The Transmembrane Domain of the E5 Oncoprotein Contains Functionally Discrete Helical Faces*

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The E5 protein of bovine papillomavirus is a 44-amino acid, Golgi-resident, type II transmembrane protein that efficiently transforms immortalized mouse fibroblasts. The transmembrane (TM) domain of E5 is not only critical for biological activity, it also regulates interactions with cellular targets including the platelet-derived growth factor receptor (PDGF-R) and the 16-kDa subunit of the vacuolar proton ATPase (V-ATPase). In order to define the specific TM amino acids essential for E5 biological and biochemical activity, we performed scanning alanine mutagenesis on 25 of the 30 potential TM residues and genetically mapped discrete α-helical domains which separately regulated the ability of E5 to bind PDGF-R, activate PDGF-R, and to form oligomers. Alanine substitutions at positions 17, 21, and 24 (which lie on the same helical face) greatly inhibited E5 association with the PDGF-R, suggesting that this region comprises the receptor binding site. PDGF-R activation also mapped to a specific but broader domain in E5; mutant proteins with alanines on one helical face (positions 8, 9, 11, 16, 19, 22, and 23) continued to induce PDGF-R tyrosine phosphorylation, whereas mutant proteins with alanines on the opposite helical face (positions 7, 10, 13, 17, 18, 21, 24, and 25) did not, indicating that the latter helical face was critical for mediating receptor transphosphorylation. Interestingly, these “activation-defective” mutants segregated into two classes: 1) those that were unable to form dimers but that could still form higher order oligomers and transform cells, and 2) those that were defective for PDGF-R binding and were transformation-incompetent. These findings suggest that the ability of E5 to dimerize and to bind PDGF-R is important for receptor activation. However, since several transformation-competent E5 mutants were defective for binding and/or activating PDGF-R, it is apparent that E5 must have additional activities to mediate cell transformation. Finally, alanine substitutions also defined two separate helical faces critical for E5/E5 interactions (homodimer formation). Thus, our data identify distinct E5 helical faces that regulate homologous and heterologous intramembrane interactions and define two new classes of biologically active TM mutants.

The 44-amino acid E5 oncoprotein of bovine papillomavirus type 1 is the smallest known oncoprotein. The first 30 amino acids are believed to constitute an α-helical transmembrane domain, and the C-terminal 14 amino acids are generally hydrophilic, containing two cysteines that stabilize homodimer formation via disulfide bonds (1–3). Immunoelectron microscopy studies have demonstrated E5 to be a type II Golgi polypeptide with its C terminus predominantly facing the Golgi lumen (4).

The transformation of mouse fibroblasts by E5 has been attributed mainly to its ability to bind and induce the autophosphorylation of PDGF-R (5–9), although E5 can also interact with the epidermal growth factor receptor (10) and transform immortalized keratinocytes (11). Other cellular proteins that associate with E5 include the 16-kDa subunit of the vacuolar proton pump (16K) and a 125-kDa α-adaptin like protein. Mutagenic analysis indicates that E5/16K binding may have an important role in cellular transformation and E5, 16K, and PDGF-R co-precipitate in transfected cells (12–15).

Specific interactions between transmembrane α-helices are important for the structure and function of many integral membrane proteins. For example, interactions between two components of the T-cell receptor complex, TCRα and CD3δ, are mediated through transmembrane domains and allow for the functional assembly of the complex (16, 17). Similarly, dimerization of glycophorin A has been shown to be dependent upon the amino acid sequence of its transmembrane domain (18, 19) and the assembly of major histocompatibility complex class II molecules appears to be mediated by interactions between the transmembrane domains of the α and β chains (20). Furthermore, the mutagenic analyses of glycophorin A have recently been substantiated on a molecular level by the determination of the structure of glycophorin A dimers using heteronuclear nuclear magnetic resonance (21). The association between E5 and the 16K V-ATPase subunit is also mediated by transmembrane interactions, specifically by an interaction between glutamine in the E5 transmembrane domain and glutamic acid in the fourth TM domain of 16K (22). In addition, we have previously demonstrated the ability of the isolated E5 TM domain to bind the PDGF-R, suggesting that specific binding interactions between the TM regions of these molecules might facilitate their interaction (12). Recent data has defined a threonine residue in the TM region of PDGF-R that might facilitate hydrogen bond formation with the E5 TM glutamine residue (position 17) (23).

