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

(Received for publication, October 7, 1999, and in revised form, January 4, 2000)

Char-Chang Lai, Carl Henningson‡, and Daniel DiMaio§

From the Department of Genetics, Yale University School of Medicine, New Haven, Connecticut 06510

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 Cγ, 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 complexes. 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 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 Cγ, 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 hemophosphorylated 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.

Transmembrane receptor tyrosine kinases (RTKs) 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 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 Cγ (PLCy), 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 to 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 in 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 PLCγ 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 coworkers (22) reported that microvillar fractions of rat mammary carcinoma cells contain a very large (>2 × 10⁶ Da) heterogeneous transmembrane complex or signal transduction particle that contains microfilaments, the activated RTK p185<sup>Src</sup>, serine/threonine kinases including enzymes of the mitogen-activated protein kinase signaling pathway, phosphotyrosine phosphatases, p60<sup>Src</sup>, p120<sup>Cbl</sup>, 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

* This work was supported in part by National Institutes of Health NCI Grant R37-CA37157. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported in part by a Medical Scientist Training Program grant from the National Institutes of Health. Present address: University of Pennsylvania School of Medicine, Philadelphia, PA 19106.

§ To whom correspondence should be addressed. Tel.: 203-785-2684; Fax: 203-785-7023; E-mail: daniel.dimaio@yale.edu.

1 The abbreviations used are: RTK, receptor tyrosine kinase; PDGF, platelet-derived growth factor; PLC, phospholipase C; GAP, GTPase activating protein; PI, phosphoinosit; BPV, bovine papilloma virus; SH, Src homology; MOPS, 4-morpholinepropanesulfonic 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 PDGFB are structurally dissimilar, suggesting that the mechanism of PDGFB β receptor activation by these two proteins is quite different. Indeed, the ligand-binding domain of the PDGFB β receptor is not required for activation by the viral protein, demonstrating that E5-induced PDGFB β receptor activation is ligand-independent (28, 32). Co-immunoprecipitation studies showed that the E5 protein, like PDGFB, binds to the receptor; however, unlike PDGFB, binding of the E5 protein occurs to the transmembrane and extracellular juxta-membrane region of the receptor (28, 32–37). We have shown that stable complex formation between the E5 protein and the PDGFB 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 PDGFB 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 PDGFB-treated cells, we have physically separated stable complexes containing activated PDGFB β receptor from the inactive receptor. Our results demonstrated that only a small fraction of PDGFB 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 PDGFB β receptor and provided insight into the assembly and composition of multi-protein signaling complexes containing activated PDGFB 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 interleukin-3. Ba/F3 cell lines expressing various combinations of exogenous genes were described previously (38, 39) and were maintained

**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

**RESULTS**

**Sedimentation Profile of the Endogenous PDGFB β Receptor in C127 Fibroblasts Transformed by the E5 Protein**—To analyze the endogenous PDGFB 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 PDGFB receptor-specific antibody was used to analyze total PDGFB β receptor, as shown in the top panel of Fig. 1, the great majority of PDGFB 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 PDGFB 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 200-kDa receptor 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 PDGFB β receptor in untransformed cells in the absence of PDGFB treatment, and PDGFB treatment of these cells induced the appearance of ma-
ture tyrosine-phosphorylated PDGF \( \beta \) 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 \( \beta \) 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 \( \beta \) 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 \( \beta \) receptor, and associated signaling proteins.

**Sedimentation Profile of PDGF \( \beta \) Receptor Activated by the E5 Protein or by PDGF—**We also examined the sedimentation of the PDGF \( \beta \) receptor in murine Ba/F3 cells engineered to co-express the full-length wild-type human PDGF \( \beta \) receptor and the E5 protein. These cells do not express endogenous PDGF \( \beta \) receptor, and we previously showed that co-expression of the human PDGF \( \beta \) 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 \( \beta \) receptor prior to Western blotting to provide further evidence that the bands detected were in fact PDGF \( \beta \) receptor, showed that most of the PDGF \( \beta \) receptor in these cells sedimented slowly with a peak in fraction 5, whereas both the mature and precursor tyrosine-phosphorylated receptor were detected by immunoblotting of each fraction. In the absence of PDGF treatment, total PDGF receptor, it did induce the appearance of the kinase-inactive full-length PDGF \( \beta \) receptor (with the mutation marked by the X) and the kinase-active truncated receptor.

