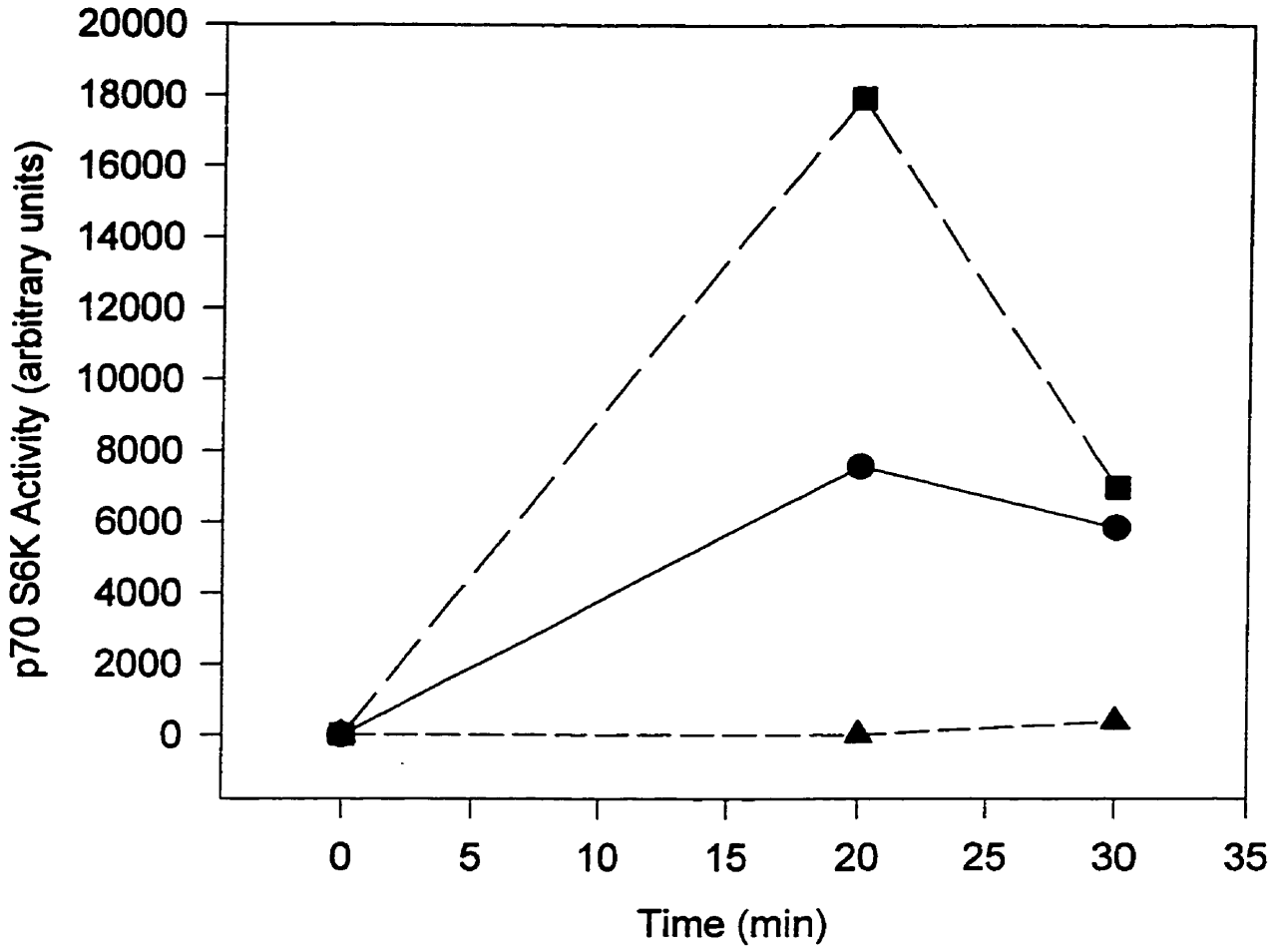


FIGURE 32: Effect of (*R*)- and (*S*)-TDB-PC on p70 S6 Kinase Activity

Quiescent MCF-7 cells were incubated with either (*R*)-TDB-PC (circles), (*S*)-TDB-PC (triangles) (30 μM, 4 h) or left untreated (squares) followed by stimulation with insulin (1 μg/ml). The cells were harvested and p70 S6K was immunoprecipitated from the lysates (1 mg). The immunoprecipitates were washed and assayed for p70 S6 kinase activity with Crosstide as described in Section 3.14. Two independent experiments with different cell preparations showed similar results.

4.19 Direct Effect of ALPs on p70 S6 Kinase Activity

Although the results of the studies above suggest that inhibition of phosphorylation of p70 S6K was responsible for the inhibition of the enzyme activity, we cannot preclude a direct effect of the drugs on p70 S6 kinase catalytic activity. To determine whether the ALPs could directly affect p70 S6K activity, p70 S6 kinase assays were performed using p70 S6K immunoprecipitated from MCF-7 cells stimulated with insulin for 20 minutes. Different concentrations of ET-18-OCH_3, (*R*)-TDB-PC, or (*S*)-TDB-PC were added to the kinase reaction mixture, and the phosphorylation of Crosstide was used as a measure of p70 S6K activity. When the kinase reaction mixture contained 15 or 30 μM of ET-18-OCH_3, (*R*)-TDB-PC or (*S*)-TDB-PC, there was no significant inhibition of p70 S6 kinase activity (**Figure 33**) indicating that the drugs did not directly inhibit p70 S6 kinase activity.

