



Identification of the transmembrane dimer interface of the bovine papillomavirus E5 protein

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We have developed a genetic method to determine the active orientation of dimeric transmembrane protein helices. The bovine papillomavirus E5 protein, a 44-amino acid homodimeric protein that appears to traverse membranes as a left-handed coiled-coil, transforms fibroblasts by binding and activating the platelet-derived growth factor (PDGF) β receptor. A heterologous dimerization domain was used to force E5 monomers to adopt all seven possible symmetric coiled-coil registries relative to one another within the dimer. Focus formation assays demonstrated that dimerization of the E5 protein is required for transformation and identified a single preferred orientation of the monomers. The essential glutamine residue at position 17 resided in the dimer interface in this active orientation. The active chimera formed complexes with the PDGF β receptor and induced receptor tyrosine phosphorylation. We also identified E5-like structures that underwent non-productive interactions with the receptor. *Oncogene* (2001) 20, 3824–3834.

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Introduction

Membrane-spanning domains of transmembrane proteins play essential roles in determining the orientation, oligomerization state, and activity of these proteins. In the case of receptor tyrosine kinases, the relative orientation of paired transmembrane domains appears to determine the activity of the linked cytoplasmic kinase domains (Burke and Stern, 1998; Bell *et al.*, 2000). Structural analysis of transmembrane domains has been complicated by technical difficulties inherent in studying these hydrophobic protein sequences. Therefore, to identify the active conformation of a small dimeric viral transforming protein, we have developed a genetic method to constrain dimeric transmembrane proteins to adopt specific orientations.

The E5 gene of bovine papillomavirus type 1 (BPV) induces morphologic and tumorigenic transformation of murine fibroblasts (DiMaio *et al.*, 2000). The E5 gene encodes a 44-amino acid, very hydrophobic, type II transmembrane protein that exists in cells as a disulfide-linked dimer of identical monomers localized primarily in the membranes of the Golgi apparatus and the endoplasmic reticulum (Burkhardt *et al.*, 1989; Schlegel *et al.*, 1986; Surti *et al.*, 1998). In transformed cells, the E5 protein forms a stable complex with the endogenous cellular platelet-derived growth factor (PDGF) β receptor, inducing ligand-independent receptor oligomerization, tyrosine phosphorylation and activation, which leads to cell transformation (Klein *et al.*, 1998; Lai *et al.*, 1998; Nilson and DiMaio, 1993; Petti *et al.*, 1991; Petti and DiMaio, 1992).

Because the E5 protein does not resemble PDGF, the natural ligand of the PDGF receptor, there is considerable interest in establishing the structural basis for receptor activation by the viral protein. Lysine 499 in the extracellular juxtamembrane region of the PDGF β receptor and threonine 513 in the transmembrane domain are required for complex formation with the E5 protein, E5-induced receptor activation, and cell transformation (Petti *et al.*, 1997). Saturation mutagenesis of the E5 protein at position 17 demonstrated that several polar residues, but no apolar residues, are able to mediate productive interaction with the PDGF receptor, suggesting that hydrogen bonding between glutamine 17 and threonine 513 on the receptor contributes to complex stability and receptor activation (Klein *et al.*, 1998; Meyer *et al.*, 1994; Sparkowski *et al.*, 1996). In addition, dimerization of the E5 protein was impaired by several apolar substitutions at position 17 (Klein *et al.*, 1998). The E5 protein also requires a negatively charged amino acid at position 33, implying the existence of a salt-bridge between aspartic acid 33 and lysine 499 of the receptor (Klein *et al.*, 1999; Meyer *et al.*, 1994). Finally, mutation of the E5 cysteines at positions 37 and 39 severely impairs cell transformation and PDGF β receptor binding and activation, implying that dimerization of the E5 protein is required for biological activity (Horwitz *et al.*, 1988; Meyer *et al.*, 1994; Nilson *et al.*, 1995).

Spectroscopic analysis of synthetic E5 dimers incorporated into lipid bilayers indicate that the E5 protein adopts a transmembrane α -helical conforma-

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tion (Surti *et al.*, 1998). Computational searches of potential helix–helix conformations predicted two low-energy structures of the E5 dimer, both of which were symmetric left-handed coiled-coils (Surti *et al.*, 1998). The preferred E5 structure (designated cluster 2 in Surti *et al.*, 1998) positions the critical glutamine and aspartic acid residues so that they are capable of the molecular interactions predicted by the genetic analysis. In this model, each E5 monomer contributes Ala₁₄, Gln₁₇, Leu₂₁, and Leu₂₄ to the interface of the E5 dimer (Figure 1). At this position, the glutamine can form hydrogen bonds across the helical dimer interface to the other E5 monomer, as well as with the transmembrane threonine of the PDGF β receptor. We further proposed that the structure of the active complex is an E5 dimer bound to two molecules of the PDGF β receptor, in which each receptor molecule contacts one E5 monomer via a threonine/glutamine interaction and contacts the other E5 monomer via a lysine/aspartic acid interaction.

A recent study involving scanning alanine mutagenesis of the E5 protein suggested an alternative model for E5 dimerization and receptor binding (Adduci and Schlegel, 1999). This study suggested that the E5 protein contains two distinct dimerization motifs (one comprised of Leu₇, Leu₁₈, Leu₂₅, the other of Val₁₃, Met₁₆, Leu₂₀), such that each E5 monomer contributes different residues to the helix–helix interface, and that a third face of the E5 protein (residues Gln₁₇, Leu₂₁, and Leu₂₄) comprises the binding site for the PDGF β receptor. In this model, E5 dimerization is required for receptor binding and activation, but within the complex each molecule of the PDGF β receptor binds a single E5 monomer.

To determine the orientation of the α -helices within the E5 dimer, we wished to test the activity of dimers in which the monomers were forced to adopt a variety of orientations relative to one another. Several approaches have been used to force transmembrane segments to dimerize in predictable orientations. Burke and Stern replaced consecutive juxtamembrane amino acids of p185^{Neu} with cysteine, thereby forcing the

formation of a series of dimers in which different dimer interfaces were specified by the positions of novel disulfide bonds (Burke and Stern, 1998). Similarly, Donoghue and colleagues (Bell *et al.*, 2000) varied the position of glutamic acid in the transmembrane domain of p185^{Neu} to vary the relative orientation of this domain in the dimer. We were concerned that novel disulfide bonds in the carboxyl-terminal segment of the E5 protein or substitutions in its transmembrane segment might perturb elements of the protein required for transformation. Therefore, we generated a series of chimeras in which a heterologous dimerization domain was appended to the non-essential amino terminus of the E5 protein, which is located in a different cellular compartment from the elements required for transformation. This domain forced the E5 segments to assume various orientations in the different chimeras, so that in aggregate all seven possible symmetric left-handed coiled-coil registries were generated. Analysis of these chimeras demonstrated that transformation requires dimerization of the E5 protein and identified a symmetric helix–helix dimer interface compatible with high transforming activity.

