Roles and Regulation of NF-xB in Platelet-Derived Growth Factor Transformed Cells

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ROLES AND REGULATION OF NF-κB IN PLATELET-DERIVED GROWTH FACTOR TRANSFORMED CELLS

by

Takeshi Shimamura

A Dissertation
Submitted to the
Faculty of The Graduate College
in partial fulfillment of the
requirements for the
Degree of Doctor of Philosophy
Department of Biological Sciences

Western Michigan University
Kalamazoo, Michigan
December 2002
Platelet-derived growth factor (PDGF) is overexpressed in various cancer cells and the overexpression may be correlated with the prognosis of several types of cancers. Binding of PDGF to its receptors induces receptor dimerization and subsequent auto-phosphorylation of tyrosine residues in the receptor's cytoplasmic domains. The phosphorylated tyrosine residues interact with the secondary signaling molecules to initiate signaling cascades that trigger cellular changes by affecting downstream effectors. In our recent publication, we have shown that a NF-κB mediates the transformation of mouse fibroblast cells overexpressing PDGF B chain. Though there is a significant correlation between PDGF stimulation and NF-κB activity, it is still unclear what secondary signaling molecules and downstream targets regulate NF-κB activity in the cell. In the glioblastoma cell line U87-MG which is known to overexpress the PDGF B chain and the PDGF beta receptor, NF-κB activity is significantly increased. A dominant negative mutant of PDGF beta receptor missing five intracellular tyrosine residues, which is unable to activate multiple secondary signaling molecules significantly, decreased NF-κB activity when it was introduced into U87-MG cells. When one of the five tyrosine residue is added back so that the
negative PDGF beta receptor can only activate PI3-K pathway, NF-κB activity returned to the level that was normal for U87-MG the cells. In addition, treating U87-MG cells with chemical inhibitors of the phosphatidylinositil 3 kinase (PI3-K) pathway resulted in the significant inhibition of NF-κB activity in a dose-dependent manner. The use of short interfering RNA (siRNA) against p110 kinase of PI3-K to suppress the expression of p110 kinase also resulted in the inhibition of PI3-K pathway activity and NF-κB activity. The study presented here demonstrates that the PI3-K pathway is one of the pathways that controls NF-κB activity in U87-MG cells. Elucidating the exact signaling cascades that mediate NF-κB activity upon PDGF stimulation will unveil a significant mechanism of PDGF induced transformation, which may lead to the discovery of novel molecular targets to suppress tumors with high PDGF activity.
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ACKNOWLEDGEMENTS

I wish to express my high regard and appreciation to my advisor and mentor, Dr. Bruce E. Bejcek for his guidance and help throughout this study. I am thankful to Dr. Alexander J. Enyedi, Dr. John R. Geiser, Dr. Silvia Rossbach, and Dr. Bart O. Williams for taking the time to be a part of my dissertation committee. I am also thankful to my master’s degree mentor Dr. Karim Essani for continuous support throughout this study. Thanks also to all faculty, staff, and graduate students in department of Biological Sciences, who provided me with unconditional support when it was necessary. I wish to thank the Graduate College and Western Michigan University for providing me dissertation fellowship to help complete my dissertation. I also appreciate the warm support from my other student colleagues, James McGee, Scott Cross, Santiago Navarro, Kelly Murphy, Patti White, Denise Smith, Thomas Wilson, and Stephanie Barbera. Finally, I would like to acknowledge everyone I have met throughout my life in the United States of America who has all been cordial hosts during this period. I also wish to thank my parents for their continued financial and moral support throughout my graduate studies. I dedicate this dissertation to my mother who, while courageously battling with breast cancer, continued her strong support of my education.

Takeshi Shimamura
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INTRODUCTION

According to the American Cancer Society 555,500 Americans are expected to die from cancer this year which means one out of every four deaths in the U.S. is related to cancer. As cancer clearly poses a serious threat to the public health, both the U.S. government and America’s pharmaceutical companies are putting considerable resources and efforts to discover cures for cancer. In the year 2001 alone, 402 medicines are in different stages of clinical trials for cancer treatment.

Many medicines are designed to target biological mechanisms induced by growth factors, as growth factors are known to play essential roles in many human cancers. Platelet-derived growth factor (PDGF) is a growth factor involved in a variety of normal developmental and physiological processes, but its over-expression has also been correlated with the development of several types of cancers such as glioblastomas, breast cancers, Wilms tumor, and colon cancer (Bronzert et al., 1987; De Jong et al., 1998; Hsu et al., 1995; Nister et al., 1991; Rozengurt et al., 1985). The expression of PDGF appears to be involved in the actual pathology of the tumor and not a fortuitous event. A reduction in tumor cell growth and/or a change in tumor cell morphology has been observed when they are treated by inhibitors of the PDGF receptor tyrosine kinase or made to express dominant negative mutants of the PDGF receptors and PDGF (Shamah et al., 1993; Strawn et al., 1994; Vassbotn et al., 1994). Due to the central role that PDGF plays in many tumors, it has become of increasing
interest to understand what secondary molecules PDGF stimulates to achieve these undesired results. Although several laboratories have investigated what secondary signaling molecule might be involved, there have been no studies investigating the role that NF-κB might play.

Growing evidence suggests that NF-κB and the signaling pathways that are regulating its activation are important for tumorigenesis (Karin et al., 2002). NF-κB has been recognized as an inducible transcription factor that targets a specific sequence of genes involved in the inflammatory and immune responses (Baeuerle, 1991). NF-κB is a family of proteins that belongs to reticuloendotheliosis (REL) family and the discovery that the p50 subunit of NF-κB shows high homology to an oncoprotein of the REL retrovirus (REV-T) (Gilmore, 1999a, 1999b) suggested that NF-κB might participate in the development of cancer. Cancer cells generally lose the ability to control proliferation and the ability to force the cell to the programmed cell death. NF-κB may play a role in bolt of these processes as it has been shown to be involved in cell proliferation by inducing cell cycle protein cyclin D1 expression (Cao et al., 2001; Guttridge et al., 1999; Hinz et al., 1999) and inhibit apoptosis by upregulating the transcription of cellular inhibitor of apoptosis (cIAP), caspase8/FAS associated death domain like IL-1β-converting enzyme inhibitory protein (cFLIP), and members of BCL-2 family (Beg & Baltimore, 1996; Karin & Lin, 2002; Liu et al., 1996; Van Antwerp et al., 1996; C. Y. Wang et al., 1999a; C. Y. Wang et al., 1999b).
The most well-studied NF-κB is a heterotrimer of the proteins NFKB1 (p50), RelA (p65), and IkBα, which primarily exists in the cytoplasm as inactive complex (Baeuerle, 1991). This complex remains in the cytoplasm as the IkB family of proteins masks the nuclear localization signal sequence of the p50/p65 dimer (Ganchi et al., 1992). Upon an appropriate stimulus, two serine residues on IkBα become phosphorylated by one of several different kinases including IkB kinase β (IKK-β), followed by its degradation by the 26S proteasome. Dissociation of IkB from the complex uncovers the nuclear localization signal sequence of the p50/p65 dimer and allows the translocation of the dimer to the nucleus (Finco & Baldwin, 1995; Rice & Ernst, 1993). The p50/p65 dimer binds to its target genes and regulates their transcriptions.

In our recent publication, we have demonstrated that the activity of a transcription factor, NF-κB, was significantly increased in mouse fibroblast cells transformed with the PDGF-B chain (Shimamura et al., 2002). Acetylsalicylic acid (ASA) and salicylic acid (SA), which are known to inhibit the dissociation of a regulatory protein IkB from NF-κB complex in the cytoplasm, significantly reduced NF-κB activity and also reduced focus formation in mouse fibroblasts transformed by the PDGF B chain gene. The inhibition was highly specific, as other non-steroidal anti-inflammatory drugs (NSAIDs), that do not have an effect on NF-κB activity, did not affect focus formation. Furthermore, a similar inhibition on NF-κB activity was observed by the expression of a dominant negative mutant of IkBα that could not be
phosphorylated and hence formed stable complexes with p50/p65, or a mutant of p65, p65AC, that lacked the carboxy terminal transactivating domain. The expression of either the IxBα mutant or p65AC resulted in a reduction of NF-κB activity. The reduction of NF-κB activity was correlated with the decrease in PDGF B chain induced transformation in mouse fibroblasts.

Our results demonstrate that a NF-κB plays a key role in the transformation of mouse fibroblast cells by the PDGF B chain, but the signaling mechanisms to transduce signals from PDGF receptors to NF-κB have not been elucidated in this system and in glioblastoma cell lines dependent on PDGF B chain stimulation.

In order to determine what secondary signaling molecules and downstream pathways are involved in the signaling between the PDGF β receptors (PDGFR) and NF-κB, NF-κB gene reporter assays were performed with the presence of different chemical inhibitors of known pathways including PI3-K inhibitors LY294002 and Wortmannin, a P44/42 Mitogen Activated Protein Kinase (MAPK) inhibitor U0126, and a p38 MAPK inhibitor SB203580. Results have shown that the presence of phosphatidylinositol 3 kinase (PI3-K) inhibitors, LY294002 and Wortmannin, suppressed NF-κB activity in U87-MG cells known to be PDGF B chain dependent for growth and prognosis. The results suggest that PI3-K pathway is involved in the regulation of the NF-κB activity in the U87-MG cells.

Based on the findings, two other experimental approaches were used to test whether PI3-K pathway is important for controlling NF-κB activity in glioblastoma cells. First, RNA interference (RNAi) was used to specifically suppress the
expression of the p110 catalytic subunit of PI3-K enzyme. Short interference RNAs (siRNAs) specifically designed to inhibit the catalytic subunit of the PI3-K pathway were used to identify if suppressing the PI3-K pathway would have an effect on NF-κB activity in glioblastoma cells. Second, dominant negative PDGFβRs that are unable to trigger the signaling through PI3-K were transfected into the cells to examine their effects in regulating NF-κB activity.

Identifying signaling cascades responsible for inducing NF-κB activity upon PDGF stimulation can provide insight to new therapeutic targets for combating cancers. Therapeutic agents to suppress the signaling cascades from activated PDGF receptors to the activation of NF-κB could give a rise to a new array of therapeutic agents for cancer treatments as it may suppress proliferation of cancer cells showing PDGF dependency. This study presented provides insight into the biology of PDGF-dependent cancer cells and creates practical approaches to develop a highly specific anti-cancer agent.
REVIEW OF LITERATURE

Platelet-derived Growth Factor and Tumorigenesis

Cancer cells are characterized mainly by their uncontrolled proliferation and the capability to colonize the body parts composed by normal tissue (Alberts, 1994). Cancer cells arise from normal cells due to the cumulative alterations in a somatic cell's DNA sequence, which can be triggered by many known factors such as exposure of the host to chemicals, ionizing radiation, and viruses (Alberts, 1994).

According to Hanahan and Weinberg, tumorigenesis entails six critical alterations to the normal cells (Hanahan & Weinberg, 2000). These alterations include self-sufficiency in growth signals, insensitivity to growth inhibition, avoidance of apoptosis, immotalization of the cell, sustained angiogenesis, tissue invasion, and metastasis. Platelet-derived Growth Factor (PDGF) fulfills some but not all of these. PDGF is a growth factor that can stimulate cell growth by autocrine, paracrine, and internal autoactivation mechanisms (Bejcek et al., 1989; C. H. Heldin & Westermark, 1999), which is known to prevent apoptosis. Several different types of mutations are responsible for the overexpression of PDGF and the sustained stimulation of cell growth by PDGF can be a critical step in the cellular transformation leading to tumorigenesis. Thus, studying the biology of growth factors such as PDGF has relevance in providing insight in prevention of tumorigenesis and cancer.
Although PDGF is involved in a variety of normal developmental and physiological processes, its over-expression has been correlated with the development of several types of cancers such as glioblastomas, breast cancers, Wilms tumor, and colon cancer (Bronzert et al., 1987; De Jong et al., 1998; Hsu et al., 1995; Nister et al., 1991; Rozengurt et al., 1985). The expression of PDGF may be involved in the actual pathology of the tumor and not an accidental event. PDGF over-expression in mouse fibroblast NIH3T3 cells results not only in focus formation but also in increased proliferation rates (Clark et al., 1993; Wahl et al., 1989). The v-sis oncogenes in two acutely transforming simian sarcoma virus and feline sarcoma virus have been identified as identical to the PDGF gene (Devare et al., 1983). Both v-sis and its cellular counterpart c-sis have been found to transform cells when they are overexpressed in the cells that express PDGF receptors (Bauer et al., 1985) and the sis genes cause tumors in animals (C. H. Heldin & Westermark, 1999). By using inhibitors of PDGF receptor tyrosine kinase activity or dominant negative mutants of the PDGF receptors and PDGF, it has been shown that a reduction in tumor cell growth and/or a change in tumor cell morphology can be induced (Shamah et al., 1993; Strawn et al., 1994; Vassbotn et al., 1994).

Platelet-derived Growth Factor

As isolated from platelets, PDGF exists as 30 kDa hetero- or homodimers of two structurally related but genetically distinct polypeptides designated A and B chains (C.-H. Heldin et al., 1989). The A chain is derived from a gene with seven
exons located on chromosome 7 and includes two differentially spliced variants with 125 or 110 amino acid (aa) residues (Betsholtz et al., 1986; Bonthron et al., 1988; Rorsman et al., 1988). The difference between the long and short forms comes from the result of alternative exon usage, with the long form using 18 aa residues from exon 6, but none from exon 7, and the short form using 3 aa residues from exon 7, but not from exon 6 (Bonthron et al., 1988). It is known that the short form is more abundant in normal cell (Matoskova et al., 1989). The B chain gene consists of 6 exons on chromosome 22 and is identical to the human c-sis gene, which is also homologous to the monkey v-sis (simian sarcoma) oncogene (Johnsson et al., 1984). c-sis encodes for a precursor polypeptides of 241 amino acid residues, which is 27kDa in size. This precursor is proteolytically processed to a 16 kDa, 160 amino acid residue polypeptide (Betsholtz et al., 1986; N. E. Heldin et al., 1993), and finally a 12kDa mature polypeptide with 109 amino acid residues (Collins et al., 1987; Collins et al., 1985; N. E. Heldin et al., 1993; Johnsson et al., 1984; Ostman et al., 1992). PDGF is a potent mitogen for a variety of cells in tissue culture and is closely involved in the process of tumor development (Bronzert et al., 1987; De Jong et al., 1998; Hsu et al., 1995; Nister et al., 1991; Rozengurt et al., 1985). A possible N-linked glycosylation site is known to exist in the mature A chain, but not the B chain (Betsholtz et al., 1986).

Recently, two new PDGF chains have been described. Both the C and D chains are secreted from the cell with a CUB domain in the N-terminus. The CUB domain contains binding sites for extra cellular matrix molecules and the CUB
domain needs to become cleaved off for the C and D chains to be biologically active. The C chains form homodimers that are expressed at the cell surface as transmembrane proteins from which a soluble hormone is secreted upon proteolytic cleavage (X. Li et al., 2000). Although the D chain appears to form soluble secreted homodimers, these homodimers must also be proteolytically activated (Bergsten et al., 2001; LaRochelle et al., 2001). In contrast to C and D chains, the A and B chains are able to form both soluble hetero and homodimers without proteolytic activation after secretion (C.-H. Heldin et al., 1989; C. H. Heldin & Westermark, 1999).

