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Impact of protein transmembrane domains on membrane organization

Vliv proteinových transmembránových domén na organizaci membrány

Bachelor`s thesis

Supervisor: Mgr. Marek Cebecauer, Ph. D.

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Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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Abstract

Plasma membrane is not a static, but rather a dynamic structure that constantly changes its form and local properties. Interactions between building blocks of plasma membrane and external factors are responsible for those changes. In this thesis, I summarize the literature which describes interactions between transmembrane proteins and lipid membranes as well as its consequences. I discuss membrane thickness, respectively thinning, protein sorting and clustering shown to be dependent on the properties of transmembrane domains. Furthermore, the role of proteins in various model of the plasma membrane organization are indicated. Finally, I report on currently discovered impact of the surface roughness of TMDs on local mobility and organization of lipids. All these data indicate importance of detailed understanding of TMDs, their properties and relation to surrounding lipid membranes.

Key words: membrane, protein, transmembrane domain, hydrophobic mismatch, protein sorting, models of the plasma membrane

Abstrakt

Plazmatická membrána není statická, ale dynamická struktura, stále měnící svoji organizaci a lokální vlastnosti. Interakce mezi stavebními prvky plazmatické membrány a vnějšími faktory jsou zodpovědné za její dynamičnost. Pracuji s literaturou, která popisuje interakce mezi transmembránovými proteiny a lipidovými membránami a jejich důsledky. V práci diskutujii vliv délky a sekvencí transmembránových domén proteinů na navýšení, respektive snížení, tloušťky plazmatické membrány, nebo třídění a shlukování proteinů. Dále je naznačena role proteinů v různých modelech organizace plazmatické membrány. Na závěr poukazuji na nedávno objevený vliv hrubého povrchu TMD na lokální mobilitu a organizaci lipidů. Všechna shrnutá data jsou důkazem významu detailního porozumění TMD, jejich vlastností a vztahu k okolním lipidům membrán.

Klíčová slova: membrána, protein, transmembránová doména, hydrofobní neshoda, třídění proteinů, modely organizace plazmatické membrány

List of abbreviations

CD	cluster of differentiation
CFTR	cystis fibrosis transmembrane conductance regulator
DLPC	dilauroylphosphatidylcholine
DMPC	dimyristoylphosphatidylcholine
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
GA	Golgi apparatus
LAT	linker for activation of T cells
TMD	transmembrane domain

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Introduction

All living systems can be divided in to two groups, prokaryotes and eukaryotes. Prokaryotic and eukaryotic cells differ primarily in their complexity. In regards to prokaryotes, the cell is not internally compartmentalized by membranes and the plasma membrane is the sole structure formed by lipid bilayer. Prokaryotic plasma membranes generally lack sterols, but it contains high amount of proteins that are vital to the existence of these cells. For example, the important part of respiratory and photosynthetic chain is located to the plasma membrane of prokaryotes. On the contrary, eukaryotic cells have an extensive system of intracellular membranes enclosing various organelles. This facilitates a higher level of organization and compartmentalization of specific functions within the cell. For example, respiratory and photosynthetic machinery is located in specialized organelles – mitochondria – separated from the rest of the cell. Membranes of mitochondria and plastids is two-layered, as is the nuclear membrane (also called karyomembrane). The internal membrane of the karyomembrane shapes the nucleus, the external is connected to the ER membrane system. Membranes for each compartment are specific in their content and have their unique functions. One of those functions is separating those compartments from the rest of the cytoplasm, so they could create an environment with specific chemical composition, different pH, etc.

The plasma membrane is a thin, continuous "coat" covering the cell, giving it its shape and firmness, protecting it but also connecting it to the extracellular space and neighbouring cells. It enables controlled exchange of ions and nutrients between the cell and surrounding environment. It also organises molecules involved in signal transduction and some other cellular processes.

In order to perform these functions the plasma membrane exhibits high complexity in terms of morphology and chemical composition. Its lipid bilayer is densely packed with membrane proteins. They affect the organization of the plasma membrane in many ways. The aim of this thesis is to explore what changes can proteins, more precisely their transmembrane domains, cause by their interaction with the lipid environment of mammalian cellular membranes.

Plasma membrane composition and structure

Lipids and proteins are the basic building blocks of cellular membranes. Both can have saccharides attached.

Lipids

The eukaryotic plasma membrane is composed of three types of amphipathic lipids: glycerophospholipids, sphingolipids and sterols. The percentage of each of these components varies between different cell types but the major species are glycerophospholipids.

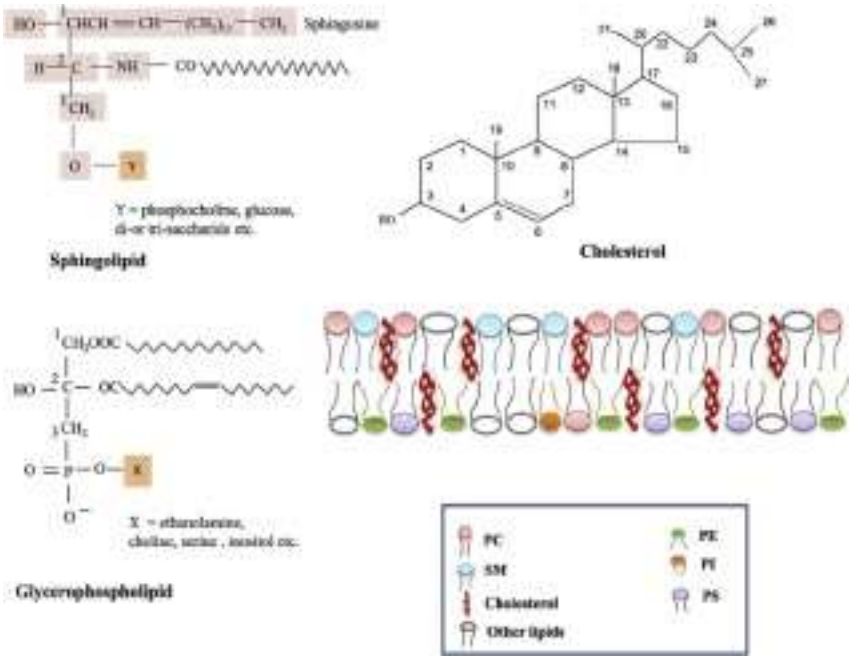

Figure 1: Chemical structure of sphingolipids, glycerophospholipids and cholesterol. Schematic representation of the lipid bilayer (Arish et al., 2015)

