The importance of membrane proteins cannot be underestimated in sequenced genomes in eukaryotes and prokaryotes. They account for 25–30% of all proteins (ORFs) identified as they receive much attention because of their great functional and pharmacological importance, such as G protein-coupled receptors possessing 7 TM segments. Although they represent roughly half of all membrane proteins, bitopic proteins (with only 1 TM helix) have so far been less well characterized. Though they include many essential families of receptors, such as adhesion molecules and receptor tyrosine kinases, many of which are excellent targets for biopharmaceuticals (peptides, antibodies, et al.). A growing body of evidence suggests a major role for interactions between TM domains of these receptors in signaling, through homo and heteromeric associations, conformational changes, assembly of signaling platforms, etc. Significantly, mutations within single domains are frequent in human disease, such as cancer or developmental disorders. This review attempts to give an overview of current knowledge about these interactions, from structural data to therapeutic perspectives, focusing on bitopic proteins involved in cell signaling.

Key words: bitopic membrane proteins, transmembrane domains, transmembrane signaling, helix-helix interactions, receptors

Abbreviations: NRP1, neuropilin-1; RTK, receptor tyrosine kinase; TCR, T-cell receptor; TM, transmembrane

Membrane proteins are critical in a diverse array of cellular functions, such as cell-cell communication, detection of environmental changes, transport of substances inside and outside of cells, energy transduction, enzymatic activities. They also have a structural role in maintaining cell shape, size and polarity. In multi-cellular eukaryotes, membrane proteins are also more specialized as receptors for many extracellular signals (hormones, growth factors, neurotransmitters), recognition molecules in the immune system and adhesion molecules. Other important classes of membrane-interacting peptides such as amphipathic helices, antimicrobial peptides and cell-penetrating peptides will not be discussed here.

The long-standing structural view of biological membranes known as the fluid-mosaic model was proposed by Singer and Nicolson almost 40 y ago. This model gave an initial vision where low concentrations of proteins were embedded in a fluid “sea” of lipids. This view has now become more complex to take into account the importance of protein-protein and protein-lipid interactions, the crowding of proteins at the cell surface and the existence of complex specialized “domains” associating specific proteins and lipids and assuming discrete functions. Indeed, it was recognized long ago that TM and membrane-associated proteins occupy environments in the cell membrane where the protein concentration in two dimensions is quite large and can exert a large influence on clustering and thus functions. This evolution of the model reflects also the highly dynamic nature of intramembrane interactions. The contribution of lipids to the complexity of membrane organization and the regulation of membrane bioactivities is now well recognized and described by the lipid raft concept.

Concerning the major problem of protein insertion in the membrane lipid bilayer, the two-stage model was proposed 20 y ago. Its main tenets, the insertion of independently stable helices across the membrane, followed by helix-helix interactions to form higher order structures still hold true. The model has been more recently extended to a third stage which considers events such as folding of extracellular loops, insertion of peripheral domains and quaternary structure formation.

In this model, the lateral association of TM helices within the lipid bilayer is the second stage in the folding of membrane proteins and lipids and assuming discrete functions.9 Indeed, it was recognized long ago that TM and membrane-associated proteins occupy environments in the cell membrane where the protein concentration in two dimensions is quite large and can exert a large influence on clustering and thus functions.10 This evolution of the model reflects also the highly dynamic nature of intramembrane interactions.7,11,12 The contribution of lipids to the complexity of membrane organization and the regulation of membrane bioactivities is now well recognized and described by the lipid raft concept.13,14

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proteins. Dimerization of TM helices is the simplest example of such lateral association, and has been much studied in the context of polytopic membrane protein assembly. But, it may also play a role in signaling across cell membranes by associating two similar or different proteins in an active (or inactive) dimer or by creating larger oligomeric structures.\textsuperscript{7} Many examples of such membrane complexes are known, e.g., the photosynthetic apparatus,\textsuperscript{18} ATPases,\textsuperscript{39} virus assembly machinery,\textsuperscript{20} immune signaling,\textsuperscript{21,22} G protein-coupled receptors,\textsuperscript{24,25} etc.