4.20 Contribution of PI3K to PKB and p70 S6K

It is known from the literature that PKB and p70 S6K can be activated by PI3K (Burgering and Coffer, 1995; Franke *et al.*, 1995). Since we saw an inhibition of both PKB and p70 S6K, we wanted to verify that the activation of PI3K is upstream to the activation of both PKB and P70 S6K in MCF-7 cells. We treated MCF-7 cells with two different inhibitors of PI3K, wortmannin and LY294002. Cells were treated with these inhibitors and the phosphorylation of PKB and p70 S6K was investigated using Western blot analysis with the phospho-specific antibodies. Cells treated with LY294002 and wortmannin resulted in decreased phosphorylation

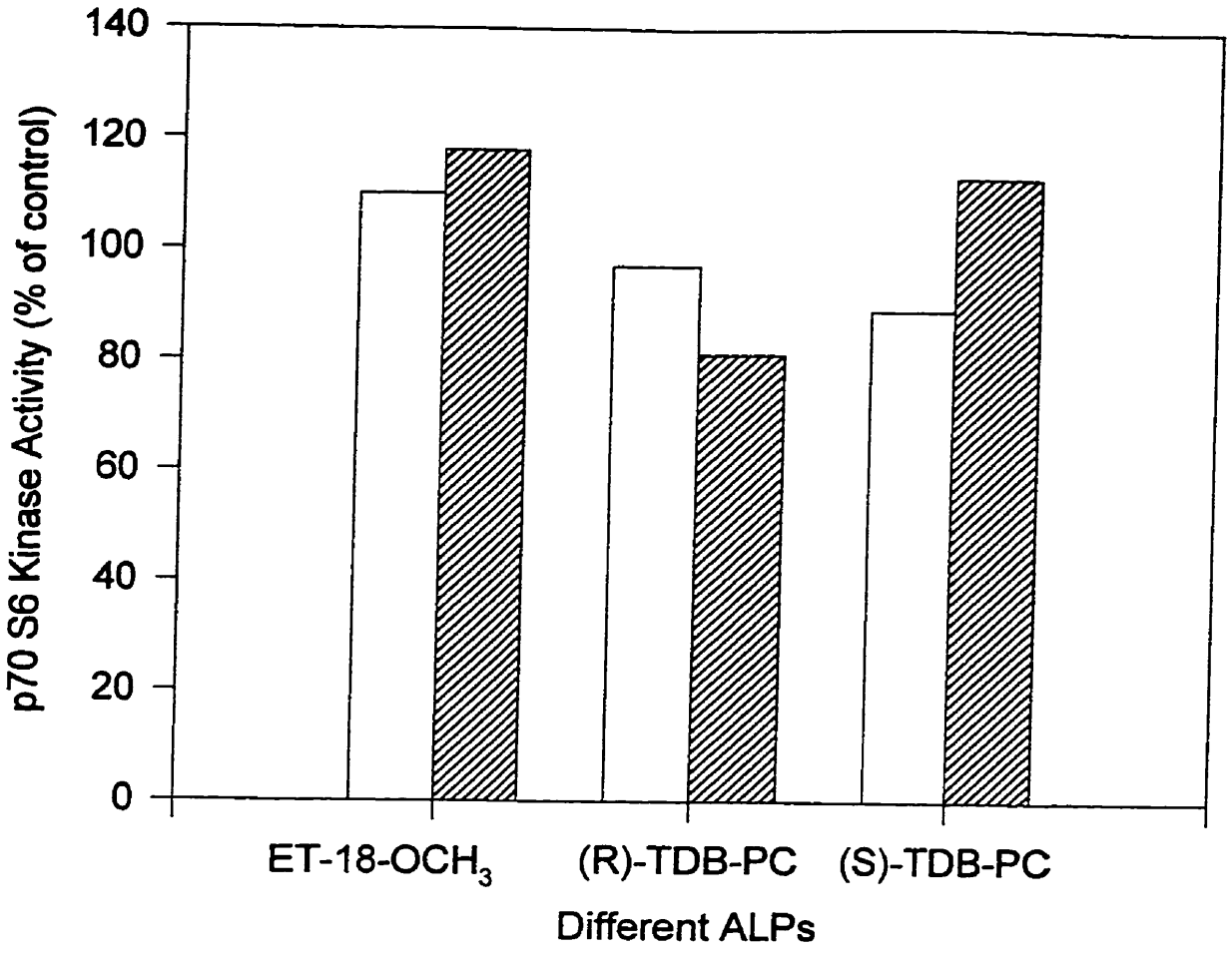


FIGURE 33: Direct Effect of ALPs on p70 S6 Kinase Activity

Quiescent MCF-7 cells were stimulated with insulin (1 μg/ml) for 30 minutes and harvested. p70 S6K was immunoprecipitated from the lysates and incubated in a reaction mixture containing either 0, 15 (open bars) or 30 μM (hatched bars) of ET-18-OCH_3, (*R*)-TDB-PC or (*S*)-TDB-PC. p70 S6 kinase activity was measured using Crosstide as substrate as described in Section 3.14. The results are expressed as a percentage of the controls without any drugs and is representative of two separate experiments.

