JOURNAL OF VIROLOGY, Sept. 1993, p. 5303–5311
0022-538X/93/095303-09$02.00/0

Vol. 67, No. 9

Transformation-Specific Interaction of the Bovine Papillomavirus E5 Oncoprotein with the Platelet-Derived Growth Factor Receptor Transmembrane Domain and the Epidermal Growth Factor Receptor Cytoplasmic Domain

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Received 30 March 1993/Accepted 24 May 1993

The bovine papillomavirus E5 transforming protein appears to activate both the epidermal growth factor receptor (EGF-R) and the platelet-derived growth factor receptor (PDGF-R) by a ligand-independent mechanism. To further investigate the ability of E5 to activate receptors of different classes and to determine whether this stimulation occurs through the extracellular domain required for ligand activation, we constructed chimeric genes encoding PDGF-R and EGF-R by interchanging the extracellular, membrane, and cytoplasmic coding domains. Chimeras were transfected into NIH 3T3 and CHO(LR73) cells. All chimeras expressed stable protein which, upon addition of the appropriate ligand, could be activated as assayed by tyrosine autophosphorylation and biological transformation. Cotransfection of E5 with the wild-type and chimeric receptors resulted in the ligand-independent activation of receptors, provided that a receptor contained either the transmembrane domain of the PDGF-R or the cytoplasmic domain of the EGF-R. Chimeric receptors that contained both of these domains exhibited the highest level of E5-induced biochemical and biological stimulation. These results imply that E5 activates the PDGF-R and EGR-R by two distinct mechanisms, neither of which specifically involves the extracellular domain of the receptor. Consistent with the biochemical and biological activation data, coimmunoprecipitation studies demonstrated that E5 formed a complex with any chimera that contained a PDGF-R transmembrane domain or an EGF-R cytoplasmic domain, with those chimeras containing both domains demonstrating the greatest efficiency of complex formation. These results suggest that although different domains of the PDGF-R and EGF-R are required for E5 activation, both receptors are activated directly by formation of an E5-containing complex.

Bovine papillomavirus type 1 (BPV) induces the proliferation of infected epithelia and underlying dermal fibroblasts, resulting in the production of benign fibropapillomas (14). The virus can also induce the in vitro transformation of mouse fibroblast cell lines (7). The major transforming gene of the BPV genome is E5, which encodes a 44-amino-acid protein that is composed of an N-terminal 30-amino-acid hydrophobic domain and a C-terminal 14-amino-acid hydrophilic domain (23, 24). By immunoelectron microscopy, the E5 protein in mouse fibroblasts was localized to the Golgi vesicles and plasma membrane, with the C termini of the majority of the molecules oriented toward the lumen of the Golgi vesicles or the exterior of the cell (4).

The E5 hydrophilic domain contains a number of amino acids that are conserved among the papillomaviruses that induce fibropapillomas, and these amino acids are important for the transformation of rodent fibroblasts by E5 (12). These critical amino acids include two conserved cysteine residues which are required for homodimer formation (3). Although there is little sequence specificity for the amino acids within the hydrophobic domain, a glutamine at position 17 is required for the transforming activity of E5 (12, 13). E5 mutants that lack this conserved glutamine do not form a stable complex with the 16-kDa protein (16K) which is the pore-forming component of the vacuolar proton ATPase that functions in the acidification of subcellular compartments (9, 11). Nontransforming mutants of E5 that contain substitutions or deletions in the hydrophilic C terminus are still able to associate with this cellular protein, reinforcing the conclusions that the membrane domain of E5 specifically interacts with 16K but that this interaction is not sufficient for biological activity (10).

BPV E5 is thought to function through the activation of growth factor receptors. Previous studies have demonstrated that E5 can activate the epidermal growth factor receptor (EGF-R) and the platelet-derived growth factor receptor (PDGF-R) by ligand-independent mechanisms (18, 20). It is unclear how E5 can activate two receptors that are so dissimilar. The EGF-R binds a monomeric ligand, contains cysteine-rich clusters in the extracellular domain, and contains an uninterrupted kinase domain, while the PDGF-R binds a dimeric ligand, contains immunoglobulin-like structures in the extracellular domain, and has a split kinase domain. The activation of the two receptors by E5 appears to be associated with different characteristic features. E5-mediated activation of the EGF-R is associated with a decrease in the down regulation of the activated receptor at the cell surface (18, 30); this alteration in receptor metabolism has not been demonstrated for the PDGF-R. Conversely, the PDGF-R appears to be activated by E5 prior to reaching the plasma membrane since underglycosylated forms of the receptor are phosphorylated on tyrosine in

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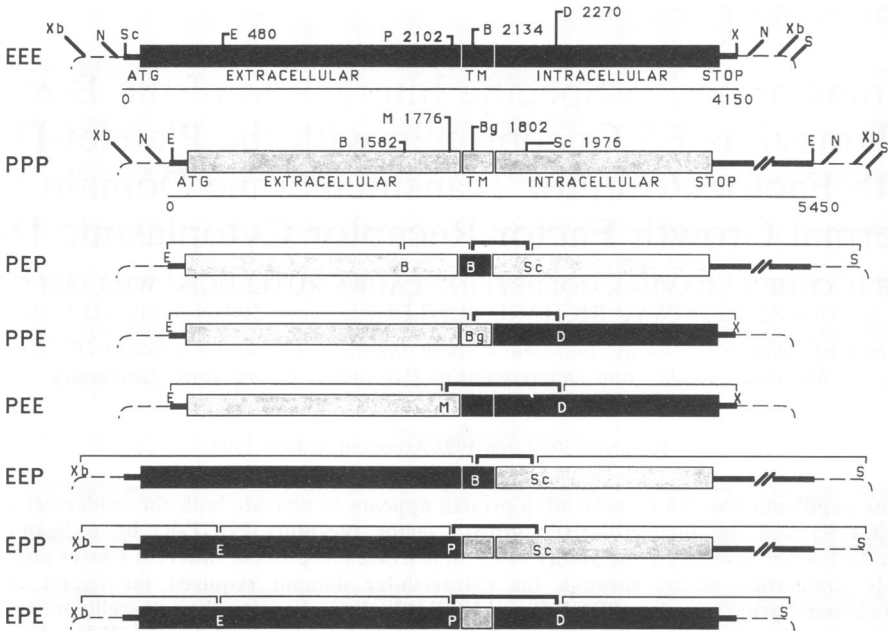


