E5 Oncoprotein Transmembrane Mutants Dissociate Fibroblast Transforming Activity from 16-Kilodalton Protein Binding and Platelet-Derived Growth Factor Receptor Binding and Phosphorylation

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Papillomaviruses are small, double-stranded DNA viruses that infect a wide variety of vertebrate species and induce epithelial cell proliferation which, in some cases, can progress to the malignant state (2, 24, 37). Bovine papillomavirus type 1 (BPV-1) induces fibropapillomas on cattle and foci on immortalized murine cell lines and has been used as a model system for studying papillomavirus transformation in vitro (9, 18, 36). The predominant in vitro transforming activity of BPV-1 is attributable to its E5 gene product (7, 9, 16, 20, 30, 32). The E5 protein consists of 44 amino acids with two distinct structural features: a hydrophobic 30-amino-acid N-terminal region which functions as a transmembrane domain and a hydrophilic 14-amino-acid C-terminal region which contains two cysteine residues essential for homodimer formation (3, 17, 31). E5 is oriented asymmetrically in cellular endomembranes with its C terminus facing the lumen of the Golgi apparatus (3), and recent studies indicate that E5 accumulation in the trans Golgi apparatus is critical for its mitogenic signalling (34).

E5 induces cellular transformation apparently via the modulation of tyrosine kinase growth factor receptor signalling pathways. For example, E5 cooperates with exogenously transfected epidermal growth factor receptor (EGF-R) in the transformation of NIH 3T3 cells and induces receptor hyperphosphorylation as well as altered downregulation (21). E5 also induces the autophosphorylation of endogenous platelet-derived growth factor receptor (PDGF-R) in fibroblasts (27) and can cooperate with PDGF-R in the transformation of cells which normally lack this receptor (12, 22). Finally, E5 coprecipitates with PDGF-R in transformed cells (25, 26) as well as with the 16-kilodalton subunit (16K subunit) of the vacuolar ATPase (V-ATPase), suggesting that physical interactions may mediate the ability of E5 to activate a given receptor complex. Recent studies indicate that the E5 transmembrane domain mediates interaction with both PDGF-R and the 16K protein (10). More specifically, E5-16K protein interactions appear to be mediated by polar or charged amino acid residues of their respective transmembrane domains (1). In contrast, E5-EGF-R interactions appear to be mediated by the E5 C-terminal domain (4) and may reflect recently described interactions between E5 and an alpha-adaptin-like protein (5).

Although the mechanism of receptor activation by E5 has not been identified, several distinct but nonexclusive possibilities exist, including the following: (i) dimeric E5 protein may facilitate the cross-linking of receptors and thereby initiate receptor transphosphorylation and activation and (ii) E5 may interfere with the function of the V-ATPase (via 16K protein binding) and alter the downregulation and/or processing of receptors at either plasma membrane or Golgi apparatus locations, inducing a prolonged mitogenic response. Indirect mechanisms of receptor activation are also a possibility.

This study examines the correlation between the ability of E5 to bind (i.e., form stable complexes with) either endogenous PDGF-R or 16K protein in fibroblasts and its ability to transform cells. We have also evaluated E5–PDGF-R interactions in a cell line, 32D, which was stably transfected with PDGF-R. Specifically, we have utilized a series of E5 transmembrane mutants (altered at position 17) which were recently shown to exhibit variant transforming activity on fibroblasts (33). Since the transmembrane domain of E5 appears to mediate its binding to cell proteins, we hypothesized that it might be possible to utilize these mutants to determine whether the 16K protein or PDGF-R was the predominant
target for E5 binding and consequent mitogenic signalling. Our results indicated that there was no absolute correspondence between the ability of E5 to bind either PDGF-R or the 16K protein and its ability to transform fibroblasts. Indeed, in fibroblasts, all position 17 mutants showed greatly reduced binding to PDGF-R, despite the finding that most of the transforming E5 proteins were able to induce receptor phosphorylation.

