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Exploratory study about the topology of transmembrane proteins in *Saccharomyces cerevisiae*

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Transmembrane (TM) proteins participate in many important biological processes ranging from small-molecule transport to complex signalling pathways. However, the elucidation of their structure by crystallography is challenging and not always feasible. Thus, experimentally confirmed information about the precise membrane topology of many integral membrane proteins is limited. The focus of this thesis was to develop crosslinking-based approaches to determine the topology of resident proteins of the endoplasmic reticulum (ER), using *Saccharomyces cerevisiae* as a eukaryotic model system.

Initially, the membrane impermeable crosslinker DTSSP (3,3′-dithiobis(sulfosuccinimidyl propionate)), which reacts with primary amines was selected to probe cytosolically exposed lysine residues of microsomal proteins. However, using different well-characterized membrane proteins as controls, it became clear that DTSSP not only reacted with cytosolically exposed lysines but also with luminal ones. These observations led us to conclude that the crosslinker DTSSP compromised the integrity of the ER membrane. Based on this, we then tested whether DTSSP would react with phospholipids containing primary amines, such as phosphatidylethanolamine (PE) or phosphatidylserine (PS). Therefore, we generated a double mutant yeast strain in which PS production was blocked, and PE production decreased. Using microsomes generated from this double mutant (*cho1Δ dpl1Δ*) we indeed observed that DTSSP at concentrations higher that 1.5 mM reacted with phospholipids and hence compromised membrane integrity. In search for a more hydrophilic and chemically more bulkier crosslinker, we tested whether a polyethylene glycol (PEG)-based crosslinker, containing a dithiol succinimide ester as reactive group, could be employed as a topology probe. After crosslinking, the 5000 Da PEG could be eliminated by reduction of the disulfide bond and the modified lysines identified by mass spectrometry. However, even with this bulky and hydrophilic crosslinker, both cytosolic as well as ER luminal lysines were modified, thus membrane integrity still got compromised.

Finally, we tested a crosslinker that can modify both lysine and cysteine residues, Sulfo-EMCS (6-maleimidocaproic acid sulfo-*N*-succinimidyl ester). In this approach, cysteines in the membrane proteins were first protected by derivatization with iodacetamide. After crosslinking of microsomal proteins with Sulfo-EMCS, a biotinylated peptide containing a single free cysteine residue was added to react with the succinimide group of Sulfo-EMCS. The resulting biotinylated membrane proteins were then enriched using avidin-agarose beads and analysed by Western blotting. This approach finally allowed to differentiate between ER luminal and cytosolically exposed lysine residues and hence Sulfo-EMCS can be used as a topological probe.

In parallel to the development of these crosslinking probes, software tools for the analysis of the resulting crosslinked peptides were evaluated. Among the different packages tested, Sim-XL was identified as providing the most sensitive algorithm, whereas pLink provided the most precise results, i.e., the fewest false positives.

In the final part of this thesis, we examined whether protein ubiquitination could be explored as a topological indicator. Therefore, ubiquitination profiles of membrane proteins from wild-type cells were compared to those of a triple mutant cells lacking the canonical ubiquitin ligases (*hrd1Δ doa10Δ asi1Δ*). Consistent with a cytosolic action of these ubiquitin ligases, we found that mostly cytosolically exposed lysine residues were ubiquitinated in microsomal membrane proteins from wild-type cells. In the triple mutant cells, we found an abundance of ubiquitinated permeases, which were mostly ubiquitinated in their N-terminal loop. Taken together, the work presented in this thesis serves as a first step towards the establishement of experimental tools to probe and characterize the topology of integral membrane proteins, *in vitro* through chemical crosslinking, and *in vivo* through the analyis of ubiquitinated peptides.

Jury:

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