FIG. 1. Schematic representation of PDGF-EGF chimeric receptors. EGF-R regions are in black, and PDGF-R regions are in white. The restriction enzymes and corresponding nucleotide sites used for the constructions are represented in the wild-type PDGF-R (PPP) and EGF-R (EEE) constructions (B, *Bst*XI; Bg, *Bgl*I; D, *Dra*III; E, *Eco*RI; M, *Mse*I; N, *Not*I; P, *Pfl*MI; Sc, *Sst*II; S, *Sal*I; Sb, *Sba*I; X, *Xho*I). TM corresponds to the transmembrane domain.

E5-expressing cells (20). Activation of immature forms of EGF-R by E5 has not been demonstrated. The copurification of the PDGF-R and E5 in immunoprecipitates has recently been demonstrated, and this complex also appears to contain 16K (8, 19). An association between E5 and the EGF-R has not been reported.

The activation of a growth factor receptor normally occurs through the binding of its physiological ligand to the extracellular domain of the receptor. Several viruses encode proteins that activate receptors by mechanisms that mimic the interaction of ligand with its receptor. Poxviruses produce an EGF-like molecule which activates the EGF-R and thereby promotes epithelial cell growth (2). The simian sarcoma virus expresses the v-Sis protein, which is a homolog of PDGF-B, that can activate the PDGF-R (6, 29). It has been speculated that BPV E5, which shares limited sequence homology with PDGF-B and which binds to the PDGF-R, may act as a ligand for the PDGF-R (20). However, it is not known whether the activation of growth factor receptors by BPV E5 occurs through an interaction with receptor extracellular domains or via the hydrophobic transmembrane domain as described for E5-16K interactions (10).

The apparent differences in the characteristic features of E5 stimulation of the EGF-R and PDGF-R suggest that they might be activated by different mechanisms. To determine whether the ligand binding domains or other domains are specifically required for E5-induced activation, we synthesized chimeric PDGF-R and EGF-R and examined their activation by E5. These chimeras were constructed so that the extracellular, transmembrane, and intracellular domains from one receptor were substituted with those of the other receptor in every possible combination. From our identification of the specific domains of the PDGF-R and EGF-R required for activation by and interaction with E5, it appears that the E5 viral oncoprotein does not act as a ligand analog but rather can activate growth factor receptors by two different mechanisms.

MATERIALS AND METHODS

Plasmid constructions. (i) Chimeric receptors. The sequences encoding the chimeric receptors were derived from cDNAs (5) of the human PDGF-R in pSV7D (26) or the human EGF-R cDNA (27) from pMI12, using standard molecular cloning techniques. pMI12 expressing the EGF-R cDNA is derived from the Harvey murine sarcoma virus DNA cloned in pBR322 (pCO6-HX) (28) in which human EGF-R has been inserted in place of the *ras* gene at the *Sst*II-*Xho*I sites. The diagram in Fig. 1 identifies fragments used to construct the chimeric gene, which were first cloned into a modified pUC19 vector (pUCMCR). pUCMCR is lacking the *Eco*RI site and contains the 1.2-kb *Xba*I fragment from pCO6-HX. All restriction endonuclease fragments are identified by brackets in Fig. 1. All sequences identified by the brackets in bold were synthesized by either the polymerase chain reaction or synthetic oligonucleotides, and their sequences have been confirmed directly. All subclones in pUCMCR were then cloned into pMI-Not, a pMI12 derivative lacking the EGF-R cDNA sequences which contains an engineered *Not*I site at the *Sac*II-*Xho*I junction. The transmembrane fragment of construct PEP (see Results for an explanation of nomenclature) was a polymerase chain reaction product derived from the EEP chimera.

(ii) COS cell expression vectors. The chimeric receptors were cloned from the pMI vector to the pSVL COS cell expression vector (8) by *Not*I digestion and fragment ligation. The wild-type EGF-R (EEE) was modified by the addition of a six-amino-acid epitope derived from the BPV L1 protein (16) which was inserted just before the stop codon. This sequence is recognized by monoclonal antibody

AU5. The E5 expression plasmid, pTA6, has been described previously (8) and contains the E5 gene with an NH_2-terminal HA1 epitope for facilitating detection by antibody 12CA5.

Cells and tissue culture. CHO(LR73) (22) cells were a gift from M. Gottesman. These cells and NIH 3T3 clone 7 cells were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum (GIBCO) at 37°C in 5% CO_2. These cells contain approximately 100,000 PDGF-Rs and 10,000 to 20,000 EGF-Rs. Cell cultures expressing the receptors were produced by pooling G418-resistant colonies from cells cotransfected with the pSV2neo plasmid and maintained in 500 μg of G418 per ml. Transfections using calcium phosphate and focus assays were performed as previously described (28). At the end of the focus assay (2 to 4 weeks), cells were stained with 0.16% carboyl fuchia–0.5% methylene blue in methanol. Soft agar experiments were done as previously described with the addition of 100 nM cholera toxin in the media (28). Transfection and transient expression of the pSVL expression vector into COS cells have been described previously (9).

