Packing contacts can mediate highly specific interactions between artificial transmembrane proteins and the PDGFβ receptor

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Communicated by Donald M. Engelman, Yale University, New Haven, CT, May 29, 2007 (received for review December 1, 2006)

We used proteins with randomized transmembrane (TM) domains to explore the role of hydrophobic amino acids in mediating specific interactions between transmembrane helices. The 44-aa bovine papillomavirus E5 protein, which binds to the TM domain of the PDGFβ receptor (PDGFβR) was used as a scaffold to construct a library encoding small dimeric proteins with randomized, strictly hydrophobic TM domains, and proteins were selected that induced focus formation in mouse C127 cells by activating the PDGFβR. Analysis of these proteins identified a motif of two hydrophobic residues that, when inserted into a 17-residue polyleucine TM domain, generated a protein that activated the PDGFβR and transformed cells. In addition, we identified transforming proteins that activated the wild-type PDGFβR but did not activate a series of PDGFβR TM point mutants that were efficiently activated by the E5 protein, indicating that these proteins were more specific than the E5 protein. Our results implied that multiple van der Waals interactions distributed along the entire length of the TM domains were required for productive interaction between the PDGFβR and some small proteins lacking hydrophilic TM residues. Our results also suggested that excluding hydrophilic residues from small TM proteins and peptides is a strategy to increase the specificity of heteromeric TM helix–helix interactions.

helix interactions | receptor activation | ES protein

Many essential cellular processes require proteins that are anchored in cell membranes. Most membrane-spanning domains cross membranes as α-helices, which can engage in highly specific side-by-side interactions with one another (1, 2). Transmembrane (TM) helix–helix interactions can mediate oligomerization of TM proteins, control their activity, and assist the proper folding of multipass TM proteins. Thus, understanding the basis for specific interactions between TM helices will provide considerable insight into the structure and function of cellular TM proteins.

Detailed analysis of homodimerization of the TM domain of the major red blood cell protein, glycophorin A, suggested that the precise geometry of van der Waals interactions between hydrophobic side-chains is a major determinant of specificity and identified a GlyXXXGly motif important for dimer formation (3–5). Leucine zippers or multiple serine and threonine residues can also drive homodimer formation (6, 7). Studies with hydrophobic peptides and TM proteins showed that hydrogen bonding or charge-charge interactions between strongly polar residues can induce the formation of homooligomers and to a large extent obviate the requirement for specific packing interactions between hydrophobic side-chains (8–11). Strong interactions between hydrophilic side-chains can also mediate heteromeric associations between TM helices (12–14), but the role of packing interactions in driving the formation of specific TM helix heterooligomers has not been systematically studied.

We developed a system to study heteromeric TM helix–helix interactions in mammalian cells, based on the 44-aa E5 protein of bovine papillomavirus (BPV) (15). The E5 protein is the major BPV protein responsible for cell transformation and appears to span intracellular membranes as a symmetric, disulfide-linked homodimer (16). The E5 dimer transforms cells by binding directly in an anti-parallel fashion to the TM domain of two molecules of the PDGFβ receptor (PDGFβR), a tyrosine kinase (16–18). E5 binding induces PDGFβR dimerization and transphosphorylation, which results in sustained mitogenic signaling and cell transformation (16, 19). Hydrogen bonding involving Gln-17 in the E5 protein and salt-bridge formation between Asp-33 of the E5 protein and Lys-499 of the PDGFβR are essential for complex formation, although packing interactions also appear important (20, 21). The E5 protein does not bind to or activate several other growth factor receptors, including the PDGFα receptor (22).

We generated libraries encoding many different small proteins in which the central segment of the E5 protein was replaced with random sequences of predominantly hydrophobic amino acids (23, 24). Small TM proteins that induced focus formation in murine C127 fibroblasts were selected from these libraries and characterized. In libraries containing the hydrophilic residues important for the E5-PDGFβR interaction, $\approx$10% of the clones induced focus formation (23), demonstrating that many different TM sequences permitted cell transformation if essential hydrophilic interactions were retained. All of the active small TM proteins recovered from the libraries transformed cells by activating the PDGFβR via interactions involving the TM domain of the receptor.

Here, we explored the basis for specific heteromeric interactions between TM helices that lack residues previously implicated in TM interactions, namely glycines, strongly polar or charged residues, or multiple serines or threonines. We constructed a library in which the central 20 amino acids of the E5 protein were replaced with random hydrophobic amino acids and recovered proteins that activated the PDGFβR and transformed cells. Some of these proteins were more specific than the E5 protein, and engagement of these proteins with the PDGFβR appeared to require multiple van der Waals interactions distributed along the TM domains.

