Membrane receptor activation mechanisms and transmembrane peptide tools to elucidate them

Published, Papers in Press, December 25, 2019, DOI 10.1074/jbc.REV119.009457

Justin M. Westerfield and Francisco N. Barrera[1]

From the Department of Biochemistry & Cellular and Molecular Biology, University of Tennessee, Knoxville, Tennessee 37996

Edited by Karen G. Fleming

Single-pass membrane receptors contain extracellular domains that respond to external stimuli and transmit information to intracellular domains through a single transmembrane (TM) α-helix. Because membrane receptors have various roles in homeostasis, signaling malfunctions of these receptors can cause disease. Despite their importance, there is still much to be understood mechanistically about how single-pass receptors are activated. In general, single-pass receptors respond to extracellular stimuli via alterations in their oligomeric state. The details of this process are still the focus of intense study, and several lines of evidence indicate that the TM domain (TMD) of the receptor plays a central role. We discuss three major mechanistic hypotheses for receptor activation: ligand-induced dimerization, ligand-induced rotation, and receptor clustering. Recent observations suggest that receptors can use a combination of these activation mechanisms and that technical limitations can bias interpretation. Short peptides derived from receptor TMDs, which can be identified by screening or rationally developed on the basis of the structure or sequence of their targets, have provided critical insights into receptor function. Here, we explore recent evidence that, depending on the target receptor, TMD peptides cannot only inhibit but also activate target receptors and can accommodate novel, bifunctional designs. Furthermore, we call for more sharing of negative results to inform the TMD peptide field, which is rapidly transforming into a suite of unique tools with the potential for future therapeutics.

Membrane receptors transmit information about extracellular stimuli into the cytoplasm. This is achieved through a combination of changes in conformation and oligomeric state by membrane proteins with a single transmembrane domain (TMD).[2] The factors that govern receptor oligomerization remain poorly understood yet are critical for obtaining a complete description of receptor function. The existence of TMD dimers shows that self-assembly information is encoded by the TMD. Recently, studies using short peptides composed of the TMDs of receptors have provided unique insights into receptor function. TMD peptides have generally been thought to interact with the TMD of the target receptor and inhibit its dimerization. However, in recent years, TMD peptides against some receptor targets have been found to promote receptor oligomerization. This suggests that interactions between TMD peptides and their targets may provide insights on receptor-activation mechanisms. This insight and the recent development of the first bi-functional TMD peptide, TYPE7, prompt our review of the subject.

The proteins discussed in this review are single-pass receptors, which contain one α-helical TMD and an extracellular ligand-binding domain. We have chosen to omit multispanning receptors from this review, as they may also undergo large changes in their transmembrane tertiary structure, which is beyond the scope of this review. A large superfamily of single-pass receptors—the receptor tyrosine kinases (RTKs)—possesses an intracellular kinase domain. Other non-RTK receptors discussed include the T-cell receptor (TCR), which contains multiple separate subunits with one TMD each. A large number of single-pass receptors have been implicated in disease due to their roles in cell proliferation and differentiation. Therefore, gaining a better understanding of these receptors is of critical importance. Here, we review how TMD peptides can be used as tools to better understand TMD-mediated self-assembly. We direct the reader elsewhere for complementary reviews focused on progress toward therapeutic TMD peptides (1–5), design of TMD peptides (6, 7), or prior reviews of similar focus (8, 9).

Role of the transmembrane domain in activation of single-pass membrane proteins

The plasma membrane offers both challenges and opportunities for membrane proteins. Unlike soluble proteins, which can diffuse and rotate in three dimensions, membrane proteins are anchored to the membrane by their TMD. Because single-pass receptors are constrained to the bilayer, they must func-

This work was supported by National Institutes of Health Grant R01GM120642. The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

This article contains Table S1.

[1] To whom correspondence should be addressed. Tel.: 865-974-4496; E-mail: fbarrera@utk.edu.

[2] The abbreviations used are: TMD, TM domain; TM, transmembrane; LIR, ligand-induced rotation; VEGFR, vascular endothelial growth factor receptor; RTK, receptor tyrosine kinase; TCR, T-cell receptor; LID, ligand-induced dimerization; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; GHR, growth hormone receptor; HGH, human growth hormone; EpoR, erythropoietin receptor; GpA, glycophorin A; MHC, major histocompatibility complex; pMHC, peptide-major histocompatibility complex; PTPRJ, protein-tyrosine phosphatase receptor J; CHAMPS, computed helical anti-membrane proteins; βcR, β common subunit; InsR, insulin receptor; PDB, Protein Data Bank; CAT, chloramphenicol acetyltransferase; IL-3, interleukin 3; CP, core peptide; MTP, membrane-targeting peptide; FDA, Food and Drug Administration; InsR, insulin receptor; PIP_2, phosphatidylinositol 4,5-bisphosphate.

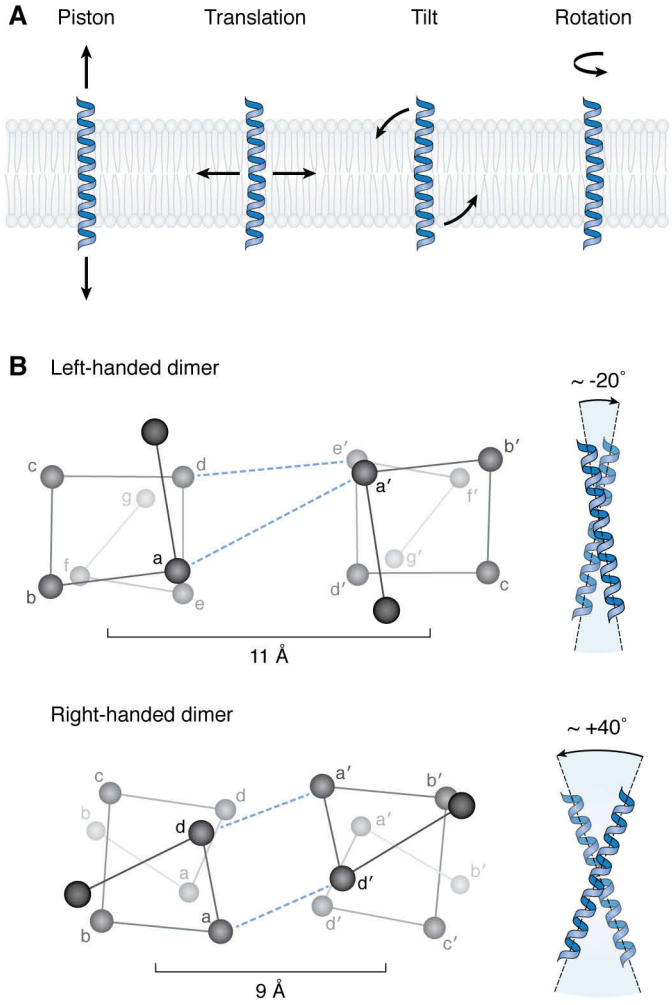

Figure 1. Common structural motifs of TMD dimers. *A,* isolated transmembrane α-helices are constrained to four principal motions: piston movement, translation, tilt, and rotation. *B,* common characteristics of interhelical interaction motifs. The left-handed TMD dimer is typically found at an approximate −20° crossing angle. Similar to soluble coiled coils, the interaction interface can be described in a heptad repeat nomenclature, *abcdefg*. Interhelical hydrogen bonding (*cyan*) is commonly found between *a*–*a*′ and *d*–*e*′. The average interhelical distance is 11 Å (58). The right-handed TMD dimer typically forms at an approximate +40° crossing angle. Right-handed dimers are best described by a tetrad repeat nomenclature, *abcd*. At the *a* and *d* positions, small residues such as glycine are enriched, which create an angled groove where helices may pack tightly together. Interhelical hydrogen bonding is commonly found between residues at positions *a* and *d*′. The average interhelical distance is ~9 Å (58). Representative structures shown are as follows: left-handed is EphA2 (PDB code 2K9Y), and right-handed is glycophorin A (PDB code 5EH4).

tion while their TMD is restricted to four principal motions: translation, piston, pivot, and rotation (Fig. 1*A*) (10). Furthermore, their secondary structure is severely constrained, as the energetic cost of unfolding a peptide backbone in the membrane is a staggering +4 kcal mol^{-1} per amino acid (11). For perspective, this means that inserting just two unfolded amino acids into the bilayer would have an energetic cost similar to phosphorylating one molecule of ADP into ATP. With such constraints, how could a signal be effectively transmitted through a membrane with minimal secondary and tertiary structural change? For many proteins, the answer is assembly. Receptor homo- or hetero-association allows a given receptor to activate phosphorylation cascades only when an extracellular stimulus is present. Receptor associations rely at least in part on the interactions of receptor TMDs. Thus, the same lipid bilayer that hosts them and restricts their conformation, also ensures that they are in an optimal position to encounter another receptor. In this section, we will explore TMD interactions that facilitate receptor activation.

Not surprisingly, studies on diverse single-pass receptors show that the TMD can strongly affect receptor functionality. This is demonstrated by substitution studies in which the TMD of a receptor is replaced with that of a constitutively active receptor. For example, substituting the TMD of the muscle-specific kinase, MuSK, for that of Neu (V664E) (an oncogenic mutant of the Neu/ErbB2 receptor) created a receptor that constitutively self-assembles into signaling-active receptor clusters (12).

Mutations in the TMD that alter dimerization can also lead to disease. TMD mutations cause aberrant activation in many receptor families. Half of the 20 classes of RTKs have members with such mutations. There are documented examples of activating or inactivating transmembrane mutants for epidermal growth factor receptor (13), ErbB2 (13), insulin receptor (14), platelet-derived growth factor receptor (15), vascular endothelial growth factor receptor (VEGFR) (16), fibroblast growth factor receptor (17–20), nerve growth factor receptor (21), Eph receptor (22), discoidin domain receptor (23), RET (24), and ROS (25). Other non-RTK single-pass membrane proteins suffer these activating mutations as well. For example the T-cell receptor ζ chain TMD contains a central aspartic acid which, when mutated to valine, abrogates dimerization resulting in inactivation of the TCR complex (26). Yet there are yet other receptors, such as multispanning and nonsignaling membrane proteins, that are aberrantly activated by TMD mutations. For example, mutating an aspartic acid in the TMD of the nine-pass protein presenilin (1 or 2) reduces heterodimerization and promotes β-amyloid production (27, 28). Note that not all of the mutations discussed above are naturally occurring, but nevertheless they demonstrate that TMDs are critical for the proper function of membrane proteins in a variety of contexts.