Cell extractions and immunoprecipitations. CHO and NIH 3T3 cells (grown in 100-mm-diameter dishes) were washed once with phosphate-buffered saline and then extracted on ice with 800 ml of lysis buffer (1% Nonidet P-40 [NP-40], 150 mM NaCl, 50 mM Tris HCl [pH 7.4], 10 mM $MgCl_2$, 1 mM ammonium vanadate) with occasional rocking for 15 min. Those cells stimulated with ligand first were incubated with 100 ng of EGF or PDGF per ml for 5 min at 37°C and then washed and lysed as described above. Ten percent of the total extract was either boiled directly in sodium dodecyl sulfate sample buffer or immunoprecipitated with 40 ml of protein A-Sepharose and 10 ml of anti-human PDGF-R GR14 or anti-EGF-R GR01 (Oncogene Science). Boiled samples were then analyzed by antiphosphotyrosine immunoblotting using antibody 05-321 (UBI) followed by ^{125}I-labeled goat anti-mouse Fab. The levels of ligand-stimulated receptor per unit extract, as measured by antiphosphotyrosine immunoblotting, were similar in all cell lines tested. COS cell immunoprecipitations have been described previously (8). Briefly, COS cells were transfected with pSVL chimera and epitope addition E5 vectors; 48 to 72 h posttransfection, the cells were labeled with [^{35}S]methionine, extracted with a modified radioimmunoprecipitation assay buffer, and immunoprecipitated with either antireceptor antibodies or anti-epitope E5 antibodies (12CA5). Specifically, the receptors PPP, PEP, and EEP were immunoprecipitated with the C-terminal PDGF-R antibody 06-131 (UBI), receptor EPE was immunoprecipitated with anti-EGF-R antibody (Amersham), receptors PPE and PEE were immunoprecipitated with N-terminal PDGF-R antibody GR-14 (Oncogene Science), and EEE was immunoprecipitated with antiepitope monoclonal antibody AU5 (16). Washed immunoprecipitates were analyzed by polyacrylamide gel electrophoresis (PAGE).

RESULTS

Chimeric receptor construction. Both PDGF-R and EGF-R can be divided into three functional domains: extracellular, membrane, and intracellular. We therefore constructed a total of six chimeric receptor clones representing each combination of analogous domains. The chimeras were designed so that each domain was intact and derived from a single receptor, thereby eliminating the possibility of confounding effects due to partial duplication or deletion of domains. The six chimeras, as well as the wild-type PDGF-R and EGF-R, were inserted into pMI, a retroviral expression vector. The nomenclature of the receptors was designated according to their extracellular, membrane, and intracellular domains, respectively. For example, PPE consists of the extracellular domain of the PDGF-R (P), the transmembrane domain of the PDGF-R (P), and the intracellular domain of the EGF-R (E) (Fig. 1).

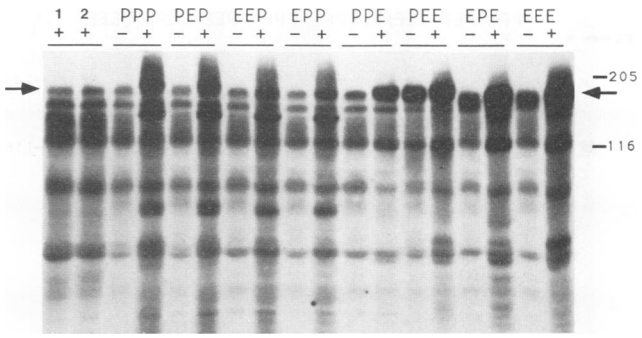


FIG. 2. Autophosphorylation of chimeric receptors is stimulated by ligand. Serum-starved CHO cell cultures were stimulated with (+) or without (−) ligand and lysed. Total NP-40-soluble extracts were analyzed by PAGE, transferred to a polyvinylidene difluoride membrane, and probed with antiphosphotyrosine antibody. Arrows mark locations of the tyrosine-phosphorylated chimeric receptors. The first two lanes reflect parental CHO cells stimulated with EGF (lane 1) and PDGF (lane 2). Sizes are indicated in kilodaltons.

Ligand activation of the chimeras. The chimeras and pSV2neo were cotransfected into CHO (LR73) cells, which lack endogenous PDGF-R and EGF-R. G418-selected mass cultures were grown to confluence and acutely stimulated with the appropriate ligand specific for the extracellular domain of the chimera. Cells were then extracted with NP-40, and the soluble fractions were analyzed for tyrosine phosphorylation in response to ligand (Fig. 2). In comparison with extracts from the unstimulated transfectants, the extracts from all the cultures contained more tyrosine-phosphorylated receptors after ligand stimulation, indicating that all of the chimeras functioned as ligand-inducible tyrosine kinases. Phosphorylation of other proteins within the extract was also apparent, with their specificity being largely dependent on the kinase domain of the chimeric receptor. Addition of the ligands to untransfected CHO cells did not induce protein tyrosine phosphorylation, demonstrating the lack of endogenous PDGF-R or EGF-R in these cells (data not shown).

The level of endogenous (ligand-independent) tyrosine kinase activity of most receptors that contained an EGF-R kinase domain (PEE, EPE, and EEE) was unexpectedly high, irrespective of the membrane or extracellular domain (Fig. 2). The only exception to this observation was the receptor PPE, which had a lower basal phosphorylation level. The relatively high level of phosphotyrosine, and therefore presumably high level of autokinase activity, of the PEE, EPE, and EEE receptors was not influenced by serum starvation or the density of the cell monolayer (data not shown). The basis for this higher activity is unclear, but these results imply that CHO cells have a cellular mechanism to activate the EGF-R cytoplasmic domain in the absence of ligand.

The biological activity of the chimeric receptors was tested by growth in soft agar. G418-selected mass cultures

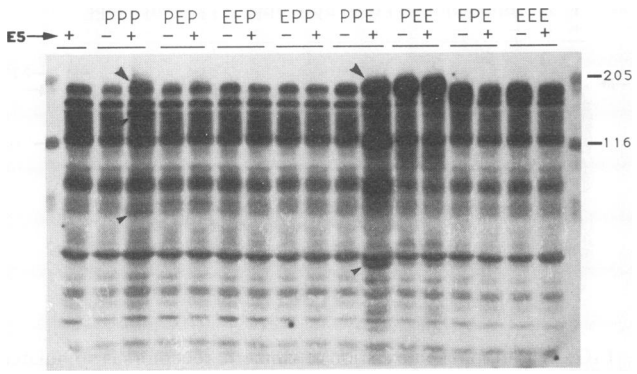


FIG. 3. BPV E5 activation of chimeric receptors in CHO cells. CHO cell lines expressing the chimeras with or without E5 were grown to confluence and lysed. Total NP-40-soluble extracts were analyzed by PAGE, transferred to a polyvinylidene difluoride membrane, and probed with antiphosphotyrosine antibody. Large arrowheads identify the activated tyrosine-phosphorylated receptors, and small arrowheads identify hyperphosphorylated proteins within the extract.

