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# 1

# Structure-Functional Insight Into Transmembrane Helix Dimerization

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## 1. Introduction

Membrane proteins, constituting ~30% of proteins encoded by whole genomes (Krogh et al., 2001), are heavily implicated in all fundamental cellular processes and, therefore, represent up to 60% of targets for all currently marketed drugs (Overington et al., 2006). Nevertheless, in spite of their significance, only few tens of spatial structures of membrane proteins have been obtained so far, while design of new types of drugs targeting membrane proteins requires precise structural information about this class of objects. Hydrophobic α-helices represent a dominant structural motif found in membrane-spanning domains of proteins, excluding membrane β-barrels. So, a membrane part a large variety of membrane proteins is formed by α-helical bundle (polytopic proteins) or just by single α-helix (bitopic proteins) (Fig. 1). Besides structural switching, oligomerization of helical membrane proteins forms the basis for various functions in the living cell including reception of extracellular signals, signal transduction, ion transfer, catalysis, energy conversion and so on (Ubarretxena-Belandia & Engelman, 2001). The mechanisms, by which helical membrane proteins fold into native structures and functionally oligomerize, are beginning to be understood from a confluence of structural and biochemical studies. Folding determinants of a membrane protein can be partially understood by dissecting its structure into pairs of interacting transmembrane (TM) helices, which, together with the connecting loops and extramembrane domains, comprise the overall structure. Obviously, the fold of helical membrane proteins along with their biological activity is largely determined by proper interactions of membrane-embedded helices. Either destroying or enhancing such helix-helix interactions can result in many diseases (developmental, oncogenic, neurodegenerative, immune, cardiovascular, and so on) related to dysfunction of different tissues in the human body.

Activity regulation of bitopic proteins that have only single-spanning TM domain is mostly associated with their lateral dimerization in cell membranes. Bitopic proteins are a broad class of biologically significant membrane proteins including the majority of receptor protein kinases, immune receptors and apoptotic proteins, which are involved in development regulation and homeostasis of multicellular organisms. Homo- and heterodimerization of bitopic proteins was earlier thought to involve mostly their extracellular and cytoplasmic domains, but recent studies have been making it increasingly

clear that the single-spanning TM domains are also critical for their dimerization and modulation of biological function. Upon bitopic protein activation, ligand-dependent or not, significant intramolecular conformational transitions result in rearrangement of the receptor domains and following receptor dimerization or switching from one dimerization state to another, e.g. ligand-dependent transition from preformed inactive dimeric state into active dimer of ErbB receptor tyrosine kinase (Schlessinger, 2000; Moriki et al., 2001; Fleishman et al., 2002; Mendrola et al., 2002). The so-called "rotation-coupled" and "flexible rotation" activation mechanisms (Moriki et al., 2001; Fleishman et al., 2002; Mendrola et al., 2002), which were initially proposed for receptor tyrosine kinases and imply active involvement of TM domains in dimerization and activation of the receptors via proper TM helix-helix packing and rearranging, are possibly widespread among bitopic proteins. However, if biological functions are carried out using only one homo- or heterodimeric state of bitopic protein TM domains, the TM helix-helix interaction can be strong, as in the case of permeabilization of the outer mitochondrial membrane by proapoptotic protein BNip3 in the course of hypoxia-acidosis induced cell death. Furthermore, amino acid polymorphisms and mutations in the TM domain of bitopic proteins have been implicated in numerous human pathological states, including many types of cancers, Alzheimer's disease, tissue dysplasias and abnormalities (Li & Hristova, 2006; Selkoe, 2001). It was shown that the mutations affect both the behavior of the isolated TM domains in model lipid bilayers, and the behavior of the full length receptors in the plasma membrane. Most probably, the effects are exerted via yet unknown mutation-induced changes in dimeric structure of the TM domains. Importantly, it was found that isolated TM domains revealed ability not only to homo- and heterodimerize in membrane-like environment, but also to specifically inhibit biological activity of bitopic proteins in cell membrane (Li & Hristova, 2006; Bennasroune et al., 2004; Rath et al., 2007). So, membrane-spanning segments of bitopic proteins represent a novel class of pharmacologically important targets, whose activity can be modulated by natural or specially designed molecules. Among the most perspective candidates for these purposes are artificial hydrophobic helical peptides, the so-called peptide "interceptors" (Bennasroune et al., 2004) or "computer helical antimembrane proteins" (CHAMPs) (Caputo et al., 2008), which are capable of specifically recognizing the target wild-type TM segments of bitopic proteins and interfering with their lateral association in cell membrane. Therefore, understanding the factors that drive packing of α-helices in membranes has attracted considerable interest of researchers from both scientific and medical communities. Nevertheless, in spite of their significance, only few spatial structures of the homo- and heterodimeric single-span TM domains have been obtained so far, notwithstanding that design of new types of drugs targeting bitopic proteins requires precise structural information about this class of objects.

At the present stage of development of the structural biology methods, obtaining high-resolution structure of a full-length bitopic protein is a scientific challenge. Issues with crystallization of membrane proteins are inherent to X-ray techniques, whereas NMR cannot effectively handle large protein-lipid complexes. The crystallographic methods, which recently allowed obtaining high-resolution structure of such multi-span TM receptors as G-protein coupled receptors (Cherezov et al., 2007), cannot be directly translated to multiple-domains flexible receptors like receptor kinases and immune system receptors. Therefore,

the structural-dynamic properties of the extracellular, cytoplasmic and intramembrane parts of such bitopic proteins are still studied separately. Extensive structural studies of extracellular and cytoplasmic domains in different functional states of the bitopic proteins are closely followed by detailed analysis of their TM domain dimerization in membrane environment. Apparently, thorough understanding of all the aspects of TM helix-helix interactions in bitopic proteins can only be achieved with multi-disciplinary approach based on a comprehensive set of modeling, biochemical and biophysical tools. The already available information about structural-dynamic properties of the dimeric TM domains of bitopic proteins along with the biophysical and biochemical data provides useful insights into the protein functioning in the human organism on the atomistic scale. This review will discuss the applicable methods, from purely theoretical approaches to direct experimental techniques, which recently allowed describing high-resolution dimeric TM domain structure for several bitopic proteins and understanding some aspects of structure-function relations and their biological activity.

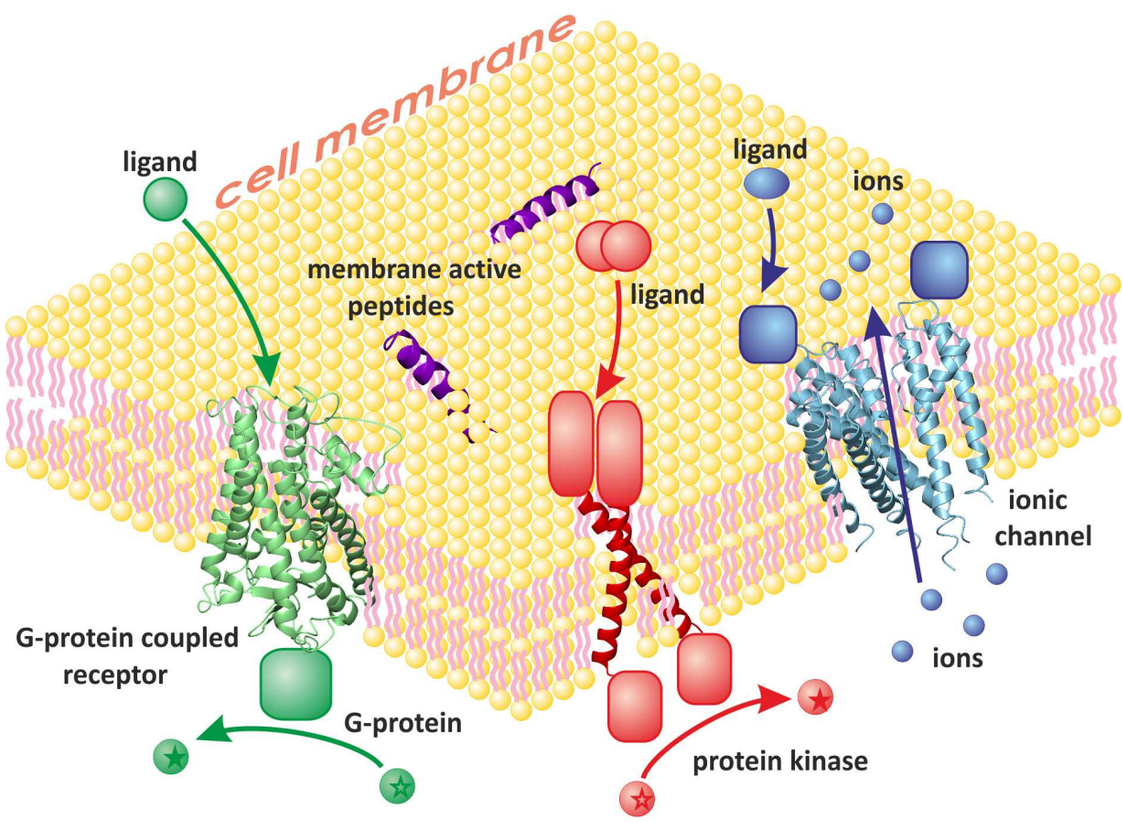


Fig. 1. Representatives of bitopic and polytopic helical TM proteins.

