

ABSTRACT

WANG, CHUN-CHAO. Crosstalk between Phosphoinositide 3-Kinase and Extracellular Signal-regulated Kinase Pathways in Platelet-derived Growth Factor Receptor-mediated Signaling. (Under the direction of Jason M. Haugh)

Cells control their behavior by maintaining a balance between signal transduction pathways. The disruption of this balance has been implicated in the evolution of normal cells into cancer cells. People expected to inhibit or activate a specific pathway to achieve the cure of cancer. However, signaling pathways are usually woven together through so-called cross-talk interaction and it is almost impossible to design a drug specifically targeting a pathway. Thus, the fundamental understanding of the intracellular signaling processes is important on medicine and biotechnology.

Platelet-derived growth factor (PDGF) is a potent stimulator of cell growth and motility. PDGF-induced phosphoinositide 3-kinase (PI 3-kinase)/Akt and Ras/extracellular signal-regulated kinase (Erk) pathways are thought to play central roles in the regulation of cell survival and proliferation. Crosstalk between PI 3-kinase/Akt and Ras/Erk pathways has provided important hints for how PDGF receptor activation may control cellular function. The apparently contradictory findings among recently studies suggest that the cross-talk mechanisms are not fully understood. In this study, we aim to systematically analyze this signaling network, comprised of Ras/Erk and PI 3-kinase/Akt pathways, stimulated by PDGF. Molecular level control over the signaling network, through genetic and pharmacological intervention, will allow these complex regulatory interaction to be isolated. We assessed the

phosphorylation of Akt and Erk as the outputs of PI 3-kinase/Akt and Ras/Erk pathways respectively, and detected DNA synthesis and cell numbers to analyze the effect of these two pathways on the resulting life and death decisions. Accumulating evidence has demonstrated that differences in the strength of stimulus, and the duration and magnitude of activity of signal molecules, determine signaling specificity. We offer a quantitative approach – crosstalk titration – to analyze the effect of our intervention strategies on the signaling network.

Our results demonstrated that Ras signals are required for full activation of PI 3-kinase/Akt, consistent with the idea that the PDGF receptors and Ras-GTP modulate PI 3-kinase activity in a cooperative fashion. The relevance of PI 3-kinase for Erk activation is complicated. We observed that PI 3-kinase may play multiple role in regulation of the Erk pathway, both positive and negative, dependent on the magnitude of PDGF and the strength and duration of signaling molecular. The extent to which PI 3-kinase activity influences Ras/Erk signaling is still uncertain. Additionally, we found that PDGF-induced DNA synthesis involved the functions of Ras, PI 3-kinase, and MEK. The relevance of Akt on PDGF-induced DNA synthesis is not fully understood, leading the questions open for further investigation.

**CROSSTALK BETWEEN PHOSPHOINOSITIDE 3-KINASE AND
EXTRACELLULAR SIGNAL-REGULATED KINASE PATHWAYS IN
PLATELET-DERIVED GROWTH FACTOR RECEPTOR-MEDIATED SIGNALING**

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BIOGRAPHY

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TABLE OF CONTENTS

	Page
LIST OF FIGURES	vi
CHAPTER 1. INTRODUCTION	1
1.1. Background.....	1
1.2. Platelet-derived Growth Factor Receptor (PDGFR).....	2
1.3. PI 3-kinase/Akt Pathway and Cell Survival.....	6
1.3.1. PI 3-kinase.....	6
1.3.2. PI 3-kinase inhibitors: wortmannin and LY294002.....	7
1.3.3. Akt.....	8
1.3.4. Akt and cell survival.....	10
1.4. Ras/Erk Pathway and Biological Functions.....	13
1.4.1. Ras and its regulatory proteins.....	13
1.4.2. Raf/MEK/Erk cascade.....	15
1.4.3. ERK and biological functions.....	17
1.5. Cross-talk between PI 3-kinase/Akt and Ras/Erk Pathways.....	19
1.5.1. Evidence that PI 3-kinases are also Ras effectors.....	19
1.5.2. Evidence that PI 3-kinase contributes to Erk activation.....	21
1.5.3. Evidence that PI 3-kinase negatively regulates Erk.....	22
1.6. Aims of This Study.....	25

CHAPTER 2. MATERIALS AND METHODS.....	27
2.1. Reagents and antibodies.....	27
2.2. Plasmid and retroviral infection.....	27
2.3. Cell culture and lysate preparation.....	28
2.4. Enzyme-linked immunosorbent assay (ELISA) of Akt phosphorylation.....	29
2.5. Quantitative immunoblotting.....	29
2.6. DNA synthesis assay.....	30
2.7. Cell proliferation assay.....	30
CHAPTER 3. RESULTS AND DISCUSSION.....	31
3.1. PDGF-stimulated Akt and Erk phosphorylation in control cells.....	31
3.2. Ras signals are required for full activation of PI 3-kinase/Akt.....	33
3.3. Erk stimulation, at low but not high concentrations of PDGF, is inhibited by PI 3-kinase-inhibitory drug.....	35
3.4. MEK activity is required for PDGF-induced Erk phosphorylation but does not significantly affect PDGF-induced Akt phosphorylation.....	38
3.5. Transient and sustained Akt and Erk activation in response to different PDGF-BB concentrations.....	40
3.5.1. Evaluation of the coupled kinetics of PI 3-kinase/Akt and Ras/Erk responses in control cells.....	40
3.5.2. Effect of constitutively active Ras on kinetics of PI 3-kinase/Akt and Ras/Erk responses.....	43
3.5.3. Effect of dominant-negative Ras on kinetics of PI 3-kinase/Akt and	

Ras/Erk responses.....	46
3.5.4. Summary of the effect of changes in Ras-GTP level on kinetics of PI 3-kinase/Akt and Ras/Erk pathways.....	49
3.6. The mechanisms of Akt on Erk phosphorylation are still poorly understood...	49
3.7. PDGF-BB induces DNA synthesis and enhances cell proliferation.....	52
3.8. PI 3-kinase/Akt and Raf/MEK/Erk pathways are required for PDGF-induced DNA synthesis.....	54
CHAPTER 4. CONCLUSIONS.....	58
CHAPTER 5. REFERENCES.....	61

LIST OF FIGURES

	Page
Figure 1.1. Interaction between PDGF β -receptor and SH2 domain-containing signal transduction molecules.....	5
Figure 1.2. A model for PI 3-kinase/Akt pathway.....	12
Figure 1.3. A model for Ras/Erk pathway.....	18
Figure 1.4. Cross-talk between Ras/Erk and PI 3-kinase/Akt pathways.....	24
Figure 1.5. Molecular level control over the signaling network in this study to analyze the cross-talk between Ras/Erk and PI 3-kinase/Akt pathways.....	26
Figure 3.1. PDGF-stimulated Akt and Erk phosphorylation in control cells.....	32
Figure 3.2. Cooperative activation of PI 3-kinase/Akt by PDGF receptors and Ras.....	34
Figure 3.3. LY294002 blocked Akt phosphorylation and partially reduced Erk phosphorylation induced by low concentration of PDGF.....	37
Figure 3.4. PD098059 blocked PDGF-induced Erk phosphorylation but had no significant effect on the PDGF-induced Akt phosphorylation.....	39
Figure 3.5. The kinetics of Akt and Erk phosphorylation in control cells.....	42
Figure 3.6. The kinetics of Akt and Erk phosphorylation in Ras G12V cells.....	45
Figure 3.7. The kinetics of Akt and Erk phosphorylation in Ras S17N cells.....	48
Figure 3.8. The kinetics of Akt and Erk phosphorylation in control cells.....	51
Figure 3.9. PDGF-BB induces DNA synthesis and enhances cell proliferation.....	53
Figure 3.10. The effect of Ras and Akt in PDGF-induced DNA synthesis.....	56
Figure 3.11. The effect of LY294002 and PD098059 on PDGF-induced DNA synthesis.....	57

CHAPTER 1. INTRODUCTION

1.1. Background

In multicellular organisms, intercellular communication is required for cell growth and differentiation as well as a systemic response to wounding. Cells synthesize and release extracellular signaling molecules, such as growth factors. Growth factors are high-affinity ligands for transmembrane receptors belonging to the family of receptor tyrosine kinases (RTKs). Binding of a growth factor to the extracellular domain of the receptor influences the target cell by triggering signaling cascades inside the cytoplasm. This process of converting extracellular signals into cellular responses is termed *signal transduction* (Fedi and Aaronson, 2000; Medico and Comoglio, 2000).

The importance of the study in this area resides in that many cellular functions and phenotypes are results of *signal transduction*. Cells control their behavior by maintaining a balance between signal transduction pathways, which regulate the order and timing of the cell cycle transitions and ensure that critical events, such as DNA replication, are completed with high fidelity. The disruption of this balance has been implicated in the evolution of normal cells into cancer cells (Elledge, 1996). From the therapeutic point of view, people expected to inhibit or activate a specific signaling pathway to achieve the cure of cancer. Unfortunately, the cell signaling pathways are usually woven together through so-called cross-talk interaction. This makes it almost impossible to design a drug specifically targeting a signal pathway (Hunter, 1997; Ballif and Blenis, 2001; Gong and Zhang, 2005). Thus, the fundamental understanding of the intracellular signaling processes is important on medicine and biotechnology.

1.2. Platelet-derived Growth Factor Receptor (PDGFR)

Platelet-derived growth factor (PDGF) is a potent stimulator of cell growth and motility. Four PDGF polypeptide chains, A, B, C, and D, have been identified. These polypeptide chains are linked by disulfide bonds to form five known dimeric isoforms: PDGF-AA, -AB, -BB, -CC, and -DD (Heldin et al., 1998; Heldin et al., 2002; Pietras et al., 2003).

The PDGF isoforms exert their effects on target cells through PDGF receptors (PDGFRs). PDGFRs are receptor tyrosine kinases consisting of an extracellular ligand-binding domain, a single transmembrane region, and an intracellular intrinsic kinase domain. There are two structurally related PDGF receptors, PDGF α - and β -receptor. Because PDGF is a dimeric molecule, it can bind two receptors simultaneously, and thus induce receptor dimerization on the cell surface. The general model holds that inactive receptor monomers are in equilibrium with active receptor dimers, and that ligand binding stabilizes the active, dimeric form. Dimerization can take place between two identical receptors (homodimerization), or between different members of the same receptor family (heterodimerization) (Lemmon and Schlessinger, 1994). PDGF-AA, -AB, -BB, and -CC can induce $\alpha\alpha$ receptor homodimers, PDGF-AB, -BB, -CC, and -DD can induce $\alpha\beta$ receptor heterodimers, and PDGF-BB and -DD can induce $\beta\beta$ receptor homodimers (Heldin and Westermark, 1999; Li et al., 2000; Bergsten et al., 2001; Gilbertson et al., 2001; LaRochelle et al., 2001).

Ligand-induced receptor dimerization juxtaposes the intracellular portions of the PDGF receptors, causing phosphorylation of specific tyrosine residues (Tyr-849 in the α -receptor and Tyr-857 in the β -receptor) in trans between the two receptors. Receptor transphosphorylation leads to an increase in the catalytic efficiencies of the intrinsic kinases

(Kaziauskas and Cooper, 1989) and creates docking sites for signal transduction molecules containing Src homology 2 (SH2) domains (Pawson and Scott, 2005).

SH2 domains are conserved motifs of approximately 100 amino acid residues that are not required for kinase activity *per se* but that regulate the function of the kinase domain and its interactions with targets (Sadowski et al., 1986). A large number of SH2 domain-containing signal transduction proteins have been reported to bind to different autophosphorylation sites in PDGF receptors (Fig. 1.1). Some of these are enzymes, such as phosphoinositide 3-kinases (PI 3-kinase), phospholipase C (PLC)- γ , Src family tyrosine kinases, and Ras GTPase activating protein (RasGAP). Others, such as Grb2 (growth factor receptor-bound 2) and Shc (Src-homology-2-containing), have adaptor functions, linking the receptor with downstream catalytic molecules. For example, Grb2 forms a complex with Sos, a nucleotide exchange factor for Ras (Heldin and Westermark, 1999; Pawson et al., 2001).

The different SH2 domain-containing signaling proteins bind to individual phosphorylated tyrosine residues in the intracellular tail of the PDGF receptor and initiate different signal pathways leading to cell growth, migration, and differentiation. Two pathways stimulated by PDGF thought to play a central role in the regulation of cell survival and proliferation are phosphoinositide 3-kinase (PI 3-kinase)/Akt and Ras/extracellular signal-regulated kinase (Erk) pathways.

The signaling pathway comprising Raf, MEK (mitogen-activated protein kinase (MAPK)/Erk kinase), and Erk lies downstream of Ras and mediates several apparently conflicting cellular responses, such as proliferation, apoptosis, growth arrest, differentiation, depending on the duration and strength of the external stimulus and on cell type. Another downstream effector of Ras is PI 3-kinase, which can either synergize with

(Rodriguez-Viciana et al., 1997) or oppose to (Hu et al., 1996; Kohn et al., 1996) the Erk pathway. To evaluate the contribution of the Erk and PI 3-kinase pathways to the phenotypic modulation of NIH 3T3 cells, we have studied the coordination of PDGF receptor signaling to Ras/Erk and PI 3-kinase/Akt cascades and their cross-regulation.

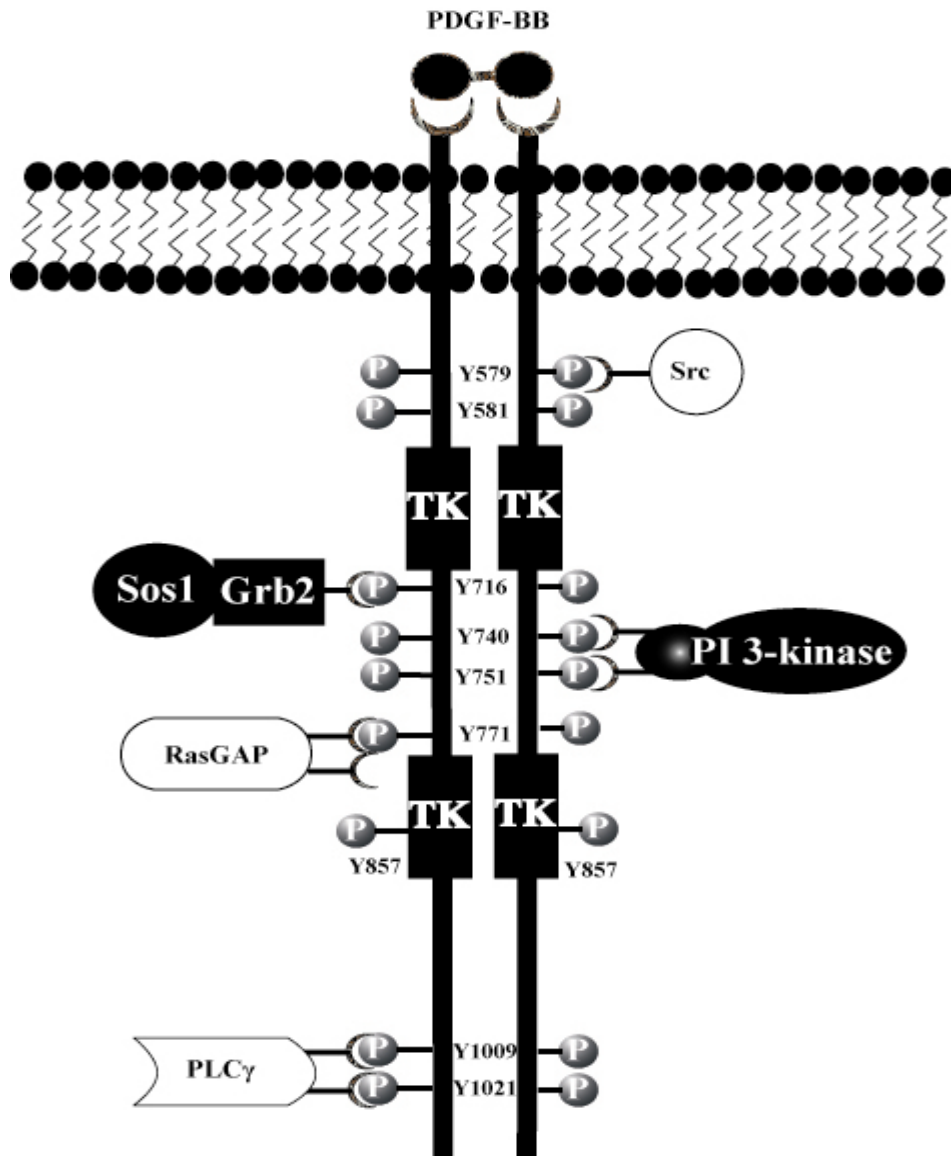


Figure 1.1. Interaction between PDGF β -receptor and SH2 domain-containing signal transduction molecules. The intracellular proteins of homodimeric PDGF β -receptor complexes are depicted, each containing dual tyrosine kinase (TK) domains and several autophosphorylation sites. Signaling proteins interact with these phosphotyrosine motifs, most often through modular SH2 (Src homology 2) domains (Pawson and Scott, 2005). Among the proteins that associate with PDGF receptors directly are: Src, a non-receptor tyrosine kinase; Grb2, an adaptor that recruits Sos for elevation of Ras-GTP; RasGAP, which accelerates the hydrolysis of Ras-GTP back to Ras-GDP; the regulator subunit of phosphoinositide 3-kinase (PI 3-kinase); and phospholipase C- γ (PLC- γ), which catalyzes lipid hydrolysis (leading to a rise in cytosolic Ca^{2+}). Many of these proteins are themselves tyrosine phosphorylated, often impacting protein function.

