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REVIEW

# Peptide Probes for Protein Transmembrane Domains

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**ABSTRACT** Much current interest in chemical biology focuses on the transmembrane domains of proteins, which have emerged as targets for the development of novel diagnostics and therapeutics. Integral membrane proteins are a group of important biomolecules that play pivotal roles in many cellular activities. Previous studies primarily focused on the extra- and/or intracellular domains of membrane proteins. However, the importance of transmembrane regions in the regulation of protein complexes is beginning to emerge. As such, a number of methods for designing and testing novel exogenous peptides that recognize transmembrane targets and modulate cellular functions have been developed. This Review outlines current methodologies for developing these transmembrane probes that may provide useful tools to study a variety of biological phenomena in the membrane.

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The wide array of functionalities demonstrated by integral membrane proteins makes them important targets in biochemical and biomedical research, evidenced by the fact that >60% of pharmaceuticals on the market target membrane proteins (*1*). The design of exogenous agents to specifically recognize and modulate the activity of naturally occurring transmembrane (TM) sequences is a complex problem for chemical biologists. Exogenous peptides must be hydrophilic to prevent aggregation in solution, yet hydrophobic to ensure delivery into the membrane. Once these basic challenges have been met, the tougher tasks of ensuring membrane insertion, proper folding, and targeting can be considered. Despite these design hurdles, there have been a number of successful studies in the regulation and probing of TM proteins. A majority of these studies have focused on native TM regions with simple modifications to manipulate a targeted cellular function; relying on nature's design bypasses many of the hazards associated with TM protein design. However, a number of innovative methods utilizing synthetic scaffolds and rational design have enjoyed modest success in manipulating a variety of biological targets, including pharmacologically relevant cell surface receptors. Moreover, screening methods for the TM regions of proteins, both *in silico* and *in vivo*, provide another option for anti-TM peptide development. This Review will focus on recent developments in the design and application of exogenous peptides as tools to probe and alter membrane protein function through TM interactions.

**Design Principles and Methodology for Investigating TM Peptides.** Although the detailed process of designing these peptides is described elsewhere in great depth, two fundamental aspects of this process should be discussed (*2*). The development of a mol-

ecule designed to specifically interact with a target TM sequence must begin with an understanding of the target. This is often a hurdle when dealing with membrane proteins as relatively few high-resolution structures have been solved. Although a high-resolution structure may not be available for the target TM protein of interest, standard biochemical methodology, in conjunction with homology modeling, can often yield a general framework of the target structure (*3*).

The first critical aspect of the design process is determining the site(s) of interaction between the anti-TM peptide and the target. The design of this interaction site is driven by the sequence of the target that designates a specific topology, charge distribution, and directionality. Complementing these motifs in 3D space and placing them at the proper location on the α-helical backbone are critical for the affinity and specificity of anti-TM peptides. In particular, small residues (glycine, alanine, serine) tend to appear frequently at TM−TM interaction sites, and polar residues can drive the association of TM helices (*4–7*). Russ and Engelman's studies of glycophorin A indicate that a structural motif based on small residues (GXXXG) is heavily involved in TM−TM interactions. The small glycine residues allow two TM helices to come into close proximity. The three random interfacial residues (XXX) add specificity by forming a distinct binding ridge or cleft that is complementary between the helix pair. Additional specificity comes from the depth of the GXXXG motif in the membrane: two GXXXG motifs that are not at the same depth in the membrane cannot interact. These discoveries make GXXXG and similar motifs, (S,A)XXX(S,A) and (V,L,I)XXX(V,L,I), an excellent starting place for anti-TM peptide design (*8*).

The second important aspect of design relates to the choice of the noninteracting residues. Interacting residues must be surrounded by a balanced mixture of hydrophobic groups to drive membrane binding and insertion, hydrophilic groups to aid aqueous solubility for delivery, anchoring residues to ensure the proper TM register, and an inherent asymmetry to ensure proper directional insertion into the target membrane. A number of studies have thoroughly analyzed the propensities for individual amino acid partitioning into the hydrophobic core of the lipid bilayer, amino acid occurrence at given positions throughout a TM α-helix, and the ability of aromatic groups to act as anchors for TM α-helices when these residues can interact with the lipid headgroup region of the membrane (*9–13*). This information can be applied when filling in the noninteracting amino acids to help maximize the probability of proper insertion and orientation.

