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The Bovine Papillomavirus Type 1 E5 Transforming Protein Specifically Binds and Activates the β-Type Receptor for the Platelet-Derived Growth Factor but Not Other Related Tyrosine Kinase-Containing Receptors To Induce Cellular Transformation

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The 44-amino-acid E5 protein of bovine papillomavirus type 1 is a highly hydrophobic protein which appears to transform cells through the activation of growth factor receptors. To investigate the specificity of E5-growth factor receptor interactions required for mitogenic signaling, we utilized a nontumorigenic, murine myeloid cell line (32D) which is strictly dependent on interleukin-3 (IL-3) for sustained proliferation in culture. This IL-3 dependence can be functionally substituted by the expression of a variety of surrogate growth factor receptors and the addition of the corresponding ligand. Several receptor cDNAs for the α- and β-type platelet-derived growth factor receptors [α PDGFR and β PDGFR], the epidermal growth factor receptor, and the colony-stimulating factor 1 receptor) were transfected into 32D cells constitutively expressing the E5 protein to test for IL-3-independent growth. Only β PDGFR was capable of abrogating the IL-3 dependence of 32D cells. The proliferative signal induced by the coexpression of β PDGFR and E5 was accompanied by stable complex formation between these proteins, constitutive tyrosine phosphorylation of the receptor, and tumorigenicity in nude mice. The lack of cooperative interaction between E5 and the epidermal growth factor receptor, the colony-stimulating factor 1 receptor, and the highly related α PDGFR was paralleled by the inability of E5 to bind to these receptors and failure to increase receptor tyrosine phosphorylation. Thus, these data indicate that the ability of E5 to induce sustained proliferation and transformation of 32D cells is a direct consequence of specific interaction between the E5 protein and the β PDGFR signaling complex and the subsequent stimulation of receptor tyrosine phosphorylation.

Papillomaviruses are a group of DNA viruses that induce the proliferation of epithelial cells in their natural hosts. Bovine papillomavirus type 1 (BPV-1) induces tumors consisting of both epithelial and fibroblast cells and has thus been classified as a fibropapillomavirus. The primary in vitro transforming protein of BPV-1 is encoded by the E5 gene (8, 9, 16, 22, 37, 39). This 44-amino-acid protein efficiently induces the transformation of several murine fibroblast cell lines. It is a highly hydrophobic protein that localizes to cellular endomembrane compartments, predominantly the Golgi apparatus (1), and consists of two distinct domains: an amino-terminal two-thirds, which is predicted to traverse the cellular membrane, and a hydrophilic, 14-amino-acid carboxyl-terminal region, which contains two cysteine residues that covalently link two E5 proteins in a homodimer (17, 38).

Strong biochemical evidence indicates that E5 transforming activity is mediated through the activation of cellular growth-regulatory proteins. In particular, several studies suggest that the E5 protein functionally cooperates with several protein tyrosine kinase-containing (PTK) growth factor receptors (4, 23, 29, 32, 34), including the epidermal growth factor receptor (EGFR), the colony-stimulating factor 1 receptor (CSF-1R), and the β-type receptor for platelet-derived growth factor (β PDGFR). Moreover, recent evidence suggests that E5-induced receptor activation and transforming activity in rodent fibroblasts involve the formation of physical complexes with two of these receptors: β PDGFR and EGFR (4). Nilson and DiMaio recently demonstrated that the E5 protein can transform a murine mammary epithelial cell line (NMuMG) only if these cells express β PDGFR as a result of transfection (29). Although these cells express readily detectable levels of endogenous EGFR, E5 cellular transformation was not achieved without cotransfection with β PDGFR. This finding suggests that the mitogenic and transforming activities induced by E5 are a consequence of stimulation of β PDGFR tyrosine kinase activity. These results are in apparent contradiction with previous studies using immortalized rodent fibroblasts (4, 23) and a study demonstrating that E5 transforms epidermal keratinocytes which express EGFR but not PDGFR (20). Consequently, it remains unclear whether the mitogenic and transforming activities of the E5 protein are a direct result of activation of a single growth factor receptor or whether E5 exhibits the ability to induce transformation through the activation of multiple receptor targets.

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To define the components of E5-mediated transformation and to identify which growth factor receptors functionally interact with E5, we used a nontumorigenic hematopoietic progenitor cell line, 32D, which is strictly dependent upon interleukin-3 (IL-3) for sustained proliferation in culture (15). 32D cells do not normally express PTK receptors for many growth factors (e.g., EGF, PDGF, CSF-1, Met, and Kit), thus eliminating complications of previous studies which evaluated E5 effects on signal transduction pathways in cells that express multiple PTK growth factor receptors. The IL-3 dependence for mitogenic signal transduction in these cells can be substituted by the expression of specific growth factor receptors and the addition of the appropriate ligand to the culture medium. This system has been used previously to study signal transduction pathways of numerous PTK receptors (18, 25, 30, 35, 36). For example, an expression vector for EGFR was introduced into the 32D cell line, and the expression of the receptor conferred the ability to utilize EGF for the transduction of both a mitogenic and differentiation signal in these myeloid progenitor cells (36). Similarly, the introduction of expression vectors for either α or β PDGFR into naive 32D cells revealed that PDGF stimulation of either receptor could independently mediate mitogenic and chemotactic signaling through intracellular pathways inherently present in these cells (25). In the study presented here, we used the 32D cell system to examine the ability of the BPV-1 E5 oncoprotein, when coexpressed with various growth factor receptors, to induce growth factor independence and cellular transformation. Unexpectedly, only β PDGFR was able to interact and cooperate with E5 to induce indefinite proliferation in the absence of IL-3 or PDGF BB.

MATERIALS AND METHODS

Plasmid constructions. All growth factor receptor genes were cloned into a previously described eukaryotic expression vector (LTR-2 [7]) containing the transcriptional initiation sequences of the Moloney murine leukemia virus long terminal repeat (LTR) along with the *Escherichia coli gpt* selectable marker, which confers resistance to mycophenolic acid (26). The expression of the E5 protein was also accomplished by cloning the E5 gene downstream of the retroviral LTR. The E5 gene was excised from plasmid pPava-2 (40) by using restriction enzymes *Xho*I and *Bgl*II and inserted into unique *Xho*I and *Bam*HI sites of plasmid LTR-2neo, which contains the gene conferring resistance to geneticin (G418).

