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Structural rearrangements of PDGFRβ in the membrane upon activation by the viral oncoprotein E5

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The transmembrane protein E5 from bovine papillomavirus is the shortest naturally occurring oncoprotein. As a dimer, it activates the platelet-derived growth factor receptor β (PDGFRβ) in a ligand-independent manner by specific helix–helix interactions within the lipid bilayer. For both proteins, we determined the detailed orientations of their transmembrane helices in aligned membrane samples. Solid-state ^{15}N-NMR was used to study either protein segment alone, as well as in the heterocomplex. Remarkably, the assembly of E5 with PDGFRβ led to structural rearrangements of both partners. Binding of E5 triggers a rotation of the PDGFRβ helices around their axes. At the same time, the E5 helices rotate in the membrane to expose Gln17 as well as Asp33, allowing their respective interaction with Thr513 and Lys499 on PDGFRβ. Based on these distinct membrane orientations, we present structural models of the heteromeric E5-PDGFRβ complex, and a mechanism for oncogenic receptor activation.

transmembrane protein | helix–helix interactions | receptor tyrosine kinases | solid-state NMR spectroscopy | oncoprotein E5

Significance

This work uncovers the molecular details by which the short viral oncoprotein, E5 from bovine papillomavirus, hijacks the platelet-derived growth factor receptor β (PDGFRβ) without any ligand. Using solid-state ^{15}N-NMR spectroscopy we determined the transmembrane helix orientations of E5 and PDGFRβ individually and in their heterodimer. Binding of E5 forces a rotation of both helices, exposing Gln17 and Asp33 of E5 to contact Thr513 and Lys499 of PDGFRβ, thereby locking the receptor in an active conformation. The resulting structural models define a membrane-embedded activation interface that can be targeted by small-molecule or peptide inhibitors, opening avenues for antiviral and anticancer therapeutics.

Signal transduction by receptor tyrosine kinases (RTK) generally requires the interaction of two single-pass membrane-spanning receptor subunits. These are usually activated by extracellular ligands, either via receptor dimerization or via the structural rearrangement of a preformed inactive dimer. In this process, the transmembrane helices play an active role and are responsible for establishing the correct molecular orientation of the two receptor subunits (1–4).

The platelet-derived growth factor receptor β (PDGFRβ) is a well-studied, representative member of the RTK family. It is involved in the differentiation of mesenchymal cells during embryogenesis, central nervous system formation and angiogenesis (5–7). Kinase activation by its PDGF-ligand requires not only receptor dimerization per se, but also a proper positioning of the two interacting TMHs relative to one another. The actual helix rearrangement within the membrane thus seems to be the allosteric switch that drives the cytosolic kinase domains into a functional signal transduction complex, whereas wrongly oriented TMHs and kinase domains remain inactive (1).

Notably, PDGFRβ can also be activated—in a ligand-independent manner—by the oncogenic E5 protein from bovine papillomavirus-1 (BPV-1) (8–17). BPV-1 causes skin fibropapillomas in cattle, which are formed by extensive proliferation of infected dermal fibroblasts and epidermal keratinocytes (18).

E5 is a short hydrophobic transmembrane protein with only 44 amino acids. It can control the activity of PDGFRβ through highly specific transmembrane helix–helix interactions. This mechanism prominently highlights the importance of the TMH–TMH interplay in the RTK activation process. E5 occurs as a disulfide-bridged homodimer with a type-II transmembrane orientation. It recognizes PDGFRβ (a type-I integral membrane protein) via two specific side chains. An antiparallel E5–PDGFRβ complex is formed via Asp33 and Gln17 on E5, and Lys499 and Thr513 on PDGFRβ (13, 19–24), leading to ligand-independent activation. Both, E5 and the receptor have unusually long hydrophobic domains, which implies significant hydrophobic mismatch in typical lipid bilayers. Therefore, their assembly as a length-matched bundle seems to contribute further to the stability of the heterocomplex, not just in the relatively thick plasma membrane, but especially in the much thinner Golgi membranes of an infected cell (25, 26). In order to better understand the general principles of RTK regulation—i.e., canonical signal transduction by PDGFRβ, as well as its noncanonical activation by E5—it is critically important to elucidate the detailed structures and membrane orientations of the constituent TMH partners, both in their homodimeric as well as hetero-oligomeric forms.

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A low-resolution model of full-length PDGFRβ in complex with its natural ligand PDGF-B has been obtained by negative-stain electron microscopy at 27 Å resolution (27). Structural calculation and in vivo studies of the isolated PDGFRβ-TMH segment revealed its strong tendency to form dimers and trimers via a leucine-zipper-like motif (28). The first steps toward a structure analysis of E5 and, more recently, of the E5–PDGFRβ complex have been based on molecular dynamics (MD) simulations (29, 30). However, the exact structure and even the stoichiometry of the E5-PDGFRβ complex have not yet been experimentally elucidated nor verified. Various models have been proposed, based principally on mutation experiments and computational studies. One such early model suggests that an E5 dimer interacts with two PDGFRβ-TMHs, in which a trans or a cis PDGFRβ-TMH arrangement in a 2:2 complex was proposed. In the *cis* geometry, both receptor-TMHs are located as a pair on the same face of the E5 dimer, whereas in the trans geometry, the E5 dimer is flanked on both sides by a single PDGFRβ-TMH. According to this model, the activation of PDGFRβ is triggered by an E5-mediated recruitment of two PDGFRβ monomers (29). The most recent model, on the other hand, proposes a hexameric complex with a stoichiometry of two E5-dimers and one PDGFRβ dimer (30). This geometry is essentially based on MD simulations and Rosetta modeling. The energetic comparison with the alternative tetrameric complex shows that the hexameric complex is more stable, in particular due to stronger van der Waals interactions and lower conformational fluctuations. Furthermore, in this model the two intracellular tyrosine kinase domains would be positioned asymmetrically, because the PDGFRβ-dimer is arranged asymmetrically in the hexamer (30). Both hetero-oligomeric models include the critical hydrogen-bond between Gln17 of E5 and Thr513 of PDGFRβ, as well as the salt bridge between Asp33-Lys499 in the juxtamembrane region. However, the functionally relevant structures of the two proteins in a proper membrane environment, other than in silico, have yet to be confirmed experimentally.

In earlier studies, we had optimized the reconstitution of E5 and some truncated analogues in macroscopically aligned lipid bilayers, in order to measure the helix orientation in the membrane in terms of its tilt angle. Solid-state ^{15}N-NMR and oriented circular dichroism (OCD) can provide this orientational information, provided that the proteins are reconstituted in macroscopically aligned membrane samples (26, 31, 32). We found that E5 has an intrinsic tendency to oligomerize, even without the two native disulfide bridges. The unusually long transmembrane helix of E5 could be reconstituted only in very thick bilayers, where it adopted a slightly tilted orientation. Upon decreasing the acyl chain length of the lipids, the protein became increasingly tilted and started to aggregate in a disorderly manner (26).

Using the same solid-state ^{15}N-NMR and OCD approach, we also analyzed the helix alignment of PDGFRβ-TMH in the same set of lipid systems. Similar to E5, the long PDGFRβ-transmembrane helix was found to assume an almost upright orientation in very thick bilayers, but its helix tilt angle gradually increased with decreasing bilayer thickness (25, 26). Unlike E5, PDGFRβ was able to tilt much farther without aggregation, i.e., it could compensate for hydrophobic mismatch in the thinner bilayers. This behavior would be expected either for a monomer, or for a homodimer that can adjust its helix–helix crossing-angle within the membrane. Remarkably, the massive aggregation of E5 on its own in thin bilayers could be reversed by co-reconstitution with PDGFRβ-TMH. From this observation, it was concluded that a tight assembly of the length-matched transmembrane helices of E5 and PDGFRβ leads to a mutual stabilization of all helices in a compact bundle, which can now withstand the hydrophobic mismatch of a thin lipid bilayer (26, 33). This interpretation appears to be biologically relevant, when considering the increase in bilayer thickness upon passing from the endoplasmic reticulum to the Golgi and finally to the plasma membrane (34–37). When PDGFRβ is activated by its usual ligand, the soluble PDGF hormone, the receptor is embedded in the relatively thick plasma membrane, where dimerization of its long transmembrane helices can take place without any penalty from hydrophobic mismatch. In contrast, E5 can activate PDGFRβ already in the much thinner membranes of the ER and/or Golgi, suggesting that this heterocomplex formation can compensate for hydrophobic mismatch (34–37).

