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Repression of Platelet-Derived Growth Factor β-Receptor Expression by Mitogenic Growth Factors and Transforming Oncogenes in Murine 3T3 Fibroblasts

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Platelet-derived growth factor BB (PDGF-BB) is an important extracellular factor for regulating the G_0-S phase transition of murine BALB/c-3T3 fibroblasts. We have investigated the expression of the PDGF β receptor (PDGFβR) in these cells. We show that the state of growth arrest in G_0, resulting from serum deprivation, is associated with increased expression of the PDGFβR. When the growth-arrested fibroblasts are stimulated to reenter the cell cycle by the mitogenic action of serum or certain specific combinations of growth factors, PDGFβR mRNA levels and cell surface PDGF-BB-binding sites are markedly downregulated. Oncogene-transformed 3T3 cell lines, which fail to undergo growth arrest following prolonged serum deprivation, express constitutively low levels of the PDGFβR mRNA and possess greatly reduced numbers of cell surface PDGF receptors, as determined by PDGF-BB binding and Western blotting (immunoblotting). Nuclear runoff assays indicate the mechanism of repression of PDGFβR expression to be, at least in large part, transcriptional. These data indicate that expression of the PDGFβR is regulated in a growth state-dependent manner in fibroblasts and suggest that this may provide a means by which cells can modulate their responsiveness to the actions of PDGF.

Platelet-derived growth factor (PDGF) is a potent mitogen and chemotactic factor for a variety of mesenchyme-derived cells. The biologically active ligand exists as a 32-kDa molecule consisting of two disulfide-linked PDGF monomers. There exist two PDGF genes, encoding related but distinct polypeptides, PDGF-A and PDGF-B. Thus, the active PDGF ligand can exist as a homodimer (AA or BB), or an AB heterodimer (19, 47). Two PDGF receptor (PDGFR) subtypes have been described; the PDGF α receptor (PDGFαR), and the PDGF β receptor (PDGFβR). The PDGFRs are encoded by separate genes. The mature receptors each exist as transmembrane proteins containing a heavily glycosylated extracellular ligand-binding region, a single membrane-spanning region, and an intracellular region containing a tyrosine kinase domain (reviewed in references 19 and 55). The PDGFαR is thought to bind all three PDGF isoforms with high affinity. By contrast, the PDGFβR binds PDGF-BB with high affinity and PDGF-AB with low affinity but does not interact with PDGF-AA (19). Both receptors are able to mediate a mitogenic response to PDGF in appropriate cell lines; however, only the PDGFβR has been shown to stimulate actin reorganization and chemotaxis when activated (19).

PDGF binding induces receptor dimerization and activation of the tyrosine kinase activity that is intrinsic to the intracellular region of the receptor molecule (50). Substrates for the activated PDGFR and other ligand-activated receptor tyrosine kinases include the receptors themselves as well as various other intracellular proteins that associate with the tyrosine-phosphorylated receptors (10, 50). Proteins that associate with the ligand-activated growth factor receptors include enzymes that synthesize second messenger molecules (phospholipase C, phosphatidylmositol 3-kinase), protein kinases (Src and Raf), and phosphatases (PTP-1D), adaptor proteins such as Shc that are thought to link activated receptors to the Ras signaling pathway (32), and other proteins of unknown function. The biochemical properties of these effector proteins are believed to be modified as a consequence of association with and phosphorylation by the receptor. Thus, ligand-activated growth factor receptors initiate a cascade of intracellular events involving second messenger synthesis, protein phosphorylation, and gene induction, which mediate the physiological responses to extracellular factors.

Murine BALB/c-3T3 fibroblasts have provided a valuable model system for the study of the mitogenic effects of PDGF (34, 43). These cells express large numbers of PDGFRs, the majority of which (>80%) are of the β subtype (14). Stimulation of growth-arrested (G_0) cells with a competence factor such as PDGF-BB (but not PDGF-AA [12]) enables entry into the G_1 phase of the cell cycle. The competent state is achieved within 30 min of PDGF treatment and decays with a half-life of 8 to 13 h. However, PDGF is unable to promote further progress through G_1; in order to traverse G_1 and enter S phase, competent BALB/c-3T3 cells require additional components usually present in serum or in platelet-poor plasma. These additional factors, termed progression factors, may be mimicked by epidermal growth factor (EGF) and insulin-like growth factor 1 (IGF-1). Progression through the first 6 h of G_1 requires treatment with EGF, and IGF-1 is the only growth factor required during late G_1 phase (9, 27, 28, 38). In addition to stimulating entry into and progression through the cell cycle, PDGF in the presence of EGF plus IGF-1 (and to some extent PDGF alone) is able to prevent cytolysis that results following serum deprivation of BALB/c-3T3 cells (44). The anticytolytic effects of these factors are distinct from their abilities to stimulate proliferation, as evidenced by the differential sensitivity of these effects to inhibitors of transcription and protein synthesis (48).

Clearly, the ability of a cell to respond to a growth factor is dependent on the presence of the appropriate receptor on the surface of that cell. Regulated expression of receptors provides

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a potential means by which a cell can modulate its responsiveness to appropriate ligands. A feature common to many receptors, including members of the tyrosine kinase growth factor receptor family (e.g., the PDGFR and c-Kit), as well as certain G-protein-coupled receptors (e.g., muscarinic acetylcholine receptors), is the phenomenon of receptor downregulation following acute agonist stimulation. Receptor downregulation can result from internalization and intracellular degradation of the ligand-activated receptor (22) and may provide a mechanism for desensitizing the cells to subsequent stimulation by that ligand. However, mechanisms other than degradation of internalized receptor complexes may also play a role in receptor downregulation; prolonged stimulation by appropriate agonists can induce decreases in the steady-state levels of mRNA encoding several receptors, including those for colony-stimulating factor 1 (CSF-1) (16), lutropin/choriogonadotropin (51), muscarinic acetylcholine receptors (26), and α-adrenergic receptors (21). These regulated decreases in mRNA levels are likely to contribute to the reduced sensitivity of cells following chronic exposure to agonist. The regulation of the PDGFR has not been studied extensively in this context. We report here that the expression of the PDGFβR in murine fibroblasts is markedly repressed following prolonged stimulation of these cells with mitogenic growth factors or in response to certain transforming oncogenes.

MATERIALS AND METHODS

Cells and culture. BALB/c-3T3 cells, NIH 3T3 cells, and KBALB cells were obtained from the American Type Culture Collection (Rockville, Md.). srcBALB cells were generated by infection of BALB/c-3T3 cells with a recombinant, replication-competent virus consisting of the v-*src* gene inserted into the Moloney murine leukemia virus genome (a generous gift of Bernard Mathey-Prevot). All fibroblast cell lines were grown at 37°C in Dulbecco modified Eagle medium (DMEM) containing 10% heat-inactivated donor calf serum supplemented with glutamine and penicillin-streptomycin. In some experiments, cells were placed in DMEM containing 0.5% serum for the indicated times in order to induce growth arrest.

