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*Topical Review*

# Molecular Recognition within the Membrane Milieu: Implications for the Structure and Function of Membrane Proteins

**Y. Shai**
Department of Biological Chemistry, The Weizmann Institute of Science, Rehovot 76100 Israel

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## Introduction

Elucidating the principles that govern the interaction between proteins and membranes as well as understanding the molecular forces underlying peptide-peptide interactions within the lipid environment are major goals in biology. Both types of interactions play key roles in numerous biological events that take place in the cell membrane. Several examples include energy metabolism, muscle contraction, nutrient absorption, signal transduction, killing of bacteria, infection by viruses and a variety of other processes. Proteins that have evolved to participate in these events can be classified into three major groups: (i) water-soluble proteins that undergo substantial conformational changes to allow them to interact and insert into the membrane (e.g., pore-forming toxins), (ii) integral membrane proteins that can fold into active forms within the membrane (e.g., ion channels, transporters, and receptors), and (iii) proteins containing both integral parts and water/membrane soluble regions (e.g., envelope proteins of viruses).

Knowing the structure of membrane proteins is a major step toward understanding their function. Sequencing membrane proteins, similarly to water-soluble proteins, has become relatively straightforward by cDNA cloning. In addition, hydropathy plots and related algorithms [38, 100, 155, 192, 193, 203], computational models [24, 64], as well as site-directed mutagenesis combined with functional studies have been used to predict the topology of membrane proteins, i.e., the segments that traverse the membrane. In addition, NMR studies [197] and electron microscopy [90, 188] have been utilized to obtain important information, although mostly limited, on the structure and organization of membrane proteins. However, whereas high-resolution structures are available for a myriad of soluble proteins, three-dimensional structures were obtained for only a few membrane proteins. The list includes bacteriorhodopsin, halorhodopsin, rhodopsin, the photosynthetic reaction center, the light-harvesting complex, photosystem I, porin, and the nicotinic acetylcholine receptor (reviewed in [19, 103, 161, 189, 202, 203]). Recently, the structure of the nicotinic acetylcholine receptor was resolved at 4.6 Å resolution using electron microscopy [121]. X-ray diffraction was used to determine the structure of the light-driven chloride pump halorhodopsin [87], an NK cell immunoglobulin-like receptor in complex with its class I MHC ligand [21], and a calcium pump from the sarcoplasmic reticulum [186]. Other studies reported the structure of only membrane-inserted domains of membrane proteins. These include the X-ray structures of the pore-forming domains of a potassium channel from *Streptomyces lividans* crystallized from micelles [36], the NMR structure of the M2 channel-lining segments from nicotinic acetylcholine and NMDA receptors [122], and the NMR structure of the transmembrane helix of glycophorin A [109]. The limited number of known 3-D structures from membrane proteins is mainly due to difficulties in crystallizing membrane proteins in their native environment. As a result, the mechanism by which membrane proteins function, e.g., transmit solutes, ions or specific information through the bilayer of the cell, is in general not known.

Spectrofluorimetric approaches have also been used for many years to get insight into the structure, organi-

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zation and dynamics of membrane proteins. In these studies, specific amino acids were labeled with fluorescent probes and energy transfer was recorded. Such studies were conducted with both water/membrane-soluble proteins such as colicins [32, 212], and integral membrane proteins such as potassium channels [25, 65].

A promising approach, which has been utilized in the last decade to get insight into the structure and function of membrane proteins, is based on studying noncovalently linked fragments of membrane proteins. Many of such studies were carried out showing that transmembrane segments of membrane proteins can coassemble in a specific manner and that these interactions can be used to predict the functional structure of membrane proteins. Detailed studies revealing important information on the forces underlying peptide-peptide interaction within the membrane milieu were conducted predominantly on self-association of single transmembrane helices derived from type I integral membrane proteins or model peptides. Studies along this line were summarized in many reviews [3, 103, 104, 146, 166, 193, 203] and, therefore, will be only partially discussed in the present review. This review will focus mainly on recent studies demonstrating the specific hetero-assembly of membrane-bound segments derived from integral and water/membrane soluble proteins, which led to a better prediction of the correct organization of these proteins in the membrane-bound state. In addition, topics such as the role of the lipid head group charge, and the chirality of the peptide, on peptide-lipid and peptide-peptide interaction within the membrane milieu, as well as the potential application of such information for interfering with the function of membrane proteins will be discussed. Dissection of complex proteins into fragments is a promising approach that, in combination with both experimental and theoretical studies on the full-length proteins, provide important information regarding the structural and functional organization of membrane-interacting proteins.

## Peptide-Peptide Interaction within the Membrane Milieu

Integral membrane proteins are composed of extramembraneous and membrane-embedded domains and can be classified into two major groups: those that function as monomers, and those that function as homo- or hetero-oligomers. It is assumed that the folding of the extramembraneous domains follows the same principles as those of soluble proteins about which extensive literature exists [63].

Several integral membrane proteins have been shown to maintain tertiary structures when the peptide backbone was cleaved, and substrate binding and/or catalytic activity was retained. These studies revealed that forces between different domains within the membrane-inserted regions of a protein are able to maintain the three-dimensional structure when the peptide backbone is not intact. Most intriguing were the observations that isolated transmembrane helices of membrane proteins can assemble in vitro within a bilayer environment into functional proteins. Examples are: bacteriorhodopsin [79, 107, 112, 144], lactose permease of *Escherichia coli* [16, 159], the $b_2$-adrenergic receptor [86], a voltage-gated anion channel [162], and the anion transport domain of human red cell band 3 (AE1) [67]. These studies strengthen the notion that fragments of membrane proteins can adopt their native structure and, therefore, support a proposed "two-stage" model for membrane protein folding and oligomerization [143, 144]. The model suggests that the final structure in membranes results from the packing of smaller elements, each of which reaches thermodynamic equilibrium with the lipid and aqueous phase before packing. The model excludes structures incorporating transmembrane segments that are not individually stable in the membrane, such as β-sheets (reviewed in [104, 145, 146, 193]). Indeed, many studies with transmembrane domains of proteins supported this theory. However, most of these studies were focused on the forces underlying the dimerization or homo-oligomerization of hydrophobic transmembrane helices derived from type I integral membrane proteins. The list includes glycophorin A [20, 104], T-cell receptors [18, 111], the aspartate sensory receptor of *E. coli* [108], the tyrosine kinase receptor family [78, 178], the IsK ion channel regulator [9, 11], and phospholamban [3]. Another group includes native membrane-interacting amphipathic peptides (hydrophobic and hydrophilic amino-acid side chains are segregated at opposite surfaces of a helix) such as antimicrobial and cytolytic peptides, as well as membrane-inserted domains of pore-forming proteins [29, 48, 49, 51, 53, 54, 62, 124, 153, 165–167, 169, 179].