To understand these equilibria, and for a comprehensive 3D structural characterization of the E5 and PDGFRβ complexes, it is obviously not enough to know the helix tilt angles τ in the membrane, which had been determined earlier using uniformly ^{15}N-labeled samples. It is essential now to resolve also the corresponding azimuthal rotation angle ρ of each participating helix, using specifically ^{15}N-labeled proteins. We have thus measured τ and ρ for all interacting partners within the three different complexes encountered here (E5–E5, PDGFRβ–PDGFRβ, and E5–PDGFRβ). Based on this complete set of orientational data, we shall then present an approach of helix–helix docking within the bilayer, which has not been described before in the literature. In this context it is important to realize that the azimuthal rotation angle ρ is a structural parameter that essentially describes the direction of tilt, i.e., toward which face the helix is inclined within the membrane (see *SI Appendix*, Fig. S1 for the full angular definition, and helical wheel representation). Once the full orientation is known for a transmembrane helix, say, in a homodimer, the dimerization interface can be deduced by simply positioning the helix into a membrane in its proper orientation (τ and ρ) and appropriate depth (given by its hydrophobic length and possible anchoring residues). When picturing the lipid bilayer as a horizontal sheath, any tilted helix can interact with another tilted helix only via its two "vertical" sides to form a crossed homodimer (i.e., left or right relative to the direction of tilt, see *SI Appendix*, Fig. S1*B*). For a virtually upright helix, the whole circumference of the helix is freely available, but in the case of a tilted helix, homodimer formation via the slanted sites (in or opposite to the direction of tilt) would require a heterotopic interaction interface or the formation of an asymmetric complex if we were to stack two tilted helices in the membrane. A stack with a heterotopic interaction interface would really only be favorable as an infinite straight line or as a large circle when curved and closed up into a ring. That is because any unoccupied heterotopic interaction interface (especially carrying a charged or polar side chain) would result in an energetically unfavorable situation where the unoccupied polar or charged side chain is exposed to the hydrophobic membrane interior. An asymmetric complex, on the other hand, would result in two signal populations of our single ^{15}N-labeled peptides, which we do not observe in NMR. This geometrical argument reduces the vast number of possible dimerization interfaces, which would usually have to be probed systematically by a helix docking algorithm over a 360° rotational search in 2D, or even in 3D space. Based on the determined membrane orientation of the helix and using this simple but effective approach, any conspicuous side chains (e.g., charged, polar, H-bonding, leucine-zipper, aromatic) can be identified that could be involved in dimerization. If a suitable motif is found on either side of the tilted helix, even the handedness of the resulting dimer can be discriminated. In an analogous manner, heterocomplexes can be assembled by inspecting the properly tilted helices to see which of the side chains are positioned such that they are able to interact with the other

partner(s) in the oligomer (however, the above-mentioned limitations must be kept in mind).

Here, we have performed a careful orientational τ and ρ analysis for both, the transmembrane helices of E5 and of PDGFRβ in lipid membranes, in order to determine the interaction interfaces in the respective homodimers. Furthermore, we analyzed the E5–PDGFRβ heterocomplex and found that both types of helices underwent a significant rearrangement upon assembly of the bundle, compared to their respective homodimeric state. Based on these models, we propose a mechanism of the E5-induced PDGFRβ activation.

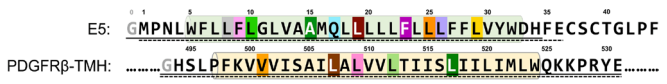

Fig. 1. Amino acid sequences of full-length E5 (1-44) and PDGFRβ-TMH (494-531). The cylinders depict the membrane-inserted regions of both constructs (Trp5-Trp32 for E5, and Phe498-Trp524 for the PDGFRβ-TMH). The underlined sequences depict the biosynthetic constructs, whereas the dashed underlined sequences depict the constructs prepared by peptide synthesis. An additional glycine remains from the hydroxylamine cleavage site at the N terminus (shown in light gray). For determining the rotation angle ρ, we produced ten analogues of ΔE5 and five analogues of PDGFRβ-TMH, each carrying a single ^{15}N-labeled amino acid at one specific position (color coded).

Results

Definition of Structural Terms and ^{15}N-Labeling Strategy. In this study, we aim to reveal the detailed membrane orientation of both E5 and of the PDGFRβ-TMH in their homomeric and heteromeric states, hoping to get insights into the oncogenic activation of PDGFRβ by E5. Static solid-state ^{15}N-NMR spectroscopy can reveal the membrane orientation of helical peptides in their quasi-natural membrane environment. When reconstituted in macroscopically aligned lipid bilayer samples, the helix tilt can be roughly estimated from simple 1D spectra. A more refined alignment can be obtained from 2D ^{1}H-^{15}N dipolar coupling/^{15}N-CSA correlations. In particular, PISEMA or SAMMY spectra (38–42) provide so-called "PISA wheels," from which the tilt angle τ, as well as the rotation angle ρ, can be determined. The tilt angle τ of a transmembrane helix is defined as the angle between the bilayer normal and the helix axis. The local azimuthal rotation angle ρ_i of any specified amino acid i describes the rotational position of its C_α-atom around the tilted helix with respect to the actual direction of the tilt, which we define as ρ = 0° (*SI Appendix*, Fig. S1). [Footnote: Please note that in some of our previous work on membrane-bound peptides we have denoted their orientation in terms of their "global azimuthal rotation angle ρ," which had been arbitrarily defined to always refer to amino acid number 12 (i.e., ρ_{12}), because the 12th residue is generally located in the middle of a typical α-helix.]

Uniformly ^{15}N-labeled peptides in macroscopically aligned membrane samples give rise to complete PISA wheels, whose size and position in the 2D spectrum directly reflects the tilt angle τ of the α-helix, with an accuracy of a few degrees. Selectively labeled peptides allow an assignment of the PISA signals, which furthermore enables the determination of the rotation angle ρ, leading to the full description of the helix alignment in the membrane. Therefore, in our study, both E5 and PDGFRβ TMH peptides were synthesized first as uniform ^{15}N-labeled analogues to obtain the full PISA wheels (and as unlabeled analogues for CD spectroscopy) by heterologous expression in *Escherichia coli*. Thereafter, several ^{15}N-selectively labeled analogues were produced by chemical peptide synthesis, in order to assign the wheels and determine the direction of tilt via the analysis of ρ.

In the case of E5, we used the same shortened construct lacking the C-terminal disulfide bridge as in our previous study (32). This truncation facilitates purification and reconstitution without changing structurally relevant sequence properties (conformation, orientation, and dimerization potency). ΔE5 construct covers the E5 sequence from Met1 to His34 in the recombinant peptide, or from Met1 to Glu36 in the synthetic peptide (where we had to add two amino acids C-terminally, because His as a starting amino acid in the peptide synthesis decreased the yield). The ΔE5 construct derived by biosynthesis carries instead an additional Gly residue at the N terminus, that remained from hydroxylamine cleavage. We can demonstrate that both ΔE5 constructs adopt the same secondary structure (*SI Appendix*, Fig. S2), therefore both constructs (1-34 and 1-36) will be abbreviated with ΔE5.

In principle, the rotation angle ρ could be obtained from a single selectively ^{15}N-labeled sample, provided that the peptide is folded as a uniform α-helix. However, to be certain in our analysis, we wanted to examine the entire length of the TMH with regard to any potential curvature or kink (which would have made our visual docking approach less reliable). Therefore, we produced and analyzed a total of 10 different selectively ^{15}N-labeled ΔE5 peptides, carrying the respective nonperturbing ^{15}N-amino acid in the position of either Leu8, Phe9, Leu10, Ala15, Gln17, Leu19, Phe23, Leu25, Leu26, or Leu29 (Fig. 1 and *SI Appendix*, Table S1).

In the case of PDGFRβ-TMH, we used the sequence from His_{494} to Glu_{531} (plus an additional N-terminal glycine), as in our earlier structure analysis in detergent micelles by solution-state NMR (25). At this point it is important to mention that the TMH sequence of the human and bovine PDGFRβ is identical (only the sequence numbering is shifted by one amino acid). It has been further shown that the E5 protein from bovine papillomavirus can also bind to and activate the human PDGFRβ (43–45). To be consistent with the existing E5/PDGFRβ literature, we used the sequence numbering of the human PDGFRβ in our study. For determining the rotation angle ρ we produced five different PDGFRβ-TMH peptides with a selective ^{15}N-label at the position of either Val501, Leu507, Leu509, Leu512, or Leu517 (Fig. 1 and *SI Appendix*, Table S2). In both E5 and PDGFRβ, the label positions were chosen to be evenly distributed along and around the TMH, to enable a comprehensive analysis of the membrane alignment along the entire helix. The intact α-helical folding of all used ΔE5 and PDGFRβ-TMH peptides was confirmed by circular dichroism measurements (*SI Appendix*, Fig. S2).

Determination of the Azimuthal Rotation Angle of the E5 Helix. In our earlier PISA wheel analysis of uniform ^{15}N-labeled recombinant ΔE5, we already determined the tilt angle of this helix, but not its azimuthal rotation angle due to the lack of assignment. We found that the peptide adopted a slightly tilted orientation in thick lipid bilayers, but aggregated in all conventionally used thinner membranes due to hydrophobic mismatch (26). In thick DErPC (di-C22:1) bilayers, the uniformly ^{15}N-labeled ΔE5 was optimally reconstituted. It yielded a well-resolved PISA wheel, indicating a uniform α-helical folding with a helix tilt angle of 13° and an order parameter (S_{mol}) of 0.98. Such a high value of S_{mol} close to 1.0 suggests that the E5 helices assemble into an oligomeric structure (26, 32). We can now use the same conditions to determine the rotation angle ρ of the E5 helix in the membrane using selectively ^{15}N-labeled peptides.

