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Ligand-Independent Activation of the Platelet-Derived Growth Factor β Receptor: Requirements for Bovine Papillomavirus E5-Induced Mitogenic Signaling

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The E5 protein of bovine papillomavirus type 1 binds to and activates the endogenous platelet-derived growth factor (PDGF) β receptor in fibroblasts, resulting in cell transformation. We have developed a functional assay to test the ability of PDGF β receptor mutants to mediate a mitogenic signal initiated by the E5 protein. Lymphoid Ba/F3 cells are strictly dependent on interleukin-3 for growth, but coexpression of the wild-type PDGF β receptor and the E5 or v-*sis*-encoded protein generated a mitogenic signal which allowed Ba/F3-derived cells to proliferate in the absence of interleukin-3. In these cells, the E5 protein bound to and caused increased tyrosine phosphorylation of both the mature and the precursor forms of the wild-type PDGF β receptor. The tyrosine kinase activity of the receptor was necessary for E5-induced receptor tyrosine phosphorylation and mitogenic activity but not for complex formation with the E5 protein. In contrast, the PDGF-binding domain of the receptor was not required for complex formation with the E5 protein, E5-induced tyrosine phosphorylation or mitogenic activity, demonstrating that E5-mediated receptor activation is ligand independent. Analysis of receptor mutants lacking various combinations of tyrosine phosphorylation sites revealed that the E5 and v-*sis*-encoded proteins display similar requirements for signaling and suggested that the wild-type PDGF β receptor can generate multiple independent mitogenic signals. Importantly, these mutants dissociated two activities of the PDGF β receptor tyrosine kinase, both of which are required for sustained mitogenic signaling: (i) receptor autophosphorylation and creation of binding sites for SH2 domain-containing proteins and (ii) phosphorylation of substrates other than the receptor itself.

The 44-amino-acid E5 protein of bovine papillomavirus type 1 (BPV) causes morphologic and tumorigenic transformation of bovine and rodent fibroblasts in culture (7). The transformation of fibroblasts by the membrane-associated E5 protein appears to be mediated by a cellular target, the platelet-derived growth factor (PDGF) β receptor. In E5-transformed mouse C127 cells, both the mature and immature precursor forms of the endogenous PDGF β receptor are constitutively phosphorylated on tyrosine residues, the PDGF β receptor displays increased in vitro tyrosine kinase activity, and the E5 protein is present in a stable complex with the activated PDGF β receptor (34, 36). Elevated levels of tyrosine phosphorylation of the endogenous PDGF β receptor have been detected in E5-transformed rat and bovine fibroblasts as well (35, 36).

Functional studies demonstrating the importance of the PDGF β receptor activation for E5 action have also been carried out. C127 cell variants that have an impaired response to PDGF respond poorly to the E5 protein (37). Furthermore, N-MuMG epithelial cells, which do not express the endogenous PDGF β receptor gene, cannot be transformed by the E5 protein. However, if an exogenous PDGF β receptor gene is introduced and coexpressed with the BPV E5 gene in these cells, they are then able to form tumors in nude mice (29). In addition, the murine myeloid 32D cell line, which is dependent on interleukin-3 (IL-3) for sustained growth in culture, is able to proliferate in the absence of IL-3 upon coexpression of the E5 protein and the PDGF β receptor but not other growth factor receptors (13).

The mechanism of PDGF β receptor activation by the E5 protein presumably involves complex formation between these two proteins, but the nature of this interaction is unclear and the features of the receptor required for signaling in response to the E5 protein have not been systematically explored. The PDGF β receptor is normally activated by PDGF binding, which induces dimerization of the receptor molecules and the activation of their intrinsic tyrosine kinase activity. This results in autophosphorylation of tyrosine residues in the PDGF receptor intracellular domain as well as phosphorylation of other substrates (48). Receptor autophosphorylation creates binding sites for SH2 domain-containing proteins, which are often tyrosine phosphorylated by the receptor and participate in the transduction of the PDGF signal to the more downstream effectors (4, 16). Tyrosine 857 is a major autophosphorylation site of the PDGF β receptor (19), but phosphorylation of this tyrosine has not been shown to generate a binding site for cellular proteins.

A number of features of the PDGF β receptor have been identified as being essential for acute mitogenic signaling in response to PDGF. The extracellular domain of the receptor is required for PDGF binding. Similarly, the intrinsic tyrosine kinase activity of the receptor is absolutely required for PDGF-induced signaling, presumably to generate binding sites for SH2 domain-containing proteins and/or to phosphorylate downstream substrates (9, 19). Finally, a number of specific sites of tyrosine phosphorylation are important for acute signaling in response to PDGF treatment, presumably by generating binding sites for SH2 domain-containing proteins. In

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addition, mutation of tyrosine 857 or tyrosines 579 and 581 has been reported to inhibit and abolish ligand-stimulated tyrosine kinase activity, respectively (11, 21, 28). It is not known which, if any, of these features of the PDGF β receptor are also involved in transformation by the E5 protein and whether the receptor-mediated intracellular events are similar following E5- or PDGF-induced receptor activation.

To carry out a systematic evaluation of the mechanism of PDGF β receptor signaling in response to the E5 protein, we used the mouse lymphoid Ba/F3 cell line. These cells do not express the endogenous PDGF β receptor and are strictly dependent on the presence of IL-3 in the medium for survival and growth in culture (33). Expression of the erythropoietin (EPO) or IL-2 receptor has been previously shown to eliminate the IL-3 requirement for growth of Ba/F3 cells if the cognate cytokines are supplied in the medium (25, 41, 50). In addition, the Friend spleen focus-forming virus gp55 glycoprotein, which is able to bind to and activate the EPO receptor, allows Ba/F3 cells expressing this receptor to grow in the absence of IL-3 and EPO (25). We reasoned that coexpression of the E5 protein and the PDGF β receptor in Ba/F3 cells might provide a mitogenic signal, thereby eliminating the IL-3 requirement for their growth. We demonstrate here that the PDGF β receptor can form a complex with the E5 protein and mediate its action in Ba/F3 cells. We have used this functional assay to determine the features of the PDGF β receptor required to mediate a sustained proliferative signal in response to the proteins encoded by the BPV E5 and v-*sis* genes.

MATERIALS AND METHODS

DNAs and retroviral stocks. Stable cell lines producing ecotropic retroviruses from pRV-Hyg^R recombinant constructs expressing the v-*sis* or BPV E5 oncogene have been previously described (29, 37). Cell lines producing amphotropic retroviruses expressing the wild-type human PDGF β receptor, and the mutants R634, F5, F579/81 (Src$^-$), F857, F5/579/81 (F5/Src$^-$) and F5/857, were obtained as previously described (19, 43, 45). All of the retroviral producer cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics. Retroviral stocks were harvested in Dulbecco's modified Eagle's medium supplemented with 2% fetal calf serum and antibiotics.

To construct the gene encoding the CP84-ΔX receptor, the 1.2-kb *Xma*I-to-*Sph*I fragment was deleted from the human PDGF β receptor cDNA in RR3/pUC13 (19) and replaced with a double-stranded oligodeoxyribonucleotide that restored the original reading frame, resulting in the construct RR5/pUC13. Standard subcloning procedures were used to subclone the CP84-ΔX mutant from RR5/pUC13 and the TPR mutant from pSM4-SV4-3 (kindly provided by Lena Claesson-Welsh, Ludwig Institute for Cancer Research) into the retroviral vector pLXSN to generate the pLXSN-DDBCP84-ΔX and pRVTPR constructs, respectively. The CP84-ΔX receptor lacks amino acids 38 to 442, whereas the TPR receptor lacks amino acids 38 to 530 of the human PDGF β receptor (data not shown). Transient retroviral stocks were prepared from transfected PA317 cells (27) and used to infect Ψcre-3 cells (6), from which stable lines producing ecotropic retroviruses were obtained.

Cell lines and tissue culture. The murine lymphoid precursor line Ba/F3 (33) was obtained from Alan D'Andrea (Dana-Farber Cancer Institute) and maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 10% WEHI-conditioned medium as a source of IL-3, 0.05 mM β-mercaptoethanol, and antibiotics (RPMI/IL-3).

Infection of Ba/F3 cells and its derivatives was performed as follows. Approximately 10^5 CFU of retrovirus was added to 5×10^5 to 5×10^6 cells in 10 ml of RPMI/IL-3 containing 4 μg of Polybrene per ml. After 1 or 2 days, drug selection was initiated by transferring 1 to 2.5 ml of infected cells into 10 ml of RPMI/IL-3 containing 1 mg of G418 per ml and/or 1,000 U of hygromycin B per ml. During selection, cells were passaged every few days or when they reached density of approximately 10^6/ml. Ba/F3-derived cells lines were usually maintained in RPMI/IL-3 medium with drugs at the same concentration used for selection.

To test the ability of Ba/F3-derived cell lines to grow in the absence of IL-3, cells growing in RPMI/IL-3 with drugs were allowed to reach a density of 10^5 to 10^6/ml. Five milliliters of cells was pelleted, washed once with 10 ml of phosphate-buffered saline (PBS), and resuspended in 5 ml of RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum and 0.05 mM β-mercaptoethanol and antibiotics but lacking IL-3 (RPMI without IL-3). One-fifth of the cells were transferred to a T-25 flask containing 10 ml of RPMI without IL-3 and incubated at 37°C. To obtain growth curves, cells were seeded in 10 to 30 ml of RPMI without IL-3 at 10^5 cells per ml and counted periodically.