In order to determine the contribution of amino acids in the E5 transmembrane domain to its biological and binding prop-

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The abbreviations used are: PDGF-R, platelet-derived growth factor receptor; PDGF, platelet-derived growth factor; TM, transmembrane; DMEM, Dulbecco's modified Eagle's medium; NGS, normal goat serum; PBS, phosphate-buffered saline; D-PBS, Dulbecco's phosphate-buffered saline; wt, wild-type; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.
E5 Alanine Scanning Mutagenesis

The presence of a series of alanine mutations was constructed. Twenty-five residues between positions 4 and 31 of E5 were individually replaced by alanine, and the resulting proteins were examined for their ability to: 1) associate with PDGF-R, 2) induce the phosphorylation of PDGF-R, 3) form homodimers, 4) localize to the Golgi apparatus, and 5) transform NIH3T3 mouse fibroblast cells. Alanine was used in the substitutions since it neither significantly alters protein conformation nor imposes electrostatic or steric effects (24).

Our results indicate that the mutants that perturb E5 activities (e.g. binding to PDGF-R, activation of PDGF-R, homodimer formation) cluster within the TM domain in a pattern that is consistent with this domain being in an α-helical conformation. Thus, E5 mutants that activate the PDGF-R align on one predicted helical face and E5 mutants that fail to activate PDGF-R align along the opposite helical face. E5 mutants that fail to bind PDGF-R represent a clustered subset of mutants that fail to activate the receptor. Most surprisingly, only two mutants were transformation-defective (positions 17 and 18). Finally, there were several E5 mutants that retained transforming activity but failed to bind or activate PDGF-R, suggesting alternative mechanisms for cellular transformation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Plasmid Constructions**—Cos-1 and NIH3T3 cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum.

The expression vector pS55 was constructed by adding a polylinker region to pS55 (Stratagene) and has been described previously (25). All alanine point mutants were synthesized via a two-step polymerase chain reaction (PCR) using oligonucleotides synthesized by Life Technologies, Inc. In the first step of each PCR reaction, two fragments were generated. The first corresponded to the 5' end of the E5 mutant and was constructed with a 5' oligonucleotide (5'-ATGATCAGTGCGGCACATCCATTGAATCTCAGGCTGTAGTAGG-3'; L24A, 5'-CAACAGCTGCGCATTATATAC-3; L26A, 5'-GAAAAAACGCTAGTATTATTTGGCATAGGATCTGAA-3'). The second fragment, generated by a 3' oligonucleotide complementary to the 3' oligonucleotide used in the above reaction as well as a 3' oligonucleotide complementary to the last four codons of E5 which included a BamHI recognition site downstream of the translational stop site (5'-ATAGCTGGATCCCTTTAAAGGGCGACGAC-3'). The 5' oligonucleotides are as follows: L4A, 5'-CAATATACCAAATGGTACGCTGCAGCAATTAG-3'; W5A, 5'-TGTATACCAACAAAAAGCTAGGTTCC-3'; F9A, 5'-CAATATAACAAATGGGTAGGGTAC-3'; F6A, 5'-GAAGGCTAAAGATTATATAC-3'; F3A, 5'-TTTTGCTGTATACTGGGAATT-3'; F27A, 5'-GAAAAACAAGGTAAGAAGTCGATG-3'; L7A, 5'-CAACATGCTGCGCATTATATAC-3'.

For detecting tyrosine-phosphorylated PDGF-R, two 15-cm diameter plates at 90% confluence were washed twice with D-PBS and incubated with serum-free DMEM for 14 to 16 h at 37 °C. Prior to harvesting, cells were washed once with PBS containing 100 μM NaVO3. The cells were then extracted in 750 μl of lysis buffer containing 1% Triton X-100, 50 mM HEPES (pH 7.5), 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 10 mM NaPPi, 1 mM NaVO3, 0.5 mM phenylmethylsulfonyl fluoride (Sigma), 10 mg/ml aprotinin (Boehringer Mannheim), and 10 μg/ml leupeptin (Boehringer Mannheim). The procedures for immunoprecipitation were similar to those described above. PDGF-R was isolated in the second immunoprecipitation using 2 μl of the monoclonal antibody PY-20 (Transduction Laboratories, Lexington, KY), while associated PDGF-R was isolated in the second immunoprecipitation using 2 μl of the polyclonal antibody 06-131 (Upstate Biotechnology Inc. (UBI), Lake Placid, NY). Proteins were separated on either 7.5% (for analysis of PDGF-R) or 14% (for analysis of E5 and 16K) sodium dodecyl sulfate (SDS)-polyacrylamide gels. All gels were subsequently fixed in 30% methanol, 10% acetic acid, enhanced with Enlightening (NEN Life Science Products), dried, and exposed to Kodak XAR-5 film for 1–28 days at 70 °C. Densitometry measurements were made on a PDI Discovery Series model DNA 35 scanner.