Solid-state 2D ^{1}H-^{15}N-NMR SAMMY spectra (Fig. 2*A*) were recorded from macroscopically aligned membrane samples in the usual experimental set-up, where the bilayer normal is parallel to the static magnetic field. A single resonance was observed for every ^{15}N-labeled analogue, confirming a homogeneous reconstitution

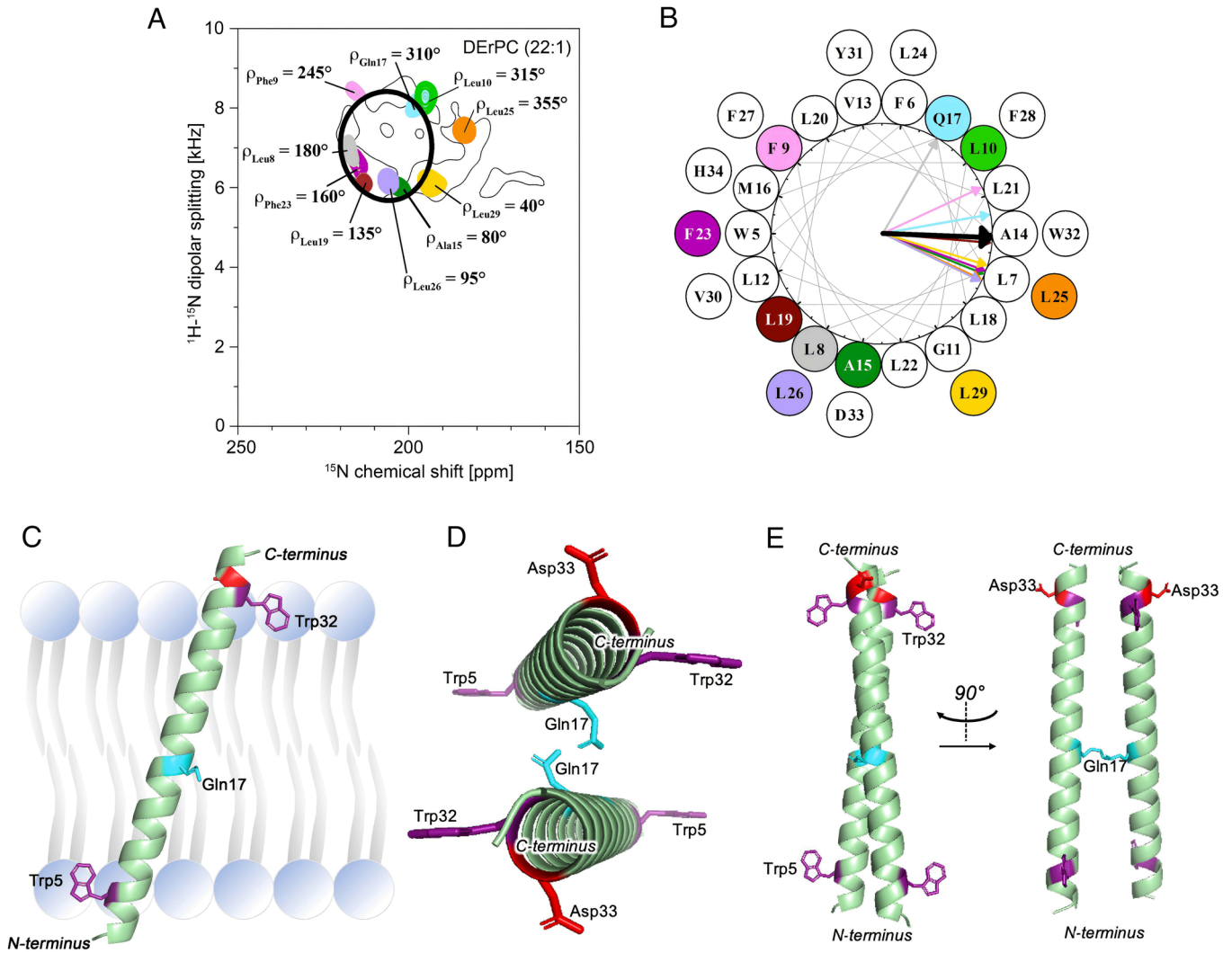

Fig. 2. Membrane orientation of the E5 protein. (*A*) Overlay of the solid-state 2D-NMR ^{1}H-^{15}N SAMMY spectra (colored contours) of the selectively ^{15}N-labeled E5 peptides reconstituted in DErPC. The spectrum of the uniform ^{15}N-labeled peptide is shown as a black contour, as published in (26), and superimposed is the corresponding simulated PISA wheel (τ = 13°, S_{mol} = 0.98, thick black line). For every single ^{15}N-labeled peptide, a single resonance was observed (colored), from which the respective ρ_i for each labeled position i could be determined. (*B*) Helical wheel plot of the E5-TMH, showing all helix tilt vectors (ρ = 0) that were calculated from the ten different ^{15}N-labeled analogues (colored arrows, colors refer to the respective label). The resulting averaged helix tilt vector is shown as a bold black arrow, pointing toward Ala14/Trp32, which thus defines the overall helix tilt direction. (*C*) The resulting membrane orientation of E5 is depicted with respect to the lipid bilayer plane. (*D*) Top view of the left-handed E5 homodimer from the C-terminal side, and (*E*) two side views.

and a high-quality membrane orientation throughout. All signals have a ^{15}N chemical shift between 180 to 220 ppm, and a ^{1}H-^{15}N dipolar splitting between 5 to 9 kHz, as expected for an essentially upright transmembrane helix (*SI Appendix*, Table S3). All resonances lie on the PISA wheel that had been obtained from uniformly ^{15}N-labeled ΔE5 (black contour in Fig. 2*A*). These data confirm that the synthetic ΔE5 peptides have the same secondary structure and orientation in DErPC bilayers as the recombinant ΔE5 peptide, even though their termini differ slightly. It is remarkable to see that the position of nearly every single resonance on the PISA wheel corresponds one-to-one to their rotational position on the helical wheel plot. This observation suggests that the TMH of E5 predominantly adopts an α-helical conformation in DErPC bilayers.

The local azimuthal rotation angle ρ_i was obtained for every ^{15}N-labeled amino acid by fitting a simulated signal to the measured one. Thus, it was possible to determine the direction of the helix tilt (global azimuthal rotation ρ = 0°) for each of the ten selectively ^{15}N-labeled samples. In Fig. 2*B*, these helix tilt vectors are plotted onto a helical wheel. Nearly all point toward a narrow 45° sector between Leu7 and Leu21. An exception is the vector of Leu8, which may well be explained by a conformational perturbation at the N terminus. The global helix tilt vector, as an average over all positions, thus points toward the direction of Ala14 and Trp32.

By inspecting the resulting membrane orientation (Fig. 2*C*), it is obvious that Trp5 and Trp32 may serve as membrane anchors to maintain the helix in this particular azimuthal orientation. Such flanking aromatic residues are well known for their ability to stabilize a discrete rotation angle of a TMH (46). In this orientation, as defined uniquely from our NMR data, there exist only two possibilities where helix–helix interactions can take place to form a crossed dimer, namely at the two vertical sides of the tilted helix (i.e., at the top or bottom of the helical wheel in Fig. 2*B*), but not via its slanted faces. Interestingly, the side chain of Gln17 is seen to point sideways relative to the direction of tilt where it is ideally available for polar interactions with Gln17 of a second E5 helix. Given the known tilt vector of the helix, this dimerization interface leads to a left-handed helix crossing, with an angle of 26° (i.e., twice the tilt angle). Our independent NMR data thus confirm

the existing model of a left-handed parallel E5 homodimer, that had been modeled with a polar interaction via Gln17 (24, 29) (Fig. 2 *D* and *E*). It can be concluded that our structural approach has worked out well, to first i) measure the helix orientation in terms of τ and ρ, and then ii) logically restrict the geometrically accessible interaction interfaces to the two sides of the tilted helix, and finally iii) to look for polar side chains on either side, which finalizes the model and determines the sidedness of the dimer crossing angle. Interestingly, Asp33 in our resulting model, which is proposed to play an important role for the interaction with PDGFRβ (19, 47), is positioned on the opposite side of the putative dimer interface and, hence, is free for interacting with other helices.

Determination of the Azimuthal Rotation angle of the PDGFRβ-TMH. Our earlier 1D solid-state NMR analysis of uniformly ^{15}N-labeled recombinant PDGFRβ-TMH had shown that the helix tilt angle increases upon going from thick DErPC (di-C22:1) to thinner DOPC (di-C18:1) bilayers, indicating that the protein can tilt to compensate for hydrophobic mismatch (26). Beyond that, in even thinner membranes, the (isolated) PDGFRβ transmembrane helix begins to aggregate nonproductively. This behavior has also been confirmed here in the 2D ^{1}H-^{15}N-NMR SAMMY spectra, where we found a helix tilt angle of 12° in DErPC (Fig. 3), 14° in DEiPC and 17° in DOPC bilayers (*SI Appendix*, Fig. S3). Interestingly, the values of S_{mol} decreased slightly from 0.95 in thick DErPC to 0.80 in thinner DOPC bilayers, which might be a sign that the homo-oligomeric state of PDGFRβ is influenced by the membrane thickness. Namely, a rigid dimeric state might not adjust its tilt angle to hydrophobic mismatch as easily as a monomer. It is thus conceivable that the dimerization equilibrium could shift toward a more mobile monomeric state in thinner bilayers. Unlike E5, there are no disulfide bridges between PDGFR monomers, and the isolated TMH segments do not show such a high oligomerization tendency on SDS–PAGE as seen for ΔE5 (*SI Appendix*, Fig. S10). Using the selectively ^{15}N-labeled PDGFRβ-TMH peptides, we can now assign these PISA wheels to determine the direction of tilt.