Results

Experimental design

To determine the relative orientation of the E5 monomers in the active dimeric structure, we constructed a series of fusion proteins in which the dimerization motif of Put3 was fused to the N-terminus of the E5 protein at seven consecutive residues (Figure 2a). Put3 is a *S. cerevisiae* transcription factor that contains a leucine zipper dimerization motif that forms a symmetric dimer consisting of a parallel left-handed coiled-coil (Swaminathan *et al.*, 1997; Walters *et al.*, 1997). Assuming that the chimeric Put3/E5 fusion proteins form continuous α -helices, for each chimera the two E5 monomers should adopt a different symmetric rotational orientation relative to one

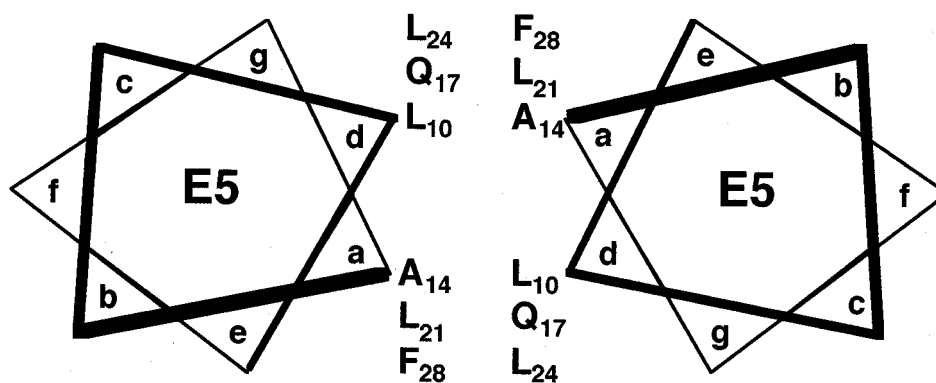


Figure 1 Diagram of dimeric E5 protein. This is a helical wheel diagram of the E5 protein as a dimeric left-handed coiled-coil in the orientation predicted for cluster 2 in Surti *et al.* (1998). Amino acids lining the dimer interface in the vicinity of glutamine 17 are shown

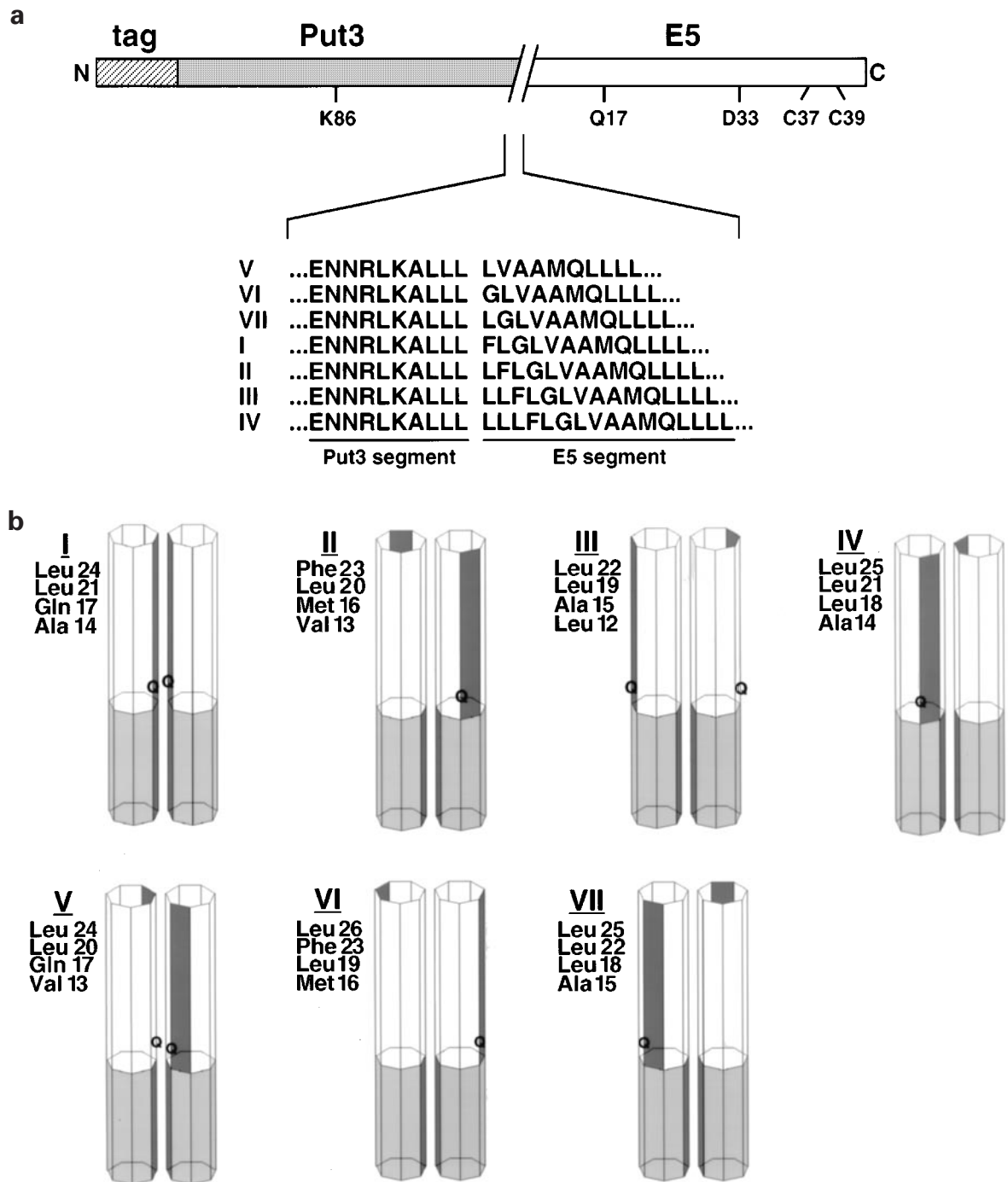


Figure 2 (a) Organization of the Put3/E5 fusion proteins. (Top) Tag indicates the amino terminal epitope tag used in some constructs, Put3 indicates the leucine zipper domain of Put3, and E5 indicates the E5 protein. The discontinuity represents the junction between the Put3 and E5 segments. Critical amino acids are numbered according to their positions in the native E5 or Put3 protein: K, lysine; Q, glutamine; D, aspartic acid; C, cysteine. (Bottom) Amino acid sequences of the chimeras at the point of fusion. (b) Predicted orientation of the monomers in the Put3/E5 dimers. The relative orientation of the monomers within the dimers is shown, based on the assumption that dimeric fusion proteins form continuous symmetric left-handed coiled-coils in the orientation specified by the Put3 domain. The heptagonal prisms represent α -helical monomers, with the N-terminus at the bottom. The Put3 segment is represented by the faces shaded in light gray, with the dimer interface specified by the leucine zipper shown in dark gray. The E5 segment is represented by the white faces, with the dimer interface of chimera I shown in dark gray. Because of the different points of fusion in the various chimeras, the relative positions of the amino acids of the E5 segment rotate relative to the fixed interface of the Put3 segment. These drawings do not illustrate the supercoiling of the paired helices. The amino acids lining the central portion of the interface of each chimera are also listed

another, so that in aggregate all seven possible orientations are generated. This assumption allowed us to predict the relative position of the E5 residues in the dimer, based on the point of fusion and the known structure of the Put3 coiled-coil. Schematic diagrams of the chimeras, including the positions of the critical glutamine residues, are shown in Figure 2b, as are the amino acids predicted to line each dimer interface. Each of the chimeras was cloned into a retrovirus vector and used to infect murine C127 fibroblasts, which express endogenous PDGF β receptor. Infected cells were subjected to quantitative focus formation assays and were selected for a co-transduced hygromycin resistance gene to generate stable cell lines for biochemical analysis.

