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REGULATION of PLATELET-DERIVED GROWTH FACTOR SIGNALING by ACTIVATED $p21^{Ras}$

Ligaya L. Stice, Cyrus Vaziri and Douglas V. Faller

Cancer Research Center, Boston University School of Medicine, Boston, MA

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1. ABSTRACT

Elucidating the molecular mechanisms regulating transduction of growth control signals and the discovery of the subversion of these pathways by oncogenes has proven critical in unraveling the biochemical factors leading to cellular transformation. One such line of investigation has been study of the effects of transforming $p21^{Ras}$ on platelet-derived growth factor type-beta receptor (PDGF-betaR) signaling. Platelet-derived growth factor is an important extracellular factor regulating the G_0-S phase transition of mesenchymal cells. Expression of activated, oncogenic Kirsten- or Harvey-$p21^{Ras}$ in cells influences PDGF-betaR signaling at multiple levels. At least two separate mechanisms account for defective PDGF-betaR signaling in activated $p21^{Ras}$-expressing cells: (i) transcriptional down-regulation of PDGF-betaR expression, and (ii) inhibition of ligand-induced PDGF-betaR phosphorylation by a factor which is present in the cellular membrane fraction of fibroblasts expressing activated $p21^{Ras}$. The state of growth arrest in G_0 is associated with increased expression of the PDGF-betaR, and oncogene-transformed cell lines, which fail to undergo growth-arrest following prolonged serum-deprivation, express constitutively low levels of the PDGF-betaR mRNA, and possess greatly reduced numbers of PDGF-BB-binding sites. This repression of PDGF-betaR expression by $p21^{Ras}$ is, at least in large part, transcriptional. The membrane-associated factor induced by oncogenic $p21^{Ras}$ provides a connection between cell morphology and cytoskeletal elements and control of ligand-dependent PDGF-betaR autophosphorylation. Reversion of the transformed phenotype results in the recovery of PDGF-betaR kinase activity. Conversely, disruption of the actin cytoskeleton of untransformed fibroblasts leads to the loss of PDGF-betaR function. These studies define two potential mechanisms for feedback control of PDGF-betaR function by downstream elements in the PDGF signaling pathway. In addition, the connection between cell morphology and the function of the PDGF-betaR established by these studies provides a new mechanistic link between the organization of the cytoskeleton, the Ras-related small G proteins, and the activity of membrane-bound receptor tyrosine kinases.

2. INTRODUCTION: THE ROLE OF ONCOGENES IN SIGNAL TRANSDUCTION

It has been approximately thirty years since retrovirologists first noted that the *src* gene carried by Rous sarcoma virus was actually a derivative of a sequence present in the avian host cells that had been captured and incorporated into the viral genome. This observation gave rise to the concept of proto-oncogenes, and introduced the genetic paradigm that now virtually dominates current cancer research.

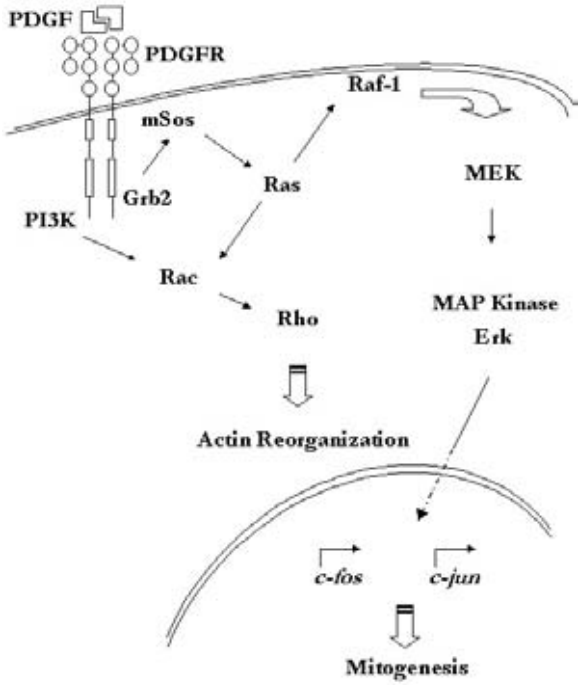

Figure 1. Signaling pathways from the PDGF-betaR. The binding of PDGF ligand to its cell surface receptor induces a wide range of cellular responses including proliferation and cytoarchitectural changes. Depicted in this figure are the pathways identified for PDGF-BB binding to the type-beta receptor.

A role for proto-oncogenes in tumorigenesis stems from the finding that their protein products are important components in normal growth regulation. Virtually all known oncoproteins so far described are constituents of growth factor signal transduction pathways (1,2). An understanding of the role of growth factors in normal development and differentiation provides insights into tumorigenesis, and conversely, the study of oncogenes and how they function in transformed cells reveals much about the regulation of normal growth control. This review will focus on one of the best-studied systems of growth factor signaling, that of the platelet-derived growth factor (PDGF) and the role that the oncoprotein p21^{Ras} plays in control of this pathway.

2.1. PDGF-R signal transduction

PDGF is a powerful mitogen for mesenchymal cells including smooth muscle cells, 3T3 cells and glial cells (3-5). In addition to its growth promoting activities, PDGF is also a potent chemotactic factor for vascular smooth muscle cells, monocytes, neutrophils and fibroblasts (6-8). The dual mitogenic and chemotactic properties of PDGF underscore its importance in the genesis of pathological fibroproliferative processes such as atherosclerosis, pulmonary fibrosis, keloid formation, myelofibrosis, glomerulonephritis and carcinogenesis (9,10). PDGF is an approximately 30 kDa disulfide-linked dimer of two homologous polypeptide chains, designated A and B (11). The A and B chains combine to form three different PDGF isoforms - homodimeric PDGF-AA or -BB, and heterodimeric PDGF-AB. PDGF-AB is the most abundant form in human platelets and was the first PDGF isoform to be identified (12). These PDGF isoforms bind with varying affinities to three different PDGF receptors.

The cell surface receptor for PDGF is an approximately 180 kDa transmembrane glycoprotein which belongs to a family of receptors that includes the colony-stimulating factor-1 receptor (CSF-1R, *fms*) and the stem cell factor receptor (*kit*). This class of receptors is similar to the insulin receptor and the epidermal growth factor receptor (EGF-R), in that they contain a single transmembrane domain and an intrinsic tyrosine kinase activity (13,14). The distinctive structural characteristics of this family include an extracellular region organized into five large disulfide bonded immunoglobulin-like domains and a region known as the kinase insert which interrupts the functional tyrosine kinase (15). The binding of PDGF at the cell surface induces either homo- or heterodimerization of receptor monomers, which subsequently become covalently linked (16,17). Since PDGF A chain interacts only with alpha receptors, while the B chain can bind both alpha and beta receptors, PDGF-AA induces only homodimers of alpha receptors while PDGF-BB induces all combinations of receptor dimers. Stimulation with PDGF-AB forms alpha-beta receptor heterodimers and alpha-alpha receptor homodimers (18). Complexes composed of alpha-alpha receptor homodimers are designated type-alpha receptors, as beta-beta homodimers are specified as type-beta.

