Molecular Interactions Position Mso1p, a Novel PTB Domain Homologue, in the Interface of the Exocyst Complex and the Exocytic SNARE Machinery in Yeast

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In this study, we have analyzed the association of the Sec1p interacting protein Mso1p with the membrane fusion machinery in yeast. We show that Mso1p is essential for vesicle fusion during prospore membrane formation. Green fluorescent protein-tagged Mso1p localizes to the sites of exocytosis and at the site of prospore membrane formation. In vivo and in vitro experiments identified a short amino-terminal sequence in Mso1p that mediates its interaction with Sec1p and is needed for vesicle fusion. A point mutation, T47A, within the Sec1p-binding domain abolishes Mso1p functionality in vivo, and *mso1T47A* mutant cells display specific genetic interactions with *sec1* mutants. Mso1p coimmunoprecipitates with Sec1p, Sso1/2p, Snc1/2p, Sec9p, and the exocyst complex subunit Sec15p. In *sec4-8* and *SEC4I133* mutant cells, association of Mso1p with Sso1/2p, Snc1/2p, and Sec9p is affected, whereas interaction with Sec1p persists. Furthermore, in *SEC4I133* cells the dominant negative Sec4I133p coimmunoprecipitates with Mso1p–Sec1p complex. Finally, we identify Mso1p as a homologue of the PTB binding domain of the mammalian Sec1p binding Mint proteins. These results position Mso1p in the interface of the exocyst complex, Sec4p, and the SNARE machinery, and reveal a novel layer of molecular conservation in the exocytosis machinery.

INTRODUCTION

Evolutionarily conserved molecular machinery regulates transport vesicle targeting, tethering, and fusion in eukaryotic cells. In yeast *Saccharomyces cerevisiae* this machinery involves the activity of the eight-subunit (Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70p, and Exo84p) tethering complex, the exocyst, a rab family small GTPase Sec4p, and the exocytic SNARE complex (Snc1/2p, Sec9p, and Sso1/2p) thought to drive the actual lipid bilayer fusion (Jahn *et al.*, 2003). The exocyst subunit Sec15p has been shown to act as an effector for Sec4p (Guo *et al.*, 1999). In addition, *sec4-8* mutant cells are defective in Snc1/2p–Sec9p–Sso1/2p SNARE complex assembly (Carr *et al.*, 1999). Despite these and numerous other studies, the regulatory mechanisms for SNARE complex formation are still poorly understood. The Sec1/Munc18 (SM) protein family members represent central regulators of SNARE complex function. These proteins perform an essential, albeit currently poorly understood function in SNARE complex regulation (Gallwitz and Jahn, 2003; Toonen and Verhage, 2003; Kauppi *et al.*, 2004).

Yeast *S. cerevisiae* possesses four Sec1p-family proteins: Sec1p mediates vesicle fusion at the plasma membrane (Novick *et al.*, 1981; Carr *et al.*, 1999), Sly1p mediates vesicle fusion at the endoplasmic reticulum–Golgi interface (Ossig *et al.*, 1991), Vps33p is required for endosome to vacuole

transport and vacuole maintenance (Banta *et al.*, 1990), and Vps45p mediates Golgi-to-vacuole transport (Piper *et al.*, 1994; Cowles *et al.*, 1994). Sec1p is the closest homologue of the mammalian Munc18-1 that has been shown to associate with syntaxin 1 and to regulate the syntaxin 1–synaptobrevin–SNAP-25 SNARE complex assembly (Misura *et al.*, 2000; Kauppi *et al.*, 2004). Munc18-1 association with syntaxin 1 has been proposed to maintain syntaxin 1 in a closed conformation and inhibit it to enter the SNARE complex (Misura *et al.*, 2000). However, overexpression of SM genes in different cell systems has both positive and negative effects on secretion (for review, see Kauppi *et al.*, 2004). Due to these partially conflicting results and the lack of detailed understanding of their regulation, the function of SM proteins is still unsolved.

Yeast cells defective for Sec1p function accumulate post-Golgi vesicles, indicating a positive role for Sec1p in membrane fusion (Novick *et al.*, 1980, 1981). It seems that the binding mode of the yeast Sec1p differs from the Munc18 proteins because at least a pool of Sec1p can associate with Sso1/2p–Snc1/2p and Sec9p-containing SNARE complexes (Carr *et al.*, 1999). Recently, Sec1p was shown also to interact with the exocyst complex or even its subcomplexes lacking some of the exocyst subunits (Wiederkehr *et al.*, 2004). In the same study, overexpression of Sec4p or Sec1p was shown to increase the amount of SNARE complexes in exocyst-defective cells. The results suggest a role for Sec1p in the molecular interface in between the exocyst complex and the SNARE machinery. An additional layer of regulation in transport vesicle fusion is provided by phosphorylation and dephosphorylation of SNARE components. Yeast Sso1p and Sso2p proteins are phosphorylated by protein kinase A in vegetatively grown cells. This phosphorylation event inhib-

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its SNARE complex assembly and suppresses endo- and exocytosis (Marash and Gerst, 2001). Dephosphorylation of the t-SNARE Sso1p by a ceramide activated type 2A protein phosphatase again promotes association of Sso1p into SNARE complexes (Marash and Gerst, 2001). Based on these and other results, it is evident that phosphorylation-dephosphorylation cycles are important regulators of SNARE complex function (for review, see Weinberger and Gerst, 2005).

Presently, only a few SM binding proteins are known. In mammalian cells, the Mint1 and 2 and the Doc2 proteins have been shown to interact with Munc18-1 (Okamoto and Sudhof, 1997; Verhage *et al.*, 1997). In addition to Munc18-1, Doc2 interacts also with a syntaxin interacting protein Munc13 (Verhage *et al.*, 1997). Mint1 and 2 are neuronally expressed proteins and Mint1 has been shown to interact with CASK and Veli proteins in metazoan cells (Borg *et al.*, 1998; Butz *et al.*, 1998). Mint 1 was originally characterized as a protein interacting through its PTB domain with β -amyloid precursor protein (APP), which has been implicated in Alzheimer's disease development (Borg *et al.*, 1996; King and Turner, 2004). Metazoan Mint-defective cells have reduced neurotransmission, supporting an active role for these proteins in neurotransmission (Ho *et al.*, 2003). The third Mint protein Mint3, which lacks the Munc18 binding domain, is ubiquitously expressed and rather poorly characterized (Okamoto and Sudhof, 1998). Based on their molecular interactions, Mint proteins have been suggested to link different regulatory molecules to the neuronal exocytosis process (Okamoto and Sudhof, 1997; Butz *et al.*, 1998; Ho *et al.*, 2003; King and Turner, 2004).

In yeast, three SM interacting proteins, Mso1p, Sso2p, and Vac1p, have been identified. Vac1p is a phosphatidyl-inositol-3-phosphate binding protein that interacts with Vps45 and is required for proper vacuole maintenance (Weisman and Wickner, 1992). The t-SNARE Sso2p was shown previously to interact with Sec1p in the yeast two-hybrid assay (Brummer *et al.*, 2001). The Sec1p interacting protein Mso1p was identified in a multicopy suppressor screen for *sec1-1* temperature-sensitive mutant (Aalto *et al.*, 1997). Deletion of *MSO1* is not lethal in vegetatively growing cells, but it leads to vesicle accumulation at the site of cell growth, implicating a positive role for Mso1p in exocytosis. Previously, we observed that deletion of Mso1p completely blocked sporulation (Jantti *et al.*, 2002), a process in which four new haploid cells are formed within the diploid mother cell at the end of the second meiotic division through de novo plasma membrane generation. Assembly of these so-called prospore membranes occurs at the cytoplasmic side of the spindle pole bodies (SPBs) and requires the function of a meiosis-specific structure of the SPBs, the meiotic plaque (Taxis and Knop, 2004). Genetic interaction of *MSO1* deletion with *sec1* and *sec4* mutations link *MSO1* functionally to the vesicle docking and SNARE complex assembly (Aalto *et al.*, 1997).

To shed light on Mso1p function and regulation of exocytosis, we have analyzed in detail association of Mso1p with the molecular machinery regulating membrane fusion in yeast cells. Our study positions Mso1p functionally in close association with Sec4p and the exocytotic SNARE complex. In addition, our results suggest a novel molecular link between the exocyst complex subunit Sec15p and the exocytotic SNARE complex. We furthermore show that Mso1p displays homology with PTB domains of mammalian Sec1p binding Mint proteins, suggesting the existence of a novel layer of conservation that regulates the membrane fusion machinery.