## 2. Thermodynamical aspects of helix-helix interaction in membrane

The balance of forces driving association of proteins in lipid membranes, in particular the helix-helix interaction of transmembrane domains, differs fundamentally from the case of protein interaction in aqueous solutions to the such degree that in some cases might seem counterintuitive. For the sake of clarity, folding of an α-helical membrane protein can be

conceptualized as a process that occurs in two thermodynamically distinct steps, involving the formation of independently stable TM helices and the subsequent specific TM helix-helix interactions giving rise to higher-order structures (Popot & Engelman, 1990), in which TM helices are usually more or less tilted with respect to the membrane plane. The former step, similarly to the case of water soluble proteins, is controlled by hydrophobic interactions, profile of which changes essentially once the helices are inserted into the lipid bilayer and the hydrophobic side chains can be exposed to hydrophobic environment without energy penalties. Lipid-protein interactions are also most likely involved, though indirectly, in driving the association of TM helices in the form of the entropy term (Helms, 2002; Schneider, 2004). Although the formation of higher ordered helix oligomers decreases the entropy of the proteins, the entropy of the lipids is greatly increased. Every TM helix is surrounded by a "coat" of lipids more or less tightly associated with it. After interaction of individual TM helices, a part of these "frozen" or anyhow correlated lipids (Morrow et al., 1985) is released into the membrane lipid pool. Therefore, TM helix oligomerization would decrease the area of protein-lipid interface and thereby increase the overall entropy of the system, thus contributing to stabilization of the protein-protein complex. In addition, adjustments of local lipid composition of the membrane and matching the hydrophobic thickness of lipid bilayer with the hydrophobic length of TM proteins can regulate lipid-protein and protein-protein interactions, e.g. resulting in cooperative lipid-mediated protein-protein lateral association into signaling platforms in biomembranes (Lee, 2004; Nyholm et al., 2007; Sparr et al., 2005; Marsh, 2008; Vidal & McIntosh, 2005; de Meyer et al., 2008).

Specific helix-helix interactions require precise mutual orientation of TM helices, imposing certain restrictions on their tilt angle and tilt direction between dimer axis and normal to the membrane, therefore proper hydrophobic matching may influence the specific TM domain association. Depending on the specifics of the protein, this would result in sorting different biologically relevant states of dimeric bitopic proteins between lipid phases and microdomains of cell membrane or in shifting the occupancies of the resultant conformation of the pair of TM helices and proteins depending on the surrounding lipid phase or microdomain (Nyholm et al., 2007; Sparr et al., 2005). Even when helices do not exhibit any tendency for specific association (Lee, 2004; Nyholm et al., 2007), helix-helix association could still occur as a result of poor packing between the lipids and helices, or of a favorable change in entropy due to the release of helix-bound lipids upon helix association. In these cases, helix association is primarily driven by lipid-protein interactions rather than strongly favorable protein-protein interactions. However, while entropy considerations and hydrophobic matching or mismatching could partly explain the formation of higher ordered TM structures in the membrane, it cannot serve the sole explanation of the specificity of TM helix interactions.

Protein-lipid interaction is not the only noncovalent force involved in the formation of TM helix oligomers, van-der-Waals forces and polar interactions also play important roles (Senes et al., 2004; Curran & Engelman, 2003). Association of TM helices often proceeds through a "ridge-into-groove" or a "knob-into-hole" packing (Langosch & Heringa, 1998; Walther et al., 1996). The ridges or knobs on the surface of one TM helix fit well into grooves or holes on the complementary helical surface. Such a complementarity of contacting adjacent TM ensures most favorable polar and van-der-Waals interactions. Electrostatic interactions also cannot be excluded from consideration despite relative rarity of occurrence of charged residues in the TM segments and play a specific role in membrane protein

folding (Zhou et al., 2000, Zhou et al., 2001; Choma et al., 2000; Adamian et al., 2003; Gratkowski et al., 2001), since the strength of such interactions increases with a decreasing dielectric constant of the environment. Electrostatic interactions stabilize folded membrane structures via polar backbone-backbone, backbone-side chain, or side chain-side chain interactions resulting in hydrogen bond formation between adjacent TM helices. Contribution of amino acid residues into interaction energy in the hydrophobic environment is a function of their polarity. Weakly polar amino acids, like glycine, alanine, serine, and threonine are characterized by a relatively small electrostatic component of the interaction energy and a complex nature of interaction. In addition to forming electrostatic interactions, these polar residues with small side chains also allow two TM helices to come into close contact and to tightly pack without significant entropy loss of side chain rotamers upon dimer formation (MacKenzie et al., 1997). This does not only facilitate the interhelical hydrogen bonding with participation of polar side chains of serine or threonine, but also enables van-der-Waals interactions between surrounding residues. In addition to polar side chains, the CαH groups of such tightly packed residues are capable of participating in non-canonical hydrogen bonding, e.g. with the opposite carbonyl groups across the helix-helix interface (Senes et al., 2001). In other words, the marginal polarity of the Cα proton might be sufficient to serve as a hydrogen bond donor in a highly hydrophobic environment. However, although the slightly polar residues could form hydrogen bonds with an adjacent TM helix, they are able to contribute significantly to the specific helix-helix interactions only consisting in an amino acid context, which promotes association of TM helices, e.g. by proper packing (Gratkowski et al., 2001; Dawson et al., 2002; Schneider & Engelman, 2004; Arbely & Arkin, 2004; Mottamal & Lazaridis, 2005).

Presence of highly polar residues, like histidine, asparagine, aspartic acid, glutamine, glutamic acid, arginine or lysine in the membrane environment can apparently drive noncovalent association of TM helices through more specific strong hydrogen bonding and salt bridge formation, resulting in very stable helix oligomers. These residues are rarely found in membrane proteins (Arkin & Brunger, 1998), but it has been shown that the presence of a single asparagine, aspartic acid, glutamine, or glutamic acid in a TM helix is sufficient to drive stable oligomerization (Gratkowski et al., 2001; Zhou et al., 2000; Zhou et al., 2001). While highly polar residues can contribute significantly to the stability of the helix-helix interaction, several problems arise when these residues are present in a membrane. Transfer of highly polar residues into a membrane is thermodynamically unfavorable, and only very few of these residues can be tolerated in a single TM helix. Furthermore, in membrane environment, the ionizable side chains of these residues prefer uncharged state and their pKa values can vary substantially depending on numerous parameters, such as local hydrogen bond network, membrane composition, transmembrane potential, and juxtamembrane environment (Smith et al., 1996; Bocharov et al., 2008a). Since highly polar residues could interact with any potential binding partner for hydrogen bonding or salt-bridge formation, which create the danger of non-specific helix-helix association and misfolding (Schneider, 2004), the polar substitutions are apparently the most common pathogenic mutations in membrane proteins that cause different human diseases (Li & Hristova, 2006; Moore et al., 2008). On the other hand, for the polar residues located at the level of the lipid headgroups where solubility of charged groups is higher than in the hydrophobic core but the electrostatic shielding is accordingly more effective, the individual interactions are not so formidable and can be modulated by external ligands (Lau et al.,

2009). In addition, arginine and lysine residues are frequently found at the ends of TM helices, where they have a tendency to participate in direct or water-mediated polar–polar interactions with phospholipid headgroups (Arkin & Brunger, 1998; Wallin et al., 1997; Adamian et al., 2005) and can modulate the helix-helix dimerization strength (Peng et al., 2009).

A separate important class of participants of specific TM helix association processes are π-π and cation-π aromatic interactions arising either between two aromatic residues or between a basic and an aromatic residue, respectively (Johnson et al., 2007; Unterreitmeier et al., 2007; Sal-Man et al., 2007). Interactions of aromatic rings of tryptophan, phenylalanine, tyrosine, and histidine residues and their self-association or interaction with protonated cation side chains of arginine, lysine, and histidine residues have been proposed to consist of van-der-Waals and electrostatic forces complemented by correct packing geometry and interactions with the aromatic ring quadrupole moment. Besides, the indole, phenol, and imidazole group of the aromatic residues can participate in hydrogen bonding across TM helix packing interface. Even though weak, CαH-π interactions enhanced in the low dielectric membrane environment can be considered as additional interactions supporting specific TM helix association (Unterreitmeier et al., 2007). In addition, aromatic residues have a strong propensity to face phospholipids in the headgroup region and are thought to act as anchors for a membrane protein, influencing on helix tilting and hydrophobic matching in the membrane (Adamian et al., 2005). Cation-π interactions occurring at the headgroup levels are often contributed or mediated by additional interaction with water molecules.

## 3. Common motifs employed for helix-helix interaction in membrane

The helical configuration of TM segments imposes certain limitations and regularities on the amino acid sequences that are suitable for forming intermolecular contacts. The TM helix-helix association modes can be roughly grouped on the basis of sequence patterning and interhelical geometry. Since N- and C-termini of α-helical TM domains of bitope proteins are usually exposed to extracellular and cytoplasmic sides of membrane respectively, such proteins specifically associate into homo- and heterodimers in a parallel manner, in the so-called ''head-to-head'' orientation. Both right- and left-handed variants of parallel helix-helix dimers with most frequently occurring helix-helix crossing angles near -40° and 20°, respectively, and the distance of 7-9 Å between helix axes appear to be quite common for TM helix packing in membrane (Walters & DeGrado, 2006). The interfaces of TM helices crossing at negative angles are often formed by [abcd]n tetrad repeats, in which a and b correspond to interfacial residues (Langosch et al., 2002). Right-handed packing of helix pairs is most often characterized by an i, i+4 separation of "small" residues, such as glycine, alanine, serine and threonine, along the TM sequence, which is alternately termed "small-xxx-small" or GG4-like motif first exemplified by self-assembling TM domain of glycophorin A (MacKenzie et al., 1997). Small residues in this motif create a shallow weakly polar groove that complements the surface of an adjacent helix and allows the helices to approach closely. The association is stabilized by van-der-Waals contacts resulting from the excellent geometric fit and weak polar interactions, which can contribute to non-canonical hydrogen bonding between CαH and carbonyl groups across helix-helix interface (Senes et al., 2001). Two GG4-like motifs in tandem form the so-called "glycine zipper" motif, which is statistically overrepresented in membrane proteins (Kim et al., 2005). The geometry of left-handed pairs of TM helices characterized by positive crossing angles requires longer [abcdefg]n heptad minimal repeat motifs, where e and g positions are

located at the periphery of these helix–helix interfaces and side-chains at a and d positions interdigitate repeatedly (Langosch & Heringa, 1998). Such a heptad pattern was originally identified in water soluble "leucine zipper" interaction domains and gives rise to "knobs-into-holes" packing of side-chains (Lupas, 1996). The left-handed TM helix pairings are mostly stabilized along heptad repeats by van-der-Waals contacts of large side chains of valine, leucine and isoleucine residues, while slightly polar interactions of interfacial residues having small side chains, like glycine, alanine, and serine, are also important for left-handed oligomerization (Lear et al., 2004; Ruan et al., 2004; North et al., 2006). In addition, the TM helix-helix dimerization via both tetrad and heptad repeat motifs can be enhanced by π-π, cation-π and CαH-π interactions across helix packing interface with participation of aromatic side chains (Johnson et al., 2007; Unterreitmeier et al., 2007). Furthermore, interhelical hydrogen bonding with participation of polar residues can work in concert with other helix packing interactions to strongly stabilize both right- and left-handed motifs, which appear to be essential for proper alignment of the polar side chains required for formation of hydrogen bonds (Moore et al., 2008).

