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Mutational Analysis of the Interaction between the Bovine Papillomavirus E5 Transforming Protein and the Endogenous β Receptor for Platelet-Derived Growth Factor in Mouse C127 Cells

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The bovine papillomavirus E5 protein is a 44-amino-acid membrane-associated protein that forms a stable complex with the endogenous platelet-derived growth factor (PDGF) β receptor in rodent and bovine fibroblasts, resulting in sustained receptor activation and cell transformation. We report here that high-level expression of the E5 protein caused a reduction in the level of the mature form of the PDGF β receptor in acutely and stably transformed mouse C127 cells. To explore in more detail the interaction of the E5 protein and the PDGF β receptor, we tested the abilities of various E5 point mutants to bind the PDGF receptor, to induce PDGF receptor down-regulation and tyrosine phosphorylation, and to transform cells. A transformation-competent mutant, like the wild-type E5 protein, bound the receptor and induced receptor tyrosine phosphorylation and down-regulation. Transformation-defective E5 proteins either failed to interact with the endogenous PDGF β receptor in mouse fibroblasts or underwent an aberrant interaction with the receptor. Mutation of glutamine at position 17, aspartic acid at position 33, or both carboxyl-terminal cysteine residues required for E5 homodimerization interfered with stable complex formation with the PDGF receptor, tyrosine phosphorylation and down-regulation of the receptor, and cell transformation. Point mutations at several other carboxyl-terminal positions generated transformation-defective E5 proteins that formed a complex with the PDGF receptor and induced receptor tyrosine phosphorylation but did not induce PDGF receptor down-regulation. Either PDGF receptor activation is not sufficient for transformation of C127 cells or the receptors that are tyrosine phosphorylated in response to these mutant E5 proteins are not fully activated and therefore are not able to deliver a mitogenic signal.

The bovine papillomavirus (BPV) E5 protein induces morphologic and tumorigenic transformation of rodent and bovine fibroblasts, and considerable evidence indicates that the cellular platelet-derived growth factor (PDGF) β receptor is an important mediator of this transforming activity (reviewed in reference 9). The PDGF β receptor is a transmembrane receptor tyrosine kinase that is normally activated when its physiological ligand, PDGF, binds to the extracellular domain of the receptor, thereby inducing receptor dimerization and stimulation of its intrinsic protein tyrosine kinase activity (reviewed in reference 16). These events result in receptor autophosphorylation on at least nine tyrosine residues, association with cellular SH2 domain-containing proteins, and mitogenic signaling (5). Similarly, the BPV E5 protein forms a stable complex with the endogenous PDGF β receptor in transformed fibroblasts and induces sustained activation of the receptor as assessed by increased receptor tyrosine kinase activity, autophosphorylation on tyrosine, and association with cellular SH2 domain-containing signal transduction proteins (10, 15, 29–31). Both the mature PDGF β receptor and an intracellular precursor form of the receptor containing immature carbohydrate side chains are tyrosine phosphorylated in E5-transformed cells (10, 15, 27, 30, 31). Furthermore, the integrity of the endogenous PDGF signaling pathway appears to be required for efficient transformation of mouse C127 cells by the E5 protein (32). In gene transfer experiments, introduction of a PDGF β receptor cDNA, but not cDNAs encoding a variety of other growth factor receptors, is sufficient to enable a cell to respond to the E5 protein (6, 10, 15, 27). In such experiments, the tyrosine kinase activity of the receptor is required for E5-induced proliferation (10). These results provide compelling evidence supporting the model that expression of the E5 protein causes sustained activation of the PDGF β receptor, resulting in mitogenic stimulation and cell growth transformation. Complex formation also occurs between the E5 protein and two other cellular proteins, a 16-kDa subunit of the vacuolar H^+-ATPase and a protein related to α-adaptin (7, 14).

The mechanism by which the E5 protein activates the PDGF β receptor is incompletely understood. Receptor activation has been proposed to result from the formation of the complex between the E5 protein and the PDGF receptor (6, 13, 29, 30). However, biochemical and genetic studies indicate that the E5 protein does not mimic the normal interaction of PDGF with its receptor (6, 10, 13, 26). The monomeric E5 protein is only 44 amino acids long and exists largely as a homodimer composed of 7-kDa subunits joined by a pair of disulfide bonds formed by carboxyl-terminal cysteine residues (2, 19, 35). The amino-terminal two-thirds of the E5 protein is extremely hydrophobic and is thought to serve as a membrane anchor that localizes the protein to intracellular membranes, where it appears to be a type II transmembrane protein with its hydrophilic carboxyl-terminal segment displayed in the lumen of the endoplasmic reticulum and Golgi apparatus (3, 4). In contrast, PDGF is a soluble 30-kDa protein composed of two identical or similar hydrophilic subunits (16). Furthermore, studies with chimeric receptor molecules suggest that the transmembrane

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domain of the PDGF receptor is the primary site of interaction with the E5 protein, and analysis of receptor mutants indicates that most of the extracellular, PDGF-binding domain of the receptor is dispensable for mitogenic signaling in response to the E5 protein (6, 10). These results indicate that activation of the receptor by the E5 protein does not result from receptor recognition by PDGF but instead is ligand independent.