Analysis of the Put3/E5 chimeras

We first determined the transforming activity of the seven Put3/E5 chimeras. Retroviruses encoding the wild-type E5 protein and each of the AU1-tagged chimeras were used to infect cells, which were then incubated at confluence to select foci. To correct for differences in viral titer, the number of foci generated by each chimera was normalized to the number of drug-resistant colonies that formed. As shown in Figure 3, only two of the seven Put3/E5 chimeras displayed measurable focus forming activity. AU1-Put3/E5-I showed the highest activity, approximately three times the activity of the non-chimeric E5 protein. AU1-Put3/E5-V displayed less than 25% of the activity of AU1-Put3/E5-I, and the other chimeras were essentially inactive. Similar results were obtained for the untagged versions of the chimeras (data not shown). The two active chimeras are the only two in which the transmembrane glutamine is predicted to

reside in the dimer interface (Figure 2b). Strikingly, the predicted interface of Put/E5-I, the most active chimera, is identical to the interface of the favored structure revealed by the computational modeling (Surti *et al.*, 1998).

Stable cell lines generated with each of the retroviruses were analysed to test whether the tagged chimeras were expressed and whether both the Put3 segment and the E5 segment were intact. Cell extracts were immunoprecipitated with the E5 antibody which recognizes the extreme C-terminus of the chimeras, and the immunoprecipitates were analysed by SDS-polyacrylamide gel electrophoresis under reducing conditions and Western blotting with an antibody directed against the AU1 epitope at the extreme N-terminus. As shown in Figure 4a, top panel, all chimeras except AU1-Put3/E5-VII were intact and expressed at high levels (in other cell lines, chimera III was abundantly expressed (data not shown)). Thus, the transforming activity of chimeras I and V was not due to the removal of the Put3 domain. Chimera VII was reproducibly present at lower levels (although the untagged versions of all the chimeras were abundantly expressed (data not shown)). We focused on AU1-tagged chimeras for the remainder of this study, because these results indicated that all but chimera VII were intact and expressed at a similar level.

We next examined the oligomeric state of the chimeras. Following immunoprecipitation with an antibody directed against the E5 protein, electrophoresis was carried out under non-reducing conditions so that disulfide bonds remained intact, and immunoblotting was performed with the AU1 antibody. As shown in Figure 4a, bottom panel, the chimeras migrated at the position predicted for dimeric molecules, except for AU1-Put3/E5-VI which formed primarily monomers.

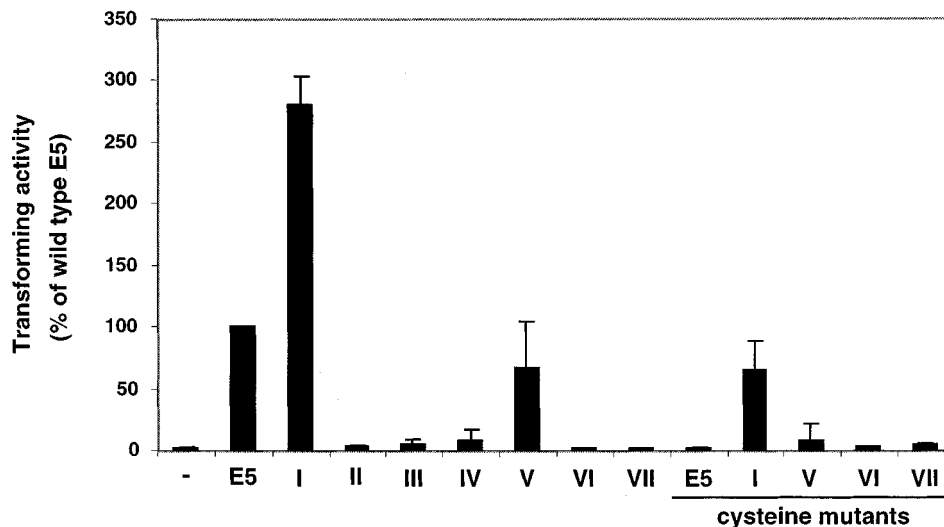


Figure 3 Transforming activity of Put3/E5 fusion proteins. Normalized focus forming activity, expressed as a percentage of the activity of the non-chimeric E5 protein, is shown for the empty vector (–), the non-chimeric E5 protein (E5), and each of the AU1-tagged chimeras with or without the cysteines, as indicated. The non-chimeric E5 protein typically induced approximately 15 foci/1000 drug resistant colonies

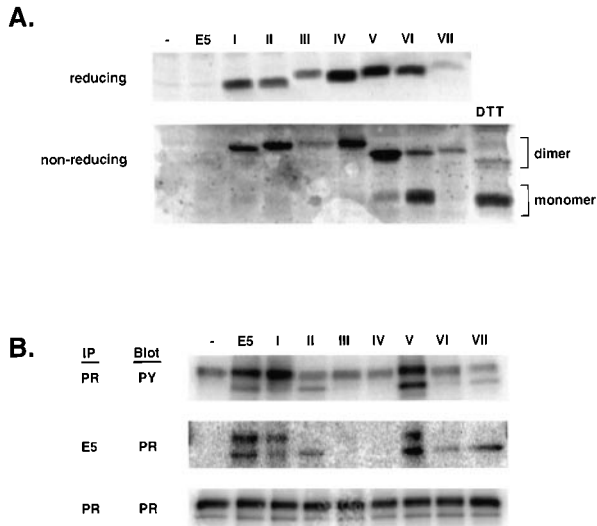


Figure 4 (a) Expression of the Put3/E5 chimeric proteins. Extracts of cells expressing the control viruses or the AU1-tagged chimeras (indicated by the Roman numerals) were immunoprecipitated with anti-E5 antibody, electrophoresed in the presence (top panel) or absence (bottom panel) of reducing agent, transferred to membranes, and probed with anti-AU1 antibody. Lane labeled DTT contains immunoprecipitated chimera I treated with dithiothreitol to demonstrate the mobility of monomer. The position of monomeric and dimeric E5 proteins are indicated at the right of the bottom panel. (b) Biochemical analysis of cells expressing Put3/E5 proteins. Cell extracts were immunoprecipitated (IP) with anti-PDGFR β receptor (PR) or anti-E5 antibody as indicated, and probed with anti-phosphotyrosine antibody to detect tyrosine phosphorylated PDGF receptor (top panel) or with anti-PR antibody to detect PDGF receptor associated with the E5 protein (middle panel) or total PDGF receptor (bottom panel)

Analysis of Put3/E5 chimeras lacking the cysteines

The Put3 segment of the chimeras is predicted to drive dimerization of the fusion proteins in the orientations shown in Figure 2b, but the E5 segment is predicted to drive dimerization in the orientation of the native E5 protein. Transforming activity is presumably highest when the Put3 orientation and the E5 orientation are in alignment, and lower when these two orientations differ. To simplify the analysis of a subset of the chimeras, both of the C-terminal cysteines were mutated to serine, which is structurally similar to cysteine. This mutation permitted a more meaningful prediction of the dimer interface of the E5 segment in the chimeras, because it eliminated the tendency of the cysteines to drive dimerization in the wild-type E5 orientation.

