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Review

Membrane topology of transmembrane proteins: determinants and experimental tools

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ABSTRACT

Membrane topology refers to the two-dimensional structural information of a membrane protein that indicates the number of transmembrane (TM) segments and the orientation of soluble domains relative to the plane of the membrane. Since membrane proteins are co-translationally translocated across and inserted into the membrane, the TM segments orient themselves properly in an early stage of membrane protein biogenesis. Each membrane protein must contain some topogenic signals, but the translocation components and the membrane environment also influence the membrane topology of proteins. We discuss the factors that affect membrane protein orientation and have listed available experimental tools that can be used in determining membrane protein topology.

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Abbreviations: GFP, green fluorescent protein; ER, endoplasmic reticulum; TM, transmembrane; TEV protease, tobacco etch virus protease.

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1. Introduction

The majority of membrane proteins are estimated to be alpha helical bundle types which contain at least one transmembrane (TM) domain that traverses the membrane. Soluble domains of a membrane protein thus reside on one or the other side of the membrane. Membrane topology refers to where the soluble domains are oriented relative to the plane of the membrane and how many TM domains are in the membrane, and it offers guidance as to structure and function studies of membrane proteins.

In referring to membrane protein topology, "in" indicates the cytoplasm, originated from bacteria, and "out" indicates the non-cytoplasmic side; periplasm in prokaryotes and luminal side of the endoplasmic reticulum (ER), Golgi, endosomes, lysosomes or extracelluar matrix side of the plasma membrane in eukaryotes.

In this article, we review the determinants of membrane topology, protein components and membrane environment that influence the membrane orientation of proteins, the global view of membrane topology of polytopic membrane proteins, and summarize currently available experimental tools that are used in determining membrane topology of proteins.

2. Membrane topology determinants

2.1. Positive inside rule

It has been observed that positively charged residues in flanking loops of TM domains in membrane proteins are predominantly found in the cytoplasmic side of the membrane, and this phenomenon is so-called the "positive inside rule" [1–3]. For bacterial membrane proteins, the positive inside rule is well preserved, so that it is rare to find positively charged residues in the soluble loops facing the periplasm [1]. For membrane proteins destined to the secretory pathway in the eukaryotic cell, the soluble domain that contains the net positive sum of charged flanking residues of the TM segment is oriented to the cytoplasmic side [4,5].

Some explanations for the positive inside rule are as follows (Fig. 1). Positively charged residues in a nascent chain seem to be less translocatable across the membrane and are left in the side of the membrane where protein translation has occurred (*cis* side). Since membrane proteins are co-translationally translocated and membrane inserted, positively charged residues exposed to the *cis* side of the membrane during translation/translocation may interact with negatively charged lipid head groups and are not translocated to the *trans* side. It also could be that the translocation machinery is less accommodating for translocation of positively charged residues. Lastly, the negative membrane potential in the cytoplasmic side of the membrane may interact with positively charged residues and/or the positive membrane potential in the periplasmic side repulses positively charged residues, thus preventing translocation of positively charged residues across the membrane.

The reason that bacterial membrane proteins exhibit stronger positive inside rule compared to the membrane proteins destined to the secretory pathway in the eukaryotic cell may be attributed to the membrane potential differences in the two membranes.

Interestingly, while all membrane proteins show the positive inside rule, it has been observed that nuclear-encoded mitochondrial inner membrane proteins do not exhibit a noticeable positive inside rule, meaning that positively charged residues are equally found in the intermembrane space and the matrix [6]. These proteins are translocated from the cytosol/intermembrane space (*cis* side), thus positively charged residues may be left in the intermembrane space by interacting with lipid head groups. But, since the inner membrane potential is negative in the matrix side, electrophoretic force may facilitate the translocation of some positively charged residues across the inner membrane. Of note, the distribution of negatively charged Glu residues is found over-presented in the intermembrane space side for nuclear-encoded mitochondrial inner membrane proteins [6].

