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Adaptation of low-resolution methods for the study of yeast microsomal polytopic membrane proteins: a methodological review

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Abstract

Most integral membrane proteins of yeast with two or more membrane-spanning sequences have not yet been crystallized and for many of them the side on which the active sites or ligand-binding domains reside is unknown. Also, bioinformatic topology predictions are not yet fully reliable. However, so-called low-resolution biochemical methods can be used to locate hydrophilic loops or individual residues of polytopic membrane proteins at one or the other side of the membrane. The advantages and limitations of several such methods for topological studies with yeast ER integral membrane proteins are discussed. We also describe new tools that allow us to better control and validate results obtained with SCAM (substituted cysteine accessibility method), an approach that determines the position of individual residues with respect to the membrane plane, whereby only minimal changes in the primary sequence have to be introduced into the protein of interest.

Introduction

Integral membrane proteins having two or more TMs (transmembrane domains) represent at least 10% of the approximately 6000 predicted genes of *Saccharomyces cerevisiae*. Although more than half of these polytopic proteins are functionally characterized and many carry out essential enzymatic reactions, it is often difficult to integrate them into metabolic flux diagrams as long as it is not known on which side of the membrane their catalytic site is located, whether substrates or products have to cross a membrane and hence whether the metabolic flux diagram should also include membrane transporters. Although protein crystallization and NMR are the methods of choice for obtaining high-resolution structures for membrane proteins, these methods are very labour intensive and even crystal structures may be distorted due to purification and crystallization constraints [1]. Protein crystallization also yields static structures, and is not well suited to follow the dynamic structural changes imposed, for example, by a changing lipid environment during vesicular transport, by post-translational modifications such as phosphorylation, by substrate binding, by association with other proteins or by changes in the membrane potential. Moreover, some proteins have been found to be inserted in several orientations in the membrane, a situation that also cannot be appreciated by crystallization studies alone (for review, see [2]). Fortunately, lower-resolution biochemical methods are available that allow for locating extra-membranous domains and even single residues with regard to the plane of the membrane and greatly facilitate the understanding of the dynamic molecular interactions and transitions a particular protein is undergoing. Indeed, even for proteins that have been crystallized, such low-resolution methods are used to understand their biogenesis and other dynamic aspects [3]. In the present review, we concentrate on the most popular methods in order to verify the position of extra membranous loops or particular residues. They include: fusion of C-terminally truncated target proteins to topology reporters; insertion of proteolysis sites; insertion of antigenic peptide tags; insertion of glycosylation motifs into the target protein; and SCAM™, a variation of the SCAM (substituted cysteine accessibility method), as applied for determining transmembrane segment orientation of polytopic membrane proteins [2,4,5]. Also, the occupation of natural glycosylation sites by N- or O-glycans, the detection of phosphorylated amino acids (phosphosites) and other post-translational modifications can sustain the establishment of membrane protein topology.

The choice of sites, i.e. where to add a topology reporter or to change/introduce amino acids or tags is usually guided by various algorithms that predict, on the basis of the primary structure, a likely membrane topology for a given protein. Based on the comparison of predictions with experimentally verified structures, the most recent of these algorithms usually correctly predicts 75–80% of the structures, but in many cases different algorithms produce different predictions. For instance, for the 1-acyl-glycerol-3-phosphate acyltransferase Slc1p of yeast, having very few TMs, the various algorithms used by TOPCONS [6], the most recent predictor, produce different topologies as shown in Figure 1. This indicates the

Key words: cysteine accessibility, dual topology reporter, N-ethyl-maleimide, tag insertion.
Abbreviations used: AGPAT, 1-acyl-*sn*-glycero-3-phosphate acyltransferase; DTR, dual topology reporter; endo H, endoglycosidase H; ER, endoplasmic reticulum; HA, haemagglutinin; NEM, *N*-ethylmaleimide; NES, *N*-ethylsuccinimide; PEG, poly(ethylene glycol); mPEG, methoxy-PEG; SCAM, substituted cysteine accessibility method; TM; transmembrane; UBI-mal, ubiquitin maleimide.
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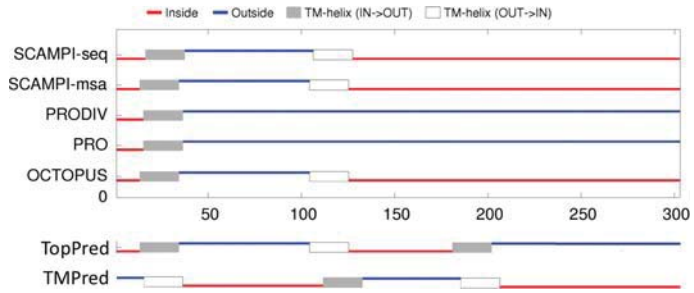
Figure 1 | Predicted topology of Slc1p