Cell lines and transfections. The murine IL-3-dependent hematopoietic cell line 32D (15) was cultured in RPMI 1640 medium (GIBCO, Grand Island, N.Y.) supplemented with 15% fetal bovine serum and 5% WEHI-3B-conditioned medium as a source of murine IL-3 (American Type Culture Collection, Rockville, Md.). DNA transfections of 32D cells were done by electroporation as previously described (36). Populations of stably transfected cells were selected by growth in medium containing 80 μM mycophenolic acid and hypoxanthine, aminopterin, and thymidine (1× HAT; GIBCO) for receptor-expressing transfectants (36) or geneticin (750 μg/ml; GIBCO) for E5-expressing transfectants. Cell transfectants expressing β PDGFR and E5 were routinely passaged in RPMI 1640 medium lacking IL-3. All other cell lines were propagated in medium supplemented with IL-3. Incorporation of [^{3}H]thymidine was used to quantify proliferation of 32D cell transfectants. Triplicate samples of exponentially growing cells (2×10^5 cells per ml) were washed twice and aliquoted in RPMI 1640 containing 15% dialyzed fetal bovine serum with no added factors, 5% WEHI-conditioned medium, or 100 ng of PDGF BB (UBI, Inc., Lake Placid, N.Y.), EGF (UBI), or CSF-1 (a kind gift of Steve Clark, Genetics Institute) per ml in 24-well Costar plates (1 ml per well). Samples were incubated for 24 h at 37°C, and 1 μCi of [^{3}H]thymidine was added per well for the final 4 h of the incubation period. Cells were harvested on an automated cell harvester (Skatron, Vienna, Va.), and samples were counted in a Beckman Beta counter.

Immunoprecipitation and immunoblot analysis. E5 expression in 32D cell transfectants was confirmed by metabolic labeling with [^{35}S]methionine/cysteine labeling mix (Amersham). Briefly, 5×10^6 cells were washed twice with phosphate-buffered saline (PBS) and incubated for 1.5 h in 2 ml of Dulbecco modified Eagle medium (DMEM) lacking methionine and cysteine; 300 μCi of labeling mix was added, and cells were incubated for an additional 4 h. Cells were then harvested, washed twice with PBS, and lysed with 1 ml of modified radioimmunoprecipitation assay (RIPA) buffer (14). Lysates were clarified by centrifugation in a microcentrifuge. To immunoprecipitate the E5 protein, 5 μl of an anti-E5 antiserum generated against the carboxyl-terminal 16 amino acids of E5 (32) and 50 μl of a 1:1 suspension of protein A-Sepharose CL-4B (Pharmacia, Piscataway, N.J.) were added to the clarified supernatants. Extracts were incubated for 1.5 h at 4°C, at which time the Sepharose beads were washed four times with 1 ml of RIPA buffer. The beads were finally resuspended in 80 μl of sodium dodecyl sulfate (SDS)-gel loading buffer and boiled for 4 min prior to loading on a 14% polyacrylamide gel.

32D cell transfectants were assayed for phosphotyrosine-containing receptors as follows. Cells were washed in DMEM (serum free), starved in DMEM with 50 μM Na_3VO_4 for 2 h, and resuspended in a small volume of medium (2×10^7 to 4×10^7 cells per ml) for growth factor triggering. After stimulation with 250 ng of PDGF BB, EGF, or CSF-1 per ml for 10 min at 37°C, cells were immediately treated with cold PBS containing 100 μM Na_3VO_4. The cells were then pelleted and lysed in a lysis buffer containing 50 mM hydroxyethylpiperazine ethanesulfonic acid (HEPES), 1% Triton X-100, 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 2.5 mM EDTA, 50 mM NaF, 10 mM $NaPP_i$, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride (Sigma), 10 μg of aprotinin (Boehringer Mannheim, Indianapolis, Ind.) per ml, 10 μg of leupeptin (Boehringer Mannheim) per ml, and 4 mM diisopropyl fluorophosphate (Sigma, St. Louis, Mo.).

The total protein content of cell lysates was determined by the Bio-Rad protein assay. Routinely, equal amounts of clarified cell lysates (2 to 5 mg) were used for immunoprecipitations using 20 μl of agarose-conjugated antiphosphotyrosine (anti-Ptyr) antibodies (4 mg/ml of settled beads; UBI) or with a receptor-specific antiserum plus protein G-coupled Sepharose (Pharmacia). The immunoprecipitates were washed three times with lysis buffer (minus diisopropyl fluorophosphate), solubilized with Laemmli buffer, boiled for 4 min, and resolved on SDS–8% polyacrylamide gels. For detection of total cell receptor levels, 300 μg of cell lysates was directly separated by SDS-polyacrylamide gel electrophoresis (PAGE). Separated proteins were transferred to Immobilon-P membranes (Millipore, Bedford, Mass.) in Tris-glycine buffer containing 20% methanol. The membranes were then treated for 1 to 2 h with 3% nonfat dry milk in TTBS (20 mM Tris [pH 7.5], 154 mM NaCl, 0.05% Tween, 0.5% NaN_3), incubated with antibodies (2 μg of anti-Ptyr [05-321; UBI] per ml, 1:200 of anti-β PDGFR [06-131; UBI], and 1:750 anti-α PDGFR [rabbit antiserum generated against a peptide containing amino acids 959 to 973 of the human α PDGFR {2}]) in TTBS containing 0.5% bovine serum albumin (BSA) for 1 to 2 h, and incubated with [^{125}I]protein A (3×10^5 cpm/ml) in TTBS-BSA for 1 h. All

incubations were carried out at room temperature, and blots were washed extensively with TTBS following each treatment. After the final wash, the membranes were air dried and placed on film for autoradiography with intensifying screens at −70°C.

For detection of protein complexes, cell lysates were prepared with either RIPA buffer (for immunoprecipitations with anti-E5 antibodies) or Triton X-100 lysis buffer (for immunoprecipitations with receptor antibodies) from 3×10^7 cells. One milliliter of clarified extracts was incubated with anti-E5 antiserum (5 μl), an anti-β PDGFR (10 μl of 06-131; UBI), anti-α PDGFR (5 μl of antibody described in reference 2), or anti-α/β PDGFR (10 μl of 06-132; UBI) antibody, an anti-EGFR monoclonal antibody (15 μl of Ab-1 [Oncogene Sciences] or 5 μl of antibody RPN.513 [Amersham]), or an anti-CSF-1R antiserum (20 μl), plus 50 μl of protein A-Sepharose beads (Pharmacia) or protein G-linked agarose (for immunoprecipitations with anti-EGFR antibodies; UBI) for 1 to 2 h. Beads were then washed three times with appropriate detergent buffer, and final pellets were resuspended in SDS-gel loading buffer and boiled for 4 min. Immunoprecipitated proteins were electrophoretically separated on either 7.5% (to resolve receptor proteins) or 15% (to resolve E5 proteins) polyacrylamide and transferred to polyvinylidene difluoride membranes (Bio-Rad). Membranes were washed twice with PBS and processed according to the Tropix chemiluminescence kit protocol (Tropix, Bedford, Mass.). The E5 protein was detected on membranes by using one of two anti-E5 antisera: one derived against the carboxyl-terminal 16 amino acids (previously described in reference 12; 1:500 dilution; Fig. 4), or another derived against the carboxyl-terminal 14 amino acids of E5 (1:5,000; Fig. 5). PDGFRs were detected on membranes by using anti-PDGFR antibodies (1:500 dilution of 06-132 [UBI] or 1:750 dilution of anti-α PDGFR) for 1 h. EGFR was detected by using an anti-EGFR antiserum (1:1,000 dilution; kind gift of P. Di Fiori), and CSF-1R was detected by using an anti-CSF-1R antiserum (1:200) (35). Proteins were visualized by using an alkaline phosphatase-conjugated goat anti-rabbit antibody for 1 h and disodium 3-(4-methoxyspiro-4-*yl*)phenyl phosphate (CSPD) substrate for 5 min. Membranes were then exposed to film for between 30 s and 5 min.