Numerous mutation studies show that addition or deletion of positively charged flanking residues strongly affects the orienta-

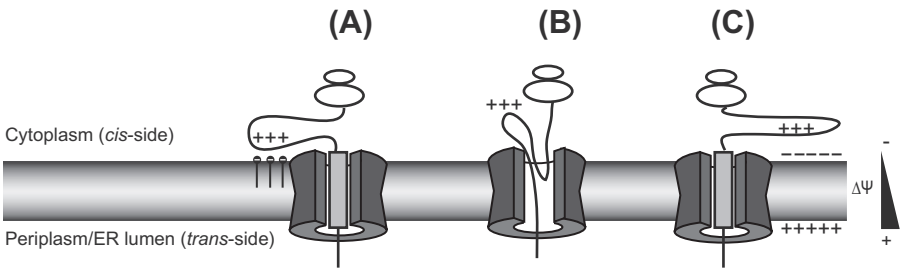

Fig. 1. Some explanations for the positive inside rule. During co-translational translocation and membrane insertion from the *cis* side of the membrane, the exposed positively charged residues of a nascent chain may interact with negatively charged phospholipid head groups (A) or the translocation machinery may disfavor translocation of positively charged residues (B). The negative membrane potential in the cytosolic side of the membrane may also influence retaining positively charged residues of a nascent chain (C).

tion of the TM domain according to the positive inside rule, demonstrating that positively charged amino acids in the flanking regions of the TM domain are prominent "inside" topogenic signals [7–12].

2.2. Hydrophobicity of the TM segment

In addition to the flanking charged residues, the hydrophobicity and the length of a signal sequence or TM domain influence the N- and C-terminal translocation of single-spanning membrane proteins [8,9,13–15]. In these studies, tendency for higher N-terminal translocation, thus N_{out}–C_{in} orientation has been observed as the hydrophobicity and the length of signal sequence/TM segment increases. It has been speculated that more hydrophobic TM segments exit the translocon faster by interacting with the hydrophobic core of the lipids: thus, they may have a lower chance to invert their orientation after entering the translocon the N-terminus first (from N_{out} to N_{in}), whereas moderately hydrophobic TM segments linger around the translocon longer and thus have a higher chance to invert and acquire an N_{in}–C_{out} membrane orientation. When the hydrophobicity gradient was given within the TM segment, more hydrophobic side was preferentially translocated to the luminal side of the membrane [16].

2.3. N and C-terminal domains

For membrane insertion of a signal anchor protein, both models: the N-terminal first then flip [9,13–15,17] and insert as a hairpin have been proposed [18,19]. Studies show that the longer the N-terminus and the higher the folding efficiency of the N-terminus, the more likely the nascent chain is to acquire an N_{in}–C_{out} membrane orientation. When the N-linked glycosylation site in the N-terminus becomes glycosylated, the N-terminus is fixed in the luminal side of the ER, thus N_{out} membrane orientation is assumed. A recent study by Kocik et al. shows that depending on the length of the N-terminus preceding the TM domain, the C-terminal translocation is differently influenced [20]. For a single-spanning membrane protein with an N-terminus shorter than ~20 residues, the C_{out} orientation was promoted as the C-terminus lengthened. However, when the N-terminus was longer than 20 residues, the effects of the C-terminal length and the hydrophobicity of the TM domain were lost. Hence, the N- and C-terminal translocations are influenced by the length and folding status of proteins.

In sum, the charged flanking residues, the length and folding status of the N-terminal soluble domain preceding the TM segment/signal sequence, the hydrophobicity of the TM segment/signal sequence, and the length of the C-terminal domain may additively contribute to the final topology of proteins in a given membrane environment.

3. Protein components that mediate orientation of membrane proteins

A yeast genetic screen to select genes responding weakly to the strong flanking charge difference of the TM segment was done using a fusion protein containing the first TM segment of Ste2p, an α-factor receptor and β-lactamase or invertase and identified two proteins [21]. One was *SPF1* that encodes a P-type ATPase in the ER or Golgi and the other was *STE24* that encodes a membrane zinc metalloprotease in the ER. Whether these proteins influence the membrane orientation of other membrane proteins and how these proteins affect topogenesis of Ste2p remain unclear.

Membrane proteins are mostly co-translationally inserted into the membrane, thus it is assumed that the TM segments acquire proper membrane orientation before or during membrane insertion. Hence, it is reasonable to expect that the translocation machineries play a role in orienting TM segments of membrane proteins during an early stage of membrane insertion (Fig. 2).

