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# Mutational Analysis of the β-Type Platelet-Derived Growth Factor Receptor Defines the Site of Interaction with the Bovine Papillomavirus Type 1 E5 Transforming Protein

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**The E5 polypeptide of bovine papillomavirus type 1 is a small membrane-bound protein which induces the transformation of immortalized fibroblasts, apparently via the formation of a ternary complex with the platelet-derived growth factor receptor (PDGFR) and the 16-kDa V-ATPase protein. This interaction seems to be mediated, at least in part, by their respective transmembrane domains. E5 also cooperates with transfected β PDGFR to induce interleukin-3 (IL-3)-independent growth of a mouse myeloid precursor cell line (32D) which normally lacks expression of most known tyrosine kinase growth factor receptors. Cell proliferation induced by β PDGFR and E5 is also highly specific, since the highly conserved α PDGFR and other related receptors did not physically or functionally interact with E5 in these cells. In the current study, analysis of chimeric α and β PDGFRs confirmed that a short region encompassing the β PDGFR transmembrane domain was sufficient for complex formation with E5, receptor autophosphorylation, and sustained proliferation of 32D cells in the absence of IL-3. Furthermore, a deletion mutant lacking the entire extracellular domain efficiently bound E5 and induced IL-3-independent growth. These data provide direct evidence that the interaction between E5 and the β PDGFR involves amino acids 531 to 556 of the receptor transmembrane region and that this specific interaction is critical for activation of the PDGFR signalling complex.**

Papillomaviruses induce benign papillomas in humans and animals and have been linked to the development of malignant neoplasms, including cervical cancer (16, 23). To study transformation by papillomaviruses in mammalian cells, bovine papillomavirus type 1 (BPV-1) has been a useful model system because of its ability to induce foci on monolayers of immortalized rodent fibroblasts (7, 18). E5 is the major transforming gene of BPV-1 and encodes a protein which consists of only 44 amino acids, representing the smallest known viral transforming protein (2, 5, 28, 30). E5 has a 30-amino-acid, hydrophobic N-terminal domain which appears to function as a membrane-associating or transmembrane (TM) domain (30) and a 14-amino-acid, hydrophilic C-terminal region which contains two cysteine residues that are essential for homodimerization and cellular transformation (2, 15, 29).

It has been reported that E5 cooperates with growth factor receptors of the protein tyrosine kinase family to induce mitogenesis. These receptors include the epidermal growth factor receptor (EGFR), the colony-stimulating factor 1 receptor (CSF-1R), and the platelet-derived growth factor receptor (PDGFR) (3, 19, 22, 24, 25). Previous experiments have shown that the TM domain of E5 is sufficient for association with the PDGFR (10) and that a glutamine residue within this domain may be critical for interaction with PDGFR as well as with another E5-associated cellular protein, the 16-kDa V-ATPase protein (11). E5, PDGFR, and the 16-kDa protein appear to form a ternary complex in cells which is dependent upon their respective transmembrane domains (10). In addition, mutation of this glutamine residue can significantly alter the biological activity of E5 (15, 31). Furthermore, studies with receptor chimeras have suggested that the β PDGFR transmembrane domain may be essential for mitogenic signalling by E5 (3).

To further analyze the molecular requirements for E5-mediated signal transduction via the PDGFR, independent of the intrinsic expression of other tyrosine kinase receptors, we used a nontumorigenic murine myeloid precursor cell line (32D) which is strictly dependent on interleukin 3 (IL-3) for growth (12). This IL-3 dependence can be functionally abrogated by the ectopic expression of growth factor receptors and addition of the appropriate ligand (20, 26, 27). Coexpression of the β PDGFR and E5 protein induces sustained proliferation and receptor autophosphorylation in the absence of IL-3 and PDGF, indicating that E5 activates this receptor complex by a ligand-independent mechanism. Cell proliferation induced by PDGFR and E5 is also highly specific, since the highly conserved α PDGFR and other tyrosine kinase receptors cannot functionally interact with E5 in these cells (9). To identify the domains of the β PDGFR which are required for physical and functional interaction with the E5 protein, we generated a series of chimeras consisting of various combinations of ectodomains, TM domains, and cytoplasmic domains of the α and β PDGFRs. Analysis of these chimeric PDGFRs in 32D cells revealed that a short region encompassing the β PDGFR TM domain and juxtamembrane residues were required for E5 binding, receptor activation, and induction of IL-3-independent growth. To test directly whether E5-induced PDGFR activation was completely independent of the external ligand-binding domain, a β PDGFR mutant lacking the extracellular region (ΔPDGFR) was evaluated for its ability to functionally interact with E5. Our results suggest that specific residues within the TM and juxtamembrane domains of β PDGFR are

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required for interaction with E5 and induction of IL-3-independent growth of 32D cells.

## MATERIALS AND METHODS

**Construction of α and β PDGFR chimeric molecules.** The wild-type α and β PDGFR cDNAs were expressed from a previously described eukaryotic expression vector, designated pLTR-2 (4). To generate the chimeric α and β receptors, a combination of available restriction sites, site-directed mutagenesis, and PCR-directed mutagenesis was employed. To make the αββ and βαα constructs, a unique *Hpa*I site was introduced into the β PDGFR at the corresponding position of the unique *Hpa*I site of the α receptor (nucleotide 1368) by oligonucleotide site-directed mutagenesis. The mutation was generated by using the oligonucleotide 5′-CCTCTCCTTCCAGTTAACGATCAATGTCCCT-3′ on an mp19 clone of the β PDGFR *Xma*I-*Sph*I restriction fragment (nucleotides 467 to 1679). The mutated fragment was isolated from the replicative form of the phage by digestion of the mp19 clone with *Xma*I and *Sph*I enzymes. This fragment was ligated into the previously described pUC18BSbPR plasmid (14) which had been cleaved with *Xma*I and *Sph*I and purified from the *Xma*I-*Sph*I receptor fragment. The chimeras αββ and βαα were then generated by fragment switching between the pUC18BSαPR, which contains the wild-type α PDGFR, and pUC18BSβPR, which contains the β PDGFR with the engineered *Hpa*I site. Both plasmids were cleaved with *Hpa*I and *Xba*I (which cleaves at the 3′ end of both inserts). Chimeric receptors were isolated as *Sal*I fragments and then inserted into the *Xho*I site of the pLTR-2 expression vector. The resulting chimeric receptors contain the first four immunoglobulin (Ig)-like domains of one receptor fused to the fifth Ig-like domain, the TM domain, and the cytoplasmic domain of the other receptor.

The ββα chimera was generated by a two-step PCR method. In one amplification on a β PDGFR template, two oligonucleotides were used to amplify a 1.6-kb fragment encoding the entire ectodomain and TM domain of the β receptor. The outside oligonucleotide was designed to anneal to the 5′ end of the β PDGFR cDNA and contained an *Xho*I site at its end. The 3′ oligonucleotide was a 40-mer, 20 bp of which annealed to the last 20 bp of the β TM domain and an additional 20 bp representing the first nucleotides of the α intracellular region (5′-TCATACCTCGGTTTCTGTTT/CCAAAGCATGATGAGGATGA-3′). A second amplification with an α PDGFR as a template was carried out with an oligonucleotide which annealed to the 3′ end of the α PDGFR cDNA (with *Bam*HI end) and an oligonucleotide representing the fusion of the cytoplasmic region of the α PDGFR to the TM domain of the β PDGFR (5′-TCATCCT CATCATGCTTTGG/AAACAGAAACCGAGGTA-3′). A PCR amplification with these oligonucleotides generated a fragment of 1.6 kb. The two PCR fragments were gel purified and used as templates for a subsequent PCR amplification with both outside oligonucleotides. A 3.4-kb fragment corresponding to a fusion of the two products of the first PCRs was generated. This fragment was restricted with *Xho*I and *Bam*HI enzymes and cloned in the *Sal*I and *Bam*HI sites of pLTR-2. The fusion between the β domains to the α intracellular domain was confirmed by dideoxynucleotide sequencing.

The ααβ construct was generated by fusing the α receptor ectodomain and TM domain to the cytoplasmic region of the β PDGFR. The α receptor ectodomain and TM domain were purified from pUC18BSαPR as a 1.9-kb *Sal*I-*Spe*I fragment (*Spe*I cleaves at position 1915 within the juxtamembrane region of the α PDGFR). The β cytoplasmic domain was generated by PCR. The 5′ oligonucleotide used for the PCR amplification had an *Spe*I site linked to 14 bp corresponding to the β juxtamembrane sequence (nucleotide position 2157), where the *Spe*I site is found in the α receptor (nucleotide position 1915). The 3′ oligonucleotide annealed to the 3′ end of the β PDGFR gene and had a *Sal*I site at the 5′ end. The PCR product was cleaved with *Spe*I and *Sal*I, gel purified, and mixed with the 1.9-kb *Sal*I-*Spe*I α receptor fragment in a three-part ligation into the pLTR-2 plasmid which had been linearized with *Xho*I.