Although TMDs play a role in receptor activation, we are still learning the structural and sequence requirements that account for the observed specific TMD interactions. It is also unclear how membrane proteins regulate these interactions to avoid spurious activation in the absence of ligand stimulation. In the following sections, we will discuss specificity of TMD interactions and how TMD interactions contribute to receptor activation mechanisms.

Specificity of transmembrane helical interactions

In a way, the discovery of specific TMD interactions resembles the discovery of specific soluble receptor–ligand interactions in the early 1900s (29). In the latter case, it was thought that with the great diversity of proteins in the body, it would be extremely unlikely that a ligand could specifically bind to just one receptor type. Similarly, specific TMD interactions were widely thought to be unlikely, given the similar hydrophobicity of TMDs and the expected frequency of stochastic membrane protein collisions. In both cases, controversy and indirect evidence abounded, but key evidence was lacking until experi-

ments were designed to systematically address the questions. A major breakthrough came with the first high-resolution crystal structure of an integral membrane protein complex, the photosynthetic reaction center. Although suggested before, this structure unambiguously showed tight transmembrane contacts, indicating that independent helices specifically associate in the membrane (30).

This was further directly tested by Popot *et al.* (31) when they cleaved bacteriorhodopsin into two inactive fragments and successfully reconstituted them into vesicles, where bacteriorhodopsin regained its fold and function. This was taken a step further by cleaving bacteriorhodopsin into its seven constitutive helices and studying them independently, a study that was complicated by some of the peptides being too soluble or aggregation-prone to readily fold into the membrane (32). This research led to the two-stage model, which posits that transmembrane helices are independently folding units that can then further assemble with one another (33).

Pioneering work on glycophorin A (GpA) provided the first structural details of a specific TMD interaction. Previously, it was thought that interhelical interactions would only be driven by the hydrophobic effect. Polar residues would aggregate together within the bilayer as nonpolar residues do in solution. In other words, nonpolar residues were thought to contribute only weakly, if at all. Eventually, alanine-scanning mutagenesis of the GpA TMD showed that certain residues, including hydrophobic ones, contribute more to dimer formation than others and revealed a key concept with deep ramifications: TMDs have preferred interaction interfaces (34, 35). Furthermore, the residues that contribute the most to the dimerization of GpA, when put into a polyleucine sequence, also confer dimerization propensity (36). This was the first discovery of a sequence motif that could confer dimerization specificity to a transmembrane α-helix. Contrary to the hydrophobic effect model, the critical region of the interface is composed of glycines and valines (LI*XX*GV*XX*GV*XX*T). Even a single methyl introduction on one of the interfacial glycines (*e.g.* Gly to Ala) is enough to disrupt the dimer (34). The authors reasoned that valine residues from one helix formed a ridge that packed into a groove created by glycine residues on the other (*i.e.* knobs-into-holes). This suggests that it is not only hydrophobicity but side-chain packing that determines specific interhelical association.

When the structure of the GpA TMD was solved, it corroborated earlier conclusions. Specifically, van der Waals–induced TMD interaction is indeed possible (37), and the dimer is strong. GpA dimerizes with a free energy of dissociation of 9.1 kcal mol^{-1} (38). Additionally, it was found that noncanonical hydrogen bonding between the α-hydrogen and carbonyl oxygen strongly contributes to the dimerization (39). Still, GpA shows us that transmembrane sequence motifs can result in specific interactions. In the next section, we will discuss such sequence motifs. For a more thorough historical review of the specificity of GpA interactions, we suggest Ref. 40.

Common transmembrane interaction motifs—The structure of GpA shows that, as predicted (34), the transmembrane interface contains two critical glycine residues separated by three amino acids (37). This so-called G*XXX*G sequence motif is highly over-represented in transmembrane α-helices compared with soluble helices (41, 42). Later, meta-analyses of the solved structures of helical dimers revealed additional interaction motifs. Just four different structural motifs account for two-thirds of all the helical pairs (43, 44). Indeed, most RTKs contain at least one predicted dimerization motif in the TMD (45). It should be noted that any such analysis is subject to the limited number of solved membrane protein structures and that there are likely more motifs that will be discovered as more TMD interactions are studied. In general, single-pass receptor TMDs form parallel interactions (*i.e.* the N termini of the TMDs are on the same side of the membrane) rather than anti-parallel interactions (46). For this reason, we will only discuss the two parallel motifs in this review. For a thorough review of transmembrane helical interaction motifs, including the two antiparallel motifs not discussed here, we suggest Ref. 47.

The G*XXX*G motif first discovered in GpA belongs to the largest class of motifs, the GAS_{right} motif (43, 44). The "right" in GAS_{right} refers to the crossing of the two helices, where the top of the helix closest to the viewer is pointing right. They cross approximately at a right-handed 40° angle (Fig. 1*B*). The "GAS" of this motif refers to the small amino acids glycine, alanine, and serine, which, when placed four residues apart, form an angled groove down the helix. When opposed, two helices with the same groove will pack tightly, and the association will be stabilized by van der Waals forces as well as Cα-H—O=C noncanonical backbone hydrogen bonding, which contributes 0.88 kcal mol^{-1} per bond (39, 41, 48, 49). Further analysis shows that this motif is precisely optimized for stabilization of these noncanonical hydrogen bonds (50). This suggests a causal link between the prevalence of G*XXX*G motifs and strong noncanonical hydrogen bonds.

Another major class of parallel motifs is the left-handed dimer (Fig. 1*B*). These typically have a smaller crossing angle, around 15–20° (44). These left-handed interactions are often stabilized by heptad repeats, repeating units of seven amino acids (*abcdefg*) (51) where the interfacial residues are often at the *a, d, e,* or *g* positions (52). This motif is based on the remarkably accurate 1953 prediction by Francis Crick that two soluble, symmetric α-helices could tightly coil around each other with their residues packing in a knobs-into-holes manner, but only if they were offset by 20° and their *a* and *d* positions were polar (53). Later, similar periodic repeats were found in many soluble proteins (54–56), and finally, a crystal structure confirmed the first knobs-into-holes–mediated soluble coiled coil (57). As mentioned above, the same periodicity that allows knobs-into-holes packing can be found in transmembrane α-helices, which tend to have two to three heptad repeating units. Meta-analysis confirmed a preference for *a*–*a*′ hydrogen bonding in TMD interactions (58). However, multiple studies have failed to find site-specific preference for amino acid type in these motifs (44, 52), and they have led to speculation that the apparent motif may simply be a consequence of helix packing (*i.e.* the interacting residues will appear periodic) and that left-handed sequence motifs may be a red herring (59).

Exceptions and unanswered questions—Despite earlier evidence that motifs drive association, TMD dimers have been found that contain no discernable sequence motif (59). Fur-

thermore, sequence motifs do not always confer dimerization propensity. For example, only very weak dimerization of the TMD of protein tyrosine kinase 7 occurs, despite the presence of a G*XXX*G motif (60). Similarly, the RTKs EphA1 (61) and EphA2 (62) form dimers in bicelles, but apparently prefer a left-handed interface over their G*XXX*G motifs. Furthermore, there are many favorable geometries that have no apparent sequence motif, lending more support to the idea of structural classes of interactions (*e.g.* left-handed parallel), rather than sequence motifs (58). In fact, the presence of a G*XXX*G motif is not a strong predictor of dimerization (63), and predicting interaction with G*XXX*G motifs often involves "idealizing" the helices into straight rods, ignoring helical kinks (48). This point was demonstrated by a double-mutant screen of GpA, which found that G*XXX*G is neither necessary nor sufficient for dimerization in the GpA TMD (64). Although in some contexts sequence and structural motifs are correlated, it seems that targeting a desired structure is more likely to yield predictable structural outcomes than relying on sequence motifs, as we will see under "Structure-based design."

Structural contribution of transmembrane domains to activation mechanisms

Understanding the activation mechanisms of single-pass receptors has remained tantalizingly out of reach despite decades of intense research. Small victories only serve to pose more questions, and a full description of receptor-mediated signaling remains elusive. Regardless, several clues have been unearthed, and as our understanding grows, so does the complexity of the models required to describe the mechanisms. Some receptors require simple descriptions, whereas others require more complex models.

As we study the TMDs of single-pass receptors, a pattern is emerging. Most receptor TMDs, including all of the RTKs, and toll-like receptors, have a tendency to self-associate (65, 66). This associative property contributes to the overall dimerization of the protein and is typically balanced with the associative and dissociative properties of soluble domains. For example, the extracellular ligand-binding domain of EphA2 promotes dimerization (67), whereas the intracellular sterile α-motif domain inhibits it (68, 69). Each receptor likely maintains a unique balance of forces such that only the right conditions will tip the scales in favor of receptor oligomerization and activation.

In this section, we will discuss three mechanistic hypotheses for receptor activation in order of increasing complexity. We propose that there is no universal model for receptor activation and that receptors may use more than one mechanism to achieve full activation.

Ligand-induced dimerization (LID)—Early work on the epidermal growth factor receptor (EGFR) suggested that the receptor is more active in the dimeric form than in the monomeric form (70, 71). This led to the LID hypothesis, which posits that ligand binding induces dimerization, which is sufficient for activation (Fig. 2) (72–74). Most of the early arguments for LID were based on observations that bivalent ligands may "cross-link" receptor monomers together (75).

After the initial observation of EGFR dimerization, a study on the human growth hormone receptor (GHR) provided the first strong evidence for LID. In this thorough analysis, Cunningham *et al.* (76) show that human growth hormone (HGH) forms a 1:2 complex with the extracellular domain of GHR; two GHR receptors bind to a single HGH molecule. This provided a mechanistic model for how receptor "cross-linking" might occur. The major determinants for activation would thus be in the extracellular ligand-binding domain. Another study on the erythropoietin receptor (EpoR) provided evidence to support this finding, showing that a cysteine mutation in the extracellular region of EpoR caused it to become a constitutively-active dimer by allowing the formation of intermolecular disulfide bonds (77).

In the simple LID hypothesis, the TMD could either be passive or active. Early experimental evidence seemed to support the passivity of the TMD (78, 79). Thus, it was thought that the extracellular domain would drive the receptors together, and the TMD would transmit that proximity information through the membrane to the intracellular domains. However, as discussed later, the TMDs of RTKs form dimers by themselves, which suggests an active role for the TMD. These studies collectively support the LID hypothesis, where a ligand stabilizes the dimeric form of the receptor, which is signaling-active. However, this hypothesis has difficulty explaining some observations, as we will see in the next section.