Extensive mutational analysis has been carried out on the E5 protein (2, 8, 19, 20, 22, 26, 37, 38). These genetic studies indicate that large segments of the E5 protein can be removed or replaced without interfering with its transforming activity. The glutamine at position 17 (in the otherwise hydrophobic central segment of the protein) and a cluster of conserved amino acids in the hydrophilic carboxyl-terminal segment, including the cysteines that mediate disulfide bond formation, have been identified as being required for acute and stable transformation of mouse C127 cells (19, 22). To explore the mechanism of ligand-independent PDGF receptor activation by the E5 protein, we have characterized the abilities of mutant E5 proteins to interact with endogenous PDGF β receptors in mouse C127 fibroblasts.

Effect of BPV E5 expression on PDGF β receptor levels. When the PDGF receptor is activated by PDGF treatment, it undergoes rapid internalization and degradation resulting in decreased receptor levels, a phenomenon referred to as receptor down-regulation (1, 17, 28, 34). In contrast, C127 cells stably transformed by a recombinant retrovirus expressing the E5 gene under the control of a Moloney murine leukemia virus long terminal repeat contained approximately the same amount of PDGF β receptor as did nontransformed cells (29, 31). However, cells transformed by a BPV mutant that overexpresses the E5 protein (33) contained severalfold less PDGF β receptor than control cells (18). These results suggested that increased levels of the E5 protein may result in reduced levels of the PDGF β receptor in transformed C127 cells. To determine whether the E5 protein, like PDGF, influenced the level of PDGF β receptor, we used immunoblotting to measure PDGF receptor levels in cells infected with a BPV-simian virus 40 (SV40) recombinant virus (designated Pava 1). We refer to this system as an acute transformation system since the E5 gene is rapidly delivered into an entire population of cells, resulting in morphologic transformation within 24 to 36 h (36). It therefore allows biochemical analysis of the direct effects of the E5 protein without the complication of delayed or secondary responses that may occur during derivation or extended passage of cell lines.

C127 cells were infected at several different multiplicities, either with Pava 1, which expresses the wild-type E5 gene, or with Pava-E5d29, a control virus which expresses an E5 frameshift mutant (36). At the time of harvest 38 to 39 h after infection, cells infected with the mutant virus remained flat, whereas cells infected with the virus expressing the wild-type E5 gene displayed dose-dependent morphologic transformation (data not shown). Cells infected at high multiplicity with the wild-type virus expressed severalfold more E5 protein than cells stably transformed by the retrovirus expressing the E5 gene (data not shown). To determine levels of PDGF receptor in these acutely transformed cells, extracts were prepared in radioimmunoprecipitation assay (RIPA) buffer, immunoprecipitated with a PDGF β receptor antiserum, and immunoblotted with the same antiserum as previously described (23, 30) (Fig. 1A). Cells infected with a range of multiplicities of the control virus contained the same amount of PDGF β receptor as did mock-infected cells. In contrast, in cells infected with the virus expressing the wild-type E5 gene, the amount of the mature form of the PDGF β receptor decreased with increasing multiplicity of infection. The level of the precursor form of

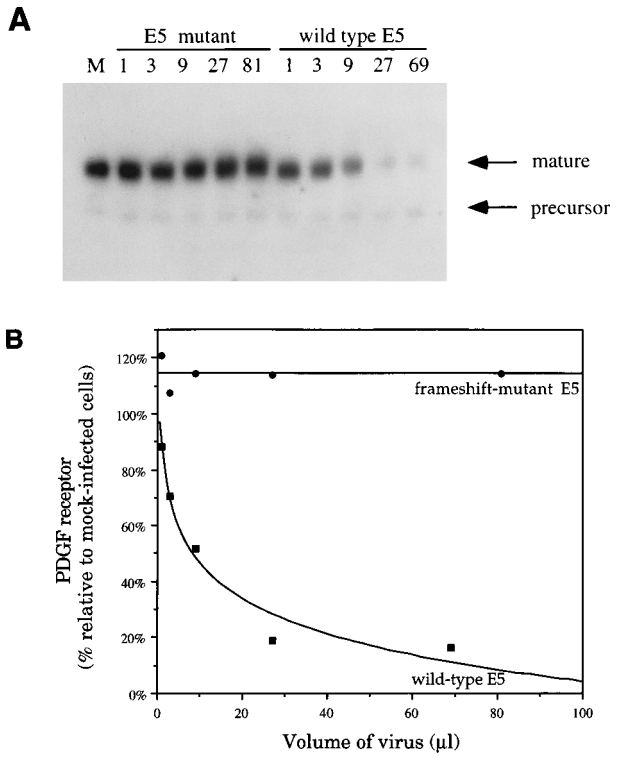

FIG. 1. Down-regulation of the PDGF β receptor by the wild-type BPV E5 protein. (A) C127 cells were infected with the indicated amounts (in microliters) of a high-titer virus stock of Pava 1-E5d29 (E5 mutant) or Pava 1 (wild type). We estimate that a multiplicity of infection of ~50 was attained at the highest dose of virus. At 39 h after infection, RIPA buffer extracts were prepared. PDGF receptor was visualized by immunoprecipitation from 50 μg of extracted protein, gel electrophoresis, and immunoblotting with antiserum α-PR4 as described previously (23, 30). Lane M, mock-infected cells. The positions of the mature and precursor forms of the PDGF β receptor are indicated. (B) The filter shown in panel A was quantitated by PhosphorImager analysis. The intensity of the band corresponding to the mature form of the PDGF β receptor is expressed as a percentage of the intensity in the sample from mock-infected cells.