MATERIALS AND METHODS

Strains

The yeast strains used are shown in Table 1. Yeast cells were grown as described previously (Sherman, 1991). Disruptions of *MSO1* gene in H304 and H973 yeast strains were done by PCR amplification of the hphMX4 cassette from pAG32 followed by transformation into the corresponding strains. The H2937, H2955, and H2785 strains were generated by integrating *StuI* cut YlpProtA-*MSO1* or the YlpProtA-*mso1*(59-210) or pRS406, respectively, into the *ura3-52* locus of H2658. To obtain strains H2820, H2821, and H2855, the *MSO1* deleted strains H2658 and H2661 were transformed with *StuI*-linearized plasmids YlpMSO1U, YlpMSO1-T47A, or the empty vector pRS406. Hemagglutinin (HA), and 4GFP tagging of genes (*MSO1* [H2657, YJM2], *SEC1* [YNR60-3, YNR53-1], *SEC2* [YNR27]) at the authentic chromosomal locus was performed for each of the genes using the PCR tagging strategy (Janke *et al.*, 2004). Strains expressing *SEC4-N34* and *SEC4-I133* mutants from *GALI* promoter were generated by integrating the *ClaI* linearized plasmids NRB571 and NRB598 (Collins *et al.*, 1997) into the *leu2* locus. H3331 (*sec4-8 MSO1-3HA-kanMX*) was generated by crossing H1128 and H2657 followed by tetrad dissection.

Plasmids

The plasmids used are listed in Table 2. To generate the constructs used in the yeast two-hybrid assay, DNA fragments encoding amino- or carboxy-terminal deletions of Mso1p were generated by PCR with oligos containing the *NcoI* and *XhoI* sites. These fragments were then ligated into *NcoI/XhoI* cut pEG202 (Golemis *et al.*, 1998). The construct with the wild-type (wt) *MSO1*, YEpMSO1-bait, and YEpSEC1-fish have been described previously (Brummer *et al.*, 2001). To obtain constructs for constitutive overexpression from *ADHI* promoter of wt *MSO1* or different *MSO1* deletion mutants, DNA fragments encoding the full-length and amino- or carboxy-terminal deletions of Mso1p were generated by PCR with oligos containing the *XhoI/XbaI* sites and cloned into *XhoI/XbaI* cut pVT102U vector (Vernet *et al.*, 1987). To generate YCpMSO1U and YlpMSO1U, a genomic fragment encoding *MSO1* promoter, open reading frame and terminator (400 base pairs upstream of ATG, 300 base pairs downstream of stop) was amplified by PCR and cloned as an *XhoI-SpeI* fragment to the BluescriptSK⁻ to yield pMSO1Gen. From this plasmid, genomic *MSO1* was transferred as an *XhoI/SpeI* fragment to pRS406 and pRS416 (Sikorski and Hieter, 1989).

The point mutations T43A, T46A, and T47A were generated using the site-directed mutagenesis (QuikChange; Stratagene, La Jolla, CA) and the plasmid pMSO1-TOPO as template. For yeast-two hybrid, T43A, T46A, and T47A mutants were amplified by PCR from the mutagenized plasmids and cloned as *NcoI/XhoI* fragments into pEG202 to yield MSO1T43A-bait, MSO1T46A-bait, and MSO1T47A-bait. YEpmsolT43A, YEpmsolT46A, and YEpmsolT47A were generated by PCR and cloned as a *XbaI/XhoI* fragment into pVT102U. To obtain YlpmsolT47A, the mutagenesis was carried out by using pMSO1Gen as the template. After site-directed mutagenesis, the T47A mutant and the wt fragments were transferred to *XhoI/SpeI* cut pRS406 (Sikorski and Hieter, 1989). Ylpmsol(59-210) with endogenous *MSO1* promoter was generated by PCR with an 5' oligo containing *NdeI* site and lacking the coding sequence for the 58 first amino acids. This oligo was used to amplify the *msol*(59-210) coding region and the terminator sequence and cloned into the *NdeI/SpeI* cut YlpMSO1U. The pMAX54 [MBP-*MSO1*(38-84)] was generated by PCR and ligated as *NcoI/HindIII* fragment into pETM40 vector (http://www.embl-heidelberg.de/ExternalInfo/geerloff/draft_frames/index.html). This leads to the expression of an Mso1p fragment spanning amino acids 38–84 fused to the carboxy-terminus of MBP. All constructs generated by PCR were verified by sequencing. The oligonucleotides used for the generation of all constructs are available upon request. Plasmids pJM146 (from James McNew, Rice University, Houston, TX) and pT-GroE (from Shunsuke Ishii, RIKEN Tsukuba Institute, Ibaraki, Japan) for bacterial expression of His₆-Sec1p and GroE, respectively, have been described previously (Yasukawa *et al.*, 1995; Scott *et al.*, 2004).

Two-Hybrid Assay

EGY48 strain was transformed with a dual reporter system responsive to transcriptional activation through the *LEU2* gene and the *lacZ* fusion gene in vector pSH18-34. Plasmids pRFHM1 and pSH17-4 were used as controls for the assay. The two-hybrid assay was performed essentially as described previously (Golemis *et al.*, 1998). For each interaction, at least three individual transformants were tested. The induction of *LEU2*-gene was assayed by growth on SCGAL-ura-trp-leu + 1% raffinose and the *lacZ*-genes as a blue color on SCGAL-ura-trp-leu+x-gal + 1% raffinose agar plates.

Genetic Methods

The *sec1-1* (H305) and *sec1-11* (H306) mutants were transformed with plasmids overexpressing different mutant versions of *MSO1* from pVT102U. The suppression test was done by spotting 10⁵ cells and three 10-fold dilutions thereof on selective plates and incubating the plates at different temperatures for 3 d. To test synthetic lethal interactions between *msol*-T47A and *sec1-1*, *sec1-11*, *sec2-48*, and *sec4-8* mutants, the strains H2784, H2855, and H2786 were crossed with H305 and

Table 1. Yeast strains used in this study

Name	Genotype	Source
NKY289	<i>Mata ura3 lys2 ho::hisG</i> (SK-1 background)	Alani <i>et al.</i> , 1987
NKY292	<i>Mata ura3 lys2 leu2::hisG ho::LYS2</i> (SK-1 background)	Alani <i>et al.</i> , 1987
YKS32	diploid obtained by crossing NKY289 and NKY292	Knop and Strasser, 2000
ESM356-1	<i>MATa ura3-52 lys2-801 trp1Δ63 his3Δ200 leu2Δ1</i> (S288C background)	Knop and Schiebel, 1998
YKS65-1	<i>MATa/ Mata ho::LYS2/'' lys2/'' Δmpc70::kanMX4/'' Δmpc54::kanMX4/''</i> <i>DON1-eGFP-kanMX4/''</i> (SK-1 background)	Knop and Strasser, 2000
MBY13-1-1	<i>MATa/ Mata ho::LYS2/lys2 LEU2/leu2 DON1-eGFP-kanMX4/''</i> <i>Δmso1::hphMX4/''</i> (SK-1 background)	This study
YMK799	NKY289/292 with <i>MSO1-4GFP-kanMX4/MSO1-4GFP-kanMX4</i>	This study
YAM274-2	NKY289/292 with <i>MSO1-ProtA-kanMX4/MSO1-ProtA-kanMX4</i>	This study
YJM2	ESM356-1 with <i>MSO1-4GFP-kanMX4</i>	This study
YNR27	ESM356-1 with <i>SEC2-3HA-KanMX4</i>	This study
YNR60-3	<i>MATa ura3-52 lys2-801 trp1Δ63 his3Δ200 leu2Δ1 Δmso1::hphMX</i> <i>SEC1::SEC1-3HA-kanMX</i>	This study
YNR53-1	<i>MATa ura3-52 lys2-801 trp1Δ63 his3Δ200 leu2Δ1 SEC1::SEC1-3HA-kanMX</i>	This study
H304	<i>MATa ura3-52 leu2-3,112</i>	P. Novick (NY179)
H305	<i>MATa sec1-1 ura3-52</i>	P. Novick (NY3)
H306	<i>MATa sec1-11 ura3-52</i>	P. Novick (NY24)
H973	<i>MATa ura3-52 his4-619</i>	P. Novick (NY15)
H1127	<i>MATa sec2-41 ura3-52 leu2-3,112</i>	P. Novick (NY770)
H1128	<i>MATa sec4-8 leu2-3,112 ura3-52</i>	P. Novick
H2657	<i>MATa leu2-3,112 ura3-52 MSO1::MSO1-3HA-kanMX</i>	This study
H2658	<i>MATa ura3-52 leu2-3,112 Δmso1::hphMX4</i>	This study
H2661	<i>MATa ura3-52 his4-619 Δmso1::hphMX4</i>	This study
H2784	<i>MATa ura3-52::pRS406-URA3 MSO1 his4-619 Δmso1::hphMX4</i>	This study
H2785	<i>MATa ura3-52::pRS406-URA3 leu2-3,112 Δmso1::hphMX4</i>	This study
H2786	<i>MATa ura3-52::pRS406-URA3 his4-619 Δmso1::hphMX4</i>	This study
H2820	<i>MATa ura3-52::pRS406-URA3 MSO1 leu2-3,112 Δmso1::hphMX4</i>	This study
H2821	<i>MATa ura3-52::pRS406-URA3 mso1T47A leu2-3,112 Δmso1::hphMX4</i>	This study
H2855	<i>MATa ura3-52::pRS406-URA3 mso1T47A his4-619 Δmso1::hphMX4</i>	This study
H2856	<i>MATa MATa ura3-52::pRS406-URA3 mso1T47A his4-619 Δmso1::hphMX4</i>	This study
H2937	<i>MATa ura3-52::pRS406-URA3 ProtA-MSO1 leu2-3,112 Δmso1::hphMX4</i>	This study
H2955	<i>MATa ura3-52::pRS406-URA3 ProtA-mso1(59-210) leu2-3,112 Δmso1::hphMX4</i>	This study
H3331	<i>MATa sec4-8 leu2-3,112 ura3-52 MSO1::MSO1-3HA-kanMX</i>	This study
EGY48	<i>MATa ura3 trp1 his3 6lexAop-LEU2</i>	E. Golemis

H306 and strains H2820, H2821 and H2785 with H1127 and H1128. After tetrad dissection, the genotypic combinations of the resulting spores were analyzed.