Ligand-induced rotation (LIR)—Since the birth of the LID hypothesis, evidence began to appear against it. Receptors can be found as unliganded dimers particularly at high protein densities in the membrane. This list includes RTKs such as EGFR (80), EphA2 (81, 82), and the insulin receptor (83), as well as cytokine receptors such as the EpoR (84). A full list can be found in Ref. 85.

Several studies have further shown that dimerization alone is not sufficient for receptor activation. For example, some cysteine mutations in the membrane-proximal region of ErbB2 induce dimerization, but not transforming activity (86), nor is dimerization sufficient for activation of TCR (87). The confusion was further compounded by the apparent observation of an inactive dimer structure of the EGFR intracellular kinase domain (88). Furthermore, varying degrees of self-association have been observed for all of the RTK TMDs, which indicates that some regulatory mechanism must be in place to prevent spontaneous activation (65). This evidence points to allosteric, rather than oligomeric, regulation of receptor activity. It should be noted that this was observed via the bacteria-based TOXCAT assay (89). In this assay, TMDs are tagged with ToxR, a transcription factor that induces expression of chloramphenicol acetyltransferase (CAT) upon dimerization. The levels of CAT are determined by an enzymatic assay and compared with a control condition. TOXCAT can detect very weak interactions but measures only relative, not thermodynamic, strength of association.

Allostery is an obvious alternative to dimerization (90). However, classical allosteric models rely on globular domain conformational changes. How could changes of the dimeric extracellular domains of receptors lead to rearrangement of the intracellular domains into an active state? This would be a con-

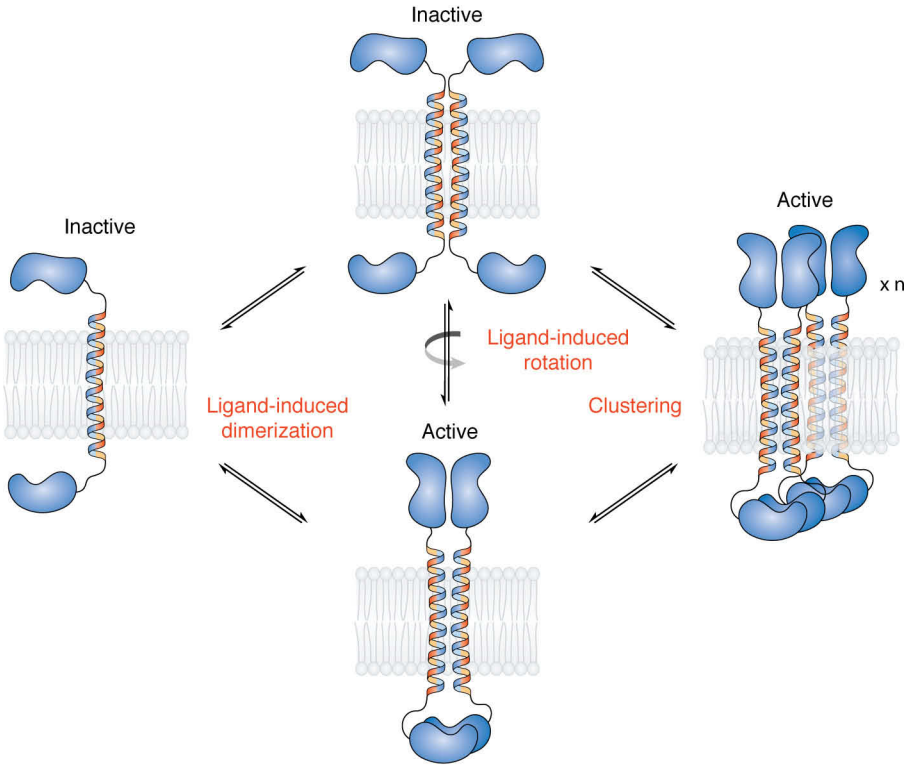

Figure 2. Role of TMD in receptor activation mechanisms. The LID hypothesis posits that ligand binding to the extracellular domains of the receptor brings receptor monomers together into a dimer that is signaling-competent. The LIR hypothesis assumes that an inactive dimer exists and that ligand binding induces a rotation of the receptor to bring the intracellular kinase domains into the active configuration for signaling. Clustering occurs when receptors are stabilized as large higher-order oligomeric signaling complexes. These mechanisms are not necessarily mutually exclusive, and some receptors may use a combination of them. *Blue* and *orange* represent different TMD interfaces. Domains are not to scale, and the ligand is not shown for clarity.

strained structural change, as the TMD would have to remain an α-helix as discussed above. A possible solution to this conundrum was proposed in Ref. 80. If the dimeric extracellular domains were to rotate upon ligand binding, then the transmembrane and intracellular domains would also rotate without sacrificing their dimeric state. This hypothesis was dubbed the LIR mechanism (Fig. 2).

A strong test of the LIR hypothesis was performed on the Neu and platelet-derived growth factor β-receptors (91). In this study, the TMD of the target receptors was replaced with a polyvaline sequence that is unable form a dimer on its own. The receptors only dimerized upon addition of two glutamic acids at positions *a* and *e* in the TMD sequence. Although dimerization occurred when the glutamic acids were in any position, activation only occurred periodically—when the glutamic acids were on one "face" of the helix. A similar observation was made on ErbB2; Burke and Stern (92) added cysteines at several positions in the juxtamembrane domain and determined that this region contains an additional dimerization interface, which may explain the rotational coupling of the TMD for activation. Both interfaces must line up face-to-face. For example, GHR forms a constitutive dimer yet is inactive in the absence of ligand. By blocking the "inactive" transmembrane interface with alanine mutations, the receptor can be activated in the absence of ligand (93).

Another approach for testing the coupling of TMD rotational conformation to receptor activation consists of using the Put3 domain (94). The soluble coiled-coil Put3, when shortened to varying degrees, can be used as a scaffold for rotating the dimerization interface. Using this approach, Matsushita *et al.* (95) found that the juxtamembrane domain of ErbB2 associates with the membrane more favorably in certain orientations of the transmembrane dimer. Similarly, Mohan *et al.* (96) found that synthetic ligands activate EpoR depending on the distance and angle that they induce between the extracellular domains. This might provide a mechanism for how LIR results in activation: certain orientations may favor dissociation of the juxtamembrane domain from the membrane interface, relieving autoinhibition of the kinase domain.

Structural studies suggest that motif switching might be a critical aspect of the LIR hypothesis. The difference between what is considered a strong transmembrane dimer (GpA) and a weak one (GpA destabilized by the G83I mutation) is only on the order of <2 kcal mol^{-1} (97). Therefore, weak or nonideal TMD interfaces may still be able to mediate dimerization in an active state. This may be the case for VEGFR. When two glutamic acids are introduced into the transmembrane domain, the interaction interface is rotated 180°, resulting in constitutive activation (98). Therefore, a weak TMD dimer in the active state may actually be preferred in order to prevent spontaneous signaling (99). TMDs that have so far been observed "switching" conformation are EGFR (100) and GHR (101). The effect has also been predicted for others such as EphA2 (22, 62).

The evidence above strongly suggests that for some receptors (*a*) there is a preferred TMD dimer orientation for activation,

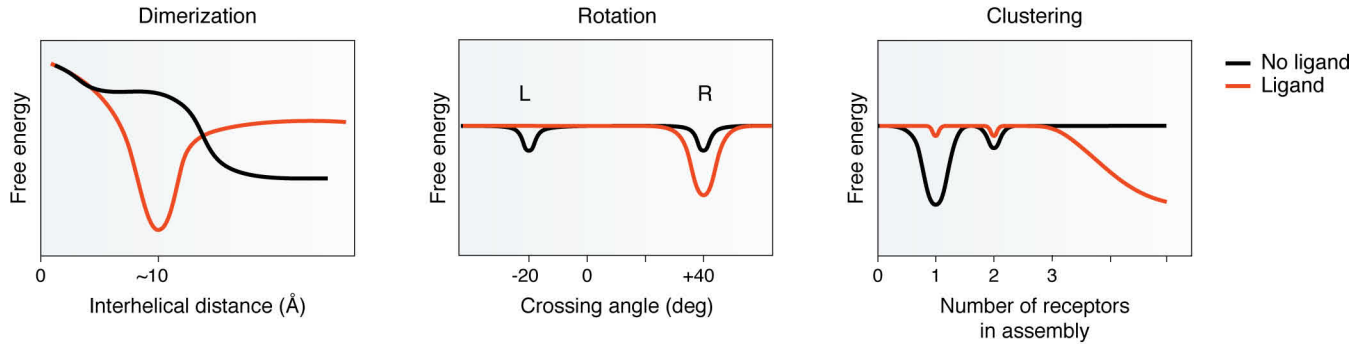

Figure 3. Receptor activation can rely on different processes. LID requires that receptors come into close contact upon ligand binding. The *left panel* shows that in the presence of ligand, a free energy minimum appears when receptors are in contact (10 Å is the approximate distance from helix centers (58)). In the absence of ligand, receptors will be separated by some context-dependent average distance between monomers. LIR requires reorientation of TMDs. The *central panel* shows that receptor TMDs may have more than one permitted crossing angle, whereas only one is favored in the presence of ligand. This example shows a ligand-induced shift toward the right-handed (*R*) conformation. Clustering requires that ligand binding induces formation of larger oligomers. The *right panel* shows that in the absence of ligand, high-order oligomers are energetically unfavorable, whereas the presence of ligand stabilizes clusters. These processes are not mutually exclusive and may all occur in the same receptor.

(*b*) dimerization is not sufficient for activation, (*c*) the TMD plays a critical role in the LIR mechanism, and (*d*) the juxtamembrane domain may play a regulatory role in dimerization.

> *Receptor clustering*
>
> *"We now know that the receptor for insulin is only one of a much larger family of structurally-related cell-surface receptors, and there are persuasive data to demonstrate that this whole family of growth factor and related receptors uses ligand-induced aggregation as a primary control mechanism" (102).*

The LIR and LID hypotheses for receptor activation invoke a type of allosteric regulation of signaling. In both mechanisms, the receptor is considered inactive until stimulation by a ligand induces a conformational change that stabilizes the signaling-competent active conformation (Fig. 3). This requires only dimerization. However, some receptors form tetramers, and even large-scale oligomers (*i.e.* clusters) after stimulation. The LID and LIR hypotheses ignore this common phenomenon, perhaps because it is a problem of a different scale. Receptors that cluster become only fully active when they are at high local densities in the membrane. This makes kinases more likely to encounter their substrates, especially when the substrate is another receptor. Here, we will discuss how clustering activates and regulates receptors, and the role that the transmembrane domain may play in this phenomenon (Fig. 2).

