A Single Amino Acid Substitution Converts a Transmembrane Protein Activator of the Platelet-derived Growth Factor β Receptor into an Inhibitor

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Receptors for PDGF play an important role in cell proliferation and migration and have been implicated in certain cancers. The 44-amino acid E5 protein of bovine papillomavirus binds to and activates the PDGF β receptor (PDGFβR), resulting in oncogenic transformation of cultured fibroblasts. Previously, we isolated an artificial 36-amino acid transmembrane protein, pTM36-4, which transforms cells because of its ability to activate the PDGFβR despite limited sequence similarity to E5. Here, we demonstrated complex formation between the PDGFβR and three pTM36-4 mutants: T21E, T21Q, and T21N. T21Q retained wild type transforming activity and activated the PDGFβR in a ligand-independent manner as a consequence of binding to the transmembrane domain of the PDGFβR, but T21E and T21N were severely defective. In fact, T21N substantially inhibited E5-induced PDGFβR activation and transformation in both mouse and human fibroblasts. T21N did not prevent E5 from binding to the receptor, and genetic evidence suggested that T21N and E5 bind to nonidentical sites in the transmembrane domain of the receptor. T21N also inhibited transformation and PDGFβR activation induced by v-Sis, a viral homologue of PDGF-BB, as well as PDGF-induced mitogenesis and signaling by preventing phosphorylation of the PDGFβR at particular tyrosine residues. These results demonstrated that T21N acts as a novel inhibitor of the PDGFβR and validated a new strategy for designing highly specific short transmembrane protein inhibitors of growth factor receptors and possibly other transmembrane proteins.

Many aspects of cell behavior are regulated by cell surface receptors. Receptors for PDGF are transmembrane tyrosine kinases, which initiate signaling pathways that affect the proliferation, motility, and survival of fibroblasts, vascular smooth muscle cells, capillary endothelial cells, and neurons. There are two different forms of the PDGF receptor, α and β, which differ in their ligand binding affinities and downstream signaling effects (1). When PDGF binds to the extracellular domains of two receptor molecules, it promotes receptor dimerization, which in turn results in autophosphorylation of key tyrosine residues in the cytoplasmic domain of the receptor (2). These phosphorylated tyrosines then recruit specific signaling or adaptor proteins containing Src homology 2 (SH2) domains to the receptor (3). Once bound to the receptor, these proteins are phosphorylated and initiate intracellular signaling cascades culminating in cell proliferation, migration, or survival.

Uncontrolled activation of PDGF receptors has been associated with several cancers including glioblastomas, fibrosarcomas, hematological malignancies, and gastrointestinal stromal tumors (reviewed in Ref. 4). Receptor activation in these tumors is typically driven by activating mutations in the receptor or by an autocrine mechanism in which the receptor and/or ligand is overexpressed. In addition, PDGF produced by tumor cells can act in a paracrine manner and promote the proliferation of tumor blood vessels and stromal cells, which can contribute to tumor growth. Therefore, understanding how PDGF receptors and their downstream signaling pathways are regulated may allow the design of novel therapies for these cancers.

Studies of the 44-amino acid bovine papillomavirus (BPV) E5 oncoprotein demonstrated that the PDGFβ receptor (PDGFβR) could be modulated by proteins that target its transmembrane domain. E5 is a dimeric transmembrane protein that interacts with the transmembrane domain of the PDGFβR and induces its activation by promoting receptor dimerization (5–10). Sustained activation of the PDGFβR by E5 results in tumorigenic transformation

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†1 This article contains supplemental Figs. S1–S3.

2 The abbreviations used are: SH2, Src homology 2; BPV, bovine papillomavirus; PDGFβR, PDGFβ receptor; HFF, human foreskin fibroblast; TPR, truncated human PDGFβR.
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of mouse fibroblasts and partial transformation of mortal human fibroblasts (10–12). Extensive mutational analysis combined with molecular modeling suggested that the two proteins interact through a salt bridge between Asp³³ of E5 and Lys⁹⁹ of the PDGFβR, hydrogen bonding between Gln¹⁷ of E5 and Thr²¹² of the PDGFβR, and presumably packing interactions (8, 13–21). In addition, covalent dimerization of E5 mediated by two C-terminal cysteine residues is required for interaction with the receptor (18, 22), although the E5 transmembrane domain itself has intrinsic dimerization potential (23, 24).

By screening retroviral libraries expressing hundreds of thousands of small proteins with randomized transmembrane domains, we identified artificial small transmembrane proteins with limited sequence identity to E5 that activate the PDGFβR and transform cells (25–28). One such 36-amino acid protein, pTM36-4, possessed a polyhistidine tag at the N terminus followed by 7 consecutive amino acids of E5 and a random 19-amino acid sequence consisting mostly of hydrophobic residues (see Fig. 1A) (25). Importantly, this protein lacked the specific amino acids of E5 that are required for activation of the PDGFβR (namely Gln¹⁷, Asp³³, Cys⁷⁷, and Cys³⁹), indicating that there are multiple different avenues to generate small transmembrane activators of the PDGFβR. pTM36-4 induced morphologic transformation and focus formation of C127 mouse fibroblasts and growth factor independence in BaF3 cells in a manner that required the transmembrane domain of the PDGFβR (25). Although pTM36-4 was able to homodimerize, we were unable to detect a physical interaction between pTM36-4 and the PDGFβR. Nevertheless, we speculated that pTM36-4, like E5, binds to the transmembrane domain of the PDGFβR.

By analogy to Gln¹⁷ in E5, we postulated that one of the hydrophilic amino acids in the transmembrane domain of pTM36-4 might interact with Thr²¹² in the PDGFβR. In this study, we showed that Thr²¹ is required for pTM36-4 activity and that replacing this residue with a large hydrophilic residue allowed us to detect a specific physical interaction between the pTM36-4 mutants and the transmembrane domain of the PDGFβR. One of these mutants, T21N, was not only severely defective for cell transformation, but actually inhibited PDGFβR activation and its associated signaling, mitogenic, and transforming effects. This study is the first to report a transmembrane protein inhibitor of the PDGFβR and establishes the feasibility of a genetic approach to isolate similar inhibitors of growth factor receptors and possibly other transmembrane proteins.