PDGF Receptors

The effects of these isoforms are mediated by two different cell surface receptors, the type α and type β PDGF receptors (PDGFαR and PDGFβR) (Bergsten et al., 2001; Bywater et al., 1988; Deuel, 1988; C.-H. Heldin et al., 1989; C. H. Heldin & Westermark, 1999; LaRochelle et al., 2001). Both receptors are single polypeptides composed of an extracellular region with immunoglobulin-like domains, a single transmembrane region, and an intracellular domain that possesses protein tyrosine kinase activity (Deuel, 1988; C.-H. Heldin et al., 1989). The type α receptor is a 170 kD protein consisting of 1066 amino acids and the type β receptor is a 190 kD protein consisting of 1074 amino acids.

While the type α receptor can bind to the A, B, and C homodimers and also the AB heterodimer, the type β receptor exhibits a more restricted binding pattern being only able to bind to the AB heterodimer and the B and D homodimers (Figure

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1) (Bergsten et al., 2001; C.-H. Heldin et al., 1989; LaRochelle et al., 2001; X. Li et al., 2000). Upon binding of the appropriate ligands, both receptor types dimerize which results in auto-phosphorylation of specific intracellular tyrosine residues by the receptors' tyrosine kinase activity (Bergsten et al., 2001; C. H. Heldin & Westermark, 1999; LaRochelle et al., 2001; X. Li et al., 2000). These tyrosine residues on the receptors serve as docking sites for many proteins through interactions with the src homology 2 (SH2) domains of secondary signaling molecules (Arvidsson et al., 1994; Escobedo et al., 1991a; Escobedo et al., 1991b; Kashishian et al., 1992; Kazlauskas et al., 1993; Kypa et al., 1990; W. Li et al., 1992; Mori et al., 1993; Ronnstrand et al., 1992; Yokote et al., 1996). It has to be noted that homodimeric αα- and ββ receptor complexes trigger different effects in the cells (C. H. Heldin & Westermark, 1999).
Figure 1. Interactions Between PDGF Isoforms and PDGF Receptors.

While PDGF A and B chains are secreted in the biologically active forms, PDGF C and D chains are secreted as inactive forms that require proteolytic cleavage in the proximity of the extracellular matrix. A chain and B chain can form either homo- or hetero-dimers while C and D chain can from only homo dimmers. α and β receptors can form either homo- or hetero- dimers upon ligand (PDGF) binding.
The rat *neu* gene product NEU has been found to be identical to the epidermal growth factor receptor (Weiner et al., 1989). The receptor tyrosine kinase NEU can be oncogenic if a single amino acid in the transmembrane domain is mutated (Bargmann et al., 1986a, 1986b; L. M. Petti et al., 1998; L. M. Petti et al., 1997). The single amino acid substitution was later found to cause receptor dimerization, which ultimately caused constitutive receptor tyrosine kinase activity (Smith et al., 1996; Weiner et al., 1989). A substitution of the insulin transmembrane domain with the mutant c-NEU transmembrane domain (NEU*) was shown to constitutively activate the insulin receptor kinases (Yamada et al., 1992). It has been shown that a chimeric PDGF β receptor including the transmembrane domain of constitutively activated NEU (erb-2) not only constitutively phosphorylated the receptor tyrosine residues but also transformed Ba/F3 cells to growth factor independence and morphologically transformed mouse C127 cells (L. Petti et al., 1991) and p185neu* transmembrane domain in the PDGFβR has been demonstrated to facilitate receptor homodimerization, thus inducing tyrosine autophosphorylation followed by association with important secondary signaling molecules and possible transforming activity (L. M. Petti et al., 1998).

In addition, naturally occurring chimeric PDGF β receptors have been identified in chronic myelomonocytic leukemia cells (Golub et al., 1994). In the chimeric receptors, the cytosolic and transmembrane domains were fused to the dimerization domain of the transcription factor TEL (Golub et al., 1994) or Huntingtin interacting protein 1 (Ross et al., 1998). These naturally occurring
chimeric PDGF receptors are shown to constitutively phosphorylated on their tyrosine residues on the intracellular domains resulting growth factor independent proliferation of hematopoietic cells (Carroll et al., 1996; Ross et al., 1998).

Secondary Signaling Molecules and the PDGF Receptor

Several secondary signaling molecules have previously been shown to be important for PDGF to induce either focus formation or colony formation in soft agarose. By introducing PDGF receptors that contained multiple point mutations in tyrosine residues into cells from the Patch mouse that lack PDGF type α receptors, it was demonstrated that either phosphatidylinositol 3-kinase (PI3-K) or Phospholipase C γ (PLC-γ) were sufficient for colony formation (DeMali et al., 1997). However, it has also been demonstrated that dominant negative mutants of both the Ha-ras protein and protein kinase Cδ (PKCδ) interfere with the ability of PDGF to stimulate foci and soft agar colony formation in NIH3T3 cells (W. Li et al., 1996). Furthermore, the PKCδ mutant did not interfere with Ha-ras mediated stimulation of mitogen activated protein kinase (MAPK) but did inhibit stimulation of the transcription of genes that were responsive to 12-O-Tetradecanoylphorbol 13-acetate (TPA) (W. Li et al., 1996).

Once intracellular tyrosine residues of PDGF receptors are phosphorylated by PDGF binding to the receptor, these residues are able to associate with a variety of secondary signaling molecules including GRB2 (Arvidsson et al., 1994), GRB7 (Yokote et al., 1996), NCK (W. Li et al., 1992), ras GTPase activating protein (rasGAP) (Kashishian et al., 1992), phosphatidylinositol 3 kinase (PI3K) (Escobedo 13

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et al., 1991b; Kashishian et al., 1992), members of the src family (Kypta et al., 1990; Mori et al., 1993), SHP-2 (Kazlauskas et al., 1993), phospholipase Cγ (PLCγ; Ronnstrand et al., 1992), and receptor-like protein tyrosine phosphatase DEP-1 (Persson et al., 2002). The specific tyrosine residues on PDGF β receptors that have been reported to associate with the secondary signaling molecules mentioned above are summarized in both Table 1 and Figure 2. Signaling subsequent to the PDGF receptor activation is modulated intracellularly through interactions with various signaling cascades.
Table 1

Secondary Signaling Molecules Associate Tyrosine Residues on PDGF β Receptor

<table>
<thead>
<tr>
<th>Secondary Signaling Molecule</th>
<th>Tyrosine Residues on PDGF β Receptor</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRB2 and GRB7</td>
<td>716/775</td>
<td>(Yokote et al., 1996) (Schlessinger, 1993) (Arvidsson et al., 1994)</td>
</tr>
<tr>
<td>Nck</td>
<td>751</td>
<td>(W. Li et al., 1992; Nishimura et al., 1993)</td>
</tr>
<tr>
<td>Stat5</td>
<td>579/581/775</td>
<td>(Novak et al., 1996)</td>
</tr>
<tr>
<td>Stat3</td>
<td>?</td>
<td>(Y. Z. Wang et al., 2000)</td>
</tr>
<tr>
<td>ras GTPase activating protein (GAP)</td>
<td>771</td>
<td>(Kazlauskas et al., 1992) (Kashishian et al., 1992)</td>
</tr>
<tr>
<td>phosphatidylinositol 3 kinase (PI3K)</td>
<td>740/751</td>
<td>(Escobedo et al., 1991a; Fanti et al., 1992; Kashishian et al., 1992; Kazlauskas et al., 1992)</td>
</tr>
<tr>
<td>src family (Src)</td>
<td>579/581</td>
<td>(Escobedo et al., 1991b) (Kypta et al., 1990) (Mori et al., 1993)</td>
</tr>
<tr>
<td>Jak family</td>
<td>?</td>
<td>(Valgeirsdottir et al., 1998)</td>
</tr>
<tr>
<td>CrkII</td>
<td>?</td>
<td>(Matsumoto et al., 2000)</td>
</tr>
<tr>
<td>SHP-2</td>
<td>1009/763</td>
<td>(Kazlauskas et al., 1993) (Klinghoffer &amp; Kazlauskas, 1995)</td>
</tr>
<tr>
<td>Phospholipase Cγ (PLCγ)</td>
<td>1021/1009</td>
<td>(Kashishian &amp; Cooper, 1993; Ronnstrand et al., 1992; Valius et al., 1993)</td>
</tr>
<tr>
<td>Shc</td>
<td>579/740/751/771</td>
<td>(Roche et al., 1996) (Yokote et al., 1994)</td>
</tr>
<tr>
<td>Fer</td>
<td>?</td>
<td>(Kim &amp; Wong, 1995)</td>
</tr>
<tr>
<td>DEP-1</td>
<td>1021</td>
<td>(Persson et al., 2002)</td>
</tr>
<tr>
<td>APS</td>
<td>1021</td>
<td>(Yokouchi et al., 1999)</td>
</tr>
</tbody>
</table>

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Studies elucidating secondary signaling molecules that associate with PDGF receptors utilize mutant PDGF receptors whose tyrosine residues are mutated to different amino acids. It has been shown that mutations in PDGF β receptor tyrosine residues 740/751/771/1009/1021 (F5Y) inhibited association of PI-3K, GAP, SHP-2, and PLC-γ to the receptor, which resulted in the inhibition of PDGF stimulated DNA synthesis and sphingosine kinase activity (Olivera et al., 1999). Substitution of tyrosine residues on the cytoplasmic domain with different amino acids has been used to identify secondary signaling molecules associating with PDGF receptors. Lysine residue 634 in PDGF β receptor was substituted with arginine and the mutant receptor was unable to activate its kinase upon PDGF stimulation (Olivera et al., 1999). In addition, the F5Y mutant was unable to associate with PLC-γ, PI-3K, GAP, and SHP-2, and failed to activate sphingosine kinase and DNA transcription upon PDGF stimulation (Olivera et al., 1999). In the following sections, signaling pathways known to be downstream of PDGF β receptors are discussed in detail.

**Phospholipase C-γ**

Phospholipase C-γ (PLC-γ) is known to associate with tyrosine 1021 and 1009 residues of active PDGFBR (Kazlauskas et al., 1993; Klinghoffer & Kazlauskas, 1995). PLC-γ is known to use Phosphatidylinositol (PtdIns)-4,5-P₂ as substrate and produces inositol-1,4,5-P₃ and diacylglycerol. PLC-γ also stimulates the release of Ca²⁺ from internal storage to activate protein kinase C (PKC) (Berridge, 1993). It has
been shown that PDGF mediated activation of PLC-γ is not directly responsible for
cell growth or cell motility in most cell types, but PLC-γ has been shown to
upregulate cell motility and growth in some cells (Kamat & Carpenter, 1997).

**Src**

Src is known to bind tyrosine residues 579 and 581 of active PDGFβR
(Escobedo et al., 1991b; Kypta et al., 1990; Mori et al., 1993). Src family tyrosine
kinases possess a src homology 3 (SH3) domain, a SH2 domain, and a catalytic
center. The association of the SH2 domain with autophosphorylated PDGF receptor,
dephosphorylation of C-terminal tyrosine phosphorylation, and phosphorylation of
other tyrosine residues causes the activation of Src, which seems to play essential role
in PDGF-mediated mitogenesis (Erpel & Courtneidge, 1995).

**Grb2/Sos1**

Grb2 is an adaptor molecule known to associate with activated PDGFβR
tyrosine residues 716 or 775 via its SH2 domain (Arvidsson et al., 1994;
Schlessinger, 1993; Yokote et al., 1996). Grb2 also contains two SH3 domains that
provide binding sites for a guanidine nucleotide exchange factor Sos known to
activates Ras (Schlessinger, 1993). As Ras has been reported to bind serine/threonine
kinase Raf which activate the mitogen-activated protein kinase (MAPK) pathway,
Grb2/Sos seems to take a part in PDGF stimulation of cell growth, migration, and
differentiation (Hu et al., 1995; Rodriguez-Viciana et al., 1994).
Fer

Fer belongs to the c-Fer and c-Fes family of tyrosine kinases that contain SH2 domain (C. H. Heldin et al., 1998). C-Fer is known to co-immunoprecipitate with PDGF β receptor, but its function and regulation have not been elucidated (Kim & Wong, 1995).

Stat

Stat isoforms have been reported to play roles in cytokine mediated signal transduction and Stat 1, 3, and 5 are known to associate with PDGF β receptor (Choudhury et al., 1996; Valgeirsdottir et al., 1998; Vignais et al., 1996; Yamamoto et al., 1996). Upon binding to phosphotyrosine, Stat isoforms have been shown to be activated and translocated to the nucleus to bind specific target sequences as a homo- or hetero-dimers (Novak et al., 1996). Although the exact role of how PDGF activates Stat has not been elucidated, Stat in conjunction with Jak kinases have been shown to play a role in PDGF-mediated mitogenesis (Novak et al., 1996).

SHP-2

SHP-2 is an universally expressed tyrosine phosphatase and known to bind to activated PDGFβR phosphotyrosine residues with different affinity. SHP-2 may aid in PDGF mediated MAPK activation (Shi et al., 1998) and activation of Ras by
binding to Grb2 (W. Li et al., 1994), but it has also been shown that SHP-2 expression can suppress the PDGF induced transformation (Jazayeri et al., 2000).

GAP

RasGAP contains two SH2 domains and that binds to phosphorylated tyrosine 771 on PDGF β receptor (Fantl et al., 1992; Kashishian et al., 1992; Kazlauskas et al., 1992) and it has not been shown to associate with PDGF α receptor (C. H. Heldin et al., 1998). GAP is a GTPase to activate Ras but its function has not been fully elucidated. It has been known that PDGF-mediated Ras activation is much more efficient in the cells with GAP activity suppressed than normal cell (van der Geer et al., 1997) and it has been known that expressing PDGF β receptor with several tyrosine mutations except tyrosine 771 in the cells is known to decrease PDGF mediated mitogenesis (Valius et al., 1995). The evidence suggests that PDGF activated GAP somehow negatively controls PDGF-mediated Ras activation.

Phosphatidylinositol 3-Kinase

The PI3-K pathway utilizes phospholipids as secondary signaling molecules to transduce signals from the periphery of the cell to the interior of the cell (Fruman et al., 1998; Vanhaesebroeck et al., 1997a). The membrane lipid phosphatidylinositol (PtdIns) is composed of fatty acids, triglycerides, and a head group with five hydroxyl groups (Figure 3). The hydroxyl groups are subject to phosphorylation by PI3-K family of kinases (Fruman et al., 1998; Vanhaesebroeck et al., 1997a). So far, three
classes of PI3-K have been identified and classified based on sequence homology and 
substrate preference (Domin & Waterfield, 1997). Class I PI3-K phosphorylates 
PtdIns-4,5-bisphosphate in vivo and also phosphorylate PtdIns-4-P in vitro (Rameh et 
al., 1997a; Rameh et al., 1997b; Zhang et al., 1997). Class II PI3-K is known to 
phosphorylate PtdIns and PtdIns-4-phosphate (Rameh et al., 1997a; Rameh et al., 
1997b; Zhang et al., 1997). Class III PI3K uses PtdIns as substrate. The term D-3 
phosphoinositides is used for the lipid products produced by PI3-K (Rameh et al., 
1997a; Rameh et al., 1997b; Zhang et al., 1997). D-3 inositides work as secondary 
signaling molecules whose functions include the recruitment of cytoplasmic proteins 
to the cell membrane and the alteration of the activity of the cytoplasmic proteins 
(Fruman et al., 1998; Vanhaesebroeck et al., 1997a).
Figure 3. The Structure of Phosphatidylinositol.