2.1.1. Cellular responses to PDGF

Binding of PDGF ligand at the cell surface induces receptor dimerization and transphosphorylation of receptor monomers on specific tyrosine residues within the cytoplasmic domain. A series of intracellular phenomena follow, culminating in DNA synthesis and cell division. Figure 1 is a schematic of some of the signals transduced *via* the PDGF type-beta receptor (PDGF-betaR). The most well-characterized pathway is the MAPK arm, thought to be particularly important in the PDGF mitogenic response. This serine/threonine phosphorylation cascade ultimately results in the phosphorylation of Erk, which then translocates into the nucleus and induces the transcription of growth-related genes such as c-*jun* and c-*fos*. An early branch-point of the p21^{Ras} pathway leading to actin reorganization involves other members of the Ras superfamily, the small GTP-binding proteins Rho and Rac (19,20). Phosphotidylinositol-3'-kinase (PI3K) is also activated by PDGF stimulation and has itself been shown to be an upstream regulator of the Rho/Rac cascade (21). The signaling molecules inositol triphosphate (IP_3) and diacylglycerol (DAG) can be generated by PDGF-betaR activation, *via* the stimulation of phospholipase C-gamma 1 (PLC-gamma 1). This is a highly simplified schematic, as PDGF-betaR signaling is known not to proceed in a linear fashion. There exists redundancy and cross-talk between these pathways, providing for fine regulation of the PDGF signal (10,22).

Biochemical responses to PDGF include fluctuations of pH and Ca^{2+}levels , activation of PLC-

gamma 1, increases in hydrolysis of phosphoinositide (PI), increases in the amount of GTP bound to p21^{Ras}[26] and induction of growth related genes such as c-*myc* and c-*fos* (23-28). A majority of these responses are mediated by signaling proteins containing src homology 2 (SH2) domains, which bind to the newly-tyrosine phosphorylated sites on the PDGF-betaR. The specificity of these interactions is dictated by short, very highly conserved amino acid sequences immediately surrounding the phosphotyrosine residue on the activated receptor (29).

Associations between the activated PDGF-betaR and signaling molecules were first demonstrated using receptor mutants lacking large domains (*e.g.*, the entire kinase insert or the cytoplasmic tail) of the receptor. Using enzymatic activity assays and co-immunoprecipitation experiments, the above mentioned physiologic responses induced by PDGF were shown to involve PI3K, the GTPase-activating protein (p120-GAP) of p21^{Ras}, the Src family kinases (Src, Fyn and Yes) and PLC-gamma 1 (24,31-34). By systematic mutation of each tyrosine residue in the cytoplasmic domain of the PDGF-betaR, specific tyrosine docking residues for the Src family kinases (Y579 and Y581), the adaptor molecule growth factor receptor-bound protein 2 (Grb2, Y716), the p85 regulatory subunit of PI3K (Y740 and Y751), p120-GAP, the GTPase activating protein of p21^{Ras} (Y771), PLC-gamma (Y1009 and Y1021) and Syp (Y1009) have been identified (35-40).

2.2. p21^{Ras} in mitogenesis and differentiation

p21^{Ras} is a central molecular switch in signal transduction pathways originating from receptor tyrosine kinases (RTKs) that control cell growth and differentiation. A requirement for p21^{Ras} function in serum-stimulated mitogenesis of NIH 3T3 fibroblasts was first suggested by microinjection experiments using anti-p21^{Ras} antibodies. Following exposure to serum, microinjected cells were unable to enter the S phase of the cell cycle and initiate DNA synthesis (41). A dominant-inhibitory mutant of p21^{Ras} in these same cells blocks the mitogenic response to serum, epidermal growth factor (EGF) and PDGF (42). Neurite formation, a marker for differentiation normally induced by nerve growth factor (NGF), was inhibited by microinjection of p21^{Ras} antibody into PC12 pheochromocytoma cells (43). Experiments using similar techniques have proven p21^{Ras} to be vital for signaling pathways initiated by fibroblast growth factor (FGF), interleukin-2, -3 and –6 (IL-2, -3 and –6), and granulocyte-macrophage colony stimulating factor (GM-CSF) (44,45).

Overexpression of normal p21^{Ras}, or introduction of oncogenic p21^{Ras} have both been shown to be able to substitute for the activation of p21^{Ras} *via* a growth factor or serum-induced signal. Low levels of normal N-Ras expression can induce DNA synthesis in the absence of any growth signal. Higher levels of expression, 20 to 50-fold above the normal cellular content of p21^{Ras} protein, produce complete cellular transformation. Introduction of oncogenic H-ras was shown to induce morphologic differentiation in PC12 pheochromocytoma cells (46). Microinjection of oncogenic H-Ras protein, but not the proto-oncogenic form, led to the acquisition of a flattened, polygonal shape and to neurite outgrowth in these cells, features normally associated with NGF stimulation. Thus, cellular differentiation or mitogenesis normally brought about by growth factor treatment can be stimulated by the direct introduction of activated p21^{Ras} or by overexpression of proto-oncogenic p21^{Ras}.

2.2.1. Regulation of p21^{Ras} activity

The p21^{Ras} proteins, H-, K-, and N-Ras, are members of a family of membrane-associated G-proteins that have the ability to bind GTP and to catalyze its hydrolysis to GDP. p21^{Ras}-GTP complexes are biologically-active and stimulate downstream effectors. Deactivation occurs upon hydrolysis of bound GTP to GDP. Although p21^{Ras} has some intrinsic GTPase activity, hydrolysis to GDP can be greatly enhanced by the action of GTPase-activating proteins (GAPs) that act as negative regulators by returning p21^{Ras} to its GDP-bound state. Replacement of bound GDP with GTP and consequent activation of p21^{Ras} is catalyzed by guanine-nucleotide exchange or releasing factors (GRFs or GEFs), such as mammalian Son of Sevenless (mSos). The relative activities of GAPs versus GRFs therefore determine the activation state of p21^{Ras} (47-49).

p21^{Ras}-mediated transformation of cells can occur by ectopic constitutive activation of endogenous cellular p21^{Ras} or *via* a mutated endogenous or exogenous (viral) *ras* gene product. Some transformed cell lines, such as NIH 3T3 fibroblasts expressing activated Src or Abl, exhibit very high constitutive levels of p21^{Ras}-GTP, seemingly independent of an exogenous growth signal (50). It has been shown that a particular class of p21^{Ras} mutations discovered first in human tumors can lock the protein into its GTP-bound state. These oncogenic mutants allow GAP to bind, but do not allow it to perform its normal GTP hydrolysis function, thereby maintaining a high level of p21^{Ras} –GTP (51). The resultant chronic activation of p21^{Ras}-mediated signaling events is thought to contribute to the aberrant growth of tumor cells.