EXPERIMENTAL PROCEDURES

**Cells and Cell Culture**—C127 murine fibroblasts and human 293T cells were maintained in DMEM supplemented with 10% FBS, 2 mM l-glutamine, 20 mM Heps, and penicillin-streptomycin as antibiotics (DMEM-10). Early passage normal diploid human foreskin fibroblasts (HFFs) were obtained from the Yale Skin Diseases Research Center and maintained in DMEM-10 or minimal essential α-medium supplemented with 10% FBS and antibiotics (MEM α-10). Murine BaF3 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 7% WEHI-3B cell-conditioned medium (as a source of IL-3), 0.05 mM β-mercaptoethanol, 0.5 μg/ml amphotericin B, and antibiotics.

**Plasmid Constructs and Mutagenesis**—The original pTM36-4 ORF was isolated in the pT2H-F13 retroviral plasmid (25). We inserted an in-frame C-terminal HA tag (YPYDVPDYA) onto pTM36-4 in the MSCVhyg retroviral plasmid. T21L, T21A, and T21N substitutions were introduced into the original pTM36-4-p2TH construct by site-directed mutagenesis using the QuikChange method (Agilent Technologies), and T21Q, T21E, and T21N substitutions were introduced into the pTM36-4-HA-MSCVhyg construct. HA-tagged T21Q, T21N, and T21E were also subcloned into the MSCVpuro vector for expression in BaF3 cells. A codon-optimized version of the T21N-HA mutant was cloned into the pBabe puro retroviral vector and designated T21N-HA* (details available from authors on request). pBabe puro constructs expressing E5 or v-Sis and the rat Neu*-LXSN retroviral construct were described previously (11), as was the RV-HYG R (RVY) retroviral vector expressing E5 (29). LXSN constructs expressing wild type or mutant (I506A and T513L) PDGFβR were generated previously (8, 16). The S516L PDGFβR mutant in LXSN was created by site-directed mutagenesis. The ßβ chimera receptor was expressed from LXSN and contains the transmembrane region of the human PDGFα receptor (amino acids 524–528) flanked by extracellular and intracellular domains of the murine PDGFβR (25). A truncated form of the human PDGFβR (TPR), which lacks most of the extracellular domain (amino acids 38–530) was expressed from LXSN and described previously (30).

**Stable Expression of Foreign Genes in Cells**—Recombinant retrovirus was produced by co-transfecting 293T cells with a retroviral plasmid encoding the gene of interest and the packaging plasmids pCL-Eco and pVSVG, which encodes the vesicular stomatitis virus G protein (Imgenex), as previously described (25). Retrovirus was used to introduce pTM36-4, PDGFβR, E5, v-Sis, or their derivatives into C127, HFFs, or BaF3 cells as described (11, 15). After 7–10 days of drug selection, cell lines stably expressing the desired transgene were established. Cells co-expressing two transgenes were established by sequential retroviral infection and selection.

**Focus Forming Assay**—Parental C127 cells or HFFs or C127 cells stably expressing T21N, T21N-HA, T21E-HA, or control retrovirus were seeded in 60-mm dishes. When the cells reached 60–85% confluence, they were infected with control or recombinant retrovirus expressing pTM36-4 or its derivatives, E5, v-Sis, or Neu* at a low multiplicity of infection in the presence of 4 μg/ml Polybrene. The next day, the cells were split 1:3 or 1:2 and then maintained at confluence with bi-weekly medium changes using DMEM-10 for C127 cells and MEM α-10 for HFFs. At 2–3 weeks after infection, cell monolayers were fixed in methanol and stained with a 5% dilution of a modified Giemsa solution (Sigma-Aldrich) for the visualization of foci. For the experiments shown in Fig. 1B and supplemental Fig. S1, the number of stained foci was normalized for virus titer (determined by counting drug-resistant colonies in parallel cultures).

**IL-3 Independence Assay**—To determine whether BaF3-derived cell lines could proliferate in the absence of IL-3, 5 × 10⁵ cells were washed twice in PBS and then resuspended in 10 ml
of RPMI medium containing 1% FBS, 0.05 mM β-mercaptoethanol, and antibiotics but lacking IL-3. Cells were then transferred to a T25 flask and incubated at 37 °C. At various times thereafter, live cells were counted using a hemacytometer.

DNA Synthesis Assay—C127 cells expressing T21N-HA* or control cells harboring the pBABE-puro vector were seeded into a 24-well dish at 3 × 10⁴ cells/well. After reaching confluence, the cells were starved by replacing the medium with serum-free DMEM. Two days later, the medium in triplicate wells was replaced with DMEM (untreated) or DMEM containing 2.5 or 5 ng/ml PDGF-DD (R&D Systems) or 10% or 2% FBS. Approximately 24 h later, the medium was replaced with DMEM containing 1.5 μCi/ml [³H]thymidine (PerkinElmer Life Sciences; specific activity, 70–90 Ci/mmol) in the presence or absence of PDGF-DD or FBS. Five to six hours later, cellular nucleic acid was precipitated by washing three times in cold 10% trichloroacetic acid and then solubilized by heating in 3% perchloric acid. Immune complexes were then precipitated using protein A-Sepharose beads, washed, and eluted in 2× Laemmli sample buffer as described previously (11). In Fig. 9B, radioimmunoprecipitation assay extracts were mixed 1:1 with 2× sample buffer and analyzed directly by immunoblotting.

For PDGF receptor, phosphotyrosine, p85-PI3K, or SHP-2 blotting, samples were boiled, electrophoresed on a SDS-7.5% gel, and transferred to nitrocellulose in transfer buffer (25 mM Tris base, 192 mM glycine, and 20% methanol) containing 0.1% SDS for 1.5 h at 100 V. For phospho-SHP-2, phospho-AKT or phospho-ERK1/2 blotting, samples were electrophoresed on a SDS-10% gel and transferred without SDS to 0.2-μm PVDF for 1 h. The blots were blocked in blocking buffer (5% nonfat dry milk in TBST (10 mM Tris-HCl, pH 7.4, 167 mM NaCl, 1% Tween 20)) for 1–2 h and incubated in primary antibody overnight. Anti-PDGF receptor, -E5, and -HA antibodies were diluted 1:250, 1:250, and 1:500, respectively, in blocking buffer. All other primary antibodies were diluted in blocking buffer according to the recommendations of the manufacturer. The blots were washed five times in TBST and incubated in a 1:7000–1:10,000 dilution of HRP-conjugated anti-rabbit or anti-mouse secondary antibody (Jackson ImmunoResearch Labs) or HRP-protein A (GE Healthcare). The blots were washed as above and then detected by ECL. In some cases blots were stripped using Restore Western blot stripping buffer (Thermo) and then reprobed with a different antibody.