TM helix interactions are mostly driven and stabilized by a broad spectrum of forces caused by protein-protein interactions via such motifs as well as interactions of the helices with the membrane environment. The precise interplay of all these forces is unique for each system and warrants individual detailed analysis since it often defines the functionality of interacting membrane proteins. Currently, many unique sequence motifs that are responsible for specific helix-helix association have been identified on the basis of tetrad and heptad repeats, which play primarily a permissive role for close helix-helix interactions (for a review see refs. Moore et al., 2008; Walters & DeGrado, 2006; Langosch & Arkin, 2009; Mackenzie, 2006). The relative importance of the sequence motifs in stabilizing helix-helix interactions depends on the specific combination of residues and location of the interacting surfaces relative to the N- and C-termini of α-helical TM segments (Johnson, 2006). Besides, the affinity of TM helix association can be modulated by flanking and non-interfacial residues (Zhang & Lazaridis, 2009).

One or a few potential dimerization motifs can be usually identified in each TM region of bitopic proteins that participate in two broad categories of helix-helix interactions (Moore et al., 2008). In the first of them, the TM domains form relatively static contacts that might be necessary e.g. for the assembly of a functional protein complex or for proper folding and export from endoplasmic reticulum. In other cases, the TM domains can undergo dynamic conformational changes between alternative dimerization modes important e.g. for signaling process that can involve a change in association state and/or lateral, vertical, and rotational motions in the membrane. Such triggering interactions cannot play a thermodynamically dominant role in overall protein conformational transitions, but are quite capable of fine-tuning the system energetics, leveraging TM coupling and restricting the pool of the allowable conformations of the full length bitope proteins in the course of their biological activity.

## 4. Predicting spatial structure of dimeric transmembrane helices by molecular modeling

Molecular modeling is a reasonably quick and efficient tool for quantitative assessment of the possible modes of helix association in membranes, especially when direct structural

methods fail to provide the necessary insights or are prohibitively resource-consuming. Moreover, relative simplicity and stability of homo- and heterodimers of TM domains of bitopic proteins facilitates development and application of computational techniques for assessing the helix-helix interactions in membranes. Though only a few experimental spatial structures of TM helical dimers are available so far, molecular modeling offers quite reasonable atomic-scale models of dimeric structures.

Adequate molecular modeling of TM protein-protein interactions is impossible without a proper representation of the membrane. Three generic techniques have been developed for representing the membrane environment for the purpose of membrane protein simulations. The simplest option is to model the effect of heterogeneous membrane environment implicitly by means of some potential of mean force. This is commonly achieved by adding special terms to the potential energy function of a protein in the framework of so-called implicit or "hydrophobic slab" membrane models (Efremov et al., 2004; Feig & Brooks, 2004). Though this kind of representation can not provide atomistic details of protein-membrane interactions, it adequately mimics the basic membrane properties, such as membrane transversal hydrophobicity, thickness, curvature, and transmembrane voltage. These approaches are quite computationally effective and allow fast sampling of the protein configurational space and reasonably guessing the key trends of protein behavior in membrane (spatial structure in the membrane-bound state, geometry of binding, etc.). The second group of modeling techniques employs explicit membrane representation. The simulations are carried out for full-atom hydrated lipid bilayers or detergent micelles with imposed periodic boundary conditions (Forrest & Sansom, 2000). This class of models is capable of providing the most reliable dimeric structures of TM peptides. Unfortunately, due to large size of the systems (up to 106 particles), such calculations are very time- and resource-consuming. Finally, the third class of membrane models, so-called "coarse-grain" (CG) models, is a reasonable trade-off between the simplicity of the former and accuracy of the latter approach (Sansom et al., 2008). In CG-models, standard groups of atoms are replaced with "grains", thus reducing considerably the number of degrees of freedom in the protein-membrane systems.

The approaches commonly employed for such studies can be subdivided into three major categories: molecular docking, Monte Carlo and molecular dynamics simulations. A group of docking techniques is intended for fast identification of homo- and heterodimeric states of bitopic protein TM domains based on their amino acid sequence (Casciari et al., 2006). Usually, one of the TM monomers is considered as a target, and the other as a ligand, the conformational lability being limited for one or both of the monomers defined with the parameters of the backbone and side chains typical for α-helical TM segments. The membrane is either ignored or modeled implicitly. This method allows quick scanning for spatially complementary surfaces with optimally matched geometrical, hydrophobic/hydrophilic, and electrostatic properties of the interacting TM helices, and thus predicts potential dimerization interfaces and intermonomer hydrogen bonds. However, due to restrictions imposed on the TM helix mobilities and due to many physical factors of protein-protein and protein-lipid interactions being ignored, docking methods are typically used only for initial characterization of the specific helix-helix packing, to be subsequently supplemented by other methods.

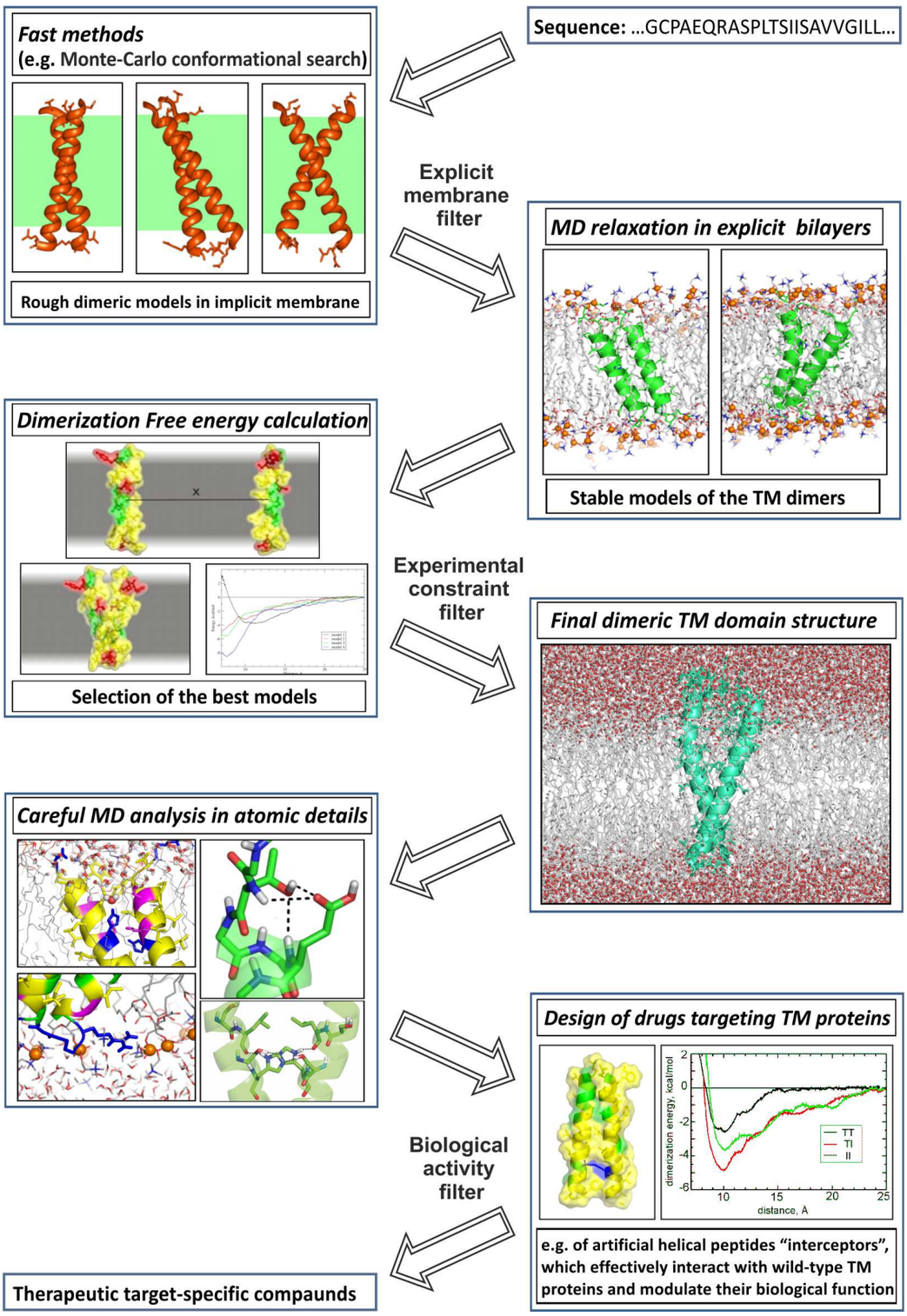


Fig. 2. Scheme of the spatial structure elucidation of homo- and heterodimeric TM domains of bitopic proteins and the subsequent molecular design of drugs targeting TM proteins with the aid of computer simulations techniques.

In Monte Carlo conformational search, both monomers are flexible permitting more careful scanning of the conformational space and thus potentially yielding more credible calculated structures. Clearly, these approaches allow the membrane to be more accurately taken into account, using either implicit or explicit representation. With implicit membrane representation, more extensive scanning of conformational space becomes feasible due to its lower computational cost, and therefore the chance of missing a realistic helix-helix configuration decreases greatly. On the other hand, the predicted dimeric structures can be graded more accurately with explicit membrane models. For acceleration of the Monte Carlo conformational search it was often assumed a priori that the TM helices adopt a proper TM orientation and their backbones were considered "rigid", and hence, common occurrence of local distortions in TM helices, like kinks and bends, was not taken into account. Under such assumptions, the effects of membrane environment on the secondary structure formation and/or stabilization, along with the events accompanying insertion of the peptides, also can not be assessed. However, Monte Carlo protocols without imposing any restraints on the secondary structure and a priori knowledge of the mode of membrane binding for the peptides were recently developed (Efremov et al., 2006; Vereshaga et al, 2005). Often, Monte Carlo algorithms operate in dihedral angles space, thus reducing dimensionality of the computational task. Usually, Monte Carlo simulations help in delineation of a limited number of low-energy conformational states of TM helical dimers (Vereshaga et al, 2005). Subsequent analysis of these families of conformers results in very few "native-like" structures, thus facilitating selection of the final models.