Nuclear DNA profile analysis. Fibroblast monolayers were harvested by trypsinization (0.2% trypsin–0.5 mM EDTA, 5 min at room temperature), washed in DMEM, fixed in 35% ethanol–65% DMEM, and stored at 4°C for up to 2 days. Fixed cells were washed in phosphate-buffered saline (PBS) and resuspended in PBS at approximately 10^6 cells per ml. Following addition of RNase A (8 μg/ml) and propidium iodide (18 μg/ml), samples were incubated in the dark for 30 min at room temperature and analyzed with a Becton Dickinson FACScan instrument.

[^{3}H]thymidine incorporation assays. Fibroblasts growing in 24-well plates were starved overnight in DMEM containing 0.5% serum. Two microcuries of [^{3}H]thymidine was added directly to the starvation medium, with or without serum, or purified growth factors at the indicated concentrations. After 20 h, the medium was aspirated, and monolayers were fixed and then washed once with 1 ml of 5% trichloroacetic acid. The fixed monolayers were solubilized in 1% sodium dodecyl sulfate (SDS)–0.3 N NaOH. Incorporated radioactivity was determined by scintillation counting.

RNA isolation and Northern (RNA) blot analysis. Total cellular RNA was prepared by the single-step method of Chomczynski and Sacchi (11). RNA samples were electrophoresed on 1% agarose gels in the presence of formaldehyde and were transferred to nitrocellulose filters with 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). RNA was UV cross-linked to the filters by using a Stratalinker device (Stratagene). Filters were hybridized with random-primed ^{32}P-labeled cDNA probes in 50% formamide–5× SSC–1% SDS–5× Denhardt's solution–100 μg of salmon sperm DNA per ml. Washes were carried out under stringent conditions, and autoradiography was performed at −70°C with enhancing screens.

cDNA probes. The murine PDGFβR probe was a 5.1-kb *Eco*RI fragment comprising the entire PDGFR cDNA (57). The fibroblast growth factor (FGF) receptor probe was a 2.1-kb *Xho*I fragment from the murine type 1 FGF receptor cDNA (58). Actin expression was probed with a 660-bp *Pst*I fragment from the mouse β-actin cDNA.

Nuclear runoff analysis. Nuclei were prepared, and in vitro transcription was allowed to proceed in the presence of [^{32}P]UTP as described previously (62). Labeled transcripts were hybridized for 3 days with filters containing immobilized DNA samples. Hybridized filters were washed at high stringency and RNase treated prior to autoradiography.

Quantitation of cell surface PDGFRs. Saturation binding analysis was performed as described by Bowen-Pope and Ross (6), using commercially available high-specific-radioactivity [^{125}I]PDGF-BB (New England Nuclear) and recombinant PDGF-BB (v-Sis) (R&D Diagnostics) as a binding competitor.

Preparation of cell membranes. After starvation for 24 h in medium containing 0.5% donor calf serum, fibroblast monolayers were rinsed twice with PBS and then scraped into ice-cold lysis buffer (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.4], 5 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamidine). The detached cells were disrupted with 10 strokes of a hand-held Teflon-glass homogenizer. The resulting homogenate was centrifuged at 1,000 × *g* for 5 min to remove nuclei and unbroken cells. The supernatant was removed and spun at 20,000 × *g* for 10 min. The resulting membrane pellet was resuspended in a small volume of lysis buffer containing 0.5% SDS and was stored at −70°C for up to 2 weeks prior to determination of protein content and Western blotting (immunoblotting).

Immunoblot detection of PDGFβR expression. Total membrane proteins were denatured in SDS reducing buffer, separated on SDS–6.5% polyacrylamide gels, and transferred to nitrocellulose filters according to standard protocols. PDGFβR antisera were generated by immunization of rabbits with a peptide corresponding to the external domain of the PDGFβR (amino acids 934 to 951). Filters containing immobilized proteins were probed with the antipeptide antisera at a 1/1,000 dilution. Bound antibodies were detected with an alkaline phosphatase-coupled goat anti-rabbit secondary antibody (Bio-Rad).

RESULTS

Growth arrest in G_0 is associated with elevated expression of the PDGFβR. To analyze receptor expression in proliferating or quiescent cells, replicate plates of BALB/c-3T3 fibroblasts were maintained in medium containing different serum concentrations (as described below) and were then processed for cell cycle analysis by FACScan or were used to prepare total cellular RNA. BALB/c-3T3 fibroblasts growing exponentially in medium containing 10% serum enter a state of quiescence, or growth arrest, when placed in low (0.5%) serum-containing medium. Nuclear DNA profile analysis of serum-starved cells (Fig. 1b) indicated greatly reduced numbers of cells in S phase and in G_2/M phase of the cell cycle compared with exponentially growing cells (Fig. 1a). However, the growth arrest was reversed by placing the starved cells in medium containing 10% serum, as evidenced by increases in the populations of cells in S phase and G_2/M phase (Fig. 1c).

RNA samples extracted from proliferating and quiescent cells were separated on agarose gels, blotted to nitrocellulose, and hybridized with a radiolabeled PDGFβR cDNA probe. The serum-starved cells expressed higher levels of PDGFβR mRNA (Fig. 1d, lane 2) than the exponentially growing fibroblasts (lane 1). Moreover, stimulation of the growth-arrested cells with 10% serum reversed the increase in expression of the PDGFβR mRNA (lane 3) concomitantly with entry into the cell cycle. NIH 3T3 fibroblasts were subjected to similar experimental conditions. As with BALB/c-3T3 cells, serum starvation increased expression of PDGFβR mRNA in NIH 3T3 cells relative to levels found in proliferating fibroblasts (data not shown). Analysis of the time course of PDGFβR mRNA downregulation in response to serum indicated that significant changes required at least 10 h of serum stimulation. However, the PDGFβR mRNA has a half-life of 4 to 6 h, and any serum-induced changes in its expression would likely require at least one half-life to become apparent (data not shown).