Results and Discussion

Isolation of Transforming Proteins That Lack Hydrophilic TM Amino Acids. To investigate the role of hydrophobic amino acids in heteromeric interactions between TM domains, we generated a retroviral library (designated JBF13) of small TM proteins lacking hydrophilic residues in the TM domain. Amino acids

Author contributions: J.B.P. and D.D. designed research; J.B.P., A.P.B.E., and L.L.F.-C. performed research; J.B.P. and D.D. analyzed data; and J.B.P., L.L.F.-C., and D.D. wrote the paper.

The authors declare no conflict of interest.

Abbreviations: PDGFβR, PDGFβ receptor; TM, transmembrane.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0704348104/DC1.

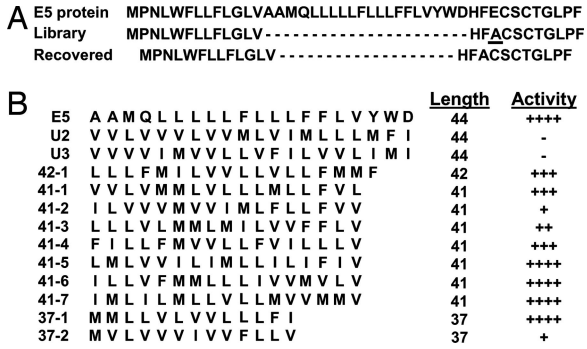

Fig. 1. Isolation of small hydrophobic proteins that transform cells. (*A*) The amino acid sequence of the bovine papillomavirus E5 protein (*Top*) was used to design the JBF13 library (*Middle*). The dashed line indicates residues 14–33, which were replaced with randomized hydrophobic amino acids, and the Glu36Ala mutation is underlined. The transforming proteins selected from the library contained randomized segments shorter than expected (*Bottom*). (*B*) TM sequences of the E5 protein, two nontransforming (U2 and U3) and transforming library proteins. The total number of amino acids in each clone and its focus forming activity are shown. ++++ indicates 76–100% wild-type E5 activity; +++, 51–75%; ++, 26–50%; +, 15–25%; and 0 = −, <15%.

14–33 of the E5 protein, including Gln-17 and Asp-33 (which are required for the E5–PDGFβR interaction) were replaced with random hydrophobic amino acids, and Glu-36 was mutated to alanine (Fig. 1*A*). The remaining E5 amino acids, including the cysteines required for homodimerization, were retained. The library contained TM domains composed of 20 randomized codons encoding exclusively hydrophobic residues, with the predicted ratio of 3L:2V:1M:1I:1F. The composition of the library was verified by isolating and sequencing individual randomly picked library clones. We estimated that JBF13 encoded ≈500,000 different proteins, a tiny fraction of the 10^{14} proteins possible given the design of the library.

The JBF13 library was exhaustively screened in murine C127 fibroblasts, which express the endogenous PDGFβR. Infected cells were incubated at confluence to select for focus formation, and genomic DNA was harvested from cell lines expanded from individual transformed foci. The library insert was amplified from this genomic DNA and recloned into the retroviral vector, and individual clones were assayed for their ability to induce foci in C127 cells.

Ten unique proteins with transforming activity were identified, including multiple independent isolates of several clones, indicating that ≈0.002% of the library proteins induced foci. Thus, C127 cells can be efficiently transformed by individual TM proteins that lack the hydrophilic amino acids normally required for activity, but such proteins are very rare in the library. Fig. 1*B* lists the TM domain sequences and the focus-forming activity of the E5 protein, two representative nontranforming proteins picked at random from the library, and the 10 proteins that induced focus formation. Although 20 codons were randomized during library construction, the randomized segment was shorter in all of the transformation-competent clones isolated: 17 (seven clones), 13 (two clones), or 18 residues (one clone) (Fig. 1*B*), yielding proteins 42, 41, or 37 aa long. The transforming proteins formed homodimers, as demonstrated by nonreducing polyacrylamide gel electrophoresis, and focus formation required the cysteines in the small TM proteins (data not shown), presumably reflecting a requirement for covalent dimerization, as is the case for the E5 protein.