The most informative E5 point mutant (serine 17 mutant) was unable to associate with PDGF-R or induce receptor autophosphorylation, despite being able to transform NIH 3T3 and C127 cells efficiently. In 32D cells expressing PDGF-R, serine 17 mutant E5 protein was incapable of generating cellular transformation or associating with and inducing auto-phosphorylation of PDGF-R, further demonstrating a lack of cooperative interaction between serine 17 mutant E5 protein and PDGF-R. The simplest explanation for these findings is that serine 17 mutant E5 protein functionally interacts with a distinct or additional cellular target to induce fibroblast transformation.

MATERIALS AND METHODS

Cell culture. COS-1, NIH 3T3, and C127 cell lines were grown in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal calf serum. The murine interleukin 3 (IL-3)-dependent hematopoietic cell line 32D (15) was cultured in medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 15% fetal bovine serum (Biofluids, Rockville, Md.) and 5% WEHI 3B-conditioned medium as a source of murine IL-3 (14).

Plasmid constructions. Construction of the expression vector pSS55, a modified pHG plasmid (Stratagene), and corresponding E5 glutamine 17 substitution mutants has been described previously (33). These constructs were used for expression in COS-1 and mouse fibroblast cells.

For analysis of BPV-1 E5 oncoproteins in 32D cells, constructs were made using expression vector pLTR-neo (gift from D. Goldstein) (6, 28). Construction of the expression vector pBS55, a modified pSG plasmid (Stratagene), and corresponding E5 glutamine 17 substitution mutants has been described previously (33). These constructs were used for expression in COS-1 and mouse fibroblast cells.

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and mutant E5 proteins and independence of PDGF-R.

To dissect the interaction of wt E5 with various mutants, phorylation and induce 32D cells to become independent of factor receptors present on fibroblasts. It has already been shown that wild-type (wt) E5 can induce IL-3-independent growth (33). These constructs vary in focus- ing ability on mouse fibroblast cell lines C127 and NIH 3T3 and include hypertransforming as well as defective forms. In order to facilitate protein detection and isolation, all E5 constructs contained the six-amino-acid epitope AU1 at the N terminus. This small epitope does not interfere with either E5 biologic activity or intracellular localization (33).

Studies with 32D hematopoietic cells. We have previously noted that the expression of a given E5 point mutation in C127 and NIH 3T3 fibroblasts induces different phenotypes (33). For example, while the aspartic acid mutant was hyperactive in C127 cells, it was defective in NIH 3T3 cells. To evaluate the biological behavior of these E5 mutants in an additional and very different cell type and assay system, we utilized 32D hematopoietic cells. These cells allowed us to evaluate the cooperative interaction of the E5 mutants with β PDGF-R without the potential interference of additional tyrosine kinase growth factor receptors present on fibroblasts. It has already been shown that wild-type (wt) E5 can induce β-PDGFR autophosphorylation and induce 32D cells to become independent of IL-3 for growth in vitro (12). In addition, we have also used this system to dissect the interaction of wt E5 with various mutants of PDGF-R (35).

(i) Two E5 mutants (glutamic acid and lysine) confer IL-3 independence. The plasmid pLTR was used to express the wt and mutant E5 proteins and β PDGF-R in 32D cells (6, 28). A PDGF-R-expressing cell line was first generated by transfecting naive 32D cells with the β-PDGF-R vector pLTR/PR. This line was then subjected to a second round of transfection with each of the E5 constructs. Transfected cells were then screened for their ability to confer IL-3-independent growth by resuspending cells in IL-3-deficient medium, plating the cells in 100-well trays, and scoring for the number of growing colonies after 3 weeks. Results from three assays demonstrated that only the glutamic acid and lysine E5 mutants were able to induce IL-3-independent growth (Fig. 2). The ability of the E5 glutamic acid mutant to induce IL-3-independent growth in 32D cells parallels its strong transforming activity in NIH 3T3 and C127 cells. However, while the lysine mutant was relatively active in 32D cells (approximately 40% of wt levels), it was less active in NIH 3T3 cells (7% of wt levels). Other mutants which exhibited transformation efficiencies in NIH 3T3 cells similar to that of the lysine mutant (e.g., aspartic acid mutant) produced very little transformation in 32D cells. Most significantly, the serine mutant, which was relatively active in NIH 3T3 cells (40% of wt levels) and hypertransforming in C127 cells (300% of wt activity), was completely defective in 32D cells, indicating a lack of cooperative interaction between E5 and β PDGF-R.