Many receptors cluster upon ligand stimulation. Examples include the following: immune receptors such as the B-cell (103–107) and T-cell receptors (108); RTKs such as EGFR (109); the platelet-derived growth factor β-receptor (PDGFβR) (110–112); GHR (76); and Eph receptors (113). Clustering may also modulate the activation of integrins (114). With the exception of GHR, each of these receptors will be discussed in detail below. This is not meant to be a comprehensive list, as there are yet other receptors not discussed here that form clusters. Rather, it is meant to highlight that clustering is a common feature of many receptor activation mechanisms.

A key difference in the examples mentioned above is the effect of ligand stimulation on how receptors associate into a cluster. For example, EGFR forms, on average, tetrameric complexes upon stimulation (115–118), whereas Eph receptors form much larger micron-sized clusters (119–122). Quantitatively assessing cluster stoichiometry in cells is difficult; therefore, there are still only a few studies that report it. However, it is clear that clustering plays an important role in signaling and deserves further investigation. Special emphasis should be placed on testing a wide range of receptors in native conditions.

The clustering model may explain why overexpression of many receptors leads to malignancy. Simply concentrating the receptors is enough to induce clustering and activate signaling, as modeled in Ref. 107. As examples, malignancy is induced by overexpression of EphA2 (123, 124), EGFR (125), Axl (an RTK) (126), hepatocyte growth factor receptor (127), and many others (128). The concentration effect can also be studied by presenting cells with artificial substrates coated with ligands at fixed intervals (129). Receptors that rely on clustering will only be activated by these substrates when the density of ligands is high enough. Eph receptors, such as EphA2 (130) and EphB2 (131), have been activated in this way, suggesting that Eph receptors rely on clustering for activity. TCR also responds more efficiently to dense clusters of peptide–major histocompatibility complex (pMHC) ligands than sparse ones (132). Various applications and findings of ligand nanopatterning studies are reviewed in Ref. 133.

In the LID and LIR hypotheses, dimerization in the correct conformation is theoretically sufficient for full-signaling activation (Fig. 2). However, some studies show that there are receptors for which dimerization does not cause maximal signaling. For example, almost a century ago, Karl Landsteiner (134) could generate an allergic response with polyvalent but not monovalent antigens against what was later found to be the immunoglobin E receptor. If dimerization were sufficient, some concentration of ligand should be able to produce a full response, even if the receptor/ligand stoichiometry differs from 2:1. The effect was also observed in the unrelated insulin receptor, where polyvalent but not monovalent antibodies could elicit cellular responses (135). As expected, clustering of ligands is also especially important for Eph receptor activation: only ligands "pre-clustered" with an antibody fragment can fully induce signaling, whereas monomeric ligands are only partial

agonists (119, 120, 136). This effect can also be replicated by allowing monovalent ligands to diffuse laterally in a bilayer. The T-cell receptor forms clusters upon activation with a diffusive pMHC but not with an immobilized pMHC (137). Not surprisingly, mobile bilayer-bound ligands also activate EphA2 (136, 138–140). Various receptors cluster upon activation, and there is evidence that the TMD is important for this process.

Transmembrane domain-induced clustering—Clustering induced by TMDs does not require a sophisticated model. According to the fluid mosaic model, proteins and lipids diffuse randomly in the lipid bilayer (141). As discussed above, this is clearly an oversimplification. However, even without including suborganization, the fluid mosaic model already goes far enough as to suggest how proteins may be prone to clustering. Confinement of diffusion to two dimensions greatly increases the effective concentration. Thus, the membrane enhances interactions simply by restricting the diffusive space (142). Aggregation is promoted by the concentration of proteins on the membrane by the "volume exclusion effect" (102, 143). Furthermore, membrane properties can directly affect TMD dimerization. For example, oligomerization is enhanced by the ordering of the bilayer caused by cholesterol (144, 145) or by a transition to the gel phase (146). Oligomerization also relies on matching of the hydrophobic thickness of the TMD and the surrounding lipid. If the lipid bilayer is too thin or too thick, oligomerization can be reduced in model membranes (145, 146). For a more thorough review of the role of the lipid bilayer on single-pass membrane protein oligomerization, we suggest Ref. 147.

Receptor clustering has profound effects on signaling efficiency and sensitivity (148). For example, the B-cell receptor co-clusters with its downstream effector Lyn, while excluding the phosphatase CD45 (107). Likewise, TCR complex clustering results in exclusion of CD45, prolonging the phosphorylation and thus signaling of receptors in the phospho-protected cluster (149). In another recent example, a TMD peptide targeting the single-pass protein-tyrosine phosphatase receptor J (PTPRJ) disrupts receptor dimerization and enhances its activation, reducing EGFR phosphorylation even though no direct PTPRJ–EGFR interaction has been observed (150). Sub-localization of molecules in clusters may be explained by interactions with the cytoskeleton, where proteins that interact more strongly with actin migrate more effectively toward the center of the cluster (151). This effect may be further enhanced by the suborganization of the membrane.

There is mounting evidence that the plasma membrane can be segregated laterally into subdomains, sometimes referred to as lipid rafts, although this topic is still debated. Lipid mixtures can phase-separate into liquid ordered and disordered domains (152, 153). Some membrane proteins have a preference for different membrane subdomains (156). It has been hypothesized that the thickness of the bilayer is a key determinant for protein sorting to lipid domains (157). However, transmembrane length does not appear to affect the clustering of receptors (78, 158). It has been recently demonstrated that, in cell membranes, protein TMDs encode preferential partitioning information based on their lipid-accessible surface area, not in their hydrophobic length (159). This disagrees with studies in model membranes, suggesting compensatory forces that will be discussed below. Lipid subdomain partitioning further increases the effective concentration of receptors to such a point that transient clustering and activation might be expected. However, full activation requires more than transient clustering. A cluster must be sustained for a set period of time before a signal is produced (160). Despite this fact, multiple factors acting in concert may be able to induce receptor activation (*e.g.* strong localization to these domains *and* interaction with other receptors). These can be brought about by seemingly minor changes in some cases. For example, a single TMD mutation induces constitutive clustering and activation of the Neu receptor (99). The membrane is thus a critical factor in understanding receptor activation (147).

Implications for understanding receptor activation—If there is a single, unified picture of single-pass receptor activation, it is a blurry one. Receptors bind ligands, and this triggers a signal. This much is clear. But what happens in-between is still poorly understood. Over the years, different hypotheses have been proposed. The LID hypothesis assumes that receptors are monomers and only signal upon dimerization and that close contact is the only requirement (Fig. 2). The LIR hypothesis assumes that receptors can form inactive dimers and only activate upon allosteric rotation to the correct intracellular conformation. So far, the transmembrane domain interface (*i.e.* crossing angle) has been implicated as a possible mechanism. Using only these two hypotheses, Paul and Hristova (161) recently proposed a general model of RTK activation. Although it is currently unclear how broadly applicable this model is, these studies help to synthesize common elements of receptor activation. The clustering hypothesis assumes that receptors somehow prevent maximal signaling until forming large clusters. It is important to keep in mind that these hypotheses are not necessarily mutually exclusive.

Each receptor likely has a unique set of factors that regulate activity. Receptors may in principle use all three of the activation mechanisms discussed above (Fig. 3). One can imagine a monomeric receptor that transiently forms inactive dimers, but only upon ligand binding does it dimerize in the correct configuration. Then, factors such as the cytoskeleton and membrane suborganization limit its diffusion until receptor dimers are recruited into a large signaling cluster and an optimal signal is generated. In fact, all of the above phenomena have been observed for a single receptor, EGFR (70, 100, 109, 162).

Other regulating factors may provide receptor specificity as well. Let us briefly review the influences discussed. There are extracellular influences, such as the distribution of ligands on opposing cells or surfaces (136). Similarly, extracellular matrix components such as fibronectin can bind to specific receptors to concentrate them around features such as focal adhesions (163). As mentioned before, lipid order favors oligomerization (144, 145). Hydrophobic matching of the TMD and lipid bilayer also favors oligomerization (145, 146). Specific helix–lipid interactions, in contrast, may hinder oligomerization (164). Lipid lateral organization may also play a role. As an example, the juxtamembrane domain of EGFR binds to PIP_2 with a free energy of -0.9 kcal mol^{-1} (165) and could thus be expected to localize to regions enriched with PIP_2. Of course, regulation of

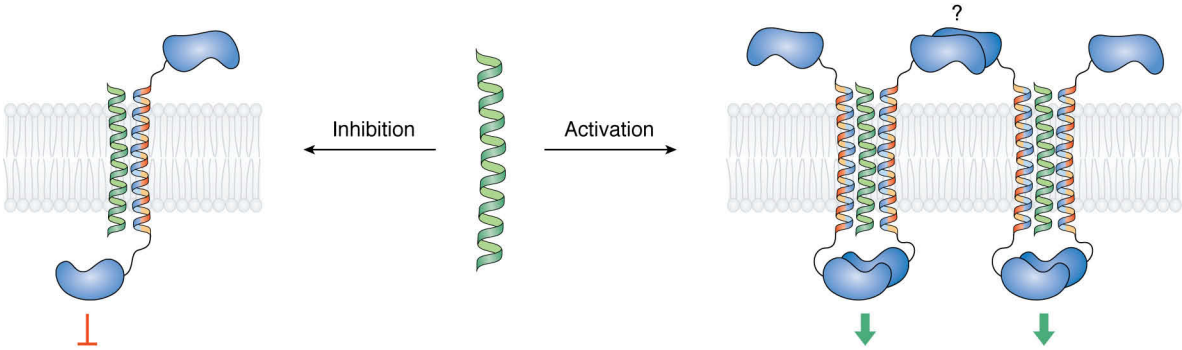

Figure 4. TMD peptide functional consequences. TMD peptides can have different functional effects based on the receptor activation mechanism. TMD peptides (*green*) may competitively inhibit receptor dimerization, leading to reduced signaling or, alternatively, increased signaling in the case of integrins. Receptor complexes may also be stabilized by TMD peptides such as the traptamers and TYPE7, leading to activation, although how this occurs structurally is still unclear. TMD peptides interact preferentially with a specific interface of the TMD shown in *blue*.

protein levels also affects oligomerization; simply increasing the levels of receptors in the membrane will make transient collisions between proteins more likely (107). Finally, intracellular factors may promote specific protein oligomerization. For example, the actin cytoskeleton limits diffusion (151), and scaffolding proteins provide substrates on which receptors can specifically associate (166).

