

Bovine Papillomavirus E5 Protein Induces the Formation of Signal Transduction Complexes Containing Dimeric Activated Platelet-derived Growth Factor β Receptor and Associated Signaling Proteins*

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The bovine papillomavirus E5 protein binds to the cellular platelet-derived growth factor (PDGF) β receptor, resulting in constitutive activation of the receptor and cell growth transformation. By subjecting extracts from E5-transformed or PDGF-treated cells to velocity sedimentation in sucrose gradients, activated PDGF β receptor complexes were separated from monomeric, inactive receptor. Rapidly sedimenting activated complexes contained oligomeric (apparently dimeric), tyrosine-phosphorylated PDGF β receptor, the E5 protein, and associated cellular signaling proteins including the p85 subunit of phosphoinositol 3'-kinase, phospholipase C γ , and Ras-GTPase activating protein. These signaling proteins made the major contribution to the increased sedimentation rate of the activated receptor complexes. Pairwise analysis of components of these complexes indicated that multiple signaling proteins and the E5 protein were simultaneously present in the activated complexes. Our results also showed that the E5 protein and PDGF activated only a small fraction of the total PDGF β receptor, that not all receptor molecules associated with the E5 protein were tyrosine-phosphorylated, and that signaling proteins could bind to hemiphosphorylated receptor dimers. On the basis of these results, we propose a model for the assembly of multiprotein, activated PDGF β receptor complexes in response to the E5 protein.

the intrinsic kinase activity of the receptor, apparently by removing inhibitory constraints on substrate or ATP binding (6–9). Numerous other tyrosines in the cytoplasmic domain of the receptor are also phosphorylated following PDGF treatment, thereby generating specific binding sites for a variety of cellular signaling and regulatory molecules that contain SH2 domains, including members of the Src family of tyrosine kinases, the 85-kDa regulatory subunit (p85) of phosphoinositol 3'-kinase (PI 3'-kinase), phospholipase C γ (PLC γ), the phosphatase SHP-2, STAT factors, Ras-GTPase activating protein (RasGAP), and a number of SH2-containing adaptor proteins (10, 11). Additional signaling proteins appear to bind the receptor indirectly upon ligand stimulation (*e.g.* Refs. 12–19). Protein binding and activation of downstream signaling pathways are required for the cellular responses to PDGF, but the relative importance of the different cellular proteins and pathways depends upon the particular cells used and phenotype measured (*e.g.* Refs. 13–19).

The existence of signal transfer particles consisting of an activated RTK and associated signaling proteins was postulated by Ullrich and Schlessinger 10 years ago (20). However, despite extensive analysis of RTK function, there have been relatively few attempts to purify such signaling complexes intact and characterize them. Numerous studies have used co-immunoprecipitation to demonstrate that particular signaling proteins are stably associated with activated RTKs, and ternary complexes containing activated PDGF β receptor, PI 3'-kinase, and PLC γ can assemble *in vitro* (21). In addition, velocity sedimentation in sucrose gradients has been used to separate dimeric PDGF β receptor from monomeric receptor, and this dimeric fraction displayed increased tyrosine kinase activity *in vitro* (1). However, other proteins, if any, in the dimeric fraction were not characterized. Carraway and co-workers (22) reported that microvillar fractions of rat mammary carcinoma cells contain a very large ($>2 \times 10^6$ Da) heterogeneous transmembrane complex or signal transduction particle that contains microfilaments, the activated RTK p185^{neu}, serine/threonine kinases including enzymes of the mitogen-activated protein kinase signaling pathway, phosphotyrosine phosphatases, p60^{c-src}, p120^{c-abl}, a retroviral Gag-like protein, and various other cellular glycoproteins (22). Recently, two-dimensional gel electrophoresis and column chromatography of extracts of colony stimulatory factor 1-stimulated macrophages have been used to detect high molecular weight complexes containing colony stimulatory factor 1 receptor and numerous signaling proteins (23, 24).

The bovine papillomavirus (BPV) E5 protein is a very short, dimeric transmembrane oncoprotein that specifically activates the PDGF β receptor (25–27). The E5-activated receptor is a constitutively active tyrosine kinase *in vitro*, is constitutively

Transmembrane receptor tyrosine kinases (RTKs)¹ play important roles in many aspects of cell growth and behavior. One of the best studied RTKs is the PDGF β receptor, which is normally activated when the dimeric ligand, PDGF-BB, binds to its extracellular domain. This results in homodimerization of the receptor and *trans*-phosphorylation of the two receptor molecules in the dimeric receptor complex on multiple tyrosine residues (Refs. 1–4; reviewed in Ref. 5). One of the major sites of receptor autophosphorylation is in the “activation loop” of the kinase domain, and phosphorylation at this site increases

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¹ The abbreviations used are: RTK, receptor tyrosine kinase; PDGF, platelet-derived growth factor; PLC, phospholipase C; GAP, GTPase activating protein; PI, phosphoinositol; BPV, bovine papilloma virus; SH, Src homology; MOPS, 4-morpholinopropanesulfonic acid.

phosphorylated on tyrosine, and is constitutively bound to downstream signaling substrates (25, 28). Moreover, cells that normally do not express the PDGF receptor and are non-responsive to the E5 protein are rendered susceptible to E5-induced transformation by introduction of a gene encoding the PDGF β receptor, but not by genes encoding other RTKs (28–30). Recently, we showed that treatment of E5-transformed cells with a specific inhibitor of the PDGF receptor tyrosine kinase resulted in rapid, reversible receptor dephosphorylation and inhibition of the transformed phenotype (31). Taken together, these experiments provide compelling evidence that the E5 protein activates the PDGF β receptor, resulting in cell transformation.

The E5 protein and PDGF are structurally dissimilar, suggesting that the mechanism of PDGF β receptor activation by these two proteins is quite different. Indeed, the ligand-binding domain of the PDGF β receptor is not required for activation by the viral protein, demonstrating that E5-induced PDGF β receptor activation is ligand-independent (28, 32). Co-immunoprecipitation studies showed that the E5 protein, like PDGF, binds to the receptor; however, unlike PDGF, binding of the E5 protein occurs to the transmembrane and extracellular juxtamembrane region of the receptor (28, 32–37). We have shown that stable complex formation between the E5 protein and the PDGF β receptor caused receptor activation by inducing receptor dimerization and *trans*-phosphorylation (38). On the basis of extensive mutational analysis, computational studies and other considerations, we have proposed a model of the interaction between the PDGF β receptor and dimeric E5 protein that explains how complex formation results in receptor dimerization and activation (27).

Here, by using sucrose gradient velocity sedimentation of non-ionic detergent extracts of E5-transformed and PDGF-treated cells, we have physically separated stable complexes containing activated PDGF β receptor from the inactive receptor. Our results demonstrated that only a small fraction of PDGF β receptor is activated in these cells and that the increased sedimentation rate of the activated complexes is due to receptor dimerization and the stable association of the activated receptor with multiple signaling proteins. These studies revealed several features of the activated PDGF β receptor and provided insight into the assembly and composition of multi-protein signaling complexes containing activated PDGF β receptor.