To show that the biological defect in the E5 mutant proteins was not due to protein instability or an inability to dimerize, 32D cell lines were screened for oncprotein expression. Figure 3 demonstrates that all E5 position 17 mutants were expressed at similar levels and that they formed homodimers. In general, there was no correlation between apparent efficiency of dimer formation and transforming activity. While the transformation-defective phenylalanine mutant formed dimers poorly, other transformation-defective mutants (e.g., histidine and serine mutants) had a greater dimer/monomer ratio than the transformation-proficient glutamic acid mutant.

(ii) Although both the glutamic acid and the lysine E5 mutants induce PDGF-R autophosphorylation, the lysine mutant fails to associate with the receptor. To evaluate whether E5
PDGF-R binding was predictive of transformation in 32D cells, we also determined the amount of PDGF-R which was coprecipitated with an antibody against the E5 protein, and the immunoprecipitates were resolved on a 14% polyacrylamide gel in the absence of reducing agent in the sample buffer. Proteins were transferred to PVDF membranes and then immunoblotted with the same E5 antibody. Detection was performed with chemiluminescence. Positions of E5 monomers and dimers are shown on the right. Molecular mass markers (in kilodaltons) are shown on the left. IP, immunoprecipitation; Ab, antibody.

FIG. 3. E5 glutamine mutants are stably synthesized and form homodimers in 32D cells. E5-expressing 32D cells were lysed in a modified RIPA buffer, the lysates were immunoprecipitated with an antibody against the E5 protein, and the immunoprecipitates were resolved on a 14% polyacrylamide gel in the absence of reducing agent in the sample buffer. Proteins were transferred to PVDF membranes and then immunoblotted with the same E5 antibody. Detection was performed with chemiluminescence. Positions of E5 monomers and dimers are shown on the right. Molecular mass markers (in kilodaltons) are shown on the left. IP, immunoprecipitation; Ab, antibody.

Studies with NIH 3T3 cells. While there was a complete correlation between the abilities of E5 mutants to transform 32D cells and to induce β-PDGFR autophosphorylation, we questioned whether these same mutants might display different phenotypes in fibroblasts (the relevant target cell for tumorigenesis). Fibroblasts express a variety of tyrosine kinase growth factors on their cell surface and might be expected to exhibit a more complex response to the expression of E5. In addition, analysis of E5 mutants in fibroblasts allows for the evaluation of E5-16K protein interactions which cannot be determined in 32D cells because of the inability to effectively label these cells with [35S]methionine or [35S]cysteine.

(i) Binding of E5 mutant proteins to 16K protein does not correlate with their biological activities. To determine whether the E5 mutant proteins exhibited altered interaction with the 16K protein, we performed the following immunoprecipitation study. Neomycin-selected NIH 3T3 cell lines (which had been stably transfected with each of the indicated E5 mutant constructs [see Materials and Methods]) were labelled with [35S]methionine and immunoprecipitated with the monoclonal antibody recognizing the AU1 epitope, and the samples were resolved on SDS-polyacrylamide gels. The results in Fig. 6 confirmed that all position 17 mutants could be expressed from stable cell lines (33) and that the differences in their biological activity were not due to the inability of an E5 protein to be synthesized in mouse fibroblasts. In addition, numerous experiments indicated that there were no gross differences in the level of E5 expression that could account for variations in biological activity (data not shown).

Analysis of E5 immunoprecipitates from these cell lines revealed that there was a profound difference in coprecipitated 16K protein (bottom panels of Fig. 6). E5 proteins with negatively charged amino acids at position 17 exhibited greatly reduced 16K protein binding (e.g., glutamic acid and aspartic acid), despite the observation that the former showed strong transforming activity (136% of the wt level). E5 with a polar serine residue was also dramatically reduced in 16K protein binding despite retaining 39% of wt transforming activity. In contrast, E5 mutants containing positively charged amino acids were nontransforming E5 mutants were unable to facilitate receptor autophosphorylation.