RESULTS

pTM36-4 Requires Thr²¹ for Activity—pTM36-4, like BPV E5, requires the transmembrane domain of the PDGFβR for its transforming activity (25). Alignment of the amino acid sequences of pTM36-4 and E5 revealed that Thr²¹ in pTM36-4 aligns with the essential Gln¹⁷ in E5 (Fig. 1A), which is thought to hydrogen bond to Thr⁵¹³ of the PDGFβR (8, 13, 17). To test whether Thr²¹ is important for the transforming activity of pTM36-4, we used site-directed mutagenesis to replace Thr²¹ with alanine, leucine, glutamine, glutamic acid, or asparagine to generate T21A, T21L, T21Q, T21E, and T21N, respectively. These mutants were tested for their ability to induce focus formation in C127 cells. As shown in Fig. 1B, T21E and T21N were nearly completely defective for inducing focus formation. The T21A and T21L mutants were also defective (supplemental Fig. S1A). In contrast, T21Q was ~80% as active as pTM36-4. These results indicated that Thr²¹ of pTM36-4, like Gln¹⁷ of E5, plays an essential role in its transforming activity.

We also tested whether the Thr²¹ mutants activated the PDGFβR. Tyrosine phosphorylation of the endogenous PDGFβR was assessed in C127 cells stably expressing wild type pTM36-4, one of the Thr²¹ mutants, or no transgene (vector control). PDGF receptors were immunoprecipitated from cell extracts and then subjected to immunoblotting using an anti-phosphotyrosine antibody. Like E5, wild type pTM36-4 induced tyrosine phosphorylation of two forms of the PDGFβR, the mature, 190-kDa, cell surface form and a faster migrating, intracellular
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FIGURE 1. Role of Thr21 in pTM36-4 activity. A, amino acid sequences of pTM36-4 and the BPV E5 protein aligned according to the nonrandomized portion of pTM36-4 (shaded regions). The open box highlights Thr21 of pTM36-4 and Gln17 of E5. B, focus forming activities of Thr21 mutants of pTM36-4. The ability of wild type and mutant pTM36-4 to induce focus formation in C127 cells was assessed. The graph shows the results of multiple trials expressed as the percentage of the number of foci relative to that induced by wild type pTM36-4 (corrected for virus titer), with standard error of the mean. C, Thr21 mutants differ in their ability to induce tyrosine phosphorylation of the PDGFβR. The PDGFβR was immunoprecipitated from extracts of C127 cells stably expressing wild type pTM36-4, the indicated Thr21 mutant, or empty vector. Immunoprecipitates were subjected to anti-phosphotyrosine (PY) or anti-PDGF receptor (PR) immunoblotting, as indicated. D, ability of the T21Q and T21N mutants to cooperate with the PDGFβR to induce IL-3 independence in BaF3 cells. The murine PDGFβR was stably expressed with T21Q, T21N, or empty vector in BaF3 cells. Cells were then cultured in the absence of IL-3 for 7 days, and viable cells were counted. The graph shows results from two trials expressed as the percentage of the number of live cells relative to that in T21Q-expressing cultures, with standard error of the mean. E, T21Q but not T21N activates the PDGFβR in BaF3 cells. PDGFβR immunoprecipitates from extracts of BaF3 cells expressing the murine PDGFβR with T21Q, T21N, or empty vector were immunoblotted using an antibody recognizing phosphorylated Tyr857 of the PDGFβR (PY-857). The blot was then stripped and reprobed for total PDGF receptor (PR). In C and E, the numbers on the left indicate the size of molecular mass markers in kilodaltons, and arrows point to the mature (m) and precursor (p) forms of the PDGFβR. C-terminally HA-tagged pTM36-4 constructs were used in all experiments shown.

precursor form (Fig. 1C). For the E5 protein and its mutants, tyrosine phosphorylation of the precursor form of the receptor is a reliable measure of receptor activation (18, 31). Compared with wild type pTM36-4, the T21N and T21L mutants were impaired in their ability to induce tyrosine phosphorylation of both mature and precursor forms of the PDGFβR, whereas the T21E and T21A mutants were defective for inducing tyrosine phosphorylation of primarily the precursor (Fig. 1C and supplemental Fig. S1B). In contrast, T21Q induced robust tyrosine phosphorylation of the PDGFβR precursor (Fig. 1C), presumably accounting for the transforming activity of this mutant. These results indicated that the ability of pTM36-4 to activate the PDGFβR requires a threonine or glutamine at position 21.

We also assessed the activity of two of the hydrophilic Thr21 mutants, T21N and T21Q, in BaF3 cells, hematopoietic cells that do not express endogenous PDGF receptors. These cells require IL-3 for growth, but exogenous expression of the PDGFβR and an activating protein such as E5, pTM36-4, or the PDGF-B homologue v-Sis can substitute for IL-3 to induce proliferation (25, 30). Here, we expressed the murine PDGFβR in the presence or absence of T21N or T21Q in BaF3 cells and then assayed the cells for IL-3-independent growth. T21Q but not T21N was able to cooperate with the PDGFβR to confer substantial IL-3-independent proliferation of BaF3 cells (Fig. 1D). Moreover, in BaF3 cells, T21Q but not T21N induced substantial phosphorylation of the PDGFβR at Tyr857, which is required for optimal receptor kinase activity (3) (Fig. 1E). Thus, T21Q but not T21N was able to activate the PDGFβR to induce growth of these cells, consistent with the activities of these proteins in C127 cells.

Thr21 Mutants Form a Stable Complex with the PDGFβR—Because the transmembrane domain of the PDGFβR is specifically required for pTM36-4 activity, it seemed likely that pTM36-4 either directly or indirectly binds to the transmembrane domain of the receptor (25). However, co-immunoprecipitation analysis failed to detect an interaction between pTM36-4 and the PDGFβR. We postulated that the interaction between pTM36-4 and the PDGFβR is not stable enough in detergent extracts to be detected by this method and that replacing Thr21 with a larger hydrophilic amino acid might strengthen the interaction, thereby allowing its detection. Therefore, we assessed the ability of the hydrophilic substitution mutants of pTM36-4 to form a complex with the PDGFβR.

