

# Specific Locations of Hydrophilic Amino Acids in Constructed Transmembrane Ligands of the Platelet-Derived Growth Factor $\beta$ Receptor

Lisa L. Freeman-Cook<sup>1</sup>, Anne P. B. Edwards<sup>1</sup>, Ann M. Dixon<sup>2</sup>  
Kristin E. Yates<sup>1</sup>, Lara Ely<sup>1</sup>, Donald M. Engelman<sup>2</sup> and Daniel DiMaio<sup>1\*</sup>

<sup>1</sup>Department of Genetics  
Yale University School of  
Medicine, New Haven, CT  
06510, USA

<sup>2</sup>Department of Molecular  
Biophysics & Biochemistry  
Yale University, New Haven  
CT 06510, USA

The 44 amino acid E5 transmembrane protein is the primary oncogene product of bovine papillomavirus. Homodimers of the E5 protein activate the cellular PDGF  $\beta$  receptor tyrosine kinase by binding to its transmembrane domain and inducing receptor dimerization, resulting in cellular transformation. To investigate the role of transmembrane hydrophilic amino acids in receptor activation, we constructed a library of dimeric small transmembrane proteins in which 16 transmembrane amino acids of the E5 protein were replaced with random, predominantly hydrophobic amino acids. A low level of hydrophilic amino acids was encoded at each of the randomized positions, including position 17, which is an essential glutamine in the wild-type E5 protein. Library proteins that induced transformation in mouse C127 cells stably bound and activated the PDGF  $\beta$  receptor. Strikingly, 35% of the transforming clones had a hydrophilic amino acid at position 17, highlighting the importance of this position in activation of the PDGF  $\beta$  receptor. Hydrophilic amino acids in other transforming proteins were found adjacent to position 17 or at position 14 or 21, which are in the E5 homodimer interface. Approximately 22% of the transforming proteins lacked hydrophilic amino acids. The hydrophilic amino acids in the transforming clones appear to be important for driving homodimerization, binding to the PDGF  $\beta$  receptor, or both. Interestingly, several of the library proteins bound and activated PDGF  $\beta$  receptor transmembrane mutants that were not activated by the wild-type E5 protein. These experiments identified transmembrane proteins that activate the PDGF  $\beta$  receptor and revealed the importance of hydrophilic amino acids at specific positions in the transmembrane sequence. Our identification of transformation-competent transmembrane proteins with altered specificity suggests that this approach may allow the creation and identification of transmembrane proteins that modulate the activity of a variety of receptor tyrosine kinases.

© 2004 Elsevier Ltd. All rights reserved.

**Keywords:** cell transformation; oncogene; bovine papillomavirus E5 protein; receptor tyrosine kinase

\*Corresponding author

## Introduction

Receptor tyrosine kinases are typically activated by ligands that bind to the extracellular domain of the receptor. Ligand binding induces receptor

dimerization, transphosphorylation, and activation of downstream signaling cascades. Some viral proteins that do not resemble the natural ligands have adopted an unusual mechanism to activate receptor tyrosine kinases.<sup>1</sup> For example, the gp55 protein of the Friend murine leukemia virus binds to the transmembrane domain of the erythropoietin receptor, and the E5 protein of bovine papillomavirus binds to the transmembrane domain of the platelet-derived growth factor (PDGF)  $\beta$  receptor to induce ligand-independent receptor dimerization

Abbreviations used: PDGF, platelet-derived growth factor.

E-mail address of the corresponding author:  
[daniel.dimaio@yale.edu](mailto:daniel.dimaio@yale.edu)

and activation.<sup>1</sup> Thus, transmembrane proteins can influence receptor activity *in trans*.

The homodimeric 44 amino acid E5 protein is the primary oncogene of BPV, and it induces transformation of rodent fibroblasts in tissue culture.<sup>2,3</sup> Disulfide bonds in the C terminus of the protein stabilize E5 homodimer formation, which is required for transformation.<sup>3-7</sup> A glutamine residue at position 17 in the transmembrane domain is also important for homodimerization, presumably by forming an interhelical hydrogen bond between the two E5 monomers.<sup>5,8,9</sup> The transforming activity of the E5 protein is primarily mediated by its interaction with the cellular PDGF  $\beta$  receptor tyrosine kinase.<sup>10-13</sup> E5 homodimers stably bind to the transmembrane domain of two PDGF  $\beta$  receptor molecules to induce receptor dimerization, transphosphorylation of both receptor molecules in the dimeric complex, constitutive association with cellular SH2 domain-containing signaling proteins, and subsequent activation of downstream signaling cascades.<sup>8,10,13-20</sup>

Specific E5 sequences upstream of position 14 and downstream of position 33 are not required for transformation,<sup>2,5</sup> indicating that the ability of the E5 protein to activate the PDGF  $\beta$  receptor and transform cells is mediated largely by its hydrophobic transmembrane segment. We proposed that the E5 homodimer is a symmetrical left-handed coiled-coil with a homodimer interface containing Leu14, Gln17, Leu21, Leu24, and Phe28, and that the glutamine residue at position 17 forms a hydrogen bond with a threonine residue at position 513 of the PDGF  $\beta$  receptor transmembrane domain.<sup>6,8,9,21</sup> We also proposed the existence of a juxtamembrane salt bridge between an aspartic acid at position 33 of the E5 protein and a lysine at position 499 of the PDGF  $\beta$  receptor.<sup>5,7,21,22</sup> Mutational analysis suggests that there are additional transmembrane contacts between the E5 protein and the PDGF  $\beta$  receptor<sup>15,23-26</sup> (L.E., D. Mattoon & D.D., unpublished results).

To test the hypothesis that it is possible to design and select small transmembrane proteins that bind the transmembrane domain of a cellular receptor tyrosine kinase and induce its activation, we used the E5 protein as a dimeric scaffold to construct a library of novel small transmembrane proteins in which the glutamine at position 17 was retained and 15 transmembrane amino acids were replaced with random hydrophobic sequences.<sup>25</sup> Approximately 10% of the random library clones bound and activated the PDGF  $\beta$  receptor, resulting in cellular transformation, even though more than one-third of the E5 protein had been randomized. A consensus sequence distinct from the wild-type E5 protein was identified in these experiments and conferred transforming activity to a non-transforming poly-leucine transmembrane sequence.<sup>25</sup> Thus, diverse transmembrane sequence motifs can induce PDGF  $\beta$  receptor activation.

Although the vast majority of amino acids in protein transmembrane domains are hydrophobic,

these domains also contain hydrophilic amino acids. Indeed, the transmembrane domain of approximately 25% of single-span transmembrane proteins contains at least one strongly polar residue, which can contribute to the proper assembly of protein complexes.<sup>27-29</sup> Furthermore, insertion of hydrophilic residues into model peptides or native proteins can drive the dimerization of otherwise monomeric hydrophobic domains by forming interhelical hydrogen bonds across the dimer interface.<sup>30-36</sup> However, the rules that govern the ability of hydrophilic amino acids to participate in specific interhelical interactions are complex and poorly understood, and hydrophilic amino acids have the potential to induce non-physiologic interactions because they can drive strong helix associations in the absence of specific packing.<sup>27,30-33</sup>

In this study, we examined the role of transmembrane hydrophilic residues in PDGF  $\beta$  receptor binding and activation. The initial library of transmembrane proteins was designed to maintain the essential glutamine residue at position 17.<sup>25</sup> Here, we constructed a library that did not maintain glutamine 17 but instead included hydrophilic residues at a low level at each of 16 randomized transmembrane positions. We then selected and sequenced library proteins that induced cellular transformation. Our results highlighted the importance of a hydrophilic residue at position 17 and a limited number of other positions. Furthermore, some of the mutants demonstrated different specificity than the wild-type E5 protein. These results provide insights into the basis for specific recognition between transmembrane helices and suggest that constructed small transmembrane proteins have the potential to bind to a variety of cellular transmembrane proteins.

## Results

### Experimental strategy

In order to investigate the role of hydrophilic amino acids in transmembrane proteins that bind and activate the PDGF  $\beta$  receptor, we designed a library in which 16 transmembrane amino acids of the dimeric 44 amino acid E5 protein were replaced with random, predominantly hydrophobic amino acids. The carboxyl-terminal cysteine residues that mediate E5 homodimerization were retained in the invariant portion of the library proteins. Degenerate oligonucleotides were synthesized to encode a random mix of Leu, Val, Ile, Met, Phe, and a lower level of 11 other amino acids. The library was designed so that each transmembrane domain contained a small number of hydrophilic residues that were predicted to occur with equal frequency at each of the 16 randomized positions, including position 17. The randomized oligonucleotides were converted to double-stranded form, amplified using PCR, and used to replace the transmembrane domain of the E5 gene in a retroviral vector.

Murine C127 fibroblasts, which express endogenous PDGF  $\beta$  receptor, were infected at low multiplicity with the library and incubated at confluence to select for focus formation. Approximately  $3 \times 10^5$  viruses were screened for transforming activity, which represent a small fraction of possible sequences given the design of the library. Individual transformed foci were picked and expanded, and the retrovirus was rescued from the cells by using a helper virus to supply viral replication functions. The rescued virus was then used to infect naïve C127 cells to confirm transforming activity. The retroviral insert was amplified from genomic DNA isolated from these transformed cells, and the insert was subcloned, sequenced, and reintroduced into naïve C127 cells to confirm focus formation activity and to generate stable cell lines expressing the transmembrane proteins. Because each cell contains a single provirus and hence expresses a single exogenous protein, homodimers but not heterodimers of the transmembrane proteins can form.

### Sequence analysis of the library clones

Approximately 1% of the library clones induced foci in C127 cells. We recovered and sequenced 31 clones with transforming activity. We also sequenced 30 unselected clones that do not transform cells. Figure 1 lists the transmembrane sequence of the 30 non-transforming and 31 transforming library clones along with the relative

focus forming activity of the transforming clones. The hydrophilic residues in each clone are shaded. The unselected library clones appeared to have a random distribution of the encoded amino acids. Most of the amino acids were present at approximately their expected frequency, but Val and Glu were over-represented, and Phe and Tyr were under-represented, apparently due to an unintended bias toward G in the first and third codon positions in the randomized segment.