MATERIALS AND METHODS

Cell Culture—C127 and Ba/F3 cells were maintained as described previously (28, 38). Briefly, C127 fibroblasts were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Ba/F3 hematopoietic cells were maintained in RPMI supplemented with 10% heat-inactivated fetal bovine serum, β -mercaptoethanol, and interleukin-3. Ba/F3 cell lines expressing various combinations of exogenous genes were described previously (38, 39) and were maintained in 1 μ g/ml puromycin, 500 μ g/ml G418, and/or 500 units/ml hygromycin. For the experiment shown in Fig. 3, Ba/F3 cells expressing the wild-type human PDGF β receptor were treated with 50 ng/ml recombinant human PDGF BB (Life Technologies, Inc.) for 7 min at room temperature or left untreated. The following PDGF β receptor mutants were used: Δ PR, which has a deletion of most of the extracellular ligand binding domain, and R634, which is a full-length human PDGF β receptor with a mutation that eliminates tyrosine kinase function (39).

Velocity Sedimentation in Sucrose Gradients—Ba/F3 cells or serum-starved C127 cells were washed once with ice-cold phosphate-buffered saline (137 mM NaCl, 3 mM KCl, 1.0 mM KH_2PO_4 , 8.0 mM Na_2HPO_4) and lysed in TG buffer (1 \times phosphate-buffered saline, 10% glycerol, 1% Triton X-100, and 0.01% sodium azide) containing 1 mM phenylmethylsulfonyl fluoride, 2 mM sodium vanadate, 10 μ g/ml leupeptin, and 10 μ g/ml aprotinin. The final concentration of extract was made up to 6 mg/ml. 0.5 ml of extract was layered on the top of a 5-ml linear 5–25% sucrose gradient in TG buffer formed with a gradient maker (Auto

Densi-Flow IIC, Buchler Instruments) in a Polyallomer Centrifuge tube (Beckman no. 326819). After ultracentrifugation at 46,000 rpm in a SW50.1 rotor (Beckman) for 16 h at 4 $^\circ\text{C}$, 13 0.4-ml fractions were collected from the top by using a Gilson FC205 fraction collector.

Trichloroacetic Acid Precipitation—For some experiments, trichloroacetic acid precipitation was used to precipitate total proteins before SDS-PAGE. 100% trichloroacetic acid was added to each fraction to final concentration 7.5%, and the samples were incubated on ice for 5 min. The samples were centrifuged in a microcentrifuge for 5 min at 4 $^\circ\text{C}$, and the pellet was washed with cold acetone, dried in a SpeedVac (Savant), and dissolved in 2 \times Laemmli sample buffer containing dithiothreitol and 3% β -mercaptoethanol.

Immunoprecipitation and Western Blotting—For immunoprecipitation in the absence of velocity centrifugation, cells were harvested by low speed centrifugation and washed once with cold phosphate-buffered saline. The cells were lysed in radioimmune precipitation/MOPS buffer (20 mM MOPS, pH 7.0, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1% deoxycholate, and 0.1% SDS) containing 1 mM phenylmethylsulfonyl fluoride, 2 mM sodium vanadate, 10 μ g/ml leupeptin, and 10 μ g/ml aprotinin. Protein concentration was measured by Bradford reagent (Pierce no. 1856209). Six μ l of BPV α E5 antiserum, 4 μ l of PDGF receptor antiserum B3a (designated here α PR) (which recognizes both full-length and truncated forms of the PDGF β receptor), 12–25 μ l of α PLC γ (Santa Cruz, SC-81), 4 μ l of α PI 3'-kinase (Upstate Biotechnology, Inc., 06-195), or 12–25 μ l of RasGAP (Santa Cruz, SC-63) antibodies were added to 600, 300, 800–2500, 400, or 800–2500 μ g of extracts, respectively. For immunoprecipitating samples from sucrose gradients, 12 μ l of α E5, 8 μ l of α PR, or 15 μ l of human PDGF extracellular region mouse monoclonal antibody (α PRex) (R&D, 1263-00) were added to equal volumes of each fraction, typically 800–1200, 500–800, or 1000 μ l, respectively. After incubation at 4 $^\circ\text{C}$ for 2–18 h, 3–5 μ l of rabbit anti-mouse antiserum was added if the primary antibodies were monoclonal. Immunoprecipitates were collected on protein A-Sepharose beads and processed for electrophoresis as described (39). Electrophoretic separation, protein transfer, immunoblotting, and phosphatase treatment were carried out exactly as described previously (39), except for E5 detection following sucrose gradient analysis, in which case ECL Plus (Amersham Pharmacia Biotech) was used. For Western blotting of PDGF β receptor and PI 3-kinase from sucrose gradient fractions without precipitation, 15 and 10 μ l of each fraction, respectively, were loaded on the gel directly. In some cases, filters were stripped of antibody and reprobbed with a different antibody as described (39).

RESULTS

Sedimentation Profile of the Endogenous PDGF β Receptor in C127 Fibroblasts Transformed by the E5 Protein—To analyze the endogenous PDGF β receptor in C127 mouse fibroblasts transformed by the BPV E5 protein, cells were lysed in TG buffer, which contains 1% Triton X-100 to solubilize membranes. Extracts were layered onto a linear 5–25% sucrose gradient in TG buffer and subjected to velocity sedimentation. Fractions were collected and analyzed by precipitation with trichloroacetic acid, gel electrophoresis, and immunoblotting. When a PDGF receptor-specific antibody was used to analyze total PDGF β receptor, as shown in the *top panel* of Fig. 1, the great majority of PDGF β receptor sedimented slowly, with a peak in fractions 3–5. The peak of receptor isolated from untransformed, serum-starved C127 cells sedimented at the same position (data not shown), implying that the PDGF β receptor in these fractions represents inactive, monomeric receptor (Fig. 2, *diagram A*).

A quite different sedimentation pattern was observed when the fractions from the gradient of E5-transformed cell extract were analyzed by Western blotting with a monoclonal antibody that recognized phosphotyrosine. Both the \sim 200-kDa receptor species with mature carbohydrates and the \sim 165-kDa precursor species with immature carbohydrates sedimented much more rapidly than monomeric receptor, in a broad distribution with a peak in fractions 9 and 10 (Fig. 1, *bottom panel*). There was virtually no tyrosine-phosphorylated PDGF β receptor in untransformed cells in the absence of PDGF treatment, and PDGF treatment of these cells induced the appearance of ma-

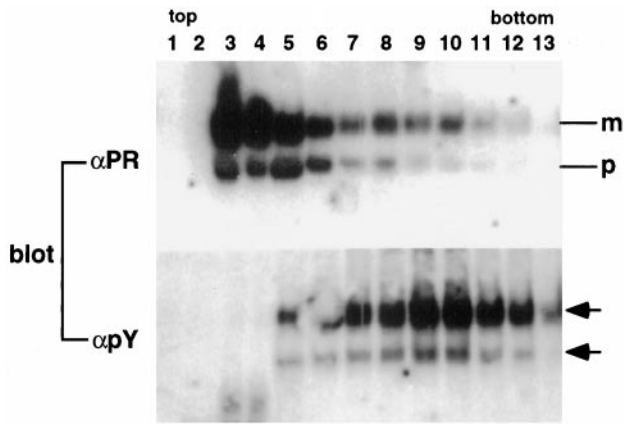


FIG. 1. Sedimentation of endogenous PDGF β receptor. E5-transformed mouse C127 fibroblasts were lysed and subjected to velocity sedimentation. Individual fractions (labeled from 1 at the top of the gradient through 13 at the bottom) were collected. Proteins were precipitated with trichloroacetic acid, subjected to gel electrophoresis, and immunoblotted with α PR to detect total PDGF β receptor (*top panel*) or with 4G10 (α pY) to detect tyrosine-phosphorylated PDGF β receptor (*bottom panel*). The positions of mature (*m*) and precursor (*p*) forms of the PDGF β receptor are shown in the *top panel*. In the *bottom panel*, the *top* and *bottom* arrows indicate the tyrosine-phosphorylated mature and precursor forms of the receptor, respectively.