the receptor was not affected by E5 expression. The relative levels of the PDGF β receptor were quantitated by PhosphorImager analysis, revealing a sevenfold reduction in the levels of the mature PDGF β receptor at high multiplicity of infection (Fig. 1B). Decreased PDGF β receptor levels were also detected when an antiserum recognizing a different PDGF β receptor epitope was used for immunoprecipitation and immunoblotting (data not shown). Therefore, high levels of expression of the wild-type E5 protein in this acute assay system caused a reduction or down-regulation in the amount of mature PDGF β receptor present in the cells.

Interaction between E5 mutants and the PDGF β receptor during acute transformation of C127 cells. To investigate further the interaction between the E5 protein and the PDGF β receptor, we examined the effect of mutant E5 proteins on the PDGF receptor. We focused on mutants that either behaved like the wild type or displayed a clear-cut transformation defect, as summarized in Table 1. In this section, we describe the analysis of cells acutely infected with recombinant SV40-based viruses expressing the E5 gene. As described in the next section, we also analyzed cells selected for the stable expression of a biochemical marker following introduction of the E5 gene by using retroviral vectors.

C127 cells were infected at high multiplicities with a series of BPV-SV40 recombinant viruses expressing E5 proteins con-

TABLE 1. Properties of E5 constructs analyzed in this study

E5 gene[a]	Transforming activity	PDGF receptor down-regulation	PDGF receptor tyrosine phosphorylation	PDGF receptor-E5 complex formation
Frameshift	−	−	−	−
Wild type	+	+	+	+
H34Q/E36D/G41A[b]	+	+	+	+
Q17L	−	ND[c]	−	−
D33V	−	−	−	−
C37S/C39S	−	−	−	−
W32S	−	−	+	+
V30L/F35S	−	−	+	+
C39S	−	−	+	+
C37S	−	ND	+	+

[a] All E5 mutants were described previously (19, 22, 37). The identity of each mutant is designated by a number indicating the position of the substitution in the E5 amino acid sequence, preceded by a letter representing the wild-type amino acid in the single-letter code and followed by a letter representing the mutant amino acid. If more than one position is substituted in a single mutant, each affected residue is separated by a slash.

[b] Mutant H34Q/E36D/G41A was tested in the acute assay system, and mutant H34Q/E36D was tested in the stable cell lines. Both mutants efficiently transform C127 cells and induce PDGF receptor tyrosine phosphorylation.

[c] ND, not determined.

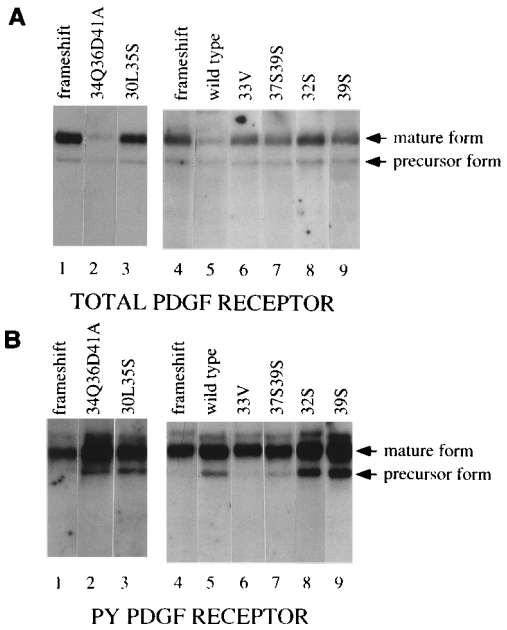

FIG. 2. PDGF β receptor down-regulation and tyrosine phosphorylation in acutely infected cells. (A) C127 cells were infected at a multiplicity of ~50 with recombinant BPV-SV40 viruses expressing the wild-type E5 gene, the E5 frameshift mutant E5d29, or the indicated amino acid substitution mutants (note that the letters identifying the wild-type amino acids have been omitted to simplify the figure). At 48 h after infection, detergent extracts containing 100 μg of protein were prepared and immunoprecipitated with antiserum α-PR4. Immunoprecipitates were subjected to gel electrophoresis and immunoblotting with antiserum α-PR4 to visualize total PDGF receptor (A) or with monoclonal antibody 4G10 (Upstate Biotechnology, Inc.) to visualize tyrosine-phosphorylated (PY) PDGF receptor (B). Lanes 1 to 3 represent samples from one experiment, and lanes 4 to 9 represent samples from an independent experiment. The positions of the mature and precursor forms of the PDGF β receptor are indicated.