We were able to record a series of SAMMY spectra for each of the five selective ^{15}N-labeled PDGFRβ-TMH peptides, reconstituted in thick DErPC bilayers (Fig. 3*A*), in thinner DEiPC, and in DOPC (*SI Appendix*, Fig. S3). For all samples, each selective ^{15}N-labeled analogue gave rise to a single NMR signal, which coincided well with the corresponding PISA wheel of the uniformly labeled peptide. All resonances had a ^{15}N chemical shift between 170 to 220 ppm and 4 to 8 kHz ^{1}H-^{15}N dipolar splitting, indicating proper folding and a transmembrane alignment (*SI Appendix*, Table S4). In all spectra of the five selectively labeled PDGFRβ analogues, the position of each single resonance on the PISA wheel corresponds one-to-one to its rotational place on the helical wheel plot. The resulting local azimuthal rotation angle ρ_i for every labeled position was calculated and used to determine the average helix tilt angle. This analysis was done for the three types of lipid samples with different bilayer thickness. We found

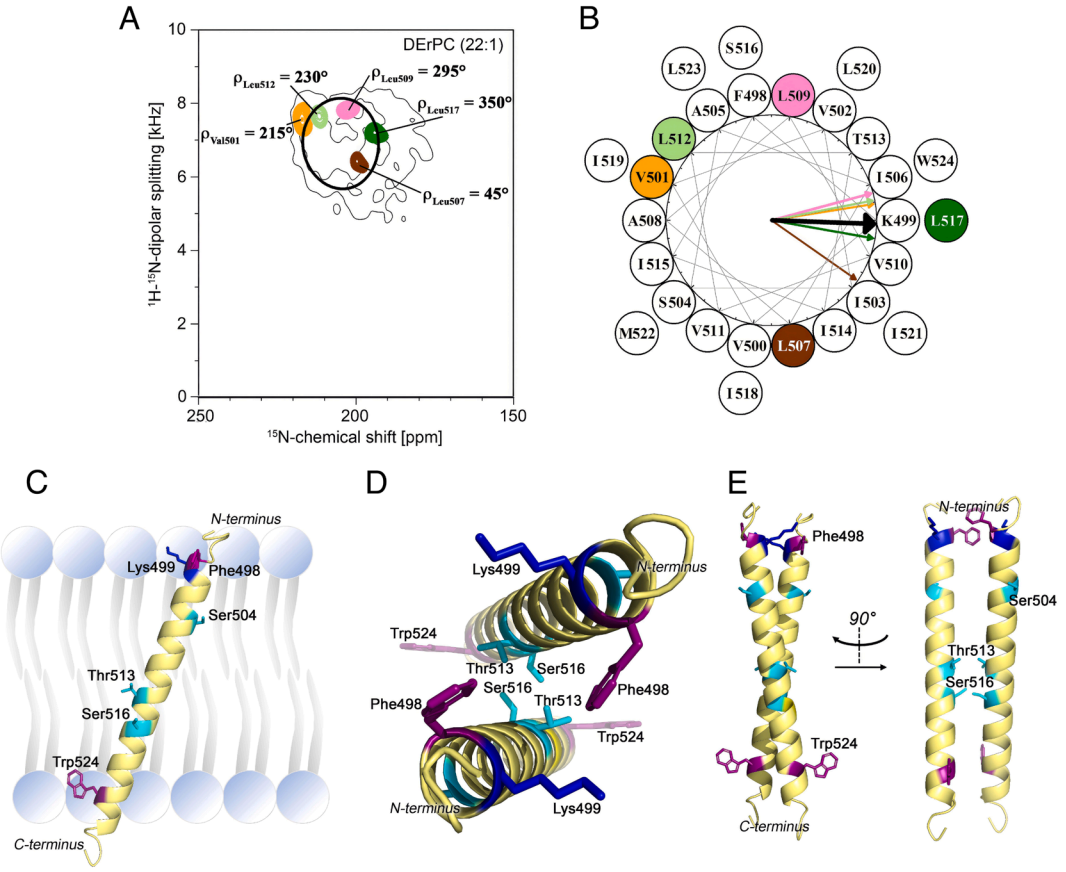

Fig. 3. Membrane orientation of the PDGFRβ-TMH. (*A*) Overlay of the solid-state 2D-NMR ^{1}H-^{15}N SAMMY spectra of all selectively ^{15}N-labeled PDGFRβ-TMH peptides (colored contours) with the spectrum of the uniformly ^{15}N-labeled construct (shown as a black contour) reconstituted in DErPC. A simulated PISA wheel (τ = 12°, S_{mol} = 0.95) is overlaid, and the respective ρ_i of each selective label is given. (*B*) Helical wheel plot of PDGFRβ-TMH, showing the helix tilt vectors (ρ = 0°) that were calculated from the five different selectively ^{15}N-labeled analogues (colored arrows, referring to the respective labels). The resulting averaged helix tilt direction is shown as a bold black arrow which points toward the direction of Lys499/Leu517 and thereby defined the tilt direction of the helix. (*C*) The resulting membrane orientation of PDGFRβ-TMH is depicted with respect to the lipid bilayer plane. (*D*) Top view of the resulting left-handed PDGFRβ-TMH homodimer, and (*E*) two side views illustrating the proposed dimerization interface with Ser516 and Thr513.

that in all lipid systems (DErPC, DEiPC, DOPC) the helix tilt vectors derived from the selectively ^{15}N-labeled samples point toward the same narrow 45° sector between Ile503 and Ile506. This means that on average the PDGFRβ helix is consistently tilted along the direction of Lys499/Leu517 (Fig. 3*B*), independent of bilayer thickness. At this point it must be mentioned that PDGFRβ is inserted in the opposite directionality (N terminus outside) into the membrane compared to E5; consequently, the PDGFRβ-TMH is actually inclined away from (rather than toward) Lys499/Leu517.

Notably, the tilt direction does not change upon going from thick to thinner bilayers; hence we can conclude that the PDGFRβ-TMH gets tilted to compensate for hydrophobic mismatch as expected, but it does not change its rotation angle when inserted into bilayers with different thicknesses. The resulting three-dimensional membrane orientation shows that Trp524 near the C-terminus and Lys499 near the N terminus (which can snorkel toward the headgroup region) turn out to be in ideal positions to serve as membrane anchors, stabilizing this particular azimuthal orientation. In this structure, the central polar amino acids Ser516 and Thr513 are oriented sideways on the helix (i.e., parallel to the axis of tilting), and are therefore available for dimerization (Fig. 3 *D* and *E*). This motif would produce a left-handed PDGFRβ dimer with a helix crossing angle of about 24° in DErPC. There are some further conspicuous aromatic and polar residues (Phe498 and Ser504) in the TMH sequence, but they seem to have no apparent function in our model, given their inappropriate azimuthal orientations. However, they will play a prominent role in the E5-PDGFRβ heterocomplex discussed below.

Membrane Orientation of E5 and PDGFRβ–TMH in the E5–PDGFRβ Heterocomplex. As a next step, we determined the orientation of both peptides in the heteromeric complex. To observe just one of the two partners, uniformly ^{15}N-labeled E5 was reconstituted together with a twofold excess of unlabeled PDGFRβ-TMH, and a complementary sample was made vice versa. In these experiments, the tilt angles of both peptides were found to be the same as in the isolated components (*SI Appendix*, Fig. S4). At first sight, this observation could mean that there is no E5-PDGFRβ interaction at all in our setup, and/or that the tilt angles are dominated by hydrophobic mismatch rather than by any specific helix–helix contacts. On the other hand, an assembly of the two peptides into a heteromeric complex might be detectable by a change in the azimuthal rotational angles of the participating helices, because their specific contact sites are expected to become rearranged upon recognition and binding.

To examine changes in the azimuthal rotation angles, we prepared five samples with different selectively ^{15}N-labeled E5 peptides in the presence of a twofold excess of unlabeled PDGFRβ-TMH, and vice versa, in DErPC bilayers. The 2D-NMR SAMMY spectra showed a dramatic change in the azimuthal rotation angles of both, E5 and PDGFRβ-TMH. Therefore, it can be concluded that in our experimental setup, the transmembrane helices of E5 and PDGFRβ interact with one another and mutually adjust each other's rotational angle. On this basis, we can proceed now to determine the 3D membrane orientations of the participating helices and build a model of the heterocomplex.

All samples with selectively ^{15}N-labeled E5 in the presence of unlabeled PDGFRβ-TMH gave single signals (Fig. 4*A* and *SI Appendix*, Fig. S5), which coincided well with the corresponding PISA wheel of the uniformly labeled peptide. All resonances had a ^{15}N chemical shift between 180 to 220 ppm, and a 6 to 9 kHz ^{1}H-^{15}N dipolar splitting, indicating proper folding and a transmembrane alignment (*SI Appendix*, Table S5). The resulting local azimuthal rotation angles ρ_i were determined for each ^{15}N-labeled analogue, and their average was used to calculate the direction into which the helix is tilted. We found that the tilt vector of E5 in the heteromeric complex is now pointed toward Leu22 (Fig. 4*B* and *SI Appendix*, Fig. S7*A*), as opposed to Ala14 in the isolated E5 homodimer. Consequently, each E5 helix is found to rotate by about −65° upon forming the E5-PDGFRβ heterocomplex.

