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Activation of the PDGF β receptor by a persistent artificial signal peptide

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Abstract

Most eukaryotic transmembrane and secreted proteins contain N-terminal signal peptides that mediate insertion of the nascent translation products into the membrane of the endoplasmic reticulum. After membrane insertion, signal peptides typically are cleaved from the mature protein and degraded. Here, we tested whether a small hydrophobic protein selected for growth promoting activity in mammalian cells retained transforming activity while also acting as a signal peptide. We replaced the signal peptide of the PDGF β receptor (PDGFβR) with a previously described 29-residue artificial transmembrane protein named 9C3 that can activate the PDGFβR *in trans*. We showed that a modified version of 9C3 at the N-terminus of the PDGFβR can function as a signal peptide, as assessed by its ability to support high level expression, glycosylation, and cell surface localization of the PDGFβR, and it retains its ability to interact with the transmembrane domain of the PDGFβR and cause receptor activation and cell proliferation. Cleavage of the 9C3 signal peptide from the mature receptor is not required for these activities. However, signal peptide cleavage does occur in some molecules, and the cleaved signal peptide can persist in cells and

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Declaration of Interest

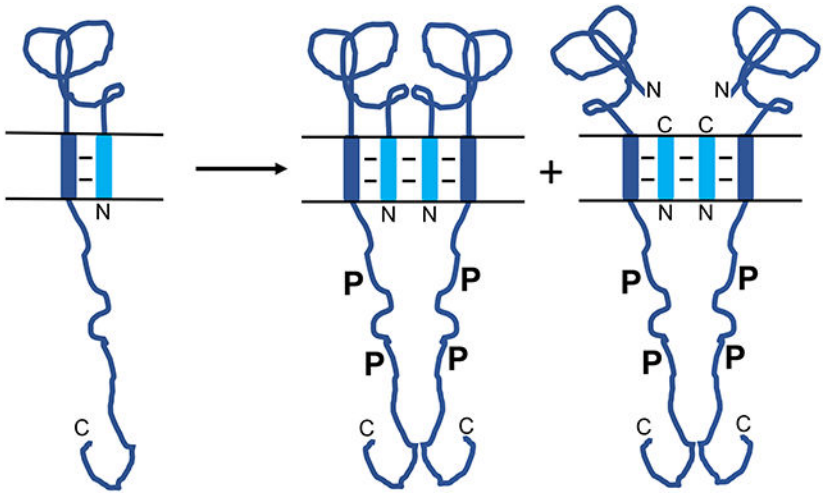
None.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

activate a co-expressed PDGFβR *in trans*. Our finding that a hydrophobic sequence can display signal peptide and transforming activity suggest that some naturally occurring signal peptides may also display additional biological activities by interacting with the transmembrane domains of target proteins.

Graphical Abstract



Keywords

Traptamer; Receptor tyrosine kinase; Transmembrane protein; E5 protein; Transmembrane domain

Introduction

Transmembrane (TM) proteins comprise up to 30% of all proteins in eukaryotic cells and serve critical roles in many biological processes [1]. For example, many receptors, transport channels, enzymes, and adhesion molecules are TM proteins. Most TM proteins are anchored in membranes by hydrophobic membrane-spanning segments known as TM domains (TMDs), which often adopt an α-helical conformation. In addition to this anchoring function, TM helices can undergo specific side-by-side interactions that mediate protein oligomerization, complex formation, and, in the case of proteins that span the membrane multiple times, proper folding [2].

The ability of TMDs to carry out such functions is illustrated most vividly by small biologically active TM proteins that lack any significant segments outside of the membrane. Many of these proteins, such as the bovine papillomavirus (BPV) E5 protein, are of viral origin [3]. E5 is a 44-amino acid TM protein that oncogenically transforms fibroblasts by binding directly to the TMD of the platelet-derived growth factor (PDGF) β receptor (PDGFβR) and promoting constitutive ligand-independent dimerization and activation of the receptor [4–11]. The PDGFβR is a type I TM protein, *i.e.*, it spans the membrane once and contains a cytoplasmic C-terminal domain. PDGFβR is typically activated when soluble PDGF binds to its extracellular domain [12]. PDGF binding induces receptor dimerization

and trans-phosphorylation of tyrosine residues in the cytoplasmic domain of the other monomer. The phosphorylated tyrosine residues serve as docking sites for other proteins, which are often subsequently phosphorylated by the receptor and trigger various signaling pathways, leading to cell proliferation, survival, and motility.

Highly specific artificial small TM proteins modeled on BPV E5, termed “traptamers” for *tra*nsmembrane *p*rotein *aptamers*, bind to the TMDs of cellular proteins and modulate their expression or activity. We have isolated and characterized traptamers that specifically target the PDGFβR, the erythropoietin receptor, or the chemokine and HIV receptor CCR5, as well as traptamers that inhibit infection by human papillomaviruses [13–20]. Some active traptamers have no amino acid sequence similarity to naturally occurring proteins and have an extremely simple hydrophobic amino acid composition [20–24].

Naturally occurring, small cellular TM proteins that function like BPV E5 or traptamers may be stably expressed in eukaryotic cells, but they have been largely overlooked because of their small size and unusual amino acid composition. For example, only open reading frames greater than 100 codons in length are typically annotated as protein-coding genes, and thus genes encoding smaller proteins are grossly underrepresented in genomic databases.

In considering possible sources of biologically active small TM proteins, we postulated that some signal peptides, N-terminal hydrophobic segments of the primary translation products of most TM and secreted proteins, might have activities in addition to their canonical role in protein translation and membrane targeting. The primary function of the signal peptide in eukaryotic cells is to target the nascent polypeptide to the endoplasmic reticulum (ER) membrane during translation so that the mature protein is anchored in the membrane or translocated into the ER lumen and eventually secreted [25–29]. During protein synthesis, the signal peptide emerges from the ribosome and binds to the signal recognition particle, which transports the nascent chain-ribosome complex to the ER membrane [30, 31]. The signal peptide then inserts into the ER translocation pore (the translocon) with its N-terminus facing the cytoplasm [32–36]. As translation proceeds, the signal peptide of secreted proteins and type I TM proteins such as the PDGFβR is cleaved from the rest of the protein by the ER-resident signal peptidase (SPase) and subsequently degraded [37, 38]. The newly synthesized protein is either released into the ER lumen or anchored in the membrane via a TMD. In addition, N-terminal hydrophobic domains of type II TM proteins and most multi-pass TM proteins such as G protein-coupled receptors serve as uncleaved “signal-anchor” or “stop-transfer” sequences that retain these proteins in the membrane [39, 40].

Signal peptides are typically 16–30 amino acids long and consist of three domains: a hydrophilic segment at the N-terminus, usually with a positive charge, a central hydrophobic segment, and a polar C-terminal segment that contains the SPase recognition site [41–44] (reviewed in [45]). The positive charge allows the signal peptide to bind to negatively charged RNA in the signal recognition particle, which then delivers the hydrophobic segment of the signal peptide to the translocon (reviewed in [46]). Mutations in the signal peptide, including loss of the positive charge, typically result in mislocalization and/or reduced expression of the secreted or TM protein [47–51]. The sequence requirements

for each of these domains are relatively permissive, leading to a high sequence diversity across known signal peptides [43]. It is possible that this sequence diversity in part reflects selection for different, currently unknown biological activities in addition to membrane insertion activity [46].

As well as playing a role in membrane targeting, several signal peptides appear to perform other functions. The signal peptides of the CD18 subunit of β2 integrins in ruminants [53] and a TM form of the prion protein [54] are not cleaved nor degraded, but it is unclear if these retained signal peptides have additional biological activities. A single nucleotide gain-of-function polymorphism in the human neuropeptide Y (NPY) signal peptide is associated with various metabolic abnormalities that could be recapitulated by injection of the mutant signal peptide into mice together with mature NPY [55], but the mechanism of this effect is not known. The signal peptide of class I human leukocyte antigens (HLA) undergoes proteolysis in the membrane and a peptide fragment binds to the extracellular domain of HLA-E and protect cells from immune attack [56, 57]. In addition, certain long signal peptides of viral glycoproteins are cleaved and remain in the membrane, where they associate with the mature protein in the virus particle (reviewed in [45]). For example, the signal peptide of glycoprotein C (GP-C) of arenaviruses such as Lassa virus and lymphocytic choriomeningitis virus is cleaved by SPase and associates with the remainder of GP-C, an interaction essential for the post-translational processing of GP-C into its mature subunits [45, 58–62]. Similarly, the signal peptide of the foamy virus envelope protein is cleaved by furin-like proteases and subsequently interacts with the envelope protein complex and affects virus egress [63–65].

Because many cellular signal peptides resemble traptamers in length, hydrophobicity, and at least transient membrane association, we wondered whether some signal peptides possess biological activities like those of traptamers. To begin to address this question, we tested whether a short, hydrophobic protein sequence inserted at the N-terminus of the PDGFβR could not only activate the receptor but also function as a signal peptide. We replaced the signal peptide of the PDGFβR with a modified version of 9C3, a previously described traptamer originally selected because it binds to the TMD of the PDGFβR and activates the receptor when expressed *in trans* as a free-standing protein [21]. We report here that modified 9C3 at an N-terminal position in the PDGFβR permits expression of the glycosylated receptor at the cell surface, thereby functioning as a signal peptide. However, unlike most signal peptides for type I TM proteins, 9C3 is not cleaved from a large fraction of PDGFβR molecules. Uncleaved 9C3 induces constitutive activation of the intracellular form of PDGFβR by interacting with the receptor TMD. In addition, the 9C3 signal peptide that is cleaved from receptor molecules can activate a co-expressed PDGFβR *in trans*. These results suggest that some naturally occurring signal peptides may similarly persist after cleavage and possess additional functions by interacting with the TMD of a target TM protein.

Results

Activation of the PDGF β receptor by replacement of its signal peptide with a traptamer

To test whether a hydrophobic oncoprotein would retain growth promoting activity while also functioning as a signal peptide, we replaced the N-terminal 27 amino acids of the murine PDGFβR with the 27 N-terminal amino acids of the 9C3 traptamer, a 29-amino acid stand-alone protein that binds to the TM domain of the PDGFβR, causing receptor activation [21] (Fig. S1A). In preliminary experiments, this chimeric receptor, designated 9C27-PR, was expressed at a much lower level than the wild-type PDGFβR (Fig. S1C). This result suggests that the signal peptide of the PDGFβR is required for proper receptor expression and that unmodified 9C3 cannot efficiently provide this activity. This is consistent with the observation that proteins with impaired signal peptide function are often poorly expressed [47, 48, 50, 51].