To perform co-immunoprecipitation analysis, we took advantage of the influenza HA epitope tag inserted at the C terminus of pTM36-4, T21E, T21N, and T21Q. Detergent extracts were prepared from C127 cells stably expressing HA-tagged pTM36-4, T21E, T21N, or T21Q or from control cells harboring an empty expression vector. Wild type or mutant pTM36-4 was immunoprecipitated using an antibody recognizing phosphorylated Tyr857 of the PDGFβR (PY-857). The blot was then stripped and reprobed for total PDGF receptor (PR). In Fig. 2A, the intracellular precursor form of the PDGFβR co-precipitated with T21Q-HA but not with wild type pTM36-4-HA (compare lanes 2 and 5). No PDGFβR co-precipitated if the HA antibody was blocked with a peptide
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FIGURE 2. Interaction of pTM36-4 mutants with the PDGFβR. A, T21E, T21N, and T21Q pTM36-4 mutants form a stable complex with the precursor form of the PDGFβR in C127 cells. Extracts of C127 cells expressing HA-tagged wild type pTM36-4, the indicated Thr21 mutant, or empty vector were immunoprecipitated with an anti-HA antibody, and the immunoprecipitates were subjected to anti-PDGFR receptor (PR) or anti-HA immunoblotting, as indicated. In lane 6, the HA antibody was preincubated with a peptide comprising the HA epitope. B, BaF3 cells expressing mPRβ or the βαβ chimeric receptor with T21Q-HA (black bars), v-Sis (light gray bar, tested in βαβ-expressing cells only), or empty vector (dark gray bars) were assessed for IL-3-independent growth. The graph shows results from two trials expressed as the percentage of five cells relative to cells present in pTM36/H9252-HA-expressing cultures, with standard error of the mean. C, extracts of BaF3 cells co-expressing the wild type murine PDGFβR (mPRβ) or the βαβ chimeric receptor with wild type pTM36-4-HA, T21Q-HA, T21N-HA, or the empty retroviral vector were analyzed as in A. In A and C, numbers indicate the sizes of molecular mass markers in kilodaltons, and arrows point to the mature (m) and precursor (p) forms of the PDGFβR.

comprising the HA epitope (Fig. 2A, lane 6) or if the cells did not express an HA-tagged protein (Fig. 2A, lane 1). Thus, the presence of the PDGFβR in T21Q-HA immunoprecipitates is not due to nonspecific binding of the receptor to the HA antibody or beads. Instead, these results indicated that T21Q and the precursor form of the PDGFβR exist in a stable complex. Similarly, the precursor form of the PDGFβR co-precipitated with T21E-HA and T21N-HA (Fig. 2A, lanes 3 and 4), showing that these mutants also formed a stable complex with the PDGFβR, even though they did not transform cells. We infer that wild type pTM36-4 also interacts with the PDGFβR in transformed cells and that the T21Q, T21E, and T21N mutations either stabilize the interaction or facilitate its detection in some other way.

To determine whether the transmembrane domain of the PDGFβR is required for the interaction with T21Q-HA and T21N-HA, we tested whether these mutants interacted with a chimeric receptor containing the extracellular and cytoplasmic domains of the PDGFβR and the transmembrane domain of the PDGFα receptor (25). First, the chimeric receptor (designated βαβ) or the wild type murine PDGFβR was expressed in BaF3 cells. The βαβ receptor was functional in these cells, because it was able to respond to v-Sis and induce IL-3-independent growth (Fig. 2B). Next, HA-tagged pTM36-4, T21Q, T21N, or empty vector was also expressed in these cells and then immunoprecipitated from detergent extracts with the HA antibody. PDGF receptor immunoblotting of the HA immunoprecipitates revealed that the wild type PDGFβR co-precipitated with T21Q-HA and T21N-HA but not with pTM36-4-HA, showing that T21Q and T21N form a stable complex with the PDGFβR in BaF3 cells as well as in C127 cells (Fig. 2C, lanes 3 and 7). In contrast, the βαβ receptor was not detected in T21Q-HA or T21N-HA immune complexes (Fig. 2C, lanes 6 and 8), indicating that this chimeric receptor could not form a stable complex with either T21Q or T21N, even though both proteins were abundantly expressed in these cells (Fig. 2C, bottom panels). Because the βαβ receptor differs from the wild type receptor by only the sequence of the transmembrane domain, these results indicated that the transmembrane domain of the PDGFβR is specifically required for a stable complex formation with T21Q and T21N. Consistent with these results, T21Q cooperated with the PDGFβR but not with the βαβ receptor to allow BaF3 cells to proliferate in the absence of IL-3 (Fig. 2B). Therefore, the transmembrane domain of the PDGFβR is required for T21Q to productively interact with the receptor.

Interaction between the PDGFβR and pTM36-4 Mutants Is Ligand-independent—To determine whether the interaction between the PDGFβR and T21Q or T21N requires the ligand-binding domain of the receptor, we assessed the ability of these mutants to interact with a truncated form of the human PDGFβR, TPR, which lacks most of the extracellular domain (30). TPR or the wild type human PDGF receptor was expressed with or without HA-tagged T21Q or T21N in BaF3 cells. PDGFβR immunoblotting showed that TPR was abundantly expressed in these cells and migrated as a doublet of ~75 kDa, which is the expected size of this truncated receptor (Fig. 3A, left panel). T21Q-HA induced abundant tyrosine phosphorylation of TPR, as assessed by anti-phosphotyrosine blotting of PDGFβR immunoprecipitates (Fig. 3A, right panel). Anti-HA immunoprecipitation followed by PDGFβR immunoblotting revealed that TPR co-immunoprecipitated with T21Q-HA as well as if not better than the wild type receptor (Fig. 3B). T21N-HA also co-immunoprecipitated with the truncated receptor, but detection of this interaction required the use of a codon-optimized version of T21N and a longer exposure time (Fig. 3B). Finally, T21Q-HA cooperated with TPR to induce robust IL-3-independent proliferation of BaF3 cells, whereas the transformation defective T21N was markedly impaired in this activity (Fig. 3C). Because T21Q was able to engage a receptor lacking the extracellular ligand-binding domain, its ability to productively interact with the PDGFβR must be ligand-independent.