Predictions given by five TOPCONS algorithms, SCAMPI-seq, SCAMPI-msa, PRODIV, PRO and OCTOPUS algorithms, are shown on top with cytosolic loops in red, luminal ones in blue, TMs as white or grey rectangles [6]. The OCTOPUS prediction is identical with the TOPCONS integrated prediction, which latter takes into account these five algorithms plus the ΔG_{mi} and ZPRED algorithms (not shown). Less recent algorithms such as TMHMM, HMMTOP and PSORT II predict the same topology as PRODIV; SOSUI, TopPred and TMPred predict either the same as OCTOPUS, or the alternative topologies shown at the bottom.



need for experimental confirmation. In the present article, we will discuss the respective advantages and disadvantages of these low-resolution methods.

Critical evaluation of different methods

Insertion of a *SUC2-HIS4C* DTR (dual topology reporter)

Insertion of a *SUC2-HIS4C* DTR into a predicted hydrophilic loop of a target protein of the secretion pathway, thereby truncating the C-terminal sequences coming after the insertion site, can reveal whether this hydrophilic loop resides in the cytosol or the extracytosolic space [7]. Its invertase (Suc2p) fragment is N-glycosylated only when localized in the ER (endoplasmic reticulum) lumen, its His4Cp fragment complements the His auxotrophy of his4Δ cells only when located in the cytoplasm. Its HA (haemagglutinin) tag allows for detection on Western blots. After the transfection of the constructs into his4Δ cells, one can determine the His prototrophy of transfectants and monitor the mobility of the HA-tagged target protein before and after removal of N-glycans using endo H (endoglycosidase H). Topologies for many membrane proteins such as Sec61p, Pmt1p, Der3/Hrd1p, Lcb4p, Dpp1p, Lpp1p, Doa10p and Teb4 have been established using this approach [8]. For mammalian cells, one may also attach a simpler reporter with only N-glycosylation sites at the C-terminal truncation site. One may also choose to translate the mRNAs of such constructs *in vitro* in the presence or absence of rough microsomes [9]. As long as the target protein does not have a cleavable N-terminal signal sequence, the addition of micrososomes to the *in vitro* translation system will lead to a reduced mobility on SDS/PAGE/Western blot because of the addition of N-glycans, but only if the C-terminal reporter has been translocated to the luminal side. However, for many eukaryotic polytopic proteins, not all TMs are immediately inserted into the membrane during the translation/translocation process, so that the location of C-terminal reporters added to truncated target proteins is not necessarily indicative of the final location of the corresponding hydrophilic loop, but rather suggests its potential temporary position during membrane insertion of the protein [10]. Indeed, the three-dimensional crystal structures of multispan membrane proteins demonstrate that TMs can even have positive free energy changes for membrane insertion (ΔGs_{mi}) and suggest that certain TM domains may depend on interactions with other parts of the protein for proper partitioning into the membrane [10–17]. Nevertheless, DTR remains a very valuable tool for predicting the location of loops placed downstream of a TM having a relatively high overall hydrophobicity and sufficient length to span the entire thickness of the membrane [14]. For instance, DTR correctly predicted the position of loops after the strongly hydrophobic TMs 1, 3, 4, 6 and 8 of Sec61p, but made wrong predictions for loops following TMs of lower hydrophobicity [18,19].

Although the truncation required for the DTR approach usually destroys the normal function of a membrane protein, it is of paramount importance for all the other methods discussed below to only analyse modified alleles that are fully functional, since normal functionality attests that the structure of the modified allele is similar to the physiological structure.