We next mutated the 9C3 segment to improve its ability to function as a signal peptide (Figs. 1a, 1b, and S1B). A lysine-glycine-glycine (KGG) sequence was inserted in-frame two amino acids after the initiating methionine to introduce an N-terminal positive charge. We also inserted the sequence glycine-proline-glutamine (GPQ) C-terminal to the 9C3 segment to fully reconstruct the native signal peptidase cleavage site. We refer to the full-length chimeric receptor with this doubly modified 9C3 segment at its N terminus as 9C-PR. Unlike 9C27-PR, 9C-PR was expressed at the same level as wild-type PDGFβR (Fig. S1D). To determine if the positive charge or improved SPase cleavage was responsible for the enhanced expression of 9C-PR, we separately removed the KGG sequence to construct 9C-GPQ or introduced two leucine substitutions into the SPase cleavage site of 9C-PR to generate 9C-LL (Figs. 1a, 1b, and S1B). These leucine substitutions are predicted to eliminate signal peptide cleavage [43, 66, 67]. Removal of the positive charge inhibited receptor expression (Fig. S1D, lane 5), whereas the mutations in the cleavage site did not (Fig. S1D, lane 4).

Taken together these results show that inserting KGG into the 9C3 segment at the signal peptide position, a mutation predicted to enhance signal peptide activity, allowed stable expression of the receptor in the absence of the native signal peptide. This finding implies that high level expression of the PDGFβR is dependent on signal peptide function, which can be provided by the modified 9C3 sequence at the N terminus of the receptor. All the rest of the experiments in this paper involve receptor constructs containing the inserted KGG sequence. To simplify the presentation, we refer to the segment consisting of the modified 9C3 sequences up to the SPase cleavage site but lacking the rest of the receptor as 9C-sp.

DNA encoding the different receptors was cloned into the LXSN retroviral vector and introduced by retroviral-mediated gene transfer into mouse BaF3 hematopoietic cells. These cells do not endogenously express the PDGFβR and are strictly dependent on IL-3 for their survival and proliferation, but expression of 9C3 or BPV E5 with the PDGFβR with allows the cells to grow in the absence of IL-3 or PDGF [21, 68]. As shown in Fig. 2, BaF3 cells transduced with the empty LXSN vector or the wild-type PDGFβR did not grow in the absence of IL-3 and PDGF, as expected. In contrast, cells transduced with tagged or untagged 9C-PR proliferated rapidly after five days of culture in the absence of growth

factors. To test whether cleavage of the 9C-sp segment is required for IL-3 independence, 9C-LL and its FLAG-tagged version, F-9C-LL, were tested. Both mutant receptors induced IL-3-independent growth in BaF3 cells to the same if not to a greater extent as parental 9C-PR (Fig. 2). These results show that modified 9C3 at an N-terminal position activates the PDGFβR and that cleavage of the signal peptide is not required for this activity.

Since the TMD of the PDGFβR is required for the transforming activity of the stand-alone 9C3 traptamer [21], we determined whether the PDGFβR TMD was also required for 9C-PR activity. To address this question, we first replaced the TMD of 9C-PR with the TMD of the PDGF α receptor, which does not respond to 9C3, to construct 9C-βαβ (Figs. 1a and 1b) [21]. As shown in Fig. 2, cells expressing 9C-βαβ did not grow in the absence of growth factors, suggesting that the TMD of 9C-PR is required for its activity. Since the 9C3 traptamer specifically requires the threonine in the middle of the TMD of the PDGFβR for receptor binding and activity (Figs. S2A and S2B), we introduced a threonine-to-leucine substitution at position 513 into F-9C-PR to construct F-9C-TL (Figs. 1a and 1b). As shown in Fig. 2, the T513L mutation abolished the ability of F-9C-PR to induce growth factor independence. When inserted into the PDGFβR, neither the PDGF α receptor TMD nor the T513L mutation impairs the response to PDGF [69, 70]. The requirement for this threonine confirms the importance of the PDGFβR TMD in the ability of 9C-sp to activate 9C-PR and demonstrates the high specificity of the 9C-sp segment.

9C3 signal peptide activates an immature form of PDGF β receptor

The expression and tyrosine phosphorylation of 9C-PR and its derivatives were also examined. BaF3 cells transduced with the various receptor constructs or the empty LXSN vector were lysed in detergent, and receptors were immunoprecipitated from protein extracts using an antibody recognizing an epitope at the C-terminus of the PDGFβR retained in all the constructs. Immunoprecipitated samples were analyzed by immunoblotting using the same PDGFβR antibody. As expected, the untagged and FLAG-tagged wild-type PDGFβR were detected as a slow migrating (~200kDa) form and a less abundant, faster migrating form, which are the mature receptor with complex carbohydrates and an intracellular precursor form with immature carbohydrates, respectively (Fig. 3a, top panels, lane 2) [8]. Tagged and untagged 9C-PR were each expressed as two forms with similar electrophoretic mobilities as wild-type PDGFβR, and their overall levels of expression were comparable to that of the wild-type receptor (Fig. 3a, top panels, lane 3). The cleavage site mutants, 9C-LL and F-9C-LL, showed a similar pattern of expression, although in both cases there was less of the mature receptor species, and the precursor form displayed a slightly faster electrophoretic mobility than the wild-type precursor (Fig. 3a, top panels, lane 4).

Anti-phosphotyrosine western blotting of receptor immunoprecipitates was performed to assess receptor activation in the absence of PDGF treatment. The rapidly migrating, presumed precursor forms but not the mature forms of 9C-PR, 9C-LL, and the corresponding FLAG-tagged versions showed elevated tyrosine phosphorylation compared to wild-type PDGFβR (Fig. 3a, bottom panels, compare lane 3 and 4 to lane 2). In contrast, there was little or no increase in tyrosine phosphorylation of either the mature or the precursor form of the 9C-βαβ chimeric receptor or the F-9C-TL mutant (Fig. 3a, bottom

panels, lane 5). Taken together, these results suggest that the precursor form of 9C-PR is constitutively activated and that its activity requires 9C-sp and the PDGFβR TMD but not signal peptide cleavage.

To verify that the faster migrating, tyrosine phosphorylated form of 9C-PR is a glycosylated precursor form, we tested its sensitivity to endoglycosidase H (Endo H), which removes high-mannose sugars from glycoproteins with immature carbohydrates. Receptors were immunoprecipitated from cell extracts, and immunoprecipitates were either left untreated or treated with Endo H and then subjected to SDS-PAGE and anti-PDGFβR immunoblotting. As shown in Fig. 3b (top panel), Endo H converted the faster migrating band of the wild-type PDGFβR, 9C-PR, and 9C-LL to an even faster migrating form, indicating that this band is indeed a form of the receptor with immature sugars. The slower migrating band was not affected by Endo H treatment, consistent with its assignment as the mature receptor with complex carbohydrates as reported previously (*e.g.*, [8]). Anti-phosphotyrosine blotting revealed that the Endo H-sensitive form of 9C-PR and 9C-LL was tyrosine phosphorylated (Fig. 3b, bottom panel, lanes 5 and 7). These results confirm that 9C-sp can direct 9C-PR into the Golgi apparatus for addition of high-mannose sugars and that the form of 9C-PR with immature carbohydrates is constitutively activated. The BPV E5 protein also preferentially activates the precursor form of the PDGFβR [8].

To determine whether 9C-PR was localized at the cell surface, we tested whether the mature form of 9C-PR was sensitive to trypsin. Intact BaF3 cells expressing FLAG-tagged wild-type PDGFβR, F-9C-PR, or LXSN were either treated with trypsin or left untreated and then lysed. Receptor levels were examined by PDGFβR immunoprecipitation followed by PDGFβR western blotting with the antibody recognizing the cytoplasmic epitope on the PDGFβR. As shown in Fig. 3c, in cells expressing either wild-type PDGFβR or F-9C-PR, trypsin treatment greatly reduced the levels of mature receptor and induced the appearance of faster migrating receptor cleavage products, whereas the precursor form, which is presumed to be intracellular, was not affected by trypsin. Susceptibility of the mature form of 9C-PR to trypsin digestion indicates that it is present at the cell surface, providing direct evidence that 9C-sp can act as a signal peptide that directs 9C-PR into the cell surface trafficking pathway.

The 9C signal peptide physically interacts with the PDGF receptor TMD

Since the 9C3 traptamer interacts with the TMD of the PDGFβR ([21]; Fig. S2A) and the induction of IL-3 independence by 9C-PR requires the PDGFβR TMD, it seems likely that 9C-sp also interacts with the PDGFβR TMD in 9C-PR. To test this biochemically, we introduced a specific protease cleavage site upstream or downstream of the TMD of 9C-PR and determined if the N- and C-terminal cleavage products associated with each other after they are delinked by proteolytic digestion. A PreScission™ protease cleavage site was introduced immediately upstream of the TMD of FLAG-tagged wild-type PDGFβR and F-9C-PR to generate sp-F-PR-X and F-9C-X, respectively (Figs. 4a and S3A). In sp-F-PR-X, the FLAG tag is downstream of the native signal peptide so that it is retained in the mature protein after signal peptide cleavage (Figs. 1b, 4a, and 4b). We also inserted the T513L mutation into the PDGFβR TMD of F-9C-X to generate F-9C-TL-X. Finally,

we inserted the PreScission protease cleavage site immediately downstream of the TMD of F-9C-PR to generate F-9C-DX (Figs. 4a and S3A). None of these proteins contains an endogenous PreScission™ cleavage site. F-9C-X and F-9C-DX induced IL-3 independence in BaF3 cells, showing that the PreScission™ cleavage site insertion did not inhibit biological activity, whereas as expected sp-F-PR-X and F-9C-TL-X did not induce IL-3 independence (Fig. S3B).

To determine receptor expression, BaF3 cells expressing the various receptors were lysed in detergent, and extracts were treated with PreScission™ protease or left untreated. Samples were then immunoprecipitated and immunoblotted with the anti-PDGF receptor antibody that recognizes an epitope on both the uncleaved receptor and the C-terminal PreScission™ cleavage products (Figs. 4b, 4c, and 4d). In the absence of PreScission™ protease treatment, the PDGFβR constructs that contain the upstream PreScission™ protease cleavage site co-migrated with the intracellular precursor form of the parental construct, and no mature form was detected (Fig. 4c, right panel, lanes 3, 5, and 7), suggesting that insertion of the cleavage site at this position prevents maturation of PDGFβR carbohydrates. In contrast, mature F-9C-DX was observed (Fig. 4d, lane 11), showing that insertion of the cleavage site downstream of the TMD did not prevent mature glycosylation. Treatment of each of these receptors with the PreScission™ protease resulted in the appearance of faster migrating bands with electrophoretic mobilities corresponding to the predicted molecular weights of the C-terminal cleavage products, indicated by arrows (Fig. 4c, right panel, lanes 4, 6 and 8; Fig. 4d, lanes 10 and 12). In addition, a substantial fraction of the receptor remained uncleaved in all cases. Thus, all constructs containing the inserted cleavage site were partially cleaved by PreScission™ protease.