Identification of PDGFβR Amino Acids Required for Interaction with pTM36-4 Mutants—To explore the basis for PDGFβR recognition by the pTM36-4 mutants, we next tested whether the same amino acids in the transmembrane domain of the PDGFβR were required for a stable interaction with the pTM36-4 mutants and E5. E5, T21Q-HA, or T21N-HA was co-expressed in BaF3 cells with PDGFβR mutants containing an IS506A, T513L, or S516L substitution in the transmembrane domain, and complex formation was assessed by anti-E5 or anti-HA immunoprecipitation followed by PDGFβR immuno-
blotting. Compared with the wild type PDGF/βR, the T513L mutant receptor was defective not only for binding to E5 as expected, but also for interacting with T21Q-HA and T21N-HA (Fig. 4A). This indicated that Thr513 in the PDGF/βR is required for the interaction with the pTM36-4 mutants, consistent with our proposal that position 21 of pTM36-4 is functionally equivalent to position 17 of E5. On the other hand, the I506A receptor mutant, which was defective for an interaction with E5 as previously reported (16), formed a stable complex with T21Q-HA and T21N-HA (Fig. 4A), suggesting that Ile506 plays a role in binding to E5 but not in binding to T21Q or T21N. Conversely, the S516L receptor mutant interacted with E5 but not with T21Q-HA or T21N-HA (Fig. 4A), suggesting that Ser516 plays a role in binding T21Q and T21N but not E5. Consistent with these data, T21Q cooperated with the I506A but not the S516L or T513L receptor mutant to confer IL-3-independent growth of BaF3 cells, whereas E5 cooperated with the S516L mutant but poorly with the other two receptor mutants (Fig. 4B). These results provide strong genetic evidence that the ability of E5 and T21Q to confer growth factor independence is dependent on their ability to bind to the transmembrane domain of the PDGF/βR. Furthermore, the PDGF/βR amino acid requirements for binding the pTM36-4 mutants and E5 differ, suggesting that pTM36-4 and E5 bind to overlapping but not identical sites in the transmembrane domain of the PDGF/βR.

T21N Inhibits E5-induced Focus Formation and Activation of the PDGF/βR in Fibroblasts—Although the T21E and T21N mutants interacted with the PDGF/βR, they were defective for inducing PDGF/βR tyrosine phosphorylation and cellular transformation, suggesting that these mutants interacted nonproductively with the receptor. If this is the case, the interaction of these mutants with the PDGF/βR might inhibit its ability to respond to E5 or PDGF.

To test whether T21N and T21E inhibited the activity of E5, we assessed their effect on E5-induced focus formation. We stably expressed T21N-HA and T21E-HA in normal HFFs and C127 cells and then infected these cells with control or E5-expressing retrovirus. Cells were maintained at confluence and monitored for the appearance of transformed foci. As expected, numerous foci appeared in naive HFFs and C127 cells when infected with the E5-expressing virus but not with the control virus (Fig. 5A). Strikingly, the E5 retrovirus induced few if any foci in HFFs or C127 cells expressing T21N-HA and T21E-HA. (These results are quantified in Fig. 5B.) Thus, T21N and T21E inhibited the ability of the E5 protein to induce focus formation in human and murine fibroblasts. Because T21N displayed greater inhibitory activity, the remainder of these studies focused on this mutant.

Next, we examined the effect of T21N on E5-induced activation of the PDGF/βR. The E5 gene was stably expressed in control and T21N-HA-expressing HFFs and C127 cells, and tyro-
sine phosphorylation of the receptor was assessed by PDGFβR immunoprecipitation followed by phosphotyrosine immunoblotting. As expected, the PDGFβR was abundantly tyrosine-phosphorylated in cells expressing E5 alone, but not in cells lacking E5 expression (Fig. 5C). T21N-HA expression caused a substantial reduction in E5-induced PDGFβR tyrosine phosphorylation in both cell types without affecting overall expression of the receptor (Fig. 5C). Thus, T21N inhibited the ability of E5 to induce tyrosine phosphorylation of the PDGFβR, as well as focus formation.

T21N Does Not Inhibit the Expression of E5 or the Interaction of E5 with the PDGFβR in Fibroblasts—To test whether T21N affects E5 expression, extracts were prepared from C127 cells expressing E5 with or without T21N-HA. The extracts were immunoprecipitated with an antibody that recognizes the E5 protein, and immune complexes were immunoblotted with the same antibody. As shown in Fig. 6A (middle panel), T21N expression did not inhibit E5 expression. Thus, decreased E5 expression is not responsible for the inhibition of E5-induced focus formation or PDGFβR tyrosine phosphorylation. In addition, probing the E5 immunoprecipitates with the HA antibody failed to detect T21N-HA (Fig. 6A, bottom panel, lane 4), suggesting that the E5 protein and T21N-HA do not exist in a stable complex. This conclusion was verified in the reciprocal experiment in which anti-HA immunoprecipitates did not contain the E5 protein, although they did contain T21N-HA and the PDGFβR precursor, as expected (supplemental Fig. S2, lane 8).

To test whether T21N-HA impairs the binding of the E5 protein to the PDGFβR, we compared the ability of E5 to co-immunoprecipitate with the PDGFβR in the presence and absence of T21N-HA. E5 was immunoprecipitated from extracts of control and T21N-expressing C127 cells, and E5 immune complexes were analyzed by PDGFβR immunoblotting. As expected, both mature and precursor forms of the receptor could be detected in E5 immunoprecipitates from extracts of cells expressing E5 alone but not of cells lacking E5 (Fig. 6A, upper panel, lanes 1 and 2). Abundant amounts of both forms of the receptor were also co-precipitated with E5 from cells that co-expressed T21N-HA (Fig. 6A, upper panel, lane 4). Similar results were observed in HFFs (data not shown). Therefore, T21N did not inhibit the ability of E5 to interact with the receptor. In fact, the amount of receptor present in E5 immune complexes was reproducibly greater in cells expressing T21N-HA (Fig. 6B).