Hydroxyl group on the third position of the head group is subject to phosphorylation by PI3-Kinase. Phosphate joins the head group with fatty acid tail.
The activation of growth receptors is known to activate the phosphatidylinositol 3-kinase (PI3-K) pathway. The current model for the PDGFβR mediated activation of the class IA PI3-K pathway is that the phosphorylated tyrosine 740 and 751 directly or indirectly associates with the SH2 domains of the regulatory protein p85 (Escobedo et al., 1991a; Fantl et al., 1992; Kashishian et al., 1992; Kazlauskas et al., 1992). This association triggers the dissociation of p110 catalytic subunit allowing p110 to migrate to the plasma membrane to contribute in the production of D-3 phosphoinositides (Fruman et al., 1998; Vanhaesebroeck et al., 1997a). In addition to the growth factor receptor mediated induction of PI3-K activity, several secondary signaling molecules are known to stimulate PI3-K pathway. Activated G proteins are known to activate class I PI3K pathways (Rodriguez-Viciana et al., 1994). Among the G proteins, the small G protein Ras is known to directly associate with p110 catalytic subunit of class IA and class IB PI3-Ks (Fruman et al., 1998; Vanhaesebroeck et al., 1997a). In addition, the βγ subunits of G proteins have been shown to activate class IB PI3K (L. Stephens et al., 1994; Stoyanov et al., 1995; X. Tang & Downes, 1997).

Among the three classes of PI3-K, Class I PI3-K is the best characterized. The functional class I PI3-K exists as a heterodimer that consists of a p110 catalytic subunit and a regulatory subunit. Furthermore, the class I PI3-K is subdivided into classes IA and IB based on the type of regulatory subunit that is present (Songyang et al., 1993). The mammalian class IA PI3-K is composed of one p110 catalytic subunit α, β, and δ that associates with a regulatory subunit that contains two src-homology 2
(SH2) domains in the carboxy (C) terminus (Domin & Waterfield, 1997). Several reports indicate that the δ isoform is abundant in leukocytes (Domin & Waterfield, 1997; Vanhaesebroeck et al., 1997b), therefore the δ isoform is presumably absent in the cells of astrocyte origin and fibroblasts. There are three genes that are known to encode five different regulatory subunit of class IA PI3-K (Domin & Waterfield, 1997). Five isoforms of the regulatory subunit are derived from three genes and they are named as p85α, p85β, p55α, p55γ, and p50α depend on the size of the regulatory protein (Vanhaesebroeck et al., 1997a). Among the isoforms, p85α contains not only two SH2 domains but also a N-terminus src-homology 3 (SH3) domain, two proline rich regions, and a RhoGAP homology domain. The p55α and p50α isoforms lack the N-terminal SH3 domain, one proline rich domain, and a RhoGAP homology domain (Vanhaesebroeck et al., 1997a). A domain located between the two SH2 domains is inter SH2 domain, that participates in binding of the p110 catalytic subunit of class IA PI3K (Vanhaesebroeck et al., 1997a). All catalytic subunits of class IA p110 include a domain for Ras binding, a phosphatidylinositol kinase (PIK) domain, a catalytic domain and a domain for binding to regulatory adapter proteins (Domin & Waterfield, 1997). The PIK domain is unique to the kinases that belong to the PI3-K family and it is thought to be involved in substrate presentation (Domin & Waterfield, 1997). The regulatory protein of class IA PI3K is known to increase lipid kinase activity by a few fold upon activation and contributes for the recruitment of lipid kinase to the proximity of the cell membrane (Backer et al., 1992; Carpenter et al., 1993).
The class Ib PI3K includes the catalytic subunit p110 γ and a regulatory subunit of 101kDa that lacks any homology to the regulatory subunit with SH2 domains (L. R. Stephens et al., 1997). It has been reported that the small G protein Ras regulates class Ib PI3-K (Rubio et al., 1997).

The role of PI3K is to increase the cellular concentration of PtdIns-3,4-P\(_2\) and PtdIns-3,4,5-P\(_3\), which are scarce in the normal cells that are rich in PtdIns-4-P, PtdIns-4,5-P\(_2\), and PtdIns-3-P (L. R. Stephens et al., 1991; Toker et al., 1995). The activation of the PI3-K pathway, causes an increase in PtdIns-3,4-P\(_2\) and PtdIns-3,4,5-P\(_3\) concentration in the cell, where they work as secondary messengers (L. R. Stephens et al., 1991; Toker et al., 1995). Targets of PtdIns-3,4-P\(_2\) and PtdIns-3,4,5-P\(_3\) include proteins that contain pleckstrin-homology (PH) domains (Lemmon et al., 1996; Rameh et al., 1997a; Touhara et al., 1994), SH2 domains (Bae et al., 1998; Rameh et al., 1995), protein kinase C (PKC) (Newton, 1997), clathrin adaptor proteins (Rapoport et al., 1997), and actin regulatory proteins (Hartwig et al., 1996; Sohn et al., 1995; Yu et al., 1992).

Among the downstream effectors of PI3-K signaling, the Akt kinase has been extensively studied due to its roles in activating molecules essential for cell survival (Burgering & Coffer, 1995; Franke et al., 1995). The Akt kinase contains a PH domain that is required to receive the PI3-K signaling (Franke et al., 1995; Klippel et al., 1996). The full activation of Akt kinase seems to require not only the binding of D-3 inositides to the PH domain, but also the phosphorylation of threonine 308 and serine 473 by phosphoinositide-dependent kinase -1 (PDK-1) (Alessi et al., 1996).
PtdIns-3,4-P$_2$ and PtdIns-3,4,5-P$_3$ have been shown to potentiate the ability of PDK-1 to phosphorylate the Akt kinase (Alessi et al., 1997; Stokoe et al., 1997). The activated Akt kinase has been shown to activate Bad responsible for the inhibition of apoptosis (S. R. Datta et al., 1997; del Peso et al., 1997), Ikk $\alpha$ and $\beta$ responsible for phosphorylating I\(\kappa\)B\(\alpha\) to activate NF-\(\kappa\)B (Factor et al., 2001; Madrid et al., 2000; Ozes et al., 1999; Romashkova & Makarov, 1999), glycogen synthase 3 $\alpha/\beta$ (GSK3 $\alpha/\beta$) to inhibit gluconeogenesis (Lochhead et al., 2001; Pap & Cooper, 1998), mammalian target of rapamycin (mTOR) to initiate DNA and protein synthesis (Peyrollier et al., 2000; Somwar et al., 1998), and Forkhead Drosophila homolog 1 rhabdomyosarcoma (FKHR) to initiate transcription of genes required for the inhibition of apoptosis (Brunet et al., 1999; Shin et al., 2001; E. D. Tang et al., 1999; Uddin et al., 2000) (Figure 4). PDGF B chain stimulation of swiss NIH3T3 cells has been shown to cause Akt activation and subsequent NF-\(\kappa\)B activation through Ikk-I\(\kappa\)B\(\alpha\) activation (Romashkova & Makarov, 1999).

Among the four p110 isoforms, the $\alpha$ and $\beta$ isoforms have been well-described in these literature and the two isoforms are known to be most catalytically active among the isoforms. Growing evidence suggests that p110$\alpha$ and p110$\beta$ have different roles in the cells, which is supported by the finding that p110$\alpha$ knockout is lethal despite the fact that p110$\beta$ is expressed (Bi et al., 1999). An mRNA sequence comparison between human phosphatidylinositol-3-kinase alpha subunit (PIK3CA, gi 20535400) and human phosphatidylinositol-3-kinase beta subunit (PIK3CA, gi
5453893) with BLAST search shows very low homology. p110β but not p110α synergistically associates with Gβ subunit for activation (Kurosu et al., 1997). p110α seems to be involved in cell growth as overexpressing p110α in 3T3-L1 adipocytes increases mitogenesis (Frevert & Kahn, 1997). In addition, suppressing p110α results in the decrease in growth factor mediated mitogenesis in several cell types (Roche et al., 1998; Siddhanta et al., 1998; Vanhaesebroeck et al., 1999). PDGF mediated mitogenesis does not seem to require p110β (Roche et al., 1998).
Figure 4. PI3-K Pathway is a Downstream Pathway of Growth Factor Receptors.

In a case of PDGFβ receptor, phosphorylation of tyrosines 740 and 751 activates the PI3-K pathway. Downstream effectors includes kinases (gray) and transcription factors (dark gray), which leads to cell growth and survival.
PDGF-Induced Gene Expression

In order to determine the PDGF β receptor activated signaling pathways and the transcriptional induction of immediate early genes (IEGs), Fambrough et al. have used gene expression array to find 66 IEGs induced by PDGF β receptor signaling in mouse fibroblast NIH3T3 cells (Fambrough et al., 1999). The F5Y mutant PDGF β receptors lacking tyrosine residues known to be used for binding and activation of the PLCγ, PI3K, SHP2, and RasGAP pathways still managed to induce 64 of these IEGs (Fambrough et al., 1999). Elimination of the Grb2-binding site further reduced induction of IEGs. When a mutant PDGF β receptor whose RasGAP-binding site was added back induced genes that were normally induced by interferons (Fambrough et al., 1999). Among the IEGs, many transcription factors were induced within 20 minutes to 2 hour post-stimulation of the receptors. Among the transcription factors, c-jun, JunB, and c-fos gene expressions was induced within 20 minutes post-stimulation and NF-κB related gene expression was upregulated within 2 hours post stimulation (Fambrough et al., 1999). This finding, together with the fact that NF-κB activity is high in several different types of tumor cells that overexpress PDGF B chain including glioblastoma (Bhat-Nakshatri et al., 2002; Murphy et al., 2001; Sliva et al., 2002), suggests that NF-κB may play essential roles in PDGF B chain and PDGFβR mediated transformation.
Transcription Factor NF-κB

NF-κB belongs to the NF-κB/Rel family of transcription factors that includes p50 (NFκB1), p65 (RelA), p52(NFκB2), c-Rel, and RelB. The NF-κB/Rel family of protein share approximately a 300 amino acid N-terminal Rel homology domain (RHD) that contains DNA binding and dimerization domains and the nuclear translocation signals (NLS) (Verma et al., 1995). NF-κB binds to the κB motif of a target gene promoter region to initiate transcription of the gene. The consensus κB motif has been elucidated as GGGRNNYYC (R = purines, Y = pyrimidines, and N = any nucleotide) (Baeuerle, 1991) and the κB motif exists in the regulatory part of genes responsible for immune responses, regulatory functions of NF-κB, growth control, cell metabolism and cell adhesion (Verma et al., 1995). Binding of NF-κB binding sequence is also dependent on sequences surrounding the binding site.

Most members of the NF-κB/Rel family can form homo- or hetero- dimers except RelB that only forms heterodimers with either p50 or p52 (Verma et al., 1995). Although the p65-p50 heterodimeric complex of the NF-κB molecule is the most abundant in nuclei, existence of homodimer p50-p50 or p65-p65 has been reported (Baeuerle, 1991). p50-p50 or p52-p52 homodimers can translocate into the nucleus due to the presence of a nuclear translocation signal on p50 and p52 molecules (Baeuerle, 1991) and can bind to the 5' GGG of NF-κB binding motif (Urban & Baerle, 1990), but the homodimers are unable to initiate transcription due to a lack of transcriptional activation (trans-activation) domain (Verma et al., 1995).
Several reports indicate that p50 and p52 subunits associate with nuclear IκB Bcl-3 to enhance transcription initiation in several cell types (Bundy & McKeithan, 1997; Cogswell et al., 2000; Dechend et al., 1999; Heissmeyer et al., 1999). c-Rel and p65 contain both RHD and transcription activation domains and the homodimeric forms of these proteins are capable of binding to κB motif of target genes and initiating transcription of the target genes.

As shown in Figure 5, a current model suggests that inactive NF-κB exists in the cytoplasm primarily as a heterotrimer of the proteins p50, p65 and IκBα. This complex remains in the cytoplasm due to the masking of the nuclear localization signal sequence of the p50/p65 dimer by the IκB family of proteins that are rich in ankyrin repeats (Ganchi et al., 1992). Upon an appropriate stimulus, IκBα becomes phosphorylated at serine 32 and 36 by one of several different kinases including IκB kinase β (IKK-β), causing its ubiquitination and eventual degradation by the 26S proteosome (Verma et al., 1995). When IκB dissociates from the p50/p65 complex, the active p50/p65 dimer translocates to the nucleus, binds to its target sequences, and regulates transcription (described in Figure 5) (reviewed in (Finco & Baldwin, 1995; Rice & Ernst, 1993)).

IKK-β seems to be a key regulator of NF-κB as acetylsalicylic acid (ASA) has been shown to inhibit cell growth by inhibiting IKK-β which results in the decrease in activation of NF-κB (Yin et al., 1998) (Figure 6). Epidemiological studies have indicated that nonsteroidal anti-inflammatory drugs (NSAIDs) such as ASA can
decrease mortality 40%-50% in individuals with colorectal cancers (Smalley & DuBois, 1997) and reduce colon cancer cell growth (Tsujii et al., 1998). ASA have several known functions within cells. Both can irreversibly acetylate both isoforms of prostaglandin synthase (PGHS) (Bhattacharyya et al., 1995; Mancini et al., 1997; Roth et al., 1983) causing a reduction in the synthesis of prostaglandins (Castano et al., 1997; Shier & Durkin, 1982).
Figure 5. A Current Model of the p50-p65 NF-κB Regulation.

Upstream signals from growth receptors initiate the dissociation of Ikkγ from an Ikkα and Ikkβ complex. Ikkα and Ikkβ phosphorylate serine 42 on a regulatory protein IκBα. IκBα then releases the p65-p50 heterodimer of NF-κB. Active NF-κB translocates into the nucleus and initiates transcription.
Figure 6. Inhibition of the NF-κB Activity by Aspirin.

Aspirin is known to inhibit an upstream kinase, Ikkβ, responsible for phosphorylating the IκBα regulatory protein causing it to release the p65-p50 heterodimer.
Nuclear Factor -κB (NF-κB) is a transcription factor that regulates the expression of a variety of cellular genes (Perkins et al., 1992) and is involved in focus formation by the Ha-ras and the abl oncogene. It is also activated in breast tumors, glioblastomas, and non-Hodgkins lymphomas (T. S. Finco et al., 1997; Murphy et al., 2001). It has recently been reported that NF-κB can stimulate the expression of the Cyclin D1 gene and that this is a central feature of movement from G1 into S phase (Guttridge et al., 1999; Hinz et al., 1999). Several reports have indicated that NF-κB can be activated by PDGF (Olashaw et al., 1992; Romashkova & Makarov, 1999). Although NF-κB plays a central role in transformation induced by Ha-ras and Bcr-abl (T. Finco et al., 1997; Reuther et al., 1998), nothing is known concerning its role in transformation by PDGF.

Post-transcriptional gene silencing (PTGS)

Post-transcriptional gene silencing (PTGS) is also referred as "co-suppression" in plants, "RNA interference" in Drosophila and C. elegans, and "quelling" in fungi. The finding of the RNA interference phenomenon stems from a rather interesting accident (Gura, 2000). Researchers were trying to overexpress a gene coding for an enzyme responsible for the pigmentation of the petunias in an attempt to produce purple petunias. Strangely, the overexpression resulted in the production of petunias with virgin white or variegated flowers (Gura, 2000). Interestingly enough, some of the phenotype could not be passed down to the generations suggesting the phenomenon pertained to RNA modification (Gura, 2000).

It is known that dsRNAs less than 30 nucleotides in length do not induce the PKR kinase pathway. As 21- to 22- nucleotide long siRNAs can induce RNA interference in *Drosophila* embryo lysates (Elbashir *et al.*, 2001b), introduction of siRNAs to mammalian cells was performed to see if it could induce gene-specific silencing (Elbashir *et al.*, 2001a). siRNAs transiently transfected to mammalian cultured cells resulted in up to 90% reduction in target RNA and protein levels (Caplen *et al.*, 2001; Elbashir *et al.*, 2001a; Holen *et al.*, 2002). In addition, the experiments revealed that the most effective siRNAs are 21 nucleotide dsRNAs with 2 nucleotide 3' overhangs. It was discovered that siRNA activity is very sequence specific such that single base pair mismatches between the siRNA and a target mRNA significantly reduce silencing (Elbashir *et al.*, 2001a; Elbashir *et al.*, 2001c).
It must be noted that not all siRNAs are always effective in silencing the homologous target genes and the reason is unclear (Elbashir et al., 2001c; Holen et al., 2002).