Proteins Recovered from the Library Activate the PDGFβR. To determine whether the small TM proteins transformed cells by activating the PDGFβR, we first tested whether an inhibitor of PDGFβR tyrosine kinase activity, AG1295, reversed the transformed cell morphology induced by these proteins. Stable C127 cell lines expressing the empty vector, the activated p185^{neu*} oncogene product (which is unrelated to the PDGFβR), the E5 protein, or the 10 transforming library proteins were grown in the presence or absence of AG1295 (representative example shown in Fig. 2*A*). Control C127 cells remained flat after reaching confluence, whereas cells expressing the proteins selected from the library acquired a transformed morphology. When cultured in the presence of AG1295, each of the transformed cell lines except *neu** reverted to a nontransformed morphology. Thus, sustained signaling by the PDGFβR was required to maintain the transformed phenotype induced by the JBF13 proteins.

We also assessed whether the JBF13 transforming proteins bound and activated the PDGFβR. Detergent protein extracts were harvested from C127 cell lines and analyzed by immunoblotting. All of the cell lines expressed similar levels of endogenous PDGFβR (Fig. 2*B Top*), and each of the library proteins was expressed at levels equal to or greater than the E5 protein (Fig. 2*B Bottom*). The vector control and two representative nontransforming proteins failed to induce tyrosine phosphorylation of the PDGFβR. In contrast, the E5 protein and each of the transforming proteins induced PDGFβR tyrosine phosphorylation (Fig. 2*B*, second blot), demonstrating that these proteins activated the receptor.

To determine whether the transforming proteins stably interacted with the PDGFβR, CHAPS detergent extracts were immunoprecipitated with an antibody directed against a constant segment of the E5 protein. The immunoprecipitates were then immunoblotted with the PDGFβR antibody (Fig. 2*B*, third blot). The E5 protein formed a stable complex with the mature and precursor forms of the PDGFβR, whereas neither of the nontransforming proteins stably bound the receptor. The mature form of the PDGFβR coimmunoprecipitated with one of the transforming clones, JBF13-41-5 (hereafter 41-5), indicating that this protein also formed a stable complex with the receptor, even though all of the side-chains in the randomized segment of 41-5 are hydrophobic. In contrast, only background levels of the receptor coimmunoprecipitated with the other transforming proteins, suggesting that most small TM proteins lacking the potential to make strong hydrophilic TM contacts did not bind the PDGFβR tightly enough to withstand our method of protein extraction and analysis, even though they induced tyrosine phosphorylation of the PDGFβR.

We used a transient reporter gene assay to examine whether the transforming proteins induced PDGFβR signaling in CV1 monkey kidney cells, which do not express endogenous PDGFβR. CV1 cells were cotransfected with four plasmids: a reporter plasmid that expresses firefly luciferase under the control of three copies of the STAT1-inducible enhancer (SIE3) element that is stimulated by PDGFβR signaling; a plasmid expressing *Renilla* luciferase as an internal transfection control; a plasmid expressing the E5 protein, v-*sis* (a homolog of the ligand, PDGF), or one of the JBF13 proteins; and a plasmid expressing the wild-type PDGFβR or a chimeric βαβ receptor. βαβ was generated by replacing the TM domain of the PDGFβR with that of the PDGFα receptor (PDGFαR) (Fig. 2*C*), which is not activated by the E5 protein (24). The results of this transient signaling assay, expressed as relative luciferase expression levels, are shown in Fig. 2*D*. In the absence of receptor expression, the TM proteins induced minimal signaling. Similarly, the nontransforming JBF13 proteins activated neither the wild-type PDGFβR nor βαβ. As expected, both the E5 protein and v-*sis* activated the wild-type PDGFβR, and only v-*sis* activated βαβ. Most of the library proteins that transformed C127 cells activated the wild-type PDGFβR to varying degrees, but none activated βαβ, demonstrating that they recognized the TM