It is becoming increasingly clear that protein interactions, both transient and stable, are far more extensive than originally appreciated. These protein complexes can present a very wide range of stabilities and form highly coordinated networks that govern biological processes. This is true for soluble proteins, but also for membrane proteins. Thus, in this context, the role attributed to membrane-spanning helices has changed dramatically over the past 10 years. Once mostly regarded as mere membrane anchors, TM domains are now recognized as full-time actors of protein-protein interactions. These interactions may be of exquisite specificity in mediating assembly of stable membrane protein complexes from cognate subunits. Further, they can be reversible and regulatable by external factors to allow for dynamic changes of protein conformation in biological function. Finally, these helices are increasingly regarded as dynamic domains. Many aspects of this topic have recently been reviewed in excellent papers.\textsuperscript{7,11,12,26-41} A brief summary of current knowledge, obtained through biophysical, biochemical, cell biological and genetic studies can be put forward:

1. TM helices are usually 20–23 amino-acids long, with a large over-representation of hydrophobic residues. Polar residues are rare, especially negatively charged ones, whereas single-spanning segments are more hydrophobic than multi-spanning helices.\textsuperscript{30,42}

2. Analysis of polytopic membrane structures and studies on model or natural single TM peptides has provided many insights on packing “rules” for TM helices. Sequence and geometric motifs have been found to drive these interactions, in a similar way as small modular domains (such as leucine zippers) that mediate soluble proteins interactions. The majority of these motifs can be classified in a rather limited number.\textsuperscript{31,38} The geometry of these packing motifs is quite variable with right-hand or left-hand crossing motifs, and crossing angles as large as 40°. Surprisingly, Walters and DeGrado found out that about two thirds of these motifs pack small chain amino-acids (Gly, Ala and Ser).\textsuperscript{18} In these motifs, the small amino-acids are separated by three other residues, thus been usually called Gx\textsuperscript{3}G or GX\textsuperscript{2}G motif or GAS motif. This motif was first recognized and much studied in the case of glycoporin A (see below) and has been characterized now in many other examples of interacting TM helices. Other characteristic motifs contain polar residues which contribute hydrogen bonds or charged residues which make static interactions.\textsuperscript{53,54}

A word of caution: the existence of one such short interaction short motifs in a TM sequence does not necessarily imply a significant interaction. For instance, about one third of Gx\textsuperscript{3}G motifs in a non-redundant database of membrane protein structures are not interacting with another helix (Duneau JP, unpublished results). And, it has to be stressed that residues adjacent to a sequence motif also contribute to the interactions.\textsuperscript{45-48}

3. Associations between helical TM domains are dynamic by nature. Different types of motion of TM α helices have been described: lateral translation, piston, rotation parallel to the membrane (pivot or tilt) and rotation perpendicular to the membrane.\textsuperscript{15,12} The dynamic character of these interactions is of prime importance for cell signaling, which absolutely requires the capacity to be regulated and reversibility.\textsuperscript{17}

### Single-Spanning Membrane Proteins: A Very Large “Family” with Very Diverse Functions

In 1992, Bormann and Engelman concluded their review entitled, “Intramembrane helix-helix association in oligomerization and transmembrane signaling”\textsuperscript{49} with this prediction: “An oligomerization/conformational change model would predict that new sites of close contact would occur between the domains of the receptor molecule, some of which may be between the transmembrane helices. Therefore, experimenters should be able to generate peptides or small molecules that can specifically interfere with either the oligomerization or generation of new close-contact sites involved in the conformational change of the receptor that leads to signaling. In this way, specific receptors might be targeted for inhibition or possibly activation using binding events inside the bilayer.”

This somewhat prophetic view has been slow to materialize, but recent years have seen much progress in our knowledge of structural as well as functional aspects of helix-helix interactions in membranes. As several excellent reviews have recently been published on many facets of these interactions (see above), this review will focus on a sub-class of membrane proteins, the single-spanning or bitopic proteins. Strikingly, this is the most abundant membrane protein class, representing more than half of all membrane protein in analyzed genomes.\textsuperscript{2,50,51} Comparative genomics have allowed for structural/functional classifications of membrane proteomes. Unsurprisingly, it was verified that eukaryotes have a higher proportion of proteins for communication since multicellular organisms require a strict control of cell-interactions.\textsuperscript{52} Figure 1 shows the comparative distribution of bitopic proteins from human and E. coli genomes.