Molecular dynamics (MD) is one of the most informative methods, since besides providing the spatial structure it allows estimation of dynamic parameters of interaction, identification of the most important residues, etc. Membrane models of any degree of complexity can be used in MD calculations. It comes at a price of great computational intensity, therefore selection of the starting state becomes a real issue due to limited capabilities for scanning conformational space, making it virtually impossible to obtain correct structure starting from an essentially wrong one. This problem is especially significant in case of calculations in the explicit bilayer. One of the ways to resolve it is based on generating a set of initial states with different geometries of the dimer packing. Though providing most detailed scanning of the conformational space, this method is often impractical due to unacceptable computational resource requirements, and is essentially limited to implicit membrane calculations. For explicit membranes, the starting structures can be obtained as a result of Monte Carlo search in an implicit membrane (or docking) with subsequent relaxation in the explicit bilayers. An alternative approach consists in preliminary investigation of the dimerization by the CG representation. In this case, the molecules are represented by "grains" (e.g. each of which roughly corresponds to 4 heavy atoms) that substantially improves the calculation time, so the intervals of up to ~1 microsecond can be investigated. As was shown Psachoulia et al., 2009, this time scale is sufficient for obtaining a realistic model of the TM dimer, which after MD relaxation in the full atomic representation correlates well with the NMR structure.

There is a number of examples of extensive application of computer modeling methods for investigation of specific TM dimerization of several bitopic proteins, including the wild type and mutated TM domains of glycophorin A (Lemmon et al., 1992), bacteriophage M13 major coat protein (Melnyk et al., 2002), proapoptotic protein BNip3 (Sulistijo et al., 2003),

erythropoietin receptor (Constantinescu et al., 2001), amyloid precursor protein APP (Scheuermann et al., 2001), and ErbB receptor tyrosine kinases (Schlessinger, 2000). Most of the methods of molecular modeling of the TM helix specific dimerization have been developed and successfully tested on the TM domain of glycophorin A protein, homodimeric conformation of which was first obtained with high resolution (MacKenzie et al., 1997). Although a number of successful in silico predictions of TM helix-helix complexes have been reported, the uncertainty of the energy estimate of the final state, which is a measure of certainty of selection of the correct conformation, is still relatively high. Therefore, without employment of additional data it is usually very difficult to choose between several alternative models with close energies, having substantially different geometries. Moreover, if several dimerization modes are actually realized for a protein, computational methods provide little or no information about population and relative stability of the possible modes of helix-helix associates, which can be affected by modeling assumptions in silico as well as by variations of membrane environment and ligand binding in vivo. Partially, such a hypothesis is corroborated by somewhat vague results of mutagenesis studies (Lemmon et al., 1992), as well as by NMR (Gratkowski et al., 2002) and MD (Im et al., 2003; Petrache et al., 2000) data that demonstrate the importance of media effects for stability of helical oligomers and provide examples of their multi-state equilibrium in lipid bilayers and membrane mimics. In real biological membranes, the situation may be more complex due to inhomogeneous composition of lipid bilayers, their domain structure, variations of physico-chemical characteristics, presence of small molecules (e.g., cholesterol), etc.

Some conformations obtained by modeling are artificially introduced by computational assumptions, and they cannot be readily discriminated from those really occurring in cellular membrane without additional experimental information, in particular about the TM dimerization interface, see Fig. 2. Such information can be obtained by solid state NMR, site-specific infrared dichroism, mutagenesis in combination with the techniques permitting assessment of dimerization degree (SDS electrophoresis, bioassays in ToxR systems, FRET), Cys scanning (insertion of cysteine residues and analysis of the extent of disulphide bridges formations), and so on (for a review see refs. Rath et al., 2007; MacKenzie, 2006; Li et al., 2009; Schneider et al., 2007). Experimental limitation can be either imposed at the stage of calculations, e.g. in the form of limitations on the distances between atoms in different monomers, or used for assessing appropriateness of the predicted structures after completion of calculations. Such a combination of experimental and modeling techniques provides important advantages, substantially narrowing the search of dimeric TM structures and simplifying membrane representation and hence significantly accelerating the analysis. Compared to direct structural methods that usually identify only one conformation, this approach gives better credit for a conformational diversity of homo- and heterodimeric TM domain structures, which can occur in vivo during biological activity of a bitopic proteins.

Effectiveness of such a combination of computational methods with various biophysical and biochemical techniques was proved by its successful applications in a number of studies several of which are presented below. Selection of the proper dimeric structure of glycophorin A TM helix in Adams et al., 1996, was done based on mutagenesis data superposed on the set of structures obtained by global conformational search in vacuum.

The proposed model of the dimer was in good agreement with the spatial structure obtained by means of NMR spectroscopy in detergent micelles (MacKenzie et al., 1997). This method was later used for analysis of the glycophorin A TM domain dimerization in lipid bilayers, where the conformational search was done with the distance restraints from solid-state NMR spectroscopy (Smith et al., 2001; Smith et al., 2002a). Beevers et al., 2006, obtained spatial structure of the TM domain of the oncogenic mutant of rat receptor tyrosine kinase Neu by MD calculations in the explicit bilayer with different possible orientations of the monomers. Correctness of the resulting 'consensus' structures was assessed based on the information about orientation of the CO groups determined by site-specific infrared dichroism. Vereshaga et al., 2007, calculated the spatial structure of TM segment dimer of human proapoptotic protein Bnip3. In this case, Monte Carlo conformational search in an implicit membrane with subsequent MD relaxation of the best models in the full-atom DMPC bilayer was used for identification of the potential structures. Dynamically unstable models were screened out at the stage of MD relaxation. Correctness of the remaining models was assessed via comparison with the mutagenesis data. As a result, one of the final models consistent with the mutagenesis data was also in good agreement with the NMR-derived structure of dimeric Bnip3 TM domain in lipid bicelles (Bocharov et al., 2007). Volynsky et al., 2010, used modeling methods in combination with ToxR assays to study dimerization of TM segments of ephrin receptor EphA1. A set of spatial structures of the dimer proposed based on Monte Carlo simulations in implicit membrane followed by MD relaxation in explicit lipid bilayer were employed for rational design of wild-type and mutant genetic constructions for ToxR assays. Such a combined, self-consistent, application of modeling and experimental techniques allowed defining the factors important for dimerization of the TM segment of the EphA1 receptor, providing unambiguous spatial model consistent with the NMR-derived structure (Bocharov et al., 2008a) of the EphA1 TM dimer in lipid bicelles. Moreover, alternative conformations of the dimer were proposed. Metcalf et al., 2009, reported the models of integrin aIIbβ3 TM heterodimers obtained using a Monte Carlo algorithm that selects conformations by a geometrical filter based on mutagenesis data. The Monte Carlo search for integrin aIIbβ3 TM heterodimers was also carried out with an additional energy term using distance restraints obtained from cysteine-scanning mutagenesis bioassay data (Zhu et al., 2009). In both cases the proposed heterodimeric structures were in good agreement with recently obtained NMR structure of heterodimeric integrin $a_{IIb}\beta3$ TM complex embedded in lipid bicelles (Lau et al., 2009).

## 5. Determination of high-resolution structure of dimeric transmembrane helices by NMR spectroscopy

Over a number of recent years, structural biology has witnessed a race of rapidly developing experimental methods matched closely by increase of complexity of the experimental objects. Nowadays, obtaining high resolution structure of entire membrane proteins or functionally essential fragments thereof has become a reality. Isolation, purification, and handling of membrane proteins in their ''native-like'' conformations are still associated with enormous difficulties and often require expanding the limits of the modern experimental techniques. Besides, tertiary and quaternary structures of membrane proteins are only moderately stabilized and transitions are often observed between

conformational substates. Multiple conformations and dynamics considerably complicate characterizing the structure of membrane proteins and their oligomers. For this reasons, despite recent increases in the number of high resolution structures of membrane proteins solved annually, the gap between soluble and membrane protein structures continues to increase. Even among the membrane proteins of known structure, specific oligomeric complexes of small membrane-spanning proteins such as TM domains of bitopic proteins are underrepresented.

Heteronuclear NMR spectroscopy proved an effective tool for investigating the systems of oligomeric α-helical TM domains of membrane proteins. Solid-state NMR has been successfully employed to obtain highly resolved spectra of membrane-bound peptides and proteins in lipid bilayer model systems, such as liposomes, which can have composition, thickness, surface tension and curvature similar to those of native lipid bilayers and thus adequately mimic cell membranes. Solid-state NMR techniques for membrane protein samples are rapidly evolving, and the structures of several small proteins in lipid bilayers have been already obtained with the aid of these methods (Opella & Marassi, 2004; Andronesi et al., 2005). There are two ways of obtaining high-resolution solid-state NMR spectra, either by performing magic angle spinning (MAS) in order to mimic the rapid tumbling that would naturally occur for a small molecule in solution for averaging the anisotropic interactions in solid-state, or by observing uniformly aligned molecules. Smith and co-workers have used $^{13}C$–$^{13}C$ rotational resonance and $^{13}C$–$^{15}N$ rotational echo double resonance MAS experiments to measure interhelical distances in the α-helical TM domain dimers of human glycophorin A (Smith et al., 2001; Smith et al., 2002a), human amyloid precursor protein (Sato et al., 2008) and rat receptor tyrosine kinase Neu (homologue of human ErbB2 receptor) with its constitutively activate Val664Glu mutant (Smith et al., 2002b). That allowed developing the structural models for the helix–helix packing interactions in lipid bilayer for these bitopic proteins. The tilt angle and rotational angle of TM helices can be estimated by analysis of the position, shape, and size of the so-called PISA wheels obtained from polarization inversion with spin exchange at the magic angle (PISEMA) experiment acquiring for oriented $^{15}N$-labeled membrane proteins (Opella & Marassi, 2004).

