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Identification of Amino Acids in the Transmembrane and Juxtamembrane Domains of the Platelet-Derived Growth Factor Receptor Required for Productive Interaction with the Bovine Papillomavirus E5 Protein

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The bovine papillomavirus E5 protein forms a stable complex with the cellular platelet-derived growth factor (PDGF) β receptor, resulting in receptor activation and cell transformation. Amino acids in both the putative transmembrane domain and extracytoplasmic carboxyl-terminal domain of the E5 protein appear important for PDGF receptor binding and activation. Previous analysis indicated that the transmembrane domain of the receptor was also required for complex formation and receptor activation. Here we analyzed receptor chimeras and point mutants to identify specific amino acids in the PDGF β receptor required for productive interaction with the E5 protein. These receptor mutants were analyzed in murine Ba/F3 cells, which do not express endogenous receptor. Our results confirmed the importance of the transmembrane domain of the receptor for complex formation, receptor tyrosine phosphorylation, and mitogenic signaling in response to the E5 protein and established that the threonine residue in this domain is required for these activities. In addition, a positive charge in the extracellular juxtamembrane domain of the receptor was required for E5 interaction and signaling, whereas replacement of the wild-type lysine with either a neutral or acidic amino acid inhibited E5-induced receptor activation and transformation. All of the receptor mutants defective for activation by the E5 protein responded to acute treatment with PDGF and to stable expression of v-Sis, a form of PDGF. The required juxtamembrane lysine and transmembrane threonine are predicted to align precisely on the same face of an α helix packed in a left-handed coiled-coil geometry. These results establish that the E5 protein and v-Sis recognize distinct binding sites on the PDGF β receptor and further clarify the nature of the interaction between the viral transforming protein and its cellular target.

The fibropapillomavirus E5 proteins are very short, membrane-associated proteins that cause cell transformation by inducing receptor tyrosine kinase activation (11). The bovine papillomavirus (BPV) E5 protein forms a stable complex with the endogenous cellular platelet-derived growth factor (PDGF) β receptor in rodent and bovine fibroblasts and induces ligand-independent tyrosine phosphorylation and activation of this receptor, resulting in cell transformation (11, 34, 35, 37). This interaction appears to be quite specific, in that the E5 protein does not associate with or activate other receptor tyrosine kinases, including the closely related PDGF α receptor (15, 30, 34). The 44-amino-acid BPV E5 protein is thought to be a type II transmembrane protein localized largely to endoplasmic reticulum and Golgi apparatus membranes (4), and it is structurally quite dissimilar from PDGF, a much larger secreted protein (3, 4, 38). Mutational analysis demonstrated that the very hydrophobic central core and specific carboxyl-terminal amino acids of the E5 protein are required for transformation of mouse C127 and NIH 3T3 cells (19, 20, 29, 39). We previously showed that mutation of the glutamine at position 17 in the hydrophobic domain of the E5 protein, the aspartic acid at position 33, or both carboxyl-terminal cysteines involved in E5 homodimer formation inhibited stable complex formation between the E5 protein and the PDGF β receptor in C127 cells (31). These residues are also required for receptor activation and for cell transformation, and they are absolutely conserved among all fibropapillomavirus E5 proteins (20, 29).

The required aspartic acid in the carboxyl-terminal segment of the type II E5 protein is presumably situated in the intralumenal space, where it might recognize residues in the ligand-binding domain of the receptor, which is inserted into the membrane in the orientation opposite to that of the E5 protein. Meyer et al. (29) have speculated that the aspartic acid might interact with a conserved lysine residue in the extracellular juxtamembrane domain of the receptor (amino acid 499 of the murine PDGF β receptor). However, it should be pointed out that it is not yet known whether the E5 protein and the PDGF β receptor contact one another directly or whether this interaction is mediated by a third protein. Although other cellular proteins can also interact with the E5 protein, there is no compelling evidence that these interactions are important for transformation of rodent fibroblasts in culture (8, 13, 14).

The hydrophobic nature of the E5 protein and the presence of the essential glutamine in the putative E5 transmembrane segment suggested that the transmembrane domain of the receptor might participate in interactions involved in complex formation. On the basis of studies of chimeric receptors, it was concluded that the transmembrane domain but not the extracellular ligand binding domain of the PDGF β receptor was specifically required for E5 binding and receptor activation and that sequences in or near the receptor transmembrane domain were sufficient to allow the E5 protein to discriminate between PDGF α and β receptors (7, 43). Furthermore, receptor mu-

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tants lacking almost all of the extracellular ligand binding domain were able to productively interact with the E5 protein, implying that this domain is not required for the interaction (12, 43).

Previous studies that used chimeric and mutant receptors to determine the requirements for productive interaction with the E5 protein were not definitive for several reasons. First, the truncation mutants retained the juxtamembrane lysine (12, 43). In addition, this lysine and the PDGF receptor transmembrane domain were substituted as a single element in many of the chimeras (43). For these reasons, it was not possible to clearly define the domains of the receptor required for the interaction with the E5 protein (12, 43). Second, in other cases, complex formation between the E5 protein and the PDGF β receptor was assessed in COS monkey epithelial cells engineered to overexpress both proteins (7, 13), a situation that appears to favor the formation of nonspecific complexes that do not occur in stably transformed cell lines (34). In fact, the carboxyl-terminal amino acids of the E5 protein required for cell transformation and for complex formation with the PDGF β receptor at normal levels of expression are not necessary for complex formation in COS cells; rather, the hydrophobic domain of the E5 protein is sufficient to drive complex formation in this overexpression system (13). Third, endogenous receptor tyrosine kinases including the PDGF β receptor were expressed in the cells used for some of these analyses, a situation that may lead to receptor cross talk, thereby complicating the interpretation of these experiments (7). Finally, it has been reported that the intracellular domain of the epidermal growth factor (EGF) receptor contains an independent binding site for the E5 protein, further complicating the analysis of receptor chimeras containing this domain (7). Therefore, we decided to analyze receptor point mutants to identify specific amino acids in the PDGF β receptor involved in the interaction with the E5 protein in an attempt to better delineate the nature of the interaction between these two proteins. Here, we identify specific amino acids in both the transmembrane domain and the extracellular, juxtamembrane domain of the PDGF β receptor that are required for complex formation with the E5 protein, receptor activation, and E5-induced mitogenic signaling.

MATERIALS AND METHODS

Construction of chimeric and mutant receptors. The chimeric receptors ERTM and NeuTM were constructed by replacing the predicted transmembrane domain of the murine PDGF β receptor with that of the human EGF receptor or rat p185^{neu} (Neu). First, standard recombinant PCR mutagenesis was used to generate pPRDTM, in which a DNA fragment encoding the transmembrane domain and 13 to 14 flanking bp was deleted from a wild-type murine PDGF β receptor cDNA construct, pSVR1 (gift from L. T. Williams, University of California, San Francisco), thereby creating a unique *Nde*I site. pPRDTM was adapted for directional cloning by inserting a linker containing a unique *Age*I site into the *Nde*I site, generating pPRDTMAN. To construct the ERTM chimera, the appropriate synthetic double-stranded oligonucleotide cassettes were inserted into pPRDTMAN between the *Nde*I and *Age*I sites. To construct the NeuTM chimera, we first constructed a receptor chimera in which the precise transmembrane domain of the PDGF β receptor was replaced with that of the transforming form of Neu (containing a point mutation in the transmembrane domain) by inserting the appropriate synthetic double-stranded oligonucleotide cassettes into the *Nde*I site of pPRDTM, thereby generating pNTM. With pNTM as a template and internal primers containing the sequence of the wild-type Neu transmembrane domain, PCR mutagenesis was then used to construct pNeuTM.