Protein extracts and immunoprecipitations. Ba/F3-derived cell lines were grown in 50 to 100 ml of RPMI/IL-3 with drugs to a density of approximately 10^6 cells per ml. In some cases, IL-3-independent lines were grown in RPMI without IL-3 (for two passages) instead. Cells were collected by centrifugation and washed with 10 ml of cold PBS containing 1 mM phenylmethylsulfonyl fluoride (PMSF). Cells were lysed in 1 to 1.5 ml of radioimmunoprecipitation assay (RIPA) buffer (20 mM morpholinepropanesulfonic acid [MOPS; pH 7.0], 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1% deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) containing 1 mM PMSF and 2 mM Na_3VO_4 and incubated for 20 min on ice. Lysates were cleared by centrifugation at $14,000 \times g$ for 10 min at 4°C and stored at −70°C. For some of the anti-PDGF receptor immunoprecipitations, cells were lysed in EBC buffer (50 mM Tris-HCl [pH 8.0], 120 mM NaCl, 0.5% Nonidet P-40) containing 1 mM PMSF and 2 mM Na_3VO_4 instead.

For anti-PDGF receptor immunoprecipitations, 1 to 10 μl of α-PR-B3a, a rabbit antiserum raised against the carboxy-terminal 13-amino-acid peptide of the human PDGF β receptor, was added to 100 to 1,000 μg of protein extract. After incubation on ice for 2 h or overnight, 60 μl of a 1:1 suspension of protein A-Sepharose beads (Pharmacia) in Tris-buffered saline (10 mM Tris-HCl [pH 7.4], 165 mM NaCl) containing 10% (wt/vol) bovine serum albumin (BSA) was added, and the mixture was rotated for 45 min at 4°C. The beads were pelleted, washed five times with cold NET-N buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) containing 1 mM PMSF and resuspended in protein sample buffer. For anti-E5 immunoprecipitations, 10 μl of α-E5, a rabbit antiserum raised against the 16 carboxy-terminal amino acids of the BPV E5 protein (3), was added to 1,000 to 1,500 μg of extract. For anti-phosphatidylinositol 3-kinase (PI3-kinase) immunoprecipitations, 3.5 μl of the rabbit polyclonal antibody α-PI3K (catalog no. 06-195; Upstate Biotechnology, Inc.) was added to 1,000 μg of extract. For anti-GTPase-activating protein (GAP) immunoprecipitations, 5.8 μl of the mouse anti-RAS-GAP monoclonal antibody B4F8 (catalog no. sc-63; Santa Cruz Biotechnology) was added to 1,500 μg of extract. For anti-phospholipase C-γ (PLC-γ) immunoprecipitations, 10 μl of the anti-PLC-γ mixture of mouse monoclonal antibodies (catalog no. 05-163; Upstate Biotechnology, Inc.) was added to 2,000 μg of extract. The remaining procedure for these immunoprecipitations was as described above.

Electrophoresis and immunoblotting. Immunoprecipitates in sample buffer were boiled for 5 min and electrophoresed on an SDS–7.5% acrylamide–0.17% bisacrylamide gel (for anti-PDGF receptor or antiphosphotyrosine immunoblotting) or on an SDS–15% polyacrylamide gel (for anti-E5 immunoblotting). For antiphosphotyrosine or anti-PDGF receptor Western blotting (immunoblotting), gels were soaked for 5 min in transfer buffer (25 mM Tris-HCl, 192 mM glycine, 20% [vol/vol] methanol, 0.1% SDS) and transferred electrophoretically to nitrocellulose membranes at 4°C at 50 V overnight or at 100 V for 2 to 3 h. Blots were stained with 10% Ponceau S (Sigma) to visualize size markers (Bio-Rad), except for E5 blots, for which rainbow molecular weight markers (Amersham) were used.

For phosphotyrosine immunoblots, membranes were incubated in blocking buffer (3% BSA, 10 mM Tris-HCl [pH 7.4], 165 mM NaCl, 0.01% NaN_2) for 1 to 2 h at 25°C. A 1:500 dilution of the antiphosphotyrosine monoclonal antibody 4G10 (catalog no. 05-321; Upstate Biotechnology, Inc.) in blocking buffer was added to blots and incubated for 2 to 3 h at 25°C. Immunoblots were washed sequentially for 10 min with rinsing buffer (10 mM Tris-HCl [pH 7.4], 165 mM NaCl) (twice), rinsing buffer containing 0.05% Triton X-100 (once), and rinsing buffer (twice). After incubation for 30 min at 25°C with filtered blocking buffer containing 1 μCi of [^{125}I]protein A (ICN) per ml, blots were washed again as described above.

For anti-PDGF receptor immunoblots, blots were incubated in 5% milk–TNE-T (5% [wt/vol] Carnation nonfat dry milk in 20 mM Tris-HCl [pH 7.5]–5 mM EDTA–100 mM NaCl–0.1% [vol/vol] Tween 20 [TNE-T]) for 1 to 2 h at 25°C. A 1:500 dilution of α-PR-C3a, another rabbit antiserum raised against the carboxy-terminal 13 amino acids of the mouse PDGF β receptor, in 5% milk–TNE-T, was incubated with blots for 2 to 3 h at room temperature. Immunoblots were washed three times for 10 min with TNE-T. After incubation with [^{125}I]protein A, washes were repeated. Blots in Fig. 3B and 4B were instead subjected to enhanced chemiluminescence detection as specified by the manufacturer (Amersham).

For anti-E5 Western blotting, gels were soaked for 15 min in transfer buffer without SDS (25 mM Tris-HCl, 192 mM glycine, 20% [vol/vol] methanol) and transferred electrophoretically to an Immobilon polyvinylidene difluoride membrane (Millipore) in the cold at 50 V for 3 to 5 h. Blots were incubated in 5% milk–TNE-T for 1 to 2 h at room temperature. A 1:500 dilution of α-E5 antiserum was incubated with blots for 2 to 3 h at room temperature. Washes and incubation with [^{125}I]protein A or enhanced chemiluminescence detection were performed as described above for the anti-PDGF receptor blotting.

In vitro tyrosine kinase assays. For kinase assays, 1,000 μg of RIPA protein extracts was immunoprecipitated with 10 μl of α-PR-B3a antiserum as described above. The protein A-Sepharose beads complexed with immunoprecipitates were washed twice with cold RIPA buffer containing 1 mM PMSF and 2 mM Na_3VO_4, twice with cold PAN [20 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 7.0), 100 mM NaCl, 1 mM PMSF, 2 mM Na_3VO_4], and twice with cold PAN containing 20 μg of aprotinin per ml. Beads were then resuspended in 100 μl of the latter buffer. One-half microliter of the substrate gluta-

TABLE 1. IL-3-independent growth of Ba/F3-derived cell lines

Cell line class[a]	PDGF receptor	BPV E5	v-*sis*	IL-3-independent lines[b]
Ba/F3-hyg/neo	None	−	−	0/4
Ba/F3-E5	None	+	−	0/7
Ba/F3-hyg-WT	WT	−	−	1/15
Ba/F3-E5-WT	WT	+	−	14/15
Ba/F3-sis-WT	WT	−	+	8/8
Ba/F3-hyg-R634	R634	−	−	0/11
Ba/F3-E5-R634	R634	+	−	0/13
Ba/F3-sis-R634	R634	−	+	0/6
Ba/F3-hyg-CP84	CP84-ΔX	−	−	0/4
Ba/F3-E5-CP84	CP84-ΔX	+	−	9/11
Ba/F3-sis-CP84	CP84-ΔX	−	+	0/7
Ba/F3-hyg-TPR	TPR	−	−	2/5
Ba/F3-E5-TPR	TPR	+	−	4/5
Ba/F3-sis-TPR	TPR	−	+	2/5

[a] Designated according to the genes introduced: neo or hyg, neomycin or hygromycin vector; E5, BPV E5; sis, v-*sis*; WT, wild-type PDGF β receptor; R634, tyrosine kinase-negative mutant; CP84, mutant lacking all of four and part of the fifth immunoglobulin-like domains of the extracellular region of the PDGF β receptor; TPR, receptor mutant containing large amino-terminal truncation with only six amino acids left in the extracellular region (see text for more detailed information).

[b] Number of independently derived IL-3-independent cell lines as a fraction of the total number of lines tested. Cell lines were considered IL-3 independent if growth in the absence of IL-3 was observed within one (for v-*sis*-expressing lines) or two (for BPV E5-expressing lines) weeks of IL-3 deprivation. Cell lines scored as negative failed to grow during a period of 4 weeks.