![Diagram of PDGF \( \beta \) receptor complexes inferred to be present in E5-transformed cells](http://www.jbc.org/). The vertical lines represent the full-length and truncated PDGF \( \beta \) receptor inserted into cell membranes. The dimeric E5 protein is also shown in diagrams C–H. \( \alpha \)PRex is the antibody that recognizes the extracellular domain of the full-length PDGF \( \beta \) receptor. \( P \) represents sites of tyrosine phosphorylation, and associated SH2 domain proteins are represented by the various shapes. Diagram E shows the hemi-phosphorylated complex containing the kinase-inactive full-length PDGF \( \beta \) receptor (with the mutation marked by the X) and the kinase-active truncated receptor.

Dependent proliferation of these cells. Following detergent extraction and velocity sedimentation, total PDGF \( \beta \) 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 \( \beta \) 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 \( \beta \) 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 \( \beta \) Receptor by the E5 Pro-**

---

\(^2\) P. M. Irusta and D. DiMaio, unpublished results.
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 implies 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 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 αPR (middle and bottom panels). Total and tyrosine-phosphorylated PDGF β receptor were detected by immunoblotting with αPR and αPY, respectively, as indicated. The bottom and middle panels are the same filter that was sequentially probed with αPY, stripped, and reprobed with αPR. The positions of phosphorylated (ΔPR-P) and unphosphorylated (ΔPR) 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 (−ΔPR) (bottom panel) or together with the truncated PDGF β receptor (+ΔPR) (top four panels) were lysed and subjected to velocity sedimentation. After immunoprecipitation with αE5 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 αPR, stripped, and reprobed with αPY.

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 αE5 (or with αPI 3′-kinase; see Fig. 10) compared with immunoprecipitation with αPR. 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 (αPRex) that recognizes the full-length receptor but not the truncated mutant. In cells expressing both receptor species, αPRex 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.
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 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 F1-3′-kinase, PLCγ, 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.
Thus, kinase-active 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 αPR. A small amount of primarily unphosphorylated receptor was precipitated from the slowly sedimenting fractions (which contained most of the phosphorylated 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 αPLCγ antibody or the αGAP antibody, and then immunoblotted with antibody recognizing the p85 subunit of PI 3'-kinase. As shown in Fig. 11, the αPLCγ and the αGAP 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 PLCγ, 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). Efficient 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 existed 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 phosphorylated 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 PLCγ 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 our 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 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.

Acknowledgment—We thank David Stern for valuable guidance during the early phases of this work.