The exact mechanisms of PTGS are still not clearly understood, but models describing the steps involved in PTGS are beginning to emerge. The first step appears to be the amplification of the double-stranded RNA in the cell. Depending on the cell type, the double-stranded RNA is digested into short stretches of 21-25 nucleotides by the enzyme named DICER, which is a member of the RNase III family of dsRNA specific ribonucleases that progressively cleaves dsRNA in an ATP-dependent manner (Hutvagner & Zamore, 2002; Sharp, 2001). It seems that amplified and digested RNA is around 25 nucleotides in length in plants (Hamilton & Baulcombe, 1999) and between 21 and 23 nucleotides long in Drosophila cells (Zamore et al., 2000) due to further processing of the dsRNA to yield 19-21 bp with each with 2-nucleotide 3' overhangs (Bernstein et al., 2001; Zamore et al., 2000).

Those short dsRNAs are small interfering RNAs (siRNAs) or guide RNAs (Hutvagner & Zamore, 2002; Sharp, 2001). The siRNA duplexes binding to a nuclease complex to form the RNA-induced silencing complex (RISC). An ATP dependent separation of the siRNA duplex seems to be required for activation of the RISC, which targets and cleaves the homologous mRNA approximately 12 nucleotides from the 3' end of the siRNA (Hammond et al., 2001; Hutvagner & Zamore, 2002; Nykanen et al., 2001; Sharp, 2001). Although the exact mechanisms of cleavage have not been fully elucidated, it has been proposed that each RISC is composed of a single siRNA and an RNase that seems to be distinct from Dicer.
(Hutvagner & Zamore, 2002). In addition to the two steps described above, an amplification step by RNA-dependent RNA polymerase (Gura, 2000) has also been observed in some cells.

**U87-MG and U118-MG Cell**

U87-MG and U118-MG glioblastoma (astrocytoma) cells were used to study the role of PI3-K pathway in the PDGF mediated tumorigenicity. There are at least three major advantages of using U87-MG to study the roles of PI3-K. First, the cell has been shown to endogenously express PDGF-B chain and the cell growth is dependent on PDGF expression and its autocrine loop (Chin et al., 1997; Kilic et al., 2000; Pollack et al., 1990; Schilling et al., 1998). Consequently, it provides an ideal model to study the relationship between PDGF mediated activation of PI3-K and NF-κB activity without the exogenous addition of PDGF-B chain. In addition, the observation can be made in a real tumor cell line. Second, the cell does not endogenously express PTEN (phosphatase and tensin homologue deleted on chromosome 10), which is capable of dephosphorylating phosphatidylinositol 3,4,5-triphosphate (Laemmli, 1970), the product of PI3-K (Downes et al., 2001; D. M. Li & Sun, 1998; J. Li et al., 1997). Therefore, the PI3-K activity can be monitored without inhibition by PTEN. The third advantage of using U87-MG cell is that our laboratory has shown that U87-MG exhibit constitutively high NF-κB activity (Murphy et al., 2001).
MATERIALS AND METHODS

Cell Lines

NIH3T3 fibroblast cells and U87-MG cells were obtained from the American Type Culture Collection. NIH3T3 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies, Carlsbad CA) supplemented with 10% calf serum (CS) and antibiotics (penicillin 100 U/ml and streptomycin 100μg/ml) unless otherwise noted. U87-MG cells were maintained in Minimal Essential Medium (MEM; Life Technologies, Carlsbad CA) supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin 100 U/ml and streptomycin 100μg/ml) unless otherwise noted. Lipofections were performed using Lipofectamine, Lipofectamine Plus (Life Technologies, Carlsbad CA), GenePorter (Gene Therapy Systems, San Diego CA), or Fugene 6 (Roche, Indianapolis, IN) according to manufacturer's specifications. A ratio of 2 μg of DNA per 10 μl of lipofection reagent or 2 μg per 6 μl (Fugene 6 only) was used throughout these experiments.

Morphological transformation and transformation inhibition assays were performed as follows. NIH3T3 cells alone or clones of NIH3T3 cells that had been transfected with the PDGF- B chain expressing plasmid and selected for their expression of the PDGF-B chain protein by Western blotting were seeded on 12 well plates at 1×10^4 cells/well or 4×10^4 cells per 60mm dish. When inhibitors were used, they were added in fresh medium immediately after the medium with the DNA was...
removed from the cells. Cells were grown in DMEM supplemented with 10% CS and antibiotics until they were at least 90% confluent. The medium was then changed to DMEM supplemented with 2% CS and antibiotics (with or without inhibitors), and cells were fed every 2-3 days for a period of 10 days. Cells were fixed in either 100% methanol or 100% ethanol before staining with 1% crystal violet.

To determine the effects of IκB67CJ, a dominant negative mutant of IκBα, and p65ΔC, a dominant negative mutant of p65, on PDGF-B chain transformation, plasmids expressing these genes were transfected into NIH3T3 cells expressing the PDGF-B chain. Briefly, NIH3T3 cells were seeded on 8 well plates (4×10⁴ cells/well), and cells were co-transfected with the indicated amounts of the different plasmids as described above. Cells were then grown, fed, and stained as described above.

For Western blotting, NIH3T3 cells and U87-MG were seeded on 60mm dishes (6×10⁴ cells/dish). The NIH3T3 cells were co-transfected with plasmids that expressed PDGF-B chain and p65, p65ΔC, or IκB67CJ. U87-MG cells were transfected with small interference RNA (siRNA) and reporter vector as indicated in sections below.

Inhibitors and Chemicals

Concentrations of inhibitors and chemicals were chosen based on previous work by others (Conte et al., 1997; Goppelt-Struebe et al., 1996; Kopp & Ghosh, 1994; Tsujii et al., 1998). NS398 (Calbiochem, San Diego, CA) was dissolved in
100% DMSO. Prostaglandin E₂ (PGE₂; Biomol, Plymouth Meeting, PA), indomethacin, acetylsalicylic acid (ASA; Sigma, St. Louis, MS), and salicylic acid (SA; Sigma, St. Louis, MS) were dissolved in 100% ethanol. When aspirin was dissolved in cell culture medium, the pH of the medium was adjusted to 7.4.

Antibodies

Several antibodies were used throughout this study. Anti NF-κB p65 CT rabbit polyclonal antibody (Upstate, Lake Plasid, NY) at 2µg/ml for western blot and 5µg for electromobility shift assay (EMSA), anti NF-κB p50 rabbit polyclonal antibody (Upstate, Lake Plasid, NY) at 5µg for EMSA, anti IκBα rabbit polyclonal antibody (Upstate, Lake Plasid, NY) at 0.5µg/ml for western blot, anti p110α mouse monoclonal antibody (Santa Cruz, Santa Cruz, CA) at 1:125 for western blot, anti p110β mouse monoclonal antibody (Santa Cruz, Santa Cruz, CA) at 1:125 for western blot, anti PDGFB receptor rabbit polyclonal antibody (Upstate, Lake Plasid, NY) at 2µg/ml for western blot, anti IκBα rabbit polyclonal antibody (Upstate, Lake Plasid, NY) at 2µg/ml for western blot and at 5µg/ml for immunoprecipitation, anti p110α rabbit polyclonal antibody (Upstate, Lake Plasid, NY) at 4µg/ml for immunoprecipitation, and anti p110β rabbit polyclonal antibody (Upstate, Lake Plasid, NY) at 5µg/ml for immunoprecipitation.
Plasmid Constructs

A fragment of the cDNA of the PDGF-B chain that contained the complete coding region was obtained by digestion of the plasmid pSM-1 (Ratner et al., 1985) with BamHI and then cloned into pCDNA3.I(-) (Invitrogen, Carlsbad, CA) digested with the same restriction endonucleases. The pNF-κBLuc reporter vector with four tandem repeats of the invariant chain li, NF-κB consensus binding site (GGGAATTCC) fused to a TATA-like promoter region from the Herpes simplex virus thymidine kinase gene was obtained (BD Clontech, Palo Alto, CA). A clone that lacked the four tandem repeats of the NF-κB consensus binding site was created by digestion of the original plasmid with KpnI and BgII, followed by treatment with E.coli DNA polymerase I and ligation. The wild type NF-κB p65 gene and a dominant negative mutant of p65, p65ΔC, were the kind gifts of Drs. Alain Israel and Nancy Rice (Kieran et al., 1990; Rice & Ernst, 1993). The pRcCMV vector containing the IkBα mutant, pMEIkB67CJ, expressed an IkBα protein that can not be phosphorylated and therefore forms highly stable complexes with the p50/p65 heterodimers (Whiteside et al., 1995). The p65ΔC gene encodes a p65 subunit that is able to dimerize with the p50 subunit of NF-κB but is unable to stimulate transcription due to the lack of c-terminal activation domain (Grimm & Baueuerle, 1994). RVY and RVY-E5, which can express the bovine papilloma virus E5 gene, were the kind gifts of Dr. Daniel DiMaio (Riese & DiMaio, 1995).
NF-κB Reporter Assays

NIH3T3 Cells and Luciferase Reporter Assay

NIH3T3 cells were seeded on 8 well plates (6×10^4 cells/well). Sixteen to 24 hours after seeding, cells were co-transfected with the indicated amount of the reporter plasmid with the NF-κB binding sequences (NF+) or the plasmid that lacked the NF-κB binding sequences (NF-) and other plasmids as the indicated ratios as described above. Four hours post-transfection, DMEM supplemented with 10% CS and antibiotics was added to the cells, and the cells were incubated for an additional 16-24 hours. Medium was then replaced with fresh DMEM supplemented with 10% CS and antibiotics and incubated another 24 hours, after which the medium was replaced with DMEM supplemented with 1% CS. At 72 hours post-transfection, cells that had been transfected with the growth factor expressing constructs were harvested.

Cells were harvested by washing 2-3 times in phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4·7H_2O, 1.4 mM KH_2PO_4, pH 7.4) and lysed in luciferase assay cell culture lysis buffer (Promega, Madison, WI). Lysates were centrifuged at 10,000 X g for 20 seconds and the pellets were discarded. The lysates were assayed immediately or frozen at -80°C until use. Protein concentrations were determined using the BCA assay (Pierce, Rockford, IL) with bovine serum albumin (Sigma, St. Louis, MO) as a standard. Equal amounts of protein were used for luciferase assays with the Luciferase assay system (Promega, Madison, WI) or LucLite (Packard Bioscience, Boston, MA) according to manufacturers’
recommendations. Assays were performed at least three times, and results were statistically analyzed using analysis of variance (ANOVA) and the Student-Newman-Keuls (SNK) tests.

**NIH3T3 Cells and Secreted Alkaline Phosphatase Assay**

When reporter vectors with secreted alkaline phosphatase were used, NIH3T3 cells were seeded on 24 well plates (2x10^4 cells/well) and allowed to grow in DMEM supplemented with 10% CS until 70% confluent. Cells were transfected with 1μg of the pNF-κBSEAP or pTAL, a negative control plasmid that lacks a NF-κB binding sequence, and pMEIkB67CJ as indicated. The amount of plasmid used for transfections was equalized between treatments by addition of the appropriate amount of pRcCMV. Four hours post-transfection, DMEM supplemented with 10% CS and antibiotics was added to the cells, and the cells were incubated for an additional 24 hours. Medium was then replaced with fresh DMEM supplemented with 10% CS and antibiotics and incubated another 24 hours, after which the medium was replaced with equal amounts of serum free DMEM. At 72 hours post-transfection, supernatants from cells were harvested. The supernatants were centrifuged at 10,000 X g for 20 seconds and the pellets were discarded. The supernatants were assayed immediately or frozen at -80°C until use. To measure the activity of the secreted alkaline phosphatase (SEAP), equal amounts of supernatants were used for SEAP assays with the Great EscAPE SEAP assay system (BD Clontech, Palo Alto, CA) according to the manufacturer's recommendations. Assay results were normalized.
for both protein and βgal to take difference in cell density and transfection efficiency into account. Assays were performed multiple times, and results were statistically analyzed using analysis of variance (ANOVA) and the Student-Newman-Keuls (SNK) tests.

**U87-MG Cells and Secreted Alkaline Phosphatase Assay**

When reporter vectors with secreted alkaline phosphatase were used for measuring NF-κB activity in U87-MG cells, U87-MG cells were seeded on 8 well plates (6×10⁴ cells/well) and allowed to grow in MEM supplemented with 10% FBS until 80% confluent. Sixteen to 24 hours after seeding, cells were triple-transfected with the indicated amount of the reporter plasmid with the NF-κB binding sequences (NF) or the plasmid that lacked the NF-κB binding sequences (pTAL), plasmid coding for β-galactosidase and other plasmids or short interfering RNA (siRNA) as the indicated ratios as described. When U87-MG cells stably transfected with PDGFβR were used, cells were seeded on 8 well plates (8×10⁴ cells/well) and allowed to grow in MEM supplemented with 10% FBS for 24 hours. The cells were co-transfected with the indicated amount of the reporter plasmid with the NF-κB binding sequences (NF) or the plasmid that lacked the NF-κB binding sequences (pTAL) and plasmid coding for β-galactosidase. Four hours post-transfection, MEM supplemented with 10% FBS and antibiotics was added to the cells, and the cells were incubated for an additional 24 hours. Medium was then replaced with fresh MEM supplemented with 10% FBS and antibiotics and incubated another 24 hours.
At 48 hours post-transfection, supernatants from cells were harvested. The supernatants were centrifuged at 10,000 X g for 20 seconds and the pellets were discarded. The supernatants were assayed immediately or frozen at -80°C until use. To measure the activity of the secreted alkaline phosphatase (SEAP), equal amounts of supernatants were used for SEAP assays with the Great EscAPE SEAP assay system (BD Clontech, Palo Alto, CA) according to the manufacturer's recommendations. Assay results were normalized for both protein and βgal to take difference in cell density and transfection efficiency into account. Assays were performed multiple times, and results were statistically analyzed using analysis of variance (ANOVA) and the Student-Newman-Keuls (SNK) tests.

Western Blotting

Western blots were performed as described below. Briefly, 80% confluent monolayers of cells were rinsed 2 times with 1X PBS and then lysed in lysis buffer (20 mM Tris HCl pH 7.5, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium vanadate, 1 mM PMSF, 1 μg/ml leupeptin). Lysates were recovered and centrifuged at 12,000 X g for 10 minutes and the pellets discarded. The protein concentrations in the lysates were measured using the BCA assay (Pierce, Rockford, IL) and equal amounts of protein samples were resolved on a 10 % SDS-PAGE gel (Laemmli, 1970). After electrophoresis, the proteins were transferred to a PVDF membrane. The membrane was blocked with blocking buffer, 0.25 % I-Block (Tropix, Bedford,
MA) in Tris buffered saline with Tween 20 (TBS-T; 19.98mM Tris HCl pH 7.6, 136.75mM NaCl, 0.01 % Tween 20). After 2 hours at room temperature, the membrane was incubated with an antibody directed against target protein in blocking buffer. The membrane was washed twice for 5 minutes in TBS-T followed by a wash for 10 minutes in TBS-T. The membrane was then incubated with anti-rabbit antibody conjugated to horseradish peroxidase (Amersham Biosciences, Piscataway, NJ). The blot was then washed as described above before incubation with the ECL reagent (Amersham Biosciences, Piscataway, NJ) and exposed to Kodak X-Omat film (Eastman Kodak, Rochester, NY).