To assess the association between the signal peptide and the TMD, we determined if the anti-FLAG antibody, which recognizes the FLAG tag on the N terminus of the receptors, immunoprecipitated the C-terminal cleavage product, with the rationale that the two fragments would co-precipitate if they associated non-covalently (see schematic in Fig. 4b). The C-terminal cleavage product was detected by immunoblotting with the PDGFR antibody recognizing this segment of the protein. Anti-FLAG antibody immunoprecipitated the uncleaved receptors in all cases, confirming that the FLAG tag is retained on at least some receptor molecules (Figs. 4c and 4d, left panels). Anti-FLAG immunoprecipitation of protease-treated F-9C-X also pulled down the C-terminal cleavage product (Figs. 4c and 4d, left panels, lane 4), whereas the C-terminal cleavage product of sp-F-PR-X, which lacks the 9C3 segment, was not co-immunoprecipitated (Fig. 4c, left panel, lane 6), suggesting that the N- and C-terminal cleavage products interact with each other in receptors containing 9C-sp but not in its absence. Moreover, the C-terminal cleavage product of the F-9C-TL-X mutant was not present in anti-FLAG immunoprecipitates (Fig. 4c, left panel, lane 8), indicating that threonine 513 in the receptor TMD is required for the interaction with 9C-sp.

Finally, we tested the ability of anti-FLAG to co-immunoprecipitate the C-terminal fragment of F-9C-DX, in which the protease cleavage site is downstream of the TMD (thereby removing the TMD from the C-terminal cleavage product) (Fig. 4b). Co-immunoprecipitation was markedly reduced compared to the corresponding fragment of F-9C-X, in which the TMD is present on the C-terminal cleavage product (Figs. 4b and

4d, left panel, compare lane 6 to lane 4). After normalization for the greater amount of the F-9C-DX C-terminal cleavage product detected in the anti-PDGFβR immunoprecipitate, co-immunoprecipitation between the N-terminal and C-terminal cleavage products was reduced by greater than 10-fold by moving the cleavage site from upstream of the TMD to downstream. Therefore, the interaction between the two cleavage products of F-9C-X requires both 9C-sp and a sequence between the upstream and downstream cleavage sites, which consists primarily of the TMD. Taken together, these results provide strong evidence that the interaction between the N- and C-terminal domains of 9C-PR is mediated by an interaction between specific sequences in 9C-sp and the PDGFβR TMD.

The 9C-PR signal peptide can act *in trans*

To determine whether 9C-sp could activate 9C-PR *in trans* by interacting with the TMD of a separate receptor molecule, we tested whether 9C-PR could activate a truncated PDGFβR (PRΔEX) containing a large in-frame deletion of the extracellular domain (Figs. 5a and 5b). Since PRΔEX retains the TM and cytoplasmic domains of the PDGFβR, it should interact with 9C-sp. PRΔEX was expressed alone in BaF3 cells or co-expressed with 9C-PR or with the wild-type PDGFβR as a control. Because of the deletion, western blotting with anti-PDGFβR detected PRΔEX as bands migrating above the 100-kDa marker (Fig. 5c, left panel, lane 2). Although the molecular weight of PRΔEX without post translational modification is predicted to be 82 kDa, the observed electrophoretic mobility is presumably due to glycosylation of the portion of the extracellular domain that is retained in the molecule. We next examined tyrosine phosphorylation of PRΔEX by performing PDGFβR immunoprecipitation followed by anti-phosphotyrosine western blotting. No tyrosine phosphorylation of PRΔEX was detected when it was expressed alone or with the wild-type PDGFβR (Fig. 5c, right panel, lanes 2 and 3). In contrast, co-expression of PRΔEX with 9C-PR resulted in substantial tyrosine phosphorylation of PRΔEX (as well as of the precursor form 9C-PR itself) (Fig. 5c, right panel, lane 4) without increasing PRΔEX expression (Fig. 5c, left panel, lane 4). (A background band with mobility similar to mature PDGFβR was detected by anti-phosphotyrosine immunoblotting in all samples including cells not expressing the receptor [asterisk, Fig. 5c, right panel, lanes 1 and 2]). Since 9C-PR and PDGFβR differ only in their signal peptide, this result shows that the signal peptide of 9C-PR can act *in trans* to induce tyrosine phosphorylation of PRΔEX.

To determine if the kinase activity of 9C-PR mediates phosphorylation of PRΔEX, we tested whether PRΔEX was activated by a 9C-PR mutant containing a lysine 602-to-arginine (K602R) mutation that inactivates its tyrosine kinase activity (designated 9C-KR in Figs. 5a and b). As expected, when expressed alone, 9C-KR was not tyrosine phosphorylated (see Fig. 7c, right panel, lane 4), demonstrating that 9C-PR activity requires a functional kinase domain. Strikingly, 9C-PR and 9C-KR induced the same level of tyrosine phosphorylation of PRΔEX (Fig. 5c, right panel, lanes 4 and 5), showing that the kinase activity of 9C-PR is not required for the ability of 9C-sp to activate a receptor molecule *in trans*. We note that 9C-KR itself was not phosphorylated in cells that express PRΔEX (Fig. 5c, right panel, lane 5), suggesting that 9C-sp does not assemble a 9C-KR/PRΔEX hetero-oligomeric complex in which PRΔEX phosphorylates 9C-KR. Rather, we propose that 9C-sp directly interacts with the TMD of PRΔEX and promotes its dimerization and trans-phosphorylation.

We also demonstrated the *in trans* activity of 9C-sp in an IL-3-independence assay. 9C-PR induced IL-3-independence of BaF3 cells in the presence and absence of PRΔEX (Fig 5d). As expected, neither PRΔEX nor 9C-KR alone induced IL-3 independence. However, co-expression of 9C-KR and PRΔEX conferred IL-3 independence, albeit to a lesser extent than induced by 9C-PR. Thus, 9C-sp can act *in trans* to activate PRΔEX to induce proliferation.

The 9C-PR signal peptide is cleaved from a fraction of molecules

We next used FLAG-tagged receptors to determine whether 9C-sp is cleaved from some molecules of the receptor (Fig. 6a). If the signal peptide with the N-terminal FLAG tag is not cleaved, anti-FLAG antibody would detect the full-length receptor on a western blot. In contrast, if the signal peptide is released and escapes degradation, the tag would be present on a small cleavage product. First, anti-FLAG immunoprecipitates from BaF3 cells expressing F-9C-PR or FLAG-tagged PDGFβR containing the wild-type signal peptide were subjected to anti-PDGFβR western blotting. Full-length PDGFβR was not detected in the anti-FLAG immunoprecipitates (Fig. 6b, lane 2) even though a substantial amount of this receptor is expressed (Fig. 6c, top panel, lane 2). Thus, the signal peptide is quantitatively cleaved off the PDGFβR, as expected (Fig. 6a, left diagram). In contrast, both the immature and the mature form of F-9C-PR were detected in the anti-FLAG immunoprecipitates (Fig. 6b, lane 4), indicating that the FLAG tag and 9C-sp are present on at least some F-9C-PR molecules (Fig. 6a, second diagram from left). Furthermore, most of mature FLAG-tagged 9C-PR was removed by trypsinization of intact cells, demonstrating that it is at the cell surface (Fig. 6b, lane 5).

We also performed anti-FLAG western blotting on receptor immunoprecipitates from cells expressing F-PR, F-9C-PR, or F-9C-LL. The anti-FLAG antibody detected the immature form of F-9C-PR and the F-9C-LL signal peptidase cleavage site mutant (Fig. 6c, middle panel, lanes 3 and 4), confirming that the signal peptide is retained on at least some of these receptors. The wild-type PDGFβR was not detected, consistent with the quantitative removal of the native signal peptide. (We could not assess the mature receptor in this experiment because a background band with mobility similar to mature PDGFβR was detected with the anti-FLAG antibody in all samples including cells not expressing the receptor [asterisk, Fig. 6c, middle panel, lane 1]). Taken together, these data indicate that 9C-sp remains uncleaved on at least some of the cell surface form of 9C-PR with mature carbohydrates as well as on the immature form. We note that the anti-FLAG antibody detected more of the immature form of the F-9C-LL mutant compared to F-9C-PR even though the absolute amounts of this form were similar for F-9C-PR and F-9C-LL (Fig. 6c, upper and middle panels, lanes 3 and 4). This result suggests that cleavage of the FLAG-tagged signal peptide from the F-9C-LL mutant is impaired relative to F-9C-PR with the wild-type SPase cleavage site, as expected (Fig. 6a, right diagram).

To determine whether signal peptide cleavage occurs in some receptor molecules, anti-FLAG immunoprecipitates from cells expressing F-PR, F-9C-PR, or F-9C-LL were electrophoresed on a high percentage polyacrylamide gel and subjected to western blotting with the anti-FLAG antibody. No small cleavage product was detected in extracts of cells expressing the FLAG-tagged wild-type receptor (Fig. 6c, bottom panel, lane 2), showing that

its signal peptide was completely degraded after cleavage. In contrast, a ~11 kDa protein containing the FLAG tag was detected in extracts of cells expressing F-9C-PR as a persistent form of the cleaved signal peptide (Fig. 6c, bottom panel, lane 3). The amount of cleaved signal peptide was greatly reduced in cells expressing F-9C-LL (Fig. 6c, bottom panel, lane 4), consistent with the increased amount of the full-length mutant as noted above (Fig. 6c, middle panel, lane 4). Thus, the leucine mutations in the signal peptidase cleavage site caused a partial defect in signal peptide cleavage (Fig. 6a, right-most diagram). These results show that the signal peptide of 9C-PR is cleaved from some receptor molecules and, unlike the wild-type signal peptide, escapes degradation (Fig. 6a, third diagram from left).

The cleaved 9C-PR signal peptide can activate a receptor *in trans*.