T21N Inhibits v-Sis-induced PDGFβR Activation—Because T21N inhibits E5 from activating the PDGFβR without preventing E5 from associating with the receptor, we hypothesized that the inhibitory activity of T21N is not specific to E5 and that T21N would also inhibit the effects of the natural ligands of the PDGFβR. To test this possibility, we first determined whether T21N inhibited focus formation by v-Sis, the viral homologue of PDGF-BB (32, 33). C127 cells stably expressing T21N or control C127 cells harboring an empty vector were infected with retrovirus expressing E5, v-Sis, or Neu*, an activated receptor tyrosine kinase unrelated to the PDGFβR. Cells were maintained at confluence and monitored for the appearance of transformed foci. As shown in Fig. 7A, few foci were induced by the pBabe retrovirus lacking a transgene. As expected, the E5 retrovirus induced numerous foci in the control cells but not in cells expressing T21N (Fig. 7, A and B), confirming that T21N inhibited E5-induced focus formation. Strikingly, in cells expressing T21N, focus formation by v-Sis was inhibited by ~10-fold (Fig. 7, A and B). In contrast, the Neu*-expressing retrovirus induced similar numbers of foci in the control cells and cells expressing T21N, demonstrating that T21N did not

FIGURE 4. Identification of the PDGFβR amino acids required for an interaction with pTM36-4 mutants. A, extracts of BaF3 cells co-expressing the wild type mouse PDGFβR (mPRβ) or the indicated receptor mutants in the presence of T21N-HA, T21Q-HA, or E5 as indicated on the left, were immunoprecipitated with anti-HA or anti-E5 antibodies, as appropriate. Immunoprecipitates were then subjected to immunoblotting for the PDGFβR and the numbers indicate the sizes of molecular mass markers in kilodaltons. B, BaF3 cells co-expressing the wild type or indicated mutant PDGFβR with T21Q-HA (light gray bars), E5 (dark gray bars), or empty vector were assessed for IL-3-independent growth. The graph shows results from three trials expressed as the average percentage of live cells relative to that in mPRβ/E5-expressing cultures after deducting the number of live cells present in cultures expressing mPRβ with vector only. The error bars represent standard error of the mean.
inhibit the ability of C127 cells to undergo transformation. These results indicated that T21N specifically inhibits focus formation mediated by the PDGFβR.

The effect of T21N on v-Sis-induced PDGFβR activation was also assessed. As expected, the PDGFβR was heavily tyrosine-phosphorylated in cells expressing either E5 or v-Sis alone but not in the control cells lacking a transgene (Fig. 7C). In contrast, T21N substantially reduced PDGFβR tyrosine phosphorylation in cells expressing E5 or v-Sis without affecting the total level of the receptor (Fig. 7C). We also examined the recruitment of an important signaling substrate, the p85 regulatory subunit of PI3K, to the PDGFβR in response to v-Sis or E5. As shown in Fig. 7C (bottom panel), E5 or v-Sis increased co-immunoprecipitation of p85-PI3K with the PDGFβR in control cells, demonstrating that E5 and v-Sis caused the receptor to recruit this substrate. However, E5- or v-Sis-induced recruitment of p85-PI3K to the PDGFβR was eliminated in cells expressing T21N. Taken together, these results indicated that T21N inhibits v-Sis-induced activation of the endogenous PDGFβR in C127 cells.

**T21N Inhibits PDGF-induced PDGFβR Activation**—We next asked whether T21N inhibited DNA synthesis induced by soluble PDGF-DD. This form of PDGF binds specifically to the PDGFβR and not the PDGFe receptor (34, 35). To maximize expression levels of T21N, we constructed a codon-optimized version encoding T21N with a C-terminal HA tag (T21N-HA*). C127 cells stably expressing T21N-HA* or control cells harboring an empty vector were grown to confluence, serum-starved for 2 days, and then treated with PDGF-DD or FBS or left untreated. Approximately 24 h later, DNA synthesis was evaluated by measuring [3H]thymidine incorporation into DNA. As shown in Fig. 8A, treatment with 2.5 or 5 ng/ml of PDGF-DD
caused a substantially greater dose-dependent increase in DNA synthesis in control cells than in cells expressing T21N-HA*. In multiple trials, PDGF-DD-induced DNA synthesis in the T21N-HA*-expressing cells was on average 40–60% of that in the control cells, a statistically significant decrease (Fig. 8B). In contrast, FBS treatment induced DNA synthesis to a similar extent in control and T21N-HA*-expressing cells (Fig. 8). Thus, T21N specifically inhibits the DNA synthesis response to PDGF-DD.

Finally, we determined whether T21N inhibited tyrosine phosphorylation of the PDGFβR and/or its downstream signaling effectors in response to PDGF. Control and T21N-HA*-expressing C127 cells were either left untreated or treated with 2.5 ng/ml PDGF-DD. After 30 min, cells were lysed, and tyrosine phosphorylation of the PDGFβR was assessed as described above. As shown in Fig. 9 (top panels), PDGF-DD increased PDGFβR tyrosine phosphorylation to a similar level in the control and T21N-HA*-expressing cells. Thus, although T21N inhibited mitogenesis in response to PDGF, global PDGF-induced PDGFβR tyrosine phosphorylation was not inhibited by T21N. This result also implied that T21N-HA* did not impair expression of cell surface, PDGF-accessible PDGFβR. We next determined whether T21N affected PDGF-induced phosphorylation of specific tyrosine residues on the receptor, namely Tyr1009, a SHP-2-binding site (36, 37), and Tyr751, a PI3K-binding site (38, 39). For this purpose, phospho-specific antibodies...
that recognize these sites were used to probe immunoblots of PDGF receptor immunoprecipitates. Phosphorylation at the Tyr1009 site was induced to a similar level by PDGF treatment in both the control and T21N-HA*-expressing cells (Fig. 9A). In contrast, phosphorylation at the Tyr751 site was substantially reduced in the T21N-HA*-expressing cells with or without PDGF treatment. Accordingly, the amount of PI3K recruited to the PDGF/H9252R was also reduced in the T21N-HA*-expressing cells (Fig. 9A). Thus, T21N specifically inhibited phosphorylation of Tyr751 on the PDGF/H9252R and the subsequent recruitment of PI3K to the receptor.

To assess the consequences of this differential phosphorylation on PDGF/H9252R signaling, cell extracts were subjected to immunoblotting using phospho-specific antibodies recognizing the phosphorylated forms of AKT, ERK1/2, and SHP-2, downstream effectors in PDGF/H9252R signaling pathways. As shown in Fig. 9B, PDGF-DD induced similar levels of SHP-2 phosphorylation in the control and T21N-HA*-expressing cells, consistent with the analysis of Tyr1009 phosphorylation described above. In contrast, although PDGF-DD induced abundant phosphorylation of AKT and ERK1/2 in the control cells, it failed to induce substantial phosphorylation of these substrates in the T21N-HA*-expressing cells (Fig. 9B). The overall abundance of AKT, ERK1/2, and SHP-2 was not affected by T21N-HA*. T21N-HA* also inhibited the phosphorylation of specific tyrosine residues in the PDGF/H9252R and thus inhibits specific ligand-induced PDGF/H9252R signaling events, namely PI3K pathways involving AKT and ERK1/2 phosphorylation, that lead to cellular DNA synthesis.