Antibodies

The HA (12CA5) and the myc-tag antibodies (9E10) were purchased from Roche Diagnostics (Indianapolis, IN). The proteins were separated either in SDS-PAGE using the buffer system of Laemmli (Laemmli, 1970) with acrylamide concentrations from 7.5–15% or by using NU-PAGE 4–20% gradient gels (Invitrogen, Carlsbad, CA) for Coomassie staining as appropriate for the proteins analyzed. For Western analysis, the protein were transferred onto nitrocellulose membranes. Bound antibodies were visualized with the ECL detection system (GE Healthcare, Piscataway, NJ). Anti-Sec1p antibodies used were either the one described previously (K6) (Aalto *et al.*, 1997) or anti-Sec1p (#57) (from James McNew, Rice University; Scott *et al.*, 2004). Anti-Sec9p-CT antibodies have been described previously (Brennwald *et al.*, 1994). Anti-Sec4p (R232), Sec15p (R233), Sso1/2p (K8), Sso1p (K6916), Sso2p (K6906), Mso1p (R285), tubulin, and Ady3p antibodies have been described previously (Aalto *et al.*, 1997; Moreno-Borchart *et al.*, 2001; Jantti *et al.*, 2002; Toikkanen *et al.*, 2003). Anti-Sec8p antibody was obtained from Wei Guo (University of Pennsylvania, Philadelphia, PA). Anti-Snc1p and Snc2p specific antibodies were generated in rabbits by using cytosolic domains (GST-Snc1-TMD and His₆-Snc2-TMD) of the corresponding proteins as antigens.

Immunofluorescence and Electron Microscopy

Immunofluorescence and electron microscopy of sporulating yeast cells have been described previously (Moreno-Borchart *et al.*, 2001).

Pull-Down Experiments and Immunoprecipitations

His₆-Sec1p Pull-Downs. The expression of soluble His₆-Sec1p in *Escherichia coli* was obtained by coexpression of pT-GroE and pJM146 as reported previously (Yasukawa *et al.*, 1995; Scott *et al.*, 2004). The same growth conditions were used for the control with the empty plasmid. Cells were harvested, lysed by sonication followed by centrifugation at 100,000 × g for 60 min at 4°C. To

produce MBP-Mso1p(38-84) or MBP alone, BL21DE3 pRIL *E. coli* cells were transformed either with pMAX54 [MBP-Mso1p(38-84)] or pETM40 vector. Cells were grown at 25°C to OD₆₀₀ 0.8 and induced with 0.2 mM isopropyl β-D-thiogalactoside at the same temperature for 15 h. Cells were harvested and lysed by sonication. The lysates were then centrifuged at 15,000 × g. Equal amounts of His₆-Sec1p and MBP-Mso1p(38-84) lysates were mixed and incubated for 5 h with mild agitation at 4°C. Amylose (E8021L; New England Biolabs, Beverly, MA) or Ni-NTA resin (30210; QIAGEN, Valencia, CA) was added and incubated overnight at 4°C. After centrifugation at 2000 rpm, resins were washed three times with buffer A (20 mM Tris, pH 7.2, 150 mM NaCl, 10 mM imidazole). Proteins were eluted with 20 mM maltose in 10 mM Tris, pH 7.2, or 300 mM imidazole in buffer A, respectively. Loading buffer was added to the samples and SDS-PAGE followed by Western blot detection for Mso1p and Sec1p was performed. The same procedures were applied to all controls.

Protein A Pull-Downs. Strains H2937 (ProtA-MSO1), H2955 [ProtA-mso1(59-210)], and H2785 (Δmso1) were grown on liquid YPD medium to a density of OD₆₀₀ 1–2. The cells were centrifuged at 3000 × g for 10 min at 4°C and washed once with water. The cells were then resuspended in 10% volume in water and dropped into the liquid nitrogen to create small pellets. The pellets were kept at –70°C until further use. For cell lysis, 5 g of each pellet was resuspended at a ratio of 1:1 (wt/vol) in ice-cold TEA-buffer (10 mM tetraethylammonium [TEA], pH 7.5, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 50 mM NaF) supplemented with protease and phosphatase inhibitors (Complete Protease inhibition cocktail [Roche Diagnostics], 4 mM 4-(2-aminoethyl)benzenesulfonyl fluoride [AEBSE; Roche Diagnostics], 40 mM β-glycerolphosphate, 5 mM benzimidazole, 5 mM Pefablock). The cells were broken by vortexing in the presence of glass beads with repeated intervals of 20–30 s until ~80% of the cells were broken. After lysis the cell suspension was adjusted to 1% (vol/vol) NP-40, kept on ice for 30–45 min, centrifuged at 3000 × g for 10 min at 4°C, where after the supernatants were transferred to new tubes. The supernatants were then centrifuged at 10,000 × g for 10 min at 4°C and used for pull-downs.

Table 2. Plasmids used in this study

Plasmid	Type	Promoter	Fragment	Marker	Source
YEpmSO1(1-210)-bait	2 μ	<i>ADH1</i>	<i>MSO1</i> wt	<i>HIS3</i>	This study
YEpmSO1(1-163)-bait	2 μ	<i>ADH1</i>	<i>mso1</i> (1-163)	<i>HIS3</i>	This study
YEpmSO1(1-132)-bait	2 μ	<i>ADH1</i>	<i>mso1</i> (1-132)	<i>HIS3</i>	This study
YEpmSO1(1-94)-bait	2 μ	<i>ADH1</i>	<i>mso1</i> (1-194)	<i>HIS3</i>	This study
YEpmSO1(1-78)-bait	2 μ	<i>ADH1</i>	<i>mso1</i> (1-78)	<i>HIS3</i>	This study
YEpmSO1(1-58)-bait	2 μ	<i>ADH1</i>	<i>mso1</i> (1-58)	<i>HIS3</i>	This study
YEpmSO1(1-37)-bait	2 μ	<i>ADH1</i>	<i>mso1</i> (1-37)	<i>HIS3</i>	This study
YEpmSO1(23-210)-bait	2 μ	<i>ADH1</i>	<i>mso1</i> (23-210)	<i>HIS3</i>	This study
YEpmSO1(38-210)-bait	2 μ	<i>ADH1</i>	<i>mso1</i> (38-210)	<i>HIS3</i>	This study
YEpmSO1(59-210)-bait	2 μ	<i>ADH1</i>	<i>mso1</i> (59-210)	<i>HIS3</i>	This study
YEpmSEC1-Fish	2 μ	<i>GAL1</i>	<i>SEC1</i> wt	<i>TRP1</i>	Brummer <i>et al.</i> , 2001
YEpmSO1(1-210)U	2 μ	<i>ADH1</i>	<i>MSO1</i> wt	<i>URA3</i>	This study
YEpmSO1(1-188)U	2 μ	<i>ADH1</i>	<i>mso1</i> (1-188)	<i>URA3</i>	This study
YEpmSO1(1-163)U	2 μ	<i>ADH1</i>	<i>mso1</i> (1-163)	<i>URA3</i>	This study
YEpmSO1(1-132)U	2 μ	<i>ADH1</i>	<i>mso1</i> (1-132)	<i>URA3</i>	This study
YEpmSO1(1-94)U	2 μ	<i>ADH1</i>	<i>mso1</i> (1-94)	<i>URA3</i>	This study
YEpmSO1(1-78)U	2 μ	<i>ADH1</i>	<i>mso1</i> (1-78)	<i>URA3</i>	This study
YEpmSO1(1-58)U	2 μ	<i>ADH1</i>	<i>mso1</i> (1-58)	<i>URA3</i>	This study
YEpmSO1(38-210)U	2 μ	<i>ADH1</i>	<i>mso1</i> (38-210)	<i>URA3</i>	This study
YEpmSO1(59-210)U	2 μ	<i>ADH1</i>	<i>mso1</i> (59-210)	<i>URA3</i>	This study
YEpmSO1T43A	2 μ	<i>ADH1</i>	<i>mso1</i> T43A	<i>URA3</i>	This study
YEpmSO1T46A	2 μ	<i>ADH1</i>	<i>mso1</i> T46A	<i>URA3</i>	This study
YEpmSO1T47A	2 μ	<i>ADH1</i>	<i>mso1</i> T47A	<i>URA3</i>	This study
YCpmSO1U	<i>CEN</i>	<i>MSO1</i>	<i>MSO1</i>	<i>URA3</i>	This study
YIpMSO1U	Interg.	<i>MSO1</i>	<i>MSO1</i>	<i>URA3</i>	This study
YIpProtA-MSO1	Interg.	<i>MSO1</i>	ProtA-MSO1	<i>URA3</i>	This study
YIpProtA-mso1(59-210)	Interg.	<i>MSO1</i>	ProtA-mso1(59-210)	<i>URA3</i>	This study
YIpmsol(59-210)	Interg.	<i>MSO1</i>	<i>mso1</i> (59-210)	<i>URA3</i>	This study
YIpmsolT47A	Interg.	<i>MSO1</i>	<i>mso1</i> T47A	<i>URA3</i>	This study
YIpsec4-N34	Interg.	<i>GAL1</i>	<i>sec4-23</i>	<i>LEU2</i>	P. Novick
YIpsec4-I133	Interg.	<i>GAL1</i>	<i>sec4-53</i>	<i>LEU2</i>	P. Novick
pVT102U	2 μ	<i>ADH1</i>		<i>URA3</i>	T. Vernet
pJG4-5	2 μ	<i>GAL1</i>		<i>TRP1</i>	E. Golemis
pEG202	2 μ	<i>ADH1</i>		<i>HIS3</i>	E. Golemis
pRFHM1	2 μ	<i>ADH1</i>	Bicoid	<i>HIS3</i>	E. Golemis
pSH17-4	2 μ	<i>ADH1</i>	LexA-Gal4	<i>HIS3</i>	E. Golemis
pSH18-34	2 μ	-	8 ops-lacZ	<i>URA3</i>	E. Golemis
pJM146	ORI	T7	<i>His₆-SEC1</i>	amp	J. McNew
pMAX54	ORI	T7	<i>MBP-MSO1</i> (38-84)	Kan	This study
pETM40	ORI	T7		Kan	EMBL
pT-GroE	ORI	T7	GroE	Cla	S. Ishii