Insertion of proteolysis sites into epitope-tagged target proteins

Insertion of proteolysis sites into epitope-tagged target proteins allows to probe accessibility of these sites by treating microsomes or other topologically defined membrane vesicles with protease and to evaluate if cleavage had occurred using Western blotting. Proteases used in this context are Factor Xa (specific for the sequence IEGR), TEV [tobacco etch virus specific for EXXYXQ(G/S)] [3,20] or chymotrypsin, which cleaves the E1 epitope from corona virus [21]. Cleavage of a microsomal target protein in absence of membrane permeabilization signifies a cytosolic location of

the protease site. The membrane topology of Gap1p, Lcb1p, Lag1p, Tsc13p, Sec61p, aquaporin or Gwt1p was investigated using this technology [18,21–26]. In particular, some incorrect topologies indicated by DTRs inserted after TMs of low hydrophobicity could be corrected using insertions of Factor Xa sites [18,19]. It is obvious that only a positive result, i.e. the efficient cleavage of a site by vectorially added protease is informative, whereas inefficient cleavage may simply be due to an insert that is not surface exposed or in a conformation that is not recognized by the protease. The relative small size of inserted protease sites allows for the study of not only the final membrane topology, but also the structure of folding and insertion intermediates of proteins [3,27].

Antigenic peptide tags

Antigenic peptide tags of various sizes have been inserted into target proteins, e.g. a 30-amino-acid long epitope from coronavirus E1 glycoprotein, FLAG, Myc, 1×, 2× or 3×HA, VSV-G (vesicular stomatis virus glycoprotein) tags and others [8,21,28]. A multitude of protocols can be employed for detection of tags. Amazingly, the insertion of large peptide sequences in many cases leaves the function of the protein intact. If the protein resides in the plasma membrane of mammalian cells, tags can be visualized using fluorescent antibodies on intact cells or cells permeabilized with low concentrations of detergent [28]. For target proteins residing in the ER or other organelles, paraformaldehyde-fixed cells can be permeabilized with digitonin (1–5 μg/ml) that selectively permeabilizes the plasma membrane or with a slightly stronger detergent (0.01–1% Triton X-100 or 0.1% saponin), which additionally permeabilizes the internal membranes [29–33]. In this way, light microscopy can distinguish tags residing in cytosolic loops, visible already after digitonin treatment, from tags in luminal loops, detectable only after Triton X-100 permeabilization. In mammalian as well as yeast cells, the position of tags can also be verified after proteolytic digestion of intact cells, microsomes or other organelles, followed by immunodetection of the remaining tagged fragments using Western blotting [8,21].

Insertion of glycosylation motifs without any truncation of the target protein

Insertion of glycosylation motifs without truncation of the target protein was used successfully to determine the topology of Gap1p [22], Lcb1p [23], Lag1p and Lac1p [24], Tsc13p [25], a human AGPAT (1-acyl-*sn*-glycero-3-phosphate acyltransferase) [34] and Gwt1p [26] and others. These studies used either a 53-amino-acid-long fragment of invertase containing three N-glycosylation sites, the so-called S2A-cassette [23–26], or just the insertion of an Asn-Xaa-Ser/Thr N-glycosylation consensus sequence [29,34]. The glycosylation of the thus modified protein can be observed by treating the extract with endo H, which removes the N-glycans and thereby causes a mobility shift on SDS/PAGE/Western blot of the target protein. Again, the insertion of large peptides in many cases leaves the function of the protein intact.

A theoretical criticism of this approach derives from the fact that during translocation certain internal hydrophobic sequences can invert the in/out orientation of a preceding TM helix, and that the frequency of such an inversion is strongly reduced if an N-glycan is added to the loop lying between the two hydrophobic sequences [35,36]. Thus addition of N-glycoslation sites theoretically may induce abnormal topology, which may not be recognized as such if it occurs in parts of the proteins that are not functionally important.

Another possible difficulty is the following: one would expect that the insertion of a 53-amino-acid-long fragment containing well-characterized glycosylation sites would yield clear cut 'all or none' answers, but this often is not the case [22,24–26]. This difficulty, however, is encountered with all methods discussed in the present article. For all instances where it is possible, one has to be careful to make sure that the biological function is truly wielded by the subpopulation of target proteins having the prevalent topology revealed by the biochemical method. The behaviour of control proteins of known topology also can help in the interpretation of ambiguous results.

Of course, when naturally glycosylated asparagine residues are identified, one can assume that one obtains unambiguous topological information about the loop orientations.