Serum comprises an incompletely characterized mixture of growth-stimulatory components. We investigated whether stimulation of growth-arrested fibroblasts with defined growth factors would induce a similar downregulation of PDGFβR expression. As discussed above (see the introduction), the mitogenic actions of PDGF, EGF, and IGF-1 have been particularly well studied in BALB/c-3T3 cells. We studied the effects of these factors on the expression of PDGFβR mRNA. Fibroblasts were maintained in medium containing 0.5% serum overnight to induce growth arrest and elevation of PDGFβR

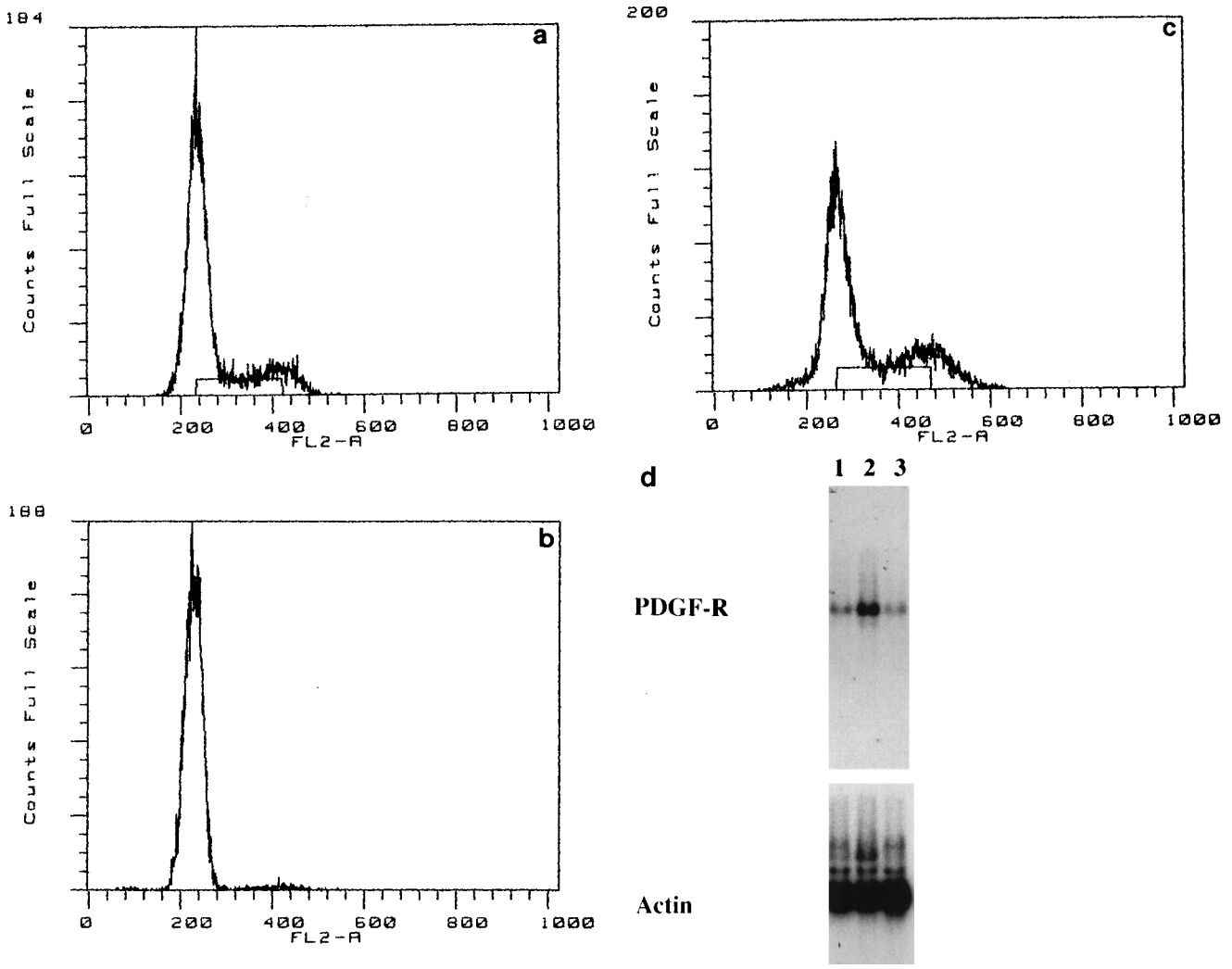

FIG. 1. Increased expression of PDGFβR mRNA during growth arrest. Replicate plates of exponentially growing BALB/c-3T3 cells were placed in DMEM containing 0.5% serum overnight to induce growth arrest. The growth-arrested cells were then stimulated to reenter the cell cycle by the addition of fresh medium containing 10% serum for 24 h. The cells were analyzed for growth state by FACScan, or for PDGFβR mRNA expression by Northern blotting, after each serum treatment. (a to c) Nuclear DNA profiles of exponentially growing BALB/c-3T3 cells (a), growth-arrested cells (b), and growth-arrested cells following 24-h restimulation with serum (c). (d) Twenty-microgram samples of total RNA from exponentially growing BALB/c-3T3 cells (lane 1), growth-arrested cells (lane 2), and growth-arrested cells following 24-h restimulation with serum (lane 3) were separated on a 1% agarose gel, transferred to nitrocellulose, and hybridized with a ^{32}P-labeled PDGFβR cDNA probe. The same blot was also stripped and reprobed for actin expression as a control for equivalent loading.

mRNA expression. Then, various factors were added to the starvation medium for 18 h, after which time RNA was extracted, and analyzed for expression of the PDGFβR transcript by Northern blotting. Stimulation of quiescent cells with PDGF-BB alone (Fig. 2, lane 6) or with a combination of EGF and IGF-1 (lane 5) had little effect on the levels of PDGFβR mRNA expressed. However, treatment with PDGF in the presence of EGF and IGF-1 resulted in marked downregulation of PDGFβR mRNA (lane 7). In some experiments, PDGF alone induced some downregulation of PDGFβR mRNA (see, for example, Fig. 3). However, in experiments in which PDGF alone was ineffective (e.g., Fig. 2), the downregulation was consistently induced by the coaddition of EGF and IGF-1. Therefore, the ability of PDGF to induce PDGFβR mRNA downregulation in the absence of added EGF plus IGF-1, in some experiments, was likely the result of the presence of residual levels of progression factors in the starvation medium. Indeed, BALB/c-3T3 fibroblasts have been reported to synthesize IGF-1 (a progression factor) under certain circumstances (37).

The ability of PDGF to elicit maximal suppression of PDGFβR mRNA expression in the presence of progression factors is similar to the reported synergy between PDGF and progression factors when inducing a mitogenic response. Therefore, we next determined whether downregulation of the PDGFβR transcript specifically required signaling through the PDGFR or, rather, could result from stimulation with growth factors capable of moving cells out of G_0. The actions of basic FGF (bFGF) have not been studied extensively in the context of the BALB/c-3T3 cell cycle. However, prolonged (>12-h) stimulation of quiescent fibroblasts with this factor was recently reported to induce entry into S phase (60). The mitogenic action of bFGF is thought to be mediated via the type 1 FGF receptor in BALB/c-3T3 cells. In agreement with the findings of Zhan et al. (60), bFGF elicited a strong DNA synthetic response (Fig. 3a) in BALB/c-3T3 cells. Stimulation