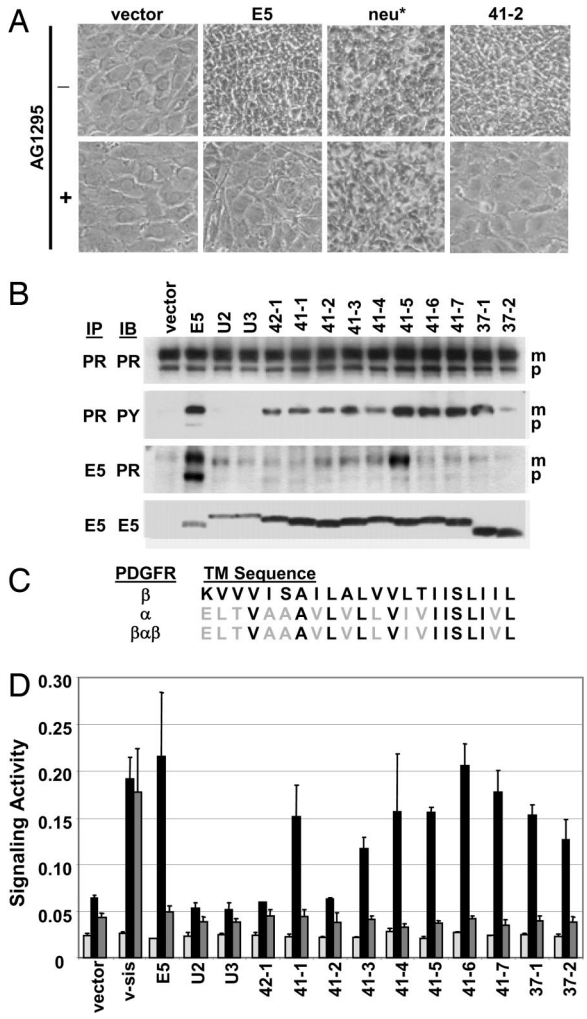

Fig. 2. Transforming proteins activate the PDGFβR. (*A*) Morphologic transformation requires PDGFβR kinase activity. Photomicrographs of C127 cells expressing the empty vector, the E5 protein, p185^{neu*} (designated *neu**) or JBF13–41-2 cultured for 7 days in the absence (*Upper*) or presence (*Lower*) of AG1295. (*B*) Biochemical analysis of cells expressing the empty vector or the proteins listed in Fig. 1*B*. C127 cell extracts were immunoprecipitated (IP) and immunoblotted (IB) with the indicated antibodies (PR, PDGFβR; PY, phosphotyrosine; E5, E5 protein) to detect expression of the endogenous PDGFβR (*Top*), tyrosine phosphorylation of the PDGFβR (second blot), stable complex formation between the small TM proteins and the PDGFβR (third blot), and expression of the small TM proteins (*Bottom*). The mature (m) and precursor (p) forms of the PDGFβR are indicated. (*C*) TM sequences of the wild-type PDGFβR, PDGFαR, and the βαβ chimera. PDGFαR residues in common with PDGFβR are shown in black. (*D*) Transient luciferase reporter assay for signaling induced by the empty vector or the indicated proteins when cotransfected with the wild-type PDGFβR (black), the βαβ chimera (dark gray), or when expressed alone (light gray). Cells also received the SIE-containing reporter plasmid and a plasmid expressing *Renilla* luciferase.

domain of the PDGFβR but not of the PDGFαR (Fig. 2*D*). Interestingly, two of the transforming proteins, JBF13-42-1 and -41-2, did not induce the SIE3 reporter, even though they induced PDGFβR tyrosine phosphorylation and their transformed morphology was reverted by the PDGFβR inhibitor. These results indicated that these two clones did not activate STAT1 signaling, even though they induced PDGFβR-mediated cell transformation.

Identification of Sequence Motifs That Confer Activity. Particular residues are preferentially found at specific positions within the

	Activity
pL	-
pL-M	+/-
pL-V	-
pL-MV	+++
pL-VM	-
pL-MFV	++++
pL-VMFV	+++++
pL-VIFV	+++++
pL-MVVIFV	+++++
pL-VIIMFV	+++++
pL-VIIIFIV	+++++
pL-VIFV+I25	+++++
pL-VIFV+I27	+++++
pL-VIFV+I29	+++++
41-5	++++

Fig. 3. Identification of a simple sequence motif that supports transformation. TM amino acid sequence and focus-forming activity of 41-aa proteins containing a 17-residue TM domain (positions 14–30) composed of leucine (gray) and amino acids that were preferentially present in the active 41-aa JBF13 library proteins (black). E5 amino acids 34–44 were fused at the C terminus of the polyleucine stretch. +++++, >100% wild-type E5 activity.

TM domains of the 41-aa JBF13 transforming proteins. The most striking bias is toward methionine at position 19 (counting from the N terminus of the protein), which is present in six of the seven transforming proteins, even though <18% of the residues in the randomized segment of the unselected clones are methionine. Using Student's *t* test to compare the frequencies of residues at specific positions in the transforming clones to their abundance in the randomized segment of the unselected clones, a notable bias was evident at Met-19 ($P \leq 0.004$), Val-30 ($P \leq 0.02$), Leu-16 ($P \leq 0.02$), Phe-28 ($P \leq 0.05$), and Val-17 ($P \leq 0.09$).