Solution NMR became a major method to determine structures of water-soluble proteins and their complexes (Wüthrich, 1986). In addition to elucidation of their structures, NMR also offers unique opportunities to probe dynamical processes in them. However, membrane proteins embedded into lipid bilayers cannot be studied by means of solution NMR techniques because their rotations in these environments are slow and highly anisotropic, which leads to unfavorable relaxation and very wide or undetectable resonance lines. An alternative approach to solving high-resolution spatial structures and obtaining dynamical information on membrane proteins is to extract the proteins from their host membranes and disperse them in non-denaturing membrane-mimicking detergent/lipid systems such as micelles, bicelles, and nanodiscs, which tumble fast enough to give well-resolved resonance lines when using solution NMR methods. Since resulting supramolecular membrane protein–detergent/lipid complexes are usually still large on the scale of protein structures that are routinely solved by NMR, the most advanced solution NMR techniques and spectrometers operating at high magnetic fields and equipped with highly sensitive cryoprobes are typically employed to solve high-resolution structure of the membrane proteins. These include labeling the proteins with two or three low-abundant

isotopes $^{2}H$, $^{13}C$ and $^{15}N$, deuterating of detergents and lipids at least on hydrophobic tails, using transverse relaxation-optimized spectroscopy (TROSY) (Pervushin et al., 1997), and obtaining structural restraints in addition to those typically obtained from nuclear Overhauser effects (NOE) and chemical shifts, such as restraints obtained from residual dipolar couplings (RDC) and paramagnetic relaxation enhancements (PRE), which can drastically improve both quality and throughput of membrane protein structure determination (for comprehensive review see ref. Kim et al., 2009). The accuracy of determining the protein structure is controlled by many factors, including the dynamical properties of the protein itself, as well as the nature and quantity of the experimentally obtained restraints. In case of dimeric TM α-helical proteins, if a well defined structure of monomers is known (particularly the side chain conformations and helix bending), just a few restrains can fully determine the structure provided that they are, in a broad sense, independent enough. However, since every restraint has an experimental error associated with the precision of measurements and with the accuracy of assignment in case of NOE contacts, having larger number of independently derived consistent restraints greatly increases confidence in the structure of individual TM helices and of the dimer as a whole. In case of underdetermined structures where there are substantial ambiguities in the NMR-derived structural information with only few reliable restraints defining global dimer structure, molecular modeling can allow making a choice in favor of the most physically justifiable model of the dimer. Obviously, this process directly depends on the accuracy of the underlying physical assumptions, i.e. the force fields used in the modeling of the membrane proteins. Given the limited amount of structures obtained in the membrane-mimicking environments, each new experimental structure is of utmost practical and methodological importance.

The smallest among membrane mimicking particles – micelles, which are formed of soft detergents, short-chain lipids or lysolipids, are optimal from the standpoint of NMR relaxation, allowing recording spectra with narrow lines and rather good chemical shift dispersion (Gautier et al., 2008; Krueger-Koplin et al., 2004) A lot of membrane-penetrating peptides, membrane associated peptides and fragments of membrane proteins were studied in micellar solutions by NMR spectroscopy (Kim et al., 2009; Krueger-Koplin et al., 2004; Sanders & Sönnichsen, 2006). Most of the structures of helical membrane proteins resolved with NMR spectroscopy were determined in micelles of different types, indicating that there is no universal detergent, applicable for every membrane protein. Therefore, extensive detergent screening is usually made to find a proper environment (Krueger-Koplin et al., 2004; Page et al., 2006; Maslennikov et al., 2007). Although majority of the membrane proteins maintain native-like structures in micelles and some retain activity, sometimes the detergent providing the best appearance of NMR spectra does not provide proper folding, and the protein dissolved in it remains inactive. Micelles have some disadvantages associated with high curvature of their spherical surfaces. Curvature effects are occasionally observed with small peptides, and the absence of specific phospholipids or mixtures of phospholipids may cause amphiphillic peptides interacting with the membrane surface to have distorted structures in micelles environment (Lindberg, 2003, Chou, 2002). Integral membrane proteins, especially those having structural element in the lipid headgroup region, can also have distorted structure and poor spectrum appearance in micellar solutions. Both the headgroup region and the hydrocarbon core in a highly curved micelle are packed less orderly and exhibit greater dynamics than in a planar or near-planar lipid

bilayer (Lindberg, 2003, Chou, 2002). The shielding effect of the interfacial headgroup region is less pronounced, and water molecules can penetrate more easily into the micellar core, resulting in distortions of TM helix structure (Bordag & Keller, 2010). Importantly, addition of a very modest amounts of phospholipids to micelles can result in dramatic enhancements of NMR spectral quality for some integral membrane proteins (Sulistijo & Mackenzie, 2010). This lipid dependence appears to reflect the requirement of some membrane proteins for specific or semi-specific lipid-protein interactions, which cannot be satisfied by detergents only. So, detergent micelles with some amounts of phospholipids offer a viable compromise for investigating TM peptides in membrane-mimetic systems, combining ease of use and good dissolving properties with anisotropic environment. Nevertheless, many detergents exert a denaturating effect on membrane proteins and peptides by abrogating helix-helix interactions (Melnyk et al., 2001; Therien & Deber, 2002). These problems could be overcome by using membrane mimicking particles with elements of flat surface, such as bicelles and nanodiscs.

Nanodiscs are similar to high-density lipoprotein particles and consist of fairly large patches of planar lipid bilayers (~160 lipid molecules) surrounded by the rim formed by apolipoprotein A-I (Borch & Hamann, 2009; Nath et al., 2007; Ritchie et al., 2007). The particles have the diameter of about 12 nm and thickness of 4 nm with the overall rotational correlation time of about 80 ns (Lyukmanova et a., 2008), which is rather high for structural NMR studies, but with TROSY (Pervushin et al., 1997) and CRINEPT (Riek et al., 1999) techniques one can record a readable heteronuclear spectrum and compare it to the one recorded in micelles or bicelles. Nanodiscs have only been applied in NMR spectroscopy for a couple of years and but few membrane protein were studied in this environment so far. However, they proved useful for verifying that other membrane mimicking media provide proper tertiary structure of membrane proteins (Shenkarev et al., 2010); they also have high potential for various bioassay applications (Borch & Hamann, 2009).

A reasonable compromise between micelles and nanodiscs – small isotropic bicelles are binary mixed micelles, consisting of two types of molecules: long-chain lipids (with long hydrophobic tails) and short-chain lipids or detergents, e.g. dimyristoylphosphatidylcholine (DMPC) mixed with dihexanoylphosphatidylcholine (DHPC) or zwitterionic bile sole derivative CHAPSO (Kim et al., 2009). As such, they represent the most convenient environment with excellent bilayer-mimicking properties for NMR structural studies of small membrane protein and their complexes (Kim et al., 2009; Poget & Girvin, 2007). A number of bicelle systems have been developed and characterized for their unique liquid-crystal phase behavior. It was shown that bicelles at some conditions have discoidal shape with a bilayer formed by long-chain lipids and a rim of short-chain lipids (Vold et al., 1997; Lee et al., 2008; van Dam et al., 2004; Glover et al., 2001; Luchette et al., 2001). The shape of the particles is controlled by three parameters: the molar ratio q of long- and short-chain lipid (or detergent) concentrations (adjusted for concentrations of free lipids in the bicellar suspension), total lipid concentration $c_L$, and temperature T; and it can be either disc or perforated bilayer the dependence being rather complex (Vold et al., 1997). At q between 0.25 and 0.5 bicelles are tumbling fast, are almost isotropic and can be used for high-resolution structure determination (Kim et al., 2009; Prosser et al., 2006). The hydrophobic thickness of the aggregates can be controlled by the choice of long-chained lipids, and it was also shown that charged lipids, e.g. with either negative serine or glycerol headgroups, can be incorporated into such particles without loss of stability (Lind et al., 2008; Struppe et al.,

2000). A number of publications report smaller distorting effect of bicellar media on the structure of membrane proteins (Kim et al., 2009). Recent determination of the structure of the heterodimeric TM domain of the platelet integrin $a_{IIb}\beta_3$ in bicelles provides an elegant example of using this medium to solve an important structural biology problem that proved elusive when conventional micelles were used (Lau et al., 2008; Lau et al., 2009). Detergent micelles destabilize the heterodimer to the point where interaction cannot be detected, while the environment provided by bicelles allows at least partial retention of native-like heterodimer avidity. Typical size of the particles consisting of fast-tumbling bicelles (e.g. DMPC/DHPC bicelle of ~80 lipid molecules, q of 0.25, $c_L$ of 3%, at 40 °C) with two embedded bitope protein TM fragments (~40 residues including hydrophobic TM segment flanked by polar N- and C-terminal regions) is ~5 nm corresponding to overall rotational correlation time of ~18 ns and the effective molecular weight of ~50 kDa. Therefore, extensive capabilities of solution heteronuclear NMR technique can be readily employed for investigating structural-dynamic properties of membrane proteins (Bocharov et al., 2008a).