of p70 S6K on Ser411 (**Figure 34**) indicating that the activation of p70 S6 kinase activity in these cells was due to the activation of PI3K. In addition, cells treated with LY294002 and wortmannin also resulted in a decreased phosphorylation of PKB on Ser473 indicating that the PKB activity was also due to the activity of PI3K. This verifies previous studies that the activation of PI3K is required for the activation of PKB and p70 S6K.

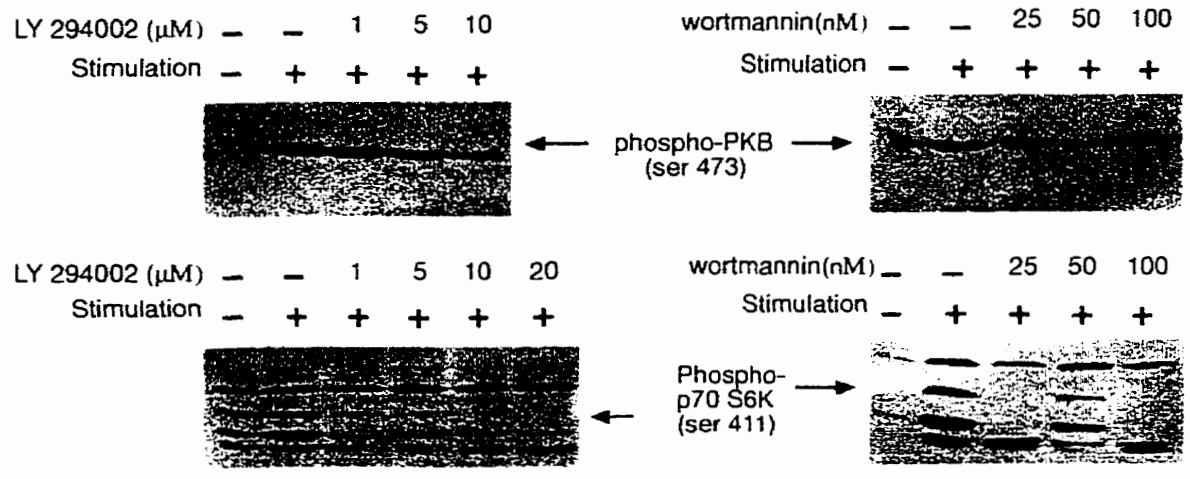


FIGURE 34: Effect of PI3K Inhibitors on PKB and p70 S6K Phosphorylation

Quiescent MCF-7 cells were treated with different concentrations of LY294002 for 20 minutes or with different concentrations of wortmannin for 60 minutes followed by stimulation with insulin (1 μg/ml) for 20 minutes. After harvesting, equal amounts of protein (65 μg) were resolved on 10% SDS gels, transferred to nitrocellulose membranes and probed with either anti-phospho-PKB (Ser 473) or with anti-p70 S6K (Ser 411) antibodies. Two independent experiments with different cell preparations showed similar results.

5 DISCUSSION

5.1 Introduction

ALPs are anticancer drugs that selectively inhibit the growth of cancer cells over normal cells. The exact mechanism by which they exert their antiproliferative effects are not entirely known. The treatment of cells with ALPs have been shown to elicit a number of different cellular responses including an increase in intracellular-free calcium levels (Seewald *et al.*, 1990; Brinkmeier *et al.*, 1996), alteration in lipid metabolism (Unger *et al.*, 1987; Weider *et al.*, 1995), and inhibition of activation of signalling molecules such as PI3K and PI-PLC (Berggren *et al.*, 1993; Powis *et al.*, 1992). Evidence that these responses correlate with inhibition of cell proliferation is lacking. Therefore, studies in our laboratory primarily focused on determining the mechanism by which ET-18-OCH_3 inhibits the growth of MCF-7 cells. It was discovered that changes in lipid metabolism were not the primary cause for the inhibition of MCF-7 cell growth (Zhou and Arthur, 1995). Further studies determined that ET-18-OCH_3 inhibits the activation and phosphorylation of MAPK (Zhou *et al.*, 1996), an enzyme shown to be sufficient to initiate cell proliferation. This inhibition of MAPK was due to an effect of ET-18-OCH_3 on Raf-1 association with the membrane (Zhou *et al.*, 1996). It is known that ET-18-OCH_3 inhibits Raf-1 association with the membrane in EGF-stimulated cells which leads to inhibition of the MAPK pathway (Zhou *et al.*, 1996), but what is not known is whether the drug inhibits the activation of other signalling molecules which

could also contribute to inhibition of cell growth. Since there are a number of signal transduction pathways in the cell, one could propose that ET-18-OCH_3 also inhibits other signalling molecules which would also contribute to the decreased cell growth. The objectives of this project were to determine if additional signalling molecules were being perturbed by ET-18-OCH_3 that could also contribute to inhibition of cell proliferation.