To determine whether these residues could support PDGFβR activation, they were inserted into a nontransforming 41-aa protein containing a 17-residue polyleucine (polyL) TM domain, and the resulting clones were tested for focus formation in C127 cells. Although neither Met-19 nor Val-30 alone allowed significant transforming activity, the insertion of Met-19 and Val-30 together generated a transformation-competent protein (Fig. 3). Swapping the position of these residues to Val-19 and Met-30 eliminated activity, and introduction of Phe-28 and Val-17 further increased transforming activity. Because 41-5 contained isoleucine instead of methionine at position 19, isoleucine was inserted together with Val-17, Phe-28, and Val-30 to generate polyL-VIFV, which also displayed robust transforming activity (Fig. 3). The transformed phenotype of cell lines expressing these clones was reverted by the PDGF receptor kinase inhibitor (data not shown). In addition, there was a good correlation between the ability of these proteins to form a stable complex with the mature form of the PDGFβR (Fig. 4*A Middle*), to induce tyrosine phosphorylation of the receptor (Fig. 4*A Top*) and to trigger signaling by the wild-type PDGFβR but not by the βαβ chimera (Fig. 4*B*). All of the small polyleucine proteins were expressed at similar levels (Fig. 4*A Bottom*). These experiments indicated that small TM proteins containing simple sequence motifs can interact with the TM domain of the PDGFβR, resulting in receptor activation and cell transformation.

Specificity of Small TM Proteins. To systematically assess the ability of small proteins recovered from the JBF13 library to discriminate between closely related TM domains, we used the transient luciferase assay to test the ability of these proteins to induce signaling by a panel of mutants containing single point mutations that span the TM domain of the PDGFβR (A.P.B.E., L. Ely-Bowers, D. Mattoon, and D.D., unpublished work). Importantly, the ability of the recovered JBF13 proteins to activate the wild-type PDGFβR and transform cells strongly suggested that

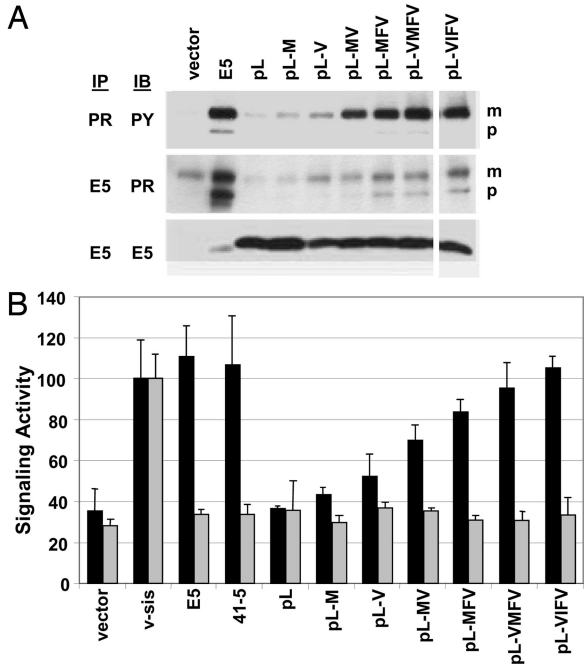

Fig. 4. Analysis of transforming proteins containing simple motifs. (*A*) Biochemical analysis of C127 cells expressing the empty vector, the E5 protein, or the indicated polyL proteins. Extracts were immunoprecipitated and immunoblotted as described in the legend to Fig. 2*B*. (*B*) Transient assay for signaling induced by the empty vector or the indicated proteins when coexpressed with the wild-type PDGFβR (black) or ββ (gray), expressed as percent activation of each receptor by v-*sis*.

they were stable proteins that inserted into cell membranes, assumed an α-helical conformation, and formed homodimers in the proper orientation to recognize the PDGFβR (25). Therefore, the inability of a protein to activate a PDGFβR TM mutant reflects a specific defect in the recognition of that particular mutant and not an overall defect.

The E5 protein induced signaling by most of the receptor point mutants, indicating that few of the tested substitutions affected the interaction between the E5 protein and the PDGFβR (Fig. 5). Strikingly, although 41-5 induced signaling by the wild-type PDGFβR, it failed to induce signaling by several point mutants that efficiently signaled in response to the E5 protein. The location of such disruptive mutations throughout the PDGFβR TM domain suggests that the ability of 41-5 to induce signaling by the PDGFβR TM domain required the existence of multiple, intimate packing contacts along its entire length. Although we tested numerous PDGFβR point mutants, we failed to identify any that signaled in response to 41-5 and not to the wild-type E5 protein. Thus, 41-5 appeared more specific than the E5 protein. Several other transforming JBF13 library proteins also displayed increased specificity compared with the E5 protein, but none were as dramatic as 41-5 (data not shown).