Tumorigenic analysis of 32D cell transfectants. Cells (10^6) were washed with PBS and injected subcutaneously into NFR nude mice in a final volume of 300 μl. Tumors arose after a mean of 40 days postinjection.

RESULTS

E5 and receptor expression in 32D cell transfectants. To determine if the E5 protein could be properly synthesized in the hematopoietic cell line 32D, the E2-E5 region of BPV-1 (positions 2440 to 4450) was cloned into a Moloney murine retroviral vector (LTR-2 [7]) which is designed to transcribe genes from the Moloney murine leukemia retroviral LTR. This plasmid, termed pLTR-E5, also expresses the *neo* gene conferring geneticin resistance. This plasmid was used to electroporate naive 32D cells that were propagated in RPMI 1640 growth medium supplemented with 15% fetal calf serum and 5% conditioned medium from WEHI-3 cells as a source of murine IL-3 (36) and selected for resistance to G418. A selected cell line, designated 32D:E5, was next tested for E5 expression by metabolic labeling with [^{35}S]methionine/cysteine and immunoprecipitation with an anti-E5 antiserum generated against the carboxyl-terminal 16 amino acids (32). Figure 1 demonstrates the expression of E5 protein in 32D:E5 cells. We have shown previously that the E5 protein binds to the 16-kDa (16K) component of the vacuolar proton ATPase in E5-transformed NIH 3T3 cells (14). A coprecipitated 16-kDa protein which we believe is the E5-associated 16K component of the ATPase enzyme complex was also detected. This result demonstrates that the E5 protein can be expressed in 32D cells and that it presumably binds to a cellular protein shown to be a target for the E5 protein in transformed mouse fibroblasts.

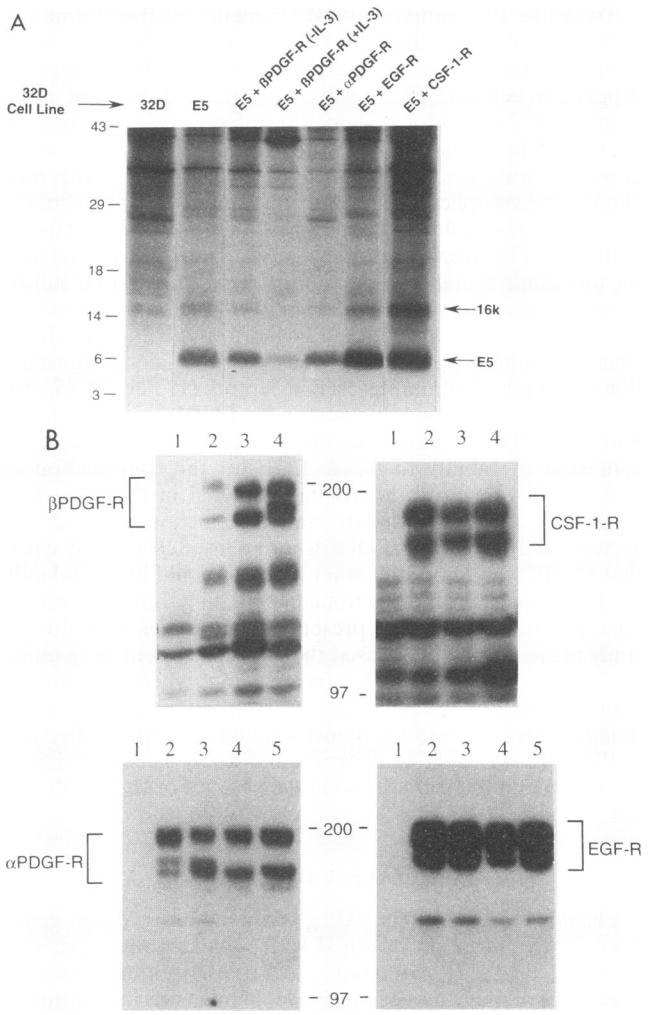

FIG. 1. E5 and growth factor receptor expression in 32D cell transfectants. (A) E5 expression. The cell lines indicated at the top were grown in the presence or absence of IL-3, metabolically labeled with [^{35}S]methionine/cysteine, and following lysis in RIPA buffer, immunoprecipitated with an anti-E5 antiserum. Precipitated proteins were separated on an SDS–14% polyacrylamide gel and visualized by fluorography. The positions of E5 and the coprecipitated 16K protein are indicated on the right, and positions (in kilodaltons) of molecular weight standards are shown on the left. (B) Growth factor receptor expression. Three hundred micrograms of protein from extracts prepared with a Triton X-100 lysis buffer was separated on 8% gels and transferred to nitrocellulose membranes, and membranes were probed with antibodies directed against each receptor. Receptors were visualized by using [^{125}I]protein A and autoradiography. Lanes: 1, 32D cells expressing E5 alone; 2, lines expressing receptor alone; 3 to 5, multiple isolates of 32D cell lines expressing E5 and the receptor indicated at either the left or right. The positions (in kilodaltons) of molecular weight standards are shown in the center.