For pull-downs 300 μ l of IgG-coated Dynabeads was used. Incubation with the lysate was done at 4°C by end-over rotation for 2–4 h where after the tubes were fixed to a magnetic collector and the supernatants were discarded. The IgG-coated beads were washed five times at 4°C for 5–10 min: once with TEA-buffer with protease inhibitors and 1% NP-40, one to two times with TEA without inhibitors in a presence of NP-40, one to two times with wash buffer: TEA buffer supplemented with 5 mM ammonium acetate, 5 mM MgCl₂. The beads were collected and eluted twice with 50 μ l with 1% SDS at 65°C for 5 min. The eluted fractions were collected and concentrated in the vacuum to a final volume of 20–30 μ l followed by mixing with 30 μ l of urea-SDS sample buffer (HU-buffer (Knop *et al.*, 1999), heating at 65°C for 10 min, and centrifugation for 5 min before loading onto the SDS-PAGE gels. For small-scale ProtA pull-downs, cells were treated as in immunoprecipitations except that IgG-coupled beads were used for precipitation (see below)

Immunoprecipitations. Cells were grown to early logarithmic growth phase and adjusted to 20 mM NaF. Thereafter, 300–400 OD₆₀₀ of cells were collected immediately by centrifugation and washed once with ice-cold water supplemented with 20 mM NaF followed by centrifugation and a wash with ice-cold lysis buffer (50 mM HEPES, pH 7.5, 150 mM KCl, 5 mM EDTA, 5 mM EGTA, 20 mM NaF, 2 mM NaVO₄). Cells were then harvested at 3000 rpm/4°C and resuspended in 4 ml of lysis buffer supplemented with the Complete Protease inhibition cocktail (Roche Diagnostics) and 4 mM AEBSF (Roche Diagnostics). The cells were lysed by vortexing together with acid-washed glass beads six to 10 times for 15 s with a 30-s pause on ice. Then, 0.5% NP-40 was added to the lysates followed by a 30-min incubation on ice. The lysates were centrifuged 10,000 $\times$ g 10 min at 4°C, and protein concentration of the supernatant was measured using the Bradford reagent (Bio-Rad, Hercules, CA). Equal amounts of protein (6–12 mg) were used for immunoprecipitations.

The lysates were precleared by incubation with magnetic protein G-coupled beads (DynaL Biotech, Oslo, Norway) for 30 min at 4°C followed by centrifugation at 10,000 $\times$ g for 10 min. Precleared lysate were subjected to immunoprecipitations for 1 h at 4°C with identical amounts of either anti-HA (12CA5) or anti-myc (9E10) monoclonal antibodies covalently coupled to protein G magnetic beads. Antibody coupling was performed according to the manufacturer's instructions (DynaL Biotech). The beads were washed three times with 1 ml of lysis buffer. The precipitated proteins were released into 60 μ l of elution buffer (HU buffer without dithiothreitol [DTT]), heated for 5 min at 95°C. The elutes were adjusted to 100 mM DTT, heated as described above, and subjected to SDS-PAGE and Western blotting.

Other Methods

Sequence alignments were performed both at <http://npsa-pbil.ibcp.fr/with/ClustalW> (Thompson *et al.*, 1994) and with ClustalX (1.83) with default settings.

RESULTS

Mso1p Is Essential for Membrane Fusion during Prospore Membrane Formation

Deletion of *MSO1* is not lethal, but results in a moderate vesicle accumulation phenotype in vegetatively grown yeast cells (Aalto *et al.*, 1997). Our previous observation that deletion of *Mso1p* completely inhibited sporulation of diploid yeast cells

Figure 1. Initiation of de novo plasma membrane biogenesis at the SPBs is blocked in $\Delta mso1$ mutant cells. (A) Wide field immunofluorescence pictures of representative cells of wt (YKS32), $\Delta mpc54 \Delta mpc70$ (YKS65-1), and $\Delta mso1$ (MBY13-1-1) strains in metaphase (top) and in anaphase of meiosis II (bottom). DAPI staining of DNA (blue), Ady3p (red), and tubulin (green) are shown. Some cytoplasmic and SPB-associated precursor structures (white open triangles in metaphase cells, yellow arrows in anaphase cells) and the leading edge protein coats (white arrows) are indicated. In anaphase of meiosis II (A, bottom), Ady3p accumulates at the spindle pole in the $\Delta mso1$ cells (yellow arrows), whereas in wild-type cells it localizes to the leading edge protein coat of the prospore membranes (outlined with yellow dotted lines). In $\Delta mpc54 \Delta mpc70$ cells, only marginal accumulation of Ady3p at the SPB is observed. Bar (A), 2 μm . (B) Electron microscopy analysis of PSM formation in wt, $\Delta mpc54 \Delta mpc70$, and $\Delta mso1$ mutants. White arrows point to the inner side of the SPBs, and asterisks mark the meiotic plaque of the SPB. White triangles outline assembled prospore membranes. $\Delta mpc54 \Delta mpc70$ cells are devoid of the meiotic plaque, and only a few vesicles are found in the vicinity of the SPB. A population of homogenous unfused vesicles (open triangles) is found attached to the meiotic SPBs in $\Delta mso1$ mutants.

(Jantti *et al.*, 2002) prompted us to study in detail this sporulation defect by immunofluorescence and electron microscopy. As controls, we used wild-type diploid cells and cells deleted for Mpc54p and Mpc70p, two components of the meiotic spindle pole body that are necessary for the initiation of prospore membrane (PSM) formation, which occurs at the cytoplasmic side of the SPB early during meiosis II (Taxis and Knop, 2004). Based on the 4,6-diamidino-2-phenylindole (DAPI) labeling, *MSO1* deletion did not impair entry into and progression through the meiotic divisions (our unpublished data). However, analysis of different stages of PSM assembly, by using Ady3p as a marker for prospore membrane (Moreno-Borchart *et al.*, 2001), revealed that prospore membranes did not assemble in the $\Delta mso1$ mutant and that the Ady3p marker localized to clusters at the meiotic SPBs (Figure 1A). The phenotype was reminiscent to the one observed in the $\Delta mpc54 \Delta mpc70$ mutant. However, in the $\Delta mso1$ mutant a larger fraction of the precursor structures was detected at the SPBs compared with the Δmpc -control, suggesting that the failure of PSM assembly was due to a block slightly later in the process. This result suggests that Mso1p is required for prospore membrane formation in sporulating cells.