Remarkably, proteins associated with signaling (receptors and ligands), cell structure and adhesion, represent about 50% of the total in the human genome. It should be stressed that such estimations have to be considered with care because of the difficulty of predicting precisely TM segments vs. hydrophobic signal sequences. Nevertheless, it can be noted that many of these bitopic proteins do participate in the regulation of cell adhesion, cell migration and cell proliferation and differentiation. A short list includes receptor tyrosine kinases (RTKs), many of their ligand precursors (e.g., EGF family), immunoglobulin superfamily receptors, integrins, plexins, syndecans, neuropilins, cadherins and so on.

The presence in that list of adhesion proteins and receptors is quite intriguing since they have very tight functional connections
To date more than 300 publications dealing with helical TM domain interactions of membrane proteins can be found, not counting studies of de novo designed sequences. Some extensive lists can be found in recent reviews. In Table 1, we present a selective list of such TM peptides associations limited to those bitopic proteins playing a role in cell adhesion and migration, and more loosely in cell signaling. In this table we give some indications on the techniques used, with reporter assay representing the Toxcat method and its derivatives, and cell assay meaning all biochemical and functional techniques used in intact cells. Molecular dynamics simulations and the numerous physicochemical methods used in many cases are not mentioned, as much more detail can be found in the review by Bordag and Keller. TM sequence data with motifs are to be found in Rath et al. Some features of this summary table can be underlined. First, many examples come from the RTK family. This is not surprising in view of the great importance of this family of receptors in the control of cell proliferation and differentiation and its roles in pathologies such as cancer or skeletal disorders. Also, one of the first characterized disease-causing mutations in a TM segment was found in Neu/erbB2, which was initially identified as an oncogene by NIH/3T3 transfection analysis of cDNA from ethynitrosourea-induced rat neuroblastomas. This also led to the delineation of an interaction motif resembling that of glycoporphin

**Experimental Evidence for TM Domain Importance**

Early evidence for the importance of helix-helix interactions in integral membrane proteins assembly and oligomerization came in the late 80s from the first crystal structures (bacteriorhodopsin and photosynthetic reaction centers) and mutational analysis of polytopic and bitopic proteins. Highly specific interactions between TM helices had already been demonstrated, the most prominent examples being glycoporphin A and M13 phage coat protein, both forming SDS resistant dimers, as well as phospholamban which forms pentamers. Glycoporphin A was the first protein for which a specific role of TM helices interactions was evidenced, and became the paradigm for these intramembrane interactions. Many more examples of such interactions have since been described, although a comprehensive understanding of structure-function relationships has yet to be achieved.

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Some features of this summary table can be underlined. First, many examples come from the RTK family. This is not surprising in view of the great importance of this family of receptors in the control of cell proliferation and differentiation and its roles in pathologies such as cancer or skeletal disorders. Also, one of the first characterized disease-causing mutations in a TM segment was found in Neu/erbB2, which was initially identified as an oncogene by NIH/3T3 transfection analysis of cDNA from ethynitrosourea-induced rat neuroblastomas. This also led to the delineation of an interaction motif resembling that of glycoporphin.
Table 2 presents disease-associated mutations in the TM domains of bitopic proteins. Again, RTKs are over-represented. The most salient feature is that most mutations represent the replacement of an hydrophobic residue by a polar one. It has been shown that in TM domains, mutations involving polar residues, and ionizable residues in particular (notably arginine), are more often associated with protein malfunction than in soluble proteins.

To summarize this brief overview, a large body of recent and less recent experimental evidences concur to demonstrate that intramembrane interhelical interactions are definitely more widespread than previously considered for bitopic proteins. The functional effects of mutations in the TM domains of these proteins in disease confirm a pivotal role for these interactions. The next section will survey the current structural understanding of inter-helix interactions in bitopic proteins.

Structural Data: NMR and Modelling

Despite their obvious importance, the effectiveness of tools to study the structure of integral membrane proteins lags far behind that of water-soluble proteins. This is due to several interlinked reasons: the difficulties to solubilize and purify these hydrophobic proteins, the disordered nature of their lipid environment and the complex nature of many membrane proteins which are oligomers of one or several polypeptides.