FIG. 4. Only wt and glutamic acid 17 mutant E5 proteins associate with immature PDGF-R in 32D cells. 32D cell lines coexpressing PDGF-R and each E5 construct were lysed in Triton X-100 buffer. Ninety percent of each extract was immunoprecipitated with an anti-E5 antibody, and the remainder was immunoprecipitated with an anti-PDGF-R antibody. Samples were resolved on 7.5% polyacrylamide gels and transferred to PVDF membranes which were subsequently immunoblotted with the anti-PDGF-R antibody. Detection was performed with chemiluminescence. m, mature; i, immature PDGF-R; IP, immunoprecipitation; Ab, antibody. Molecular mass markers (in kilodaltons) are shown on the left.
(e.g., arginine, histidine, and lysine) showed strong 16K protein binding although their transforming activities were uniformly low (7, 3, and 2%, respectively). Noncharged residues (i.e., glycine) interfered with both 16K protein interaction and cell transformation, corroborating the results of an earlier study (11). These binding studies are also in agreement with a previous genetic analysis demonstrating that the glutamine residue in the E5 transmembrane domain interacts with the negative

FIG. 5. Abrogation of IL3-dependence for growth correlates with E5-induced autophosphorylation of immature PDGF-R in 32D cells. 32D cell lines coexpressing PDGF-R and each E5 construct were lysed in Triton X-100 buffer. Ten percent of each lysate was immunoprecipitated with an anti-PDGF-R antibody, and the remainder was immunoprecipitated with the antiphosphotyrosine antibody. Immunoprecipitates were resolved on 7.5% polyacrylamide gels in the presence of reducing agents and transferred to PVDF membranes which were subsequently immunoblotted with the anti-PDGF-R antibody. Signals were detected by chemiluminescence. Positions for the mature and immature PDGF-R (m PR and i PR, respectively) are shown on the right. IP, immunoprecipitation; Ab, antibody. Molecular mass markers (in kilodaltons) are shown on the left.

FIG. 6. Glutamine 17 mutants dissociate transforming ability from binding to 16K protein and βPDGF-R in NIH3T3 cells. NIH3T3 cell lines expressing each of the E5 constructs were metabolically labelled with [35S]methionine as described in Materials and Methods. Cell lysates were first immunoprecipitated with antibody against the epitope-tagged E5 protein (AU1). An aliquot was removed for E5 analysis on a 14% polyacrylamide gel, and the remainder of the immunoprecipitate was subjected to a second immunoprecipitation using antibodies specific for the βPDGF-R. Proteins from the second immunoprecipitation were resolved on a 7.5% polyacrylamide gel. Positions for the mature and immature PDGF-R (m PR and i PR, respectively), 16K protein, and E5 are shown on the right. Molecular mass markers (in kilodaltons) are shown on the left.
atively charged glutamic acid residue in the fourth transmembrane domain of the 16K protein (1). Thus, it would be anticipated that the substitution of negatively charged residues into position 17 of E5 would interfere with 16K protein binding and that substitution with positively charged residues would augment interaction.

Overall, there were three classes of E5 mutants with regard to 16K protein association and cellular transformation: (i) those that bound 16K protein and were transformation defective, (ii) those that did not associate with 16K protein but were biologically active, and (iii) those that did not associate with 16K protein and also did not transform cells. While extensive mutagenesis of E5 residue 17 has clearly dissociated transforming activity from 16K protein binding, these results probably represent the complex interaction of the E5 transmembrane domain not only with cellular target proteins but also with adjacent E5 transmembrane domains (homodimer formation). (ii) All E5 position 17 mutants exhibit markedly reduced interaction with β-PDGFR. While there have been numerous studies implicating PDGFR as the target for E5 (10, 12, 22, 25–27, 29), there are limited data on the ability of various transforming and nontransforming E5 transmembrane mutants to associate with this receptor. In order to pursue a systematic evaluation of such mutants, we evaluated E5 immunoprecipitates of the fibroblast lines described above for coprecipitated β-PDGFR. As indicated in the upper panels of Fig. 6, a second round of immunoprecipitation with antibodies against β-PDGFR confirmed that wt E5 associated predominantly with the immature form of PDGFR (25), although the mature form also coprecipitated. Regardless of amino acid substitution, all position 17 mutants showed greatly reduced PDGFR association, indicating that PDGFR association was strictly dependent upon the E5 glutamine residue at position 17. Interestingly, the glutamic acid substitution mutant (which transforms NIH 3T3 cells more efficiently than wt E5 [Fig. 1] [34]) exhibited one of the lowest levels of association with PDGFR (Fig. 6, lane Glu). Transformation-defective mutants, such as the arginine substitution mutant, exhibited higher levels of PDGFR association than the hypertransforming glutamic acid mutant. The serine mutant also showed very low levels of receptor association despite its being one of the few mutants which retained significant transforming activity. Binding studies (to show levels of associated 16K protein and PDGFR) were carried out three times, and densitometry was also performed to quantitate the amount of immature receptor bound to either wt or mutant E5. All E5 mutants demonstrated binding of 13% or less compared with the level of binding of wt E5, including the hyperactive glutamic acid mutant. Clearly, E5-receptor association does not predict E5 transforming activity, and the glutamic acid E5 mutant illustrates convincingly that it is not necessary for E5 to associate stably with PDGFR in order to transform NIH 3T3 cells.