There were three striking differences between the transforming and non-transforming clones. First, the number of hydrophilic residues per clone was significantly greater in the non-transforming clones ( $p = 1 \times 10^{-6}$ ). The transforming clones had at most one hydrophilic residue, whereas the non-transforming clones had an average of 2.3 and as many as five hydrophilic residues. It is possible that some of the non-transforming proteins with multiple hydrophilic amino acids were unstable or did not properly insert into the membrane. Second, the non-transforming clones contained eight of the nine expected hydrophilic residues, whereas more than 90% of the hydrophilic amino acids in the transforming clones were glutamine and glutamic acid, and the remainder were aspartic acid. Finally, the distribution of the hydrophilic residues along the length of the randomized segment was dramatically different in the two sets of clones. The hydrophilic residues appeared with essentially equal frequency at each position of the non-transforming clones ( $p = 0.97$ ), whereas there was a

### Non-transforming Clones

NT1 LVFVMVIVVMMMLLL  
 NT2 DMMVLV**E**KMLIVVVLV  
 NT3 V**F**E**V**ELLMV**H**V**I**NVMV  
 NT4 L**V**E**V**VLVLV**K**VIVVVV  
 NT5 LVVIVVMV**E**MLLI**E**VV  
 NT6 V**N**VVMVL**Q**LVV**M**EMI  
 NT7 VLLVVLVMMVLLLMF  
 NT8 V**D**VVVVVVLVLLILL  
 NT9 VVLLLLLLVVLV**K**LN**M**  
 NT10 VFIMVLFV**E**VLM**E**VV  
 NT11 D**L**VLFVVMALVLM**E**M  
 NT12 IFVLFIMV**F**VVL**Q**VVM  
 NT13 MVLV**Q**ELMV**D**MVLM  
 NT14 VVMLVLLLVIL**V**IVM  
 NT15 **Q**LVLVV**D**LFLLLVLLM  
 NT16 V**V**E**L**VMVVVLLLLLL  
 NT17 MVVMVM**E**ILVLLMA  
 NT18 LVVVLV**V**D**V**KV**N**MDVM  
 NT19 VLVMMVMVMM**K**VE  
 NT20 LLLV**K**YVVLFILMLM  
 NT21 V**V**H**K**VVMVL**K**MLIVV  
 NT22 VVML**T**MVL**H**MV**K**MLIV  
 NT23 VVVVVVV**F**E**E**V**E**ILL  
 NT24 VVL**K**VVAMVLL**E**VL  
 NT25 VVVL**E**FIV**K**KVL**D**V**D**V  
 NT26 MVL**D**LIVFVV**K**LVVLV  
 NT27 VMVV**E**VLALLL**M**N  
 NT28 V**N**M**F**M**N**MLLMV**E**EDV  
 NT29 VMLVLVVLV**M**KLVLV  
 NT30 VLVI**V**HLLVMLLVLLV

### Transforming Clones

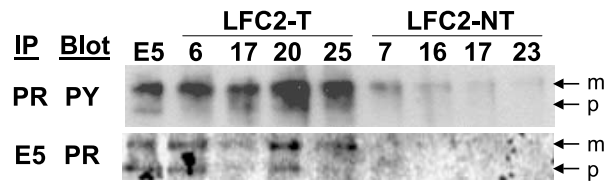
		relative activity
E5	AAM <b>Q</b> LLLLLFFLLFFL	+++
T1	LVV <b>Q</b> LVVLVVLIVLV	+++
T2	LLI <b>Q</b> LLVLMVLLLVL	+++
T3	AVV <b>Q</b> VVMLMVLMLFM	++
T4	MVM <b>Q</b> VVLLVMLLLM	++++
T5	VVM <b>Q</b> LLVLLIVLVLML	+++
T6	LLL <b>Q</b> LLMVLLVLLVLL	++++
T7	MLI <b>Q</b> LFIFLVLFVVV	+++
T8	LVI <b>Q</b> LFVLIIVVVLM	++++
T9	LVM <b>Q</b> LVVLVVVLMVM	++++
T10	LVV <b>Q</b> EMVLLLVLVVVV	++++
T11	LVV <b>Q</b> EMVFLMVIIVVV	++++
T12	LI <b>Q</b> ILVMLLVVLLFVL	++++
T13	VVLM <b>Q</b> MLVVVVMV	+++
T14	VLM <b>Q</b> MVVLVLLVLLV	++
T15	VVM <b>Q</b> LIIVVLLVVLV	++++
T16	VVVL <b>E</b> VVMIVMLVLLM	+++
T17	VIV <b>E</b> VLMVVFIVVF	+++
T18	VVV <b>E</b> VVLLIMVLLM	++++
T19	VLV <b>D</b> VLLIVMLLVV	++++
T20	MMLVLL <b>E</b> ILVVLV	+++
T21	VVLLIM <b>E</b> IVLLIMV	++++
T22	<b>Q</b> IVMVVMVLLVLLV	++++
T23	<b>E</b> LVMLLLLLIVLVMML	++++
T24	<b>E</b> VIMLVLLLFVVVLM	++++
T25	LVLM <b>I</b> ILLVVVLMV	++
T26	MVIMAVV <b>F</b> VLLVVV	++++
T27	LMVLMV <b>L</b> VVVMVLM	++++
T28	MLLVMI <b>V</b> VVMLIVMV	++++
T29	VVVVM <b>L</b> IVMLMVV	++++
T30	LVLV <b>L</b> ILLVMLVIVV	++++
T31	LFMVLL <b>L</b> MVVMIMMF	++

**Figure 1.** Transmembrane sequences of non-transforming (left) and transforming (right) library proteins with the hydrophilic residues highlighted. The far right column lists the relative focus-forming activity of the transforming proteins. ++++ corresponds to >130% of wild-type E5 activity, +++ to 70–130%, and ++ to 30–70% of wild-type E5 activity. The arrow indicates position 17.

strong positional bias of the hydrophilic amino acids in the transforming clones ( $p = < 10^{-8}$ ) (Figure 2). 26% of the transforming clones had a glutamine at position 17, and 35% had either glutamine, aspartic acid or glutamic acid at this position, indicating that there was a strong selection for a hydrophilic residue at position 17 in the active library proteins. The only other positions that contained hydrophilic amino acids in the transforming clones were either immediately adjacent to position 17 (primarily at position 18) or at positions corresponding to the E5 homodimer interface (at positions 14 and 21).<sup>6,9</sup> Seven of the transforming clones did not contain any hydrophilic amino acids, in contrast to only two non-transforming clones.

**The transforming proteins bind and activate the PDGF  $\beta$  receptor**

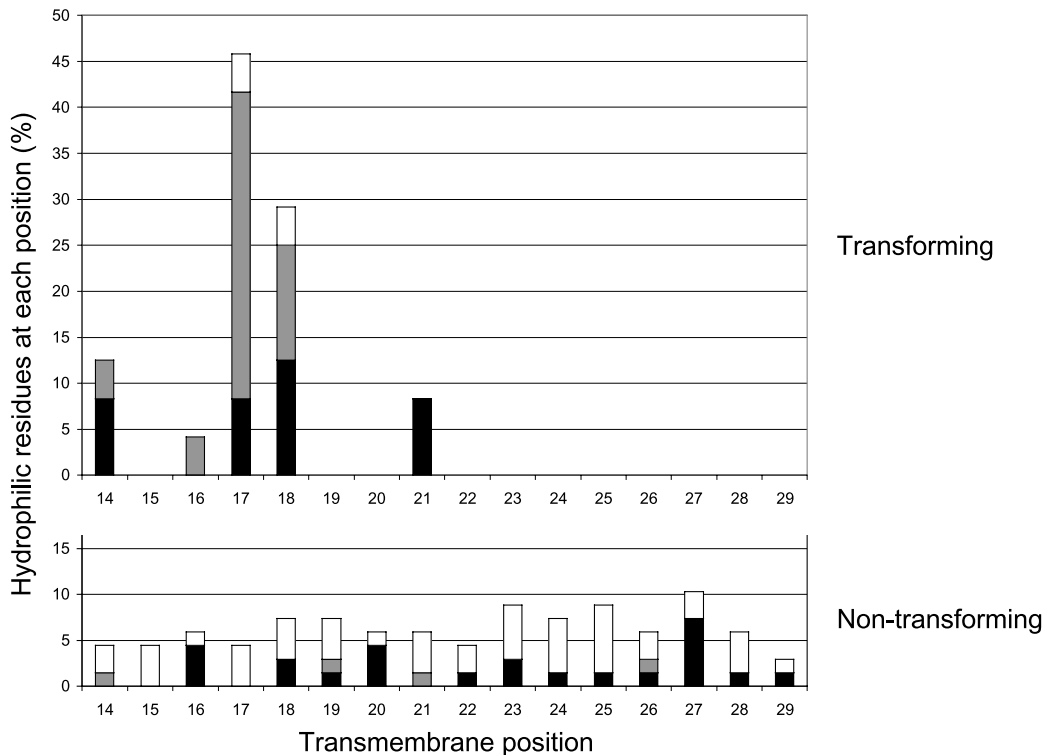
We determined whether the transforming library proteins bound and activated the endogenous PDGF  $\beta$  receptor. Stable C127 cell lines that expressed representative transforming or non-transforming library proteins were analyzed biochemically. All cell lines expressed similar levels of the PDGF  $\beta$  receptor (not shown). Protein extracts from these cells were immunoprecipitated with an antibody that recognizes the PDGF  $\beta$  receptor and then immunoblotted with a monoclonal antibody that recognizes phosphotyrosine (Figure 3, top



**Figure 3.** Biochemical analysis of the small transmembrane proteins. Cell extracts from C127 cells expressing the wild-type E5 protein (E5) or the indicated transforming (LFC2-T) or non-transforming (LFC2-NT) library proteins were immunoprecipitated and blotted with the indicated antibodies to detect receptor tyrosine phosphorylation (top) or association between the library proteins and the PDGF  $\beta$  receptor (bottom). The antibodies used for immunoprecipitation (IP) and Western blotting (blot) are: E5, E5 C-terminus; PR, PDGF  $\beta$  receptor; PY, phosphotyrosine. Bands corresponding to the mature (m) and precursor (p) forms of the receptor are indicated.

panel). The wild-type E5 protein and each of the transforming proteins tested (left) induced marked tyrosine phosphorylation of the PDGF  $\beta$  receptor. In contrast, the four non-transforming proteins tested (right) induced only weak phosphorylation of the receptor.