1.3. PI 3-kinase/Akt Pathway and Cell Survival

Phosphatidylinositol (PI) is a major phospholipid component of the plasma membrane. The acute phosphorylation of PI at the 3' position of the inositol head group puts in motion a coordinated set of events leading to cell growth, cell migration, and cell survival. PI 3-kinase is primarily responsible to phosphorylate PI at the 3' position in response to growth factor (Fruman et al., 1998). Nearly 10 years ago it was recognized that PDGF receptor-mediated activation of PI 3-kinase provides cells with a survival signal that prevented cell apoptosis (Fig. 1.2) (Yao and Cooper, 1995; Cantley, 2002). Indeed, more recent evidence has shown that the contributions of other PDGF β -receptor-associated signaling molecules, such as Src, PLC- γ , and RasGAP, are far less important for PDGF-stimulated survival (Vantler et al., 2005).

1.3.1. PI 3-kinase

Type I PI 3-kinases are heterodimers consisting of catalytic and regulatory subunit. Class I PI 3-kinases are subdivided into type I_A and I_B. In the case of type I_A PI 3-kinases, three mammalian p110s (p110 α , β , and δ) associate with a regulatory subunit (p85 α or β) that has two SH2 domains and one Src homology 3 (SH3) domain (Escobedo et al., 1991; Otsu et al., 1991; Skolnik et al., 1999). The only class I_B PI 3-kinase is the p110 γ catalytic subunit, which complexes with a 101 kDa regulatory protein (p101) (Fruman et al., 1998; Vanhaesebroeck and Waterfield, 1999).

The SH2 domains of p85 bind preferentially to the motif -pY-X-X-M-, where pY represents a phosphorylated tyrosine residue (Songyang et al., 1993). Two tyrosines, Tyr-740 and Tyr-751, in the PDGF β -receptor have been shown to be autophosphorylation sites that

bind p85 (Fig. 1.1) (Fantl et al., 1992; Kazlauskas et al., 1992; Kashishian et al., 1992), and both are conserved in the α -receptor, at Tyr-731 and Tyr-142 (Yu et al., 1991). The binding of the p85 SH2 domains to PDGF receptor induces a conformational change, which is transmitted to the p110 and regulates enzymatic activity by an allosteric mechanism (Shoelson et al., 1993).

In quiescent cells, p85 maintains the p110 in a low activity state. Upon growth factor stimulation, p85 directly interacts with RTKs or adaptor proteins leading to activation of p110. Activated type I PI 3-kinase converts phosphatidylinositol-4,5-bisphosphate (PI(4,5)P₂) to phosphatidylinositol-3,4,5-trisphosphate (PI(3,4,5)P₃), the second messenger. Kinetic studies in the ³²P-labeled cells suggest that the typically delayed generation of phosphatidylinositol-3,4-bisphosphate (PI(3,4)P₂) may be produced in part by the action of inositol 5-phosphatases, like SHIP1 and SHIP2 (SH2-containing inositol phosphatase-1 and -2), on PI(3,4,5)P₃ (Stephens et al., 1991); PI(3,4,5)P₃ and PI(3,4)P₂ largely overlap with respect to their intracellular functions.

1.3.2. PI 3-kinase inhibitors: wortmannin and LY294002

Wortmannin and LY294002 [2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one] are two cell-permeable, low molecular weight compounds commonly used to elucidate cellular functions of PI 3-kinases (Djordjevic and Driscoll, 2002).

Wortmannin, a fungal metabolite originally isolated from *Penicillium wortmannii*, was shown to be a potent inhibitor of PI 3-kinase (Arcaro and Wymann, 1993; Yano et al., 1993; Ui et al., 1995). The crystal structure of wortmannin complexed to p110 γ indicates a close complementarity between the active site of p110 γ and wortmannin. Wortmannin binds

irreversibly to the active sites by forming a covalent interaction with Lys833 residue and induces a conformational change in p110 γ (Walker et al., 2000). Wortmannin can also inhibit other, PI 3-kinase-related enzymes, but with 20- to 50-fold higher 50% inhibitory concentration (IC₅₀) than for PI 3-kinase inhibition (around 5nM) (Vanhaesebroeck et al., 2001).

LY294002 is a synthetic compound that was designed as a PI 3-kinase inhibitor (Vlahos et al., 1994). LY294002 is a competitive inhibitor of ATP binding and effects no significant change on the conformation of the binding-site residues (Djordjevic et al., 2002). Although the reported IC₅₀ of LY294002 (around 1 μ M) is much higher than that of wortmannin, LY294002 is widely used as a specific PI 3-kinase inhibitor because it is much more stable in solution than wortmannin and thus better suited for long-term studies (Jones et al., 1999).

1.3.3. Akt

PI(3,4,5)P₃, the product of PI 3-kinase, exhibits a high affinity for proteins containing pleckstrin-homology (PH) and certain other modular domains (Pawson and Scott, 1997; Klarlund et al., 2000; Pawson et al., 2002; Pawson and Scott, 2005). Signaling proteins with PH domains accumulate at sites of PI 3-kinase activation by directly binding to PI(3,4,5)P₃. Of particular interest in this study are the protein serine/threonine kinases Akt (also known as protein kinase B (PKB)) and phosphoinositide-dependent kinase 1 (PDK1). PI(3,4,5)P₃ mediated membrane translocation brings these kinases into close proximity, facilitating phosphorylation of Akt by PDK1 (Lawlor and Alessi, 2001).

Akt belongs to the AGC superfamily (protein kinase A (PKA)/protein kinase G/protein kinase C-like). As with other AGC members, Akt is regulated by upstream second

messengers and secondary, activating enzymes. In mammals, there are three isoforms of Akt (Akt1, 2, and 3). All three isoforms are composed of three functional distinct regions: a N-terminal PH domain, a central catalytic domain, and a C-terminal hydrophobic motif. PH domain binding to PI(3,4,5)P₃ or PI(3,4)P₂ results in Akt recruitment from the cytosol to the plasma membrane, where the enzyme is thought to undergo a conformational change and become activated through phosphorylation of two residues (Schael and Woodgett, 2003).

Akt, like other AGC members, contain a flexible loop (T-loop). In unstimulated cells, a threonine residue at position 308 (Thr308) in Akt1 (209 in Akt2 and 305 in Akt3) in the T-loop is largely unphosphorylated, acting to inhibit kinase function. When phosphorylated, the T-loop is modified, allowing access to ATP and the kinase substrate (Schael and Woodgett, 2001). Active Akt phosphorylates proteins containing the consensus motif -R-x-R-x-x-S/T-F/L-. It is now clear that Thr308 in Akt1 is phosphorylated by PDK1 (Stokoe et al., 1997; Stephens et al., 1997; Alessi et al., 1997a,b), and this appears to be the major input required for the activation of Akt. PDK1 also contains a PH domain at its C-terminus; PI(3,4,5)P₃ and PI(3,4)P₂ also interact with the PH domain of PDK1. The rate of Akt phosphorylation by PDK1 is greatly enhanced by the inclusion of PI(3,4,5)P₃ and PI(3,4)P₂, which concentrate both Akt and PDK1 (Currie et al., 1999).

The other important phosphorylation event associated with Akt activation is at Ser473 (in Akt1), situated in the hydrophobic motif. Phosphorylation of Ser473 is required for Akt activity (Schubert et al., 2000), but the mechanism is not completely understood. There is evidence implicating both Akt autophosphorylation (Toker and Newton, 2000) and phosphorylation by other serine kinases, like the integrin-linked kinase (ILK) (Persad et al., 2001), although contrary results suggest that pSer473 kinase activity is distinct from ILK and

PDK1 (Hill et al., 2002). A recent study has provided persuasive evidence that a rapamycin-insensitive form of the mammalian target of rapamycin (mTOR) protein kinase, in complex with Rictor:GβL mediates the phosphorylation of Akt at Ser473 (Sarbasov et al., 2005).

1.3.4. Akt and cell survival

Recent evidence indicates that Akt is the principal mediator of cell survival (Doenward, 2004). One of the major functions of Akt is to promote growth factor-mediated cell survival and to block apoptosis directly. Akt phosphorylates the Bcl-2 family pro-apoptotic BH3 only protein (BAD) on Serine 136 (Zha et al., 1996; Datta et al., 1997). As a result of phosphorylation, BAD interacts with 14-3-3 proteins and is prevented from blocking Bcl-2, an anti-apoptotic protein.

Akt also regulates cell survival through transcriptional factors that are responsible for modulating pro- as well as anti-apoptosis genes. Akt phosphorylates the three members of the FoxO subfamily of Forkhead transcription factors, FoxO1, 2, and 3 (previously known as FKHR, FKHL1 and AFX) (Biggs III et al., 1999; Brunet et al., 1999a; Kops et al., 1999). Upon phosphorylation by Akt at up to three regulatory sites, FoxO binds to 14-3-3 proteins. This results in translocation of FoxO to the cytosol from the nucleus and consequently inactivation of its function as a transcription factor. Akt binds and phosphorylates E3 ubiquitin ligase Mdm2 (murine double minute 2), which inhibits activation of p53, a major regulator of cell death (Gottlieb et al., 2002). Thus, Akt could result in promotion of the inactivation and degradation of p53. Additionally, in most cases, the activation of the transcription factor nuclear factor-κB (NF-κB) is dependent on the phosphorylation of IκB

kinase (IKK) and degradation of I κ B, an inhibitor of NF- κ B. Akt has been shown to regulate IKK activity by both direct and indirect mechanisms, leading to the nuclear translocation and activation of NF- κ B and transcription of NF- κ B-dependent, pro-survival genes (Song et al., 2005).

Another major physiological function of Akt is the regulation of cell metabolism. An interesting hypothesis has been proposed that the effects of Akt on cell survival are connected with its effects on cell metabolism. In insulin treated cells, glycogen synthase kinase 3 (GSK3) is inhibited upon phosphorylation by Akt, with the result that the storage of glucose as glycogen is promoted. GSK3 is also involved in other signaling pathways, and the inhibition of GSK3 is protective against apoptosis in many circumstances (Song et al., 2005).

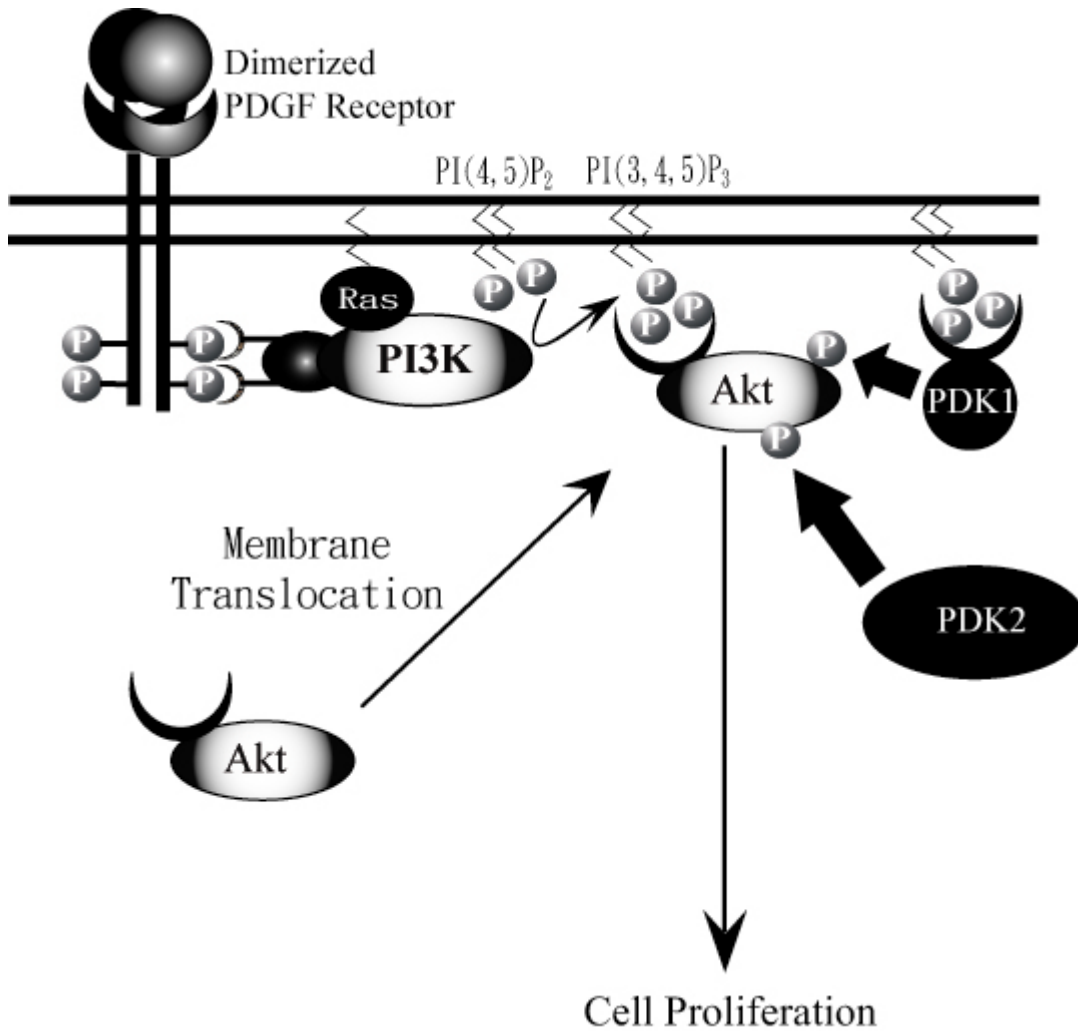


Figure 1.2. A model for PI 3-kinase/Akt pathway. PI 3-kinase binds to phosphorylated receptors through its p85 regulatory subunit, activating the p110 catalytic subunit. The active PI 3-kinase generates PI(3,4,5)P₃ that recruit cytosolic Akt and PDK1 through their PH domains. Akt is phosphorylated and activated at the plasma membranes by PDK 1/2.

1.4. Ras/Erk Pathway and Biological Functions

The mitogen-activated protein kinase (MAPK) pathway is another primordial pathway controlling fundamental cellular processes. In mammalian cells, some distinguishable MAPK modules have been identified. These include the c-Jun N-terminal kinase (JNK) and p38 cascades, which function in stress response, and the Erk-1/2 cascade, which regulates cell growth and differentiation. The arrangement of MAPK cascades includes a G-protein working upstream of three kinases: a MAPK kinase kinase (MAPKKK) that phosphorylates and activates a MAPK kinase (MAPKK), which in turn activates MAPK. Here we focus on the regulation of Erk pathway, which features Ras as G-protein, Raf as MAPKKK, MEK (MAPK/Erk kinase) as MAPKK, and Erk as MAPK.

The small GTP-binding protein Ras is a crucial downstream effector of the PDGF-stimulated signal transduction pathways. RTK stimulation by growth factors induces Ras to convert from an inactive GDP-bound to an active GTP-bound state (Schlessinger, 1993; Gale et al., 1993). Activation of Ras is a necessary step for PDGF receptor to activate the Erks. The Ras/Raf/MEK/Erk pathway is at the heart of signaling networks that govern proliferation, differentiation and cell survival. Curiously, Erk activation has been associated with both stimulation and inhibition of cell proliferation (Kolch, 2000). Further study is required to fully elucidate the complexity of these pathway and its connections to cell behaviors.

1.4.1. Ras and its regulatory proteins

Grb2 (growth factor receptor-bound 2) is an adaptor molecule consisting of two SH3 domain and one SH2 domains. The SH2 domain of Grb2 can bind directly to two sites in

autophosphorylated PDGF β -receptors (Tyr716 and Tyr775) (Fig. 1.1) (Arvidsson et al., 1994; Ruusala et al., 1998). Grb2 can also bind indirectly to PDGF β -receptors, i.e. to the adaptor Shc (Src-homology-2-containing) (Yokote et al., 1994) or to the tyrosine phosphatase SHP-2 (Li et al., 1994) after these molecules have bound to the PDGF β -receptors and become phosphorylated. The SH3 domains of Grb2, which recognize proline-rich sequences, mediate binding of Sos1 (son of sevenless 1), a guanine nucleotide exchange factor (GEF) for Ras, which converts inactive Ras-GDP to active Ras-GTP (Schlessinger, 1993; Chardin et al., 1993). This binding promotes the translocation of Sos1 to the plasma membrane and positions it in direct proximity to membrane-bound Ras. RasGAP is a protein with two SH2 domains that binds to Tyr771 of the activated PDGF β -receptor (Fig. 1.1.) (Fantl et al., 1992; Kazlauskas et al., 1992). RasGAP is important for negative control of Ras activation because it converts Ras-GTP to Ras-GDP, by accelerating the intrinsic GTP hydrolysis activity of Ras.

Three isoforms of Ras (as known as p21^{ras}) protein, H-Ras, N-Ras, and K-Ras, are expressed in mammalian cells. The G-domain of Ras (amino acids 1-166) is >95% conserved between isoforms and comprises the regions that bind GTP or GDP, the switch 1 and 2 regions that undergo the major conformational changes upon GDP-GTP exchange and offer binding surfaces for effectors, GEFs, and GAPs. In contrast, the Ras c-terminal HVR (hyper-variable region) is poorly conserved between isoforms. The HVR comprises the sequences that direct post-translational processing, plasma membrane anchoring and trafficking of newly synthesized and processed Ras from ER (endoplasmic reticulum) to the plasma membrane (Prior and Hancock, 2001; Hancock, 2003; Hancock and Parton, 2005).