Single amino acid substitutions of lysine 499 of the PDGF β receptor were made either by PCR mutagenesis (generating the PRK499D and PRK499E mutants) or by site-directed mutagenesis using the QuikChange system (Stratagene) (generating the PRK499A and PRK499R mutants). Both methods involved using a wild-type mouse PDGF β receptor cDNA clone as the template and mutagenic primers in which GAC, GAA, GCA, or AGA replaced the AAA codon at nucleotide 1726 of the wild-type sequence (45), to create the change of lysine 499 to aspartic acid (PRK499D), glutamic acid (PRK499E), alanine (PRK499A), or arginine (PRK499R), respectively. A threonine 545-to-leucine substitution was introduced into the human PDGF β receptor by using the unique-site elimination (10) method of site-directed mutagenesis and a mutagenic primer containing CTC replacing the ACC codon at nucleotide 1989 of the human PDGF β receptor cDNA sequence (5, 6). This mutation was constructed in the human PDGF receptor in an attempt to avoid the background level of delayed interleukin-3 (IL-3)-independent growth that was caused by the NeuTM and PRK499E mutants, which were constructed in the context of the murine receptor (see Results). For each mutant receptor or chimera, the desired DNA sequence in the segment altered by site-directed mutagenesis or replaced by the oligonucleotide insertion or PCR amplification was confirmed.

Standard subcloning procedures were used to subclone the wild-type mouse PDGF β receptor gene from pSVR1 into the retroviral vector pLXSN to generate pMPRRV-3. The wild-type PDGF β receptor gene in pPMPRRV-3 was replaced with the mutant gene encoding NeuTM, ERTM, PRK499D, or PRK499E to generate pNNTMRV-9, pERTMRV, pPRK499D2, or pPRK499E44, respectively. The wild-type human PDGFβ receptor cDNA subcloned into LXSN, pLXSN-AKHPR, was a gift from A. Kazlauskas. The PRK499A, PRK499R, and PRT545L mutants were generated by site-directed mutagenesis of the PDGF β receptor cDNA in the context of the LXSN vector. Stable cell lines producing high-titer ecotropic retrovirus stocks were obtained after selection for G418 resistance as described previously (24).

Establishment of stable cell lines. Ba/F3 cells (32) were obtained from Alan D'Andrea (Dana Farber Cancer Institute) and maintained as previously described (12) in RPMI 1640 (RPMI) supplemented with 10% heat-inactivated fetal bovine serum, WEHI conditioned medium as a source of IL-3, 0.05 mM β-mercaptoethanol, and antibiotics (RPMI/IL-3). Stable Ba/F3 cell lines expressing the various receptor constructs in the absence or presence of the BPV E5 gene or v-*sis* were established in most cases by using recombinant retroviruses to infect BaD5RVY, BaD5E5 (which stably express E5), or BaD5v-sis (which stably express v-*sis*) cells, respectively, as previously described (12), with minor modifications. Briefly, approximately 2×10^6 CFU of retrovirus was added to 10^6 to 5×10^6 cells in 10 ml of RPMI/IL-3 containing 4 μg of Polybrene per ml. After 2 days, 1 ml of infected cells was transferred into 10 ml of RPMI/IL-3 containing G418 (1 mg/ml) and hygromycin B (1,000 U/ml; Calbiochem). Cells were passaged during drug selection when they reached a density of approximately 10^6 cells/ml, and after three to five passages, stable cell lines were established. In some cases (e.g., Fig. 6B), the receptor genes were introduced first into Ba/F3 cells, which were then infected with viruses encoding v-Sis or E5 or with an empty viral vector. Stable cell lines established with the genes introduced in either order displayed similar properties, and the data shown in Table 1 and 2 combine results obtained from cells derived in either way.

Assay for IL-3-independent growth. The ability of Ba/F3 cells to proliferate in the absence of IL-3 was assessed as described previously (12), with some modifications. Drug-resistant cells were grown to a density of approximately 10^6 cells/ml, pelleted, and resuspended in an equal volume of RPMI formulated as described above except that WEHI conditioned medium was omitted and the serum concentration was reduced to 1% (RPMI/IL-3−). Approximately 5×10^5 to 7.5×10^5 cells were seeded into 10 ml of RPMI/IL-3− and incubated at 37°C, and then total or viable cells were counted in a hemocytometer at various times periods thereafter. Cells that proliferated at least 20-fold during a 2-week incubation period were scored as IL-3 independent. The final columns in Tables 1 and 2 list the number of IL-3-independent cell lines/total number of cell lines tested.

Immunoprecipitation and immunoblotting. Extracts of Ba/F3 cells were prepared in cold EBC or radioimmunoprecipitation assay buffer as described previously (12). The PDGF β receptor was immunoprecipitated as previously described (12) by adding 1 to 1.5 μl of antibody α-PR-C3a or α-PR-B3a (both of which recognize the C-terminal 13 amino acids of the PDGF β receptor) per 100 μg of protein extract. Immunoprecipitation of the E5 protein and associated proteins was performed as described previously (34) by adding approximately 1 μl of an anti-E5 antiserum (which recognizes the 16 C-terminal amino acids of the E5 protein) per 100 μg of protein extract. For the experiment shown in Fig. 2C, anti-E5 immune complexes were eluted from the protein A-Sepharose beads by boiling in elution buffer (10 mM Tris-HCl [pH 8.0], 1% sodium dodecyl sulfate [SDS]), diluted to 0.1% SDS in EBC buffer, and incubated for 2 h with 100 μl of a 1:1 suspension of wheat germ lectin-Sepharose (Pharmacia) in EBC buffer to precipitate glycoproteins. Washed immunoprecipitates were boiled in 2× Laemmli sample buffer, electrophoresed on an SDS–7.5 or 15% polyacrylamide gel (for PDGF receptor or E5 immunoblotting, respectively), and transferred to nitrocellulose at 50 V overnight or 100 V for 2 h. Phosphotyrosine, PDGF β receptor, and E5 immunoblotting was performed as described previously (12, 34) by using a 1:500 dilution of antiphosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology, Inc.), a 1:250 dilution of α-PR-C3a, or a 1:500 dilution of anti-E5 antiserum, respectively. Proteins were detected with ^{125}I-protein A (ICN) or enhanced chemiluminescence using a protein A-horseradish peroxidase conjugate (Amersham).

For the experiment shown in Fig. 5, cells were incubated with 50 ng of recombinant human PDGF BB (Collaborative Biomedical Products) per ml for 15 min at room temperature. Extracts were prepared in EBC buffer, and 500 μg of extracted protein was immunoprecipitated with 9 μl of anti-PDGF receptor antibody and blotted with antiphosphotyrosine antibody.