To determine if the transforming proteins physically interacted with the PDGF  $\beta$  receptor, cell extracts were immunoprecipitated with an



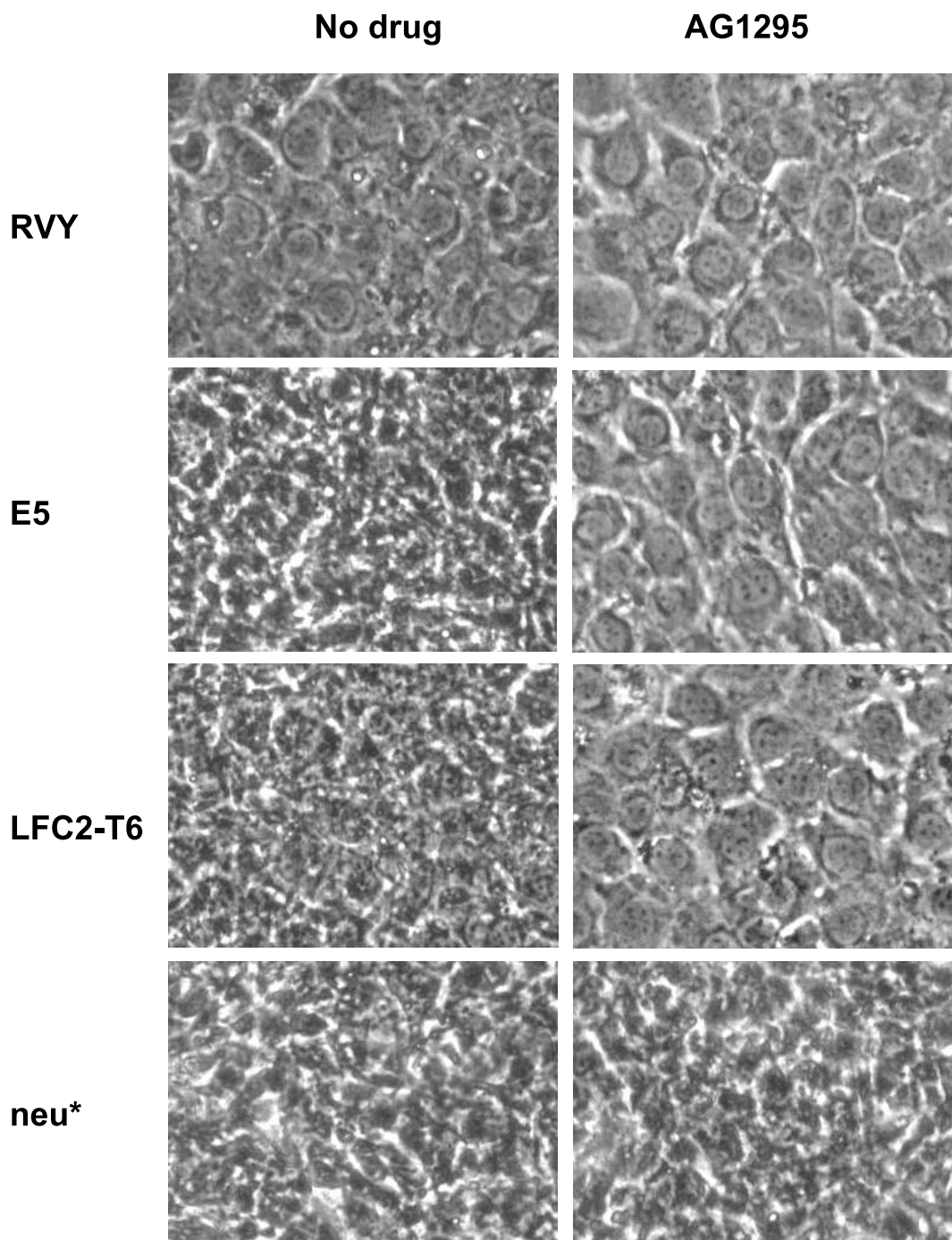
**Figure 2.** Distribution of hydrophilic residues in the transforming (top) and non-transforming (bottom) library clones. The height of the bars represents the percent of the total number of hydrophilic residues that are present at each position. The bars are subdivided to indicate the percent of glutamic acid (black), glutamine (gray) or other (white) hydrophilic amino acids at each position.

antibody that recognizes the C-terminal segment of the E5 protein which was not randomized in the library clones. The immunoprecipitates were then immunoblotted with the PDGF  $\beta$  receptor specific antibody (Figure 3, bottom panel). The wild-type E5 protein and to varying degrees each of the transforming proteins formed a stable complex with the mature and immature forms of the PDGF  $\beta$  receptor. In contrast, none of the four tested non-transforming proteins bound the receptor. These results demonstrated that the transforming but not

the non-transforming proteins stably bound and activated the PDGF  $\beta$  receptor.

#### The role of the PDGF $\beta$ receptor in transformation

To further examine the role of the PDGF  $\beta$  receptor in the transformed phenotype induced by the active library proteins, we used a specific inhibitor of PDGF receptor tyrosine kinase activity, AG1295 (Figure 4). Stable C127 cell lines expressing



**Figure 4.** Effect of the PDGF receptor kinase inhibitor AG1295. Photomicrographs of C127 cells stably expressing an RVY empty vector, the wild-type E5 protein, the LFC2-T6 transforming protein, or the *neu* oncogene are shown in the absence of AG1295 (left) and after four days of treatment with the drug (right).

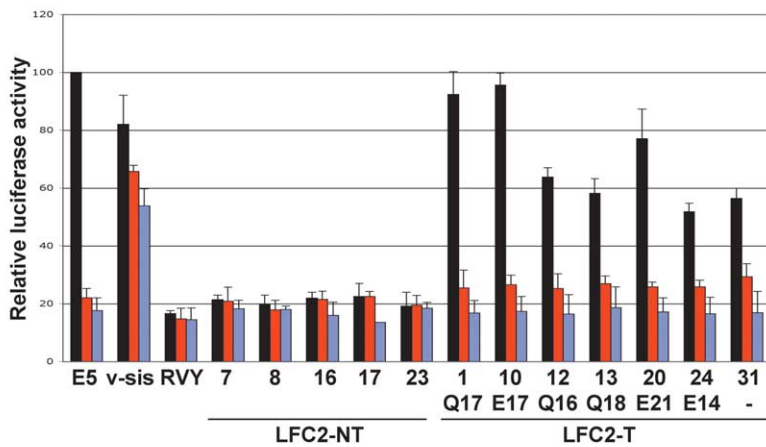
transforming or non-transforming library proteins were incubated in the presence of AG1295. In cells expressing the wild-type E5 protein and each of 13 transforming proteins tested, the cells continued to grow after reaching confluence and acquired a transformed morphology (LFC2-T6 is shown as a representative example). Notably, AG1295 reverted each of the transformed cell lines to a non-transformed morphology, indicating that continuous PDGF  $\beta$  receptor kinase activity was required for transformation. The drug had no effect on cell lines expressing the RVY empty vector (top panel) or non-transforming library proteins (not shown), and it did not revert the transformed morphology of cells transformed with an unrelated oncogene (*neu*, bottom panel).

Finally, we used a transient transfection assay to determine the ability of the library proteins to activate the PDGF  $\beta$  receptor. In this assay, four plasmids were co-transfected into CV1 cells, which do not express PDGF  $\beta$  receptor endogenously: a *Renilla* luciferase plasmid that serves as a transfection control; a reporter plasmid that expresses

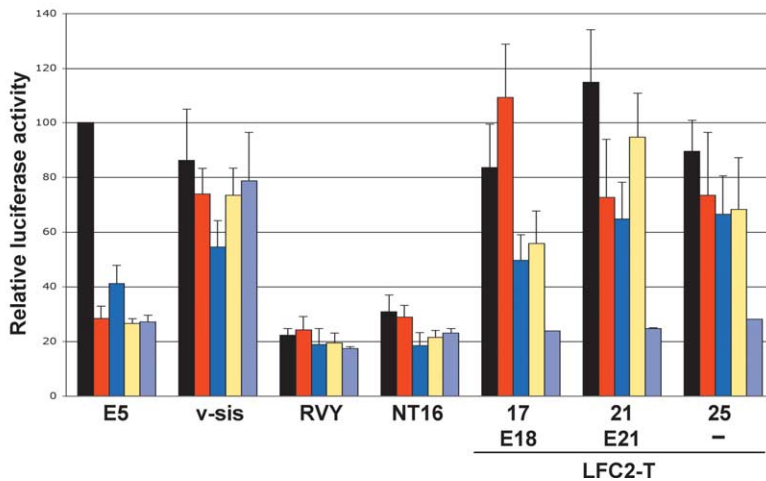
firefly luciferase under the control of a GAS STAT-responsive promoter that is activated by PDGF  $\beta$  receptor signaling; a plasmid expressing the wild-type or mutant PDGF  $\beta$  receptor; and a plasmid expressing the E5 protein, one of the library clones, or the *v-sis* PDGF homolog. Firefly luciferase expression was measured two days after transfection and was expressed as the percentage of the activity seen in response to co-expression of the wild-type PDGF  $\beta$  receptor and the wild-type E5 protein.