For constructing the β PDGFR deletion mutant lacking the extracellular domain (ΔPDGFR), a PCR product representing amino acids 531 to the C terminus of the β PDGFR was generated. The oligonucleotides used for this PCR amplification included the restriction sites for *Eco*RI (5′) and *Bam*HI (3′) at their 5′ ends. This fragment was gel purified and subcloned into a pUC18 plasmid containing the 5′ end of the α PDGFR cDNA through the unique *Eco*RI site at nucleotide 262. This region encodes the amino-terminal 43 amino acids of the α PDGFR, including its signal sequence. The *Sal*I fragment containing the entire mutant cDNA was then subcloned into the *Xho*I site of the expression vector pLTR-2.

**Cell lines and transfections.** The murine IL-3-dependent hematopoietic cell line 32D (12) was cultured in RPMI 1640 medium (GIBCO, Grand Island, N.Y.) supplemented with 15% fetal bovine serum (FCS) 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 (27). Populations of stably transfected receptor-expressing transfectants were selected by growth in medium containing 80 mM mycophenolic acid plus hypoxanthine, aminopterin, and thymidine (1× HAT; GIBCO). E5-expressing transfectants were selected in geneticin (750 μg/ml; GIBCO). Cell transfectants expressing wild-type β PDGFR or PDGFR chimeras containing β TM domains plus E5 were routinely passaged in RPMI 1640 medium lacking IL-3. All other cell lines were passaged in medium supplemented with IL-3.

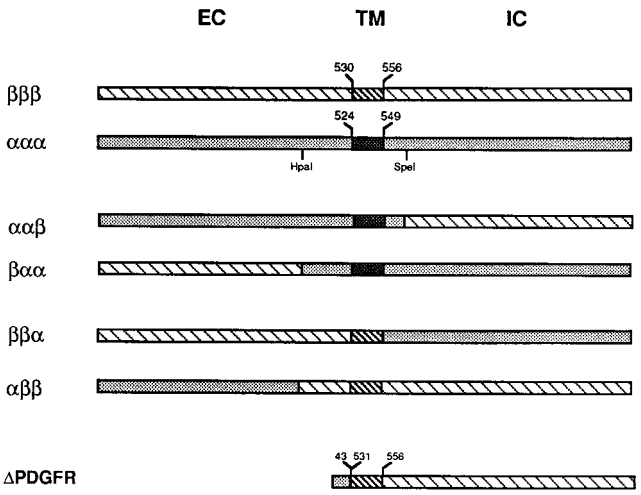



FIG. 1. Construction of α/β PDGFR chimeras and a deletion mutant of the β PDGFR. Four different chimeras of the α and β PDGFRs and a deletion mutant of the β PDGFR were generated by using available restriction sites or PCR methods (see Materials and Methods). This schematic representation of the modified receptors shows the different combinations of extracellular (EC), TM, and intracellular (IC) domains. The chimeras and the wild-type α and β PDGFRs were subcloned into the pLTR-2 expression vector.

**Growth curve of 32D cell transfectants.** Cells were seeded at $3 \times 10^5$ cells per ml in RPMI medium supplemented with 15% FCS but without IL-3 and transferred at a 1:10 dilution every 2 days. Cell number was determined daily after testing for cell viability by trypan blue exclusion.

**Immunoprecipitation and immunoblot analysis.** 32D cell transfectants were assayed for phosphotyrosine-containing receptors as previously described (9). Lysates were first immunoprecipitated with 10 μl of an anti-PDGFR antiserum that recognizes both α and β species (UBI; 06-132). Protein A-Sepharose CL-4B (50 ml of a 1:1 suspension; Pharmacia, Piscataway, N.J.) was 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 lysis 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 7.5% polyacrylamide gels. Immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in duplicate, transferred to Immobilon-P membranes (Millipore, Bedford, Mass.), and immunoblotted with either an antiphosphotyrosine antibody (1:500 dilution of 4G10; UBI) or an anti-PDGFR antibody (1:500 dilution of 06-132). Proteins were visualized by chemiluminescence with the Tropix kit (Tropix, Bedford, Mass.). Membranes were typically exposed to film for between 30 s and 10 min.

For detection of E5 and growth factor receptor complexes, cell lysates were analyzed as previously described (9) with either an antiserum directed against the 15 carboxyl-terminal amino acids of E5 (5 μl) or an anti-β PDGFR polyclonal antibody (10 μl of 06-132; UBI) plus 50 μl of protein A-Sepharose beads (Pharmacia) per ml of lysate. Immunoprecipitated proteins were electrophoretically separated on either 7.5% (to resolve receptor proteins) or 14% (to resolve E5 proteins) polyacrylamide gels and transferred to Immobilon-P. Membranes were washed twice with phosphate-buffered saline and processed according to the Tropix chemiluminescence kit protocol. E5 protein was detected with the anti-E5 antiserum at a 1:5,000 dilution. The receptors were detected with the antiserum that recognizes both α and β species (UBI; 1:500 dilution).

## RESULTS

Our previous studies demonstrated that the ability of the BPV-1 E5 protein 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 (9). In this study, we exploited the ability of the E5 protein to differentially bind and activate the α and β PDGFR species to identify the region(s) of the β PDGFR which governs these processes.