3.1. Sec61

When two positively charged Arg residues in the plug domain and one negatively charged Glu in the TM8 of yeast Sec61 were mutated to the opposite charge residues (Arg to Glu and Glu to Arg), the membrane orientation of a model single-spanning membrane protein inserted according to the positive inside rule became less pronounced [22]. Further, screening of yeast Sec61 mutants that altered the orientation of a model single-spanning membrane protein identified residues in Sec61 across the entire protein [23]. These mutants also showed increased translocation and membrane insertion of proteins carrying the defective signal sequence or TM domain [23]. Our recent study also identified mutants in the gating helices of Sec61 that cause increased membrane insertion of a model single-spanning membrane protein as an N_{in}–C_{out} orientation [24]. Thus, these studies show the correlation between increased translocation/membrane insertion and altered membrane topology of proteins. It remains to be seen whether particular residues in the Sec61 are critical for orienting a TM domain of membrane proteins or the changed open/closed state of the Sec translocon due to mutations influences topogenesis of membrane proteins.

3.2. YidC/Oxa1

YidC is present in the cytoplasmic membrane of *Escherichia coli* and mediates membrane insertion of proteins by itself or with the SecY translocon [25]. Its eukaryotic homolog, Oxa1 resides in the mitochondrial inner membrane and mediates membrane insertion of proteins from the matrix.

Studies have shown that moderately hydrophobic TM domains and negatively charged residues in periplasmic domains of a membrane protein may be an important determinant for YidC dependent membrane insertion [26]. Proteins that are dependent on Oxa1 for membrane insertion in the mitochondrial inner membrane have also shown to have a tendency of higher net negatively charged residues in the intermembrane space side [27]. Therefore, these studies suggest that YidC and Oxa1 play a role in facilitating the translocation of soluble loops that contain negatively charged residues across the membrane.

3.3. Sec62

The Sec62/Sec63 complex has been known to mediate post-translational translocation of a subset of secretory proteins in yeast [28,29]. However, Sec71, a non-essential subunit of the Sec62/Sec63 complex was originally found from a genetic screen rescuing the defect of membrane protein translocation across the ER mem-

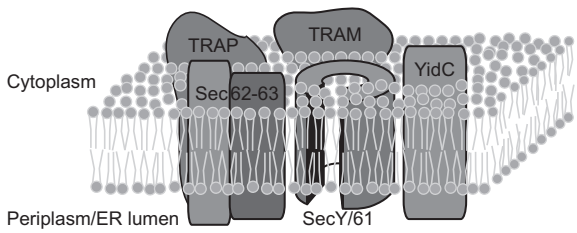

Fig. 2. Translocation components involved in the membrane protein topogenesis. The Sec61/SecY translocon associates with the Sec62-Sec63 complex in yeast, the TRAM and TRAP complexes in mammalian cells, and YidC in *E. coli* and mediate membrane protein topogenesis.

brane, and was thus implicated the involvement of the Sec62/Sec63 complex in membrane protein insertion [30]. Recently, Sec62 has been shown to recognize moderately hydrophobic TM domains and mediate membrane insertion of single-spanning membrane proteins in an N_{in}–C_{out} orientation [31]. Further, our recent work shows that the Sec62/Sec63 complex recognizes moderately hydrophobic TM domains in multi-spanning membrane proteins and facilitates the C-terminal translocation (Jung et al., submitted for publication).

3.4. TRAM and TRAP

The translocating chain associated membrane protein (TRAM) and the translocon associated protein (TRAP) complexes are associated with the Sec61 complex and mediate co-translational translocation and membrane insertion of proteins in the mammalian cell [32–41]. The TRAM is required to translocate and membrane insert a subset of proteins, and it has been observed that the TRAM dependent proteins tend to have a short N-terminal domain upstream of the signal sequence or the TM domain [32]. Thus, it has been implicated that the TRAM may mediate the positioning of the signal sequence or the TM domain of a protein that contains a short N-tail during translocation and membrane insertion.

Sommer et al. reported that when TRAP expression was silenced by small interfering RNA (siRNA), the topogenesis of a model single-spanning membrane protein according to the positive inside rule became moderate, suggesting the involvement of TRAP in the topogenesis of membrane proteins in the mammalian cell [41].