Figure 5(a) shows the normalized luciferase activity induced by the wild-type E5 protein (far left), *v-sis*, RVY empty vector, and representative transforming and non-transforming library proteins, when co-expressed with the wild-type PDGF  $\beta$  receptor (black) or two receptor mutants that are unable to interact with the wild-type E5 protein. One of the receptor mutants has a threonine to leucine mutation at position 513 (T513L, red),<sup>21</sup> and in the other mutant ( $\beta\alpha\beta$ , lavender) the transmembrane domain of the PDGF  $\beta$  receptor was replaced with the PDGF  $\alpha$  receptor

(a)



(b)



**Figure 5.** Transient reporter gene assay for signaling by the PDGF  $\beta$  receptor. The graphs show the relative firefly luciferase activity induced by the wild-type E5 protein (E5), a viral PDGF homologue (*v-sis*), an empty vector control (RVY), and representative transforming (LFC2-T) and non-transforming (LFC2-NT) library proteins co-expressed with the wild-type PDGF  $\beta$  receptor or PDGF  $\beta$  receptor mutants. The activity of each combination is expressed as a percentage of the activity induced by co-expression of the wild-type E5 protein and the wild-type PDGF  $\beta$  receptor. Three replicates of each transfection were performed for each experiment, and the average of at least three experiments is shown, with error bars representing the standard deviation. (a) The activity of five non-transforming proteins and seven transforming proteins that specifically activate the wild-type PDGF  $\beta$  receptor are shown. The proteins were co-expressed with the wild-type PDGF  $\beta$  receptor (black), the T513L mutant receptor (red), or the  $\beta\alpha\beta$  chimeric receptor (purple). Hydrophilic amino acids present in the transmembrane domain of the transforming proteins are indicated. (b) Relative activity of the controls listed above, one non-transforming protein (LFC2-NT16)

and three transforming proteins (LFC2-T) showing altered specificity are shown. The proteins were co-expressed with the wild-type PDGF  $\beta$  receptor (black), the  $\beta\alpha\beta$  chimeric receptor (lavender), and the T513L (red), L520A (blue) and L512V (yellow) mutant receptors.

transmembrane domain (L.E., D. Mattoon & D.D., unpublished results). As expected, the wild-type E5 protein activated the wild-type PDGF  $\beta$  receptor but neither of the mutant receptors, and the *v-sis* positive control activated all three receptors. Similar to the RVY empty vector, the five non-transforming library proteins tested did not activate any of the receptors. Each of the transforming proteins shown here activated the wild-type PDGF  $\beta$  receptor to varying degrees but did not activate either of the mutant receptors. The transforming library proteins included representative clones with either no hydrophilic residue or a hydrophilic residue at position 14, 16, 17, 18, or 21. These results indicate that transforming proteins with widely varying sequences all activated the PDGF  $\beta$  receptor and not a receptor mutant lacking a crucial amino acid in the transmembrane domain.

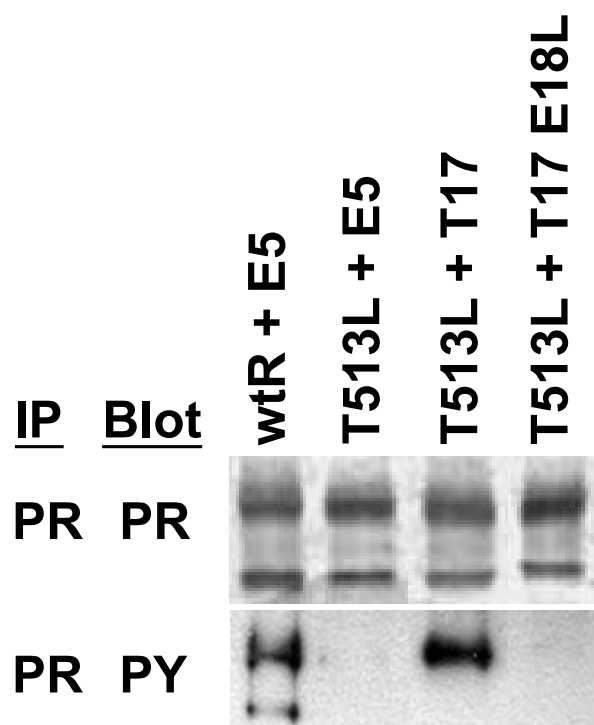
### Some transforming library proteins display altered specificity

We reasoned that some of the transforming library proteins that did not contain a glutamine at position 17 might no longer require a threonine at position 513 of the PDGF  $\beta$  receptor for interaction. We used the transient reporter assay described above to test this possibility. Plasmids expressing the wild-type E5 protein, *v-sis*, RVY, one non-transforming library protein (LFC2-NT16), and three transforming library proteins (LFC2-T17, 21, and 25) were transfected into CV1 cells along with the luciferase reporter construct. The cells were co-transfected with the wild-type PDGF  $\beta$  receptor (black) and four mutant receptors that are not activated by the wild-type E5 protein: Thr513 replaced with leucine (T513L, red),<sup>21</sup> leucine 520 replaced with alanine (L520A, blue),<sup>15</sup> leucine 512 replaced with valine (L512V, yellow), and the chimeric receptor containing the PDGF  $\alpha$  transmembrane domain ( $\beta\alpha\beta$ , lavender) (L.E., D. Mattoon, & D.D., unpublished results).

Figure 5(b) shows the normalized luciferase expression induced by these combinations of plasmids. As expected, the wild-type E5 protein activated only the wild-type receptor, the empty vector and the non-transforming library protein did not activate any of the receptors, and the *v-sis* control activated all of them. Unlike the wild-type E5 protein, LFC2-T17, which contains glutamic acid at position 18, strongly activated both the wild-type receptor and the T513L mutant receptor. LFC2-T17 also activated the L520A and L512V mutant receptors, albeit to a lesser extent than the wild-type receptor. LFC2-T21 and -T25 also demonstrated altered specificity, activating the wild-type receptor as well as the T513L, L520A, and L512V mutants. None of the library proteins activated the  $\beta\alpha\beta$  chimeric receptor. All of the transforming proteins were tested for their ability to activate the T513L mutant. In addition to the three mutants shown in Figure 5(b), LFC2-T14, -T15, -T16, -T18, -T19, -T22, and -T29 also activated the T513L

receptor to varying degrees (data not shown). Thus, six of the seven proteins with a hydrophilic residue at position 18 did not require Thr513 in the PDGF  $\beta$  receptor transmembrane domain, whereas all 11 proteins with a hydrophilic amino acid at position 17 required Thr513.

We used a stable gene transfer strategy to determine whether the LFC2-T17 transforming protein induced tyrosine phosphorylation of the T513L mutant receptor. Ba/F3 mouse hematopoietic cells do not endogenously express the PDGF  $\beta$  receptor. Cell extracts from Ba/F3 cells co-expressing the wild-type or T513L mutant receptor and the wild-type E5 protein, the LFC2-T17 transforming protein, or an LFC2-T17 mutant in which Glu18 was replaced with leucine were analyzed by immunoblotting for tyrosine phosphorylation of the PDGF  $\beta$  receptor. The wild-type E5 protein induced tyrosine phosphorylation of the wild-type receptor but not the T513L mutant receptor, as expected (Figure 6). Strikingly, the LFC2-T17 transforming protein induced robust phosphorylation of the T513L mutant receptor. The LFC2-T17 E18L mutant did not induce phosphorylation of the mutant receptor, indicating that Glu18 is required for this interaction. Taken together, these results demonstrated that we have identified small transmembrane proteins that



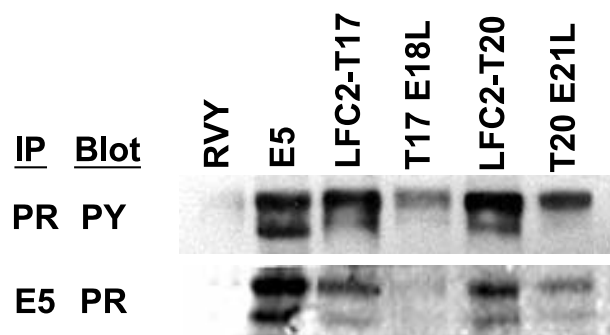
**Figure 6.** Biochemical analysis of a library protein with altered specificity. Cell extracts from Ba/F3 cells with the wild-type receptor (wtR) or the T513L mutant receptor co-expressed with wild-type E5 (E5), the LFC2-T17 transforming protein (T17), or the LFC2-T17 E18L mutant protein (T17 E18L) were immunoprecipitated and blotted to detect receptor expression (top) and receptor tyrosine phosphorylation (bottom).

cannot only bind and activate the PDGF  $\beta$  receptor, but that can also activate mutant receptors that the wild-type E5 protein does not recognize.

### Role of hydrophilic residues in the transforming library proteins

The wild-type E5 protein requires Gln17 for dimerization and receptor binding,<sup>8</sup> and many of the transforming proteins have a hydrophilic residue at position 17 that presumably plays a similar role. We wanted to determine if the hydrophilic residues at positions other than 17 were required for the transforming activity of the library proteins. The glutamic acid residues present at positions 18 and 21 in LFC2-T17 and -T20, respectively, were mutated to leucine. Although the parental LFC2-T17 and -T20 proteins transformed C127 cells efficiently, the LFC2-T17 E18L mutant had no focus-forming activity, and the LFC2-T20 E21L mutant had only 13% of wild-type E5 activity (data not shown). Tyrosine phosphorylation of the endogenous PDGF  $\beta$  receptor in stable C127 cell lines expressing these proteins is shown in Figure 7 (top panel). The wild-type E5 protein and the LFC2-T17 and -T20 proteins induced robust phosphorylation of the mature and immature forms of the PDGF  $\beta$  receptor. The LFC2-T17 E18L and the LFC2-T20 E21L mutants induced substantially less receptor tyrosine phosphorylation, consistent with the reduced ability of these mutants to transform C127 cells. As noted above, the LFC2-T17 E18L mutant also failed to induce phosphorylation of the T513L receptor mutant in Ba/F3 cells (Figure 6). Further, the LFC2-T17 E18L and LFC2-T20 E21L mutants displayed severely reduced ability to bind the PDGF  $\beta$  receptor as assessed by co-immunoprecipitation (Figure 7, bottom panel).