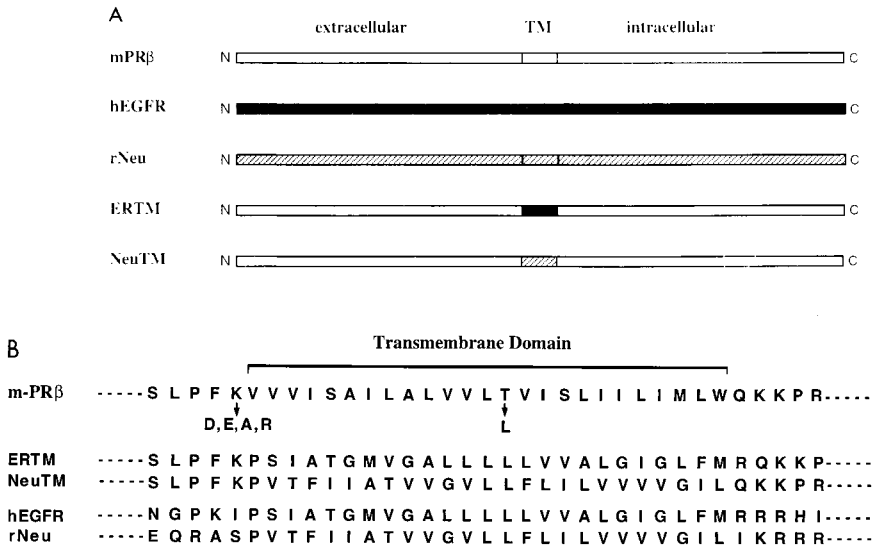

FIG. 1. Structures of the chimeric and mutant receptors. (A) Schematic diagram of the wild-type murine PDGF β receptor (mPRβ), the human EGF receptor (hEGFR), rat p185^{neu} (rNeu), and the chimeric receptors. The transmembrane (TM) domain of the murine PDGF β receptor was replaced with that of the human EGF receptor or rat p185^{neu} to generate the chimeric receptors ERTM and NeuTM, respectively. Segments derived from the PDGF β receptor, the EGF receptor, and p185^{neu} are represented as open, filled in, and hatched boxes, respectively. (B) Amino acid sequence of the transmembrane region of ERTM and NeuTM in comparison with that of the wild-type murine PDGF β receptor, the EGF receptor, or p185^{neu}. Amino acid substitutions introduced at the juxtamembrane lysine (K) and transmembrane threonine (T) are indicated.

RESULTS

Structure of the chimeric and mutant receptors. To identify specific domains and amino acids in the PDGF β receptor required for a productive interaction with the E5 protein, we examined a variety of chimeric and mutant receptors in murine Ba/F3 cells. These cells do not express endogenous PDGF receptor or other receptor tyrosine kinases proposed to interact with the E5 protein (7, 12, 27), and hence they allow analysis of PDGF β receptor mutants in the absence of these confounding factors. Ba/F3 cells are normally dependent on IL-3 for survival and proliferation. We previously showed that coexpression of the BPV E5 protein and the wild-type human PDGF β receptor in Ba/F3 cells resulted in complex formation between these two proteins, constitutive tyrosine phosphorylation of the receptor, and IL-3-independent proliferation (12). Therefore, to assess the effects of receptor mutations, we determined the ability of the E5 protein to form a complex with mutant receptors expressed in Ba/F3 cells, to induce tyrosine phosphorylation of these mutants, and to support IL-3-independent proliferation.

Figure 1A shows schematically the structures of the chimeric receptors in comparison with those of the wild-type PDGF β receptor, the wild-type EGF receptor, and wild-type Neu. The predicted transmembrane domain of the wild-type murine PDGF β receptor was replaced with that of either the EGF receptor or Neu to generate the chimeras ERTM and NeuTM, respectively. Sequence analysis confirmed the replacement of the transmembrane domain of the PDGF β receptor (amino acids 500 to 524) and the retention of the lysine at position 499 in all cases (Fig. 1B). The positions of single amino acid substitution mutations introduced into the juxtamembrane and transmembrane domains of the PDGF β receptor are also shown in Fig. 1B. Lysine 499 located in the extracellular juxtamembrane region of the murine PDGF β receptor was replaced with either aspartic acid, glutamic acid, alanine, or arginine, to generate the mutants PRK499D, PRK499E, PRK499A, and PRK499R, respectively. In addition, threonine 545 within the transmembrane domain of the human PDGF β receptor was replaced with leucine, generating the mutant PRT545L. Threonine 545 of the human PDGF β receptor corresponds to threonine 513 in the murine PDGF receptor sequence indicated in Fig. 1.

Requirement for the transmembrane domain of the PDGF receptor. Previous analysis suggested that the transmembrane domain of the PDGF receptor plays an important role in the productive interaction between the PDGF receptor and the E5 protein (7, 43). To reexamine this question, Ba/F3 cells expressing the BPV E5 gene, v-*sis*, or no exogenous genes were infected with retrovirus vectors carrying genes encoding chimeric receptors and subjected to selection for a cotransduced G418 resistance gene. The resulting stable cell lines were analyzed for expression and tyrosine phosphorylation of the PDGF receptor. As shown in Fig. 2A, the chimeric receptors were expressed at levels similar to that of the wild-type receptor (and similar to that of the endogenous PDGF β receptor in mouse fibroblasts [data not shown]). Two forms of foreign PDGF receptor were expressed in these cells: a cell surface, slowly migrating form with mature carbohydrates and a less abundant, more rapidly migrating precursor form (12). The E5 protein was also expressed at similar levels in the appropriate cell lines (data not shown). To determine the state of tyrosine phosphorylation of the PDGF β receptor, PDGF receptor was immunoprecipitated with a specific antibody, electrophoresed on an SDS-polyacrylamide gel, and then immunoblotted with an antibody that recognized phosphotyrosine. As shown in Fig. 2B, there was no receptor tyrosine phosphorylation in the absence of a viral oncogene, and all of the receptors displayed significant tyrosine phosphorylation in response to v-*sis*, a transduced version of the gene encoding the B chain of PDGF, indicating that the chimeras were not defective for ligand-dependent autophosphorylation. The wild-type receptor was also tyrosine phosphorylated in response to the E5 protein, demonstrating that the viral protein can activate the wild-type receptor. In contrast, there was no detectable tyrosine phos-

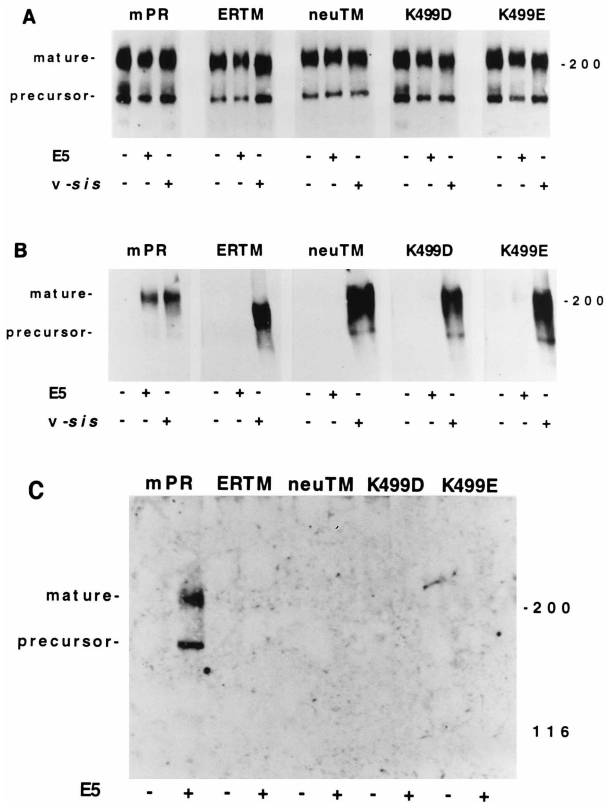

FIG. 2. Biochemical analysis of the chimeric and mutant PDGF receptors. Ba/F3 cells expressing either the wild-type mouse PDGF β receptor (mPR), the PRK499D or PRK499E mutant, or the ERTM or NeuTM chimera in the absence (−) or presence (+) of E5 or v-Sis were lysed in EBC buffer. As described in Materials and Methods, PDGF receptor immunoprecipitated from cell extracts was subjected to either anti-PDGF receptor immunoblotting to detect total receptor amounts (A) or antiphosphotyrosine immunoblotting to detect tyrosine-phosphorylated receptor forms (B). In panel C, glycoproteins immunoprecipitated with an anti-E5 antiserum were subjected to anti-PDGF receptor immunoblotting to detect PDGF β receptor that was complexed with the E5 protein. Each lane represents 100, 1,900, and 2,800 μg of extracted protein in panels A, B, and C, respectively. The positions of the mature and precursor forms of the PDGF receptor are indicated on the left, and sizes of marker proteins in kilodaltons are indicated on the right.

phorylation of either of the two chimeric receptors in response to the E5 protein, indicating that both chimeric receptors were defective for activation by the E5 protein.