Ras proteins function as regulated GDP/GTP molecular switches that control diverse signaling networks (Sato et al., 1992). The GTP-bound conformation of Ras is active since

it can interact with effector proteins, whereas the GDP-bound conformation can not. Ras becomes activated by the nucleotide exchange reaction. The dissociation of GDP from Ras is rate-limiting, with nucleotide-free Ras immediately binding to GTP. GEFs, such as Sos1, is important in Ras functions because GEFs increase the dissociation rate of GDP bound to Ras (Mitin et al., 2005). Ras-bound GTP is hydrolyzed to GDP by the intrinsic GTPase of Ras, which is very slow and must be catalyzed by GAPs (Bourne et al., 1991).

The use of Ras molecules with point mutations has been exploited to identify the specific residues that mediate Ras-effector interactions. More recently, Ras mutants have been used to correlate effector interaction with biological function, providing further evidence of branchpoints in Ras signaling (Rodriguez-Viciano et al., 1994; Rodriguez-Viciano et al., 1996; Klinghoffer et al., 1996). In this study, we use the constitutively activated mutant Ras G12V and dominant negative mutant Ras S17N.

1.4.2. Raf/MEK/Erk cascade

The best characterized Ras-dependent signaling pathway is the Raf/MEK/Erk cascade. Mammals possess three Raf serine/threonine kinases: Raf-1, A-Raf, and B-Raf. The ubiquitously expressed Raf-1 is certainly the best studied (Qi and Elion, 2005).

In quiescent cells, Raf-1 exists in an inactive state in the cytosol. Typically, the activation of Raf-1 is initiated by its interaction with Ras, which leads to the relocalization of Raf-1 to the plasma membrane. Critical evidence connecting this kinase cascade to Ras was the observation that active Ras-GTP, but not inactive Ras-GDP, forms a high affinity complex with Raf-1 (Van Aelst et al., 1993; Moodie et al., 1993; Zhang et al., 1993; Vojtek et al., 1993). Ras actually binds to two sites on Raf-1. Ras interaction with Ras-binding domain

(RBD; amino acids 55-131) of Raf-1 alone is sufficient for the recruitment of Raf-1 from the cytosol to the plasma membrane, but Ras interaction with the cysteine-rich domain (CRD; amino acids 139-184) of Raf-1 is essential for efficient activation of Raf-1 (Brtva et al., 1995; Roy et al., 1997). Ras binding promotes conformational changes that relieve Raf-1 autoinhibition and facilitate the phosphorylation of activating sites (Terai and Matsuda, 2005). These activating sites are found in the Raf-1 catalytic domain and include Ser338, Tyr341, Thr491 and Ser494 (Dougherty et al., 2005).

All three Raf isoforms share MEKs as the only commonly accepted downstream substrates. The interaction of MEKs with Raf is dependent on a proline-rich sequence unique to MEKs and not found in other MAPKKs. Raf kinases have shown to selectively phosphorylate and activate MEK-1 and MEK-2. A-Raf preferential activates MEK-1, whereas Raf-1 activate both MEK-1 and MEK-2 equally well; B-Raf activates MEK-1 better than MEK2 (Schaeffer and Weber, 1999).

PD098059 [2-(2'-amino-3'-methoxyphenyl)-oxanaphthalen-4-one] is a synthetic inhibitor that selectively blocks the activation of MEK-1 and, to a lesser extent, the activation of MEK-2. PD098059 does not inhibit the stress-and-IL-1 stimulated JNK/SAPK and the p38 pathways, demonstrating its specificity for the Erk pathway (Alessi et al., 1995; Dudley et al., 1995).

MEKs belong to the rare breed of dual-specificity kinases that can phosphorylate both threonine and tyrosine residues. Both MEK isoforms can activate the downstream Erk-1 and Erk-2 (also called p44 and p42 MAPK respectively based on the molecular weight: 43/44 and 41/42 kDa) via phosphorylation of a -Thr-Glu-Tyr- motif in the activation loop (Thr202 and Tyr204 in Erk-1 and Thr183 and Tyr185 in Erk-2) (Kolch, 2000). Erk-1 and Erk-2 are

proline-directed protein kinases that phosphorylate -Ser/Thr-Pro- motifs in the consensus sequence -Pro-Xaa_n-Ser/Thr-Pro-, where Xaa is any amino acid and n = 1 or 2 (Reuter et al., 2000).

1.4.3. ERK and biological functions

Erk is a highly conserved serine/threonine kinase that phosphorylates various substrates, including many enzymes, transcription factors and cytoskeletal proteins. Erk regulates growth factor-stimulated cell cycle progression, cell differentiation, migration, and survival. It remains uncertain how the activation of this one protein transduces multiple signals from extracellular stimuli to specific cell responses; however accumulating evidences have demonstrated that differences in duration, magnitude and subcellular compartmentalization of Erk activity generate variations in signaling output (Ebisuya et al., 2005).

In PC12 cells, NGF (nerve growth factor)-mediated sustained activation of Erks can lead to a differential cellular response and transient activation lead to proliferation; however, PDGF-induced proliferation requires sustained Erk activity in fibroblasts (Weber et al., 1997). Sustained Erk activation is associated with translocation of Erks to the nucleus, whereas transient activation does not lead to nuclear translocation. Therefore, transient activation will have different consequences for gene expression compared with sustained activation, because nuclear accumulation of active Erk will result in phosphorylation of transcription factors (Marshall, 1995; Wellbrock et al., 2004).

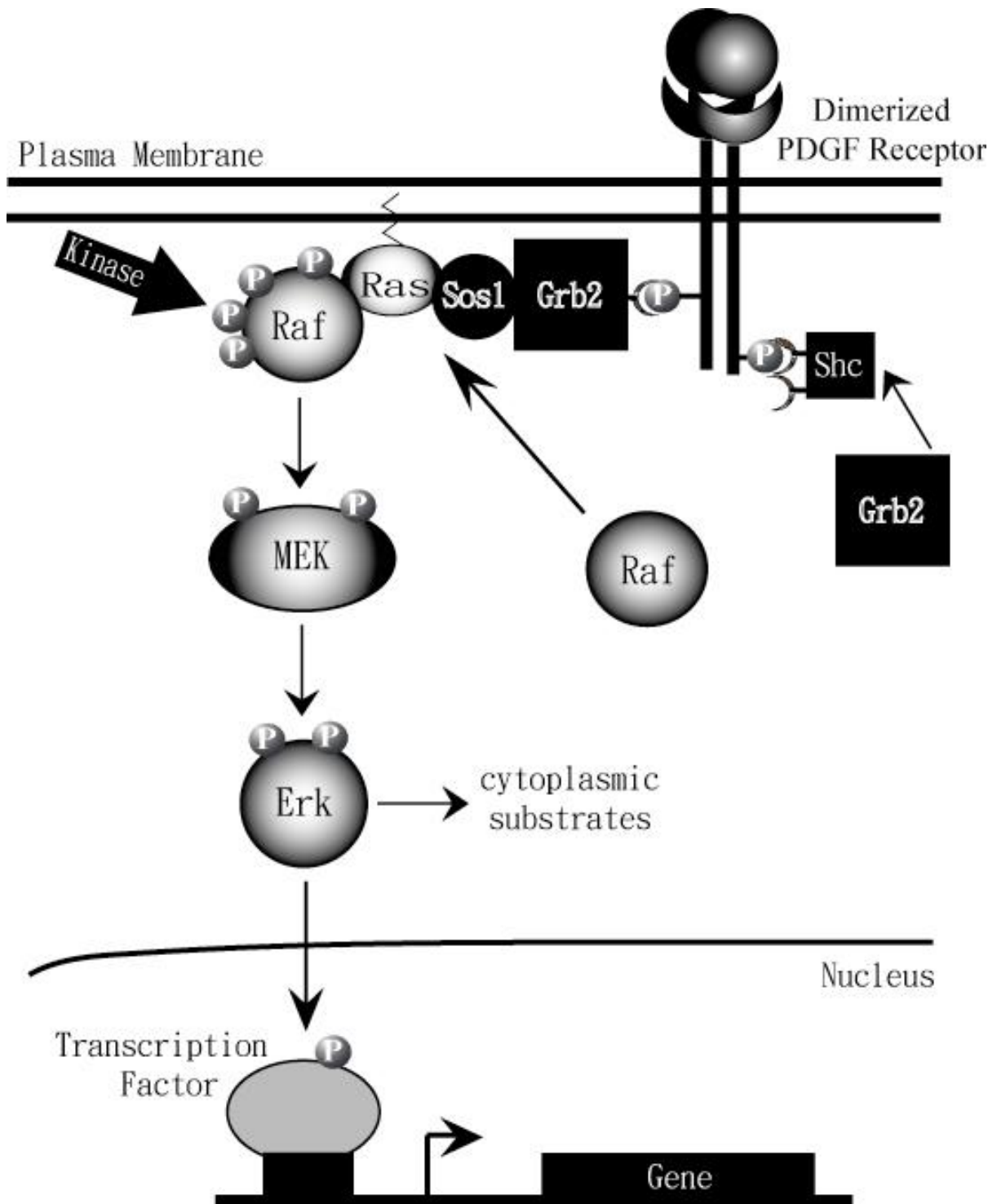


Figure 1.3. A model for Ras/Erk pathway. PDGF dimer binds to PDGF receptor that initiates this signaling pathway. The Grb2-Sos1 complex, which contains guanine nucleotide exchange factor activity, binds to the receptor and elevates Ras-GTP level. Ras-GTP recruits Raf, that phosphorylates and activates MEK, which in turn activates Erk and cell proliferation.

1.5. Cross-talk between PI 3-kinase/Akt and Ras/Erk Pathways

A complex network of signaling pathways is activated in cells in response to various cytokines and growth factors. We are interested in the relationship between the PDGF-induced activation of PI 3-kinase/Akt and Ras/Erk pathways (Fig. 1.4).

1.5.1. Evidence that PI 3-kinases are also Ras effectors.

There is no good evidence that components of the Ras/Erk pathway are involved in activation of Akt, except for the demonstration that Ras-GTP can bind to and activate the p110 α catalytic subunit of PI 3-kinase *in vitro*. The Ras-binding site on the p110 α of PI 3-kinase was shown to require amino acid residues 133 and 134 (Rodriguez-Viciana et al., 1994). Thus, Ras acts as an upstream positive effector of both the Raf/MEK/Erk pathway and the PI 3-kinase/Akt pathway (Katz and McCormick 1997). However, The weakness of this hypothesis is the attempt to detect a stable Ras G12V/p110 α (and P110 γ) complex *in vivo* was still a failure, even when both are transiently overexpressed (Rubio et al., 1997).

Since PI(3,4,5)P₃ is a product of PI 3-kinase, many researchers measure PI(3,4,5)P₃ levels to assess PI 3-kinase activity. Transfection of both Ras G12V and p85/p110 together in to COS cells gives a greater than additive elevation of PI(3,4,5)P₃ levels, while dominant-negative Ras S17N inhibits the receptor-mediated activation of PI 3-kinase (Rodriguez-Viciana et al., 1994; Rodriguez-Viciana et al., 1996). Klinghoffer et al. also showed that PDGF-stimulated PI(3,4)P₂ accumulation was consistently decreased by 50 to 60% in NIH 3T3 cells expressing Ras S17N (Klinghoffer et al., 1996). Therefore, the interaction between Ras and PI 3-kinase traces a putative pathway through Ras and PI 3-kinase to Akt (Kauffmann-Zeh et al., 1997).

Akt phosphorylation is a sensitive output of PI 3-kinase/Akt pathway and many researchers detect levels of Akt phosphorylation to assess PI 3-kinase activity. Franke et al. reported that in NIH 3T3 cells, transfection of both Ras S17N and HA-Akt (hemagglutinin (HA) epitope-tagged Akt) together partially blocks the PDGF-induced activation of Akt, but Ras G12V was not sufficient to activate Akt at all in the absence of PDGF in NIH 3T3 cells (Franke et al., 1995). In contrast, Klippel et al. shown that, in COS-7 cells, Ras S17N did not interfere with PDGF-mediated Akt activation. However, Ras G12V caused a 5-fold increase in Akt activation in the same cell line and the activation of Akt by Ras G12V is wortmannin sensitive, implying that Ras-induced Akt activation required PI 3-kinase activity. (Klippel et al., 1996). These studies also implied that Ras G12V and Ras S17N might mediate Akt activation in distinct manners.

Ras G12V alone in the absence of EGF (epidermal growth factor) and EGF alone in the absence of Ras G12V were inefficient in PI 3-kinase/Akt activation (Chan et al., 2002), suggesting that signals transduced via small GTPases and RTKs regulate PI 3-kinase activity cooperatively. Indeed, more recent evidence has shown that the PDGF receptors and Ras-GTP recruit PI 3-kinase to the plasma membrane in a cooperative fashion, through the formation of a receptor/PI 3-kinase/Ras complex. The magnitude of the cooperativity is limited by the availability of activated PDGF receptors and PI 3-kinase at low and high PDGF concentrations, respectively (Kaur et al., 2005).

However, other study showed that they did not detect significant changes in cellular (or plasma membrane associated) PI 3-kinase activity in Ras G12V transformed IEC-6 cells (Karasarides et al., 2001), implying the Ras-PI 3-kinase cross-talk appears to be dependent on the system of study. Moreover, the extent to which Ras influences PI 3-kinase/Akt

signaling is still uncertain. The implication of PI 3-kinase for Erk activation is another area of conflicting reports and required further study.

1.5.2. Evidence that PI 3-kinase contributes to Erk activation.

The relevance of PI 3-kinase for activation of Erk has been controversial.

It has been suggested that the PI 3-kinase pathway can lead to Erk activation. The PI 3-kinase inhibitor wortmannin completely blocked the effects of insulin on Erk in rat adipocytes and inhibition of PI 3-kinase function interferes with IL-2-dependent Erk activation at the level of MEK (Standert et al., 1995; Karnitz et al., 1995; Hu et al., 1996).

Expression of activated mutant p110* of PI 3-kinase has been reported to stimulate Erk activation in NIH 3T3 cells (Hu et al., 1995), whereas other studies found that p110* was not able to activate Erk in COS-7 cells (Klippel et al., 1996) and Rat-1 fibroblasts (Kauffmann-Zeh et al., 1997). Similarly, Frevert et al. showed that the elevation of PI 3-kinase activity by coexpression of iSH2 (a 102-amino acid fragment of the inter-*Src* homology region 2 of the p85 regulatory subunit of PI 3-kinase) and p110 elicits no effect on the activation of Erk in 3T3-L1 cells (Frevert and Kahn, 1997). In addition, expression of dominant negative p85 (Δ p85) led to consistent decrease in the ability of IL-3 (Interleukin 3) to induce MEK and Erk-1/2 activation in BaF/3 cells but not in FDC-P1 cells (Sutor et al., 1999; Craddock et al., 2001).

In trying to reconcile these apparently contradictory findings, it is important to better understand the role that PI 3-kinase plays in the activation of Erk in response to different types and magnitudes of stimulus. Duckworth and Cantley found that wortmannin blocks PDGF-dependent activation of Raf-1 and MAP kinase cascade in Chinese hamster ovary

(CHO) cells, which express PDGF receptors at low levels, but has no effect on Erk activation in Swiss 3T3 cells, which have high levels of PDGF receptor, however, wortmannin blocks Erk activation in Swiss 3T3 cells at lower, physiological concentration of PDGF (Duckworth and Cantley, 1997). Consistent with this, Wennstrom and Downward reported that activation of both Erk2 and Ras in COS cells by low but not high, concentration of EGF is suppressed by PI 3-kinase inhibitors (Wennstrom and Downward, 1999). Studies suggest but do not definitively prove the existence of the PI 3-kinase level cross-talk, leading the question open for further investigation.

1.5.3. Evidence that PI 3-kinase negatively regulates Erk

Another potential mode of cross-talk between the PI 3-kinase and Erk pathways is on the interaction of Akt with Raf. Exposure of MCF-7 cell to insulin-like growth factor induces cell proliferation as a result of activation of the PI 3-kinase/Akt pathway and transient activation of the Erk pathway. However, prolonged activation of the Raf cascade inhibits growth in the same cells (Dufourny et al., 1997; Alblas et al., 1998).

The kinase activity of Raf is regulated by phosphorylation of a highly conserved serine residue (Ser259) in the amino-terminal regulatory domain. Phosphorylation of Ser259 mediates binding of 14-3-3 protein, resulting in Raf sequestration (Morrison and Cutler Jr, 1997). Zimmermann and Moelling reported that Akt antagonizes Raf activity by direct phosphorylation Raf on Ser259, inhibiting activation of the Raf/MEK/Erk pathway and shifting the cellular response from cell cycle arrest to proliferation (Rommel et al., 1999; Zimmermann and Moelling, 1999). Interestingly, in the same studies, the interaction between Akt and Raf was observed in differentiated myotubes but not in the undifferentiated myoblast

cells, implicating other regulatory events controlling the phosphorylation; Akt-mediated inhibition of Raf may only occur in specific cell types and may also be dependent on the stage of cell differentiation (Brazil et al., 2002).

