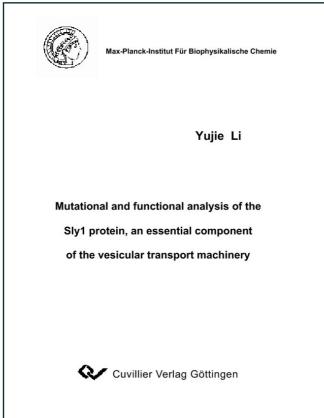


Yujie Li (Autor) Mutational and functional analysis of the Sly1 protein, an essential component of the vesicular transport machinery



https://cuvillier.de/de/shop/publications/2235

Copyright: Cuvillier Verlag, Inhaberin Annette Jentzsch-Cuvillier, Nonnenstieg 8, 37075 Göttingen, Germany Telefon: +49 (0)551 54724-0, E-Mail: info@cuvillier.de, Website: https://cuvillier.de

CONTENTS

1 INTRODUCTION	1
1.1 Vesicular transport in eukaryotic cells	1
1.2 Bi-directional protein transport between the ER and the Golgi	3
1.2.1 COPII vesicles (Table 1.1)	4
1.2.2 COPI vesicles (Table 1.2)	4
1.3 Budding	7
1.3.1 Coat assembly	7
1.3.2 Cargo selection	9
1.4 Tethering, docking and fusion	10
1.4.1 Tethering factor	11
1.4.2 Ypt/Rab GTPases	11
1.4.3 SNAREs	14
1.4.4 Sec1/Munc18 family (Table 1.3)	17
1.4.3.1 Yeast Sly1p	19
1.4.3.2 Conserved structures of SM proteins	20
	20
2 MATERIALS	24
2.1 Bacterial E.coli strains (Table 2.1)	24
2.2 Yeast strains (Table 2.2)	24
2.3 Plasmids	25
2.3.1 Yeast vectors (Table 2.3)	25
2.3.2 SLY1 constructs (Table 2.4)	26
2.3.3 Protein expression constructs (Table 2.5)	28
2.4 Oligonucleotides	29
2.5 Antibodies	36
2.6 Growth media	37
2.6.1 Media components	37
2.6.2 Bacterial media	37
2.6.3 Yeast media	38
2.7 Frequently used buffers and solutions.	39
2.8 Chemicals	40
2.9 Enzymes and Kits	41
2.10 Disposable supplies	41
2.11 Laboratory gadgets	42
3 METHODS	44
3.1 Bacteria and yeast culture techniques	44
3.2 DNA preparation, manipulation, amplification and analysis	44
3.2.1 Bacterial plasmid DNA preparation	44
3.2.2 Yeast genomic and plasmid DNA preparation	45
3.2.3 Spectrophotometric estimation of DNA purity and quantitation	45
3.2.4 Enzymatic treatment of DNA	46
3.2.5 E. coli transformation	46

1

a) Preparation of competent cells and transformation by heat shock	46
 b) Preparation of competent cells and transformation 	
by electroporation	48
3.2.6 PCR amplification of DNA	48
3.2.7 Construction of pRS316-SLY1-LEU2	49
3.2.8 In vitro mutagenesis	49
3.2.9 DNA-Sequencing	52
3.3 Yeast genetics and cell biology methods	52
3.3.1 S. cerevisiae transformation	52
3.3.2 Analysis of growth properties	53
3.3.3 Invertase assay	53
3.3.4 Pulse-chase experiments	54
3.3.5 Kar2p/BiP secretion assay	57
3.3.6 Proteolytic processing of α-factor-tagged Sec22p	57
3.3.7 GFP fluorescence microscopy	58
3.4 Biochemical methods	58
3.4.1 Polyacrylamide gel electrophoresis (PAGE)	58
a) Denaturing polyacrylamide gel electrophoresis (SDS-PAGE).	59
b) Non-denaturing PAGE	59
3.4.2 Western blotting and immunological detection of proteins	00
on nitrocellulose filters	60
3.4.3 Lumilmafer analysis	61
3.4.4 Protein quantification	61
3.4.5 Protein extraction	61
a) Protein extraction from bacteria	61
	61
b) Protein extraction from yeast	63
3.4.6 Expression of proteins and recombinant proteins	63
a) Fusion tags in this study	
b) Expression of proteins in bacteria	64
3.4.7 Protein purification	64
a) 10xHis-fusion protein purification	64
b) GST-fusion protein purification	65
3.4.8 Affinity binding assay with GST-fusion proteins	66
3.4.9 Immunoprecipitation	67
4 RESULTS	68
4.1 Directed mutagenesis of SLY1 yields a collection of mutants	68
4.1.1 Experimental strategy for mutant generation	68
4.1.2 Growth phenotypes of <i>sly1</i> mutants (Table 4.1)	72
4.2 Phenotypic analysis of novel temperature-sensitive <i>sly1</i> mutants	76
4.2.1 Location of substitution in 5 novel temperature-sensitive	
<i>sly1</i> mutants.	76
4.2.2 Growth properties of different <i>sly1</i> mutants and the stability of	
the mutant proteins	78
4.2.3 Binding affinities to Sed5p of different <i>sly1</i> mutant proteins	78
4.2.4 Protein trafficking defects in newly identified sly1 temperature-sens	itive

mutants 4.2.5 Golgi-to-ER retrograde traffic is defective in some <i>sly1</i> mutants	80 82
 4.2.6 Sly1p directly binds to nonsyntaxin ER-SNAREs and is associated with SNARE complexes on ER membranes 4.2.7 Inefficient coprecipitation of Sly1-5p with Ufe1p and ER-nonsyntaxin 	88
SNAREs from $s/y1-5$ mutant cell lysates 4.2.8 Genetic interactions between $s/y1-5$ and mutants defective in Golgi-	91
to-ER retrograde transport 4.2.9 Sly1p cycles through the ER	92 94
4.3.1 Identification of novel <i>SLY1</i> alleles bypassing the essential	94 96
YPT1 function 4.3.2 The SLY1-10, SLY1-15, and SLY1-20 mutant cells lacking Ypt1p are	96
defective in growth and in protein transport to the Golgi 4.3.3 The mutant Sly1 proteins still bind to Golgi- and ER-SNAREs	100 101
 4.3.4 ypt1 ts mutant cells are defective in transport but do not affect SNARE assembly 4.3.5 Mutant Sly1 proteins have a reduced apparent affinity for Golgi- and 	102
ER-SNAREs in <i>SLY1-15</i> and <i>SLY1-20</i> cells lacking Ypt1p 4.3.6 <i>SLY1-10, SLY1-15 and SLY1–20</i> bypass the function of <i>YPT6</i>	104 105
5 DISCUSSION	107
5.1 Site-directed substitution of amino acid residues in the Sly1 protein and their effect on cell growth	107
5.2 Evidence for an essential role of Sly1p in retrograde Golgi-to-ER transport 5.3 A functional connection of Sly1p and Ypt/Rab GTPases	111 114
6 SUMMARY	117
7 ABBREVIATIONS	119
8 REFERENCES 8.1 Original papers 8.2 Book chapters	122 122 132