To assess complex formation between the E5 protein and the chimeric receptors, protein extracts were immunoprecipitated with antibody recognizing the E5 protein, and the immunoprecipitates were examined by immunoblotting for the PDGF receptor. As shown in Fig. 2C, the E5 antibody immunoprecipitated both the mature and precursor forms of the wild-type PDGF β receptor from cells coexpressing the receptor and the E5 protein but not from cells expressing the receptor in the absence of the E5 protein. Thus, as was the case for the human PDGF β receptor, the E5 protein and both forms of the wild-type murine PDGF β receptor exist in a stable complex in Ba/F3 cells. In contrast, the chimeric receptors were not immunoprecipitated with the E5 antibody, indicating that they were defective for complex formation with the E5 protein.

A number of independently derived cell lines of each genotype were incubated in the absence of IL-3 to determine whether they proliferated in an IL-3-independent manner. The results of these experiments are summarized in Table 1. In the great majority of cases, cell lines either were defective for growth in the absence of IL-3 (cell densities remained less than 2.5×10^5 cells/ml) or proliferated efficiently under these conditions (cell densities reached approximately 2×10^6 cells/ml) (see Fig. 5 for examples of growth curves of IL-3-dependent and IL-3-independent cell lines). As expected, cells not expressing receptor or expressing receptor without coexpression of a viral oncogene remained IL-3 dependent for proliferation. All 14 cell lines that coexpressed the E5 protein and the wild-type PDGF receptor underwent sustained proliferation in the absence of IL-3. In contrast, neither chimeric receptor supported efficient IL-3-independent proliferation when coexpressed with the E5 protein. A fraction of cell lines expressing NeuTM underwent limited IL-3 proliferation after a long delay, regardless of whether they coexpressed the E5 protein (Table 1 and data not shown). Finally, both chimeric receptors were able to support IL-3-independent proliferation when coexpressed with v-*sis*, indicating that they were functional, ligand-dependent receptor tyrosine kinases capable of delivering a proliferative signal.

These results establish that the wild-type murine PDGF β receptor, like its human counterpart, can form a complex with the BPV E5 protein and can undergo tyrosine phosphorylation

TABLE 1. IL-3 tests of cell lines expressing the murine PDGF receptor

Cell line	PDGF receptor[a]	Expression		IL-3-independent cell lines/total tested
		BPV E5	v-Sis	
G418	None	−	−	0/15
G418-E5	None	+	−	0/14
G418-sis	None	−	+	0/10
mPR	Murine wt	−	−	1/17
mPR-E5	Murine wt	+	−	14/14
mPR-sis	Murine wt	−	+	10/10
ERTM	ER chimera	−	−	1/6
ERTM-E5	ER chimera	+	−	0/6
ERTM-sis	ER chimera	−	+	5/6
NeuTM	Neu chimera	−	−	0/9[b]
NeuTM-E5	Neu chimera	+	−	0/5[c]
NeuTM-sis	Neu chimera	−	+	4/5
PRKD	Lys to Asp	−	−	0/8
PRKD-E5	Lys to Asp	+	−	0/8
PRKD	Lys to Asp	−	+	5/5
PRKE	Lys to Glu	−	−	0/7[b]
PRKE-E5	Lys to Glu	+	−	0/7[b]
PDKE-sis	Lys to Glu	−	+	5/5
PRKA	Lys to Ala	−	−	0/7
PRKA-E5	Lys to Ala	+	−	0/8[d]
PRKA-sis	Lys to Ala	−	+	7/7
PRKR	Lys to Arg	−	−	1/7
PRKR-E5	Lys to Arg	+	−	11/12
PRKR-sis	Lys to Arg	−	+	6/6

[a] wt, wild type; ER, EGF receptor.
[b] Three of these cell lines underwent markedly delayed proliferation in the absence of IL-3.
[c] Two of these cell lines underwent markedly delayed proliferation in the absence of IL-3.
[d] Four of these cell lines underwent markedly delayed proliferation in the absence of IL-3.

and deliver a proliferative signal in Ba/F3 cells in response to the E5 protein. Furthermore, sequence information required for complex formation, receptor autophosphorylation, and mitogenic signaling in response to the E5 protein resides in the transmembrane domain of the PDGF β receptor but not in the transmembrane domain of other receptor tyrosine kinases.

Requirement of the lysine in the extracellular juxtamembrane domain of the PDGF receptor. The aspartic acid at position 33 of the E5 protein is required for transformation of C127 cells and for complex formation between the E5 protein and the PDGF β receptor in these cells. This aspartic acid also is important for transformation of NIH 3T3 cells. It has been proposed that there might be a direct interaction between the negatively charged aspartic acid and a positively charged juxtamembrane lysine in the PDGF receptor, the only charged residue in this domain of the receptor (29).

To determine whether lysine 499 of the murine PDGF β receptor is required for the interaction with the E5 protein, PDGF β receptor mutants containing amino acid substitutions at this position were constructed and tested for the ability to interact with the E5 protein. Lysine 499 was replaced with one of two acidic residues, aspartate or glutamate, a neutral residue, alanine, and a basic residue, arginine, to generate the mutants PRK499D, PRK499E, PRK499A, and PRK499R, respectively (Fig. 1B). The ability of these mutants to interact with the E5 protein was assessed in Ba/F3 cells.

Immunoblotting analysis demonstrated that all the mutant receptors were expressed at similar levels in the cell lines tested and that the mutations had no detectable effect on receptor processing (Fig. 2A and data not shown). As was the case for the wild-type PDGF receptor, none of the mutants were significantly tyrosine phosphorylated in the absence of a viral oncoprotein (Fig. 2B and 3A). To confirm that the mutant receptors were expressed normally and biologically active, Ba/F3 cells expressing various receptors were treated acutely with PDGF, which activates only receptors accessible in the correct orientation at the cell surface. PDGF receptor was immunoprecipitated from protein extracts prepared 15 min after PDGF treatment and immunoblotted with an antiphosphotyrosine antibody. As shown in Fig. 4, PDGF induced markedly increased levels of tyrosine phosphorylation of the wild-type receptor and all of the receptor mutants. This result established that the mutations at position 499 in the juxtamembrane domain did not interfere with expression of the receptors at the cell surface. Furthermore, it demonstrated that the wild-type and mutant receptors exhibited comparable increases in tyrosine autophosphorylation upon ligand stimulation, indicating that the mutants were functional tyrosine kinases.