A major question is, what are the underlying forces that dictate the specific assembly of membrane bound/inserted polypeptides? In the case of amphipatic α-helical peptides, in which the hydrophilic face of the helices is oriented toward the interior of the bundle, hydrogen bonds and/or salt bridges are the predominant forces, as has been shown with the M2 channel-lining segments from nicotinic acetylcholine and NMDA receptors, by NMR spectroscopy [122]. Other examples include: (i) the pore-forming amphipatic helix pardaxin [152], in which neutralization of the three positive charges by acetylation preserved the ability of the molecule to specifically assemble within the membrane, similarly to the native molecule [152]; and (ii) the amphipatic antimicrobial peptide dermaseptin: substituting serine with aspartic acid in its hydrophilic face caused assembly of the mutated peptide in model phospholipid membranes

[179]. On the other hand, in hydrophobic transmembrane helices, Van der Waals interactions have been gaining favor as the dominant forces, but increasing evidence has revealed that hydrogen bonding is also crucial in a membrane environment. The following examples will elaborate this point: (i) a model membrane-soluble peptide was designed and found to associate in a monomer-dimer-trimer equilibrium, in which the trimer predominates at the highest peptide/detergent ratios. Interestingly, the oligomers are stabilized by a buried Asn side chain. Mutation of this Asn to Val essentially eliminates oligomerization of the membrane-soluble peptide. Thus, within a membrane-like environment, interactions involving a polar Asn side chain provide a strong thermodynamic driving force for membrane helix association [28]. (ii) A model transmembrane helix was designed based on the GCN4 leucine zipper. It was found that in both detergent micelles and biological membranes, helix association is driven strongly by hydrogen bonding between asparagines, independent of the rest of the hydrophobic leucine and/or valine sequence. Accordingly, hydrogen bonding between membrane helices provides stronger associations than the packing of surfaces in glycophorin A helices [214]. (iii) NMR studies of the dimeric transmembrane domain of glycophorin A (GpA) solubilized in aqueous detergent micelles demonstrated that Van der Waals interactions alone can mediate stable and specific associations between transmembrane helices [109]. (iv) Transmembrane domains exhibiting high-affinity homo-oligomerization were selected from a randomized sequence library based on the right-handed dimerization motif of glycophorin A. The most frequent motif isolated, GxxxG, occurs in more than 80% of the isolates. However, flanking residues were found to act in concert with the GxxxG motif, and the size complementarity was found to be maintained at the interface, consistent with the notion that the identified sequence patterns represent packing motifs [158]. (v) Deber et al., [35] have shown that Val→Ala mutations within the effective transmembrane segment of a model single-spanning membrane protein, namely the 50-residue major coat (gene VIII) protein of bacteriophage M13, have a sequence-dependent impact on the stabilization of membrane-embedded helical dimeric structures in SDS-PAGE.

Specific self-association within the membrane environment occurs also with peptides that do not predominantly form an α-helical structure. For example, the 33-amino acid N-terminal fusion peptide of HIV-1 envelope glycoprotein (gp41) adopts a predominantly β-sheet structure in phospholipid membranes [81, 148]. This peptide self-associates in phospholipid membranes and forms trimers in SDS. Substitution of Val at position 2 with Glu abolished the trimerization and only dimers could be observed [81]. Similar results were obtained with a Phe→Gly mutation at position 11 [148]. Other examples will be discussed in detail in the following sections.

## The Role of the Charge of the Membrane Head Group and Peptide Chirality on Peptide-Membrane and Peptide-Peptide Interaction within the Membrane Milieu

Both zwitterionic and negatively charged phospholipids are present in biological membranes. In most cells there is an asymmetric distribution of the phospholipid head groups such that the internal surface of the cell is highly negatively charged whereas the outer surface contains predominantly zwitterionic head groups [191]. Electrostatic interactions therefore play an important role in the process of membrane binding. In the case of peptides with charge opposite to that of the membrane, the first step in the binding process depends on electrostatic attraction. The peptide concentration at the bilayer-water interface is related to the membrane-surface charge density and can be estimated based on the Gouy-Chapman model ( for a review *see* McLaughlin, 1989 [117]). The difference in the chemical standard potential of free and bound peptide, expressed by a partition coefficient, and the effective charge of the peptide can be derived from this model [176]. After the initial binding, the peptide can insert deeper into the membrane. The extent of penetration varies, depending on both the charged and hydrophobic groups present in the peptide and the membrane lipidic composition. Whereas positively charged peptides may remain close to negatively charged head groups, deeper insertion can be observed in zwitterionic bilayers [204]. In addition, electrostatic forces are also important in determining the transmembrane orientation of integral membrane proteins, as expressed by the "positive-inside" rule for the distribution of basic residues on the cis relative to the trans side of the membrane-spanning alpha-helices [192]. In contrast, many studies revealed that peptide-peptide interactions within the membrane are usually independent of the charge of the phospholipid head group, although the energy of binding might be different. Several examples include: (i) The pore-forming lytic peptide pardaxin binds, organizes and permeates similarly both zwitterionic and negatively charged phsopholipids [152, 153]; (ii) the amphipatic helix, α5, of the pore-forming protein insecticide *Bacillus thuringiensis* δ-endotoxin self-associates and permeates similarly zwitterionic and negatively charged membranes [48, 52]. (iii) Matsuzaki et al. [116] have shown that the antimicrobial peptide magainin can insert and assemble when bound to phosphatidylglycerol but stays unassembled on the surface of phosphatidylserine. (iv) Fisher et al., [44] used Forster resonance energy transfer to measure dimerization of the glycophorin A transmem-

brane helix in detergent micelles. Interestingly, they observed that the structure was the same but the $K_d$ was at least two orders of magnitude weaker in sodium dodecyl sulfate than in zwitterionic detergents. In contrast to these examples, self-assembly of peptides that bind onto the surface of the membrane has been shown to be strongly dependent on the charge of the phospholipid head group. For example, the α-helical antimicrobial peptide dermaseptin [62, 147, 179], and the helix S4 of the *Shaker* potassium channel [153] could self-associate only on the surface of negatively charged membranes.