SCAM™

Of all the methods discussed in the present paper, cysteine chemistry has the highest resolution in that water accessibility of individual cysteine residues can be determined. Since its introduction by Koshland and co-workers [4], site-directed thiol chemistry has been exploited in many ways, such as to identify the residues that line the pathway taken by solutes across a membrane pore [5,37,38] and the amino acids forming the charge and solute specificity filters as well as the widths of these filters in such pores [5,39]; to monitor the binding of substrates to enzymes and transporters and the ensuing conformational changes; to study the topology of membrane proteins in different physiological states, for instance in different lipid environments [40]; to identify the residues involved in protein–protein interactions [41]; and to follow the transit of the passenger domain through the C-terminal β barrel of bacterial autotransporters [3]. Maybe most spectacularly, SCAM has allowed following of the translocation process of proteins into the ER, i.e. the exit of a peptide from the ribosomal protein tunnel, later from the Sec61p translocation pore, the steric conformation of the Sec61p pore in different detergents, the shielding of the nascent chain by the ribosome, the dependence of this shielding on peripheral membrane proteins and the inversion of a signal sequence inside the translocon [42]. The reactivity of a given cysteine in the target protein indicates that it is accessible to the water-soluble alkylating agent used, it is not buried inside the water-excluding compact parts of the folded protein, it is not covered by interacting proteins, it

is not bonded by disulfide bridges and that the pK_a value of the thiol group allows deprotonation, as the reaction usually involves a nucleophilic attack by an -S^- anion at the alkylating agent. Information is gleaned both when substrate–ligand complexes block access of the alkylating reagent to a given cysteine, or when the alkylation of a given cysteine blocks substrate–ligand binding. When membrane proteins are studied, reactivity also means that there is no membrane barrier blocking the access of the hydrophilic alkylating agent to the cysteine under investigation. Most experiments use biotinylated, radioactive, fluorescent or mass-tagged derivatives of NEM (*N*-ethylmaleimide) as alkylating reagents [2]. For the elucidation of membrane topologies, they may be used in combination with highly impermeant, non-tagged maleimide derivatives, which are used as blocking agents.

Adaptations of SCAM™ for yeast microsomes

SCAM™ has also been used to determine the topology of polytopic proteins located in the vacuole and the endoplasmic reticulum [32,43–46] and our laboratory has recently employed these techniques to assay the topology of yeast microsomal acyltransferases [8]. SCAM classically distinguishes three categories of cysteine residues in membrane proteins: those at the water accessible surfaces on either the cytosolic or the luminal side of the membrane, and those that are 'buried', in other words, do not react because they are embedded in the water-excluding interior of the lipid bilayer or because they are hidden in the water-free interior of the protein. Cysteine residues may also be hidden by peripheral membrane proteins interacting with the target protein, but such proteins can be removed by washing the membrane in 0.5 M salt or 2.5 M urea, apparently without destroying the orientation and tightness of microsomal vesicles [42,44]. Ideally, proteins with only one cysteine residue should be analysed. This can be achieved in many cases by generating a version of the target protein wherein all cysteine residues have been replaced by alanine or serine and which retains full activity. Into such a pseudo wild-type [5], one then can substitute cysteine residues for amino acids at selected positions. However, it is not always possible to remove all cysteine residues without affecting the protein function. In those cases one still can perform SCAM if functionality is preserved in alleles that only have buried, non-reactive cysteine residues and in which residues of interest can then be replaced by cysteine residues [5,45]. The detection rate of substituted cysteine can be increased by choosing residues that have a high probability of surface exposure as calculated by NetSurfP (http://www.cbs.dtu.dk/services/NetSurfP/).

To distinguish luminal from cytosolic cysteine residues, microsomes (which typically have their cytosolic side outwards) are treated in two conditions, one allowing access of the alkylating reagent from only the cytosolic side, the other from both sides. Several methods have been used to generate this differential access in microsomes. One is to open the membrane barrier with non-denaturing detergent [43–45]. Especially when one works with proteins having more than one cysteine, one has to worry that detergent may not only remove the membrane barrier, but also induce subtle changes in the protein conformation, thereby giving access to cysteine residues that are buried when the protein is in its natural habitat, the membrane. An alternative way is to add the pore-forming peptide melittin [47], but it is much less efficient than non-denaturing detergent and its effects on protein structure also are not predictable [42]. Another approach utilizes permeable and impermeable alkylating agents as well as a denaturing detergent in combinatorial ways. An example is shown in Table 1 [32,46]: alkylation in four different conditions can result in three different patterns, which are indicative of whether a given cysteine is buried, exposed at the cytosolic or at the luminal surface. Alternatively, one may first treat with either impermeant or permeant untagged alkylating agents, thus blocking accessible cysteine residues either from one or both sides of the membrane. Then the protein is denatured with SDS and mPEG {methoxy-PEG [poly(ethylene glycol)]} is added to derivatize remaining cysteine residues [45].