One major consequence of these technical hurdles is the under-representation of membrane protein structures in the PDB database. At the end of 2008 and 2009, only 217 unique structures of membrane proteins were available (http://blanco.biomol.uci.edu/Membrane_Proteins_xtal.html). Although progress is rapid, this to be compared with about 20,000 known structures.
The first one was Glycophorin A, followed later by a few TM homodimers from RTKs (erbB2, EphA1 and EphA2), sub-unit ζ of the TCR, Bcl2 (an apoptosis regulator acting on mitochondrial membranes) and only one heterodimer of integrin subunits. These structures are depicted in Figures 3 and 4, and a summary of the experimental conditions is given in Table 3.

A few facts are striking about the structures depicted in Figure 3, besides their very small number and the fact that all but one were determined in the past four years. The dimers are generally right-handed, with the exception of the TCR ζ-ζ (zeta-zeta) dimer and the EphA2 receptor. Both of these structures, however, are atypical as far as can be told based on such a limited sample. The ζζ TM dimer is disulfide-bridged at its N-terminal end, and its other dimer interactions are mostly polar, with the exception of a Leu9-Leu9' self-contact (which is not obvious based on the published models). The EphA2 receptor structure is unique within this limited set, as it features the classic GX3G dimerization for soluble proteins. Also for many membrane proteins, only the structure of soluble sub-domains is known, which leads to incomplete understanding of structure-function relationships in these usually complex proteins. The situation is somewhat worse for bitopic membrane proteins, for which less than ten structures are available, be they monomeric or dimeric. This neglect is certainly due to several factors. The usual difficulties to express, purify and obtain suitable environment for crystallization or NMR are possibly worsened for bitopic proteins which can be less stable. Also, the prevalent belief until recently that these single helices are mere membrane anchors made them unworthy targets for structural studies.

### Structural data

For bitopic proteins, the very few available structures were obtained by NMR. Some are monomeric structures, such as M13 major coat protein or sarcolipin (www.drorlist.com/nmr/MPNMR.html). Only seven structures of dimers of TM helices, to date, have been deposited in the PDB. The first one was Glycophorin A, followed later by a few TM homodimers from RTKs (erbB2, EphA1 and EphA2), sub-unit ζ of the TCR, Bcl2 (an apoptosis regulator acting on mitochondrial membranes) and only one heterodimer of integrin subunits. These structures are depicted in Figures 3 and 4, and a summary of the experimental conditions is given in Table 3.

There are a few facts about the structures depicted in Figure 3 that are noteworthy. All the dimers are generally right-handed, except for the TCR ζ-ζ (zeta-zeta) dimer and the EphA2 receptor. The ζζ TM dimer is disulfide-bridged at its N-terminal end, and its other dimer interactions are mostly polar, with the exception of a Leu9-Leu9' self-contact. The EphA2 receptor structure is unique within this limited set, as it features the classic GX3G dimerization for soluble proteins.