When the cysteines were removed, AU1-Put3/E5-I-CS retained significant transforming activity, approximately 80% that of the wild-type E5 protein (Figure 3). In contrast, insertion of the cysteine mutations into the non-chimeric E5 protein totally abolished focus-forming activity. The transforming activity of Put3/E5-I-CS was reduced compared to its counterpart containing the cysteines, perhaps because the absence of the cysteines introduced subtle structural perturbations

into the dimeric structure or because the cysteines made a minor contribution to transformation unrelated to their role in dimerization. The transforming activity of chimera V was severely impaired by removal of the cysteines. Indeed, in the majority of experiments, AU1-Put3/E5-V-CS failed to induce foci. Chimeras VI and VII remained transformation-defective following mutation of the cysteines. Thus, when the E5 cysteines no longer influenced dimerization, chimera I was the only one with significant transforming activity.

As shown in Figure 5a, top panel, each of the cysteine double mutant chimeras was expressed and intact. Because AU1-Put3/E5-I-CS cannot form disulfide bonds, we used co-immunoprecipitation to examine dimerization of this fusion protein. A gene encoding Put3/E5-I-CS containing an N-terminal influenza virus hemagglutinin (HA) epitope tag rather than the AU1 tag was constructed in a retroviral vector encoding G418 resistance. HA-Put3/E5-I-CS induced foci in C127 cells with similar efficiency as the non-chimeric E5 protein (Figure 5b). Cells expressing AU1-Put3/E5-I-CS were super-infected with the retrovirus expressing HA-Put3/E5-I-CS, and pooled stable cell lines co-expressing both chimeras were selected with G418. Co-expression of the HA-tagged and AU1-tagged chimeric proteins was verified by immunoprecipitating cell extracts with an antibody directed against the C-terminus of the E5 protein, followed by immunoblotting with an antibody directed against either the HA tag or the AU1 tag (Figure 5b). To assay for the presence of AU1/HA heterodimers in cells co-expressing these chimeras, cell extracts were immunoprecipitated with the anti-AU1 antibody and immunoblotted with the anti-HA antibody. As shown in Figure 5b (bottom panel), the AU1 antibody immunoprecipitated HA-Put3/E5-I-CS from cells co-expressing the two chimeras (fifth lane from the left), but not from cells expressing only the HA-tagged version (third lane from the left). Thus, Put3/E5-I-CS formed oligomers, presumably Put3-mediated dimers, even though this chimera is unable to form disulfide bonds.

To determine if Put3-mediated dimerization of chimera I was required for transformation, we inserted a proline in place of a lysine in the dimer interface of the Put3 segment to generate HA-Put3/E5-I-CS-KP. This mutation, which is predicted to disrupt dimerization of the Put3 coiled-coil because proline introduces kinks into α -helices (Williams and Deber, 1991), abolished transformation by HA-Put3/E5-I-CS-KP (Figure 5b). We analysed cells co-expressing AU1-Put3/E5-I-CS and HA-Put3/E5-I-CS-KP to assay the effect of the proline mutation on dimer formation. C127 cells expressing AU1-Put3/E5-I-CS were super-infected with retrovirus encoding HA-Put3/E5-I-CS-KP, and stable cell lines co-expressing the two chimeras were selected. Each cell line expressed readily detectable levels of the appropriate chimeric proteins, although the KP mutants were expressed at somewhat lower levels than their counterparts with a wild-type Put3 domain (Figure 5b). The co-immunoprecipitation

experiment in Figure 5b (bottom panel, lane on the far right) showed that, as expected, the proline mutation in the Put3 domain disrupted heterodimer formation.

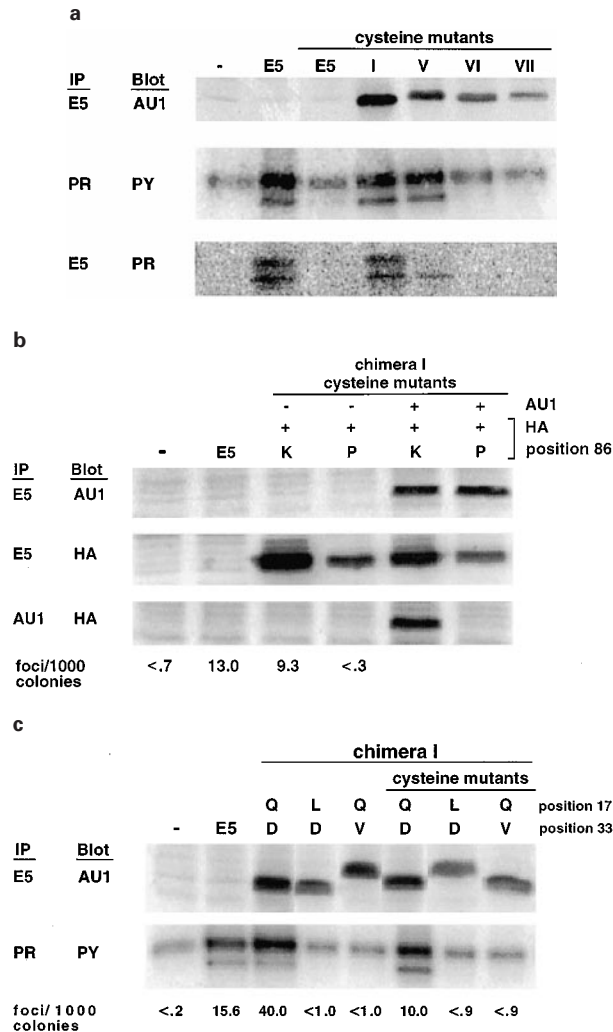


Figure 5 (a) Biochemical analysis of cysteine-mutants. Cell extracts were immunoprecipitated and blotted with the indicated antibodies. In the proteins designated ‘cysteine mutants’, the two C-terminal cysteine residues were mutated to serine. (b) Expression and dimerization of chimeric proteins with an intact or disrupted Put3 domain. Cell extracts were immunoprecipitated with the indicated antibody, and probed with anti-AU1 or anti-HA antibody to assay for expression of chimeric proteins containing the appropriate epitope tag, or probed with anti-HA to assay complex formation between the differentially tagged chimeras (bottom panel). The four right-most lanes contain chimera I proteins with both C-terminal cysteines mutated to serine. Cells expressing (+) or not expressing (-) an AU1-tagged or HA-tagged chimera are indicated. The HA-tagged chimeras contained either wild-type lysine (K) or proline (P) at position 86. Normalized focus forming activity of the proteins is shown at the bottom. (c) Analysis of glutamine and aspartic acid chimera I mutants. Cell extracts were immunoprecipitated and probed with the indicated antibodies. Control samples and samples from cells expressing chimera I with either the wild-type cysteines or the serine mutations were analysed. The letters above each lane indicate the amino acid at position 33 or position 17: Q (glutamine), D (aspartic acid), L (leucine), V (valine). Normalized focus forming activity of the proteins is shown at the bottom

Therefore, in the absence of the cysteine residues, Put3-mediated dimerization of the Put3/E5-I-CS fusion protein was required for cell transformation.