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Kodak XAR-1 film for 7–14 days.

In order to determine the competence of E5 mutants to dimerize, two 15-cm plates of the designated cell lines (at 90% confluence) were harvested in RIPA buffer as described previously. Following immunoprecipitation with 5 μl of anti-AU1 antibody, the immunoprecipitates were washed three times with RIPA buffer and solubilized in 30 μl of sample buffer without 2-mercaptoethanol. These proteins were separated on 14% polyacrylamide gels and then transferred to Immobilon-P transfer membranes (Millipore, Bedford, MA) in Tris-glycine buffer, either overnight at 20 V or for 2 h at 80 V. Immunoblotting was performed with a Tropix (Bedford, MA) Western Light protein detection kit according to the described procedures. The anti-E5 polyclonal antiserum (1:5000 dilution) was used to detect E5 on the membranes.

Immunofluorescence Assays—COS-1 cells were grown on glass coverslips and at 60% confluence were transfected with the pJS55 constructs as described above. Twenty-four hours following glycerol shock, the cells were washed twice with PBS and fixed in 3.7% formaldehyde for 20 min. The coverslips were then washed three times with PBS and incubated for 20 min with 10% normal goat serum (NGS), 0.1% saponin in PBS. Following two more washes in PBS, cells were incubated for 1 h with AU1 antibody that was diluted 1:5000 dilution was used to detect E5 on the membranes.

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RESULTS

Construction of Epitope-tagged E5 Transmembrane Mutants—In order to probe the E5 transmembrane domain for residues or domains important in the interactions and functions of E5, 26 of the 30 residues in the putative TM domain of E5 were changed independently to alanine (Fig. 1). The immunological detection and isolation of the mutant E5 proteins was facilitated by the addition of an N-terminal, six-amino acid AU1 epitope, which has been shown to have no effect on E5 biological activity (25). Each of the E5 mutants was cloned into pJS55, a modified pSG5 vector (Stratagene) that contains an inserted polylinker and utilizes the SV40 early promoter for gene expression. The fidelity of the mutant E5 genes was verified in the final plasmid constructs by DNA sequencing.