ture tyrosine-phosphorylated PDGF β receptor that sedimented more rapidly than the bulk receptor (data not shown; see also Fig. 3). Thus, the E5 protein caused a dramatic alteration in the physical properties of the murine PDGF β receptor, resulting in the far more rapid sedimentation of the activated species. The clear separation of the activated species from most of the receptor in E5-transformed cells demonstrated that the E5 protein activated only a small fraction of PDGF β receptor in transformed fibroblasts, and the shift to higher sedimentation rate suggested that the activated receptor is present in a much larger multiprotein complex. As described below and diagrammed schematically in Fig. 2G, this species contains the E5 protein, dimeric PDGF β receptor, and associated signaling proteins.

Sedimentation Profile of PDGF β Receptor Activated by the E5 Protein or by PDGF—We also examined the sedimentation of the PDGF β receptor in murine Ba/F3 cells engineered to co-express the full-length wild-type human PDGF β receptor and the E5 protein. These cells do not express endogenous PDGF β receptor, and we previously showed that co-expression of the human PDGF β receptor and the E5 protein resulted in complex formation between these two proteins, receptor dimerization and activation, and growth factor-independent cell proliferation (28, 34). These experiments, in which we used an antibody to immunoprecipitate PDGF β receptor prior to Western blotting to provide further evidence that the bands detected were in fact PDGF β receptor, showed that most of the PDGF β receptor in these cells sedimented slowly with a peak in fraction 5, whereas both the mature and precursor tyrosine-phosphorylated receptor activated by the E5 protein sedimented rapidly with a peak in fractions 10–12 (data not shown). Thus, E5 expression caused a marked change in the physical behavior of the constitutively active murine as well as human PDGF β receptor, and in both cases only a small fraction of the total PDGF β receptor expressed in cells was activated.

The sedimentation profile of the PDGF β receptor in response to PDGF treatment was also determined. Ba/F3 cells expressing the human PDGF β receptor without the E5 protein were treated with 50 ng/ml PDGF-BB or left untreated. This concentration of PDGF is sufficient to allow interleukin-3-in-

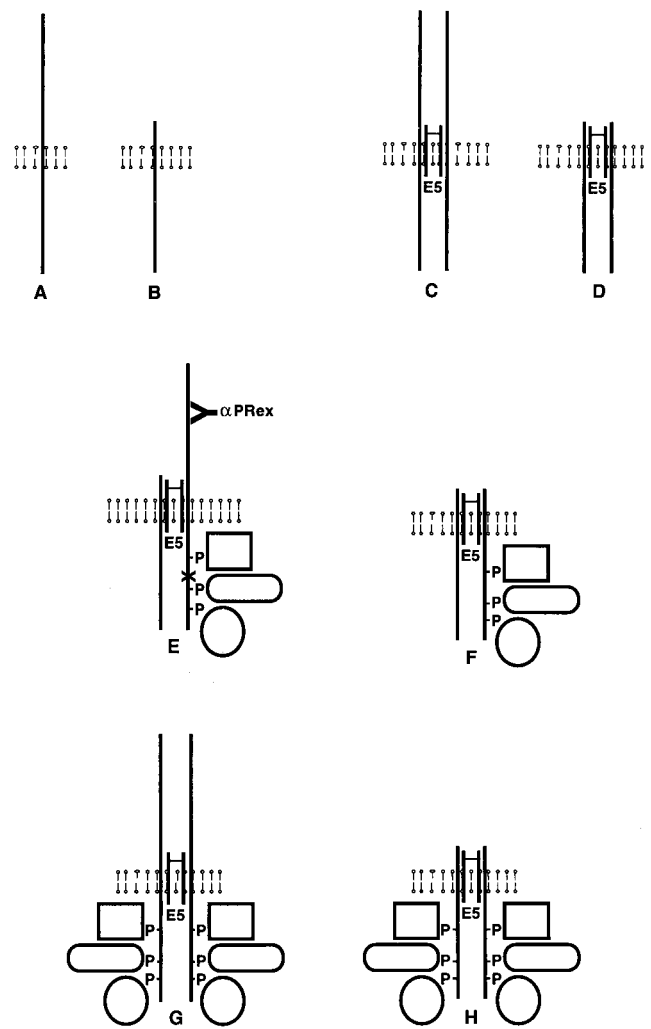


FIG. 2. Diagram of PDGF β receptor complexes inferred to be present in E5-transformed cells. The vertical lines represent the full-length and truncated PDGF β receptor inserted into cell membranes. The dimeric E5 protein is also shown in *diagrams C–H*. α PRex is the antibody that recognizes the extracellular domain of the full-length PDGF β receptor. P represents sites of tyrosine phosphorylation, and associated SH2 domain proteins are represented by the various shapes. *Diagram E* shows the hemiphosphorylated complex containing the kinase-inactive full-length PDGF β receptor (with the mutation marked by the X) and the kinase-active truncated receptor.

dependent proliferation of these cells.² Following detergent extraction and velocity sedimentation, total PDGF β receptor and tyrosine-phosphorylated receptor were detected by immunoblotting of each fraction. In the absence of PDGF treatment, total receptor sedimented with a peak in fraction 5, and there was no tyrosine-phosphorylated receptor (Fig. 3A). Although PDGF treatment caused no change in the sedimentation pattern of the total PDGF receptor, it did induce the appearance of readily detectable tyrosine-phosphorylated mature PDGF β receptor, which sedimented with a peak in fraction 8 (Fig. 3B). Similar results were obtained if PDGF treatment was carried out at 4 °C (data not shown). Thus, under our conditions, PDGF treatment results in the activation of only a small fraction of the total PDGF β receptor in cells. However, only the mature, cell-surface form of the receptor is activated by PDGF treatment, and this activated receptor sediments more slowly than E5-activated receptor.

Activation of a Truncated PDGF β Receptor by the E5 Pro-

² P. M. Irusta and D. DiMaio, unpublished results.