E5 interacts biologically only with β PDGFR. Though expressing the E5 protein, 32D:E5 cells failed to proliferate in the absence of IL-3 (Table 1), suggesting that 32D cells lack

TABLE 1. Coexpression of the BPV-1 E5 protein and β PDGFR induces IL-3-independent growth

DNA	Cell line transfected	IL-3-independent growth[a] (no. of positive wells/24 wells plated)
E5	32D	0
	32D:β PDGFR	13
	32D:α PDGFR	0
	32D:EGFR	0
	32D:CSF-1R	0
β PDGFR	32D	0
	32D:E5	20
EGFR	32D:E5	0
CSF-1R	32D:E5	0

[a] Cells were electroporated with 5 μg of each DNA as previously described. Following electroporation, cells were cultured overnight in RPMI 1640 growth medium supplemented with 15% fetal bovine serum and 5% WEHI-3B-conditioned medium as a source of IL-3. After this overnight incubation, cells were washed two times in RPMI 1640 medium supplemented with 15% fetal bovine serum without WEHI-conditioned medium and plated at 10^5 cells per ml in 24-well plates. Wells in which viable cells emerged and were still proliferating at day 14 were scored as positive. Factor-independent lines could be readily established from positive wells in all cases. Data are the results of one representative experiment out of three experiments attempted.

other components necessary for E5-induced mitogenesis. To determine if the failure of E5 to induce a proliferative signal in the absence of IL-3 was due to lack of expression of certain growth factor receptors, 32D:E5 cells were supertransfected with expression vectors containing individual receptor genes (Table 1). EGFR, CSF-1R, and PDGFRs have been implicated in mediating transformation by E5. Moreover, 32D cells have been shown to possess the components required to allow efficient signal transduction through each of these receptors. Thus, pLTR expression plasmids carrying these five receptor genes were transfected into 32D:E5 cells. These plasmids also contained the *gpt* gene conferring resistance to mycophenolic acid and HAT, thereby allowing selection of stable cell lines expressing receptor genes in combination with E5. Transfected cells were either selected for drug resistance or directly selected for the ability to grow in the absence of IL-3. Only E5/β PDGFR-cotransfected (32D:E5/β PDGFR) cells were capable of sustained proliferation following direct abrogation of IL-3 dependence (Table 1). Whereas all other cell lines perished within 24 h, 32D:E5/β PDGFR cells grew indefinitely under these conditions. All cell lines were also tested in a less stringent assay for IL-3-independent growth. Rather than exposing transfected cell lines directly to growth medium lacking IL-3, we first selected lines in IL-3- and mycophenolic acid/HAT-containing medium. After the transfectants could be stably propagated in selection medium, IL-3 was then withdrawn from the cultures. Again, only E5/β PDGFR transfectants grew under these conditions. Lastly, we performed a reciprocal experiment in which receptor-expressing cell lines were transfected with the E5-expressing plasmid and then tested for growth in the absence of exogenously added factors. Transfection of only β PDGFR-expressing cell lines with the E5 expression vector induced factor-independent growth (Table 1).

The ability of E5 to cooperate only with β PDGFR to induce factor-independent growth was also verified by measuring the ability of 32D cell transfectants to incorporate [^{3}H]thymidine in a mitogenic assay. Figure 2 shows that all cell lines expressing E5 and growth factor receptors induced efficient DNA synthesis in the presence of IL-3. In addition, all cell lines expressing growth factor receptors were capable of eliciting significant mitogenic responses when the appropriate ligand was added to medium lacking IL-3, indicating that the cell lines expressed functional receptors. However, whereas E5/α PDGFR-, E5/EGFR-, and E5/CSF-1R-coexpressing 32D cells failed to induce detectable DNA synthesis in the absence of added growth factors, E5/β PDGFR-coexpressing cells displayed constitutive DNA synthesis in the absence of added factors (Fig. 2A).

To confirm that the failure of all other receptors to cooperate with E5 to induce proliferation in the absence of IL-3 was not due to either loss of E5 expression or inefficient expression of receptor proteins, multiple-drug-resistant clones of E5/receptor-expressing 32D cell lines were isolated in IL-3-containing medium and characterized for E5 and receptor protein expression by either immunoprecipitation or Western blotting (immunoblotting). The continued expression of the E5 protein following dual selection was confirmed by metabolic labeling and immunoprecipitation using an anti-E5 antiserum (Fig. 1A; data not shown for multiple isolates). To demonstrate the efficient expression of receptor proteins in various transfectants, immunoblot analysis was performed. All E5/receptor transfectants were shown to express high levels of the different receptor proteins (Fig. 1B). Taken together, these results suggest that the inability of E5 to cooperate with EGFR, CSF-1R, or α PDGFR was not due to lack of expression of either E5 or functional receptors. Furthermore, these data strongly indicate that only β PDGFR can cooperate with the E5 protein to induce proliferation of 32D cells in the absence of IL-3 and exogenously added ligand.

E5 activates only β PDGFR. To determine if the factor-independent growth induced in the β PDGFR/E5 transfectants was due to the ability of E5 to specifically activate this particular receptor in coexpressing 32D cells, coexpressing cell lines were examined for the presence of tyrosine-phosphorylated receptors by immunoprecipitation with an anti-Ptyr monoclonal antibody followed by immunoblot analysis with either anti-PTyr or receptor antibodies. To demonstrate that the various receptors possess functional tyrosine kinase activities, transfectants were also exogenously stimulated with appropriate ligands. As shown in Fig. 3, E5 clearly stimulated the constitutive basal phosphorylation of β PDGFR on tyrosine residues in the absence of exogenously added PDGF BB. Two species of activated receptor were detected: a slower-migrating species, which presumably represents the fully processed form, and a faster-migrating species, which represents the immature, metabolic precursor of the receptor (19). Consistent with their inability to proliferate in the absence of IL-3, there was no appreciable increase in receptor phosphorylation on tyrosine residues in 32D transfectants coexpressing E5 and α PDGFR (Fig. 3B), EGFR (Fig. 3C), or CSF-1R (data not shown) compared with untreated lines expressing receptor alone. However, the addition of either PDGF BB (Fig. 3B), EGF (Fig. 3C), or CSF-1 (data not shown) to E5/receptor or receptor transfectants induced readily detectable tyrosine phosphorylation of the receptors. These results confirmed that the inability of E5 to couple with either α PDGFR, EGFR, or CSF-1R to induce a mitogenic effect was clearly not due to lack of expression of functional receptors and that the sustained proliferative signal elicited by the coexpression of the E5 protein and β PDGFR correlated with a dramatic increase in basal receptor tyrosine phosphorylation.

E5 forms a complex with β PDGFR but not with α PDGFR, EGFR, or CSF-1R. To determine if E5 and β PDGFR physically associated in a stable complex in IL-3-independent 32D transfectants, a coimmunoprecipitation experiment was