taining amino acid substitutions (37). Cells were assessed for morphologic transformation at the time of harvest, and all mutants elicited the previously observed morphologic response (37). Cell extracts were immunoprecipitated with PDGF receptor antiserum, and the immunoprecipitates were electrophoresed and immunoblotted with either a PDGF receptor antiserum or an antiphosphotyrosine antibody. As shown in Fig. 2A, cells infected with the wild-type virus (lane 5) or with a virus expressing the transformation-competent mutant H34Q/E36D/G41A (lane 2) contained markedly lower levels of mature PDGF β receptor than did cells infected with the E5 frameshift mutant (lanes 1 and 4). Strikingly, the transformation-defective mutants V30L/F35S, D33V, C37S/C39S, W32S, and C39S did not induce down-regulation of the PDGF β receptor (Fig. 2A, lanes 3 and 6 to 9; Fig. 3, top panel). We carried out immunoblot analysis with an anti-E5 antiserum (4, 10) to confirm that the lack of PDGF receptor down-regulation in cells infected with representative transformation-defective E5 mutants did not reflect low levels of the mutant E5 proteins. As shown in Fig. 3, the W32S and C39S mutant E5 proteins did not induce receptor down-regulation (top panel), although they were expressed at a level equal to or higher than the level of the wild-type protein sufficient to induce down-regulation (bottom panel). These results indicated that the inability of these mutants to induce PDGF receptor down-regulation was not due to inefficient expression of the E5 mutant proteins. Thus, the wild-type E5 protein and the transformation-competent E5 mutant induced down-regulation of the PDGF β receptor, whereas the transformation-defective E5 mutants were defective for this activity.

Tyrosine phosphorylation of the PDGF β receptor was examined by immunoblotting PDGF receptor immunoprecipitates with an antiphosphotyrosine antibody. In the experiments reported here, we observed high basal tyrosine phosphorylation of the mature form of the PDGF β receptor in mock-infected cells and in cells infected with the E5 frameshift mutant. We observed this degree of basal phosphorylation of the mature PDGF β receptor when several different batches of C127 cells and fetal calf serum were tested, and similar basal phosphorylation of this receptor form was found previously in another cell type (27). Therefore, we were unable to determine reliably whether the E5 mutants induced tyrosine phosphorylation of the mature receptor species. In contrast, as previously reported, tyrosine phosphorylation of the precursor form of receptor was consistently low or undetectable unless the E5 protein was expressed (27, 31). Therefore, tyrosine phosphorylation of the precursor species of receptor was examined as a measure of E5 activity. As shown in Fig. 2B, the wild-type E5 protein and the transformation-competent mutant induced tyrosine phosphorylation of the precursor form of the receptor (lanes 2 and 5). Two transformation-defective mutants, D33V and C37S/C39S, had little effect on tyrosine phosphorylation of the PDGF receptor (lanes 5 and 6) and thus appear defective for PDGF receptor activation. Surprisingly, a number of the transformation-defective E5 mutants, specifically V30L/F35S, W32S, and C39S, induced significant levels of tyrosine phosphorylation of the immature form of the PDGF β receptor (Fig. 2B, lanes 3, 8, and 9; Fig. 3, middle panel). Therefore, in cells acutely expressing these mutants, tyrosine phosphorylation of PDGF β receptor precursor occurs in the absence of cell transformation and receptor down-regulation. Nevertheless, the inability of the transformation-defective E5 mutants to induce down-regulation of the PDGF β receptor indicated that they do not interact normally with the receptor.

Interaction between the E5 protein and the PDGF β receptor in stable C127 cell lines. To examine the interaction of the E5 protein and the PDGF β receptor in more detail, each of

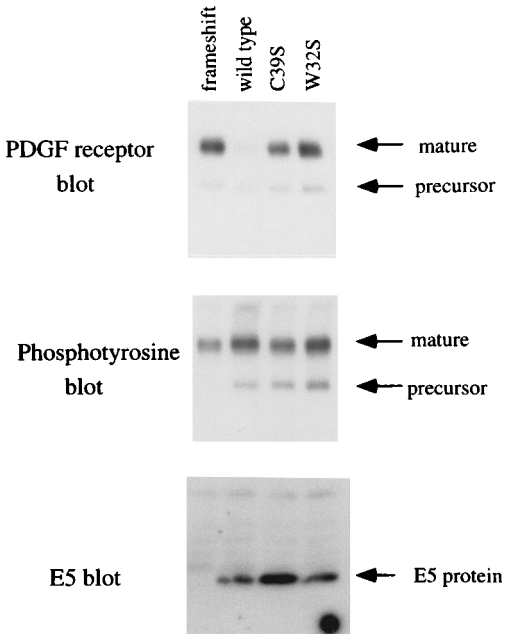

FIG. 3. Status of the E5 protein and PDGF receptor in acutely infected cells. C127 cells were infected at high multiplicity with recombinant BPV-SV40 recombinant viruses expressing the E5 frameshift mutant, the wild-type E5 protein, or E5 mutant C39S or W32S as indicated. RIPA buffer extracts were prepared 39 h after infection. Extracts were immunoprecipitated with antiserum α-PR4 (50 μg of extracted protein in the top panel; 300 μg of extracted protein in the middle panel) or antiserum α-E5 (4) (bottom panel; 100 μg of extracted protein). After gel electrophoresis and transfer, filters were probed with antiserum α-PR4 to visualize total PDGF β receptor (top panel), monoclonal antibody 4G10 to visualize tyrosine-phosphorylated PDGF β receptor (middle panel), and antiserum α-E5 to visualize E5 protein (10) (bottom panel). The positions of the E5 protein and the PDGF receptor are indicated.