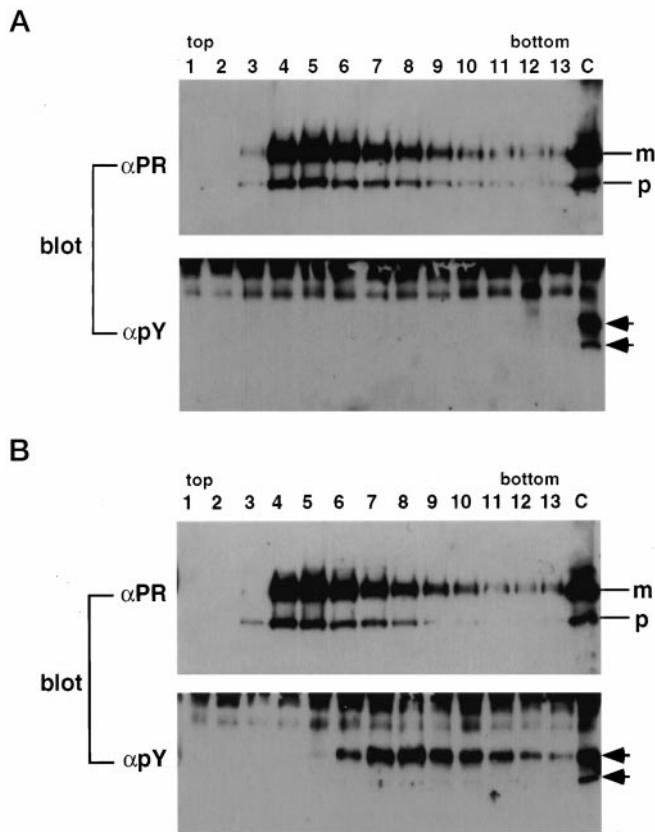


FIG. 3. Sedimentation of ligand-stimulated PDGF β receptor. Ba/F3 cells containing the human PDGF β receptor were stimulated with PDGF (panel B) or left unstimulated (panel A). After extraction and sedimentation, total PDGF β receptor was detected by immunoblotting (top panels) or tyrosine-phosphorylated receptor was detected by immunoprecipitation with α PR followed by immunoblotting with 4G10 (α PY) (bottom panels). The lane labeled C is unfractionated PDGF β receptor from Ba/F3 cells co-expressing the receptor and the E5 protein. The figure is labeled as in Fig. 1.

tein—We next analyzed Ba/F3 cells expressing a mutant human PDGF β receptor lacking most of its extracellular ligand-binding domain. The E5 protein can still bind and activate this truncated receptor via interactions involving the transmembrane and juxtamembrane domains of the receptor (39). We chose to examine this mutant because it is possible to separate phosphorylated and unphosphorylated forms of the truncated receptor by gel electrophoresis (38), as illustrated in the top panel of Fig. 4. In cells not expressing the E5 protein, the truncated PDGF β receptor migrated as a single band in an SDS-polyacrylamide gel (lane 1). Expression of the E5 protein caused a small fraction of the truncated receptor to migrate more slowly (lane 2), even though most of the receptor migrated at the same position as in cells not expressing the E5 protein. This slowly migrating species was tyrosine-phosphorylated (data not shown), and it was eliminated by phosphotyrosine phosphatase treatment (lane 3). This result implied that the E5 protein caused the tyrosine phosphorylation of only a small fraction of the PDGF β receptor, in agreement with the sedimentation analysis described above, and that this fraction can be directly visualized on the basis of its altered electrophoretic mobility.

These two forms of the truncated PDGF β receptor and their interaction with the E5 protein were further analyzed in a co-immunoprecipitation experiment (Fig. 4, bottom panel). An antiserum recognizing the E5 protein failed to immunoprecipitate the PDGF β receptor from untransformed cells, as expected (lane 1). However, in cells expressing the E5 protein, the

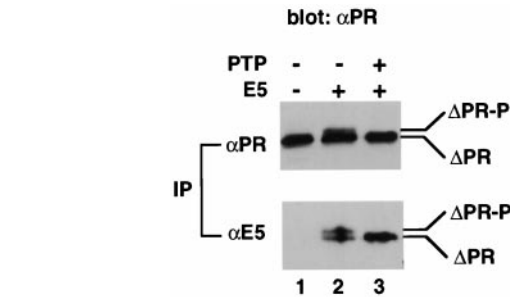


FIG. 4. Separation of phosphorylated and unphosphorylated truncated PDGF β receptor by gel electrophoresis. Ba/F3 cells expressing truncated PDGF β receptor alone or together with the E5 protein (E5) were immunoprecipitated with α PR or α E5, as indicated, subjected to electrophoresis, and immunoblotted with α PR. In lane 3, samples were treated with phosphotyrosine phosphatase (PTP) prior to electrophoresis. The positions of phosphorylated (Δ PR-P) and unphosphorylated (Δ PR) truncated receptor are shown.

E5 antiserum co-immunoprecipitated both the slowly migrating tyrosine-phosphorylated species and the more rapidly migrating unphosphorylated species of the receptor in approximately equimolar amounts (lane 2). This result indicates that, even though the E5 protein activated only a small fraction of the total receptor in the cell, this activated fraction preferentially associated with the E5 protein. However, not all of the PDGF β receptor molecules associated with the E5 protein were tyrosine-phosphorylated.

Sedimentation Profile of Truncated PDGF β Receptor in Response to the E5 Protein—Detergent extracts were prepared from Ba/F3 cells expressing the truncated PDGF β receptor in the presence or absence of the E5 protein. Following velocity sedimentation, gradient fractions were analyzed for the presence of PDGF β receptor by immunoprecipitation and immunoblotting. As shown in Fig. 5 (top panel), for cells not expressing the E5 protein, the truncated receptor sedimented as a single prominent species with a peak in fraction 5, and it migrated as a single band upon gel electrophoresis with the mobility of the unphosphorylated species. Thus, this represents monomeric, inactive receptor (Fig. 2, diagram B). In contrast, when extracts from cells co-expressing the truncated PDGF β receptor and the E5 protein were analyzed, the distribution of the receptor in the gradient was no longer simple (Fig. 5, middle panel). The majority of receptor sedimented slowly with a peak in fraction 5, exhibited the same electrophoretic mobility as did the receptor from untransformed cells, and was not tyrosine-phosphorylated. However, the extracts also contained a second, less abundant species of truncated PDGF β receptor that sedimented rapidly (peak in fractions 11 and 12) and displayed the lower electrophoretic mobility characteristic of the tyrosine-phosphorylated form. Blotting with the anti-phosphotyrosine antibody confirmed that the truncated receptor in the rapidly sedimenting fractions was in fact tyrosine-phosphorylated (Fig. 5, bottom panel). Thus, as is the case for the full-length receptor, expression of the E5 protein induced a striking increase in the sedimentation rate of the activated truncated PDGF β receptor, consistent with it being assembled in a multiprotein signaling complex (Fig. 2H).

Gradient fractions were also analyzed for the presence of the E5 protein by Western blotting. The bottom panel of Fig. 6 shows that when the E5 protein was expressed in the absence of the PDGF β receptor, all of the E5 protein sedimented very slowly, consistent with its extremely small size. When the E5 protein was isolated from cells that also expressed the PDGF β receptor, most of it still sedimented very slowly, but there was also a less abundant species of E5 protein that sedimented in the same fractions as the rapidly sedimenting activated PDGF

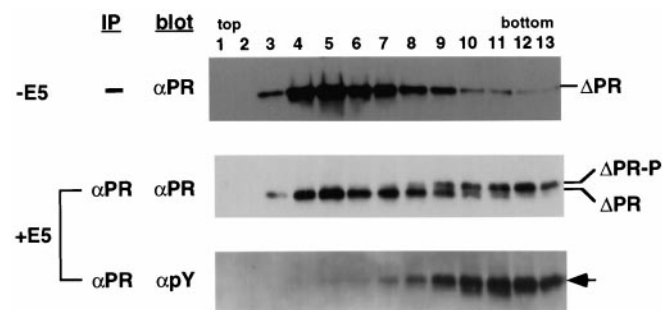


FIG. 5. Effect of the E5 protein on sedimentation of truncated PDGF β receptor. Ba/F3 cells expressing the truncated PDGF β receptor alone (*top panel*) or together with the E5 protein (*middle and bottom panels*) were lysed and subjected to velocity sedimentation. Individual gradient fractions were analyzed by electrophoresis either directly (*top panel*) or after immunoprecipitation with α PR (*middle and bottom panels*). Total and tyrosine-phosphorylated PDGF β receptor were detected by immunoblotting with α PR and α pY, respectively, as indicated. The *bottom and middle panels* are the same filter that was sequentially probed with α PY, stripped, and reprobed with α PR. The positions of phosphorylated (Δ PR-P) and unphosphorylated (Δ PR) truncated receptor are shown. The *arrow* shows tyrosine-phosphorylated truncated PDGF β receptor.