PDGF β receptor binding and activation by the Put3/E5 chimeras

To determine which of the chimeras induced tyrosine phosphorylation of the PDGF β receptor, the receptor was immunoprecipitated from cell extracts and immunoblotted with a monoclonal antibody that recognizes phosphotyrosine (Figure 4b, top panel). The transformation-competent chimeras AU1-Put3/E5-I and -V, as well as the non-chimeric E5 protein, induced high-level tyrosine phosphorylation of the mature form of the PDGF β receptor and of an immature form of the receptor with incompletely processed carbohydrates. In contrast, the non-transforming constructs AU1-Put3/E5-II and -VII induced tyrosine phosphorylation of the immature receptor form only. The other chimeras did not induce tyrosine phosphorylation of the PDGF β receptor. As shown in Figure 4b, bottom panel, each of these cell lines expressed equal levels of receptor.

To test whether the chimeric E5 proteins formed a stable complex with the PDGF β receptor, cell extracts were immunoprecipitated with an antibody directed against the E5 protein and immunoblotted with an antibody specific for the PDGF β receptor (Figure 4b, middle panel). The wild-type E5 protein and the transforming constructs AU1-Put3/E5-I and -V formed stable complexes with both the mature and immature forms of the PDGF β receptor. In contrast, the non-transforming constructs failed to associate with the receptor (AU1-Put3/E5-III and -IV) or associated with the immature form of the receptor only (AU1-Put3/E5-II, -VI and -VII). Cells expressing the untagged chimeras displayed a similar pattern of PDGF receptor binding and tyrosine phosphorylation (data not shown).

We also examined cells expressing the chimeras lacking the cysteines. As shown in Figure 5a (middle panel), high-level receptor tyrosine phosphorylation was induced by the non-chimeric E5 protein and by AU1-Put3/E5-I-CS and AU1-Put3/E5-V-CS. In contrast, the transformation-defective chimeras AU1-Put3/E5-VI-CS and AU1-Put3/E5-VII-CS failed to induce tyrosine phosphorylation of the PDGF receptor over background levels. An equal amount of receptor was expressed in each of the cell lines (data not shown). Co-immunoprecipitation experiments demonstrated that the non-chimeric E5 protein and AU1-Put3/E5-I-CS formed comparable levels of complex with the mature and immature forms of the PDGF β receptor (Figure 5a, bottom panel). AU1-Put3/E5-V-CS formed a lower level of complex with the immature form of the receptor only, and AU1-Put3/E5-VI-CS and AU1-Put3/E5-VII-CS failed to form stable complexes. As expected, HA-Put3/E5-I-CS induced PDGF β receptor tyrosine phosphorylation, which was abolished by the proline mutation that disrupted dimerization (data not shown).

Analysis of Put3/E5-I chimeras containing mutations at position 17 or position 33

To determine if glutamine 17 and aspartic acid 33 were required for transformation by chimera I, the glutamine was replaced with leucine or the aspartic acid was replaced with valine in AU1-Put3/E5-I and AU1-Put3/E5-I-CS. In the native E5 protein, these mutations prevent PDGF β receptor binding, activation, and cell transformation. C127 cells were infected with retrovirus encoding each of these mutants and analysed for focus formation. Although these mutants were abundantly expressed in C127 cells, they were transformation-defective (Figure 5c). Thus, the transmembrane glutamine and juxtamembrane aspartic acid were required for the transforming activity of chimera I in the presence or absence of the cysteines. Biochemical analysis demonstrated that the mutations at the glutamine and aspartic acid in AU1-Put3/E5-I and AU1-Put3/E5-I-CS reduced tyrosine phosphorylation of the PDGF β receptor to background levels (Figure 5c, bottom panel).

Models of the Put3/E5 dimers

Previous modeling studies suggested that the transmembrane helices of the E5 dimer form left-handed coiled-coils (Surti *et al.*, 1998). To assess whether the Put3/E5 fusion proteins were able to form continuous helices and pair in such a coiled-coil, models were constructed and subjected to energy minimization. Two coiled-coil proteins of known structure, tropomyosin and cortexillin, were used as templates for the construction of the models (Burkhard *et al.*, 2000; Whitby and Phillips, 2000). The positions of the main chain atoms were harmonically restrained to their starting positions, ensuring maintenance of the coiled-coil architecture during the molecular dynamics. In all cases, for a given Put3/E5 construct, the different starting models converged to essentially identical structures (rms differences in C α positions typically ≤ 1 Ångstrom). Chimeras I and V both gave well-packed dimer interfaces with favorable overall van der Waals energies and no close contacts (Figure 6a and data not shown). Strikingly, the structure of the E5 segment of the chimera I model was the same structure as the preferred possible packing model identified in the previous study of Surti *et al.* (1998). Many of the other constructs also gave reasonable dimer interfaces, which is perhaps not surprising, given the large number of leucines found in the transmembrane segment of the E5 protein. However, in all chimeras except I and V, the side chain of glutamine 17 faced into the interior of the lipid bilayer, in orientations where no hydrogen bonding partners could be identified; such orientations are expected to be energetically unfavorable for the polar glutamine side chain.

In chimeras I and V, the modeling suggested reasonable mechanisms by which the two glutamine 17 side chains could form hydrogen bonds connecting the two monomers across the dimer interface. In the

most favorable configuration identified for chimera I, the two glutamine side chains both adopt χ^1 and χ^2 values of approximately -60° and 180°C , respectively. This allows the formation of a hydrogen bond between the side chain nitrogen of glutamine from one monomer and the side chain carbonyl oxygen of glutamine from the other monomer. In addition, the glutamine side chain nitrogen from both monomers can form a hydrogen bond to the alanine 14 backbone carbonyl oxygen on the other monomer. A similar configuration for the glutamine side chains was proposed by Surti *et al.* (1998). This arrangement does not display strict twofold symmetry, because different atoms on each glutamine side chain participate in these hydrogen bonds. For chimera V, it is possible for each glutamine 17 side chain to hydrogen bond to the backbone carbonyl of valine 13 on the opposite monomer. This requires the glutamine side chains from the two monomers to adopt different dihedral angles: for one side chain, $\chi^1 = -60^\circ\text{C}$ and $\chi^2 = 180^\circ\text{C}$, while for the other, $\chi^1 = +60^\circ\text{C}$ and $\chi^2 = 180^\circ\text{C}$. This configuration does not appear to allow the two glutamines to interact with each other. The models for both active chimeras suggested that one functional group of each glutamine side chain remains available to hydrogen bond to another protein, such as the PDGF β receptor.