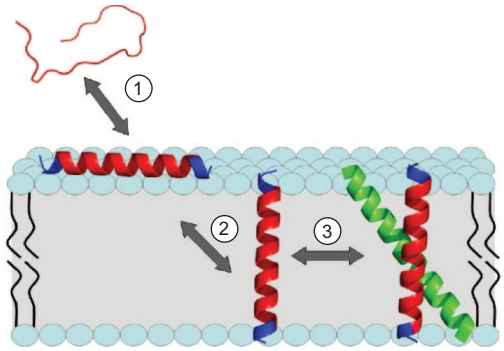


**Figure 1. Schematic of TM peptide insertion and interaction in three steps. 1) TM sequence binds the lipid bilayer; 2) bound TM sequences insert into the lipid bilayer and adopt the desired membrane-spanning orientation, and anchoring residues (blue) hold the peptide in the lipid bilayer; 3) TM sequences associate with the target TM helix (green).**

Upon design of a candidate molecule, the functionality and specificity of this molecule should be examined using both *in vitro* and *in vivo* methods. The process of transitioning from fully soluble to membrane protein interacting states can be simplified into three transitional steps, related to the two-stage model proposed by Popot and Engelman (Figure 1) (*14–16*). The steps in this process are the transition from a fully soluble to a membrane-bound conformation, the transition from the membrane-bound to TM orientation, and finally the association of the exogenous peptides with their target protein.

*Step 1. Binding to Lipid Bilayers.* The first step in determining if the designed peptide will behave as anticipated is to confirm its ability to bind to model lipid membranes without nonspecific aggregation. This is often a difficult task considering that the numerous hydrophobic groups required in these molecules to facilitate a TM orientation can result in aggregation. The incorporation of polar and ionizable groups flanking the TM segment has been shown to enhance the solubility of the molecule without affecting the desired properties of membrane binding and insertion (*17–19*). In addition, pH changes may also facilitate the binding and insertion of TM peptide sequences into the membrane (*20–23*).

Characterizing membrane binding events is accomplished through a variety of techniques, including fluorescence spectroscopy (*24–27*), isothermal titration calorimetry (*28–32*), and surface plasmon resonance (*33–36*). After the designed peptide is shown to bind model membranes, it is critical to ensure that the peptide is not causing instability or deformations in the target membrane. A large number of small, α-helical peptides have evolved as defensive or toxic agents and act by binding to and disrupting the integrity of a target cell membrane. Fluorescent dye leakage assays are a well-characterized and broadly applicable method for monitoring increases in the permeability of a liposomal membrane upon binding of the designed peptide (*37*).

*Step 2. Inserting into Lipid Bilayers in the Desired TM Orientation.* Once bound, the peptide must spontaneously adopt a TM orientation to allow the intended helix−helix association. The ability of the peptide to adopt a TM conformation is directly related to the amino acid sequence or, more specifically, the hydrophobicity of the membrane spanning region (*38*). The TM segment is primarily composed of hydrophobic or nonpolar amino acids, but several *in vitro* and *in vivo* studies have shown that very hydrophilic residues such as aspartic acid or lysine can be tolerated near the center of a TM α-helix (*39–43*). In addition, the asymmetry in the flanking polar groups will direct the insertion of the TM peptide into the bilayer in the intended orientation (*44, 45*). A number of spectroscopic techniques, including fluorescence quenching (*22, 29, 35, 46*), polarized attenuated total reflectance infrared spectroscopy (*37, 44, 47*), hydrogen−deuterium exchange infrared spectroscopy (*47, 48*), and oriented circular dichroism spectroscopy (*18, 49–52*) can be employed to discriminate between TM and bilayer surface associated topologies in model membranes. Moreover, discrimination between surface-bound and TM-inserted peptides has been demonstrated by the differential quenching of fluorescence by aqueous and membrane bound quenchers (*24, 38, 53*).

*Step 3. Peptide–Target Interaction.* The final stage in the process is to confirm peptide association with the target. There are many well-characterized methods for qualitatively and quantitatively determining the oligomerization of heterologous membrane proteins and peptides, including equilibrium analytical ultracentrifugation (*54–58*); fluorescence methods, including fluorescence polarization (*44, 59*) and FRET (*59–64*); chemical- or photo-cross-linking (*65–67*); and electron paramagnetic resonance spectroscopy (*68, 69*).

This section is far from a complete overview of techniques for investigating the *in vitro* properties of rationally designed TM peptides (for a comprehensive review on α-helical TM proteins, see ref 70). Choice of technique will be inextricably linked to the specific system being examined, which will pose inherent limitations and advantages for certain modes of investigation.