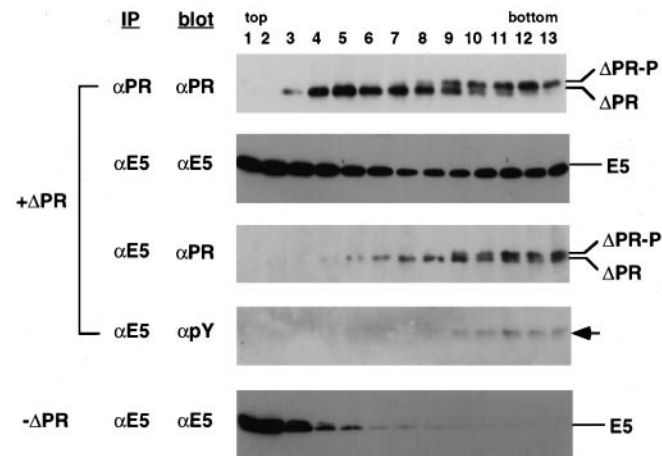


FIG. 6. Analysis of E5 protein/PDGF β receptor complexes by sedimentation. Ba/F3 cells expressing the E5 protein alone ($-\Delta$ PR) (*bottom panel*) or together with the truncated PDGF β receptor ($+\Delta$ PR) (*top four panels*) were lysed and subjected to velocity sedimentation. After immunoprecipitation with α E5 and gel electrophoresis, the E5 protein, the PDGF β receptor, and tyrosine-phosphorylated PDGF β receptor were detected by immunoblotting. The *top panel*, taken from a gradient run under identical conditions, is reproduced from Fig. 4 and is shown as a reference. The *third and fourth panels* from the *top* are the same filter that was sequentially probed with α PR, stripped, and re-probed with α pY.

β receptor (Fig. 6, *second panel*). The co-sedimentation of activated PDGF β receptor and a subpopulation of the E5 protein suggested that the two proteins were present in molecular complexes that can be separated by sedimentation from most of the PDGF β receptor and E5 protein in the cell extracts.

To determine whether the rapidly sedimenting activated PDGF β receptor and E5 protein were physically associated, co-immunoprecipitation experiments were carried out. Individual gradient fractions from extracts prepared from cells co-expressing the two proteins were immunoprecipitated with the anti-E5 antibody, and total (Fig. 6, *third panel*) and tyrosine-phosphorylated (Fig. 6, *fourth panel*) PDGF β receptor were detected by Western blotting. The E5 antiserum co-immunoprecipitated both electrophoretic forms of the truncated receptor, although the resolution between phosphorylated and unphosphorylated receptor forms was poorer following co-immunoprecipitation with α E5 (or with α PI 3'-kinase; see Fig.

10) compared with immunoprecipitation with α PR. Little, if any, monomeric PDGF β receptor was co-immunoprecipitated by the E5 antiserum. The sedimentation rate of the E5-associated receptor mirrored the state of receptor tyrosine phosphorylation, with complexes having a high proportion of phosphorylated receptor sedimenting more rapidly. The tyrosine-phosphorylated form of the receptor in complex with the E5 protein sedimented rapidly, with the peak in fraction 11 (*third and fourth panels*). In contrast, the unphosphorylated receptor form in complex with the E5 protein sedimented between the peak of monomeric receptor and the rapidly sedimenting phosphorylated receptor. Fraction 10 contained similar amounts of E5-associated phosphorylated and unphosphorylated receptor (*third panel*). These results indicated that the E5 protein induced the formation of a heterogeneous set of complexes that sedimented more rapidly than monomeric receptor. These complexes contained the E5 protein itself and PDGF β receptor that was tyrosine-phosphorylated to varying extents (Fig. 2, *diagrams D, F, and H*). The correlation between sedimentation rate and extent of receptor tyrosine phosphorylation implies that phosphorylation played a direct role in assembling rapidly sedimenting complexes.

Analysis of Cells Co-expressing Full-length and Truncated Receptor—The rapid sedimentation of the tyrosine-phosphorylated receptor could be a consequence of receptor dimerization or of complex formation between the receptor and signaling proteins, or both. To explore the sedimentation behavior of receptor dimers, we examined cells co-expressing the truncated PDGF β receptor and a full-length receptor with an inactive kinase domain. We previously used these two mutants to demonstrate that the E5 protein induced dimerization of the PDGF β receptor (39). Furthermore, in cells expressing these three proteins, only the precursor form of the full-length receptor was stably associated with the E5 protein, and only this form of the full-length receptor underwent E5-induced *trans*-phosphorylation. The inability of the truncated PDGF β receptor to *trans*-phosphorylate the mature form of the full-length receptor appears to reflect the localization of the mature form to an endoglycosylase H-resistant compartment, in contrast to the truncated receptor and the precursor form of the full-length receptor, which are localized in an endoglycosylase H-sensitive compartment³ (25).

Extracts of cells co-expressing the two receptor mutants and the E5 protein were analyzed by velocity sedimentation. The full-length and truncated receptor were largely monomeric and sedimented slowly (data not shown). However, a minority of truncated receptor sedimented rapidly and was phosphorylated, and some of the precursor of the full-length kinase-negative receptor was tyrosine-phosphorylated and sedimented in an intermediate range with a peak in fractions 8–10 (data not shown). To examine the sedimentation profile of oligomeric receptor complexes, we carried out immunoprecipitation with an antiserum (α PRex) that recognizes the full-length receptor but not the truncated mutant. In cells expressing both receptor species, α PRex co-immunoprecipitates the truncated receptor only if it is in complex with the full-length receptor. The bulk of the full-length receptor sedimented with a peak in fraction 5, regardless of E5 expression (Fig. 7, *top two panels*). In the absence of E5 expression, the full-length specific antibody did not co-immunoprecipitate any truncated receptor, confirming that receptor oligomerization was not detectable in untransformed cell extracts (Fig. 7, *top panel*). When the E5 protein was expressed together with the two receptors, the antiserum specific for the full-length receptor co-immunoprecipitated the

³ C. C. Lai and D. DiMaio, unpublished results.