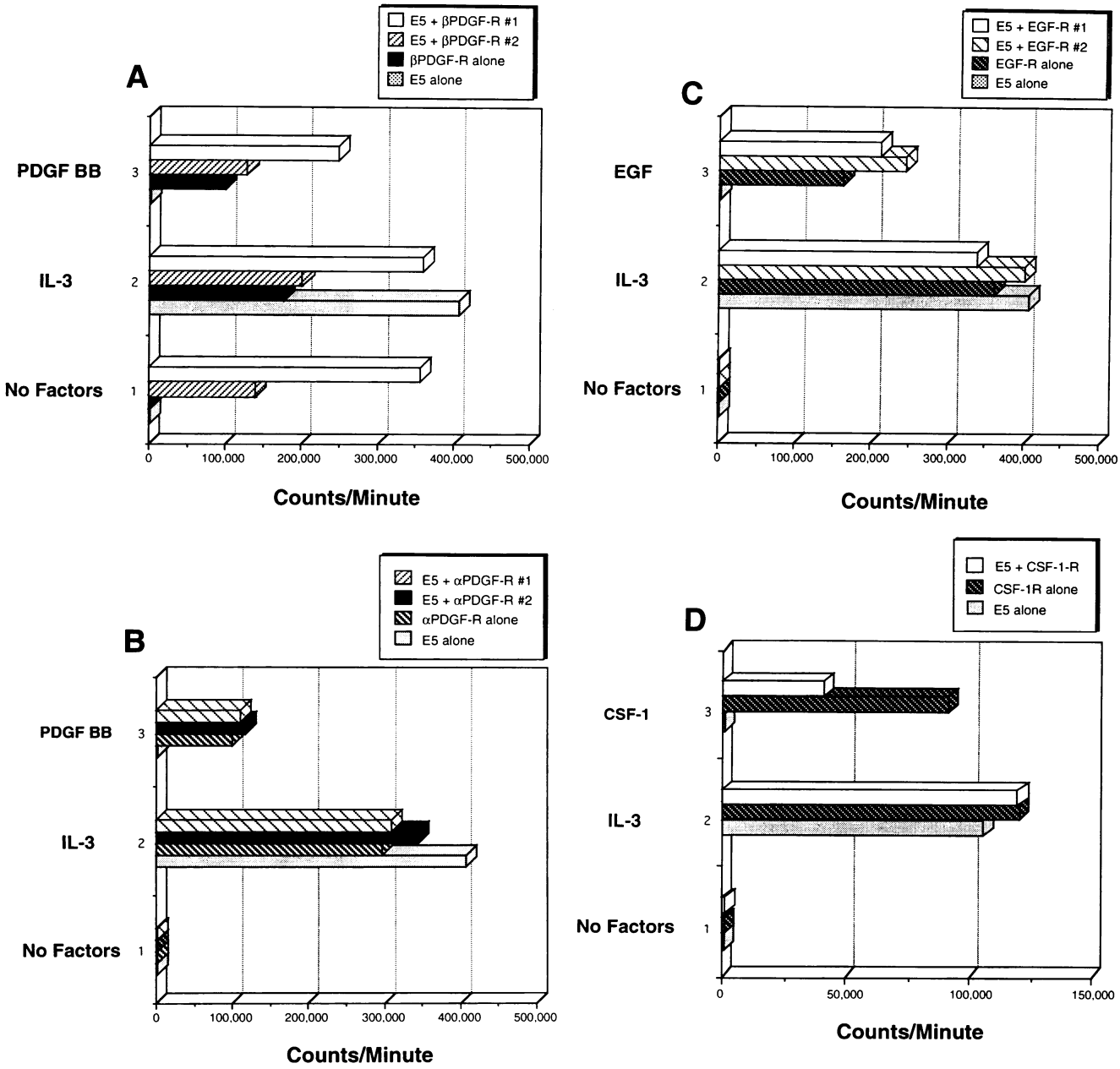

FIG. 2. Only E5/β PDGFR-coexpressing 32D cells induce mitogenic activity in the absence of added factors. Mitogenic potential of 32D transfectants (β PDGFR-expressing lines [A], α PDGFR-expressing lines [B], EGFR-expressing lines [C], and CSF-1R-expressing lines [D]) were carried out by measuring the ability of cells to incorporate [^{3}H]thymidine in the presence and absence of IL-3. As a positive control for the expression and inducibility of the appropriate growth factor receptor, incorporation was also measured when cells were grown in the presence of 100 ng of PDGF BB, EGF, or CSF-1 per ml. Data are expressed as counts per minute of [^{3}H]thymidine incorporated and are averages of duplicate samples.

carried out (Fig. 4). Cells coexpressing E5 and β PDGFR (grown in the absence of IL-3) and cells expressing each protein individually (grown in the presence of IL-3) were immunoprecipitated with either anti-β PDGFR or anti-E5 antiserum. Immunoprecipitated proteins were subjected to SDS-PAGE, and proteins were transferred to membranes and subsequently immunoblotted with either anti-E5 (Fig. 4A) or anti-β PDGFR (Fig. 4B) antibodies. Stable complex formation between β PDGFR and E5 was clearly demonstrated in three different isolates of coexpressing 32D cells that were immunoprecipitated with anti-β PDGFR antiserum and blotted with anti-E5 serum (Fig. 4A, lanes 4, 6, and 8). This anti-β PDGFR antiserum (which detects equally both α and β PDGFR species) did not nonspecifically immunoprecipitate E5 from a cell line highly expressing E5 (α PDGFR/E5 cells), confirming the specificity of the anti-PDGFR coprecipitation of the β PDGFR-E5 complex (Fig. 5A, lane 6). When anti-E5 immunoprecipitates were analyzed for the presence of associated receptor protein by immunoblotting with the anti-β PDGFR-specific antiserum (Fig. 4B), all cell lines demonstrated readily

