Characterization of temperature-sensitive mutations in the yeast syntaxin 1 homologues Sso1p and Sso2p, and evidence of a distinct function for Sso1p in sporulation

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Accepted 11 October 2001
Journal of Cell Science 115, 409-420 (2002)

SUMMARY

The duplicated genes *SSO1* and *SSO2* encode yeast homologues of syntaxin 1 and perform an essential function during fusion of secretory vesicles at the plasma membrane. We have used in vitro mutagenesis to obtain a temperature-sensitive *SSO2* allele, *sso2-1*, in which a conserved arginine has been changed to a lysine. A yeast strain that lacks *SSO1* and carries the *sso2-1* allele ceases growth and accumulates secretory vesicles at the restrictive temperature. Interestingly, the strain also has a pronounced phenotype at the permissive temperature, causing a defect in bud neck closure that prevents separation of mother and daughter cells. The same mutation was introduced into *SSO1*, producing the *sso1-1* allele, which also has a temperature-sensitive phenotype, although less pronounced than *sso2-1*. A screen for high copy number suppressors of *sso2-1* yielded three genes that are involved in the terminal step of secretion: *SNC1*, *SNC2* and *SEC9*. The *sso1-1* mutation interacts synthetically with a disruption of the *MSO1* gene, which encodes a Sec1p interacting protein. Interestingly, we further found that both *MSO1* and *SSO1*, but not *SSO2*, are required for sporulation. This difference is not due to differential expression, since *SSO2* expressed from the *SSO1* promoter failed to restore sporulation. We conclude that a functional difference exists between the Sso1 and Sso2 proteins, with the former being specifically required during sporulation.

Key words: Vesicular transport, *MSO1*, *SSO1*, *SSO2*, Secretion, SNARE, Sporulation, Syntaxin

INTRODUCTION

In eukaryotic cells, intracellular membrane transport involves vesicle formation, transport and finally fusion with the target membrane. The docking and fusion of transport vesicles is a complicated process that is mediated by a large number of components. Key players are the v- and t-SNAREs (soluble NSF attachment protein receptors), which are thought to form a core complex during the fusion event (Rothman, 1994; Jahn and Südhof, 1999). The v-SNAREs, which reside on the transport vesicle interact with t-SNAREs on the target membrane. In the case of synaptic vesicle fusion, the v-SNARE synaptobrevin (VAMP) and the t-SNAREs syntaxin and SNAP-25 form a ternary complex that may bring the two lipid bilayers sufficiently close for the membrane fusion to proceed. In the yeast *Saccharomyces cerevisiae* the synaptobrevin homologues Snc1p and Snc2p, the syntaxin 1 homologues Sso1p and Sso2p, and the SNAP-25 homologue Sec9p form an analogous complex that mediates secretory vesicle fusion with the plasma membrane. The structures of the yeast and mammalian ternary complexes have been resolved (Fiebig et al., 1999; Sutton et al., 1998).

Both the *SNC* and *SSO* genes are duplicated, and the encoded proteins are highly homologous. Thus, Snc1p and Snc2p are 77% identical and 84% similar, whereas Sso1p and Sso2p are 74% identical and 86% similar. All four genes are expressed during vegetative growth, and genetic, morphological and biochemical data has so far suggested that each pair of proteins is functionally redundant (Gerst et al., 1992; Protopopov et al., 1993; Aalto et al., 1993). This raises the question why would the yeast cell express two highly homologous proteins with identical functions. It should be noted that all the other essential genes that are involved in the same vesicle docking event (e.g. *SEC1*, *SEC2*, *SEC4*, *SEC5*, *SEC6*, *SEC8*, *SEC10*, *SEC15*, *EXO70* and *EXO84*) are unique. It is therefore conceivable that the duplication of the *SSO* and *SNC* genes could reflect different functions for Sso1p and Sso2p (and perhaps also for Snc1p and Snc2p) in a processes where membrane fusion is required. Interestingly, one example of such a functional differentiation was recently found in sporulation. Thus, the essential *SEC9* gene, which encodes a homologue of the mammalin SNAP-25 protein, is not required for prospore formation (Neiman, 1998). This function is instead provided by the *SPO20* gene, which encodes another SNAP-25 homologue that is specifically expressed during meiosis and sporulation (Neiman, 1998; Chu et al., 1998).

We have now isolated a temperature-sensitive allele of

SSO2, *sso2-1*, and made an *SSO1* allele, *sso1-1*, carrying the same mutation. Cells that have either *sso1-1* or *sso2-1* as their only expressed *SSO* gene are temperature sensitive for both growth and secretion. In addition, *sso2-1* cells (but not *sso1-1* cells) have a pronounced phenotype at the permissive temperature, with a partial defect in bud neck closure and cytokinesis. We further show that the *MSO1* and *SSO1* genes, but not *SSO2*, are required for sporulation. We conclude that the cellular functions of Sso1p and Sso2p are not fully redundant, with Sso1p (but not Sso2p) being essential for sporulation.

Materials and Methods

Yeast strains

The yeast strains used are listed in Table 1. To construct the *sso1Δ sso2-1* yeast strain H603, we started with H440, a W303 congenic strain that carries an *sso1-Δ1::URA3* disruption and has a *GAL1-SSO1,HIS3* cassette inserted into the *SSO2* gene. This strain was transformed with a linear *Sph*I-*Hin*dIII fragment containing the *sso2-1* allele together with a replicating *TRP1* plasmid, selecting for the latter. Transformants were screened for temperature sensitivity and loss of the *HIS3* marker, indicating that the *sso2-1* fragment had replaced the *GAL1-SSO1,HIS3* cassette at the *SSO2* locus. The genotype of one such transformant was verified in Southern blots, after which the *TRP1* plasmid was eliminated by plating the cells without selection. The resulting strain was transformed with an *sso1-Δ1::HIS3* fragment and plated in the absence of histidine, to obtain a strain in which the *sso1-Δ1::URA3* disruption has been replaced by *sso1-Δ1::HIS3*. A strain of mating type **a**, H603, was finally obtained by crossing this strain to W303-1A and picking a spore with the desired genotype.