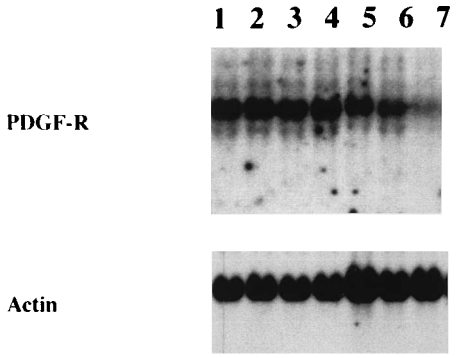

FIG. 2. Repression of PDGFβR mRNA by purified growth factors. Replicate plates of exponentially growing BALB/c-3T3 cells were placed in medium containing 0.5% serum for 24 h to induce growth arrest. Then, purified factors (or an equal volume of PBS for controls) were added to the starvation medium for a further 18 h, at which time the cells were harvested and used to prepare total RNA. Twenty-microgram samples of RNA were separated on 1% agarose gels, transferred to nitrocellulose, and probed for PDGFβR expression. The same filter was stripped and reprobed for actin expression to confirm equivalent loading of RNA. Lanes: 1 and 2, RNA samples extracted from cells immediately after 24 h of serum starvation; 3 and 4, control (PBS-treated) cells; 5, EGF–IGF-1-stimulated cells; 6, PDGF-treated cells; 7, PDGF–EGF–IGF-1-treated cells. Growth factors were used at the following final concentrations: PDGF, 10 ng/ml; EGF, 10 ng/ml; and IGF-1, 100 ng/ml.

of [^{3}H]thymidine incorporation was dose dependent, with a 50% effective concentration of approximately 3 ng/ml, and maximal DNA synthesis was attained at approximately 30 ng/ml. These parameters are consistent with previously reported pharmacological properties of the FGF receptor in 3T3 cells (33). When used to stimulate serum-starved cells, a maximally mitogenic dose of bFGF (30 ng/ml) was highly effective at repressing PDGFβR mRNA (Fig. 3b, lane 4) relative to the control untreated cells (lane 1). Interestingly, bFGF was even more effective at downregulating PDGFβR mRNA than serum (lane 2), PDGF alone (lane 3), or PDGF in combination with EGF and IGF-1 (lane 5). Furthermore, the ability of bFGF to downregulate PDGFβR mRNA did not depend upon simultaneous addition of progression factors (lane 5).

We analyzed the binding of [^{125}I]PDGF-BB to quiescent and bFGF-treated BALB/c-3T3 cells. At concentrations of PDGF-BB sufficient to saturate all available cell surface receptors, the bFGF-treated cells exhibited approximately 60% fewer PDGF-binding sites than the control fibroblasts (Fig. 3c). Scatchard analysis (not shown) of the data in Fig. 3c indicated a decrease in the number of PDGF-binding sites from 90,000 to 45,000 per cell as a consequence of bFGF treatment, with no significant change in the affinity of the receptors for PDGF. The effect of bFGF on subsequent PDGF-BB binding was dose dependent (Fig. 3d), with a 50% effective concentration of approximately 3 ng/ml; maximal downregulation of PDGF-binding sites occurred at 30 to 100 ng of bFGF per ml. These parameters are similar to the values obtained for maximal [^{3}H]thymidine incorporation in response to bFGF (Fig. 3a). Thus, there is good correlation between the ability of bFGF to downregulate PDGFβR expression and its ability to stimulate proliferation.

Although PDGF-BB can bind to both PDGFαR and PDGFβR, the decreased numbers of PDGF-BB-binding sites following bFGF treatment was most likely due to loss of the PDGFβR, since we have found that the expression of PDGFαR mRNA is constitutively low and unresponsive to growth factor treatment in BALB/c-3T3 cells (data not shown). Indeed, previous workers have also shown that BALB/c-3T3 cells express predominantly (>80%) the β-type receptor (14).

Effect of transforming oncogenes on PDGFβR expression. Many proto-oncogenes (such as c-*ras* and c-*src*) encode cellular proteins that are postulated to be downstream components of the mitogenic signal transduction pathways, normally activated by ligand-occupied growth factor receptors. Both c-Src and c-Ras have been implicated as cellular effectors of activated growth factor receptors, including the receptors for PDGF (24, 42) and EGF (8, 31). Indeed, Src may play a role in exit from G_0 and completion of G_1 (56), and Ras is thought to be necessary for the traverse of G_1 in BALB/c-3T3 cells (30). By contrast with their cellular proto-oncogene counterparts, v-*src* and v-*ras* encode mutant proteins that deliver constitutive mitogenic signals, even in the absence of growth factor receptor activation. Thus, a feature common to cell lines transformed by v-*ras*, v-*src*, and many other oncogenes is their failure to undergo growth arrest when placed in medium containing low levels of serum growth factors. Since elevated expression of the PDGFβR is associated with arrest in G_0, we predicted that transformed cell lines, which fail to undergo growth arrest following serum deprivation, may express constitutively low levels of PDGFβR mRNA. Accordingly, we examined the expression of the PDGFβR mRNA in BALB/c-3T3 cell lines transformed by the v-*src* (referred to here as srcBALB cells) and v-Ki-*ras* (referred to as KBALB cells) viral oncogenes.

We confirmed the ability of srcBALB and KBALB cells to proliferate in medium containing low concentrations of serum growth factors, using incorporation of [^{3}H]thymidine into DNA as an index of cell growth. Serum-starved BALB/c-3T3 cells entered G_0 and incorporated only low levels of [^{3}H]thymidine yet displayed a large relative increase in DNA synthesis following stimulation with 10% serum (Fig. 4a). By contrast, srcBALB and KBALB cells continued to incorporate high levels of thymidine into DNA when placed in medium containing reduced (0.5%) serum. Moreover, serum stimulation did not further increase the rates of DNA synthesis of the transformed cells. DNA profile analysis of srcBALB and KBALB lines demonstrated significant numbers of cells present in S and G_2/M phases, even following 72 h of serum deprivation (not shown).

Northern analysis of total RNA from the v-*src*- and v-*ras*-expressing cell lines showed that serum-starved srcBALB (Fig. 4b, lane 2) and KBALB (lane 3) cells expressed reduced levels of PDGFβR mRNA relative to untransformed BALB/c-3T3 fibroblasts (lane 1). Although the PDGFβR mRNA was detectable in samples from the transformed cell lines following more prolonged exposure of the autoradiographs shown in Fig. 4b, we estimate that srcBALB and KBALB lines expressed 5- to 10-fold less PDGFβR mRNA than the parental BALB/c-3T3 cell line under conditions of serum starvation.

The numbers of functional cell surface PDGFRs decreased in parallel with the levels of PDGFβR mRNA transcripts as a consequence of cellular transformation, as shown by equilibrium binding analysis using [^{125}I]PDGF-BB. At saturating concentrations of PDGF-BB, srcBALB and KBALB cells bound significantly fewer PDGF molecules per cell (Fig. 4c) than did untransformed fibroblasts. Scatchard analysis (not shown) of the data in Fig. 4c indicated that the srcBALB and KBALB cells expressed 10,000 and 12,000 receptor sites, respectively, compared with 78,000 sites per cell in the untransformed fibroblasts. Scatchard plots of these data indicated no significant change in the ligand affinity of the PDGFRs in the transformed cells compared with untransformed fibroblasts. As discussed previously, the decreased numbers PDGF-BB-binding sites are unlikely to reflect changes in the expression of the PDGFαR.