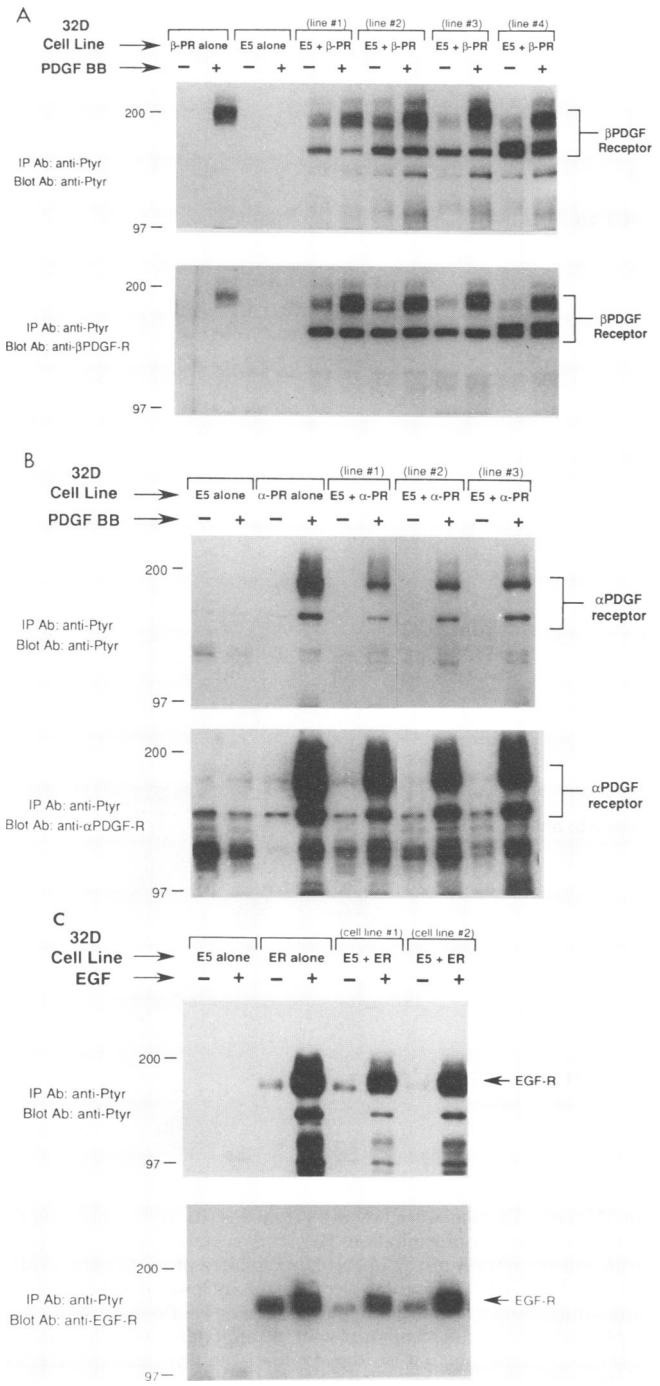

FIG. 3. The E5 protein stimulates the constitutive tyrosine phosphorylation of only β PDGFR in 32D cell transfectants. 32D cell transfectants, as indicated above each panel, were analyzed for tyrosine-phosphorylated receptors by using combined immunoprecipitation-immunoblot analysis. Multiple isolates of 32D cells expressing E5 plus β PDGFR (β-PR; A), α PDGFR (α-PR; B), or EGFR (ER; C) were extracted in a Triton X-100-based lysis buffer as described in Materials and Methods and immunoprecipitated (IP) with anti-Ptyr antibodies (Ab). Cells expressing E5 or receptor alone were grown in parallel and were used for comparison as a negative control. As a positive control for the inducibility of receptor phosphorylation, all cell lines were triggered prior to cell lysis by the addition of the appropriate ligand for 10 min at 37°C (+ lanes). Precipitated proteins were separated by SDS-PAGE (8% gel), transferred to nitrocellulose, and immunoblotted with either anti-Ptyr (upper panels) or antireceptor (lower panels) antisera as indicated. Precipitated proteins were then detected with [^{125}I]protein A and autoradiography. Phosphorylated receptor species are indicated on the right, and positions (in kilodaltons) of molecular weight markers are indicated on the left.

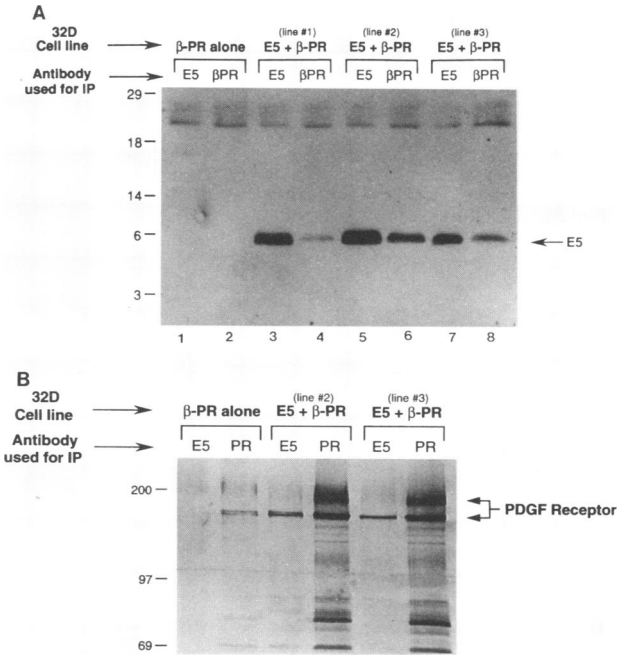

FIG. 4. The E5 protein forms a stable complex with β PDGFR. Coimmunoprecipitation of independently isolated 32D cell transfectants expressing E5 and β PDGFR (β-PR; grown in the absence of IL-3) and cells expressing β PDGFR alone (grown in the presence of IL-3) were extracted in RIPA buffer and immunoprecipitated (IP) with the antibodies indicated at the top. Immunoprecipitated proteins were separated on either a 14% (A) or 7.5% (B) polyacrylamide gel and transferred to polyvinylidene difluoride membranes. Membranes were immunoblotted with either anti-E5 (A) or anti-β PDGFR (B) antisera, and precipitated proteins were detected by using alkaline phosphatase-conjugated secondary antibodies and a chemiluminescence detection kit (Tropix). The positions of E5 and PDGF proteins are indicated on the right, and positions (in kilodaltons) of molecular weight standards are indicated on the left.

detectable E5-receptor complex formation (data shown only for two cell lines). No E5 was detected in precipitations using the anti-E5 antiserum from extracts of a transfectant expressing only β PDGFR (Fig. 4, lane 1).

To provide evidence that physical association between E5 and the growth factor receptors is critical for IL-3 independence in 32D cells, and to determine if failure of α PDGFR, EGFR, and CSF-1R to cooperate with the E5 protein to abrogate IL-3 dependence was due to lack of E5 affinity for these receptors, a series of coimmunoprecipitations was performed on E5/receptor-coexpressing cells, using antibodies to either E5 or receptor proteins (Fig. 5). As a positive control for coprecipitation, a 32D cell line expressing E5 and β PDGFR was analyzed in parallel. Precipitated proteins were transferred to membranes and probed with antibodies specific to either each receptor or E5. As shown in Fig. 4, E5 complex formation could easily be demonstrated with use of either anti-β PDGFR antibodies (Fig. 5A, lower panel) or anti-E5 antiserum (Fig. 5A, upper panel). In striking contrast, no complex formation