Discussion

To identify the dimer interface of the biologically active E5 protein, we tested the transforming activity of seven fusion proteins, each of which was predicted to form a different symmetric dimer. Several results indicated that the Put3 domain drove dimerization of these proteins in specific orientations. First, the activity of different fusion proteins varied markedly, even though they differed only by the point of fusion to the E5 sequence. Only chimera I and chimera V displayed transforming activity, with tagged chimera I being more active than the native, non-chimeric E5 protein. Second, when disulfide bond formation was eliminated, chimera I retained significant transforming activity, whereas transformation by the non-chimeric E5 protein was abolished. Third, chimera I lacking the C-terminal E5 cysteines dimerized, and transformation by this chimera was abolished by a mutation that prevented Put3-mediated dimerization. Taken together, these results demonstrated that dimerization is required for transforming activity and that the Put3 domain in chimera I induced dimerization in the proper orientation. Moreover, the cysteines did not play an essential role in transformation other than driving homodimer formation, since they could be replaced by a heterologous dimerization motif.

The disulfide bonds, hydrogen bonds, and packing interactions involving the E5 transmembrane segment tended to drive dimerization of the fusion proteins in the native E5 orientation, and the leucine zipper provided by the Put3 domain drove dimerization in

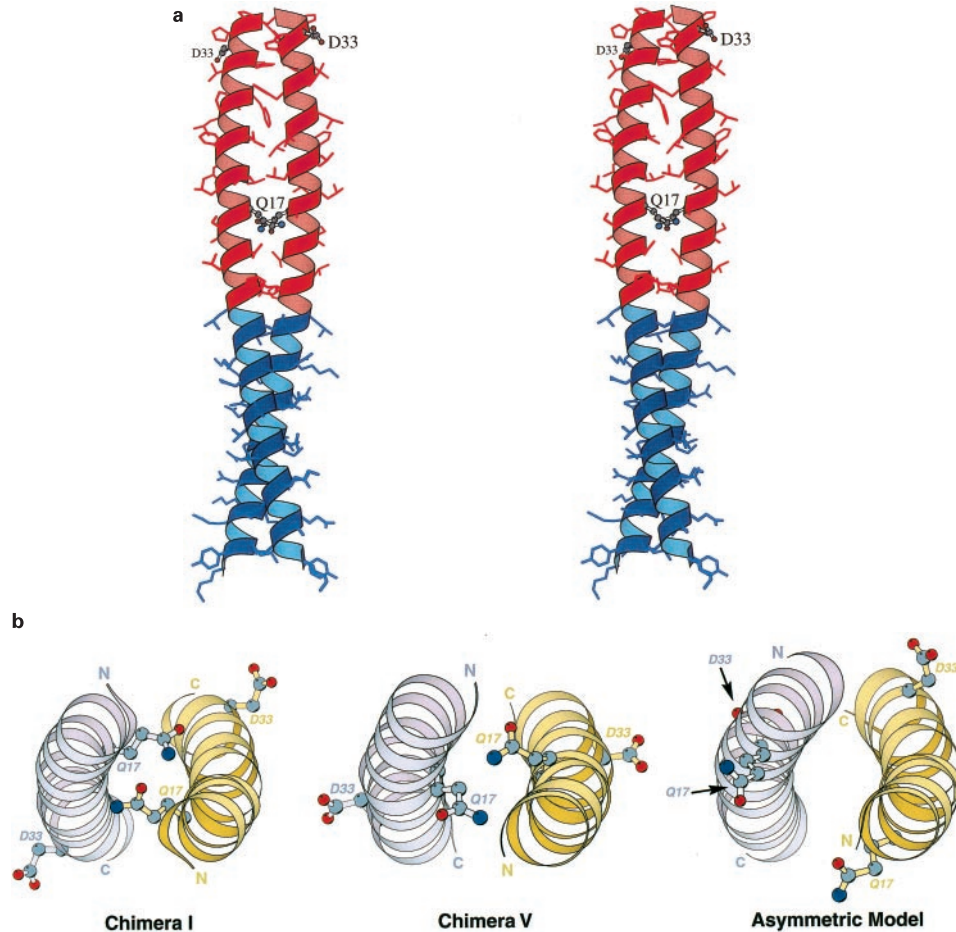


Figure 6 (a) Stereo view of chimera I. Divergent stereo view of the model of Put3/E5 chimera I. The portion of the chain derived from Put3 is shown in blue, and the portion corresponding to the E5 sequence is shown in red. The amino terminus of both chains is at the bottom. The Gln-17 and Asp-33 side chains are drawn as ball-and-stick figures. The remainder of the side chains are drawn as stick representations. (b) Relationship of the two monomers within postulated dimers. The models for chimeras I and V were derived as described in the text. The asymmetric model was prepared assuming a canonical tropomyosin-like left-handed coiled-coil configuration for the backbone; the relative orientation of the two helices was obtained by placing residues 7, 18, and 25 from one monomer and residues 13, 16, and 20 from the other monomer into the dimer interface (Adduci and Schlegel, 1999). The helices are viewed approximately end-on. The N- and C-termini are labeled with the C-termini extending below the plane of the page, away from the reader. One monomer is colored gray, and the other gold. For the sake of clarity, only the side chains of Gln-17 and Asp-33 are shown. For chimeras I and V, the Gln-17 side chains lie in the dimer interface, while for the asymmetric model, both Gln-17 side chains lie on the same face of the dimer and the Asp-33 side chains lie on the opposite face of the dimer

the orientation specified by the point of fusion with the E5 protein. Because chimera I invariably displayed the highest activity in numerous transformation assays involving untagged, tagged, and cysteine mutant forms, we infer that the E5- and the Put3-mediated interfaces coincide in this chimera (Figure 7). In addition, chimera I was less severely impaired than was chimera V by elimination of the cysteines, a mutation predicted to favor the orientation specified by the Put3 segment. Based on the known structure of Put3, the residues lining the dimer interface in chimera I (and by inference lining the interface of the wild-type E5 dimer) are Leu₁₀, Ala₁₄, Gln₁₇, Leu₂₁, Leu₂₄, the same residues that were predicted to line the E5 dimer interface by our previous modeling and mutational analysis (Klein *et al.*, 1998, 1999; Surti *et al.*, 1998).