Studies in yeast, *Drosophila,* and *Caenorhabditis elegans* have demonstrated a highly-conserved pathway leading from receptor tyrosine kinases through p21^{Ras} to the induction of growth or differentiation pathways. In all cases, p21^{Ras} is thought to be linked to receptor activation *via* the recruitment of an adaptor protein associated with a GRF. In mammalian fibroblasts, EGF-induced receptor phosphorylation leads to formation of a growth factor receptor-Grb2-mSos complex. The GRF, now in proximity of the plasma membrane, can then positively modulate p21^{Ras} activity (52). p21^{Ras}, in turn, serves to localize the serine/threonine kinase, Raf, to the plasma membrane, where initiation of the mitogen-activated protein kinase (MAPK) cascade occurs. Activation of this signaling mechanism is also evolutionarily conserved, and leads to the eventual induction of growth-related or cell specific genes (48,53).

3. PDGF RESPONSE IN CELLS TRANSFORMED BY p21^{Ras}

Approximately ten years ago, it was noted by our

laboratory and by others that fibroblasts expressing transforming p21^{Ras} exhibited anomalies in PDGF signaling events (54-58). In a series of early studies by Gorman and colleagues, NIH-3T3 cells expressing the *ras* oncogene isolated from the EJ human bladder carcinoma, were found to have defects in two G-protein-regulated systems - hormone-stimulated adenylate cyclase and PDGF-stimulated phospholipase activities (57,58). Prostaglandin E_2 (PGE_2) release and PI turnover, events downstream of PLC activation were decreased in EJ-*ras*-containing cells *vs.* normal controls. Further studies revealed virtually no PDGF-mediated increase in intracellular Ca^{2+}, another marker of phospholipase activity, in the vast majority (90%) of EJ-*ras*-transfected cells, and a marked difference in the localization of Ca^{2+} increases in the small population that did respond with very small, transient fluxes (59). This series of studies showed that PDGF ligand binding in EJ-*ras* transformed cells was uncoupled from an entire sequence of events downstream of and including phospholipase activity, PI turnover and Ca^{2+} mobilization. The decrease in these PDGF-stimulated events was not due to a downregulation of PDGF-betaR, as the EJ-*ras* transformed cells were able to bind at least 70% as much ^{125}I-labelled PDGF as the control cells. The diminished PI turnover in p21^{Ras}-transformed cells in response to PDGF was confirmed by others. These studies showed that cells transformed with either activated H- or Ki-p21^{Ras} had decreased PDGF-stimulated PI hydrolysis, demonstrating that this effect was not peculiar for H-v-p21^{Ras}. Again, PDGF-betaR levels were near normal and could not account for the decreased response in these cells (60,61).

Research done in our laboratory and that of Lin *et al.* looked further downstream at PDGF-induced immediate early gene expression in p21^{Ras}-transformed cells. Lin, *et al.* observed a reduction in steady-state mRNA levels of c-*fos* in EJ-*ras*-transformed cells compared to wild-type controls (62). In Ki-v-p21^{Ras}-transformed Balb/c-3T3 fibroblasts, Zullo and Faller demonstrated aberrations in mRNA regulation for the immediate early genes c-*myc*, c-*fos* and *JE* (55). The introduction of v-*ras* into exponentially growing Balb cells by retroviral infection caused c-*myc* mRNA levels to fall by approximately 30-fold compared to uninfected Balb controls. Following infection, the cells continued in their exponential growth phase, despite the lack of induction of growth-related genes. In quiescent Balb cells only two hours after infection with Ki-MSV, stimulation with PDGF-BB failed to produce the expected increase in c-*myc* mRNA. These studies were important because they were able to focus on PDGF signaling events very early after introduction of the Ki-v-*ras* gene, allowing the investigators to attribute their observations to v-p21^{Ras} itself, rather than to the more complex events needed to establish a frankly-transformed cell.

Experiments were then undertaken in our laboratory to try to determine where specifically the signal was impeded in the cascade of PDGF-induced events. Rake *et al*. demonstrated that Ki-v-p21^{Ras} transformed Balb cells (KBalb) displayed little increase in tyrosine phosphorylation of the PDGF-betaR following ligand stimulation, indicating that the block to these PDGF-mediated events occurred very early, essentially at the level of PDGF-R autophosphorylation (54). An important finding in this report was that, in addition to a lack of ligand-induced PDGF-betaR autophosphorylation in KBalb cells, receptor phosphorylation could be dominantly-inhibited in parental Balb fibroblasts. When either detergent-solubilized supernatants or membrane preparations of KBalb and Balb cells were mixed together and then subjected to a PDGF-BB-dependent *in vitro* kinase assay, autophosphorylation of the PDGF-betaR present on Balb cells was inhibited.

The inhibitory activity associated with KBalb membranes had no effect when mixed with a pre-phosphorylated PDGF-betaR from Balb cells, demonstrating that this phenomenon was not due to simple dephosphorylation of the receptor by enhanced tyrosine phosphatase activity. Pretreatment of KBalb membranes with 1 mM Na_3VO_4, a known tyrosine phosphatase inhibitor, prior to the *in vitro* kinase reaction also had no effect on the level of PDGF-betaR phosphorylation in KBalb membranes following PDGF-BB stimulation. Finally, the lack of ligand-dependent PDGF-betaR phosphorylation was also not due to the presence of a constitutively-active receptor, as very little phosphotyrosine could be detected in unstimulated KBalb cell lysates.

3.1. p21^{Ras} revertants as a tool for studying PDGF-betaR signaling

The blockade to PDGF-betaR signaling was further explored in our laboratory by the use of KBalb revertants. Exposure of p21^{Ras}-transformed cells to cAMP analogs or cholera toxin restores a normal phenotype and permits a partial return of Ca^{2+} mobilization and PI turnover (63). Expression of *Krev-1*, which codes for another member of the Ras superfamily of GTP-binding proteins, p21^{rap1}, can also revert p21^{Ras}-transformed cells to a normal morphology (64). Both of these approaches were employed in KBalb fibroblasts to see which, if any, of the PDGF-induced phenomena could be restored. The KBalb revertants showed morphologic and growth characteristics similar to those of the parental Balb cells. Activation of PLC, Ca^{2+} mobilization, and induction of c-*fos*, c-*myc* and *JE* were restored when revertant cells were treated with PDGF however, suppression of receptor phosphorylation was not reversed. Thus, phosphorylation of the PDGF-betaR remained uncoupled from some, but not all, downstream events (56,65).