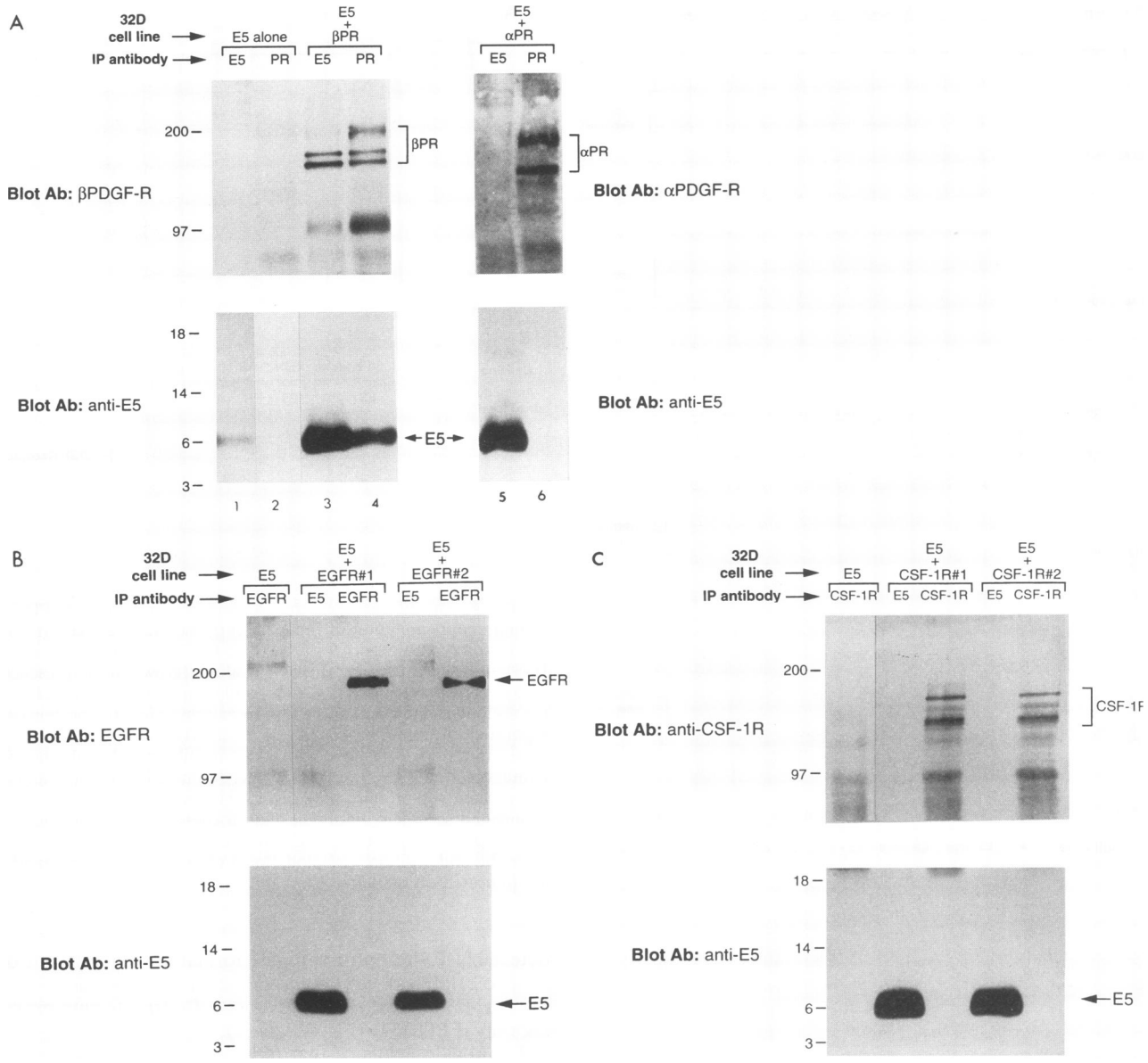

FIG. 5. The E5 protein does not bind to α PDGFR, EGFR, or CSF-1R in 32D cells. Extracts were prepared from 3×10^7 cells expressing the proteins indicated above the lanes, using either the Triton X-100-based lysis buffer (for immunoprecipitations [IP] with receptor-specific antibodies [Ab]; see Materials and Methods) or RIPA buffer (for immunoprecipitation with anti-E5 antiserum). Samples of precipitated proteins were divided equally and separated on either a 7.5% gel (to resolve receptor proteins; upper panels) or a 15% gel (to resolve E5 protein; lower panels). Proteins were transferred to polyvinylidene difluoride membranes, immunoblotted with antibodies to receptors (upper panels) or E5 (lower panels), and visualized by chemiluminescence. Cells lines expressing either E5 alone or E5 plus receptor were precipitated with the antibodies indicated directly above each lane. The upper blots were probed with antireceptor antibodies to demonstrate the expression levels and amount of receptor coprecipitated with anti-E5 antiserum. The lower blots were probed with anti-E5 antiserum to determine the levels of E5 expression and amount of E5 coprecipitated with antireceptor antibodies. (A) Cell lines expressing E5 and β PDGFR (βPR; left) or E5 plus α PDGFR (αPR; right). (B) Cell lines expressing E5 and EGFR. (C) Cell lines expressing E5 and CSF-1R. Precipitated receptor and E5 proteins are indicated to the right of each blot. Positions (in kilodaltons) of molecular weight markers are indicated to the left of each blot.

could be detected between E5 and α PDGFR, EGFR, or CSF-1R with use of either antireceptor antibodies (Fig. 5, lower panels) or anti-E5 antibodies (Fig. 5, upper panels). Even prolonged exposures did not reveal E5-receptor interactions (data not shown). Similar negative results were obtained from several additional, independently isolated E5/receptor-coexpressing cell lines (data not shown). The lack of ability to detect complex formation between E5 and these receptors does not appear to be due to insufficient synthesis of either E5 or receptor proteins, since E5 and receptor proteins were readily detected in all cell lines tested (Fig. 5). To minimize the possibility that the antibodies used to precipitate the E5-receptor complex may have interfered with complex formation, similar precipitations were carried out (on α PDGFR- and EGFR-expressing cells) with other available antireceptor antibodies; once again, no complex formation could be detected (data not shown). These experiments strongly suggest that the E5 protein displays preferential binding to the β PDGFR and

that the binding to this receptor is critical for both stimulation of tyrosine kinase activity of the receptor and the induction of mitogenic signaling.

E5 and β PDGFR coexpression induces tumorigenic conversion of 32D cells. To determine the tumorigenic potential of cell transfectants, 10^6 cells were subcutaneously injected into nude mice at five distinct sites. No tumors were detected in mice injected with cells expressing E5 or receptor alone or cells expressing both E5 and EGFR, α PDGFR, or CSF-1R. However, cells expressing E5 and the β PDGFR consistently induced tumors of between 30 and 60 mm in diameter at all five sites injected within 30 to 40 days following injection (data not shown). These results demonstrate that the coexpression of E5 and β PDGFR leads to tumorigenic conversion of 32D cells in athymic nude mice.

DISCUSSION

To dissect the components of the signal transduction pathways activated by the BPV-1 E5 oncoprotein, we have used an IL-3-dependent nontumorigenic hematopoietic progenitor cell line, designated 32D. The distinct advantage offered by this cell system is that these cells normally lack expression of many PTK growth factor receptors that are endogenously expressed in cells commonly used to study E5-induced cellular transformation (e.g., fibroblasts and epithelial cells). Thus, this approach allows for the direct examination of the effects of E5 on individually expressed growth factor receptor genes. In addition, the factor-independent survival of transfected cells is dependent on continued expression of E5 and receptor genes, thereby providing a growth selection for only those cells exerting a mitogenic stimulus through E5 and individually expressed growth factor receptors.