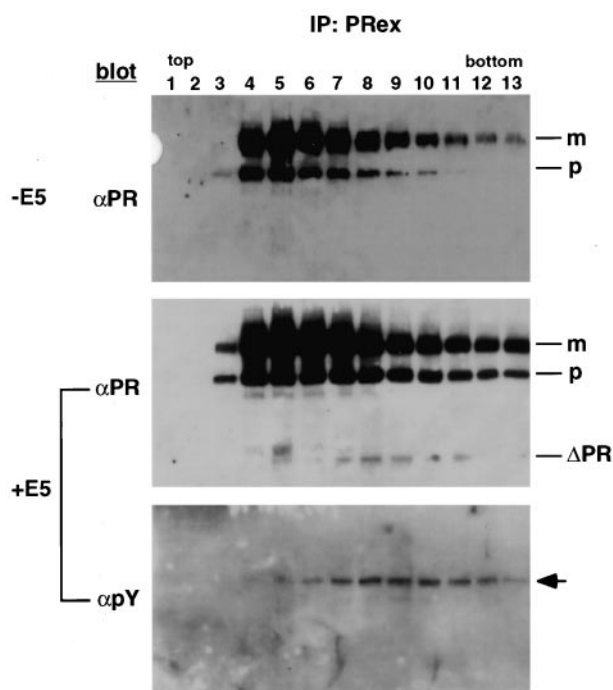


FIG. 7. Sedimentation analysis of oligomeric PDGF β receptor. Ba/F3 cells co-expressing the kinase-negative full-length PDGF β receptor and the truncated receptor in the presence (*middle and bottom panels*) or absence (*top panel*) of the E5 protein were lysed and subjected to velocity sedimentation. After immunoprecipitation of individual gradient fractions with α PRex, which recognizes the full-length receptor and associated proteins, total and tyrosine-phosphorylated PDGF β receptor were detected by immunoblotting. The positions of mature (*m*) and precursor (*p*) forms of the PDGF β receptor are shown in the *top two panels*. The *arrowhead* in the *bottom panel* indicates the tyrosine-phosphorylated form of the full-length receptor. The *middle and bottom panel* are the same filter that was sequentially probed with α pY, stripped, and reprobed with α PR.

truncated receptor from intermediate fractions 7–11 (Fig. 7, *middle panel*), indicating that heteromeric complexes of the full-length and truncated receptors sedimented at this intermediate position (Fig. 2, *diagram E*). In confirmation of our published results (39), the truncated receptor in complex with the kinase-negative full-length version was not phosphorylated, as assessed by electrophoretic mobility (*middle panel*) and phosphotyrosine blotting (*bottom panel*). Phosphorylated forms of the truncated receptor in the heterodimer were not detectable in longer expanses of the gels shown here or upon analysis of an independent gradient (data not shown). Notably, the tyrosine-phosphorylated precursor form of the full-length receptor (*bottom panel*) and the unphosphorylated truncated receptor in complex with the full-length form (*middle panel*) co-sedimented, providing further evidence that *trans*-phosphorylation was catalyzed by the kinase-active truncated receptor in the heteromeric complex.

Co-immunoprecipitation experiments with the anti-E5 antiserum were carried out to determine which of these receptor species were physically associated with the E5 protein (Fig. 8). The slowly sedimenting monomeric forms of the full-length and truncated PDGF β receptors were not co-immunoprecipitated with the E5 antiserum. E5-associated truncated receptor was broadly distributed in the intermediate and rapidly sedimenting fractions. The full-length PDGF β receptor precursor was also co-immunoprecipitated by the anti-E5 antiserum from the intermediate fractions that contained heteromeric receptor complexes (Fig. 8, *top panel*). There was little E5-associated full-length receptor in the rapidly sedimenting fractions near the bottom of the gradient, indicating that this kinase-negative

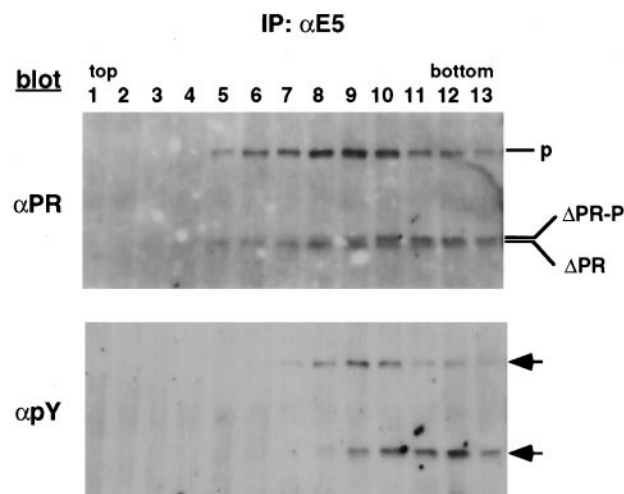


FIG. 8. Sedimentation analysis of the E5-associated PDGF β receptor in cells co-expressing the full-length and truncated receptor forms. Ba/F3 cells co-expressing the E5 protein, the full-length kinase-negative PDGF β receptor, and the truncated PDGF β receptor were lysed and subjected to velocity sedimentation. Individual fractions were immunoprecipitated with α E5, and co-immunoprecipitated total and tyrosine-phosphorylated PDGF β receptor were detected by immunoblotting. The position of the precursor (*p*) form of the full-length receptor is shown. In the *bottom panel*, the *top and bottom arrowheads* indicate the tyrosine-phosphorylated precursor form of the full-length receptor and the truncated receptor, respectively. *Both panels* are the same filter that was sequentially probed with α pY, stripped, and reprobed with α PR.

receptor mutant was not able to assemble into the most rapidly sedimenting complexes. Taken together, these results indicated that, in cells expressing the E5 protein and both receptor species, the E5 protein induced the formation of a receptor complex that displayed an intermediate sedimentation rate and contained the E5 protein, unphosphorylated truncated receptor, and tyrosine-phosphorylated kinase-negative full-length receptor (Fig. 2, *diagram E*).

The cells also contained E5-associated, tyrosine-phosphorylated truncated receptor that sedimented with a peak in fractions 10–12 (Fig. 8, *bottom panel*). This rapidly sedimenting truncated receptor was not associated with the full-length receptor and evidently represented complexes containing homomeric truncated receptor (Fig. 3, *diagram H*). Notably, this complex containing the smaller truncated receptor sedimented several fractions more rapidly than the heteromeric complex containing the truncated receptor and the full-length *trans*-phosphorylated receptor. This can be seen most clearly when E5 immunoprecipitates were analyzed by anti-phosphotyrosine blotting (Fig. 8, *bottom panel*). This implies that much of the increased sedimentation rate displayed by the activated receptor complexes was not due solely to the molecular weight of the receptor subunits themselves, but also to the presence of additional proteins in the complex.

Association of Signaling Proteins with Activated PDGF β Receptor Complexes—We previously reported that the full-length E5-activated PDGF β receptor was constitutively bound to the SH2 domain-containing proteins PI 3'-kinase, PLC γ , and RasGAP (28). Here, we examined the signaling proteins bound to the truncated receptor mutant. Extracts from cells expressing the truncated PDGF β receptor in the presence or absence of the E5 protein were immunoprecipitated with antisera recognizing these proteins and then analyzed by immunoblotting. These antisera immunoprecipitated equivalent amounts of the cognate signaling proteins whether or not cells expressed the E5 protein (data not shown). As shown in Fig. 9, these antisera did not co-immunoprecipitate significant amounts of PDGF β

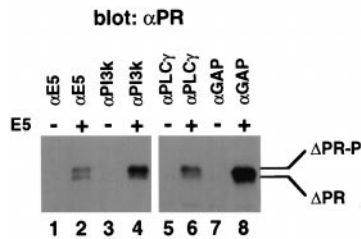


FIG. 9. Association of signaling proteins with truncated PDGF β receptor. Extracts of Ba/F3 cells expressing the truncated PDGF β receptor in the presence or absence of the E5 protein were immunoprecipitated with the indicated antibodies, and total PDGF β receptor in the immunoprecipitate was detected by immunoblotting with α PR.

receptor from extracts of cells that did not contain the E5 protein (lanes 3, 5, and 7). Upon prolonged exposure of the filters, faint background bands corresponding to unphosphorylated receptor were visible in these lanes (data not shown). In contrast, the antisera to the signaling proteins co-immunoprecipitated readily detectable amounts of both the phosphorylated and the unphosphorylated truncated receptor from extracts of cells containing the E5 protein (lanes 4, 6, and 8). Because receptor tyrosine phosphorylation is required for SH2 protein binding and co-immunoprecipitation, the presence of the unphosphorylated receptor in these immunoprecipitates implies that some complexes contain at least two receptor molecules, one phosphorylated and the other not (Fig. 2, diagram F).