4. Membrane environment

4.1. Membrane potential

Many studies have shown the effects of the membrane electrochemical potential on the translocation of charged residues of soluble domains of membrane proteins [42–45]. Especially, translocation of negatively charged residues in the loops of the membrane protein is shown to be highly sensitive to the membrane potential in *E. coli*. These studies suggest that the positive electrophoretic potential in the periplasmic side of the membrane may pull the negatively charged residues adjacent to the TM domain, thus influencing the proper orientation and membrane insertion of a protein. Further, electrostatic interaction between the negative cytoplasmic side membrane potential and positively charged residues of membrane proteins explains the pronounced positive inside rule observed for bacterial membrane proteins.

4.2. Lipids

Small multidrug resistance (SMR) proteins that work in pairs tend to a have minimal charge difference and distribution between loops in the cytoplasm and periplasm, thus, it is speculated that this family of proteins work as dual topology protein oligomers [46]. In order to keep a dual topology, either the charge distribution of soluble domains in the "in" or "out" side of the membrane should be neutral or the lipid composition should have compensatory effects. Examining these features across bacterial species, a bioinformatics analysis showed a correlation between biased charged residue distribution of SMR proteins and lipid composition, illustrating that lipid composition influences membrane topology [47].

Extensive studies led by Dowhan and his colleagues suggest that the balance between anionic and zwitterionic lipids in the bacterial membrane influences membrane topology by the electrostatic interactions between polar head groups of the lipids and charged residues in the soluble loops of membrane proteins and/or by mediating the assembly of membrane proteins [48–51]. The effects of lipids on the membrane topology of proteins are summarized in detail in a recent review [51].

5. Global view on topology of polytopic membrane proteins

A combination of experimentally verified C-terminal orientation and bioinformatics prediction has generated large data sets on the topology of polytopic membrane proteins in both *E. coli* and *Saccaromyces cerevisiae* [52,53]. These data show that the even number TM proteins are overwhelmingly more prevalent than the odd number TM proteins and that N_{in}–C_{in} orientations are more predominant than N_{out}–C_{out} orientations in both organisms. These observations suggest that a helix–loop–helix, a hairpin structure may be a common membrane insertion motif, in turn implicating that TM helix interactions may be important for membrane protein insertion and topogenesis.

6. Experimental tools to determine the membrane topology of proteins

Numerous bioinformatics programs are available for the prediction of membrane protein topology [54–57], however, the topology needs to be experimentally verified. There are a number of experimental tools, and most of these methods include a fusion of reporter proteins. Membrane proteins are tagged with a reporter, such as a fluorescent protein or an enzyme, whose activity depends on its subcellular location. Thus, the compartment-specific activity of the reporter and the accessibility of the reporter domain by antibodies, chemical labels, proteases indicate the location of a reporter fused to the part of the membrane protein. Caution should be taken that a fused reporter may influence targeting and folding of proteins, especially when fused in the N-terminus or internally. Although the C-terminal tagging also can cause the same problems, it is less detrimental, thus the C-terminus is a good choice to start with.

6.1. Fluorescence-based assays

6.1.1. GFP-PhoA or LacZ-PhoA fusion in bacterial cells

In bacteria, alkaline phosphatase A (PhoA) along with green fluorescent protein (GFP) or LacZ has been employed to deduce membrane protein topology [52,58–62]. PhoA is active only in the periplasm where it forms two disulfide bonds required for proper activity [63]. In contrast, GFP folds properly and fluoresces only in the cytosol [64]. β-Galactosidase (LacZ), similar to GFP, is active in the cytosol but not in the periplasm [65]. Thus, the measurement of PhoA activity and fluorescence intensity of GFP/LacZ activity gives complementary information on the membrane protein topology of a protein that is tagged with them. This method has been successfully used to screen the membrane topology of *E. coli* inner membrane proteome and secondary transporter proteins [52,59].