### Table 1

| Protein (UniProtKB ID) | Function | Method(s) | Reference(s) |
|------------------------|----------|-----------|--------------|
| **I. Receptors**       |          |           |              |
| All RTKs (n = 58)      | RTK, receptor tyrosine kinase | Reporter assay | 108          |
| EGFR family (erbB 1-4) | RTK      | Reporter assay | 74, 75, 109  |
| (P00533, P04626, P21860, Q15303) | | FRET | 79, 110      |
| FGFR 1-4, fibroblast growth factor receptors | RTK | Cell and reporter assays, FRET | 114–121 |
| (P11362, P21802, P22607, P22455) | | Cell assay | 122          |
| VEGFR-2 (P35968)       | RTK      | Cell assay | 122          |
| PDGFR (P16234, P09619) | RTK      | Cell assay | 59–62, 123   |
| RET (P07949)           | RTK      | Cell assay | 124          |
| Erythropoietin receptor (P19235) | Cytokine receptor | Cell assay | 125–129 |
| PRL-R, prolactin receptor (P16471) | Cytokine receptor | Cell assay | 130          |
| EphA1, Ephrin type-A receptor 1 (P21709) | RTK | FRET | 131 |
| Insulin & IGF-1 receptors (P06213, P08069) | RTK | Cell assay, FRET | 132–134 |
| Neurilpin 1 (P97333)   | Co-receptor for semaphorins and VEGF | Cell and reporter assays, FRET | 103, 136 |
| major histocompatibility complex class II | Immune response | Cell and reporter assays, FRET, Flow cytometry | 137–139 |
| (alpha and beta chains) | Immune response | Cell assay | 140          |
| **II. Adhesion molecules** | Cell-Adhesion receptors | Cell and reporter assays | 93, 141–146 |
| Syndecans (P18827, P34741, O75056, P31431) | Cell surface adhesion co-receptors | Reporter assay | 147, 148 |
| Myelin protein P0 (P25189) | Myelin adhesion | Reporter assay | 149 |
| Cadherins (P12830, O15943) | Calcium-dependent cell adhesion molecules | Cell and reporter assays | 150, 151 |
| Synaptobrevin-2 (P63027) | Targeting and fusion of transport vesicles | Cell and reporter assays | 152, 153 |
| **III. Others**        | Unknown  | Cell and reporter assays, FRET, etc., | 40, 67, 154–157 |
| Glycophorin-A (P02724) | Apoptosis inducer | Reporter assay | 72, 158 |
| APP, amyloid precursor protein (P05067) | Synapse formation regulator | Cell assay | 159–162 |

From left to right, columns give the name of protein with its ID in the UniProt Protein knowledgebase (www.uniprot.org/), function of the protein, methods used and references.
Table 2. Selected examples of disease-associated mutations in the TM domains of bitopic proteins

| Protein (OMIM ID) | Function | Disease(s) | Mutation(s) | Reference(s) |
|-------------------|----------|------------|-------------|--------------|
| **I. Receptors**  |          |            |             |              |
| erbB 2 (*164870)  | RTK      | Neuroblastoma (rat) Breast cancer polymorphism | V664E | 111, 163 164 |
| FGFR family       | RTK      | Dysplasias: Osteoglophonic dysplasia | C379R, etc., | 165 |
| FGFR1 (*136350)   | RTK      | Beare-Stevenson Cutis gyrata syndrome | Y375C | 166 |
| FGFR2 (*176943)   | RTK      | Achondroplasia, Crouzon syndrome with Acanthosis nigricans | G380R, A391E, etc., | 167–169 |
| FGFR3 (*134934)   | RTK      | Cancers | G388R | 170 |
| FGFR4 (*134935)   | Receptor for interleukin-2 | Visceral leishmaniasis | G245R | 171 |
| **II. Adhesion molecules** |          |            |             |              |
| Myelin Protein P0 (*159440) | Major structural protein of peripheral myelin | Charcot-Marie-Tooth disease, Dejerine-Sottas syndrome | I162M, G163R, G167R | 172–174 |
| TACI (*604907)    | Tumor necrosis factor receptor | Immunodeficiency | A181E | 175 |

From left to right, columns give the name of protein with its ID in the OMIM (Online Mendelian Inheritance in Man) database (www.ncbi.nlm.nih.gov/omim/), function of the protein, nature of the associated disease(s), mutation(s) and references.

Figure 3. Views of TM helix dimer structures from NMR. For each dimer, two views are presented, one showing the crossing angle and the second, rotated by 90 degrees around the (pseudo)-symmetry axis, showing the structure of the interface. Structures are: (A) Glycophorin A (1afo), (B) ζ-ζ dimer of T cell receptor (2 hac), (C) Receptor tyrosine kinase EphA1 (2k1k), (D) Integrin alphaIIb-beta3 TM complex (2k9i), (E) Receptor tyrosine kinase EphA2 (2k9y), (F) BNip3 TM domain dimer (in mitochondrial outer membrane) (2ka1), (G) TM domain of growth factor receptor ErbB2 (2jwa). Properties fo the structures are summarized in Table 1. Residues belonging to dimerization motifs or participating in dimer contacts are outlined in space-filling or stick representation and colored by amino acid type: Gly (yellow), Ala (brown), Val (tan), Leu (green), Ile (green), Glu/Asp (purple), Ser (orange), Thr (mauve), Pro (gray), Tyr (blue-gray), Cys (lime). Molecular graphics rendered with VMD.
significant sources of variation remain: the location of the dimer motif in the TM sequence, which results in the helices being pinned together at various heights in the membrane, and the deviation of the TM secondary structure from ideal $\alpha$-helices. When extracting the latter information from NMR-based models, the possible presence of arbitrary restraints on secondary structure should be kept in mind. In recent structures, such restraints would include terms in the empirical potential used to model motifs facing away from the dimer interface. Instead, dimerization is mediated by a heptad repeat motif, and the authors suggest that switching between dimerization modes involving either motif could have functional significance, as has been proposed for other families of RTKs.74-76