**T21N Minimally Inhibits PDGF/H9252R Signaling in BaF3 Cells**—We also determined whether T21N inhibited the activity of E5...
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FIGURE 10. T21N minimally inhibits E5 activity in BaF3 cells. A, T21N-HA (dark gray bars) or empty MSCVpuro (light gray bars) retrovirus was expressed in BaF3-mPγR cells expressing either E5 or empty RY7 vector as indicated. IL-3-independent growth was assessed after 7 days. The graph shows results from two independent trials expressed as the percentage of live cells relative to that in mPγR/E5/MSCVpuro-expressing cultures, with standard error of the mean. B, extracts of BaF3-mPγR cells co-expressing T21Q-HA, T21N-HA, or empty vector were immunoprecipitated with anti-HA (top two panels) or anti-PγR (bottom panel) antibodies, and immunoprecipitates (IP) were immunoblotted for PDGFβR or HA as indicated. The numbers indicate the sizes of molecular mass markers in kilodaltons, and arrows point to the mature (m) and precursor (p) forms of the PDGFβR.

in BaF3 cells. As expected, E5 cooperated with the PDGFβR to induce IL-3-independent growth (Fig. 10A). Co-expression of T21N-HA with E5 inhibited this activity by ∼30% (Fig. 10A). Moreover, T21N did not inhibit IL-3 independence induced by v-Sis or PDGF (data not shown). To explore why T21N only minimally inhibited PDGFβR signaling in BaF3 cells in contrast to the dramatic inhibition in fibroblasts expressing endogenous PDGFβR, we compared the ability of T21N and T21Q to interact with the PDGFβR. Co-immunoprecipitation analysis revealed that substantially less receptor co-precipitated with T21N than with T21Q, even though T21N and T21Q were expressed at similar levels in these cells, as was the PDGFβR (Fig. 10B). This suggests that in BaF3 cells the interaction of T21N with the PDGFβR is greatly reduced compared with the interaction of T21Q with the receptor, in contrast to the situation in C127 cells where T21N and T21Q appear to bind to the receptor with similar efficiency (Fig. 2A). Therefore, the limited ability of T21N to inhibit the PDGFβR in BaF3 cells correlates with its reduced binding to the receptor in these cells.

DISCUSSION

We report that the residue at position 21 in the transmembrane domain of pTM36-4 determines its effect on the PDGFβR and its ability to transform cells. Although the T21Q, T21E, and T21N pTM36-4 mutants all interacted with the PDGFβR, only T21Q retained near wild type transforming activity, whereas T21E and T21N were severely defective. Strikingly, T21N inhibited activation of the PDGFβR by E5, v-Sis, and PDGF itself. Therefore, a single amino acid substitution converted a small transmembrane protein activator of the PDGFβR into an inhibitor.

Although we were not able to detect an interaction between pTM36-4 and the PDGFβR, complex formation between the precursor form of the PDGFβR and several pTM36-4 point mutants was readily detectable. Furthermore, the transmembrane domain but not the extracellular domain of the PDGFβR was required for this interaction and for T21Q activity, consistent with our previous analysis of pTM36-4 activity (25). These results strongly suggest that wild type pTM36-4 and the PDGFβR also exist in a stable complex, primarily in intracellular membranes. The T21Q, T21E, and T21N mutations may directly stabilize the interaction between pTM36-4 and the PDGFβR, thereby enabling detection by co-immunoprecipitation, or these mutations may increase the propensity of pTM36-4 to oligomerize, which may indirectly stabilize the complex with the PDGFβR or increase the number of receptor molecules bound to pTM36-4. Consistent with a possible effect on homo-oligomerization, the identity of the hydrophilic amino acid position 17 in the BPV E5 protein and a similar position in the Hendra virus F protein affects the ability of these proteins to self-associate via transmembrane domain interactions (13, 40). It is also possible that the residue at position 21 of pTM36-4, like Gln17 of E5, participates in both homo-oligomerization and binding to the PDGFβR.

Analysis of the transformation competent mutant, T21Q, indicated that pTM36-4 is functionally similar to E5. Like E5, pTM36-4 and T21Q activate the PDGFβR in a ligand-independent manner and require the transmembrane domain of the PDGFβR for activity. Also, T21Q is likely to localize to intracellular membranes, because it binds and activates the intracellular precursor form of the receptor. Finally, there was an absolute correlation between the ability of T21Q to bind to various PDGFβR mutants and to induce proliferation in BaF3 cells. Despite this functional similarity, the numerous sequence differences between pTM36-4 and E5 imply that they differ in the way they interact with the transmembrane domain of the PDGFβR. In fact, overlapping but distinct sets of amino acids in the receptor transmembrane domain are required for an interaction with the pTM36-4 mutants compared with E5: only the pTM36-4 mutants required Ser516, and only E5 required Ile506. Ser516 may hydrogen bond with the hydrophilic amino acid at position 21 or another hydrophilic amino acid in pTM36-4, such as Asn18, which is also required for focus forming activity.3

The T21N mutant not only failed to activate the PDGFβR, but it actually inhibited the cellular response to E5, v-Sis, and PDGF. Decreased tyrosine phosphorylation of the receptor and a reduction in the amount of PI3K recruited to the receptor provide biochemical evidence that T21N inhibited PDGFβR activation by E5 and v-Sis. Similarly, T21N specifically inhibited PDGF-induced phosphorylation of the PI3K-binding site (Tyr751) on the PDGFβR, recruitment of PI3K to the receptor, and phosphorylation of AKT and ERK1/2, signaling effectors of PI3K. However, T21N did not inhibit PDGF-induced phosphorylation of the SHP-2-binding site (Tyr1009) or of SHP-2 itself. The inhibition of the PI3K pathway most likely contributed to the T21N-mediated decrease in PDGF-induced DNA synthesis, because PI3K stimulates a number of pathways that contribute to mitogenesis. Although T21N inhibited global phosphorylation of the PDGFβR in response to E5 or v-Sis, both of which activate intracellular forms of the receptor, it inhibited phosphorylation of only certain key tyrosine residues on the cell surface form of the receptor that binds to PDGF. Because T21N

3 L. M. Petti, K. Talbert-Slagle, M. Hochstrasser, and D. DiMaio, unpublished results.
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binds primarily to the precursor form of the receptor, it may have a more potent inhibitory effect on PDGFβR than encounters E5 or v-Sis in intracellular membranes than it does on cell surface receptors.