were plated in agar in the presence of 100 nM cholera toxin. Cholera toxin has been shown to inhibit the growth of CHO cells in agar, and this inhibition can be overcome by the activity of tyrosine kinase oncoproteins such as pp60$^{v\text{-}src}$ (22). Compared with the parental CHO cells, cells expressing the chimeras produced more and larger colonies in agar when grown in the presence of the appropriate ligand (data not shown). This result demonstrated that the ligand-induced biochemical activation of the chimera correlates with the transduction of the appropriate signals required for cell growth. Cells expressing chimeras EEE, EPE, and PEE, which demonstrated a high background autokinase activity in the absence of ligand, were able to form colonies in agar without added ligand, indicating a correspondence between receptor activation and anchorage-independent growth (data shown for EEE in Fig. 4).

BPV E5 activation of chimeras. Having shown that the chimeric receptors were active biochemically and biologically, we examined them in cells coexpressing BPV E5, with the goal to identify the receptor domains required for BPV E5 activation. The vector pMI-E5, which encodes the wild-type BPV E5 protein, was cotransfected into CHO cells with each chimera, and G418-resistant mass cultures were isolated. Cells were grown in the absence of added ligand and assayed for E5-induced receptor autophosphorylation by antiphosphotyrosine immunoblotting. As expected, wild-type PDGF-R (PPP) demonstrated an E5-dependent increase in phosphotyrosine content. Chimera PPE was also activated, but chimera PEP was not activated in association with E5 (Fig. 3), even though this chimeric receptor was stimulatable by ligand (Fig. 2). These results indicate that the transmembrane domain of the PDGF-R is required for E5 activation of this receptor. The chimeric receptor EPP had no detectable increase in tyrosine phosphorylation in E5-expressing cells. Taken together, these biochemical results implicate the extracellular and transmembrane domains but not the cytoplasmic domain in E5-induced activation of the PDGF-R.

It has previously been demonstrated that BPV E5 can activate the EGF-R in NIH 3T3 cells. However, neither the EGF-R (EEE) nor the chimeras which contained the EGF-R cytoplasmic domain (PEE and EPE) demonstrated an E5-dependent increase in receptor phosphorylation in the CHO assays. It is possible that the high background of endogenous kinase activity of these receptors in CHO cells may preclude or mask an E5-induced activation. Alternatively, E5 activation of the EGF-R may occur through a mechanism that does not function in CHO cells.

Having examined the relationship between E5 expression and biochemical activation of the chimeras, we sought to determine whether there was an associated biological response. The CHO cell lines expressing the chimeras were assayed for the ability to form colonies in soft agar in the presence or absence of E5. Cells coexpressing E5 and PPP or PPE induced ligand-independent colony formation, whereas E5 alone or receptors alone did not (Fig. 4; data not shown for E5 alone). In addition, the cells cotransfected with E5 and chimera PEP, which contains the EGF-R transmembrane domain, were unable to grow in agar. These biological results correlate with the biochemical activation of these receptors by E5. Unexpectedly, cotransfection of EPP and E5 consistently induced colony formation even though biochemical activation of EPP by E5 was not detected. A possible interpretation for this discrepancy is that the biological assay is a more sensitive measure of E5 activity and that the E5-induced increased tyrosine phosphorylation on EPP is below our means of detection. From these experiments, we conclude that the biological activation of the PDGF-R by E5 specifically requires only the receptor's transmembrane domain. Since biochemical activation was detected for PPP but not EPP, it is possible that the extracellular domain of the PDGF-R facilitates activation by E5.

It was not possible to assess the domains required for E5 activation of the EGF-R in CHO cells since those chimeras containing an EGF-R cytoplasmic domain, EEE, EPE, and PEE, were already active biochemically (Fig. 2) and biologically in the absence of E5 (data shown for EEE in Fig. 4). There was no apparent E5-induced stimulation of these receptors.

Identification of domains required for E5 activation of the EGF-R. The activation of growth factor receptors by BPV E5 was first shown in NIH 3T3 cells overexpressing the EGF-R (18). Since we were unable to determine the domains necessary for E5 activation of the EGF-R in CHO cells, we assayed the activity of all of the chimeric receptors in NIH 3T3 cells. NIH 3T3 cells contain a low level of endogenous EGF-Rs (10,000 to 20,000 per cell) and do not exhibit ligand-independent activation of transfected human EGF-R in the absence of E5. G418-selected NIH 3T3 cultures expressing the chimeric receptors and E5 were isolated, and equal numbers of ligand-stimulatable chimeric receptors (data not shown) were immunoprecipitated from NP-40-soluble extracts, using either a mammalian cell-specific anti-EGF-R or human cell-specific anti-PDGF-R antibody. Both of these antibodies recognized the external domain of the receptor. Immunoprecipitation of the transfected receptors allowed us to clearly distinguish the activation of the chimeric receptors from activation of the endogenous mouse PDGF-R. PPP and chimeras PEP, PEE, and PPE were immunoprecipitated with anti-human PDGF-R and assayed for biochemical activation by antiphosphotyrosine immunoblotting. As expected, PPP was activated by E5 (data not shown), and cells expressing those chimeras which contained an EGF-R cytoplasmic domain, PEE and PPE, exhibited ligand-independent E5-induced tyrosine phosphorylation. Chimera PEP, which has a PDGF-R kinase domain and EGF-R transmembrane domain, was negative for activation