5.2 Effect of ET-18-OCH_3 on Raf-1 association with the membrane

Since the objectives of this study were to examine other signalling pathways, we first had to choose a mitogen for MCF-7 cells that activated other signalling pathways in addition to the MAPK pathway. We decided to use insulin as it is known to activate a number of multiple diverging pathways (White and Kahn, 1994) which would be invaluable to us in examining the effect of ET-18-OCH_3 on different signalling molecules. Initially we verified that insulin was indeed a potent mitogen for our cell line (**Figure 14**) and that the mitogenic ability of insulin was inhibited by ET-18-OCH_3 (**Figure 15**).

After establishing this, the first thing we set out to do was to investigate whether the inhibition of MAPK was a common mechanism of inhibition of cell growth. If this is a universal mechanism by which ET-18-OCH_3 inhibits the growth of cells, then we would expect MAPK to be inhibited by all mitogens that activate this kinase. This is exactly what we observed; when the MCF-7 cells were treated with ET-18-OCH_3 and then stimulated with insulin, there was a decrease in both the

phosphorylation and activation of MAPK (**Figure 16**). To take this one step further, we looked at the effect of ET-18-OCH_3 on Raf-1 association with the membrane in insulin-stimulated cells. Similar to the results obtained with EGF stimulation (Zhou *et al.*, 1996), ET-18-OCH_3 inhibited this association (**Figure 18**).

Since treatment with ET-18-OCH_3 inhibited Raf-1 association with the membrane with two different mitogens, this suggests that this is a common mechanism of action of the drug and could be responsible for the inhibition of cell proliferation. We wanted to delve further into the basis for the ALP-induced decrease in Raf-1 membrane levels. The activation of Raf-1 is a very complex procedure, involving many key players. The first step in Raf-1 activation is the binding of Ras to Raf-1 thereby recruiting Raf-1 to the plasma membrane (Marshall, 1995). Since we know that Raf-1 association with the membrane is inhibited in MCF-7 cells treated with ET-18-OCH_3, it is possible that the ALP could be binding directly to Raf-1 in the Ras binding domain (RBD) therefore reducing the Raf-1 - Ras binding. If this were to occur, there would be less Raf-1 recruited to the plasma membrane. Alternately, ET-18-OCH_3 may bind to another portion of Raf-1, inhibiting the binding of another key molecule such as 14-3-3 that is necessary for Raf-1 activation. It is known that 14-3-3 proteins bind to both inactive and active Raf-1 and this is thought to keep Raf-1 in an inactive conformation in the cytosol and in an active conformation in the membrane (Tzivion et al., 1998). If ET-18-OCH_3 binds in the region of 14-3-3 binding, then perhaps Raf-1 is in a conformation that is more difficult to be activated. If it is held in an unfavourable conformation,

then Ras may not have access to its binding domains on Raf-1 and hence would be incapable of binding and recruiting Raf-1 to the membrane ultimately resulting in decreased membrane levels of Raf-1. Once Ras binds to Raf-1 and recruits it to the plasma membrane, Raf-1 remains bound to the plasma membrane independent of Ras. Therefore, ET-18-OCH_3 may bind to Raf-1 and inhibit its association with the membrane independent of Ras. It was shown that Raf-1 has binding sites for phosphatidic acid and phosphatidylserine (Ghosh et al., 1994, Ghosh et al., 1996). The discovery of the ability of phospholipids to bind directly to Raf-1 may provide a mechanism by which Raf-1 is held in the plasma membrane since these lipids are components of the plasma membrane. Since ET-18-OCH_3 is a synthetic lipid and lipids have been found to bind to Raf-1, it is conceivable that ET-18-OCH_3 may also bind to Raf-1 and inhibit its association with the membrane subsequent to translocation. In addition, once anchored in the membrane, Raf-1 becomes phosphorylated on multiple residues and these phosphorylations are necessary for Raf-1 to be active. ALPs may affect the phosphorylation state of Raf-1 by inhibiting a kinase or activating a phosphatase. If Raf-1 requires phosphorylation to be anchored in the membrane subsequent to binding to Ras, then the activation of a phosphatase that would dephosphorylate Raf-1 could result in dissociation from the membrane.

Of the above possibilities that could result in decreased membrane Raf-1 levels, we examined whether the drug has an effect on the Raf-1-Ras binding. It was previously demonstrated that ET-18-OCH_3 did not affect Ras activation (Zhou