To test if the cleaved form of 9C-sp is responsible for activation of PRΔEX *in trans*, we tested the effect of inhibiting signal peptide cleavage. We first compared the ability of 9C-PR and the 9C-PR-LL signal peptidase cleavage site mutant to induce tyrosine phosphorylation of PRΔEX (Fig. 7a, top panel). We co-expressed PRΔEX with the wild-type PDGFβR, 9C-PR, or 9C-LL and performed receptor immunoprecipitation followed by anti-PDGFβR or anti-phosphotyrosine western blotting. PRΔEX was expressed at similar levels in the absence or presence of a co-expressed full-length receptor (Fig. 7b, left panel). As shown above, tyrosine phosphorylation of PRΔEX occurred in cells co-expressing 9C-PR but not in cells co-expressing the wild-type receptor (Fig. 7b, right panel, lanes 3 and 4), confirming that 9C-sp can act *in trans*. In contrast, tyrosine phosphorylation of PRΔEX was greatly reduced when it was co-expressed with 9C-PR-LL even though tyrosine phosphorylation of 9C-LL itself was unaffected (Fig. 7b, right panel, lanes 4 and 5). Since 9C-LL is partially defective for signal peptide cleavage, this result suggests that only the cleaved form of 9C-sp can activate PRΔEX *in trans*.

To determine if cleaved 9C-sp acts independently of the remainder of 9C-PR, we constructed a 9C-PR mutant containing both the kinase-inactivating K602R mutation and the cleavage site mutations (9C-KR-LL) and assessed its ability to induce tyrosine phosphorylation of PRΔEX. BaF3 cells expressing LXSN, wild-type PDGFβR, 9C-KR, or 9C-KR-LL with or without PRΔEX were established, and receptor expression and tyrosine phosphorylation were examined. Levels of PRΔEX were unaffected by co-expression of a full-length receptor and *vice versa* (Fig. 7c, left panel). 9C-KR and 9C-KR-LL were not tyrosine phosphorylated, confirming that kinase activity is abolished by the K602R mutation. However, although 9C-KR induced tyrosine phosphorylation of PRΔEX as shown above, the leucine mutations in 9C-KR-LL substantially reduced PRΔEX phosphorylation (Fig. 7c, right panel, compare lanes 5 and 7). Thus, cleavage of 9C-sp from a kinase-dead receptor increases its ability to activate PRΔEX *in trans*, showing that cleavage allows 9C-sp to act independently from the remainder of the receptor. Indeed, the reduced ability of 9C-KR-LL to activate PRΔEX implies that the truncated receptor is preferentially activated by the cleaved form of 9C-sp (Fig. 7a, bottom panel).

Finally, we assessed the activity of 9C-sp when expressed as a stand-alone 45-residue protein designated F-9C3SRG with the identical sequence as 9C-sp after cleavage (Fig. 8a). BaF3 cell lines expressing F-9C3SRG or untagged PDGFβR or co-expressing both

proteins were established and analyzed for the ability of F-9C3SRG to bind to the PDGFβR and induce its tyrosine phosphorylation (Fig. 8b, lower left and middle panels). Anti-PDGFβR immunoprecipitation followed by anti-phosphotyrosine western blotting revealed that both the mature and precursor forms of the PDGFβR were tyrosine phosphorylated in the presence but not in the absence of F-9C3SRG (Fig. 8b, right panel). Moreover, anti-FLAG immunoprecipitation of F-9CSRG followed by anti-PDGFβR western blotting showed that the immature form and a small amount of the mature form of the PDGFβR co-immunoprecipitated with F-9C3SRG (Fig. 8b, upper left panel, lane 3), indicating that F-9C3SRG forms a stable complex with the PDGFβR. Finally, F-9C3SRG cooperated with the PDGFβR to allow IL-3 independent growth, while neither protein alone displayed this activity (Fig. 8c). These results show that F-9C3SRG associates with and activates the PDGFβR to induce a proliferative signal and imply that 9C-sp released from 9C-PR by signal peptidase action can also associate with the PDGFβR TM domain *in trans* to induce receptor activation.

Discussion

The primary function of signal peptides in eukaryotic cells is to mediate the translational insertion of nascent TM and secreted proteins into the ER membrane. Since approximately 30% of eukaryotic proteins are TM and another 9% of proteins are secreted, eukaryotic cells express >10,000 different signal peptide sequences [1, 71]. There appear to be relatively loose sequence requirements for signal peptide activity [44], implying that many hydrophobic sequences might be able to function as signal peptides, even if they had not been selected for this activity during evolution. Indeed, Kaiser *et al.* showed that random hydrophobic amino acid sequences can functionally replace the signal peptide of *Saccharomyces cerevisiae* invertase [72]. We show here that an artificial short hydrophobic protein originally selected for growth stimulatory activity can function as a signal peptide in mammalian cells when positioned at the N-terminus of a class I TM protein. Furthermore, this artificial signal peptide retained its growth promoting activity by interacting with the TMD of its host protein. The major activated form of 9C-PR, as assessed by tyrosine phosphorylation, is the immature form containing high-mannose carbohydrates. Indeed, 9C-PR-X, which exists exclusively in the immature form (Fig. 4c), is tyrosine phosphorylated and confers IL-3 independence. The basis for the preferential tyrosine phosphorylation of the precursor form is not known but has been previously observed for wild-type PDGFβR in response to BPV E5 [8].

Because the PDGFβR is a single-span type I TM protein with its TMD ~500 amino acids from its N terminus, it is predicted to be defective for membrane insertion if it lacks an N-terminal signal peptide. In support of this prediction, naturally occurring PDGFβR fusion proteins lacking the signal peptide but retaining the PDGFβR TMD and intracellular domain are cytosolic [73, 74]. Consistent with the importance of an N-terminal signal sequence for optimal PDGFβR expression, replacement of the native signal peptide with 9C3 without additional modification supported only low-level receptor expression (Fig. S1). Three pieces of evidence demonstrate that 9C-sp functions as a signal peptide for the PDGFβR. First, the ability of 9C3-derived sequences to allow receptor expression is greatly increased by insertion of an N-terminal positive charge predicted to enhance the ability of 9C3 to function

as a signal peptide. Second, as assessed by the electrophoretic mobility of the mature receptor form and the endo H sensitivity of the precursor form, 9C-PR molecules undergo glycosylation, a process that occurs the secretory pathway after membrane insertion. The reduced levels of glycosylated receptor forms when the 9C3 segment lacks the positive charge implies that unmodified 9C3 can provide signal peptide function for a minority of receptor molecules, but most receptor molecules do not successfully incorporate into the membrane and are degraded. Finally, most of 9C-PR is transported to the plasma membrane, as shown by sensitivity to trypsinization in intact cells. We also note that the ability of 9C-PR to undergo tyrosine phosphorylation and induce IL-3 independence shows that it retains its expected type I orientation with a cytoplasmic kinase domain. The ability of 9C-sp to provide signal peptide activity is consistent with previous reports that many hydrophobic sequences can function as signal peptides.

Although 9C-sp can function as a signal peptide, it is not cleaved from some 9C-PR molecules, including some of those that reach the cell surface. The leucine mutations in the SPase cleavage site inhibit cleavage but do not impair the ability of 9C-PR to induce IL-3 independence, confirming that signal peptide cleavage is not required for 9C-PR biological activity. Thus, cleavage of 9C-sp is not required for it to display signal peptide activity for a type I TM protein. Similarly, stop-transfer sequences are typically not cleaved in type II and multi-pass TM proteins. Despite the existence of 9C-PR molecules with an uncleaved signal peptide, 9C-sp is cleaved from some molecules of 9C-PR, persists in cells, and can activate a co-expressed PDGFβR *in trans*, resulting in cell proliferation. It is likely that the cleaved form of 9C-sp binds to the PDGFβR, since the sequence-identical 9C3SRG can bind productively to the receptor when expressed as a stand-alone protein (Fig. 8). The ability of cleaved 9C-sp to escape degradation and act *in trans* suggests that some naturally occurring signal peptides might persist in membranes after cleavage from their host proteins and bind to TM proteins to regulate their expression, activity, or processing. Indeed, there is a report that expression of the 58-residue signal peptide of Lassa virus GP-C as a free-standing protein can support GP-C processing [58, 62]. Thus, at least two activities have been attributed to cleaved signal peptides that can act *in trans*, receptor tyrosine kinase activation by an artificial signal peptide and modulation of proteolytic processing of a viral fusion protein by its cognate signal peptide.

How does 9C-sp activate the PDGFβR by binding to its TMD? Since PDGF induces the formation of active receptor dimers that trans-phosphorylate, we assume that the active signaling complex induced by 9C-sp likewise consists of a receptor dimer. The ability of 9C-sp to activate the PDGFβR whether or not it is cleaved from the rest of the molecule provides insight into its mechanism of action. When 9C-sp is physically linked to the rest of the PDGFβR in 9C-PR, it is likely that the 9C-sp segment and the TMD are present in a 2:2 stoichiometry in the signaling complex containing two PDGFβR monomers, because a complex of receptor molecules assembled by TMD interactions is unlikely to contain enough space for the large intracellular and extracellular domains of additional receptor molecules. In intact 9C-PR, the uncleaved 9C-sp and the receptor TMD are likely to interact in an anti-parallel fashion because the known type II orientation of signal peptides after membrane insertion is opposite to the type I orientation of the PDGFβR itself. Furthermore, a parallel interaction would require that the receptor spans the membrane multiple times

even though it contains only a single TMD (Fig. S4A). Moreover, the ability of 9C3SRG to activate the PDGFβR showed that covalent linkage of 9C-sp to the PDGFβR is not required for activity, ruling out models in which such linkage mediates receptor dimerization (Fig. S4B). Our data support a model in which anti-parallel interactions between 9C-sp and the PDGFβR TMD, possibly in concert with homotypic interactions between two molecules of 9C-sp, induce the receptor to adopt an active dimeric conformation (Fig. S4C). Although the details may differ, this is similar to the model for BPV E5 action and the recently described model of activation of the EphA2 receptor by a designed small TM peptide [4, 75].