Phospholipids have one pole formed by a hydrophilic headgroup, the other one is hydrophobic, formed by one or more acyl chains (most frequently by two; Fig. 1). Due to polarity, phospholipids can form lateral structures - bilayers. Hydrophobic poles are oriented towards each other and hydrophilic poles are on the distal poles of the bilayer. Fatty acyl chains are connected to the glycerol or sphingosine base in phospholipids. In glycerophospholipids, different peripheral substituents (such as choline or inositol) are linked to the lipid base via a phosphate bond. Acyl chains consist of an even number of carbon atoms with different levels of saturation of their carbon-carbon bonds (Fig. 1). The degree of saturation of the acyl chains of lipids is one of the factors influencing membrane fluidity (for more details see Text Box 1) (P. C. Calder, 1994). Phospholipids are insoluble in water.

Fluidity of cellular membranes is the property implemented from biophysical studies of model membranes. Lipids in model membranes can form liquid (fluid) or solid (gel) phase. Molecules are mobile in liquid phase membranes but their mobility is highly restricted in gel phase. Membranes of mammalian cells must be fluid at all times. This can be achieved, for example, by adapting phospholipids (saturation level of their acyl chains) to the changes of environment (Calder et al., 1994). Terms such as rigidity and stiffness are also used in the literature but describe similar properties of lipid membranes. I will use the term fluidity to cover these physical aspects of membranes.

Text box 1: Membrane fluidity

Cholesterol is the main sterol found in mammalian cells. It preferentially localises to the plasma membrane, parts of Golgi apparatus and endosomal vesicles. It can insert itself into the phospholipid bilayer, but cannot form a bilayer on its own. Cholesterol is insoluble in water. Its presence modulates fluidity and rigidity of the membrane (Boggs & Hsia, 1972) (see Text box 1 for more details), especially in the presence of sphingolipids with high melting point (e. g. gangliosides). Gangliosides form gel membranes in the absence of cholesterol at the physiological temperature of 37°C. Living mammalian cells actively avoid formation of gels in their membranes by the increasing cholesterol concentration.

Most lipids associate very strongly with the bilayer due to the presence of aqueous solutes on both sides of the plasma membrane. The two membrane leaflets cling tightly to each other with their hydrophobic sides in order to maximize their separation from the aqueous environment. Thickness of the bilayer is normally 4-5 nm.

Lipid bilayer of the plasma membrane is asymmetric (Morrot et al., 1986). The exoplasmic leaflet is abundant in phosphatidylcholine (PC) and sphingomyelin (SM) and the cytosolic leaflet is abundant in phosphatidylethanolamine (PE) and phosphatidylserine (PS) (Van Deenen, 1981). For example, the exoplasmic leaflet of human erythrocytes contains almost all the PC and SM and it is less fluid. The cytosolic leaflet is made preferentially of PS, PE and phosphatidylinositol (PI) and hence is more fluid (Connor et al., 1992). This asymmetry is already established during membrane formation.

Proteins

L-α-amino acids ordered in linear chains and linked by peptide bonds create big organic biomacromolecules called proteins. Each protein has a unique sequence of amino acids. They

are commonly 20 to 1000 amino acids long. Many proteins are actually enzymes catalysing biochemical reactions and are essential for the metabolism. Others, such as actin, have structural functions, e.g. by being part of the cytoskeleton. Other proteins are essential for cell signalling, adhesion, cell cycle, neuronal and immune systems. In other words, these biomolecules are involved in all vital functions of living cells. Function of proteins does not depend exclusively on its amino acid sequence. Their structure also affects their behaviour and tasks they perform. Moreover, proteins can co-operate and form stable complexes to execute their tasks more efficiently.

Primary structure of proteins is their unique amino acid sequence and the arrangement of peptide bonds. This, including the number of amino acids, varies greatly. Even the slightest change in primary structure can greatly influence the overall 3-dimensional structure and function of proteins. Amino acids can be hydrophobic, hydrophilic, aromatic, i. e. have properties which can influence protein structure.

Secondary structure is the conformation of polypeptide chain that it forms based on hydrogen bonds between carbonyl and amide groups. All the secondary structures are held together by backbone interactions with side chains. However, bonds that stabilize secondary structures are hydrogen bonds. Basic secondary structures are α-helix, β-sheet and a short β-turn in a shape of letter U. Some parts of the protein do not adopt these basic shapes, but are still stable and can be classified using extended structural motifs (e.g. random coil). Again, changes in the secondary structure of a protein may influence its function.

Tertiary structure is the global folding of the polypeptide chain. It is based on interactions between polypeptide chains remote in primary structure. This includes amino acid residues with their 3D arrangement, hydrogen bonds involving polar side chains and protein backbone and hydrophobic interactions between non-polar side chains, which stabilizes the tertiary structure in the greatest degree. Disulphide bonds have a role in decreasing flexibility of proteins by covalently linking side chains of cysteine residues apart in the primary sequence of a protein.

Quaternary structure is a spatial arrangement of polypeptides in a multi-subunit complex(es). For example, it can be found in haemoglobin, which is a hetero-multimeric protein. Subunits in such complex are linked non-covalently, in some cases by electrostatic forces or disulphide bonds.

Glycosylation

For the most part, integral membrane proteins are modified by short hydrocarbon chains. Lipids and proteins of the plasma membrane can have one or more carbohydrate chains attached. Attached carbohydrates are formed by one or more saccharides which can be variously branched. Glycosylation pattern can form large antennas that exceed the size of the substance being glycosylated. In proteins, amino acids that can be glycosylated are serine, threonine and asparagine.

Glycolipids use sphingosine to form a bond with carbohydrate chains. Carbohydrate chains of both, glycoproteins and glycolipids, are located in the exoplasmic leaflet (or lumen of exo- and endocytic membrane systems). This allows them to interact with substances found in extracellular space (and organelles). This inequality in carbohydrate distribution supports the fact that plasma membrane is asymmetric.