The cellular response of RTK signaling is influenced by the strength and the duration of Erk-1/2 phosphorylation (Marshall, 1995). Reusch et al. reported that in vascular smooth muscle (VSM) cells, PDGF induced a short-lived Erk-1/2 phosphorylation but a complete and sustained phosphorylation of Akt. Disruption of the PI 3-kinase signaling prevented the PDGF-induced Akt and Raf-Ser259 phosphorylation and elicited a more sustained MEK and Erk phosphorylation (Reusch et al., 2001). Sustained activation of Akt was able to phosphorylate Raf and terminate its kinase activity. Raf-Akt cross-talk appears to be regulated in a concentration- and ligand-dependent manner (Moelling et al., 2002).

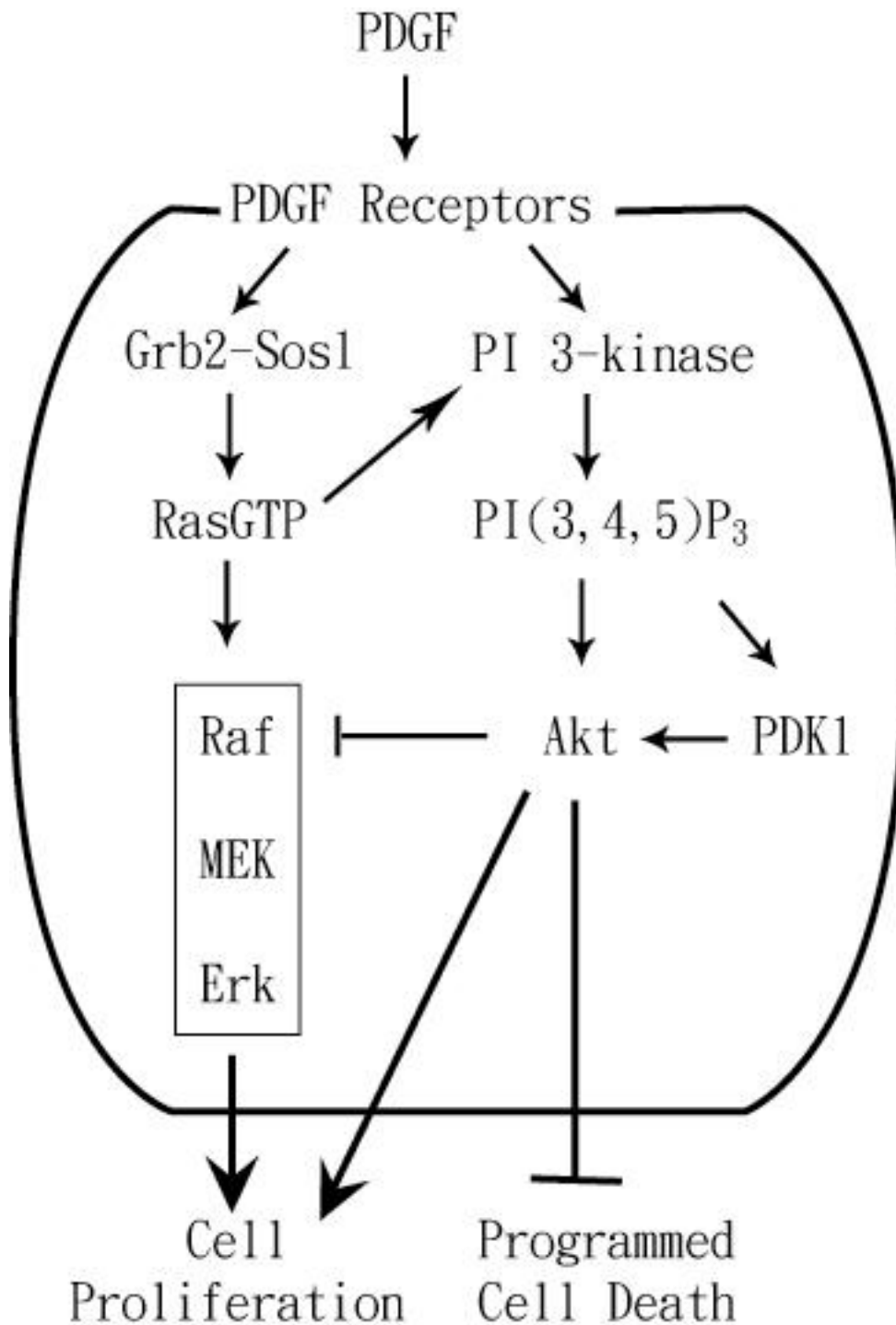


Figure 1.4. Cross-talk between Ras/Erk and PI 3-kinase/Akt pathways PI 3-kinase not only receives a signal from the receptor but also interacts with Ras-GTP. Akt, which is direct downstream of PI 3-kinase, phosphorylates and inhibits Raf. In contrast to Akt inhibition of Raf, there are still some uncertain pathways downstream of PI 3-kinase, contribute to the activation of Ras/Erk pathway.

1.6. Aims of This Study

In this study, we aim to systematically analyze a signaling network, comprised of Ras/Erk and PI 3-kinase/Akt pathways, stimulated by PDGF (Fig. 1.5). Molecular level control over the signaling network, through genetic and pharmacological intervention, will allow these complex regulatory interaction to be isolated. Constitutively active Ras G12V and dominant-negative Ras S17N was transfected into NIH 3T3 cells to modulate the levels of Ras-GTP. Over-expression of HA-Akt in cells dramatically increase total Akt amount. PI 3-kinase inhibitor, LY298004, and MEK inhibitor, PD098059, are commonly used to block cellulate functions of PI 3-kinase and MEK respectively. We assessed the phosphorylation of Akt and Erk as the outputs of PI 3-kinase/Akt and Ras/Erk pathways respectively, and detected DNA synthesis and cell numbers to analyze the effect of these two pathways on the resulting life and death decisions.

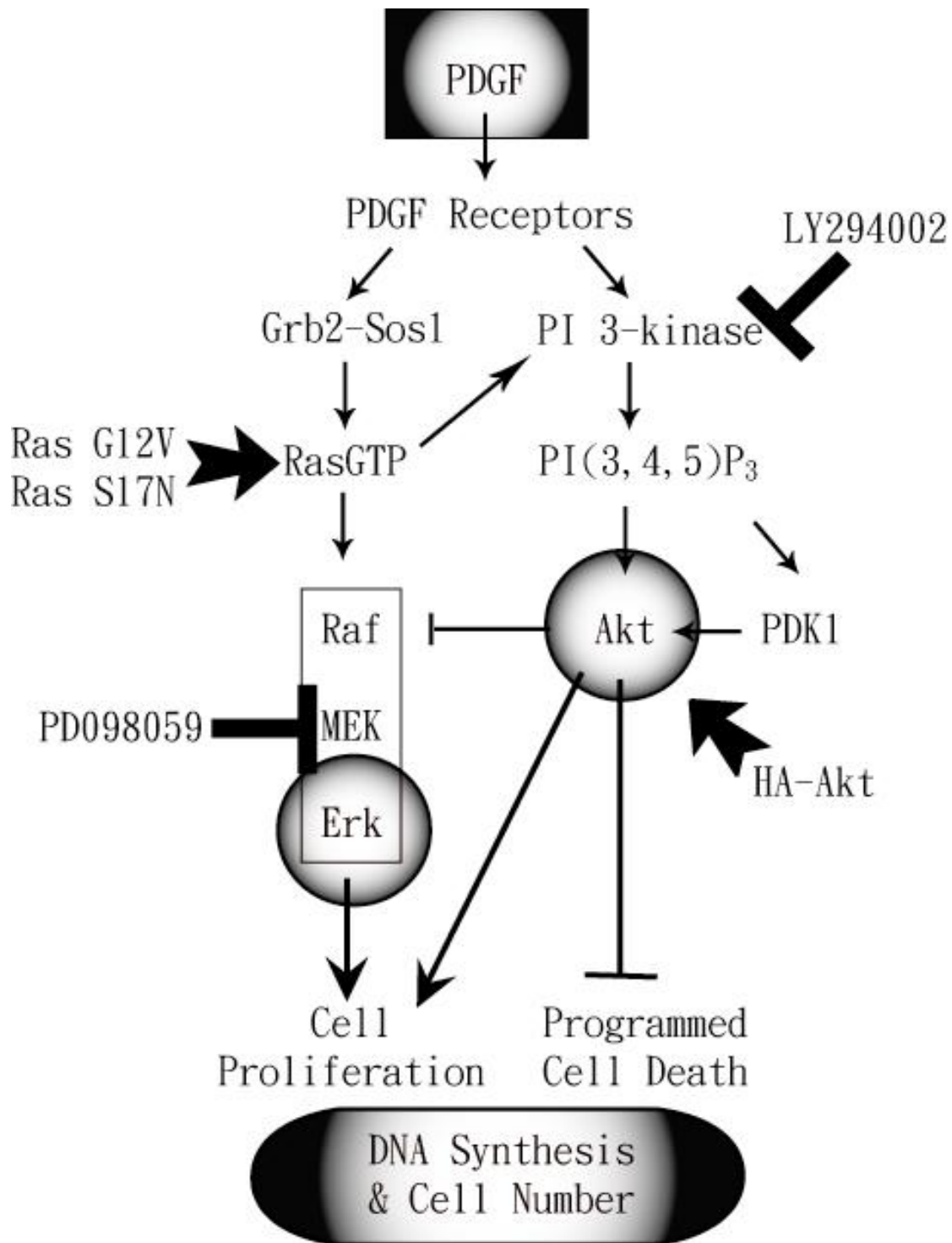


Figure 1.5. Molecular level control over the signaling network in this study to analyze the cross-talk between Ras/Erk and PI 3-kinase/Akt pathways. Through genetic (Ras G12V, Ras S17N, and HA-Akt) and pharmacological (LY294002 and PD098059) intervention, we assessed the dose response of Akt and Erk phosphorylation as the outputs of the PI 3-kinase/Akt and Ras/Erk pathways and detected the effect of these modulations on DNA synthesis and Cell number increase as the level of cell proliferation.

CHAPTER 2. MATERIALS AND METHODS

2.1. Reagents and antibodies

All tissue culture reagents were purchased from Invitrogen (Carlsbad, CA). Human recombinant PDGF-BB was from Peprotech (Rocky Hill, NJ). PD098059 and LY294002 were from Calbiochem (San Diego, CA). Antibodies against Erk-1 C terminus were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phospho-p44/p42 MAP Kinase (Thr202/Tyr204), anti-rabbit IgG HRP conjugated antibody, PathScan total Akt1 sandwich ELISA kit and PathScan phospho-Akt1(Ser473) sandwich ELISA kit were from Cell Signaling Technology (Beverly, MA). Anti-goat IgG HRP conjugated antibodies and puromycin dihydrochloride were from Sigma-Aldrich (St. Louis, MO). [methyl-³H]-thymidine was from PerkinElmer Life and Analytical Sciences (Boston, MA). Cell Counting Kit-8 was from Dojindo Laboratories (Kumamoto, Japan). Unless otherwise noted, all other reagents were from Sigma-Aldrich (St. Louis, MO).

2.2. Plasmid and retroviral infection

H-Ras plasmids and haemagglutinin epitope-tagged wild-type Akt (HA-Akt) were used as PCR templates for cloning into the Not I/BamHI sites of the retroviral vector pBM-IRES-Puro, a kind gift from Drs. Steven Wiley and Lee Opresko (Pacific Northwest National Labs). All constructs were confirmed by DNA sequencing. Packaging cells were transfected by calcium phosphate precipitation for 4 hours with 15 µg pBM-IRES-Puro construct per 100 mm plate. After replacing the medium, viral supernatants were collected at 24, 28 and 32 hours after transfection, filtered, supplemented with 5 µg/ml polybrene, and

used for serial infection of NIH 3T3 fibroblasts. These cells were plated at 2×10^5 per 60 mm dish 24 hours before infection. 24 hours after the last infection, viral supernatants were removed, and target cells were incubated with growth medium containing 2 $\mu\text{g}/\text{ml}$ puromycin and selected for two days.

2.3. Cell culture and lysate preparation

NIH 3T3 fibroblasts (American Type Culture Collection, Manassas, VA) and variant cell lines were cultured at 37°C , 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (BSA), 2mM L-glutamine, and the antibiotics penicillin and streptomycin. Where applicable, Ras or Akt expression was maintained under puromycin selection. Cells were serum-starved in DMEM containing 0.1% FBS, 2mM L-glutamine, and the antibiotics penicillin and streptomycin, and 1 mg/ml fatty acid-free BSA, for 24 hours prior to PDGF-BB stimulation. PDGF-BB was added to each plate at the final concentration indicated. The cells were washed once with ice-cold Dulbecco's phosphate-buffered saline (PBS) and then lysed in ice-cold buffer containing 50mM HEPES, pH 7.4, 100mM NaCl, 10% v/v Triton X-100, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 50mM β -glycerophosphate, pH 7.3, 5 mM sodium fluoride, 1mM EGTA, and 10 $\mu\text{g}/\text{ml}$ each aprotinin, leupeptin, pepstatin A, and chymostatin. After scraping insoluble debris and transferring to an Eppendorf tube, the lysates were vortexed briefly, incubated on ice for 20 minutes, and clarified by centrifugation. The supernatants were collected and stored frozen at -70°C .

2.4. Enzyme-linked immunosorbent assay (ELISA) of Akt phosphorylation

Phospho-Akt(Ser473) antibodies are pre-coated in the microwells of CST's PathScan phospho-Akt1 (Ser473) Sandwich ELISA Kit. After incubation with cell lysates overnight at 4°C, phosphorylated Akt proteins are captured by the coated antibodies. After extensive washing, Akt1 (2H10) monoclonal antibodies are added to detect the captured phospho-Akt1 proteins for 1 hour at 37°C. HRP-linked anti-mouse antibodies are then used to recognize the bound detection antibody for 30 minutes at 37°C. HRP substrate, TMB, is added to develop color for 30 minutes at room temperature. The magnitude of optical density is measured using spectrophometric determination, reading absorbance at 450 nm. Amounts of total Akt proteins were performed in the same way using CST's PathScan Total Akt1 (Ser473) Sandwich ELISA Kit. Phospho- and total-Akt ELISA results comparing different cell lines, various PDGF concentration, with and without inhibitors pretreatment and different stimulation times were detected in the same ELISA plate and read at the same time. The data of phosphorylated Akt are all normalized with total Akt.

2.5. Quantitative immunoblotting

Pooled cell lysates were subjected to SDS-PAGE gels using standard techniques. After gel electrophoresis, proteins were transferred to PVDF membrane (Immobilon, Millipore, Bedford, MA) and probed with the indicated antibodies. The blots were incubated with chemiluminescence substrates (Pierce, Rockford, IL) and imaged using a high sensitivity CCD camera (BioRad Fluor S-Max, Hercules, CA). Phospho- and total Erk blots comparing different conditions were performed in parallel and exposed at the same time. The data of phosphorylated Erk are all normalized with total Erk.

2.6. DNA synthesis assay

NIH 3T3 fibroblasts and variant cell lines were plated in 0.5 ml of DMEM containing 10% FBS at 7.5×10^4 cells per well in 24-well plates, and after 24 hours the cells were washed twice with 1 ml of PBS and incubated in 0.5 ml of starvation medium (DMEM, 0.1% FBS, 1 mg/ml fatty acid-free BSA) for another 24 hours. Cells were then treated with PDGF-BB in starvation medium for 14 hours followed by labeling with [methyl- ^3H]-thymidine (1 $\mu\text{Ci/ml}$) (PerkinElmer Life and Analytical Sciences, Boston, MA) for 10 hours. After labeling, the cells were washed once with 1 ml of ice-cold PBS, and DNA was precipitated with 1 ml of ice-cold 5% trichloroacetic acid for 30 minutes at 4°C . The cells were then washed with 1 ml of ice-cold PBS and solubilized in 250 μl of 0.25N NaOH for 30 minutes at room temperature. Aliquots (200 μl) were transferred into scintillation vials containing 50 μl of 6N HCl and 2.7 ml of scintillation cocktail. The incorporated radioactivity was determined by liquid scintillation counting (Wallac Microbeta).

2.7. Cell proliferation assay

NIH 3T3 fibroblasts were plated in 100 μl of DMEM containing 10% FBS at 2.5×10^3 cells per well in 96-well plates, and after 24 hours the cells were washed twice with 100 μl of starvation media (DMEM, 0.1% FBS, 1 mg/ml fatty acid-free BSA), and incubated in 100 μl of starvation media for 24 hours. After the serum starvation, the quiescent cells were treated with PDGF-BB in starvation media for 37 hours. Cell numbers were detected using the Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan), reading absorbance at 450 nm using microplate reader (Molecular Devices Corporation, Sunnyvale, CA).