The isolation of numerous traptamers with diverse activities from randomized hydrophobic libraries suggest that many different hydrophobic sequences can productively interact with the TMDs of cellular TM proteins. Similarly, there is considerable flexibility in hydrophobic sequences that can support signal peptide function. Natural selection might exploit this apparent plasticity to regulate the expression and activity of cellular TM proteins. This line of reasoning suggests that cells may repurpose signal peptides as regulatory molecules, or *vice versa*. During evolution, mutations that imparted regulatory activity to signal peptides may have conferred selective advantage and become fixed in the germline. Alternatively, genes encoding small hydrophobic regulatory proteins may have been transposed to the 5’ end of genes encoding TM or secreted proteins, where they could adopt signal peptide activity in addition to their regulatory activity. Alternatively, transposition of such sequences to the N-terminus of a soluble cytoplasmic protein could convert it into a secreted protein. If the signal peptide acts on its new host protein, such a configuration would ensure coordinate expression of the regulatory protein and its target and potentially result in a high local concentration of the regulator near its target protein. If the signal peptide is instead cleaved and acts *in trans* on a different protein, more complex combinatorial regulatory schemes are possible. Finally, genetic alterations that occur in certain diseases could mobilize this mechanism to contribute to pathogenesis. For example, during cancer development, somatic mutations in a signal peptide may allow it to persist and modulate a cellular TM protein that affects cellular growth, thereby promoting cell proliferation and/or survival.

In conclusion, we report that an artificial N-terminal hydrophobic sequence can function both as a signal peptide and as a regulator of its host TM protein. The regulatory function of this signal peptide depends on its ability to persist and interact with the TMD of the host protein either intramolecularly in its uncleaved form or *in trans* after it is cleaved. These findings suggest that naturally occurring signal peptides might possess such dual functions.

Materials and Methods

Plasmids and Mutagenesis

Traptamer 9C3 was reported previously [21]. To construct 9C-PR and its derivatives, QuikChange (Aligent) site-directed mutagenesis was first used to introduce a silent mutation at nucleotide position 171 of the murine PDGFβR gene in the LXSN retroviral vector, which generated a unique AgeI restriction site. To generate 9C27-PR, we inserted a synthetic double-stranded DNA fragment encoding the first 27 amino acids of 9C3 (gBlock®; Integrated DNA Technologies, Coralville, IA) between this AgeI site and a unique upstream SalI site in LXSN. A gBlock encoding the N-terminal 33-amino acids of the PDGFβR was

inserted between the SalI and AgeI sites to introduce unique MluI and ClaI restriction sites flanking the signal peptide region and provide a backbone for constructing 9C-PR and its derivatives. The MluI site did not affect the encoded amino acid sequence, but the ClaI site introduced an isoleucine-aspartic acid insertion after amino acid position 34 (Fig. 1b). This insertion did not affect the expression or activity of the PDGFβR (data not shown). F-PR, sp-F-PR, 9C-PR, F-9C-PR, 9C-LL, F-9C-LL, 9C-GPQ were constructed by cloning gBlocks between the MluI and ClaI sites. To construct the TMD mutants F-9C-TL and 9C-βαβ, the BsiWI to ApaI fragment containing the TMD of previously reported mouse PDGFβR mutants [76, 77] was subcloned into F-9C-PR and 9C-PR. To construct the kinase inactive mutants 9C-KR and 9C-KR-LL, the EcoRI to BsiWI fragment from 9C-PR or 9C-LL, respectively, was subcloned into PR-NK, which contains a lysine to arginine substitution at position 602 in the kinase domain of the murine PDGFβR that abolishes receptor tyrosine kinase activity [78, 79]. F-9C3SRG encoding the FLAG-tagged stand-alone 9C3 signal peptide was constructed by cloning a gBlock into the BglII and EcoRI sites of the $MSCV_{puro}$ retroviral vector.

To generate F-9C-X, DNA encoding a PreScission™ protease cleavage site was inserted 24 amino acids upstream of the TMD of F-9C-PR by using a gBlock to replace a BsiWI-BstEII fragment encompassing this region of the receptor (Fig. S3A). The gBlock also contained a mutation causing a cysteine-to-serine substitution at amino acid position 246 to prevent the formation of a possible disulfide bond between the N-terminal and C-terminal cleavage products. This segment was subcloned into sp-F-PR and F-9C-TL, generating F-9C-TL-X and sp-F-PR-X, respectively. The PreScission™ protease cleavage site was separately introduced 12 amino acids downstream of the F-9C-PR TMD by using a gBlock to replace a BsiWI-ApaI fragment to generate F-9C-DX (Fig. S3A).

PRΔEX was generated by digesting a plasmid containing the murine PDGFβR cDNA (pSVR1) with EcoRV and PmlI followed by self-ligation of the large fragment to generate an in-frame deletion of amino acids 135–467 from the extracellular domain of the PDGFβR [80]. This construct was then subcloned into the $pBabe_{puro}$ retroviral vector.

Cell culture and expression of exogenous genes in BaF3 cells

Mouse BaF3 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (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 (RPMI/IL-3 medium). 293T cells were maintained in DMEM supplemented with 10% FBS, 20 mM Hepes, 0.5 μg/ml amphotericin B, and antibiotics (DMEM-10).

Receptors were stably expressed in BaF3 cells by retroviral-mediated gene transfer. Briefly, Lipofectamine® 2000 (Invitrogen) was used to co-transfect 293T cells with a retroviral plasmid containing a receptor gene and the packaging plasmids pCL-Eco and pVSVG (Invitrogen). Two days after transfection, the culture medium containing retrovirus was collected, filtered through a 0.45μ pore membrane, and used to infect BaF3 cells at 1–2 ml per 5×10^6 cells. Two days later, the cells were split 1:5 into RPMI/IL-3 containing 1 mg/ml G418 and incubated for approximately 1 week. To establish BaF3 cells stably co-expressing a full-length receptor with PRΔEX or F-9C3SRG, cells expressing the full-length

receptor were infected with retrovirus expressing PRΔEX, F-9C3SRG, or the corresponding empty vector and then selected with 1 μg/ml puromycin. Sequential retroviral infection and selection was also used to establish BaF3 cells co-expressing F-9C3 and either the human PDGFβR or its T545L mutant [70].

IL-3 independence assay

To determine if BaF3 cells expressing a particular receptor could survive and proliferate in the absence of IL-3, 5×10^5 cells were washed twice with phosphate-buffered saline (PBS) and then resuspended in 10 ml of RPMI medium containing 2% heat-inactivated 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 hemocytometer.

Immunoprecipitation and Immunoblotting

BaF3 cells were washed once in PBS, and cell pellets were lysed in cold Triton lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100) containing protease inhibitors. Cell lysates were clarified by centrifugation at 16,000 × g for 30 min, and the protein concentration of the supernatants was measured by a bicinchoninic acid assay (Pierce/Thermo Fisher). To immunoprecipitate receptor, rabbit antiserum recognizing the C-terminal 13 amino acids of the PDGFβR was incubated with cell extracts at 1 μl of antiserum per 100 μg of extracted protein at 4°C for 2 to 16 hrs. Immune complexes were precipitated using protein A-Sepharose beads (GE Healthcare) and washed three to five times in cold Tris-Buffered Saline (TBS; 50 mM Tris-HCl (pH 7.4), 150 mM NaCl) or NET-N buffer (100 mM NaCl, 20 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, 0.5% Nonidet P-40). 2X Laemmli sample buffer was then added to immune complexes. To immunoprecipitate FLAG-tagged receptor, F-9C3SRG, or 9C3, approximately 150 μl of a slurry of anti-FLAG M2 monoclonal antibody conjugated to agarose beads (Sigma-Aldrich) were added to 2 to 4 mg of protein extract and incubated at 4°C for 4–16 hrs. The beads were then washed five times with cold TBS, and 2X Laemmli sample buffer was added.

To detect receptors, samples were heated at 90–95°C, electrophoresed on an SDS 7% polyacrylamide gel, and transferred to nitrocellulose or 0.45μ polyvinylidene difluoride (PVDF) membranes in transfer buffer (25 mM Tris base, 192 mM glycine, and 20% methanol) for 1.5 to 2 hrs at 100 V. Membranes were blocked in blocking buffer (5% nonfat dry milk in TBST [10 mM Tris-HCl (pH 7.4), 167 mM NaCl, and 1% Tween 20]) at 4°C overnight and then incubated for 2 to 3 hrs at room temperature in a 1:1000 dilution of the anti-PDGFβR antiserum. Membranes were then washed five times in TBST, incubated in a 1:8000 dilution of horse radish peroxidase (HRP)-conjugated protein A (GE Healthcare) for 1 hr, washed as above, and then processed for enhanced chemiluminescence (ECL). To detect tyrosine phosphorylated receptor, immunoblots of receptor immunoprecipitates were incubated in blocking buffer for 1 hr at room temperature, incubated with a 1:1000 dilution of anti-phosphotyrosine antibody PY100 (Cell Signaling Technology, Inc.) in TBST containing 2% nonfat dry milk overnight at 4°C, and then processed as above using HRP-conjugated anti-mouse secondary antibody (Jackson ImmunoResearch Labs). Membranes were then incubated overnight in blocking buffer containing sodium azide and reprobed with

the anti-PDGFβR antiserum. To detect FLAG-tagged receptor, membranes were blocked overnight, incubated for 2 hrs with a 1:1000 dilution of an HRP-conjugated anti-FLAG antibody (Sigma-Aldrich), washed and then processed for ECL. To detect the cleaved form of 9C-sp, 9C3SRG, or the 9C3 traptamer, samples were electrophoresed on a 17% or 19% polyacrylamide gel without SDS in the gel, transferred to 0.2μ PVDF membrane for 1 hr and processed using the anti-FLAG-HRP antibody as above.

Detection of cell surface mature and intracellular precursor forms of PDGFβR and 9C-PR

To identify the intracellular form of receptors with immature carbohydrates, we used Endoglycosidase H (Endo H), which cleaves immature N-linked high mannose oligosaccharides on glycoproteins in the Golgi and ER. Briefly, receptors were immunoprecipitated from lysates of BaF3 cells by incubating approximately 2 mg of protein extract with 13 μl of anti-PDGFβR antiserum for 3 hrs. Immune complexes were precipitated with protein A sepharose and eluted by mixing the beads in 100 μl of elution buffer (50 mM Tris-HCl [pH 6.8], 1% SDS, 0.1M β-mercaptoethanol, and protease and phosphatase inhibitors) and heating at 90°C for 10 min. After centrifugation, the eluate was divided into two 50 μl aliquots. After adding 6 μl of 50 mM sodium acetate to each aliquot, 2 μl of Endo H (1000 units, New England Biolabs) were added to one of the aliquots, and both were incubated at 37°C for 2 hrs. Approximately 20 μl of 5X Laemmli sample buffer was added to each sample, which were then heated and subjected to gel electrophoresis and anti-PDGFβR and anti-phosphotyrosine immunoblotting.