The response of the receptors to stable expression of v-Sis and the E5 protein was also assessed. All of the mutants except PRK499A displayed abundant tyrosine phosphorylation in response to v-Sis, confirming that these mutations did not impair the ability of the normal ligand to stimulate receptor autophosphorylation (Fig. 2B and 3A). Although PDGF induced abundant tyrosine phosphorylation of PRK499A, tyrosine phosphorylation of PRK499A in response to v-Sis was variable. Some cell lines coexpressing PRK499A and v-Sis displayed a low level of tyrosine phosphorylation (e.g., Fig. 3A), whereas others displayed abundant tyrosine phosphorylation (data not shown). One PRK499A/v-*sis* cell line displayed abundant tyrosine phosphorylation when tested initially and then displayed reduced tyrosine phosphorylation when independent extracts were prepared and tested (data not shown). Despite this apparently impaired biochemical response of PRK499A to v-Sis, this mutant receptor was able to deliver a proliferative signal in response to v-Sis, as described below.

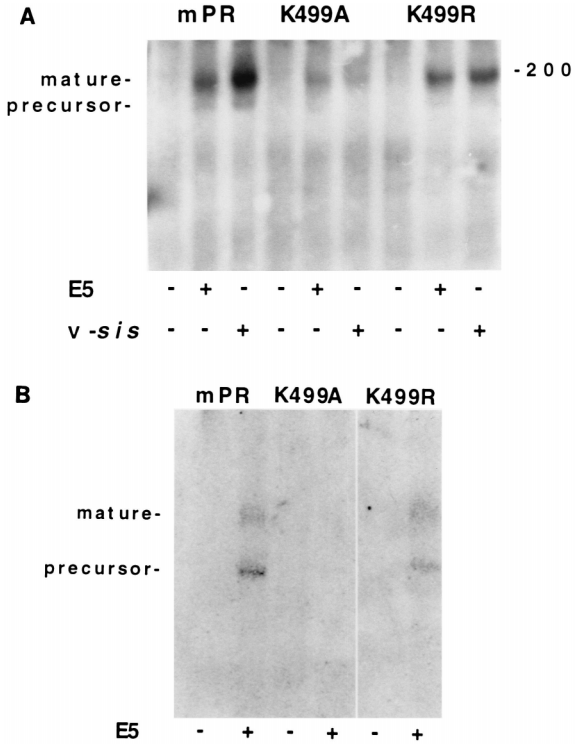

FIG. 3. Biochemical analysis of the PRK499A and PRK499R mutants. Ba/F3 cells expressing the wild-type murine PDGF β receptor (mPR) or the PRK499A or PRK499R mutant alone (−) or in the presence (+) of the E5 protein or v-Sis were lysed in EBC (A) or radioimmunoprecipitation assay (B) buffer. As described in Materials and Methods, PDGF receptor was immunoprecipitated from cell extracts and then immunoblotted to detect tyrosine-phosphorylated PDGF receptor (A). In panel B, the E5 protein was immunoprecipitated from cell extracts with anti-E5 serum, and immune complexes were subjected to anti-PDGF receptor immunoblotting to detect PDGF β receptor in a complex with the E5 protein. Each lane represents 420 μg (A) or 500 μg (B) of extracted protein. The positions of the mature and precursor forms of the PDGF receptor are indicated on the left. The size of the marker is indicated in kilodaltons on the right.

The response of some of the mutant receptors to the E5 protein was markedly different from their response to v-Sis or

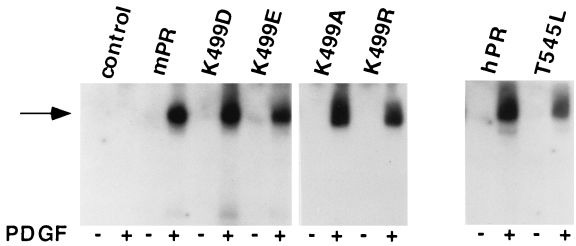

FIG. 4. Cell surface expression and ligand responsiveness of PDGF receptor point mutants. Ba/F3 cells expressing no exogenous PDGF receptor, the wild-type PDGF β receptor, or the indicated PDGF receptor mutants were treated with PDGF (+) or left untreated (−). Protein extracts were prepared 15 min after PDGF addition, and PDGF receptor immunoprecipitates were subjected to immunoblotting with antiphosphotyrosine antibody. Each lane represents 500 μg of extracted protein. Pairs of lanes show the results for the indicated receptors: no PDGF receptor (control), wild-type murine PDGF β receptor (mPR), PRK499D, PRK499E, PRK499A, PRK499R, wild-type human PDGF β receptor (hPR), and PRT545L. Arrow, the mature form of the PDGF receptor that is activated by PDGF addition.

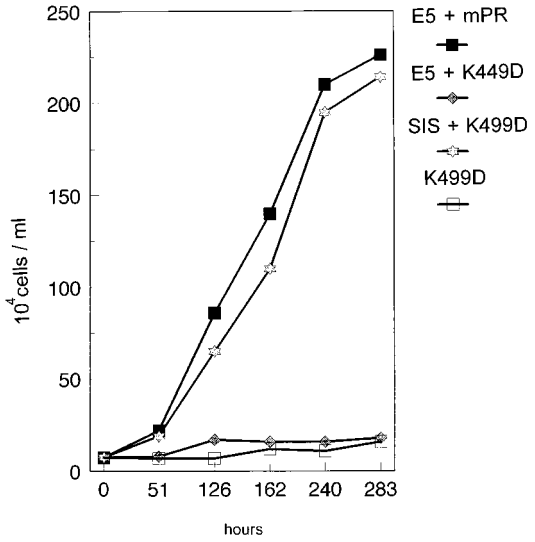

FIG. 5. IL-3 dependence of Ba/F3 cells expressing the PRK499D mutant receptor in the absence or presence of the E5 protein or v-Sis. Ba/F3 cells or Ba/F3 cells expressing E5 or v-Sis were infected with recombinant retrovirus containing the wild-type PDGF β receptor gene (mPR) or PRK499D, as indicated. After selection for G418 resistance in the presence of IL-3, 7.5×10^4 cells/ml were incubated in the absence of IL-3 for the indicated times and counted.

PDGF. As is the case for the wild-type receptor, the E5 protein induced tyrosine phosphorylation of the PRK499R mutant (Fig. 3B). Strikingly, E5 expression did not induce significant tyrosine phosphorylation of the PRK499D or PRK499E receptor mutant (Fig. 2B) and induced markedly reduced tyrosine phosphorylation of the PRK499A mutant (Fig. 3A). The E5 protein was expressed at similar levels in cells expressing each of these mutants (data not shown). These results indicate that the PRK499D, PRK499E, and PRK499A mutants were severely impaired in the ability to be activated by the E5 protein, whereas all of the mutants appeared to respond normally to PDGF treatment and the mutants containing a negative charge also appeared to respond normally to the v-Sis protein. In contrast, the PRK499R mutant, which retains the juxtamembrane positive charge, was not impaired in its response to the E5 protein.