We recently modified these classical protocols in an attempt to increase the probability of correct topological assignments of residues to the ER lumen or the cytosol. These modifications include: the introduction of Gpi8p instead of Kar2p as a luminal control protein, the exploration of the influence of NEM concentration in the combinatorial approach shown in Table 1, and the development of a new mass-tagged maleimide derivative.

Kar2p/BiP (immunoglobin heavy-chain-binding protein) is a soluble chaperone of the ER lumen. It is classically used as a control to show that supposedly permeant alkylating agent does have access to luminal cysteine residues, and that the supposedly non-permeant alkylating agent does not, i.e. that microsomes are not leaky [46]. In our hands, using the method described in Table 1, it was often impossible to show that NEM reaches the cysteine residue of Kar2p when added to intact microsomes. As shown in Figure 2(A), NEM at 5 or 10 mM, at 0°C, could not prevent subsequent derivatization of Kar2p with mPEG after SDS denaturation (lanes 4 and 7), arguing that NEM does not penetrate our microsomes or that it takes denaturation of Kar2p to make its cysteine accessible. When the NEM incubation was carried out at 25°C (following the protocol described in [32]), NEM still could not block the cysteine residues of Kar2p quantitatively (Figure 2A, lanes 8 and 9). Gpi8p seems to be a better control. Gpi8p is the core protease of the five-subunit GPI transamidase complex [48]. It is a type I 50-kDa membrane glycoprotein having all of its four cysteine residues on the luminal side of the ER membrane and only 14 amino acids in the cytosol [49]. Up to three of the four cysteine residues can be derivatized with mPEG in presence of SDS at room temperature, i.e. in conditions where the membrane is dissolved and the protein is partially denatured (Figure 2B, lane 3). Figure 2B demonstrates that

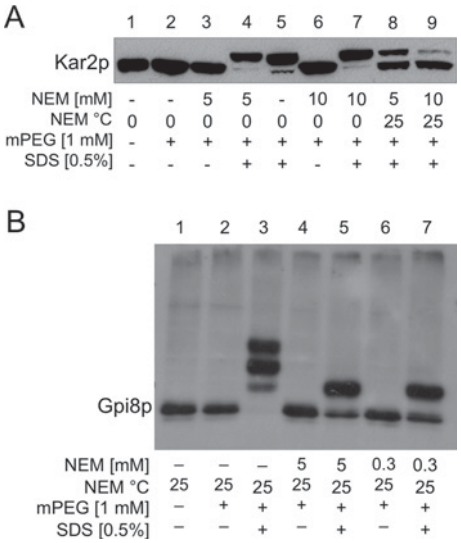
Table 1 | **Workflow of the combinatorial approach [32,46]**

Four tubes with microsomes are successively incubated with or without NEM, washed, then incubated with mPEG in the presence or absence of SDS. The expected mass shift for differently located cysteines is indicated at the right-hand side.

	Experimental work flow				Expected result		
Sample	Incubation 1 permeable (NEM)	Wash microsomes	Incubation 2 Non-permeable mPEG	SDS in Incubation 2	Cytosolic cysteine	Lumenal cysteine	Buried cysteine
1	–	+	+	–	Shift	No shift	No shift
2	+	+	+	–	No shift	No shift	No shift
3	–	+	+	+	Shift	Shift	Shift
4	+	+	+	+	No shift	No shift	Shift