Notably, two substantially different modeling strategies, which began from different starting assumptions, led to essentially the same model. Surti *et al.* (1998) docked canonical helices as rigid bodies and made no assumptions about the arrangement of the helical backbones or the identity of the residues present in the dimer interface. The modeling carried out here assumed a specific coiled-coil backbone structure with the residues in the interface determined by the Put3 fusion partner. The structure predicted by Surti *et al.* (1998) to be most energetically favorable and most consistent with the mutational analysis is the same structure predicted for the most active chimera. Further support for this model is provided by recent solid-state NMR studies performed on synthetic E5 dimers in lipid bilayers, which demonstrated that the

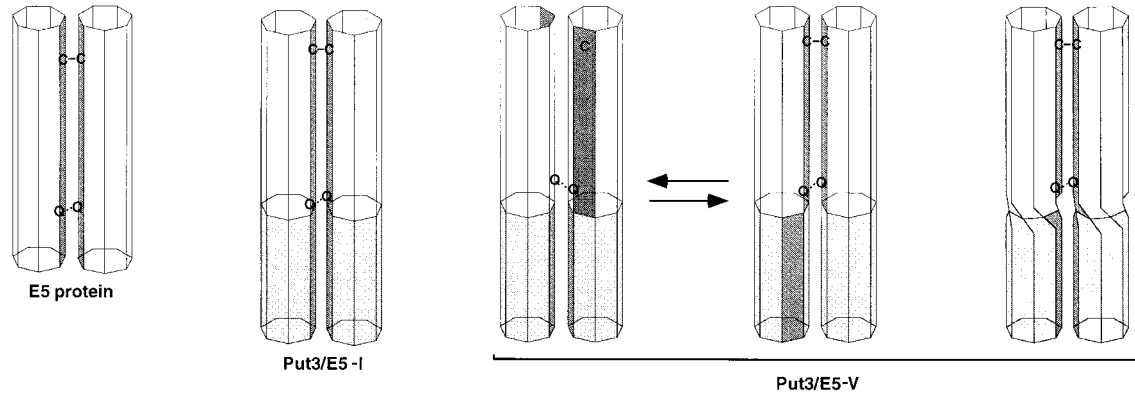


Figure 7 Diagram of the E5 protein and the active Put3/E5 chimeras. The diagrams use the same format as Figure 2b. The left dimer shows non-chimeric E5 protein and the second dimer shows chimera I. The three right-most dimers show potential active configurations of Put3/E5-V. The left-most chimera V dimer shows the orientation as specified by the Put3 segment, in which the amino acids that line the interface of the E5 segment in chimera I are rotated out of the dimer interface. This dimer may be in equilibrium with the middle chimera V dimer, in which the E5 segment retains the same interface as does chimera I, and the Put3 leucine zipper is rotated out of the dimer interface. The chimera V dimer on the right represents a structure in which the E5 segment adopts the native E5 interface and the Put3 segment adopts the interface specified by the leucine zipper. In this case, this drawing does not attempt to specify the precise nature of the distortion in the vicinity of the point of fusion. The disulfide bonds involving the cysteines are shown as solid lines, and the interhelical hydrogen bonds involving the glutamines are shown by the dotted lines. Because the organization of the disulfide bonds is not known, only a single cysteine per monomer is shown

glutamine side chains were in close proximity to each other and may form a hydrogen bond across the dimer interface, and further found that each glutamine formed a hydrogen bond across the interface to the peptide backbone of the other monomer at the position of alanine 14 (S Smith and D DiMaio, submitted). This hydrogen bonding pattern is identical to that predicted by our modeling studies of chimera I. In fact, only chimera I adopts the orientation in which both alanine 14 and glutamine 17 are in the dimer interface so that these interactions can take place. The inter-helical hydrogen bonds formed by each glutamine are expected to contribute to dimer stability, in agreement with our previous findings that dimer formation was reduced by residues at position 17 unable to form such hydrogen bonds (Klein *et al.*, 1998). This conclusion is also consistent with studies in other systems demonstrating that hydrophilic residues can drive the specific association of transmembrane helices via formation of interfacial hydrogen bonds (Zhou *et al.*, 2000; Choma *et al.*, 2000; Smith *et al.*, 1996).

In the other chimeras, the Put3- and E5-driven interfaces do not coincide, and with the exception of chimera V, these chimeras are severely impaired for transformation. It can be argued that chimeras I and V are the only two proteins likely to form dimers in the membrane, since they are the only ones predicted to remove the glutamine side chain from the energetically unfavorable interior of the lipid bilayer into the dimer interface. If chimera I adopts the orientation of the non-chimeric E5 protein, why is chimera V active, albeit at a reduced level? Even though the Put3 domain is predicted to drive the E5 segments into different orientations in chimeras I and V (Figure 7), both orientations may lead to productive outcomes,

although the nature of the interaction may differ for the two chimeras. In fact, the molecular modeling suggests that glutamine 17 and aspartic acid 33 from different monomers are on the same face of the chimera I dimer, where they are in a position to contact a single PDGF β receptor molecule (Figure 6b). In contrast, for the chimera V dimer, glutamine 17 and aspartic acid 33 from the same monomer are on a single face of the dimer (Figure 6b). However, the finding that removal of the cysteines from chimera V eliminated transforming activity suggested alternative models. For example, chimera V dimers with the Put3-driven interface may be in equilibrium with those with a different interface driven by the E5 segment (Figure 7), and only those molecules exhibiting the E5-mediated interface are active. Alternatively, both the Put3 dimer interface and the native E5 dimer interface may be formed simultaneously by chimera V, with the different registries of the two sections of the fusion protein accommodated by distortion of the helices and/or looping out of residues (Figure 7). These events may occur with chimera V, and not with the transformation-defective chimeras, because the Put3-mediated orientation of chimera V is stabilized by interhelical hydrogen bonds and is only one rotational position away from the preferred orientation of chimera I.

We also explored the interaction between the E5 protein and the PDGF β receptor. The transformation competent chimeras formed stable complexes with the PDGF β receptor and induced tyrosine phosphorylation of the receptor, and transformation by these chimeras was blocked by mutations at the glutamine and aspartic acid that interfered with receptor activation. In addition, morphologic transformation induced by these chimeras was reversed by treatment

with a kinase inhibitor specific for the PDGF receptor (data not shown), indicating the PDGF β receptor activation is required for transformation. However, the interaction between the E5 protein and the PDGF receptor is complex. Chimeras II and VII induced PDGF β receptor phosphorylation, but failed to transform C127 cells, a phenotype similar to that displayed by some C-terminal E5 point mutants (Nilson *et al.*, 1995). These fusion proteins may form Put3-mediated dimers with structures that bind the receptor in an aberrant fashion, which may cause tyrosine phosphorylation of the receptor at inappropriate sites or with inappropriate stoichiometry, so that the mitogenic signaling cascade is not initiated. Thus, simple measures of E5/PDGF receptor complex formation or E5-induced receptor tyrosine phosphorylation may not give an accurate measure of the ability of E5 proteins to activate the receptor.