| WT E5 | mpnLWLLLFLGLVAAMQLLLLLLLLLLFFLVYwdfhecsctg1pf |
|-------|----------------------------------------------|
| L4A   | mpnLWLLLFLGLVAAMQLLLLLLLLLLFFLVYwdfhecsctg1pf |
| W5A   | mpnLWLLLFLGLVAAMQLLLLLLLLLLFFLVYwdfhecsctg1pf |
| F6A   | mpnLWLLLFLGLVAAMQLLLLLLLLLLFFLVYwdfhecsctg1pf |
| L7A   | mpnLWLLLFLGLVAAMQLLLLLLLLLLFFLVYwdfhecsctg1pf |
| L8A   | mpnLWLLLFLGLVAAMQLLLLLLLLLLFFLVYwdfhecsctg1pf |
| F9A   | mpnLWLLLFLGLVAAMQLLLLLLLLLLFFLVYwdfhecsctg1pf |
| L10A  | mpnLWLLLFLGLVAAMQLLLLLLLLLLFFLVYwdfhecsctg1pf |
| G1A   | mpnLWLLLFLGLVAAMQLLLLLLLLLLFFLVYwdfhecsctg1pf |
| V13A  | mpnLWLLLFLGLVAAMQLLLLLLLLLLFFLVYwdfhecsctg1pf |
| M16A  | mpnLWLLLFLGLVAAMQLLLLLLLLLLFFLVYwdfhecsctg1pf |
| Q17A  | mpnLWLLLFLGLVAAMQLLLLLLLLLLFFLVYwdfhecsctg1pf |
| L18A  | mpnLWLLLFLGLVAAMQLLLLLLLLLLFFLVYwdfhecsctg1pf |
| L19A  | mpnLWLLLFLGLVAAMQLLLLLLLLLLFFLVYwdfhecsctg1pf |
| L20A  | mpnLWLLLFLGLVAAMQLLLLLLLLLLFFLVYwdfhecsctg1pf |
| L21A  | mpnLWLLLFLGLVAAMQLLLLLLLLLLFFLVYwdfhecsctg1pf |
| L22A  | mpnLWLLLFLGLVAAMQLLLLLLLLLLFFLVYwdfhecsctg1pf |
| F23A  | mpnLWLLLFLGLVAAMQLLLLLLLLLLFFLVYwdfhecsctg1pf |
| L24A  | mpnLWLLLFLGLVAAMQLLLLLLLLLLFFLVYwdfhecsctg1pf |
| L25A  | mpnLWLLLFLGLVAAMQLLLLLLLLLLFFLVYwdfhecsctg1pf |
| L26A  | mpnLWLLLFLGLVAAMQLLLLLLLLLLFFLVYwdfhecsctg1pf |
| F27A  | mpnLWLLLFLGLVAAMQLLLLLLLLLLFFLVYwdfhecsctg1pf |
| F28A  | mpnLWLLLFLGLVAAMQLLLLLLLLLLFFLVYwdfhecsctg1pf |
| L29A  | mpnLWLLLFLGLVAAMQLLLLLLLLLLFFLVYwdfhecsctg1pf |
| V30A  | mpnLWLLLFLGLVAAMQLLLLLLLLLLFFLVYwdfhecsctg1pf |
| Y31A  | mpnLWLLLFLGLVAAMQLLLLLLLLLLFFLVYwdfhecsctg1pf |

**FIG. 1.** Scanning alanine mutagenesis of the E5 transmembrane domain. Each amino acid in the proposed transmembrane domain of E5 was individually converted to alanine by a two-step PCR technique as described under "Experimental Procedures." The wt E5 sequence is indicated at the top of the figure with the TM region delineated in capital letters. The position of the mutant alanine residues is indicated in bold capital letters. Residues 14 and 15 were not mutagenized since they were already alanine. The PCR products were cloned into pJS55, a modified pSG5 vector (Stratagene) that contains an inserted polylinker and utilizes the SV40 early promoter for gene expression. The fidelity of the mutant E5 genes was verified in the final plasmid constructs by DNA sequencing.
minus (27). The immunoprecipitated proteins were then separated by PAGE, transferred to nitrocellulose membranes, and detected by Western blot analysis using antiserum generated against the C terminus of E5 (28).

Fig. 2A shows an SDS-PAGE gel of the E5 alanine mutant proteins that were immunoprecipitated and blotted from NIH3T3 cell lines. All of the mutant proteins were stably expressed in these cell lines, although there was some variation in the absolute level of E5 protein. While the majority of the wild-type E5 (wt E5) was present in cells as a dimer, alanine substitutions at positions 7 (Leu), 13 (Val), 18 (Leu), 20 (Leu), 25 (Leu), and 26 (Leu) generated mutants that failed to form dimers and preferentially formed tetramers. These tetramers were not observed when the samples were treated with reducing agent, indicating that disulfide linkages were responsible for tetramer stabilization (data not shown). When the dimerization-defective E5 mutants were plotted as a helical net diagram (Fig. 2B), they sorted into two distinct zones or faces of the predicted E5 helix (residues 7, 18, and 25, and residues 13, 16, and 20), indicating that two non-contiguous regions regulated the interaction of adjacent E5 molecules in dimers. In addition, E5 mutants at positions 16 (Met) and 31 (Tyr) formed an abundance of higher order oligomers, up to and including hexamers.