et al., 1996). We loaded a recombinant GST-Ras protein with GTP-γ-S to simulate active Ras which binds to Raf-1 in the cytosol and recruits it to the membrane. We found that when the cells were treated with ET-18-OCH_3, there was a 50% decrease in the ability of cytosolic Raf-1 from the cells to bind to the activated Ras (**Figure 19**). Upon examination of other lysolipids, we found that the inhibition of Raf-1 to the GST-Ras-GTP-γ-S was limited to ET-18-OCH_3 and was not due to nonspecific inhibition (**Figure 20**). When the other lipids were added to the MCF-7 cells, there was no inhibition of Ras-Raf-1 binding. Although all these molecules have the glycerol backbone and are similar structurally, the other lipids, unlike ET-18-OCH_3 can be metabolized in the cells. ET-18-OCH_3 was originally synthesized as an analogue of lysophosphatidylcholine which would have a longer half-life. The ester functions of the glycerol backbone in positions one and two were replaced with ethers and were therefore not susceptible to phospholipases. Also, many of these lipids are naturally occurring in the cells and are components of biological membranes or could be converted to components of membranes. Hence when added to the cells, they have no effect on Ras- Raf-1 binding. We also found decreased binding when the ALP was added directly to the cell lysates (**Figure 19**) indicating that ET-18-OCH_3 inhibited this binding directly. Although this is only shown *in vitro*, using a purified Ras construct, it does provide evidence that ET-18-OCH_3 may be inhibiting the binding of GTP-Ras to Raf-1 in the intact cell. Our studies suggest that ET-18-OCH_3 is able to bind directly to Raf-1 and thereby inhibit the binding of Ras although it does not preclude that the drug binds to other

molecules necessary for Raf-1 activation. If this were to occur, these molecules may be unable to bind to Raf-1 and therefore result in decreased Raf-1 translocation to the membrane. Further studies will have to be done to confirm that ET-18-OCH_3 is able to bind to Raf-1, and if it is, whether this binding is in the regions where Ras is known to bind to Raf-1. If binding of the ALP does occur in the RBD, then the inhibition of Ras binding to Raf-1 would result in a decreased association of Raf-1 with the membrane. To further confirm that ET-18-OCH_3 inhibits Ras binding with Raf-1, Raf-1 with a CAAX tag is targetted to the membrane and bypasses the need for Ras (Leevers et al., 1994). If the drug is inhibiting this binding, then Raf-1 with the tag should be insensitive to ET-18-OCH_3 since it is already in the plasma membrane and does not need to bind to GTP-Ras. If the ET-18-OCH_3 is found to bind to Raf-1 but not in the regions of Ras binding, then the drug may be disrupting the binding of a protein required to hold Raf-1 in a conformation favourable for activation and binding to Ras. The exact mechanism by which ET18-OCH_3 inhibits Raf-1 membrane levels still remains to be elucidated

5.3 Effect of ET-18-OCH_3 on insulin-stimulated signalling molecules

We have demonstrated that the inhibition of Raf-1 association with the membrane is a common mechanism by which ET-18-OCH_3 inhibits MAPK activity. The activation of MAPK has been shown to be necessary for cell growth but whether this inhibition is solely responsible for the inhibition of MCF-7 cell growth

cells which might also contribute to its antiproliferative effects. As stated in the introduction, it is known that ET-18-OCH_3 inhibits different processes in the cell including lipid metabolism and PI-PLC activity but the contribution of these events to cell proliferation has been questioned.

One of the initial steps in the insulin pathway is the tyrosine phosphorylation of IRS-1. Once IRS-1 becomes activated, it binds to many SH2-domain containing proteins which culminates in a multitude of signals in the cell. Since the tyrosine phosphorylation of IRS-1 is important, we started our studies on the insulin pathway by investigating the ability of ET-18-OCH_3 to affect this phosphorylation. We found that ET-18-OCH_3 had no effect on the tyrosine phosphorylation of IRS-1 (**Figure 17**). We next investigated whether the drug has an effect on the association of PI3K with IRS-1. Once again, ET-18-OCH_3 had no effect on the binding of PI3K to the activated IRS-1 (**Figure 21**). Once the 85 kDa subunit of PI3K binds to IRS-1, it activates the catalytic activity of the 110 kDa subunit (Giorgetti *et al.*, 1993). ET-18-OCH_3 also had no effect on PI3K activity subsequent to its association with IRS-1 (**Figure 22**). On the other hand, we found that ET-18-OCH_3, when added directly to the kinase assay decreased the amount of PIP produced by 50% (**Figure 23**). In a study by Berggren *et al.* (1993), it was found that, in an *in vitro* assay, different conditions caused different degrees of inhibition of PI3K activity by ET-18-OCH_3. When the cells were treated with ET-18-OCH_3 and the lipids extracted, and used as a source of substrate for the PI3K activity, the IC_{50} value was less than if exogenous PI was added to the reaction mixture. This indicates that the substrate

as a source of substrate for the PI3K activity, the IC_{50} value was less than if exogenous PI was added to the reaction mixture. This indicates that the substrate composition greatly influences the activity of the enzyme thereby making it unreliable to extrapolate the *in vitro* assay to the effect of the drug on the enzyme in the cell. Since we added exogenous PI to our kinase assay, the results we obtained in the *in vitro* assay do not indicate whether an inhibition of PI3K occurs in the cell. In order to determine whether an inhibition of PI3K occurs in intact cells, we decided to look at the effect of ET-18-OCH_3 on molecules downstream of PI3K. It is possible that the drug could inhibit PI3K activity (could have a direct effect) but is needed to be there to exert its effect. If this is the case then we would expect molecules downstream of PI3K to be affected by ET-18-OCH_3.