REFERENCES

1. Bishayee, S., Majumdar, S., Khire, J., and Das, M. (1989) J. Biol. Chem. 264, 11699–11705
2. Heldin, C.-H., Ernlund, A., Rorsman, C., and Rönstrand, L. (1989) J. Biol. Chem. 264, 8905–8912
3. Kelly, J. D., Haldeman, B. A., Grant, F. J., Murray, M. J., Seifert, R. A., Bowen-Pope, D. F., Cooper, J. A., and Kazlauskas, A. (1991) J. Biol. Chem. 266, 8987–8982
4. Seifert, R. A., Hart, C. E., Phillips, P. E., Forstrom, J. W., Ross, R., Murray, M. J., and Bowen-Pope, D. F. (1989) J. Biol. Chem. 264, 8771–8778
5. Heldin, C.-H., Ostman, A., and Rönstrand, L. (1998) Biochim. Biophys. Acta 1378, F79–F113
6. Escobedo, J. A., Barr, P. J., and Williams, L. T. (1988) Mol. Cell. Biol. 8, 26–31
7. Hubbard, S. R., Wei, L., Ellis, L., and Hendrickson, W. A. (1994) Nature 372, 746–754
8. Kazlauskas, A., Durden, D. L., and Cooper, J. A. (1991) Cell Regul. 2, 413–425
9. Mohammadi, M., Schlessinger, J., and Hubbard, S. R. (1996) Cell 86, 577–587
10. Koch, C. A., Anderson, D., Moran, M. F., Ellis, C., and Pawson, T. (1991) Science 252, 668–674
11. Kashishian, A., Kazlauskas, A., and Cooper, J. A. (1992) EMBO J. 11, 1573–1582
12. Li, W., Nishimura, R., Kashishian, A., Batzer, A. G., Kim, W. J., Cooper, J. A., and Kazlauskas, A. (1994) Mol. Cell. Biol. 14, 509–517
13. Brasse, M. A., and Hunter, T. (1996) J. Biol. Chem. 271, 16798–16806
14. DeMali, K. A., and Kazlauskas, A. (1998) Mol. Cell. Biol. 18, 2014–2022
15. DeMali, K. A., Whiteford, C. C., Ulug, E. T., and Kazlauskas, A. (1997) J. Biol. Chem. 272, 9011–9018
16. Klinghofer, R. A., Sachermaier, C., Cooper, J. A., and Soriano, P. (1999) EMBO J. 18, 2459–2471
17. Roche, S., McGlade, J., Jones, M., Gish, G. D., Pawson, T., and Courtneidge, S. A. (1996) EMBO J. 15, 4940–4948
18. Twamley-Stein, G. M., Pepperkok, R., Anzorge, W., and Courtneidge, S. A. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 7696–7700
19. Valius, M., and Kazlauskas, A. (1993) Cell 73, 321–334
20. Ullrich, A., and Schlessinger, J. (1990) Cell 61, 203–212
21. Herbst, R., Shearman, M. S., Sallal, R., Schlessinger, J., and Ullrich, A. (1995) Biochemistry 34, 5971–5979
22. Juang, S.-H., Carvajal, M. E., Whitney, M., Liu, Y., and Carothers Carraway, C. A. (1996) Oncogene 12, 1033–1042
23. Kanagasundaram, V., Jaworowski, A., Byrne, R., and Hamilton, J. A. (1999) Mol. Cell. Biol. 19, 4079–4092
24. Yeung, Y.-G., Wang, Y., Einstein, D. B., Lee, P. S. W., and Stanley, E. R. (1998) J. Biol. Chem. 273, 17128–17134
25. Pettij, L., Nilson, L., and DiMaio, D. (1991) EMBO J. 10, 845–855
26. Schlegel, R., Wade-Glass, M., Rabson, M. S., and Yang, Y.-C. (1986) Science 233, 464–467
27. Sartti, T., Klein, O., Aschemn, K., DiMaio, D, and Smith, S. O. (1998) Proteins Struct. Func. Genet. 33, 601–12
28. Drummond-Barbosa, D., Vaillancourt, R. L., Kazlauskas, A., and DiMaio, D. (1995) Mol. Cell. Biol. 15, 2570–2583
29. Goldstein, D. J., Li, W., Wang, L.-M., Heidaran, M. A., Aaronson, S. A., Shinn, R., Schlegel, R., and Pierce, J. H. (1994) J. Virol. 68, 4432–4441
30. Nilson, L. A., and DiMaio, D. (1993) Mol. Cell. Biol. 13, 4157–4145
31. Klein, O., Polack, G. W., Sartti, T., Kegler-Ebo, D., Smith, S. O., and DiMaio, D. (1998) J. Virol. 72, 8921–8932
32. Staehler, A., Pierce, J. H., Brazinski, S., Heidaran, M. A., Li, W., Schlegel, R., and Goldstein, D. J. (1995) J. Virol. 69, 6507–6517
33. Cohen, B. D., Goldstein, D. J., Butelidge, L., Vass, W. C., Lowy, D. R., Schlegel, R., and Schiller, J. (1993) J. Virol. 67, 5303–5311
34. Goldstein, D. J., Andresson, T., Sparkowski, J. W., and Schlegel, R. (1992) J. Virol. 66, 4851–4859
35. Pettij, L. M., Reddy, V., Smith, S. O., and DiMaio, D. (1997) J. Virol. 71, 7318–7327
36. Nilson, L. A., Gottlieb, R., Polack, G. W., and DiMaio, D. (1995) J. Virol. 69, 5869–5874
37. Pettij, L., and DiMaio, D. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 6736–6740
38. Palacios, R., and Steinmetz, M. (1985) Cell 41, 727–734
39. Lai, C. C., Henningson, C., and DiMaio, D. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 15241–15246
40. Burke, C. L., and Stern, D. F. (1998) Mol. Cell. Biol. 18, 5371–5379
Bovine Papillomavirus E5 Protein Induces the Formation of Signal Transduction Complexes Containing Dimeric Activated Platelet-derived Growth Factor β Receptor and Associated Signaling Proteins

Char-Chang Lai, Carl Henningson and Daniel DiMaio

J. Biol. Chem. 2000, 275:9832-9840.
doi: 10.1074/jbc.275.13.9832

Access the most updated version of this article at http://www.jbc.org/content/275/13/9832

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 40 references, 26 of which can be accessed free at http://www.jbc.org/content/275/13/9832.full.html#ref-list-1