Finally, we analyzed the ability of the mutants to

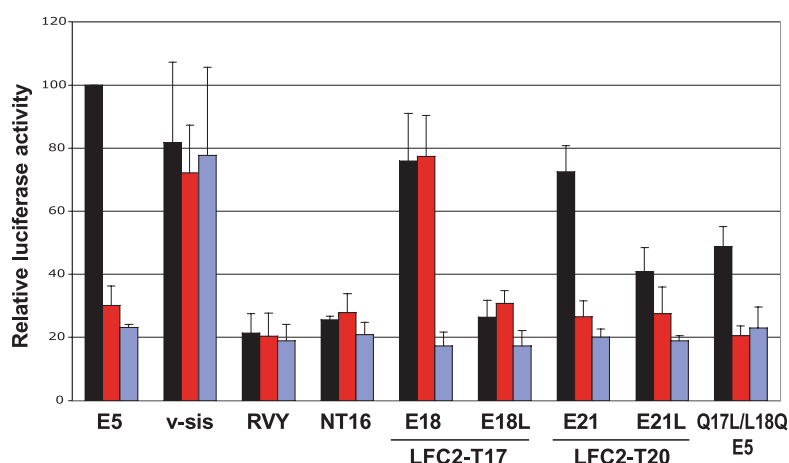


**Figure 7.** Biochemical analysis of mutant library proteins. Cell extracts from C127 cells with an empty vector control (RVY) or expressing the wild-type E5 protein (E5), the LFC2-T17 or -T20 transforming proteins, or the LFC2-T17 E18L or -T20 E21L mutant proteins were immunoprecipitated and blotted to detect receptor tyrosine phosphorylation (top) or association between the library proteins and the PDGF  $\beta$  receptor (bottom) as in Figure 3.

activate the PDGF  $\beta$  receptor in the transient luciferase assay (Figure 8). As noted above, *v-sis* activated the wild-type and the T513L and  $\beta\alpha\beta$  mutant receptors, and the RVY empty vector and the LFC2-NT16 non-transforming protein failed to activate any receptor. Similar to the wild-type E5 protein, both LFC2-T17 and -T20 activated the wild-type PDGF  $\beta$  receptor but not the  $\beta\alpha\beta$  mutant, and as described above, LFC2-T17 but not LFC2-T20 also activated the T513L mutant receptor. Transient signaling was completely abolished by the E18L mutation in LFC2-T17 and severely reduced by the E21L mutation in LFC2-T20. Thus, the hydrophilic residues present in these proteins are required for transforming activity and PDGF  $\beta$  receptor-mediated transient signaling.

### The library proteins are expressed and dimerize

Extracts from representative stable C127 cell lines were immunoprecipitated and immunoblotted with the E5 antibody to determine whether the transforming and non-transforming proteins were expressed (Figure 9(a)). The wild-type E5 protein and one of the three tested transforming proteins expressed readily detectable protein. Two of the three non-transforming proteins tested were also highly expressed, whereas one was not detectable. The fact that two of the transforming proteins, including LFC2-T17, showed little to no signal despite their robust transforming activity and clear interaction with the PDGF  $\beta$  receptor (see Figure 3) suggested that some of the proteins are difficult to detect by immunoblotting. To further examine the expression levels and dimerization state of the library proteins, C127 cells expressing the wild-type E5 protein, the LFC2-T17 protein, or the LFC2-T17 E18L mutant were metabolically labeled with [<sup>14</sup>C]leucine, and membrane fractions were immunoprecipitated with the E5 antibody. The immunoprecipitates were electrophoresed on polyacrylamide gels under both reducing and non-reducing conditions and detected using Phosphor-Imager analysis (Figure 9(b)). The wild-type E5 protein and the LFC2-T17 protein were both highly expressed, and the LFC2-T17 E18L mutant was expressed at a lower but readily detectable level. Electrophoresis under non-reducing conditions demonstrated that the LFC2-T17 protein homodimerized at least as well as the wild-type E5 protein. As described in the next section, molecular modeling of an E5 mutant containing a glutamine at position 18 suggested that Gln18 is in the dimer interface. Therefore, we tested whether removing Glu18 from LFC2-T17 affected dimerization. Approximately 50% of the LFC2-T17 E18L protein was monomeric under non-reducing conditions, suggesting that Glu18 in LFC2-T17 plays a role in mediating dimerization and that the transformation defect of LFC2-T17 E18L is at least partly due to a homodimerization defect.

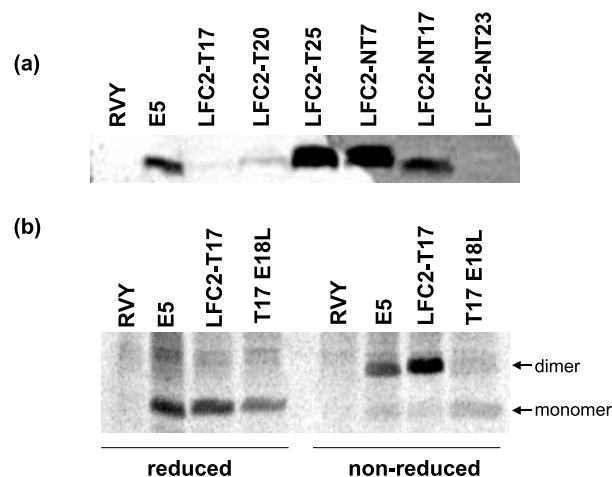


**Figure 8.** Transient reporter gene assay for transmembrane mutants. This Figure shows relative activity of the controls and non-transforming protein shown in Figure 5(b), the LFC2-T17 and -T20 transforming proteins, the LFC2-T17 E18L and -T20 E21L mutant proteins, and the Q17L/L18Q E5 mutant. The proteins were co-expressed with the wild-type PDGF  $\beta$  receptor (black), the T513L mutant receptor (red), or the  $\beta\alpha\beta$  chimeric receptor (lavender), and assayed as described in the legend to Figure 5.

### The wild-type E5 protein with a glutamine at position 18 transforms cells

Although most transforming library proteins had a hydrophilic amino acid in the wild-type E5 dimer interface, more than 20% of the active proteins had a hydrophilic residue at position 18, outside of the wild-type E5 dimer interface. To simplify the analysis of the role of hydrophilic amino acids that lie outside the wild-type dimer interface, we determined whether cells could be transformed by an otherwise wild-type E5 protein with a glutamine at a position other than 17. We constructed a series of double mutants in which the glutamine at

position 17 was replaced by a leucine, and a residue at another position was replaced by a glutamine. The single mutant lacking Gln17, Q17L, is transformation defective and does not undergo productive interaction with the PDGF  $\beta$  receptor.<sup>8</sup> The Q17L/L18Q E5 protein with a glutamine at position 18 had robust focus-forming activity in C127 cells, the Q17L/L21Q E5 protein had low but detectable transforming activity, and the other double mutants were transformation-defective (Figure 10(a)). The transformation-competent Q17L/L18Q E5 protein formed a stable complex with both the mature and immature forms of the PDGF  $\beta$  receptor in transformed cells and induced tyrosine phosphorylation of both receptor forms (Figure 10(b)). Furthermore, the transformed phenotype induced by this mutant was reverted by the AG1295 PDGF receptor kinase inhibitor (data not shown), demonstrating that PDGF  $\beta$  receptor kinase activity is required for the transforming activity of the Q17L/L18Q E5 protein. Finally, the Q17L/L18Q E5 protein induced signaling by the wild-type PDGF  $\beta$  receptor but not by the T513L or  $\beta\alpha\beta$  mutant receptors in the transient reporter assay (Figure 8, last series). Thus, in the context of otherwise wild-type E5 amino acids, a glutamine substitution at position 18 allowed productive interaction with the PDGF  $\beta$  receptor.

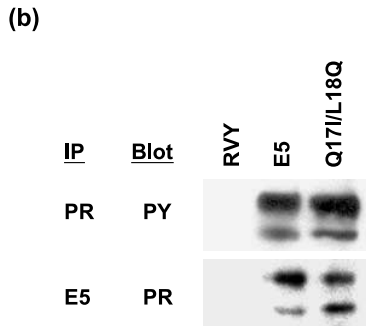


**Figure 9.** Expression of the library proteins. (a) Cell extracts from C127 cells containing the RVY empty vector or expressing the wild-type E5 protein or the indicated transforming or non-transforming library proteins were immunoprecipitated and blotted with the C-terminal E5 antibody to detect expression of the library proteins. (b) C127 cells containing the RVY empty vector or expressing the wild-type E5 protein, the LFC2-T17 transforming protein, or the LFC2-T17 E18L mutant protein were metabolically labeled with [<sup>14</sup>C]leucine, immunoprecipitated with the E5 antibody, and electrophoresed under reducing (left) or non-reducing (right) conditions. Arrows indicate the position of the monomer and dimer forms of the proteins.

We used the CNS searching of helix interactions (CHI) from the crystallographic and NMR system (CNS) to generate structural models of the wild-type E5 protein and the Q17L/L18Q E5 protein.<sup>37–39</sup> We chose to model the Q17L/L18Q E5 dimer because the position of the hydrophilic residue is the only change from the wild-type E5 protein, allowing us to directly compare the results with the well-defined wild-type model. The CHI simulation was performed on two parallel  $\alpha$ -helices containing the transmembrane sequence of the wild-type or Q17L/L18Q E5 proteins. Although the models shown in Figure 11 indicated that the transmembrane domains of both proteins formed left-handed coiled-coils, the homodimer interface was different in the two models. The wild-type E5 interface consisted of well-packed residues at positions 14,

(a)		focus formation	luciferase activity
E5	AAMQ <sup>1</sup> LLLLLFLFLFFL	+++	+++
Q17L	AAMLLLLLFLFLFFL	-	-
Q17L/L18Q	AAML <sup>1</sup> QLLFLFLFFL	++	++
Q17L/L21Q	AAMLLLLL <sup>1</sup> FLFLFFL	- <sup>1</sup>	+
Q17L/F23Q	AAMLLLLL <sup>1</sup> QLLFFL	-	-
Q17L/L24Q	AAMLLLLL <sup>1</sup> FLFFL	-	-
Q17L/L25Q	AAMLLLLL <sup>1</sup> FLFFL	-	-
Q17L/L29Q	AAMLLLLL <sup>1</sup> FLFFL <sup>1</sup>	-	-

<sup>1</sup>Although this protein has little to no focus forming activity, colonies of C127 cells expressing this protein have a transformed morphology



**Figure 10.** Analysis of mutant E5 proteins with glutamine residues at positions other than position 17. (a) Transmembrane sequence of E5 mutants. The glutamine residues are highlighted. The wild-type E5 sequence is shown at the top followed by the Q17L non-transforming E5 mutant. The focus formation column lists the relative focus-forming activity of the proteins, according to the scale in Figure 1. The luciferase activity column lists the relative luciferase activity induced by the proteins when co-expressed with the wild-type PDGF  $\beta$  receptor. (b) Biochemical analysis of the Q17L/L18Q E5 protein. Cell extracts from C127 cells containing the RVY empty vector or expressing the wild-type E5 protein or the Q17L/L18Q E5 mutant protein were immunoprecipitated and blotted to detect receptor tyrosine phosphorylation (top) or association between the library proteins and the PDGF  $\beta$  receptor (bottom) as in Figure 3.