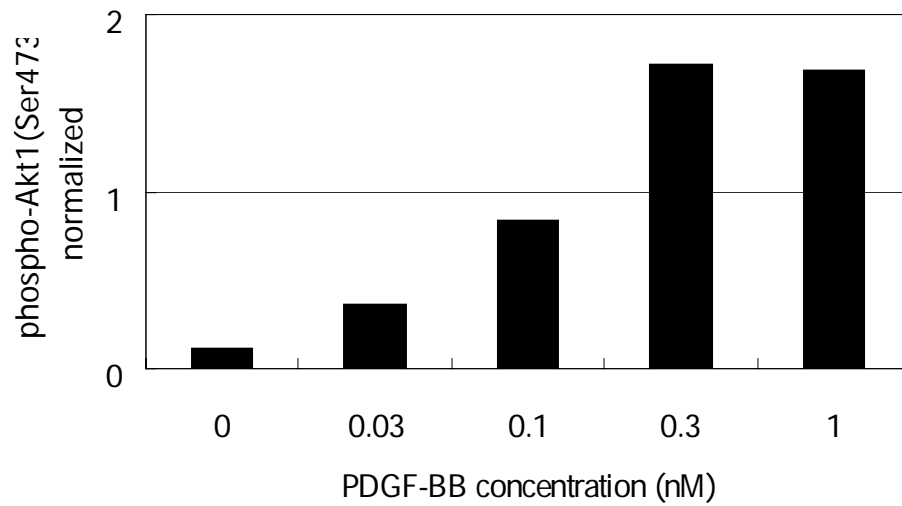
CHAPTER 3. RESULTS AND DISCUSSION

3.1. PDGF-stimulated Akt and Erk phosphorylation in control cells.

Initially we examined the phosphorylation of Akt (Ser473), a simple and sensitive readout of the PI 3-kinase/Akt pathway, at 5 minutes after exposure of our control cell line, NIH 3T3 cells transfected with empty pBM-IRES-Puro vector, to various concentrations of PDGF-BB. Total and phosphorylated Akt in cell lysates were quantified by quantitative sandwich ELISA. As reported previously (Park et al., 2003; Kaur et al., 2005), stimulation of serum-starved cell with PDGF-BB caused an increase in Akt phosphorylation on Ser473, with saturation of the response at PDGF concentrations above ~0.3 nM (Fig. 3.1A). The levels of phosphorylated Akt are normalized by total Akt, measured in parallel, which was not significantly affected by PDGF stimulation.

The phosphorylation of Erk is the output of the Raf/MEK/Erk pathway. Here, we examined the PDGF dose response of Erk phosphorylation. Lysates were prepared from control cells exposed to the indicated concentration of PDGF-BB for 5 minutes, subjected to SDS-PAGE, and probed with anti-total-Erk or anti-phospho-Erk (pThr202/pTyr204) antibodies. As reported previously (Kaur et al., 2005), stimulation of serum-starved cells with PDGF-BB results in an increase in phosphorylation of both Erk1 and Erk2 (Fig. 3.1B). The amounts of the total Erk protein were approximately the same in treated and non-treated control cells as determined by using anti-pan Erk antibody, which recognizes both the phosphorylated and non-phosphorylated forms of Erk1/2. The data of phosphorylated Erk are normalized by total Erk.

A.



B.

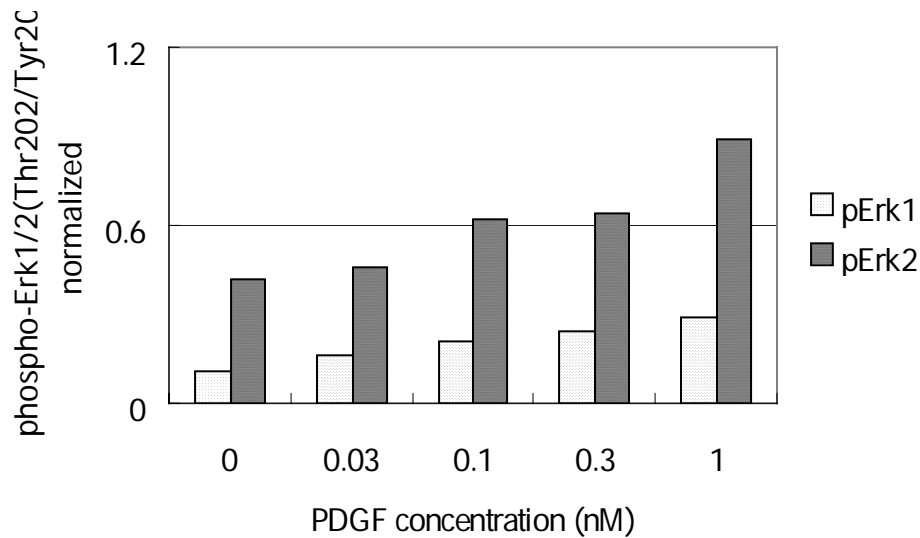


Figure 3.1. PDGF-stimulated Akt and Erk phosphorylation in control cells. NIH 3T3 cells transfected with empty pBM-IRES-Puro vector (control cells) were serum-starved for 24 hours, and then stimulated with indicated concentrations of PDGF (nM) for 5 minutes. A. Akt1 phosphorylation. Cell lysates (10 μ l) were quantified by quantitative sandwich ELISA, probed with anti-total-Akt or anti-phospho-Akt (Ser473) antibodies. B. Erk1/2 phosphorylation. Cell lysates (30 μ l) were subjected to SDS-PAGE, and probed with anti-total-Akt or anti-phospho-Erk (pThr202/pTyr204) antibodies. The levels of phosphorylated Akt and Erk are all normalized by total Akt and Erk respectively.

3.2. Ras signals are required for full activation of PI 3-kinase/Akt.

To ascertain the degree of cooperation between PDGF receptors and Ras in the PI 3-kinase/Akt pathway, we modulated the levels of Ras-GTP in our cells through the expression of constitutively active (G12V) H-Ras or dominant-negative (S17N) H-Ras, introduced by retroviral infection and puromycin selection; controls cells were infected with empty pBM-IRES-Puro vector.

Previous experiments in our laboratory confirmed that Ras-GTP levels followed the prospective trend (S17N<control<<G12V) (Kaur et al., 2005). PDGF-stimulated Erk phosphorylation, as the output of the Raf/MEK/Erk pathway, was inhibited by Ras S17N, while Erk phosphorylation in Ras G12V cells was elevated in the absence of PDGF and insensitive to PDGF stimulation (Kaur et al., 2005).

To confirm that changes in Ras-GTP level are significant for modulating the activity of PI 3-kinase, in this study we assessed the dose responses of Akt phosphorylation as the output of PI 3-kinase/Akt pathway (Fig. 3.2). Akt phosphorylation of control, Ras G12V, and Ras S17N cells were quantified by quantitative sandwich ELISA. Compared to the dose response in control cells, PDGF-stimulated Akt phosphorylation was severely reduced by Ras S17N. The degree of inhibition varied with PDGF dose consistent with previous findings (Kaur et al., 2005). Ras G12V cells exhibited elevated Akt phosphorylation in both the absence of PDGF (~2.5-fold compared with control cell) and PDGF treatment. Our result is consistent with the idea that Ras signals are required for full PI 3-kinase/Akt activation. PI 3-kinase is a likely effector molecule of the Ras proteins (Chan et al., 2002; Kaur et al., 2005).

Ras S17N cells did not completely inhibit Akt phosphorylation, suggesting endogenous

Ras may be modestly activated in the Ras S17N-expressing cells. Interestingly, Ras G12V cells expressed 2-fold higher total Akt, whereas Ras S17N cells expressed the same level of Akt as control cells did, suggesting the protein expression profile was somehow changed because of the G12V protein after several cell cycles.

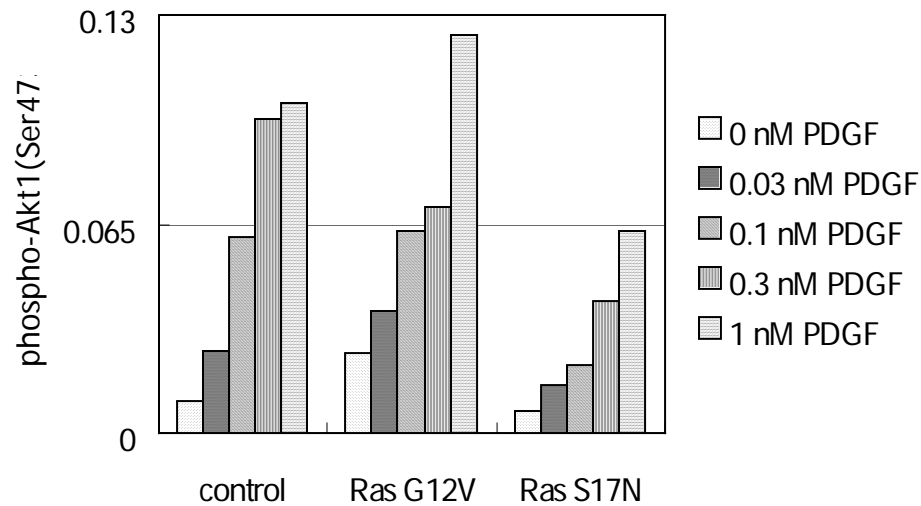


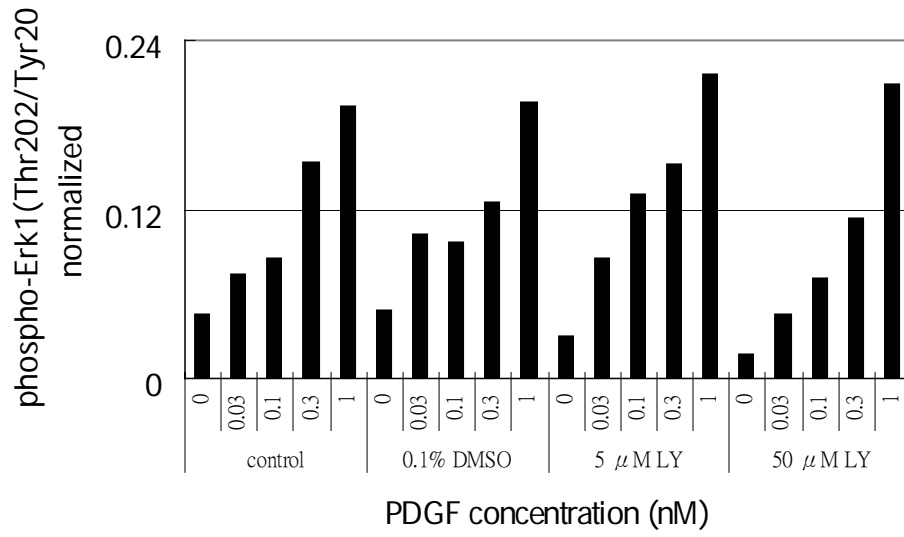
Figure 3.2. Cooperative activation of PI 3-kinase/Akt by PDGF receptors and Ras. We modulated the levels of Ras-GTP in our cells through the expression of constitutively active (G12V) H-Ras or dominant-negative (S17N) H-Ras, introduced by retroviral infection and puromycin selection; controls cells were infected with empty pBM-IRES-Puro vector. Dose responses of Akt phosphorylation (pSer473) at 5 minutes of PDGF stimulation were measured by quantitative sandwich ELISA for the control, Ras G12V, and Ras S17N cells.

3.3. Erk stimulation, at low but not high concentrations of PDGF, is inhibited by PI 3-kinase-inhibitory drug.

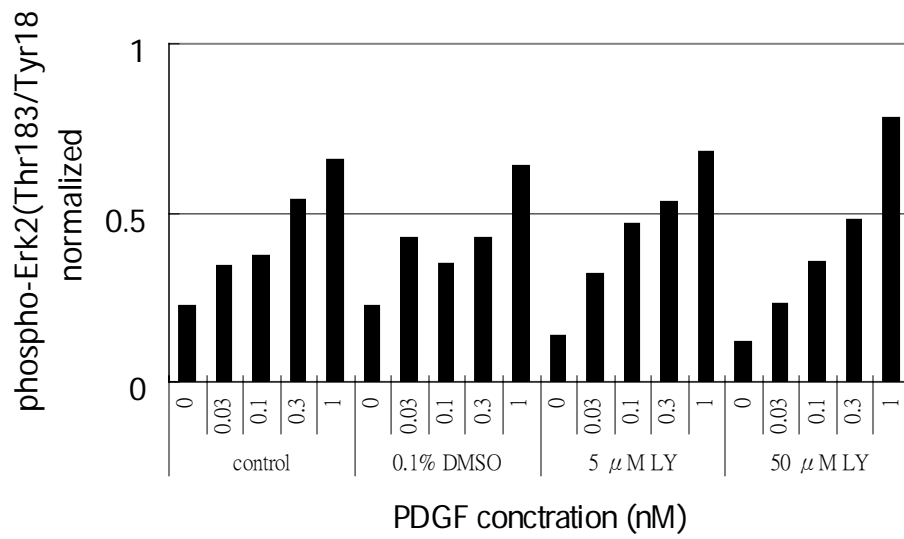
To confirm that changes level of PI 3-kinase activity were significant for modulation of downstream signaling, we assessed the dose responses of Akt phosphorylation as the output of the PI 3-kinase/Akt pathway. As expected, PDGF-stimulated Akt phosphorylation was effectively blocked by LY294002 (Fig. 3.3C). High concentration LY294002 even completely inhibits PDGF-BB-induced phosphorylation. A small amount of basal phosphorylation on Akt Ser473 is present even in serum-starved, unstimulated cells and this phosphorylation remained even when cells were pretreated with 50 μ M LY294002.

As outlined in the introduction, confusion has arisen recently about the role of PI 3-kinase in the regulation of the Ras/Erk pathway. To investigate whether PDGF-induced activation of Erk is promoted or inhibited as a result of PI 3-kinase/Akt activation, control cells were serum-starved for 24 hours, pretreated with the PI 3-kinase inhibitor, LY294002 for 30 minutes, and then challenged with different concentrations of PDGF (Fig. 3.3AB). Erk1 and Erk2 phosphorylation were partially reduced by a high dose (50 μ M) of LY294002, in unstimulated cells or at lower concentrations (0.03, 0.1, and 0.3 nM), but were insensitive to LY294002 at higher concentrations (1 nM) of PDGF. A lower dose (5 μ M) of LY294002 did not significantly effect Erk-1 or Erk-2 phosphorylation. These results are consistent with those reported by other groups. Lower PDGF concentrations, near the level needed for mitogenesis, cause activation of Erk that is more sensitive to PI 3-kinase inhibition (Duckworth and Cantley, 1997; Wennstrom and Downward, 1999) Clearly, further investigations are needed to clarify the modes of PI 3-kinase regulation of Erk activation.

A.



B.



C.

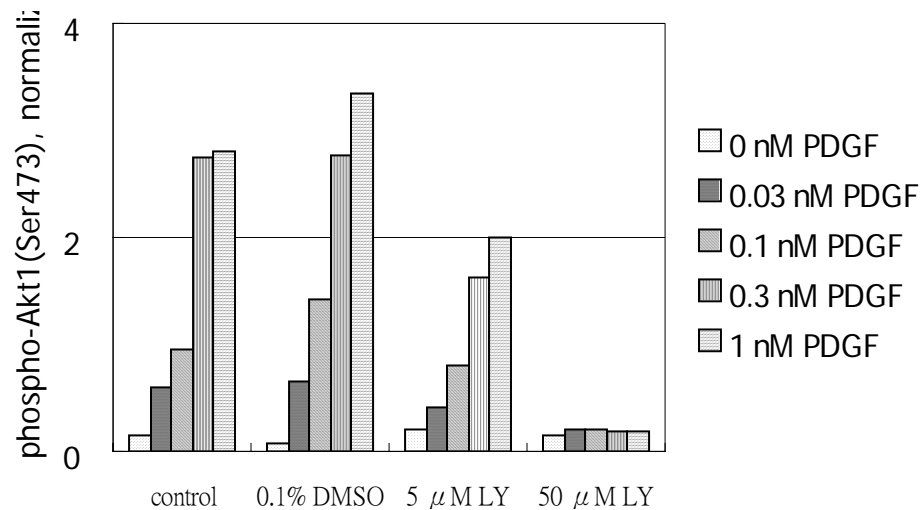
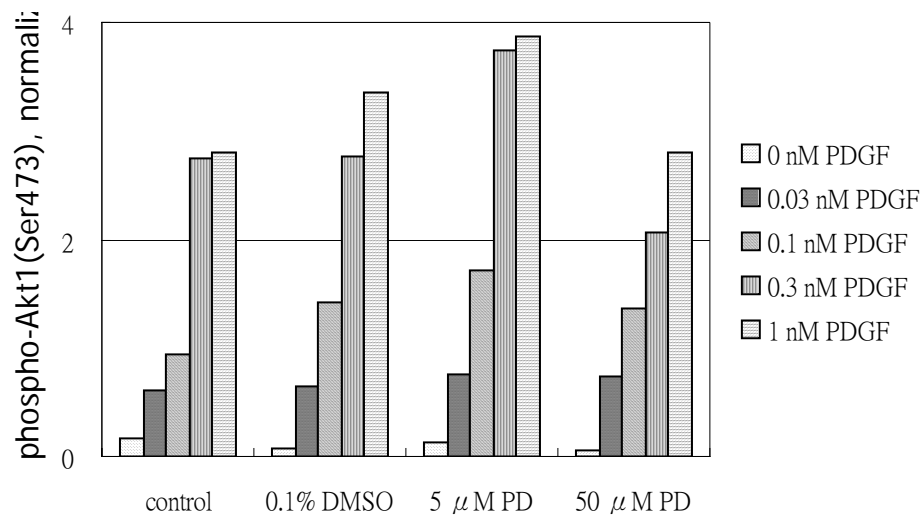


Figure 3.3. LY294002 blocked Akt phosphorylation and partially reduced Erk phosphorylation induced by low concentration of PDGF. Control cells were serum-starved for 24 h, pretreated with the low (5 μM) or high (50 μM) concentration of LY294002 for 30 minutes and then challenged with indicated concentration of PDGF for 5 minutes. A. B. Erk1/2 phosphorylation. Cell lysates (25 μl) were subjected to SDS-PAGE, and probed with anti-total-Akt or anti-phospho-Erk (pThr202/pTyr204) antibodies. C. Akt1 phosphorylation. Cell lysates (20 μl) were quantified by quantitative sandwich ELISA, probed with anti-total-Akt or anti-phospho-Akt (Ser473) antibodies. The levels of phosphorylated Akt and Erk are all normalized by total Akt and Erk respectively.