Another important factor that has been investigated is the role of peptide chirality on peptide-lipid and peptide-peptide interaction within the membrane. Natural phospholipids are all L-forms and therefore provide the biological membrane interface with stereospecificity. It has been shown that cell surfaces and phospholipid monolayers can stereospecifically recognize each other [17, 69]. In addition, peptide chirality has been shown to be crucial for peptide-peptide interaction in solution. Examples include the ribonuclease S-peptide/S-protein complex [31], HIV-1 protease/substrate complex [119] and the coiled coil formed by the heptad repeats derived from the F1 fusion protein of Sendai virus [58]. Should stereospecific peptide-lipid and peptide-peptide recognition within the membrane exist, it is expected that during the biochemical process that involves such interactions, two peptide enantiomers will function differently. To test this hypothesis the fusion peptide of HIV-1 has been used as a model [148]. The fusion peptide is an hydrophobic N-terminal domain of the envelope glycoprotein gp41 of HIV-1 that shares high homology with equivalent domains of other enveloped viruses [47]. This peptide is directly involved in the fusion of the viral and cell membranes and its synthetic version can induce fusion of phospholipid membranes (*see* details in the section on membrane fusion). It was found that a synthetic fusion peptide of HIV-1 gp41 and its enantiomeric (all D-amino acid) analogue bind equally to phospholipid membranes and have equal potencies in inducing membrane fusion. These findings indicate that the stereospecificity of the fusion peptide is not important for peptide-lipid interaction during the fusion process [148]. That peptide chirality is not a prerequisite for peptide-lipid interaction has been shown in other cases as well. Enantiomers of amphipatic α-helical lytic peptides such as the bee venom melittin and the antimicrobial peptides cecropin and magainin, possess lytic activity indistinguishable from that of the parent molecules [14, 118, 195]. These enantiomers preserved the amphipatic α-helical structure of the wild-type peptides, a structure proposed to be prerequisite for their function. Since the biological function was preserved, the enantiomeric peptides should be organized in the membrane similarly to their parent all L-amino-acid peptides. Similar results were obtained with all D-amino acid Androctonin, a β-sheet antimicrobial peptide [71].

Studies with intact HIV-1 virus and synthetic fusion peptides derived from HIV-1 and Sendai virus revealed that fusion peptides can specifically self-associate in the membrane and thus assist in the oligomerization of the envelope proteins [46, 81, 149, 154]. Surprisingly, with the fusion peptide of HIV-1, it was demonstrated that peptide chirality is not necessary for peptide-peptide recognition within the membrane, since the all D-amino-acid HIV-1 fusion peptide could coassemble with the native all L-amino-acid fusion peptide when inserted into model phospholipid membranes. More interesting were the findings that both the native and the enantiomeric synthetic fusion peptides could inhibit cell-cell fusion mediated by HIV-1 gp41/gp120 and their receptors, because of their ability to associate with the analogous domain in the intact fusion protein and to interfere with its action [81, 148].

Recent studies with diastereomers (containing both D- and L-amino acids in the same peptide) of amphipatic α-helical peptides revealed that the membrane environment can impose on them a helical structure and preserve their activity, although the structure can be locally altered [157, 171, 205]. Moreover, this family of peptides can discriminate between lipids containing different head groups (reviewed in [127, 169]). For example, diastereomers of melittin and pardaxin bind negatively-charged lipids better than zwitterionic lipids [123, 125, 126, 170]. Furthermore, the NMR structure of a diastereomer of melittin in lipid micelles has a helical structure similar to the native all L-melittin [171] and they are organized similarly in negatively charged membranes [126]. Interestingly, a short (14 amino acids) and highly positively charged amphipatic α-helical peptide could form dimers in SDS-PAGE [128]. In the same study it was shown that replacing 30% of the L-amino acids with their D-amino acids preserved a similar structure in the membrane, but the resulting diastereomer did not form dimers. Cyclization of the all L-peptide also prevented its ability to self-associate in the membrane [128].

## Heteroassembly of Peptides within the Membrane Milieu

Compared to extensive studies on the dimerization and homo-oligomerization of membrane-inserted peptides, only limited studies were done to show direct evidence for specific heteroassembly within the membrane milieu. The following sections will summarize studies done with synthetic peptides derived from three representative membrane proteins: (i) ion channels as representatives for integral membrane proteins; (ii) the pore-forming protein *B. thuringiensis* δ-endotoxin as a representative

for water/membrane soluble membrane proteins; and (iii) envelope proteins of viruses which contain both integral and water/membrane soluble regions.

## Studies on Peptide-Peptide Interaction Between Membrane-Inserted Domains of Voltage-Activated Ion Channels Agree with Site-Directed Mutagenesis and X-Ray Data

Ion channels are abundant integral membrane proteins that allow the passage of specific ions through the phospholipid membrane barrier, an essential step in many cellular processes [72]. Voltage-activated $K^+$ and $Na^+$ channels belong to a large and diverse group within the family of voltage-activated ion-conducting channels. They comprise four polypeptide monomers of about 70 kD each in the case of the $K^+$ channel or four homologous subunits in a single-chain protein in the case of the $Na^+$ channel [4, 72]. Sequence analysis of the $K^+$ channel monomers and the $Na^+$ subunits suggests that each of them consists of six hydrophobic segments (S1–S6), each long enough to form a transmembrane helix, and a pore region (P-region) proposed to form part of the lumen of the channel. They also include long N- and C-terminal domains. One of the helices, the S4 segment, is highly conserved in the *Shaker*-like potassium channels, and also exists in each of the four subunits of the sodium and calcium channels. S4 has a basic residue at every third or fourth position. Because of this unique structure it was proposed that the S4 can serve as the voltage sensor of the channel [15, 160, 184]. Whereas the four P-regions of the $K^+$ channel are the same, those of the $Na^+$ channel vary considerably in length. Despite extensive studies, it is not clear which structural components are involved in the folding of the monomers/subunits, in their assembly to form functional channels, or in the specific recognition needed for this "discriminative" assembly process. Subunit interactions within the hydrophobic core region were reported in the assembly process [7, 130, 131, 164, 172, 173], as well as sites in the intracellular N-terminal domains of the channels [106], which seem to be crucial for the process of "discriminative" assembly. Furthermore, interactions between segments and subunits of the channel have functional importance in the assembled channel as well, as was shown for the P-region [80, 181] and the voltage sensor S4 segments of the *Shaker* $K^+$ channel [132]. Thus, the voltage-gated ion channels are an interesting and relevant system to examine interactions between membrane-embedded segments.

Studies along this line were carried on with the *Shaker* $K^+$ channel, the *Electrophorus electricus* (eel) $Na^+$ channel, and the inward rectifier $K^+$ channel.