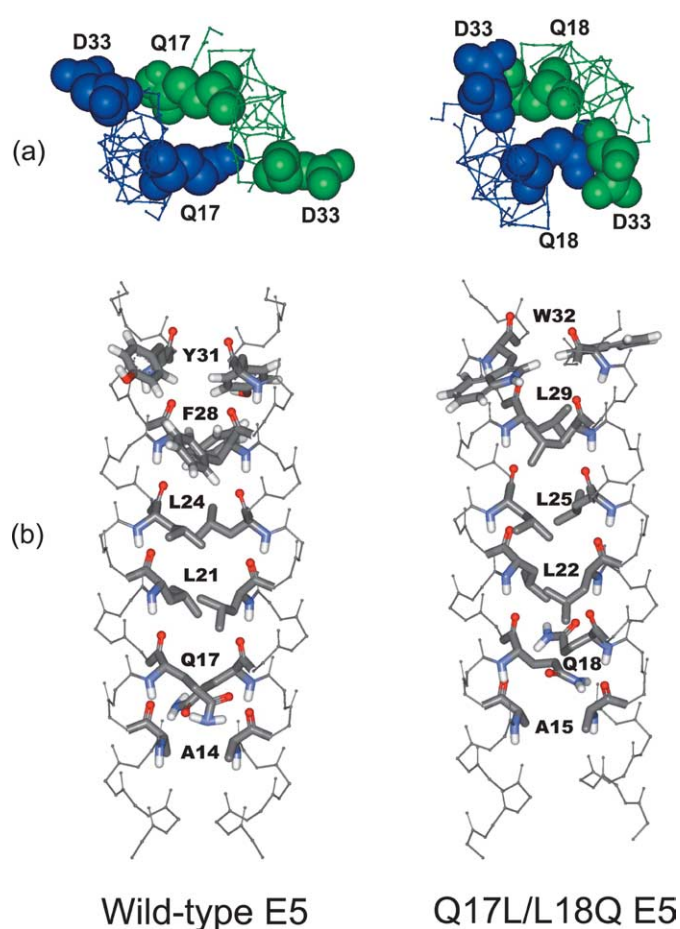
21, 24 and 28 as well as the glutamine at position 17, consistent with previously published results.<sup>9,25</sup> The homodimer interface of the Q17L/L18Q E5 protein was shifted one position and consisted of the glutamine at position 18 as well as residues at positions 15, 22, 25, and 29 (Figure 11(b)). Thus, the model of the Q17L/L18Q E5 protein had a novel homodimer interface that contained the hydrophilic glutamine at position 18. Further, the Q17L/L18Q E5 dimer model contained an interhelical hydrogen bond involving Gln18, analogous to the interhelical bond involving Gln17 of the wild-type E5 protein. Importantly, the side-chain of Asp33 was present on the exterior of the Q17L/L18Q E5 dimer model, where it may be accessible to interact with Lys499 of the PDGF  $\beta$  receptor (Figure 11(a)). As is the case for the wild-type E5 homodimer<sup>9</sup>, the glutamine of one monomer and the aspartic acid of the other monomer are on the same face of the homodimer. These results suggest that a hydrophilic residue located outside the canonical E5 homodimer inter-

face can induce the monomers to rotate to create a new interface that includes the hydrophilic residue and still allows binding and activation of the PDGF  $\beta$  receptor.

## Discussion

Here, we selected and characterized transformation-competent small transmembrane proteins from a library of proteins that contained a variety of hydrophilic amino acids at a low level at each of 16 randomized positions. This approach allowed us to determine not only what sequences were capable of activating the PDGF  $\beta$  receptor but also to examine the importance of the location of transmembrane hydrophilic residues. Although only approximately 1% of the library proteins induced transformation, transmembrane proteins with diverse sequences were transformation-competent. Despite their sequence diversity, all of the tested transforming proteins bound and activated the PDGF  $\beta$  receptor and required receptor activity for signaling and transformation. We note that there is not a perfect correlation between the relative focus-forming activity of the selected proteins and their activity in the transient assay. For example, LFC2-T24 was more active than the wild-type E5 protein in the focus formation assay but less active in the transient assay. The transient assay scores signaling by a single pathway, whereas focus formation is the result of multiple biochemical and cellular events. These results suggest that some of the transmembrane proteins differentially activate certain signaling pathways compared to the wild-type E5 protein.

Transmembrane hydrophilic residues play important roles in stabilizing some naturally occurring transmembrane protein complexes.<sup>27-29,40</sup> In addition, hydrophilic residues can induce dimer formation and are localized to the dimer interface when they are introduced into otherwise monomeric hydrophobic model peptides or naturally occurring transmembrane domains.<sup>30-36</sup> Furthermore, we have proposed that an essential hydrogen bond forms between Gln17 in the wild-type E5 protein and Thr513 in the transmembrane domain of the PDGF  $\beta$  receptor.<sup>5,8</sup> Therefore, we predicted that hydrophilic residues in the random transforming proteins would be present in the dimer interface or at a position where they can interact with the receptor threonine. There was indeed a strong selection for specific placement of the hydrophilic residues, with 35% of the transforming library proteins containing a hydrophilic residue at position 17. This result strongly supports the importance of an interaction between Gln17 of the wild-type E5 protein and Thr513 of the PDGF  $\beta$  receptor. In addition, in some clones lacking a hydrophilic amino acid at position 17, hydrophilic residues were present at position 14 or 21, which, like position 17, lie in the homodimer interface and



**Figure 11.** Structural models of transmembrane dimers. CHI structural models of protein dimers are shown for the wild-type E5 protein (left) and the Q17L/L18Q E5 mutant protein (right). (a) An end-on view of the two helical monomers with the Asp33 residues and the interfacial glutamine residues shown as space-filling models. The residues from one monomer are colored blue and from the other monomer are colored green. (b) A side view of the dimers with the residues predicted to lie in the interface of the protein dimers highlighted and labeled.

thus may be important for E5 homodimer formation.<sup>6,8,9</sup>

Several transforming proteins had hydrophilic amino acids at positions 16 or 18, which lie outside of the wild-type E5 coiled-coil homodimer interface. It was difficult to analyze the structures of these transforming proteins because they contain many amino acid differences relative to the wild-type E5 protein. Therefore, we constructed an E5 mutant protein that lacked Gln17 and instead contained a glutamine at position 18 in an otherwise wild-type E5 context. This Q17L/L18Q E5 protein was transformation-competent, and bound and activated the PDGF  $\beta$  receptor. Molecular modeling indicated that the Q17L/L18Q E5 monomers rotated relative to one another to generate a new homodimer interface containing position 18, and that the Asp33 residues were still exposed to the surface of the dimer in a position that may allow them to interact with Lys499 of the receptor. These modeling studies suggest that random transforming library proteins with hydrophilic amino acids at position 18 may also adopt novel homodimerization interfaces that include the hydrophilic amino acid. Consistent with this interpretation, removal of Glu18 from LFC2-T17 impaired homodimerization. The transforming library protein with a hydrophilic residue at position 16 may also rotate to form a new homodimerization interface. Thus, it is possible that

all of the transformation-competent library proteins contain a hydrophilic amino acid in the homodimer interface.

The hydrophilic residues in the transforming proteins may be important for receptor binding as well as homodimerization. For example, receptor activation by LFC2-T20, which contains a glutamic acid at position 21, and LFC2-T24, which contains a glutamic acid at position 14, required a threonine residue at position 513 in the transmembrane domain of the receptor. Packing interactions involving Thr513 may be important for the interaction between these transforming proteins and the PDGF  $\beta$  receptor. Alternatively, the glutamic acid side-chains at the interfacial positions 14 and 21 may be able to “reach” up or down the dimerization interface to form a hydrogen bond with Thr513 of the receptor. To further address the role of hydrophilic amino acids in the transforming proteins, we replaced the hydrophilic residue at position 18 or 21 with a leucine in LFC2-T17 and LFC2-T20, respectively, and tested their ability to transform cells. Both mutants displayed severely decreased transforming activity that correlated with decreased ability to bind and activate the PDGF  $\beta$  receptor. An interaction with Thr513 was not essential for transformation by LFC2-T17, suggesting that the homodimerization defect of the LFC2-T17 E18L mutant protein may be responsible for its inability

to activate the receptor. In contrast, the LFC2-T20 protein required Thr513 for signaling, and the LFC2-T20 E21L mutant protein was capable of homodimerization in bacterial membranes (unpublished results). Thus, for the LFC2-T20 protein, the essential glutamic acid residue at position 21 appears more important for receptor recognition than for homodimerization.

Inspection of the sequences of the transforming proteins revealed that all seven transforming proteins with a hydrophilic residue at position 18 contained a valine residue four positions upstream at position 14. Similarly, the wild-type E5 protein and the active library proteins with a hydrophilic residue at position 17 contained a valine four positions upstream at position 13, which is in the segment that was not randomized. It is possible that a hydrophilic residue four amino acids downstream of a valine may contribute to a structure that is particularly conducive to PDGF  $\beta$  receptor binding and activation. In addition, 22 of the 24 hydrophilic amino acids in the transmembrane domains of the transformation-competent proteins are glutamine or glutamic acid residues, even though this is true for only 26 of the 68 hydrophilic residues in the non-transforming proteins. Glutamine and glutamic acid contain an additional methylene unit relative to the structurally related asparagine and aspartic acid, implying that the extra length or flexibility of the glutamine and glutamic acid side-chains may facilitate the formation of hydrogen bonds in a wider variety of sequence contexts.

Several of the transforming clones did not contain hydrophilic transmembrane residues. These clones may rely solely upon hydrophobic packing interactions to stabilize both the E5 homodimer and the interaction with the PDGF  $\beta$  receptor. Interestingly, six of the seven active clones without a hydrophilic amino acid contain a methionine residue at position 16, 17, or 18. Furthermore, when the wild-type E5 protein is mutated to contain a methionine at position 17, the mutant protein retains approximately 50% transforming activity.<sup>8</sup> Although methionine has substantial hydrophobic character, it is able to form hydrogen bonds through its side-chain sulfur atom, such as the hydrogen bond between a methionine and a threonine residue in myohemerythrin.<sup>41</sup> The ability of methionine to form a hydrogen bond may contribute to the ability of library proteins lacking a hydrophilic amino acid to bind and activate the PDGF  $\beta$  receptor.