To study the defect in the $\Delta mso1$ cells in meiosis in more detail, the formation of the prospore membrane was examined by electron microscopy in wt, $\Delta mpc54 \Delta mpc70$, and the $\Delta mso1$ mutants. In wild-type cells, the prospore membrane surrounds the newly formed haploid nucleus (Figure 1B). In *MSO1*-deleted cells ($\Delta mso1$), no prospore formation occurred, but instead numerous 60- to 70-nm vesicles accumulated at the SPB (Figure 1B). These vesicle were frequently seen lined along the cytoplasmic side of the SPB, which at this stage of meiosis is modified with the meiotic plaque composed of Mpc54p, Mpc70p, and Spo74p (Knop and Strasser, 2000; Nickas and Neiman, 2002). This indicates that these vesicles have been docked to the scaffold of the meiotic SPB, but they seem to have failed to fuse and form a prospore membrane. This phenotype was observed at several SPBs ($n > 10$). In $\Delta mpc54 \Delta mpc70$ cells devoid of the meiotic plaque, only a few vesicles were detected

adjacent to SPB (Figure 1B). These results show that Mso1p is essential for membrane fusion in sporulating cells and support the previously proposed role for Mso1p in regulation of membrane fusion. In addition, these results suggest that prospore membrane biogenesis is initiated by formation of a homogeneously sized population of vesicles and their subsequent homotypic fusion.

Mso1-GFP Localizes to Sites of Exocytosis in Mitosis and to the Prospore Membranes in Meiosis

Morphological data indicated that Mso1p acts in membrane fusion at the site of exocytosis and PSM formation. To analyze Mso1p localization, a cassette encoding four copies of GFP was inserted in to the *MSO1* locus. This resulted in carboxy-terminal tagging of Mso1p with GFP (Mso1-4GFP) and enabled expression of Mso1-GFP from endogenous *MSO1* promoter. Mso1p is a low abundance protein in yeast cells (our unpublished data), and therefore multiple GFP tags were required to visualize the protein in living cells. The fusion proteins were functional *in vivo*, because they did not impair spore formation (our unpublished data). In vegetatively grown haploid cells, Mso1-GFP localized to sites of vesicle fusion at the plasma membrane, at the bud-tip and bud-neck, but was not detectable on migrating vesicles (Figure 2A). In sporulating diploid cells, Mso1-GFP was found to localize to areas around the SPBs in early stages of meiosis II (Figure 2B). Faint staining was visible all along the extended prospore membranes throughout the growth of the membranes during anaphase of meiosis II (Figure 2B, top, metaphase; bottom, anaphase). Ady3p was visualized by immunofluorescence to detect the leading edge of the growing prospore membranes (Moreno-Borchart *et al.*, 2001). Previously, Sec1p has been localized by GFP tagging to a restricted area in the bud and in the neck region of dividing vegetatively grown mitotic cells (Carr *et al.*, 1999). In addition to this localization, Sec1p also has been localized by indirect immunofluorescence with anti-Sec1p antibody-

Figure 2. Localization of Mso1-4GFP in mitotic and meiotic cells. Vegetatively growing living haploid cells (YJM2) (A) and fixed sporulating diploid cells (YMK799) in meiosis (B) expressing chromosomally encoded *MSO1-4GFP* at endogenous expression levels were investigated by fluorescence microscopy. (A) Mso1-4GFP localized to the area of bud formation in an unbudded cell (arrow) and at the tip of the bud (open triangle) and the bud neck (arrow heads) in budded cells. (B) Immunofluorescence localization of Mso1-4GFP to small prospore membranes in fixed meiotic cells in metaphase (top) and in anaphase (bottom). The size of the prospore membranes is indicated by codetection of Ady3p at the leading edge of the prospore membrane, whereas tubulin staining depicts the positions of the meiotic spindles. Bar, 1 μ m (A) and 2 μ m (B).

ies to other areas of plasma membrane in the mother cell (Scott *et al.*, 2004). The Mso1-GFP localization data are in agreement with the proposed role for Mso1p in Sec1p binding.

Mapping of Mso1p Interaction with Sec1p

Previously, we showed that the full-length Mso1p interacts with Sec1p (Aalto *et al.*, 1997). To map in more detail the Sec1p-binding domain in Mso1p, we prepared amino- and carboxy-terminal deletion series of Mso1p and investigated their interaction with full-length Sec1p in the yeast two-hybrid assay. Deletion of Mso1p from the carboxy terminus up to amino acid E58 gave similar growth on Leu⁻ plates and generated β -galactosidase activity comparable with that of wt Mso1p (Figure 3A). When Mso1p was reduced to only the 37 amino-terminal amino acids, no Sec1p interaction was detected. The role of the amino terminal Mso1p fragment in Sec1p binding was further mapped by deletions of the amino terminal part of Mso1p. Amino acids 1–38 could be deleted without any significant decrease in Sec1p binding. However, when the first 58 amino terminal amino acids were deleted, Sec1p interaction was lost (Figure 3A). This assay localizes the minimal Sec1p binding domain between amino acids E38 and E58. All the constructs used resulted in similar expression of the encoded wt and mutant proteins (our unpublished data).

To verify the role of the amino-terminal part of the Mso1p in Sec1p binding, yeast strains were prepared in which the endogenous *MSO1* was replaced by an amino-terminally protein A-tagged full-length Mso1p or a mutant version lacking the first 58 amino acids [ProtA-Mso1(59–210)]. When lysates prepared from these cells were subjected to precipitation experiments with IgG-conjugated magnetic beads followed by SDS-PAGE and Western detection with anti-Sec1p and Mso1p antibodies, it was evident that the ProtA-Mso1p efficiently brought down Sec1p, whereas ProtA-Mso1p(59–210) did not bind Sec1p (Figure 3B).

To verify that the interaction of Sec1p with Mso1p is direct, the putative Sec1p-binding domain fused to MBP, MBP-Mso1p(38–84), and His₆-tagged Sec1p were produced in *E. coli*. Clarified lysates were mixed together and incubated for 5 h at 4°C followed by binding to either Nickel-NTA or amylose resins. In pull-downs with Ni-NTA resin, specific binding between His₆-Sec1p and MBP-Mso1p(38–84) was observed, whereas no binding was detected in samples containing His₆-Sec1p and MBP or MBP-Mso1p(38–84) and the empty plasmid control (Figure 3C). Pull-downs performed with amylose resin revealed the same specific interactions between His₆-Sec1p and MBP-Mso1p(38–84) (our unpublished data).

The Sec1p Binding Domain Is Necessary, but Not Sufficient, for Mso1p In Vivo Function

MSO1 was identified in a genetic screen for multicopy suppressors of *sec1-1* temperature-sensitive mutant (Aalto *et al.*, 1997). We made use of this genetic interaction to test the role of the observed Sec1p binding domain for in vivo Mso1p function. Multicopy plasmids expressing wt *MSO1*, different mutant version of the gene, or the empty vector were transformed to *sec1-1* and *sec1-11* temperature-sensitive mutants, and the growth of the transformants was tested at different temperatures. The wt *MSO1* was able to efficiently rescue the growth of *sec1-1* and *sec1-11* mutant cells at 37°C (Figure 4A). The mutant version of *MSO1* encoding amino acids 1–132 was still able to rescue the growth of both *sec1-1* and *sec1-11* mutants. Additional deletion of *MSO1* resulted in decreased suppression capacity, and an Mso1p fragment spanning amino acids 1–94 no longer was able to rescue the growth of *sec1-1* at 37°C and displayed also reduced rescue of the less severe *sec1-11* mutant at this temperature. This fragment, however, was still able to rescue the growth at a

Figure 3. Mapping of the Mso1p domain responsible for Sec1p interaction. (A) Table summarizing yeast two-hybrid interactions of Mso1p mutants with Sec1p. The “+” indicate the relative strength of the interaction based on β -galactosidase activity and growth on medium lacking leucine. (B) The amino terminus of Mso1p is needed for Sec1p interaction in yeast cell lysates. Protein A pull-downs with IgG-coupled beads were performed with cell lysates expressing ProtA-Mso1p (H2937), ProtA-Mso1p(59-210) (H2955), or lysates deleted for Mso1p ($\Delta mso1$) (H2785). The input and pull-down samples were analyzed by Western blotting using anti-Sec1p and Mso1p antibodies. (C) In vitro binding of His₆-Sec1p with its binding domain in Mso1p(38-84). Only in samples containing His₆-Sec1p and MBP-Mso1p(38-84) copurification is detected. In negative control samples, no His₆-Sec1p and MBP copurification is observed. Likewise, no copurification of MBP-Mso1p(38-84) with Ni-NTA resin alone is observed. Positions of molecular weight standards are indicated on the right. Antibodies to Mso1p, MBP, or Sec1p were used for protein detection.

lower, but still restrictive temperature for both mutants. Additional deletion of Mso1p beyond amino acid N78 caused loss of growth rescue in both mutants even at 35°C.

The in vivo function of the amino terminal part of Mso1p was further mapped by testing the capability of the amino terminal deletion constructs of Mso1p to rescue the growth of *sec1-1* and *sec1-11* at restrictive temperature. Mutants lacking amino acids 1–37 were still fully active in vivo (Figure 4B). However, when a segment containing the Sec1p binding domain (amino acids 1–58) was deleted, the growth rescue phenotype was completely lost in both *sec1* mutants. The mutant Mso1p containing only the amino-terminal part of the protein (amino acids 1–58), including the Sec1p binding domain, could not suppress *sec1* mutants (Figure 4B). Similar expression of the encoded mutant protein was obtained for all constructs used (our unpublished data). The essential role of the Sec1p binding domain for vesicle fusion was further verified in sporulating diploid cells expressing solely the *mso1* mutant deleted for the 57 amino-terminal amino acids. In these cells, no prospore membrane formation was observed, and the sporulating cells displayed a very similar vesicle accumulation phenotype as seen in *MSO1*-deleted cells (our unpublished data; Figure 1B). This suggests that interaction of Mso1p with Sec1p is required for prospore membrane precursor vesicle fusion and prospore membrane formation. Together, these results show that the Sec1p binding domain is necessary, but not sufficient, for full Mso1p in vivo function.