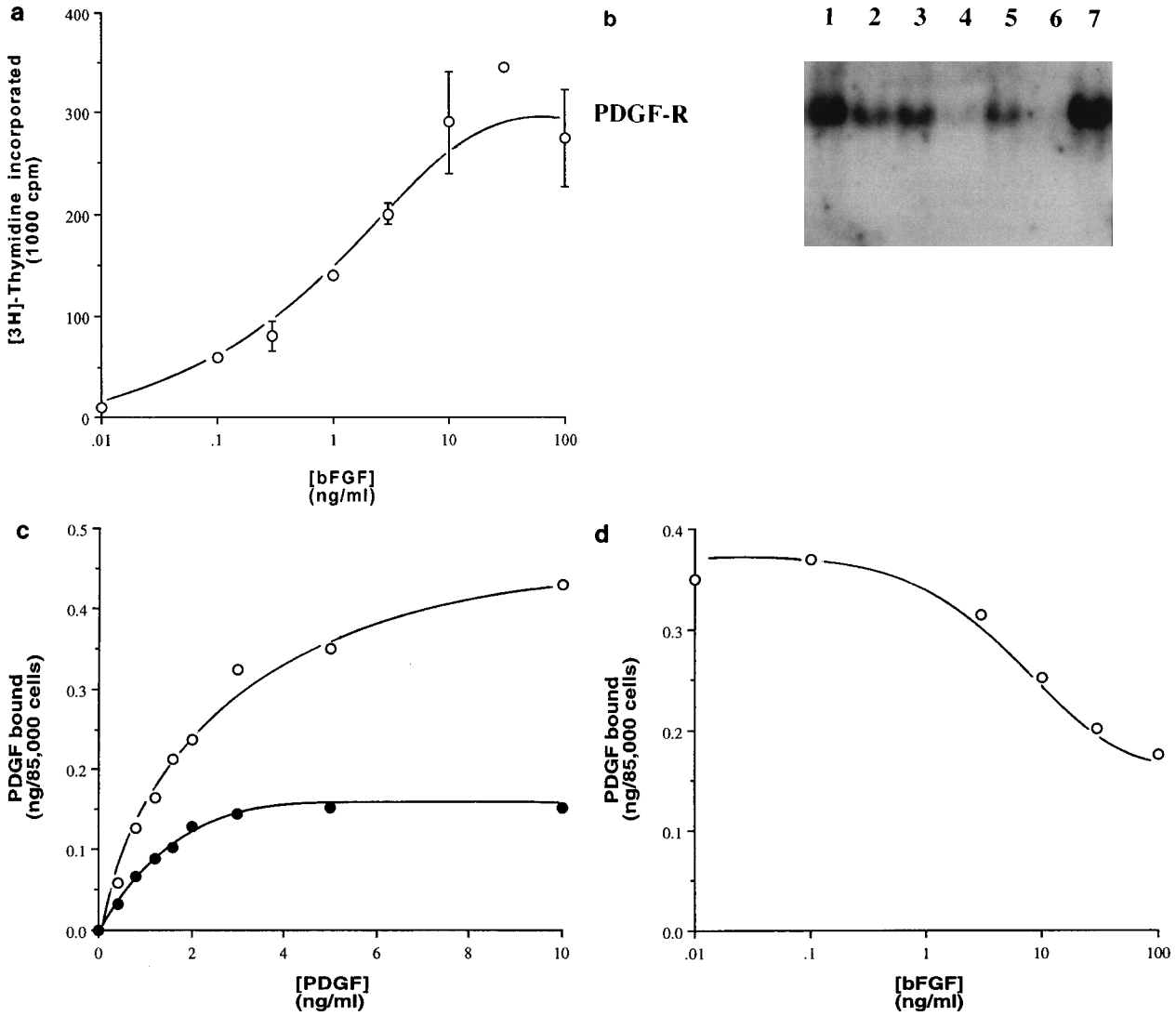

FIG. 3. Effect of bFGF on entry into cell cycle and PDGFβR expression. (a) Dose dependency of bFGF-induced DNA synthesis in BALB/c-3T3 cells. Serum-starved cells were stimulated with the indicated concentrations of bFGF in the presence of [^{3}H]thymidine. Incorporated radioactivity was determined as described in Materials and Methods. (b) Northern blot showing effect of bFGF on PDGFβR mRNA levels. Serum-starved cells were stimulated with 10% serum or purified growth factors. Total RNA from growth factor-stimulated cells was analyzed for PDGFβR mRNA expression as described in the legend to Fig. 1. Lanes: 1, no treatment; 2, 10% serum; 3, PDGF; 4, FGF; 5, PDGF, EGF, and IGF-1; 6, FGF, EGF, and IGF-1; 7, EGF and IGF-1. Growth factors were used at the following final concentrations: PDGF and EGF; 10 ng/ml, bFGF; 30 ng/ml, and IGF-1; 100 ng/ml. (c) Effect of bFGF on subsequent [^{125}I]PDGF-BB binding. Serum-starved cells were incubated for a further 18 h with (●) or without (○) bFGF at 30 ng/ml. [^{125}I]PDGF-BB binding to control and bFGF-treated cells was determined in the presence of the indicated concentrations of unlabeled PDGF-BB as described in Materials and Methods. (d) Dose dependency of bFGF-induced downregulation of PDGF-BB-binding sites. Serum-starved cells were stimulated with the indicated concentrations of bFGF for 18 h. [^{125}I]PDGF-BB binding was determined in the presence of 5 ng of unlabeled recombinant PDGF-BB per ml.

Western blot analysis showed reduced levels of PDGFβR present in plasma membrane preparations from srcBALB (Fig. 4d, lanes 2 and 5) and KBALB (lanes 3 and 6) cells relative to the untransformed BALB/c-3T3 fibroblast line (lanes 1 and 3). These results confirm the PDGF-BB binding data and correlate well with the decreased levels of the PDGFβR transcript demonstrated in the Northern analyses of RNA from oncogene-transformed cells (Fig. 4b). Similar effects of transforming oncogenes on PDGFβR mRNA and protein expression were observed in NIH 3T3-derived cell lines (data not shown), further indicating that control of PDGFβR expression by cellular growth state and oncogenes is a generalizable phenomenon.