(iii) Cell transformation by the E5 mutants correlates with PDGFR autophosphorylation except for the serine mutant. Previous studies have demonstrated a correlation between the transforming activity of E5 and its ability to induce β-PDGFR autophosphorylation (8, 12, 22, 25). More specifically, this induction of receptor autophosphorylation affects primarily the immature form of the receptor (22, 23, 25, 27). In order to determine if the level of receptor autophosphorylation paralleled the transformation efficiency of the E5 mutant proteins described above, we immunoprecipitated all cell lines with either anti-PDGFR antibodies (to determine the total amount of cellular receptor) or antiphosphotyrosine antibodies (to determine the level of PDGFR autophosphorylation). Following separation of immunoprecipitate samples on acrylamide gels, proteins were transferred to PVDF membranes and immunoblotted with the anti-PDGFR antibodies.

The results shown in Fig. 7 (upper panel) indicated that the wt E5 protein and the transforming E5 glutamic acid mutant induced phosphorylation of immature PDGFR but that all of the transformation-defective E5 mutants (i.e., glycine, phenylalanine, and asparagine mutants) failed to induce immature-receptor phosphorylation. However, the transforming serine mutant (approximately 40% of wt activity in NIH 3T3 cells and 300% of wt activity in C127 cells [33]) was defective for inducing receptor autophosphorylation. In order to rule out artifacts due to neomycin resistance selection, NIH 3T3 lines expressing either wt E5 or serine 17 mutant E5 were also generated by isolating transformed foci. Similar to findings with the drug-selected lines, only the foci expressing wt E5 (and not those expressing the serine 17 E5 mutant) showed PDGFR auto- phosphorylation (data not shown). In addition, other mutants with lower transforming activities (aspartic acid, 10%; lysine, 7%) were also defective for this induction. Evidently, phosphorylation of immature PDGFR was not a requirement for cellular transformation by the serine mutant. All E5 mutants, whether transforming or nontransforming, induced variable phosphorylation of the mature form of PDGFR, suggesting that its activation did not result in cellular transformation (27). In summary, our results indicate that the level of E5-induced phosphorylation of immature PDGFR correlated with cell transforming activity for most E5 mutants, with the exception of the serine mutant. The serine E5 mutant transformed cells without significantly binding to or inducing autophosphorylation of PDGFR.

E5 mutants also dissociate PDGFR binding and autophosphorylation from cell transformation in C127 cells. We also evaluated the biochemical properties of the E5 mutants in an additional fibroblast cell line, C127. In C127 cells, the serine 17 E5 mutant displays a hyperactive phenotype and transforms cells with 300% of the wt level of activity (33). It was important to verify whether this serine mutant was also defective for activation of PDGFR in this cell line as well as to establish whether the other E5 mutants showed a similar dissociation of receptor binding and cell transformation. The ability of the E5 mutant proteins to bind PDGFR was assayed as described above for NIH 3T3 cells. Induction of receptor autophosphorylation was determined through a double immunoprecipitation. Figure 8A shows that the transformation-defective basic substitution (arginine and lysine) mutants bound significant levels of receptor (approximately 85 and 30% of wt levels, respectively), while the transformation-competent basic histidine mutant also bound receptor (approximately 30%). In contrast, the highly transforming serine, glutamic acid, and aspartic acid mutants bound low levels of receptor (approximately 6, 13, and 10% of the wt level, respectively). It appeared, therefore, that the level of binding of PDGFR did not correlate with the ability to transform C127 cells. The 16K subunit was not obviously core precipitated with the E5 molecules in these experiments. It has been reported previously that detection of 16K protein in C127 cells is greatly reduced compared with that in NIH 3T3 cells (34).