Three E5 Mutants Define a Potential Binding Site for the PDGF-R Complex—In most cases, the ability of E5 to transform fibroblast cells has been linked to its ability to bind and induce the phosphorylation of PDGF-R (5–9) and to associate with the 16K protein (12–15). It is believed that E5-mediated activation of PDGF-R constitutively stimulates signal transduction pathways, which results in a mitogenic response and consequent cellular transformation. In order to define those residues that were important for the binding of E5 to the PDGF-R complex, immunoprecipitates of the indicated E5 mutant proteins from NIH3T3 cell lines were evaluated for the presence of co-precipitated PDGF-R (Fig. 3A). As reported previously, wt E5 predominantly associates with the immature form of the receptor (6, 8). Substitution at positions 17 (Gln), 21 (Leu), and 24 (Leu) resulted in mutant proteins that were unable to co-precipitate PDGF-R. While previous studies had identified position 17 as being critical for E5/receptor association (29), the current study identifies two additional residues (Leu-21 and Leu-24) that also regulate interactions between E5 and the PDGF-R complex. Assuming that the transmembrane domain of E5 forms an α helix with a periodicity of 3.6 residues per turn, these residues reside in a restricted area on the same face of the helix (Fig. 3B).

An Entire E5 TM Helical Face Regulates PDGF-R Tyrosine Phosphorylation—While the above experiments clearly indicated that three distinct residues of E5 modulated the association of E5 with the PDGF-R complex, it was important to determine whether these same sites affected receptor phosphorylation. Therefore, each of the alanine mutants was screened for its ability to induce PDGF-R phosphorylation in NIH3T3 cell lines. Cells were grown in the absence of exogenous PDGF ligand (serum-free media), labeled with [35S]methionine/cysteine, and the amount of total and phosphorylated PDGF-R determined by immunoprecipitation as described in Fig. 4A. Comparison of the ratio of phosphorylated PDGF-R to total PDGF-R revealed that E5 mutants at positions 7 (Leu), 10 (Leu), 13 (Val), 17 (Gln), 18 (Leu), 20 (Leu), 21 (Leu), 24 (Leu), 25 (Leu), 26 (Leu), 30 (Val), and 31 (Tyr) failed to induce significant phosphorylation of PDGF-R. When these mutants (positions 7–25) and their biological activities were plotted as a helical wheel diagram, it was apparent that all E5 mutants defective for inducing receptor phosphorylation were aligned along one helical face and those that retained biochemical activity were aligned along the opposite helical face (Fig. 4B). Mutants at residues 26, 30, and 31 did not conform precisely to this localization. However, the 19 amino acids between positions 7 and 25 are sufficient to span the plasma membrane as a TM domain and the amino acids distal to this, especially residues 30 and 31, have been proposed to constitute a non-helical, “juxtamembrane” region rather than a transmembrane domain (28).

The above experiments on receptor binding and activation indicate that there are two distinct classes of E5 mutants that are defective for inducing PDGF-R phosphorylation. First, there are those mutants that fail to bind to the receptor and therefore cannot subsequently induce receptor phosphorylation (i.e. mutants at positions 17, 21 and 24). Second, there are those mutants that can bind to the receptor but still cannot induce receptor activation (i.e. mutants at positions 7, 13, 18, 20, 25, and 26). Interestingly, this second class of mutants is incapable of forming dimers, suggesting that this might represent their defect in mediating receptor activation. Evidently both receptor binding and homodimer formation are essential for E5-induced phosphorylation of the PDGF-R complex.

TM Mutants Further Dissociate E5 Transforming Activity from PDGF-R Binding/Activation—Although the ability of E5 to bind and activate PDGF-R has been thought to be essential for fibroblast transformation (5–9), a recent study of E5 mutations at position 17 indicated that the substitution of glutamine 17 with serine resulted in a biologically active protein that failed to activate the PDGF-R (29). This suggested that PDGF-R phosphorylation was not essential for mitogenic signaling by E5. Our current study reinforces these findings. When the alanine TM mutants were evaluated for transforming activity (Fig. 5), only two mutants (at position Gln-17 and Leu-18) were transformation-defective. All other mutants were transformation-competent, most displaying transformation efficiencies above 75% wt activity. Two mutants, at Leu-7 and Val-13, had slightly reduced transforming efficiencies of 42% and 52%, respectively (Table I). Most importantly, a large number of substitutions resulted in E5 oncoproteins with higher transformation efficiencies than wt E5. Three of these mutants, substitutions at positions 24 (Leu), 25 (Leu), and 26 (Leu), did so without inducing phosphorylation of PDGF-R (Fig. 4). In addition, mutation of Leu-24 produced an E5 protein that showed little or no ability to bind PDGF-R, yet it transformed fibroblasts 3-fold better than wt E5. Thus, these studies establish several new E5 mutants that are unable to induce the phosphorylation of PDGF-R yet retain transforming activity.

