Fakultät für Biologie

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Offen im Denken

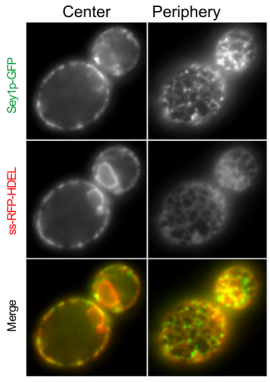
Analysis of Sey1p-Mediated Homotypic Endoplasmic Reticulum Fusion

Introduction

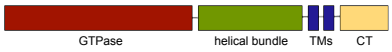
The endoplasmic reticulum (ER) is an essential membrane-bounded organelle found in all eukaryotic cells. It plays an important role in the biosynthesis, modification, quality control and transport of secretory and membrane proteins, lipid and steroid synthesis, intracellular calcium homeostasis, and cellular detoxification. The ER forms a highly dynamic network and is comprised of the nuclear envelope, peripheral ER sheets, and a network of highly curved membrane tubules.

Homotypic ER fusion is required to maintain the ER as one continuous network. In metazoans, this process appears to be mediated by the atlastins, which are dynamin-like GTPases anchored to the ER membrane. In plants and fungi, no sequence orthologs of Atlastin can be found. However, these organisms contain functional orthologs called RHD3 (root hair defective 3) *in A. thaliana* and Sey1p (synthetic enhancer of YOP1) in *S. cerevisiae*.

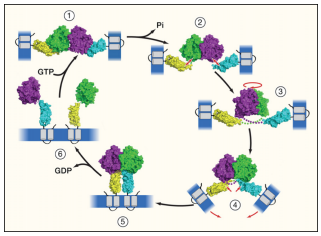
In this thesis, a purification protocol producing enzymatically active, detergent solubilized Sey1p in high purity and high yields has been developed. Sey1p was reconstituted into artificial membranes and the fusion reaction was characterized *in vitro*. The results of this thesis will help to elucidate the mechanistic details of Sey1p-mediated membrane fusion and form the starting point for the structural characterization of Sey1p.



Localization of Sey1p (Hu *et al.*, 2009)
Sey1p localizes to the tubular ER in punctae, sometimes to three-way junction of the ER tubles. Sey1p is mostly absent from the nuclear envelope.



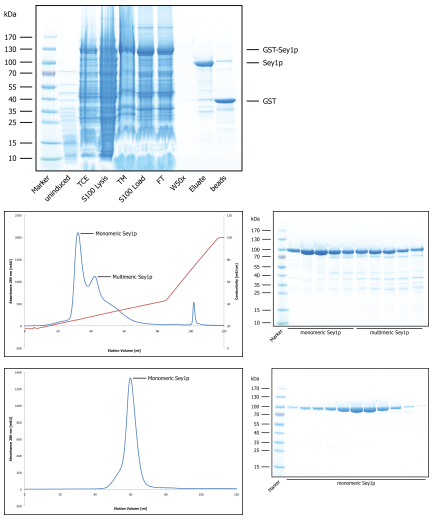
Domains of Sey1p
Sey1p consists of an N-terminal GTPase domain, a helical bundle domain, two closely-spaced transmembrane segments (TMs), and a C-terminal tail (CT). The TMs anchor Sey1p to the ER membrane with the GTPase domain, the helical bundle domain, and the CT facing the cytosol.



Model of the Homotypic Fusion Reaction Mediated by the Altastins (Hu *et al.*, 2011)
Two Atlastin molecules sitting in apposing membranes dimerize in a GTP-dependent manner (1). Upon GTP hydrolysis and phosphate release, the linker domain between the helical bundle and the GTPase domain is extended (2), allowing a rotation of the GTPase domains (3). Upon a 180° twist, the helical domain of one molecule is captured by the other molecule's GTPase domain (4), which pulls the apposing membranes together and leads to fusion (5). Upon GDP release, the Atlastin molecules dissociate and can participate in another fusion event (6).