Gln17 and Asp33 on the E5 oncoprotein are known to be essential for the activation of PDGFRβ (24, 47, 48), yet they are positioned on opposite faces of the helical wheel (Fig. 4*B*). In the homodimeric structure of E5, Asp33 faces outward, i.e., away

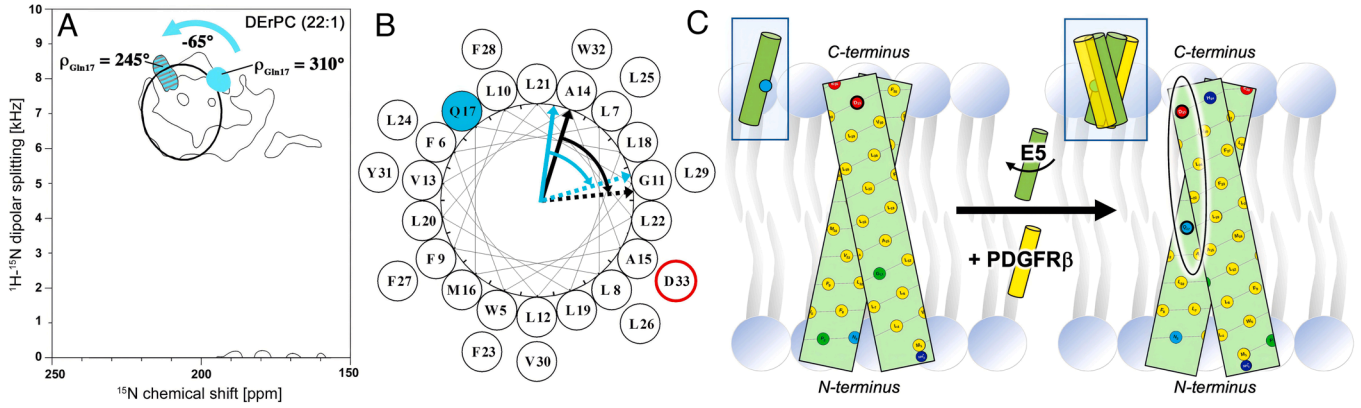

Fig. 4. Membrane orientation of the E5 protein in the heteromeric E5-PDGFRβ complex. (*A*) Solid-state 2D-NMR ^{1}H-^{15}N SAMMY spectra of selectively ^{15}N-Gln17 backbone-labeled ΔE5 (light blue), of ^{15}N-Gln17 backbone-labeled ΔE5 in the presence of a twofold excess (mol/mol) of unlabeled PDGFRβ-TMH (light blue, hatched), and of uniformly ^{15}N-labeled recombinant ΔE5 (black contour) reconstituted in DErPC bilayers. The spectra are overlaid with a simulated PISA wheel (τ = 13°, S_{mol} = 0.98). The local ρ_i of Gln17 changes from $\rho_{17} \approx 310°$ in the original ΔE5 homodimer to $\rho_{17} \approx 245°$ for ΔE5 in the E5-PDGFRβ heterocomplex. This rotation corresponds to an azimuthal rotation change of −65°. (*B*) Helical wheel plot of ΔE5, showing the helix tilt direction of the original homodimer depicted by a solid arrow, and the new orientation in the heteromeric E5-PDGFRβ complex by a dashed arrow. The local tilt vector of Gln17 (light blue arrow) was calculated from the single ^{15}N-labeled Gln17 analogue, while the overall helix tilt vector (black arrow) was obtained as an average from five selectively labeled samples (see supplementary data for the other four ^{15}N-labeled samples, *SI Appendix*, Figs. S5 and S7*A*). (*C*) Illustration of the azimuthal rotation change of the E5 helix upon switching from the homomeric E5–E5 dimer to the heteromeric E5-PDGFRβ complex. Both, Gln17 (light blue) and Asp33 (red) have become available now on the same side of the dimer (black elliptic outline), where they are in an ideal position to simultaneously interact with PDGFRβ. The color code used in the helix representation of (*C*) distinguishes amino acids according to their character: hydrophobic – yellow; polar – light blue; anionic – red; cationic – dark blue; flexible Gly/Pro – green.

from the dimer interface that is held together by Gln17 (Fig. 2 *D* and *E*). Remarkably, after the rotation of E5, these two important residues Gln17 and Asp33 become lined up on the same face of the E5 dimer, as each E5 helix contributes one side chain to this new Gln17-Asp33 binding patch (Fig. 4*C*). For symmetry reasons, obviously two such binding interfaces emerge, i.e., on either side of the E5 dimer, which are available now for interaction with PDGFRβ.

Having demonstrated the azimuthal reorientation of E5 upon forming the heteromeric complex, we next examined the response of the PDGFRβ transmembrane helix under analogous conditions. Interestingly, the reverse experimental design with selectively ^{15}N-labeled PDGFRβ-TMH mixed with a twofold excess of unlabeled ΔE5 showed two signal populations in each of the five samples with the selectively ^{15}N-labeled analogues (Fig. 5*A* and *SI Appendix*, Fig. S6). One population always remained at the same ^{15}N-chemical shift and dipolar splitting range as in the original PDGFRβ-TMH dimer. In contrast, the second signal population indicates a new azimuthal orientation of the PDGFRβ-TMH, as seen in all five selectively ^{15}N-labeled PDGFRβ samples. We note that the presence of two populations demonstrates that in the case of PDGFRβ-TMH, contrary to E5, a twofold excess of the partnering protein is not enough to fully convert all PDGFRβ-TMH into the new alignment. This shows that E5 must be present in excess in the heterocomplex (probably because it only acts in a dimeric form). All resonances of the second signal population had a ^{15}N chemical shift between 180 to 220 ppm and 6 to 8 kHz ^{1}H-^{15}N dipolar splitting, indicating proper folding and a transmembrane alignment (*SI Appendix*, Table S6). Analysis of the new signals show that the averaged tilt vector points toward Val500, as opposed to Lys499 in the original PDGFRβ dimer (Fig. 5*B* and *SI Appendix*, Fig. S7*B*). (Please note that the PDGFRβ-TMH is inserted in the opposite directionality, hence the helix is actually inclined away from Val500). We can conclude from our ab initio NMR analysis that the PDGFRβ transmembrane helix gets rotated by about −95° on average when forming the heterocomplex with E5. Due to this rotation, the residues Thr513 and Lys499, which are known to be involved in the activation by E5 (13, 49), become exposed on the vertical sides of the tilted helix. They had been previously inaccessible on the slanted surface, but in this newly exposed position they are now well positioned to interact with Asp33 and Gln17 of E5 (Fig. 5*C*).

Structure of the E5-PDGFRβ Heterocomplex. Knowledge of the accurate membrane orientation of the E5 and PDGFRβ transmembrane helices in the heterocomplex now enables us to build a structural model by helix docking. Please note that E5 and PDGFRβ are arranged in an antiparallel fashion, because E5 has an N-in and PDGFRβ an N-out topology. Here, we take it that E5 remains a parallel homodimer during heterocomplex formation. This assumption is justified by the high structural stability of the E5 dimer, even of the truncated ΔE5 construct without disulfide bridges, demonstrated in earlier studies (21, 32, 50, 51). On this basis, two models of the E5-PDGFRβ heterocomplex can be constructed from the NMR-derived helix orientations, namely i) a four-helix-model consisting of two PDGFRβ helices bridged by an E5-dimer, and ii) a six-helix-model with a PDGFRβ-dimer clamped between two E5-dimers (Fig. 6). In both models, a hydrogen bond between Gln17 (E5) and Thr513 (PDGFRβ), as well as a salt bridge between Asp33 (E5) and Lys499 (PDGFRβ) can be formed. Earlier mutational analyses have shown that these particular residues are essential for complex formation (13, 19, 20, 22). Our experimentally determined azimuthal orientations of E5 and PDGFRβ in the heterocomplex are not only compatible with the postulated interactions of these four residues. In fact, had there been no previous knowledge about the E5 PDGFR interactions, our models would have predicted precisely these two pairs of critical side chains, interacting by H-bonding and charge in a nontrivial manner (i.e., some within the same helix pair and some across such pair). Considering the role of membrane anchoring residues (i.e., bilayer-inserted aromatic side chains, and/or snorkeling Lys), we found that Trp524 and Lys499 served as azimuthal anchors of the PDGFRβ helix in its original state. Upon