DISCUSSION

Previous mutagenic studies of the E5 oncoprotein have suggested several cellular targets that may be important for the biological activity of E5, including the PDGF-R (5–9), the epidermal growth factor receptor (10), the 16K V-ATFase subunit (12–15), and an α-adaptin-like protein (30). Earlier studies with E5 indicated that its C-terminal region might be a critical determinant of its biological activity and that the E5 TM domain could be replaced with other hydrophobic residues, suggesting that it did not confer specificity for target interactions or cell transformation (3, 31). Since the E5 C terminus was suggested to show homology to PDGF, it was hypothesized that E5 stimulated cell proliferation via mimicry of this mitogenic ligand (8). However, more recent studies have demonstrated that the E5 TM domain is a critical regulator of binding to target proteins (including PDGF-R) and biological activity (12) and that there are residues within the PDGF-R and E5 TM domains that can alter these interactions (23). In this study we have performed scanning alanine mutagenesis of the E5 TM
domain in order to define those residues that were critical for regulating E5 transforming activity and ability to interact with target proteins. Our results demonstrate for the first time that the E5 TM domain contains functionally distinct domains that separately regulate homologous (E5/E5) interactions and heterologous (E5/PDGF-R) interactions. In addition, our studies define two new classes of mutants, which transform by a mechanism that is independent of PDGF-R phosphorylation, significantly extending our previous studies with mutants at position 17 (29).

Translocation of E5 to the Golgi Complex Is Not Dependent upon Any Single TM Residue—None of the E5 alanine mutants generated in this study was defective for Golgi localization, suggesting that there is no single residue within the TM domain which is required for proper intracellular translocation. However, this does not eliminate the possibility that a series or combination of TM amino acids regulates this processing. Several other possibilities might explain Golgi localization (via retention or recycling): (a) The first is the length of the E5 TM domain. Variation in the length of transmembrane α-helical domains has been proposed as a mechanism of differentially regulating membrane protein flow from the Golgi to other compartments such as the plasma membrane (32). Since there is a gradual, cholesterol-dependent increase in the thickness of membranes during the progression from the ER to the Golgi to the plasma membrane, a membrane embedded protein with a long TM α-helix could make the full transit from the ER to the plasma membrane. Proteins with shorter TM domains would become selectively sequestered in intermediate compartments such as the Golgi depending upon the length of their TM domain (33). If this hypothesis were correct, only a subset of the 30 amino-terminal hydrophobic residues of E5 could be involved in forming a membrane-spanning α-helix. This would be consistent with our experimental results suggesting that only 19 amino acids of E5 (residues 7–25) constitute the transmembrane domain. (b) The second possibility is oligomer formation. Another hypothesis for Golgi retention proposes that the formation of oligomers interferes with normal protein traffic through the various membrane compartments, thereby restricting access to the plasma membrane (34, 35). E5 certainly has the ability to form both homo-oligomers and well as hetero-oligomers, which could specify its retention in the Golgi. However, our finding that mutant E5 proteins that are incapable of binding PDGF-R still accumulate in the Golgi indicates that E5/PDGF-R hetero-oligomer interactions cannot be required for this sequestration. (c) The third possibility is non-TM sequences. A linear sequence might exist outside the TM domain, in the C-terminal hydrophilic region, which is responsible for Golgi localization.

Three E5 TM Residues Are Important for the Association with PDGF-R—Only 3 of the 25 alanine scanning mutants failed to associate with PDGF-R in NIH3T3 lines. These three residues (positions 17, 21, and 24) lie on the same face of the α-helix and include the glutamine at position 17, previously shown to be important for receptor association (15, 29, 31). In addition, alanine mutants at positions 21 and 24 were still able to transform mouse fibroblast cells, indicating that the association of E5 with PDGF-R cannot be the sole determinant of E5 transforming activity. Most likely, residues 17, 21, and 24 represent components of a specific binding site for PDGF-R. Alternatively, these three residues may be essential for the structure of