4. MOLECULAR BASIS OF ABERRANT PDGF-betaR SIGNALING

4.1. Repression of PDGF-betaR expression in p21^{Ras} - expressing cells

In recent years, studies from our laboratory and others, have established that at least two separate mechanisms may contribute to impaired PDGF receptor signaling in p21^{Ras}-transformed cell lines. First, fibroblasts expressing activated p21^{Ras}, or certain other transforming

oncogenes, express reduced levels of PDGF-betaR mRNA and protein relative to parental untransformed cell lines. Moreover, a factor present in membrane preparations from oncogenic p21^{Ras}-expressing cells is able to act in *trans* to inhibit ligand-dependent autophosphorylation of the PDGF-betaR.

Modulation of growth factor receptor expression appears to be common in transformed cell lines. Transformation of Balb cells by SV40, or by Harvey, Kirsten or Moloney sarcoma viruses, decreases the density of insulin receptors (66). EGF-R expression is decreased in SV40-transformed 3T3 cells, yet dramatically increased in A431 cells (67-69). Decreased PDGF-betaR expression in cells transformed both by chemical means and by retroviral infection has been noted by other investigators (70,71). Other studies examining fibroblasts transformed by p21^{Ras} however, have not shown any consistent changes in PDGF-betaR expression levels (58-60,62).

During the study of PDGF signaling in transformed cells, we observed that 3T3 cell lines chronically expressing oncogenic p21^{Ras} expressed reduced levels of PDGF-betaR protein relative to parental untransformed fibroblasts. To quantify the changes in PDGF-R levels resulting from *v-ras* expression, we compared the numbers of [^{125}I]-PDGF-BB-binding sites on untransformed and *v-ras*-containing 3T3 fibroblasts by Scatchard analysis of ligand-binding. Parental Balb 3T3 fibroblasts expressed approximately 78,000 PDGF-BB-binding sites per cell, compared to approximately 12,000 PDGF-BB receptor sites per cell transformed with Ki-v-p21^{Ras}. p21^{Ras} expression had no effect on the ligand-binding affinity of the PDGF-R. Our results were similar to those of Grotendorst, who had previously shown reduced numbers of PDGF-binding sites in Kirsten sarcoma virus-expressing NIH-3T3 cells, relative to the untransformed parental fibroblasts (72). We extended these observations by showing that the steady-state levels of PDGF-betaR mRNA in Ki-v-p21^{Ras}-expressing fibroblasts were 5-10 fold lower than those expressed in untransformed cells. Nuclear run-off analyses indicated that changes in rates of transcription of the *PDGF-betaR* gene could account for the reduced levels of PDGF-betaR expression in v-p21^{Ras}-expressing fibroblasts.

Procedures which are known to antagonize v-p21^{Ras} activity and revert the transformed phenotype of ras-transformed cells (*e.g.*, treatment with cell-penetrant cAMP analogues or stable expression of the *k-rev* gene) restored normal levels of PDGF-R expression upon v-p21^{Ras}-containing cells (73). Therefore, the decreases in PDGF-R expression seen in v-p21^{Ras}-containing fibroblasts were unlikely to result merely from selection of PDGF receptor-deficient cells during transformation in culture. Instead, these data suggested either that a v-p21^{Ras}-induced signal might negatively regulate transcription of the *PDGF-betaR* gene, or that transcriptional events secondary to the transformed phenotype resulted in down-regulation of PDGF receptor expression.

To discriminate between these possibilities we tested the effects of other transforming oncoproteins on PDGF-betaR expression in 3T3 cells. Ectopically-expressed oncogenes other than *v-ras* (including *v-src*, *v-abl*, and the Human Papilloma Virus *E7* gene) also conferred a transformed phenotype, and repressed PDGF-R expression (73). Our findings were corroborated by the studies of Zhang *et. al*. who showed that expression of *v-src*, or of the Polyoma virus middle T antigen, in NRK (rat kidney fibroblasts) resulted in 7-fold decreases in PDGF-binding sites (74). Similarly, Wang and colleagues reported that PDGF-beta (as well as –alpha) receptors were down-regulated at the level of mRNA in SV 40-transformed human fibroblasts (75). Overall, these results suggested that the effect of activated p21^{Ras} on PDGF-betaR expression was unlikely to be mediated by p21^{Ras}-specific signal transduction events. Instead, there appeared to be a correlation between the transformed phenotype and reduced expression of PDGF-betaR.

4.1.1. Cell cycle influences on PDGF-betaR expression

Transformed cells frequently display constitutive and growth factor-independent activation of mitogenic signal transduction events. Thus, oncogene-transformed fibroblasts fail to exit the proliferative cell cycle in response to environmental signals, such as growth factor deprivation or high confluence, which otherwise elicit growth arrest. We hypothesized that PDGF-betaR repression in oncogene-expressing fibroblasts may be a consequence of the constitutive proliferative state of chronically-transformed cells. To test this hypothesis, we examined PDGF-betaR levels during the course of the normal cell cycle in untransformed 3T3 fibroblasts. The state of growth arrest (G_0 or quiescence) was associated with high levels of PDGF-betaR mRNA expression relative to exponentially-growing fibroblasts. Moreover, treatment of growth-arrested 3T3 cells with mitogenic factors such as serum, or a combination of PDGF, EGF and IGF-1, resulted in repression of PDGF-betaR mRNA levels concomitant with re-entry into the cell cycle. Thus, the PDGF-betaR is preferentially expressed during growth arrest, and is suppressed following entry into the cell cycle. Interestingly, the PDGF-betaR was recently identified during a screen for growth arrest-specific genes (76). As we had found for the PDGF-betaR, this study demonstrated that PDGF-betaRs were preferentially expressed in quiescent cells, and that receptor expression was suppressed in response to mitogens or oncogenes.

These data provided a molecular basis for earlier observations demonstrating an inverse relationship between the chemotactic response to PDGF and the rate of proliferation (6). Quiescent NIH 3T3 fibroblasts were found to exhibit a twenty five-fold greater chemotactic response to PDGF relative to exponentially-growing cells. Moreover, NIH-3T3 cell lines which had been transformed by SV-40 or by the Kirsten sarcoma virus lost their ability to respond to PDGF as a chemoattractant. These results were attributed to reduced numbers of PDGF-binding sites in proliferating and transformed cultures relative to quiescent cells.