Membrane proteins

Not only are lipids distributed asymmetrically in the plasma membrane, but one can also consider that proteins and ions contribute to this asymmetry by orientating different domains and ion species, respectively, towards the two sides of this membrane. In contrast to lipids and ions, a flip-flop across the membrane has never been detected for proteins. This kind of movement would demand enormous amount of energy. That is why transmembrane domains of proteins obtain and keep their asymmetric topology throughout their lifespan. Even though moving the hydrophilic amino acid residue through the hydrophobic core is disadvantageous, individual transmembrane segments of a transmembrane protein domain can alter their orientation, for example G protein $\beta_1\gamma_2$ subunits reorient in order to dock G-protein coupled receptor kinase 2 (Boughtona et al., 2011).

Mammalian proteins can exist as soluble individual units or as parts of complex structures. Moreover, proteins are part of cellular membranes. Indeed, approximately half of the membrane mass is made of proteins (Dupuy & Engelman, 2008). If the bilayer would only be composed of lipid molecules, it would be impermeable to most of substances soluble in water (insoluble in fat). Therefore, the membrane is densely occupied with proteins that play variety of roles; e.g. receptors, transport channels, signalling molecules, etc.
There are three different approaches how proteins associate with membranes (Fig. 2).

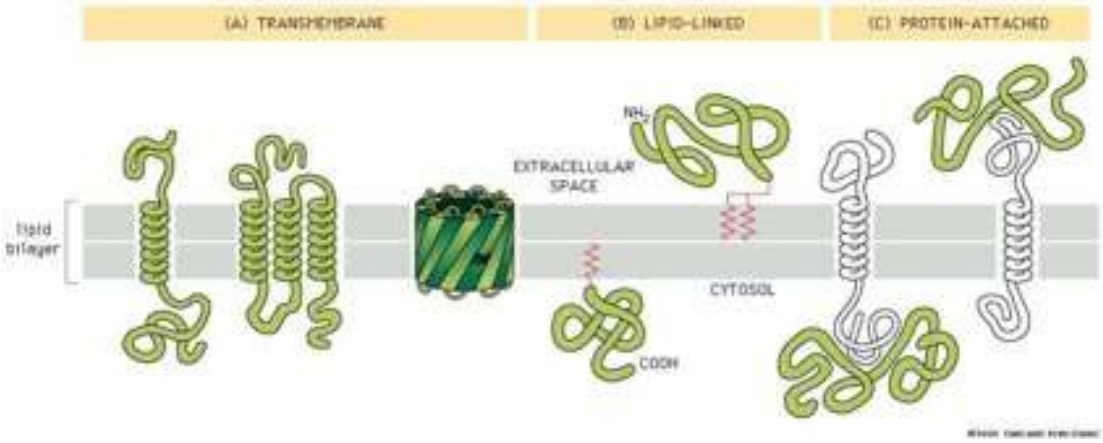

Figure 2: Association of proteins with membranes (Alberts, 1998)

Integral membrane proteins penetrate the membrane and partially protrude the cytosol and extracellular matrix. They are also called transmembrane proteins. They are crucial for a number of vital cellular processes, including the flow of nutrients and signalling from the environment. Transmembrane segment(s) (domain(s)) is hydrophobic and is anchored in the membrane by side chain interactions with acyl chains in the phospholipid bilayer. The solute protruding parts are more hydrophilic.

Lipid-anchored (peripheral) membrane proteins use lipid-anchors to associate with one or the other side of the plasma membrane. Cytosolic face is reserved for proteins anchored to the membrane by fatty acids or isoprenoid groups. Post-translational addition of fatty acids such as myristic or palmitic acid is a process called acylation of proteins. GPI anchors allow proteins to localise at the extracellular face of the plasma membrane.

Membrane-associated proteins are not covalently attached to but are associated with membranes by non-covalent and transient interactions. There are two types of association with the plasma membrane: i) by the interaction of amino acid side chains of proteins with function groups (e.g. inositol or serine) of phospholipids, or ii) by binding to integral or lipid-anchored membrane proteins. For example, electrostatic attraction between phospholipids of the membrane and positively charged basic residues of hydrophobic patch in cytochrome c is what holds them together (Dickerson, 1971).

Classification of integral proteins by topology

"Topology of membrane proteins is defined by the number and sequential position of membrane spanning segments and the localization of sequence segments between them relative to the membrane (cytosolic or extracytosolic)."[1] There are four major types according to Von Heijne (1988): I, II, III, which are single-pass transmembrane proteins, and IV, which is reserved for multi-pass transmembrane proteins (Fig. 3). Types I, II and III are distinctive in the position of C- and N-terminal domains and the presence/absence of the signal sequence.

Type I proteins have a single transmembrane helix that anchors them to the bilayer. Their N-terminal domains are in the lumen of the ER (or other organelles of the exo/endosomal system) or the extracellular space, if the protein is destined to be in the plasma membrane. The N-terminus (signal peptide) is cleaved off and not translocated across the membrane during the process of co-translational insertion of these proteins into the membrane of the ER.

[1] Tusnády, G. E., & Simon, I. (2001). Topology of membrane proteins. *Journal of chemical information and computer sciences*, *41*, 364-368.

This group includes many growth factor and hormone receptors, proteins involved in cell adhesion (Meighan-Mantha et al., 1999), (Aguet et al., 1988) or antigen recognition, enzymes (Charbonneau et al., 1988).

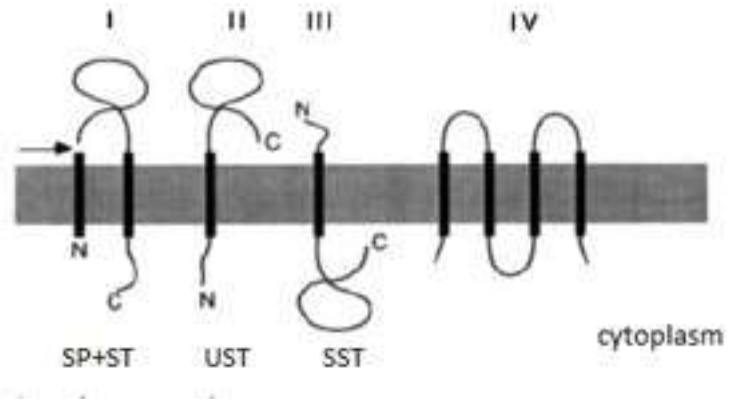

Figure 3: Classification of integral membrane proteins

Type II proteins have non-translocated N-terminus. Signal peptide is uncleavable and identical with the TMD of the protein. Longer C-terminus is involved in enzymatic reactions such as glycosylation or in binding of proteins in the exterior of the cell, for example human transferrin receptor (Schneider et al., 1984).