The Mso1p T47 Is Essential for Mso1p Function and the T47A Mutation Causes Specific Genetic Interactions with *sec1* Mutations

We next proceeded to further characterize the Sec1p binding domain. Threonine residues were selected for the initial targets in mutagenesis. The wt *MSO1* was mutagenized to yield three different mutant versions of *MSO1*, *mso1T43A*, *mso1T46A*, and *mso1T47A*. These mutants and the wt *MSO1* were overexpressed in *sec1-1* and *sec1-11* cells and their capability to suppress the ts phenotype was scored. In contrast to wt and the T43A and T46A mutants, the T47A was unable to rescue the growth at the restrictive temperature (Figure 5A). The role of T47 in Sec1p binding was tested by the yeast two-hybrid system. The T47A mutation reduced, but did not inhibit, Sec1p binding when compared with T43A and T46A mutants (our unpublished data). For all constructs used, similar expression of the encoded mutant protein was verified by Western blotting (our unpublished data).

MSO1 deletion in combination with *sec1-1*, *sec1-11*, *sec2-41*, or *sec4-8* mutation is lethal (Aalto *et al.*, 1997). This suggests that Mso1p function may be functionally closely associated not only with Sec1p, but also Sec2p and Sec4p. We therefore tested synthetic interactions of the Sec1p binding-deficient mutant form of *mso1T47A* in combination with *sec1* mutants or with *sec2-41* or *sec4-8*. For this, the haploid *mso1T47A* mutant was crossed with the corresponding haploid *sec* mutants. The diploids were sporulated, and after tetrad dissection the genotypes of the viable spores were scored (Figure 5B). The *mso1T47A* showed synthetic lethality with both *sec1* alleles. Interestingly, *mso1T47A* was not synthetically lethal with *sec2-41* or *sec4-8* mutants. This suggests that in contrast to the complete loss of Mso1p, the Sec1p-binding deficient form of Mso1p is still capable of supporting cell growth in cells compromised for the Sec2p or Sec4p function. This indicates that the carboxy-terminal part of Mso1p may contain determinants functionally associated with Sec2p and Sec4p.

Figure 4. The Sec1p binding domain is necessary, but not sufficient for Mso1p *in vivo* function. Temperature-sensitive *sec1-1* (H305) and *sec1-11* (H306) mutants were transformed with high-copy plasmids harboring wt *MSO1* or fragments of *MSO1*. The growth of the transformants was scored at different temperatures. (A) The effect of carboxy-terminal truncations of Mso1p. (B) The effect of amino-terminal truncations of Mso1p. The amino acids encoded by the fragments are indicated by numbers in the figures. The growth of serial 10-fold dilutions of transformed cells was monitored at the temperatures indicated in the figures.

Mso1p Coimmunoprecipitates with *Sec1p*, the *Sso1/2–Sec9–Snc1/2* SNARE Complex, and the Exocyst Subunit *Sec15p*

To study whether Mso1p could be found in association with other proteins than Sec1p, we performed immunoprecipitations in a yeast strain in which triple HA-tagged Mso1p (Mso1p-3HA) expression (the sole copy of *MSO1* in these cells) was under control of the endogenous *MSO1* promoter to retain wild-type expression levels of the protein. Immunoprecipitates were analyzed by immunoblotting using antibodies specific for Sec1p, Sso1/2p, Sec9p, Snc1/2p, Sec15p,

and HA. Anti-HA antibody coupled magnetic beads efficiently precipitated Mso1p-HA, in contrast to the negative control beads in which identical amount of anti-myc antibody was coupled to (Figure 6A). In addition to Mso1p-HA, Sec1p, Sso1/2p, Sec9p, Snc1/2p, and Sec15p coimmunoprecipitated reproducibly with Mso1p-HA (Figure 6A). No coprecipitation of Sec4p or the exocyst subunit Sec8p was observed with Mso1-HA under the experimental conditions used (our unpublished data).

We next asked whether Mso1p is necessary for the observed coimmunoprecipitation of Sec15p, Sec1p, and the SNARE complex components. For this, anti-HA and anti-myc control immunoprecipitations were performed in two strains in which the sole genomic copy of *SEC1* contained a carboxy-terminal triple HA tag and *MSO1* was either deleted or not. Immunoprecipitates were analyzed by immunoblotting using antibodies specific for Sso1/2p, Sec9p, Snc1/2, Sec15p, and HA. In both strains, efficient coimmunoprecipitation of the same proteins was observed with Sec1p-HA (Figure 6B). These results show that deletion of Mso1p does not significantly affect the coimmunoprecipitation of Sec1p-HA with Sso1/2–Sec9–Snc1/2 SNARE complex components or Sec15p in vegetatively grown cells. It is thus likely that Mso1p performs a nonessential, regulatory function in association with Sec15p, Sec1p, and the Sso1/2–Sec9–Snc1/2 complex in these cells.

To address the stoichiometry of the interaction between Mso1p and Sec1p, we purified Mso1p using protein A tagging. This revealed that protein A-Mso1p and Sec1p were present in a complex in almost stoichiometric amounts, whereas no SNARE or Exocyst components could be detected under these rather stringent purification conditions, either by mass spectroscopy or Western blotting. This suggests that Mso1p can form a stable stoichiometric complex with Sec1p (Figure 6C).

Figure 5. The *mso1T47A* mutation abolishes Mso1p *in vivo* function and displays specific genetic interactions. (A) The point mutation T47A within the Sec1p binding domain abolishes the *sec1* mutant suppression activity of Mso1p. (B) Table summarizing synthetic lethal interactions between different *mso1*-mutants and *sec1*, *sec2*, and *sec4* mutants. A “+” indicates a synthetic lethal interaction, and “–” indicates a viable combination of the mutations.

Mso1p Displays Reduced Binding with the SNARE Complex in Different *sec4* Mutants

MSO1 deleted cells display synthetic genetic interactions with *sec4-8* mutation (Aalto *et al.*, 1997) (Figure 5B). To evaluate the effect of *sec4* mutations on molecular interac-

Figure 6. Immunoprecipitation analysis of Mso1p associating protein complexes. (A) Mso1p coimmunoprecipitates with Sec1p, the Snc1/2p-Sec9p-Sso1/2p SNARE complex subunits and Sec15p. Anti-HA immunoprecipitations were carried out from yeast cell (H2657) lysates where the sole copy of cellular Mso1p is triple HA-tagged at the carboxy terminus. Analysis of the immunoprecipitates was carried out by Western blotting with antibodies to HA, Sec1p, Sso1/2p, Sec9p, Snc1/2p, and Sec15p. (B) Effect of Mso1p deletion on the Sec1p, Snc1/2p, Sec9p, Sso1/2p, and Sec15p coimmunoprecipitation. Anti-HA immunoprecipitations were carried out in yeast strains expressing Sec1p-3HA in the presence (YNR53-1) or absence (YNR60-3) of Mso1p. The precipitates were analyzed for the presence of SNARE molecules and Sec15p with specific antibodies indicated on the left. Deletion of Mso1p does not affect the composition of the complex. (C) Sec1p copurifies in equimolar manner with ProtA-Mso1p (Coomassie staining). Protein A complex purification from cells expressing Protein A-Mso1p (YAM274-2). wt, cells expressing untagged Mso1p (YKS32); no ext., only IgG-beads without cell extract. Positions of molecular weight standards are indicated on the right. Asterisk marks the position of the copurifying, nonspecific virus L-A coat protein.

tions of Mso1p, we performed anti-HA immunoprecipitations from vegetatively grown cells that express Mso1p-HA or Sec2p-HA from their endogenous promoters. In addition, these cells expressed dominant negative mutant forms of Sec4p under the inducible *GAL1* promoter. The Sec4p-S34N mutant is analogous to Ras N17 mutation and presumably results in Sec4p arrested in an intermediate state between GDP and GTP bound forms, whereas the N133I mutation results in impaired guanine nucleotide binding of Sec4p (Walch-Solimena *et al.*, 1997; Collins *et al.*, 1997). The expression of *sec4* mutants was induced by shifting the cells to a galactose containing growth medium for 2 h. The cells were then lysed and processed for immunoprecipitations. Immunoprecipitates were analyzed by immunoblotting using antibodies specific for HA, Sec1p, Sec4p, Sso1p, and Sso2p. In cells expressing wt Sec4p, coimmunoprecipitation of Sec1p and Sso1p and Sso2p proteins with Mso1p-HA was observed (Figure 7A). These proteins did not coprecipitate with Sec2p-HA. Both mutant forms of Sec4p coprecipitated with Sec2p-HA, supporting the previously detected in vitro interaction (Walch-Solimena *et al.*, 1997). In addition, small amounts of the endogenous wild-type Sec4p were detected. No Mso1p was found to precipitate together with Sec2p-HA. Interestingly, however, Sec4p-N133I, but not Sec4p-S34N, mutant coprecipitated weakly, but specifically, with Mso1p (Figure 7A). In addition, the presence of Sec4p-N133I, and to a lower extent the Sec4p-S34N, mutant specifically reduced the amount of the coprecipitated Sso1p and Sso2p.