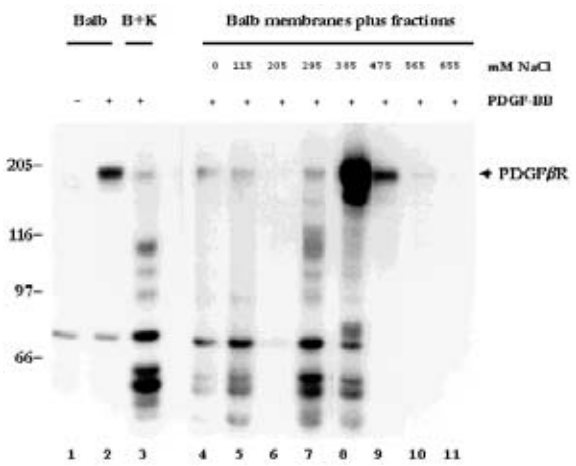

Figure 2. Fractionation of cell membranes from Balb cells expressing oncogenic p21^{Ras} (Kbalb cells) on a MonoQ anion-exchange column. Approximately 15 mg of membrane protein prepared from serum-starved KBalb cells were applied to a MonoQ column and eluted with a 0-0.9 M NaCl gradient. Fractions were assayed for PDGF-betaR inhibitory activity by addition of a 40μL aliquot to an *in vitro* kinase mixing experiment with Balb cell membranes. Anti-phosphotyrosine immunoprecipitates were separated by 7.5% SDS-PAGE. Lane 1, Control Balb cell membranes. Lane 2, Balb membranes stimulated with PDGF. Lane 3, Balb mixed with KBalb membranes stimulated with PDGF. Lane 4, Balb membranes mixed with the column starting material (denoted fraction 0) stimulated with PDGF. Lanes 5-11, Balb membranes mixed with aliquots from various column fractions. The inhibitory activity localizes to a salt concentration of approximately 205 mM. The PDGF-betaR elutes at 385 mM NaCl. The figure is an autoradiogram. Molecular weight markers are to the left.

Overall these studies have shown that mesenchymal cells express highest levels of PDGF receptors during growth arrest, and that entry into the cell cycle due to mitogens or ectopically-expressed oncogenes results in down-regulation of both PDGF-betaR and -alphaR.

PDGF is an important factor in regulating entry of quiescent mesenchymal cells into the cell cycle. It is likely that the induction of PDGF-R expression during growth arrest serves to 'prime' cells for potential stimulation by PDGF. Conversely, the reduced expression of PDGF-R following entry into G_1 appears to serve as a negative-regulatory mechanism which renders proliferating cells refractory to the actions of (all three isoforms of) PDGF. Cells generally exert negative controls upon signal transduction systems. Such homeostatic mechanisms quench signaling events in the face of continuing stimulation by extracellular agonists (*e.g.*, hormones, polypeptide growth factors). Although numerous mechanisms are now known to participate in negative regulation of ligand-activated signaling pathways, attenuation is often achieved by regulation of receptor function. Collectively, the studies described above indicate that transcriptional down-regulation of receptor expression during proliferation provides an additional mechanism for modulating cellular responsiveness to extracellular ligands.

In addition to PDGF-Rs, other growth factor receptors are known to be subject to regulation at the level of mRNA expression. For example, c-*kit* transcripts are suppressed in mast cells following treatment with hematopoietic growth factors (*e.g.*, IL-3, GM-CSF, and EPO), and long-term NGF treatment downregulates EGF receptors in PC12 cells (77,78). Thus, transcriptional regulation of receptors is likely to provide a general mechanism for modulating cellular responses to polypeptide growth factors.

4.1.2. Transcriptional regulation of PDGF-betaR expression

To elucidate the mechanisms of transcriptional regulation of PDGF-R expression, we have cloned the 5' promoter region of the murine *PDGF-betaR* gene. Heterologous promoter-reporter gene plasmids into which putative promoter sequences derived from this region are inserted upstream of bacterial CAT or firefly luciferase reporter genes have been constructed. These putative promoter sequences confer high level expression of the reporter genes when the chimeric plasmids are transiently expressed in mesenchymal cells (79). Sequence analysis of the minimal promoter region revealed a TATA-less promoter with consensus binding sites for known transcription factors including Sp1, GATA, CREB and NF-1. Funa and colleagues, who cloned the *PDGF-betaR* gene independently, have suggested that a single NF-Y site 60 bp upstream of the transcriptional start site is critical for basal promoter activity (80). Studies are currently underway in our laboratory to identify the putative *cis*-acting elements and *trans*-acting factors that mediate regulated changes in PDGF-R expression. The results from these studies will eventually permit elucidation of the signal transduction pathways that integrate changes in PDGF-R expression with the proliferative state of cells.

4.2. Identification of a p21^{Ras}-induced inhibitor of PDGF-betaR activation

Aside from the decreased levels of PDGF-betaR expression described above, a second mechanism involving the presence of an inhibitory factor in the membranes of p21^{Ras}-transformed cells may operate to blunt PDGF-induced events. As mentioned previously, this inhibitory factor functions in *trans* to suppress PDGF-betaR autophosphorylation and does not appear to be a phosphatase.

In an effort to further characterize the PDGF-betaR inhibitory factor present in p21^{Ras}-transformed cells, we partially-purified this factor from membranes prepared from KBalb cells by anion-exchange chromatography (81). The inhibitory activity could be separated from the PDGF-betaR, as the receptor eluted at a much higher salt concentration (figure 2). The partially-purified PDGF-betaR from KBalb cells could be stimulated with PDGF-BB and displayed a normal autophosphorylation response when separated from the inhibitory activity. The inhibitory activity also co-eluted with several signaling molecules known to interact directly or indirectly with the PDGF-betaR: Grb2, Syp and p21^{Ras}.

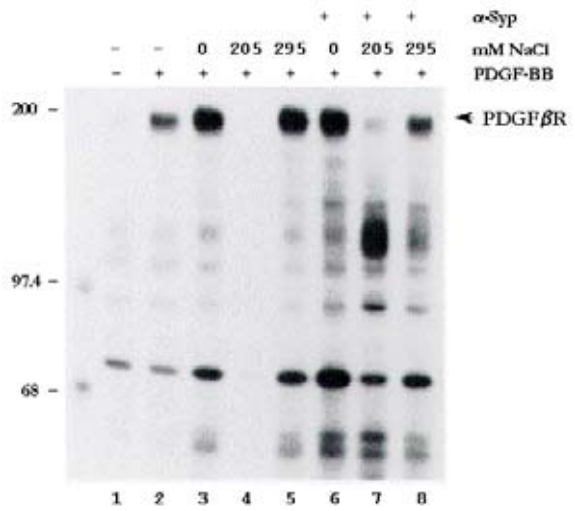

Figure 3. Immunodepletion of Syp from a purified fraction containing the PDGF-betaR inhibitor, derived from Balb cells expressing oncogenic p21^{Ras}. Forty μL of the column fractions described in Figure 2 legend were immunoprecipitated with 2 μg of an affinity purified anti-Syp antibody, before assaying for inhibitory activity in an PDGF-BB dependent *in vitro* kinase mixing experiment. Anti-phosphotyrosine reactive proteins were separated by SDS-PAGE on a 7.5% gel. Lane 1, Control Balb cell membranes. Lane 2, PDGF-BB-stimulated Balb cell membranes. Lanes 3-5, Balb cell membranes mixed with column fractions before immunoprecipitation with anti-Syp. Lanes 6-8, Balb cell membranes mixed with column fractions after removal of Syp by immunodepletion. The figure is an autoradiogram. Molecular weight markers are shown on the left.