As it is becoming clear that different receptors use different activation mechanisms, high-resolution descriptions of individual receptors would be useful. The relative contributions of dimerization, rotation, and clustering for individual receptors should be the focus of future studies.

Transmembrane peptides to modulate receptor oligomerization

Single-pass receptor activation mechanisms rely on TMDs, at least as an anchor and at most as a participant in stabilizing oligomeric states. The role of the TMD can be studied directly with peptides that target the TMD (*i.e.* TMD peptides) (Table S1). TMD peptides may either be synthesized or expressed in cells, a point that may affect their efficacy and will be discussed later. Interactions between receptors and TMD peptides can inform on the receptor activation mechanism. For example, if a receptor requires TMD-mediated dimerization to function, then a TMD peptide targeting that receptor should be able to competitively inhibit receptor dimerization. For some receptors, this simple case is observed. Others require more complex models (Fig. 4). The various effects of TMD peptides have much to teach us about the diversities and similarities between single-pass receptor activation mechanisms. In this section, we will discuss the different approaches to designing TMD peptides to modulate receptor function and how the resulting peptides can be used to study activation.

Structure-based design

An obvious, yet technically challenging approach to designing TMD peptides is to design a TMD peptide based on the structure of the target. The challenge of this approach lies in being able to accurately predict the forces that drive TMD association. TMD interactions can be stabilized by electrostatic interactions, polar side-chain hydrogen bonds (167, 168), backbone hydrogen bonds (39), van der Waals interactions (169), or a combination of these forces. As backbone hydrogen bonding and van der Waals interactions rely on packing of helices and side chains, respectively, structural complementarity is, in principle, sufficient for helix association.

The process of designing TMD peptides based on structure was developed by Yin *et al.* (170). Briefly, a "template" dimer structure is populated with the amino acids of the target sequence on one helix and a random sequence on the other. Next, a Monte Carlo repacking algorithm varies the side chains on the random helix in order to generate nonclashing combinations. Finally, an energy function determines optimal residues for each position. The resulting peptides are called computed helical anti-membrane proteins (CHAMPs).

Integrin receptors detect extracellular matrix components to control cell adhesion. Unlike many receptors, the TMDs of α- and β-integrin heterodimers *dissociate* when activated by agonist-induced extracellular domain rearrangements (172). For integrin heterodimers containing a β_3-subunit, this results in platelet aggregation. The simple dimer–monomer activation mechanism of integrins offered a convenient platform to test whether TMD peptides could be designed against them.

Before designing peptides based on structure, Yin *et al.* (170) first designed a TMD peptide based on the sequence. This rational design approach allowed them to first test the feasibility of targeting the integrin transmembrane domain. Site-directed mutagenesis had already suggested that disruption of the $\alpha_{IIb}\beta_3$ helical dimer would induce signaling (173). However, it was unclear whether a synthetic TMD peptide could produce the same effect. Thus, the authors synthesized a peptide composed of the α_{IIb} TMD (α_{IIb}-TM) (Table S1) with hopes that it would bind to the β_3-subunit and disrupt the $\alpha_{IIb}\beta_3$ TMD interaction (174). Indeed, α_{IIb}-TM binds to and activates the $\alpha_{IIb}\beta_3$ integrin, demonstrating that a TMD peptide can disrupt TMD dimer formation and lead to integrin activation.

CHAMP peptides, unlike α_{IIb}-TM, are not required to have homology with any native sequence. CHAMPs were first designed to disrupt the $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ TMD interactions (170). Using the side-chain repacking method described above, the authors designed sequences that were predicted to structurally complement the α-subunit TMD. The resulting peptides (*i.e.* anti-α_v and anti-α_{IIb}) (Table S1) activate their target

receptors by competing with the integrin transmembrane domain heterodimer (175). Anti-α_{IIb} induces platelet aggregation, whereas anti-α_v induces platelet adhesion to osteopontin-coated plates (a feature of $\alpha_v\beta_3$ activation) (170), but neither induces the other effect. This demonstrates not only the validity of the CHAMP approach, but that TMD peptides can be specifically targeted.

A key advantage of the CHAMP design approach is that the target sequence is the only input. This means that studying the effects of disrupting heterodimeric interactions is possible, even if a native interacting partner is unknown. It also means that peptides can be composed of noncanonical amino acids (171). Another advantage is that CHAMP peptides do not need to replicate complex native interactions. For example, the structure of the $\alpha_{IIb}\beta_3$ heterodimer contains an arginine–aspartate salt bridge that is not possible with anti-α_{IIb}, yet anti-α_{IIb} activates $\alpha_{IIb}\beta_3$ regardless (176).

The CHAMP design method assumes that tight helical packing will lead to interhelical associations. However, site-directed mutagenesis of the $\alpha_{IIb}\beta_3$ heterodimer shows that mutation of β_3 (*i.e.* I693A) results in underpacking of the dimer interface, yet it has no effect on integrin activity (176). This is likely due to the presence of strong polar interactions (*e.g.* the arginine–aspartate salt bridge) acting on the same dimer. van der Waals-only interaction models have dramatically improved lately due to incorporation of more sophisticated prediction programs such as Rosetta membrane (177, 178). This concept has been used to accurately design a transmembrane helical pentamer using only van der Waals interactions (169). Future prediction models will benefit from incorporating polar and nonpolar interactions as well as protein–lipid and protein–solvent (*e.g.* side-chain snorkeling) interactions.

Sequence-based design

Screening

Genetic-based selections for functional TMD interactions is perhaps the most efficient way to identify new sequences that activate receptors via their TMDs. In recent years, the DiMaio and co-workers have used this strategy to discover a large number of artificial transmembrane peptides with divergent sequences that activate PDGFβR (179) or EpoR (180) or even reduce levels of the C–C chemokine receptor type 5, potentially by binding to its TMD and destabilizing the protein (181). These peptides are unusual in that they induce rather than inhibit receptor oligomerization.

Platelet-derived growth factor β receptor—Platelet-derived growth factor receptors are a small subfamily of RTKs. Both PDGFRs, α and β, have a single TMD connecting an extracellular ligand-binding domain to an intracellular kinase domain. Both receptors are also required for PDGF-induced activation (112). PDGFβR activity can also be induced by bovine papillomavirus infection. This virus oncogenically transforms fibroblasts via a short oncoprotein called E5 (182), and the transforming ability of E5 largely depends on the presence of PDGFβR (183, 184). E5 is only 44 amino acids long and is largely composed of a single TMD (185, 186) that specifically interacts with the PDGFβR TMD and activates PDGFβR signaling (187). E5 must be a dimer to interact with PDGFβR and transform cells, and dimerization is mediated by a central transmembrane glutamine and two C-terminal cysteines in E5 (188–190). For a comprehensive review of the E5–PDGFβR interaction, see Ref. 191.

Armed with the knowledge that a short transmembrane oncoprotein could activate PDGFβR, Freeman-Cook and co-workers sought to elucidate the sequence requirements for activation of PDGFβR (179). To do this, they set up a genetic screening strategy to identify novel transmembrane sequences that could activate PDGFβR. By randomizing nucleotides at certain positions, the authors generated libraries of millions of short hydrophobic peptide sequences. When constructs containing these sequences were introduced into murine fibroblasts, peptides that activate the endogenous PDGFβR caused the appearance of transformed foci. Similarly, in murine hematopoietic BaF3 cells, only cells expressing a peptide that activates PDGFβR were able to proliferate in the absence of interleukin 3 (IL-3). Thus, isolating transformed cells or cells grown in the absence of IL-3 and amplifying their DNA revealed which sequences activate PDGFβR. These peptides were named transmembrane protein aptamers (traptamers) (Table S1).

The genetic screens started conservatively. First, they only randomized the hydrophobic amino acids in the TMD, leaving the critical central glutamine intact (179). Next, they expanded the screen to include the glutamine and lengthened the peptides to include the positions of two critical cysteines. They also allowed hydrophilic amino acids to be inserted sparsely (192). When they further expanded the screen to the 20 most central TMD residues allowing only hydrophobic residues to be inserted, they found that van der Waals forces alone could generate remarkably specific interactions (193). Some interactions were even more specific for PDGFβR than is E5. Thus, each expansion of the screen led to new sequences that could activate PDGFβR, even when supposedly critical residues were mutated. This demonstrates the main advantage of screening for transmembrane sequences that activate receptors: it allows for high-throughput, side-by-side comparisons of activating ability among potential TMD peptides (194).

Screening has resulted in traptamers with extremely simple sequences. For example, some traptamers contain only two different residues, leucine and isoleucine, and are affectionately named LIL traptamers (Table S1). In contrast, the per-residue probability of finding a leucine or isoleucine at a given position in a TMD is only 26% (analysis of Table S4 from Ref. 195). The specificity of LIL traptamers can be altered by mutations that change the position of a single side-chain methyl group (196). Remarkably, one active traptamer was isolated that contains only a single isoleucine in a polyleucine sequence (197).

What can these discoveries teach us about PDGFβR activation? A recent study may provide insights. Using Rosetta, the authors generated a compelling mechanism for E5-induced PDGFβR dimerization (198). Their model predicts that the PDGFβR dimer is stabilized between two E5 dimers. Much like E5, LIL traptamers activate PDGFβR and thus may also stabilize the PDGFβR dimer. Unlike E5, however, LIL traptamers do not have polar residues with which to form hydrogen bonds.

This hints at an entirely van der Waals–stabilized complex, which may look very different from the E5–PDGFβR complex. Regardless, the simplest explanation for PDGFβR appears to be that ligand-induced dimerization is sufficient for activation. However, it is still unclear whether the PDGFβR dimer must be stabilized in a specific conformation. It would also be interesting to determine whether all LIL traptamer–PDGFβR complexes share the same hexameric stoichiometry as the E5–PDGFβR complex, which would suggest a common mechanism for dimer stabilization.

Traptamers that target other receptors have been isolated. For example, DiMaio and co-workers (196, 199) set up a screen for transmembrane sequences that induce IL-3–independent proliferation of BaF3 cells engineered to express EpoR, but not parental BaF3 cells lacking EpoR expression. This led to the discovery of traptamers that activate EpoR but not PDGFβR. Case studies on select EpoR traptamers demonstrated that they may use fundamentally different mechanisms to activate the receptor. One traptamer, ELI-3, does not require EpoR cytoplasmic tyrosines, but requires activation of a secondary receptor, the cytokine receptor β common subunit (βcR) (199), whereas another traptamer, EBC5-16, requires EpoR tyrosines, but not βcR.