Preparation of Nuclear Extract

Nuclear extracts were prepared as described previously (Murphy et al., 2001; Paulose et al., 1998). Cells were cultured in 150mm dishes with DMEM supplemented with 10% calf serum and allowed to achieve approximately 80% confluence. After washing cells with 1X PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4*7H2O, 1.4 mM KH2PO4, pH 7.4), cells were removed from the dishes by trypsin and centrifuged at 2000×g for 5 minutes at 4°C. The supernatant was discarded, and the cell pellet was resuspended in 1ml buffer A (10mM HEPES, pH7.9, 1.5mM MgCl2, 10mM KCl, 0.1% NP-40, 1mM NaF, 1mM phenylmethylsulfonylfluoride (PMSF, Sigma, St. Louis, MO) and 0.5mM DL-Dithiothreitol (DTT, Sigma, St. Louis, MO) and centrifuged again at 2,000×g for 5 minutes at 4°C. The supernatant was decanted and the cell pellet was completely
resuspended in 80μl of buffer A with 0.1% triton x-100 (Sigma, St. Louis, MO). The suspension was then incubated on ice for 30 minutes and centrifuged at 2,000×g for 5 minutes at 4°C. The supernatant was discarded, and the resultant nuclear pellet was resuspended in 60μl buffer C (20mM HEPES, pH7.9, 0.42M NaCl, 25% (v/v) glycerol, 1.5mM MgCl₂, 1mM NaF, 1mM PMSF, and 0.2mM EDTA). The suspension was incubated on ice for 30 minutes followed by centrifugation at 16,000×g for 20 minutes at 4°C. The resultant supernatant was the nuclear extract. Total protein concentration of the extract was measured immediately after preparation by using the colorimetric bicinechoninic acid (BCA) assay (Pierce, Rockford, IL), and the nuclear extract was stored at -80°C until use.

Electrophoretic Mobility Shift Assay

Electrophoretic Mobility Shift Assay (EMSA) was performed in 40μl containing 40μg of total nuclear protein with 1 pmol oligo containing a κB motif (consensus NF-κB binding sequence from mouse Ig κ light chain; 5'-AGT TGA GGG GAC TTT CCC AGG C-3') and its complement (5'-G CCT GGG AAA GTC CCC TCA ACT-3') labeled with γ-32P ATP in binding buffer (10mM HEPES, 50mM KCl, 2.5mM dithiothreitol, 0.2mM EDTA, 0.05% nonidet-P40, and 10% glycerol, pH7.9). Competition assays were performed by incubating 1pmol of 32P labeled oligo containing the κB motif together with 50 fold excess of unlabeled oligonucleotide. Samples were electrophoresed on a 7% native polyacrylamide gel in low ionic
strength Tris-glycine buffer (50mM Tris, 380mM glycine, and 0.2mM EDTA, pH8.5). Following electrophoresis, the gel was exposed to storage phosphor screen (Eastman Kodak, Rochester, NY) for an hour, and the screen was analyzed with an 860 Storm™ FluorImager (AmerishamPharmacia Biotech, Piscataway, NJ).

Fluorescent Electrophoretic Mobility Shift Assay

Fluorescent electrophoretic mobility shift assays (FEMSA) were performed as described (Murphy et al., 2001). Briefly, assays were performed in 40ul volumes containing 20μg of nuclear protein incubated with 1pmol of the FAM (492nm absorption and 515nm emission) labeled oligonucleotides (NF-κB, Research Genetics, Carlsbad CA). Oligonucleotides that contained the consensus binding sequence for NF-κB binding were used (FAM-CATGACCGGGGTTCTAACA TG-Dabcyl and its complement AATTGGAAATTCCCGGTTCC). Oligonucleotides with the same sequence that were not labeled as well as oligonucleotides that contained two point mutations that had previously been shown to abolish NF-κB binding (CATGACCGCATCAACCACAATG and its complement AATTGGAAAATGCGGGTTCC ) were used as competitive and non-competitive inhibitors for binding with the labeled oligonucleotides (Murphy et al., 2001). Assays were performed in total volume of 40 μl with binding buffer (10mM HEPES, 50mM KCl, 2.5mM DTT, 0.2mM, 0.05% NP-40, 10% glycerol, pH 7.9). Competition experiments were performed by adding the appropriate competitor oligonucleotides at a 50 fold molar excess with the labeled oligonucleotides prior to the addition of the
cell lysate. The supershift assays were performed by adding 0.5 pmol of antibody to the p65 subunit of NF-κB (Upstate, Lake Placid, NY) to reactions that contained labeled oligonucleotides without a competitor. Samples were electrophoresed on a 7% native polyacrylamide gel in low ionic strength Tris-glycine buffer (50mM Tris, 380mM glycine, and 0.2mM EDTA, pH8.5). After electrophoresis the gel was analyzed by blue-excitation fluorescence scanning with an 860 Storm™ FluorImager (Amerisham Pharmacia Biotech, Piscataway, NJ).

Akt Kinase Assay

To measure the activity of PI3-K activity, 8×10⁴ U87-MG cells were seeded on 60 mm dishes and allowed to grow for at least 24 hours to achieve 80% confluence. The cells were transfected with different amount of siRNA (including optimal amount determined from earlier experiment) and the cells were lysed with lysis buffer (20mM Tris (pH7.5), 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 2.5mM sodium pyrophosphate, 1mM β-Glycerophosphate, 1mM Sodium Orthovanadate, 1μg/ml Leupeptin) at 24 hours post transfection. Lysates from the samples were collected and sonicated briefly while the samples were kept on ice. Then the lysates were centrifuged at 14,000xg for 5 minutes at 4 °C. Then the supernatants were collected. Total protein concentration of the extract was measured immediately after preparation by using the colorimetric bincinchoninic acid (BCA) assay (Pierce, Rockford, IL), and the lysates were stored at -80°C until use. Lysates were then subject to immunoprecipitation with immobilized active Akt antibody (Ser
308 Thr 473) to capture active Akt in the lysates. The immunocomplex was washed twice in the lysis buffer then two times in kinase assay buffer (25mM Tris (pH7.5), 5mM β-glycerophosphate, 0.1mM sodium orthovanadate, 2mM DTT, 10mM MgCl₂). After the wash, the collected active Akt was subject to a kinase assay by incubating active Akt with substrate GSK-3α/β fusion proteins (1μg/sample) supplemented with 200μM ATP for 30 minutes at 30 °C. The kinase reaction was terminated by adding 3X sample preparation buffer (187.5mM Tris-HCl (pH 6.8 at 25 °C), 6% w/v SDS, 30% glycerol, 150mM DTT, 0.03% w/v bromophenolblue) and vortexing. Supernatant was then resolved on a 10% Tris-Glycine gel and Western blot was performed. In principle, active Akt phosphorylates GSK-3α/β on serine 9 and 21 and the phosphorylated serine residues can be determined by the incubation with anti-phospho-GSK-3 α/β serine 21/9 antibody on Western blot.

**Immunoprecipitation**

Immunoprecipitation of p110α and p110β was performed to determine the expression of these proteins under siRNA treatment. 150μg/200μl of lysate was precleared with 10μl of protein A/G plus agarose (Santa Cruz, San Diego, CA) before incubation with an anti p110 antibody (Upstate, Lake Placid, NY) at concentrations described in antibody section of Materials and Methods above. This mixture was incubated for 2 hours at 4 °C. 50μl protein A/G plus agarose (50% v/v) was added to the antibody and lysate mixture and the mixture was incubated for 1 hour at 4 °C on a rocker table. After the incubation, the agarose beads were collected by pulse
centrifugation and washed three times with PBS supplemented with 1mM NaF and 1mM PMSF. The collected beads-antibody-protein complex was mixed with 60µl of 2X sample buffer, vortexed, and centrifuged. The supernatant was collected and boiled for 5 minutes before resolving on an SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmili, 1970).

**Design and Transfection of siRNA**

The siRNA was designed according to the published specifications available by literatures (Elbashir et al., 2001a; Elbashir et al., 2001b; Elbashir et al., 2001c). From the AUG start codon of target mRNA, AA dinucleotide sequences were searched. The 3' adjacent 19 nucleotides were considered as potential siRNA target sites. The effective siRNA to suppress mRNA expression should be homologous to the sequence within 75 bases from start codon of the target mRNA sequence (Elbashir et al., 2001a; Elbashir et al., 2001b; Elbashir et al., 2001c). The GC content of the siRNA should be between 30 - 40%. Once the 21 nucleotide siRNA was designed, the sequence was compared against the appropriate genome database (human, mouse, rat, etc.) such as Basic Local Alignment Search Tool for nucleotide (BLASTn) from National Center for Biotechnology Information (NCBI) and some siRNA were eliminated from consideration due to significant homology to other coding sequences.
This was particularly important as siRNA against p110α should silence the expression of p110α, but it should not inhibit the expression of p110β or other essential mRNAs in the cells. In addition, negative control should be prepared.

For the p110α, the Homo sapiens phosphoinositide-3-kinase alpha catalytic subunit polypeptide PIK3CA mRNA (NM_006218) sequence from genebank was used to design the siRNA.

siRNA against p110α was designed as follows.

Sense: AUG GAA UGA UAG UGA CUU U dTdT  
Anti-sense: dTdT UAC CUU ACU AUC ACU GAA A

The Control siRNA against p110α was designed as follows.

Sense: UUU CAG UGA UAG UAA GGU AdTdT  
Anti-sense: dTdT AAA GUC ACU AUC AUU CCA U

For the p110β, Homo sapiens phosphoinositide-3-kinase alpha catalytic subunit polypeptide PIK3CB mRNA (NM_006219) sequence from genebank was used to design the siRNA.

siRNA against p110β was designed as follows.

Sense: UUG CUU CCA UAA CAU CUG CdTdT  
Anti-sense: dTdTA ACG AAG GUA UUG UAG ACG

Control siRNA against p110β was designed as follows.

Sense: GCA GAU GUU AUG GAA GCA AdTdT  
Anti-sense: dTdTC GUC UAC AAU ACC UUC GUU

All siRNAs used in this study were obtained as 2'-ACE protected (Dharmacon Research, Lafayette, CO) for stability. Equimolar concentration of each strand of siRNA was suspended in 2'-deprotection buffer (100 mM Acetic acid,
adjusted to pH 3.8 with TEMED) and combined. The mixture was incubated at 60 °C for 30 minutes followed by incubation at room temperature for 30 minutes. The resultant double stranded RNA (siRNA) was ethanol precipitated and lyophilized. The working siRNA solution was prepared in 1X annealing buffer and optical density at 260 and 280nm was measured to determine the concentration of the sample. The concentration of siRNA sample used in this study was consistently adjusted to 1μg/μl.

In order to test the effect of the siRNAs in the cells, 80% confluent U87-MG cells were transfected with different concentrations of siRNA against either p110α or p110β. 0, 1, and 2 μg siRNA per 60mm were used initially as the concentrations had been reported to be effective to suppress the expressions of luciferase gene in insect cells (Elbashir et al., 2001a; Elbashir et al., 2001b; Elbashir et al., 2001c). Control siRNA that has same number of nucleotides, but the sequence is random so that the sequence is no longer homologus to the target mRNA. Lipofections were performed using Fugene 6 (Roche, Indianapolis, IN) according to the manufacturer’s specifications. A ratio of 2 μg of DNA per 6 μl (Fugene 6 only) was used throughout these experiments. Transfected cells were harvested at 24, 32, and 48 hrs post-transfection to find an optimal time point and lysate was collected. The effect of siRNA was tested by using Western blots to determine the expression level of the target protein.
RESULTS

Activation of NF-κB in PDGF-B Chain Transformed NIH3T3 Cells

ASA Inhibits PDGF B Chain Induced Focus Formation

As some colon tumors that are known to over-express PDGF-B chain (Hsu et al., 1995) some colon cancer cells are sensitive to ASA (Rosenberg et al., 1998), we hypothesized that ASA might potentially inhibit PDGF-B chain induced transformation. NIH 3T3 cells were transfected with a plasmid that constitutively expresses the PDGF B chain (Ratner et al., 1985), and a cell line that expressed the PDGF-B chain homodimer was identified by Western blotting (data not shown). Cells were subjected to treatment with DMEM supplemented with 2% calf serum (CS) and ASA or SA in solvent (ethanol). The results (Figure 7A) demonstrated that the addition of either ASA or SA potently inhibited focus formation in these cells. The solvent control did not affect normal cell growth and resulted in much higher cell density than that of ASA or SA treated cells masking individual foci. Inhibition by ASA and SA was not due to an altered pH of the medium as the pH of the medium was re-established at 7.4 after ASA or SA addition. The concentration of ASA and SA used in this assay was not toxic to the cells as cells grew normally after removal of ASA and SA from the media (data not shown).
To determine if the inhibition of focus formation caused by ASA was due to its inhibition of prostaglandin synthesis, two inhibitors of prostaglandin synthesis were tested for their effects on focus formation. In contrast to the results obtained with ASA and SA, treatment of cells with NS398 which preferentially inhibits prostaglandin H synthase (PGHS2; COX2) (Tsujii et al., 1998) did not result in a decrease in focus formation even with the concentration known to completely inhibit PGHS2 (10μM) (Attiga et al., 2000; Q. Guo et al., 1996) (Figure 1B). In a same manner, indomethacin, which inhibits both PGHS1 and PGHS2 (Kopp & Ghosh, 1994) did not result in a decrease in focus formation even at concentrations well above those known to inhibit PGHS activity (Figure 7B).

To further confirm that the inhibition of focus formation by ASA was not due to its inhibition of prostaglandin synthesis, we performed focus formation assays in the presence of ASA and PGE$_2$. PGE$_2$ is the major prostaglandin produced by PDGF stimulation of fibroblasts and PGE$_2$ addition to medium can restore PDGF stimulated DNA synthesis that has been inhibited by ASA (Shier & Durkin, 1982). Addition of PGE$_2$ to PDGF-B chain transfected cells does not significantly affect focus formation either in the presence or absence of ASA (Figure 8).

**NF-κB Activity in PDGF B Chain Overexpressing NIH3T3 Cells**

Previous reports have indicated that ASA and SA but not other NSAIDS may also inhibit the activity of the transcription factor NF-κB (Kopp & Ghosh, 1994). We were, therefore, interested in determining if NF-κB was significantly activated in
PDGF-B chain transformed NIH3T3 cells and, if so, what effects ASA and SA had on this activation. NIH3T3 cells were transiently co-transfected with the NF-κB reporter vector containing 4 tandem repeats of invariant chain NFκB binding sequence or the reporter vector that completely lacked the NF-κB binding sequences. These plasmids were co-transfected with an empty expression vector, a plasmid that expressed the PDGF-B chain pSM-l (Ratner et al., 1985).
Figure 7. ASA Inhibits Focus Formation Caused by PDGF-B Chain Gene Expression.

Clones of NIH3T3 cells that constitutively express the PDGF-B chain were seeded on 12 well plates and treated with different inhibitors in DMEM supplemented with 2% CS and antibiotics. A) Cells treated with ASA or SA. Note that absence of ASA or SA (Control) promoted excessive cell growth that masked foci. ASA was more effective than SA at inhibiting PDGF-B chain induced focus formation. B) Cells treated with PGHS2 inhibitors NS398 or indomethacin (IND). PGHS2 inhibitors were not effective at inhibiting PDGF-B chain induced focus formation compared to ASA. As complete inhibition of PGHS2 by NS398 occurs at concentrations below 10mM, the decreased focus formation in 20 and 50 mM NS398 was not due to the inhibition of PGHS2 but probably due to secondary effects. Control indicates cells treated with solvent alone (either ethanol or dexamethasone). Cells were fixed in either methanol or ethanol before being stained with 1% crystal violet. In the tables above, numbers indicate number of foci per well.
NIH3T3 cells were transfected with the plasmid that expresses the PDGF-B chain gene (PDGF-B). Cells were then treated with DMEM supplemented with 2% CS alone or supplemented with 5mM ASA or both ASA and 500 nM PGE$_2$ or 500 nM PGE$_2$ alone. After 10 days, cells were fixed with ethanol and stained with 1 % crystal violet. In the table above, numbers of foci per plate are shown.