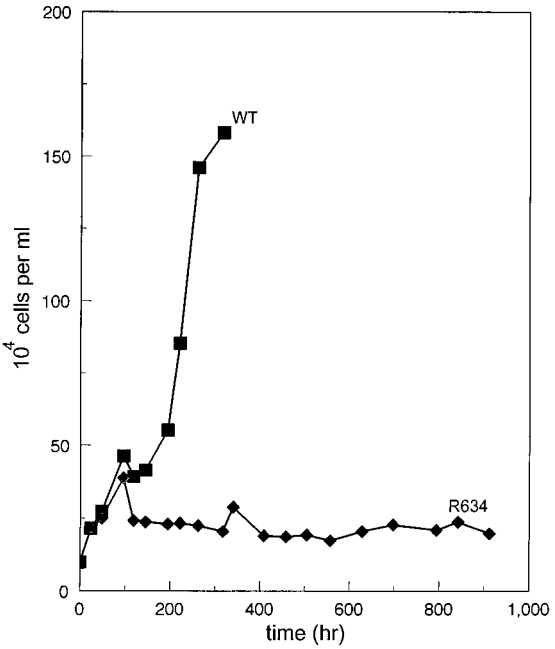

FIG. 1. IL-3-independent growth of Ba/F3-derived cell lines coexpressing the E5 protein and the wild-type or kinase-negative PDGF β receptor. The wild-type (WT) or tyrosine kinase-negative (R634) PDGF β receptor gene was introduced into a Ba/F3 cell line expressing the BPV E5 gene. The resulting cell lines were seeded in medium lacking IL-3 at a density of 10^5 cells per ml and counted periodically.

thione *S*-transferase (GST)–PLC-γ fusion protein (including amino acids 580 to 850 from rat PLC-γ) (46) (1 μg/μl in 50 mM Tris-HCl [pH 8.0]–5 mM glutathione) was added to 10 μl of resuspended beads. Kinase reactions were then performed in UKB buffer (20 mM PIPES [pH 7.0], 10 mM $MnCl_2$, 20 μg of aprotinin per ml) and 50 μM ATP in a total volume of 20 μl for 10 min at 30°C, and 40 μl of protein sample buffer was added to stop the reactions.

Kinase reaction products were electrophoresed, transferred to nitrocellulose membranes, and probed with antiphosphotyrosine antibodies as described above. To measure PDGF receptor levels in the reaction mix, 40 μl of protein sample buffer was added to 30 μl of beads resuspended in PAN containing 20 μg of aprotinin per ml, which were then electrophoresed, transferred to nitrocellulose membranes, and probed with anti-PDGF receptor antibodies as described above. Blots were subjected to PhosphorImager analysis.

RESULTS

Coexpression of the wild-type PDGF β receptor and the E5 protein provides a mitogenic signal for Ba/F3 cells. To test whether the PDGF β receptor was able to mediate a proliferative signal from the E5 protein in Ba/F3 cells, retroviral infection was used to introduce the BPV E5 and PDGF β receptor genes separately into these cells. After selection for expression of linked drug resistance genes, the cells were tested for the ability to grow in the absence of IL-3. In the great majority of cell lines tested, expression of neither the E5 protein nor the PDGF β receptor abrogated the IL-3 dependence of Ba/F3 cells (Table 1). Therefore, naive Ba/F3 cells do not appear to express endogenous proteins capable of mediating an E5 proliferative signal.

To assess the effects of coexpression of the E5 protein and the PDGF β receptor, cells expressing the E5 protein were infected with retroviruses carrying the PDGF β receptor gene, and cells expressing the PDGF β receptor were infected with retroviruses carrying the BPV E5 gene. After drug selection, the resulting cell lines expressing both genes were analyzed. The biological and biochemical properties of the cells in these experiments and in the other experiments described in this report did not differ according to the order of introduction of the BPV E5 and PDGF β receptor genes (data not shown); therefore, the data described here and subsequently combine results obtained from cells derived in either way. In contrast to the effects of expression of each protein separately, coexpression of the E5 protein and the PDGF β receptor allowed sustained IL-3-independent proliferation of 14 of 15 cell lines tested (Table 1), with the cells attaining saturation densities of over 1.5×10^6/ml, as shown in Fig. 1. Exponential IL-3-independent growth of these cells occurred after a lag period of a few days (Fig. 1; see also Fig. 6), which was not observed when cells were seeded in medium containing IL-3 (data not shown). Coexpression of the PDGF β receptor and the E5 protein did not result in IL-3 secretion and growth via autocrine stimulation, since medium conditioned by IL-3-independent cells coexpressing these proteins did not support growth of naive Ba/F3 cells (data not shown). We also introduced the v-*sis* gene, which encodes an oncogenic form of the PDGF B polypeptide (47), into Ba/F3 cells in the absence or in the presence of the PDGF β receptor. Expression of v-*sis* alone did not allow IL-3-independent growth, whereas coexpression of the PDGF β receptor and the v-*sis*-encoded protein allowed IL-3-independent proliferation (Table 1 and data not shown). The cells reached high saturation densities slightly faster than cell lines coexpressing the E5 protein and the PDGF β receptor (data not shown). Thus, both the E5 and v-*sis*-encoded proteins can signal through the PDGF β receptor in Ba/F3 cells.

For biochemical analysis of the Ba/F3-derived cell lines, protein extracts prepared from representative cell lines grown in the presence of IL-3 were examined by immunoblotting. Expression of the PDGF β receptor and of the E5 protein was readily detectable in the cell lines that received the appropriate

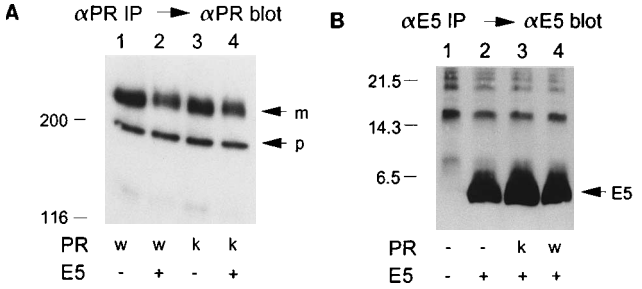

FIG. 2. Total PDGF β receptor (PR) (A) and E5 protein (B) expression in Ba/F3-derived cell lines. (A) Extracts (400 μg) made from Ba/F3-derived cell lines expressing the wild-type (w) or the kinase-negative R634 mutant (k) in the presence (+) or absence (−) of the E5 protein were immunoprecipitated with anti-PDGF receptor antibodies (αPR IP). Proteins were resolved by electrophoresis, transferred to membranes, and probed with anti-PDGF receptor antibodies for detection of total receptor levels (αPR blot). Bands corresponding to the mature (m) and precursor (p) forms of the PDGF β receptor are indicated by arrows. (B) Ba/F3 cell extracts (1,000 μg) were immunoprecipitated with anti-E5 antibodies (αE5 IP), and membranes were probed with anti-E5 antibodies for detection of total E5 protein expression (αE5 blot). The band corresponding to the E5 protein is indicated by an arrow. Migration of molecular weight markers (in kilodaltons) is shown on the left side.

genes, and the different cell lines expressed similar levels of these proteins (Fig. 2A, lanes 1 and 2; Fig. 2B, lane 4; and data not shown). These proteins were not present in cells that did not receive these genes (Fig. 2B, lane 1, and data not shown). Anti-PDGF receptor immunoprecipitates were also probed with antiphosphotyrosine antibodies. In the absence of the E5 protein, there was a low level of background tyrosine phosphorylation of the mature form of the PDGF β receptor (Fig. 3A, lane 1). When the E5 protein was present, the mature form of the receptor displayed markedly increased levels of tyrosine phosphorylation. In addition, a faster-migrating form of the PDGF β receptor, which presumably represents its precursor form with immature carbohydrate side chains, was also tyrosine phosphorylated in cells expressing the E5 protein (Fig. 3A, lane 2). Furthermore, the E5 protein formed a stable complex with the PDGF β receptor in cells coexpressing these proteins, as assessed by the ability of anti-E5 antibodies to immunoprecipitate both forms of the receptor from cell extracts (Fig. 3B, lane 2). In the absence of the E5 protein, no

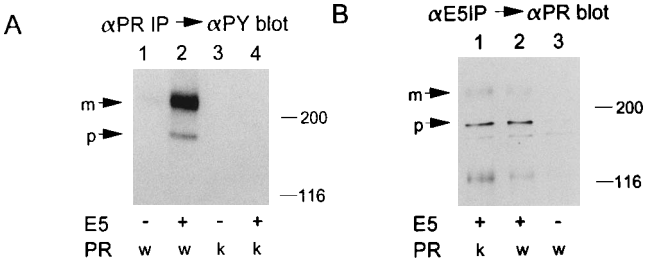

FIG. 3. Tyrosine phosphorylation of the PDGF β receptor (PR) (A) and complex formation between the E5 protein and the PDGF β receptor (B) in Ba/F3-derived cell lines. (A) Extracts (1,000 μg) made from Ba/F3-derived cell lines expressing the wild-type (w) or the kinase-negative R634 mutant (k) in the presence (+) or absence (−) of the E5 protein were immunoprecipitated with anti-PDGF receptor antibodies (αPR IP). Proteins were resolved by electrophoresis, transferred to membranes, and probed with antiphosphotyrosine antibodies for detection of tyrosine-phosphorylated receptors (αPY blot). (B) Ba/F3 cell extracts (1,500 μg) were immunoprecipitated with anti-E5 antibodies (αE5 IP), and membranes were probed with anti-PDGF receptor antibodies for detection of receptor associated with the E5 protein (αPR blot). Bands corresponding to the mature (m) and precursor (p) forms of the PDGF β receptor are indicated by arrows. Sizes of markers are indicated in kilodaltons.

significant immunoprecipitation of the PDGF β receptor with anti-E5 antibodies was observed (Fig. 3B, lane 3). Thus, the E5 protein binds to and activates both the mature and immature precursor forms of the PDGF β receptor in Ba/F3 cells, as has been previously described in fibroblasts, epithelial, and myeloid cells (13, 29, 34–36). Thus, the Ba/F3 cell system provides a functional assay in which various PDGF β receptor mutants can be tested for the capacity to interact with the E5 protein and mediate its mitogenic action.