**Peptides That Modulate Cellular Activities at the TM Region.** With the aforementioned design principles and their subsequent shortcomings in mind, we can discuss a few current strategies for designing and selecting TM peptides. Three methods in particular are quite attractive: (i) truncated native TM regions, (ii) directed evolution, and (iii) rationally designed anti-membrane proteins. Each method provides a set of tools for overcoming issues with stability and insertion. Utilizing truncated native TM regions is the simplest method of all. Short peptide fragments (~20 residues) corresponding to the native TM regions of integral membrane proteins are built with solid-phase peptide synthesis. These fragments are then utilized as inhibitors to modulate and study the TM protein from which they were designed. Clipping out native TM sequences is an expedient means to effectively borrow nature's solutions for anchoring, inserting, and targeting TM peptides. Directed evolution utilizes the bovine papilloma virus and, in particular, a small protein in the viral genome termed E5. E5 is a TM protein that targets and activates the platelet-derived growth factor β receptor (PDGFβR). More importantly, the TM region of E5 can be randomized at the genomic level, and a variety of E5 mutants can be screened for activity. Subsequent rounds of viral infection, selection of transformed cells, and recovery of the virus can evolve new anti-TM sequences. Finally, computed helical anti-membrane protein (CHAMP) provides an *in silico* method for designing *de novo* anti-TM sequences that bind their targets with high affinity. CHAMP relies on the databank of TM protein structures to select a basic TM scaffold. Residues that interact with the

**KEYWORDS**

**2-Stage model:** Thermodynamic model of transmembrane protein folding proposed by Popot and Engelman. Consists of the protein segment in question (1) adopting a TM orientation and then (2) associating with other TM structures.

**Asymmetric insertion:** The preference of a TM protein to enter the membrane in a unidirectional manner.

**Anchoring residues:** Amino acids that are thermodynamically stable in the polar headgroup region of a lipid bilayer. These residues help drive the TM peptide insertion into the bilayer and subsequently hold it in place.

target are calculated using a repacking algorithm, and noninteracting residues are optimized for insertion, anchoring, and stability. These methods have enjoyed various degrees of success and have demonstrated significant potentials in modulating a variety of clinically relevant systems (*1, 71, 72*).

**Exogenous Peptides That Correspond to Native TM Domains.** Utilizing small peptides that correspond to native TM regions represents the initial strategy to develop anti-TM peptides. Native TM regions are selected on the basis of information from a crystal structure or a prediction program. The selected TM region is built with solid-phase peptide synthesis and tested for aggregation and proper insertion into the membrane. Nonbinding residues are modified as necessary, and the affinity with which the anti-TM peptide binds to its target is tested and optimized. This strategy has been employed in a variety of studies to both examine and modulate protein function. An excellent example of regulating protein−protein interactions with native TM sequences are the studies of epidermal growth factor receptors ErbB1 and ErbB2, members of the epidermal growth factor receptor tyrosine kinase family (*73, 74*). These receptors stimulate cellular growth and division in response to epidermal growth factor and are highly overexpressed in certain types of cancers. It is well-known that TM homodimerization is critical for both ErbB proteins in signal transduction (*75, 76*). These ErbB growth factors each contain two GXXXG-like motifs (*77*) that are often observed in TM dimerization (*78*). Site II GXXXG was implicated in homodimerization of ErbB1 (*79*), but the role of the second GXXXG (site I) was not clear. Shai and coworkers built TM sequences corresponding to the TM regions of ErbB1 and ErbB2 (Table 1) with one noteworthy change: the site II GXXXG motif was omitted, allowing an unhindered study of site I. Subsequent studies identified that site I contributes to the formation of an ErbB1−ErbB2 heterodimer (*1*). These results are particularly interesting because the ErbB proteins present a unique case of TM regions with two distinct binding domains that are responsible for homo- and heterodimerization, respectively.

Beyond exploration of protein function, native anti-TM regions are also capable of altering protein function. One such example is the integrins, a family of important integral membrane proteins characterized by an α/β heterodimeric structure (*80*). Integrin αIIbβ3 regulates the process of platelet aggregation in response to trauma (*81*). Aggregation requires an α/β heterodimer to access an active conformation (*72*) and bind ligands such as fibronectin (*82*). The mechanism of activation for the αIIbβ3 heterodimer was unclear in mutagenesis studies (*72, 83–86*). An exogenous peptide derived from the αIIb TM region successfully disrupted the TM interactions between αIIb and β3, trapping the heterodimer in an active conformation (*37*). Moreover, the study implicated the TM region in the activation of αIIbβ3 and demonstrated the utility of anti-TM peptides in pharmacologically important systems.