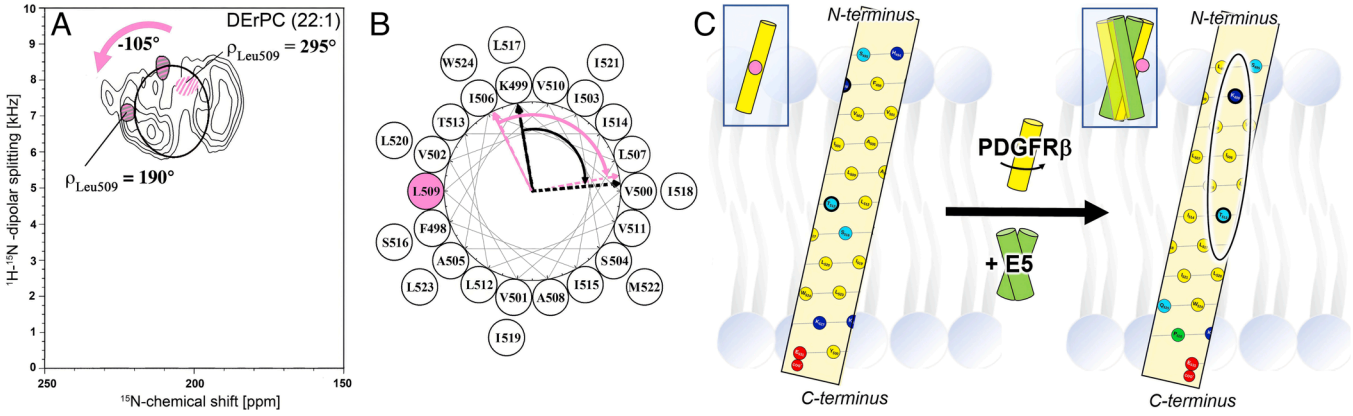

Fig. 5. Membrane orientation of PDGFRβ in the heteromeric E5-PDGFRβ complex. (*A*) Solid-state 2D-NMR ^{1}H-^{15}N SAMMY spectra of selectively ^{15}N-Leu509 labeled PDGFRβ-TMH (pink, filled), of ^{15}N-Leu509 labeled PDGFRβ-TMH in the presence of unlabeled ΔE5 (pink, hatched), and of uniformly ^{15}N-labeled recombinant PDGFRβ-TMH (black contour), reconstituted in DErPC bilayers. The spectra are overlaid with a simulated PISA wheel (τ = 12°, S_{mol} = 0.95). The selectively ^{15}N-labeled PDGFRβ-TMH mixed with a twofold excess (mol/mol) of unlabeled ΔE5, showed two signal populations: one corresponding to the orientation in the original isolated peptide, and the second to the new orientation in the heteromeric complex with E5. The local azimuthal rotation angle ρ_{509} changes from $\rho_{509} \approx 295°$ in the original structure of PDGFRβ-TMH, to $\rho_{509} \approx 190°$ for PDGFRβ in the E5-PDGFRβ-complex, which corresponds to an azimuthal rotational change of −105°. (*B*) The helical wheel plot of the PDGFRβ-TMH shows the helix tilt direction (solid arrow for the original peptide, dashed arrow for the heteromeric complex) that was calculated from the single ^{15}N-labeled Leu509 in pink. The averaged helix tilt vector obtained from five selectively labeled samples is shown in black (see supplementary for the other ^{15}N labeled analogues, *SI Appendix*, Figs. S6 and S7*B*). (*C*) The azimuthal rotational change of PDGFRβ induced by complex formation with E5 leads to an exposure of Lys499 and Thr513 on the vertical side of the helix, where they are now accessible for interaction with E5 (black outline). For helix color coding see Fig. 4*C*.

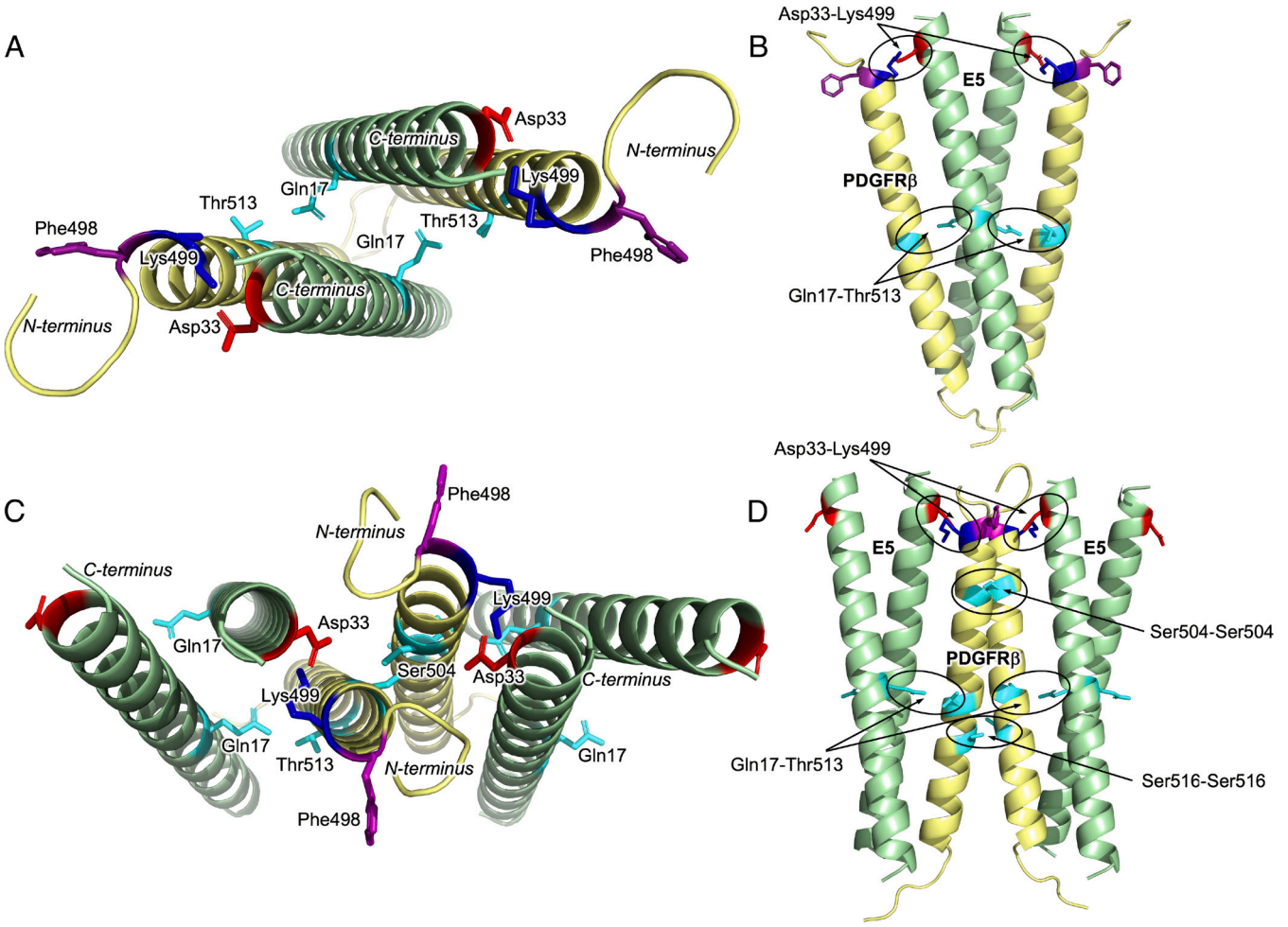

Fig. 6. Top view and side views of the two models of the E5-PDGFRβ heterocomplex derived from our structural characterization of their TMH segments. Polar amino acids are colored in light blue, anionic side chains in red, cationic ones in dark blue, and aromatic side chains are shown in purple (only important side chains for complex formation are shown). (*A* and *B*) Four-helix model: The E5 dimer (green) is flanked by two PDGFRβ-TMH (yellow). (*C* and *D*) Six-helix model: The PDGFRβ-TMH dimer is clamped between two E5 dimers.

interaction with E5, Phe498 takes over the role of a new anchoring residue in the rotated PDGFRβ helix.

Discussion

Transmembrane helices of integral membrane proteins are often much more than just simple hydrophobic membrane anchors. The function of many membrane proteins can be regulated by a realignment of their constituent transmembrane helices within the lipid bilayer (52–54). For example, by changing their TMH orientation or by rearrangement of their TMHs, receptors can alternate between an active and an inactive state (1, 54–59).

Based on our NMR-derived orientations of E5 and the PDGFRβ-TMH, we can thus postulate structural models for their homodimeric resting structures, and we present two feasible models for the functional heteromeric E5-PDGFRβ complex in a lipid bilayer matrix.

Structural Features of the E5-Dimer. Our solid-state NMR analysis of 10 different selectively ^{15}N-labeled E5 analogues confirmed the expected continuous α-helical conformation, supporting earlier qualitative results from infrared, circular dichroism and NMR spectroscopy (26, 29, 31, 32, 51, 60). Systematic analyses in different types of membranes showed that E5 can be properly reconstituted without aggregation only in very thick DErPC bilayers, where it assumes a helix tilt angle of 13°. We note that this tilt angle is significantly smaller than what would be expected from simply matching the hydrophobic bilayer thickness of DErPC [34.4 Å, calculated according to (61)] to the effective length of the TMH with its 28 hydrophobic residues (42 Å, calculated based on a 1.5 Å translation per residue). Using purely geometric arguments, a tilt angle of 28 to 35° would be predicted for such a long monomeric TMH (excluding or including the two outermost Trp residues). The reason for finding a significantly smaller tilt angle of only 13° in DErPC is very likely due to the fact that E5 occurs as a tightly assembled dimer under all conditions observed so far, even in the truncated form of ΔE5 where the disulfide bridges have been removed (26, 31, 32, 51, 60, 62). Such self-stabilization seems to render E5 less prone to adapt its tilt angle to changes in bilayer thickness, because a fixed dimer should prefer maintaining its ideal helix–helix crossing angle. This interpretation is in line with the previously observed inability of E5 to compensate for the negative hydrophobic mismatch in thin bilayers, where it is found to distort and aggregate, yielding disordered ^{15}N-SAMMY-NMR spectra (26). Only the use of thick DErPC membranes (corresponding to the actual thickness of real plasma membranes) makes it possible to reconstitute E5 without aggregation under quasi-native conditions.