The ability of the lysine 499 receptor mutants to form a stable complex with the E5 protein was assessed by coimmunoprecipitation analysis as described above for the chimeric receptors. Like the wild-type receptor, both mature and precursor forms of the PRK499R mutant could be coimmunoprecipitated with the E5 antiserum, demonstrating that this mutant receptor is capable of forming a stable complex with the E5 protein (Fig. 3B). In contrast, neither the PRK499D, the PRK499E, nor the PRK499A mutant receptor could be detected in significant amounts in E5 immunoprecipitates from cells coexpressing E5 and the mutant receptor (Fig. 2C and 3B), indicating that these mutant receptors are unable to form a stable complex with the E5 protein. Thus, the mutant containing a positive charge in the juxtamembrane domain behaved like the wild-type receptor, whereas substitution of the lysine with either a neutral or acidic amino acid inhibited its interaction with the E5 protein.

The ability of cell lines expressing the various mutants to proliferate in an IL-3-independent fashion was also determined. Table 1 summarizes the results of these experiments, and growth curves of representative cell lines are shown in Fig. 5. The receptor mutant containing an arginine replacing lysine 499, PRK499R, was not defective for cooperating with the E5 protein in Ba/F3 cells. Eleven of twelve cell lines coexpressing the PRK499R mutant and the E5 protein remained viable in the absence of IL-3 and proliferated to a high density. As expected, coexpression of v-Sis with the PRK499R mutant also promoted cell viability and growth in the absence of IL-3, whereas PRK499R expressed alone did not support cell growth. Therefore, the PRK499R mutant resembled the wild-type receptor with respect to its ability to cooperate with the E5 protein to induce a proliferative response in Ba/F3 cells.

The receptor mutant, PRK499D, which contains an aspartic acid replacing lysine 499, was completely defective for cooperating with the E5 protein in this assay (Table 1 and Fig. 5). All eight cell lines coexpressing the PRK499D mutant and the E5 protein underwent a rapid loss in cell viability and did not proliferate in the absence of IL-3. However, cells coexpressing either v-Sis and the PRK499D mutant or v-Sis and the wild-type receptor proliferated to the same extent and with similar kinetics, indicating that this mutant was not defective for normal receptor signaling.

A mutant PDGF β receptor containing a glutamic acid or an alanine in place of lysine 499 (PRK499E or PRK499A, respectively) also was defective for cooperating with the E5 protein. None of numerous independently derived Ba/F3 cell lines coexpressed the E5 protein, and these mutants proliferated efficiently in the absence of IL-3 (Table 1). As was the case with the NeuTM chimera, some cell lines expressing PRK499E exhibited IL-3-independent growth that was markedly delayed compared to that of cells coexpressing the wild-type receptor and the E5 protein (data not shown). The same proportion of the tested PRK499E cell lines displayed delayed growth in the presence and absence of the E5 protein (Table 1), indicating that the delayed growth observed with this mutant was not elicited by an interaction with the E5 protein. In the presence of v-*sis*, PRK499E supported a proliferative response comparable to that supported by the wild-type receptor, indicating that this mutant could function normally as a receptor tyrosine kinase. In the case of the PRK499A mutant, none of the eight cell lines coexpressing PRK499A and the E5 protein proliferated efficiently in the absence of IL-3 (Table 1). However, upon extended incubation, half of these cell lines eventually proliferated with a 1- to 2-week lag in comparison to cells coexpressing the E5 protein and the wild-type PDGF receptor (data not shown). Since none of the PRK499A cell lines tested in the absence of E5 expression underwent growth even after a delay, these results suggest that PRK499A was not absolutely defective in its response to the E5 protein. This interpretation is consistent with the markedly reduced but not absent ability of the E5 protein to induce tyrosine phosphorylation of this mutant receptor. Although the results of phosphotyrosine blotting indicated that the PRK499A mutant did not respond normally to v-*sis*, this mutant did couple efficiently to v-*sis* in the biological assay of supporting IL-3-independent growth. All seven cell lines coexpressing v-*sis* and PRK499A proliferated efficiently in the absence of IL-3. Therefore, the biochemical analysis and the growth properties of the cells indicated that both the PRK499E and PRK499A mutants, as well as PRK499D, were significantly deficient in their ability to functionally interact with the E5 protein.

In summary, mutations in the PDGF receptor that replaced lysine 499 with a negatively charged or neutral amino acid severely inhibited the ability of the receptor to bind to and respond to the E5 protein, whereas a mutation which retained the positive charge had no apparent effect on this interaction. Thus, a juxtamembrane positive charge appears to be required

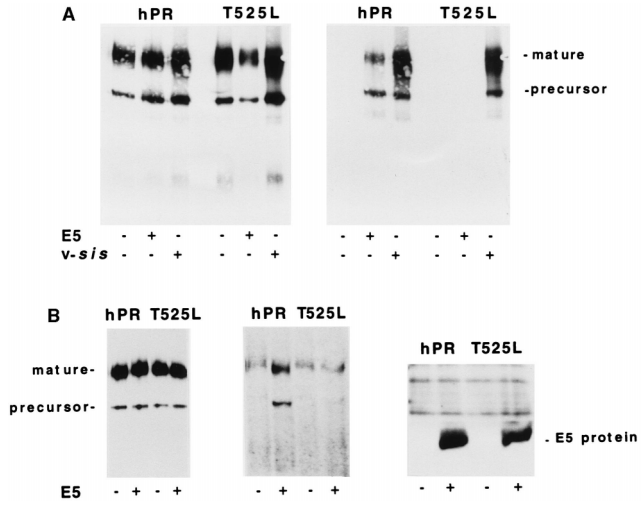

FIG. 6. Biochemical analysis of the PRTL mutant. Ba/F3 cells expressing the wild-type human PDGF β receptor (hPR) or the PRT545L mutant alone (−) or in the presence (+) of the E5 protein or v-Sis were lysed in EBC buffer. As described in Materials and Methods, PDGF receptor was immunoprecipitated from cell extracts and then immunoblotted with either anti-PDGF receptor antiserum to detect the total amount of receptor expressed in the cells (A, left; B, left) or antiphosphotyrosine antibody to detect tyrosine-phosphorylated PDGF receptor (A, right). In panel B, the E5 protein was immunoprecipitated from cell extracts, and immune complexes were then subjected to either anti-E5 immunoblotting to detect the amount of E5 protein expressed (right) or anti-PDGF receptor immunoblotting to detect PDGF β receptor in a complex with the E5 protein (middle). Each lane represents 1,000 μg of extracted protein in panel A or 50 μg (left) or 1,140 μg (middle and right) of protein in panel B. The positions of the mature and precursor forms of the PDGF receptor are indicated.

for a productive interaction between the PDGF receptor and the E5 protein.

Requirement of Thr545 within the transmembrane domain of the PDGF β receptor. Because a glutamine at position 17 in the predicted transmembrane domain of the E5 protein is required for transformation and for complex formation with the PDGF receptor, we speculated that this glutamine may interact with another polar amino acid in the transmembrane domain of the PDGF β receptor. We focused on the threonine in the transmembrane domain of the PDGF β receptor because it is not present at the corresponding position in the transmembrane domains of the PDGF α receptor, the EGF receptor, and p185^{neu}, which do not interact with the E5 protein. To test whether this threonine was required for interaction with the E5 protein, we constructed PRT545L, in which a leucine was substituted for threonine 545 of the human PDGF β receptor, which corresponds to threonine 513 of the murine receptor.