The PDGF-betaR regained its ability to autophosphorylate once physically separated from the p21^{Ras}- induced inhibitory activity, demonstrating that the receptor present on KBalb cells was not permanently modified as a result of transformation, and that the inhibition of PDGF-betaR phosphorylation is a reversible process.

4.2.1. Candidate inhibitors of the PDGF-betaR

The fraction containing the inhibitory activity also co-eluted with the adaptor molecule Grb2, the protein tyrosine phosphatase Syp, and p21^{Ras}. The ability of Syp to associate with the PDGF-betaR, the apparent general phosphatase activity present in the fraction containing the inhibitor, and the elution of an isoform of Syp in the same fraction, prompted further investigation into Syp as a candidate inhibitor (82). Immunodepletion of Syp from the inhibitory fraction, followed by an *in vitro* kinase mixing experiment with Balb membranes, recovered significant amounts of substrate tyrosine phosphorylation. However, kinase activation of the PDGF-betaR remained inhibited (figure 3). So, although Syp appears to be responsible for much of the tyrosine phosphatase activity present in the inhibitory fraction, Syp clearly is not the PDGF-betaR inhibitor.

Qualitative differences do exist, however, between the Syp protein present in normal cells and in oncogenic Ras-expressing cells. Two isoforms of Syp have been described in the literature and can be distinguished on the based on their migration on SDS-PAGE. The slower-migrating form is more heavily tyrosine-phosphorylated and is catalytically-active, whereas the faster-migrating form is hypophosphorylated and has relatively less catalytic activity (82-84). The Syp isoform detected in the inhibitory fraction from KBalb cells was the faster-migrating form and presumably less catalytically-active. However, removal of this isoform by immunodepletion allowed for substantial recovery of substrate tyrosine phosphorylation (with the exception of the PDGF-betaR) in response to PDGF-BB. The activity of oncogenic p21^{Ras} may alter the substrate specificity or activity of Syp, perhaps *via* interaction with v-p21^{Ras} itself or through the induction of a co-factor which then associates with, and influences, Syp activity.

The presence of Ras protein in the PDGF-betaR-inhibitory column fraction raised the possibility that p21^{Ras} had a direct effect on the deregulation of the PDGF-betaR signaling. Preincubation of KBalb membranes with two different monoclonal antibodies to p21^{Ras} was done prior to a PDGF-betaR *in vitro* kinase assay. The antibodies used recognized two different epitopes of the p21^{Ras} protein, and both appeared to be able to relieve suppression of phosphorylation in KBalb membranes.

These experiments demonstrated that neutralization of p21^{Ras} activity, or interference with binding of downstream effector molecules, results in reconstitution of PDGF-betaR kinase activation. Additionally, they suggest that the presence of p21^{Ras} protein is somehow necessary for the inhibitor to act on the PDGF-betaR. These findings would be in agreement with the results of the PDGF-BB-dependent kinase assay done on partially-purified receptor from KBalb cells. That is, when the PDGF-betaR is separated from the inhibitor, and also from p21^{Ras}, the receptor has the capacity to respond to ligand.

4.2.2. Mechanisms of action of the PDGF-betaR inhibitor

Although our laboratory had previously ruled out an enhanced phosphatase activity as being responsible for the inhibition of PDGF-betaR autophosphorylation, a report by Tomaska and Resnick demonstrated that the *in vivo* treatment of p21^{Ras}-transformed NIH-3T3 fibroblasts with various phosphatase inhibitors could restore kinase activity of the PDGF-betaR (85). Similar to results previously obtained in our laboratory, these investigators showed that *in vitro* treatment of membrane preparations from p21^{Ras}-transformed cells with various phosphatase inhibitors had no effect on PDGF-betaR kinase activation. However, treatment of intact cells with sodium orthovanadate or phenylarsine oxide (PAO) could allow for some recovery of PDGF-betaR kinase activity. *In vivo* treatment of p21^{Ras}-transformed cells with a combination of these phosphatase inhibitors restored wild-type levels of ligand-dependent PDGF-betaR autophosphorylation. We found that, Balb cells showed normal phosphorylation of the PDGF-betaR in response to ligand *in vivo*, both in the presence and absence of vanadate (200 microM). Other reports concerning the differential *in vitro* and *in vivo* effects of vanadate have been documented in the current literature

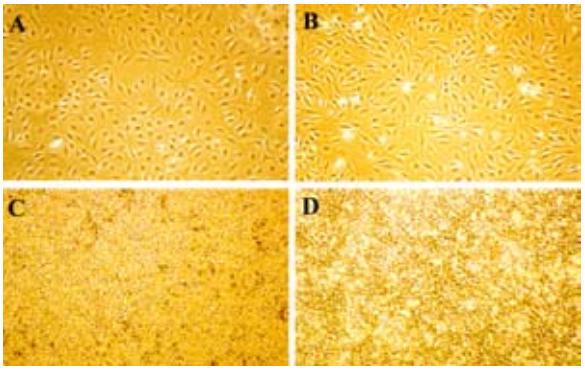

Figure 4. Morphological changes in Balb cells expressing oncogenic p21^{Ras} (Kbalb cells) upon vanadate treatment. Confluent monolayers of both Balb and KBalb cells were starved overnight in 0.5% DCS. Sodium vanadate was added to a concentration of 200 μM for one hour. Panel A, Control Balb cells. Panel B, Balb cells treated with vanadate. Panel C, Untreated KBalb cells. Panel D, KBalb cells treated with vanadate. Panels are all 100x magnification.

Table 1. Preatment of Balb and Kbalb cells with sodium vanadate prior to PDGF-BB stimulation: Correlation with PDGF-betaR kinase activity and cell phenotype

Cell type	VO_4	PDGF-bR kinase	Phenotype
Balb	-	+	Untransformed fibroblast
Balb	-	+	Untransformed fibroblast
KBalb	+	-	Transformed fibroblast
KBalb	+	+	Fibroblast-like

Interestingly, there did not appear to be any quantitative or qualitative increases in tyrosine phosphorylation in the vanadate-pretreated Balb cells after PDGF-BB stimulation. KBalb cells, in the absence of vanadate pretreatment, exhibited no detectable tyrosine phosphorylation of the PDGF-betaR after ligand stimulation. Following preincubation with vanadate however, these cells regained the ability to respond to ligand by autophosphorylation of the PDGF-betaR.

(86-90). The mechanism of action by which orthovanadate functions remains cryptic. When vanadate (V) is internalized, it is reduced intracellularly to vanadyl ion (IV), which has different substrate specificities and potency as an inhibitor, in comparison to the more oxidized state. The diverse actions of vanadate thus make it difficult to predict what effect it may have in a particular system.