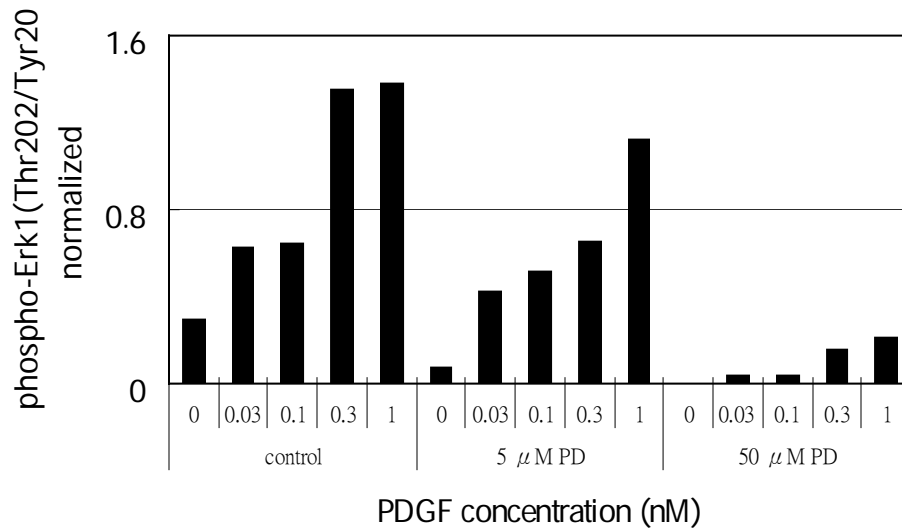
3.4. MEK activity is required for PDGF-induced Erk phosphorylation but does not significantly affect PDGF-induced Akt phosphorylation.

To ascertain the effect of MEK activity on Erk phosphorylation and PI 3-kinase/Akt pathway, we pretreated control cells with MEK inhibitor, PD098059, and assessed the dose responses of Akt and Erk phosphorylation as the output of the PI 3-kinase/Akt and Raf/MEK/Erk pathways. As expected, at the high concentration (50 μ M), PD098059 completely blocked PDGF-stimulated activation of Erk-1/2 phosphorylation (Fig. 3.4AB). Our results demonstrated that PD098059 had no significant effect on the PDGF-BB-mediated Akt phosphorylation on Ser473 (Fig. 3.4A).

A.



B.



C

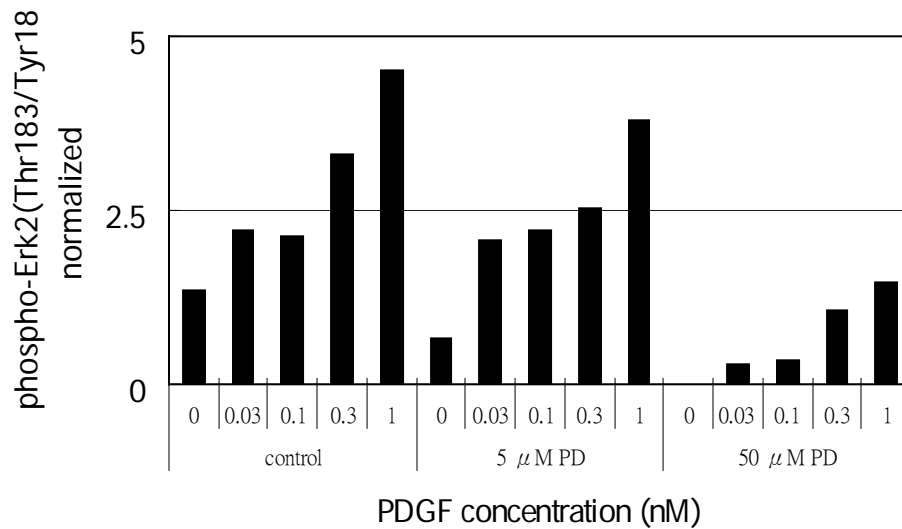


Figure 3.4. PD098059 blocked PDGF-induced Erk phosphorylation but had no significant effect on the PDGF-induced Akt phosphorylation. Control cells were serum-starved for 24 h, pretreated with the low (5 μM) or high (50 μM) concentration of PD098059 for 30 minutes prior to treatment with indicated concentrations of PDGF-BB for 5 minutes. A. Akt1 phosphorylation. Cell lysates (20 μl) were quantified by quantitative sandwich ELISA, probed with anti-total-Akt or anti-phospho-Akt (Ser473) antibodies. The levels of phosphorylated Akt and Erk are all normalized by total Akt and Erk respectively. B. C. Erk1/2 phosphorylation. Cell lysates (25 μl) were subjected to SDS-PAGE, and probed with anti-total-Akt or anti-phospho-Erk (pThr202/pTyr204) antibodies.

3.5. Transient and sustained Akt and Erk activation in response to different PDGF-BB concentrations.

The PDGF-stimulated increase in Ras-GTP is transient, due to a feedback loop mediated by downstream components. By expressing Ras G12V or Ras S17N in our cells, a perturbed Ras-GTP response is achieved (Kaur et al., 2005), and we will examine the hypothesis that duration of this signal has a corresponding effect on the time course of Akt activation.

3.5.1. Evaluation of the coupled kinetics of PI 3-kinase/Akt and Ras/Erk responses in control cells.

Control cells, NIH 3T3 cells transfected with empty pBM-IRES-Puro vector, were serum-starved for 24 h. Cell lysates were harvested at 5, 15, 30, 60, and 120 minutes post-addition of PDGF-BB and used to determine the level of Akt phosphorylation by ELISA kit and Erk phosphorylation by Western blotting.

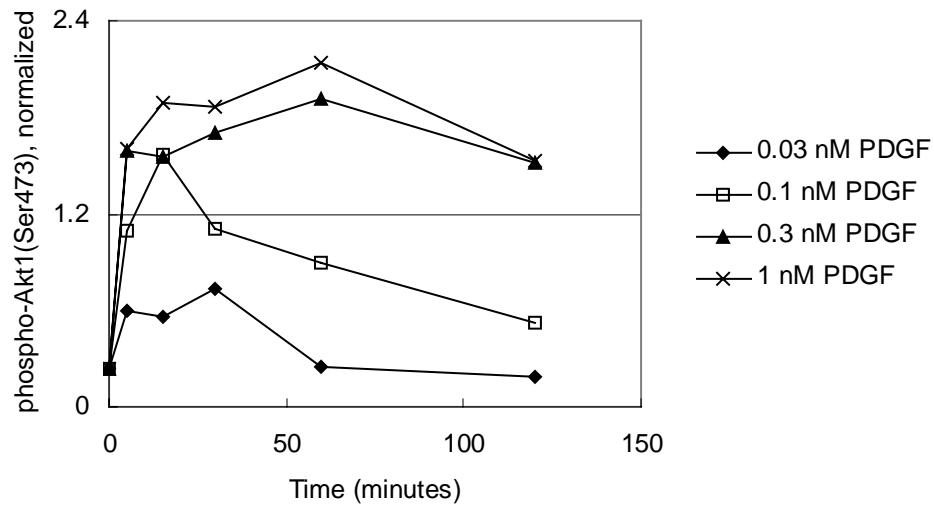
Lower concentrations (0.03, 0.1 nM) of PDGF resulted in the transient Akt phosphorylation (Fig. 3.5A). However, higher concentrations (0.3, 1 nM) of PDGF induced both rapid and sustained Akt phosphorylation. The magnitude of this effect from high concentration of PDGF-BB remained thereafter for at least 120 minutes. These observations is consistent with previous study and suggests that the ability of the cell to activate Akt is saturated in high concentration of PDGF, with respect to the number of phosphorylated PDGF receptors (Kaur et al., 2005).

Measurements of Erk-2 phosphorylation stimulated by different concentrations of PDGF-BB displays a bell-shaped dose-response curve between 5 and 60 minutes after PDGF-BB treatment (Fig. 3.5.C). Maximum stimulation is obtained with 0.1 nM PDGF at 30

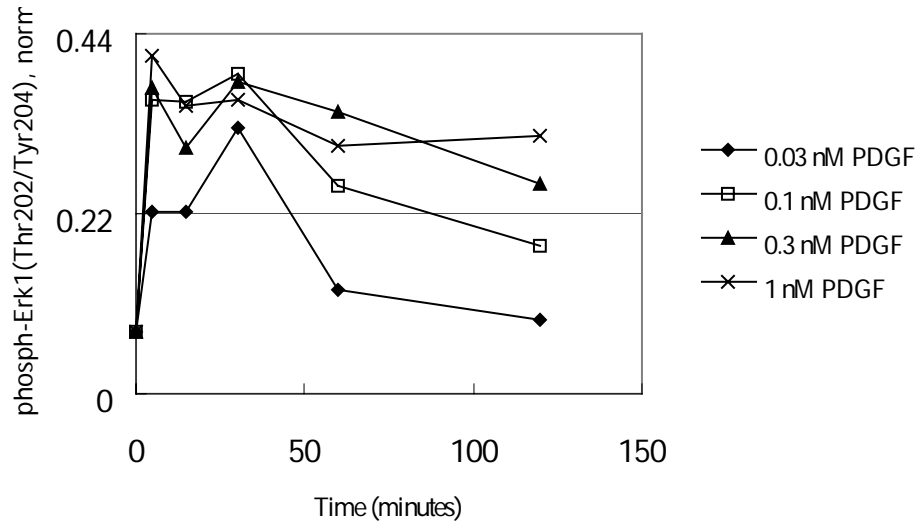
minutes. At higher PDGF concentration (0.3, and 1 nM), a weaker phosphorylation of Erk-2 was observed. These observations are consistent with a balance in signal strength between Akt and Raf-1, which exert negative (Akt) and positive (Raf-1) effects on Erk phosphorylation.

As seen for Akt phosphorylation, low concentration (0.03, 0.1 nM) of PDGF-BB only induce transient Erk phosphorylation (Fig. 3.5BC), which is at least in part due to PDGF-dependent activation of MAP kinase phosphatases that inactivate Erk. However, higher concentrations (0.3, 1 nM) of PDGF induced both rapid and sustained Erk phosphorylation.

A.



B.



C.

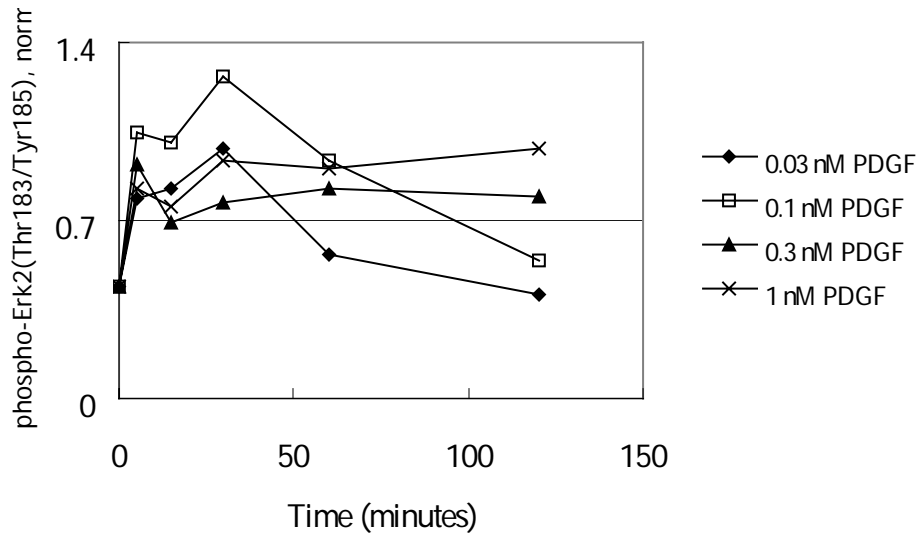


Figure 3.5. The kinetics of Akt and Erk phosphorylation in control cells. Control cells, NIH 3T3 cells transfected with empty pBM-IRES-Puro vector, were serum-starved for 24 h. Cell lysate then was harvested at 5, 15, 30, 60, and 120 minutes post-addition of PDGF-BB. A. Akt1 phosphorylation. Cell lysates (20 μ l) were quantified by quantitative sandwich ELISA, probed with anti-total-Akt or anti-phospho-Akt (Ser473) antibodies. The levels of phosphorylated Akt and Erk are all normalized by total Akt and Erk respectively. B. C. Erk1/2 phosphorylation. Cell lysates (25 μ l) were subjected to SDS-PAGE, and probed with anti-total-Akt or anti-phospho-Erk (pThr202/pTyr204) antibodies.

3.5.2. Effect of constitutively active Ras on kinetics of PI 3-kinase/Akt and Ras/Erk responses.

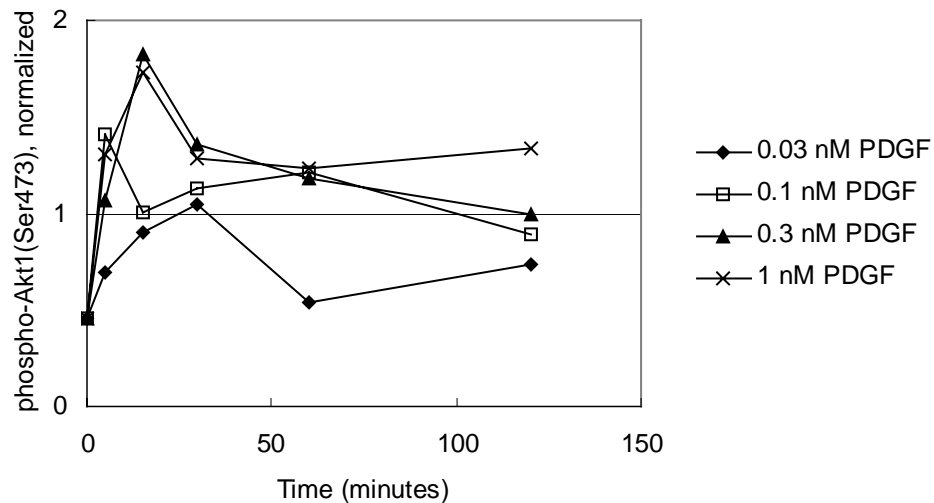
To examine whether RasGTP levels affect dynamic Akt and Erk phosphorylation, we expressed constitutively active Ras G12V and quantified Akt1 and Erk-1/2 phosphorylation at different time points after PDGF-BB treatment.

The change in Ras-GTP level by expressing Ras G12V was significant for regulation of kinetics of Akt phosphorylation (Fig. 3.6A). In Ras G12V cells, PDGF-BB induced a rapid increase of Akt1 phosphorylation in a different manner comparing with that in control cells. Lower concentration (0.1 nM) of PDGF induced slightly sustained Akt phosphorylation for at least 120 minutes, which tends to return to base line in control cells.

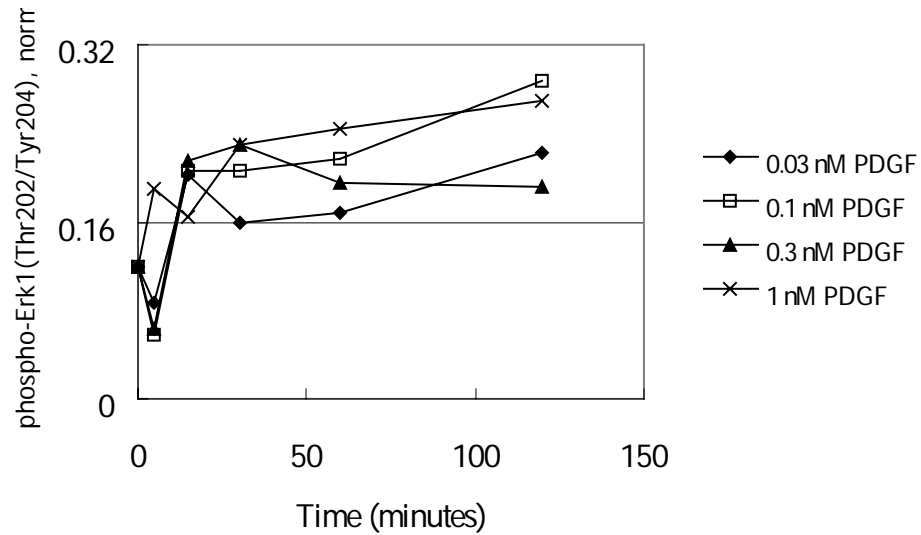
The kinetics and pattern of transient and sustained Erk phosphorylation observed in Ras G12V cells is completely distinct from the Erk phosphorylation in control cells (Fig. 3.6BC). In contrast with control cells, in which PDGF induced rapid Erk phosphorylation, PDGF-BB induced a slight reduction of Erk-1/2 phosphorylation in Ras G12V cells. This effect partially results from that the basal Erk phosphorylation is high because of the constitutively activated Ras G12V, and thus the effect of PDGF-BB on Erk phosphorylation is not obvious. This effect also suggests that another signal pathway induced by PDGF receptor exerts negative regulation on Erk phosphorylation. The possible pathway is through PI 3-kinase/Akt. This idea is readily tested in G12V cells after treated with LY294002 in further experiments. Rapid Akt phosphorylation is induced by PDGF-BB within 5 minutes and consequently inhibits the ability of Raf-1 to activate MEK. However, the highest concentration (1 nM) of PDGF treatment can overcome the initial reduction and induce slight increase in Erk phosphorylation, suggesting the initial cooperation between PI 3-kinase/Akt and Ras/Erk

pathways is also dependent on the strength of stimulus. Erk phosphorylation induced by various concentration of PDGF displays a bell-shaped dose-response curve after 30 minutes time point, as that observed in control cells. As expected, in Ras G12V cells Erk-1/2 phosphorylation remained sustained, even with low concentration PDGF (Fig. 3.6. B,C). The constitutively active Ras G12V is sufficient to promote the activity of Raf/MEK/Erk pathway and maintains sustained Erk phosphorylation. Why is the duration of an Erk signal important? Since the proliferation in response to Erk activation required its translocation to the nucleus (Brunet et al., 1999b), one possibility is that the duration of Erk activation can direct qualitative changes in gene expression which, in turn, determine cellular fate (Roovers and Assoain, 2000; Kondoh et al., 2005)

A.