Purification of Detergent Solubilized Sey1p

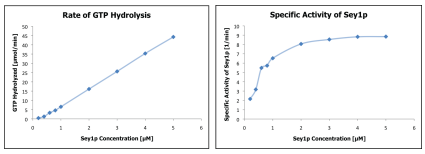
A codon-optimized construct with an N-terminal GST-tag and a SUMO-cleavage site was recombinantely expressed in *E. coli*. Upon cell lysis, membranes were purified by high speed centrifugation. The membrane protein Sey1p was solubilized in n-Dodecyl-β-D-Maltopyranoside (DDM). Sey1p was affinity purified and eluted by the addition of the SUMO protease Ulp1p. Subsequently, Sey1p was further purified by anion exchange chromatography and gel filtration, resulting in high purity and high yields.



Purification of the Membrane Protein Sey1p
Top row: SDS-PAGE gel with different steps from the affinity purification. The middle and bottom row show the chromatograms and SDS-PAGE gels of the fractions of anion exchange chromatography and gel filtration, respectively.

GTPase Activity of Sey1p

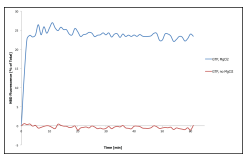
The GTPase activity of Sey1p increased in a protein concentration-dependent manner. This is not the case for Atlastin (Orso *et al.*, 2009), but assembly-stimulated increase of GTPase activity is a major hallmark of Dynamin 1 (Stowell *et al.*, 1999).



GTPase Activity of Sey1p
The GTPase activity of Sey1p was determined. 400 µM GTP were incubated with varying concentrations of Sey1p. Upon addition of $MgCl_2$, the rate of GTP hydrolysis was determined (left). The specific activity of Sey1p was calculated (right).

Fusion Activity of Sey1p

Sey1p was reconstituted into defined liposomes. Donor liposomes contained fluorophores coupled to lipids at a quenching concentration. Upon fusion with acceptor liposomes, dequenching could be measured. The fusion reaction was started by the addition of GTP. Upon addition of GTP, the fluorescence signal increased to 25% of the total NBD fluorescence. The kinetics of the reaction are very fast, as the signal plateaus after approximately 1 min. The reaction is both GTP and $MgCl_2$ dependent.

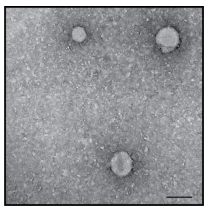


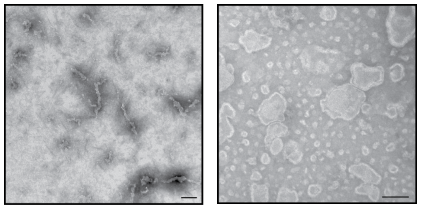
Fusion Assay of Sey1p Reconstituted into Liposomes
Sey1p was reconstituted into preformed liposomes at a molar ratio of protein:lipids of 1:200. Fusion caused dequenching of the fluorescent lipids of the donor proteoliposomes and was measured over time.

Electron Microscopy of Proteoliposomes

Liposomes, proteoliposomes containing Sey1p, and proteoliposomes after incubation with GTP and $MgCl_2$ were examined by transmission electron microscopy (TEM). Samples were negative stained using 1% uranyl acetate.

The liposomes had a more or less uniform shape and size with a diameter of approximately 100 nm. After reconstituting liposomes with Sey1p, the proteoliposomes appeared to adopt a tubular shape. The proteoliposomes had a diameter of about 20 nm and a length of 100 to 300 nm. Proteoliposomes that were incubated with GTP and $MgCl_2$ showed an unusual morphology. Some proteoliposomes seemed to have fused as there were proteoliposomes of sizes bigger than 100 nm. Besides this population of diverse, bigger proteoliposomes, many small vesicles with a diameter of 20 nm or even smaller could be observed. These small vesicles could be caused by membrane fission and could be a byproduct of the fusion reaction.





Transmission Electron Microscopy of Proteoliposomes
Liposomes (top), proteoliposomes with reconstituted Sey1p (bottom left), and proteoliposomes after incubation with GTP and $MgCl_2$ (bottom right) were stained with 1% uranyl acetate and analyzed by TEM. Scale bar: 100 nm.

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