The ability of antisera recognizing the signaling proteins to co-immunoprecipitate the phosphorylated receptor implies that signaling protein/receptor complexes were in the rapidly sedimenting fractions, which contained most of the phosphorylated receptor. To confirm this interpretation, we used the anti-p85 antiserum to carry out co-immunoprecipitation across the gradient. As shown in Fig. 10, the vast majority of the PDGF β receptor in complex with PI 3'-kinase sedimented rapidly (*middle panel*), even though most of the total PI 3'-kinase sedimented more slowly with a peak in fractions 5–8 (*bottom panel*). A small amount of primarily unphosphorylated receptor was precipitated from the slowly sedimenting fractions (which contained the peak of total unphosphorylated receptor) and apparently corresponded to the background signal noted above. Thus, activated PDGF β receptor complexes containing PI 3'-kinase (and presumably other signaling proteins) sedimented rapidly.

We also tested whether more than one signaling protein were simultaneously present in the same activated PDGF β receptor complex. Extracts were prepared from cells expressing the E5 protein, the truncated PDGF β receptor, or both proteins. These extracts were immunoprecipitated by using the α PLC γ antibody or the α GAP antibody, and then immunoblotted with antibody recognizing the p85 subunit of PI 3'-kinase. As shown in Fig. 11, the α PLC γ and the α GAP antibodies co-immunoprecipitated abundant PI 3'-kinase when the E5 protein and the PDGF β receptor were co-expressed (lanes 7 and 10). However, little PI 3'-kinase was precipitated when either the E5 protein or the receptor was expressed separately. The amount of total PI 3'-kinase was similar in the three cell lines (lanes 2–4). Thus, some activated receptor complexes contained both PI 3'-kinase and PLC γ , and some contained PI 3'-kinase and RasGAP, results that suggested that some activated complexes may contain all three signaling proteins. We also tested whether there was a ternary complex between the E5 protein, the activated PDGF β receptor, and the p85 subunit of PI 3'-kinase. As shown in Fig. 12, the E5 antibody co-immunoprecipitated abundant p85 from extracts of cells expressing the wild-type PDGF β receptor and the E5 protein (lane 2). Effi-

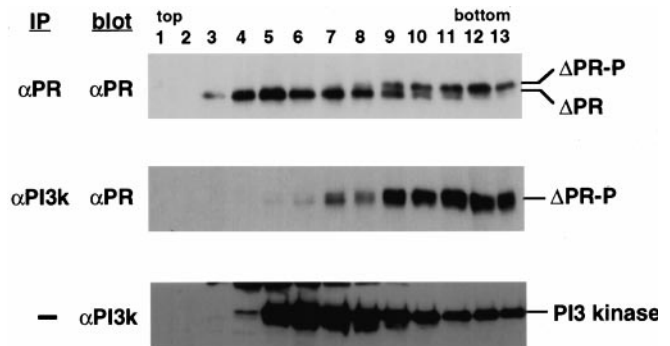


FIG. 10. Sedimentation analysis of PI 3'-kinase. Ba/F3 cells co-expressing the E5 protein and the truncated PDGF β receptor were lysed and subjected to velocity sedimentation. Individual fractions were subjected to electrophoresis directly or after immunoprecipitation with α PR or antibody recognizing PI 3'-kinase (α PI3K). Total PI 3'-kinase and PDGF β receptor in complex with PI 3'-kinase were detected by immunoblotting. The *top panel*, taken from a gradient run under identical conditions, is reproduced from Fig. 4 and is shown as a reference.

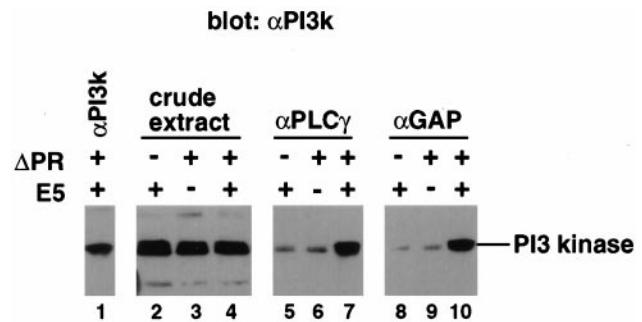


FIG. 11. Pairwise analysis of signaling proteins in activated receptor complexes. Extracts were prepared from Ba/F3 cells expressing the E5 protein, the truncated PDGF β receptor, or both proteins, as indicated. Crude extracts were electrophoresed (lanes 2–4) or subjected to immunoprecipitation with antibodies that recognize PI 3'-kinase, PLC γ , or GAP, as indicated. The p85 subunit of PI 3'-kinase was then detected by immunoblotting.

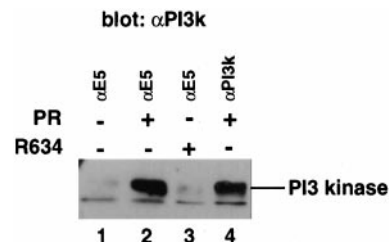


FIG. 12. Ternary complex formation in E5-transformed cells. Extracts were prepared from Ba/F3 cells expressing the E5 protein and either no PDGF β receptor, the full-length wild-type PDGF β receptor (PR), or the kinase-negative mutant (R634), as indicated. Immunoprecipitation was carried out with α E5 antiserum or antibody recognizing PI 3'-kinase, and PI 3'-kinase in the immunoprecipitates was detected by immunoblotting.

cient co-immunoprecipitation did not occur if the cells did not express receptor (lane 1) or if the receptor carried a mutation (R634) that inactivated its tyrosine kinase activity (lane 3). Thus, kinase-active PDGF β receptor was required to induce the formation of complexes containing both the E5 protein and PI 3'-kinase, implying that these three proteins formed a ternary complex. Taken together, our results indicated that the rapidly sedimenting signaling complex in cells expressing the E5 protein consisted of the E5 protein, activated PDGF β receptor, and a variety of bound signaling proteins (Fig. 2, diagrams G and H).

DISCUSSION

The BPV E5 protein causes sustained activation of the PDGF β receptor, resulting in cell transformation. Here, we used velocity sedimentation, co-immunoprecipitation, and gel electrophoresis to identify and characterize stable complexes containing the E5 protein, dimeric, tyrosine-phosphorylated PDGF β receptor, and associated signaling proteins. Our results showed that PI 3'-kinase was present in these complexes with the other signaling proteins and with the E5 protein and that the existence of these complexes was dependent upon the co-expression of the E5 protein and a kinase-active PDGF β receptor. Thus, large activated PDGF β receptor complexes exist that simultaneously contain multiple signaling proteins. The existence of multiprotein signaling complexes containing activated PDGF β receptor in ligand-stimulated cells has been inferred on the basis of co-immunoprecipitation and *in vitro* association studies, but such complexes have not been directly visualized previously.