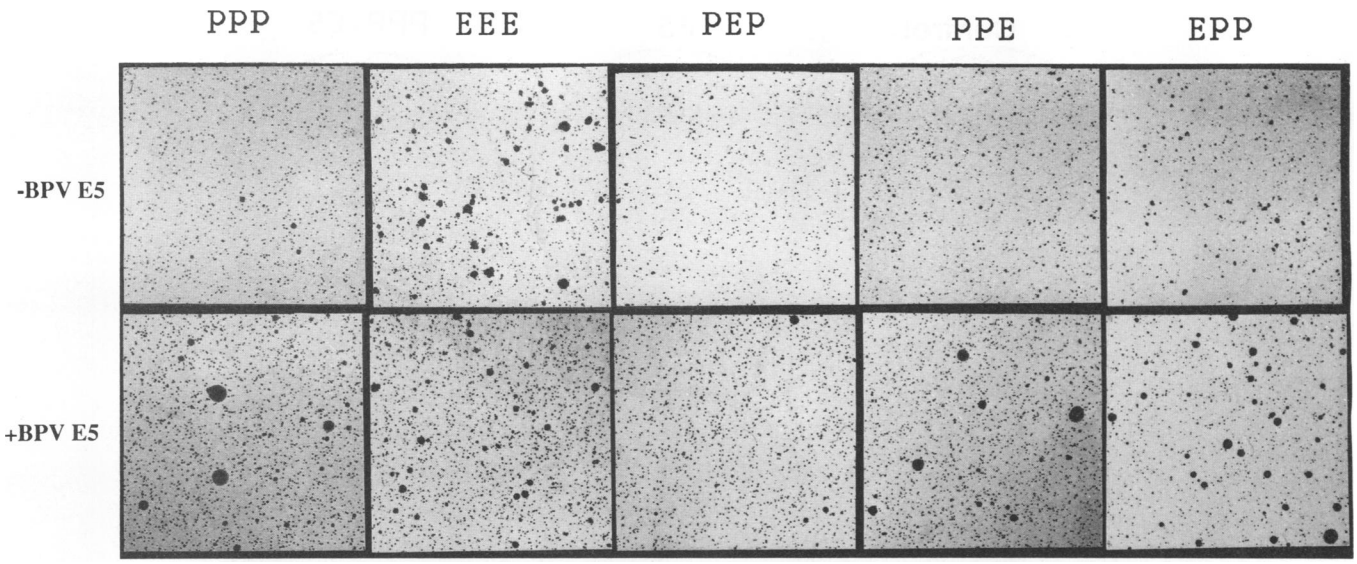


FIG. 4. Soft agar growth of CHO cells expressing E5 and chimeras. CHO cell lines expressing the chimeras with or without E5 were plated in soft agar containing 10% serum and 100 nM cholera toxin.

(Fig. 5). These results confirm the findings in CHO cells indicating the importance of the transmembrane domain of the PDGF-R in its activation by E5 and further that the cytoplasmic domain of the EGF-R is critical for its activation by E5. Results supporting this conclusion were obtained when the receptors EEE, EPE, EEP, and EPP (all containing an EGF-R extracellular domain) were immunoprecipitated with anti-EGF-R under identical conditions. EEE and EPE, which contain an EGF-R cytoplasmic domain, exhibited an increased level of phosphotyrosine when coexpressed with E5; EEP, which is lacking an EGF-R cytoplasmic domain, did not. As in the CHO cells, EPP did not exhibit an E5-induced activation although it contained a PDGF-R transmembrane domain. Together, these results identify the EGF-R cytoplasmic domain as being required for E5 activation of the EGF-R. The small amount of phosphorylated receptor detected in the EEP and EPP assays is possibly due to inefficient activation by E5 or by a

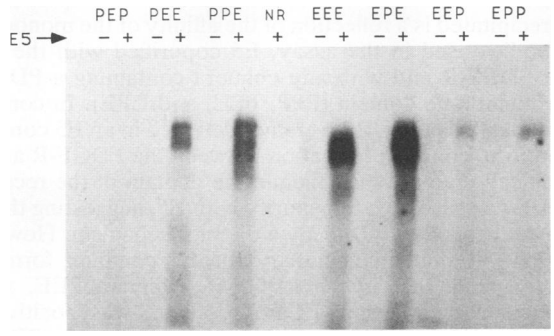


FIG. 5. BPV E5 activation of the chimeric receptors in NIH 3T3 cells. Cells expressing the chimeras with or without E5 were grown to confluence and lysed. The chimeric receptors PEP, PEE, PPE, and NR (no transfected receptor) were immunoprecipitated with anti-human PDGF-R, whereas receptors EEE, EPE, EEP, and EPP were immunoprecipitated with anti-EGF-R. Immunoprecipitates were analyzed by PAGE, transferred to a polyvinylidene difluoride membrane, and probed with antiphosphotyrosine antibody.

secondary effect due to E5 activation of the endogenous mouse PDGF-R. Although the cytoplasmic domain of the EGF-R was sufficient to confer E5 responsiveness to the chimeras, those chimeras which contained both the membrane domain of the PDGF-R and the cytoplasmic domain of the EGF-R (PPE and EPE) were more highly phosphorylated than those containing only one of these domains (Fig. 5).

To correlate the biochemical activation of the PDGF and EGF chimeras by E5 with a biological response, we measured the ability of all of the chimeras to augment E5 transforming activity in NIH 3T3 cells in a focal transformation assay (Fig. 6). The E5-expressing clone pHLB717 (23) was cotransfected with each of the chimeras into NIH 3T3 cells, and the transfected cells were allowed to form foci in monolayer culture. Since the PDGF in the serum might preferentially promote the growth properties of cells expressing the chimeras with a PDGF-R extracellular domain, we have compared the activities of chimeras with external PDGF-R domains with each other but not with those of chimeras containing the EGF-R extracellular domain. The expression of E5 alone was sufficient to induce focus formation through activation of the endogenous mouse PDGF-R. As shown by an increase in the number and size of foci, the addition of the wild-type PDGF-R (PPP) augmented E5 activity. Cooperation was not detected when chimera PEP was used, confirming the requirement of the transmembrane domain of the PDGF-R for its activation by E5. If the cytoplasmic domain of PEP was substituted with the EGF-R cytoplasmic domain to produce PEE, cooperation with E5 was detected. Furthermore, chimera PPE, which contained both the PDGF-R transmembrane domain and the EGF-R cytoplasmic domain, demonstrated the most transforming activity in the cotransfection assay (Fig. 6). Transfection of only the chimeras in the absence of E5 did not induce focal transformation (data not shown). These biological data parallel the biochemical results and demonstrate that the EGF-R cytoplasmic domain is critical for E5-induced activation of the EGF-R. This conclusion was also supported by the results obtained with the set of chimeras containing the