In order to facilitate comparisons with existing *sec* mutations, we also made an NY179 congenic strain in which *sso2-1* is the only expressed syntaxin. The strain was made using popin-popout replacement. We started with strain H900 (Table 1) which has an *sso1-Δ1::LEU2* disruption. It was transformed with pMO5, an integrating *URA3* plasmid containing the *sso2-1* allele. The plasmid was targeted for integration at the *SSO2* locus by cutting at the unique *Bsi*WI site within the *sso2-1* insert. After a popin strain had been obtained, popout events were selected by plating the cells on 5-fluoro-orotic acid (Boeke et al., 1984). The popouts were screened for temperature sensitivity. The *sso2-1* genotype of one temperature-sensitive strain, H902, was verified by PCR analysis and complementation with an *SSO2* plasmid. The W303 congenic *sso1-1 sso2Δ* strains H1241 and H1239 were made in a similar way starting with strains H835 and H836 (Table 1) which were transformed with pIsso1-1 cut at the unique *Afl*II site within the *SSO1* gene. The resulting popin strains were plated on 5-fluoro-orotic acid to select the respective popout strains.

Table 1. Yeast strains

Name	Relevant genotype*
H403	**a** *sso1-Δ1::URA3*
H404	α *sso2-Δ1::LEU2*
H440	α *sso1-Δ1::URA3 sso2-Δ1::leu2::(GAL1-SSO1, HIS3)*
H603	**a** *sso1-Δ1::HIS3 sso2-1*
H609	**a** *mso1-Δ1::LEU2*
H833	**a** *sso1-Δ1::URA3*
H835	**a** *sso2-Δ1::LEU2*
H836	α *sso2-Δ1::LEU2*
H900	**a** *sso1-Δ1::LEU2*
H902	**a** *sso1-Δ1::LEU2 sso2-1*
H1239	α *sso1-1 sso2-Δ1::LEU2*
H1241	**a** *sso1-1 sso2-Δ1::LEU2*
H1247	**a** *sso2-Δ1::LEU2 mso1-Δ1::HIS3*
H1251	**a** *sso1-1 sso2-Δ1::LEU2 mso1-Δ1::HIS3*
H1267	**a** *sso1-Δ1::HIS3 mso1-Δ1::LEU2*
H1269	**a** *sso1-1 sso2-1*
H1271	**a** *sso1-Δ1::HIS3 sso2-1 mso1-Δ1::LEU2*
D67	**a**/α *MET13/MET13 trp1-1/trp1-1*
D272	**a**/α *sso1-Δ1::LEU2/sso1-Δ1::LEU2 MET13/met13-Δ1 trp1-1/TRP1*
D273	**a**/α *sso1-Δ1::LEU2/sso1-Δ1::URA3 MET13/MET13 trp1-1/trp1-1*
D286	**a**/α *mso1-Δ1::LEU2/mso1-Δ1::LEU2*

*All strains except H900, H902 and D286 are W303 congenic (Thomas and Rothstein, 1989) and therefore carry the *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1* markers. H900, H902 and D286 are congenic to NY179 (Aalto et al., 1997) and carry the *leu2-3,112* and *ura3-52* markers.

Plasmids

Plasmid YEpSSO2 is the original *SSO2* plasmid that was isolated from a yeast cDNA library as a suppressor of *sec1-1* (Aalto et al., 1993). It is a *TRP1* 2μ plasmid that expresses the *SSO2* cDNA from the *ADH1* promoter. Plasmid YCpSSO1U has the *SSO1* gene, isolated by PCR from NY179 genomic DNA with oligos 4488 and 4490 (Table 2) cloned as a *Bam*HI/*Eco*RI fragment into the centromeric vector pRS416. YEpSSO1U has the *Bam*HI/*Eco*RI fragment from YCpSSO1U cloned into pRS426. YEpSSO2U has the *SSO2* gene, isolated by PCR from NY179 genomic DNA with oligos 4625 and 4626, cloned as a *Bam*HI/*Eco*RI fragment into pRS426. YCpMSO1U, finally, carries *MSO1* with added *Xho*I and *Spe*I sites, isolated by PCR from NY179 genomic DNA using oligos 5470 and 5471, cloned into pRS416. All PCR products were sequenced prior to use. YCpsso1-1T and pIsso1-1 have the *sso1-1* allele cloned as a *Bam*HI/*Eco*RI fragment into pRS414 and YIp5, respectively. Plasmids pSSO2, pSNC1, pSNC2 and pSEC9 were recovered in a library screen for high copy number suppressors of *sso2-1* (see below) and carry the respective genes as partial *Sau*3A inserts into the *Bam*HI site of pHR81 (Nehlin et al., 1989). Plasmid YEpsso2-1 is YEpSSO2 carrying the *sso2-1* mutation, which was recovered in our screen for temperature-sensitive *SSO2* alleles. Plasmid pMO5 is YEpsso2-1 in which the *Hin*dIII fragment carrying the 2μ DNA has been replaced by the *URA3 Hin*dIII fragment. YEpSEC1aU has been characterised previously (Aalto et al., 1993). YEpSEC4 has a genomic fragment containing *SEC4* cloned into YEp24, and was obtained from Peter Novick (Yale University, New Haven, CT).