Type III group of integral proteins have C-terminus in cytosol due to start-stop transfer sequence being on the translocated N-terminus in the absence of signal peptide.

Type IV have more than one transmembrane span, irrespective of their C- or N- terminus orientation. An example is human glucose transporter (Mueckler et al., 1985), although there are many other type IV transmembrane proteins involving receptors and transporters.

Transmembrane domains

As previously mentioned, transmembrane domains are parts of integral proteins that anchor it to the membrane. Integral proteins are not equally periodically embedded, so the density of the presence of TMDs in the membrane changes (Hoyer & Trabold, 1971). TMDs are formed by α-helix in eukaryotes (Lenard & Singer, 1966) which is embedded in the bilayer nearly perpendicularly or in a slightly tilted form. The less common form of TMD in mammalian cells is β-barrel (Granseth et al., 2005). In order to match the hydrophobic surroundings in the membrane, they must be flexible to some extent. The way membrane packs single-pass transmembrane proteins is different than the way it packs multi-pass transmembrane proteins with difference in membrane thickness and elasticity (Weiss et al., 2003).

Each α-helix can adapt a slightly different orientation. This fact has impact mainly on multispanning proteins that can adopt various (energetically more favourable) conformations due to reorganization of their TMDs.

Helices are cca 30-40Å or 18-25 amino acids long and are highly diverse in amino acid sequence. Prevailing the TM domains sequences are the hydrophobic amino acids Ile, Val, Leu, Phe, Cys, Met, Ala, Gly and Trp. Hydroxy amino acids Ser, Thr and Tyr, and also the helix-breaker, Pro, occur occasionally in these helices. Their purpose is to conserve the hydrophobic core.

There are several ways a transmembrane domain can be anchored in the bilayer (Fig. 3). Approximately 90%-95% of the amino acid residues in α-helices of TMD are hydrophobic, so the first requirement is that the polypeptide chain passing the membrane, has to form hydrogen bonds between polar main-chain atoms. This makes the helix stable in the hydrophobic environment of the lipid bilayer. The length of TMD can match the hydrophobic core of a bilayer or a hydrophobic mismatch, positive or negative, will occur between these two structures (Fig. 6). Mismatching helices can be tilted in the membrane, or the membrane itself is thinned/thickened to accommodate the TMD.

Integral proteins having one, two, four or seven TMDs passing the membrane are the most frequent ones (Fig. 4). Channels and transporters are usually formed by 12, 16 or 21

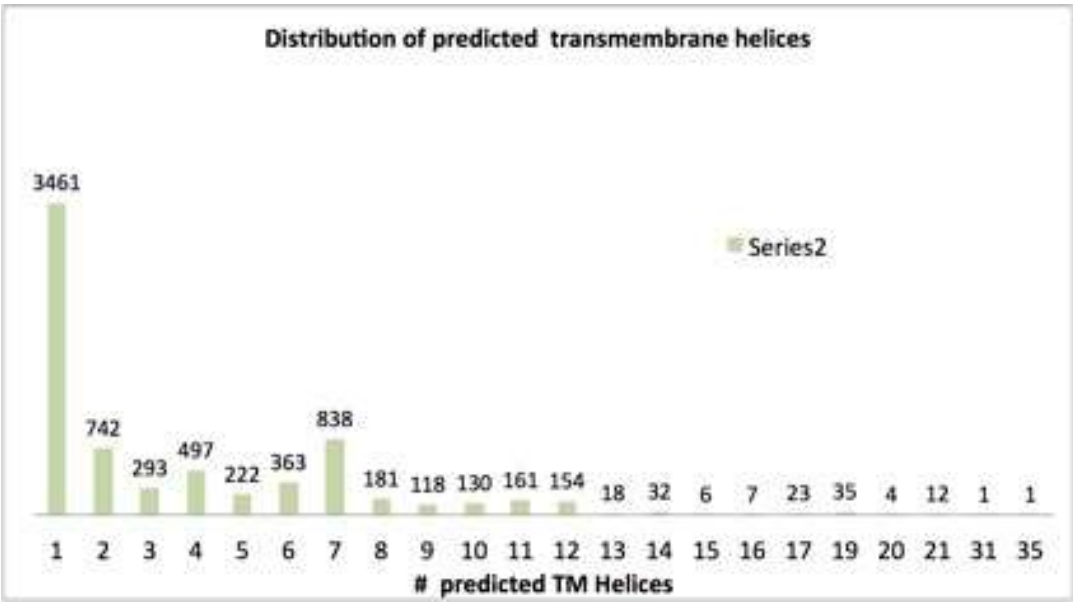

Figure 4: Distribution of predicted transmembrane helices *(Pieper et al., 2013)*

TMDs. A variety of systems biology tools can be used for the in silico analysis of a fined in the well-accepted database: Uniprot (www.uniprot.org).

Monospanners receive and pass on signals, are involved in signal recognition and adhesion. For example, majority of receptor tyrosine kinases are activated after their cytoplasmic and transmembrane domains get into proximity and dimerize upon ligand binding on the extracellular domains of the receptor. In the case of EGFR, 2 molecules of EGF are required and each has one binding site for 1 EGFR thus forming a stable 1:1 intermediate (Lemmon et al., 1997). Pituitary growth hormone, prolactin and placental lactogen have four bundled α-helices. This very asymmetric structure produces two different hormone binding sites on molecules of receptor, which allows a more precise, step-by-step regulation (Kossiakoff, 2004). Monospanners can also aggregate in a large cluster upon ligand activation like integrins and selectins do (Welf et al., 2012).

Dispanners are proteins with two membrane spans connected by a loop extracellularly. Known example is Ca^{2+} binding protein MCTP1/2, involved in signal transduction or membrane trafficking (Shin et al., 2005).