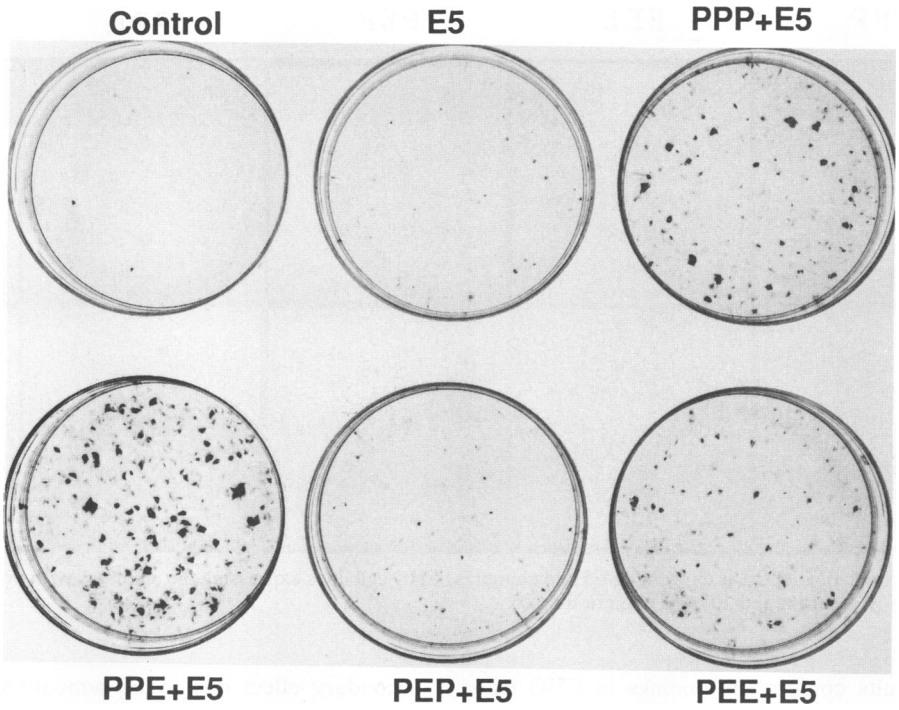


FIG. 6. Focal transformation assay of NIH 3T3 cells expressing E5 and chimeras. Cells were transfected with the chimeras and E5 and directly plated onto 60-mm-diameter dishes. Cells were maintained in Dulbecco modified Eagle medium containing 10% fetal calf serum for 16 days and then stained.

EGF-R extracellular domain. Cells expressing EEE augmented E5 activity, whereas EEP did not. Moreover, chimera EPE, which contains the PDGF-R domain responsive to E5, was more active than EEE. EPP was also able to increase E5's transforming activity, which parallels the CHO cell data in that we detected a biological but not a biochemical response (data not shown).

Coimmunoprecipitation of the EGF-R and PDGF-R with BPV E5. The foregoing results indicate that the membrane domain of the PDGF-R is required for its activation by E5, while the cytoplasmic domain of the EGF-R is required for E5 activation of this receptor, suggesting that E5 may activate these two receptors by different mechanisms. It is likely that E5 activates the PDGF-R by a relatively direct mechanism, since E5 copurifies with the PDGF-R by immunoprecipitation (19). However, the receptor domain responsible for this association has not been determined. Activation of the EGF-R also could be the direct result of complexing with E5, or it could occur through a more indirect process, such as secondary consequence to the activation of the endogenous PDGF-R in NIH 3T3 cells.

To support the biochemical and biological data suggesting that E5 activates the PDGF-R and EGF-R by distinct mechanisms, we sought to determine whether E5 also forms a complex in vivo with the EGF-R and whether the receptor domains required for that interaction were different from those required for coprecipitation of the PDGF-R with E5. To optimize the sensitivity of complex detection, we increased the expression of E5 and of the chimeric genes by cloning them into an expression vector containing a simian virus 40 (SV40) origin of replication and transfecting the constructs into COS cells, which express SV40 large T antigen. Although only about 10% of the total cell population expressed the designated plasmid(s), the presence of SV40 large T antigen induced amplification of the constructs so that the level of expression within the subpopulation of transfected cells was high, facilitating the detection of protein-protein complexes.

The chimeras and E5 were cotransfected into the COS cells, and the labeled extracts were divided and analyzed by immunoprecipitation, using either receptor-specific or E5-specific antisera (Fig. 7). Antireceptor lanes identify the mobility of the chimeric receptors. Anti-E5 immunoprecipitates were divided and analyzed on two separate gels. A low-percent gel was used to identify the receptors in the E5-receptor complex (Fig. 7A), and a high-percent gel (Fig. 7B) was used to verify the presence of E5 protein. The relatively large difference in the amount of receptor immunoprecipitated is a reflection of the affinity of the monoclonal antibodies used in the assay. E5 copurified with the wild-type PDGF-R and with any chimera containing a PDGF-R transmembrane domain (PPP, PPE, and EPE). In contrast, chimeras PEP and EEP were not detected in an E5 complex. Therefore, complex formation between the PDGF-R and E5 specifically required the membrane domain of the receptor.