The ability of the hypertransforming serine mutant to induce PDGFR autophosphorylation in C127 cells was also analyzed. For these experiments, cells expressing the indicated E5 mutant proteins were metabolically labelled with [35S]methionine and subjected to double immunoprecipitation using antibodies against β PDGFR and subsequently with antibodies against phosphotyrosine as described in Materials and Methods. Figure 8B demonstrates the induction of immature-PDGFR auto- phosphorylation by wt E5. In contrast, there were only back-
ground levels of receptor phosphorylation in cell lines expressing vector alone, the defective asparagine mutant, or the hypertransforming serine mutant. It appears, therefore, that the serine mutant induces cellular transformation independent of PDGF-R binding and activation.

All E5 position 17 mutant proteins localize to the Golgi apparatus. Although we have previously shown that each of the described E5 transmembrane mutants forms dimers normally (33), it remained a possibility that some of these mutants were transformation defective because of abnormal intracellular topology. This is particularly important to evaluate since recent findings indicate that E5 cannot mitogenically signal if it is restricted to the endoplasmic reticulum or cis Golgi region and is prevented from accumulating in the trans Golgi region (34).

To determine the intracellular localization of each of the E5 mutants, we performed immunofluorescence microscopy on COS-1 cells transfected with each of the mutant E5 construct plasmids. Cell fixation and staining with the AU1 antibody (specific for the AU1 epitope appended to the E5 N terminus) are described in Materials and Methods. As Fig. 9 demonstrates, a selected group of transforming and nontransforming E5 mutant proteins localized in the Golgi apparatus similarly to wt E5, indicating that defects in accumulation in the Golgi apparatus did not account for their inability to transform cells or to induce autophosphorylation of PDGF-R. These results also indicate that position 17, while critical for mediating the interaction of E5 with cellular proteins, was not essential for E5 translocation and retention in the Golgi apparatus. Since an E5 mutant with its C terminus truncated was also able to localize to the Golgi apparatus (unpublished results), it appears that the transmembrane domain is sufficient for targeting E5 to the Golgi apparatus.

**DISCUSSION**

Previous mutagenic analysis has shown the importance of the transmembrane glutamine residue (position 17) for the biological activity of the BPV-1 E5 protein (11, 33). While the glutamine residue is not absolutely required for focus formation on mouse fibroblasts, it is highly conserved among the fibropapillomaviruses, and most substitutions at this position result in a transformation-defective protein (11, 33). Our study involved the use of biochemical assays with E5 position 17 mutants in an attempt to identify the molecular mechanism(s) of E5 transformation (results summarized in Tables 1 and 2).

**Lack of correlation between E5–PDGF-R or E5-16K protein interactions and cell transformation.** In fibroblasts, all E5 mutants displayed reduced binding to PDGF-R, including those that transformed well, such as the glutamic acid and serine mutants. The glutamic acid mutant, in particular, exhibited an extremely low level of binding to PDGF-R.

Our analysis of a series of position 17 mutants indicates that, in NIH 3T3, C127, and 32D cells, there was no direct correlation between E5 transforming activity and binding to PDGF-R. There was, however, a direct parallel between E5 transforming activity and induction of PDGF-R autophosphorylation, with the exception of the serine 17 mutant. The E5 serine mutant was able to transform NIH 3T3 and C127 cells with levels of activity of 40 and 300% of the wt, respectively, yet was defective for both binding and inducing autophosphorylation of PDGF-R. While this may appear contradictory to several pre-
Previous reports claiming requirements for PDGF-R in E5-mediated cellular transformation (8, 12, 22), this study represents the first extensive analysis of transmembrane E5 mutants for their abilities to bind 16K protein and PDGF-R, induce PDGF-R autophosphorylation, and transform cells. Previous studies have focused predominantly on receptor requirements and receptor mutants. The current study, therefore, indicates that E5-mediated cellular transformation, particularly in mouse fibroblasts, may proceed via mechanisms independent of PDGF-R.