The renewed capacity of the PDGF-betaR for ligand-dependent phosphorylation following *in vivo*, but not *in vitro*, treatment with vanadate in the presence of oncogenic p21^{Ras} suggested a potential mechanism of action for the inhibitor. *In vitro* treatment of membranes with vanadate did not allow the PDGF-betaR to regain ligand-dependent phosphorylation. The fact that *in vivo* vanadate treatment can reconstitute PDGF-betaR kinase activity suggests that the vanadate-sensitive molecule was not contained in membrane preparations, and is not the inhibitor itself. The inhibitor may be regulated by a cytoplasmic entity that is sensitive to the actions of vanadate. When this cytoplasmic regulator is physically separated from the inhibitor, as in the process of preparing membranes, vanadate then has no effect on the inhibitory activity.

If the cytoplasmic regulator can suppress inhibitor function upon vanadate treatment, then membranes prepared from KBalb cells that have been pretreated *in vivo* with vanadate might be expected to retain the ability to phosphorylate the PDGF-betaR. This hypothesis was tested by incubating KBalb cells in 200 microM vanadate prior to harvesting the cells and making membranes. KBalb cells membranes exposed to vanadate did not regain the ability to phosphorylate the PDGF-betaR *in vitro*. The implications of this experiment are that the regulator must be present, and perhaps associated with the inhibitor, in order to allow for PDGF-betaR phosphorylation. The interactions that the regulator has with the PDGF-betaR inhibitor either are not of sufficient affinity to be retained in membrane preparations, or the regulatory activity is not stable under these circumstances.

5.CELL MORPHOLOGY AND PDGF-betaR PHOSPHORYLATION

Accompanying the recovery of PDGF-betaR ligand dependent kinase activity by *in vivo* exposure to vanadate, a striking morphologic change in the KBalb cells was noted (figure 4). KBalb cells are not normally contact-inhibited and in addition display a rounded morphology that is highly refractile under the light microscope. Within one hour of exposure to 200 microM vanadate, the KBalb cells lose their rounded shape and appear to flatten out on the tissue culture plate, looking somewhat like a confluent plate of normal Balb fibroblasts. This phenotypic reversion correlated with the recovery of PDGF-betaR ligand-dependent kinase activity in these transformed cells (see table 1 for a summary of this data).

Previous studies have compared ligand-dependent PDGF-betaR autophosphorylation of fibroblasts grown in monolayer culture versus cells grown on collagen matrices that can be either mechanically stressed or relaxed. The PDGF-betaR showed markedly decreased levels of phosphorylation (approximately 90%) when stimulated on a mechanically-relaxed matrix relative to the monolayer controls (91). Cells grown under conditions of mechanical stress or relaxation also exhibited differences with regard to actin filament organization. The relaxed state is associated with a disruption of cytoskeletal architecture (92). Treatment of cells in culture with the microfilament assembly inhibitor cytochalasin D destroys the actin cytoskeleton and causes the cells to round up on the tissue culture plate. We have found that quiescent Balb cells treated with cytochalasin D, and then stimulated with PDGF-BB, show a dramatic decrease in the ligand-induced phosphotyrosine content of the PDGF-betaR, suggesting

that the actin cytoskeleton may play a role in the ability of the cell to respond to growth factor such as PDGF-BB.

In order to further test a dependency of the ligand-dependent phosphorylation of the PDGF-betaR on attachment or cell shape, Balb cells were stimulated with PDGF-BB while in suspension, rather than as a cell monolayer. Balb cells stimulated in suspension demonstrated no deficiency in PDGF-betaR phosphorylation. Thus, cell shape as a function of attachment does not correlate with the ability of the PDGF-betaR to autophosphorylate. Rather, the integrity of certain cytoskeletal elements, particularly actin filament assembly as specifically indicated by the experiments employing cytochalasin D, may play a role in regulating PDGF-betaR activity. Confocal microscopy was used to examine the relationship between ligand-dependent PDGF-betaR phosphorylation and the arrangement of actin filaments in the cell. Balb cells treated with or without cytochalasin D, and KBalb cells treated with or without vanadate, were stained with FITC-phalloidin to detect F-actin bundles. In typical quiescent fibroblasts, actin stress fibers are observed, along with actin in the diffuse cortical network (93). Treatment of these cells with cytochalasin D dramatically disrupts actin filament organization. Untreated KBalb cells display punctate staining without any defined actin framework and are clearly distinct from Balb cells with regard to cell morphology. Other investigators also have been unable to detect actin stress fibers or actin filament bundles in p21^{Ras}-transformed fibroblasts (94). Following vanadate treatment, KBalb cells appear to redistribute actin filaments, there is more peripheral staining, and the formation of stress fibers in these cells can be seen. Thus, there appears to be a correlation between the ability of the PDGF-betaR to autophosphorylate in response to ligand and the cytoarchitecture of the cell. The integrity of the cytoskeleton and, in particular, the presence of actin stress fibers, may be important for the function of the PDGF-betaR.

6. THE CYTOSKELETON AS A LINK BETWEEN p21^{Ras}, RHO, AND RAC

The PDGF-betaR is able to undergo ligand-dependent autophosphorylation under circumstances of normal actin stress fiber assembly and organization. Formation of stress fibers, and the assembly of focal adhesion complexes in response to growth factors, is mediated by another member of the Ras superfamily of small GTP-binding proteins, Rho (20). Growth factor-induced membrane ruffling is carried out by yet another family member, Rac. Microinjection of oncogenic p21^{Ras} triggers the activation of both of these proteins (19). Their activation is usually associated with the formation of stress fibers and with focal adhesion assembly. However chronic activation of Rho and Rac, in the context of a cell transformed by p21^{Ras}, may lead instead to disruption rather than to ordered assembly of actin fibers. Overexpression of these activities may result in a loss of cytoskeletal order through the deregulation of the dynamic interaction of actin filament formation and depolymerization.

The pathways that influence actin cytoarchitecture, in particular the regulators and downstream effectors for Rho and Rac signaling, are partially elucidated. The p21-activated protein kinase (Pak) family of serine/threonine kinases are candidates for downstream effectors of Rac, as they bind specifically to, and are activated by, Rac-GTP (95). Pak is activated by, and can associate with, EGF- and PDGF-Rs following ligand stimulation (96). Inhibition of Pak activity can be achieved by PI3K inhibitors such as wortmannin, thereby placing PI3K and Rac in a signaling cascade between tyrosine kinase receptors and Pak (97).

It has been hypothesized that Pak plays a role in the turnover of focal complexes. Overexpression of constitutively-active Pak mutants causes disassembly of focal adhesions and loss of stress fibers, and appears to allow for regeneration of these components (98). Our laboratory is currently exploring the role of Pak1, its influence on cytoskeletal architecture, and its ability to affect PDGF-betaR function, through the use of both constitutively-active and dominant-negative Pak1 mutants. Overexpression of kinase-deficient Pak1 in KBalb cells for example, appears to revert cell morphology and allow for phosphorylation of the PDGF-betaR.