To determine whether the inability of 41-5 to induce signaling by selected PDGFβR point mutants was due to its inability to activate these mutants, we analyzed receptor tyrosine phosphorylation in extracts of CV1 cells expressing the receptor mutants that were most defective for signaling in response to 41-5. As shown in Fig. 6, both the E5 protein and 41-5 induced tyrosine phosphorylation of the wild-type PDGFβR but not ββ. Strikingly, the E5 protein also induced tyrosine phosphorylation of the three PDGFβR TM mutants, Ile503Asp, Ile506Val, or Val517Ala, whereas 41-5 did not induce significant tyrosine phosphorylation of these mutants. These results indicated that 41-5 was not able to activate a number of receptors containing

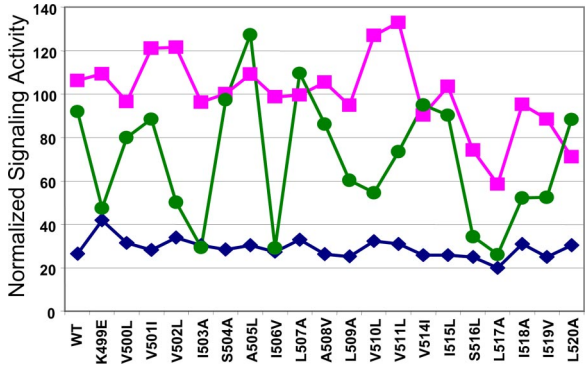

Fig. 5. Specificity of a highly specific transforming protein. Transient assay for signaling induced by the RVY empty vector (blue diamond), the E5 protein (pink square), or 41-5 (green circle) when coexpressed with either the wild-type PDGFβR or the PDGFβR point mutants indicated on the *x* axis. Receptor activation is normalized to the level of activation of that receptor by v-*sis*. The graph shows results averaged from two independent experiments.

point mutations in the TM domain, even though these mutants were activated by the wild-type E5 protein.

Mapping the Determinants of Specificity. We also used the transient luciferase assay to compare the specificity of 41-5 with polyL-VIFV, which contained the active motif from 41-5. PolyL-VIFV, like the E5 protein, induced signaling by several PDGFβR TM point mutants that were not activated by 41-5 (Fig. 7*A*). Thus, although polyL-VIFV contained the active motif from 41-5, it was not as specific. Similarly, polyL-MV, polyL-MFV, and polyL-VMFV induced signaling by both the wild-type and point mutant receptors (data not shown).

To identify the residues that confer high specificity, we substituted groups of amino acids from 41-5 into polyL-VIFV and tested focus formation and transient signaling activity. PolyL-MVVIFV, polyL-VIIMFV, and polyL-VIIIFIV efficiently induced foci in C127 cells (Fig. 3*B*) and induced robust signaling by the wild-type PDGFβR (Fig. 7*B*). PolyL-VIIMFV activated all of the PDGFβR TM point mutants. PolyL-MVVIFV displayed an intermediate phenotype, efficiently activating the PDGFβR mutants Ile503Ala, Ile506Val, and Val510Leu, but displayed reduced ability to activate Lys499Glu and Leu517Ala. PolyL-VIIIFIV most closely resembled 41-5, failing to activate the PDGFβR mutants with the exception of Val510Leu. These results indicated that the three isoleucines

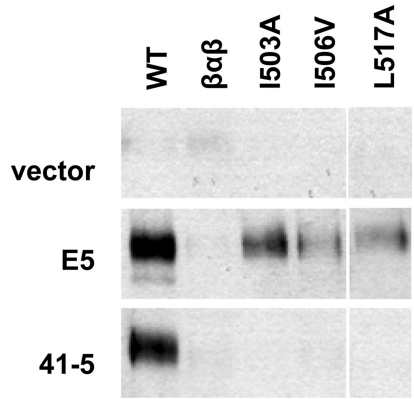

Fig. 6. JBF13-41-5 is defective for activation of selected PDGFβR TM point mutants. CV1 cells were sequentially infected with the PDGFβR construct indicated at the top and either the RVY vector, or RVY expressing the E5 protein or 41-5. CHAPS extracts were analyzed for PDGFβR tyrosine phosphorylation.