### *The* Shaker $K^+$ *Channel*

Studies with synthetic peptides comprising various putative membrane-inserted regions of the channel revealed several interesting features. First, the structure of S4 was found to be an α-helix [139] in agreement with what was reported using other methods applied on the functional channel [65]. Interestingly, it was found that the tilt angles derived for S3 and S4 are 44° and 72°, respectively. In other words, S4 is oriented almost parallel to the membrane plane in agreement with its role as a voltage sensor that can change its orientation upon transmembrane potential [139]. As for S3, its tilt angle suggests a transmembrane orientation. Furthermore, resonance energy transfer (RET) studies with donor/acceptor-labeled pairs of peptides also revealed a specific interaction between the helices S3 and S4. S3 interacts with S4 derived from the potassium channel, but does not interact with S4 derived from the eel sodium channel, despite the fact that they share high homology in their hydrophobicity and positive charges [139]. Two positively charged amino acids in S4 were shown to interact with negatively charged amino acids in S2 and S3 in the intact channel (K374 and R377 [132, 185]). Neither has positive charges in the S4 homologous to the sodium channel. Thus, it is reasonable to assume that the lack of these necessary residues prevents the coassembly of the S3 with the S4 segments of the sodium channel. In addition, it was found the P-region of the *Shaker* $K^+$ channel can self-associate when bound to the membrane, in line of its role as the channel lining segment [137, 138].

### *The* Electrophorus electricus *(Eel)* $Na^+$ *Channel*

Peptides corresponding to the P-regions of the four homologous subunits (I, II, III, and IV) of the eel $Na^+$ channel were synthesized and structurally and functionally characterized. A network of peptide-peptide interactions within the membrane was determined using RET measurements between donor/acceptor-labeled pairs of peptides. The data revealed that besides the P-regions of subunits I and III, which did not coassemble in the membrane, all other pairs coassembled but did not bind to unrelated membrane-bound peptides. Thus, the following P-region pairs were found in the membrane-bound state; I/II, II/III, III/IV and I/IV. Similar results were obtained with studies done on the intact channel. Benitah et al. [13] used site-directed mutagenesis and introduced pairs of cysteines into the P-regions of a rat skeletal muscle sodium channel. Only cysteinyl residues that were in close proximity could form disulfide bonds or metal-chelating sites. They found that cysteine in domain I spontaneously formed a disulfide bond when

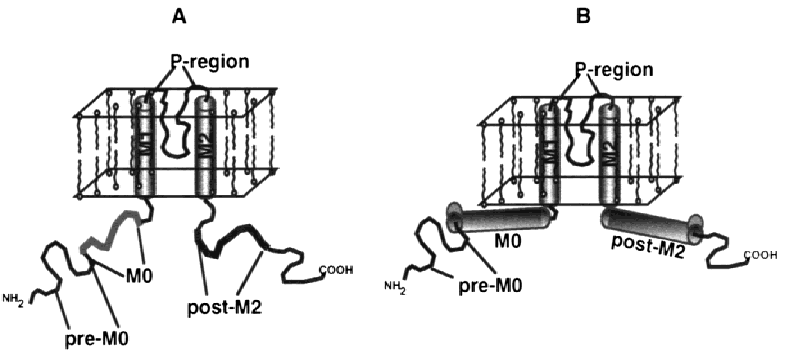


**Fig. 1.** Panel *A*, topology of the ROMK1 channel based on hydropathy analysis. Panel *B*, studies done with peptides modeled after several segments of the channel led to the identification of two additional membrane-interacting helices [10, 12].

paired with cystein in domain II, whereas the same residue, when coupled with cystein in domain IV, created a high-affinity binding site for $Cd^{2+}$ ions.

*The ROMK1 $K^+$ Channel*

ROMK1 is a member of the inwardly rectifying $K^+$ channels that conduct an inward $K^+$ current at hyperpolarizing membrane potentials. They play an important role in regulating the resting membrane potential and electrical excitability of cells in a variety of tissues, including the brain and heart [72]. Several proteins of this family have been identified and include ROMK1 [73], IRK1 [89], GIRK1/KGB [34, 88], KATP [6], RACTK1 [180], and prokaryotic KcsA [163]. Hydropathy analysis of these channels predicts only two potential transmembrane domains, namely M1 and M2 (Fig. 1*A*). Furthermore, significant similarity to the P-region of voltage-gated $K^+$ channels was found for a P-region between M1 and M2 (44% similarity for ROMK1 [73]). Hydropathy plots of the ROMK1 channel also showed that M0 exhibits intermediate hydrophobicity and that there are hydrophobic stretches in the C-terminus that are potentially membrane-associated [73]. Furthermore, experimental evidence suggests that the C-terminus contributes to the pore properties of inwardly rectifying potassium channels [182].

Synthetic peptides comprising six regions within ROMK1, namely, M0, M1, M2, P-region, pre-M0 and post-M2 were synthesized and characterized regarding their structure in the membrane and their ability to self-associate or to coassemble with each other in the membrane milieu (Fig. 1*B*) [10, 12]. A summary of the findings is listed below:

1. M0, M1, M2, P-region, post-M2, but not pre-M0 bind strongly to the membrane.
2. M1, M2, and P-region are inserted into the hydrophobic core of the membrane, whereas M0 and post-M2 bind onto the surface of the membrane.
3. M0, M1, M2, and post-M2 adopt high α-helical structures, whereas the P-region and pre-M0 are not α-helices.
4. Molecular recognition studies using donor/acceptor-labeled pairs of peptides revealed that (i) the P-region and M2 can self-associate in their membrane-bound state but M1 cannot; (ii) Self-associated P-region can coassemble with M2 but not with M1; and (iii) M1 can coassemble with M2. No coassembly was observed between any of the segments and a membrane-embedded α-helical control peptide.
5. Molecular recognition studies using donor/acceptor-labeled pairs of peptides also revealed that M0 and Post-M2 do not self associate but they coassemble with each other in their membrane-bound state. Their ability to coassemble might assist in the oligomerization of the channel.

Based on the above findings, a model for the organization of the ROMK1 potassium channel within the membrane was suggested [10, 12, 168]. If we assume that the channel is composed of tetramers, then the inner core is composed of a tetramer of P-region domains surrounded by M1 and M2 regions. Post-M2 and M0 are hypothesized to be surface-localized and to surround M1 and M2, forming interactions between them (Fig. 2*A*). The finding that Post-M2 is membrane-localized gains support from site-directed mutagenesis on the intact channel. It was shown that exchange of the C-terminus altered pore properties, suggesting that in inwardly rectifying channels certain regions of the C-terminus are likely to be in the membrane and that the C-terminus seems to make a major contribution to the pore [142, 182].