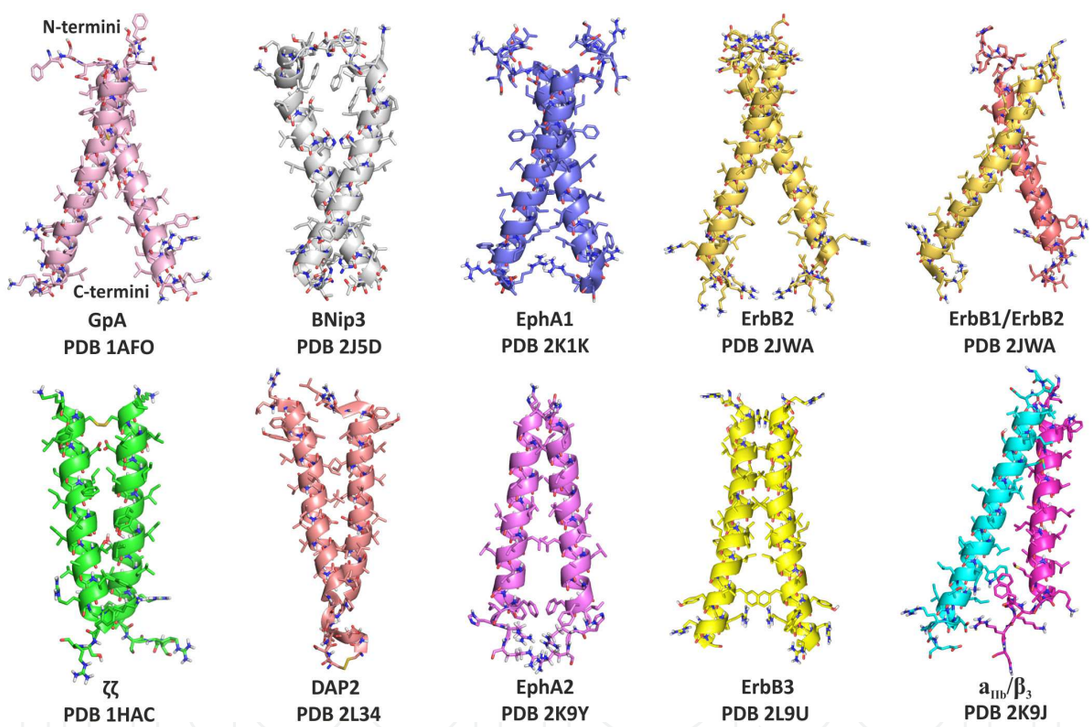


Fig. 3. High-resolution spatial structures of homo- and heterodimeric TM domains of bitopic protein obtained at present time.

A useful property of such systems is low effective ratio of detergent/lipid to protein and restricted protein mobility that can make homo- or heterodimerization effective enough even if specific interaction of TM helices are weak (e.g. in the case of receptor tyrosine kinase TM domains). Moreover, typical size of micelles and bicelles allows detecting intermolecular NOE contact network (up to ~6 Å) along TM helix-helix interface that is crucial for obtaining high-resolution structures of homo- and heterodimeric TM domains of bitopic protein. Nevertheless, one of the main problems encountered in structure determination of molecular complexes by NMR spectroscopy is to distinguish between intra- and intermolecular NOE

contacts. In case of self-association of bitopic protein TM domains, if the dimers of α-helical TM segments are symmetrical on the NMR time scale, their two monomer chains display similar chemical shifts so that inter- and intramonomeric NOE contacts are indistinguishable in the NMR spectra. Furthermore, small chemical shift dispersion inherent to α-helical structure as well as line broadening owing to large size of the supramolecular system and slow conformational exchange widespread in oligomeric complexes are additional unfavorable factors complicating unambiguous identification of intermonomeric NOE contacts also in the cases of TM heterodimers or asymmetric homodimers.

This symmetry degeneracy problem can be tackled analytically, with the aid of the so-called "ambiguous distance restraints" method (Nilges & O'Donoghue, 1998), according of which spatial structure of a symmetrical dimer is calculated in two stages, involving an initial stage the structure refinement of the monomer subunit before proceeding to the dimer. Experimentally identified NOE contacts are interpreted in a conservative manner and only those that are clearly inconsistent with the global fold of the monomer could be assigned as unambiguous intermonomeric NOE contacts. All other NOE contacts are treated as having arisen from either intra- or intermonomer cross-relaxation. Back in 1997 MacKenzie et al., successfully used this strategy in the pioneering work of determining high-resolution structure of homodimeric TM domain of glycophorin A (GpA), which was solubilized in DPC micellar media (PDB 1AFO) (Fig. 3). Glycophorin A, a surface protein marker of human erythrocytes, is widely used as a model protein in developing the experimental and theoretical methods to study the specific dimerization of TM domains of bitopic proteins. In detergent micelles, the membrane-spanning α-helices of glycophorin A self-associate in a parallel right-handed manner with crossing angle of -40° *via* tetrad repeat dimerization pattern $L^{75}IxxG^{79}VxxG^{83}VxxT^{87}$ including the so-called tandem GG4-like motif (also known as 'glycine zipper' (Kim et al., 2005)) composed of residues with small side chains allowing close approach of the helices. Along with numerous van-der-Waals interactions, four close polar CαH···O helix-helix contacts, which can be described as non-canonical hydrogen bonds across the dimer interface afforded by GG4-like motif, occur between $C\alpha H_1$ of Gly79 and Gly83 and opposite backbone carbonyls of Ile76 and Val80. The dimer structure also revealed the intramolecular hydrogen bonding of hydroxyl group of Thr87 with backbone carbonyl group of Gly79. As shown recently, the structure of the TM domain dimer of glycophorin A embedded into DMPC/DHPC lipid bicelles is similar (PDB 2KPF, Mineev et al., 2011a). Nevertheless, the formation of an intermonomeric hydrogen bond between side chain hydroxyl group of Thr87 and backbone carboxyl group of Val84 was proposed based on several dipolar interaction observed with solid state NMR using dry DMPC and POPC lipid bilayers (Smith et al., 2001; Smith et al., 2002a). The work of MacKenzie et al., 1997, was an important early accomplishment both for technical reasons and because of the insight that the glycophorin A TM domain structure provides into membrane protein folding and stability.

There is a more straightforward, experimental approach to circumventing the symmetry degeneracy problem through a direct search of intermolecular NOE contacts in dimer interface. For this purpose, an isotopic "heterodimer, consisting of $^{2}H$, $^{13}C$, $^{15}N$ isotope labeled and natural abundance monomers, is to be prepared for the NMR experiments to select NOE contacts between isotopically bound and nonisotopically bound protons. Besides the case of symmetrical homodimerization, such experiments are useful for directly obtaining interhelical spatial restraints for asymmetric TM dimers (or oligomers) as well for identifying close

intermolecular protein-lipid contacts. A simple method to distinguish intermonomer NOE contacts is to produce a $^{2}H/^{15}N$-isotopic "heterodimer", in which one subunit is $^{15}N$-labeled and fully deuterated (except NH groups) whereas the other subunit is unlabelled ($^{1}H/^{12}C/^{14}N$). This method allows directly obtaining interhelical proton-proton restrains from side chain and backbone groups of one subunit to backbone amide groups of the other. Such strategy was successfully used for determination of high-resolution NMR structure of a constitutively disulfide-linked TM domains of the T cell receptor ζζ-chain homodimer embedded into mixed 5:1 DPC/SDS micelles (PDB 2HAC, Call et al., 2006) (Fig. 3). In detergent micelles the TM ζζ-chain helices form a left-handed dimer with a crossing angle +23° *via* extended heptad repeat dimerization pattern $C^{2}xxL^{5}D^{6}xxL^{9}xxY^{12}xxxL^{16}T^{17}xxF^{20}xxV^{23}$ encompassing almost entire TM segment and making numerous interhelical side chain contacts, several of which are polar. It was shown that the side-chain hydroxyls of Tyr12 and Thr17 form a pair of interhelical hydrogen bonds that create "brackets" defining the lateral edges of the dimer interface. Structural and mutagenesis analysis revealed that two aspartic acid Asp6 situated near intersubunit Cys2-Cys2 bridge, which are required for receptor assembly, can form extensive hydrogen-bonding network with several hydrogen-bond donors and acceptors including at least one water molecule, the cysteine carbonyls, the carboxyl side chain and amide groups of aspartic acids themselves. So, the structure of the TM ζζ-chain dimer nicely demonstrated how multiple hydrogen bonding can establish a left-handed TM homodimer. A more recent study provided the structure of another, functionally homologous TM-signaling dimer, DAP12, both alone (PDB 2L34) (Fig. 3) and with a receptor TM domain, NKG2C (PDB 2L35), in an assembled trimeric complex using mixed-label ($^{15}N^{2}H+^{13}C^{1}H$)-isotopic "heterodimer" samples (Call et al., 2010). In detergent TDPC/SDS micelles the TM DAP12 helices, linked covalently through a native disulfide bond in extracellular stalk region, form a left-handed dimer with a crossing angle +18° *via* extended heptad repeat dimerization pattern $L^{9}xxI^{12}V^{13}xxD^{16}xxL^{19}T^{20}xxI^{23}xxxV^{27}$ making numerous interhelical side chain contacts, several of which are polar but without inter-helical hydrogen bonding. Assembled in immunoreceptor complex with the TM domain of type II, C-type lectin-like receptor NKG2C the DAP12 TM dimer formation of an extensive membrane-embedded electrostatic

The strategy of ILV-methyl-selective protonation (Tugarinov & Kay, 2005) was employed for high-resolution structure determination of the heterodimeric TM domain of intact $a_{IIb}\beta_3$ integrin in POPS/POPC/DHPC (q = 0.32) and deuterated DMPC/DHPC (q = 0.30) lipid bicelles (PDB 2K9J, Lau et al., 2009) (Fig. 3). The $^{1}H^{13}C_3$-Ile,Leu,Val;$^{2}H/^{13}C/^{15}N$-labeled and unlabelled 1:1 mixtures of the $a_{IIb}$ and $\beta_3$ integrin TM subunits were used for partial side-chain assignments and for identification of intermonomeric proton-proton NOE contacts between methyl groups of one subunits and any groups of the second subunit. Guided by packing interaction with three distinct glycine residues, the integrin TM helices cross at an angle of -25° and connect through tetrad repeat patterns $G^{972}xxxG^{976}xxL^{979}L^{980}xxxL^{984}$ and $V^{700}M^{701}xxI^{704}L^{705}xxG^{708}xxxL^{712}$ of $a_{IIb}$ and $\beta_3$, respectively, forming a TM heterodimer of unique structural complexity. The assembly enables strong electrostatic interactions (as detected by mutagenesis) between side chains of Arg995 and Asp723 of $a_{IIb}$ and $\beta_3$, respectively, within the relatively low dielectric environment of lipid headgroups. The reported heterodimeric TM structure along with structure-based side-directed mutagenesis of $a_{IIb}\beta_3$ integrin provides important insights into the structural basis for integrin signaling in cell membrane, revealing the structural events that underlie the transition from associated to dissociated states upon receptor activation (Lau et al., 2009).