Figure 2 | **Kar2p compared with Gpi8p as control of microsomal integrity**

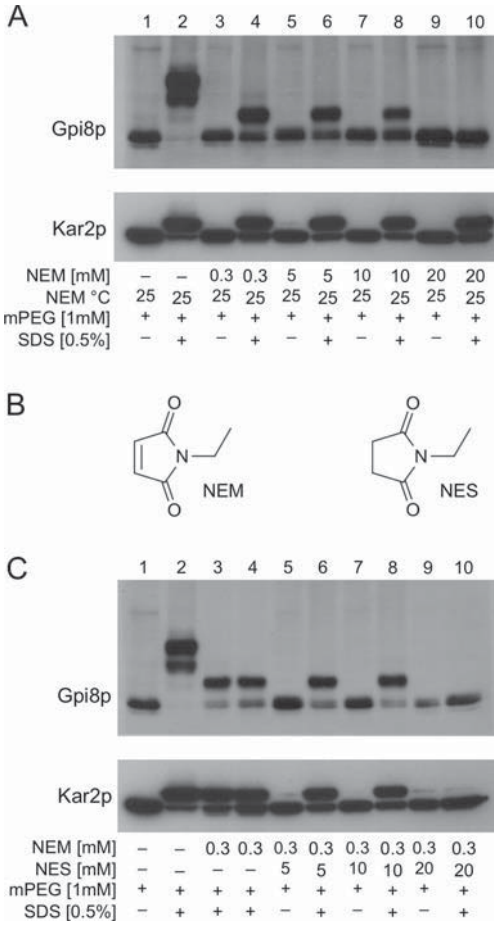
(**A** and **B**) Microsomes (100 μg) from BY4742 cells containing Gpi8p–FLAG on a centromeric plasmid were incubated for 30 min with 0–10 mM NEM and at indicated temperature in buffer A (0.2 M sorbitol, 5 mM $MgCl_2$ and 100 mM Na_2HPO_4/NaH_2PO_4, pH 7.2) containing 1 mM PMSF and a Roche EDTA-free protease inhibitors cocktail as described [8]. NEM was removed by washing the membranes twice in buffer A by sedimentation (16 000 ***g*** for 30 min at 0°C). Microsomes were then incubated for 10 min at 0°C with 1 mM mPEG, except for the negative control (lane 1). SDS was added (0.5%) as indicated, and the samples were incubated for another 25 min at room temperature for the samples with SDS, at 0°C for the others. The reaction was quenched by 40 mM dithiothreitol and reducing sample buffer and heated for 15 min at 60°C. Derivatization patterns were visualized by Western blotting with anti-Kar2p (**A**) or anti-FLAG (**B**) antibody.



NEM (0.3 mM at room temperature) reaches the ER lumen where it can completely derivatize two cysteine residues of Gpi8p, whereas the third one is only marginally blocked, even at higher NEM concentrations (lanes 4–7). The control also shows that microsomes efficiently exclude mPEG (lane 2), i.e. that they are not leaky. The third buried cysteine residue

Figure 3 | **High concentrations of NEM may cause protein denaturation**

(**A**) Microsomes were prepared and treated as above in Figure 2, using NEM concentrations up to 20 mM at 25°C. The Western blot was first probed with mouse anti-FLAG antibody to detect Gpi8p and then re-probed with rabbit anti-Kar2p. (**B**) Structures of NEM and NES. The C = C double bond of NEM reacts specifically with the thiolate anion on the cysteine, forming a stable thioether bond, whereas NES does not react. (**C**) Microsomes were prepared and incubated as above, with NEM and increasing amounts of NES added simultaneously as indicated. In lane 4, the incubation with NEM was 60 min instead of 30 min as in other lanes.



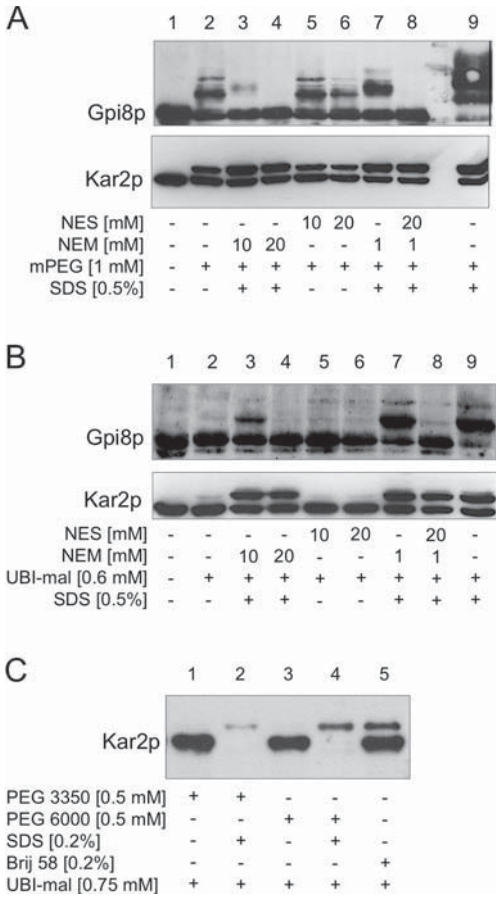
of Gpi8p can, however, be derivatized by NEM when added at 20 mM (Figure 3A, lanes 9 and 10), whereas Kar2p was still not alkylated at this concentration.