Our orientation analysis shows that the transmembrane helix of E5 is inclined toward the face of the helix where Ala14 and

Trp32 are located. Trp32, as well as Trp5 at the opposing end of the helix, are textbook examples of membrane-anchoring residues that are often found to be flanking hydrophobic TMH regions (Fig. 2*C*). These aromatic residues are well known to stabilize transmembrane segments within the amphiphilic bilayer interface, by helping them i) to maintain the proper depth of insertion, and ii) to hold the preferred azimuthal rotation angle (63–67). Trp5 and Trp32 are separated by exactly 7.5 helix turns (i.e., 27 amino acids) in E5, which places these two aromatic side chains on opposite faces of the helix (see helical wheel in Fig. 2 *B* and *C*). This geometry gives the oncoprotein an unusually long hydrophobic stretch that induces a significant helix tilt angle, which seems to further enhance the ability of the Trp side chains to control the azimuthal rotation angle in the membrane, as it was previously shown for a series of WALP peptides (46). The experimentally obtained azimuthal orientation of E5 is thus perfectly in line with the expected helix orientation according to fundamental biophysical principles.

Knowledge of the full membrane orientation of E5 further allowed us to predict the face of the helix by which homodimerization of E5 could take place, according to simple geometrical considerations. Residue Gln17, which is known to be critical for dimerization (24), is indeed suitably located on one of the two vertical sides of the tilted helix (rotated about −60° away from the tilt direction), where it is in a perfect position to form intermolecular hydrogen bonds. Even though we have no direct contact measurements for the oligomeric state of E5 in our NMR samples, the observed azimuthal rotational angle makes it very likely that E5 is present as a dimer with Gln17 located within the dimer interface. (This interpretation is supported by the high-order parameter S_{mol} that is close to 1.0). Furthermore, it is well known that E5 has a high tendency to oligomerize, even without the C-terminal disulfide bridges (32) (see also our SDS–PAGE analysis in *SI Appendix*, Fig. S10). An early MD simulation had suggested two possible dimer interface clusters (29). Given that "cluster 1" (*SI Appendix*, Fig. S8*A*, light green motif) faces toward the direction of tilt, it is unlikely that it constitutes the interface of the E5 dimer according to our NMR data. On the other hand, a couple of residues of "cluster 2" including Gln17 (*SI Appendix*, Fig. S8*A*, dark green motif), are positioned roughly orthogonally to the tilt direction and would be in a favorable position to be involved in dimer formation. Further studies, too, have confirmed that residues of "cluster 2" are involved in homodimerization (21, 50). The subtle but fundamental distinction between left-handed and right-handed helix crossing angle in the dimer can also be readily made now, as the known Gln17–Gln17 interaction and the azimuthal rotation angle determined here clearly support a left-handed helix–helix interaction in the parallel E5 dimer.

Structural Features of PDGFRβ. The PDGFRβ transmembrane helix has a similar hydrophobic length as E5, and is also found to be slightly tilted in thick DErPC bilayers. However, unlike E5, the PDGFRβ-TMH can adapt its tilt in thinner bilayers without aggregating nonproductively (26) (*SI Appendix*, Fig. S3). To follow our geometric description of the tilt direction of PDGFRβ-TMH, it is important to realize at this point that PDGFRβ has a native N-out topology (which is opposite to E5). [Footnote: As there is no control of the up/down alignment of either protein in the NMR sample, E5 and PDGFRβ can assemble freely side-by-side in their preferred mode of binding]. According to the graphic convention of drawing PDGFRβ in a cellular plasma membrane, its N terminus is displayed at the top, hence it would lean away from Lys499/Leu517 (Fig. 3). Interestingly, Trp524 near the C-terminus is positioned on the same face of the helix as Lys499/Leu517. This observation suggests that a tryptophan again determines the azimuthal rotation angle, as in the E5 helix. At the opposite end of the PDGFRβ helix, but lined up with Trp524, Lys499 is ideally situated to serve as an azimuthal membrane anchor, too. Here, its $N^{\varepsilon}H_3^+$-side chain can typically snorkel toward the lipid head group region, as it was demonstrated for a series of KALP peptides (46). Thus, both residues (Trp524 and Lys499) fix the PDGFRβ-TMH not only at a certain depth of insertion, but also stabilize the particular azimuthal orientation.

Interestingly, in our NMR-derived orientation of PDGFRβ-TMH, the polar side chains of Thr513/Ser516 are exposed sideways on the helix, where they could readily form hydrogen-bonds within a left-handed dimer. So far, it is unknown whether the inactive ligand-free state of PDGFRβ is monomeric, or whether a preformed inactive dimer exists in the native membrane environment (27, 68, 69). This question arises for many receptor tyrosine kinases (RTKs), and has essentially led to the formulation of two different activation models. One activation model is based on a ligand-mediated dimerization of monomeric receptors, whereas the other so-called "rotation-model" describes activation as a rearrangement of constitutively preformed dimeric receptors (2, 3, 70–72). Our SDS–PAGE analysis of several different transmembrane helices from various RTKs showed that PDGFRβ-TMH has a high tendency to form dimers or even higher oligomers, whereas PDGFRα-TMH and EGFR-TMH are mainly monomeric on the gel (*SI Appendix*, Fig. S10). According to our NMR-derived membrane orientation, it is quite likely that PDGFRβ is present as a dimer. Both Thr513/Ser516 are oriented in an optimal way for hydrogen-bonding and dimer formation, which would also shield these polar side chains from the hydrophobic membrane interior. Yet, this indirect support of dimerization cannot be proven from our data, and it need not be relevant for the full-length receptor protein with bulky extracellular domains. It is also conceivable that the PDGFRβ receptor in its resting state is present as a monomer-dimer equilibrium, where the dimeric state is stabilized by a range of different intermolecular interactions between the ligand-binding, transmembrane, and tyrosine kinase domains.

An earlier study had postulated that Ser516 is involved in the PDGFRβ-TMH dimerization interface, which also includes Val502, Ala505, Leu509, Leu512, and Leu523 (28). These residues occupy the "a" and "d" positions of a leucine-zipper-like packing motif (*SI Appendix*, Fig. S8*B*, orange motif). It should be pointed out, however, that the helix crossing angle in such an extended heptad motif ought to be significantly smaller than what we have observed here (24°) in the PDGFRβ-TMH dimer, which presumably involves Thr513/Ser516 as a polar contact. In an earlier liquid-state NMR study of PDGFRβ-TMH in DPC micelles, we had reported yet another interaction interface (*SI Appendix*, Fig. S8*B*, yellow motif) (25), which is consistent with a left-handed coiled-coil structure. However, our present solid-state NMR data show that this proposed interface is located on the slanted face of the helix in the direction of the tilt. Therefore, the structure observed in soft detergent micelles does not seem to represent the homodimeric resting state of PDGFRβ within a proper membrane. Compared to a flat lipid bilayer with a pronounced lateral pressure profile, the more unnatural highly curved DPC micelle could allow for a different mode of TMH assembly (33). This comparison demonstrates that the transmembrane helices of RTK should be studied in a natural membrane environment, i.e., in a proper lipid bilayer with appropriate thickness, in its hydrated liquid-crystalline phase. If PDGFRβ is present as a preassembled dimer, then our model of the dimer interface would describe the resting state of the receptor, in the absence of any PDGF ligand.

Structural Features of the E5-PDGFRβ Heterocomplex. The most important result in this study is the observation that activation of PDGFRβ by E5 involves a dramatic change in the azimuthal orientation angles of the transmembrane helices of both proteins, while there is no change in helix tilt. Both helices within the E5 dimer undergo a rotation of about −65°, consequently their original Gln17 dimerization motif is rotated away and replaced by an alternative interface. Interestingly, this new dimerization interface involves the hydrophobic residues of Ala14, Leu18, Leu21, and Leu25, which had been predicted in early MD-simulations as an alternative dimer interface for E5 (cluster 1), in which the interhelical hydrogen bond between Gln17–Gln17 is not involved (*SI Appendix*, Fig. S8*C*, light green residues) (29). Inspection of our 3D model suggests that some π–π interactions between Phe28, Trp32, and Phe35 in the C-terminal region could further contribute to the stability of the rotated E5-dimer in the heterocomplex. In this rotated E5-dimer, the Gln17 side chain ends up outside of the E5-dimer interface and therefore becomes available for interaction with Thr513 on PDGFRβ. Moreover, on the same face of this rotated E5-dimer, an Asp33 from the E5 partner helix becomes exposed in a line with Gln17. These two critical residues, $Gln17_{(a)}$ and $Asp33_{(b)}$ are clearly seen to come together to form the previously postulated interaction interface for PDGFRβ-TMH (29, 30).

We also found that the PDGFRβ transmembrane helix changes its azimuthal orientation by about −95° in the heterocomplex with E5. Here, Lys499 and Thr513, which are both crucial for interaction with E5 (13, 49), are being rotated into an orientation that is essentially perpendicular to the helix tilt direction. Hence, these two side chains now become accessible to interact with Asp33 and Gln17 on the E5-dimer. In our model, Lys499 is not perfectly oriented toward Asp33 of E5, but its long side chain should be flexible enough to find a position where it can interact electrostatically with the aspartate (Fig. 6). It is also noteworthy that in the heteromeric complex, Phe498 now takes over the role of Lys499 as an N-terminal anchoring residue of PDGFRβ-TMH (Fig. 6, purple residues). In addition, hydrophobic interactions between Leu10/Leu24 on E5 and Ile506/Leu520 on PDGFRβ have been proposed to be involved in heterocomplex formation (49, 50). With our determined helix orientation of E5 and PDGFRβ, these amino acids are perfectly aligned to interact.