The organization of M1, M2, and the P-region, as deduced from the experiments done with peptides, is in agreement with the X-ray structure of a truncated form of the homologous KcsA channel from *Streptomyces lividans*, which contained only M1, M2, and the P-region (Fig. 2*B*) [37]. In that study, X-ray analysis with data to 3.2 Å revealed four identical subunits that create an inverted teepee, or cone, cradling the selectivity filter of the pore in its outer end. In this structure only a small

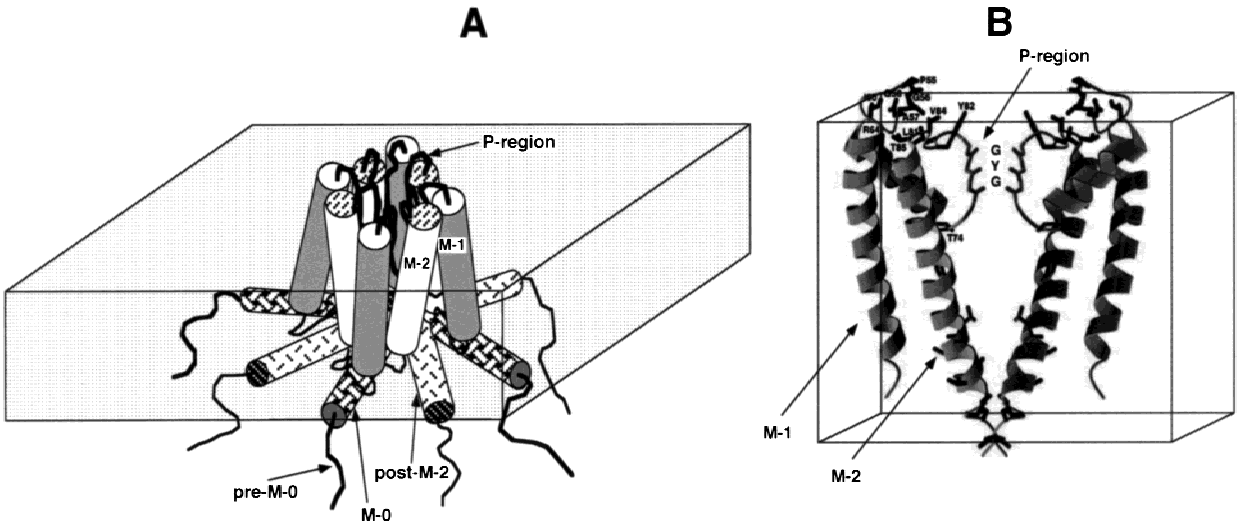


**Fig. 2.** Comparison of the structural organization of the ROMK1 channel as predicted based on studies done with model peptides [10, 12] (Panel *A*), and as determined by X-ray crystallography. (Taken with permission from Doyle et al., 1998 [36].) (Panel *B*). The protein-dissection approach agrees with the 3D X-ray structure and predicts the localization of regions of the channel that were not present in the crystals.

part of the P-region is α-helical and lines the lumen of the pore. The P-regions are surrounded by the M2 helix. M1 forms the outer ring and is associated with M2.

A recent mutagenesis study with a homologous inward-rectifying potassium channel indicated that the M2 pore-lining inner helices are surrounded by the M1 lipid-facing outer helices, arranged such that the M1 helices participate in subunit-subunit interactions. This arrangement, although revealing a similar order in the assembly of the membrane-inserted domain, seems to be different from the X-ray structure of the bacterial potassium channel in which helix M1 did not seem to participate in the subunit-subunit assembly [120].

### A Network of Helix-Helix Interactions Reveals an "Umbrella-like" Structure for the Pore-Forming Domain of *B. thuringiensis* δ-Endotoxin

The δ-endotoxins are a family of highly potent insecticidal toxins produced during sporulation by *B. thuringiensis* (for review *see* [74, 84]). They are released as protoxins, which are solubilized in the midgut of insects and activated by gut proteases. The crystal structure of two variants of δ-endotoxins in aqueous solution [66, 105] have been determined. The toxins are composed of three distinct domains (Fig. 3*A*). Domain I, the pore-forming domain, consists of a bundle of six α-helices surrounding α5, the central helix. Domain II, the receptor-binding domain, is comprised of three β-sheets, and Domain III has a two-antiparallel β-sheets sandwich structure. The pore-forming domain has a general structure similar to other bacterial toxins such as colicin [133]. Among the seven helices comprising the pore-forming domain of various families of δ-endotoxins, α5 is the most conserved helix, and α7 is conserved to a lesser extent. The pore-forming domain needs to undergo a drastic conformational change in order to bind and insert into the membrane. It is assumed that the trigger for the insertion of the pore-forming domain of the toxins into the epithelial cell membrane is a conformational change in the toxin, which occurs when the receptor-binding domain binds to a receptor present on the brush-border membranes [1, 190]. Studies with synthetic peptides corresponding to α5 and α7 from two families of δ-endotoxins [33, 48, 52, 54] suggest that α5, but not α7, oligomerizes within lipid membranes, permeates phospholipid vesicles, and forms ion channels within planar lipid bilayers. Furthermore, α5 was shown to insert into the membrane and specifically associate with the partially inserted α7 helix [54]. In addition, α5 could self-associate within the membrane in a parallel manner as expected if the pore is formed from the association of several helices.

In a more detailed study, all the seven helices of the pore-forming domain of Cry3A, a member of the δ-endotoxin family, were synthesized and compared for their membrane interaction, structure within the membrane, orientation relative to the membrane plane and the network of peptide-peptide interactions in the membrane between all pairs of peptides [50]. Attenuated total reflectance Fourier-transform infrared (ATR-FTIR) spectroscopy was used to study the structure and orientation of the helices when bound/inserted into the membrane. The results showed that all the helices, except α1, interact with lipid membranes, and only α4 and α5 are in a transmembrane orientation. RET experiments between