To determine whether receptors were expressed at the cell surface, their sensitivity to trypsin was assessed. For each cell line, approximately 5×10^7 cells were aliquoted into two tubes, washed in PBS, resuspended in 1.25 ml of PBS with or without 0.05% trypsin (Gibco), and incubated at 37°C for 10 min. Between 6 and 10 ml of RPMI containing 10% heat-inactivated FBS and 1mg/ml soybean trypsin inhibitor (Sigma Aldrich) was added to each aliquot, and the cells were incubated on ice for 5 min. Cells were then washed once with PBS, lysed in Triton lysis buffer containing protease inhibitors, and immunoprecipitated with either anti-PDGFβR antiserum or the anti-FLAG affinity matrix. Immunoprecipitates were then subjected to gel electrophoresis and immunoblotting with PDGFβR antiserum.

PreScission™ protease digestion

BaF3 cell lines expressing receptor with or without an inserted PreScission™ protease cleavage site were lysed in Triton lysis buffer without protease inhibitors and clarified, and extracts were divided into two 1.2 ml-aliquots, each containing approximately 3 mg of protein. Forty-five μl (90 units) of PreScission™ Protease (GE Healthcare) was added to one aliquot, and both aliquots were incubated at 4°C for 4 hrs. One-sixth (~500 μg) of each sample was removed and immunoprecipitated with anti-PDGFβR antiserum, while the remainder of the sample (~2.5–4.5 mg) was immunoprecipitated with the anti-FLAG affinity matrix. Immunoprecipitates were then subjected to gel electrophoresis and immunoblotting with anti-PDGFβR antiserum.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BPV	bovine papillomavirus
Endo H	endoglycosidase H
ER	endoplasmic reticulum
FBS	fetal bovine serum
GP-C	glycoprotein C
HLA	human leukocyte antigen
HRP	horseradish peroxidase
NPY	neuropeptidase Y
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
PDGFβR	PDGF β receptor
PVDF	polyvinylidene difluoride
RTK	receptor tyrosine kinase
SPase	signal peptidase
SDS-PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
TBS	tris-buffered saline
TBST	TBS containing Tween
TM	transmembrane
TMD	transmembrane domain

Glossary

Signal peptide, short, typically N-terminal protein segment that directs nascent protein translation products into cell membranes

Traptamer, artificial transmembrane protein aptamer

Transmembrane domain, hydrophobic protein segment that spans cell membranes

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Highlights

- Signal peptides can have non-canonical biological activities
- A growth-stimulatory transmembrane protein can also serve as a signal peptide
- Signal peptide and proliferative activity do not require signal peptide cleavage
- A cleaved signal peptide can persist in cells and activate a receptor *in trans*
- Naturally occurring signal peptides may possess diverse biological activities

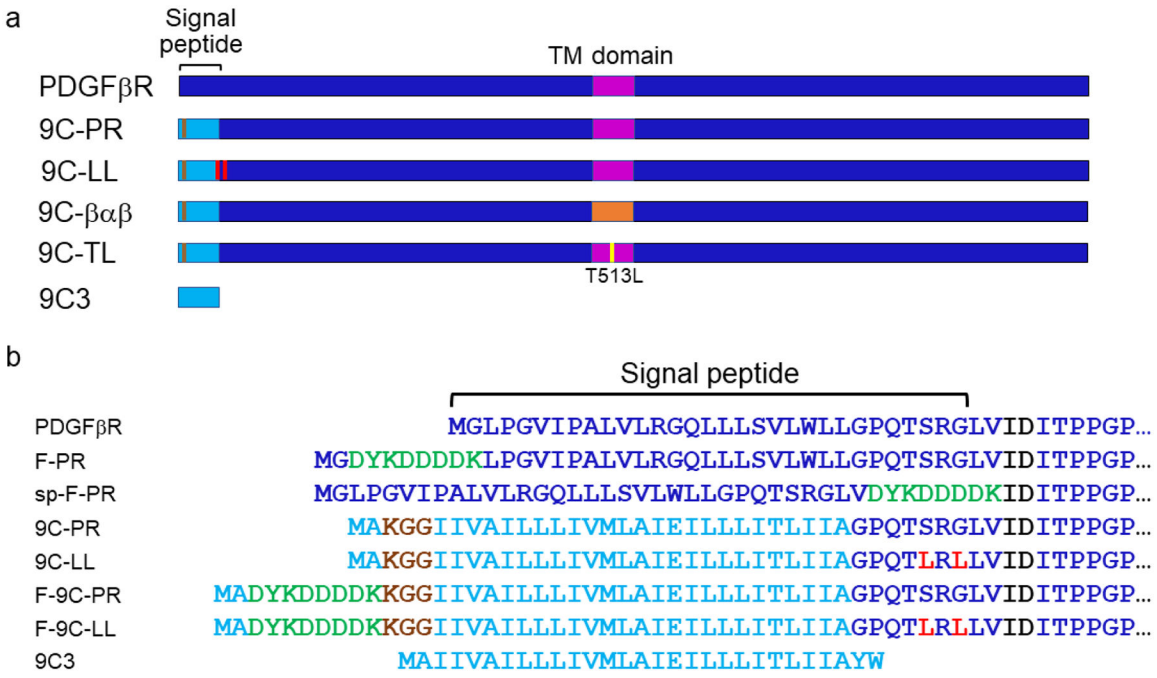

Fig. 1. Diagrams and sequences of traptamer 9C3 and chimeric PDGF β receptors.

(**a**) Diagrams of 9C3 and PDGFβR constructs. PDGFβR segments are shown in dark blue, 9C3 segments are shown in light blue, and PDGFβR TMD is shown in purple. 9C-PR is identical to the wild-type PDGFβR except most of the native signal peptide region is replaced with 9C3 containing a KGG insertion (brown line). In 9C-βαβ, the PDGFβR TMD in 9C-PR is replaced with the TMD of the PDGF α receptor (orange). In 9C-LL, leucine substitutions are introduced into the 9C-PR SPase cleavage site (red lines), and in 9C-TL, threonine 513 in the 9C-PR TMD is substituted with leucine (yellow line). **(b)** Amino acid sequences of 9C3 and the N-terminal segments of murine PDGFβR, 9C-PR, and its derivatives are shown in the single letter amino acid representation. PDGFβR sequence is shown in dark blue, with the signal peptide indicated at the top. The following sequence elements were inserted into this segment of the PDGFβR: amino acids 1–27 of the 9C3 traptamer (light blue), a lysine-glycine-glycine (KGG) sequence (brown), a FLAG epitope tag (DYKDDDK, green), and in some cases leucine mutations that inhibit signal peptide cleavage (red). All receptor constructs also contain an isoleucine-aspartic acid (ID, black) insertion immediately downstream of the signal peptide as a consequence of inserting a restriction site to facilitate cloning.

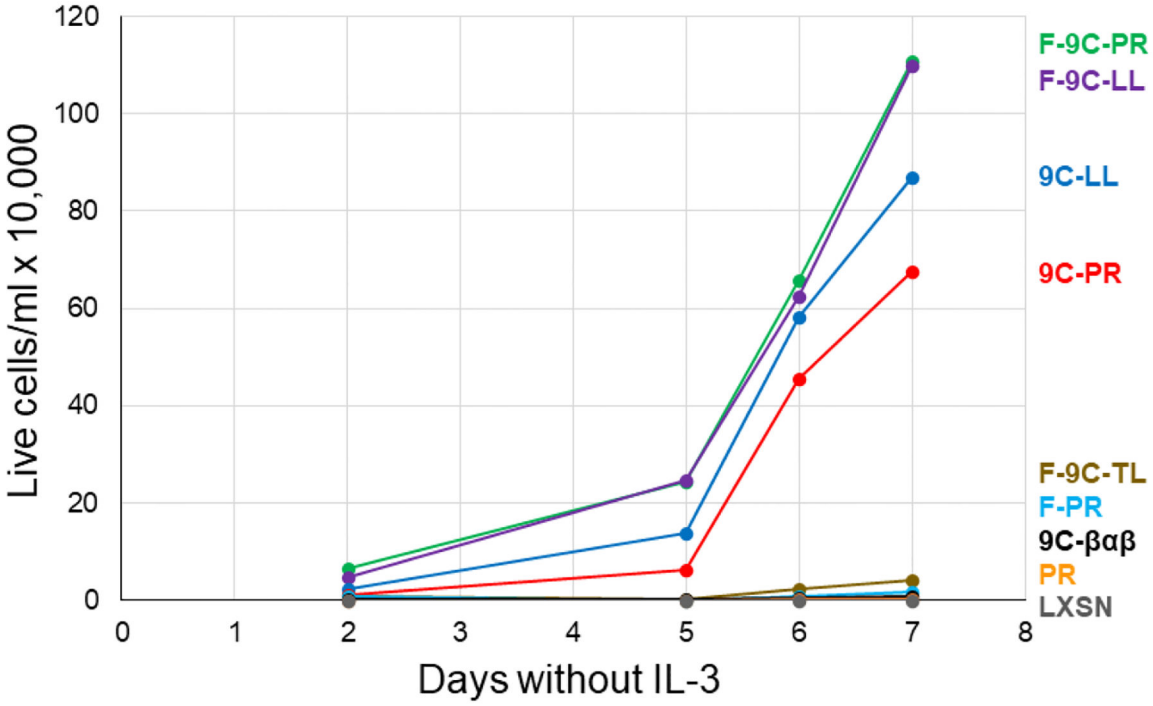

Fig. 2. 9C-PR induces IL-3-independent growth of BaF3 cells.

BaF3 cells transduced with the empty retroviral LXSN vector or a retrovirus expressing PDGFβR (PR), 9C-PR, or the indicated derivatives were subjected to G418 selection for stable retroviral transduction. Polyclonal G418-resistant cells were then incubated in medium lacking IL-3 and PDGF. Live cells were counted at various times thereafter. Data are representative of multiple independent experiments.