Although most transforming proteins displayed the same specificity as the wild-type E5 protein, several proteins with altered specificity were identified. The isolation of small transmembrane proteins with different specificity compared to the E5 protein provides strong evidence that these proteins interact directly with the PDGF  $\beta$  receptor. Interestingly, most of the proteins tested that did not require Thr513 also had a relaxed requirement for some other residues along the length of the PDGF  $\beta$  receptor transmembrane domain. However, these library proteins still maintained

considerable specificity, since they did not bind and activate the  $\beta\alpha\beta$  chimeric receptor, which shares ten transmembrane amino acid residues with the PDGF  $\beta$  receptor. Therefore, the library proteins with altered specificity require a PDGF  $\beta$  receptor-like transmembrane sequence, but the details of the interaction are likely to be different from the interaction between the wild-type E5 protein and the wild-type PDGF  $\beta$  receptor.

The proteins with altered specificity no longer required Thr513 in the PDGF  $\beta$  receptor, indicating that hydrogen bond formation with this residue is not important for receptor recognition by these transforming library proteins. Indeed, some of the proteins that displayed altered specificity lacked hydrophilic amino acids. Altered specificity was not solely due to the identity of the hydrophilic amino acid at a certain position, but the position of the hydrophilic amino acid also appears important. For example, LFC2-T15 and LFC2-T17 both displayed altered specificity but have different hydrophilic residues at position 18. On the other hand, although both LFC2-T20 and LFC2-T21 have a glutamic acid at position 21, only the latter displayed altered specificity. This difference must therefore result from one or more of the 11 differences in the hydrophobic sequences between the two proteins. Thus, hydrophobic packing interactions play a role in specific transmembrane domain recognition even for those transforming proteins that contain a strongly polar residue. Furthermore, the presence of a hydrophilic amino acid at position 18 conferred a high likelihood that the protein would display altered specificity, whereas proteins with a hydrophilic residue at position 17, including the wild-type E5 protein, required Thr513. Further experiments are required to elucidate the basis for this altered specificity, but systematic analysis of these proteins may provide novel insights into specific recognition between transmembrane helices.

In summary, we used a genetic method to identify small transmembrane proteins that bind and activate the PDGF  $\beta$  receptor to induce cellular transformation. Although diverse sequences were capable of transformation, localization of hydrophilic amino acids at specific positions within the transmembrane domain appeared to be essential for transforming activity by driving homodimerization or recognition of the PDGF  $\beta$  receptor. Notably, several transforming proteins lacked a hydrophilic transmembrane amino acid, and transforming proteins with altered specificity did not require the PDGF  $\beta$  receptor transmembrane threonine. In these cases, specific transmembrane recognition appears to be mediated by hydrophobic packing interactions. These results indicate that specific, productive interactions between the small transmembrane proteins and their intracellular target do not necessarily require an interhelical hydrogen bond between strongly polar amino acids. It should be possible to use the approach described here to identify small transmembrane proteins that

recognize diverse transmembrane domains, thereby allowing the creation of novel proteins that can modulate the activity of a variety of receptor tyrosine kinases or other cellular transmembrane proteins.

## Materials and Methods

### Library construction

A PCR-based method was used to construct a library of 44 amino acid transmembrane proteins in which rare hydrophilic amino acids were present in a random, predominantly hydrophobic transmembrane sequence.<sup>25</sup> The degenerate upstream oligonucleotide had a fixed 5' end corresponding to the wild-type E5 sequence including a SpeI restriction enzyme site, followed by 16 NXS codons where N is an equal mix of A, T, C, and G; X is a 5 : 1 : 0.1 mix of T:A:C; and S is an equal mix of G and C, and ending with a fixed 3' end derived from the wild-type E5 sequence. The non-degenerate downstream oligonucleotide contained a BamHI restriction enzyme site at its 5' end followed by the antisense sequence of the 3' end of the E5 gene. The two oligonucleotides were annealed at their complementary 3' ends and extended to create double-stranded products that contained 16 randomized codons. Short primers that annealed to the fixed ends were then used to amplify the randomized library. The purified PCR products were digested with SpeI and BamHI and subcloned into an RVY-based retroviral vector to create a library of genes in which 16 transmembrane codons of the wild-type E5 gene were replaced with a random mix of codons in the following expected ratio: 15(L):10(V):5(I,M,F):1(K,N,Y,Q,H,E,D,stop):0.2(S,T,P,A). Cloning details are available from the authors upon request.

### Cell lines and tissue culture

All cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum and antibiotics (DMEM-10). Retroviral stocks were prepared as described in GP2-293 cells.<sup>42</sup> To assay focus-forming activity, C127 cells were infected with retrovirus, and the cells were incubated at confluence for two to three weeks. In parallel, infected cells were selected in medium containing 300 µg/ml hygromycin B. Focus formation efficiency was calculated by normalizing the number of foci to the number of hygromycin B-resistant colonies arising from the same infection. Helper virus-mediated rescue of transforming library clones was as described.<sup>25</sup>

### Recovery of transforming library clones

Hygromycin-resistant colonies that grew after infection of C127 cells with the rescued virus were pooled and expanded. Genomic DNA was harvested using a DNeasy kit (Qiagen), and the retroviral insert was amplified from the genomic DNA by using primers that flanked the insert and the Expand Long Template PCR system (Roche). The PCR products were digested with SpeI and BamHI and subcloned into a retroviral vector to generate individual genes with random transmembrane sequences. The sequence of the complete DNA insert was then obtained for each individual transforming library clone and for representative clones chosen at

random from the library. The statistical methods used to quantify the positional bias in the distribution of hydrophilic residues in the transforming proteins and to determine if the average number of hydrophilic residues was different between the transforming and non-transforming clones are available from the authors upon request.

### PDGF $\beta$ receptor inhibitor studies

C127 cells were plated at ~70% confluence in 12 well plates and incubated in DMEM-10 in the presence or absence of 50 µM AG1295, a PDGF receptor tyrosine kinase inhibitor (Calbiochem).<sup>8</sup> Cells were photographed four days after plating.

### Metabolic labeling

C127 cell lines at approximately 80% confluence in 10 cm dishes were washed in PBS and incubated for one hour in leucine-free DMEM (DMEM labeling kit; Specialty Media). Cells were then incubated for five hours in 3 ml leucine-free DMEM containing 27 µCi [<sup>14</sup>C]leucine (Amersham). To isolate labeled membrane proteins, the labeled cells were rinsed with cold 0.01% (w/v) sodium citrate and incubated for ten minutes at room temperature in 5 ml of cold 0.01% sodium citrate. The cells were then scraped from the plate, transferred to a cold Dounce homogenizer, and subjected to ten strokes with a type A tight pestle. The lysed cells were centrifuged for 15 minutes at 3000 RPM at 4 °C. The supernatant was removed from the pelleted intact nuclei and centrifuged at 37,000 RPM in a Beckman SW50.1 rotor for 45 minutes at 4 °C. The pellet, which contains cell membranes and associated proteins, was dissolved in 300 µl of modified RIPA buffer and immunoprecipitated after normalizing for incorporated label, as described below. After electrophoresis, the gel was dried and visualized using a PhosphorImager (Molecular Dynamics).

### Biochemical analysis

Cell lines at near confluence were serum-starved for 24 hours. Cell extracts were made using modified RIPA buffer containing protease and phosphatase inhibitors as described.<sup>10</sup> Immunoprecipitation and immunoblotting were carried out essentially as described.<sup>8,10</sup> Samples were boiled in 2× Laemmli buffer with  $\beta$ -mercaptoethanol, except the metabolically labeled samples which were boiled in buffer with or without reducing agents as described.<sup>8</sup> For anti-phosphotyrosine blots, a 1 : 500 dilution of P-Tyr-100 (Cell Signaling Technology) or 4G10 (Upstate Biotechnology) in 5% (w/v) BSA in TBST was used.

### Transient assay for PDGF receptor signaling

CV1 cells were transfected with a pRL-SV40 *Renilla* luciferase plasmid for normalization, a pGAS-luciferase reporter plasmid, a plasmid encoding wild-type or mutant PDGF  $\beta$  receptor, and a plasmid encoding wild-type or mutant E5 protein, *v-sis*, or a small transmembrane library protein as described.<sup>25</sup> The dual-luciferase reporter assay system (Promega) was used according to the manufacturer's protocol. Samples were read using a Turner Designs TD-20/20 or Veritas luminometer.

### Computational searches using CHI

Details of the CHI computational search method have been described.<sup>37–39</sup> All structural calculations were carried out *in vacuo* on two canonical  $\alpha$ -helices containing residues 8–34 of the wild-type protein or the Q17L L18Q E5 mutant protein, using a Silicon Graphics O2 workstation. Starting geometries, molecular dynamics simulations, and energy minimization were as described.<sup>25</sup>

### Acknowledgements

We thank Yu Xia for the statistical analysis; Joshua Drumm for assistance recovering and testing non-transforming clones; Chad McCormick for assistance generating mutants and testing library clones; and Jan Zulkeski for assistance in preparation of the manuscript. L.L.F.-C. was supported by an NIH postdoctoral training grant (CA09159), A.M.D. was supported by a postdoctoral fellowship from the American Cancer Society (PFCSM-0301701), K.E.Y. was supported by a predoctoral training grant from the NIH (T32 AI55403), and D.M.E. was supported by a grant from the NIH (PO1 GM054160) and a grant from the National Foundation for Cancer Research. This work was supported by a grant from the National Cancer Institute to D.D. (CA37157).