EEE was also able to copurify with E5, suggesting that E5 also activates the EGF-R by a direct mechanism. However, the EGF-R domain necessary for E5 complex formation differed from that in the PDGF-R. Chimera PEE, which contains a cytoplasmic EGF-R domain, was positive for binding to E5, but chimera PEP, which contains the PDGF-R cytoplasmic domain, was negative, indicating the specific requirement of the EGF-R cytoplasmic domain for complex formation. Those chimeras which contained both a PDGF-R membrane domain and an EGF-R cytoplasmic domain (PPE and EPE) demonstrated greater association with E5 than did chimeras containing only one of these domains. As a control, immunoprecipitation of cells expressing only the receptors

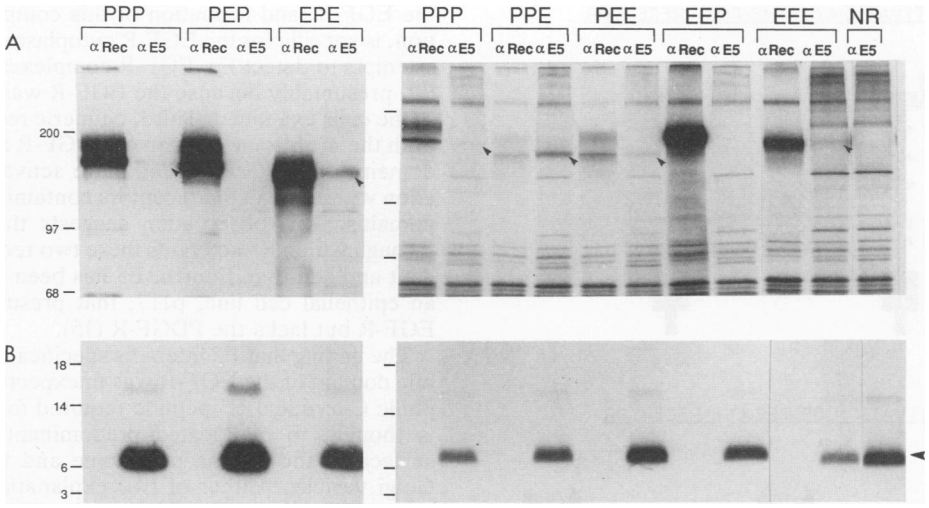
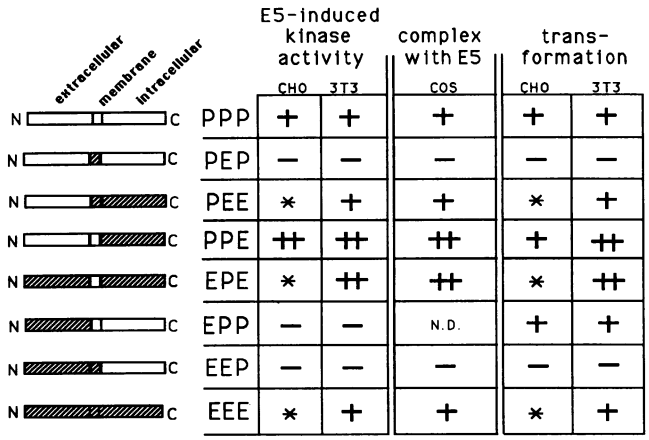


FIG. 7. E5 copurifies with the PDGF-R and EGF-R through distinct domains. COS cells transfected with the chimeras and E5 were [^{35}S]methionine labeled and lysed after 48 h, and the extracts were divided for immunoprecipitation with either an antireceptor antiserum (α Rec) or anti-E5 antibody 12CA5 (α E5). Immunoprecipitates were analyzed on either a 7.5% (to identify receptors [A]) or 15% (to identify E5 [B]) polyacrylamide gel. The small arrows identify the copurified chimeric receptors. Sizes are indicated in kilodaltons.

with the E5-specific antibodies did not identify any complex formation (data not shown). Therefore, the domains required for the biochemical and biological activation of the chimeras by E5 correlated directly with their ability to form a complex with E5 (Fig. 8).

DISCUSSION

Previous studies have shown that E5 can induce the ligand-independent activation of both the PDGF-R (20) and the EGF-R (18), which belong to different classes of transmembrane growth factor receptors. To determine the domains specifically involved in the ligand-independent activation of these growth factor receptors by E5, we have constructed a series of chimeric PDGF-R and EGF-R genes in which sequences encoding the extracellular, membrane, and intracellular domains of these two receptors were interchanged. Our results indicate that the domains specific for E5 binding and activation are different for the two receptors.



	E5-induced kinase activity		complex with E5	trans-formation	
	CHO	3T3	COS	CHO	3T3
PPP	+	+	+	+	+
PEP	−	−	−	−	−
PEE	*	+	+	*	+
PPE	++	++	++	+	++
EPE	*	++	++	*	++
EPP	−	−	N.D.	+	+
EEP	−	−	−	−	−
EEE	*	+	+	*	+

FIG. 8. Summary of biochemical and biological activation of chimeric receptors by the E5 protein. *, spontaneously activated; N.D., not determined.

This finding implies that despite being only 44 amino acids, E5 activates these two receptors by distinct mechanisms. Since the specificity of activation does not involve the extracellular domains of either receptor, neither of these mechanisms appears to mimic ligand-induced activation of the receptors.

The transmembrane domain of the PDGF-R is specifically required for its response to E5 activation. This necessity is clearly exemplified by the observation that chimera PEP, although activated by PDGF, was not biochemically or biologically activated by E5. Few biologically significant functions other than anchoring of the protein in the membrane have previously been attributed to the transmembrane peptides of growth factor receptors. One example is the activation of *erbB-2* (*neu*) proto-oncogene-encoded protein by substitution of a valine with glutamic acid within the transmembrane domain (1). This mutation enhances receptor oligomerization and subsequently activates the protein tyrosine kinase activity (25). In addition, Lonardo and coworkers demonstrated, using chimeric EGF-R–ErbB-2 receptors, that the wild-type transmembrane domain of ErbB-2 protein exerts an inhibitory effect on receptor kinase activity (17). A second example is the recent demonstration that the transforming activity of Friend spleen focus-forming virus involves an interaction of its *env* gene product, gp55, with the transmembrane domain of the erythropoietin receptor (31).