Tetraspanners include four TMDs connected by one intracellular and two extracellular loops. One of the loops is commonly larger, usually having up to 100 amino acid residues. They are present in all multicellular organisms participating in cell adhesion, proliferation, motility, morphogenesis (Zhang et al., 2002) and many others functions. For example, CD9, 53, 63, 81, 151 proteins are tetraspanners .

Heptaspanners are a large group of receptors, most of which belong to the family of G-protein coupled receptors (GPCRs). Also enlarging is the group of seven transmembrane receptors signalling in a different path than binding a G-protein. GPCRs have binding sites for neurotransmitters, hormones such as serotonin, prostaglandin E2, epinephrine (Dixon et al., 1986) and some respond to light to modulate pathways involved in the vision (Henderson & Unwin, 1975). Beginning from N-terminus in the extracellular space, seven TM domains pass the membrane up and down in a serpentine patterning and end with C-terminus in the cytosol. C-terminus can be activated by a conformational change(s) in the organization of TMDs upon ligand binding.

Multi-spanners are usually channel proteins. Large number of membrane spans provide numerous binding sites required for their action. The loops connecting TMDs are significantly involved in activities of such proteins. Proteins with 10 TMDs co-transport Cl^- and HCO_3^- and transport amino acids. Common 12 TMD protein is GABA transporter (Borden, 1996), as is dodecaspanner CFTR composed of five protein domains, two of which form the channel pore. Multi spanning domains are connected extracellularly by the regulatory domain (Riordan et al., 1989) (Sheppard & Welsh, 1999).

Non-specific interactions

Not only is plasma membrane asymmetric, but it differs significantly from probably all other cellular membranes. In contrast to ER membrane, plasma membrane is rich in sterols and sphingolipids. Membrane proteins have to adapt to distinct environments in the plasma membrane by an asymmetric distribution of residues passing the bilayer – the TMD (Sharpe et al., 2010). This means that by changing the properties of the membrane, we would be able to affect the location and activity of membrane proteins (e. g. channel and transporters).

In 1991, for the first time, it was proven that membrane-spanning domains are a major determinant whether a protein is retained in the GA or reaches the plasma membrane using vesicular stomatitis virus G protein and the α-subunit of gonadotropin as model proteins. Amino acid sequences of TMDs involved in intracellular localization are not conserved, but the interplay of TMD length and hydrophilicity leads to sorting. Replacing single TMD of vesicular stomatitis virus G protein and the α-subunit of gonadotropin with the first TMD from E1glycoprotein from an avian coronavirus caused their trapping in GA. Likewise, even one substitution of amino acid sequence in the first TMD of E1 allowed sorting of the mutant proteins to the plasma membrane (Swift Machamer, 1991).

TMDs are on average 5 amino acid residues longer in plasma membrane compared to those localized in GA (Fig. 5). Physical properties of shorter TMD causes the protein to remain in GA (Bretscher & Munro, 1993) (Munro, 1995), whereas longer TMD, as is the case with bovine β-1,4-galactosyltransferase, lets the protein proceed to its destination. Greater glycosyltranferase activity was observed in plasma membrane fractions containing longer mutants or mutants that had up to 4 substituted isoleucines (Masibay et al., 1993).

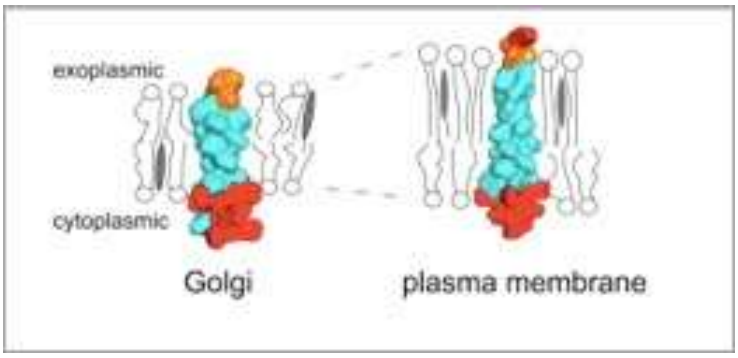

Figure 5: TMDs of Golgi-resident proteins are shorter than those of plasma membrane proteins (Sharpe, 2010)

Physical properties, including the length of the hydrophobic segment and the interactions of its side chains with the surrounding fatty acyl chains, were shown to be responsible for different sorting of proteins in cell membranes.

Surface of TMD does not have to be of the same length as the bilayer, so often there is a mismatch between them (Fig. 6). TMDs cause the surrounding lipid bilayer to match to their hydrophobic surface. Out of the two structures, the membrane is the one adjusting. Vice versa, the membrane can influence the conformation of membrane proteins or induce intramolecular interactions between proteins.

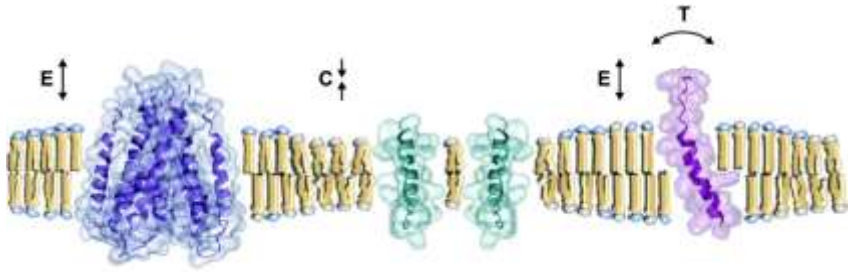

Figure 6: Hydrophobic mismatch (Mitra et al., 2004)

Mitra (2004) showed on membrane fractions of rat hepatocytes that thickness of the membrane is dependent on TMDs of proteins and that cholesterol is a marginal contributor. When up to 90 % of cholesterol was depleted from membranes, GA and apical plasma membrane retained their thickness. As for the ER and basolateral plasma membrane, the thickness decreased only up to 1.0 Å. But, when membrane proteins were depleted, depending on the membrane studied, the thickness increased or decreased up to 5 Å. Protein depletion had no effect on apical plasma membranes. The ER and basolateral membrane were thicker, while Golgi membrane thickness decreased. This change happens because some proteins are not naturally hydrophobically matched with lipids of their target membrane. By matching thickness of the membrane, the cell is avoiding energetically disadvantageous hydrophobic exposure to a hydrophilic environment. "… it is concluded that hydrophobic mismatch can strongly affect protein and lipid organization, but that the precise consequences depend on the individual properties of the proteins and lipids."[2]

TMDs modulate conformation of fatty acyls and their packing in order to be matched hydrophobically (Nezil & Bloom, 1992). The thickening of the lipid bilayer was also

[2] *Killian, J. A. (1998). Hydrophobic mismatch between proteins and lipids in membranes. *Biochimica et Biophysica Acta*, 401-416.

observed, amongst many other observations, when a model peptide synthetic analogue of gramicidin A was inserted into DMPC and DLPC bilayers (Küsel et al., 2007).