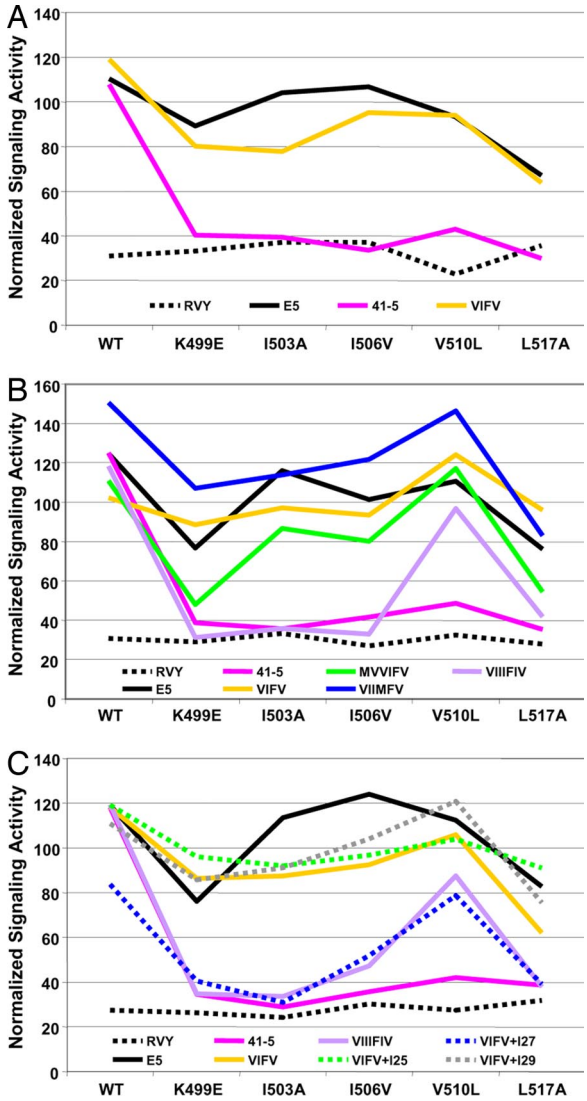

Fig. 7. Mapping determinants of specificity. Transient assay for signaling induced by small transforming proteins. Empty vector (RVY) and the indicated small TM proteins were tested for their ability to induce expression of the PDGFβR-responsive reporter after cotransfection with the wild-type PDGFβR (WT) or the PDGFβR point mutant indicated on the *x* axis. The results are expressed as a percentage of signaling of that receptor by v-*sis*. *A*, *B*, and *C* each represents a separate transfection experiment.

added to generate polyL-VIIIFIV were primarily responsible for conferring high specificity to 41-5, although Met-15 and/or Val-18 may also play a minor role.

The isoleucines at positions 25, 27, and 29 were introduced one at a time into polyL-VIFV to identify the residues responsible for increased specificity. All three of these single isoleucine add-back mutants transformed C127 cells efficiently (Fig. 3*B*) and induced signaling by the wild-type PDGFβR (Fig. 7*C*). Addition of Ile-25 or Ile-29 to polyL-VIFV did not affect specificity (Fig. 7*C*). In marked contrast, introduction of Ile-27 into polyL-VIFV eliminated its ability to activate most of the tested PDGFβR TM point mutants and generated a protein displaying a pattern of activity nearly identical to polyL-VIIIFIV (Fig. 7*C*). These results demonstrated that one conservative substitution, isoleucine for leucine at position 27, in combination with residues required for activation of the wild-type PDGFβR, was sufficient to confer a highly specific phenotype. Notably, this substitution controlled the ability of polyL-VIFV+I27 to induce signaling by mutant PDGFβRs with substitutions along the length of their TM domains, suggesting that this residue influenced the overall structure and specificity of the small TM protein.

Conclusions

This study identified the importance of packing interactions in determining the specificity of heteromeric TM helix–helix interactions. Some proteins lacking hydrophilic residues in the TM domain activated the PDGFβR with exquisite specificity, as assessed by their inability to activate numerous receptor point mutants that were activated by the E5 protein. We conclude that productive interactions between helices unable to participate in strong hydrophilic interactions may require multiple weak but highly specific van der Waals contacts along the length of the TM domain, and that loss of even a single crucial contact is sufficient to abrogate receptor activation. Interestingly, several 41 mers containing different TM sequences displayed increased specificity compared with the authentic E5 protein (unpublished results), implying there are multiple ways to generate highly specific interactions with the same target TM sequence. Furthermore, the residues responsible for activation of the wild-type PDGFβR are distinct from the residues that confer high specificity. Strikingly, a highly specific phenotype could be conferred by replacing leucine 27 with an isoleucine in a polyleucine TM domain containing a simple motif that allowed activation of the wild-type PDGFβR. Although the leucine and isoleucine sidechains are both nonpolar and have similar volumes, isoleucine, a β-branched amino acid, has less rotational freedom and may generate a TM helix with fewer possible packing modes and diminished entropic cost of engaging in heterodimer formation. Thus, a relatively conservative substitution in a small TM protein can contribute to specificity by energetically favoring the formation of multiple interhelical van der Waals contacts. This study sheds light on factors that influence TM interactions and indicates that it may be possible to select or rationally design small TM proteins and hydrophobic peptides that can discriminate between closely related TM targets. The expression of such proteins in cells or the insertion of such peptides into cell membranes may be a new approach to modulate the activity of cellular TM proteins in a highly specific manner.