A tyrosine kinase-negative PDGF β receptor mutant is not able to mediate E5 mitogenic activity. The first PDGF β receptor mutant tested in the Ba/F3 system was the tyrosine kinase-negative mutant R634, which contains a lysine-to-arginine substitution at position 634 in the ATP-binding site of the tyrosine kinase domain. This mutation destroys the catalytic activity of the receptor and therefore its ability to phosphorylate itself or other substrates. The R634 mutant is thus unable to mediate a mitogenic response to PDGF treatment (9, 21).

Ba/F3 cells expressing the R634 mutant, either in the absence or in the presence of the BPV E5 or v-*sis* gene, remained IL-3 dependent for growth (Fig. 1 and Table 1). Thus, unlike the wild-type PDGF β receptor, the tyrosine kinase-defective mutant was not able to deliver a mitogenic signal in response to either the E5 or v-*sis*-encoded protein. Western blotting analysis of these cell lines showed that the R634 mutant was expressed at levels comparable to those of the wild-type receptor and that the E5 protein was also abundantly expressed (Fig. 2A, lanes 3 and 4; Fig. 2B, lane 3). No tyrosine phosphorylation of the R634 mutant was detected, regardless of the presence of the E5 or v-*sis*-encoded protein (Fig. 3A, lanes 3 and 4, and data not shown). In cells coexpressing the E5 protein and the tyrosine kinase-negative mutant R634, these two proteins were present in a stable complex, as assessed by coimmunoprecipitation (Fig. 3B, lane 1). Therefore, the tyrosine kinase activity of the PDGF β receptor is required for E5 mitogenic activity and for the E5-induced increase in receptor tyrosine phosphorylation but not for stable complex formation between the PDGF β receptor and the E5 protein.

The ligand-binding domain of the PDGF β receptor is not required for E5 proliferative signaling. Two PDGF β receptor mutants (CP84-ΔX and TPR) containing amino-terminal truncations were also analyzed in the Ba/F3 system. The CP84-ΔX mutant contains a 405-amino-acid deletion (amino acids 38 through 442) that removes all of the first four and part of the fifth extracellular immunoglobulin-like domains of the PDGF β receptor. The TPR mutant contains a 493-amino-acid deletion (amino acids 38 through 530) and retains only 6 amino acids in the extracellular domain after cleavage of the signal peptide. Because the PDGF-binding domain is absent from both of these mutants, they are unable to bind and respond to PDGF (17).

Ba/F3-derived cell lines expressing these mutant receptors were tested for the ability to grow in medium lacking IL-3. The CP84-ΔX mutant alone or in the presence of v-*sis* was not able to support IL-3-independent growth of these cells, confirming that this mutant is not activated by PDGF. However, when the E5 protein was coexpressed with this mutant, the cells grew in an IL-3-independent fashion, reaching saturation densities comparable to those of cells coexpressing the wild-type PDGF β receptor and the E5 protein (Table 1 and data not shown). The ability of the CP84-ΔX mutant to mediate E5 action shows that the ligand-binding domain of the PDGF β receptor is not necessary for E5 mitogenic activity.

Two of five independently derived Ba/F3 cell lines expressing the TPR mutant in the absence of the E5 protein were able to grow in the absence of IL-3. This relatively high level of

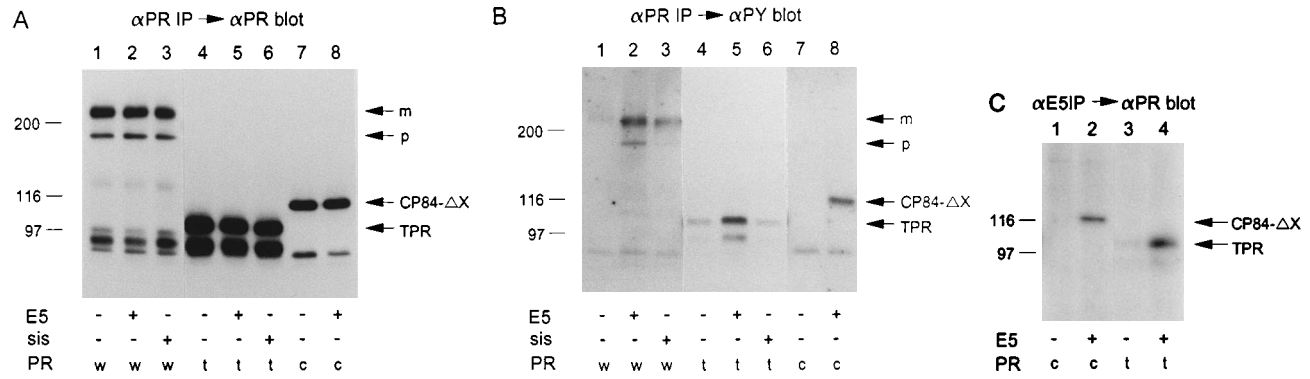

FIG. 4. PDGF β receptor (PR) expression (A), receptor tyrosine phosphorylation (B), and complex formation between receptor mutants containing amino-terminal deletions and the E5 protein (C) in Ba/F3 cells. (A) Extracts (100 μg) made from Ba/F3-derived cell lines expressing the wild-type (w), TPR mutant (t), or CP84-ΔX mutant (c) PDGF β receptor in the presence (+) or absence (−) of the E5 or v-*sis*-encoded protein were immunoprecipitated with anti-PDGF receptor antibodies (αPR IP). Proteins were resolved by electrophoresis, transferred to membranes, and probed with anti-PDGF receptor antibodies for detection of total receptor levels (αPR blot). (B) Ba/F3 cell extracts (400 μg) were immunoprecipitated with anti-PDGF receptor antibodies, and membranes were probed with antiphosphotyrosine antibodies for detection of tyrosine-phosphorylated receptors (αPY blot). Lanes 4 to 6 were exposed for 20-fold less time than the other lanes. (C) Ba/F3 cell extracts (1,500 μg) were immunoprecipitated with anti-E5 antibodies (αE5 IP), and membranes were probed with anti-PDGF receptor antibodies for detection of receptors associated with the E5 protein. Bands corresponding to the mature (m) and precursor (p) forms of the wild-type PDGF β receptor, as well as to the CP84-ΔX and TPR mutants, are indicated by arrows. Migration of molecular weight markers (in kilodaltons) is shown on the left.

background mitogenic activity suggests that this large amino-terminal truncation may result in partial activation of the PDGF β receptor. The expression of v-*sis* did not increase the level of background activity, confirming that this receptor mutant lacking the ligand-binding domain is incapable of responding to PDGF. Coexpression of the E5 protein with the TPR mutant in Ba/F3 cells increased the proportion of cell lines capable of growing in the absence of IL-3 to four of five (Table 1).

PDGF receptor immunoblotting showed that the CP84-ΔX and TPR receptor mutants were expressed at levels equivalent to or higher than those of the wild-type PDGF β receptor (Fig. 4A). The CP84-ΔX mutant displayed a low level of basal tyrosine phosphorylation when it was expressed alone (Fig. 4B, lane 7). The basal level of tyrosine phosphorylation of the TPR mutant was greater than that of the wild-type receptor or the CP84-ΔX mutant (Fig. 4B, lane 4; lanes 4 to 6 were exposed for approximately 20-fold less time than the remaining lanes of the blot), consistent with the ability of the TPR mutant alone to abrogate IL-3 dependence in a significant fraction of cell lines. When the E5 protein was coexpressed with either of these mutants, they displayed increased tyrosine phosphorylation (Fig. 4B, lanes 5 and 8), even though they lack almost the entire extracellular domain. As expected, coexpression of v-*sis* with either the CP84-ΔX or the TPR mutant did not result in increased receptor tyrosine phosphorylation relative to the basal level (lane 6 and data not shown). In addition, coimmunoprecipitation analysis demonstrated that only a very low background level of PDGF β receptor was immunoprecipitated by E5 antibodies in the absence of E5 expression (Fig. 4C, lanes 1 and 3), whereas expression of the E5 protein resulted in a dramatic increase in the amount of receptor immunoprecipitated by the E5 antibodies (Fig. 4C, lanes 2 and 4). Therefore, the E5 protein was present in a stable complex with both of these truncated mutants.

Analysis of requirements for tyrosine phosphorylation sites of the PDGF β receptor for E5 mitogenic activity in Ba/F3 cells. A series of human PDGF β receptor mutants containing phenylalanine-for-tyrosine substitutions at tyrosine phosphorylation sites were also tested for the ability to mediate E5-induced IL-3-independent growth of Ba/F3 cells (Fig. 5). The mutants tested were the F579/81 or Src$^-$ mutant, which contains phenylalanine substitutions at positions 579 and 581 and does not bind the Src family tyrosine kinases; the F5 mutant, which contains phenylalanine substitutions at positions 740, 751, 771, 1009, and 1021 and does not bind PI3-kinase, GAP, Syp, or PLC-γ; and the F857 mutant, in which tyrosine 857, a major site of tyrosine phosphorylation, is replaced by a phenylalanine (19, 28, 45). We also studied mutants containing either the F5 and the Src$^-$ mutations combined (F5/Src$^-$) or the F5 and F857 mutations combined (F5/857). The genes encoding these mutants were introduced into Ba/F3 cells either alone or together with the BPV E5 or v-*sis* gene, and the properties of the resulting cell lines were assessed.