Owing to the concentration-dependence shown in Figure 3(A), we wondered whether NEM, beyond acting as an alkylating agent, also denatures proteins because of its amphipathic properties (Figure 3B). To this end, we tried to mimic the potential detergent effect of high concentrations of NEM using NES (*N*-ethylsuccinimide), a closely related compound lacking alkylating activity (Figure 3B). Indeed, in the presence of 20 mM NES, low amounts of NEM (0.3 mM) could derivatize the third buried cysteine of Gpi8p, although NEM alone at this low concentration had no effect with regard to this third cysteine (Figure 3C, lane 10 compared with lane 3). Together, these results suggest that NEM, beyond being a permeable alkylating agent, may possibly alter the structure of target proteins. However, this is only observed with high concentrations of NEM at room temperature, so that the protocol by Liu et al. [46] using 5 mM of NEM is probably safe if the NEM incubation is performed on ice. Generally speaking, when having to decide whether a cysteine residue not reacting with mPEG in the absence of detergent is luminal or buried, it would appear that the lower the temperature and the lower the NEM concentration at which it can be blocked, the higher the probability that it truly is luminal. Indeed, many high-quality SCAM studies have observed reactions rates or have titrated the alkylating reagent in order to obtain more reliable data.

Classical SCAM uses small membrane impermeant alkylating agents, to which the mammalian ER membrane is more permeable than other cellular membranes [50]. Therefore we initially chose as a tagging agent mPEG, which does not penetrate the mammalian ER at 4°C [50]. However, in certain experiments we found that yeast ER membranes were permeable to mPEG, at least at 25°C (Figure 4A, lane 2). As PEG is soluble not only in water but also in benzene and dichloromethane, we wondered whether its linear form can permeate yeast microsomal membranes. Branched PEG molecules may behave differently, but they were not available to us. We therefore developed an alternative mass tag by coupling a maleimide with ubiquitin, a 76-amino-acid-long protein (8.5 kDa), which does not contain any cysteine residues but seven lysine residues. Its conjugation with the heterobifunctional cross-linker sulfo-EMCS [*N*-(6-maleimidocaproyloxy)sulfosuccinimide], carrying two different chemical groups, reactive towards thiol and primary amino groups respectively, yielded a highly water-soluble, entirely membrane-impermeable cysteine labelling reagent, UBI-mal (ubiquitin–maleimide) [8]. Sulfo-GMBS [*N*-(4-maleimidobutyryloxy)sulfosuccinimide], a derivative with a slightly shorter spacer yields an UBI-mal with similar properties. As can be seen in Figures 4(A) and 4(B), microsomes that were penetrated by mPEG were tight with regard to UBI-mal (lane 2). We wondered whether PEG by itself would be able to disrupt the membrane, but, as shown in Figure 4(C), this did not seem to be the case.

Figure 4 | ER membrane permeability to mPEG and UBI-mal

(**A** and **B**) Microsomes were incubated at 25°C with mPEG (**A**) or UBI-mal (**B**) and processed by Western blotting as in Figure 3. (**C**) Microsomes were preincubated for 15 min with PEG 3350 or PEG 6000 and detergents (SDS, BrijS8) at 25°C as indicated. Then UBI-mal was added and samples further incubated for 30 min at 25°C. Samples were processed as above.



In conclusion, if many different methods have been developed to determine the topology of membrane protein *in situ*, taking into account their physiological environment and dynamic state, none of them can be considered as sufficient in itself. All methods have advantages, as well as serious drawbacks. A membrane topology can be established with a satisfactory degree of certainty only through the combination of several approaches and, as shown by the comparison of Kar2p and Gpi8p, carefully controlled conditions are necessary to tailor each experiment to the protein under investigation.

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