Common features do emerge among the right-handed dimer structures. All are mediated by GpA-like small-residue-containing dimerization motifs spanning two or three helical turns. Two

---

**Table 3. Summary of experimental conditions and geometry of TM helix dimer structures represented in Figure 3**

| Structure | Experimental conditions | pH | Dimer motif | Contact pairs (bold for polar contacts) | Angle |
|-----------|-------------------------|----|-------------|----------------------------------------|-------|
| GpA (1afo) | 5% DPC micelles | 6.0 | G79X3G | I76-G80, G83-V84 | R -40° |
| $\zeta$- $\zeta$ (2hac) | DPC/SDS 5:1 micelles | 7.0 | none | C2 (disulfide), D6, L9, Y22, T27 | L |
| EphA1 (2k1k, 2k1l) | DMPC/DHPC 1:4 | 4.3 | A550X3GX3G, A560X3G | E154, A1550, V551, G554-L555, G558-A559 | R -44° |
| EphA2 (2k9y) | DMPC/DHPC 1:4 | 5.0 | G70X3G (outward-facing) | L151, I154-G159 | L 20° |
| BNip3 (2j5d) | DMPC/DHPC 1:4 | 5.0 | A550X3GX3G | S172-H173, G180, G180-I181 | R -45° |
| BNip3 (2ka1, 2ka2) | DPC/DPPC | 5.1 | A550X3GX3G | S172-H173, G180, G180-I181 | R -34° |
| ErbB2 (2jwa) | DMPC/DHPC bicelles | 5.0 | T70X5S0X3G | S656, G660 | R -42° |
for simulation-based refinement; predicted secondary structures have been shown to vary depending on the choice of potential.77 The fewer the NMR restraints, the more model refinement must rely on molecular interaction potentials. Taking this trend to its limit, purely physics-based approaches for ab initio prediction of TM dimer structures seem increasingly usable.

Another informative representation of the structure of helix dimers is provided by the projection of the helix surface in a dimer, as made with Ptuba (Fig. 4).78 Ptuba was developed to simplify the visualization of the surfaces of 3D helices as pseudo 2D projections that can be color coded to represent various aspects of their properties. Briefly, this software “unfolds” the 3D structure of an helix and draws it as a duplicated surface. In Figure 4, the unfolded surfaces are colored according to the distance between atoms of the two helixes in NMR structure. In the lower part of the figure, residues are indicated in one letter code and colored according to averaged distances between helices for Ca and all atoms of each residue in the dimer. Panel A depicts the surface of the glycophorin A helix where the interacting residues involve a relatively wide strip of surface with numerous closed contacts distributed along the interacting patch. The central panel (Fig. 4B) shows the surface of one ErbB2 helix, which in spite of a very similar geometry, shows a smaller interacting surface and less marked proximities than glycophorin A. This can be related to experimental data (FRET) showing that this homodimerization is weaker.79,80 Finally, in the TCR ζ-ζ TM domains the interface extends all along one helix face but involves only a handful of very local, short-distance contacts (Fig. 4C).