Screening to discover TMD peptides has several advantages. In theory, the approach outlined above can be applied to find TMD peptide activators of any receptor that induces cell proliferation or some other selectable phenotype. Because traptamers are not based on pre-existing, evolved sequences, they may display activities that have not evolved in nature. Also, these TMD peptides may use novel mechanisms, such as the ELI-3–induced heteroactivation of EpoR and βcR. However, this may also be a disadvantage of screening. Without secondary screens, the mechanism of activation cannot be selected. For example, selecting TMD peptides that activate PDGFβR, but only by a different mechanism, would require extra screening steps. Another disadvantage is that the screening approach described above is survival-based, and thus only selects for *activating* TMD peptides. Discovering deactivating TMD peptides would require a different approach. For example, He *et al.* (200) set up a SDS-PAGE–based screen to find ErbB2 TMD sequences that dimerize more readily than the WT sequence. One can imagine adding a second step to this screen in which peptides are exogenously added or expressed and then tested for their ability to inhibit ErbB2 activation. This approach might result in TMD peptides that deactivate their targets. In general, however, the success of screening is directly tied to the ability of the screen to detect the desired TMD peptides.

Rational design

Sequence-based rational design of TMD peptides permits testing specific hypotheses. The simplest and most common approach is to isolate the TMD of the target receptor and present it as a peptide to cells expressing the full-length receptor. To our knowledge, the first time this was achieved was with GpA. The GpA TMD peptide (201) shifts the full-length GpA from a dimer to an apparent monomer through hetero-association (202). This line of experiments represents the first demonstration that a TMD peptide can compete with a full-length membrane protein to disrupt dimerization (203, 204).

Rational design of TMD peptides has been used on diverse protein targets. For example, there are TMD peptides that disrupt hetero-association between toll-like receptors 2 and 6 (Table S1) (205, 206). Similarly, a peptide derived from the TMD of the p75 neutrophin receptor inhibits p75-tyrosine kinase receptor B association (Table S1) (207). The same principle that applies to single-pass receptors also works for G-protein–coupled receptors such as the *CXC* motif chemokine receptor 4 (208), dopamine D2 receptor (209), and β2-adrenergic receptor (Table S1) (210). As we will see in the forthcoming sections, this approach has proven successful for several other receptors, and, when coupled with studies on receptor activity, can shed light on receptor activation mechanisms.

T-cell receptor—The T-cell receptor (TCR) is found on T lymphocytes and is required for cell-mediated adaptive immune response (211). TCR activates upon binding to the MHC complex on antigen-presenting cells. This prompts a signaling cascade that results in T-cell proliferation and cytokine production. TCR activation in response to host cells, rather than pathogens, results in autoimmune disorders such as rheumatoid arthritis and type 1 diabetes. TCR is thus a promising target for therapeutic inhibition.

TCR is a complex of six different proteins (TCR α and β,and CD3 δ, ϵ, γ, and ζ), each of which possesses a single TMD. The TCR α- and β-subunits contain extracellular ligand recognition sites that bind to the MHC complex (212). Complex assembly is mediated by the TMDs (213). First, heterodimers of TCRαβ, CD3δϵ, and CD3γϵ form (214). TCRα then binds to the CD3δϵ heterodimer via a critical lysine residue, and likewise TCRβ binds to the CD3γϵ heterodimer. The two CD3ζ subunits join the complex last and are phosphorylated to initiate a signaling cascade.

Manolios *et al.* (216) hypothesized that they could inhibit TCR signaling by blocking the formation of the full TCR complex. At the time, they knew that to interact with CD3δϵ TCRα required a stretch of eight amino acids containing a critical arginine and lysine (215). Based on the central role of TCRα in complex assembly, the authors designed several short peptides based on the TCRα TMD (216). The most active TCR inhibitor was derived from the central portion of the TCRα TMD and thus named "core peptide" (CP) (Table S1). CP treatment results in inhibition of CD3ζ phosphorylation and the downstream production of interleukin 2 (217), and it inhibits TCR in human cells *in vitro* (218) and in mice (219, 220). CP can even be applied topically to inhibit allergic response in mice and in humans, and it improved symptoms of a small number of psoriasis patients (221).

CP functions by competitively inhibiting formation of the TCR complex. This is evidenced by the fact that a cross-linked TCR complex is unaffected by CP (217). For CP to function, it must contain two hydrophobic faces interrupted by two opposing positively charged residues (222), and the same sequence constraints apply even if D-amino acids are used (Table S1) (223). These two residues (*i.e.* lysine and arginine) are thought to interact with negatively-charged amino acids in the CD3δϵ

and ζζ dimers (214), whereas the hydrophobic faces are thought to interact with lipids. This suggests stringent structural requirements for signaling activation.

An often-overlooked practical aspect of TMD peptide development is membrane partitioning. Obviously, for CP to interact with the TCR TMD, it must adopt a transmembrane state. This requires delivery from aqueous solution into the cell membrane. The high hydrophobicity of most membrane peptides makes this a nontrivial step. Indeed, proper delivery is one of the determining factors for the ability of TMD peptides to act on their receptors (224). Delivery may be enhanced by post-translational modifications such as palmitoylation (216, 225–227). However, delivery systems may affect the ability of peptides to work properly. For example, CP is typically delivered to cells in DMSO, whereas *in vivo* CP is delivered in squamous oil, a less toxic hydrophobic medium. When comparing how strongly peptides inhibit TCR in different model systems, Collier *et al.* (228) found that peptides dissolved in oil before being administered to mice inhibited TCR much more effectively than peptides dissolved in DMSO before being administered to cells. Collier *et al.* (228) attribute this different response to the difference in solvent. Thus, delivery may dramatically affect the ability of TMD peptides to interact with their receptors (see under "FGFR3").

Neuropilin-1—Semaphorins are a diverse class of small extracellular proteins that regulate cell morphology by promoting or inhibiting axon guidance and motility (229). Of their many members, Sema3A in particular has been singled out as a key inhibitor of axon growth through its interactions with the single-pass membrane receptor, neuropilin-1 (NRP1) (230, 231). Although initially studied for its role in semaphorin signaling in the nervous system, misregulation of NRP1 has also been implicated in cancer via its role as a co-receptor for vascular endothelial growth factor (232). How NRP1 signals is not fully understood, as it does not possess an intracellular kinase domain or obligate partner. It is thus thought to form dynamic complexes with other co-receptors such as plexins (233, 234). The determinants for complex formation, however, remain elusive.

Roth *et al.* (235) set out to test whether a TMD peptide could inhibit NRP1 signaling and provide insights into Sema3A-induced signaling. Using a modified TOXCAT assay (89), they found that the TMD of NRP1 strongly dimerizes—a finding that agrees with simulations (236). They then synthesized a peptide composed of the NRP1 TMD called "membrane-targeting peptide," MTP-NRP1 (Table S1), to interfere with the interaction. MTP-NRP1 inhibits NRP1 by competitively inhibiting its G*XXX*G-mediated dimerization (235).

Blocking NRP1 dimerization has *in vitro* and *in vivo* effects. Sema3A induces growth cone collapse in COS cells, a fibroblast-like simian cell line, expressing NRP1. Pre-treatment with MTP-NRP1 blocks this effect, presumably because it prevents the receptor from forming dimers (235). This allows axons to grow even in the presence of the inhibitory Sema3A. Thus, treatment with MTP-NRP1 can facilitate innervation of bioprosthetics (237). Promisingly, MTP-NRP1 also inhibits the growth of tumors in mice by blocking VEGFR signaling (238, 239). The *in vivo* antiproliferative effects of MTP-NRP1 make it a promising therapeutic agent, and Bagnard *et al.* (240) have patented it for therapeutic development.

The high hydrophobicity of MTP-NRP1 required most of the experiments described above to be performed in the presence of a surfactant, lithium dodecyl sulfate. Although this surfactant is nontoxic at the concentrations tested, the need for surfactants may complicate the direct delivery of MTP-NRP1. As with CP, efficacy of MTP-NRP1 will likely benefit from enhanced clinical delivery systems.

Besides of its therapeutic promise, MTP-NRP1 offers insight into NRP1 activation. Early experiments demonstrated the necessity of the G*XXX*G motif for MTP-NRP1 function (235). This suggests that NRP1 likely activates by LID, as no rotational or higher-order clustering requirements for activation have yet been found. However, NRP1 is known to hetero-associate with other receptors such as plexins. Further studies may help elucidate whether NRP1 hetero-interactions are also mediated by the NRP1 TMD.

EGFR/ErbB1/HER1—The ErbB family of RTKs has been heavily targeted for TMD peptide development due to its members' oncogenic roles in many cancers. Although there have been no FDA-approved ErbB TMD peptides yet, many anticancer drugs have been developed to target their soluble domains, demonstrating the clinical value of understanding ErbB receptors. EGFR (ErbB1) alone has 16 FDA-approved drugs targeting it (241). Beyond its clinical value, the ErbB family has much to teach us about RTK activation mechanisms.

Despite several decades of study, a full mechanistic description of EGFR activation remains elusive. EGFR was the second RTK discovered, long before the term "RTK" was coined (242, 243). Like many RTKs, EGFR requires dimerization for signaling (244). Two EGFR extracellular domains each bind to one EGF molecule (245, 246). EGF binding then induces a conformational change in the extracellular domains to expose a "dimerization arm" (247–249) that promotes EGFR dimerization (250). However, as discussed above, studies of the soluble extracellular domains of the receptor do not always inform the full-length receptor.

An LIR mechanism was proposed for EGFR after several reports of EGFR dimers formed in the absence of ligand (80, 251, 252). The TMD of EGFR contains two G*XXX*G-mediated dimerization interfaces (*i.e.* one N- and one C-terminal), which suggests a plausible activation mechanism. EGFR could use one G*XXX*G interface for the inactive form and the second for the active form. The coupled rotation of the extracellular, transmembrane, and intracellular domains would place the kinase domains in the correct asymmetric configuration to signal upon switching transmembrane interfaces (Fig. 3) (80, 100, 253). However, some observations are inconsistent with this model. As reviewed in Ref. 254, unliganded dimers were observed in conditions with unnaturally high receptor densities. When great care is taken to observe EGFR at native expression levels and in the absence of ligand, it is ≥70% monomeric (162, 255). Furthermore, the unliganded extracellular domain dimer contacts seem to be heterogeneous and transient (250). This suggests a model in which EGFR can exist as an unliganded dimer but is not observed doing so in native conditions. If so, it

follows that the LIR mechanism for EGFR may only be relevant when the receptor is overexpressed.