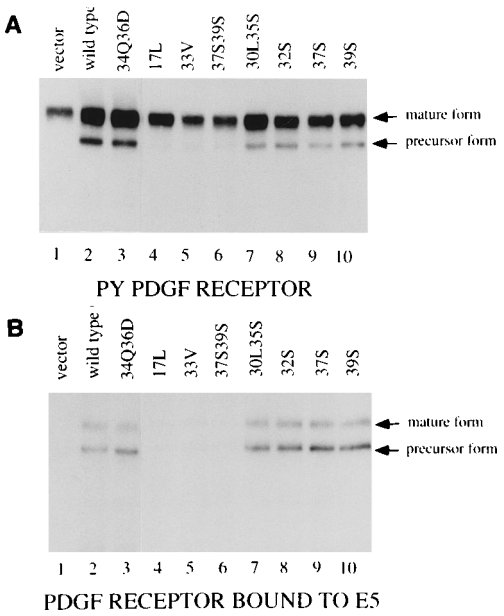

FIG. 4. PDGF receptor tyrosine phosphorylation and complex formation with the E5 protein in stable cell lines. C127 cell lines established following retrovirus infection and selection with hygromycin were serum starved overnight, and RIPA buffer extracts were immunoprecipitated with antiserum α-PR4 (A, 350 μg of extracted protein) or antiserum α-E5 (B, 1.2 mg of extracted protein). After gel electrophoresis and transfer, filters were probed with monoclonal antibody 4G10 to visualize tyrosine-phosphorylated (PY) PDGF β receptor (A) or antiserum α-PR4 to visualize PDGF β receptor in complex with the E5 protein (B). The positions of the PDGF receptor are indicated.

the mutants studied above was cloned into a retrovirus expression vector. DNA fragments containing point mutations in the E5 gene were amplified by PCR and inserted by standard subcloning procedures in place of the wild-type E5 gene in pRVYBE5, which also contains a hygromycin resistance gene (32). During PCR using the full-length BPV clone with the mutation H34Q/E36D/G41A as a template, the 3′-most mutation was covered by the 3′ primer and was therefore absent from the PCR product, resulting in the generation of mutant H34Q/E36D. DNA sequencing confirmed the identity of the mutants after subcloning and the absence of extraneous mutations. Ecotropic stocks of recombinant retroviruses were prepared from these plasmids as described previously (24, 32). Stable C127 cell lines were generated by infection with retroviruses expressing the wild-type or mutant E5 genes followed by selection for a cotransduced hygromycin resistance gene. Drug-resistant colonies (at least 100 per plate) were pooled to generate stable cell lines, which were in general analyzed within a few passages of their derivation. The resulting cell lines either displayed a transformed morphology (wild type and H34Q/E36D) or appeared morphologically normal (all of the other mutants), as predicted (19, 22) (Table 1). The cell lines expressed similar levels of the E5 protein as assessed by immunoblotting (data not shown).

There was no difference in the level of PDGF β receptor in cells infected with the control vector compared with cells expressing the wild-type protein or any of the mutant E5 proteins (data not shown), confirming our previous observation that stable expression of the E5 protein at a relatively low level from the retrovirus long terminal repeat does not induce significant down-regulation of the PDGF β receptor. As shown in Fig. 4A, the pattern of PDGF β receptor tyrosine phosphorylation in these stable cell lines was similar to the results obtained in the acute assay system. The mature form of the receptor, but not the immature form, displayed high basal tyrosine phosphorylation in all cells, including those infected with the vector not containing an insert (lane 1). The wild-type E5 protein and the transformation-competent mutant induced tyrosine phosphorylation of the precursor form of the receptor (lanes 2 and 3), and the transformation-defective mutants Q17L, D33V, and C37S/C39S did not (lanes 4 to 6). In contrast, abundant tyrosine phosphorylation of the receptor precursor was induced by stable expression of the transformation-defective mutants W32S, V30L/F35S, C37S, and C39S (lanes 7 to 10).

The ability of the E5 mutants to form a stable complex with the endogenous PDGF β receptor in the C127 cell lines was assessed by coimmunoprecipitation analysis. Cell extracts were immunoprecipitated with the E5 antiserum, electrophoresed, and immunoblotted with a PDGF receptor antiserum to detect PDGF β receptor in association with the E5 protein (Fig. 4B). As reported previously (29), the wild-type E5 protein was present in a stable complex with both the mature and precursor forms of the PDGF β receptor in stably transformed cells (lane 2). Similarly, the transformation-competent mutant, H34Q/E36D, was also stably associated with the PDGF β receptor (lane 3). Strikingly, transformation-defective mutants Q17L, D33V, and C37S/C39S, which failed to induce receptor tyrosine phosphorylation, also failed to form a stable complex with the PDGF β receptor (lanes 4 to 6). These mutants thus appear to be transformation defective because of their inability to form a stable complex with the PDGF β receptor. In contrast, the transformation-defective mutants that induced receptor tyrosine phosphorylation formed a stable complex with the PDGF β receptor in these cell lines (lanes 7 to 10). Thus, the

low transforming activity of these mutants and the lack of receptor down-regulation was not due to an inability of these mutants to form complexes with the PDGF β receptor. However, binding of these mutant E5 proteins to the receptor and induction of PDGF receptor tyrosine phosphorylation were not sufficient to initiate a signal leading to transformation.