### References

- DiMaio, D., Lai, C. C. & Klein, O. (1998). Virocrine transformation: the intersection between viral transforming proteins and cellular signal transduction pathways. *Annu. Rev. Microbiol.* **52**, 397–421.
- DiMaio, D., Guralski, D. & Schiller, J. T. (1986). Translation of open reading frame E5 of bovine papillomavirus is required for its transforming activity. *Proc. Natl Acad. Sci. USA*, **83**, 1797–1801.
- Schlegel, R., Wade-Glass, M., Rabson, M. S. & Yang, Y.-C. (1986). The E5 transforming gene of bovine papillomavirus encodes a small hydrophobic protein. *Science*, **233**, 464–467.
- Horwitz, B. H., Burkhardt, A. L., Schlegel, R. & DiMaio, D. (1988). 44-amino-acid E5 transforming protein of bovine papillomavirus requires a hydrophobic core and specific carboxyl-terminal amino acids. *Mol. Cell. Biol.* **8**, 4071–4078.
- Meyer, A. N., Xu, Y.-F., Webster, M. K., Smith, A. S. & Donoghue, D. J. (1994). Cellular transformation by a transmembrane peptide: structural requirements for the bovine papillomavirus E5 oncoprotein. *Proc. Natl Acad. Sci. USA*, **91**, 4634–4638.
- Mattoon, D., Gupta, K., Doyon, J., Loll, P. J. & DiMaio, D. (2001). Identification of the transmembrane dimer interface of the bovine papillomavirus E5 protein. *Oncogene*, **20**, 3824–3834.
- Nilson, L. A., Gottlieb, R. L., Polack, G. W. & DiMaio, D. (1995). Mutational analysis of the interaction between the bovine papillomavirus E5 transforming protein and the endogenous beta receptor for platelet-derived growth factor in mouse C127 cells. *J. Virol.* **69**, 5869–5874.
- Klein, O., Polack, G. W., Surti, T., Kegler-Ebo, D., Smith, S. O. & DiMaio, D. (1998). Role of glutamine 17 of the bovine papillomavirus E5 protein in platelet-derived growth factor beta receptor activation and cell transformation. *J. Virol.* **72**, 8921–8932.
- Surti, T., Klein, O., Aschheim, K., DiMaio, D. & Smith, S. O. (1998). Structural models of the bovine papillomavirus E5 protein. *Proteins: Struct. Funct. Genet.* **33**, 601–612.
- Drummond-Barbosa, D. A., Vaillancourt, R. R., Kazlauskas, A. & DiMaio, D. (1995). Ligand-independent activation of the platelet-derived growth factor beta receptor: requirements for bovine papillomavirus E5-induced mitogenic signaling. *Mol. Cell. Biol.* **15**, 2570–2581.
- Goldstein, D. J., Li, W., Wang, L.-M., Heidarani, M. A., Aaronson, S. A., Shinn, R. *et al.* (1994). The bovine papillomavirus type 1 E5 transforming protein specifically binds and activates the beta-type receptor for platelet-derived growth factor but not other tyrosine kinase-containing receptors to induce cellular transformation. *J. Virol.* **68**, 4432–4441.
- Nilson, L. A. & DiMaio, D. (1993). Platelet-derived growth factor receptor can mediate tumorigenic transformation by the bovine papillomavirus E5 protein. *Mol. Cell. Biol.* **13**, 4137–4145.
- Petti, L., Nilson, L. A. & DiMaio, D. (1991). Activation of the platelet-derived growth factor receptor by the bovine papillomavirus E5 transforming protein. *EMBO J.* **10**, 845–855.
- Staebler, A., Pierce, J. H., Brazinski, S., Heidarani, M. A., Li, W., Schlegel, R. & Goldstein, D. J. (1995). Mutational analysis of the beta-type platelet-derived growth factor receptor defines the site of interaction with the bovine papillomavirus type 1 E5 transforming protein. *J. Virol.* **69**, 6507–6517.
- Nappi, V. M., Schaefer, J. A. & Petti, L. M. (2002). Molecular examination of the transmembrane requirements of the platelet-derived growth factor beta receptor for a productive interaction with the bovine papillomavirus E5 oncoprotein. *J. Biol. Chem.* **277**, 47149–47159.
- Goldstein, D. J., Andresson, T., Sparkowski, J. J. & Schlegel, R. (1992). The BPV-1 E5 protein, the 16 kDa membrane pore-forming protein and the PDGF receptor exist in a complex that is dependent on hydrophobic transmembrane interactions. *EMBO J.* **11**, 4851–4859.
- Petti, L. & DiMaio, D. (1992). Stable association between the bovine papillomavirus E5 transforming protein and activated platelet-derived growth factor receptor in transformed mouse cells. *Proc. Natl Acad. Sci. USA*, **89**, 6736–6740.
- Petti, L. & DiMaio, D. (1994). Specific interaction between the bovine papillomavirus E5 transforming protein and the beta receptor for platelet-derived growth factor in stably transformed and acutely transfected cells. *J. Virol.* **68**, 3582–3592.
- Lai, C. C., Henningson, C. & DiMaio, D. (1998). Bovine papillomavirus E5 protein induces oligomerization and trans-phosphorylation of the platelet-derived growth factor beta receptor. *Proc. Natl Acad. Sci. USA*, **95**, 15241–15246.
- Lai, C. C., Henningson, C. & DiMaio, D. (2000). Bovine papillomavirus E5 protein induces the formation of signal transduction complexes containing dimeric activated platelet-derived growth factor  $\beta$  receptor and associated signaling proteins. *J. Biol. Chem.* **275**, 9832–9840.

21. Petti, L. M., Reddy, V., Smith, S. O. & DiMaio, D. (1997). 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. *J. Virol.* **71**, 7318–7327.
22. Klein, O., Kegler-Ebo, D., Su, J., Smith, S. & DiMaio, D. (1999). The bovine papillomavirus E5 protein requires a juxtamembrane negative charge for activation of the platelet-derived growth factor beta receptor and transformation of C127 cells. *J. Virol.* **73**, 3264–3272.
23. Adduci, A. J. & Schlegel, R. (1999). The transmembrane domain of the E5 oncoprotein contains functionally discrete helical faces. *J. Biol. Chem.* **274**, 10249–10258.
24. Nappi, V. M. & Petti, L. M. (2002). Multiple transmembrane amino acid requirements suggest a highly specific interaction between the bovine papillomavirus E5 oncoprotein and the platelet-derived growth factor beta receptor. *J. Virol.* **76**, 7976–7986.
25. Freeman-Cook, L., Dixon, A. M., Frank, J. B., Xia, Y., Ely, L., Gerstein, M. *et al.* (2004). Selection and characterization of small random transmembrane proteins that bind and activate the platelet-derived growth factor beta receptor. *J. Mol. Biol.* **338**, 907–920.
26. Horwitz, B. H., Weinstat, D. L. & DiMaio, D. (1989). Transforming activity of a 16-amino-acid segment of the bovine papillomavirus E5 protein linked to random sequences of hydrophobic amino acids. *J. Virol.* **63**, 4515–4519.
27. Dawson, J. P., Melnyk, R. A., Deber, C. M. & Engelman, D. M. (2003). Sequence context strongly modulates association of polar residues in transmembrane helices. *J. Mol. Biol.* **331**, 255–262.
28. Call, M. E., Pyrdol, J., Wiedmann, M. & Wucherpfennig, K. W. (2002). The organizing principle in the formation of the T cell receptor-CD3 complex. *Cell*, **111**, 967–979.
29. Manolios, N., Bonifacino, J. S. & Klausner, R. D. (1990). Transmembrane helical interactions and the assembly of the T cell receptor complex. *Science*, **249**, 274–277.
30. Choma, C., Gratkowski, H., Lear, J. D. & DeGrado, W. F. (2000). Asparagine-mediated self-association of a model transmembrane helix. *Nature Struct. Biol.* **7**, 161–166.
31. Gratkowski, H., Lear, J. D. & DeGrado, W. F. (2001). Polar side chains drive the association of model transmembrane peptides. *Proc. Natl Acad. Sci. USA*, **98**, 880–885.
32. Zhou, F. X., Cocco, M. J., Russ, W. P., Brunger, A. T. & Engelman, D. M. (2000). Interhelical hydrogen bonding drives strong interactions in membrane proteins. *Nature Struct. Biol.* **7**, 154–160.
33. Zhou, F. X., Merianos, H. J., Brunger, A. T. & Engelman, D. M. (2001). Polar residues drive association of polyleucine transmembrane helices. *Proc. Natl Acad. Sci. USA*, **98**, 2250–2255.
34. Smith, S. O., Smith, C. S. & Bormann, B. J. (1996). Strong hydrogen bonding interactions involving a buried glutamic acid in the transmembrane sequence of the neu/erbB-2 receptor. *Nature Struct. Biol.* **3**, 252–258.
35. Bell, C. A., Tynan, J. A., Hart, K. C., Meyer, A. N., Robertson, S. C. & Donoghue, D. J. (2000). Rotational coupling of the transmembrane and kinase domains of the Neu receptor tyrosine kinase. *Mol. Biol. Cell.* **11**, 3589–3599.
36. Webster, M. K. & Donoghue, D. J. (1996). Constitutive activation of fibroblast growth factor receptor 3 by the transmembrane domain point mutation found in achondroplasia. *EMBO J.* **15**, 520–527.
37. Brunger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W. *et al.* (1998). Crystallography & NMR system: A new software suite for macromolecular structure determination. *Acta Crystallog. sect. D, Biol. Crystallog.* **54**, 905–921.
38. Adams, P. D., Arkin, I. T., Engelman, D. M. & Brunger, A. T. (1995). Computational searching and mutagenesis suggest a structure for the pentameric transmembrane domain of phospholamban. *Nature Struct. Biol.* **2**, 154–162.
39. Adams, P. D., Engelman, D. M. & Brunger, A. T. (1996). Improved prediction of the structure of the dimeric transmembrane domain of glycophorin A obtained through global searching. *Proteins: Struct. Funct. Genet.* **26**, 257–261.
40. Sal-Man, N., Gerger, D. & Shai, Y. (2004). The composition rather than position of polar residues (QxxS) drives aspartate receptor transmembrane domain dimerization *in vivo*. *Biochemistry*, **43**, 2309–2313.
41. Gregoret, L. M., Rader, S. D., Fletterick, R. J. & Cohen, F. E. (1991). Hydrogen bonds involving sulfur atoms in proteins. *Proteins: Struct. Funct. Genet.* **9**, 99–107.
42. DeFilippis, R. A., Goodwin, E. C., Wu, L. & DiMaio, D. (2003). Endogenous human papillomavirus E6 and E7 proteins differentially regulate proliferation, senescence, and apoptosis in HeLa cervical carcinoma cells. *J. Virol.* **77**, 1551–1563.

G. von Heijne

(Received 24 September 2004; received in revised form 22 October 2004; accepted 24 October 2004)