PKB acts downstream of PI3K and the activation of PI3K has been shown to be necessary for its activation. Likewise, p70 S6K acts downstream of PI3K. To confirm that both PKB and p70 S6K act downstream of PI3K, we treated MCF-7 cells with two different PI3K inhibitors, wortmannin and LY294002. When the cells were treated with both of these inhibitors, the phosphorylation of both PKB (Ser473) and p70 S6K (Ser411) was inhibited (**Figure 34**), verifying that these molecules act downstream of PI3K and the activation of PI3K is necessary for their activity. When MCF-7 cells were treated with ET-18-OCH_3, both the phosphorylation (**Figure 24**) and the activation of PKB was inhibited (**Figure 25**). The same inhibition of activity and phosphorylation was seen with p70 S6K (**Figure 29** and **30**). This inhibition of both PKB and p70 S6K could likely result from the inhibition of PI3K.

5.4 *Does the inhibition of PKB and p70 S6K activity by ET-18-OCH_3 result in inhibition of cell proliferation?*

The purpose of this study was to find molecules inhibited by ET-18-OCH_3 was inhibiting and to determine whether this inhibition could result in inhibition of cell proliferation. We needed to determine whether the inhibition of PKB and p70 S6K activities we observed when ET-18-OCH_3 was added to the cells could contribute to decreased cell growth. We have two enantiomers, (*R*)- and (*S*)-TDB-PC, which provide us with a tool to investigate this question. (*S*)-TDB-PC inhibited the proliferation of a number of different cell lines relative to the (*R*)- enantiomer. (*R*)-TDB-PC had little effect on the proliferation of the same cell lines, although this compound was slightly more effective on MCF-7 cells whose growth was inhibited by 50% (Samadder, 1998). Therefore by using incubation conditions where the (*S*)-enantiomer inhibits proliferation of MCF-7 cells and the (*R*)- enantiomer does not, we can identify whether an inhibition of signalling molecules in the cell is relevant to the mechanism of growth inhibitions by ALPs.

Although PKB and p70 S6K activities have been found to be inhibited by ET-18-OCH_3, we do not know whether the inhibition of these molecules could result in decreased cell growth. In order to investigate this further, MCF-7 cells were treated with the enantiomeric analogs, (*R*)-TDB-PC and (*S*)-TDB-PC. When these ALPs were used, we observed that both PKB phosphorylation (**Figure 26**) and activity (**Figure 27**) were inhibited. The same inhibition was observed with p70 S6K (**Figure 31** and **32**). Although both these molecules were inhibited by these ALPs,

the extent of inhibition was different with the two compounds. Cells treated with (*R*)-TDB-PC had decreased phosphorylation of PKB relative to the controls but the phosphorylation in cells treated with (*S*)-TDB-PC was completely abolished. A similar inhibition of PKB activity was in cells treated with the (*S*)- enantiomer, as the inhibition of PKB was much greater compared to cells treated with the (*R*)-enantiomer. The same phenomena was observed with p70 S6K. Because of the difference in inhibition between the two enantiomers, it is possible that this may have some relevance to inhibition of cell growth. As Samadder (1998) has shown, the (*R*)- enantiomer does have some inhibitory effect on MCF-7 cell growth. It is therefore conceivable that (*R*)-TDB-PC could have some effect on molecules responsible for inhibiting cell proliferation.

Since there was a discrepancy in the results when examining the PI3K activity when ET-18-OCH_3 was added to the cells versus directly to the kinase reaction, we wanted to use the (*R*)- and (*S*)-TDB-PC and see if we could investigate this further. We examined the effect of exogenous (*R*)-TDB-PC and (*S*)-TDB-PC on PI3K activity and found that both drugs inhibited this activity to a similar extent (**Figure 23**) and the inhibition was less than that obtained with ET-18-OCH_3. Since we have shown that (*S*)-TDB-PC has a much greater effect on PKB and p70 S6 kinase activity relative to (*R*)-TDB-PC and actually completely abolishes PKB and p70 S6 kinase activity, the results we see in this *in vitro* assay are likely not reflective of what is really occurring in the cells.

5.5 *Target of ET-18-OCH_3 in the PI3K pathway*

We have demonstrated that ET-18-OCH_3 inhibits the activity of both PKB and p70 S6K and that this inhibition could likely contribute to the anti-proliferative effects of the drug. Although we know that these molecules are inhibited, the target of ET-18-OCH_3 and its analogues in the PI3K pathway remains to be discovered. Our studies suggest it is likely to be PI3K since this molecule lies upstream of both PKB and p70 S6K but we have not looked at PI3K activity in the intact cell.

There are two phosphorylation sites on PKB and these two sites have been found to be phosphorylated by two different kinases, PDK1 and PDK2 (Alessi et al., 1996). Since ET-18-OCH_3 inhibited phosphorylation of both of these sites, it is likely that the action of the drug lies upstream of both PDK1 and PDK2 although studies that demonstrate no effect of the drug on the kinase activity of the PDKs would be needed to support this conclusion. We have also shown that the ALPs do not have a direct effect on PKB (**Figure 28**) providing further evidence that the target of the drugs lies upstream. It is possible that in intact cells, the activity of PI3K may be inhibited by the drugs and there would be a decrease in the amount of cellular phosphoinositides produced. If the levels of PI 3,4 P_2 and PI 3,4,5 P_3 are decreased in the cells, then it is probable that this would result in inhibition of PKB activity as 3-phosphoinositides are implicated in the activation of PKB. This suggestion would require an examination of the effect of ET-18-OCH_3 on endogenous phosphatidylinositol 3-phosphates in the cell as results on the direct effect of ET-18-OCH_3 on PI3K *in vitro* could not be extrapolated to the *in vivo*

observations.