Based on the detailed membrane alignments of E5 and PDGFRβ-TMH in the heterocomplex, we derived two structural models by assembling the helices in their determined orientations (Fig. 6). We assume that even after the observed azimuthal rotation, E5 remains a left-handed dimer in the E5-PDGFRβ heterocomplex, which is consistent with our NMR data and with the proposed interaction interface described above. It was also shown in earlier studies that E5 dimerization is necessary for its transforming activity (21, 47, 50, 51). Our NMR-based orientational analysis restricts the number of plausible structural models essentially to two types of stoichiometry. On the one hand, the E5 dimer could recruit and bridge two separate PDGFRβ monomers to form a four-helix bundle. On the other hand, a preformed PDGFRβ dimer could be forced to reorient by being clamped from two sides by two E5 dimers, resulting in a six-helix complex. Both models involve the same Gln17-Thr513 and Asp33-Lys499 interactions. In the six-helix-model, there is an additional PDGFRβ-PDGFRβ contact, likely via interhelical hydrogen bonds between Ser504-Ser504 and Ser516-Ser516.

A closer look at these structural models shows that the Gln17 side chain spans nearly the entire diameter of the neighboring E5 partner-helix, since PDGFRβ-TMH interacts with both E5-helices of the E5-dimer at the same time (Fig. 6). Interestingly, E5 will lose its transforming activity if Gln17 is mutated to asparagine (22). Therefore, it seems that a shortening of the glutamine side chain by one methylene segment abolishes heterocomplex formation. The actual side chain length also plays a crucial role in the hydrophobic interactions of Ile506 and Leu520 on PDGFRβ with Leu24 and Leu10 on E5, which apparently need to form a snugly packed hydrophobic interface. This was shown by mutational studies in which mutants Ile506Ala and Leu520Ala inhibited the formation of the E5-PDGFRβ complex, while mutants Ile506Val and Leu520Ile did not affect complex formation (49). Hence, the E5-PDGFRβ heterocomplex is very likely stabilized by a leucine- or isoleucine-zipper-like interaction via these hydrophobic amino acids.

Functional Aspects of PDGFRβ Activation by E5. Knowledge of the membrane orientations of E5 and PDGFRβ-TMH in both, the homomeric as well as heteromeric complexes, allows us to restrict any potential activation mechanisms to two possibilities. In both models that we propose for the oncogenic activation of PDGFRβ, E5 remains a left-handed dimer (*SI Appendix*, Fig. S9). In native membranes, the inactive PDGFRβ is most likely present in a monomer-dimer equilibrium, primarily in the monomeric state (27, 28). In our first model, E5 would interact with the monomeric population of PDGFRβ. In this case, the E5-dimer acts as a scaffold for monomeric PDGFRβ, by bringing two receptor molecules close together, bridging them and enforcing a rotation of the PDGFRβ transmembrane helices. This rotation, in turn, leads to activation of the intracellular tyrosine kinase domains. This four-helix-model speaks for a dimerization-based mechanism, in which the E5 protein induces at the same time dimerization and a change in azimuthal membrane orientation of the receptor helix. Dimerization of PDGFRβ is known to be a critical step for receptor activation (27, 73). However, the simple concept of dimerization cannot fully account for the activation of PDGFRβ. It has been shown that mere cross-linking of two receptor molecules with a bivalent ligand, such as an antibody, does not suffice to activate PDGFRβ or other RTK (3, 74–77). Mutational studies on the ligand binding domain of PDGFRβ have demonstrated that PDGF-induced dimerization is not enough for receptor activation (74, 78). Notably, in both our models, the E5-induced rotation of the PDGFRβ-TMH not only brings the kinase domains into proximity but it also reorients them to facilitate transphosphorylation.

There is in fact growing evidence that a preformed dimeric inactive RTK can become activated by TMH rotations about their long axis, as illustrated for the TRKA, ErbB2, and FGFR3 receptors (1, 3, 4). Accordingly, in our second model, two E5-dimers would interact with a preformed inactive PDGFRβ-dimer, induce rotation of its transmembrane helixes, and thereby enable transphosphorylation. In this six-helix-model, a new right-handed PDGFRβ dimer is formed from the original left-handed dimer of the resting state. This activated dimer is most likely stabilized by polar interactions between the side chains of Ser504-Ser504 and Ser516-Ser516. An inversion of the relatively shallow helix–helix crossing angle (about 24° in DErPC membranes) has to take place in PDGFRβ during the process of heterocomplex formation (while the E5 dimer retains its left-handed configuration throughout). In order to keep the two receptor molecules of the preformed PDGFRβ-dimer together, its rotation and change in handedness would have to be achieved by two E5 dimers. Therefore, in our six-helix model, two E5 dimers bind to both sides of the PDGFRβ dimer and thus force its reorientation. Based on MD simulations,

a different asymmetric model with the same stoichiometry but a left-handed configuration of the PDGFRβ dimer has been postulated by Karabadzhak et al. (30).

Our six-helix model could potentially be extended further, due to symmetry reasons, if even more PDGFRβ dimers and E5 dimers are recruited at both sides of the six-helix heterocomplex, resulting in a long line of alternating dimers. Such a cluster would ultimately have a formal 1:1 stoichiometry. However, the formation of such large clusters of E5 and PDGFRβ is hard to conceive for the full-length receptor, because of its sterically demanding intra- and extracellular domains. Hence, a stoichiometry of 1:2 for PDGFRβ:E5 for this model seems to be the most reasonable suggestion.

A fundamental difference between the four- and six-helix models is the distance and the relative orientation between the C-terminal ends of the PDGFRβ-TMHs. Crystallographic studies of the ligand-bound PDGFRβ-dimer showed that the two transmembrane segments cross-over near the N termini of the helix, causing a significant separation of the C termini in the cytosol. This kind of assembly seems to provide enough flexibility for the active rearrangement of the kinase domains (27). The necessity for a certain separating distance between the C termini of the transmembrane helices was also reported for other activated receptors of the RTK family (56, 79–81). In our four-helix-model, the E5 dimer could act like a spacer to adjust the proper separation for transphosphorylation of the kinase domains, whereas the six-helix-model explains receptor activation by a repositioning of the C-terminal tyrosine kinase domains. However, the distance between the PDGFRβ-TMHs in both of our E5-induced activation models may differ from the corresponding distance in the PDGF-bound receptor dimer, which could explain the previously reported reduced efficiency of transphosphorylation in the E5-PDGFRβ complex (45).

In summary, we have used solid-state 2D-NMR spectroscopy to characterize the detailed membrane alignment of the E5 oncoprotein and of its target helix in PDGFRβ, both, in their homomeric states as well as in the heteromeric complex. Strikingly, both proteins changed their azimuthal orientation upon forming the E5-PDGFRβ heterocomplex, which allowed us to derive two models for the oncogenic activation of PDGFRβ by E5. This study provides the three-dimensional membrane alignment of an RTK-TMH in its inactive state and in its activated form, measured in a natural lipid environment. It also presents a straightforward concept for helix–helix docking, allowing to build a 3D model from simple NMR-derived orientational parameters. Even though the local molecular contacts are not seen directly, nor are the exact cross-over points of the helices fixed, these kinds of 3D models will serve as valuable starting points for all-atom molecular dynamics simulations, possibly even incorporating the given NMR constraints. Already at this stage, some new functional roles for particular side chain have been suggested, which are now open to mutational analysis. Therefore, this robust solid-state NMR approach will be highly useful for other transmembrane homo- and heterodimers, as well as for larger complexes.

Materials and Methods

Recombinant Expression and Purification of Unlabeled and Uniform ^{15}N-Labeled ΔE5 and PDGFRβ-TMH. Unlabeled and uniform ^{15}N-labeled ΔE5 and PDGFRβ-TMH peptides were produced by recombinant expression in *E. coli*. We used a truncated ΔE5 sequence (Met1 to His34, see Fig. 1) that adequately represents the entire TMH segment (32). For the PDGFRβ-TMH construct we used the same sequence (His494 to Glu531) as in our earlier structure analysis by liquid-state NMR (25). For a detailed description of the recombinant expression and purification see *SI Appendix, Materials and Methods*.

Chemical Synthesis and Purification of Selectively ^{15}N-Labeled TMH Peptides. Chemical synthesis of selectively labeled peptides was performed using Fmoc-Glu(OtBu) Wang resin (0.32 mmol/g), according to standard 9-fluorenylmethoxycarbonyl (Fmoc)/tert-butyl (tBu) solid phase peptide synthesis protocols. The ΔE5 sequence was selectively ^{15}N-labeled in the backbone at 10 different positions and the PDGFRβ-TMH was selectively ^{15}N-labeled at five different positions, resulting in 10 and 5 individual peptide analogues, respectively (Fig. 1 and *SI Appendix*, Tables S1 and S2). For a detailed description of the chemical synthesis and purification see the *SI Appendix, Materials and Methods*.

Solid-State NMR Sample Preparation and Measurements. The ^{15}N-labeled peptides were reconstituted in macroscopically aligned lipid bilayers for solid-state NMR measurements, as described earlier (26, 32). Solid-state two-dimensional (2D) ^{1}H-^{15}N-SAMMY static NMR measurements (42) were performed on a 600 MHz Bruker Avance Neo widebore spectrometer, using a home-built HX-probe equipped with a lowE-field resonator (KIT Karlsruhe, Germany, and NHMFL Tallahassee, United States) (82). For a detailed description of the solid-state NMR sample preparation and measurements see the *SI Appendix, Materials and Methods*.

Data, Materials, and Software Availability. All study data are included in the article and/or *SI Appendix*.

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