**Fig. 2.** A, expression and oligomerization of wt and mutant E5 proteins. G418-selected NIH3T3 cell lines were extracted and immunoprecipitated with AU1 monoclonal antibody and separated on 14% SDS-PAGE gels in the absence of reducing agent. The proteins were then transferred to PVDF membranes and detected using a polyclonal antiserum generated against the C terminus of E5 and a chemiluminescence detection kit (Tropix). The total expression level of wt and mutant proteins is similar, although the proportions of monomer, dimer, and tetramer vary. For wt E5, the dimeric form predominates. Several mutant E5 proteins (e.g. L7A, L25A, and L26A) fail to form dimers but still form tetramers, whereas one can only form dimers (e.g. L22A). B, helical net plot of E5 mutants that are dimerization-incompetent. The position of dimerization-incompetent E5 alanine mutants is indicated in the two shaded areas of a helical web plot of the E5 TM domain. These defective mutants segregated into two distinct domains or faces: (domain 1, residues 7, 18, and 25; domain 2, residues 13, 16, and 20).
FIG. 3. A, binding of PDGF-R by wt and mutant E5 proteins. NIH3T3 stable cell lines were metabolically labeled with [35S]cysteine/methionine, extracted, and immunoprecipitated with AU1 monoclonal antibody. 10% of the lysate was separated by 14% SDS-PAGE, while the remaining lysate was subjected to a second immunoprecipitation using a monoclonal antibody against PDGF-R and separated by 7.5% SDS-PAGE. No E5-associated PDGF-R was detected in cells transfected with the control plasmid, pJS55, whereas cells expressing wt E5 show significant amounts of co-precipitated receptor. While the majority of E5 alanine mutant proteins were able to associate with PDGF-R, two are shown that are negative (L21A, L24A). Q17A was also unable to associate with PDGF-R. B, helical net plot of the apparent E5 binding site for PDGF-R. The E5 alanine mutants that were defective for binding PDGF-R (shaded area) are displayed as a helical net plot of the E5 TM domain. The three binding-defective mutants (Q17A, L21A, and L24A) mapped to a discrete region on one face of the predicted E5 helix, the presumed binding site for PDGF-R.
E5 and the substitution of alanine at these positions could perturb normal E5 protein conformation. This seems unlikely, however, since mutants 21 and 24 retain normal transforming activity.

Phosphorylation of PDGF-R Correlates with E5 Dimerization, Not Tetramerization—Prior to this study, most experimental data regarding the role of E5 oligomerization in cell transformation relied upon the analysis of E5 mutants mutated in the two C-terminal cysteine residues. Substitution of either cysteine (in the Cys-X-Cys sequence) with methionine or arginine diminished E5 transforming activity (3). The most profound inhibition was achieved when both cysteines were simultaneously replaced with methionine. This double cysteine mutant could not form dimers, could not activate the PDGF-R, and could not transform cells (3). These previous studies, therefore, concluded that E5 disulfide bond formation was critical for E5 function.

Our current study indicates that the ability to form dimers and oligomers is strongly regulated by the TM region and perhaps may be the critical domain in initiating oligomer for-
mation. Nearly all alanine TM mutants that retain the ability to form dimers can also induce the phosphorylation of the PDGF-R (e.g., mutants at position 8, 9, 11, 16, 19, 22, and 23). As expected, alanine mutants in the PDGF-R binding domain (positions 17, 21, and 24) form dimers but cannot induce PDGF-R phosphorylation. Conversely, alanine mutants that cannot form dimers are unable to activate the PDGF-R (e.g., mutants at position 7, 13, 25, 26). Thus, it appears that the ability of E5 to form stable dimers is essential for inducing the phosphorylation of the PDGF-R. Interestingly, oligomerization

FIG. 5. Transforming activity of wt and mutant E5 proteins. Each of the E5 mutant plasmids was transfected into recipient NIH3T3 cells and evaluated for focus formation after 2 weeks as described under “Experimental Procedures.” The percentage of transforming efficiency of each mutant (compared with (wt E5) is indicated by the numbers adjacent to each amino acid in the helical wheel plot of the E5 TM region. Only 2 of the 25 alanine mutants (positions 17 and 18) showed greatly reduced transforming activity (0% and 2%, respectively) and are indicated in bold lettering.