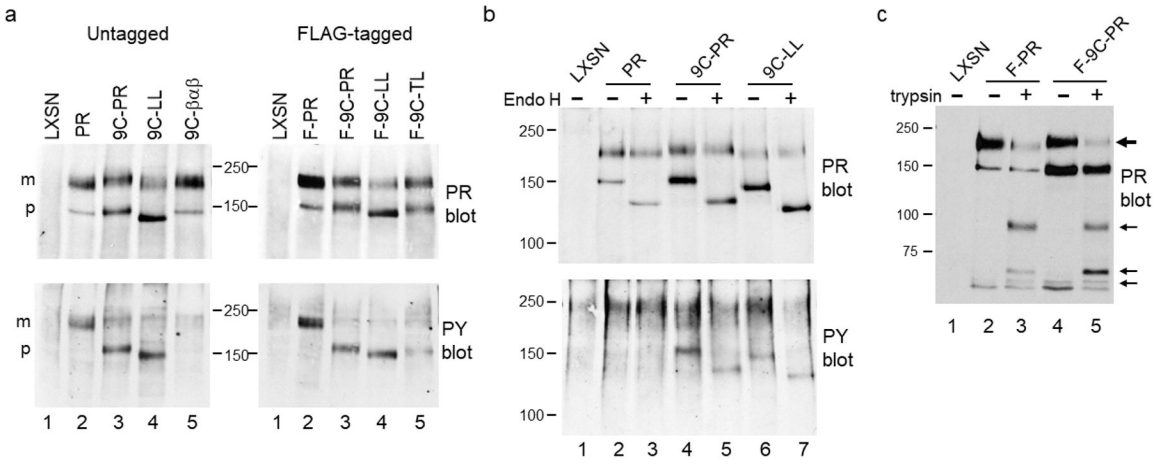

Fig. 3. Expression, trafficking and tyrosine phosphorylation of 9C-PR and its derivatives.

(a) BaF3 cells stably expressing LXSN, the indicated untagged (left panels) or FLAG-tagged (right panels) PDGFβR (PR), 9C-PR, or its derivatives were lysed in detergent buffer. Extracts were immunoprecipitated with an antibody recognizing the C-terminus of the PDGFβR, and immunoprecipitates were subjected to SDS-PAGE followed by immunoblotting with an antibody recognizing phosphotyrosine (PY, bottom panels) and then reprobing with anti-PDGFβR antibody (PR, top panels). The positions of the mature (m) and precursor (p) forms of the receptor are indicated. **(b)** BaF3 cells expressing LXSN or untagged PDGFβR (PR), 9C-PR, or 9C-LL were lysed and immunoprecipitated as in **panel a**. Immunoprecipitates were then either untreated (−) or treated with endoglycosidase H (Endo H) (+) and subjected to SDS-PAGE and immunoblotting for phosphotyrosine and PDGFβR as in **panel a**. **(c)** Intact BaF3 cells expressing LXSN, F-PDGFβR, or F-9C-PR were either untreated (−) or treated (+) with trypsin and then lysed. Samples were immunoprecipitated with an antibody recognizing the C-terminus of the PDGFβR and analyzed by SDS-PAGE and immunoblotting with the same antibody. The thick arrow shows the trypsin-sensitive cell surface form of the receptor. Faster migrating trypsin cleavage products are indicated by the thin arrows. Numbers on the side in all panels indicate the size in kilodaltons (kDa) of molecular weight markers.

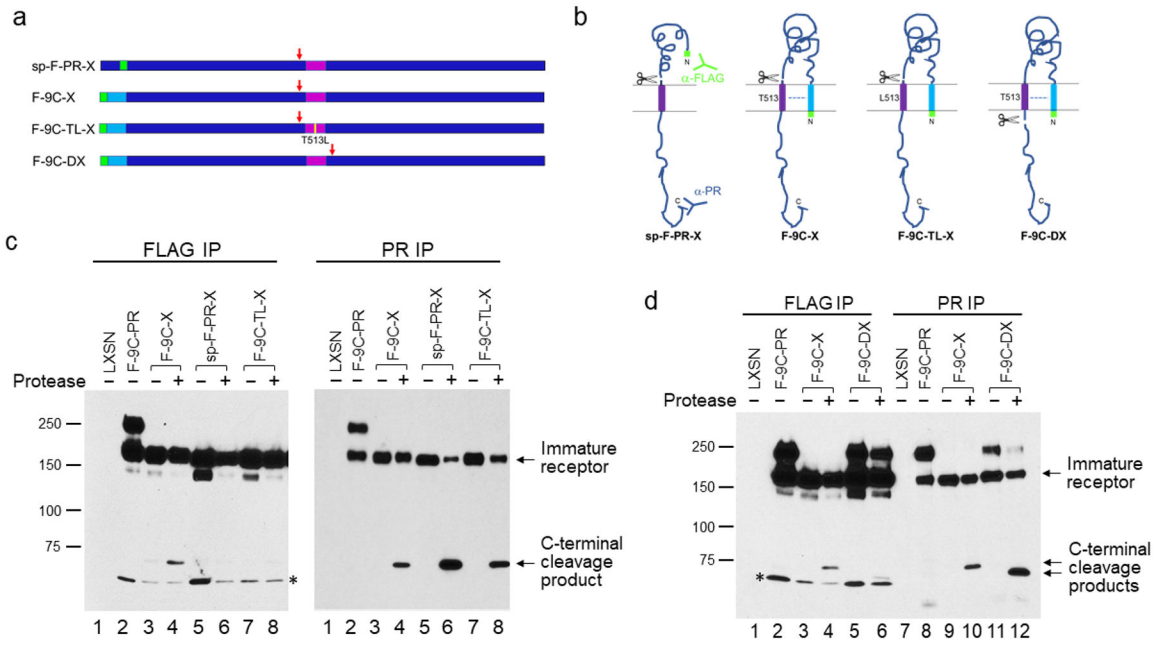

Fig. 4. The signal peptide and the TM domain of 9C-PR interact.

(a) Diagrams as in Fig. 1A of sp-F-PR-X, F-9C-X, and F-9C-TL-X constructs, all of which contain a PreScission™ protease cleavage site (indicated by the red arrows) upstream of the PDGFβR TMD, and of F-9C-DX, which contains the cleavage site downstream of the TMD. sp-F-PR-X contains the wild-type PDGFβR signal peptide and a FLAG tag downstream of the SPase cleavage site that is retained on the mature molecule after SPase cleavage. **(b)** Schematic diagrams showing how signal peptide/TMD interactions were assessed by co-immunoprecipitation. PreScission protease cleavage sites are represented by the scissors. If the signal peptide (light blue) and TMD (purple) of F-9C-X interact, an antibody recognizing the N-terminal FLAG tag (green) should co-immunoprecipitate the C-terminal cleavage product, which is recognized by the PDGFβR antibody. Co-immunoprecipitation is predicted to be inhibited by the threonine 513 to leucine substitution in the TMD the 9C-TL-X mutant. Horizontal lines represent the lipid bilayer, with the cytoplasm at the bottom, and N- and C-termini of the PDGFβR are indicated. **(c)** BaF3 cells expressing LXSN or the indicated receptor were lysed in detergent buffer. Lysates were either untreated (−) or treated (+) with PreScission™ protease and then immunoprecipitated (IP) with antibody recognizing the C-terminus of the PDGFβR (right panel) or the N-terminal FLAG epitope (left panel). Immunoprecipitates were subjected to SDS-PAGE followed by immunoblotting with an antibody recognizing the C-terminus of the PDGFβR. **(d)** BaF3 cells expressing LXSN or the indicated receptor were analyzed as described in **panel c**. In **panels c and d**, arrows on the right indicate the intracellular form of the receptor with immature carbohydrates and the C-terminal cleavage product, the asterisk indicates a non-specific band detected by the anti-PDGFβR antibody, and numbers on the left indicate the size in kDa of molecular weight markers.

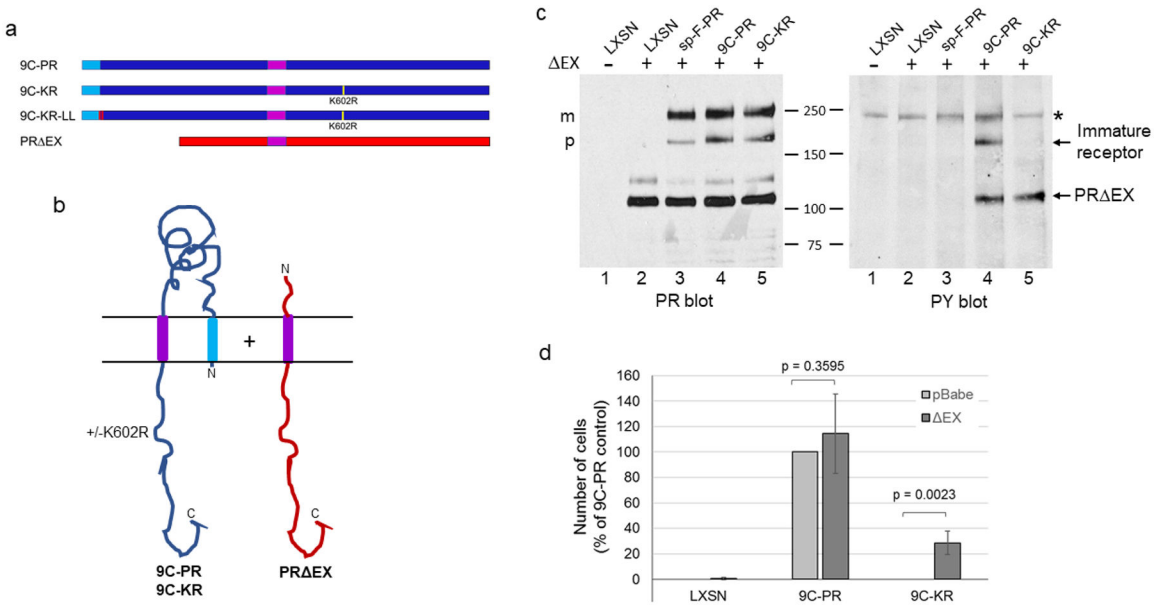

Fig. 5. The signal peptide of 9C-PR can act *in trans*.

(**a**) Diagrams as in Fig. 4a depicting 9C-PR (blue) and PRΔEX (red), a truncated PDGFβR with a deletion removing most of the extracellular domain of the receptor. A kinase-dead 9C-KR mutant contains a lysine-to-arginine substitution at position 602 in the kinase domain (yellow line). **(b)** Schematic diagram as in Fig. 4b. If the signal peptide of 9C-PR is able to act *in trans*, 9C-PR but not the wild-type PDGFβR should promote activation of PRΔEX. (**c**) BaF3 cells expressing LXSN, sp-F-PR, 9C-PR, or the K602R mutant of 9C-PR (9C-KR) in the absence (−) or presence (+) of co-expressed PRΔEX (ΔEX) were lysed in detergent buffer. Cell lysates were immunoprecipitated with an antibody recognizing the C-terminus of the PDGFβR, and immunoprecipitates were subjected to immunoblotting with the same antibody (left panel) or an antibody recognizing phosphotyrosine (right panel). Arrows indicate PRΔEX and the immature form of full-length PDGFβR. Numbers between panels indicate the size in kDa of molecular weight markers. The asterisk indicates a non-specific band detected by the anti-phosphotyrosine antibody in all samples including cells not expressing the receptor. (**d**) BaF3 cells co-expressing LXSN, 9C-PR, or 9C-KR with either the empty $pBabe_{puro}$ vector (pBabe, light gray) or PRΔEX (ΔEX, dark gray) were incubated in medium lacking IL-3 for seven days, and viable cells were counted. The graph shows the results of five independent experiments expressed as the mean percentage of viable cells relative to that in cells expressing 9C-PR alone, set at 100%. Error bars show standard error of the mean. p-values were calculated using Student's two-tailed t-test with unequal variances.