It was shown by experiments using cargo proteins SNARE that hydrophobic mismatch with local lipid environment causes proteins to cluster and that integrity of SNARE domains is dependent on cholesterol. Segregation of these proteins is most probably important for their function since they are the docking and fusion sites of secretory granules and caveolae (Ohara-Imaizumi et al., 2004), they are involved in the exocytosis (Fili et al., 2001) (Lin & Scheller, 1997).

Self-association is promoted by weak homophylic interactions between TMDs involving the SNARE motif of the cytoplasmic domain (Sieber et al., 2006). Positive charges of residues in the extracellular and intracellular domains are compensated by phosphoinositides and ions, which reduce the repulsion that stands usually in the way of protein clustering (Milovanović et al., 2015). How the clusters segregated was determined by the length of their TMDs though. TMD of the SNARE protein syntaxin 4 is longer by 1-2 amino acid residues compared to the TMD of syntaxin 1. Syntaxin 1 showed preference for artificial membrane of lower thickness composed of C16:1 PC and syntaxin 4 for artificial membrane of higher thickness composed of C18:1 PC (Milovanović et al., 2015). Thus, syntaxins can be considered as contributors to membrane patterning.

Specific protein-lipid interactions

Lipids and proteins of membranes interact in different ways, thereby influencing one another`s structure and/or function. In the next paragraph, I will shortly describe the nature of these interactions.

Lipid molecules adhere and adjust to protein surface when in vicinity. There are few modes of binding between the two: lipid “shells” of annular lipids (Anderson & Jacobson, 2002) or non-annular lipids. The former has a preference to the hydrophobic or hydrophilic surface of the protein. Therefore, the structure of TMD defines the composition of a lipid shell. They integrate protein tightly and position it vertically in the bilayer. The latter are lipids immersed in hydrophobic clefts of protein surface mostly in contact sites adjacent to TMDs. They have much lower exchange rate than annular lipids. Their intimate interaction with proteins allow them to modulate structure, localization and functions of proteins. The third type of lipids are known as “bulk” lipids. Typically, interaction with TMDs is kept on a minimum and they have a fast exchange rate at lipid-protein interface. (Contreras et al., 2011)

Modification of membrane proteins by fatty acids

The purpose of post-translation modifications is to diversify proteins of relatively short sequences in their functions. It has been shown that such modifications help in protein folding and guide them to their destination in the cell, different cellular membrane systems or to the domains of receptor proteins. Post-translational modifications can also be signals for protein degradation and senescence. Intracellular signalling is based as well on the cell's ability to modify proteins (James & Olson, 1990), and regulate their interactions with other proteins, lipids, nucleic acids and other cofactors.

A greater focus is placed here on lipidation of proteins. It is a mean of targeting proteins to cell membranes and lipid vesicles. Hydrophobicity of proteins increases upon addition of fatty acids, so does its affinity for membranes. Attachment of lipid moieties, as previously mentioned, can alter protein properties, thus having a role in protein-protein and protein-lipid interactions and membrane targeting of proteins.

GPI anchors attach proteins to the outer side of the plasma membrane. They are often found in lipid rafts (see below).

Palmitoylation

Palmitoylation is a typical membrane-targeting lipid posttranslational modification, where palmitic acid (C16) is covalently linked via labile thioester bond to cystein residues of membrane proteins. This process is reversible (Duncan & Gilman, 1996). Less frequent is the transfer of other acyl chains to proteins, such as stearate, oleate, arachidonate. The transfer is mediated by palmitoyl acyltransferases.

A consensus for palmitoylation does not exist, but certain preferences in palmitoylated cysteines have been observed. They are usually close to sites of myristoylation and prenylation. Amino acids surrounding the cysteine to be palmitoylated are basic or nonpolar. Regarding the location of these cysteines, they are close to the cytoplasm–membrane interface in proximity to the TMD or a part of the TMD itself (Roth et al., 2006).

The central role of palmitoylation is in the intracellular protein sorting. Although palmitoylation helps with the localisation of some proteins, such as LAT, TMD still primarily

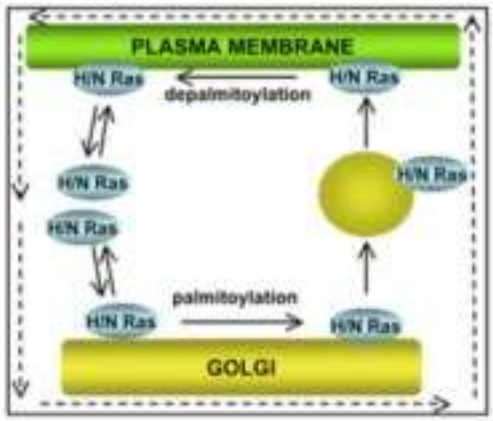

Figure 7: Model of palmitoylation dependent plasma membrane-to-GA cycling pathway for Ras proteins (Greaves & Chamberlain, 2011)

serves as an anchor to the membrane (Chum et al., 2016). In case of signalling molecules H-Ras and N-Ras, if they are palmitoylated, and previously farnesyalted, it is an exit signal to rapidly leave the GA and proceed to the plasma membrane. Depalmitoylation leads to their release from the membrane and the whole process can be repeated (Rocks et al., 2005).