**TABLE 1. Native and modified TM sequences for ErbB studies**

| TM peptide | Sequence |
|---|---|
| Site I Site II | |
| Native ErbB1[a] | I-A-**T-G-M-V-G**-A-L-L-L-L-L-V-V-**A-L-G-I-G**-L-F-M |
| Modified ErbB1[a] | S-I-A-**T-G-M-V-G**-A-L-L-L-L-L-V-V |
| Native ErbB2[a] | S-I-V-**S-A-V-V-G**-I-L-L-V-V-V-L-**G-V-V-F-G**-I-L-I |
| Modified ErbB2[a] | L-T-S-I-V-**S-A-V-V-G-**I-L-L-V-V-V |

[a]Reference *1*.

While the methodology to develop anti-TM peptides from native TM sequences is very simple and effective, the aforementioned targets present ideal cases for designing and testing anti-TM peptides. Both examples (ErbB and αIIbβ3) take advantage of binding motifs that are clearly discernible from the sequence and utilize readily observable cellular responses to help screen for the activity of their anti-TM peptides. Various TM targets would have to be tested to determine if truncating anti-TM peptides from native sequences is a broadly applicable strategy for studying and modulating cell surface receptors.

Manolios and co-workers made some efforts to generalize the model in their studies of T-cell receptors (TCR) (*87*). TCRs are cell surface receptors that recognize antigens and

**KEYWORDS**

**CHAMP:** Computed helical anti-membrane protein, an *in silico* method for designing *de novo* TM sequences which interact with a chosen target. The method relies on the data bank of TM protein scaffolds as well as a depth dependent force field for designing TM sequences.

**Directed TM evolution:** A process for designing *de novo* TM sequences with the E5 protein of the bovine papilloma virus. The TM sequence of the E5 protein can be randomized at the genomic level to generate a library. The library is used to infect cells and evolve novel TM sequences through rounds of infection, selection, and recovery.

**GXXXG motif:** A common interacting motif found in TM proteins. Glycine residues at the termini of the sequence motif allow TM helices to interact very closely. Other residues with small side chains (alanine, serine) have been shown to promote similar interactions.

**TABLE 2. Native TCR Binding Residues and Core Peptide**

| TM peptides | Sequence |
|---|---|
| TCR binding residues[a] | L-R-I-L-L-L-K-V |
| Core peptide[b] | G-L-R-I-L-L-L-K-V |

[a]Reference *90*. [b]Reference *87*.

stimulate the production of cytokines (*88, 89*). Through mutagenesis and *in vitro* studies, Manolios was able to elucidate a critical TM binding region composed of eight amino acids and including two charged residues (Table 2) (*90*). These key residues were excised from their surrounding TM scaffold and utilized in designing a potent nine-residue anti-TM peptide (Table 2) termed core peptide (CP) that hampered the formation of the TCR complex *in vitro* and *in vivo* (*87*). Interaction of CP with the TM region of the TCR complex was verified with surface plasmon resonance (*91*).

The collective studies demonstrated that TM residues critical for interaction and anti-TM activity could be revealed through mutagenesis studies. Furthermore, the entire native TM scaffold was not necessary for anti-TM activity; only the critical binding residues were necessary. This suggests that CP maintained not only its secondary structure but also the proper helical propensity for interaction with its target. This discovery leaves chemical biologists with a great degree of flexibility in anti-TM peptide design. Assuming that only a small piece of a native TM scaffold has to be conserved, the remaining residues of the scaffold can be modified to enhance water solubility, facilitate membrane insertion, and perhaps add greater binding affinity with the target. One such improvement that has already been examined is lipidation or glycosylation of the C-terminal residue of CP. Lipidating or glycosylating the terminal residue of CP helps to anchor CP to the membrane. This translates to higher levels of CP inserting into the membrane, thereby increasing its anti-TM activity. The modification should be explored for its inhibitory benefit as well as its pharmacological benefit in biodistribution (*87, 92*).