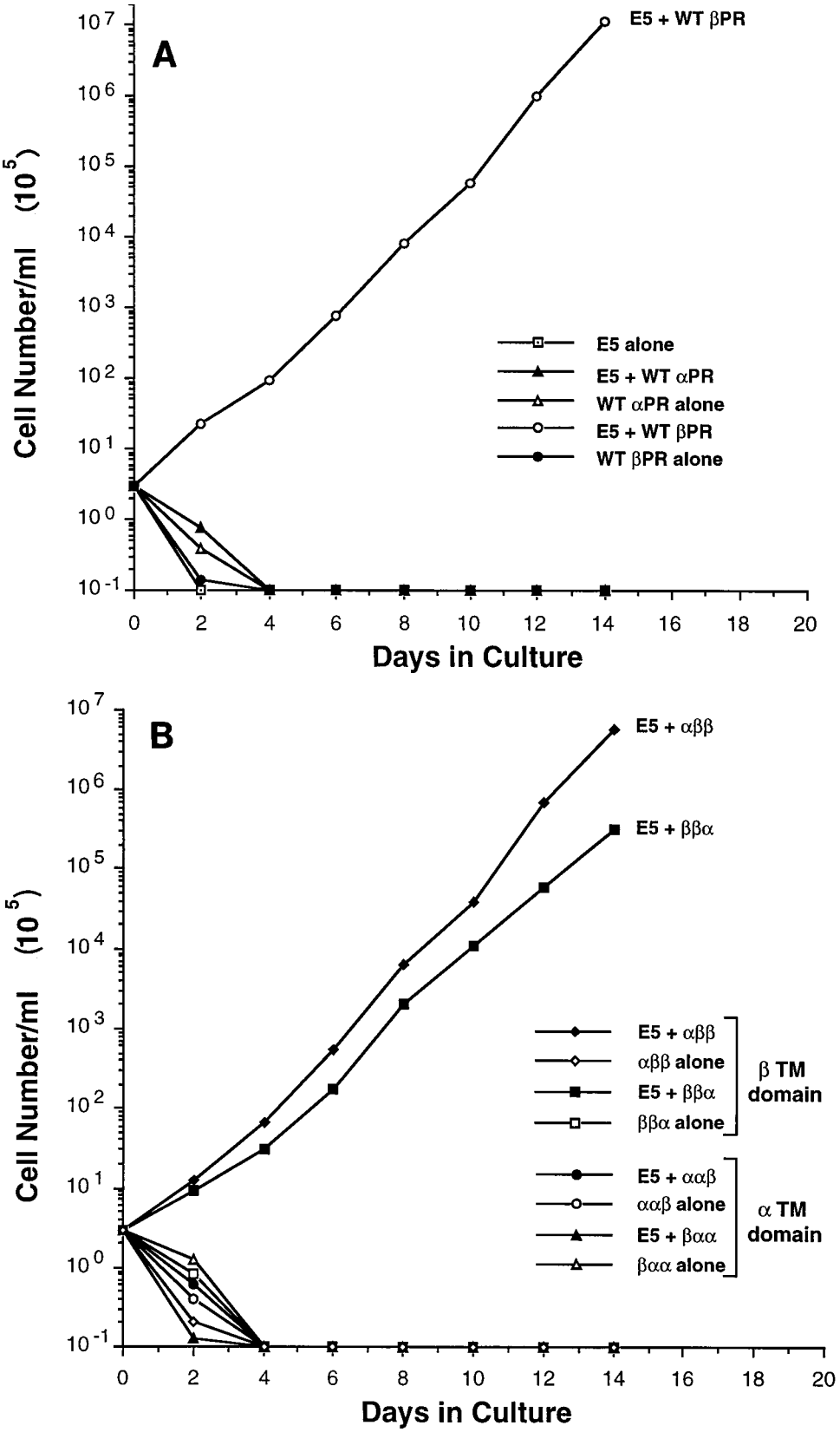



FIG. 2. β PDGFR region containing amino acids 410 to 556 is essential for E5/PDGFR-induced IL-3-independent growth of 32D cells. Different 32D cell transfectants expressing wild-type (A) and chimeric (B) PDGFR molecules in the presence or absence of E5 were tested for their ability to grow independently of IL-3. Cells were seeded at $3 \times 10^5$ cells per ml in RPMI medium supplemented with 15% FCS but without IL-3 and transferred at a 1:10 dilution every 2 days. Cell number was determined every second day after testing for cell viability by trypan blue exclusion.

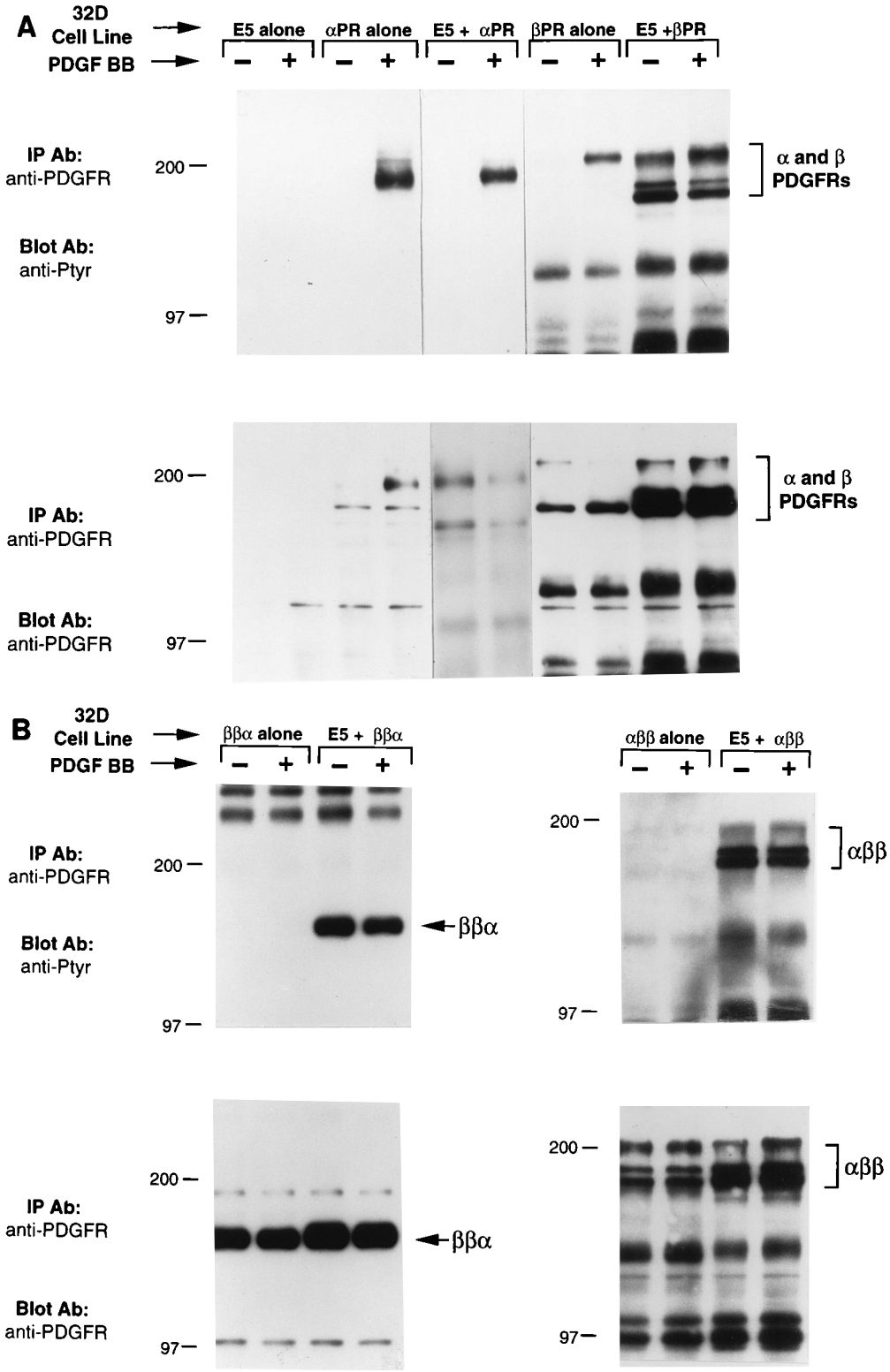



FIG. 3. E5 protein stimulates the constitutive tyrosine phosphorylation of PDGFR molecules in 32D cell transfectants only if they contain amino acids 410 to 556 of the β PDGFR. 32D cell transfectants, as indicated above each panel, were analyzed for the presence of tyrosine-phosphorylated receptors by combined immunoprecipitation-immunoblot analysis with and without prior stimulation with PDGF BB (250 ng/ml) as described previously. Analysis was performed on cell lines expressing E5 and wild-type PDGFRs (A) or E5 and chimeric receptors containing the TM domains of either β PDGFR (B) or α PDGFR (C). Cells expressing receptor or receptor chimeras alone were grown in parallel and used as negative controls. Following immunoprecipitation with anti-PDGFR antibodies, the precipitated proteins were separated by SDS-PAGE on a 7.5% gel and transferred to polyvinylidene difluoride membranes. Immunoblot detection was performed with either an antiphosphotyrosine monoclonal antibody (top panels) or antireceptor sera (lower panels). Precipitated proteins were then visualized with alkaline phosphatase-conjugated secondary antibodies and chemiluminescence. The positions of the wild-type and chimeric receptors are indicated on the right, and positions (in kilodaltons) of molecular mass standards are indicated on the left.