B.



C.

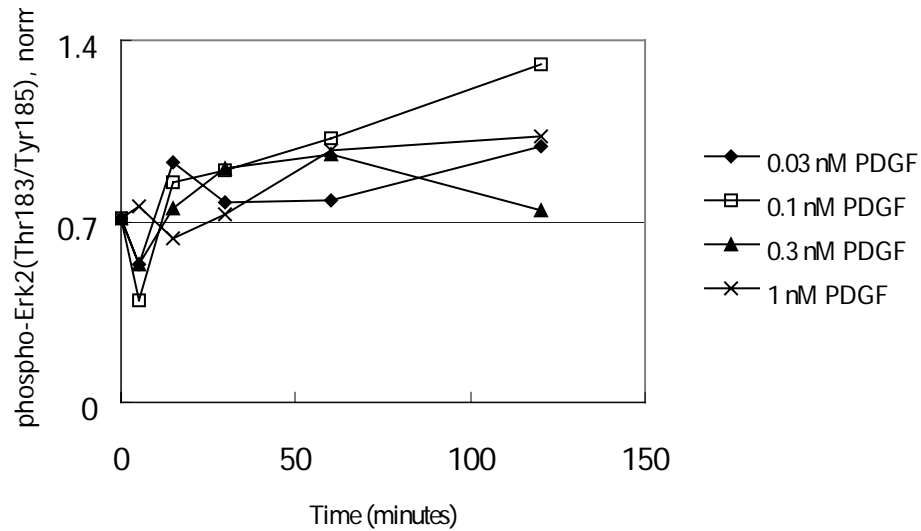


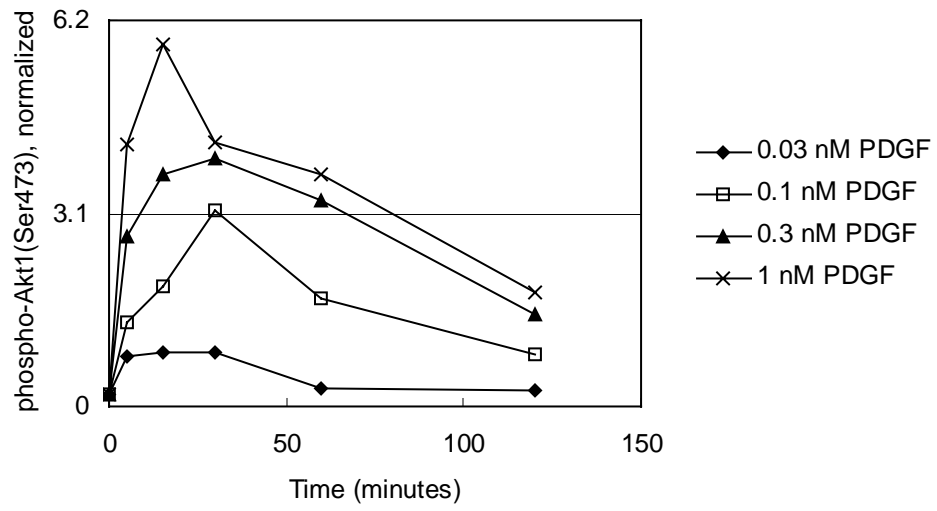
Figure 3.6. The kinetics of Akt and Erk phosphorylation in Ras G12V cells. We modulated the levels of Ras-GTP in our cells through the expression of constitutively active Ras G12V. Ras G12V cells were serum-starved for 24 h. Cell lysate then was harvested at 5, 15, 30, 60, and 120 minutes post-addition of PDGF-BB. A. Akt1 phosphorylation. Cell lysates (20 μ l) were quantified by quantitative sandwich ELISA, probed with anti-total-Akt or anti-phospho-Akt (Ser473) antibodies. The levels of phosphorylated Akt and Erk are all normalized by total Akt and Erk respectively. B. C. Erk1/2 phosphorylation. Cell lysates (25 μ l) were subjected to SDS-PAGE, and probed with anti-total-Akt or anti-phospho-Erk (pThr202/pTyr204) antibodies.

3.5.3. Effect of dominant-negative Ras on kinetics of PI 3-kinase/Akt and Ras/Erk responses.

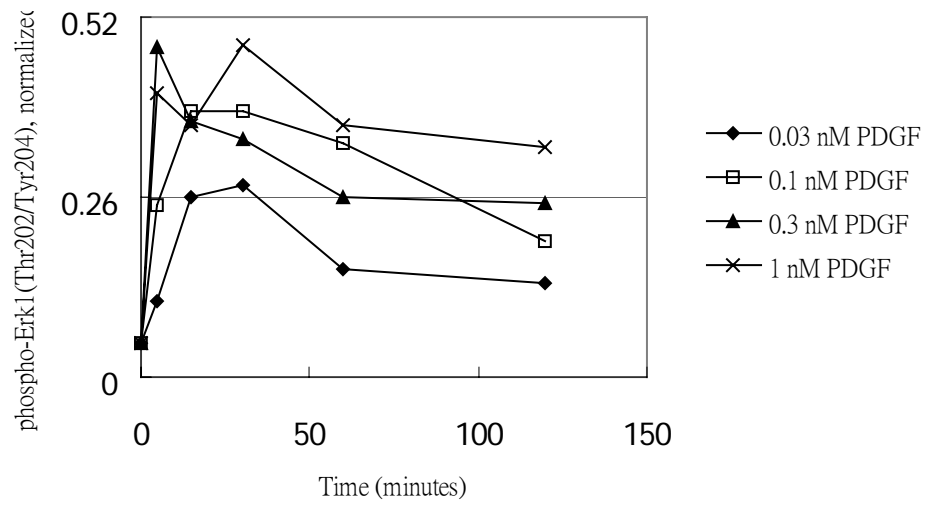
The influence of Ras S17N on kinetics of Akt phosphorylation is shown on Fig. 3.7A. In Ras S17N cells, PDGF-BB can also induce a rapid increase of Akt1 phosphorylation. However, Ras S17N postponed the Akt phosphorylation peak of lower PDGF concentration. For example, the maximum Akt phosphorylation in response to 1 nM PDGF-BB appears at 15 minutes and the peak in response to 0.3 nM PDGF is at 30 minutes. The other feature of Ras S17N cells is that Akt phosphorylation tends to return to near basal levels by 120 minutes, even at high concentration of PDGF-BB. This effect might be attributed to a well-known hypothesis that RasGTP acts as an upstream positive effector of PI 3-kinase/Akt pathway. Ras S17N protein decreased the levels of Ras-GTP and impaired the ability to maintain the sustained Akt phosphorylation in high concentration of PDGF.

Erk phosphorylation stimulated by different concentration of PDGF-BB displays a bell-shaped dose-response curve between 15 and 60 minutes, as that observed in control cells (Fig. 3.7BC). Maximum stimulation is obtained with 0.1 nM PDGF at 60 minutes. Comparing with control cells, Ras S17N cell postponed and moderated the peak of Akt phosphorylation (30 minutes), and the succeeding decrease of Erk phosphorylation was delayed, consistent with the hypothesis that Akt blocked the activity of Raf-1.

A.



B.



C.

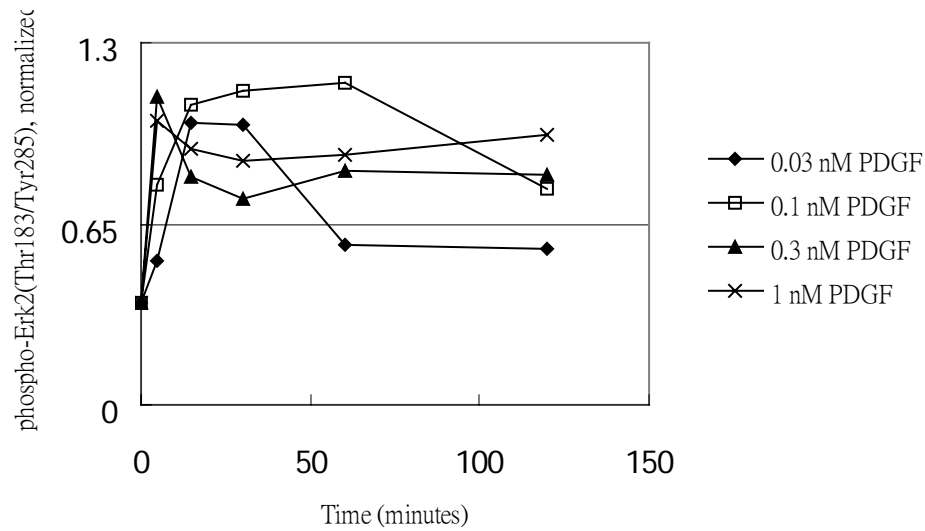


Figure 3.7. The kinetics of Akt and Erk phosphorylation in Ras S17N cells. We modulated the levels of Ras-GTP in our cells through the expression of dominant negative Ras S17N. Ras S17N cells were serum-starved for 24 h. Cell lysate then was harvested at 5, 15, 30, 60, and 120 minutes post-addition of PDGF-BB. A. Akt1 phosphorylation. Cell lysates (20 μ l) were quantified by quantitative sandwich ELISA, probed with anti-total-Akt or anti-phospho-Akt (Ser473) antibodies. The levels of phosphorylated Akt and Erk are all normalized by total Akt and Erk respectively. B. C. Erk1/2 phosphorylation. Cell lysates (25 μ l) were subjected to SDS-PAGE, and probed with anti-total-Akt or anti-phospho-Erk (pThr202/pTyr204) antibodies.

3.5.4. Summary of the effect of changes in Ras-GTP level on kinetics of PI 3-kinase/Akt and Ras/Erk pathways.

To ascertain the degree of Akt activity in the inhibition of Raf-1 activity, we compare the kinetic data of Akt and Erk phosphorylation in control cells stimulated by lower concentration (0.1 nM) of PDGF-BB, where both Akt and Erk phosphorylation were not saturated. 0.1 nM PDGF-BB induces a rapid phosphorylation of Akt (15 minutes) and Erk (15-30 minutes), and consequent severe decrease of Ras phosphorylation (30-60 minutes). However, Ras S17N postponed and moderated the peak of Akt phosphorylation (30 minutes), and the succeeding decrease of Erk phosphorylation was delayed, suggesting that at the Akt-Raf-1 interaction could be observed under low (0.1 nM), and probably more physiologically relevant, concentration of PDGF-BB.

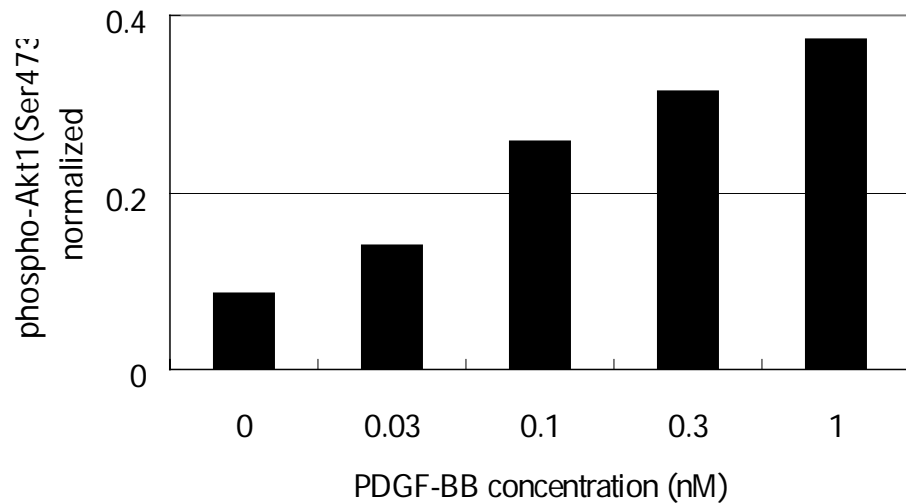
3.6. The mechanisms of Akt on Erk phosphorylation are still poorly understood.

To examine the reported role of Akt in regulating the Raf/MEK/Erk pathway, we increased the level of total Akt in our cells through the expressing of haemagglutinin epitope-tagged wild-type Akt (HA-Akt). HA-Akt cells expressed ~36-fold higher of total Akt and ~27-fold higher of phosphorylated Akt than control cells did, confirming that ectopically expressed HA-Akt is able to be phosphorylated on Ser473. PDGF caused an increase in Akt phosphorylation on Ser473 in HA-Akt cells and the ability of cell to phosphorylate Akt is not saturated as a result of Akt overexpression (Fig. 3.8A).

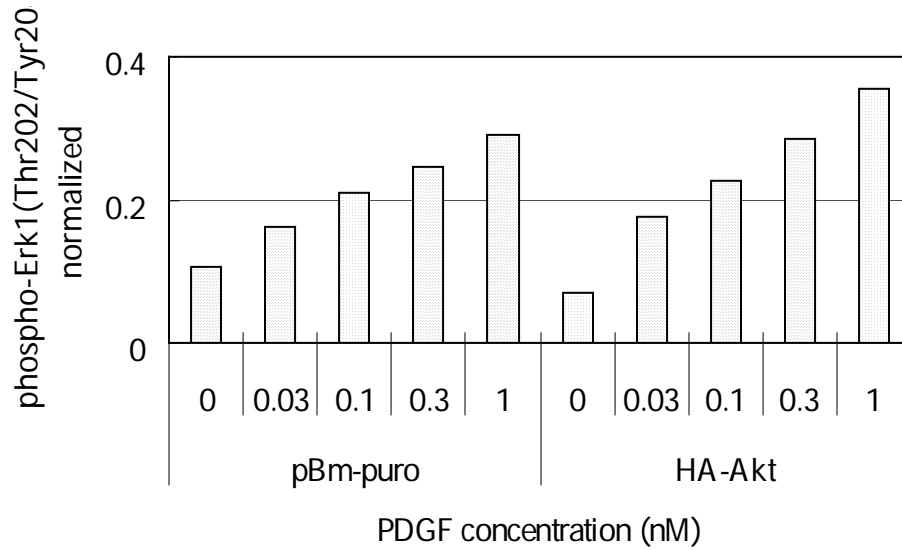
We should acknowledge that effects of Akt overexpression on Erk phosphorylation is still uncertain and this experiment may need to be repeated. In our experiment, HA-Akt cells partially decreased the basal phosphorylation of Erk in the absence of PDGF-BB, but

enhanced the PDGF-induced Erk phosphorylation (Fig. 3.8BC). This implies another possibility link between PDGFR and Erk, which can be influenced by overexpression of Akt: this construct probably acts by sequestering PI(3,4,5)P₃ and the data may not suggest a role for Akt itself in this process of Erk phosphorylation. One possible mechanism is that other competitive targets of PI(3,4,5)P₃, such as PLC/PKC pathway, could feed into the MAP kinase pathway after PDGF-BB stimulation (Corbit et al., 2003; Robin et al., 2003; Ginnan and Singer, 2005). It is current unclear how broadly relevant such mechanism might be in the regulation of the Erk pathway, leading the question open for further investigation.

A.



B.



C.