Minor structural differences in the side chains at position 21 determine the activity of the pTM36-4 mutants. Even though glutamine differs from asparagine by only an additional methylene group, glutamine dictates that pTM36-4-T21Q is an activator of the PDGFβR, whereas asparagine converts it into an inhibitor. The amino acid at position 21 may differentially affect the way in which pTM36-4 self-associates, which may alter its conformation in the membrane and/or the formation of an active receptor complex, or it might directly influence the manner in which pTM36-4 interacts with the PDGFβR, which in turn may affect the proper positioning or activity of the receptor kinase domains. Previous studies of the EGF receptor family revealed that certain transmembrane domain interactions involved in receptor dimerization reposition the kinase domains to the specific orientation required for activation (41–43). Thus, threonine or glutamine but not asparagine or glutamic acid may contact the PDGFβR in a manner that induces the proper coupling between the transmembrane and kinase domains. Biochemical and biophysical analyses of T21Q, T21N, and T21E protein complexes may help determine the basis for these differences.

Our results provide insight regarding the mechanism by which T21N inhibits PDGFβR signaling. The ability of T21N to inhibit two completely different activators (E5 and v-Sis/PDGF), which bind to distinct sites on the receptor, suggests that T21N does not compete with E5 or PDGF for binding to the receptor. In fact, because T21N binds to a truncated PDGFβR lacking the ligand-binding domain, it must bind to a different domain of the receptor than PDGF. Similarly, different amino acids in the transmembrane domain of the PDGFβR were required for the interaction with T21N or E5, and T21N did not inhibit co-immunoprecipitation of the PDGFβR with E5, suggesting that T21N does not inhibit E5 action by displacing the viral protein. Finally, T21N and T21Q showed comparable binding to the PDGFβR in C127 cells, whereas T21N showed reduced binding in BaF3 cells, correlating with the limited ability of T21N to inhibit PDGFβR-mediated proliferation in BaF3 cells. This observation suggests that the ability of T21N to bind to the PDGFβR plays a role in the mechanism of inhibition.

The forms of receptor inhibited by T21N may also provide insight into the inhibitory mechanism. T21N binds primarily to the intracellular form of the PDGFβR, but it inhibits tyrosine phosphorylation of both mature and precursor forms in response to E5 or v-Sis, and it inhibits phosphorylation of the PI3K-binding site induced by PDGF, which activates only cell surface forms of the receptor. T21N might induce a change in the PDGFβR precursor that persists in the mature form even if T21N dissociates from the receptor, or T21N-bound intracellular receptors might selectively mobilize an inhibitory signal, which acts in trans on other PDGFβR molecules, including cell surface forms. Such a negative signal must be specific for the PDGFβR, because T21N did not inhibit focus formation induced by the activated Neu+ receptor or DNA synthesis induced by FBS. Furthermore, if T21N, like the E5 protein (6), binds only a small fraction of PDGFβR molecules, a trans signaling event could explain how T21N inhibited signaling in response to PDGF, which stimulates the vast majority of receptor molecules.

Several other observations also may be important clues regarding the mechanism(s) by which binding of T21N to the PDGFβR inhibits the response to several different ligands. Expression of T21N caused an increased amount of PDGFβR to co-immunoprecipitate with E5 (Fig. 6). T21N may cause the recruitment of excess receptor molecules into the E5 complex, thereby accounting for increased co-immunoprecipitation. In such an aberrant complex, the receptor kinase domains might be inappropriately positioned or sterically hindered. However, our inability to co-immunoprecipitate E5 and T21N suggests that both proteins are not simultaneously present in the same PDGFβR complexes. It is also interesting that the interaction between T21N and the exogenous PDGFβR was reduced in BaF3 cells, suggesting that a cellular protein, expressed at low levels in BaF3 cells, may be required for the interaction and the inhibitory activity of T21N. For example, complex formation between the T21N and PDGFβR might be facilitated by another protein that is bound by T21N, such as a transmembrane protein-tyrosine phosphatase. Such an interaction might recruit the phosphatase to the receptor, where it might catalyze inhibitory dephosphorylation globally or at specific sites. Finally, it is possible that T21N locks the PDGFβR in an inactive conformation that inhibits receptor autophosphorylation. For example, recent studies of EGF receptor and Neu suggest that certain transmembrane domain interactions play a role in receptor activation by altering the positioning of the cytoplasmic juxtamembrane domain such that it dissociates from an inhibitory interaction with lipids in the membrane (44–46). Similarly, T21N might alter the conformation of the transmembrane domain of the receptor to impose an inactive conformation on the PDGFβR. In any case, the mechanism by which T21N inhibits the PDGFβR is likely to involve complex, dynamic interactions of T21N with various receptor forms, and further analysis of this phenomenon may reveal additional interesting features of PDGFβR trafficking or metabolism.

The rational design of inhibitory proteins is challenging. In some cases, it is possible to convert a naturally occurring protein into a dominant-negative version by deleting a domain of the protein required for its normal activity. For example, deletion of the C-terminal kinase domain of the transforming growth factor-β receptor and insulin-like growth factor-I receptor gives rise to a dominant-negative truncated receptor, which binds and sequesters ligand (47, 48). This approach requires a detailed understanding of the structure-function relationships of the native protein. Here, we describe an alternative approach to construct artificial inhibitory proteins. We begin with the biological selection of a small transmembrane protein activator of the target of interest. Selections for activators are often more straightforward than selections for inhibitors and are sufficiently robust to screen large libraries encoding many proteins with totally randomized transmembrane domains. This activator can then be used as a starting point to introduce a limited number of substitutions, which can be
screened on a much smaller scale to identify those that convert the activator into an inhibitor. Our results suggest that the initial selection of the activator allows the isolation of a protein that binds to the target of interest and that the mutations introduced later subtly alter the interaction between the small protein and its target to change its activity.

In conclusion, we describe an artificial 36-amino acid transmembrane protein inhibitor of the PDGFβR. Further analysis of T21N may provide new insight into the activation and signaling mechanisms utilized by the PDGFβR. Ultimately, T21N could serve as a prototype for developing hydrophobic peptidomimetic inhibitors of the PDGFβR that could be used for therapeutic purposes. We constructed this inhibitor by introducing a single amino acid substitution into an activator, an approach that may be applicable to other growth factor receptors and transmembrane proteins.

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