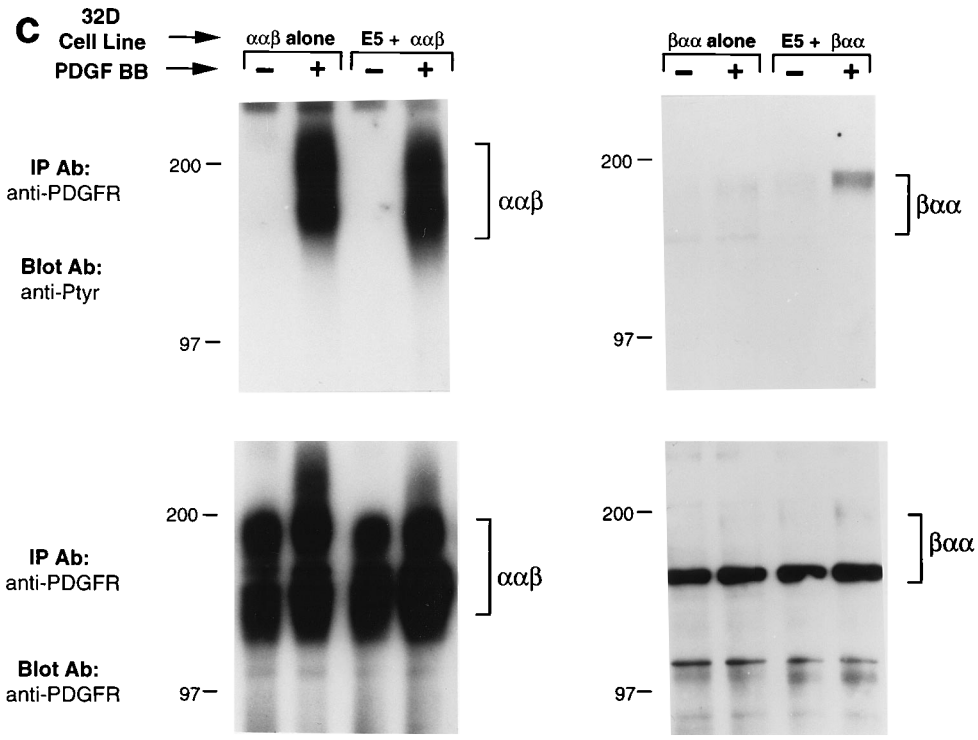


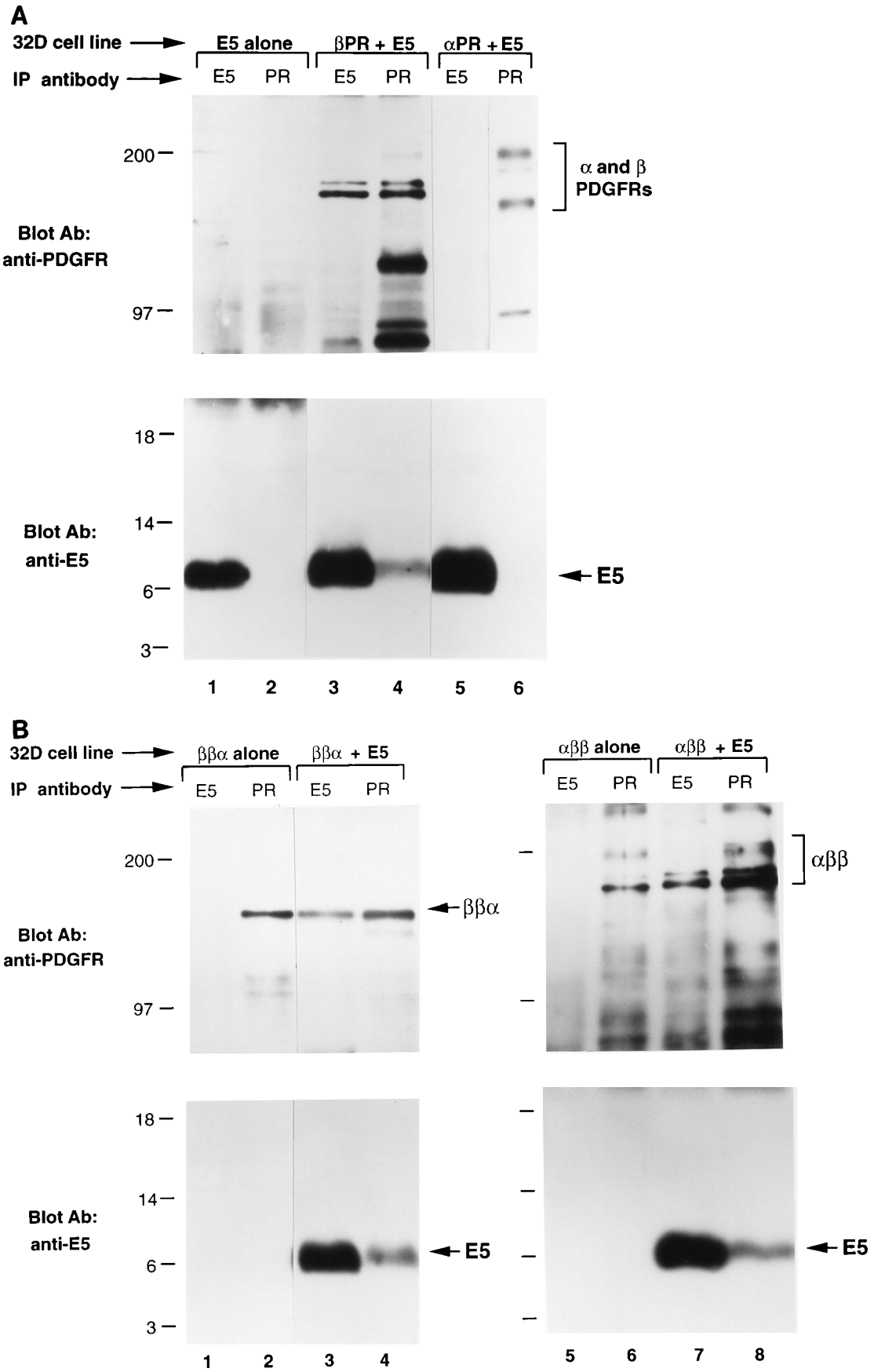

FIG. 3—*Continued.*

**Construction of α/β PDGFR chimeras.** Four different α and β PDGFR chimeras were generated by using available restriction sites or by PCR methods (see Materials and Methods). The αββ and βαα chimeras were generated by switching four of the five amino-terminal Ig-like domains within the extracellular regions (Fig. 1). The ββα construct consists of the entire β PDGFR extracellular and TM regions (amino terminus to the first intracellular juxtamembrane amino acid, Trp-556) fused to the α PDGFR intracellular domain (from the second juxtamembrane amino acid, Lys-549, to the carboxyl terminus). The ααβ chimera was generated by switching the α PDGFR intracellular region with the corresponding β PDGFR region at a unique *Spe*I site located within the α PDGFR intracellular juxtamembrane domain (see Materials and Methods for details) (Fig. 1). The cDNAs for the four chimeras, as well as the wild-type α and β PDGFRs, were inserted into the pLTR-2 expression vector, designed to transcribe genes from the Moloney murine leukemia retrovirus long terminal repeat. These plasmids also express the *gpt* gene, conferring resistance to mycophenolic acid and HAT. Plasmids encoding all four chimeric receptor genes were individually used to transfect either parental 32D cells or 32D cells expressing the E5 protein (E5:32D). Both of these cell lines required murine IL-3 for growth (9, 27).

**A region contained within amino acids 410 to 556 of the β PDGFR is essential for E5/PDGFR-induced IL-3-independent growth of 32D cells.** Cell lines expressing transfected receptors and E5 were selected for resistance to mycophenolic acid and HAT and for the ability to proliferate in the absence of IL-3. To further characterize the growth phenotype of receptor-expressing cells, cell lines expressing each receptor alone or in combination with E5 were subjected to a growth assay in medium lacking IL-3 (Fig. 2). Only those cell lines coexpressing E5 and PDGFRs containing amino acids 410 to 556 of the β PDGFR were capable of sustained proliferation in the absence of IL-3, suggesting that the receptor domain required for functional interaction with E5 encompasses the β TM domain (amino acids 531 to 556). All cell lines expressed high levels of both receptor and E5 proteins, as determined by combined immunoprecipitation and immunoblot procedures (Fig. 3). Therefore, the failure of the receptors lacking this region of the β PDGFR to induce growth of E5:32D cells was not due to low levels of either E5 or receptor proteins, but instead was due to the absence of the described region of the receptor protein.

It has been shown previously that cotransfection of NIH 3T3 cells with the β PDGFR enhanced the transforming activity of the E5 gene (3). Therefore, a cotransfection assay with all vectors carrying the wild-type and chimeric receptor cDNAs together with the E5 gene vector was also performed in these cells. Only those receptor chimeras containing amino acids 410 to 556 of β PDGFR enhanced E5 focus formation of NIH 3T3 cells. Furthermore, the size of foci was significantly augmented by the cotransfection of these constructs with E5. In contrast, the α PDGFR and chimeras containing the equivalent TM region of the α PDGFR were ineffective in increasing the number or size of E5-induced foci. Consistent with its behavior in 32D cells, therefore, E5 cooperated with only those chimeras containing the β PDGFR TM domain in a murine fibroblast cell line (data not shown).