We found that the coexpression of the E5 oncoprotein and β PDGFR was sufficient to induce the abrogation of IL-3 dependence and transformation of 32D cells. The mitogenic stimulus evoked by the coexpression of these proteins was characterized by a dramatic increase of receptor autophosphorylation on tyrosine residues, which is a direct measure of receptor kinase activity. Furthermore, the E5 and receptor proteins expressed in 32D cells were coprecipitated with antibodies directed against either protein, providing direct evidence for stable complex formation in these cells. Therefore, this study confirms that the mitogenic and tumorigenic activities induced by the E5 protein are a direct consequence of activation of β PDGFR tyrosine kinase activity.

Since previous studies indicated that E5 transforming abilities are potentiated by the expression of several different growth factor receptor genes (4, 23), we also examined the ability of E5 to induce a proliferative signal when coexpressed with α PDGFR, EGFR, and CSF-1R. E5 was unable to cooperate with any of these receptors, even though each receptor was capable of eliciting a mitogenic response after addition of the appropriate ligand. These findings were unexpected considering previously published studies which demonstrated that E5 induced the activation of EGFR and cooperated with this receptor or CSF-1R to increase transformation of rodent fibroblasts (4, 23). The inability of E5 to couple with these receptors in the 32D cell system was clearly not due to lower expression of E5 or receptor proteins (Fig. 1 and 5). Most importantly, we show that the failure of these receptors to functionally couple with E5 is likely due to lack of affinity between E5 and these receptor proteins. These results therefore strongly suggest that the specific complex formation between E5 and β PDGFR is critical for the activation of this receptor and induction of mitogenesis. These results are consistent with recent studies demonstrating that a mouse mammary epithelial cell line can be transformed by E5 only when cotransfected with β PDGFR, despite the endogenous expression of EGFR in these cells (29). Furthermore, most recent studies by Petti and DiMaio indicate that the E5 protein preferentially binds and activates the β PDGFR in cell lines expressing multiple growth factor receptors (33). These results, however, are not consistent with studies showing that E5 can transform a mouse keratinocyte cell line that presumably lacks PDGFRs but express EGFR (20). The possibility remains that E5 targets an alternative cellular protein in these cells.

The contrasting effects on β PDGFR and EGFR in rodent fibroblasts (4, 23) and 32D cells may be due to the different interactions between E5 and the two receptors. It has recently been suggested that E5 functional and physical interaction with these receptors involves two different receptor domains: whereas the interaction between E5 and β PDGFR is thought to occur within the receptor transmembrane domain, E5 interaction with the EGFR is thought to reside within the receptor cytoplasmic domain (4). It is possible that the mechanism involving the activation of the EGFR does not function in 32D cells, perhaps because of the lack of expression of an additional required factor(s). For example, these cells may not express a recently identified 125-kDa α-adaptin-like molecule which specifically binds to the carboxyl terminus of E5 in NIH 3T3 cells and which might provide a necessary bridge between E5 and EGFR (5). This family of proteins has been shown to bind specifically to the carboxyl termini of growth factor receptors within coated pits (31). This protein may also be required to mediate E5 interaction with other receptors, such as CSF-1R. It will thus be important to examine whether 32D cells express this newly identified, E5-associated adaptin-like protein.

The inability of the E5 protein to functionally couple with α PDGFR in 32D cells was surprising considering the high degree of homology between the α and β receptors. We have determined that this difference is most likely due to lack of E5 affinity for α PDGFR (Fig. 5). Our previous studies indicated that the interaction between E5 and β PDGFR presumably occurs through transmembrane interactions (11). Although there is a high degree of amino acid homology within their two tyrosine kinase domains (85% for domain 1 and 75% for domain 2), they exhibit much lower homology within their putative transmembrane domains (46%) (24). Previous studies indicate that specific charged or hydrophilic amino acids within membrane-spanning regions can contribute to the interaction between transmembrane proteins (6, 13). It is interesting to note that the β receptor has two hydrophilic residues (serine at amino acid 535 and threonine at amino acid 544) within its transmembrane domain which are hydrophobic residues (alanine and valine, respectively) in the α receptor. It remains to be determined if these residues mediate interaction with the E5 protein.

We cannot, however, exclude the possibility that other structural differences between the two PDGFR types dictate unique protein-protein interactions or receptor-specific signaling capacity. For example, the two receptors share only 31% amino acid homology within their extracellular ligand-binding domains. It is thought that the two receptors exert both common and distinct biological effects (10), which may be a result of differential binding affinities for the three isoforms of PDGF (AA, AB, and BB). Whereas α PDGFR binds all three forms with high affinity, β PDGFR binds only PDGF BB with high affinity and PDGF AB with lower affinity (3, 24). Alternatively, the structural differences in the ligand-binding do-

mains, or other receptor domains, may impart differential availability for transmembrane interactions.

Although 32D cell transformation was accomplished by coexpression of E5 and β PDGFR, it is likely that additional cellular factors comprise the E5 transformation complex. For instance, we have previously shown that E5 interaction with the 16K protein of the vacuolar proton pump may be essential for efficient cellular transformation (13). Since proton pump activity is required for several important regulatory functions that take place within cellular compartments that process growth factor receptors (27, 28), it is possible that E5 alteration of pump activity contributes to the activation of β PDGFR. Furthermore, it was found that the 16K protein may be found in a ternary complex with E5 and β PDGFR and that overexpressed 16K protein in COS cells bound to the PDGFR in the absence of E5, suggesting that 16K may mediate the interaction of E5 with the receptor (11). It will therefore be important to determine whether alterations in 16K function affect the activity of β PDGFR, or whether the 16K protein serves more of a structural role in the E5-β PDGFR complex.

Perhaps the simplest model for E5-induced cellular transformation through interaction with β PDGFR would be that E5 induces the dimerization of receptor molecules through binding to the receptor as a homodimer. The binding of PDGF to its receptor leads to the formation of both noncovalent and covalent PDGFR dimerization (21). This dimerization correlates with autophosphorylation of the receptors, which probably occurs as a *trans*-phosphorylation reaction between receptor molecules in the dimer. By interacting with two receptor molecules through hydrophobic, transmembrane sequences, the E5 homodimer may be mimicking PDGF dimers which cross-link two receptor molecules through interaction with the extracellular, ligand-binding domains of two receptor molecules.

The 32D cells should serve as a system for a thorough molecular and biochemical dissection of the components required for E5-mediated mitogenesis and transformation. By providing a growth selection (abrogation of IL-3 dependence), these cells will allow for the evaluation of functional domains of both E5 and receptor proteins which are critical for signal transduction. Furthermore, this approach may assist in the identification of additional cellular factors that are important for E5 function, as well as providing new insights into the functional differences between α and β PDGFRs.

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