Plasmid pMO11 contains the *SSO1* gene, amplified from W303-1A genomic DNA using primers 4490 and 4488 (Table 2), cloned between the *Bam*HI and *Eco*RI sites of pUC119. Plasmids pMO9 and pMO14 contain the *SSO2* gene, amplified from W303-1A genomic DNA using the primers SSO2F+BamHI and SSO2R+EcoRI, cloned between the *Bam*HI and *Eco*RI sites of pFL38 and pUC119, respectively. An *Nde*I site that overlaps with the start codon was introduced into *SSO1* using two rounds of PCR with either primers 4490 and NdeI→SSO1R or NdeI→SSO1F and 4488 in the first round and 4490 and 4488 in a second round with the PCR product from the first round as template. A *Bst*1107I-*Afl*II fragment from the PCR

Table 2. Oligonucleotides

NdeI→SSO1F	AAATCATATGAGTTATAATAATCCGTACCAGT
NdeI→SSO1R	CTCATATGATTTGTTTCTATTTTTAATTGCCT
NdeI→SSO2F	TTGCACATATGAGCAACGCTAATCC
NdeI→SSO2R	CTCATATGTGCAATATTTGTGCGTG
4488	GCATTGAATTCGATGGTATTTCAATTGGTGTTG
4490	GCATTGGATCCGAATAAATAACATATAAGAC
4528	CAATAACTCTTGGTGCTTTGCCTGGACTTCCGC
4529	GCGGAAGTCCAGGCAAAGCACCAAGAGTTATTG
4625	GCATTGAATTCGGATCGCATATAAACAAAACTCG
4626	GCATTGGATCCAGCTTCTAGACAATTCTGAAAAC
5470	GCATTCTCGAGAATAAGAAGAAATGTATCAAAAGG
5471	GCATTACTAGTAACCAAGACCTATGCATGATC
SSO2F+*Bam*HI	TAGGATCCCGTACGTAATAGTATGTGCG
SSO2R+*Eco*RI	ATGAATTCGACTAATATTAAGGGCGACA

product containing the *Nde*I site was then cloned between the same two sites in pMO11, yielding pMO30. An *Nde*I site was introduced in the same position in *SSO2* using primers SSO2F+BamHI and NdeI→SSO2R or NdeI→SSO2F and SSO2R+EcoRI in the first round of PCR, and SSO2F+BamHI and SSO2R+EcoRI in the second round. In this case, an *Eag*I-*Eco*RV fragment was cloned between the same two sites in pMO14, yielding pMO17. The promoters of pMO30 and pMO17 were then released as *Bam*HI-*Nde*I fragments and cloned into the same sites in the other plasmid, producing in pMO31 (*SSO2* promoter from pMO17 fused to the *SSO1* open reading frame in pMO30) and pMO32 (*SSO1* promoter from pMO30 fused to the *SSO2* open reading frame in pMO17). Finally, *Bam*HI-*Eco*RI fragments containing the different genes were cloned into the centromeric vector pFL38 resulting in pMO33 (*SSO1* with *Nde*I site), pMO35 (P_{SSO2}-*SSO1* fusion), pMO24 (*SSO2* with *Nde*I site) and pMO37 (P_{SSO1}-*SSO2* fusion).

Hydroxylamine mutagenesis and mutant screen

Plasmid YEpSSO2 was subjected to hydroxylamine mutagenesis (Sikorski and Boeke, 1991). Five tubes, each containing 20 μg of plasmid DNA in 1 ml of hydroxylamine solution, were incubated at 75°C for 0, 30, 60, 90 and 120 minutes. The reactions were stopped by placing the tubes on ice and the hydroxylamine was removed using NAP-25 columns (Pharmacia Biotech AB, Uppsala, Sweden). Aliquots of the mutagenized DNA were then transformed into *E. coli*, and the number of colonies obtained for each time point were counted to obtain an estimate of the mutagenic efficiency. Based on these results, transformants from the 60 and 90 minute tubes were collected, pooled, and then used to inoculate a large scale plasmid preparation. The resulting library of mutant plasmids was transformed into yeast strain H440 (Table 1) which lacks *SSO2* and has *SSO1* expressed from the *GAL1* promoter. Transformants were selected on tryptophan-less synthetic galactose plates incubated at 30°C. After colonies had formed, the galactose plates were replicated to tryptophan-less synthetic glucose plates and incubated for 3 days at 24°C. Since the plasmid library was heavily mutagenized, only about 50% of the colonies expressed a functional Sso2p protein as shown by their ability to grow on glucose. These colonies were replicated to two new tryptophan-less synthetic glucose plates, which were incubated at 24°C and 37°C, respectively. Plasmids were rescued from colonies that failed to grow at 37°C and tested by re-transformation into H440 to verify that the temperature-sensitive phenotype was linked to the plasmid DNA. Three temperature-sensitive plasmids were found, all of which contained the same R200K mutation, subsequently referred to as *sso2-1*. Finally, a corresponding mutation (R196K) was made in the *SSO1* gene using the QuickChange system (Stratagene) and the primers 4528 and 4529. The resulting *sso1-1* allele was sequenced to ensure that no other mutations had occurred.

High copy number suppressor screen and suppression analysis

For the high copy number suppressor screen, we used the temperature-sensitive strain H603 (Table 1) and the pHR81 library (Nehlin et al., 1989). Transformants were plated at 20°C and then screened for suppression of the temperature-sensitive phenotype at 32°C by replica-plating. Suppression with specific plasmids was tested in both H603 (W303 background) and H902 (NY179 background). Four transformants with each plasmid were grown on a selective plate at 22°C, and then replicated to both selective plates and YPD plates which were incubated at 22°C, 30°C, 32°C, 34°C and 36°C. The growth of the replicas were monitored for three days.

Hsp150p secretion

Yeast strains H1239 (*sso1-1 sso2Δ*), H603 (*sso1Δ sso2-1*) and the congenic wild-type W303-1A were grown at 24°C to A_{600} 0.3-0.5. Cells were collected and the cultures were divided and resuspended at the same cell density in YPD medium prewarmed either to 24°C or 38°C. Samples were removed 0, 15, 30, 60, 120 and 240 minutes after the temperature shift. NaN_3 was immediately added to a final concentration of 10 mM and the samples were cooled on ice. Cells were pelleted at 4°C and the supernatant was transferred to a new tube. A 10 μl aliquot of each sample was separated on an 8% SDS-polyacrylamide gel and analysed by western blot with anti-Hsp150p antibodies.