Palmitoylation often serves as a secondary signal for membrane association of cytosolic proteins. Dually lipidated proteins have a higher affinity binding themselves to membranes, than proteins which have only one type of modification. Some tyrosine kinases, such as Fyn, in addition to palmitoylation, need to be myristoylated in ordered to be addressed to the plasma membrane (Alland et al., 1994). In case of H- and N-Ras hydrophilic proteins, they have to be prenylated and palmitoylated to be capable of inserting into cellular membranes (see above) (Schroeder et al., 1997).

Myristoylation

On the contrary to palmitoylation, myristoylation is an essentially irreversible addition of myristic acid to cytosolic proteins. Myristoyl group (C14) is added to the α-amino group of glycine of the nascent protein (Zha et al., 2000) after cleavage of the adjacent initiating terminal methionine. Myristoylation has a strict consensus sequence MGXXXS/T. This modification occurs co- or post-translationally.

N-myristoylation attaches a weak membrane anchor to proteins, hence providing them with hydrophobicity that targets them to the plasma membrane, as well as to the mitochondrial membrane (Zha et al., 2000). Usually, it is not sufficient for a protein to be

myristoylated to be targeted to a membrane (Murray et al., 1998), but it requires a cluster of basic amino acid residues that are able to participate in electrostatic interactions with acidic phospholipids (Bentham et al., 2006) or to be palmitoylated for a more stable membrane association.

Prenylation

Adding a farnesyl (C15) or geranylgeranyl (C20) group in the proximity of C-terminus is a common process, called prenylation. This modification is not prone to hydrolysis. What defines the type of modification of the Ras proteins is the motif of 4 amino acids at C-terminus. All members of Ras superfamily need to be associated with the plasma membrane in order to transduce signals. All of them are prenylated, which is not sufficient for stable membrane anchoring. That is why H- and N-Ras also need palmitoylation or, in the case of K-Ras4B, a polybasic sequence near C-terminus stabilizes the interaction with the membrane (see above) (Hancock et al., 1990).

Plasma membrane organization

If we want to see the influence of integral proteins on membranes, we should look at how TMDs influence mobility of other components of the bilayer and at physical properties of the membrane when the protein is present. Over time, with the help of technological advancements, scientists were uncovering part by part in which way and measure this interference happens.

The Singer and Nicholson fluid mosaic model from 1972 is one of the most accepted model for cell membrane. It highlighted the fact that membranes of cells are not pure lipid structures, but that lipids and proteins are coexisting and are free to move. Membrane components are cooperating, thanks to lipid-lipid, protein-protein and lipid-protein interactions. Nowadays, it is known that membrane proteins often are incorporated into complexes or are connected to the cytoskeleton or both, so they are confined to certain areas of the membrane.

A model that was limited to integral proteins was the hydrodynamic model (Saffman & Delbrück, 1975). Integral proteins are regarded as cylindrical inclusions. Membrane components were viewed as independently moving molecules in a two-dimensional continuum whose diffusion is influenced by viscosity, their density and weakly on their size. TMDs are also found to be rigid and large compared to the bilayer, which slows down boundary lipids upon hindering (*Kahya, 2006), (Ramadurai et al., 2009).

Lipid rafts are a concept of membrane sub-compartmentalization born in 1997. The segregation of lipids and non-homogeneous distribution of membrane components was observed even earlier, but Simons and Ikonen (1997) suggested that these dynamic, mobile "patches" are enriched in cholesterol and sphingolipids and are sites of membrane trafficking and cell signalling. They are present in both leaflets of the bilayer and are coupled functionally. They can differ in stability and size in order of tens to hundreds of nanometres. It is stressed that rafts are a result of lateral segregation potential of membrane and that lipids are not plain solvent for other membrane components.
It is also thought that lipid rafts association is determined by TMD length. Longer TMDs would gather into a thicker raft due to minimizing exposure to aqueous environment. By observing wild type and mutant LAT proteins, it is suggested that structure of the wild type LAT is designed to be specific for only certain subdomain, which affects micropatterning of membranes (Diaz-Rohrer et al., 2014).

Transmembrane proteins are embedded in sphingolipid and cholesterol domains or are moving freely. However, due to the rigidity of the bilayer in lipid rafts, it is most probable that the majority of TM proteins are not part of these domains.

Thus far, this model has not been proven so far, except in silico, and should not be confused with detergent resistant membranes, which represent a biochemical tool using inefficient solubilisation of membrane lipids and proteins to study biochemical properties of these molecules.

In 1984 (Mouritsen & Bloom, 1984) and later in 1993 (*Mouritsen & Bloom, 1993) Mouritsen and Bloom proposed mattress model based on the perturbations induced by proteins in membranes. They took into consideration not only lipid-protein interactions between TMDs and lipids in hydrophobic environment of the bilayer, but also lipid-protein interactions derived from the sole mismatch in hydrophobic regions of lipids and proteins. The effect of mismatch, which was discussed in the text above (Fig. 6), was presumed to cause protein domain formation, sorting and later also accumulation of certain lipids around integral proteins. Therefore, the membrane was no longer viewed as homo-, but heterogeneous. There is no experimental proof for this theory for now probably due to technical limitations of available tools.

According to the present, most accepted model of plasma membrane organization, plasma membrane movements are strongly regulated by “fences” made of cortical actin and by binding to anchoring structures (Sako & Kusumi, 1995). Two versions of the model appeared with regards to techniques used for their discovery. Both emphasize decreased mobility of molecules due to plasma membrane segmentation thanks to actin-based cortical skeleton meshwork.

Cortical actin meshwork is the portion of the cytoskeleton closely associated with plasma membrane. It physically connects plasma membrane components amongst themselves or to the cytosol. For transmembrane proteins that have a short half-life at the surface of the cell serves as a stabilizer or for some as a positional cue when 2 cells are in contact, for example in case of E-cadherin-mediated adhesion (McNeill et al., 1990). An exchange between plasma membrane compartments separated by the meshwork, known as “hop” diffusion, occurs often because the membrane is fluid after all and membrane components do have sufficient energy to overcome the obstacles (Suzuki et al., 2005).