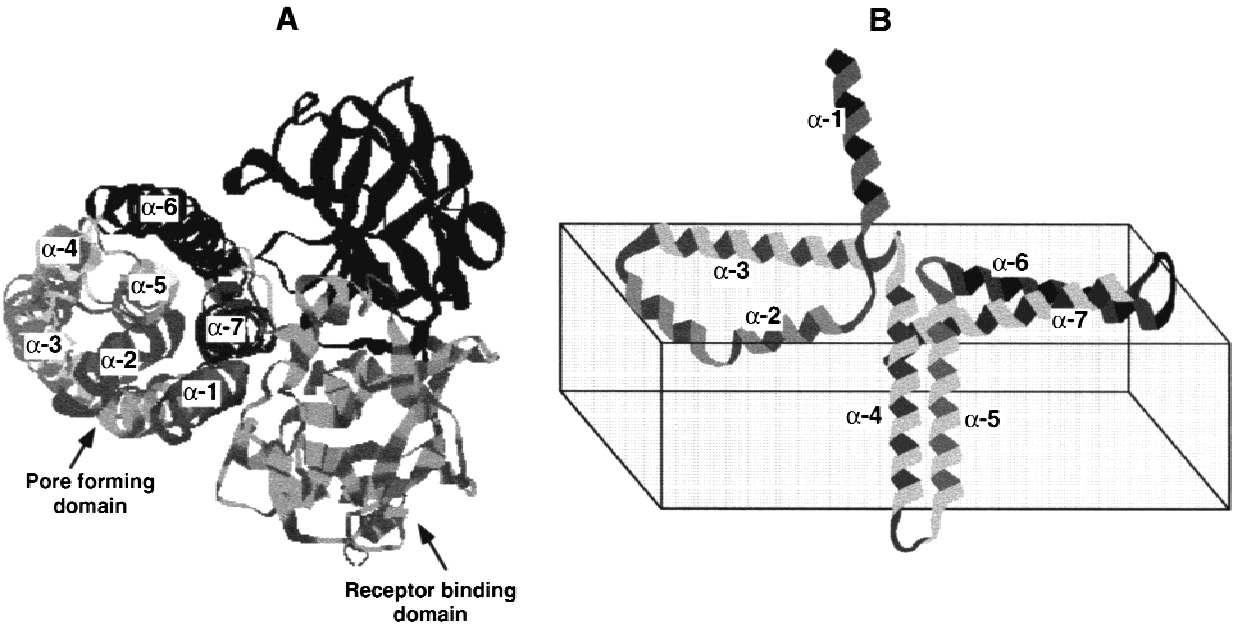


**Fig. 3.** Panel *A,* 3D X-ray structure of a δ-endotoxin in aqueous solution (modified from Grochulski et al., 1995 [66]; Li et al., 1991 [105]). Panel *B,* model of the membrane-bound form of the pore-forming domain, based on studies done with model peptides (taken with permission from Gazit et al., 1998 [50]).

donor- and acceptor-labeled pairs of peptides were carried out, enabling the mapping of the network of recognition of all the possible combinatorial pairs of membrane-bound helices. Remarkably, the RET experiments showed that only α4 and α5 self-assemble within membranes. Moreover, α4 and α5 coassemble in an antiparallel manner. This orientation would be expected if they are inserted as a hairpin. The results also showed that α4, α5, and α6 recognize α7 in the membrane-bound state. Overall these results are consistent with a model in which only helices α4 and α5 insert into the membrane as a helical hairpin in an antiparallel manner, whereas the other helices lie on the membrane surface like ribs of an umbrella (the "umbrella model" [50]). The results also support the suggestion that α7 serves as a binding sensor to initiate the structural rearrangement of the pore-forming domain. Support for the role of domain I as the pore forming domain comes from the X-ray structure [105], site-directed mutagenesis, and studies with hybrid toxins [1, 207]. This notion is further supported by studies that showed that truncated proteins, corresponding to domain I, of two members of the δ-endotoxins form ion channels similar to those formed by the intact toxins [194, 196]. The "umbrella" model is also supported by mutational analysis at various regions in the pore-forming domain [215, 27, 76].

The insertion of the α4-α5 hairpin into the membrane is also expected because of theoretical considerations, since: (1) the C-terminal of α4, the loop between α4 and α5, and the N-terminal of α5 form a hairpin that contains the least polar segment of domain I [66], and (2) the helices are joined on the side of the pore-forming domain proximal to the membrane. Therefore, their insertion into the membrane is in agreement with the hydrophobic hairpin hypothesis suggested for the insertion of proteins into membranes [39].

The helix α7 is located in the interface between the pore-forming domain and the receptor-binding domain (Fig. 3*B*). Its ability to coassemble with α5 and α6 may assist the insertion of the α4-loop-α5 hairpin into the membrane by unpacking the helical bundle that exists in the non-membrane bound form of the toxin. After receptor binding, the affinity of α7 to the membrane surface and its affinity for the helices should lead to the unpacking of the pore-forming domain and facilitate the insertion of α4 and α5 into the membrane.

Very recently, another member of the δ-endotoxin family, namely Cry1Ac, was studied [56]. In this study the complete hairpin domain, α4-loop-α5, its isolated α4 and α5 helices, as well as mutant α4 peptides, based on mutations done on the intact toxin, were synthesized and investigated. Membrane permeation studies revealed that only α5 and F9L α4, which has a mutation that increases the toxicity of wt Cry1Ac, could permeate phosphatidylcholine vesicles. This is in agreement with the finding that only these two helices self-associate in a positive cooperative manner when bound to the membrane. Interestingly, a peptide corresponding to the α4-loop-α5 hairpin was highly active, indicating the complementary role of the two helices in membrane permeation. The synergistic effect between the two helices is in agreement with the findings that α4 and α5 recog-

nize each other in the membrane as measured using RET experiments. Surprisingly, the active F9L α4 could associate with the α4-loop-α5 hairpin within the membrane and completely abolished its capacity to make pores in the membrane. In contrast, none of the other α4 peptides or α5 displayed any substantial inhibition [56]. These findings suggest that F9L α4 can interrupt this interaction in the hairpin, whereas α5 cannot, despite being more active. Thus, it suggests that α4 is positioned facing the aqueous lumen of the channel, in agreement with site-directed mutagenesis studies [115]. These findings provide the first example that a mutated helix within a pore can function as an "immunity protein" by directly interacting with the segments that form the pore. This presents a potential means of interfering with the assembly and function of other membrane proteins as well. This mode of inhibition was suggested also in the case of colicin [42, 55, 213]. It had been hypothesized that the ability of a few immunity protein molecules in the cytoplasmic membrane to confer protection against the lethal effects of a channel-forming colicin involves a complex stabilized by electrostatic or polar interactions between immunity protein and the hairpin inserted helices of the colicin channel. Cramer et al. [213] concluded that the immunity protein exerts its specific effect through rapid lateral diffusion in the cytoplasmic membrane and helix-helix recognition and interaction with at least one hydrophobic and one amphiphilic transmembrane helix of the colicin channel. Interaction with the amphiphilic helix implies that the immunity protein can react with the channel in the open state [213]. Another study indicated that the immunity protein interacts with the membrane-anchored channel domain that requires a functional membrane-inserted immunity protein but does not require the channel to be in the open state [41].