Discussion. We have investigated the effects of wild-type and mutant E5 proteins on the endogenous PDGF β receptor in mouse fibroblasts. A high level of expression of the wild-type BPV E5 protein induces down-regulation of the mature form of the endogenous PDGF β receptor in transformed mouse C127 cells, whereas the level of the intracellular, precursor form of the receptor is not affected. The lower levels of E5 expression routinely attained in stable cell lines generated by infection with recombinant retroviral constructs or by transfection with wild-type BPV DNA are not sufficient to induce detectable PDGF receptor down-regulation. In these latter transformed cell lines, only a small fraction of the PDGF β receptor is activated by the E5 protein (18). Evidently, down-regulation of such a small fraction of the receptor is not detectable in the presence of a large pool of receptor not engaged by the E5 protein.

Martin et al. (25) reported that expression of the BPV E5 protein results in the accumulation of increased levels of exogenous human epidermal growth factor receptor in E5-transformed NIH 3T3 fibroblasts and in retarded down-regulation of the receptor in response to epidermal growth factor treatment, leading them to propose that the E5 protein transforms cells by stabilizing activated growth factor receptors at the cell surface. This is clearly not the case for the endogenous PDGF β receptor in C127 cells, since expression of the E5 protein results in reduced levels of the receptor. This difference suggests that the effect of the E5 protein on various growth factor receptors may depend on the particular receptor and cell type under study.

In addition to PDGF β receptor down-regulation, expression of the wild-type E5 protein or a transformation-competent mutant results in tyrosine phosphorylation of the PDGF receptor and in the formation of an E5-PDGF receptor complex. The transformation-defective E5 mutants fall into two classes (Table 1). One class is defective for complex formation with the endogenous PDGF β receptor and for induction of receptor tyrosine phosphorylation and receptor down-regulation. Therefore, the primary cause of the transformation defect displayed by these mutants appears to be their inability to physically associate with the PDGF β receptor. The second class of transformation-defective E5 mutants retains the ability to form a complex with the PDGF β receptor. In addition, these mutants induce tyrosine phosphorylation of the precursor form of the receptor, but they fail to induce receptor down-regulation. The receptors that are autophosphorylated in response to these mutants are evidently not delivering a signal sufficient to transform C127 cells.

There are a number of possible explanations why complex formation between these mutant E5 proteins and the PDGF β receptor does not result in transformation, even though receptor autophosphorylation is induced. One possibility is that the E5 protein must activate two targets, the PDGF β receptor and an additional target, in order for transformation to occur, and that these particular mutant E5 proteins interact normally with the PDGF receptor but not with the other, putative target. The mutants in this class associate normally with the 16-kDa vacuolar ATPase subunit in COS cells (12), suggesting that the interaction with this protein is normal. It is also possible that E5-induced PDGF receptor signaling is sufficient for transformation, but that the PDGF receptors that are tyrosine phosphorylated in response to the transformation-defective E5 proteins are impaired in their ability to deliver a mitogenic signal. Indeed, the defective down-regulation of the PDGF receptor in response to these mutants suggests that the receptor is responding in an aberrant fashion to these mutant E5 proteins. These mutants may induce the receptors to assume an aberrant conformation or to be localized in an inappropriate intracellular compartment, so that a mitogenic signal cannot be propagated. Alternatively, complex formation between the receptor and such a mutant E5 protein might stimulate the tyrosine kinase activity of the receptor, resulting in autophosphorylation on several tyrosines but not on a critical tyrosine residue that must be phosphorylated for an essential SH2 domain-containing cellular signaling molecule to bind the receptor. In several cell systems, mutation of individual tyrosine residues does not affect overall receptor autophosphorylation but does block binding of important proteins to the PDGF β receptor and impairs signaling in response to PDGF treatment (e.g., reference 11). Phosphorylation of individual tyrosines is also required for PDGF-induced receptor down-regulation (21), so it is possible that the inability of a mutant E5 protein to induce phosphorylation of a crucial tyrosine residue of the receptor would impair both transformation and E5-induced receptor down-regulation, thereby accounting for the observed phenotype. Biochemical mapping of the sites of receptor tyrosine phosphorylation in response to the wild-type and mutant E5 proteins may validate this explanation.