Previous studies had demonstrated a correlation between the abilities of E5 to bind 16K protein and to transform cells (11). Substitution of glutamine 17 with either a glycine or a leucine residue resulted in a biologically inactive E5 molecule that did not associate with the membrane-spanning, 16K V-ATPase subunit. This finding led to the hypothesis that E5 protein might function by disrupting the V-ATPase proton pump. Interference with the proton pump would be predicted to alkalinate acidic compartments such as endosomes, lysosomes, and the trans Golgi apparatus and interfere with many cellular processes, including receptor downregulation and receptor processing and maturation in the Golgi apparatus. One exception to this observed correlation was a reported mutant, HR15, which generated significant focus formation on mouse fibroblasts yet did not associate with 16K protein in the same cell type (11). Since then, HR15-16K protein coprecipitation has been repeated and this E5 protein has been shown to bind to the 16K protein (unpublished data). However, since we were able to detect normal or increased amounts of 16K protein bound to nontransforming E5 position 17 mutants (Arg, His, and Lys) and significantly less 16K protein bound to active E5 mutants (Glu and Ser), there does not appear to be a direct correlation between transformation and 16K protein binding. Similar to our conclusions about E5-PDGF-R interactions for fibroblast transformation, we cannot rule out 16K protein as a potential target for wt E5. Indeed, the transforming serine mutant, which binds poorly to 16K protein and PDGF-R and fails to induce PDGF-R autophosphorylation, may simply reflect the retargeting of this particular mutant protein to another regulatory protein. It is still possible that PDGF-R and

![Fig. 8](http://jvi.asm.org/)

**FIG. 8.** E5 mutants dissociate transforming activity from PDGF-R binding and autophosphorylation in C127 cells. (A) C127 lines expressing E5 were metabolically labelled with [35S]methionine, and extracts were immunoprecipitated first with antibody AU1 against the appended E5 epitope. An aliquot of the first immunoprecipitate was used for second immunoprecipitation with the β-PDGF-R antibody. Proteins from the second immunoprecipitation were resolved on a 7.5% polyacrylamide gel. Positions for immature PDGF-R (i PR) and E5 are indicated. Molecular mass markers (in kilodaltons) are listed on the left. (B) C127 lines expressing E5 were metabolically labelled with [35S]methionine, and extracts were immunoprecipitated first with antibodies against the PDGF-R. An aliquot of the first immunoprecipitate was removed and used for determining total levels of receptor. The remainder of the immunoprecipitate was used for a second immunoprecipitation with antiphosphotyrosine antibody. Proteins were resolved on a 7.5% polyacrylamide gel. Positions for mature and immature PDGF-R (m PR and i PR, respectively) and E5 are shown on the right. Molecular mass markers (in kilodaltons) are listed on the left.
16K protein are important targets for wt E5 in several cell types, but there clearly needs to be another target(s) for the serine E5 mutant in mouse fibroblasts. It is also apparent from recent studies that mutation of the 16K protein can convert it into an oncoprotein, suggesting its potential importance in E5-mediated cell transformation (1).

Several studies have delineated β PDGF-R as the primary target for E5-mediated cell transformation (22, 25–27, 29). Indeed, in mouse hematopoietic cells, β PDGF-R appears to be a necessary and sufficient component for wt E5 to generate a mitogenic signal in the absence of IL-3 (8, 12). In addition, E5 coprecipitates with PDGF-R, suggesting that there is a functional, physical interaction between the receptor and the oncoprotein (10, 27). In the current study, glutamic acid mutant E5 but not lysine mutant E5 (both inducers of IL-3-independent growth) associated with the receptor. All transformation-defective E5 mutants also failed to associate with PDGF-R in the same cells.