## Peptide-Peptide Interactions Involved in Protein-Mediated Membrane Fusion

### *Introduction*

Membrane fusion is an essential reaction involved in many biological processes such as viral infection, endo- and exocytosis, fertilization, neurotransmission, and vesicle trafficking [177, 201]. In order to merge two opposing membranes, strong repulsive hydration, steric, and electrostatic barriers must be overcome by the action of specialized membrane proteins. In the case of infection by enveloped viruses, several "fusion proteins" were identified [200]. Fusion proteins from different viral families (e.g., Paramyxo-, Orthomyxo-, and Retroviridae) share conserved features [201]. Specifically, (i) they are type I integral membrane proteins synthesized as inactive precursors that are cleaved by host-cell proteases to become active; (ii) the newly generated N-terminus contains the fusion peptide, a hydrophobic stretch of amino acids believed to insert and destabilize the membrane, leading to fusion [37, 201]; and (iii) either a heptad repeat adjacent to the N-terminal fusion peptide folds into a protease-resistant trimeric coiled-coil at a certain step during the fusion process [177, 201], or two heptad repeats, one adjacent to the N-terminal fusion peptide and the other to the C-terminal transmembrane domain, fold into a trimer of heterodimers such that the interior contains the N-terminal heptad repeat trimer and the outer ring the C-terminal heptad repeats [8, 43, 175].

Although much is known about the three-dimensional structure of fusion proteins in the absence of membranes [23, 85, 156], the details of their interaction with the membrane are still unknown. In an effort to shed light on this complex phenomenon, the interaction between model membranes and synthetic peptides that mimic the corresponding region in the intact protein has been studied (reviewed in [37, 134]). The significance of this strategy has been demonstrated by three observations: (i) there is a direct correlation between the effects of mutations in the intact protein and the peptide analogs [46, 75, 81, 114, 141, 149, 154], (ii) the fusion activity of synthetic peptides, measured in vitro, is sensitive to factors such as pH or to the addition of inhibitory agents that affect the infectivity of the virus in vivo [140, 199], and (iii) some synthetic peptides corresponding to regions of viral fusion proteins show anti-viral activity, suggesting that they can accurately model and interact with functional domains of the viral protein [57, 60, 77, 81, 101, 102, 149, 151, 206, 208, 209].

### *Self-Association of Fusion Peptides within the Membrane Milieu*

After receptor binding, a conformational change is manifested by an increase in the exposed hydrophobicity, which is thought to be related to the exposure of the fusion peptide [174]. The fusion peptide is then inserted into the cell membrane [70, 129, 177, 187, 202], the viral membrane [199], or both [68], and has been postulated to induce local membrane dehydration [215] and to promote negative curvature in the bilayer [40]. Brasseur and co-workers [22] predicted that the N-terminal fusion peptide can insert into the membrane with an orientation oblique relative to the water-membrane interface. This orientation was found for example with the N-terminal fusion peptides of Influenza [183], SIV [30, 113], HIV-1 [81, 114], and Sendai virus [61, 154].

It has been shown that fusion peptides specifically self-associate when inserted into the membrane, and that the level of oligomerization is important for their activity. This has been demonstrated with fusion peptides that adopt both an α-helical secondary structure [59, 61,

154] or a β-sheet structure [135, 148, 149]. Interestingly, an amino-acid substitution that decreased the fusogenic activity of fusion peptides in a model system decreased the size of the oligomers in SDS-PAGE. Similarly, such mutations also decreased the activity of the intact virus or cells expressing the corresponding mutated fusion proteins. For example, mutation of the valine at position 2 in the amino-terminal domain of HIV-1 gp41 to glutamic acid resulted in an envelope glycoprotein that dominantly interfered with both syncytium formation and infection mediated by the wild-type HIV-1 envelope glycoprotein [45]. This interference was not abolished by excess of wild-type glycoprotein, suggesting that a higher-order envelope glycoprotein complex is involved in membrane fusion. In an attempt to understand this phenomenon, Kliger et al. [81] synthesized a 33-residue peptide (wild type, WT) identical to the N-terminal segment of gp41 and its V2E mutant. They showed that both peptides inhibited HIV-1 envelope-mediated cell-cell fusion and self- and coassemble in the membrane. Interestingly, the WT, but not the V2E mutant, induced liposome aggregation, destabilization, and fusion. Moreover, the V2E mutant inhibited vesicle fusion induced by the WT peptide, probably by forming inactive heteroaggregates. These data suggest that specific interactions mediated by N-terminal fusion peptides are required to form higher order oligomers necessary for membrane fusion.

### *The Protease-Resistant Coiled-Coil of Fusion Proteins Melts and Dissociates Upon Interaction with Membranes*

Binding of the fusion protein to the host-cell receptor induces a change in conformation that is believed to cause the packing of the C-terminal heptad repeats against the grooves of the coiled-coil formed by the N-terminal heptad repeats, resulting in the structure observed by X-ray crystallography or NMR [8, 23, 26, 43, 110, 198, 208]. Using electron paramagnetic resonance analysis, Shin and co-workers [150, 211] showed that cysteine-substituted peptides comprising the loop region and part of the N-terminal heptad repeat of Influenza virus fusion protein (HA) and the N-terminal heptad repeat of HIV-1 fusion protein (gp41) insert reversibly into phospholipid vesicles. Based on these results, the authors suggested that binding of the N-terminal heptad-repeat regions to the membrane could bring the viral and cell membranes closer together and facilitate fusion. Ben-Efraim et al. [12] analyzed the interaction between peptides corresponding to the N- and C-terminal heptad repeats from Sendai virus fusion protein, in the presence and in the absence of membranes. They showed that in an aqueous environment, the peptides coassemble into an α-helical complex, presumably with a structure similar to that of the core complex of the homologous SV5 fusion protein, as determined by X-ray crystallography [8]. However, in the presence of phospholipid membranes, this complex binds strongly to the membrane's surface and dissociates therein. In agreement with these observations, NMR studies showed that the N-terminal repeat of the homologous Newcastle Disease virus fusion protein adopts an α-helical structure in SDS that is consistent with the idea that it binds parallel to the bilayer as a monomer, with its hydrophobic face buried in the membrane [210]. Furthermore, similar results were obtained with HIV-1 fusion protein. Kliger et al. [82] found that recombinant proteins corresponding to the ectodomain of HIV-1 fusion protein, but lacking the fusion peptide, bind membranes and consequently undergo a major conformational change. As a result, the protease-resistant core becomes susceptible to proteolytic digestion. In agreement with this observation, Kliger et al. showed that synthetic peptides corresponding to the segments that construct this core, oligomerize in aqueous solution, but dissociate upon binding to the membrane. However, the C-terminal heptad repeat, when elongated towards the transmembrane region, self-associates in the membrane [82]. After the membrane-induced conformational change, both the N-terminal and C-terminal heptad repeats can assist in bringing the viral and cellular membranes closer, facilitating the subsequent merging. The similarity between what was found in HIV-1, a retrovirus, and in Sendai, a paramyxovirus, suggests that these distantly related viruses share common steps in their fusion mechanism. However, the ectodomain of paramyxovirus fusion proteins has certain features that result in a more complex picture. First, it is composed of more than 380 amino acids, compared to those of Influenza virus (an orthomyxovirus) and HIV-1 (a retrovirus), which are only composed of about 175 amino acids. Second, in addition to the N- and C-terminal heptad repeats, which are similar to those found in retrovirus fusion proteins, an extra leucine zipper was found in the interior of paramyxovirus fusion protein ectodomain [57]. A peptide modeled after it was able to self-associate both in solution and when bound to membranes. Interestingly, the peptide was shown to bind specifically to Sendai virions and was a highly potent inhibitor of Sendai virus-mediated lysis of human red blood cells, suggesting that it can accurately model and interact with regions of the full-length viral fusion protein.