The mutations that inhibit complex formation between the E5 proteins and the PDGF receptor include substitution Q17L in the central, extremely hydrophobic domain of the E5 protein that is thought to be embedded in the lipid bilayer (9, 35) and substitution D33V in the hydrophilic carboxyl-terminal segment that is thought to extend into the intralumenal space (3). The glutamine at position 17 may interact with another polar component in the membrane, such as a polar residue in the transmembrane domain of the PDGF receptor. Our finding that a substitution of valine for aspartic acid at position 33 disrupts PDGF receptor binding and activation provides experimental support for the speculation of Meyer et al. (26) that the aspartic acid is involved in PDGF receptor binding, perhaps by interacting with a lysine in the extracellular juxtamembrane domain of the PDGF β receptor. Alternatively, the hydrophobic valine residue may interfere with essential interactions involving the juxtamembrane domain of the receptor. Complex formation is also disrupted by substitution of both cysteine residues with serines (mutant C37S/C39S), which also prevents E5 dimerization, whereas substitution of either cysteine separately still allows E5 homodimer formation and PDGF receptor binding. These results suggest that dimerization of the E5 protein is required for formation of a stable complex with the PDGF receptor.

In COS cells, an E5 mutant containing a glycine in place of glutamine 17 was defective for binding the PDGF β receptor and the 16-kDa vacuolar ATPase subunit, whereas an E5 mutant lacking the entire carboxyl-terminal domain, including the essential aspartic acid and cysteines, was able to bind these two proteins (13). This C-terminal truncation might reposition the C-terminal main chain carboxylate group to the vicinity of that normally occupied by the side chain carboxylate group of aspartate 33, thereby mimicking the wild-type amino acid. Alternatively, in COS cells in which proteins encoded by transfected genes are greatly overexpressed, complex formation may be driven largely by relatively nonspecific hydrophobic interactions between the transmembrane segments, whereas in a physiological setting, complex formation may also require E5 dimerization and additional contacts involving the receptor juxtamembrane domain and the E5 carboxyl-terminal domain. Finally, it was suggested that the 16-kDa vacuolar ATPase subunit might link the E5 protein to the PDGF receptor (13).

However, the inability of the D33V and the C37S/C39S mutants to bind the endogenous PDGF receptor in C127 cells, despite their ability to bind the ATPase subunit (12), argues that this subunit does not mediate complex formation between the E5 protein and the PDGF receptor.

In summary, the results reported here imply that complex formation between a dimeric E5 protein and the PDGF β receptor stimulates receptor tyrosine kinase activity and receptor autophosphorylation, presumably by inducing receptor dimerization. However, the analysis of E5 mutants indicates that these events are not necessarily sufficient to generate a proliferative signal and induce receptor down-regulation. Rather, these activities appear to require specific residues in the carboxyl-terminal segment of the E5 protein that are not required for receptor binding per se. These results imply either that the E5 protein must engage an additional target or that certain E5 mutants undergo a nonproductive interaction with the PDGF receptor, perhaps by failing to induce tyrosine phosphorylation at the normal constellation of sites. Further biochemical, genetic, and structural analyses of E5-induced activation of the PDGF β receptor promise to provide new insights into receptor-mediated signal transduction and viral transformation.