It was formally possible that prolonged passage of transformed, growth factor-independent cells may have simply selected for cells expressing fewer PDGFRs. Therefore, agents which antagonize or reverse the proliferative effects of the mutant oncogenes were studied for their effects on the regulated expression of PDGFRs in transformed cells. Treatment with cell penetrant cyclic AMP (cAMP) analogs (such as dibutyryl-cAMP) or transfection with the k-*rev* gene has been used to revert the phenotype of *ras*-transformed cell lines. cAMP has recently been shown to prevent Ras from activating Raf-1 and subsequent downstream signaling events (13). The k-Rev protein, also known as Smg-1 and Rap-1, is a member of the family of Ras-like small G proteins (23). When overexpressed, k-Rev is thought to compete with v-Ras for putative downstream effector molecules, thereby antagonizing the transform-

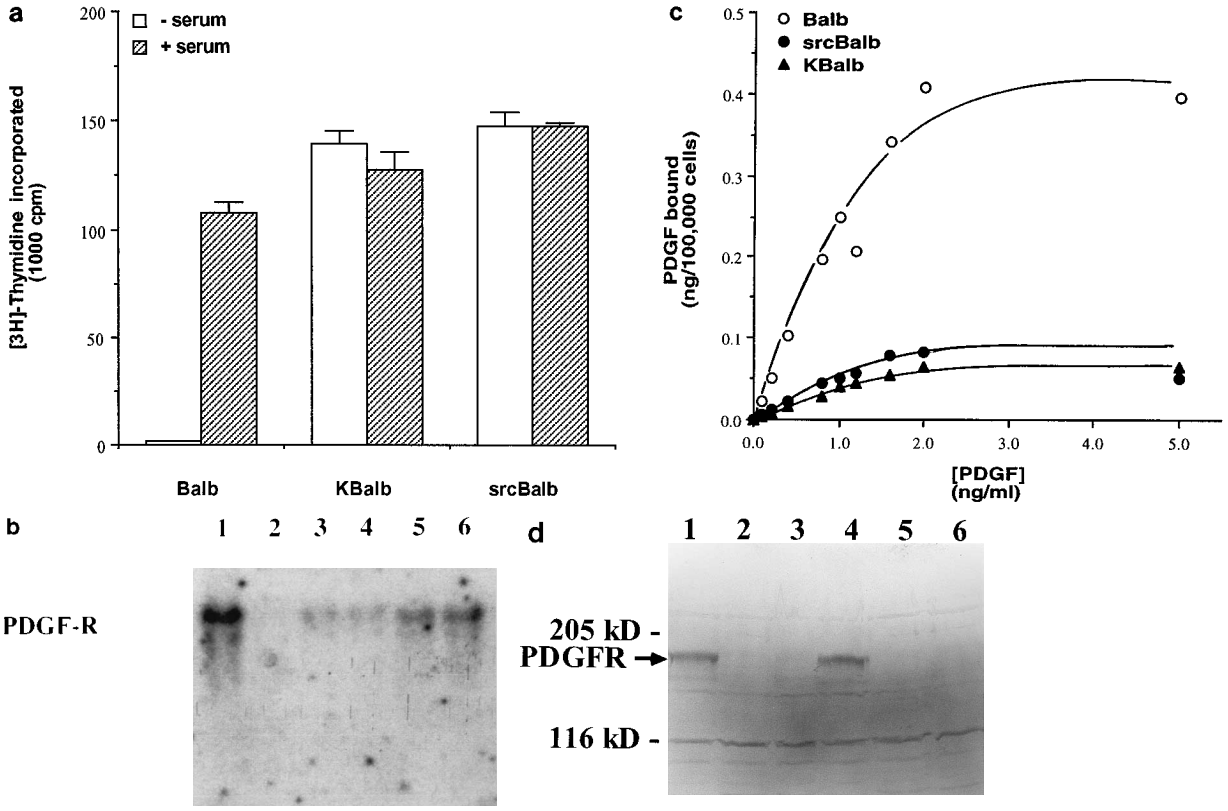

FIG. 4. Effects of viral oncogenes on proliferation and PDGFβR expression. (a) BALB/c-3T3 KBALB, and srcBALB cells were placed in 0.5% serum for 24 h. Then, [^{3}H]thymidine (2 μCi/ml) was added to the starvation medium in the presence or absence of 10% serum. After 20 h, trichloroacetic acid-insoluble radioactivity was determined as described in Materials and Methods. (b) PDGFβR mRNA levels in growth factor-stimulated and oncogene-transformed BALB/c-3T3 cells. Cells were serum starved for 24 h and then stimulated for 18 h with serum (10%) or growth factors as indicated below; then, 20-μg samples of total RNA were separated on a 1% agarose gel, blotted to nitrocellulose, and probed for PDGFβR expression. Lanes: 1, BALB/c-3T3 cells; 2, srcBALB cells; 3, KBALB cells; 4, bFGF-stimulated BALB/c-3T3 cells; 5, PDGF–EGF–IGF-1-stimulated BALB/c-3T3 cells; 6, serum-stimulated BALB/c-3T3 cells. Growth factors were used at the same concentrations described in the legend to Fig. 2. (c) [^{125}I]PDGF-BB binding to BALB/c-3T3, srcBALB, and KBALB cells was determined in the presence of the indicated concentrations of unlabeled PDGF-BB as described in Materials and Methods. (d) Western blot analysis of PDGFβR expression in BALB/c-3T3 fibroblasts and in oncogene-transformed cells. Two hundred-microgram samples of membrane protein from BALB/c-3T3 fibroblasts (lanes 1 and 4), srcBALB cells (lanes 2 and 5), and KBALB cells (lanes 3 and 6) were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with antisera to the PDGFβR as described in Materials and Methods. Lanes 1 to 3 and 4 to 6 represent membrane preparations from two separate experiments. The positions of molecular weight markers and of the PDGFβR are indicated. The identity of the nonspecific band at 116 kDa is unknown, but the band serves as a useful internal control for equivalent loading of proteins.

ing effect of mutant Ras oncoproteins (23). Previous work from this laboratory has demonstrated reversion of the transformed phenotype of KBALB cells following dibutyryl-cAMP treatment or transfection with k-*rev* (40). We investigated whether these protocols would upregulate the constitutive low expression of PDGFβR mRNA in KBALB cells. Northern analysis of mRNA derived from both cAMP- and k-*rev*-induced revertants showed large increases in the expression of PDGFβR mRNA (Fig. 5) coincident with the reverted phenotype. This result suggested that PDGFβR downregulation was indeed a direct consequence of cellular transformation. In some experiments, small increases in PDGFβR expression following dibutyryl-cAMP treatment of untransformed BALB/c-3T3 cells were noted (not shown).

Specificity of growth factor receptor mRNA repression by mitogenic stimuli. To determine whether the expression of other tyrosine kinase growth factor receptors is regulated by growth state, the RNA samples represented in Fig. 4b were probed for expression of the type 1 FGF receptor (Fig. 6). We observed no significant changes in the levels of FGF receptor mRNA as a consequence of cellular transformation (lanes 2 and 3) or in response to stimulation by purified growth factors (lanes 4 and 5) or serum (lane 6). This indicated that the growth state-dependent changes in PDGFβR expression were relatively specific for this receptor.