The finding that peptides corresponding to the C-terminal heptad repeat of HIV-1 gp41 (DP-178 or T-20) self-associate in the membrane led to the discovery of a second site for the inhibition of viral infection. Kliger et al. [83] showed that DP178 could block two steps in the gp41 conformational cascade at different affinities. The low-affinity site represents inhibition of host-cell recep-

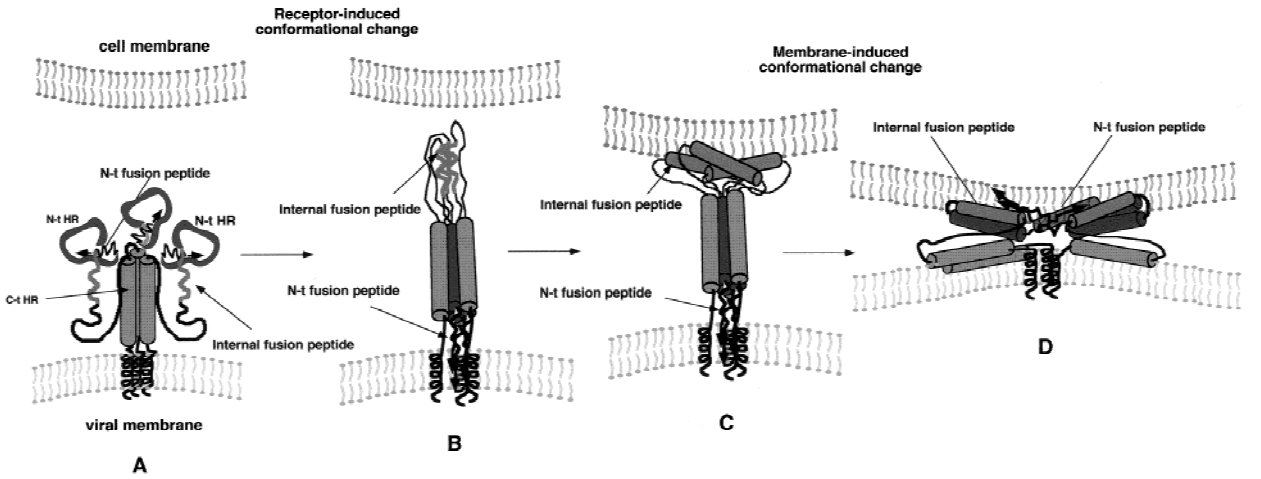


**Fig. 4.** The new "umbrella" model of paramyxovirus-induced membrane fusion (adapted from Peisajovich et al., 2000b [136]). Binding of the virus to cell receptors (Panel *A*) results in a conformational change of the fusion protein, leading to the formation of a trimeric coiled-coil (Panel *B*). The internal fusion peptide, placed on top, is able to interact with the target membrane (Panel *C*). The affinity of the N-terminal and the C-terminal heptad repeats toward the membrane causes the opening of the coiled-coil, accompanied by the binding of these regions to the membrane (Panel *D*). This membrane-induced conformational change causes the cellular and viral membranes to approach each other, facilitating the insertion and self-association of the N-terminal fusion peptide into the membrane. Subsequently, both the internal and the N-terminal fusion peptides induce the merging of the membranes. A similar model, but without the presence of an additional internal fusion peptide, can be postulated also for HIV-1 cell entry.

tor-induced conformational change, before the coiled coil binds to the membrane [82]. The high-affinity site represents inhibition of the newly postulated change in the oligomerization state of gp41: DP-178 interacts with its corresponding segment in the full-length protein, thus inhibiting the recruitment of several gp41-membrane complexes, which leads to fusion pore formation [82].

An intriguing observation was the finding that paramyxoviruses contain an additional internal fusion peptide that also self-associates in the membrane-bound state [136]. Interestingly, a synthetic peptide modeled after this region is a potent inhibitor of Sendai virus-cell fusion [60]. Based on these results, a revised model for paramyxovirus-induced membrane fusion has been postulated. According to the new "umbrella" model, binding of the virus to cell receptors (Fig. 4, panel *A*) results in a conformational change of the fusion protein, leading to the formation of a trimeric coiled-coil (Fig. 4, panel *B*). The internal fusion peptide, placed on top, is able to interact with the target membrane (Fig. 4, panel *C*). The affinity of the N-terminal and the C-terminal heptad repeats toward the membrane causes the opening of the coiled coil, accompanied by the binding of these regions to the membrane (Fig. 4, panel *D*). This membrane-induced conformational change causes the cellular and viral membranes to approach each other, facilitating the insertion and self association of the N-terminal fusion peptide into the membrane. Subsequently, both the internal and the N-terminal fusion peptides induce the merging of the membranes. The finding of two fusogenic regions within a single protein radically changes our view of the functional organization of viral fusion proteins. Further studies are required to understand the specific role played by each fusion peptide and their possible interaction during the fusion process, as well as to determine whether this finding is common to envelope glycoproteins from other viral families.

## Conclusions

Protein-membrane interaction and protein-protein recognition within the membrane milieu are of fundamental importance to understand the basic rules that govern cellular processes. However, the high complexity of the forces involved and the technical difficulties that arise when working with membrane proteins are obstacles hard to overcome for completely understanding molecular recognition within the bilayer. As reviewed here, dissection of complex proteins into fragments is a promising approach that, in combination with both experimental and theoretical studies on the full-length proteins, can provide important information regarding the structural and functional organization of membrane-interacting proteins.

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