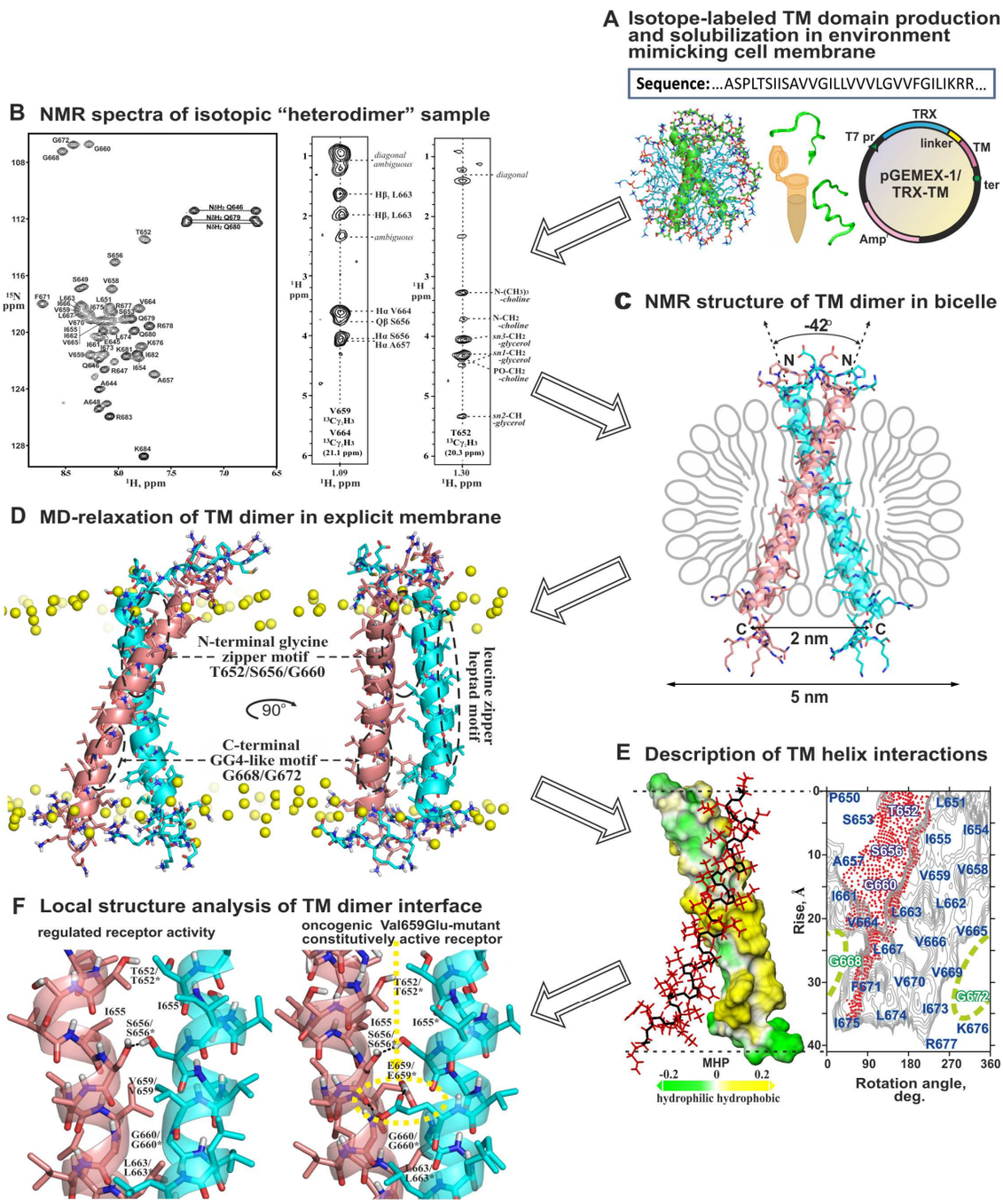


Fig. 4. Spatial structure elucidation of dimeric TM domains of bitopic proteins with the aid of heteronuclear NMR spectroscopy combined with MD-relaxation (exemplified by receptor tyrosine kinase ErbB2; Bocharov et al., 2008a).

Figure was adapted from Bocharov et al., 2010. (A) Production of the isotope-labeled TM fragments of the protein (e.g. chemical synthesis, bacterial or cell-free expression) and their subsequent solubilization in membrane mimicking environment (e.g. in detergent micelles or lipid bicelles). (B) Acquisition of NMR spectra of isotopic "heterodimer", consisting of

$^{13}C/^{15}N$-isotope labeled and natural abundance ErbB2 TM fragments (residues 641-685) embedded into DMPC/DHPC lipid bicelles. From *left* to *right*, $^{1}H$-$^{15}N$ HSQC spectrum with amide backbone resonance assignments, two representative 2D strips from the 3D $^{13}C$ F1-filtered/F3-edited-NOESY spectrum with intermolecular protein-protein and protein-lipid NOE contacts are presented. (C) Determination of high-resolution spatial structure of the right-handed ErbB2 TM homodimer in lipid bicelle using NMR-derived restraints. The obtained N-terminal association mode of the ErbB2 TM dimer via N-terminal dimerization motif corresponds to the receptor active state. (D) MD-relaxation of the ErbB2 TM homodimer in hydrated explicit DMPC lipid bilayer with imposed NMR-derived constraints. *Yellow* balls show phosphorus atoms of lipid heads. The spatial locations of the three characteristic dimerization motifs of ErbB2tm are marked by dashed ovals. (E) Analysis of interacting surfaces of the ErbB2 TM helices. In *left*, hydrophobic and hydrophilic (polar) surfaces of one TM helix in the homodimer colored in *yellow* and *green* according to the molecular hydrophobicity potential (MHP) (Efremov & Vergoten, 1995). The second monomer of the dimer is shown with *red* side chains. In *right*, hydrophobicity map for ErbB2 TM helix surface with contour isolines encircling hydrophobic regions with high values of MHP is presented with red-point area indicating the helix packing interface via N-terminal glycine zipper motif $T^{652}xxxS^{656}xxxG^{660}$. The residues composing C-terminal unemployed dimerization GG4-like motif $G^{668}xxxG^{672}$ are highlighted in green. (F) Local structure analysis of intra- and intermolecular interactions in the ErbB2 TM dimer. Comparison of intermonomeric hydrogen bonding (*black* dotted lines) in the TM helix-helix interface of ErbB2 and its constitutively active Val659Glu-mutant is presented.

A robust strategy to distinguish intermonomeric NOE contacts in protein dimers was based on producing a $^{13}C/^{15}N$-isotopic "heterodimer", in which one subunit is $^{13}C/^{15}N$-labeled and the other subunit is unlabelled. For the direct detection of the intermolecular NOE contacts in such isotopic "heterodimer", NMR pulse sequences were developed (Zwahlen et al., 1997; Stuart et al., 1999), employing so-called X-filtering elements to select NOE contacts arising between nonisotopically and isotopically bound protons. Due to fast transverse magnetic relaxation as consequence of relatively big overall correlation time of the studied supramolecular systems, the intermonomeric proton-proton contacts in the $^{13}C/^{15}N$-isotopic "heterodimer" are mainly detected from methyl groups (having smallest relaxation rates) to other groups. This approach was successfully applied in our lab for elucidation of structural-dynamic properties of homo- and heterodimeric α-helical TM domains of several biologically different human proteins, including proapoptotic protein BNip3 and representatives of receptor tyrosine kinase ErbB and Eph subfamilies. The high-resolution NMR structures of dimeric TM domains of these bitopic proteins were obtained using DMPC/DHPC (q = 0.25) bicelles consisting of lipids with deuterated hydrophobic tails and lipid/protein molar ratios of ~35. The resulting NMR structures of the TM domain dimers were subjected to energy relaxation using MD during several ns of MD trajectory in hydrated explicit lipid bilayers with the imposed NMR-derived constraints and then without constraints to study the conformational stability of the dimer in the membrane. The MD relaxation procedure provided a detailed atomistic picture of the intra- and intermolecular (protein-protein, protein-membrane and protein-water) interactions and allowed estimating the influence of amino acid substitution, including pathogenic TM mutations, on the structural-dynamic properties of bitopic proteins, see Fig. 4.

BNip3 is a prominent representative of apoptotic Bcl-2 proteins with unique properties initiating an atypical programmed cell death pathway (Chen et al., 1999). Investigation of spatial structure and internal dynamics of the homodimeric TM domain of human protein BNip3 (PDB 2J5D, Bocharov et al., 2007) revealed that in the lipid bicelles the central membrane-spanning α-helices of BNip3 cross at the angle of -45° and form a right-handed parallel symmetric dimer via tetrad repeat pattern $S^{172}H^{173}xxA^{176}xxxG^{180}xxxG^{184}$ (Fig. 3). In addition, labile *Phe*-ring hydrophobic cluster with numerous intermonomeric stacking interactions between six phenylalanine residues $(Phe^{157}/Phe^{161}/Phe^{165})_2$ was identified in the interface between short mobile N-terminal helices, flanking the central helices. According to the obtained NMR data supported by MD relaxation, a hydrophilic motif $(Ser^{172}/His^{173})_2$ in the centre of dimerization interface of BNip3 TM domain forms a water-accessible *His-Ser* node of inter- and intramonomeric hydrogen bonds decreasing apparent pK*a* of the imidazole group below 4. The C-terminal TM part of the BNip3tm dimer is stabilized by van-der-Waals side chain contacts and by weakly hydrophilic backbone contacts of the helices tightly self-associated through a glycine zipper motif, which appears to be essential for proper alignment of the side chains in the *His-Ser* node required for hydrogen bonding. In the DMPC/DHPC bicelles the *His-Ser* node undergoes slow conformational exchange with ~10% occupancy of the minor state probably associated with alternative hydrogen bonding and water permeability. Nevertheless, it was shown that an addition of long chain DPPC lipid to DPC micelles (lipid/detergent ratio of 1:50) with embedded dimeric BNiP3 TM domain allows to eliminate the conformational inhomogenity in the dimer interface (Sulistijo & Mackenzie, 2009). The revealed structural-dynamic properties of the BNip3 TM domain with a potentially switchable network of hydrogen bonds and water accessibility up to the middle of the membrane appear to enable the protein to form ion-conducting pathway across the membranes. Indeed, the TM domain was shown to induce conductivity of artificial bilayer lipid membrane in a pH-dependent manner (Bocharov et al., 2007). These findings and currently available information about phenomenology of programmed cell death allowed us to propose a mechanism of triggering necrosis-like cell death by BNip3 in case of hypoxia-acidosis of human tissues.