Yeast cell lysates

Cell lysates for the determination of the specificity of the Sso1p and Sso2p antisera were prepared from overnight cultures of H403, H404 and the congenic wild-type strain W303-1A. Cells were broken by vortexing in the presence of 0.45 μm glass beads in 2% SDS supplemented with a protease inhibitor coctail (Complete, Roche). Samples were centrifuged for 10 minutes at 20,200 ***g*** and the supernatants were then heated for 5 minutes at 95°C. The protein concentration was determined by the method of Lowry. To study the stability of the mutant Sso1-1 and Sso2-1 proteins, cells were grown identically to the Hsp150 secretion experiment (see above). At 0, 15, 30, 60 and 120 minutes after the temperature shift, 20 ml samples of the cultures were removed. These were supplemented with NaN_3 to a final concentration of 10 mM, cooled on ice and centrifuged. Cells were then lysed and protein concentrations determined as described above. Equal amounts of protein from each sample were separated on a 12% SDS-polyacrylamide gel and analysed by western blotting using anti-Sso1p and anti-Sso2p antisera.

Antisera and western blots

Polyclonal anti-Sso1p and anti-Sso2p peptide antisera were generated by immunising rabbits with peptides coupled to Keyhole Limpet hemocyanin (Sigma-Aldrich, Finland) using glutaraldehyde. The peptides (Sigma Genosys Ltd, UK) used correspond to amino acids 8-22 in both proteins, plus an additional C-terminal lysine (Sso1p: QLETPFEESYELDEGK; Sso2p: ENNNPYAENYEMQEDK). The N-terminus of the peptides was acetylated and the C-terminus amidated. The efficiency of coupling was monitored by reverse phase HPLC. The antibodies against Hsp150p (Russo et al., 1992) was a kind gift from Marja Makarow (Institute of Biotechnology, University of Helsinki). In the Western blot experiments, proteins were separated on SDS-polyacrylamide gels, blotted electrophoretically onto nitrocellulose filters, and detected by incubation with specific antisera followed by enhanced chemiluminescence (Amersham). Quantitation of Hsp150p from Western blots was done after ECL by densitometry using a GS-170 densitometer and Qantity One software (Bio-Rad).

Other methods

All recombinant DNA work was done according to published procedures (Sambrook et al., 1989). Synthetic yeast media were prepared as described (Sherman et al., 1986), but with twice the recommended amount of leucine. YPD media is 1% yeast extract, 2% peptone and 2% glucose. Diploid cells were sporulated in spor 5+ medium, which is 1% KAc, 0.1% yeast extract and 0.05% glucose. The medium was supplemented with adenine, histidine, leucine, tryptophan and uracil at standard concentrations (Sherman et al., 1986). The diploids were sporulated for 5 days at room temperature. Sporulation frequencies were determined by counting the cells under a microscope using a Bürker chamber. At least 1000 cells were counted for each data point. Electron microscopy on yeast cells was performed as previously described (Aalto et al., 1997). Determination of the secreted α-amylase activity in the growth medium was done as described by Ruohonen et al. (Ruohonen et al., 1997).

Results

Generation of temperature-sensitive *SSO1* and *SSO2* alleles

Three temperature-sensitive *SSO2* mutants were obtained by hydroxylamine mutagenesis as described in Materials and Methods. All of them contained the same G to A transition that changes the arginine at position 200 to a lysine. Since the plasmid library was amplified in *E. coli* prior to the screen in yeast, it is likely that the three clones represent the same original mutation. We will subsequently refer to the temperature-sensitive *sso2* R200K mutation as *sso2-1*. Since the Sso1 and Sso2 proteins are highly homologous, we wanted to examine if the same mutation would have a temperature-sensitive phenotype when transferred to the Sso1 protein. The corresponding arginine to lysine mutation (R196K) was therefore made in *SSO1* (see Materials and Methods) resulting in the *sso1-1* allele.

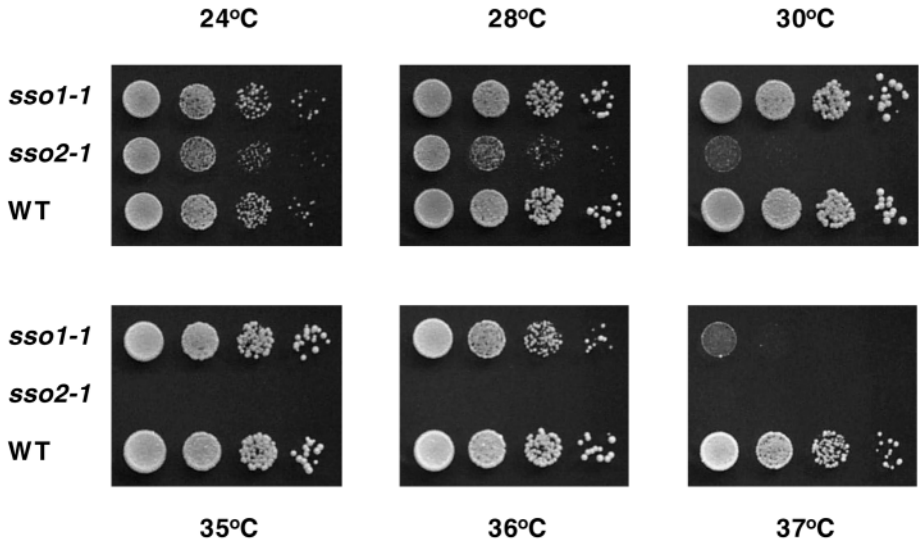

Fig. 1. Temperature sensitivity of *sso1-1 sso2Δ* (H1239) and *sso1Δ sso2-1* (H603) and congenic wild-type (W303-1A) strains. Aliquots corresponding to 6×10^4 cells and three tenfold dilutions of each strain were spotted onto YPD plates which were incubated for 2 days at the indicated temperatures.