6.1.2. Glycosylatable GFP

Glycosylatable GFP (gGFP) is a variant of GFP with an engineered N-linked glycosylation site [66]. The engineered glycosylation site itself does not interfere with the GFP fluorescence intensity, but when glycosylated, it abolishes fluorescence. Thus, gGFP is not glycosylated and fluorescent in the cytosol, but is glycosylated and non-fluorescent in the ER lumen (Fig. 3). Fluorescence measurement and assessment of glycosylated state of a gGFP fusion protein by Western blotting allow the determination

of the localization of gGFP fusion part. This method has the potential to be applicable to all eukaryotic cells.

6.1.3. Fluorescence protease protection

In fluorescence protease protection (FPP) assay, GFP is fused to a membrane protein of interest and expressed in cells [67–71]. This method utilizes selective permeabilization of the cholesterol-rich plasma membrane but not the intracellular membranes by digitonin. Thus, treatment with digitonin followed by trypsin results in the selective loss of fluorescence of the GFP moiety that is exposed to the cytosol [72,73]. In contrast, the GFP is fluorescent if the GFP fusion part is located in the luminal side of a subcellular organelle as it is shielded by an organellar membrane. Hence, the differential fluorescence pattern upon selective membrane permeabilization and protease digestion provides information about membrane protein topology in intracellular compartments in the eukaryotic cell. The strength of FPP is that it is applicable to all subcellular organelles.

6.1.4. roGFP – luciferase fusion in mammalian cells

Similar to the GFP-PhoA system in bacterial cells, the use of redox sensitive GFP (roGFP) with luciferase as a complementary topology reporter system has been demonstrated in the mammalian cell [74]. roGFP forms a disulfide bond when exposed to an oxidative environment of the ER lumen and loses fluorescence [75–78]. Luciferase, on the other hand, folds properly in an oxidative environment [79,80]. Therefore, GFP fluorescence and luciferase activity together serve as a dual topology reporter such that high-GFP fluorescence and low bioluminescence indicates cytosolic localization whereas low-GFP fluorescence and high bioluminescence indicates the ER luminal localization [74].

6.1.5. BiFC and split GFP

Bimolecular fluorescence complementation (BiFC) uses split yellow fluorescent protein (YFP) [81,82]. In the assay, YFP is split into two, N- and C-terminal halves. Both N- and C-terminal halves need to be co-expressed in the same subcellular compartment for the formation of a fluorescent protein. Thus, examination of fluorescence after the expression of the membrane protein of interest tagged with the N-terminal part of YFP along with the C-terminal part of YFP targeted to the specific subcellular compartment gives topology information [83]. This BiFC assay has been used for the characterization of plant Golgi membrane protein topologies [84].

Split GFP, also known as self-assembling GFP (saGFP), is a variant of BiFC [85]. Here, GFP is divided into two separate peptides, the first peptide containing the beta strands 1–10 and the second peptide containing the beta strand 11. The affinity of the split peptides toward each other is higher than classic BiFC. When expressed separately, split GFP is not fluorescent. However, when two fragments are expressed together in the same compartment, these fragments are capable of assembling into a functional form of protein and exhibit fluorescence. Thus, the expression of a membrane protein of interest tagged with the C-terminal part of split GFP along with the N-terminal part of split GFP targeted to the designated compartment can be used as a topology reporter. Split GFP has been used to determine membrane topology of proteins in *E. coli* and *Plasmodium falciparum* [86,87].

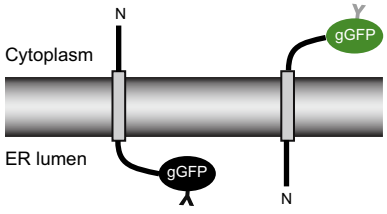

Fig. 3. Glycosylatable GFP is unglycosylated and fluorescent in the cytosol but is glycosylated and non-fluorescent in the ER lumen [66].

6.1.6. YFP binding assay

This method has been used to determine membrane topology of isoprenylcysteine carboxyl methyltransferase [88]. The rationale of the approach is as follows. YFP is tagged to a cytosolic Kras12V185V protein and a membrane protein of interest is tagged to a Ras-binding domain (RBD) of Raf-1. Two fusion proteins are co-expressed in the cell and visualized under a fluorescent microscope. When the RBD of a fusion membrane protein is exposed to the cytosolic side of the ER membrane, it interacts with Kras12V185V tagged with YFP, hence when assayed under a fluorescent microscope, the YFP fluorescence is found in the ER membrane. However, when the RBD is translocated across the membrane, YFP remains in the cytosol and the resulting fluorescence pattern resembles that of the soluble cytosolic proteins.