**Figure 8. PGE$_2$ Does Not Rescue Focus Formation Inhibited by ASA.**

<table>
<thead>
<tr>
<th>PDGF-B chain +ASA+PGE$_2$</th>
<th>PDGF-B chain +ASA</th>
<th>PDGF-B chain +PGE$_2$</th>
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</thead>
<tbody>
<tr>
<td>0</td>
<td>266.7±37.1</td>
<td>5±1</td>
</tr>
<tr>
<td></td>
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<td>206.7±35.3</td>
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or a plasmid that expressed the E5 protein from Bovine Papilloma virus. The results (Figure 9) demonstrated that both of the plasmids that expressed genes that could activate the PDGF receptors, pSM-1 and the E5 construct (Kilk et al., 1996; L. Petti et al., 1991; Riese & DiMaio, 1995), were able to cause a significant and substantial increase in NF-κB activity. The NF-κB activity was increased more than 2 (pSM-1) and 3.8 (E5) fold as compared to the NF-κB activity in either cells transfected with the reporter vectors lacking the NF-κB binding sequences or cells transfected with its empty expression vectors. Similar results were obtained when NIH3T3 cells were transfected with the NF-κB reporter vectors and the cells then stimulated with exogenous PDGF-B chain homodimer 72 hours post transfection (data not shown).

ASA to Suppress NF-κB Activity in PDGF B Chain Overexpressing NIH3T3 Cells

This activation of NF-κB in PDGF-B chain over-expressing cells was significantly reduced upon treatment of the transfected cells with ASA (Figure 10). NIH3T3 cells that were transfected with pSM-1 and either a reporter vector with the NF-κB binding sites or a reporter vector without the NF-κB binding sites before treatment with ASA. The results clearly show that, as before, pSM-1 induced a greater than 2 fold increase in NF-κB activity as compared to cells that were transfected with the reporter vector lacking the NF-κB binding sequences and a greater than 1.5 fold increase in NF-κB activity as compared to cells that had been transfected with the vector control. When compared to cells that were treated with
ASA, cells co-transfected with pSM-1 and the reporter plasmid that contained the NF-κB binding sites had a significantly higher level.
NIH3T3 cells were transfected with plasmids that express PDGF-B chain gene (PDGF-B) or the bovine Papilloma virus E5 gene (E5) or empty vector (RVY) and either the reporter plasmid that contains the NF-κB binding sequences (NF+) or the reporter plasmid that lacks the NF-κB binding sequences (NF-). Luciferase assays were performed three times, and the results were averaged. Luminescence was expressed as fold increase against control compared to the results from cells that were co-transfected with RVY and the reporter plasmid that contains the NF-κB binding sequences (RVY & NF+). Activation of NF-κB by PDGF-B (*) or E5 (**) was significantly greater than others (p < 0.05, ANOVA and SNK tests).

Figure 9. PDGF-B Chain Over-expression Promotes NF-κB Activity.
Figure 10. ASA Decreases NF-κB Activity in PDGF-B Chain Expressing Cells.

NIH3T3 cells were transfected with either the plasmid that expresses the PDGF B chain gene (PDGF-B) or the empty expression vector alone (pCDV-1) in conjunction with the reporter plasmids that either contain the NF-κB binding sequences (NF+) or lack the NF-κB binding sequences (NF-). Luciferase activity was expressed as fold increase over the control (pCDV-1 & NF+). Activation of NF-κB by PDGF-B (PDGF-B & NF+) was significantly greater than the control (pCDV-1 & NF+) and was reduced by ASA treatment to background levels (p < 0.05, ANOVA and SNK tests).
of activity than those cells that had received ASA treatment. In fact, the levels of luciferase under these conditions had been reduced to background (compare pCDV-1&NF+ and pSM-1&NF+ ASA treated).

Electrophoretic mobility shift assays (EMSA) were performed to confirm the presence of NF-κB in the nucleus. As shown in Figure 11, it was clear that NF-κB DNA binding activity was greatly increased in PDGF-B chain over-expressing cells (PDGF-B chain-ASA), and the increase was approximately 2 fold more than the NF-κB DNA binding activity observed in control cells. When PDGF-B chain over-expressing cells were treated with ASA (PDGF-B Chain+ASA), DNA binding activity was decreased approximately 50% compared to DNA binding activity observed in control cells (vector control).

**Suppression of NF-κB Activity to Inhibit PDGF B Chain Transformation**

As ASA appeared to be controlling focus formation and inhibiting NF-κB promoter and DNA binding activities in PDGF-B chain expressing NIH3T3 cells, we were interested in a more direct test of the effect of NF-κB on focus formation. Two dominant negative mutants were used in transfection assays. One was a mutant of IκBα, IκB67CJ, that can not be phosphorylated and therefore, forces the retention of NF-κB/IκB complexes in the cytoplasm. The second was a dominant negative mutant of p65, p65ΔC, that lacked the transactivation domain of p65 and, therefore, could form heterodimers that were translocated to the nucleus but can not activate transcription (Grimm & Baeuerle, 1994).
Figure 11. PDGF-B Chain Over-expression Increases NF-κB Binding Activity Which is Inhibited by Treatment With ASA.

Stable NIH3T3 cell lines that were transfected with either the plasmid that expresses the PDGF-B chain gene (PDGF-B) or the empty expression vector (Vector Control) were established. The PDGF-B chain gene transfected cells were grown in DMEM supplemented with 10% calf serum in the presence of 500 μM ASA (+ASA) or the absence of ASA (-ASA). All lanes contained 1 pmol of $^{32}$P labeled oligonucleotides that contained the κB motif. The competition lane included nuclear extract from PDGF-B chain expressing cells, labeled oligonucleotides and a 50 fold excess of unlabeled oligonucleotides.
Transfection of PDGF-B chain over-expressing NIH3T3 cells with increasing amounts of the plasmid IκB67CJ clearly demonstrated the ability of this plasmid not only to express IκBα but also for this IκBα mutant to inhibit NF-κB activity in a dose dependent manner (Figure 12). To determine the ability of PDGF-B chain to induce focus formation with these different constructs, NIH3T3 cells expressing the PDGF-B chain were transfected with the IκBα mutant (IκB67CJ) or an empty expression vector (pRcCMV), the vector that was used to express the IκB67CJ, or the wild type form of p65 or the p65 mutant (p65ΔC) (Figure 13). Transfection of NIH3T3 cells with the mutant form of IκBα (IκB67CJ) or the mutant form of p65 (p65ΔC) and PDGF-B chain resulted in significant inhibition (50% and 39% respectively) of focus formation when compared to focus formation in NIH3T3 cells that were transfected with both PDGF-B chain and empty expression vector.
Figure 12. Expression of the Mutant of IκB Decreases NF-κB Activity in PDGF-B Chain Over-expressing Cells.

PDGF-B chain over-expressing NIH3T3 cells were transfected with increasing amounts of plasmid that expressed the mutant form of IκB (pMEIκB67CJ). A Western blot was performed on cell extracts (IκB blot) using an anti-IκBα antibody. The reporter plasmid used in these experiments contained the same promoter and NF-κB binding sequences as in previous figures but used secreted alkaline phosphatase (SEAP) instead of luciferase as a reporter. Activation of NF-κB by PDGF-B (PDGF-B chain + pMEIκB67CJ 0μg) was significantly greater than the NF-κB activation in cells transfected with 5μg of pMEIκB67CJ (PDGF-B chain + pMEIκB67CJ 5μg) (p < 0.05, ANOVA and SNK tests). The total amount of plasmid transfected into PDGF-B chain over-expressing NIH3T3 cells was equalized between samples by using the empty expression vector pRcCMV.

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**Number of Foci**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Number of Foci</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGF-B chain</td>
<td>11.2±1.0</td>
</tr>
<tr>
<td>PDGF-B chain + pRcCMV</td>
<td>12.7±1.8</td>
</tr>
<tr>
<td>PDGF-B chain + p65WT</td>
<td>8.25±0.9</td>
</tr>
<tr>
<td>PDGF-B chain + pMEIkB67CJ</td>
<td>6.3±0.6</td>
</tr>
<tr>
<td>PDGF-B chain +p65ΔC</td>
<td>7.75±0.5</td>
</tr>
<tr>
<td>pCDNA3.1 (-)</td>
<td>1.25±0.3</td>
</tr>
</tbody>
</table>

Figure 13. Over-expression of IκB and p65ΔC Inhibits Focus Formation in PDGF-B Chain Over-expressing Cells.

NIH3T3 cells transfected with PDGF-B chain gene alone (PDGF-B Chain). NIH3T3 cells transfected with the PDGF B chain gene and pRcCMV vectors (PDGF-B chain + pRcCMV). NIH3T3 cells transfected with the PDGF-B chain gene and p65 wild type expressing plasmid (PDGF-B chain + p65WT). NIH3T3 cells transfected with PDGF-B chain gene and IκB mutant (PDGF-B chain +IκB67CJ). NIH3T3 cells transfected with PDGF-B chain gene and p65ΔC (PDGF-B chain +p65ΔC). NIH3T3 cells transfected with pCDNA3.1(-), the vector used for expression of the PDGF-B chain gene pCDNA3.1(-). In the table above, number of foci per cm² is shown.
NF-κB Activity in U87-MG and U118-MG Cells

U87-MG and U118-MG glioblastoma cells have been shown to endogenously express PDGF-B chain and the growth of these cells is dependent on PDGF expression and its autocrine loop (Chi et al., 1997; Kilic et al., 2000; Pollack et al., 1990; Schilling et al., 1998). The cells have been shown to exhibit high NF-κB activity (Murphy et al., 2001), thus they provide an ideal model to elucidate secondary signaling molecules that mediate signaling between PDGF stimulation and the regulation of NF-κB activity. In our laboratory, the activity of 8 transcription factors in the glioblastoma cell lines U87-MG, U118-MG, U373-MG and a nontumor derived cell line MCF10A were measured by 8 different reporter vectors containing 4 tandem repeats of consensus binding sequences for 8 different transcription factors including NF-κB, AP-1, heat shock transcription factor -1 (HSF-1), ATF-2/CREB, GR, Elk-1/SRF, Myc, and NFAT. In each reporter vector, 4 tandem repeats of binding sequences for each transcription factor are followed by a thymidine kinase promoter and a gene coding for secreted alkaline phosphatase (SEAP). Thus the amount of SEAP activity in the medium should be directly proportional to the amount of transcription factor activity in the cells. The reporter vectors were transfected into U87-MG, U118-MG, U373-MG and MCF10A cells. The reporter vectors that contained binding elements for NF-κB, AP-1, HSF-1, ATF-2/CREB, GR, Elk-1/SRF, Myc, and NFAT indicated activities of HSF-1, ATF-
2/CREB, GR, Elk-1/SRF, Myc, and NFAT transcription factors in the cells were low and comparable to the control normal epithelial cell line MCF-10A. On the other hand, transfection of the reporter vector for the NF-κB and AP-1 transcription factors exhibited significantly elevated levels of SEAP activity in U87-MG and U118-MG cells as compared not only to the control plasmid that lacked the NF-κB or AP-1 binding sequences (pTAL) but also to the glioblastoma cell line U373-MG and the MCF-10A control (Figure 14. Shimamura et al. manuscript submitted).
Figure 14. NF-κB is Activated in U87-MG and U118-MG but Not in U373-MG Cells.

Cells were transfected as described in materials and methods. The amount of SEAP in the culture media was measured by adding substrate and resultant fluorescent was measured in a fluorescent reader. Results are the averages of three independent experiments. Dark bar: pTAL Shadowed bar: pNF-κBSEAP.
The results were confirmed with fluorescent electrophoretic mobility shift assays (FEMSA) performed with nuclear extracts from U87-MG, U118-MG, U373-MG and MCF-10A. Detectable amounts of NF-κB binding to the fluorescently labeled oligonucleotide containing the consensus NF-κB binding sequence were observed in U87-MG, U118-MG, and U373-MG cells. The same extracts were incubated with an antibody directed against the human NF-κB p65 subunit to determine if the binding was due to NF-κB. Incubating lysates from U87-MG, U118-MG, U373-MG with the antibody resulted in an apparent increase in molecular weight of the band (supershift), indicating the complex that was able to bind to the consensus NF-κB binding sequence contained the NF-κB p65 subunit. These experiments demonstrated that NF-κB activity was present in all of the extracts except the MCF-10A controls (Murphy et al., 2001).

Our laboratory has also shown that a truncated mutant of the PDGFβR interferes with the physiology of the U87-MG and U118-MG cells. The mutant PDGFβR lacks both kinase domains and many of the tyrosine residues of the PDGFβR cytosolic domain that are essential in initiating receptor mediated secondary signaling. Therefore, the truncated PDGFβR serves as a dominant negative mutant in the cells. U87-MG and U118-MG cells were triple-transfected with wild-type or truncated PDGFβR, NF-κB reporter vector, and β-galactosidase reporter plasmid. SEAP activity was measured to assess NF-κB activity. The results were normalized.
with the specific activity of demonstrated that NF-κB activity was significantly decreased in the cells that expressed the truncated PDGFβR (data not shown).

**Chemical Inhibitors of PI3-K Influence NF-κB Activity**

In order to demonstrate what pathways are involved in the signaling between the PDGFβR and NF-κB, NF-κB gene reporter assays were performed in the presence of different chemical inhibitors including the PI3-K inhibitors LY294002 and Wortmannin, P44/42 Mitogen Activated Protein Kinase (MAPK) inhibitor U0126, and p38 MAPK inhibitor SB203580. Among the chemical inhibitors, PI3-K inhibitors only LY294002 and Wortmannin were able to suppress NF-κB activity in a dose dependent manner (Figure 15).

**Dominant Negative Mutant of PI3-K Pathway Adapter Protein p85**

In order to confirm that PI3-K pathway is involved in the signaling between PDGFβR and NF-κB activation, a dominant negative mutant (DNM) of the p85α regulatory subunit (Von Willebrand *et al.*, 1996) was transfected into U87-MG and U118-MG cells and NF-κB activity was measured. The particular DNM used in this experiment lacks the inter-SH2 domain in the C-terminus of the protein, which is essential for the p85 regulatory subunit activated by PDGFβR to bind to a p110 catalytic subunit of the PI3-K pathway. Therefore the DNM of the p85α regulatory subunit cannot stimulate tyrosine p110 kinase subunit of PI3-K pathway. The results indicated that
this mutant was ineffective in inhibition of NF-κB activity possibly due in part to low expression of the DNM (Figure 16).
Figure 15. Chemical PI3-K Inhibitor to Suppress NF-κB Activity in U87-MG Cells.

(A) NF-κB activity in U87-MG cells treated with 0μM, 5μM, 10μM and 25μM of LY294002 as indicated. (B) NF-κB activity in U87-MG cells treated with 0nM, 5nM, 10nM and 25nM of Wortmannin as indicated.
Figure 16. p85 Regulatory Subunit and NF-κB Activity in U87-MG Cells

Suppression of PI3-K p110 Isoform Expression in U87-MG

It has been shown that significant levels of active PI3-K in U87-MG cells, which was shown by the activity of PDK-1 (Flynn et al., 2000). In our laboratory, the use of PI3-K inhibitors on the cells exhibited significant morphological changes in the cells which were quite evident under microscopic observation.