To further investigate the effect of the temperature-sensitive mutations in vivo, we made stable integrants in which either the *SSO1* gene has been deleted and the wild-type *SSO2* gene has been replaced by the *sso2-1* allele or the *SSO2* gene has been deleted and *SSO1* gene replaced by the *sso1-1* allele. We found that such strains are viable at 24°C. This shows that expression of either mutant protein from its own promoter is sufficient for growth at the permissive temperature. However, the *sso1Δ sso2-1* strain is clearly more temperature sensitive. It shows a slightly decreased growth already at 24°C, and fails to grow above 30°C (Fig. 1). By contrast, the *sso1-1 sso2Δ* strain grows just as well as the wild-type up to 36°C (Fig. 1). We further noted that both the *sso1-1 sso2Δ* and *sso1Δ sso2-1* strains are less temperature sensitive by 1-2°C when grown on synthetic media than when grown on YPD (data not shown).

Impaired secretion in *sso1-1 sso2Δ* and *sso1Δ sso2-1* strains at restrictive temperatures

To confirm that the *sso1-1 sso2Δ* and *sso1Δ sso2-1* cells have a conditional secretion defect we assayed secretion of the heat shock protein Hsp150p (Fig. 2). Low levels of Hsp150p are expressed constitutively, but the expression is highly induced at elevated temperatures (Russo et al., 1992). Therefore, the cells were grown at 24°C until an A_{600} of 0.3 and then shifted to either 37°C (*sso2-1*) or 38°C (*sso1-1*). At indicated time points, the culture medium was collected and the amount of secreted Hsp150p was determined in western blots. Already 15 minutes after the shift, the amount of secreted Hsp150p is clearly reduced in *sso1Δ sso2-1* cells compared with *sso1-1 sso2Δ* or wild-type cells (Fig. 2), whereas at 24°C both mutant strains secreted Hsp150p with the same efficiency as the wild-type strain (data not shown). After 4 hours at the restrictive temperature, there is a clear effect in both mutant strains. Thus, the amount of secreted Hsp150p is reduced by 80-90% in *sso1Δ sso2-1* cells, and by 30-40% in *sso1-1 sso2Δ* cells. We conclude that secretion of Hsp150p is strongly impaired in the *sso1Δ sso2-1* strain and significantly but less impaired in the *sso1-1 sso2Δ* strain. This is consistent with our finding that the growth defect is more pronounced in the former strain (Fig. 1).

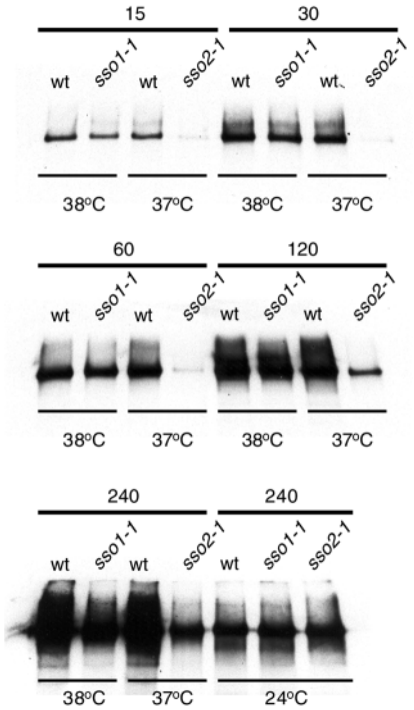

Fig. 2. Secretion of Hsp150 protein in wild-type (W303-1A) and congenic *sso1-1 sso2Δ* (H1239) and *sso1Δ sso2-1* (H603) cells. Yeast cells were grown at 24°C until they reached an A_{600} of 0.3. The cells were then shifted to either 24, 37 or 38°C. Samples were collected after 15, 30, 60, 120 and 240 minutes. Cells were removed by centrifugation and the amount of Hsp150p in a 10 μl aliquot of the growth medium was quantified by western blotting.

For the *sso1Δ sso2-1* strain, the secretion defect was verified using another reporter protein, *Bacillus* α amylase. This heterologous protein is efficiently secreted when expressed in yeast and has previously been used as a marker protein in secretion studies (Ruohonen et al., 1997). In agreement with the Hsp150p results, we found that secretion of α-amylase is strongly reduced in the *sso1Δ sso2-1* strain within 2 hours after a shift to the restrictive temperature (data not shown). This coincides with a cessation of growth, as determined by the absorbance at 600 nm. Finally, secretion of invertase is inhibited in the *sso1Δ sso2-1* strain at the restrictive temperature, thus providing further independent support for the secretion defect (Ossig et al., 2000).

Accumulation of secretory vesicles in *sso1-1 sso2Δ* and *sso1Δ sso2-1* cells at restrictive temperatures

To study the terminal phenotypes of the temperature-sensitive mutants, we used electron microscopy to examine both *sso1-1 sso2Δ* and *sso1Δ sso2-1* cells after a shift to their restrictive temperatures. As shown in Fig. 3a, *sso1-1 sso2Δ* cells that have been incubated for 3 hours at 38°C accumulate vesicles. Many cells also show incomplete septum formation. In Fig. 3b, a closeup is shown of vesicles that have accumulated at a partially formed septum. By contrast, *sso1-1 sso2Δ* cells incubated at 24°C accumulate no vesicles, but occasional cells with unusually wide bud necks are seen (Fig. 3c). In the *sso1Δ sso2-1* strain, these phenotypes are more pronounced and are seen at lower temperatures. Thus, the *sso1Δ sso2-1* cells accumulate vesicles in the bud already at 24°C (see below). At 37°C, there are numerous vesicles throughout the cells, with cell wall deposits frequently being formed in the bud neck region (Fig. 4a). As a control, we also examined congenic wild-type cells that had been incubated at either 24°C or 37°C. As shown in Fig. 4b,c, they do not accumulate vesicles or show any problems with cytokinesis at either temperature. We conclude that the vesicle accumulation phenotype is seen when the only expressed *SSO* gene is either *sso1-1* or *sso2-1*, and that it is more pronounced for *sso2-1*. This is consistent with the more severe growth and secretion phenotypes of *sso2-1*, compared with *sso1-1*.