Mechanism of repression of PDGFβR mRNA. The reduced levels of PDGFβR mRNA in actively proliferating cells may have potentially resulted from decreased transcription of the PDGFβR gene, from decreased stability of the PDGFβR transcript, or from a combination of these two mechanisms. To begin to address these possibilities, nuclear runoff assays were carried out with the mouse PDGFβR cDNA immobilized on filters to detect newly transcribed ^{32}P-labeled RNA from isolated nuclei. However, we were unable to detect strong signals with the mouse PDGFβR cDNA probe (not shown). By analogy with closely related growth factor receptor genes such as those encoding the PDGFαR, c-Kit, and c-Fms, the PDGFβR gene is likely to span 60 to 100 kb and comprise many short coding regions interrupted by large introns (17). To increase the sensitivity of our nuclear runoff assay, we isolated genomic clones encoding regions of murine PDGFβR gene. Newly transcribed RNA has higher complementarity to genomic DNA than to the corresponding cDNA and is therefore likely to generate stronger signals when used in nuclear runoff assays.

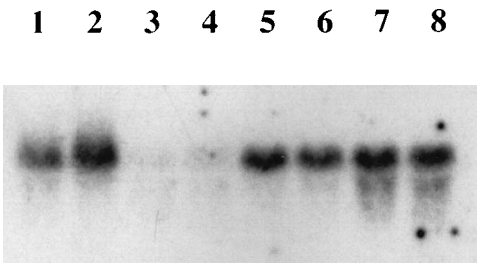

FIG. 5. Restoration of PDGFβR mRNA expression in cAMP and k-*rev*-induced KBALB revertants. cAMP- and k-*rev*-induced cells were generated from KBALB cells as previously described (40) by prolonged dibutyryl-cAMP treatment and by transfection of an expression vector containing the k-*rev* gene, respectively. Twenty-microgram samples of total RNA from serum-starved BALB/c-3T3 cells (lanes 1 and 2), KBALB cells (lanes 3 and 4), k-*rev*-induced cells (lanes 5 and 6), and cAMP-induced cells (lanes 7 and 8) were separated on 1% agarose gels, transferred to nitrocellulose filters, and hybridized with a PDGFβR cDNA probe.

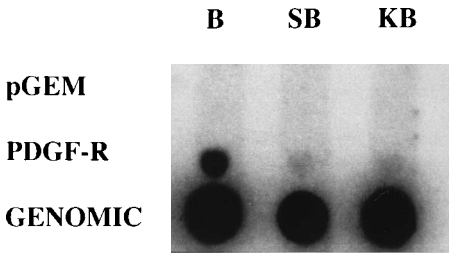

FIG. 7. Mechanism of repression of PDGFβR expression in proliferating cells. Nuclei were prepared from serum-starved BALB/c-3T3 cells, srcBALB cells, and KBALB cells, and ^{32}P-labeled runoff transcripts were isolated as described in Materials and Methods. Approximately 10^7 cpm of in vitro-labeled transcripts was hybridized with filters containing 100 ng of immobilized spots of the following DNAs: pGEM3 (negative control), pG3P2.6 (pGEM3 containing a 2.6-kb genomic fragment from the PDGFβR), and mouse genomic DNA (positive control).

The plasmid that we have designated pG3P2.6 contains a 2.6-kb genomic *Pst*I fragment, containing 346 bp of DNA upstream of the reported extreme 5′ of the mouse PDGFβR cDNA and approximately 2.2 kb of genomic sequence, cloned into the pGEM3 vector. This region of the PDGFβR gene will be described in more detail elsewhere (50a). ^{32}P-labeled runoff transcripts derived from isolated serum-starved BALB/c-3T3 nuclei hybridized strongly with the PDGFβR genomic fragment (Fig. 7). By contrast, weaker signals were obtained when runoff transcripts from growing cells were hybridized with filters containing the same immobilized genomic fragment. These data show that the mechanism of repression of the PDGFβR mRNA in proliferating cells is, at least in large part, due to decreases in the rate of transcription of the PDGFβR gene.

DISCUSSION

The data presented here show that the state of growth arrest, induced by serum deprivation, is associated with increased expression of the PDGFβR in 3T3 fibroblasts. This finding suggests that these cells can modulate their responsiveness to PDGF-BB by regulating the expression of the receptor for this particular mitogenic growth factor. An increase in the total number of cell surface PDGFRs might enable lower concentrations of ligand to achieve the threshold occupancy required to elicit a signal, thereby sensitizing the cells to PDGF. Thus, environmental conditions and growth state may be major determinants of a cell's ability to respond to growth factors. It is significant, perhaps, that the quiescent state induced following serum deprivation results in increased sensitivity to a potent survival factor and mitogen. Our findings are consistent with a recent report from Psarras et al. (39), who noted increased levels of PDGF-BB-binding sites in growth-arrested human embryo fibroblasts compared with actively proliferating cells. Other genes whose expression is increased during G_0 and reduced in proliferating cells have been described (2, 15, 45). Growth factor receptors have not been reported to be products of growth arrest-specific genes. Interestingly, however, Thomopoulos et al. (49) demonstrated elevated expression of insulin receptors in quiescent BALB/c-3T3 fibroblasts compared with growing cells.

In contrast to the increased expression of PDGF receptors resulting from quiescence, our finding that certain growth factors induce downregulation of PDGFβR expression suggests a mechanism for reducing cellular responsiveness to PDGF following stimulation by appropriate mitogenic signals that enable exit from G_0 and entry into the cell cycle. Our experiments show repression of PDGFβR expression to be an example of heterologous downregulation, whereby decreased PDGFβR expression may occur in response to multiple distinct stimuli. There are previous reports of modification of PDGF receptor expression in response to certain growth factors. For example, transforming growth factor β downregulates expression of the PDGFαR in human fibroblasts (36) and in Swiss 3T3 cells (18). Conversely, transforming growth factor β also causes an increase in PDGFβR expression in both human fibroblasts (39) and 3T3 cells (18). PDGF itself is able to modify receptors for other growth factors; PDGF stimulation of BALB/c-3T3 cells causes a decrease in the number of EGF binding sites (54) and also increases transcription and expression of interleukin-1 receptors (5). Other growth factor receptors are also modified in response to their own or other ligands; for example, both EGF (1) and FGF (20) decrease the number of EGF-binding sites in Swiss 3T3 cells.

Modified expression of growth factor receptors in response to specific ligands also occurs in cell lines other than fibroblasts. Certain hematopoietic growth factors (interleukin-3, granulocyte-macrophage CSF, and erythropoietin but not interleukin-4) suppress c-*kit* mRNA and protein expression in mast cells and stem cell progenitors (53), long-term nerve growth factor treatment downregulates EGF receptors in

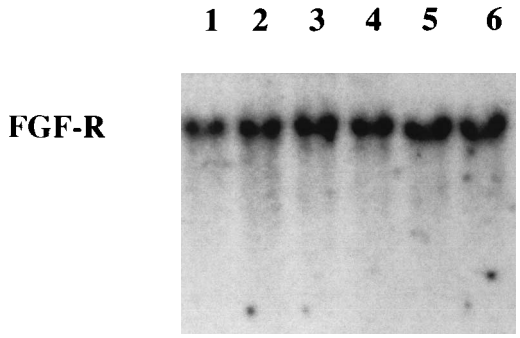

FIG. 6. Expression of FGF receptor mRNA in oncogene-transformed and growth factor-stimulated BALB/c-3T3 cells. The RNA samples described in the legend to Fig. 4b were separated on a 1% agarose gel, transferred to nitrocellulose, and hybridized with a random-primed ^{32}P-labeled FGF receptor cDNA probe. Lanes: 1, BALB/c-3T3 cells; 2, srcBALB cells; 3, KBALB cells; 4, bFGF-stimulated BALB/c-3T3 cells; 5, PDGF–EGF–IGF-1-stimulated BALB/c-3T3 cells; 6, serum-stimulated BALB/c-3T3 cells.