The p110 catalytic subunit is a key molecule in PI3-K pathway phosphorylating proteins and lipids in the cells. It is the kinase which LY294002 (Kovacsovics et al., 1995; Sanchez-Margalet et al., 1994; Vlahos et al., 1994) and Wortmannin (Nakanishi et al., 1992; Okada et al., 1994a; Okada et al., 1994b) inhibit to suppress PI3-K pathway activity in different cells. The p110 catalytic subunit of PI3-K pathway therefore provides an ideal target for modulating the biological activity of PI3-K pathway.

The p110 catalytic subunit includes four different isoforms; α, β, γ, and δ. Among the isoforms, α, β, and δ are known to associate with p85α regulatory subunit and the γ subunit is known to associate with p101 regulatory subunit in different varieties of cell types and seems to be regulated quite differently compared to α, β, and δ isoforms. Several reports indicate that the δ isoform is abundant in leukocytes (Domin & Waterfield, 1997; Vanhaesebroeck et al., 1997b), therefore δ isoform is most-likely absent in the cells of astrocyte origin such as U87-MG and fibroblast NIH3T3. Consequently, only the α and β isoforms among all four isoforms were investigated in this study using siRNA.
The experiments were designed to consider three potential problems. First, it has been reported that delivering more than optimal amount of siRNA sometimes causes detrimental effects on the cells (Gura, 2000). Second, using a suboptimal amount of siRNA may not be enough to silence the expression of the target mRNA and its expression. Third, the timing of harvesting the cells after the transfection will be critical as the silencing of protein expression by siRNA should be transient and the silencing will wear off as time goes.

siRNAs against p110α and p110β and control siRNAs were synthesized. Transfected cells were harvested at 24, 32, and 48 hours post-transfection to find the optimal time point of suppression and cell lysates were collected. The lysates were resolved on a SDS-PAGE followed by Western blot with anti-human p110α or anti-human p110β antibody. It was found that the intensity of the bands was decreased as siRNA suppresses the endogenous expression of p110α and p110β (Figure 17).
Figure 17. siRNA Suppression of p110 Expression.

Results from the Western blot performed on sample lysates from U87-MG and U118-MG cells transfected with indicated amount of p110α siRNA and control siRNA. SiRNA against p110α suppresses endogenous expression of p110α. The membrane was also blotted with anti-NF-κB p65 subunit antibody as a control. (+ve) positive control: EGF stimulated A431 cell lysate.
Suppression of p110α and β and PI3-K Pathway

After optimal dose and optimal harvesting time were determined for p110α mRNA silencing by siRNA, activity of PI3-K pathway in the U87-MG and U118-MG cells was measured by using the Akt kinase assay described in Materials and Methods. The Akt kinase assay is based on the fact that both glycogen synthase kinase-3 (GSK-3) α and β are substrates of Akt kinase (protein kinase B and Bad kinase) and are known downstream targets of PI3-K pathway (Brady et al., 1998; K. Datta et al., 1996; Franke et al., 1995; Hideshima et al., 2001). Threonine 308 and serine 473 of Akt kinase are known to be phosphorylated by 3-Phosphoinositide-dependent kinase-1 (PDK-1), which is activated by PI3-K and PIP3 produced by PI3-K. The active Akt kinase has been shown to phosphorylate serine 21 of GSK-3α and serine 9 of GSK-3β. The anti-phospho-GSK-3 α/β serine 21/9 antibody is specific and highly sensitive with near zero background.

Results show that significant PI3-K activity was present in U87-MG cells (Figure 18). The Akt kinase activity was reduced significantly in the U87-MG cell lysates treated with siRNA against p110α and siRNA against p110β in a dose dependent manner. The result indicates that silencing p110α with siRNA in U87-MG cells decreases the activity of PI3-K pathway in the cells.
Figure 18. Inhibition of PI3-K Activity by siRNA Against p110α and p110β

Results from the Akt Kinase assay performed on sample lysates from U87-MG cells transfected with the indicated amounts of p110α siRNA or p110β siRNA and control siRNA.
Suppression of p110 Isoforms and NF-κB Activity

Once the efficacy of siRNA against PI3-K p110α and p110β to suppress PI3-K pathway activity was verified, NF-κB activity in glioblastoma cell lines U87-MG and U118-MG transfected with p110 siRNA was measured by a reporter vector containing a binding sequence for a transcription factor NF-κB. The reporter vectors, optimal doses of siRNAs, and vector coding for β- galactosidase were cotransfected into U87-MG cells. The control plasmid that lacks the NF-κB binding sequences (pTAL) was also used to measure the background noise of the assay. The assay results were normalized for specific activity of β-galactosidase in order to take cell density variability and the variability in transfection efficiencies among samples into account.

The assay was carried out to determine if the PI3-K pathway plays essential roles in controlling NF-κB activity in U87-MG cells. In theory, it was anticipated that the activity of a NF-κB was directly proportional to the activity of PI3-K pathway. The inhibition of p110 isoforms should result in the failure of NF-κB activation as a regulatory subunit IκB retains NF-κB in the cytoplasm.

Results from the assay showed that inhibition of PI3-K pathway by 2 μg of siRNA against p110α and p110β reduced NF-κB activity approximately 19% and 18% respectively in U87-MG (Figure 19 and 20).
Figure 19. Suppression of NF-κB Activity by siRNA Against p110α

Results from NF-κB gene reporter assays with secreted alkaline phosphatase (SEAP). Background noise of the assay measured by pTAL control reporter vector was subtracted from the SEAP activity obtained for the lysates from each treatment. U87-MG cells treated with 2 μg of siRNA against p110α (U87+NF+p110αsiRNA2) had 20% less NF-κB activity than U87-MG cells transfected with the reporter vectors alone (U87+NF).
Figure 20. Suppression of NF-κB Activity by siRNA Against p110β

Results from NF-κB gene reporter assays with secreted alkaline phosphatase (SEAP). The activity obtained with the pTAL control reporter vector was subtracted from the SEAP activity obtained for the lysates from each treatment. U87-MG cells treated with 2 µg of siRNA against p110β (U87+NF+p110beta siRNA 2ug) shows 19% less NF-κB activity than U87-MG cells transfected with reporter vectors only (U87+NF).
FEMSA Assay Confirms the Inhibition of NF-κB with siRNAs Against p110α and β

Fluorescence electrophoresis mobility shift assay (FEMSA) assay was performed with nuclear extracts from U87-MG cells transfected with or without siRNA against p110α and p110β (Figure 21). At 24 hours post-transfection, cells were harvested and nuclear extracts were prepared. The amount of NF-κB and its binding capabilities were comparable between untreated (U87-MG) and U87-MG cells transfected with control siRNA (U87-MG Ctrl siRNA). When the consensus oligonucleotides were incubated with no nuclear extract (no EXT) there was no band. When unlabeled NF-κB consensus oligonucleotides were added in excess to the mixture of nuclear extract and non-labeled consensus NF-κB oligonucleotides (competition), the band indicating the binding of NF-κB disappeared due to the competition between labeled and unlabeled consensus NF-κB binding sequence to bind NF-κB. When the mixture was mixed with scrambled oligonucleotides, the binding of NF-κB to the NF-κB consensus oligonucleotide was present indicating the specificity of the NF-κB binding (Scramble). The amount of NF-κB and its binding capabilities were decreased when U87-MG cells were treated with 2μg of siRNA against p110α and the amount of NF-κB and its binding capabilities were completely abolished in U87-MG cells treated with 4μg of siRNA against p110α.
Figure 21. FEMSA Assay Performed with U87-MG Nuclear Extracts.

No Extract - No nuclear extract from U87-MG cells was mixed with labeled NF-κB consensus oligonucleotide and complementary sequence.

U87-MG - Nuclear extract was mixed with labeled NF-κB consensus oligonucleotide and complementary sequence.

Scramble - Nuclear extract was mixed with labeled NF-κB consensus oligonucleotide with scrambled sequence and complementary sequence.

Competition - No nuclear extract was mixed with labeled NF-κB consensus oligonucleotide and complementary sequence in the presence of excess unlabeled NF-κB consensus oligonucleotide.

U87-MG Control siRNA - Nuclear extract from U87-MG cells transfected with control siRNA was mixed with labeled NF-κB consensus oligonucleotide and complementary sequence.

U87-MG siRNA p110α 6μg - Nuclear extract from U87-MG cells transfected with 6μg of siRNA against p110α was mixed with labeled NF-κB consensus oligonucleotide and complementary sequence.

U87-MG siRNA p110α 12μg - Nuclear extract from U87-MG cells transfected with 12μg of siRNA against p110α was mixed with labeled NF-κB consensus oligonucleotide and complementary sequence. An arrow indicates binding of NF-κB on consensus oligonucleotides.
NF-κB Activity in U87-MG Cells Transfected with Mutant PDGF Receptors

In order to demonstrate that PI3-K pathway downstream of PDGFβR is responsible for controlling NF-κB activity, mouse fibroblast NIH3T3 cells were stably transfected with pLXSN vectors containing wild type PDGFβR, F5Y PDGFβR lacking five essential intracellular tyrosine residues, R634 PDGFβR whose lysine 634 residue essential for dimerization and auto kinase activity was replaced with arginine (R634), F5Y PDGFβR whose intracellular 740/751 tyrosine residues were added back (740), and F5Y PDGFβR whose intracellular 1009 tyrosine residues were added back (1009) (Figure 22). Table 2 summarizes the effects of the wild-type and mutant PDGF receptors. The cells stably expressing PDGFβR were established and the overexpression of the receptors was confirmed by Western blot using anti-PDGFβR antibody (Figure 23).

Gene reporter assays using SEAP were performed and the result indicated that overexpressing PDGF β receptor in U87-MG cells increased NF-κB activity compared to U87-MG cells without PDGF β receptor overexpressed (U87) except for a cells transfected with R634 mutant (Figure 23). U87-MG cells transfected with R634 mutant PDGF β receptor exhibited low NF-κB activity almost to the level of the negative control (U87TAL). NF-κB activity in F5Y PDGF β receptor was lower than the NF-κB activity in U87-MG cells transfected with wild-type PDGF β receptor. When 740 and 751 intracellular tyrosine residues were added back to the
F5Y (740), NF-κB activity was comparable to the one in U87-MG cells transfected with wild-type PDGF β receptor.
Figure 22. Design of Wild Type and Mutant PDGF β Receptor.

WT - Wild type. R634 - Amino Acid Lysine 643 was replaced with argine (R). F5Y - 5 tyrosine residues at positions 740/751/771/1009/1021 were replaced with Phenylalanine thus they cannot be phosphorylated upon receptor dimerization. 740/751 - Among the 5 residues replaced to phenylalanine, amino acids 740 and 751 were replaced (added back) to tyrosine. This receptor can transduce signals downstream through PI-3K pathway as 740/751 phospho-tyrosine resides provide docking sites for p85 regulatory subunit of PI3-K pathway.
Table 2

Mutations in Tyrosine Residues and Their Potential Effects on the Association of Secondary Signaling Molecules

<table>
<thead>
<tr>
<th>Human PDGF Preceptor Intracellular Domain Containing Mutations in Tyrosine Residues</th>
<th>Explanation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>No mutation. A positive control</td>
</tr>
<tr>
<td>F5Y</td>
<td>5 tyrosine residues 740/751/771/1009/1025 unable to activate PI-3K, GAP, SHP-2, and PLC-γ</td>
</tr>
<tr>
<td>R634</td>
<td>Unable to carry out kinase activity (a negative control)</td>
</tr>
<tr>
<td>740/751</td>
<td>Activates Shc, Nck, PI-3K, but not GAP, SHP-2, and PLC-γ</td>
</tr>
<tr>
<td>None</td>
<td>Vector only. Serves as a control</td>
</tr>
</tbody>
</table>
Figure 23. Overexpression of Wild Type and Mutant PDGF β Receptor.

Western blot analysis showing overexpression of PDGF β receptor. Whole cell lysates from untransfected U87-MG cells (U87-MG) and U87-MG cells transfected with PDGF β receptors (WT, F5Y, 740/751, 1009, R634) were blotted using an anti-PDGFβR antibody.
Figure 24. NF-κB Activity in U87-MG Cells Transfected With Different PDGF β Receptors.

WT - wild type PDGF β receptor, F5Y - PDGF β receptor lacking five essential intracellular tyrosine residues, R634 - PDGF β receptor whose lysine 634 residue essential for dimerization and auto kinase activity was replaced with arginine (R634), 740 - F5Y PDGF β receptor whose intracellular 740/751 tyrosine residues were added back, 1009 - F5Y PDGF β receptor whose intracellular 1009 tyrosine residues were added back. U87 - U87-MG cells transfected with empty vector and NF-κB reporter vectors. U87TAL - U87-MG cells transfected with empty vector and NF-κB control reporter vectors lacking NF-κB binding sequences.
DISCUSSION

Activation of NF-κB in PDGF-B Chain Transformed NIH3T3 Cells

Effects of Acetyl Salicylic Acid

Receptor tyrosine kinases (RTKs) such as the PDGF receptors rely on the intrinsic tyrosine kinase activities present in their cytoplasmic domains to transduce stimulatory signals to the cell interior. PDGF receptors, like many other receptors, activate multiple signaling cascades in a cell, not all of which may play a role in the myriad of responses to PDGF. In an effort to determine the pathways that are necessary for transformation of cells by PDGF, we used ASA to study the effects on the ability of PDGF to form foci and stimulate mitogenesis.

The results presented here demonstrate that ASA and SA can effectively inhibit PDGF stimulated focus formation. Both ASA and SA have several known functions within cells. Both can irreversibly acetylate both isoforms of PGHS (Bhattacharyya et al., 1995; Mancini et al., 1997; Roth et al., 1983) causing a reduction in the synthesis of prostaglandins that eventually leads to inhibition of DNA synthesis stimulated by PDGF (Castano et al., 1997; Shier & Durkin, 1982). ASA can also inhibit cell growth by inhibiting IKK-β which results in a decrease in activation of NF-κB (Yin et al., 1998).
Prostaglandins and Tumor

Prostaglandin synthesis regulated by COX2 (PGHS2) has been drawing the attention of researchers as recent studies suggest that COX-2-derived prostaglandins may play a pivotal role in the maintenance of tumor viability, growth, and metastasis (Attiga et al., 2000; Crew et al., 2000; Fosslien, 2000; Murata et al., 1999; Williams et al., 1999). To determine if the inhibition of prostaglandin synthesis is the reason for ASA's ability to inhibit focus formation in PDGF-B chain transformed cells, cells were treated with NS398 and indomethacin, NSAIDs known to inhibit PGHS2 but not NF-κB. Unlike ASA, neither of these had a significant effect on PDGF induced focus formation at concentrations known to inhibit PGHS2. At elevated concentrations (above 20mM), NS398 inhibited PDGF-induced focus formation but these concentrations are much higher than concentrations reported to completely inhibit PGHS2 (less than 10mM) (Attiga et al., 2000; Q. Guo et al., 1996). Similarly, the addition of PGE2, the primary prostaglandin produced upon PDGF stimulation of cells and the prostaglandin capable of restoring PDGF induced DNA synthesis after it has been blocked by ASA, had no effect on restoring PDGF induced focus formation. It has been reported that glioma cells with a higher rate of cell proliferation tend to have increased expression of COX-2 (Prayson et al., 2002). In our laboratory, treating glioma cells such as U87-MG and U118-MG with high concentration of ASA had no effect on NF-κB or AP-1 activity (data not shown). The evidence suggests that the involvement of COX-2 in tumor growth and development may depend on cell types.
NF-κB and Glioblastomas

Inhibition of NF-κB activation by a dominant negative mutant of IκBα or a dominant negative form of p65 that lacks c-terminus transactivation domain could effectively inhibit PDGF induced focus formation, indicating that the inhibition of focus formation by ASA was probably due to its inhibition of activation of NF-κB. The EMSA results clearly indicated that the DNA binding capability of NF-κB in PDGF-B chain over-expressing fibroblasts was significantly reduced under ASA treatment and that NF-κB promoter activity also decreased in the presence of ASA.