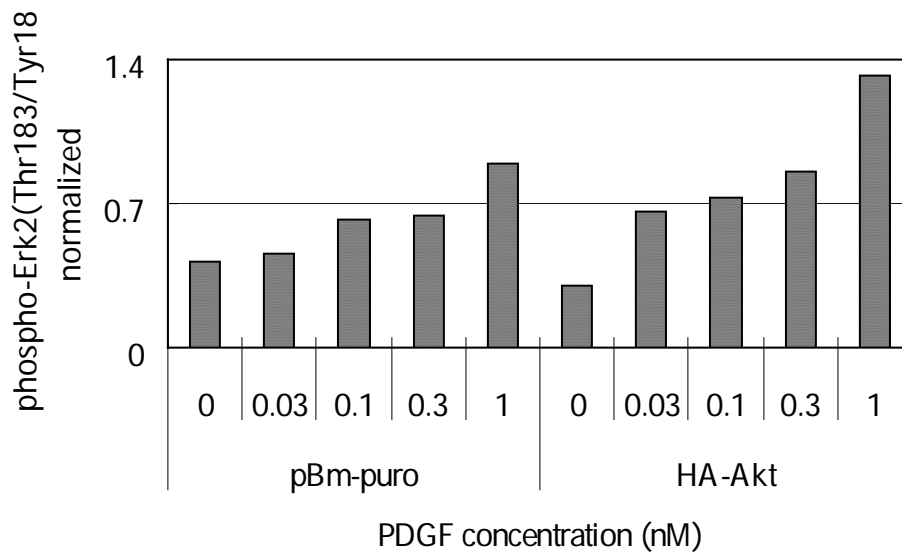


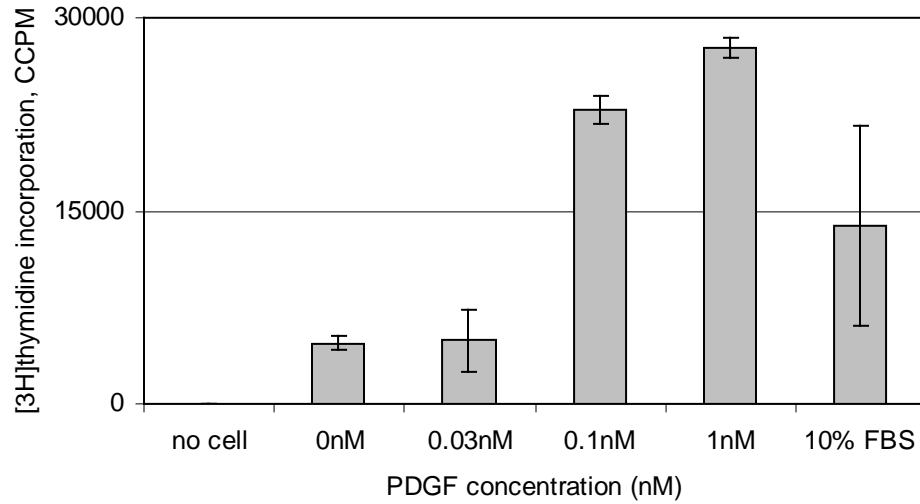
Figure 3.8. PDGF-stimulated Akt and Erk phosphorylation in HA-Akt cells. NIH 3T3 cells were transfected with haemagglutinin epitope-tagged wild-type Akt (HA-Akt). HA-Akt were serum-starved for 24 h. Cell lysate then was harvested at 5, 15, 30, 60, and 120 minutes post-addition of PDGF-BB. A. Akt1 phosphorylation. Cell lysates were quantified by quantitative sandwich ELISA, probed with anti-total-Akt or anti-phospho-Akt (Ser473) antibodies. The levels of phosphorylated Akt and Erk are all normalized by total Akt and Erk respectively. B. C. Erk1/2 phosphorylation. Cell lysates (25 μ l) were subjected to SDS-PAGE, and probed with anti-total-Akt or anti-phospho-Erk (pThr202/pTyr204) antibodies.

3.7. PDGF-BB induces DNA synthesis and enhances cell proliferation.

PDGF is a principal survival factor that inhibits apoptosis and promotes proliferation (Romashkova and Makarov, 1999). In quiescent cells, PDGF stimulation induces exit from G0 phase, progression through G1 phase, and entry into S phase (DNA synthesis) (Jones et al., 1999). Many studies have shown that when treated with PDGF, quiescent NIH 3T3 cells required a 10-hour exposure to induce DNA synthesis maximally. In treated cells, [³H]thymidine incorporation increased at 12-14 hours, with peak incorporation between 16 and 22 hours (Jones and Kazlauskas, 2001).

In our experiments, [³H]thymidine was added to cells at 14 hours and detected at 24 hours after PDGF treatment. The results show that PDGF-BB stimulates [³H]thymidine incorporation in a dose-dependent manner, with the greatest stimulation seen at a concentration of 1 nM (Fig.3.9A). To determine the effect of PDGF concentration on NIH 3T3 cells division, cell number was determined by Cell counting Kit-8 after 37 hours PDGF stimulation (Fig.3.9B). PDGF-BB increased the cell number in a dose-dependent manner, with 1 nM PDGF treatment leading to an increase in cell number of 2.3-fold.

A.



B.

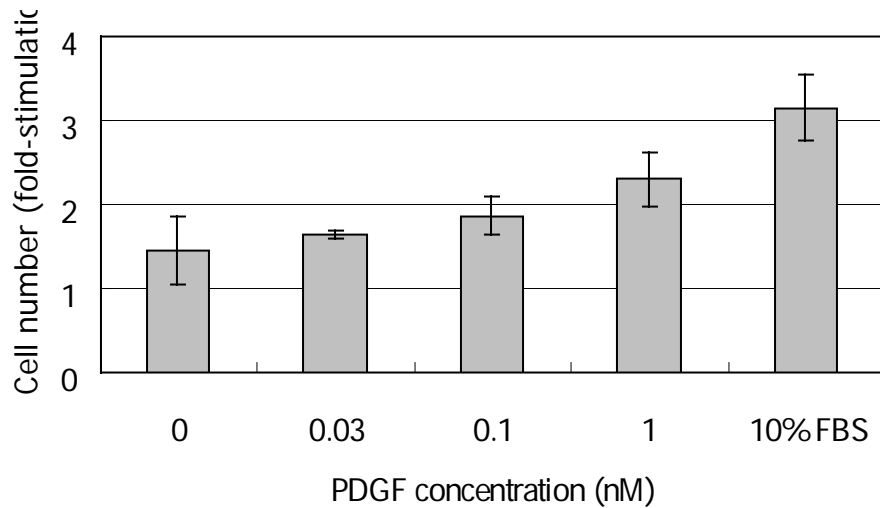


Figure 3.9. PDGF-BB induces DNA synthesis and enhances cell proliferation. NIH 3T3 cells were serum-starved with 0.1% fetal bovine serum (BSA) in DMEM for 24 hours. A. Quiescent cells were treated with PDGF-BB at the indicated concentration in the presence of 0.1% FBS for 24 hours. [³H]thymidine was added at 14 hours and detected at 24 hours after PDGF treatment. Control cells were stimulated with 10% FBS instead of PDGF. B. PDGF-BB was added to quiescent NIH 3T3 cells, and 37 hours later, the cell number was determined by Cell counting Kit-8.

3.8. PI 3-kinase/Akt and Raf/MEK/Erk pathways are required for PDGF-induced DNA synthesis.

The identification of the PDGF-BB-initiated signaling events responsible for cell cycle progression is difficult, because multiple signaling systems functioning over many hours are required to progress through the cell cycle restriction point. Both the PI 3-kinase/Akt and Raf/MEK/Erk pathways have been identified to play key roles in this process. Earlier studies have shown that whereas activation of the Erk pathway functions to drive cells out of quiescence, PI 3-kinase does not perform this function. PI 3-kinase does contribute to mitogenic signaling at a later time, as cells progress through the second half of G1 and into S phase (Taylor and Shalloway, 1996; Stacey and Kazlauskas, 2002; Dangi et al., 2003).

Romashkova and Makarov have shown a putative anti-apoptotic pathway whereby PDGF activates NF- κ B transcription factor through Ras and PI 3-kinase to Akt and the I κ B kinase (IKK), thus linking anti-apoptotic signalling with transcription machinery (Romashkova and Makarov, 1999). In our system, expression of constitutively active Ras G12V promoted DNA synthesis whereas expression of dominant negative Ras S17N strongly inhibited PDGF-induced DNA synthesis (Fig. 3.10), confirming that Ras is involved in pathways triggering DNA synthesis.

In anti-apoptotic PDGF signaling, PI 3-kinase is a proximal downstream target of Ras. Early study has supported the idea that PI 3-kinase products must accumulate hours after exposure to PDGF in order for the cells to reach S phase (Jones et al., 1999). To investigate the role of PI 3-kinase in PDGF-induced DNA synthesis, quiescent control cells were pretreated with an LY294002 for 30 minutes before challenged with various concentrations of PDGF. PDGF-stimulated Akt phosphorylation (Fig. 3.3C) and DNA synthesis (Fig. 3.11)

were effectively blocked by LY294002 treatment, confirming that PI 3-kinase is involved in PDGF-induced cell cycle progression.

As Akt is a proximal downstream effector of the Ras/PI 3-kinase pathway, we next examined the involvement of Akt. NIH 3T3 cells were transfected with HA-Akt. Surprisingly, overexpression of wild type HA-Akt completely inhibited PDGF-induced DNA synthesis in our experiments (Fig. 3.10). Akt is a critical mediator of survival signals that protect cells from apoptosis, but the mechanisms of Akt on cell proliferation are still poorly understood (Shimamura et al., 2003). Further detailed investigations are needed to clarify the modes of action of Akt on proliferation and anti-apoptosis pathways.

Activation of Ras by growth factors led to the rapid activation of Erk-1 and Erk-2. Both Taylor and Shalloway (Taylor and Shalloway, 1996) and Gille and Downward (Gille and Downward, 1999) found that Erk was active only during the initial segment of G1 phase. Jones and Kazlauskas have shown that activation of MEK activation and induction of the transcription factor c-Myc are sufficient to drive quiescent cells to G1 phase (Jones and Kazlauskas, 2001).

To investigate the role of MEK/Erk in PDGF-induced DNA synthesis, control cells were serum-starved for 24 h, pretreated with the low (5 μ M) or high (50 μ M) concentration of PD098059 for 30 minutes and then challenged with different concentration of PDGF.

DNA synthesis was inhibited by PD098059 pretreatment in a dose-dependent manner (Fig. 3.11), consistent with the idea that Erk phosphorylation is involved in PDGF-induced cell-cycle progression. The intermediate concentration (5 μ M) of PD098059 does not completely inhibit either Erk phosphorylation (Fig. 3.4BC) or DNA synthesis (Fig. 3.11) induced by the high dose (1 nM) of PDGF-BB.

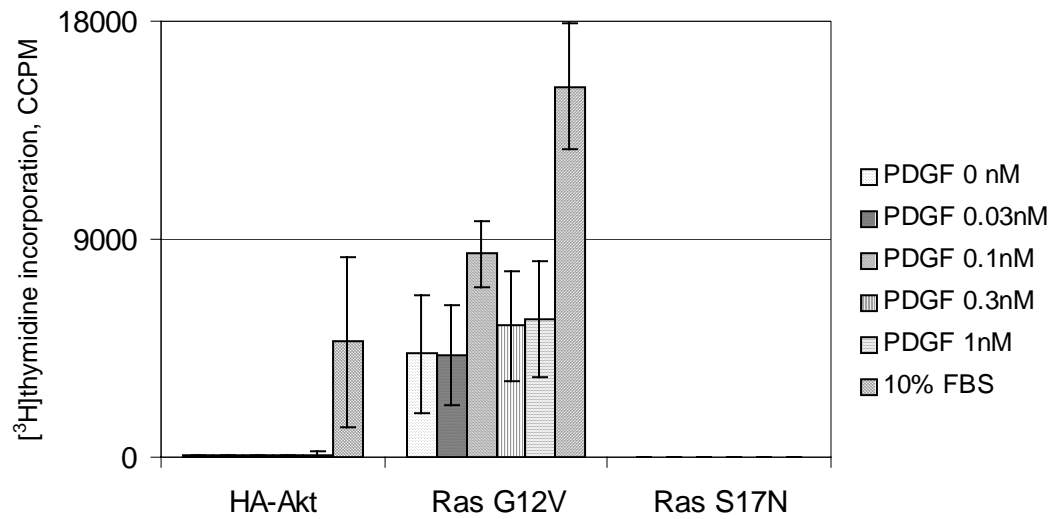


Figure 3.10. The effect of Ras and Akt in PDGF-induced DNA synthesis. HA-Akt, Ras G12V, and Ras S17N cells were serum-starved with 0.1% fetal bovine serum (BSA) in DMEM for 24 hours. Quiescent cells were treated with PDGF-BB at the indicated concentration in the presence of 0.1% FBS for 24 hours. $[^3\text{H}]$ thymidine was added at 14 hours and detected at 24 hours after PDGF treatment. Control cells were stimulated with 10% FBS instead of PDGF. This result is representative of three independent experiments.

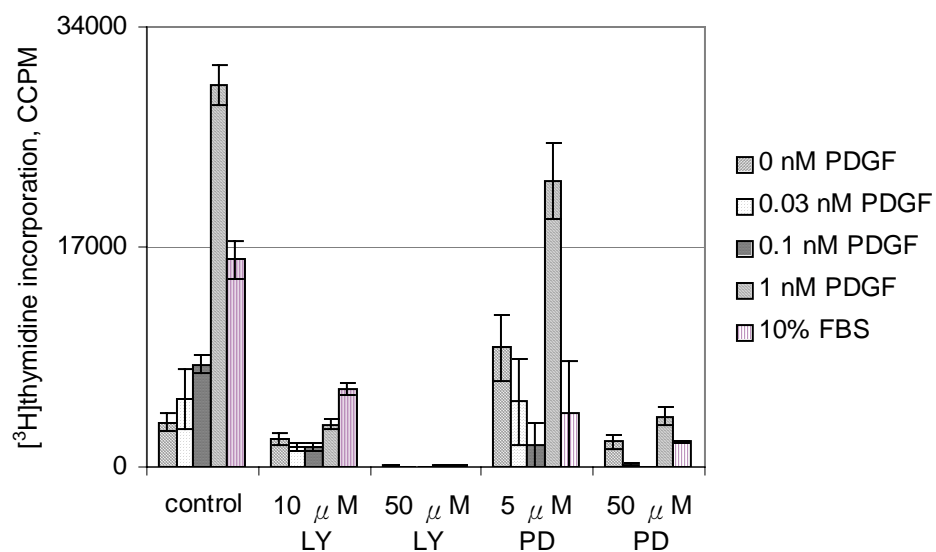


Figure 3.11. The effect of LY294002 and PD098059 on PDGF-induced DNA synthesis. NIH 3T3 cells were serum-starved with 0.1% fetal bovine serum (BSA) in DMEM for 24 hours. A. Quiescent cells were pretreated with LY294002 or PD098059 for 30 minutes and then treated with PDGF-BB at the indicated concentration in the presence of 0.1% FBS for 24 hours. $[^3\text{H}]$ thymidine was added at 14 hours and detected at 24 hours after PDGF treatment. Control cells were stimulated with 10% FBS instead of PDGF. PDGF

CHAPTER 4. CONCLUSIONS

Recent evidence for cross-talk between the PI 3-kinase/Akt and Ras/Erk pathways has provided important hints for how PDGF receptor activation may control cellular function. The apparently contradictory findings among these studies suggest that the cross-talk mechanisms are not fully understood. Accumulating evidence has demonstrated that differences in the strength of stimulus, and the duration and magnitude of activity of signal molecules, determine signaling specificity. Many studies involved experiments using only one (usually high) concentration of stimulus and at a single time point of stimulation. It will be important to explore the cellular response over time or by different strengths of growth factor. In this study, we aim to systematically analyze this signaling network, comprised of Ras/Erk and PI 3-kinase/Akt pathways, stimulated by PDGF.

The model of signal transduction contains a series of temporal events. First, PDGF triggered the tyrosine phosphorylation of PDGF receptors, which led to activation of both PI 3-kinase and Ras. Both Akt and Raf-1 translocate from the cytoplasm to the plasma membrane and become targets of other upstream activating kinases, including PDK1 and PKC. Raf-1 activity would then be expected to decline, in part because of the inhibition from Akt. Phosphorylations of Akt and Erk were respectively considered the output of PI 3-kinase/Akt and Ras/Raf/MEK/Erk pathways.

Our results are consistent with the idea that the PDGF receptors and Ras-GTP modulate PI 3-kinase activity in a cooperative fashion (Rodriguez-Viciana et al., 1994; Chan et al., 2002; Kaur et al., 2005). Constitutively active Ras G12V resulted in elevated Akt phosphorylation in both the absence of PDGF and PDGF treatment, when dominant-negative

Ras S17N severely reduced PDGF-dependent Akt phosphorylation (Fig. 3.2), suggesting Ras is required for full activation of PI 3-kinase/Akt. In our system, Ras G12V induced slightly sustained Akt phosphorylation at low (0.1 nM) PDGF concentration (Fig. 3.6A) and Ras S17N may impaired the ability to maintained Akt phosphorylation in high PDGF concentration (0.3 and 1 nM) (Fig. 3.7A).

The relevance of PI 3-kinase for Erk activation is complicated. Several studies showed that PI 3-kinase may play multiple role in regulation of the Erk pathway (Scheid and Woodgett, 2000), both positive and negative, dependent on the input signal and the strength and duration of Akt and Raf-1 (Rommel et al., 1999; Sutor et al., 1999; Wennstrom et al., 1999; Zimmermann and Moelling, 1999; Craddock et al., 2001; Reusch et al., 2001; Moelling et al., 2002). Our results demonstrated that Erk stimulation, at low but not high concentrations of PDGF, is inhibited by PI 3-kinase-inhibitory drug (Fig. 3.3).

The relevance of Akt on Raf activity is not fully understood. Transient Erk phosphorylation stimulated by different concentrations of PDGF displays a bell-shaped dose-response curve, implying a balance in signal magnitudes between Akt and Raf-1. Comparing with control cells (Fig. 3.5A), Ras S17N cells postponed and moderated the peak of Akt phosphorylation at low PDGF concentration (Fig. 3.7A), and the succeeding decrease of Erk phosphorylation was delayed (Fig. 3.7C). The balance between Akt and Erk phosphorylation may be controlled by the magnitude and duration of Akt and Raf-1. The inhibitory of Akt on Raf could be observed under low, and probably more physiologically relevant, concentrations of PDGF. High PDGF concentrations, or Ras G12V may be sufficient to maintain the sustained Erk phosphorylation and thus no significant Akt-Raf cross-talk was observed.

Our result demonstrated that PDGF-induce DNA synthesis involved the functions of Ras, PI 3-kinase, and MEK. The relevance of Akt on PDGF-induced DNA synthesis is not fully understood, leading the question open for further investigation.

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