To further investigate the role of Mso1p in association with Sec4p, we carried out immunoprecipitations of Mso1p-HA from extracts of the temperature-sensitive *sec4-8* mutant cells. These cells were grown either at 24°C or shifted to 37°C for 15 or 30 min before harvesting, where after the cells were lysed and subjected for immunoprecipitations with anti-HA-coupled or with anti-myc-coupled negative control beads. Immunoprecipitates were analyzed by Western blotting with antibodies to Sec1p, Snc1/2, Sso1/2p, Sec9p, Sec4p, Sec15p, and HA. In cells incubated at the restrictive temperature, Sec1p still efficiently coprecipitated with Mso1-HA (Figure 7B), whereas coimmunoprecipitation of Snc1/2, Sso1/2p, or Sec9p was abolished. In these immunoprecipitations, Sec15p still coprecipitated with Mso1 and Sec1p, whereas no Sec4-8p was detected. Interestingly, this was also true for the immunoprecipitation at permissive temperature (Figure 7B). This suggests that association of Mso1p with SNARE complexes is compromised in the *sec4-8* mutant. Furthermore, these results show that association of Mso1p with Sec1p and Sec15p is not affected by *sec4-8* mutation.

Mso1p Is Homologous to the PTB Domain of Mint Proteins

The molecular machinery regulating exocytosis is conserved from yeast to mammalian cells (Jahn *et al.*, 2003). However, for Mso1p no mammalian homologue has been identified so far. In different yeast species close homologues for Mso1p can be found (Figure 8A). Database searches for mammalian homologues were hampered due to the high serine content (17% of all amino acids) of *S. cerevisiae* Mso1p. Searches to a small database containing patented protein sequences without functional information, identified a homologous protein sequence in which the homology was not restricted to serine residues. When this sequence was used to search GenBank sequences, the PTB domains of the mammalian Sec1p (Munc18) binding proteins (Okamoto and Sudhof, 1997), the Mint proteins, were identified as homologues of this sequence. Direct alignment of yeast Mso1 proteins verified the

Figure 7. Immunoblot analysis of Mso1p-3HA immunoprecipitates in different *sec4* mutant expressing cells. (A) In the experiment, strains expressing either Sec2p-3HA (YNR27) or Mso1p-3HA (H2657) (both from their endogenous promoter) or no HA-tagged protein (EMS356-1) (as indicated above the blots), and their variants, where chromosomally integrated dominant alleles of *SEC4* (S34N or N133I) under the control of the *GAL1* promoter, were used. On induction of the *GAL1* promoter for 2 h, cell extracts were subjected to anti-HA immunoprecipitations. Isolated immunocomplexes were separated on 4–12% Bis-Tris NU PAGE gradient gels followed by immunoblotting with the indicated antibodies (anti-HA IP). Extracts were analyzed for the presence of the expressed *sec4*-alleles (extract). More than 90% of HA-tagged proteins present in the crude extracts were precipitated (our unpublished data). (B) The effect of *sec4-8* mutation on Mso1p protein associations. *sec4-8* cells (H3331) expressing Mso1p-3HA from *MSO1* promoter were incubated either at 24 or 37°C, lysed and subjected for anti-HA immunoprecipitations. The presence of indicated proteins was analyzed by Western blotting. Coimmunoprecipitation of Mso1p-3HA with Sso and Sec9 proteins is affected already at 24°C, whereas Mso1p association with Sec1p and Sec15p is not affected.

homology. The *Kluyveromyces lactis* Mso1p (KIMso1p) showed the highest homology with the PTB domains of Mint proteins of all known hemiascomycete yeast Mso1p homologues (Figure 8B). The homology is 46% and the identity 12%. Significantly, the similarity is not restricted to serine residues but is distributed throughout the length of Mso1p. The homology between KIMso1p with Mint PTB domains is higher than the homology between known PTB domains of human Shc, Mint1, and Numb proteins (30% similarity, 6% identity), underlining the fact that known PTB domains may share very low sequence homology but still retain a PTB-like fold (Yan *et al.*, 2002). The phylogenetic tree shows the relationship between fungal Mso1p homologues and the Mint proteins (Figure 8C). Notably, the homology between the ascomycete *Schizosaccharomyces pombe* Mso1p and the Mints is higher than the hemiascomycete Mso1p.

DISCUSSION

Sec1p protein family members play an essential but poorly understood role in regulation of membrane fusion in eukaryotic cells (Gallwitz and Jahn, 2003; Toonen and Verhage, 2003; Kauppi *et al.*, 2004). Numerous studies in different model systems have resulted in partly contradictory conclusions on their function. Here, we studied the Sec1p binding protein Mso1p, to understand its functional link to Sec1p and regulation of membrane fusion. We previously noticed that no sporulation occurred in diploid yeast cells deleted for *MSO1* (Jantti *et al.*, 2002). This prompted us to investigate in more detail the role of Mso1p in this process. In meiosis, formation of the spores occurs inside the mother cell. Thereby, the PSM, which are the precursors of the spore walls and the plasma membranes of the spores, become assembled de novo, discontinuously from the plasma membrane of the mother cell. The meiotic spindle pole bodies provide on their cytoplasmic side a specific scaffold, called the meiotic plaque, on top of which the earliest structures of the prospore membranes can be found (Moreno-Borchart and Knop, 2003). Up to now, it had been enigmatic how the initial steps of PSM assembly occur. Our observation of a class of small and homogenous vesicles in the $\Delta mso1$ mutant that seem to have docked to the SPB, but not fused, suggest that PSM formation is mediated by homotypic vesicle fusion that most likely involves secretory vesicles. This is in agreement with the previous finding that *sec1*, *sec4*, or *sec8* mutants also block prospore membrane formation, although in these cases no detailed analysis of the phenotype with regard to the processes at the SPB has been carried out (Neiman, 1998). This suggests that core exocytic machinery is involved in all steps that govern the PSM assembly pathway, including the very early assembly processes. However, during PSM initiation, the membrane fusion machinery seems to be more stringently regulated compared with constitutive exocytosis in vegetatively grown cells. This is exemplified by the fact that PSM formation depends on the presence of Mso1p, whereas in Mso1p-deleted vegetatively grown cells only a moderate accumulation of vesicles at the sites of membrane growth is visible (Aalto *et al.*, 1997). This essential function of Mso1p in PSM formation seems to be mediated through its interaction with Sec1p as mutant cells expressing a Sec1p binding defective form of Mso1p had a very similar phenotype compared with cells where the whole Mso1p was deleted.

The findings that Mso1p is essential for membrane fusion, most likely in association with Sec1p, prompted us to study in more detail the molecular interactions of Mso1p with Sec1p. Mapping the Sec1p interaction domain of Mso1p by

the yeast two-hybrid system revealed an amino-terminal peptide (aa 37–59) to be necessary and sufficient for this protein–protein interaction. This interaction is direct because it could be reproduced in vitro with bacterially expressed components. Mso1p seems to form a rather stable complex with Sec1p as Sec1p copurified in stoichiometric amounts with ProtA-Mso1p from yeast lysates under stringent conditions and in all experiments performed, an Mso1p–Sec1p complex was always observed. Within the Sec1p interacting peptide, T47 turned out to be a critical amino acid for Mso1p in vivo function and Sec1p–Mso1p interaction. The in vivo effect of the T47A mutation is presently unclear. Based on the in vitro binding results with bacterially expressed proteins it is evident that phosphorylation of T47 is not an absolute requirement for Sec1p binding. However, we cannot presently exclude the possibility that, phosphorylation of this residue may still serve as a means to regulate the Mso1p–Sec1p interaction in vivo. By using the *mso1T47A* mutant, we could show that cells expressing *mso1T47A* in combination with *sec2* or *sec4* mutations were viable, although this mutation formed a synthetic lethal combination with *sec1* mutants. This is in contrast to the previous findings where complete loss of Mso1p formed a lethal combination also with *sec2* and *sec4* mutations. This underlines the importance of the Sec1p binding domain for Mso1p in vivo function and suggests that the carboxy-terminal part of Mso1p functionally interacts with the GTPase Sec4p and its exchange factor Sec2p.