The results described here do not support an asymmetric model that two different faces of the E5 helix mediate dimer formation, leaving a third face available for PDGF β receptor binding (Adduci and Schlegel, 1999). Neither face proposed by Adduci and Schlegel (1999) to be involved in E5 dimer formation coincides with the interfaces generated by chimeras I or V, and the face they postulate forms the PDGF receptor binding site, Gln₁₇, Leu₂₁, and Leu₂₄, is the face identified here as the E5 dimer interface. The model proposed by Adduci and Schlegel (1999) is based on the disruption of E5 activities by alanine substitutions. However, it is possible that point mutations outside of the dimer interface affect the oligomeric state of mutant E5 proteins, and that mutations in the interface compromise binding to the PDGF receptor. For example, mutation of glutamine 17 is expected to impair PDGF β receptor binding due to the loss of both the interhelical hydrogen bonds and the hydrogen bond to the receptor. Mutation of the dimer interface leucines to alanine, a residue with a significantly smaller functional group than leucine, might alter the assembly or packing of the E5 dimer and the position of the residues that contact the PDGF β receptor, thus indirectly affecting complex formation. Alternatively, our molecular modeling suggests that leucine 21 and leucine 24 are not completely buried in the interface, so they may be able to participate in packing interactions with the PDGF β receptor. Finally, it is not clear how the asymmetric model addresses the role for aspartic acid 33 in receptor binding, since this residue is predicted to fall on the opposite face of the coiled-coil dimer from the postulated receptor binding site (Figure 6b, asymmetric model).

In summary, we have described a genetic method that allowed us to identify the amino acids comprising the transmembrane dimer interface of the BPV E5 protein. A similar approach was used by Cochran and Kim (1996) to generate three different dimeric orientations of a soluble version of the *E. coli* aspartate receptor. Variations of these methods may be useful for determining the relative orientation of paired α -helical protein segments in other systems. In addition, chimera

I may be a suitable substrate for structural analysis such as X-ray crystallography.

Materials and methods

Plasmid construction

The Put3/E5 chimeras were generated by using overlap extension PCR and Pfu Polymerase (Invitrogen) (Horton *et al.*, 1989). In one reaction using the Put3 template (gift of Dr Ronen Marmorstein), a 75 bp segment of DNA corresponding to residues 73–97 of the dimerization domain of Put3 was amplified. The 5' outside primer annealed to the 5' end of the Put3 cDNA and contained a *NcoI* site, a start codon, and codons encoding an Asn-Pro helix-capping sequence immediately N-terminal to residue 73 (Aurora and Rose, 1998). Each 3' primer corresponded to the 3' end of the Put3 cDNA and the first nucleotides of the appropriate E5 segment. All seven possible segments of the E5 gene, corresponding to starting residues 6–12 and extending through the C-terminus of the protein, were amplified in separate reactions using the E5 template. The 5' primers corresponded to the last nucleotides of the Put3 segment and the first nucleotides of the E5 segment. The 3' outside primer corresponded to the 3' end of the E5 gene and contained a *XbaI* site. In a third PCR reaction, equal amounts of unpurified product from each pair of reactions described above were amplified with the outside primers. During the course of mixing, denaturing, and reannealing, the DNA strands having complementary sequences at the Put3/E5 junction act as primer-templates for each other. DNA fragments from the final PCR reaction were purified by agarose gel electrophoresis, digested with *NcoI* and *XbaI*, and subcloned into the pProEX-HTb vector.

Standard PCR-based procedures were used to subclone the chimeric E5 genes into the retroviral vector pRVY (encoding hygromycin resistance) or pLXSN (encoding G418 resistance) (Klein *et al.*, 1998). The AU1 and HA epitope tags were added by performing PCR using 5' primers encoding the appropriate tag. The cysteine double mutants (designated CS), the proline disruption mutant (designated KP), and the glutamine and aspartic acid mutants (designated QL and DV, respectively) were generated using QuikChange (Stratagene) site-directed mutagenesis. The DNA sequence of the entire chimeric Put3/E5 gene was confirmed for each mutant. Details of the mutagenesis and subcloning procedures are available from the authors on request.

Cell lines and tissue culture

The retroviral packaging cell lines and C127 fibroblasts were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics (DME-10). Retroviral stocks were obtained as described previously (Klein *et al.*, 1998). To assay mutants for focus-forming activity, C127 cells were infected with various retroviruses and foci were counted three weeks after infection as described previously. Additional cells from each infection were selected in medium containing 300 μ g of hygromycin B per ml (or 1 mg G418 per ml for the HA-tagged chimeras). Cell lines were established from pools of >100 drug resistant colonies. To calculate focus-forming efficiencies, the number of foci that formed were normalized for the number of hygromycin B or G418 resistant colonies that arose in parallel in the same infection. The results shown in Figure 3 are the averages of between two and six independent infections for each mutant.

Biochemical analysis

Cell lines grown to near confluence were incubated in the absence of serum for 24 h. Cell extracts were prepared in modified RIPA buffer containing protease and phosphatase inhibitors as described previously (Drummond-Barbosa *et al.*, 1995). Immunoprecipitation and immunoblotting were carried out essentially as described (Drummond-Barbosa *et al.*, 1995; Klein *et al.*, 1998); details are available from the authors on request.

For anti-AU1 immunoprecipitations, 1 μ l of α -AU1 antibody (BAbCO, Inc.) was added to 1000 μ g of extracted protein. For anti-AU1 and anti-HA (Santa Cruz, Inc.) immunoblotting, samples were transferred electrophoretically in transfer buffer lacking SDS and the resulting membranes were processed as for anti-E5 immunoblotting (Klein *et al.*, 1998). A 1:1000 dilution of the anti-AU1 antibody or a 1:200 dilution of the anti-HA antibody was used to probe the membranes.

Put3/E5 model building

Models of constructs I and V were built using Swiss-PdbViewer (<http://www.expasy.ch>). Tropomyosin (PDB ID: 1C1G), a canonical left-handed coiled-coil, was used as a template upon which the models were built. Tropomyosin lysine residues 48 and 332 are located at the same register position as the starting lysine in the Put3 sequence. Using the mutate function in Swiss-Pdb Viewer, the amino acids of tropomyosin, starting with residues 48 and 332 on the respective chains, were changed to the amino acids

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corresponding to the sequences of the different Put3/E5 chimeras. Side-chain positions were not adjusted prior to energy minimization.

To determine if the final structure was sensitive to the starting position on the tropomyosin backbone, a model of each construct was built starting at residues 56 and 339. This frameshift was chosen in order to keep the correct registry assignment for each amino acid. Additionally, to determine the effect of using a different starting template, models were built using cortexillin I (PDB ID: 1D7M) as the base molecule. Cortexillin lysine 288, which is in register with the first amino acid of the Put3 segment, was used as the starting position on which to build the models.

All models were then subjected to simulated annealing/molecular dynamics, using CNS v. 1.0 (Brunger *et al.*, 1998). Cartesian molecular dynamics was performed, using a constant annealing schedule of 1000 steps at 0.0005 ps time steps and a starting temperature of 2000 K. The dynamics were repeated using dielectric constants of 1, 2 and 78. Figure 6 was generated using the program MOLSCRIPT (Kraulis, 1991).

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