Receptor tyrosine kinases conducting biochemical signals across plasma membrane *via* lateral dimerization play an important role in normal and in pathological conditions of human organism by providing cell signaling, maintaining cellular homeostasis and controlling cell fate (Schlessinger, 2000). Eph receptors are found in a wide variety of cells in developing and mature tissues and represent the largest family of receptor tyrosine kinases regulating cell shape, movement, and attachment (Pasquale, 2005). Because all Eph receptors and their ligand ephrins are cell surface-associated proteins, a direct cell-cell contact is required for receptor activation resulting in cytoskeletal remodeling that underlies cell adhesion, repulsion and motility in both communicating cells. Although the Eph TM segments reveal relatively low amino acid sequence homology, several dimerization motifs, including at least one explicit GG4-like motif, can be identified in each Eph TM region. Structural-dynamic properties of the homodimeric TM domains of the EphA1 and EphA2 receptors were investigated with the aid of solution NMR in lipid bicelles and MD relaxation in explicit lipid bilayers of different composition. High-resolution spatial structures of homodimeric TM domains of EphA1 (PDB 2K1K and 2K1L, Bocharov et al., 2008a) and EphA2 (PDB 2K9Y, Bocharov et al., 2010a) embedded into DMPC/DHPC bicelles (q = 0.25) revealed a right- and left-handed parallel packing of the α-helical TM domains

with crossing angle of -45° and +15°, respectively (Fig. 3). The EphA1 TM segment self-associates through the N-terminal glycine zipper motif $A^{550}xxxG^{554}xxxG^{558}$ whereas the C-terminal GG4-like dimerization motif $A^{560}X_3G^{564}$ is not employed. And vice versa, the EphA2 TM helices interact through the extended heptad repeat motif $L^{535}xxxG^{539}xxA^{542}xxxV^{546}xxxL^{549}$ assisted by intermolecular stacking interactions of aromatic rings of $(FF^{557})_2$, whereas the N-terminal glycine zipper motif $A^{536}X_3G^{540}X_3G^{544}$ remains vacant. Thus, our studies of the Eph1 and EphA2 receptors demonstrated that the TM domains of different representatives of the same receptor tyrosine kinase family can use alternative dimerization motifs in the same bicellar system, the different motifs possibly being corresponding to active and inactive dimeric state of the receptor. Moreover, in the case of EphA1 TM domain, variations of external pH and lipid composition of the bicelles initiated triggering between the alternative motifs, which can be viewed as an argument in favor to the so-called "rotation-coupled" mechanism of the receptor tyrosine kinase activation (Moriki et al., 2001; Fleishman et al., 2002; Mendrola et al., 2002). The obtained results indicated also that alternative dimeric conformations of the TM domains can influence the receptor localization in plasma membrane microdomains and signaling platform, such as rafts and caveolae (Bocharov et al., 2010a).

Four human ErbB members of epidermal growth factor receptor family form numerous homo- and heterodimer combinations, recognizing different EGF-related ligands and performing diverse functions in a complex signaling network (Warren & Landgraf, 2006). All the species of the ErbB family are activated by proper ligand-induced dimerization or by reorientation of monomers in preformed receptor dimers after ligand binding that can be widespread among receptor tyrosine kinase family (Schlessinger, 2000; Tao & Maruyama, 2008). The TM segments of all four human ErbB receptors have at least one such motif, and all except ErbB3 have two of them, located in the N- and C-terminal parts of the TM helices. So, two possible dimeric conformations of the α-helical ErbB TM segments with interfaces located either at N- or C-terminus were proposed to associate different receptor active states (Moriki et al., 2001; Fleishman et al., 2002; Mendrola et al., 2002). According to high-resolution spatial structure of homodimeric ErbB2 TM domain embedded into DMPC/DHPC lipid bicelles (PDB 2JWA, Bocharov et al., 2008b), the α-helical TM segments of ErbB2 interact with right-handed crossing angle of -42° through the N-terminal glycine zipper motif $T^{652}xxxS^{656}xxxG^{660}$ (Fig. 4). Polar contact area of this motif is shielded from lipid tails by the side chains of leucine, isoleucine, and valine residues, while slightly polar concave surface of the C-terminal GG4-like motif $G^{668}xxxG^{672}$ is exposed to hydrophobic lipid environment. In the C-terminal part of the dimeric interface, aromatic rings of the opposite Phe671 residues participate in intermolecular edge-face stacking interaction. Constrained MD relaxation of the ErbB2tm dimer structure revealed that the $(Thr652/Ser656)_2$ hydrophilic motif in the N-terminal part of the dimerization interface forms a node of switching inter- and intramonomeric hydrogen bonds mediating the ErbB2 TM helix packing. Based on the NMR-derived structure it was also shown by molecular modeling that pro-oncogenic Val659Glu mutation leads to overstabilization of the described ErbB2 TM domain conformation which was ascribed to the active state of the tyrosine kinase. Spatial structure of the heterodimeric complex formed by TM domains of ErbB1 and ErbB2 receptors was also obtained using the bicellar environment, in which the domains associate in a right-handed α-helical bundle with crossing angle of -46° through their N-terminal double GG4-like motif $T^{648}G^{649}X_2G^{652}A^{653}$ and glycine zipper motif $T^{652}X_3S^{656}X_3G^{660}$,

respectively (PDB 2KS1, Mineev et al., 2010) (Fig. 3). The described heterodimer conformation is believed to support the juxtamembrane and kinase domain configuration corresponding to the receptor active state. The capability for multiple polar interactions along with hydrogen bonding between TM segments correlates with the observed highest affinity of the ErbB1/ErbB2 heterodimer, implying an important contribution of the TM helix-helix interaction to signal transduction. Recently, an alternative left-handed homodimeric conformation was described for the ErbB3 TM domain embedded in DPC micelles (PDB 2L9U, Mineev et al., 2011b) (Fig. 3). The tight association of ErbB3tm α-helices with crossing angle of +24° is accomplished via the extended heptad-like motif $I^{649}xxL^{652}VxI^{655}FxxL^{659}xxxF^{663}LxxR^{667}$, which is similar to the motif, implemented in the dimerization of TM segments of EphA2 tyrosine kinase receptor (Bocharov et al., 2008a).

The assumption that the N-terminal association mode of the ErbB TM dimer corresponds to the receptor active state has been supported by recent structural studies of the juxtamembrane segment and kinase domain dimerization upon kinase activation of the ErbB1 receptor (Jura et al., 2009). It was shown that folding of the juxtamembrane regions of both monomers in the receptor dimer into an antiparallel helical structure, requiring the spacing between the C-termini of the TM helices to be about 20 Å, is essential for the kinase domain activation (Jura et al., 2009). The homodimeric ErbB2 TM structure we obtained has exactly the required distance between the C-termini of the TM helices (Fig. 4C), and is thus allowing proper kinase domain activation. Overall these findings enhance understanding of the functional conformational changes of receptor tyrosine kinases during activation of the signaling ligand-receptor complex in cell membranes in normal and pathologic states of human organism.

## 6. Conclusion

Information about structure and dynamic of non-covalently bonded protein oligomers in the membrane is very challenging to obtain. To date, there are only a few experimentally solved dimeric structures of the TM domains of bitopic proteins. Several strategies based on various theoretical and physicochemical methods and their combination are currently available, providing structural-dynamic information about atomic-scale details of TM helix-helix and helix-membrane interactions. Experimental high-resolution structure obtained in a particular membrane mimicking environment usually corresponds to only one of homo- or heterodimeric states of TM domains, which are apparently realized in vivo in the course of bitopic protein activity. Even if special selection of environment allows obtaining an alternative conformation for some proteins, it is impossible to stabilize every conformation of interest to live long enough for comprehensive experimental investigation by mere choice of the external conditions. Molecular modeling, in its turn, predicts all possible alternative dimerization interfaces of the bitopic protein TM domains, existence of which in vivo should be verified in experiment wherever possible. Many aspects of the specific helix-helix interactions in membranes are yet far from being completely understood and are awaiting detailed investigation, which is only possible through concerted use of various physical-chemical and biological methods supported by molecular modeling. Theoretical and experimental methods to study protein-protein and protein-lipid interactions in membrane are rapidly evolving in a correlated manner. Molecular modeling is used to support interpretation of data about specific TM helix association, whereas the theoretical modeling parameters are refined based on the experimentally obtained information. This will likely

result, within a few years to come, in detailed description of a large variety of intra- and intermolecular interactions in membranes and elucidation of the roles of the TM domains in normal and abnormal functioning of the proteins and in their proper localization in cell membranes. The most important practical implications of these studies are primarily related to molecular design of pharmaceutical compositions that can affect specific helix-helix association in cell membrane, providing a novel form of therapy of many human diseases related with abnormal activity of the bitopic proteins. Naturally, that does not diminish the current importance and topicality of obtaining the structure of full-length bitope proteins both separately and in complexes. However, at the present state of development of structural biology this remains quite an ambitious undertaking.

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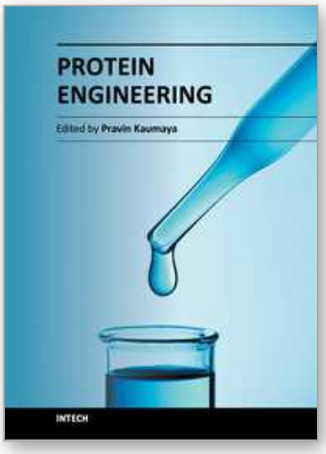


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