**E5 activates only α/β chimeras containing amino acids 410 to 556 of the β PDGFR.** To determine if the factor-independent growth induced by the coexpression of E5 and β PDGF chimeras containing amino acids 410 to 556 was due to the ability of E5 to specifically activate these receptors, coexpressing cell lines were examined for the presence of autophosphorylated receptors by a combined immunoprecipitation-immunoblot procedure. To demonstrate that all receptors possessed functional tyrosine kinase activity, receptor-expressing cells were stimulated with exogenous PDGF BB, which equally ac-


A
32D cell line
E5 alone
βPR + E5
αPR + E5
IP antibody
E5
PR
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PR
E5
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200
97
Blot Ab:
anti-PDGFR
α and β
PDGFRs
18
14
6
3
Blot Ab:
anti-E5
E5
1
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6
B
32D cell line
ββα alone
ββα + E5
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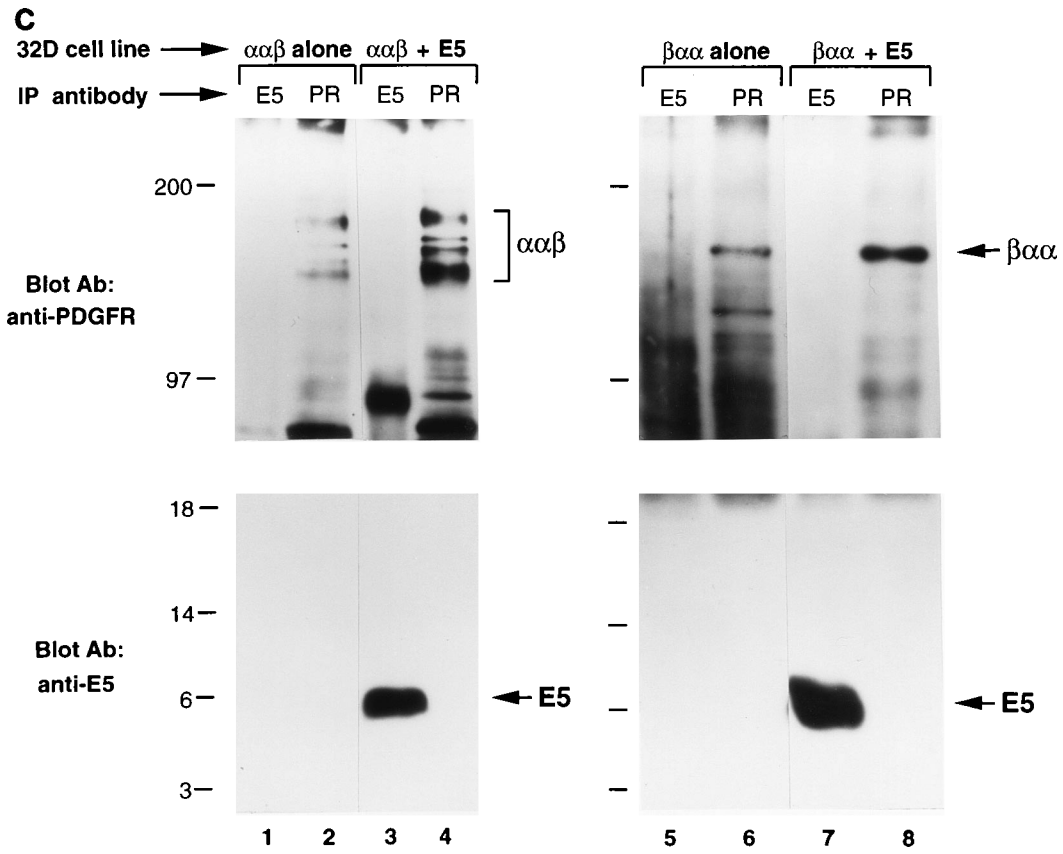



FIG. 4. E5 protein forms a complex only with α/β chimeras containing the β PDGFR TM domain. Independently isolated 32D cell transfectants expressing wild-type PDGFR (A) or chimeras containing either the β TM domain (B) or the α TM domain (C) were extracted in Triton X-100 buffer and immunoprecipitated with a polyclonal antiserum raised against the 14 C-terminal amino acids of E5 or against the intracellular domain of PDGFR. Cells expressing E5 or the respective receptor molecules alone were included as negative controls. The immunoprecipitates were separated on 7.5% (top panels) or 14% (bottom panels) polyacrylamide–SDS gels (SDS-PAGE) and transferred to polyvinylidene difluoride membranes. Western detection of immunoprecipitated proteins was performed with anti-PDGFR (1:1,000) (top) or anti-E5 (1:5,000) (bottom) antiserum, alkaline phosphatase-conjugated secondary antibodies, and a chemiluminescence detection kit (Tropix). The positions of E5 and PDGFR proteins are indicated on the right, and the positions (in kilodaltons) of molecular mass standards are indicated on the left.

tivates α and β receptors. In accordance with earlier studies (9, 25), E5 clearly stimulated the constitutive basal tyrosine phosphorylation of only the β PDGFR, independent of PDGF BB (Fig. 3A). Several species of activated PDGFR were detected: the lower-molecular-weight species presumably represents immature precursors of the larger, fully glycosylated, mature receptor (17). Constitutive autophosphorylation of receptor species was observed in cells that were selected either by IL-3 independent growth or by drug resistance in the presence of IL-3.

Cell transfectants coexpressing E5 and either αββ or ββα chimeras showed a significant increase in tyrosine phosphorylation in the absence of ligand (Fig. 3B), whereas cell lines expressing E5 and either ααβ or βαα receptors showed no detectable tyrosine-phosphorylated receptor (Fig. 3C). The E5-induced activation of PDGFR autophosphorylation therefore requires amino acids 410 to 556 of the β PDGFR. Cell transfectants expressing the ααβ chimera demonstrated readily detectable tyrosine phosphorylation of receptors in response to ligand (Fig. 3C, lanes 2 and 4), indicating that this chimera possesses functional tyrosine kinase activity. There was also an observable increase in receptor tyrosine phosphorylation in lines expressing the βαα chimera, although to a lesser extent (Fig. 3C, lanes 6 and 8). We were, however, unable to detect exogenous ligand stimulation of receptor autophosphorylation in cell lines expressing either ββα or αββ chimeras. This was most likely due to the intracellular retention of these receptors (Fig. 4B and C). Moreover, we were unable to detect these receptors on the cell surface, while α and β wild-type, ααβ, and βαα PDGFRs could be detected by fluorescence-activated cell sorting (FACS) with anti-α PDGFR and anti-β PDGFR antibodies directed against the receptor extracellular domain (data not shown). This indicated that failure to detect an increase in chimeric receptor phosphorylation in the presence of exogenously applied ligand was likely due to inaccessibility of the ligand to these internally localized chimeras.

To provide additional proof that the chimeric receptors which were not activated by E5 (ααβ and βαα) were indeed functional receptors, the c-*sis* proto-oncogene (PDGF BB gene) was cotransfected with these chimeric receptors into 32D cells. Cell transfectants were assayed for IL-3-independent growth. Whereas cells transfected with c-*sis* alone failed to grow in the absence of IL-3, both chimeras readily induced IL-3-independent cell lines when cotransfected with c-*sis*. This confirms that these chimera constructs expressed functional receptors that could be activated by internally expressed ligand and that the activation was independent of subcellular localization (data not shown).

**E5 forms a complex only with α/β chimeras containing amino acids 410 to 556 of the β PDGFR.** To determine if the ability of the E5 protein to activate only chimeras containing

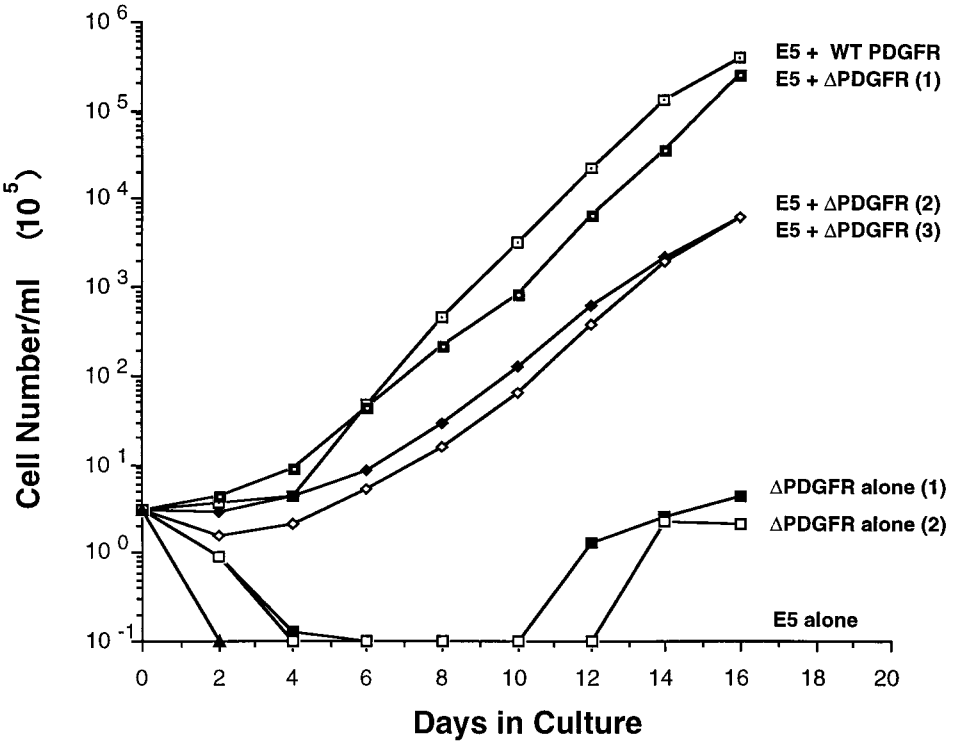



FIG. 5. E5 protein can cooperate with a β PDGFR lacking the extracellular domain to induce IL-3-independent growth of 32D cells. Independently isolated 32D cell transfectants expressing wild-type β PDGFR or the deletion mutant (ΔPR) in the presence or absence of E5 were tested for their ability to grow independently of IL-3. Cells were seeded at $3 \times 10^5$ cells per ml in RPMI medium supplemented with 15% FCS but without IL-3 and transferred at a 1:10 dilution every 2 days. Cell number was determined every second day after testing for cell viability by trypan blue exclusion. Numbers in parentheses correspond to clonal isolates.