Native TM sequences have one principal advantage: the sequence is already optimized for trafficking and insertion. Unfortunately, these sequences may aggregate in solution or enter the membrane in an erroneous direction without their hydrophilic regions to stabilize them. However, provided that a distinct binding motif or region is discernible from the sequence, simple modifications or deletions in nonbinding regions can prevent undesired aggregation and insertion, allowing for rapid adaptation of native TM sequences to anti-TM tasks. Although native TM sequences certainly provide a starting point for anti-TM peptide design, they limit the researcher in terms of the targets they can choose. All of the aforementioned examples studied oligomerization events in which TM interactions were critical to natural function. If a native TM region does not play an important role in oligomerization, it is unlikely to serve as a good lead for anti-TM design. However, given that a good lead sequence is available, the method could provide a starting point for building a library of sequences that could incorporate different binding motifs or unnatural amino acids for peptidomimetics in high throughput screens.

**Directed Evolution Approach To Identify Novel TM-Binding Sequences.** The second noteworthy approach to peptide design is directed evolution (Figure 2) (*93, 94*). This model was demonstrated in PDGFβR. PDGFβR modulates mitosis and angiogenesis in response to signals from water-soluble extracellular peptides. However, the bovine papilloma virus utilizes a small TM protein (E5) to interact with PDGFβR, producing a transformation (*95, 96*). After successive rounds of selection and recovery, Freeman-Cook *et al.* successfully generated a library of transforming TM sequences that were statistically different than wild-type E5 and identified a consensus sequence for PDGFβR activation in the library. Unfortunately, not all of the generated peptides activated PDGFβR in a separate *in vivo* experiment. However, a handful of the peptides activated PDGFβR with high efficacy, confirming the utility of directed evolution in selecting for anti-TM peptides (*93*). Furthermore, TM peptides selected for PDGFβR distinguished between the native receptor and a point mutated PDGFβR. Wild-type E5 was not capable of the same distinction (*94*).

Directed evolution has several advantages over utilizing native TM sequences. First, the method is capable of screening a large variety of sequences that are not based on a native sequence. This opens up a number of possibilities, including discovery of new TM binding motifs and identification of critical binding residues. The method also skirts around the problem of asymmetric peptide insertion and aggregation in solution by utiliz-

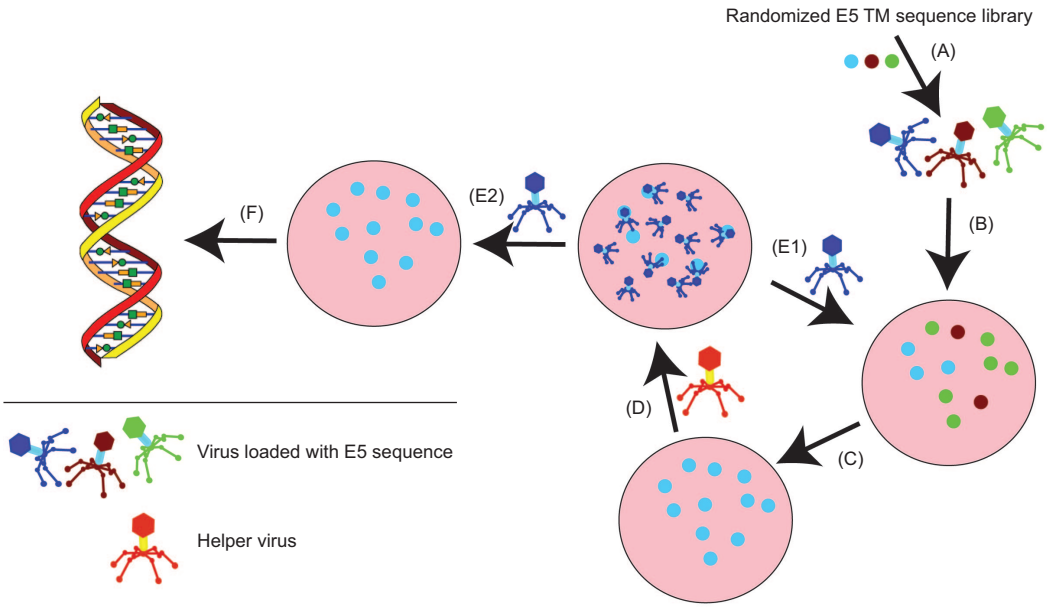


**Figure 2. Utilizing directed evolution to generate novel anti-TM peptide sequences. TM amino acid residues in the E5 scaffold are randomized, creating a large library (A). This library is loaded into viruses, and C127 murine fibroblasts are infected (B). Transformed cells are selected, and the cell line is cultured (C). A helper virus rescues the transforming sequences (D). These sequences may be subjected to successive rounds of infection and selection (E1) or they may be utilized to infect new cells (E2). DNA from the new cells is isolated, and the viral inserts can be amplified and sequenced (F).**