The *sso2-1* mutation causes a neck closure defect at the permissive temperature

Interestingly, we found that the *sso2-1* mutation has a pronounced effect at the permissive temperature. Thus, although *sso1Δ sso2-1* cells are able to grow at 24°C, they have a highly abnormal morphology that affects cytokinesis and closure of the bud neck. Initially, the cells form buds with much wider necks than normal (Fig. 5b) and cytokinesis is not completed in a normal way. In many cases, a septum is formed that separates the two cells, but closure of the neck does not occur, and as a consequence the two cells remain attached to each other (Fig. 5a). Septum formation is frequently incomplete (Fig. 5d,e). As a consequence of these cytokinetic

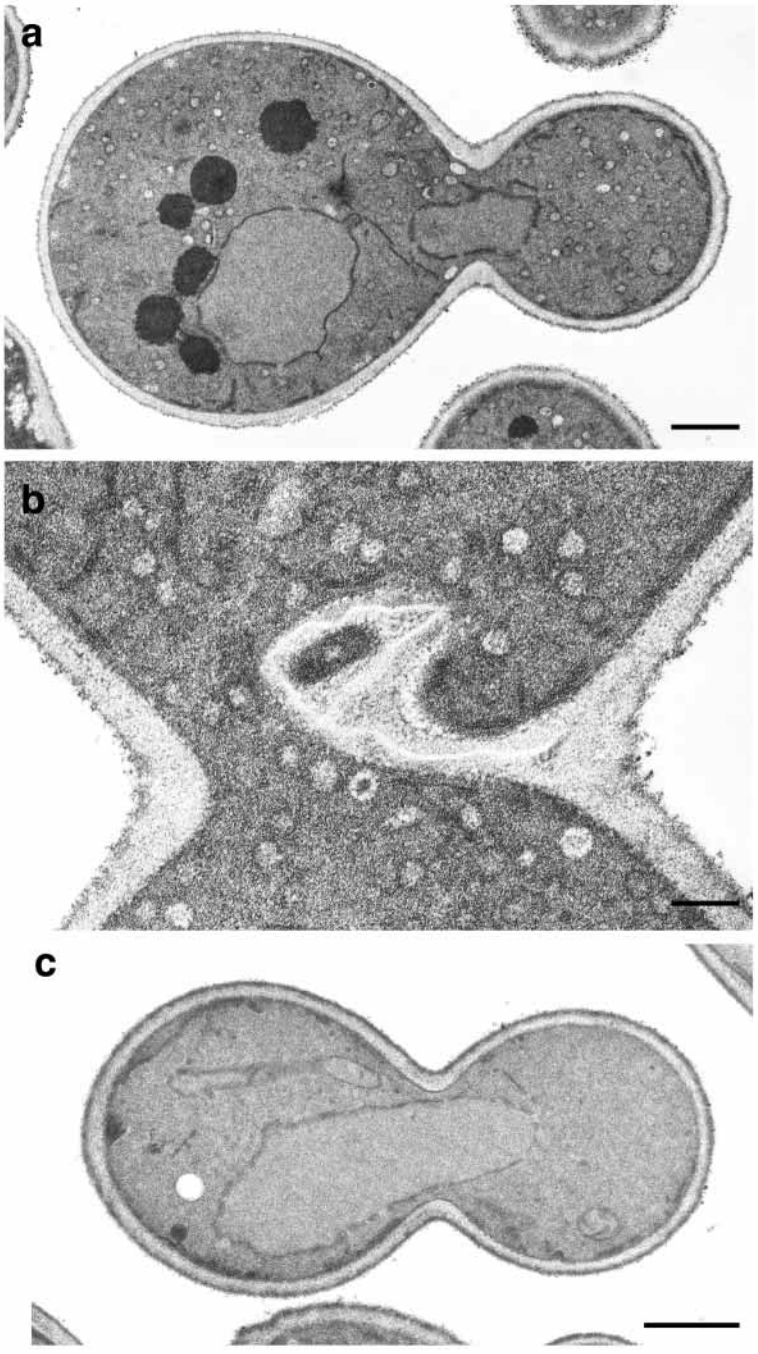

Fig. 3. Electron micrographs of the *sso1-1 sso2Δ* strain H1239 at permissive and restrictive temperatures. The cells were grown in YPD at 24°C to an A_{600} of 0.3. The cell culture was then split into subcultures grown either at 24°C or 38°C. The cells were fixed after 3 hours of growth and samples were processed for electron microscopy. (a) The *sso1-1 sso2Δ* cells accumulate transport vesicles at 38°C. (b) They also have problems with completing cytokinesis and frequently accumulate vesicles at the septum. (c) Cells grown at 24°C do not accumulate vesicles or display incomplete septum formation, but occasionally have wider bud necks than wild-type cells. Bars, 500 nm (a); 200 nm (b); 1 μm (c).

defects, the *sso1Δ sso2-1* mutant strain forms elongated branched chains of interconnected cells at the permissive temperature. This branched chain phenotype is particularly pronounced in the W303 background, where it is visible in the light microscope (data not shown). It should further be noted that the *sso1Δ sso2-1* cells grow more slowly than the wild-type and accumulate secretory vesicles already at 24°C. However, unlike the situation at the restrictive temperature, these vesicles are seen mostly in the bud region (Fig. 5b) and near the incomplete septum (Fig. 5c).