Like Rac and Pak1, two families of kinases that preferentially associate with Rho-GTP have also been identified. These include the ROKs (or ROCKs) and the PKN-related kinases (99-104). ROK alpha is a cytosolic protein that translocates to the membrane and becomes localized with actin filaments following Rho activation. Stress fiber and focal adhesion formation can be achieved by expression of ROK alpha in quiescent HeLa cells. Kinase-deficient ROK alpha mutants do not induce stress fiber formation, while constitutively-active mutants produce more stress fibers than wild-type ROK alpha consistent with a role for ROK alpha as a downstream effector of Rho (100). Studies utilizing RhoA effector mutants have further confirmed that interaction with ROCK-1 and another unidentified effector is required for stress fiber formation (105).

A balance of Pak and ROK alpha activities is likely required in the dynamic processes involving actin filament formation. As Pak seems to be involved in the reorganization and disassembly of focal complexes, perhaps an upregulation of this pathway exists in KBalb cells, ultimately resulting in a reduction in the number of adhesion complexes and stress fibers. An impairment of normal ROK- betaR activity in p21^{Ras}-transformed cells could similarly lead to failure to form stress fibers, structures that appear to correlate positively with PDGF-betaR function.

6.1. Modulation of PDGF-betaR signaling by integrins

Processes that involve cell attachment and spreading inevitably alter cell phenotype. It is well established that these functions are mediated in part by the integrin family of transmembrane receptors. These

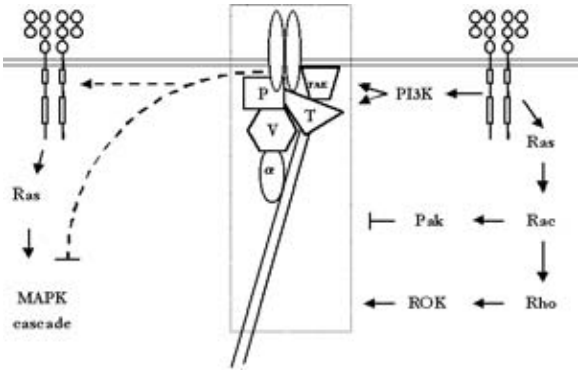

Figure 5. Cross-talk between PDGF-betaR and integrin signaling pathways. At the left are shown PDGF-BB mediated events that can be modulated by integrin engagement. Adhesion induces a transient, ligand-independent tyrosine phosphorylation of the PDGF-betaR, and is necessary for efficient PDGF-mediated signal transduction downstream of Ras, specifically the MAPK cascade. The intermediaries of these adhesion-induced phenomena have not yet been defined (dashed line). The right half of the figure shows events downstream of PDGF-betaR activation that influence actin and focal complex reorganization. In addition to stimulation of the Rac/Rho pathway, phosphorylation of the focal adhesion proteins tensin and FAK, via PI3K, is mediated by PDGF-BB. The focal adhesion complex, including the integrin receptor, focal adhesion and actin-binding proteins, and the terminal end of the actin filament, are delineated by the dotted rectangle. The abbreviations used are: FAK, focal adhesion kinase; P, paxillin; V, vinculin; T, tensin; α, α–actinin and PI3K, phosphatidylinositol-3´-kinase.

receptors co-localize with the actin-binding proteins talin, vinculin, tensin, and alpha-actinin, and are concentrated at focal adhesion sites. Focal adhesions also contain signaling molecules such as the focal adhesion kinase, p125^{FAK}, which have the potential to transmit signals from these sites. Integrins can therefore serve as mechanochemical transducers (106).

Evidence exists for cross-talk between integrins and PDGF-betaRs (figure 5). Plating of fibroblasts on collagen type I, fibronectin, or immobilized anti-integrin subunit IgG induces a transient, ligand-independent phosphorylation of PDGF-betaRs. This tyrosine phosphorylation response is specific for the beta-type PDGFR, as neither the EGFR nor the PDGF-alphaR demonstrate this effect (107). Stimulation of fibroblasts with PDGF-BB leads to tyrosine phosphorylation of tensin, paxillin, and p125^{FAK}, and to increases in steady-state mRNA levels of the collagen-binding alpha-2 integrin subunit (108-110). The activity of the beta-1 subunit, as measured by the ability to contract a collagen type I matrix, is increased by PDGF-BB treatment (111). Thus, several lines of evidence suggest that PDGF-betaRs regulate, and in turn are regulated by, cellular signals involving integrin activation. Our findings demonstrate that PDGF-betaR phosphorylation is sensitive to alterations of cell phenotype and suggest that modulation of receptor phosphorylation may involve integrin-mediated events.

Cell anchorage is an important modulating element in the growth factor signaling cascade downstream of p21^{Ras} (112). Stimulation of NIH-3T3 cells in suspension with PDGF or EGF produced efficient phosphorylation of their respective receptors and GTP-loading of p21^{Ras}. Activation of the kinases downstream of p21^{Ras}, specifically Raf-1, MEK, and MAP kinase, however, was substantially attenuated compared to cells anchored on fibronectin. It has recently been demonstrated that constitutive expression of oncogenic Ki-p21^{Ras} in HD6-4 colonic epithelial cells prevents the glycosylation, and therefore the maturation, of the integrin beta-1 chain (113). This defect prevents proper anchorage to extracellular matrix components and may influence proper polarization and differentiation of these cells as well. These results, together with the data presented above, suggest the existence of modulatory pathways between integrin signaling and the p21^{Ras}/Raf-1/MAP kinase cascade. The pathways that regulate and maintain the cellular architecture may thus provide promising possibilities for the site of action of the p21^{Ras}-induced PDGF-betaR inhibitor.

7. SUMMARY

These studies have established that activation of p21^{Ras} can regulate signaling from the PDGF-betaR in two different ways. The level of expression of the receptor is transcriptionally repressed, as a result of dysregulation of the cell cycle induced by the mitogenic activity of activated p21^{Ras}. In addition, the presence of p21^{Ras} in its activated form results in the production of a membrane-associated factor which suppresses ligand-dependent autophosphorylation of the PDGF-betaR. The common result of each of these processes is desensitization of the PDGF-mediated mitogenic pathway. As stimulation through the PDGF-R activates endogenous p21^{Ras} as a critical downstream effector, it is intriguing to speculate that this physiological activation of p21^{Ras} could serve as a feedback negative regulator to desensitize the cell to further mitogenic stimulation through the PDGF-betaR while it transits the cell cycle. In addition, the connection between cell morphology and the function of the PDGF-betaR established by these studies provides a new mechanistic link between the organization of the cytoskeleton, the Ras-related small G proteins, and the activity of membrane-bound receptor tyrosine kinases.

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Send correspondence to: Douglas V. Faller, Ph.D., M.D., Director, Cancer Research Center, Boston University School of Medicine, 80 E. Concord Street, K701, Boston, MA 02118, Tel: (617) 638-4173, Fax: (617) 638-4176, E-mail: dfaller@bu.edu

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