Materials and Methods

Library Construction. The JBF13 library of 44-aa proteins containing random exclusively hydrophobic TM domains was generated by PCR by using a degenerate oligonucleotide as template [see supporting information (SI) *Text*]. The upstream oligonucleotide consisted of a fixed 5′ end corresponding to the first 13 codons of the wild-type E5 sequence, followed by 20 NTS codons (where N is an equal mix of A, T, C, and G, and S is an equal mix of C and G), and then by a fixed end composed of the 3′ E5 sequence containing a mutation in codon 36 to encode alanine. The downstream oligonucleotide was composed of antisense sequence of the 3′ end of the E5 gene containing the complement to the mutation at codon 36. The two oligonucleotides were annealed and extended by using Pfu turbo polymerase (Invitrogen, Carlsbad, CA) to generate double-stranded products, which were subcloned into the RVY retroviral vector backbone. After transformation of DH10β bacteria, maxiprep DNA was prepared from pooled ampicillin-resistant colonies for packaging into retrovirus. The number of ampicillin-resistant colonies generated by dilutions of the culture was used to estimate the number of independent sequences in the library.

Cell Lines, Virus Stocks, and Tissue Culture. C127, CV1, and 293T cells were maintained as described (23). Retroviral stocks pseudotyped with VSV G protein were prepared in 293T cells and concentrated (see *SI Text*).

Selection and Recovery of Transforming Library Proteins. C127 cells were infected with concentrated stocks of the JBF13 library and incubated at confluence for 3 wk to select for focus formation. Library inserts were cloned from genomic DNA isolated from individual foci, and focus-forming activity of individual clones was determined as described (24). Cell lines were established from pools of drug-resistant colonies established with individual clones.

PDGFβR Inhibitor Assay. C127 cells expressing the controls or small TM proteins were plated at ≈60% confluence in 12-well plates and incubated in DMEM-10 with or without 50 μM AG1295 (Calbiochem, San Diego, CA). Media were changed every 2–3 days. Cells were photographed after 7 days.

Biochemical Analysis. C127 cells were serum starved for 24 h and then washed and lysed in either modified RIPA buffer or CHAPS buffer supplemented with protease and phosphatase inhibitors, and immunoprecipitation and immunoblotting were carried out as described with minor modifications (26) (see *SI Text*). The following antibodies were used: rabbit antiserum against the C-terminal amino acids of the human PDGFβR, rabbit antiserum against the constant 16 C-terminal amino acids of the E5 protein, and antiphosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology, Lake Placid, NY). CV1 cells were infected with high-titer LXSN retrovirus stocks expressing wild-type PDGFβR, βαβ, or various TM point mutants. After selection in G418, resistant colonies were pooled, infected with high-titer retrovirus stocks of empty vector RVY or RVY expressing the E5 protein, or 41-5, incubated for 7 days, and harvested in CHAPS buffer for phosphotyrosine immunoblotting.

Transient Luciferase Reporter Assay. CV1 cells in 24-well plates were cotransfected with the pRL-SV40 plasmid expressing *Renilla* luciferase; the pSIE3-luciferase reporter construct; an LXSN-based plasmid encoding the wild-type or mutant PDGFβR; and an RVY-based plasmid encoding v-*sis*, the E5 protein, or a small TM protein (see *SI Text*). Luciferase activity was measured 48 h later. All transfections were done in triplicate, and the results were averaged and normalized to the expression of the *Renilla* luciferase transfection control.

We thank Lara Ely-Bowers for essential reagents, Don Engelman for helpful discussions, Stacy Horner for assistance in preparing figures, members of the D.D. laboratory for critical review of this manuscript, and Jan Zulkeski for assistance with manuscript preparation. This work was supported by National Institutes of Health Grant CA37157 (to D.D.).

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