Our results do not preclude a direct effect of ET-18-OCH_3 on other molecules in the PI3K pathway. We have shown that the phosphorylation of p70 S6K on Ser411, Thr421 and Ser424 are also inhibited by ET-18-OCH_3. The kinase responsible for phosphorylating these sites has not yet been identified but is likely to be a proline-directed kinase since they all contain proline in the +1 position . ET-18-OCH_3 could inhibit the activity of this kinase.

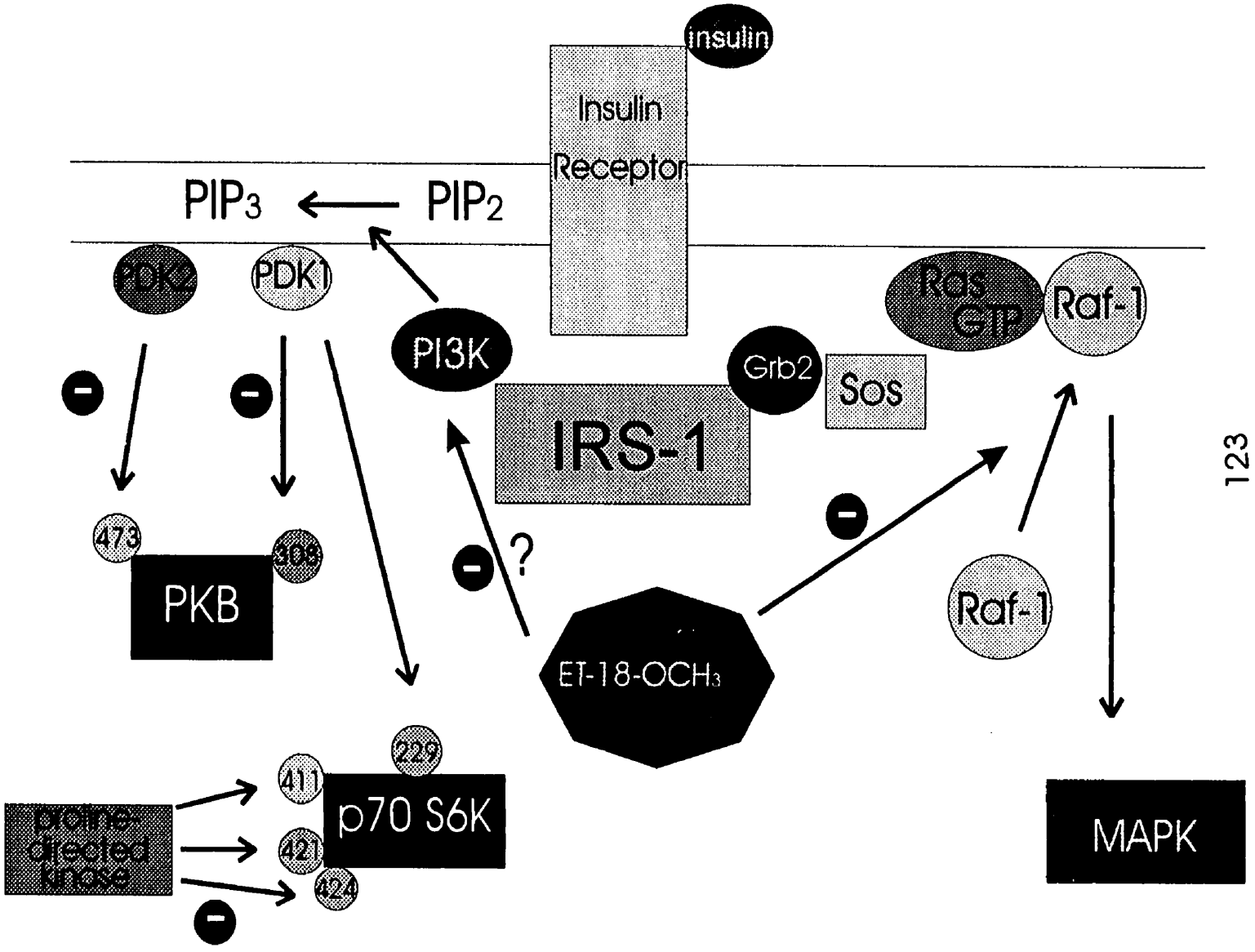
We have demonstrated that ET-18-OCH_3 inhibits the phosphorylation and activation of PKB and p70 S6K, but there are still some uncertainties in this pathway. Although PI3K, PKB, and p70 S6K have been speculated to play a role in cell growth and mitogenesis (Carpenter and Cantley, 1996; Proud, 1996), the contribution of these molecules to cell proliferation in these cells remains to be established. The studies with the (*R*)- and (*S*)-TDB-PC shed some light on this and indicate that these molecules may play some role in regulating cellular proliferation. If the activation of PKB and p70 S6K do not correlate to an increase in cell growth, then we would expect the enantiomeric ALPs to have similar effects when examining the activity of these molecules. This is not the case. The (*S*)-enantiomer, which completely inhibits cell growth also completely inhibits the phosphorylation and activation of both PKB and p70 S6K . The (*R*)- enantiomer, on the other hand, which inhibits MCF-7 cell growth to a lesser extent also inhibits the phosphorylation and activation of PKB and p70 to lesser extents.