NF-κB is a transcription factor that regulates the expression of a variety of cellular genes (Perkins et al., 1992). It has been reported that NF-κB is involved in focus formation by the Ha-ras, abl, and Her2/neu oncogene (T. S. Finco et al., 1997; Pianetti et al., 2001; Reuther et al., 1998). It has been known that NF-κB activity is increased in some breast tumors, glioblastomas, and non-Hodgkins lymphomas (T. S. Finco et al., 1997; Murphy et al., 2001).

Although it is not known how exactly NF-κB contributes to tumor formation, several reports have indicated that NF-κB can stimulate the expression of the Cyclin D1 gene and that this is a central feature of movement from G1 into S phase (Guttridge et al., 1999; Hinz et al., 1999). It remains unclear whether the NF-κB mediated Cyclin D1 gene expression is necessary for its ability to stimulate focus formation in cells.
PI3-K Pathway and the Regulation of NF-κB in PDGF Stimulated NIH3T3 Cells

It has been reported that PDGF may stimulate NF-κB through the activity of PI-3K (Olashaw et al., 1992; Romashkova & Makarov, 1999). We have not observed decreased NF-κB activity in NIH3T3 cells expressing both dominant negative mutants of the p85 PI-3K regulatory subunit and PDGF-B chain or in NIH3T3 cells over-expressing PDGF-B chain treated with drugs that inhibit PI-3K activity (data not shown). Similarly, by using PDGF receptor mutants incapable of signaling through PI-3K, we have not obtained results that indicate PI-3K is necessary for NF-κB activation in CHO cells (data not shown). This results somewhat corresponds to the observation that radiation induced NF-κB activity in human ovarian carcinoma is inhibited by adenovirus type 5 E1A expression and inhibition is not mediated by the PI-3k downstream molecule Akt but by the inhibition of IκB degradation (Shao et al., 1997; Shao et al., 2001). In contrast to these results, transformation of NIH3T3 cells with the retroviral oncogene p3k (v-p3k) of avian sarcoma virus 16 (ASV16) that codes the catalytic subunit of phosphoinositide (PI) 3-kinase (Chang et al., 1997), resulted in increased activity of NF-κB (data not shown). This conflicting evidence indicates that the induction of NF-κB activity by PDGF may involve complex cellular mechanisms that require multiple secondary signaling pathways responsible at various steps in cellular transformation. In addition, the involvement of the PI-3K pathway in PDGF-B chain mediated NF-κB may well be dependent on the cell lines used.
Possible Involvement of Transcription Factor AP-1

It has been demonstrated that ASA also inhibits the induction of activator protein-1 (AP-1) (Huang et al., 1997) and that AP-1 activation may be necessary for transformation induced by PDGF, TPA, and TNF (J. J. Li et al., 1997; Mercola et al., 1988) and in neoplastic progression in human papillomavirus (HPV) immortalized human keratinocytes (J. J. Li et al., 1998). Although our results do not exclude a role for ASA inhibition of AP-1 in the suppression of PDGF induced transformation of NIH3T3 cells, it is clear that NF-κB also must play a role.

Regulation of NF-κB in Glioblastoma Cells

NF-κB Activity in U87-MG and U118-MG Cells

U87-MG glioblastoma (astrocytoma) cells were chosen as models to study the PDGF mediated tumorigenicity, primarily because the cells were known to overexpress PDGF B chain and exhibited significantly higher NF-κB activity compared to U373 glioblastoma cells and normal epithelial cell MCF-10 (Figure 14). Results presented here strongly suggest that PDGFβR play an essential role for regulating NF-κB activity in U87-MG glioblastoma cells as indicated by the experiments using kinase-deficient PDGFβR (R634) which significantly decreased NF-κB activity in these cells (Figure 24). In addition, introducing truncated PDGFβR into U87-MG cells with resulted in significant growth inhibition and reduced NF-κB activity (data not shown).
In order to elucidate the secondary signaling molecules responsible for mediating signals from PDGF β receptor to NF-κB in U87-MG cells, NF-κB gene reporter assays were performed in the presence of different concentrations of chemical inhibitors including the PI3-K inhibitors LY294002 and Wortmannin, P44/42 Mitogen Activated Protein Kinase (MAPK) inhibitor U0126, and p38 MAPK inhibitor SB203580. Among the chemical inhibitors, only PI3-K inhibitors LY294002 and Wortmannin were able to suppress NF-κB activity in a dose dependent manner (Figure 15).

Based on the results obtained from the use of PI3-K inhibitors, the hypothesis that the activity of a transcription factor NF-κB is under the regulation by PDGF mediated PI3-K pathway activity was tested.

A dominant negative mutant of p85 PI3-K regulatory subunit (p85ΔiSH2) that is unable to bind to the p110 catalytic subunit and therefore unable to mediate signals from the PDGF receptor to the p110 catalytic subunit of PI3-K was used to inhibit PI3-K pathway activity. The use of p85ΔiSH2 did not suppress NF-κB activity as anticipated (Figure 16). There seems to be two possible explanations for these results. First, the expression of p85 dominant negative mutant may have not been enough to overcome the intrinsic expression of p85 in U87-MG cells. It has been known that an over-saturating amount of dominant negative protein is sometimes necessary to overcome the endogenous expression of a target protein. It was very
difficult to verify overexpression of wild-type p85 and p85ΔiSH2 by Western blot, as a molecular weight difference between p85 wild-type and p85ΔiSH2 was not large enough to be resolved on a gel. Second, it is possible that NF-κB activity in U87-MG cells is regulated by PI3-K pathway activity independent of an interaction between p85 regulatory subunit and p110 catalytic subunit. PDGF B chain binding of PDGFβRs results in the association of multiple secondary signaling molecules (Table 1), which include RasGAP (Rodriguez-Viciana et al., 1994) known to associate and independently stimulate the p110 catalytic subunit to initiate lipid kinase activity. Moreover, it has been reported that some tissue samples isolated from p85α knockout mouse exhibited high PI3-K activity (Suzuki et al., 1999; Terauchi et al., 1999). PI3-K inhibitors Wortmannin and LY294002 are known to inhibit not only the activity of p110 catalytic subunit of PI3-K pathway but also the activity other kinases including phosphatidylinositol 4 kinase that may potentially provide supplemental roles to the functionality of PI3-K pathway (Sorensen et al., 1998; Wong & Cantley, 1994).

p110 Suppression by siRNA and PI3-K Activity

Both Wortmannin and LY294002 are known to inhibit the catalytic activity of p110 subunit of PI3-K pathway (Sanchez-Margalet et al., 1994; Ui et al., 1995; Vlahos et al., 1994), but their specificity for the p110 isoforms are not known. Based on the finding that inhibition of p85 regulatory subunit of PI3-K pathway does not decrease NF-κB activity in U87-MG cells, selective inhibition of the p110 catalytic
subunit gene was considered to inhibit the activity of PI3-K and subsequent regulation of NF-κB. The p110 catalytic subunit includes four different isoforms, α, β, γ, and δ. Among the four p110 isoforms, α and β isoforms were chosen for targets for inhibition mainly due to the fact that the two isoforms are known to be most catalytically active among the four isoforms (Beeton et al., 2000). The use of a dominant negative mutant p110α (kinase deficient) called p110α-CAAX has been described, but it was concluded that stability of the p110α-CAAX could not be guaranteed without expressing the dominant negative mutant together with p85 regulatory subunit (Rodriguez-Viciana et al., 1994). In addition, the unavailability of p110β dominant negative mutant forced us to stay away from the use of dominant negative mutant.

RNA interference by short interfering RNA (siRNA) is a relatively new technique that utilizes double stranded RNA of 21 nucleotides in length to inhibit gene expression. The double stranded RNA corresponds to exon sequences in the mRNA and suppresses gene expression of the target gene by degrading mRNA much more efficiently than anti-sense RNA.

The experiments described above were designed to consider two important parameters; 1) optimal amount of siRNA and 2) the timing to harvest the cells after the transfection. It seemed that maximal suppression of p110α and p110β protein expression took place at 24 hrs post-transfection, though the inhibition of target protein expression by siRNAs observed at the time point was not 100%. Testing the expression of p110 catalytic subunit by Western blot was extremely difficult due in
part to the lack of good immunoblotting antibodies against p110α and p110β. Two different kinds of antibodies were used for each p110 isoform with unsatisfactory results. Apparently, this problem has been confirmed by others as well (Bi et al., 1999). Immunoprecipitations by antibodies against each p110 isoform were absolutely necessary to capture enough p110 to resolve on an acrylamide gel (Figure 17). Although Western blot is an ideal method to detect the suppression of gene expression by siRNA, quantitative RT-PCR may be necessary to detect and measure mRNA degradation.

In spite of the weak inhibition of p110 expression, the introduction of siRNA targeting p110 isoforms resulted in reduction of PI3-K activity measured by Akt kinase assay (Figure 18), but further testing by using densitometry must be performed to verify the results.

The results presented here point out the fact that important parameters need to be worked out in the future for the implementation of siRNA technique. Series of new techniques are emerging to better implement the robustness of gene silencing by siRNA. Initially, it was believed that targeting 5’ end of the target mRNA is sufficient to degrade target mRNA, but it seems that it is not always the case. Growing evidence suggests that it is better to design multiple siRNAs that target different locations of a target mRNA. Yang et al. recently reported that Escherichia coli RNase III could be used to cleave long double-stranded RNA (dsRNA) into endoribonuclease-prepared siRNA (esiRNA) that can target multiple sites within an
mRNA (Yang et al., 2002). Apparently, esiRNA is much more effective than using one siRNA to silence a target mRNA (Yang et al., 2002).

It needs to be mentioned that the duration of gene silencing effect by siRNA has not been fully elucidated. If siRNA is used against a protein with high turnover, the amount of siRNA used must be increased proportionally. Due to the lack of a system to deliver large quantity of siRNA without disrupting the physiological stability of the cell, it seems to be very difficult to suppress protein with a high turnover. The p110 molecule studied here may fall under the category of high-turnover protein. Expressing short siRNA duplexes within cells from recombinant DNA constructs has been proposed, because it allows long-term target-gene suppression in cells (Brummelkamp et al., 2002; Paul et al., 2002).

**p110 Suppression by siRNA and NF-κB Activity**

Although the use of siRNA against p110α and p110β managed to decrease PI3-K activity in U87-MG cells as measured by Akt kinase assay (Figure 18). Gene reporter assay (Figure 19 and 20) showed that the reduction in NF-κB activity caused by p110α siRNA and p110β siRNA treatments was 20 % and 19% respectively, which was not statistically significant.

The discrepancy between those results and results obtained with chemical inhibitors could be due to several reasons. Inhibiting p110 catalytic subunit with siRNA may be quite different from inhibition of PI3-K pathway with chemical inhibitors. As mentioned earlier, the chemical inhibitors Wortmannin and LY294002
may have yet unknown side effects in the cells. Thus just inhibiting p110 isoforms might fail to fully suppress PI-3K pathway as the inhibition may inadvertently activate other signaling molecules that can supplement PI3-K activity and modulate NF-κB activity. The p85 regulatory subunit of PI3-kinase has been reported to specifically associate with tyrosine-phosphorylated IκBα through its Src homology 2 (SH2) domains in vitro and in vivo after stimulation of T cells with pervanadate (Beraud et al., 1999). This might have resulted in NF-κB activation. It has been shown that cdc42 could directly associate with p85. Although cdc42 does not seem to directly activate NF-κB, cdc42 is known to interact with guanidine nucleotide exchange factors and proteins with PH domains that are known to modulate NF-κB activity (Korus et al., 2002). Thus it is possible for cdc42 to indirectly stimulate NF-κB activity.

In addition, though the PI3-K pathway plays significant roles in controlling NF-κB in the cells, PI3-K pathway may not be the only pathway to regulate NF-κB activity. The presence of other pathways besides PI3-K pathway downstream of PDGF receptor to activate NF-κB may substitute the roles normally played by PI3-K pathway as well.

It is also possible that the SEAP gene reporter assay might fail to accurately report NF-κB activity. Accumulation of SEAP in the medium usually takes 48 hours post-transfection to be optimal and detectable. As the inhibition of PI3-K pathway by p110α siRNA and p110β siRNA seemed to be at its best at 24 hrs post-transfection, PI3-K activity might slowly return to normal during the 24-48 hour time period.
(Figure 8. Gray area below parabola 24hrs to 48 hours). The increase in p110α and p110β production and subsequent increase in PI3-K activity might be sufficient for the activation of NF-κB and production of SEAP, which might accumulate during the 24-48 hours time period. The siRNA mediated silencing of mRNA was transient, which means that the inhibition could wear off as time goes. SEAP assay measures accumulation of SEAP in the medium, therefore the incomplete inhibition of p110α and p110β may hinder the suppression of SEAP production (i.e. inhibition of NF-κB) achieved at 24 hour time point. Same situation could be true to the period where siRNA starts to inhibit p110α and p110β (Figure 8. Gray area below parabola 0-24 hours time point). During this period, one would assume PI3-K activity was present and sufficient enough to cause the activation of NF-κB and production of SEAP, which might be accumulated during the 0-24 hours time period.
Figure 8. p110 Inhibition by siRNA.

siRNA is known to suppress the target gene between 24 to 48 hour post transfection in mammalian cells (Elbashir et al., 2001a). In this diagram, p110α siRNA is shown to maximally suppress p110α expression at 24 hours post transfection. PI3-K activity still exists before and after the maximal suppression by siRNA, which may be enough for NF-κB to be actively initiate transcription.
EMSAs were also performed to determine the effect of PI3-K pathway on the regulation of NF-κB activity. By harvesting nuclear protein at 24 hour from the cells treated with siRNA against the p110 catalytic subunit of PI3-K the most significant reduction in NF-κB binding should be evident. It was hypothesized that PI3-K activity was the lowest at 24 hours post transfection with siRNA as suppression by siRNA against p110α and p110β should be the greatest at the time. The result presented (Figure 21) shows that binding of NF-κB was not significantly altered by the treatment of the cells with siRNA against p110α or p110β.

Regulation of NF-κB Activity by PDGF

Although this study attempted to focus on the roles of PI3-K activity in the regulation of NF-κB in glioblastoma U87-MG cells, what this study actually revealed was the complex regulation of transcription factor NF-κB by PDGF β receptors. The results obtained from gene reporter assays in U87-MG cells transfected with wild-type and mutant PDGF β receptors are statistically insignificant, but it was a quite surprise to discover that R634 mutant (kinase deficient mutant) of PDGF β receptors significantly suppressed NF-κB activity in U87-MG cells almost to the background level. This result strongly suggests that PDGF β receptor plays pivotal role in the fate of NF-κB and U87-MG glioblastoma cells. On the contrary, it was a quite surprise to know that add-back mutants 1009 and 740/751 are capable of activating NF-κB as wild-type (Figure 24). Considering the fact that U87-MG cells transfected with the
F5Y mutant and, secondary signaling molecules that associate with tyrosine 1009, 740, and 751 are capable of turning on signaling cascade leading to the activation NF-κB.

Overall, the results obtained from this study points out that transcription factor NF-κB plays an essential roles in PDGF mediated cellular transformation. Secondary signaling cascades that connect PDGF receptor activation and NF-κB seem to be very complex. Not much has been known for crosstalks between signaling cascades, which make signal transduction a complex process.
Appendix A

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