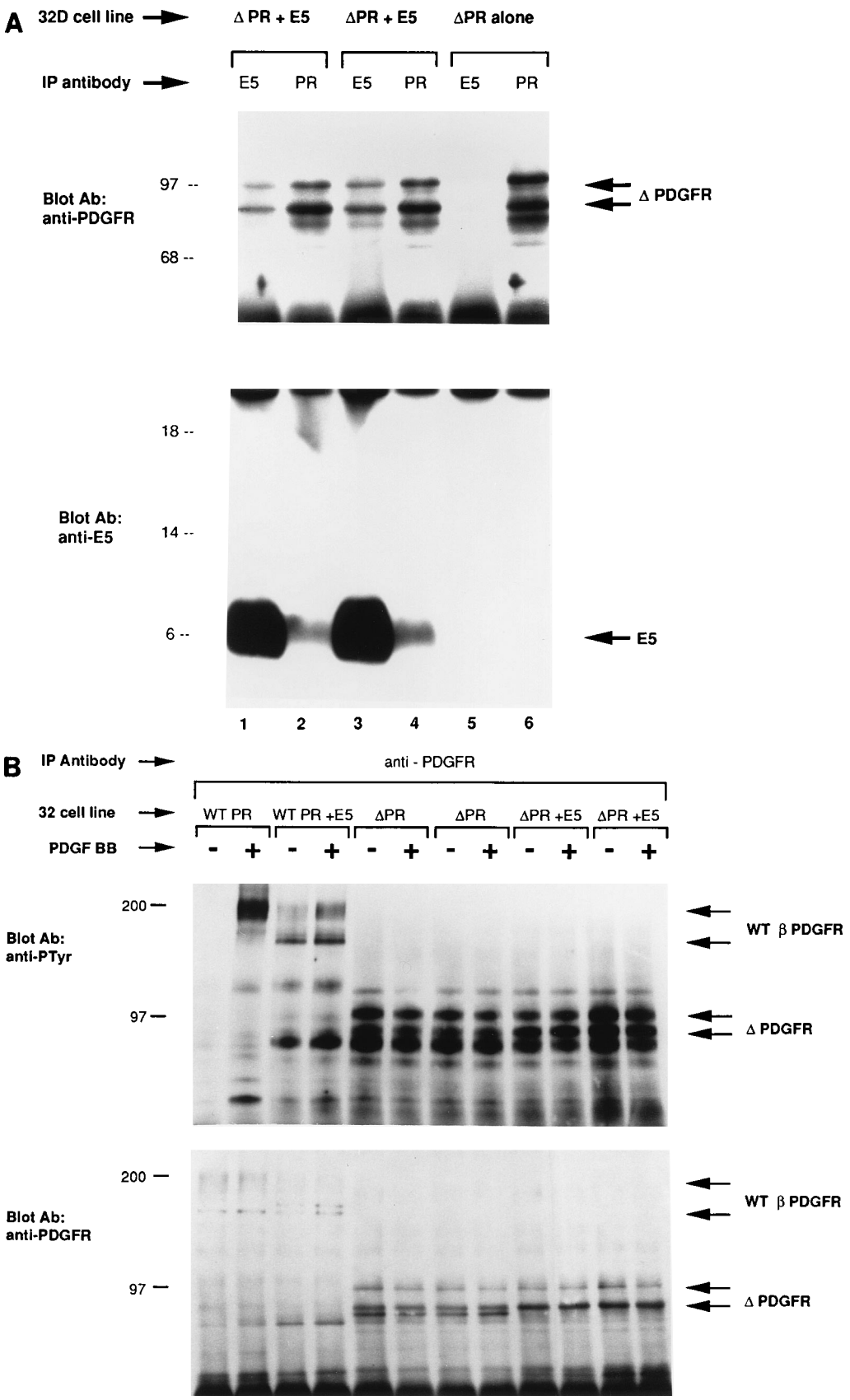
amino acids 410 to 556 of the β PDGFR was due to specific association of E5 with these particular chimeric receptors, a coimmunoprecipitation experiment was performed (Fig. 4) with lysates from cell lines expressing E5 and each wild-type or mutant receptor. Receptor proteins were readily detected in anti-E5 immunoprecipitates from E5 and wild-type β PDGFR (Fig. 4A, lane 3), ββα-, and αββ-coexpressing cells (Fig. 4B, lanes 3 and 7). In addition, E5 protein was readily detected on immunoblots of anti-PDGFR immunoprecipitates in those cell lines (Fig. 4A, lane 4, and Fig. 4B, lanes 4 and 8). In striking contrast, no association could be detected between E5 and the α PDGFR or the other chimeras, ααβ and βαα, with use of either anti-E5 antibodies (Fig. 4A, lane 5, and Fig. 4C, lanes 3 and 7) or anti-PDGR antibodies (Fig. 4A, lane 6, and Fig. 4C, lanes 4 and 8). These results strongly suggest that the portion of the receptor molecule required for E5 interaction lies between amino acids 410 and 556, containing the fifth Ig-like domain and TM region of β PDGFR.

**E5 cooperates with a β PDGFR molecule lacking the entire extracellular domain (ΔPDGFR).** Since the αββ chimera retained the fifth Ig-like domain and juxtamembrane region of the β PDGFR, we constructed a β PDGFR mutant that lacked the entire extracellular domain and evaluated its ability to interact functionally and physically with E5. To ensure proper insertion and orientation of the deletion mutant (ΔPDGFR) within cell membranes, we fused the first 43 amino acids of the α PDGFR, containing the signal sequence, to the first polar residue (Lys-531) outside the TM domain of β PDGFR. The cDNA for the deletion mutant was inserted into the same pLTR-2 expression vector that was used for the wild-type and chimeric receptors.

To determine whether ΔPDGFR could interact with E5 and transform 32D cells, we assayed different 32D cell transfectants for their ability to grow in the absence of IL-3 (Fig. 5). Cell lines expressing either β PDGFR alone (R:32D) or E5 alone (E5:32D) were unable to survive under these conditions. Cells expressing ΔPDGFR alone (ΔR:32D) were also drastically reduced in their ability to grow. However, transfection of E5 into ΔR:32D cells conferred the same IL-3-independent growth potential as in cell lines expressing E5 and the wild-type receptor. These results suggest that E5 can initiate signal transduction via the β PDGFR without the need for the entire receptor extracellular domain, including the fifth Ig-like loop.

To determine whether ΔPDGFR could also form a complex

FIG. 6. E5 protein binds to a β PDGFR mutant lacking the entire extracellular domain without increasing basal receptor tyrosine phosphorylation. (A) Different 32D cell transfectants expressing the deletion mutant (ΔPR) in the absence or presence of E5 were extracted in Triton X-100 buffer and subjected to the combined immunoprecipitation-immunoblotting procedure described in the legend to Fig. 4. The positions of E5 and ΔPR proteins are indicated on the right, and the positions (in kilodaltons) of molecular mass standards are indicated on the left. (B) Different 32D cell transfectants were analyzed for tyrosine-phosphorylated receptors by combined immunoprecipitation-immunoblot analysis with and without stimulation with PDGF BB (250 ng/ml; GIBCO BRL) as described previously (9). Cells were starved in the absence of serum for 3 h and lysed in Triton X-100 buffer with phosphatase inhibitors 10 min after stimulation with PDGF. Following immunoprecipitation with anti-PDGFR antibodies, the precipitated proteins were separated by SDS-PAGE on a 7.5% gel and transferred to polyvinylidene difluoride membranes. Immunoblot detection was performed with either antiphosphotyrosine antibodies (05-321; UBI; 1:500, top panel) or anti-PDGFR antibodies (bottom panel). The positions of the wild-type and deleted PDGFR are indicated on the right, and the positions (in kilodaltons) of molecular mass standards are indicated on the left.