To test if the neck closure defect is a dominant negative effect of the *sso2-1* mutation we also examined *sso1Δ sso2-1* cells transformed with centromeric plasmids carrying the *SSO1* and *SSO2* wild-type genes. These cells have a normal morphology, which shows that the mere presence of the Sso2-1 mutant protein is not sufficient to produce the neck closure defect. We conclude that the neck closure defect is seen if Sso2-1p is the only expressed Sso protein in the cell. A likely explanation for this finding is that the mutant protein is partially inactive already at the permissive temperature, therefore providing a reduced function. Accordingly, the neck closure defect would be the consequence of a partially impaired terminal step in secretion.

The Sso1-1 and Sso2-1 proteins are both stably expressed at the restrictive temperature

A temperature-sensitive phenotype does not necessarily reflect a temperature-sensitive function of the mutant protein. It is also possible that the mutation affects expression of the protein (e.g. by making it more prone to proteolytic degradation). To test if this was the case, we carried out a western blot analysis using anti-peptide antisera that are specific for either Sso1p or Sso2p. As shown in Fig. 6A, these antisera specifically recognize either Sso1p or Sso2p in cell lysates and detect both proteins with similar efficiency. Using purified recombinant Sso1-GST and Sso2-GST fusion proteins as standards, we estimate that Sso1p and Sso2p comprise approximately 0.05-0.1% of the total cellular protein in haploid cells grown on YPD (data not shown). To examine whether the Sso1-1 and Sso2-1 mutant proteins are poorly expressed or prematurely degraded, the levels of Sso1-1p and Sso2-1p were monitored in a temperature shift experiment where *sso1-1 sso2Δ*, *sso1Δ sso2-1* and wild-type strains were grown at 24°C and then shifted to either 24°C or 38°C (for details see Materials and Methods). Cell lysates were collected during a 2 hour period after the shift and analysed in western blots with Sso1p and Sso2p-specific antisera. As seen in Fig. 6B, we found that both Sso1-1p and Sso2-1p are present at the same level as the corresponding wild-type proteins at both the permissive and the restrictive temperature. We conclude that the temperature-sensitive phenotypes are not caused by decreased expression or increased degradation, but rather reflect a real temperature sensitivity of the mutant proteins.

The *sso2-1* mutation can be suppressed by overproduction of either Snc1p, Snc2p or Sec9p

One reason for making the *sso1Δ sso2-1* strain was that we wanted to investigate how the *sso2-1* mutation interacts genetically with other genes involved in secretion. We therefore proceeded to screen a high copy number genomic yeast library made in the 2μ vector pHR81 (Nehlin et al., 1989) for genes that can suppress the temperature-sensitive

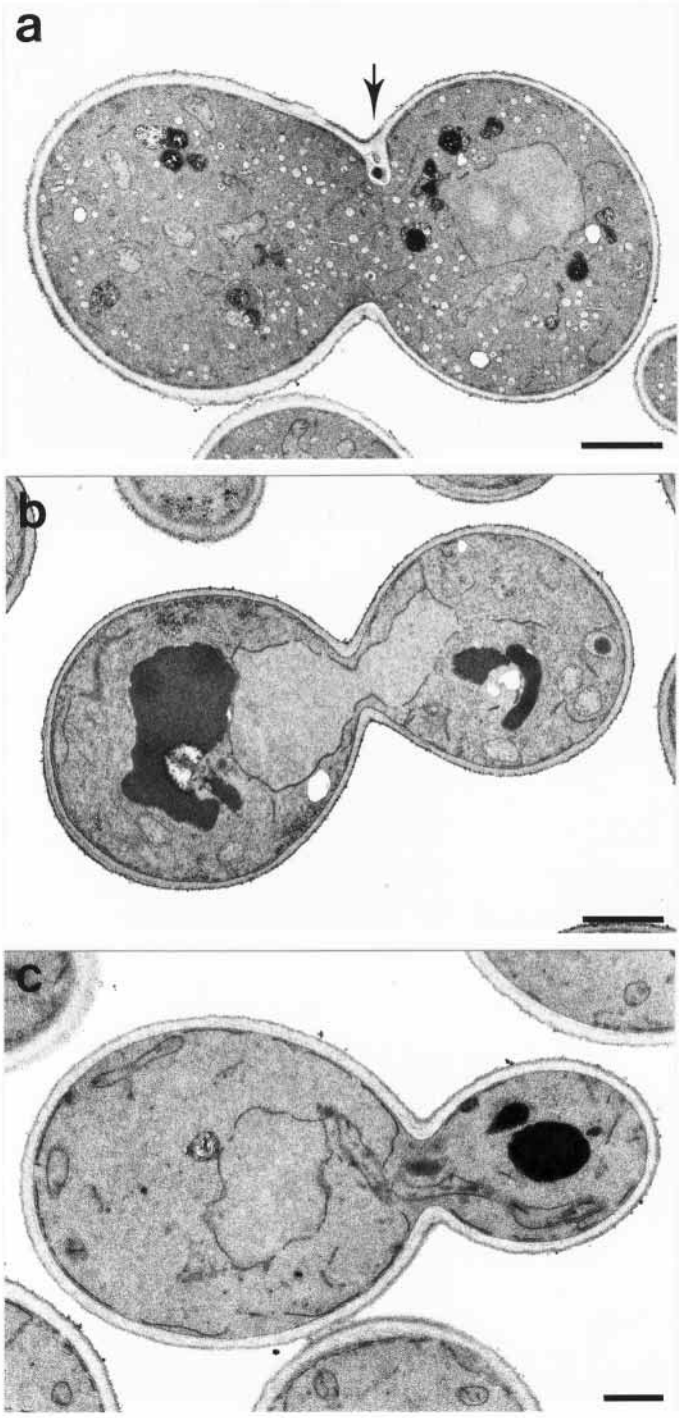

Fig. 4. Electron micrographs of *sso1Δ sso2-1* (H902) and congenic wild-type (NY179) cells at different temperatures. The cells were first grown in YPD at 24°C until an A_{600} of 1.0. An aliquot of each culture was then incubated at 37°C for 3 hours. (a) *sso1Δ sso2-1* cells at 37°C; (b) wild-type cells at 24°C; (c) wild-type cells at 37°C. The arrow in panel a points to the incomplete septum that can frequently be found in *sso1Δ sso2-1* cells at the restrictive temperature. Bars, 1 μm (a,b); 500 nm (c).