As expected, none of the tyrosine phosphorylation site mutants expressed in the absence of the BPV E5 or v-*sis* gene allowed Ba/F3 cells to proliferate in medium lacking IL-3 (Table 2). Coexpression of the E5 or v-*sis*-encoded protein with either the F5 or Src$^-$ mutant receptor allowed Ba/F3 cells to proliferate in the absence of IL-3 (Table 2 and Fig. 6A). Compared with cells coexpressing the wild-type PDGF β receptor and the E5 protein, cells coexpressing the F5 mutant consistently displayed a shorter lag period before initiating rapid growth, whereas cells coexpressing the Src$^-$ mutant displayed a longer lag period. However, all three types of cell lines displayed similar doubling times during exponential growth and attained high saturation densities (Fig. 6A). These differences in lag period may reflect the absolute amounts of PDGF β receptor expressed in the cells. The F5 mutant was expressed more abundantly than the wild-type receptor, whereas the Src$^-$ mutant was expressed in lower amounts than the wild-type receptor (Fig. 7A, lanes 1 to 4, 13, and 14; see also Fig. 8, lanes 1 to 3, and Fig. 9B, lanes 1 and 2). The relative levels of wild-type and mutant PDGF β receptors in these cell lines were confirmed by using a second PDGF receptor antibody for immunoprecipitation and by fluorescence-activated cell sorting analysis (data not shown). The basis for these differences in receptor expression is not known. Nevertheless, these results demonstrate that both the F5 and Src$^-$ receptors are able to mediate an E5- or v-*sis*-induced proliferative signal in Ba/F3 cells. Interestingly, coexpression of the E5 or v-*sis*-encoded protein with the F5/Src$^-$ mutant, which contains the same mutations described above combined in a single molecule, did not allow IL-3-independent growth of Ba/F3 cells (Table 2 and

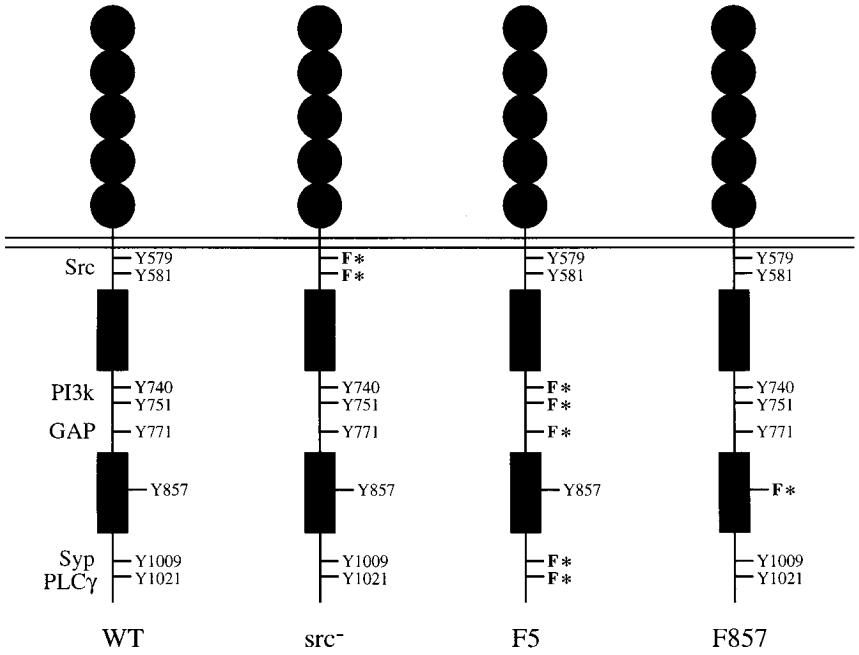

FIG. 5. Schematic representation of wild-type PDGF β receptor and tyrosine phosphorylation site mutants. Eight tyrosine phosphorylation sites of the wild-type PDGF β receptor (WT) tested in the Ba/F3 system, as well as SH2 domain-containing proteins known to bind to these phosphorylated sites, are shown. Tyrosine-to-phenylalanine mutations are indicated by F*. The Src$^-$ mutant contains phenylalanine-for-tyrosine substitutions at the juxtamembrane positions 579 and 581, important for binding to the Src family tyrosine-kinases Src, Fyn, and Yes (28). The F5 mutant lacks tyrosines 740, 751, 771, 1009, and 1021, known to bind to the p85 regulatory subunit of PI3-kinase, Nck, Ras-GAP, SH2 domain-containing phosphotyrosine phosphatase (Syp), and PLC-γ (20, 22–24, 32, 38, 44). The F857 mutant lacks tyrosine 857, which is not known to bind to any SH2 domain-containing proteins.

Fig. 6A), even though this mutant was expressed at levels comparable to those of the wild-type receptor (Fig. 7A, lanes 5 and 6).

In the absence of E5 expression, low levels of basal tyrosine phosphorylation of the Src$^-$ and F5/Src$^-$ mutants were observed (Fig. 7B, lanes 3 and 5). The level of tyrosine phosphorylation of the mature form of the receptor in the absence of the E5 protein was higher for the F5 mutant (lane 13), possibly reflecting the higher receptor amounts present in these cells. Expression of the E5 protein induced increased tyrosine phosphorylation of both the mature and immature precursor forms of the Src$^-$ mutant receptor (lane 4). The level of E5-induced

TABLE 2. IL-3-independent growth of Ba/F3-derived cell lines expressing tyrosine phosphorylation site mutants

Cell line class[a]	PDGF receptor	BPV E5	v-*sis*	IL-3-independent lines[b]
Ba/F3-hyg-WT	WT	−	−	1/15
Ba/F3-E5-WT	WT	+	−	14/15
Ba/F3-sis-WT	WT	−	+	8/8
Ba/F3-hyg-F5	F5	−	−	0/6
Ba/F3-E5-F5	F5	+	−	7/7
Ba/F3-sis-F5	F5	−	+	4/4
Ba/F3-hyg-src$^-$	Src$^-$	−	−	0/5
Ba/F3-E5-src$^-$	Src$^-$	+	−	5/6
Ba/F3-sis-src$^-$	Src$^-$	−	+	2/3
Ba/F3-hyg-F857	F857	−	−	1/4
Ba/F3-E5-F857	F857	+	−	1/5[c]
Ba/F3-sis-F857	F857	−	+	0/3[d]
Ba/F3-hyg-F5/src$^-$	F5/Src$^-$	−	−	0/4
Ba/F3-E5-F5/src$^-$	F5/Src$^-$	+	−	1/6
Ba/F3-sis-F5/src$^-$	F5/Src$^-$	−	+	2/5
Ba/F3-hyg-F5/857	F5/857	−	−	0/4
Ba/F3-E5-F5/857	F5/857	+	−	0/5
Ba/F3-sis-F5/857	F5/857	−	+	0/3

[a] Designated as in Table 1. The cell lines expressing the wild-type PDGF β receptor are the same cell lines shown in Table 1.
[b] Number of independently derived IL-3-independent cell lines as a fraction of the total number of lines tested. Cell lines were scored as described in Table 1, footnote *b*, except as noted in footnotes *c* and *d*.
[c] One of the cell lines grew like cells coexpressing the wild-type receptor and the E5 protein, while other four cell lines were markedly defective for growth (see Fig. 7B; also data not shown).
[d] Two cell lines started to grow approximately 1 week after cells coexpressing the wild-type receptor and the v-*sis*-encoded protein, and one cell line showed no growth.