PC12 cells (25), and the macrophage CSF receptor (c-*fms* proto-oncogene) is downregulated by granulocyte-macrophage CSF or multi-CSF stimulation of myeloid cells (16). Thus, cross talk between growth factor receptors appears to be a general feature of mitogenic signaling networks and is likely to provide a means whereby cells fine tune their responsiveness to external stimuli.

Most previous studies of receptor cross talk or heterologous downregulation have not addressed the proliferative state of the cell as a possible determinant of growth factor receptor function or expression. Our experiments indicate good correlation between the mitogenic potential of growth factors and their ability to repress PDGFβR expression. Numerous studies have shown that PDGF is able to render growth-arrested fibroblasts competent to respond to the mitogenic action of progression factors such as EGF and IGF-1 (9, 28, 27, 38). Thus, a competence factor such as PDGF enables the cells to exit G_0 and, in synergy with a combined dose of EGF plus IGF-1, permits progress through G_1 and entry into S phase. Our experiments show that growth factors that are unable, by themselves, to stimulate proliferation of BALB/c-3T3 cells also fail to repress PDGFβR expression. This result indicates that there exists specificity in the ability of particular growth factors to suppress PDGFβR expression and suggests that receptor downregulation cannot simply be the direct result of increased tyrosine kinase activity generated by activated growth factor receptors. Rather, the growth factor specificity appears to lie at the level of the ability of the signal to allow reentry of growth-arrested (G_0) cells into the cell cycle. We investigated whether downregulation of the PDGFβR transcript specifically required signaling through the PDGFR or, rather, could result from stimulation with growth factors capable of moving cells out of G_0. Activation of the bFGF receptor elicited a strong mitogenic response and downregulated PDGFβR expression. Furthermore, the ability of bFGF to downregulate PDGFβR mRNA did not depend upon simultaneous addition of progression factors. However, as discussed earlier, we cannot exclude the possibility that autocrine factors, or residual factors present in the starvation medium, synergize with the exogenously added growth factors to repress the expression of the PDGFβR message.

The significance of the PDGFβR downregulation concomitantly with entry into cell cycle remains unclear; perhaps this serves to desensitize cells to certain actions of PDGF following commitment to proliferation. Alternatively, the decreased PDGFR expression in cycling cells may reflect a reduced requirement for PDGF during exponential growth relative to exit from quiescence. Indeed, exponentially cycling cells require only a single factor (IGF-1) during G_1 (9), whereas three growth factors (PDGF, EGF, and IGF-1) are necessary for quiescent cells to enter and progress through G_1. The growth factor-induced changes in PDGFβR levels most likely result from changes in the proliferative state of the cells (i.e., exponentially growing versus quiescent), as opposed to cell cycle-dependent oscillations in gene expression such as have been described for some cyclins. In support of this possibility, we have been unable to observe reelevation of PDGFβR expression after the first mitosis following exit from G_0 (unpublished observations). We note, however, that it is difficult to maintain a synchronous population of cells beyond the first cell cycle subsequent to exit from G_0.

Since oncogenes have profound effects on the proliferative state of cells, we investigated the effects of transformation by v-*src* and v-*ras* on PDGFβR expression. Both transformed cell lines exhibited reduced levels of PDGFβR mRNA and cell surface receptors relative to the parent cell line. Although this observation is consistent with our finding that actively proliferating cells express less PDGFβR than cells arrested in G_0, these data do not prove that growth factors and transforming oncogenes utilize common intracellular pathways to modify PDGFβR expression. In fact, the transformed cell lines that we have studied usually display lower levels of PDGFβR expression than are attained following stimulation of untransformed cells with mitogens. However, the greater extent to which expression of PDGFβR mRNA is repressed in oncogene-containing cells may be a consequence of the more extreme and deregulated proliferation resulting from chronic transformation.

Our finding that v-*src*- and v-*ras*-transformed cells express low levels of the PDGFβR is consistent with a recent report that PDGF is less able to induce the expression of certain immediate-early genes in cells transformed by viral oncogenes (v-*src*, v-*ras*, v-*sis*, and v-*raf*) than in the parent BALB/c-3T3 cell line (59). Similarly, previous reports from this laboratory (40, 41) and others (3, 4, 29, 35) have shown significant decreases in PDGF-induced second messenger synthesis and immediate-early gene induction in v-*ras*-transformed fibroblasts relative to untransformed cells. Recent studies in this laboratory have shown that v-*ras*-transformed cells contain a potent inhibitor of PDGFβR phosphorylation (41, 61); the present study indicates that the decreased responsiveness of v-*ras*-transformed cells to PDGF may additionally result from reduced expression of the PDGFβR.

Bowen-Pope et al. (7) also noted lower numbers of PDGF-BB-binding sites in transformed fibroblasts than in parental cell lines. Our data indicate that these authors' findings may result from transformation-induced repression of PDGFβR mRNA levels. Transforming oncogenes have also been shown to modify the expression of other growth factor receptors. Thomopoulus et al. (49) found lower numbers of insulin receptors to be present on simian virus 40 (SV40)-transformed BALB/c-3T3 cells than on untransformed fibroblasts. These authors also noted decreased expression of insulin receptors on proliferating cells compared with quiescent fibroblasts and therefore attributed the decreased insulin receptor expression in SV40-transformed cells to the deregulated proliferation of this cell line. Downregulation of EGF receptors also occurs in SV40-transformed 3T3 cells (1). v-Src was also shown to inhibit the synthesis of the EGF receptor in Rat-1 cells (52). Our data raises the possibility that many of the documented effects of oncogenes on growth factor receptors represent exaggerations of events induced by receptor signaling pathways during the course of the normal cell cycle.

Thus, PDGFβR expression can be controlled by the growth state of cells and therefore is subject to regulation by growth factors or growth-promoting oncogenes. Nuclear runoff experiments indicate that the regulation of PDGFβR mRNA expression results, at least in large part, from transcriptional control. Further experiments analyzing the promoter region of the PDGFβR gene are underway to define the regulatory DNA sequences, and DNA-binding proteins, that mediate the changes in PDGFβR transcription during entry into and exit from the cell cycle.

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