Ba/F3 cell lines expressing this mutant receptor were generated and analyzed. PDGF receptor immunoblotting demonstrated that this mutant receptor was expressed at a level similar to that of the wild-type human receptor in cell lines established in parallel (Fig. 6). Antiphosphotyrosine immunoblotting of PDGF receptor immunoprecipitates demonstrated that E5 expression induced tyrosine phosphorylation of the wild-type PDGF β receptor but not of the PRT545L mutant (Fig. 6A), suggesting that this mutant is not activated by the E5 protein. However, coexpression of v-*sis* with the PRT545L mutant (Fig. 6A) and PDGF treatment of cells expressing PRT545L (Fig. 4) induced receptor tyrosine phosphorylation indistinguishable from that displayed by the wild-type human PDGF receptor. Therefore, this mutant was not defective for normal, ligand-dependent activation and was expressed at the cell surface. Coimmunoprecipitation analysis revealed the presence of the wild-type PDGF β receptor but not the PRT545L mutant in E5 immunoprecipitates from cells coexpressing receptor and the E5 protein (Fig. 6B, middle panel), indicating that the mutant is unable to form a stable complex with the E5 protein. The E5 protein was expressed at similar levels in all tested lines (Fig. 6B, right panel). These biochemical results indicate that the threonine-to-leucine substitution in the transmembrane domain of the human PDGF receptor inhibited the ability of the mutant receptor to form a complex with the E5 protein and undergo E5-induced activation.

TABLE 2. IL-3 tests of cell lines expressing the human PDGF receptor

Cell line	PDGF receptor	Expression		IL-3-independent cell lines/total tested
		BPV E5	v-Sis	
hPR	Human wt[a]	−	−	0/8[b]
hPR-E5	Human wt	+	−	8/8
hPR-sis	Human wt	−	+	5/6
PRTL	Thr to Leu	−	−	0/6
PRTL-E5	Thr to Leu	+	−	0/6
PRTL-sis	Thr to Leu	−	+	4/4

[a] wt, wild type.
[b] Three of these cell lines underwent markedly delayed proliferation in the absence of IL-3.

Cells expressing wild-type or mutant PDGF receptors were also tested for the ability to survive and proliferate in the absence of IL-3 (Table 2 and Fig. 7). None of the cell lines expressing either receptor in the absence of a viral oncogene underwent IL-3-independent proliferation. As previously demonstrated, cells coexpressing the wild-type human PDGF β receptor and the E5 protein remained viable and actively proliferated in the absence of IL-3 (Fig. 7) (12). Cells coexpressing v-Sis and either PRT545L or the wild-type receptor also proliferated efficiently in the absence of IL-3. In contrast, cells coexpressing the PRT545L mutant and the E5 protein rapidly lost viability and failed to proliferate under these conditions

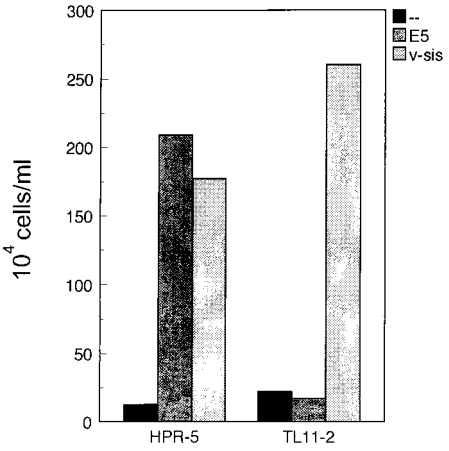

FIG. 7. IL-3 dependence of Ba/F3 cells expressing the PRT545L mutant in the absence or presence of the E5 protein or v-Sis. Ba/F3 cells (−−) or Ba/F3 cells expressing the E5 protein or v-Sis as indicated were infected with recombinant retrovirus containing the wild-type human PDGF β receptor or the PRT545L mutant. After selection for G418 resistance in the presence of IL-3, 7.5×10^4 cells/ml were incubated in the absence of IL-3 for 16 days, and total cells were counted. HPR-5 cells express the wild-type receptor, and TL11-2 cells express the PRT545L mutant.

(Fig. 7). Thus, the PRT545L mutant was defective in its ability to cooperate with the E5 protein to induce a proliferative response. In summary, the threonine-to-leucine mutation in the PRT545L mutant severely impaired the physical and functional interaction with the E5 protein, indicating that the threonine residue in the wild-type receptor plays an important role in the interaction between the E5 protein and the PDGF β receptor.

DISCUSSION

In the experiments reported here, we have confirmed that the transmembrane domain of the PDGF β receptor is required for a productive interaction between the receptor and the E5 protein. Moreover, the threonine within the transmembrane domain of the receptor is required for this interaction. Finally, we established a requirement for a positive charge located in the extracytoplasmic juxtamembrane region of the receptor, indicating that the transmembrane domain alone is not sufficient for stable complex formation, receptor activation, and mitogenic signaling. The ability of numerous receptor mutants to respond to v-Sis and PDGF, the expected pattern of carbohydrate addition as inferred from electrophoretic mobility, and the analysis of cell surface expression of receptor mutants by fluorescence-activated cell sorting (36) indicated that the mutations characterized here did not cause significant alterations in receptor function, orientation, or localization.

These results can be compared with previous attempts to localize the site(s) of interaction between the E5 protein and the PDGF β receptor. Cohen et al. (7) described a chimeric PDGF receptor very similar in structure to ERTM; as was the case with ERTM, this chimera was defective in its response to the E5 protein. However, they also reported that chimeras lacking the PDGF β receptor extracellular juxtamembrane domain were able to interact with the E5 protein, leading them to conclude that the transmembrane domain of the PDGF β receptor contained all the specific sequence information required for the interaction. Strikingly, inspection of the sequence of the EGF receptor (Fig. 1B) reveals the presence of a lysine in the extracellular juxtamembrane domain of this receptor as well, and so the presence of this amino acid in the chimeras studied by Cohen et al. may have obscured the requirement for a juxtamembrane positive charge. Moreover, complex formation was assessed by Cohen et al. in COS cells overexpressing both the E5 protein and the chimeric receptors, conditions under which the requirements for association between the E5 protein and growth factor receptors seem quite relaxed (34). Receptor mutants lacking most of the extracellular domain were also able to interact productively with the E5 protein (12, 43), but the juxtamembrane lysine was retained in these mutants as well. Chimeric receptors were also used to localize the difference between the PDGF α and β receptors (43). In those experiments, the juxtamembrane and transmembrane regions of the receptor were substituted en bloc, and so the individual contributions of these two portions of the receptor were not assessed. It is interesting that the PDGF α receptor, which does not interact with the E5 protein, contains a negatively charged glutamate at the juxtamembrane position and does not contain the threonine in the transmembrane domain. Thus, the presence of the lysine and the threonine in the PDGF β receptor may form the basis for the ability of the E5 protein to distinguish between these two receptor types.

A crucial unanswered question is whether the E5 protein and the PDGF β receptor contact one another directly, or whether another cellular protein mediates this interaction. It has been reported that a ternary complex comprised of the E5 protein, the PDGF β receptor, and a 16-kDa subunit of the vacuolar H^+-ATPase can form in COS cells overexpressing all three proteins (13). However, recent studies in mouse fibroblasts revealed a lack of any correlation between the ability of E5 mutants to bind the 16-kDa protein and to associate with the PDGF β receptor or transform cells (41). Thus, it appears unlikely that the 16-kDa protein plays an essential role in the formation of the complex between the E5 protein and the PDGF β receptor.