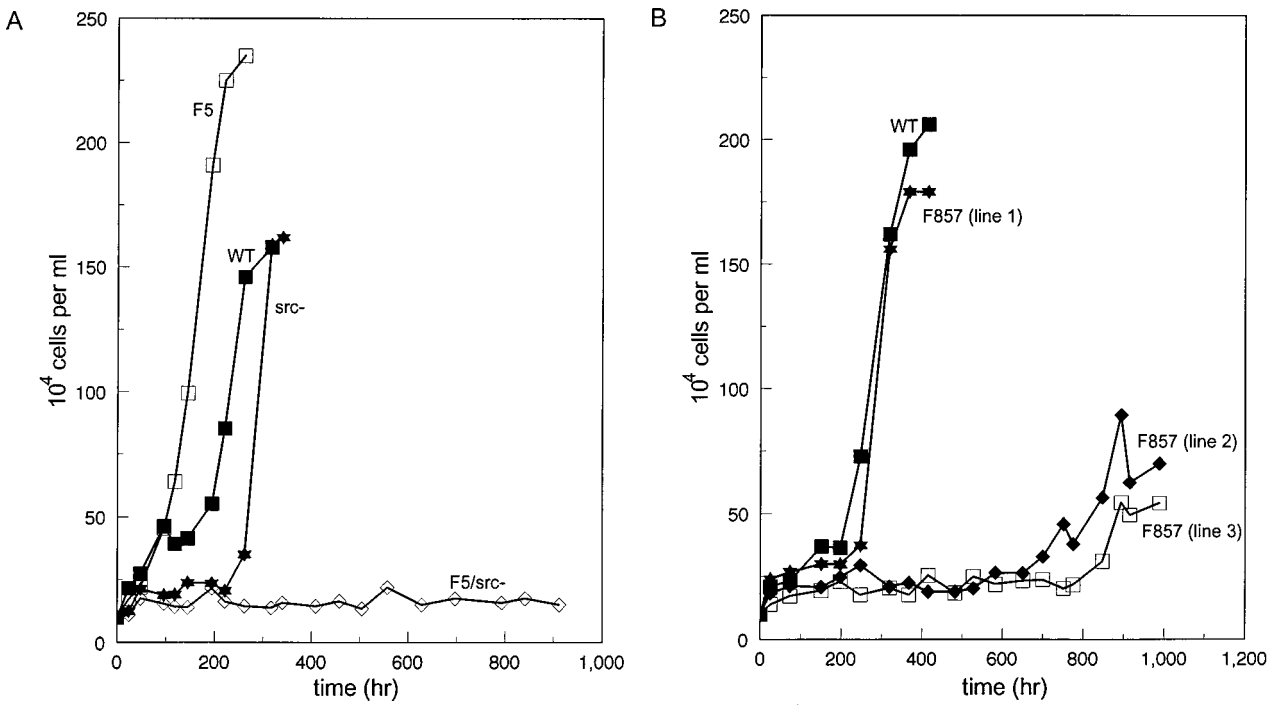

FIG. 6. IL-3-independent growth of Ba/F3-derived cell lines coexpressing the E5 protein with PDGF β receptor tyrosine phosphorylation site mutants. Representative Ba/F3-derived cell lines coexpressing the E5 protein with the wild-type PDGF β receptor (WT) or with the F5, Src$^-$, and F5/Src$^-$ mutants (A) or the F857 (B) mutant were seeded in medium lacking IL-3 at a density of 10^5 cells per ml and counted periodically. Three different cell lines expressing the F857 mutant are shown (B).

tyrosine phosphorylation of this mutant receptor was comparable to that of the wild-type receptor when normalized to amounts of receptor expression. E5 expression also resulted in increased tyrosine phosphorylation of the immature form of the F5 mutant and, to a smaller extent, of the mature form as well (Fig. 7B, lane 14). In contrast, E5-induced tyrosine phosphorylation of the F5/Src$^-$ mutant was markedly reduced relative to the wild-type level, being barely detectable (Fig. 7B, lane 6). All three PDGF β receptor mutants formed stable complexes with the E5 protein in Ba/F3 cells, as assessed by the ability of anti-E5 antibodies to coimmunoprecipitate the mutants when coexpressed with the E5 protein (data not shown).

The F857 mutant was partially defective in mediating IL-3-independent growth of Ba/F3 cells in the presence of the E5 or v-*sis*-encoded protein. When the five cell lines coexpressing the E5 protein and the F857 receptor were tested for IL-3-independent growth, one line grew like those coexpressing the wild-type receptor and the E5 protein (Fig. 6B, line 1), another three showed markedly defective growth (e.g., lines 2 and 3 in Fig. 6B), and one showed no growth at all (data not shown). Cell lines coexpressing the v-*sis*-encoded protein and the F857 receptor also showed impaired growth in the absence of IL-3 (Table 2). Thus, the F857 mutant was partially defective for mediating an E5- or v-*sis*-initiated proliferative signal, even though tyrosine 857 is not known to bind to any SH2 domain-containing proteins. The F5/857 mutant, which contains the F857 and F5 mutations combined, was unable to provide a mitogenic signal for Ba/F3 cells, since all Ba/F3 cell lines coexpressing this mutant with either the E5 or v-*sis*-encoded protein remained IL-3 dependent (Table 2). Both the F857 and the F5/857 mutants were expressed at levels comparable to those of the wild-type receptor, indicating that the proliferation defects of these cell lines were not due to reduced levels of PDGF receptor expression (Fig. 7A, lanes 7 to 10). Similarly, the E5 protein was expressed abundantly in these cells (parental cell line shown in Fig. 2B, lane 2, and data not shown), and both mutants formed stable complexes with the E5 protein (data not shown). In the absence of E5 expression, low levels of tyrosine phosphorylation of the F857 and F5/857 receptor mutants were observed (Fig. 7B, lanes 7 and 9). Expression of the E5 protein induced increased tyrosine phosphorylation of both the mature and immature precursor forms of the F857 receptor at levels comparable to those of the wild-type receptor in all cell lines tested, including those markedly defective for IL-3-independent growth (e.g., line 3 shown in Fig. 7B, lane 8). Evidently, abundant tyrosine phosphorylation of the F857 receptor does not enable efficient E5-mediated signal transduction. The F5/857 mutant showed markedly reduced levels of E5-induced tyrosine phosphorylation relative to wild-type levels (lane 10).

Binding of the wild-type PDGF β receptor and tyrosine phosphorylation site mutants to SH2 domain-containing proteins. The binding of the wild-type PDGF β receptor and of the various tyrosine phosphorylation site mutants to several SH2 domain-containing proteins was also assessed (Fig. 8 and data not shown). Cell extracts prepared from representative cell lines (for these experiments, in most cases IL-3-independent cell lines were grown in the absence of IL-3 prior to protein extraction) were immunoprecipitated with anti-PI3-kinase, anti-GAP, or anti-PLC-γ antibodies, transferred to nitrocellulose membranes after electrophoresis, and probed with anti-PDGF receptor antibodies. The wild-type PDGF β receptor did not show significant binding to any of these proteins in the absence of E5 (data not shown). However, when the receptor and the E5 protein were coexpressed, both the mature and the immature precursor forms of the wild-type receptor

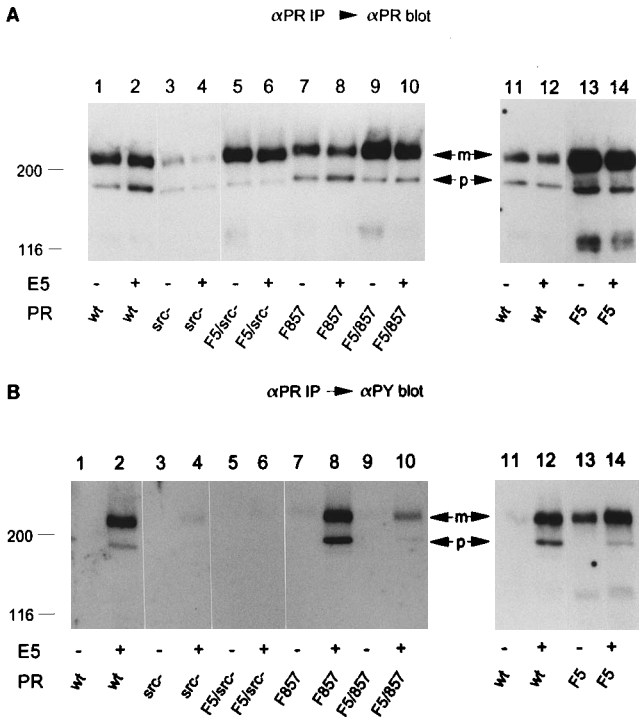

FIG. 7. Expression (A) and tyrosine phosphorylation (B) of PDGF β receptor (PR) phosphorylation site mutants. (A) The wild-type PDGF β receptor (wt) or the Src$^-$, F5, F5/Src$^-$, F857, or F5/857 mutant receptor as indicated were introduced into Ba/F3 cells in the absence (−) or presence (+) of the E5 protein; 300 μg (lanes 1 to 10) or 400 μg (lanes 11 to 14) of extracts from the resulting lines were immunoprecipitated with anti-PDGF receptor antibodies (αPR IP). Proteins were resolved by electrophoresis, transferred to membranes, and probed with anti-PDGF receptor antibodies for detection of total receptor expression (αPR blot). (B) Anti-PDGF receptor immunoprecipitates from 700 μg (lanes 1 to 10) or 400 μg (lanes 11 to 14) of Ba/F3 extracts were electrophoresed, transferred to membranes, and probed with antiphosphotyrosine antibodies for detection of tyrosine-phosphorylated PDGF receptors (αPY blot). Bands corresponding to the mature (m) and precursor (p) forms of the PDGF β receptor are indicated by arrows. Migration of molecular weight markers (in kilodaltons) is shown on the left side.