A
32D cell line
Δ PR + E5
ΔPR + E5
ΔPR alone
IP antibody
E5
PR
E5
PR
E5
PR
Blot Ab:
anti-PDGFR
97 --
68 --
Δ PDGFR
18 --
Blot Ab:
anti-E5
14 --
6 --
E5
1 2 3 4 5 6
B
IP Antibody
anti - PDGFR
32 cell line
WT PR
WT PR +E5
ΔPR
ΔPR
ΔPR +E5
ΔPR +E5
PDGF BB
- + - + - + - + - + - +
200 —
Blot Ab:
anti-PTyr
97 —
WT β PDGFR
Δ PDGFR
200 —
Blot Ab:
anti-PDGFR
97 —
WT β PDGFR
Δ PDGFR

with E5, as anticipated from its biological cooperativity with E5, coprecipitation experiments were performed with cell lines expressing ΔPDGFR and E5 or ΔPDGFR alone (Fig. 6A). As expected, ΔPDGFR was detected as a protein of approximately half the molecular weight of the full-length receptor. ΔPDGFR was detected in anti-E5 immunoprecipitates when immunoblotted with anti-PDGFR antiserum (Fig. 6A, top panel, lanes 1 and 3). No receptor molecule was precipitated by anti-E5 antibody in a cell line expressing high levels of only ΔPDGFR (lane 5). When anti-PDGFR immunoprecipitates were analyzed for the presence of E5 by immunoblotting with anti-E5 antiserum, complex formation was readily detected (Fig. 6A, bottom panel, lanes 2 and 4).

When assayed for tyrosine phosphorylation by immunoprecipitation-immunoblotting, ΔPDGFR showed elevated baseline phosphorylation even in the absence of E5 (Fig. 6B). Cotransfection with E5 did not result in any significant increase in tyrosine phosphorylation despite the obvious difference in phenotype, which is presumably the result of E5 binding to the receptor molecule. Binding studies and the capacity for IL-3-independent growth showed that E5 retained the ability to interact functionally and physically with a β PDGFR lacking the entire extracellular domain. From the results with the α/β receptor chimeras and the deletion mutant, we therefore conclude that the site necessary for the full phenotypic effect of the interaction between the β PDGFR and the E5 molecule lies between the juxtamembrane Lys-531 and Trp-556, which includes the entire TM domain plus a single extracellular lysine residue.

## DISCUSSION

Several lines of evidence suggest that E5 cooperates with growth factor receptors to induce cellular transformation. For instance, the transforming ability of the E5 protein in a mouse fibroblast transformation assay is enhanced when E5 is cotransfected with the EGFR, CSF-1R, or PDGFR (19). However, more recent studies indicate that E5 preferentially binds and activates β PDGFR but not related tyrosine kinase-containing growth factor receptors (9, 22, 24). The specificity exhibited by E5 in these later studies suggests that transformation of fibroblasts by cotransfection of E5 and receptor genes may be affected by the expression of multiple growth factor receptors in these cells. For example, it is possible that abundant expression of one receptor type by transient transfection might influence the activity of other endogenously expressed receptors.

In the present study, we used 32D cells, which enable both biological and biochemical studies in a single cell type. These cells do not express most growth factor receptors present in fibroblasts and epithelial cells, allowing us to evaluate the domains of β PDGFR which are required for interaction with the BPV-1 E5 protein. Furthermore, 32D cells are also strictly dependent on IL-3 for growth, permitting selection for only those cells exerting a mitogenic stimulus through productive E5 interaction with the exogenously expressed receptors. Therefore, we used 32D cells to analyze chimeras between the β and α PDGFRs for their interaction with the E5 protein. The latter receptor, despite extensive amino acid homology with the β PDGFR, does not interact with E5 and therefore provides an ideal partner for domain switching.

It has recently been demonstrated that the ligand-binding specificities of the PDGFRs lie within the first three Ig-like domains of the extracellular domain (13, 14). Therefore, to determine if the ligand-binding specificity of the β PDGFR played a role in the physical and functional interaction with the E5 protein, we evaluated two chimeric receptors whose first four Ig-like domains had been switched reciprocally. Despite only 31% amino acid homology within the extracellular domains, neither functional nor physical interaction of E5 with these chimeras was influenced by the domain switch. This result suggests that the ligand-binding site of the β PDGFR is not involved in interaction with E5.

Only chimeras that contained a segment encompassing the juxtamembrane region on the extracellular side and the TM domain of the β PDGFR were able to cooperate with the E5 oncoprotein for induction of IL-3 independence in 32D cells. Like wild-type β PDGFR in the presence of cotransfected E5, these chimeras were constitutively phosphorylated on tyrosine residues and bound to E5, as determined by coprecipitation. Neither wild-type α PDGFR nor chimeras containing the α PDGFR TM domain showed any of these properties.

The region in the β PDGFR required for cooperation and binding to E5 was further delineated to the TM domain, including a single lysine of the extracellular domain, by using a β PDGFR deletion mutant. This mutant (ΔPDGFR) was able to bind to E5 and cooperate for induction of IL-3 independence. Despite elevated phosphorylation levels of the receptor mutant even in the absence of E5 (Fig. 6B), the cells containing ΔPDGFR alone either underwent apoptosis or grew much more slowly than those cotransfected with E5. Studies with this mutant also underline the fact that the E5 molecule can interact with the β PDGFR TM domain independently of the extracellular domain with the exception of Lys-531. This positively charged residue might interact with the negatively charged Asp-33 of the E5 molecule, as suggested by Meyer et al. (21). These results are consistent with the recent work of Drummond-Barbosa et al., which showed that a β PDGFR lacking all but six amino acids of the extracellular domain retained the ability to interact with E5 (6). In contrast to that study, we were unable to detect an increase in levels of tyrosine phosphorylation of our deleted receptor after cotransfection with E5.

The two PDGFR types have the highest degree of homology (80%) in their cytoplasmic tyrosine kinase domains. It was therefore not unexpected that the β PDGFR cytoplasmic region, when replaced by the α cytoplasmic region (ββα), had no effect on the ability of the receptor to physically or functionally interact with the E5 protein. This suggests that any difference in substrate specificity exhibited by the tyrosine kinases of the α or β species (8) does not affect either receptor binding to E5 or the ability of the receptor to cooperate with E5 to induce IL-3-independent growth of 32D cells.

Several lines of evidence suggest that two of the PDGFR chimeras (ββα and αββ) cannot reach the cell surface. For example, we were unable to detect these receptors by FACS analysis with an antibody that recognizes the PDGFR extracellular domain (unpublished data). Second, we consistently failed to detect tyrosine phosphorylation of these chimeras following exogenous application of PDGF BB. Nevertheless, these receptor molecules were able to couple with E5 or c-*sis* to induce IL-3-independent growth. In addition, these receptors exhibited readily detectable tyrosine phosphorylation in response to E5 expression. These mutants will thus provide the opportunity to examine the signaling pathways engaged by an intracellularly localized PDGFR in response to either E5 or c-*sis* expression.

Previous data suggest that the E5 oncoprotein interacts with at least two of its known targets via its TM domain. The hydrophobic domain of E5 alone retained the ability to bind to the β PDGFR and the 16-kDa component of the vacuolar proton ATPase (10). Furthermore, mutation of the hydrophilic

amino acid Gln-17 in the center of the hydrophobic TM domain changes both binding to these two targets and the transforming potential of E5 (11, 31). It has recently been demonstrated by mutagenic analysis that the direct interaction with the 16-kDa protein is mediated by the formation of a charged intramembrane pair (1).

The interaction of E5 with the PDGFR, however, is not as well understood. Our approach in 32D cells now provides the opportunity for a detailed molecular and functional analysis of the β PDGFR TM domain without interference by intrinsically expressed receptor molecules. By correlating the biochemical properties of TM mutants with the ability to induce IL-3 independence, we will gain further insight into the molecular requirements for the cooperation between β PDGFR, E5, and the 16-kDa protein.