6.2. SDS–PAGE-based size-shift assays

A certain site of the membrane protein can be selectively modified or cleaved depending on the subcellular location. The modification or cleavage changes the size of the protein: thus, the migration pattern of the modified/cleaved protein differ from an intact protein on SDS–PAGE. Commonly used techniques are N-linked glycosylation and protease protection.

6.2.1. N-linked glycosylation in eukaryotic cells

Eukaryotic membrane proteins of the secretory pathway are co-translationally inserted into the ER membrane through the Sec61 translocon. The Sec61 translocon is associated with the oligosaccharyl transferase (OST) which recognizes an N-X-T/S (X can be any residue but proline) sequon on a nascent polypeptide and adds oligosaccharides on asparagine residue in the ER lumen [89,90]. Thus, if the N-linked consensus sequence is glycosylated, that portion of the protein must reside in the ER lumen. N-linked glycosylation of a protein can be assessed by endoglycosidase H (Endo H) treatment followed by SDS–PAGE. Each N-linked glycosylation adds approximately 2 kDa to the protein mass, thereby slowing down the migration of glycosylated proteins on the SDS-gel. As Endo H removes glycans from glycoproteins, an Endo H treated sample serves as a non-glycosylated protein control. The comparison of the gel mobility of Endo H treated and non-treated samples gives the glycosylated status of a protein, and the glycosylated soluble loop is deduced to the luminal side of the ER membrane [91–93].

N-linked glycosylation sites can easily be introduced into a protein by site-directed mutagenesis. However, it should be noted that the efficiency of glycosylation and the screening of endogenous sequons must be taken into account. The efficiency of glycosylation is known to be dependent on various factors, including the distance between the sequon and the end of the membrane, and the sequence context of the neighboring residues [94–99]. The strength of the technique is that it does not require additional tagging of a reporter protein and is applicable to all eukaryotic cells, but, this method is not applicable to mitochondrial membrane proteins.

6.2.2. Protease protection

Externally added proteases cleave only the exposed parts of a membrane protein, and the membrane embedded segments as well as the inside loops remain protected. By adapting this property of proteases, membrane protein topology can be elucidated. The non-specifically cleaving Proteinase K is widely used, but the proteases that recognize a specific cleavage sequence have the advantage of targeted proteolysis that leaves other parts (or

epitopes or reporters) of the protein intact. Such proteases are Factor Xa and TEV proteases. The cleavage sites for Factor Xa and TEV proteases are 4 and 7 residues, respectively: thus, these proteases are unlikely to disrupt the protein folding/structure, and their cleavage sites can be easily introduced by site-directed mutagenesis. The soluble loops between the TM domains in multi-spanning membrane proteins can be short and too close to the membrane, and inaccessible to proteases. Addition of a few residue cleavage sites can be useful in such a loop to increase the length of the loop, enhancing its chance of being recognized and cleaved. These proteases have been successfully used to determine topology of multi-spanning membrane proteins as well as mitochondrial protein [100–103].

6.3. Growth assays

Growth-based assays utilize compartment-specific reporters that lead to growth under selective conditions and are mainly used in microorganisms.

6.3.1. Antibiotic resistance

Bacterial cells exhibit resistance to antibiotics when they carry the resistance gene. The resistance gene product needs to be localized to the proper sub-cellular compartment to exhibit its function. For instance, β-lactamase needs to be present in the periplasm in order to support bacterial growth in the presence of ampicillin [104]. Another antibiotic resistance protein, chloramphenicol acetyltransferase supports cell growth in the presence of chloramphenicol only when it is located in the cytoplasm [105].