Sec4p has been shown to interact with Sec15p, a subunit of the exocyst tethering complex (Guo *et al.*, 1999). Furthermore, in *sec2* and *sec4* mutants no assembly of plasma membrane Sso1/2–Sec9–Scn1/2 SNARE complexes takes place and localization of Sec1p to the bud site is lost (Carr *et al.*, 1999). These results functionally link Sec4p with Sec1p and SNARE complex function. However, no physical interactions between Sec4p and the SNARE complex or the SNARE

Figure 8. Mso1p is homologous to PTB domains from Mint1 and Mint2. (A) Comparison of *S. cerevisiae* (Sc) Mso1p with homologues found in *Saccharomyces mikatae* (Sm), *K. lactis* (Kl), and *Candida albicans* (Ca). (B) Comparison of KIMso1p with PTB domains of human HsMint1 and HsMint2. (C) Phylogenetic tree of the PTB domains of *Homo sapiens* (Hs) Mint 1, 2, and 3, and Mso1p homologues in *S. pombe* (Sp), *K. lactis* (Kl), *C. albicans* (Ca), *Saccharomyces castellii* (Sca), *S. cerevisiae* (Sc), and *S. mikatae* (Sm). The scale equals the distance where 0.1 nucleotide substitutions per site have occurred. In the pileups, the asterisk (*) marks identical, colon (:), strongly similar, and period (.), similar amino acids.

complex interacting Sec1p has been reported so far. Our immunoprecipitation experiments carried out both with Mso1p and Sec1p identified coprecipitation of Mso1p, Sec1p, the Sso_p-Sec9_p-Snc_p SNARE complex subunits, and the exocyst subunit Sec15p. Under the conditions used, we did not detect Sec8p in the Mso1p-Sec1p-containing complexes. This suggests that only Sec15p and possibly some other subunits of the exocyst complex associate directly or indirectly with Mso1p. The coimmunoprecipitation of Mso1p and Sec1p with Sec15p is supported by the recent findings showing that Sec1p coimmunoprecipitates with the exocyst complex or even its subcomplexes lacking some of the subunits (Wiederkehr *et al.*, 2004). Previously, we showed that *MSO1* deletion in combination with conditional mutations in exocyst subunits (*sec3-2*, *sec5-24*, *sec6-4*, *sec8-9*, *sec10-2*, and *sec15-1*) and the t-SNARE component *sec9-4* formed a harmful combination for the cells (Aalto *et al.*, 1997). At the same time, no synthetic interaction was detected when combining *MSO1* deletion and *sec18-1*, a mutation affecting the disassembly of the SNARE complexes. These results support the close functional association of Mso1p with vesicle tethering and SNARE complex assembly. It has previously been proposed that exocyst complex assembly takes place during vesicle docking at the plasma membrane (Wiederkehr *et al.*, 2004). Although Sec8p was not detected in our immunoprecipitations, it is possible that Mso1p interacts with the vesicle associated exocyst complex at the early stages of vesicle tethering. Exocyst is a large protein complex (Hsu *et al.*, 1998), and it is likely that the complex has to be at least partially disassembled or displaced before SNARE assembly. Therefore, Mso1p also could facilitate formation of the t-SNARE complexes in a timely manner with the exocyst complex disassembly. The timing of these associations could be regulated through the association of Mso1p with Sec4p.

A functional association of Mso1p with Sec4p is supported by the findings that the combined effect of *MSO1* deletion and *sec4-8* mutation is lethal even at the permissive temperature. This lethality is not observed when only the Sec1p binding domain of Mso1p is mutated indicating that the Sec4p interaction is undisturbed by this mutation. We observed coimmunoprecipitation of Sec4I133 protein, a dominant negative mutant of Sec4p, with Mso1p, whereas under the conditions used, we could not observe coimmunoprecipitation with wt Sec4p, sec4-8p, or Sec4N34p. In *sec4-8*, *SEC4N34* and *SEC4I133* mutant cells association of Mso1p with Sec1p persisted, whereas at the same time in *sec4-8* and *SEC4I133* cells, interaction with the SNARE complex subcomponents was impaired. Our results thus suggest that the association of Mso1p with Sec1p is not regulated by the GTPase activity of Sec4p. Sec4-8p binds poorly GTP, Sec4N34 binds preferentially GDP, whereas Sec4I133p is presumably defective both in GDP and GTP binding (Walworth *et al.*, 1989). Although, Sec4N34p and I133 mutants bind similarly the Sec4p function regulating proteins Sec2p and Dss1p (Collins *et al.*, 1997; Walch-Solimena *et al.*, 1997), these mutants are likely to possess slightly different conformations due to their different nucleotide binding properties. This could affect their binding to Mso1p or to possible, presently unknown proteins that may mediate this interaction. We speculate that GTPase cycle defective Sec4I133 protein is locked into a Sec1p and Mso1p containing complex and that this interaction impairs Mso1p–Sec1p association with the SNARE machinery and thus assembly of functional SNARE complexes. On GTP hydrolysis, Sec4p may normally be rapidly released from Sec1p and Mso1p and thus allow assembly of the t-SNARE complexes. A role for Mso1p as a protein facilitating Sec1p and Sec4p interaction with the SNARE machinery could explain the apparently positive role for Mso1p in membrane fusion as deletion of it or inhibition of its interaction with Sec1p impairs membrane fusion. Localization of Mso1-GFP to the bud tip and the site of PSM generation suggest that Mso1p–Sec1p–Sso1/2p interactions take place at sites of active membrane fusion. Because of the nature of immunoprecipitation experiments, we do not know whether all proteins coimmunoprecipitated here are part of a large protein complex or whether several smaller Mso1p containing complexes with different compositions have been retrieved. Clearly, to fully understand Mso1p function and the detailed composition of the protein complexes it associates with, further biochemical and genetic studies are needed. These results nevertheless for the first time position Mso1p to the molecular interface of the putative vesicle tethering machinery and the SNARE machinery.

In analogy to the exocyst, the HOPS protein complex is required for vesicle tethering during homotypic vacuole fusion. Within this complex, both the SM protein Vps33p and the Rab-family small GTPase Ypt7p were found in association with the SNARE machinery (Wurmser *et al.*, 2000; Price *et al.*, 2000). Our results together with the previous results with the HOPS complex imply that in different membrane fusion events, there exists a molecular machinery that links the vesicle tethering machinery with the SNARE machinery, possibly through a small GTP binding protein. In exocytosis, Mso1p and Sec1p seem to possess molecular interactions to fulfill such a task.

Database searches identified Mso1p as a homologue of the PTB domains of mammalian Sec1p binding Mint proteins. In mammalian cells, three Mint proteins exist. Mint1 and Mint2 proteins bind to Munc18 (Okamoto and Sudhof, 1997), the neuronal Sec1p homologue, whereas the ubiquitously ex-

pressed Mint3 has not been shown to interact with Munc18 (Okamoto and Sudhof, 1998). Mint proteins are multidomain proteins, which contain, in addition to the PTB domain, two PDZ domains and a separate Munc18 interaction domain (Okamoto and Sudhof, 1997). The PTB domain of Mint proteins has been reported to bind to the YENPTY peptide present in Alzheimer's disease-linked APP and to regulate cellular APP levels (Biederer and Sudhof, 2000; King and Turner, 2004). In addition to its association with Munc18, Mint1 protein has been shown to be part of a tripartite protein complex containing Mint1 (LIN-10), CASK (LIN-2), and Veli (LIN-7) and also to bind presenilins that are components of the APP processing enzyme γ -secretase (Borg *et al.*, 1998; Butz *et al.*, 1998; Lau *et al.*, 2000). Based on a mouse knockout study, Mint1 and 2 may display partially redundant neuronal functions (Ho *et al.*, 2003). Importantly, this study showed a clear role for Mint1 in neurotransmitter release. Although the detailed cellular function of Mint proteins remains obscure, the existing data support their role as adapter proteins that may form protein scaffolds with diverse downstream effectors in neuronal exocytosis. Although the expression of the Mint PTB domains could not rescue the loss of Mso1p in yeast cells (our unpublished data), the analogy of Sec1p binding and the sequence homology support the idea that Mso1p and Mint proteins are functional homologues. The interaction of Mso1p with Sec1p is confined to a short amino-terminal region of Mso1p, which makes it likely that the remaining part of the Mso1p has additional interaction partners. The Mint PTB domain also has been shown to bind phosphoinositol-4,5-bisphosphate and other phosphoinositol phosphates that are required for exocytosis (Okamoto and Sudhof, 1997; Odorizzi *et al.*, 2000; Cockcroft and De Matteis, 2001). Our attempts to detect a similar interaction of Mso1p with PIPs were not successful. This leaves the identification of the ligand for the putative PTB-pocket of Mso1p to future work.

Together, our results demonstrate that vesicle fusion at the SPB in meiosis is a highly regulated membrane fusion process that bears evolutionarily conserved features with regulated vesicle fusion processes in higher eukaryotes. The functional importance of Mso1p for membrane fusion in yeast meiosis indicates that tight control of Sec1p via Mso1p has evolved as a distinct way how to mediate timed vesicle fusion. This unexpected novel layer of functional conservation in exocytosis regulation in yeast indicates an important additional role for the PTB-domain like protein Mso1p in conjunction with Sec1p, the exocyst subunit Sec15p, and the small GTPase Sec4p.

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