From sequences to structures. Molecular modeling and computer simulations are increasingly used for simulations of lateral association and oligomerization of TM helices. Predictions of TM interaction can rely on two distinct approaches (reviewed in Punta et al. 2007).41 Homology modeling and ab initio or de novo design. The first approach mainly concerns cases where 3D templates are available, and obviously this is not the case for the association of single-spanning TM proteins. However, the glycophorin A TM NMR structure has been used as a modeling template each time a GAS motif was thought to be involved in interactions. However, all GAS motifs do not necessarily lead to unique packing geometry.31,38

The other method used to predict structural organization of membrane proteins relies on de novo (knowledge based) or ab initio (thermodynamics) approaches. The principle may be formulated very simply: one has just to find pairwise interactions between TM domains. The early success of the primary algorithms are nowadays always present in strategies that concern all membrane proteins. They comprise three main tasks: first, the prediction of TM domain stretches from sequence; second, the prediction of the orientation of the TM segment relatively to the membrane and the remainder of the protein and third, the prediction of contact maps between the distinct helices. Early approaches for the prediction of TM stretches relied on propensity scales to describe the partition of the protein sequence in membrane.82 They quickly attained relatively good precision results. Today, best “modern” algorithms are able to reach 80% in accuracy at the topology level for membrane proteins. This high precision was thought to be definitively attained only by Hidden Markov Models (TMHMM)83 or Neural Networks approach (PHDhtm).84 More recently a first principle method based on the partition energetics of the translocon machinery was developed (TopPred85),86 which performs equally as the previous algorithms. An additional improvement of the predictor using HMM (SCAMPI) rises further the performances for correct topology prediction.85

This kind of de novo algorithm is of prime interest for single spanning TM domains which are more conserved than for polytopic proteins,34 and for which the usefulness of statistics coming from polytopic membrane proteins remains to be assessed. Also, importantly for bitopic proteins, efforts have been made to discriminate signal peptides which can be mistaken from authentic TM segments and thus lead to errors in topology.2,86 Prediction of membrane protein structure needs also a correct estimation of the orientation of helix faces relative to the membrane protein interior and exterior. Early developments involved the calculation of hydrophobic moment with physically based approaches. However the hydrophobicity difference is by far less pronounced in membrane proteins than in soluble ones. Such an approach was not proved as very useful even for multiple spanning membrane proteins.87

In recent years progress was made towards the prediction of contact maps between TM segments by including residue conservation (LIPS)88 and co-evolution.89 The accuracy for lipid exposed surface prediction reached 88% with LIPS. Contact map predictors were implemented using Neural Networks89 and Support Vector Machine (Tmhit).90 Their accuracy was argued to be between 31–57%. To our knowledge, those recently developed methods have not been applied to the prediction of associations of single spanning TM proteins. Probably, algorithms could be adapted to that situation; however, applying those methods to single-spanning TM domains is not obvious since divergence in sequences could not be sufficient to detect such correlated mutations. The fact that TM domains of bitopic membrane proteins are more conserved than others precludes the practical use of such methods. In fact, for a long time, prediction of interactions between single transmembrane domain have been based on de novo approach using sophisticated molecular mechanics methods.91 The ability of such modeling to achieve good predictions has been demonstrated92 and verified experimentally (CHAMP).93 The complexity of such protocols currently precludes genome wide analysis of potential single TM associations.

In conclusion, biases in structural databases toward polytopic membrane proteins and difficulties of interpretation of evolutionary data for bitopic proteins limits the usefulness of ab initio approaches. In addition, the de novo strategy suffers from inherent complexity and, overall, a deficit of available experimental validation, particularly at the structural level. So more work needs to be done to measure structural and environmental constraints that specifically apply on single TM assemblies. NMR and other experimental data, including biological assays, mutational scanning and biophysical measurements should feed strategies to
develop new kinds of prediction algorithms optimized towards this class of proteins.

**Perspectives and Questions**

Together with other reviews in this focus issue, this overview of TM-TM interactions in bitopic proteins signaling demonstrates their functional importance. Nevertheless, it is clear that many questions remain to be answered, and much technical progress is needed before we get a comprehensive knowledge of all the structural and dynamic aspects of interactions (homologous and heterologous) between intramembrane helices. This understanding is necessary to describe protein-mediated information transfer across the membrane during cell signaling.