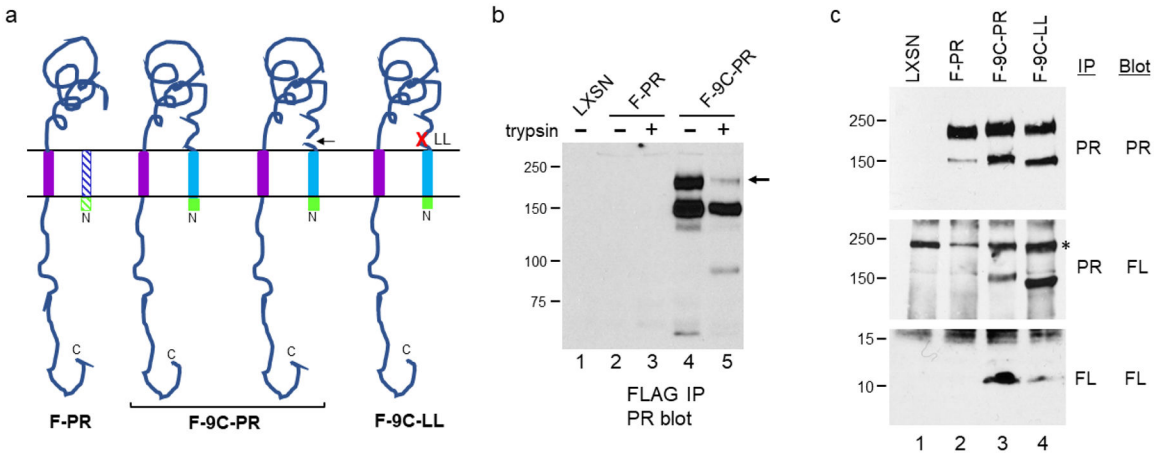

Fig. 6. The signal peptide of 9C-PR undergoes limited cleavage and escapes degradation.

(**a**) Schematic diagrams as in Fig. 4b depicting the FLAG-tagged wild-type PDGFβR (F-PR) after signal peptide cleavage (the released FLAG-tagged signal peptide is shown in the membrane but is rapidly degraded in the cell, represented by hatching), F-9C-PR with a uncleaved (left) or cleaved (right) signal peptide, or uncleaved F-9C-LL mutant. **(b)** Intact BaF3 cells expressing LXSN, F-PR, or F-9C-PR were either left untreated (−) or treated (+) with trypsin and then lysed. Detergent extracts were immunoprecipitated with an anti-FLAG antibody, and immunoprecipitates were subjected to SDS-PAGE and immunoblotting with the antibody recognizing the C-terminus of the PDGFβR. Arrow indicates the trypsin-sensitive cell-surface form of F-9C-PR. **(c)** BaF3 cells expressing LXSN or the indicated receptor were lysed in detergent buffer, then immunoprecipitated (IP) with anti-FLAG (FL) or anti-PDGFβR (PR) antibodies and subjected to anti-FLAG or anti-PDGFβR western blotting (Blot), as indicated. An asterisk indicates a background band with mobility similar to mature PDGFβR detected with the anti-FLAG antibody in all samples including cells not expressing the receptor. Numbers on the left of each image in **panels b** and **c** indicate the size in kDa of molecular weight markers.

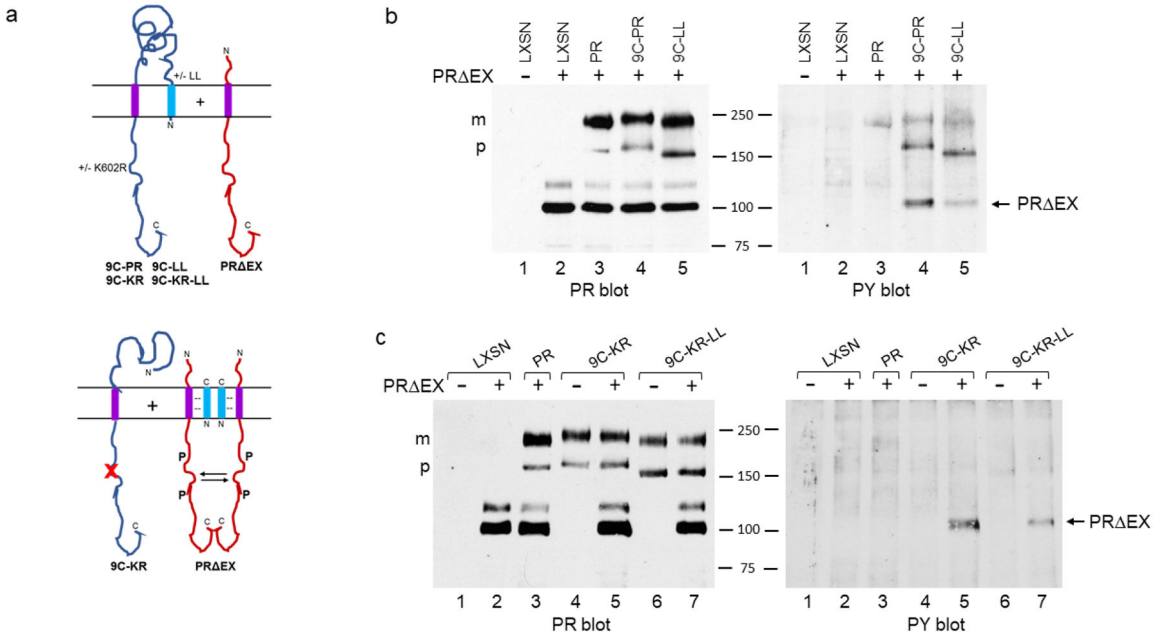

Fig. 7. The cleaved signal peptide of 9C-PR acts *in trans*.

(**a**). Schematic diagrams as in Fig. 4b. Top panel. shows 9C-PR with or without the kinase-inactivating K602R mutation and/or the signal peptidase cleavage site mutations (LL) co-expressed with PRΔEX. Bottom panel shows 9C-sp cleaved from 9C-KR acting *in trans* to activate PRΔEX. (**b & c**) BaF3 cells expressing the indicated full-length receptor in the absence (−) or presence (+) of PRΔEX were lysed, and extracts were immunoprecipitated with an antibody recognizing the C-terminus of the PDGFβR. Immunoprecipitates were subjected to SDS-PAGE followed by immunoblotting with anti-PDGFβR (PR) or anti-phosphotyrosine (PY). Numbers between images in **panels b** and **c** indicate the size of molecular weight markers in kDa.

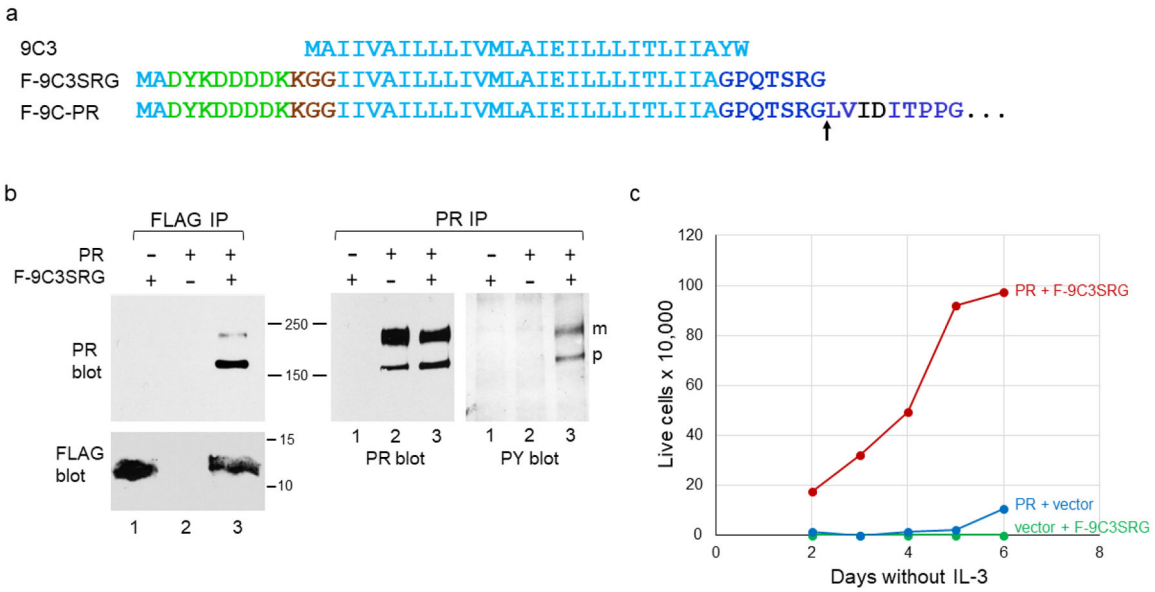

Fig. 8. 9C-sp expressed as a stand-alone protein acts *in trans* by binding to the PDGFβR.

(**a**) Amino acid sequence of stand-alone F-9C3SRG aligned with the original 9C3 traptamer (light blue) and the N-terminal domain of F-9C-PR. N-terminal FLAG tag of F-9C3SRG and F-9C-PR is shown in green, PDGFβR sequence is dark blue, and KGG after the FLAG tag is brown. Predicted site of SPase cleavage is shown with black arrow. (**b**) BaF3 cells expressing F-9C3SRG the untagged PDGFβR (PR), or both as indicated were lysed in detergent buffer, and extracts were immunoprecipitated (IP) by using anti-FLAG or anti-PDGFβR (PR) antibodies as indicated. Immunoprecipitates were then analyzed by SDS-PAGE followed by western blotting using anti-FLAG, anti-PDGFβR (PR), or anti-phosphotyrosine (PY), as indicated. Numbers between panels indicate the size in kDa of molecular weight markers. **(c)** BaF3 cells expressing F-9C3SRG, PDGFβR (PR), or both were incubated in the absence of IL-3, and viable cells were counted at various times thereafter.