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## REFERENCES

1. **Andresson, T., J. Sparkowski, D. J. Goldstein, and R. Schlegel.** 1995. V-ATPase mutants transform cells and define a binding site for the papillomavirus E5 oncoprotein. J. Biol. Chem. **270:**6830–6837.
2. **Burkhardt, A., D. DiMaio, and R. Schlegel.** 1987. Genetic and biochemical definition of the bovine papillomavirus E5 transforming protein. EMBO J. **6:**2381–2385.
3. **Cohen, B. D., D. J. Goldstein, L. Rutledge, W. C. Vass, D. R. Lowy, R. Schlegel, and J. T. Schiller.** 1993. Transformation-specific interaction of the bovine papillomavirus E5 oncoprotein with the platelet-derived growth factor receptor transmembrane domain and epidermal growth factor receptor cytoplasmic domain. J. Virol. **67:**5303–5311.
4. **Di Fiore, P. P., J. H. Pierce, T. P. Fleming, R. Hazan, A. Ullrich, C. R. King, J. Schlessinger, and S. A. Aaronson.** 1987. Overexpression of the human EGF receptor confers an EGF-dependent transformed phenotype to NIH 3T3 cells. Cell **51:**1063–1070.
5. **DiMaio, D., D. Guralski, and J. T. Schiller.** 1986. Translation of open reading frame E5 of bovine papillomavirus is required for its transforming activity. Proc. Natl. Acad. Sci. USA **83:**1797–1801.
6. **Drummond-Barbosa, D., R. R. Vaillancourt, A. Kazlauskas, and D. DiMaio.** 1995. Ligand-independent activation of the platelet-derived growth factor β receptor: requirements for bovine papillomavirus E5-induced mitogenic signaling. Mol. Cell. Biol. **15:**2570–2581.
7. **Dvoretzky, I., R. Shober, S. K. Chattopadhyay, and D. R. Lowy.** 1980. A quantitative *in vitro* assay for bovine papilloma virus. Virology **103:**369–375.
8. **Eriksson, A., A. Siegbahn, B. Westermark, C.-H. Heldin, and L. Claesson-Welsh.** 1992. PDGF alpha- and beta-receptors activate unique and common signal transduction pathways. EMBO J. **11:**543–550.
9. **Goldstein, D. J., W. Li, L.-M. Wang, M. A. Heidaran, S. Aaronson, R. Shinn, R. Schlegel, and J. H. Pierce.** 1994. The bovine papillomavirus type 1 E5 protein specifically binds and activates the β-type receptor for the platelet-derived growth factor but not other tyrosine kinase-containing receptors to induce cellular transformation. J. Virol. **68:**4432–4441.
10. **Goldstein, D. J., T. Andresson, J. J. Sparkowski, and R. Schlegel.** 1992. The BPV-1 E5 protein, the 16 kDa membrane pore-forming protein and the PDGF receptor exist in a complex that is dependent on hydrophobic transmembrane interactions. EMBO J. **11:**4951–4859.
11. **Goldstein, D. J., R. Kulke, D. DiMaio, and R. Schlegel.** 1992. A glutamine residue in the membrane-associating domain of the bovine papillomavirus type 1 E5 oncoprotein mediates its binding to a transmembrane component of the vacuolar $H^+$-ATPase. J. Virol. **66:**405–413.
12. **Greenberger, J. S., M. A. Sakakeeny, R. K. Humphries, C. J. Eaves, and R. J. Eckner.** 1983. Demonstration of permanent factor-dependent multipotential (erythroid neutrophil basophil) hematopoetic progenitor cell lines. Proc. Natl. Acad. Sci. USA **80:**2931–2935.
13. **Heidaran, M. A., D. Mahadevan, and W. J. Larochelle.** 1995. Chimeras demonstrate that human PDGFR Ig-like domains 1-3 are sufficient for high affinity PDGF BB binding. FASEB J. **9:**140–145.
14. **Heidaran, M. A., J. H. Pierce, T. Matsui, and S. A. Aaronson.** 1990. Chimeric alpha- and beta-platelet-derived growth factors define three immunoglobulin-like domains of the alpha-PDGF receptor that determine PDGF-AA binding specificity. J. Biol. Chem. **265:**18741–18744.
15. **Horwitz, B. H., A. Burkhardt, R. Schlegel, and D. DiMaio.** 1988. 44-amino-acid E5 transforming protein of bovine papillomavirus requires a hydrophobic core and specific carboxyl-terminal amino acids. Mol. Cell. Biol. **8:**4071–4078.
16. **Howley, P. M., and R. Schlegel.** 1987. Papillomavirus transformation. Plenum Press, New York.
17. **Keating, M. T., and L. T. Williams.** 1987. Processing of the platelet-derived growth factor receptor. J. Biol. Chem. **262:**7932–7937.
18. **Lowy, D. R., I. Dvoretzky, M. F. Law, L. Engel, and P. M. Howley.** 1980. In vitro tumorigenic transformation by a defined subgenomic fragment of bovine papilloma virus. Virology **103:**369–375.
19. **Martin, P., W. C. Vass, J. T. Schiller, D. R. Lowy, and T. J. Velu.** 1989. The bovine papillomavirus E5 transforming protein can stimulate the transforming activity of EGF and CSF-1 receptors. Cell **59:**21–32.
20. **Matsui, T., J. H. Pierce, T. P. Fleming, J. S. Greenberger, W. J. LaRochelle, M. Ruggiero, and S. A. Aaronson.** 1989. Independent expression of human alpha or beta platelet-derived growth factor receptor cDNAs in a naive hematopoietic cell leads to functional coupling with mitogenic and chemotactic signaling pathways. Proc. Natl. Acad. Sci. USA **86:**8314–8318.
21. **Meyer, A. N., Y.-F. Xu, M. K. Webster, A. S. Smith, and D. J. Donoghue.** 1994. Cellular transformation by a transmembrane peptide: structural requirements for the bovine papillomavirus E5 oncoprotein. Proc. Natl. Acad. Sci. USA **91:**4634–4638.
22. **Nilson, L. A., and D. DiMaio.** 1993. Platelet-derived growth factor receptor can mediate tumorigenic transformation by the bovine papillomavirus E5 protein. Mol. Cell. Biol. **13:**4137–4145.
23. **Orth, G.** 1987. Epidermodysplasia verruciformis, p. 199–243. *In* N. P. Salzman and P. M. Howley (ed.), The Papovaviridae. Plenum Press, New York.
24. **Petti, L., and D. DiMaio.** 1994. Specific interaction between the bovine papillomavirus E5 transforming protein and the β receptor for platelet-derived growth factor in stably transformed and acutely transfected cells. J. Virol. **68:**3582–3592.
25. **Petti, L., L. Nilson, and D. DiMaio.** 1991. Activation of the platelet-derived growth factor receptor by the bovine papillomavirus E5 transforming protein. EMBO J. **10:**845–855.
26. **Pierce, J. H., E. Di Marco, G. W. Cox, D. Lombardi, M. Ruggiero, L. Vaserio, L. M. Wang, G. G. Choudhury, A. Y. Sakaguchi, P. P. Di Fiore, and S. A. Aaronson.** 1990. Macrophage-colony-stimulating factor (CSF-1) induces proliferation, chemotaxis, and reversible monocytic differentiation in myeloid progenitor cells transfected with the human c-fms/CSF-1 receptor cDNA. Proc. Natl. Acad. Sci. USA **87:**5613–5617.
27. **Pierce, J. H., M. Ruggiero, T. P. Fleming, P. P. Di Fiore, J. S. Greenberger, L. Varticovski, J. Schlessinger, G. Rovera, and S. A. Aaronson.** 1988. Signal transduction through the EGF receptor transfected in IL-3-dependent hematopoietic cells. Science **239:**628–631.
28. **Schiller, J. T., W. C. Vass, K. H. Vousden, and D. R. Lowy.** 1986. E5 open reading frame of bovine papillomavirus type 1 encodes a transforming gene. J. Virol. **57:**1–6.
29. **Schlegel, R., and M. Wade-Glass.** 1987. E5 transforming polypeptide of bovine papillomavirus. Cancer Cells **5:**87–91.
30. **Schlegel, R., M. Wade-Glass, M. S. Rabson, and Y.-C. Yang.** 1986. The E5 transforming gene of bovine papillomavirus encodes a small, hydrophobic polypeptide. Science **233:**464–467.
31. **Sparkowski, J., J. Anders, and R. Schlegel.** 1994. Mutation of the bovine papillomavirus E5 oncoprotein at amino acid 17 generates both high- and low-transforming variants. J. Virol. **68:**6120–6123.