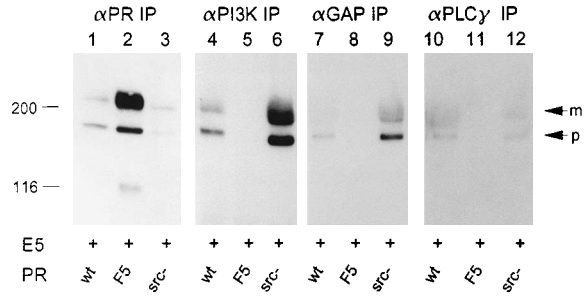

FIG. 8. Binding of wild-type PDGF β receptor (PR) and tyrosine phosphorylation site mutants to PI3-kinase, GAP, and PLC-γ. Extracts (250 μg for anti-PDGF β receptor [αPR] immunoprecipitation [IP], 1,000 μg for anti-PI3-K [αPI3K] IP, 1,500 μg for anti-GAP [αGAP] IP, 2,000 μg for anti-PLC-γ [αPLCγ]IP) made from Ba/F3-derived cell lines (grown in the absence of IL-3) expressing the wild-type PDGF β receptor (wt) or the F5 or Src$^-$ receptor in the presence (+) of the E5 protein were immunoprecipitated with anti-PDGF receptor, anti-PI3-kinase, anti-GAP, or anti-PLC-γ antibodies. Proteins were resolved by electrophoresis and transferred to membranes, which were probed with anti-PDGF receptor antibodies for detection of total receptor expression (lanes 1 to 3) and of receptor associated with the various substrates (in lane 12, half of the usual amount of protein was immunoprecipitated). Lanes 4 to 9 were exposed 8-fold as long as lanes 1 to 3, and lanes 10 to 12 were exposed 20-fold as long as lanes 1 to 3. Bands corresponding to the mature (m) and precursor (p) forms of the PDGF β receptor are indicated by arrows. Migration of molecular weight markers (in kilodaltons) is shown on the left side.

were coimmunoprecipitated with anti-PI3-kinase, anti-GAP, or anti-PLC-γ antibodies (lanes 4, 7, and 10). These results demonstrated that the E5 protein induces complex formation between the PDGF β receptor and the SH2 domain-containing proteins PI3-kinase, GAP, and PLC-γ in Ba/F3 cells, presumably as a consequence of tyrosine phosphorylation of the receptor at the cognate sites. Analysis of the various tyrosine phosphorylation site mutants yielded the predicted results. For example, in the presence of the E5 protein, the F5 mutant displayed markedly reduced binding to PI3-kinase, GAP, or PLC-γ (lanes 5, 8, and 11), whereas, as predicted, the E5-activated Src$^-$ mutant was not defective for binding to these proteins (lanes 6, 9, and 12). The Src$^-$ mutant bound the analyzed SH2 domain-containing proteins more efficiently than did the wild-type receptor, and this was most striking in the case of PI3-kinase. As described in the figure legend, different amounts of extracts were immunoprecipitated and the membrane was exposed to film for different lengths of time in these experiments. Taking these factors into account, approximately 7% of the wild-type receptors were coimmunoprecipitated by the PI3-kinase antibodies, whereas roughly 40% of the Src$^-$ mutant receptors were present in a complex with PI3-kinase. Consistent with its normal levels of tyrosine phosphorylation, the F857 mutant displayed normal binding to PI3-kinase and GAP when the E5 protein was present (data not shown). These results confirmed that the tyrosine-to-phenylalanine substitutions do, in fact, inhibit binding of the mutant receptors to the predicted SH2 domain-containing proteins.

Despite extensive efforts, we were unable to detect binding of Src family members to the wild-type PDGF β receptor in Ba/F3 cells. Indeed, it is in general more difficult to detect the PDGF β receptor associated with Src family members than with PI3-kinase, GAP, or PLC-γ (40). To confirm the identity of the Src$^-$ mutant, we extracted genomic DNA from cells expressing this mutant, amplified the region of interest by using primers specific for the human PDGF β receptor gene, and sequenced the PCR products. The sequencing confirmed that tyrosines 579 and 581 were substituted by phenylalanines in the Src$^-$ mutant.

Levels of intrinsic tyrosine kinase activity of the wild-type PDGF β receptor and tyrosine phosphorylation site mutants. To measure the levels of intrinsic kinase activity of the wild-type PDGF β receptor and of the Src$^-$, F857, F5/Src$^-$, and F5/857 mutants, protein extracts were immunoprecipitated with anti-PDGF receptor antibodies. A GST–PLC-γ fusion protein was added to the immunoprecipitates as a substrate, and a kinase reaction was performed in the presence of unlabeled ATP. The reaction products were subjected to electrophoresis, transferred to membranes, and analyzed by probing with antiphosphotyrosine antibodies. All of the receptors were capable of phosphorylating the substrate on tyrosine residues, although to various extents (Fig. 9A). To determine the absolute amounts of receptor present in the reactions, a fraction of each immunoprecipitate was immunoblotted with anti-PDGF receptor antibodies (Fig. 9B).

To compare the specific tyrosine kinase activities of the various mutant receptors, PhosphorImager analysis was used to quantitate the relative level of substrate tyrosine phosphorylation and of expression of both the mature and precursor forms of the receptor. Although receptor immunoprecipitated from cells expressing the Src$^-$ mutant exhibited reduced tyrosine kinase activity compared with cells expressing the wild-type receptor, there was less receptor present in cells express-

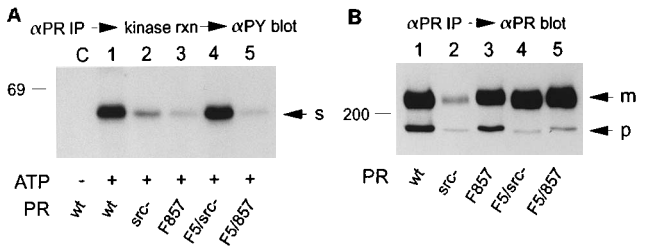

FIG. 9. In vitro tyrosine kinase assay of wild-type and tyrosine phosphorylation site mutant PDGF β receptors (PR). (A) Extracts (1,000 μg) made from Ba/F3-derived cell lines expressing the wild-type (wt), Src$^-$, F857, F5/Src$^-$, or F5/587 mutant PDGF β receptor as indicated were immunoprecipitated with anti-PDGF receptor antibodies (αPR IP). One-tenth of each immunoprecipitate was incubated with buffer containing ATP and $MnCl_2$(+) and the substrate GST–PLC-γ fusion protein (including amino acids 580 to 850 of rat PLC-γ). Kinase reaction (rxn) products were resolved by electrophoresis, transferred to membranes, and probed with antiphosphotyrosine antibodies for detection of tyrosine-phosphorylated substrate (αPY blot). As a negative control (lane C), buffer containing ATP and $MnCl_2$ was not added (−) to the immunoprecipitate. Bands corresponding to the substrate (s) are indicated by an arrow. (B) To measure PDGF receptor levels in the reaction mix as described for panel A, 3/10 of each immunoprecipitate was electrophoresed, transferred to membranes, and probed with anti-PDGF receptor antibodies (αPR blot). Bands corresponding to the mature (m) and precursor (p) forms of the PDGF β receptor are indicated by arrows. Migration of molecular weight markers (in kilodaltons) is shown on the left side.

ing this mutant (Fig. 9A and B, lanes 1 and 2). After normalization for the total amount of receptor present, the Src$^-$ mutant showed twice as much intrinsic tyrosine kinase activity as did the wild-type receptor. The partially defective F857 mutant, on the other hand, displayed approximately 40% of the tyrosine kinase activity of the wild-type receptor (Fig. 9A and B, lanes 3). Strikingly, the defective F5/Src$^-$ mutant displayed higher tyrosine kinase activity than wild-type (Fig. 9A and B, lanes 4), whereas the F5/857 mutant exhibited only about 30% of the tyrosine kinase activity of the wild-type receptor (Fig. 9A and B, lane 5). Coexpression of the E5 protein did not affect the kinase activity of any of the receptors (data not shown), probably because the assay conditions appear to fully activate the receptors, thus revealing their intrinsic kinase activities (23). These results suggest that the F857 mutant receptor, although displaying normal levels of autophosphorylation, has a reduced ability to tyrosine phosphorylate other substrates, whereas the tyrosine kinase activity of the F5/Src$^-$ mutant appears intact.

DISCUSSION

These experiments have shown that coexpression of the E5 protein and the wild-type human PDGF β receptor generates a mitogenic signal which allows lymphoid Ba/F3 cells to grow in an IL-3-independent manner. Although the E5 protein influences the metabolism and activity of a variety of growth factor receptors in NIH 3T3 cells, the PDGF β receptor appears to be the preferred target in bovine fibroblasts, mouse C127 cells, N-MuMG cells, and 32D cells (13, 25a, 29, 34–37). Nevertheless, it is possible that E5 activity involves complex interplay among a variety of growth factor receptors. Since Ba/F3 cells do not express endogenous epidermal growth factor receptors (41), the epidermal growth factor receptor does not contribute to the proliferation supported by the E5-PDGF β receptor interaction in these cells. It is also possible that proteins not normally involved in PDGF signaling participate in E5 signal transduction. However, the results presented here and elsewhere establish that such components, if they exist, are not sufficient to initiate E5 transformation unless the PDGF β receptor is also present (13, 29). In Ba/F3 cells, the E5 protein forms a complex with the PDGF β receptor and induces increased tyrosine phosphorylation of both the mature and precursor forms of the receptor. Interestingly, when activated by the E5 protein, both forms of the receptor associate with several SH2 domain-containing proteins. Therefore, these substrates are accessible to the intracellular precursor form of the receptor. Association between SH2 domain-containing proteins and the precursor form of the PDGF β receptor, which contains immature, endo-β-*N*-acetylglucosaminidase H-sensitive carbohydrate side chains and is presumably localized in the endoplasmic reticulum, has also been observed in E5-transformed rodent fibroblasts (30). The v-*sis*-encoded protein was also able to activate the wild-type PDGF β receptor and generate a mitogenic response in Ba/F3 cells.

E5 mitogenic activity requires the intrinsic tyrosine kinase activity of the PDGF β receptor, confirming a central feature of the model that E5 signaling occurs through activation of the PDGF β receptor signal transduction pathway. Furthermore, E5-induced increase in tyrosine phosphorylation of the wild-type receptor is dependent on its intrinsic kinase activity and is likely due to receptor autophosphorylation and not to its phosphorylation by different tyrosine kinases. However, complex formation between the E5 protein and the PDGF β receptor does not require kinase activity or tyrosine phosphorylation of the receptor.