It is much more difficult to explain the activity of the serine E5 mutant without invoking PDGF-R-independent mechanisms. The serine mutant was hypertransforming in C127 cells (300% of wt activity), mildly reduced in NIH 3T3 cells (40% of wt activity), and completely inactive in 32D cells, despite the presence of PDGF-R in all of these cell lines. Most importantly, the serine mutant did not bind or induce PDGF-R.

### TABLE 1. Characterization summary for BPV-1 E5 glutamine 17 mutants in 32D cells

<table>
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<tr>
<th>Residue at position 17</th>
<th>Dimerization</th>
<th>IL-3 abrogation</th>
<th>Immature β PDGF-R: Association</th>
<th>Tyrosine phosphorylation</th>
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<td>Histidine</td>
<td>++</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Lysine</td>
<td>++</td>
<td>+</td>
<td>–</td>
<td>++</td>
</tr>
<tr>
<td>Arginine</td>
<td>++</td>
<td>–</td>
<td>–</td>
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</tr>
<tr>
<td>Nonpolar</td>
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<tr>
<td>Phenylalanine</td>
<td>+</td>
<td>–</td>
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*Results are presented as very positive (+++), positive (+), or negative (–) for each characteristic.*
autophosphorylation in either NIH 3T3 or C127 cells. Unlike the case for the glutamic acid mutant, we cannot postulate that the apparent lack of binding reflects an increased rate of dissociation from E5-receptor complexes, because the serine mutant also fails to induce autophosphorylation of the receptor. It would be necessary to speculate that the serine mutant also induced increased dephosphorylation of PDGF-R in order to explain its lack of demonstrable phosphorylation on tyrosine residues. How then is the serine mutant able to transform cells in the absence of receptor autophosphorylation? Two possibilities are discussed below.

(i) The serine 17 mutant still induces signal transduction through PDGF-R without inducing receptor autophosphorylation. Induction of signal transduction without induction of receptor phosphorylation has been reported previously for a v-sis recombinant protein (19). When v-sis is fused to the transmembrane domain of the vesicular stomatitis virus G protein, it associates with cellular membranes. This membrane-bound form of v-sis is expressed on the cell surface and induces a mitogenic response and even promotes focus formation on NIH 3T3 cells. Surprisingly, the membrane-bound v-sis protein does not induce detectable levels of PDGF-R autophosphorylation, suggesting that v-sis need not necessarily induce this state to transmit a mitogenic signal. It is possible that the serine E5 mutant might also signal without the need for receptor autophosphorylation. In addition, serine mutant E5 may also signal indirectly through PDGF-R in the absence of a direct association between the two molecules.

(ii) The serine 17 mutant generates a mitogenic signal via a different cellular target. The finding that a single amino acid change in E5 can decrease its association with the 16K protein and PDGF-R suggests that the serine mutant might also display an altered (possibly increased) association with other cellular proteins (i.e., other growth factor receptors or possibly other receptor-associated signalling proteins). Possible alternate targets would include the closely related α PDGF-R, which contains 46% identical amino acids in its transmembrane domain. However, other receptor proteins such as EGF-R are also potential candidates.

It is unclear why all E5 mutants retain the ability to induce the phosphorylation of the mature form of PDGF-R, although it is clear that this activity is not sufficient to convey a continued mitogenic response which would induce focus formation. It is possible that the dimeric E5 mutant proteins might still associate sufficiently well with mature receptor to induce their cross-linking and activation but that surface-associated receptor cannot function as a continued mitogenic source. Stable cellular transformation may require receptor signalling from the cell interior, a location where activated receptors may not be as readily downregulated.

Perhaps the greatest insight into E5 biology is provided by the serine mutant. This mutant transformed NIH 3T3 and C127 cells despite its inability to bind and induce autophosphorylation of PDGF-R, suggesting that it can transform cells via a mechanism independent of PDGF-R. Compatible with this hypothesis is the finding that the E5 serine mutant cannot transform 32D cells expressing PDGF-R alone. Regardless of the ultimate explanation for the activity of the E5 serine mutant, further analysis of its biochemical and biological activities should permit the definition of new regulatory mechanisms in the receptor transformation-signalling pathway, including potential new insights into wt E5 function.

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