That EGFR can weakly dimerize in membranes without ligand is important, given the strong lateral proximity effects imposed by the membrane. This leads to a correlation between the membrane density of EGFR and activation (256). Despite this, it is still not fully understood whether activation involves simple dimerization or formation of larger oligomers. Spatially confining EGFR is enough to induce activation (257, 258). This is corroborated by reports that show that EGFR probably tetramerizes upon activation (117, 259, 260). These lines of evidence hint that EGFR clustering is likely to play a role in its activation.

Depending on the technique used, EGFR *appears* to have different mechanisms of activation. For example, to our knowledge, glutaraldehyde cross-linking followed by SDS-PAGE has never shown species larger in size than EGFR dimers, whereas single particle tracking experiments suggest tetrameric species. In other words, in some conditions EGFR appears to activate by dimerizing, whereas in other cases it appears to require rotation or clustering. It is possible that a combination of the three mechanisms (*i.e.* dimerization, rotation, and clustering) is required for full activation *in situ*. It is also possible that some observations are artifactual. Therefore, it is critically important to determine which factors are most influential in native conditions. Additional tools are needed to distinguish these different mechanisms, and TMD peptides can be useful in this regard, as we will discuss below.

To test whether EGFR activates by a strict LID mechanism, Bennasroune *et al.* (261) created an EGFR TMD peptide (Table S1). They hypothesized that if dimerization were necessary and sufficient for receptor activation, then the peptides would competitively inhibit dimerization by interacting with the TMD. Thus, the authors expressed constructs containing the TMD of EGFR in human adenocarcinoma A431 cells. They observed that the EGFR TMD peptide reduced phosphorylation of EGFR and the downstream effector, extracellular signal-related kinase. This effect was concomitant with decreased EGFR dimerization. This suggests that, as predicted, dimerization of EGFR *can* be competitively inhibited by TMD peptides.

Similar results were obtained independently by Gerber *et al.* (262), who found that EGFR TMD peptides disrupt EGFR dimer formation. In these experiments, the peptide was tagged with four lysines for solubility, a so-called host–guest system, which did not inhibit its ability to interact with its target (263). By mutating the two transmembrane G*XXX*G motifs in turn, Tzahar *et al.* (264) were able to propose a plausible TMD-based explanation for the previous finding that the ErbB receptors extensively heterodimerize. The authors proposed that the N-terminal G*XXX*G motif of EGFR mediates EGFR–ErbB2 heterodimerization, whereas the C-terminal motif mediates EGFR homodimerization.

In a follow-up study, Bublil *et al.* (265) targeted the C-terminal heterodimerization motif, but this time they used ErbB2 as a template, not ErbB1. Although they sought to inhibit the heterodimer of ErbB2–ErbB3, they inhibited all ErbB heterodimerization with ErbB2, including ErbB1, ErbB3, and ErbB4. This had a marked effect on reducing cell proliferation in the presence of EGF and the ErbB3 ligand, neuregulin. The best-performing peptide, B2C-D, contained D-amino acids to prevent proteolysis and reduced cell proliferation of multiple cancer cell lines. The effect was also observed in mice: xenografted tumors were smaller when mice were co-treated with B2C-D and the chemotherapeutic drug gemcitabine than in mice treated with gemcitabine alone.

The EGFR TMD peptide studies demonstrate that dimerization is necessary for activation. However, this does not rule out a more complex model, such as LIR or clustering. Structural studies on EGFR provide a potential mechanism for activation that involves rotation of receptor dimers to an active conformation (100), a conformation in which tetramers can then form (116). In the study by Bennasroune *et al.* (261), the authors observed that EGFR TMD inhibited EGFR phosphorylation only at the lowest concentration tested. At high concentrations, the authors reasoned, the EGFR TMD peptide oligomerizes and therefore reduces its own ability to interact with the receptor. When expressing peptides in cells, this does not appear to be the case, as the isolated EGFR TMD does not induce EGFR oligomerization by itself (116). In any case, more research is required to determine how EGFR is activated in cells, and it seems likely that EGFR utilizes a complex mechanism that involves dimerization, rotation, and clustering.

ErbB2/Neu/HER2—As an orphan receptor, ErbB2 has no known ligands (266). As such, ErbB2 must associate with other receptors such as ErbB1, ErbB3, and ErbB4 to undergo transphosphorylation and initiate signaling cascades. As with many RTKs, misregulation of ErbB2 can be pro-oncogenic.

Early research on rat glioblastoma led to the discovery of the *neu* proto-oncogene, an ortholog of the later discovered human ErbB2 (267). When Bargmann *et al.* (13) compared the sequences of the oncogenic and proto-oncogenic isoforms of *neu*, they discovered that a single nucleotide was consistently mutated in the oncogene. The resulting valine–to–glutamic acid mutation in the Neu TMD causes increased self-association of receptors, constitutive activation, and therefore increased cell proliferation and tumor formation (99). A glutamic acid substitution in position 663 or 665 does not induce activation, demonstrating that Neu likely requires association of specific interhelical interfaces to activate. This suggests that a complete description of ErbB2 activation requires dimerization and rotation, as with ErbB1.

Lofts *et al.* (268) hypothesized that a TMD peptide could competitively inhibit the oncogenic self-association of Neu (V664E). They thus created constructs corresponding to the TMDs of Neu and Neu (V664E) (Table S1) and expressed them in mouse neuroblastoma cells expressing the Neu (V664E) receptor. Not surprisingly, the Neu (V664E) TMD peptide inhibits Neu more than the WT Neu TMD peptide does (268). They observed stark inhibition of cell growth, colony growth, and tumor growth in mice. A similar study by He *et al.* (269) showed that this is likely due to decreased phosphorylation of Neu (V664E).

Taking a slightly different approach, Arpel *et al.* (270) synthesized a Neu (V664E) TMD peptide (MTP-NeuNT) (Table S1). MTP-NeuNT forms dimers with all murine ErbB TMDs as well as the human ErbB2 TMD and thus reduces phosphoryla-

tion of the Neu (V664E) receptor. In a breast cancer mouse model, MTP-NeuNT inhibits metastasis and improves survival. The strong antiproliferative effects of peptides targeting ErbB2 have led to increased therapeutic interest. Greene *et al.* (271) have patented a transmembrane peptide to target the Neu receptor.

The studies on the ErbB family demonstrate that TMD peptides offer insights into receptor activation mechanisms. Simple mutation and competition experiments suggested key interfaces for heterodimerization that could be exploited for broad inhibition of ErbB2-induced cell proliferation. However, the mechanistic studies above, mainly based on TOXCAT assays, could not distinguish between dimerization, rotation, and clustering. High-resolution studies (*e.g.* single-molecule bleaching or FRET) may give insight into the mechanisms by which the TMD peptides can interrupt signaling.

Insulin receptor—The insulin receptor (InsR) is an RTK that induces cellular uptake and storage of glucose. Disruption of InsR signaling therefore leads to insulin resistance and type 2 diabetes. Despite its critical role in human health, many features of insulin receptor activation remain enigmatic. Each InsR monomer is actually a heterodimer composed of proteolytically cleaved α- and β-subunits. The extracellular α-subunit is disulfide-bonded to the extracellular domains of the single-spanning β-subunit (272). The α-subunits of two InsR heterodimers also form disulfide bonds, making an $(\alpha\beta)_2$ homodimer irrespective of ligand (273).

A maximum of four insulin molecules can bind to an InsR $(\alpha\beta)_2$ homodimer (274). Insulin binding induces a conformational change in the extracellular domains. A recently solved structure of the liganded extracellular domains of InsR shows that the TMDs of the β-subunits are separated in the unliganded complex but swing toward each other upon ligand binding. This may be why multiple mutations of the InsR TMD have no effect on InsR activation (275); even if TMD binding is disrupted, they are still held close to each other by the extracellular domains.

As with their studies on EGFR, Bennasroune *et al.* (276) sought to determine whether InsR could be controlled by a TMD peptide. The InsR TMD only weakly dimerizes, and as discussed above, the TMDs are thought to be separated in the receptor dimer. Not surprisingly, the InsR TMD peptide (Table S1) had no effect on insulin-induced phosphorylation of InsR. The authors thus reasoned that replacing the TMD of InsR with one that dimerizes more strongly would make the receptors susceptible to TMD peptides. Thus, they created a chimeric InsR containing an EGFR TMD and expressed it in Chinese hamster ovary cells. When an EGFR TMD peptide was expressed in the same cells, it inhibited insulin-induced phosphorylation of the chimeric InsR–EGFR receptor. Similar effects were observed when the chimeric InsR TMD was replaced with that of the oncogenic mutant ErbB2 (*i.e.* V664E). A separate study found that a synthetic InsR TMD peptide increases phosphorylation of InsR on its own (277). What is the cause of these discrepancies? The synthetic TMD peptide (277) activated InsR, whereas the expressed and tagged TMD peptide did not (276). This may suggest that the soluble tags of the expressed construct clashed with the receptor soluble domains, preventing close contact with the correct interface.

FGFR3—The FGFR3 is part of a small RTK family (278). FGFR3 negatively regulates bone growth (279), so it is not surprising that activating TMD mutations are found in several skeletal diseases (280). For example, the Crouzon syndrome-related A391E mutation of the FGFR3 TMD stabilizes dimerization (17). Other mutations in FGFR3 stabilize dimer formation but lead to completely different phenotypes. For example, several cysteine mutations that enhance TMD dimerization cause skeletal dysplasias (18). Yet another TMD mutation involved in the skeletal dysplasia achondroplasia, G380A, causes constitutive activation (19, 20) without altering dimerization of the TMD (281). This mutation is potent: if the FGFR3 G380A TMD is introduced into the Neu receptor, the resulting chimera is also constitutively active (19). That FGFR3 is so sensitive to TMD mutations suggests an important role for the TMD in activation.