Our results indicated that the E5 protein and PDGF activated only a small fraction of the PDGF β receptor, in confirmation of our previous studies with chemical cross-linkers and E5-activated receptor (39). Our results also demonstrated the specific nature of the E5-activated PDGF β receptor complex. Although most of the E5 protein, the PDGF β receptor, and the p85 subunit of PI 3'-kinase sedimented slowly, PDGF receptor complexes containing these proteins sedimented rapidly. Thus, complex formation was the result of specific interactions occurring within cells and not the result of nonspecific interactions occurring in gradient fractions containing high concentrations of the three proteins. In addition, activated receptor complexes were not huge, nonspecific aggregates that pelleted upon centrifugation.

By studying the truncated PDGF β receptor, we showed that the E5 protein formed complexes with both phosphorylated and unphosphorylated receptor molecules. The ratio of these two receptor forms in the E5 complex varied continuously across the gradient. Some relatively slowly sedimenting E5-associated complexes contained exclusively unphosphorylated receptors, whereas rapidly sedimenting ones appeared to contain primarily phosphorylated receptors. In addition, the co-immunoprecipitation of both receptor species by antibodies that recognize signaling proteins indicated that some of the E5-induced receptor complexes consisted of an unphosphorylated receptor molecule together with a tyrosine-phosphorylated receptor bound to SH2 signaling proteins. This latter result implied that tyrosine phosphorylation of a single receptor chain in a dimer is sufficient to recruit signaling proteins. We conclude that there are several classes of E5-associated PDGF β receptor complexes, those in which all receptor subunits are unphosphorylated, those in which they are phosphorylated, and mixed complexes containing both phosphorylated and unphosphorylated receptors (Fig. 2, *diagrams D, F, and H*). Thus, not only does the E5 protein bind to and activate a small fraction of the PDGF β receptor in cells, but even the fraction of receptor that is associated with the E5 protein is not completely phosphorylated.

The high sedimentation rate of the activated complexes induced by the E5 protein appeared to be due both to dimerization of the PDGF β receptor and to recruitment of additional proteins into the complex. Three lines of evidence indicated that the E5 protein induced the formation of PDGF β receptor oligomers that sedimented more rapidly than monomeric receptor: 1) truncated receptor was co-immunoprecipitated from the intermediate fractions by the antibody specific for the full-length receptor, 2) *trans*-phosphorylated kinase-negative full-length receptor was present in these fractions, and 3) unphosphorylated receptor was co-immunoprecipitated by antibodies that recognize signaling proteins that bind only phosphoryl-

ated receptor. The lack of phosphorylated truncated PDGF β receptor in association with the kinase-negative full-length receptor strongly suggested that there were only two molecules of the receptor in the activated complex, since transphosphorylation between multiple truncated receptors would be expected to occur within higher order oligomers. Furthermore, this result indicated that there was limited exchange of receptor subunits between complexes.

Phosphorylated complexes containing exclusively truncated receptors sedimented more rapidly than heteromeric complexes containing full-length and truncated receptors, implying that the size of the receptor molecules themselves was not the major determinant of the sedimentation rate of the activated complex. It seems unlikely that the more rapid sedimentation of the homomeric complex containing the truncated receptor reflected a higher order oligomeric state of the receptor in this complex compared with mixed complexes. Rather, we conclude that all activated receptor complexes contain two molecules of receptor and that the associated signaling proteins played an important role in determining sedimentation rate. The simplest explanation for the more rapid sedimentation of the fully phosphorylated receptor dimers is that they bind two molecules of each signaling protein, in contrast to hemiphosphorylated dimers, which can bind at most one molecule of each (Fig. 2, compare *diagrams G and H* with *diagram E*). Alternatively, it is possible that fully-phosphorylated receptor dimers bind a more complete set of signaling molecules than do hemiphosphorylated dimers. The correlation between the extent of phosphorylation of E5-associated truncated receptor and sedimentation rate (Fig. 6, *middle panel*) suggested the existence of unphosphorylated receptor dimers not bound to signaling proteins, fully phosphorylated dimers bound to the complete complement of signaling proteins, and hemiphosphorylated dimers bound to half as many signaling proteins. In further support of these models, co-immunoprecipitation experiments suggested that the kinase-negative receptors in the hemiphosphorylated receptor heterodimers were in fact able to bind signaling proteins.³ It is interesting that PDGF-activated receptor complexes do not sediment as rapidly as E5-activated complexes. We speculate that PDGF-activated receptor complexes dissociated into monomeric receptor molecules bound to signaling proteins, although we have not ruled out that these complexes contain dimeric receptor bound to only a subset of signaling molecules.

Because of the presumably asymmetric shape of the PDGF β receptor complexes and the effect of detergent binding (which influences sedimentation behavior), we have not attempted to assign molecular weights to the complexes described here. However, we note that a complex containing two molecules of the truncated receptor, an E5 dimer, and two molecules each of p85 PI 3'-kinase, RasGAP, and PLC γ is predicted to have a higher molecular weight than a complex containing one truncated and one full-length receptor, an E5 dimer, and one molecule of each signaling protein, consistent with the sedimentation results.

We do not know if the hemiphosphorylated receptor complexes are capable of delivering a mitogenic signal. Cells expressing these complexes are interleukin-3-independent, but they also express fully phosphorylated homodimers of the truncated receptor. In other cell systems, co-expression of kinase-negative receptors can inhibit ligand-stimulated signaling. The apparent lack of a dominant-negative effect in our system may reflect some specific feature of the E5 protein or Ba/F3 cells, such as the precise structure or composition of the signaling complexes or the relative levels of homodimeric and heterodimeric receptors.

Essentially all E5-associated PDGF β receptor molecules sedimented more rapidly than the peak of monomeric receptor. Thus, the E5 protein was bound exclusively to receptor dimers and/or receptors associated with signaling proteins. The existence of E5-associated receptor dimers that are not phosphorylated (e.g. in fractions 5–8 in Fig. 6) coupled with the apparent absence of monomeric, tyrosine-phosphorylated receptor, implied that E5 binding and receptor dimerization preceded receptor tyrosine phosphorylation. Taken together, these results imply that the following sequence of events occurs during E5-mediated receptor activation: E5 dimers form and bind to a small fraction of the PDGF β receptors, resulting in near-quantitative conversion of bound receptors into receptor dimers. This is followed by a less efficient step, resulting in *trans*-phosphorylation of one or both receptor subunits in the dimer and in association of the phosphorylated subunits with downstream signaling molecules. We speculate that the receptor subunits in the E5-induced complex are not in optimal alignment for efficient *trans*-phosphorylation, thereby accounting for the existence of unphosphorylated receptor molecules in these complexes. This possibility is consistent with the finding that the level of tyrosine phosphorylation of constitutively dimerized ErbB2 mutants depends on the relative orientation of receptor subunits within the dimer (40).

The results reported here demonstrated that velocity sedimentation in sucrose gradients can be used to separate activated PDGF β receptor complexes from the inactive receptor and revealed several new features of the interaction between the E5 protein and its cellular target. Further characterization of multiprotein, activated PDGF β receptor complexes promises to provide new insight into a novel mechanism of viral transformation and to uncover new aspects of growth factor receptor signaling.

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