The transmembrane peptide of E5 has been implicated in the association of E5 with the 16K subunit of the vacuolar ATPase (9). Mutation of glutamine at position 17 within the transmembrane domain of E5 abolishes this interaction as well as the transforming activity of E5 (11–13). Since the PDGF-R appears to be the primary growth factor receptor activated in murine fibroblasts, this observation suggests that 16K binding may be important in the activation of the PDGF-R by E5. Recent work in one of our laboratories indicates that 16K also interacts with the PDGF-R in the absence of E5 and that E5, the PDGF-R, and 16K can be detected in the same complex (8). Taken together, these observations suggest a model (Fig. 9a) for E5 activation of

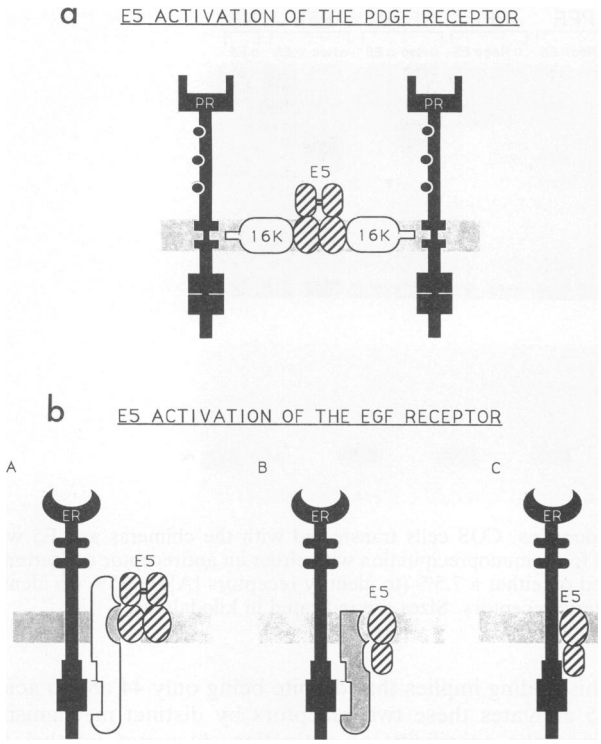


FIG. 9. Potential mechanisms of E5 activation of the PDGF-R and EGF-R. (a) E5 dimers activating the PDGF-R by inducing receptor dimerization through 16K. (b) Model A depicts E5 dimers activating the EGF-R by inducing receptor dimerization through an unknown accessory protein. Activation of the EGF-R could also occur with E5 in the reverse orientation, allowing the C-terminal portion to interact indirectly (B) or directly (C) with the cytoplasmic domain of the EGF-R.

the PDGF-R in which a dimer of E5 would induce receptor dimerization and activation through the interaction of the membrane domains of E5, the PDGF-R, and 16K.

In contrast to the PDGF-R, the cytoplasmic domain of the EGF-R is specifically required for E5-induced activation by this receptor. This conclusion is based on our finding that EEE and PEE are activated in a ligand-independent manner by E5, but EEP and PEP are not. Since E5 activation of transfected EGF-R was assayed in NIH 3T3 cells, which also contain E5-activated endogenous PDGF-R, the possibility existed that activation of the EGF-R kinase domain was a secondary event that was the result of E5 activating PDGF-R. This might occur via the *trans*-phosphorylation of the EGF-R by the PDGF-R (a phenomenon which has not been shown to occur) or via an even more indirect mechanism. We believe that E5-induced activation of the EGF-R is mechanistically independent of PDGF-R activation for several reasons. First, activation of the PDGF-R by PDGF in NIH 3T3 cells does not result in activation of the transfected EGF-R, ruling out a simple *trans*-phosphorylation mechanism (our unpublished results). In addition, our previous finding that the E5-induced pattern of EGF-R phosphorylation was the same as that induced by EGF provided evidence that, in response to E5, the EGF-R becomes a fully active autokinase (18). However, there was no evidence of additional phosphorylations as would be expected if E5 induced EGF-R to become a specific substrate for another kinase. Second, we have now shown that E5 forms a complex with the EGF-R, and formation of this complex, like E5 activation, is specific for the EGF-R cytoplasmic domain. Previous attempts to detect E5–EGF-R complexes were unsuccessful (8), presumably because the EGF-R was not overexpressed in the cells examined. Third, chimeric receptors that contain both the membrane domain of PDGF-R and the cytoplasmic domain of EGF-R exhibited more activation by and association with E5 than did receptors containing only one of these domains. This observation suggests that the mechanisms through which E5 activates these two receptors are independent and additive. Fourth, E5 has been shown to transform an epithelial cell line, p117, that presumably contains the EGF-R but lacks the PDGF-R (15).

The finding that E5 interacts specifically with the cytoplasmic domain of the EGF-R was unexpected, since the hydrophilic C-terminal E5 peptide required for E5 transformation is thought to be located predominantly on the external surface of the plasma membrane and the lumenal side of Golgi vesicles. Either of two explanations might reconcile this discrepancy. Activation of the EGF-R could be mediated through a transmembrane cellular protein that interacts with the dimerized E5 C-terminal domains and with the EGF-R cytoplasmic domain (Fig. 9b, bottom model A). Alternatively, E5 might be able to assume the reverse orientation in the membrane so that the C-terminal portion is in the cytoplasm, where it would presumably exist as a monomer due to the reducing conditions within the cell. In this conformation, the E5 C-terminal domain could interact directly with the cytoplasmic domain of the EGF-R or indirectly through an intervening cytoplasmic protein (Fig. 9b, models B and C). These models can be tested experimentally.

It is interesting that HPV16 E5 appears to cooperate with the EGF-R but not with the PDGF-R to induce cellular transformation (21). Therefore, activation of the EGF-R through its cytoplasmic domain may be a common mechanism whereby papillomavirus E5s contribute to the cellular proliferation associated with papilloma formation. Activation of the PDGF-R may be an additional E5 function limited to those viruses, such as BPV, that are able to induce fibropapillomas.

ACKNOWLEDGMENTS

We thank Michael Gottesman for kindly providing the CHO cells and technical advice, Thorkell Andresson for assistance with constructing the EGF-R COS cell expression vector, and Lena Claesson-Welsh for providing the PDGF-R cDNA.

B.D.C. is a National Institutes of Health fellow (IRTA grant TA-CA-B046).

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