To inhibit the FGFR3 A391E mutant receptor, He *et al.* (269) designed a TMD peptide against it (Table S1). However, their study was confounded because the peptides, FGFR3/WT and FGFR3/A391E, did not localize to the cell surface. Therefore, activation of FGFR3 A391E was unaffected (269). The study by He *et al.* (269) is one of few that report a negative result on a TMD peptide, although there are surely more that go unreported, thus hindering progress. Knowing how and why TMD peptides fail not only informs the community how to improve TMD peptide design, but also may provide additional mechanistic insights. In the study above, the authors blamed trafficking for the failure of FGFR3/WT. Yet, in the same study TMD peptides based on the Neu receptor properly localized to the membrane. Clearly the sequence of the TMD plays a role in trafficking and localization. Understanding other factors that govern endogenous TMD peptide trafficking will surely aid future research endeavors.

EphA2—Eph receptors are the largest family of RTKs (282). In various contexts, they mediate cell– cell contact by binding to their membrane-bound ephrin ligands. EphA2 is of particular interest due to its active role in various cancers (283). Paradoxically, EphA2 can act either as an oncoprotein in the absence of ligand (284) or as a tumor suppressor when activated (285). In the absence of ligand, EphA2 exists in a monomer–dimer equilibrium (286). Upon stimulation by its membrane-bound ephrinA ligands, large, sometimes micron-sized clusters lead to full activation. These clusters grow to encompass even unliganded receptors (113). This demonstrates that EphA2 uses a cluster seeding mechanism, in which bound receptors recruit unbound receptors to amplify signaling.

Seemingly contradictory findings complicate a full understanding of Eph receptor clustering. Activation of EphA2 was initially thought to require clustering. Monomeric ephrinA1 could not activate the receptor. However, antibody clustered or membrane-bound ephrinA1 could activate it (119). It was later found that antibodies targeting the extracellular domain of EphA2 can activate the receptor without any ligand (287). A conflicting report showed that monomeric, soluble ephrinA1 is indeed functional and activates EphA2 (288). Further studies show that monovalent ligands can be engineered to activate

EphA2 (289, 290). What is cause of this conflict? Might EphA2 have multiple independent activation states? The related EphB1 receptor has a unique response to monovalent, bivalent, and tetravalent ligands, respectively, suggesting different intermediate pre-clustered states (291). Might this be the case for EphA2? In one study EphA2 does appear to have distinct oligomeric states depending not only on the valency of the ligand, but on the concentration of the receptor (122). Perhaps the cellular context is the key to determining whether a given ligand valence will be able to activate EphA2.

Alves *et al.* (292) employed a new rational design strategy to develop a TMD peptide targeting EphA2. This approach was based on the strategic addition of glutamic acid residues to the TMD of EphA2 to create the TYPE7 peptide (Table S1). As a result, the isolated TMD of EphA2 became water-soluble, while having the ability to insert into membranes in a controllable way. There are examples of hydrophobic sequences that contain the acidic residues aspartic acid and glutamic acid, such as the pHLIP (293) and ATRAM peptides (294). These residues impart solubility to the peptides at neutral pH, where they are negatively charged. In these conditions, the peptides bind reversibly to the surface of membranes, while a pH decrease titrates the acidic groups, causing membrane insertion in the form of a helical TMD (295). Whereas pHLIP and ATRAM are thought to be essentially inert in the membrane, their unidirectional insertion (296, 297) can be used to translocate cargo into the cytoplasm of target cells. This property can have important practical applications, as aggressive solid tumors typically have acidic extracellular environments (298, 299). As a result, pHLIP and ATRAM can be used for the targeted delivery of drug-like cargoes into tumors (300, 301). The rational approach for the design of a pH-responsive membrane peptide succeeded at reproducing the biophysical properties of ATRAM and pHLIP. However, it also laid a foundation that allowed an additional functionality, binding to EphA2 (292). The basis for the interaction with the TMD of EphA2 is that TYPE7 retains the membrane dimerization interface, as the glutamic acids were added to a different helical phase. Simple competition for this interface would allow TYPE7 to engage the TMD of EphA2.

TYPE7 activates EphA2 by interacting with the TMD and juxtamembrane domain. The EphA2 juxtamembrane domain, as with all RTKs, contains highly-conserved, positively-charged residues. It is possible that an electrostatic interaction between the negatively-charged residues of TYPE7 and the positively-charged residues of EphA2 facilitate the interaction. As discussed above, EphA2 forms large clusters upon activation; however, TYPE7 induces the formation of smaller clusters of EphA2. How this occurs is not fully understood, and it may involve a combination of TMD and juxtamembrane interactions. It remains to be seen whether TYPE7 inhibits EphA2 more efficiently at low pH. Downstream, TYPE7-induced EphA2 activation results in decreased phosphorylation of Akt and inhibition of cell migration. Furthermore, TYPE7 is remarkably specific for EphA2 activation over other RTKs (292). These observations make TYPE7 a promising potential therapeutic peptide. The idea that pH sensitivity could be combined with a TMD peptide was proposed previously (7). However, to our knowledge TYPE7 is the first pH-sensitive peptide that, upon controlled insertion, specifically binds a TMD and modulates the activity of a membrane protein.

Future directions

Despite the various design techniques, most TMD peptides are equivalent in their main technical aspects. They can be expressed in cells or added to cellular media via a stock solution in organic solvent, detergent, or buffer. However, the choice of delivery method can greatly affect the efficacy of peptides. Proper delivery of peptides to membranes is critical for TMD peptides to interact with receptors (224, 269). For example, synthetic InsR TMD peptides delivered in detergent-activated InsR, whereas expressed InsR TMD peptides did not (276, 277). Beyond delivery, peptides must also remain stable long enough to reach their target receptors (*e.g.* by capping the termini of the peptides). A recent study shows that improved stability may also be achieved through TMD peptide stapling without loss of interference activity (302). It is also imperative that future experiments use delivery systems that do not alter membrane properties (*e.g.* DMSO at low concentrations) or potentially interfere with physiological receptor expression levels. For this reason, we recommend that peptides be designed to maximize solubility in aqueous media. Furthermore, testing on native expression levels is especially important given the direct correlation between receptor expression and dimerization.

What determines whether a given TMD peptide will specifically interact with the target receptor is not clear. Sequence motifs (59), hydrogen bonding, and side-chain packing (169) all contribute. Some TMD peptides are highly specific for their target receptors, as is the case with TYPE7, whereas others, such as MTP-NeuNT, dimerize with several receptors of the same family. The design of specific peptides will benefit from studies that screen candidate peptides against libraries of known or predicted TMD structures. More research is needed before a recommendation can be made on the best way to maximize the specificity of TMD peptides. A publication bias toward "working" TMD peptides may account for the slow development of this field. To expedite development, we encourage authors to publish their studies on TMD peptides that (*a*) are insoluble/undeliverable, (*b*) do not interact with their target receptors, (*c*) interact with but do not activate their target receptors, and (*d*) have off-target effects. This information will aid in understanding which design approaches are most likely to result in highly-specific TMD peptides.

The majority of TMD peptides function similarly: they inhibit receptor self-assembly by competitively blocking the TMD helix–helix interface (Fig. 4). This results in inactivation of receptors that require association of their TMDs (*e.g.* EGFR, Neu, NRP1, and PDGFR), and activation of those that require separation of their TMDs (*e.g.* integrin). In some cases, the TMD peptides have no effect (*e.g.* FGFR3, InsR), possibly due to mislocalization or other undefined reasons.

Some TMD peptides stabilize receptor oligomers (*e.g.* TYPE7 and LIL traptamers). For example, TYPE7 associates with the TMD of its target, EphA2, and activates it, although EphA2 is not expected to require dissociation of its TMDs for activation. Rather than reducing receptor self-assembly like other TMD peptides, TYPE7 induces the formation of small receptor olig-

omers (292). How this is achieved mechanistically is still unclear. Similarly, traptamers can induce PDGFβR dimerization and activation. The oncogenic E5 protein activates PDGFβR by "sandwiching" a TMD dimer between two E5 TMD dimers. We hypothesize that a similar stabilization mechanism is occurring between TYPE7 and EphA2. Future studies will be needed to elucidate how these TMD peptides stabilize receptor oligomeric states. A simple approach for using TMD peptides to understand receptor activation mechanisms is as follows: TMD peptides can be designed to interact with specific interfaces of receptors, starting with the most likely (*e.g.* if the protein contains a transmembrane G*XXX*G motif). If the receptor employs an LID mechanism, then the peptide will inhibit the interaction and lead to decreased receptor activity. If the receptor activates via an LIR mechanism, then the TMD peptide will inhibit receptor activation only if it interacts with the active interface but promotes it at others by competitively inhibiting the ability of the receptor to utilize the inactive receptor interface. A small library of TMD peptides designed to each interact with a different interface could accomplish this objective. Finally, if the receptor activates by clustering, the peptide will activate the receptor only if it induces oligomerization. As mentioned above, it remains to be understood how TMD peptides stabilize receptor oligomerization. For now, these peptides can be discovered by screening. Future structural studies on oligomerization-inducing peptides may open opportunities for intentional rational design of oligomerization stabilizers or interrupters.

Many of these peptides have resulted in patents, although there are currently no FDA-approved TMD peptides. This should not be considered discouraging or stifling. Rather, this shows that researchers have a unique opportunity to understand potential future therapeutics—and, more importantly, their targets—before they go to market.

Single-pass receptors have been studied for the better part of 40 years, yet a full mechanistic description of the activation mechanism remains elusive. The TMDs of most receptors interact specifically, and TMD mutation and substitution studies demonstrate that the TMD clearly plays a role in many receptor activation mechanisms. However, in many cases it is still unclear how the TMD is involved mechanistically. Three major hypotheses have been proposed for receptor activation: dimerization, rotation, and clustering. Some receptors seem to require the simplest case, LID, whereas others probably use a combination of mechanisms, each with their own energetic requirements (Fig. 3). Future research should focus on elucidating which mechanisms regulate activation in the most native-like conditions possible and consider alternative activation mechanisms. TMD peptides can be used as tools to study these mechanisms. As TMDs encode specific associations, TMD peptides can induce specific biological effects. Most TMD peptides to date seem to competitively inhibit dimerization by interacting with the TMD of the target; however, there are some (*e.g.* LIL traptamers and TYPE7) that appear to enhance oligomerization by interacting with the TMDs. High-resolution information about how TMD peptides alter receptor function will contribute to understanding complex receptor activation mechanisms.

Acknowledgments—We thank Daniel DiMaio (Yale University), Adam Smith (University of Akron), and Katherine Stefanski, Yujie Ye, Andrew Dixson, and Jennifer Schuster (University of Tennessee) for insightful comments on the manuscript.

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