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REFERENCES

1. **Bowen-Pope, D. F., and R. Ross.** 1982. Platelet-derived growth factor. II. Specific binding to cultured cells. J. Biol. Chem. **257:**5161–5171.
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. **Burkhardt, A., M. Willingham, C. Gay, K.-T. Jeang, and R. Schlegel.** 1989. The E5 oncoprotein of bovine papillomavirus is oriented asymmetrically in Golgi and plasma membranes. Virology **170:**334–339.
4. **Burnett, S., N. Jareborg, and D. DiMaio.** 1992. Localization of bovine papillomavirus type 1 E5 protein to transformed keratinocytes and permissive differentiated cells in fibropapilloma tissue. Proc. Natl. Acad. Sci. USA **89:**5665–5669.
5. **Cantley, L. C., K. R. Auger, C. Carpenter, B. Duckworth, A. Graziani, R. Kapeller, and S. Soltoff.** 1991. Oncogenes and signal transduction. Cell **64:**281–302.
6. **Cohen, B. D., D. J. Goldstein, L. Rutledge, W. C. Vass, D. R. Lowy, R. Schlegel, and J. Schiller.** 1993. Transformation-specific interaction of the bovine papillomavirus E5 oncoprotein wtih the platelet-derived growth factor receptor transmembrane domain and the epidermal growth factor receptor cytoplasmic domain. J. Virol. **67:**5303–5311.
7. **Cohen, B. D., D. R. Lowy, and J. Schiller.** 1993. The conserved C-terminal domain of the bovine papillomavirus E5 oncoprotein can associate with an α-adaptin-like molecule: a possible link between growth factor receptors and viral transformation. Mol. Cell. Biol. **13:**6462–6468.
8. **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.
9. **DiMaio, D., L. Petti, and E.-S. Hwang.** 1994. The E5 transforming proteins of the papillomaviruses. Semin. Virol. **5:**369–379.
10. **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.
11. **Fantl, W. J., J. A. Escobedo, G. A. Martin, C. W. Turck, M. del Rosario, F. McCormick, and L. T. Williams.** 1992. Distinct phosphotyrosines on a growth factor receptor bind to specific molecules that mediate different signaling pathways. Cell **69:**413–423.
12. **Goldstein, D., 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.
13. **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:**4851–4859.
14. **Goldstein, D. J., M. E. Finbow, T. Andresson, P. McLean, K. Smith, V. Bubb, and R. Schlegel.** 1991. Bovine papillomavirus E5 oncoprotein binds to the 16K component of the vacuolar H^+-ATPase. Nature (London) **352:**347–349.
15. **Goldstein, D. J., W. Li, L.-M. Wang, M. A. Heidaran, R. Shinn, S. A. Aaronson, R. Schlegel, and J. H. Pierce.** 1994. The bovine papillomavirus type 1 E5 transforming protein specifically binds and activates the β-type receptor for the platelet-derived growth factor but not other related tyrosine kinase-containing receptors to induce cellular transformation. J. Virol. **68:**4432–4441.
16. **Heldin, C.-H.** 1992. Structural and functional studies on platelet-derived growth factor. EMBO J. **11:**4251–4259.
17. **Heldin, C.-H., A. Wasteson, and B. Westermark.** 1982. Interaction of platelet-derived growth factor and its fibroblast receptor. J. Biol. Chem. **257:**4216–4221.
18. **Henningson, C., and D. DiMaio.** Unpublished results.
19. **Horwitz, B. H., A. L. 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.
20. **Horwitz, B. H., D. L. Weinstat, and D. DiMaio.** 1989. Transforming activity of a sixteen-amino-acid segment of the bovine papillomavirus E5 protein linked to random sequences of hydrophobic amino acids. J. Virol. **63:**4515–4519.
21. **Joly, M., A. Kazlauskas, F. S. Fay, and S. Corvera.** 1994. Disruption of PDGF receptor trafficking by mutation of its PI-3 kinase binding sites. Science **263:**684–687.
22. **Kulke, R., B. H. Horwitz, T. Zibello, and D. DiMaio.** 1992. The central hydrophobic domain of the bovine papillomavirus E5 transforming protein can be functionally replaced by many random hydrophobic sequences containing a glutamine. J. Virol. **66:**505–511.
23. **Kypta, R. M., Y. Goldberg, E. T. Ulug, and S. A. Courtneidge.** 1990. Association between the PDGF receptor and members of the *src* family of tyrosine kinases. Cell **62:**481–492.
24. **Leptak, C., S. Ramon y Cajal, R. Kulke, D. R. Riese, B. H. Horwitz, G. P. Dotto, and D. DiMaio.** 1991. Tumorigenic transformation of mouse keratinocytes by the E5 genes of human papillomavirus type 16 and bovine papillomavirus type 1. J. Virol. **65:**7078–7083. (Authors' correction **66:**1833, 1992).
25. **Martin, P., W. Vass, J. T. Schiller, D. 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.
26. **Meyer, A. N., Y.-F. Xu, M. K. Webster, A. E. 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.
27. **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.
28. **Nilsson, J., J. Thyberg, C.-H. Heldin, B. Westermark, and A. Wasteson.** 1983. Surface binding and internalization of platelet-derived growth factor in human fibroblasts. Proc. Natl. Acad. Sci. USA **80:**5592–5596.
29. **Petti, L., and D. DiMaio.** 1992. Stable association between the bovine papillomavirus E5 transforming protein and activated platelet-derived growth factor receptor in transformed mouse cells. Proc. Natl. Acad. Sci. USA **89:**6736–6740.
30. **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.
31. **Petti, L., L. Nilson, and D. DiMaio.** 1991. Activation of the platelet-derived growth factor receptor by the bovine papillomavirus E5 protein. EMBO J. **10:**845–855.
32. **Riese, D. J., and D. DiMaio.** 1995. An intact PDGF signaling pathway is required for efficient growth transformation of mouse C127 cells by the bovine papillomavirus E5 protein. Oncogene **10:**1431–1439.
33. **Riese, D. J., J. Settleman, K. Neary, and D. DiMaio.** 1990. Bovine papillomavirus mutant displays a high-copy-number phenotype and enhanced transforming activity. J. Virol. **64:**944–949.
34. **Rosenfeld, M. E., D. Bowen-Pope, and R. Ross.** 1984. Platelet-derived growth factor: morphologic and biochemical studies of binding, internalization, and degradation. J. Cell. Physiol. **121:**263–274.
35. **Schlegel, R., M. Wade-Glass, M. S. Rabson, and Y.-C. Yang.** 1986. The E5 transforming gene of bovine papillomavirus encodes a small hydrophobic protein. Science **233:**464–467.
36. **Settleman, J., and D. DiMaio.** 1988. Efficient transactivation and morphologic transformation by bovine papillomavirus genes expressed from a bovine papillomavirus/simian virus 40 recombinant virus. Proc. Natl. Acad. Sci. USA **85:**9007–9011.
37. **Settleman, J., A. Fazeli, J. Malicki, B. H. Horwitz, and D. DiMaio.** 1989. Genetic evidence that acute morphologic transformation, induction of cellular DNA synthesis, and focus formation are mediated by a single activity of the bovine papillomavirus E5 protein. Mol. Cell. Biol. **9:**5563–5572.
38. **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.