The identification of specific required residues in the transmembrane and juxtamembrane regions of the receptor suggests some possible models for their role in complex formation. Dimer formation between membrane helices can be mediated by van der Waals interactions between hydrophobic residues, electrostatic interactions between oppositely charged residues, or hydrogen bonding interactions involving polar side chains (9, 22, 23, 26, 44). In many cases, transformation by the E5 protein is not inhibited by extensive hydrophobic substitutions within its putative transmembrane domain if the glutamine is retained (21, 29), implying that the hydrophobic residues in this segment contribute little sequence specificity to complex formation. Furthermore, none of the residues in the predicted membrane-spanning segment of the PDGF β receptor can participate in electrostatic interactions. Rather, we propose that hydrogen bonds involving the essential transmembrane threonine residue in the PDGF β receptor and the essential glutamine at position 17 in the predicted transmembrane segment of the E5 protein may occur and contribute to complex formation. This model is consistent with the observation that replacement of the glutamine with nonpolar amino acids disrupts complex formation and inhibits receptor activation and transformation, whereas a variety of polar substitutions are tolerated (20a, 31, 41, 42). Moreover, the threonine of the PDGF β receptor and the glutamine of the E5 protein are likely to lie at roughly the same position in the membrane relative to the membrane surface. T513 of the murine receptor (corresponding to T545 of the human version) is 14 residues away from K499, the first charged amino acid in the extracellular domain of the PDGF β receptor, while Q17 of the E5 protein is 16 residues away from D33, the first charged amino acid in the extracytoplasmic domain of the E5 protein. However, it remains possible that the interaction between the E5 protein and the PDGF receptor is indirect, in which case the threonine may allow the transmembrane domain of the receptor to directly contact another cellular protein that in turn recognizes the hydrophobic segment of the E5 protein.

The requirement for a positive charge in the extracellular juxtamembrane region of the PDGF β receptor may indicate the existence of an electrostatic interaction between the lysine in the receptor and an acidic residue in the hydrophilic carboxyl terminus of the E5 protein or in the extracellular domain of a cellular protein that mediates complex formation. If there is a direct contact between the lysine and the E5 protein, it is most likely that the lysine interacts with the essential aspartate at position 33 in the E5 protein, as was initially suggested by Meyer et al. (29). The lysine and the aspartate are both thought to lie at the border between the extracytoplasmic juxtamembrane domain and the transmembrane domain of the interacting proteins. Furthermore, with the exception of cysteines involved in E5 homodimer formation and the aspartic acid, mutations at other amino acids in the carboxyl terminus of the E5 protein do not impair the ability of the mutant E5 protein to form a stable complex with the PDGF β receptor (31). In fact, if the aspartate and E5 homodimer formation are maintained, these other residues are dispensable for transformation of NIH 3T3 cells (29). Glutamic acid, the other acidic

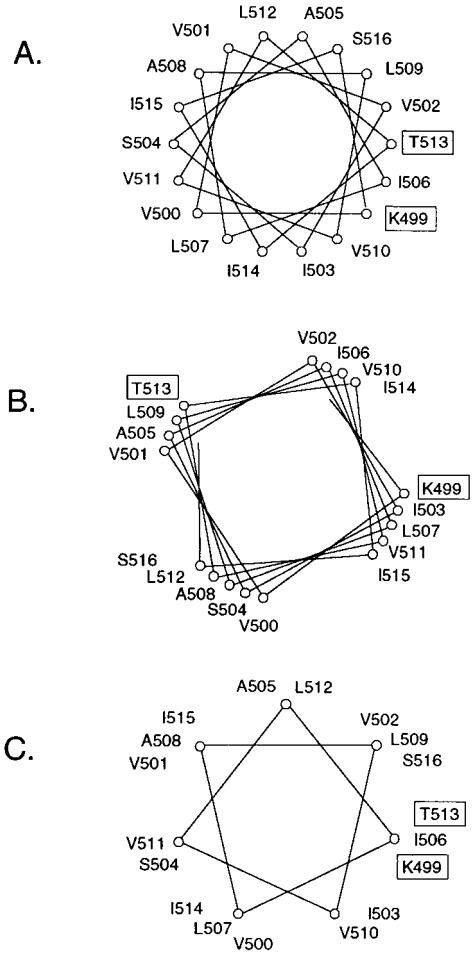

FIG. 8. Helical wheel diagrams of the murine PDGF β receptor transmembrane domain from K499 to S516. Helical wheel diagrams of the murine PDGF β receptor transmembrane domain are shown for a canonical α helix (A), a helix in a right-handed coiled coil (B), and a helix in a left-handed coiled coil (C). Note that the required transmembrane threonine (T513), corresponding to T545 of the human receptor, and juxtamembrane lysine (K499) align precisely in the left-handed coiled-coil geometry, which would allow the receptor to interact with the E5 protein along a single interface.

residue in the BPV E5 protein, is not conserved in related E5 proteins and is not required for C127 cell transformation.

Although both the transmembrane threonine and the juxtamembrane lysine of the PDGF β receptor are required for complex formation with the E5 protein, neither of these sites by itself appears to be sufficient, since mutation at either position disrupts the interaction. Independent weak interactions involving the receptor transmembrane and juxtamembrane regions may both contribute to the stability of the complex with the E5 protein, whereas the affinity of each separate interaction is not sufficient to allow stable complex formation. Helical wheel diagrams of potential geometries of the PDGF β receptor transmembrane domain are shown in Fig. 8. A canonical helical wheel diagram having 3.6 residues per turn (Fig. 8A) shows that the required transmembrane threonine (T513) and juxtamembrane lysine (K499) are spaced 40° apart on roughly the same face of the helix and are separated by four helical turns. However, paired membrane-spanning helices typically pack in either a right- or a left-handed coiled-coil geometry. The relative geometries of helices packed in this manner can be represented by helical wheel diagrams having 3.9 residues per turn for a right-handed coiled coil (Fig. 8B) and 3.5 residues per turn for a left-handed coiled coil (Fig. 8C). We note that K499 and T513 are predicted to align precisely along a single interface in a left-handed coiled-coil complex with another membrane helix, and thus we favor this geometry for a complex between the transmembrane domains of the PDGF β receptor and the E5 protein.

The results presented here as well as previously published results establish that the E5 protein interacts with the PDGF β receptor in a manner different from that of the normal ligand, PDGF. In particular, the specific transmembrane and juxtamembrane amino acids of the receptor identified as being important for an interaction with the E5 protein are not required for PDGF binding and PDGF-induced receptor activation, implying that the E5 protein (or an intermediary protein in a ternary complex) contacts very different regions on the receptor than does PDGF. Although the mechanism by which the E5 protein induces receptor activation is still obscure, biochemical evidence suggests that the E5 protein, like v-Sis, induces receptor dimerization (16, 17). We have also recently found that a PDGF β receptor chimera containing the transmembrane domain of the oncogenic form of p185^{neu} is constitutively activated in the absence of the E5 protein or PDGF (33). This mutation in p185^{neu} appears to stimulate dimer formation and receptor activation by allowing hydrogen bond formation involving glutamic acid residues on adjacent transmembrane helices (1, 2, 40, 44). In addition, the gp55 oncoprotein of the Friend erythroleukemia virus binds to and activates the erythropoietin receptor via interactions that involve the transmembrane domains of these proteins (18, 25, 46). Taken together, these results emphasize that a variety of interactions involving growth factor receptor transmembrane and juxtamembrane regions have the potential to induce receptor dimerization and activation. The E5 protein is emerging as a prototype of a class of small, transmembrane proteins that can activate growth factor receptors and may serve as a model for the rational design of oligopeptides and other small molecules with similar effects on receptor function.

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