6.3.2. His4C in yeast

HIS4C gene encodes a catalytic domain of histidinol dehydrogenase which catalyzes the conversion of histidinol to histidine when located in the cytosol. When it is translocated to the ER lumen, it does not support growth of the *his4*Δ strain (His4 gene deletion strain) on the medium lacking histidine and containing histinol [106,107]. For the assay, membrane proteins are fused to His4C and transformed into a *his4*Δ yeast strain. Yeast transformants are assayed for growth on the selection plate containing histidinol but lacking histidine. This growth based assay has been successfully used to study the global membrane protein topology in *S. cerevisiae* and also genetic screening of genes involved in the ER membrane protein insertion and translocation [53,106].

6.4. Other methods

The other membrane topology assays, in general, take advantage of the selective permeability of the lipid bilayer. Antibody accessibility and specific residue labeling by chemical reagents belong to this category.

Antibodies and membrane impermeable chemical reagents only recognize the residues exposed to the antibody/chemical treated side of the membrane, whereas membrane permeable chemical reagents recognize residues in soluble loops on both sides of the membrane. These methods are followed by immunofluorescence and/or mass spectrometry for detection.

6.4.1. Antibody accessibility

The antibody accessibility test utilizes the binding of antibodies to the endogenous or tagged epitope of the protein. As antibodies cannot cross the lipid bilayer, they only recognize the epitopes exposed to the outside of the membrane. Epitope recognition is then followed by immunofluorescence for detection [108–110].

6.4.2. Cysteine accessibility assay

The cysteine accessibility assay, also known as the substituted cysteine accessibility method (SCAM), utilizes the modification of the thiol group on cysteine residues with different thiol-reactive labeling reagents [111–114]. The pre-requisite for the method is the generation of the cysteine-free version of a protein of interest. A cysteine residue is then engineered into designed site of a protein. Once the protein is expressed in the cell, thiol-reactive labeling reagents with different membrane permeabilities can be treated to label a test protein. Reagents such as 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS) and polyethyleneglycol maleimide (PEG-mal) do not cross the membrane, therefore only modify cysteines exposed outside of the membrane. Reagents that can cross the membrane such as *N*-ethylmaleimide (NEM) modify all cysteins except the ones embedded in the membrane. Thus, comparison of cysteine labeling patterns by two different labeling reagents gives detailed information on the locations of transmembrane regions and the positions of loops relative to the membrane. Typically, NEM is added to samples after pre-incubation with AMS. NEM cannot modify cysteine residues already labeled with AMS, therefore results in a different labeling pattern. The drawback to this assay is that the experimental procedure can be laborious and the removal of native cysteine residues may influence the protein structure. Cysteine labeling has allowed detailed examination of the effects of the lipid composition on membrane protein topology in combination with *E. coli* strains of different lipid compositions [112].

6.4.3. Oxidative labeling and mass spectrometry

Similar to the cysteine labeling assay, hydroxyl radicals (·OH) are employed to react with residues exposed to the solvent accessible environment. Hydroxyl radicals generated by photolysis of H_2O_2 react efficiently with sulfur atom on methionine and cysteine residues, resulting in 16 Da increase in peptide mass and can be detected by mass spectrometry [115,116]. Only the residues embedded in the membrane remain unmodified. This method has been used to verify membrane protein topology predictions [117].

7. Concluding remarks

Experimental determination of membrane topology is important in understanding membrane protein structure and function. A plethora of membrane topology reporter systems described here offers the guidance to the assessment of membrane protein topology in different model organisms and sub-cellular compartments of the eukaryotic cell. These methods can be used in combination with bioinformatics prediction programs to further enhance the accuracy of the membrane topology predictions.

Each membrane protein may contain different combinations of topogenic determinants (the number of charged flanking residues, the hydrophobicity and length of the TM domains, and the length and folding properties of soluble domains), and these features cooperate differently with the translocation components in a given membrane environment to finalize its membrane topology. It must occur during early stages of membrane insertion for the majority of membrane proteins. However, exceptions have been shown. Modulation of phosphatidylethanolamine content in the proteoliposomes caused changes in LacY membrane topology post-insertionally [118]. For EmrE, the entire protein topology was reoriented by a single positively charged residue in the C-terminal end of the protein [119]. Further, human aquaporin 1 protein was shown to be inserted as a 4 TM protein in the ER but acquired a final 6 TM topology at a later stage [120]. Therefore, these studies illustrate the plasticity of membrane protein topologies and dynamics of membrane protein topogenesis.

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