Membrane-skeleton “fence” model (Sako & Kusumi, 1995) (Kusumi, 2012) proposes that while the actin-based skeleton is in immediate proximity to the inner membrane leaflet transmembrane proteins temporary corral in the skeleton meshwork upon collision of

cytoplasmic domains with the skeleton. When the meshwork is in sufficient distance from the membrane or when it dissociates (Tomishige et al., 1998) integral proteins can diffuse further to neighbouring compartments.

Anchored transmembrane protein "picket" model (Sako & Kusumi, 1995) (Kusumi, 2012) is based on lipids in both leaflets being regulated by the skeleton meshwork, that is on the cytoplasmic face of the membrane. Transmembrane proteins align next to the meshwork fence, act as pickets upon encounter of the meshwork and cytoplasmic domains of transmembrane proteins, by binding to anchoring structures such as cytoskeleton or coated structures. Friction of the surface of corralled proteins prevents free diffusion of boundary lipids and other integral proteins.

According to Sheetz (1980) membrane becomes more viscous in the corralled parts of the membrane, due to the restricted mobility. Indeed, reduced mobility of lipids in vicinity of TMDs was shown by in silico (Niemelä et al., 2010) and experimentally (Olšinová et al., 2016). Current research (Olšinová et al., 2016) shows that the increase in viscosity is caused by trapping of acyl chains of the annular lipids in the rough surface of TMDs made by amino acid side chains. Indeed, all published 3D structures of proteins with TMDs exhibit strong roughness of the surface of these domains (Olšinová et al., 2016). Furthermore, cholesterol avoids contact with TMDs due to differences in shapes of their molecules leading to reorganization of molecules within a membrane. Altogether, this causes significant decrease in mobility of membrane components, similar to that observed in cells (Frick et al., 2007).

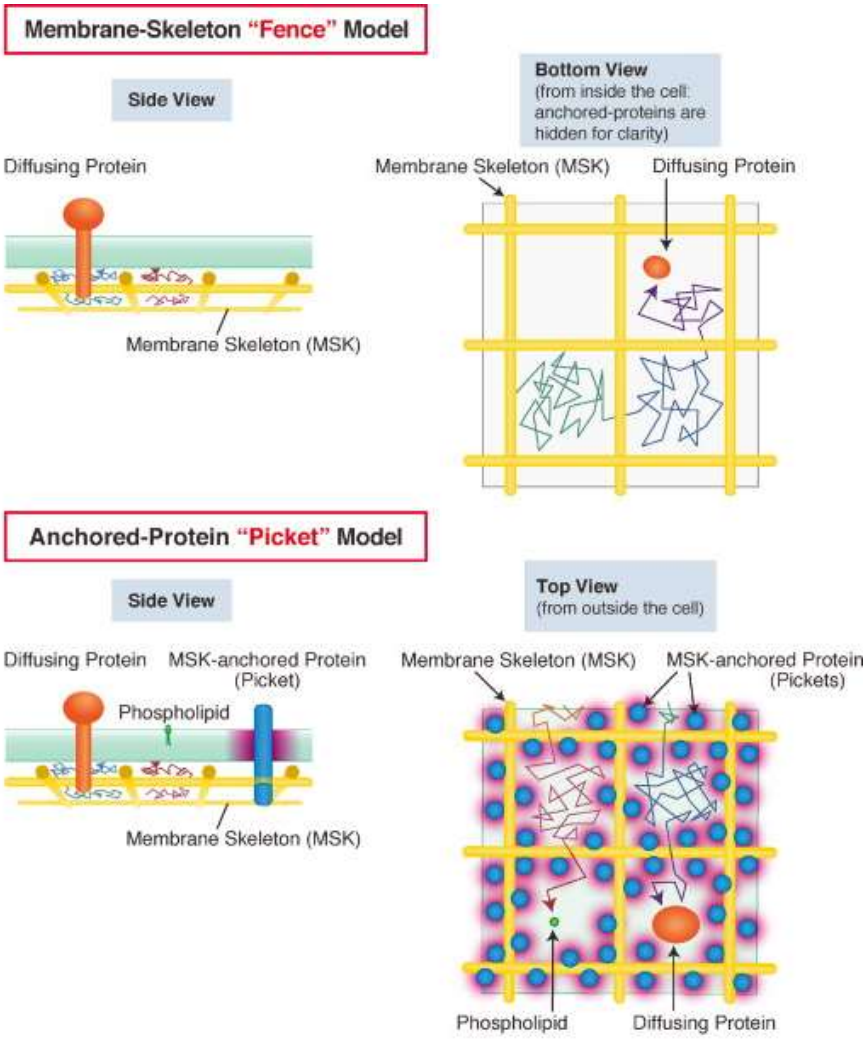

Figure 8: The membrane-skeleton fence model and the anchored TM protein picket model. The left column shows the side view (longitudinal cross-section) of the membrane, while the right column shows the bottom view (from the cytoplasm) and the top view (from outside the cell). *(Kusumi et al., 2012)*

Conclusion

To be able to fully understand how the plasma membrane works we must comprehend the interplay between all components of the membrane. Transmembrane proteins make up for about 50% of the membrane, but their influence is still poorly understood.

From the time when it was thought that the membrane was made mostly from lipids to nowadays, when the knowledge about the membrane is vast, we have learned that the plasma membrane would not exist in the form we know it without integral proteins.

TMDs by inserting themselves into membranes, contribute to the bilayer asymmetry. Also, they play a significant role in modulation of membrane thickness. TMDs can also be membrane-targeting signals, a defining factor of which membrane is the protein destined to be a part of. Besides targeting the protein to a specific membrane, it was shown that sometimes the TMDs prefer certain domains in the membrane itself. However, universal signal for protein sorting has not been found.

Recent findings show that the presence of TMDs cause a decrease in mobility of surrounding molecules and cholesterol segregation, which causes increased viscosity in TMDs' vicinity and possibly supports forming of domains low in protein.

Effects caused by TMDs in membranes could be useful in controlling activities of transport proteins and channels. Knowing how a protein is addressed to a certain membrane or its compartment could be used in prevention of their mislocalization and possibly loss-of-function or degradation.

The detailed understanding of all processes in which TMDs are involved will expand our knowledge of events in hydrophobic environment of the membrane on molecular scale. Our insight in TMDs and interactions they are involved in is still far from complete and there is much more to learn about how the domains of proteins influence the membrane.

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