TABLE I
Summarized biological properties of mutant E5 proteins

| MPN | L | W | F | L | L | F | L | G | L | V | A | A | M | Q |
|-----|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
|     |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| Residue number | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 |
| Focus Formation | 415 | 364 | 42 | 90 | 122 | 316 | 238 | 52 | 304 | 0 |
| PR binding | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Forms w/o bME | 1,2 | 1,2 | 1,2 | 1,2 | 1,2 | 1,2 | 1,2 | 1,2 | 3,4 | 1,2 |
| Phosphorylation | ++++ | ++ | +++ | + | +++ | + | +++ | - | ++ | - |

TABLE II
Helical wheel plot of the E5 TM region.
of E5 molecules is clearly insufficient for promoting PDGF-R phosphorylation since mutants at positions 25 and 26 can readily form tetramers but cannot activate PDGF-R. Since these tetrameric forms of E5 still bind PDGF-R, it appears that their inability to induce receptor phosphorylation may be the result of inappropriate spatial configurations, which do not allow appropriate receptor trans-phosphorylation.

**E5 Tetramers Can Transform Cells**—E5 alanine mutants that exist as stable tetramers (i.e., mutants at positions 25 and 26) are transformation-competent but cannot induce the phosphorylation of PDGF-R. Clearly this revises previous conclusions that only the dimeric form of E5 has transforming activity. Potentially tetrameric E5 might signal through the PDGF-R in a phosphorylation-independent mechanism. More likely, however, tetrameric forms of E5 might have distinct biological activities. Indeed, wt E5 also exists in tetrameric forms, although it is the minority of total E5 protein. The observation that E5 can form biologically active oligomers is suggestive that it might display membrane pore-forming activities. The M2 protein of influenza protein is a similar, small hydrophobic polypeptide, which is localized in the Golgi apparatus, assembles into tetramers, and forms proton pores in the membrane (36–40). To date, however, no pore-forming activity has been ascribed to E5. It will also be important in future experiments to evaluate the functional consequences of E5/16K interactions; it is possible that the transformation-competent E5 mutants that cannot activate PDGF-R might still bind 16K protein and interfere with V-ATPase activity.

**Substitution of Alanine along One Side of the E5 Helix Interferes with Activation of the PDGF-R**—Glutamine 17 in the E5 TM region has previously been identified as being critical not only for transforming activity but also for binding to cellular target proteins; it is essential for binding to the 16K V-ATPase subunit (15) and the PDGF-R (23, 29). It is also required for the induction of PDGF-R phosphorylation (15, 29, 31). When viewed in the context of the helical wheel plot in Fig. 4 and the helical net plot in Fig. 3, it is evident that this glutamine residue lies near the middle of the E5 helical face along which alanine substitutions perturb PDGF-R phosphorylation. Alanine substitutions along the opposite helical face have no effect on the ability of E5 to induce PDGF-R activation. The residues surrounding the glutamine residue are also critical for regulating PDGF-R binding and E5 dimerization.

**Preliminary Model for E5 TM Interactions**—Collation of our experimental results suggests a preliminary model for explaining the various activities of the mutant E5 proteins (Fig. 6). The model is based upon the hypothesis that the E5 TM domain forms homo- and hetero-oligomers via intermediates of increasing stability. Thus, when two E5 TM domains first come in contact, the preliminary complex does not have the lowest energy level possible. Only after the two TM domains realign and optimize binding interactions (including the formation of hydrophobic interactions, hydrogen bonding, charge interactions, and disulfide bonds) is a complex of low energy state achieved. It is also postulated that the accessibility of binding sites for 16K and PDGF-R in the TM domain varies with the structure of the various energy state intermediates. In the case of wt E5 interactions, therefore, the formation of a stable dimeric E5 complex would present a binding site for PDGF-R with appropriate structure to allow for receptor transphosphorylation. On the other hand, mutation of the helical domain involved in dimer formation would result in an alternate low energy state in which further interactions with E5 would be favored over those with PDGF-R, resulting in tetramer formation and the loss of appropriate conformation to facilitate PDGF-R transphosphorylation. Further dissection of these E5 alanine mutants will assist in defining a new mechanism for fibroblast transformation as well as provide a general model for specific intramembrane α-helical interactions.
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