ing the E5 scaffold. Collectively, these positives make the method very attractive for applications where critical binding residues are not easily discerned from the native TM sequence. Unfortunately, it is not clear whether the E5 scaffold will bind and modulate TM protein targets other than PDGFβR. The TM residues of E5 could certainly be modified at the genomic level to preferentially bind other TM proteins, and because E5 is quite small, with about half of its 44 residues contributing to the TM region, there is a low likelihood that an extracellular or intracellular interaction will hamper the binding event. A description of some of the potential targets for directed evolution is detailed elsewhere (*97*).

**Computational Methods To Develop Novel Anti-TM Sequences.** CHAMP is a computational method that designs exogenous TM proteins to interact with the TM region of a target protein (*44*). The CHAMP design starts with a two-helix bundle that is chosen from a database of naturally occurring TM structures. One helix of the chosen helix bundle is then threaded with the target helix. A repacking algorithm (*98*) generates a *de novo* target-binding sequence and an empirically designed, depth-dependent program assigns noninteracting amino acids that help the peptide bind, insert, and remain anchored in the membrane (*9*). Any unassigned noninteracting sites are filled in with random lipophilic residues, including leucine, isoleucine, alanine, phenylalanine, and valine. CHAMP peptides were designed to target the aforementioned αIIbβ3 and αVβ3 integrins, thereby driving them into their active conformations by

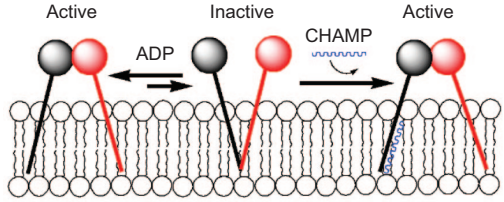


**Figure 3. CHAMP peptide that regulates the protein–protein interactions at the TM regions of integrin αIIbβ3. Activation of the integrin αIIbβ3-mediated signal transduction requires the presence of ADP, which induces the separation of the TM domains of the α- and the β-subunits. CHAMP peptides interrupt TM interactions between the α/β subunits, activating integrin αIIbβ3 (right).**

disrupting the protein−protein interaction between TM domains of the α and β subunits (*44*) (Figure 3). A handful of CHAMP peptides tested *in vitro* demonstrated excellent selectivity for their targets in the presence of other homologous integrins. CHAMP peptides also successfully induced αIIbβ3 and αVβ3 activation in mammalian cells.

CHAMP is an effective method for designing *de novo* anti-TM peptides. More importantly, the program selects highly specific anti-TM peptides that insert properly into the membrane. This makes CHAMP quite attractive for chemical biologists unfamiliar with TM peptides. In addition, any solubility issues can be easily addressed by appending lysine or polyethylene glycol to the ends of the CHAMP peptide as detailed by the designers (*44*). It has to be tested whether or not CHAMP will work for a wide array of targets. Furthermore, choosing a scaffold from a library of TM protein structures is only as good as the library itself, which is currently very small. However, a number of generalized scaffolds have already been identified, and the size of the protein structure library is constantly growing.

Recently, Baker and colleagues used the Rosetta program (*99, 100*) to generate rough TM scaffolds that were optimized using an energy function that carefully examined highly detailed and orientation specific hydrogen-bonding (*101*) in the prediction of protein TM structures. Another important addition is an atomic solvation term (*102*), which was carefully attenuated for TM prediction. The model was highly successful at predicting side chain interactions, native amino acid sequence, and *de novo* structures of small protein domains (*103*).

**Summary.** The rational design of peptides targeting protein TM domains encompasses a variety of challenges, including solubility in aqueous and lipid phases, asymmetric insertion of the peptide sequence, and high-affinity/high-specificity binding with the target sequence. Major strides have been made in developing techniques that facilitate such designs for the researcher. These techniques include truncating native TM regions, directed evolution with the E5 protein, and computational designs with CHAMP and Rosetta. These methods have been successfully applied to modulate a variety of biologically and clinically relevant systems. These techniques will likely play a major role in designing peptides to study molecular recognition in the membrane, signaling pathways, and TM protein structure.

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