Taken together these results suggest that inhibition of PKB and p70 S6K

activities by ET-18-OCH3 and its ALP analogues likely contribute to the antiproliferative effects of the drugs. Thus we postulate that inhibition of both the MAPK and PI3K pathways by ALPs are responsible for inhibition of cell proliferation. A model showing the effects and likely target(s) of ET-18-OCH_3 in the MAPK and PI3K pathways is shown in **Figure 35**.

FIGURE 35 Scheme of ET-18-OCH_3 Action

Insulin binds to and activates its receptor, which then phosphorylates IRS-1 on multiple tyrosine residues. Grb2 binds to IRS-1 and leads to the activation of Ras. ET-18-OCH_3 inhibits the translocation of Raf-1 to the plasma membrane by inhibiting the binding of Ras-GTP to Raf-1. This, in turn, results in inhibition of MAPK activation. PI3K also binds to IRS-1. PI3K phosphorylates PIP_2 to PIP_3. The accumulation of PIP_3 in the plasma membrane leads to the activation of PDK1 and PDK2. PDK2 phosphorylates Ser473 on PKB which is inhibited by ET-18-OCH_3. Likewise, ET-18-OCH_3 also inhibits the phosphorylation of PKB on Thr308 by PDK1. Also, upon insulin stimulation, a proline-directed kinase becomes activated that phosphorylates p70 S6K on Ser411, Thr421 and Ser424. The phosphorylation of these sites is inhibited by ET-18-OCH_3. The activities of both PKB and p70 S6K are also inhibited by ET-18-OCH_3. Since both PKB and p70 S6K are inhibited, and the phosphorylation due to both PDK1 and PDK2 are also inhibited, this suggests that the target of ET-18-OCH_3 lies upstream and is likely PI3K. (See Figure on page 123)


insulin
Insulin Receptor
PIP3
PIP2
PDK2
PDK1
PI3K
Grb2
Sos
IRS-1
Ras GTP
Raf-1
473
303
PKB
?
ET-18-OCH3
Raf-1
229
411
421
424
p70 S6K
Proline-directed kinase
MAPK

123

6 CONCLUSION

The antiproliferative effects of ALPs have been studied extensively in many laboratories. In our laboratory, studies on the mechanism of action of ET-18-OCH_3, the prototype ALP, led to the discovery that ET-18-OCH_3 perturbs the association of Raf-1 with the membrane. This results in inhibition of MAPK which leads to inhibition of cell growth. In this thesis, we set out to explore the affect of ET-18-OCH_3 on Raf-1 and other signalling molecules activated following insulin stimulation.

The conclusions that can be drawn from our studies are:

[1] ET-18-OCH_3 inhibits insulin-stimulated MAPK activity and Raf-1 association with the membrane in MCF-7 cells, as in EGF-stimulated cells.

[2] ET-18-OCH_3 inhibits the binding of Raf-1 to a GST-Ras construct loaded with GTP-γ-S providing evidence that a similar inhibition may occur in intact cells.

[3] ET-18-OCH_3, (*R*)-TBD-PC and (*S*)-TDB-PC have a direct effect on PI3K activity but this is likely not representative of what is occurring in the cell and may therefore be nonspecific effects of the *in vitro* assay.

[4] ET-18-OCH_3, (*R*)-TDB-PC and (*S*)-TDB-PC inhibit the phosphorylation and activation of PKB. There was a differential effect of the (*R*)-TDB-PC and the (*S*)-TDB-PC where the (*R*)-enantiomer inhibits the activity relative to the control but the activity was completely abolished with the (*S*)-enantiomer.

[5] ET-18-OCH_3, (*R*)-TDB-PC and (*S*)-TDB-PC inhibit the phosphorylation and

activation of p70 S6K. As with the PKB, there was a differential effect of the two enantiomers where there was inhibition with the (*R*)-TDB-PC but the activity was completely abolished with the (*S*)-TDB-PC

In conclusion, the studies in this thesis have further confirmed that the inhibition of Raf-1 association with the membrane is a primary target of ET-18-OCH_3 in inhibiting cell growth. Also, evidence suggests that this may occur by inhibiting the binding of activated Ras to Raf-1. Furthermore, we have shown that ALPs could also be inhibiting cell proliferation by inhibiting the PI3K pathway.

Further studies to elucidate the exact mechanism by which ET-18-OCH_3 inhibits Raf-1 activation and also to unequivocally determine whether the ALPs inhibit cell proliferation by inhibiting the PI3K pathway should lead to the rational use of these compounds in cancer therapy.

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