The results obtained with the mutants containing large amino-terminal truncations showed that the E5 protein interacts with the PDGF β receptor in a manner distinct from the PDGF interaction and that E5-mediated receptor activation does not involve binding of PDGF to its receptor. Therefore, the E5 protein activates the PDGF β receptor in a ligand-independent fashion and not by inducing PDGF expression or by increasing the sensitivity of the receptor to limiting amounts of PDGF present in the media. The biochemical analysis of the TPR mutant indicated that no more than six amino acids from the extracellular region of the receptor are required for E5 binding or E5-induced increase in receptor tyrosine phosphorylation. These results are consistent with previous studies, which suggested that the transmembrane region of the PDGF β receptor and the hydrophobic domain of the E5 protein are largely responsible for the interaction between these two proteins (5, 12). In addition, E5 transformation of NIH 3T3 cells does not appear to require most of the hydrophilic carboxy-terminal portion of the E5 protein (26), which is thought to be on the same face of the membrane as the ligand-binding domain of the PDGF β receptor (2). However, specific carboxy-terminal amino acids in the E5 protein appear important for C127 cell transformation and mitogenesis (15, 18). Taken together, these results suggested that activation of the PDGF β receptor by the E5 protein involves interactions occurring in the transmembrane and juxtamembrane regions of the receptor.

The high background of tyrosine-phosphorylation and mitogenic signaling displayed by the TPR mutant suggested that removal of the extracellular domain results in partial activation of the PDGF β receptor. An amino-terminal truncation activates the epidermal growth factor receptor (8), but mutational activation of the PDGF β receptor has not been previously reported. A chromosomal translocation involving the fusion of a cellular Ets-related protein to the transmembrane and cytoplasmic domains of the PDGF β receptor has been described in human chronic myelomonocytic leukemia, but it is not known whether this event results in receptor activation (14).

In addition to the extracellular domain and tyrosine kinase activity of the PDGF β receptor, several sites of receptor

tyrosine phosphorylation are required for transduction of the PDGF signal. The tyrosine phosphorylation sites required for acute PDGF signaling differ in different cell types. In most cell types tested, at least one of the sites missing from the F5 mutant is required for mitogenic signaling (1, 10, 38, 39, 45). In Ba/F3 cells, mouse PDGF β receptor mutants lacking either the PI3-kinase-binding site, the GAP-binding site, or the PLC-γ-binding site are still able to mediate ras activation by PDGF, suggesting that these cells have relatively relaxed requirements for PDGF signaling (39).

To investigate which sites are required for sustained E5 and v-*sis* mitogenic signaling in Ba/F3 cells, tyrosine phosphorylation site mutants were tested. The F5 mutant, which is defective for binding to PI3-kinase, GAP, PLC-γ, and Syp, was able to provide a mitogenic signal to Ba/F3 cells when coexpressed with the BPV E5 or v-*sis* gene. Moreover, the E5 protein bound to the F5 mutant and induced increased levels of tyrosine phosphorylation of both forms of this mutant receptor. Coimmunoprecipitation experiments confirmed that signaling can occur in the absence of significant levels of binding of PI3-kinase, GAP, and PLC-γ in Ba/F3 cells. Although the Src$^-$ mutant has been previously reported to lack kinase activity in response to acute ligand stimulation (28), our results showed that tyrosines 579 and 581 are not required for PDGF β receptor association with the E5 protein, tyrosine autophosphorylation, tyrosine-kinase activity, or mitogenic signaling in Ba/F3 cells. However, unlike the studies cited above, we analyzed long-term proliferation in response to chronic receptor activation rather than Ras activation or DNA synthesis in response to acute stimulation by ligand addition. There are a number of plausible explanations for the increased binding observed of the Src$^-$ mutant to SH2 domain-containing proteins. It is possible that when tyrosines 579 and 581 are missing, there is a more efficient phosphorylation of other sites, resulting in binding of these SH2 domain-containing proteins to a larger proportion of receptor molecules. Alternatively, binding of the Src family members to the wild-type receptor might sterically hinder the binding of other SH2 domain-containing proteins; receptors lacking tyrosines 579 and 581 would thus bind these proteins more efficiently.

The results obtained with the F5 and Src$^-$ mutants showed that none of the tyrosine phosphorylation sites tested which are known to bind to SH2 domain-containing proteins are absolutely required for E5 or PDGF signaling in Ba/F3 cells. However, the F5/Src$^-$ mutant receptor, in which the F5 and Src$^-$ mutations are combined on the same molecule, was not able to mediate a mitogenic signal in response to the E5 or v-*sis*-encoded protein. This mutant was not defective for tyrosine phosphorylation of an exogenous substrate, implying that the protein is not grossly misfolded. However, it displayed very low levels of autophosphorylation, presumably because it lacked most of its tyrosine phosphorylation sites. Since the tyrosine kinase activity of the F5/Src$^-$ receptor is high and it forms a complex with the E5 protein, its inability to mediate a mitogenic signal is probably due to impaired binding to many SH2 domain-containing proteins. Binding of these proteins may be required to localize them to an essential membrane compartment or to bring them into proximity of the receptor tyrosine kinase so that they can be phosphorylated. Alternatively, binding to the tyrosine-phosphorylated receptor may induce conformational activation of some SH2 domain-containing proteins, as has been reported for the p85 regulatory subunit of PI3-kinase (42).

The fact that the F5 and Src$^-$ mutants are functional, whereas the F5/Src$^-$ mutant is defective, suggests that at least two sets of receptor tyrosine phosphorylation sites and cognate binding proteins can independently provide mitogenic signals to Ba/F3 cells. This may occur, for example, if different sets of proteins bind to the Src sites and the F5 sites, yet either set is sufficient to deliver a proliferative signal. Alternatively, a single essential signaling molecule may bind independently to both the Src sites and the F5 sites, and binding to either set of sites may be sufficient for signaling. Such putative signaling molecules include GRB2 and Shc, both of which bind multiple sites on the receptor (1, 49). Previous work has also suggested that multiple mitogenic signals are generated upon acute stimulation of the PDGF β receptor by its ligand (39, 45).

The F857 mutant was markedly defective for mitogenic signaling, even though extensive analysis by several laboratories has failed to detect a protein that binds to tyrosine 857. This mutant formed a complex with the E5 protein, showed E5-induced tyrosine autophosphorylation levels similar to those of the wild-type receptor in vivo, and was able to bind to SH2 domain-containing proteins. On the other hand, the F857 mutant exhibited markedly reduced in vitro kinase activity on an external substrate. Similarly, in other cell types, the human F857 receptor mutant and the analogous mutant of the mouse PDGF β receptor have been shown to be competent for receptor autophosphorylation but defective for in vitro kinase activity as well as in vivo tyrosine phosphorylation of cellular SH2 domain-containing proteins in response to acute PDGF stimulation (11, 21). Therefore, we favor the interpretation that this mutant is defective because of its reduced ability to tyrosine phosphorylate substrates other than the receptor itself. In fact, preliminary results indicated that in vivo tyrosine phosphorylation of GAP was markedly reduced in Ba/F3 cells coexpressing the E5 protein and the F857 mutant relative to cells coexpressing the E5 protein and the wild-type PDGF β receptor (data not shown). Since tyrosine phosphorylation of some PDGF β receptor substrates, such as PLC-γ, results in enzymatic activation (31), impaired phosphorylation of such substrates would blunt the receptor-mediated response.

The analysis of the PDGF β receptor mutants suggests the dissociation of two activities of the PDGF β receptor tyrosine kinase in mitogenic signaling, namely, receptor autophosphorylation and tyrosine phosphorylation of other substrates, both of which are required to generate a proliferative signal. The F5/Src$^-$ mutant displays normal in vitro tyrosine kinase activity but reduced in vivo autophosphorylation; therefore, its inability to signal appears to be due to reduced binding to SH2 domain-containing proteins. Conversely, the F857 mutant displays normal levels of in vivo autophosphorylation but has markedly defective in vitro kinase activity, suggesting that its signaling defect results from impaired ability to phosphorylate substrates other than the receptor itself. The fact that these activities were dissociated for the PDGF β receptor upon activation by distinct mechanisms, either PDGF binding to the extracellular domain of the receptor or the E5 protein acting in a ligand-independent fashion (presumably by binding to the transmembrane and juxtamembrane domains of the receptor), indicates that these are likely to be general features of PDGF β receptor signaling. The E5 protein-PDGF β receptor interaction also appears to mediate transformation of dermal fibroblasts, one of the natural host cells of BPV infection, and it may be possible to carry out experiments similar to those described here in dermal fibroblasts derived from PDGF β receptor knockout mice (42a) in order to explore E5 signaling in this cell type.

In summary, the E5 protein activates the PDGF β receptor in a ligand-independent manner. Nevertheless, mitogenic signaling in response to both the E5 and v-*sis*-encoded proteins requires receptor tyrosine kinase activity, which has two essen-

tial consequences: (i) receptor autophosphorylation and recruitment of effectors (SH2 domain-containing signaling proteins) to the receptor and (ii) tyrosine phosphorylation of effectors. Furthermore, the tyrosine phosphorylation site requirements for signaling by the E5 and v-*sis*-encoded proteins are strikingly similar in Ba/F3 cells, suggesting that both proteins initiate similar intracellular signal transduction cascades.

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