It must be stressed that the common view that bitopic membrane receptors activation or inactivation is due to ligand-activated homodimerization (or heterodimerization) is certainly oversimplistic. Very briefly, the case of the EGFR/ErbB family receptor illustrates the limits of the “divide and conquer” strategy for multi-domains membrane proteins. Structures of nearly all the parts of ErbB receptors have now been solved through crystallography or NMR, namely extracellular, transmembrane, juxta-membrane and kinase domains. Nevertheless, it has become clear that the sum of these parts does not fully account for receptor properties, including their allosteric regulation. Moreover, the existence of large oligomers or aggregates of ErbB receptors at the cell surface has been widely documented. Elucidation of the role of TM domains interactions in the assembly of such large ErbB structures, and in the transfer of conformational changes through the lipid bilayer, certainly represent major challenges.

An interesting point about the interaction TM motifs evoked in the introduction is that they usually lie on a well defined “face” of the helix. Other motifs can exist on other sides, opening the possibility for higher order interactions with other TM domains. In this way, interactions between TM helices could serve as “adapter domains” participating in the assembly of dynamic and evolving multimeric complexes with new functions.

Short hydrophobic peptide mimics of TM segments have already proven useful as tools to decipher the role of interactions between TM helices, notably in cultured cell models (see Table I). But could such peptides be considered as drug templates? In general, contrary to early disdain towards the use of peptides or short proteins, much emphasis is now put on the interest of biologicals, in general, and peptides in particular. Furthermore, inhibition of protein-protein interactions is beginning to hold its promises. A spectacular example is the 36-amino-acid peptide enfuvirtide (Fuzeon®, Roche), which targets the HIV-1 envelope protein and inhibits CD4 receptor binding thereby preventing HIV-1 entry into the host T cell.

Finally, very few examples of TM peptide activity in animal models are found in the literature. One very recent example is the demonstration by some of us that a synthetic peptide mimicking the transmembrane domain of Neuregulin-1 (NRP1) blocks the biological functions of this Plexin and VEGFR coreceptor. The sequence of this TM domain contains two adjacent GxxG motifs, and various biochemical and functional assays have revealed the remarkable specificity of the strategy. In particular, FRET analysis showed the lack of hetero-interactions between wild type and mutant version of the peptide (3 glycines of the motif replaced by 3 valines). At the cellular level, this peptide blocked Semaphorin-3A induced differentiation of PC12 cells while not affecting NGF-induced PC12 differentiation, thereby demonstrating a selective inhibition of NRP1-dependent pathways. These interesting properties pushed us to examine how this peptide could be used as a novel therapeutic agent in a pathological context. To this end, we have explored the migratory and proliferative capacity of brain tumor cells in its presence. This choice was motivated by the major role of NRP1 in brain tumor progression and tumor associated angiogenesis, a key step for cancer progression. Strikingly, we found that the peptide blocked VEGF-induced endothelial and tumor cell migration and proliferation in vitro. Moreover, our data demonstrate in orthotopic and heterotopic graft models of brain tumors that the growth of rat and human glioma is strongly reduced (up to 80%) in the presence of the peptide. Thus, this preclinical study suggests that targeting TM domain interactions possibly represents a clear alternative to current protein inhibitors.

Indeed, much progress has recently been made in developing techniques that will help designing molecules targeting protein TM domains. These include truncating native TM regions such as the core peptide, directed evolution with the E5 protein and computational design (CHAMP) with integrins. It is also interesting to note that some derivatives of TM peptides, containing D-amino acids or modified with hydrophobic moieties, are also active. These examples all demonstrate that it is possible to design small peptides or peptidomimetics that specifically modulate much larger target membrane proteins by acting within cell membranes. While several questions related to the stability, biodistribution or toxicity of such TM peptides have yet to be addressed, these developments represent the first steps towards a new generation of peptide drugs.

In conclusion, we certainly have only seen the tip of the iceberg. More studies using a wide variety of methods, from single TM helices to integrated views in biochemical (e.g., lipid vesicles) and cellular contexts are required to describe TM-TM interactions, together with contribution of the increasing power and pertinence of computational methods. A better understanding of structure-function relationships of these interactions is necessary to apprehend such fundamental biological processes as membrane biogenesis, membrane protein folding and assembly in the plane of the membrane, as well as their contribution to the “vertical” information transfer across the membrane.

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