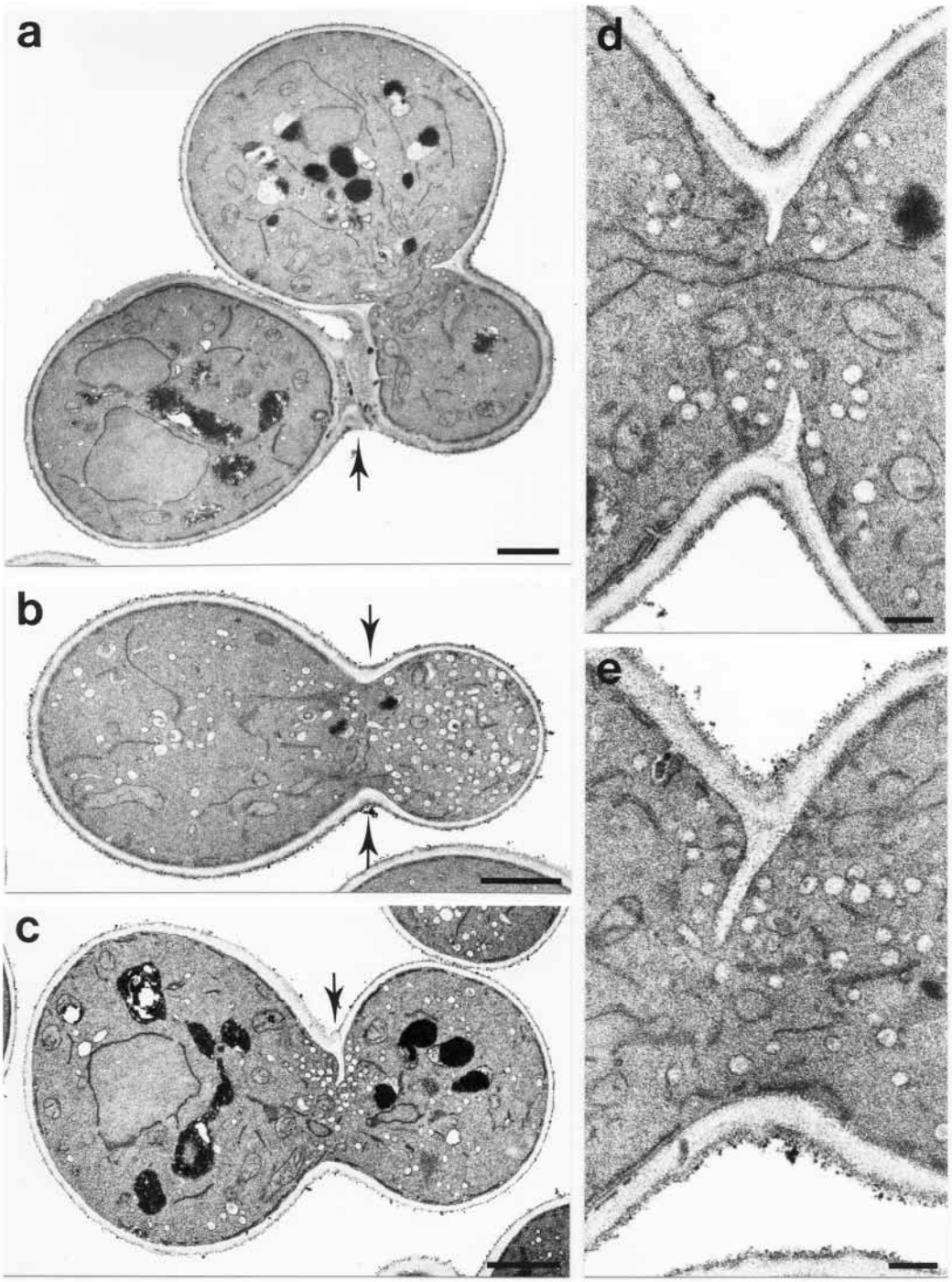

Fig. 5. Electron micrographs of *sso1Δ sso2-1* cells (H902) grown at the permissive temperature (24°C). The panels were chosen to illustrate the following morphological defects: (a) incomplete cell separation; (b) accumulation of vesicles in the bud and unusually wide bud neck; (c-e) incomplete septum formation and accumulation of vesicles in the bud neck. The arrows point to the relevant structures. Bars, 1 μm (a,b,c); 200 nm (d,e).

phenotype of *sso1Δ sso2-1* cells. In addition to the *SSO2* gene itself, three suppressor plasmids were recovered, which were found to contain *SNC1*, *SNC2* and *SEC9*, respectively. As shown in Fig. 7, the genes differ in their ability to suppress *sso2-1* at different temperatures. The poorest suppressor is *SNC2*, which can suppress *sso2-1* well at 30°C and poorly at 32°C. *SNC1* and *SEC9* suppress *sso2-1* well at 32°C and poorly at 34°C. As expected, the *SSO2* gene itself on either a high copy number or a single copy plasmid can complement *sso2-1* at all temperatures tested.

Several other genes that were not recovered in the suppressor screen were tested for their ability to suppress *sso2-1*. As expected, we found that *SSO1* can suppress *sso2-1* when present on either a high copy number or single copy number plasmid. By contrast, high copy number plasmids containing the *SEC1* (Aalto et al., 1991), *SEC3* (Haarer et al., 1996), *SEC4* (Salminen and Novick, 1987), *SEC15* (Salminen and Novick, 1989) or *MSO1* gene (Aalto et al., 1997) were all unable to suppress the mutation (data not shown). The *sso2-1* mutant allele on a high copy number plasmid, YEpsso2-1 was also tested, but did not suppress the temperature sensitivity at any temperature tested. We conclude that overproduction of the

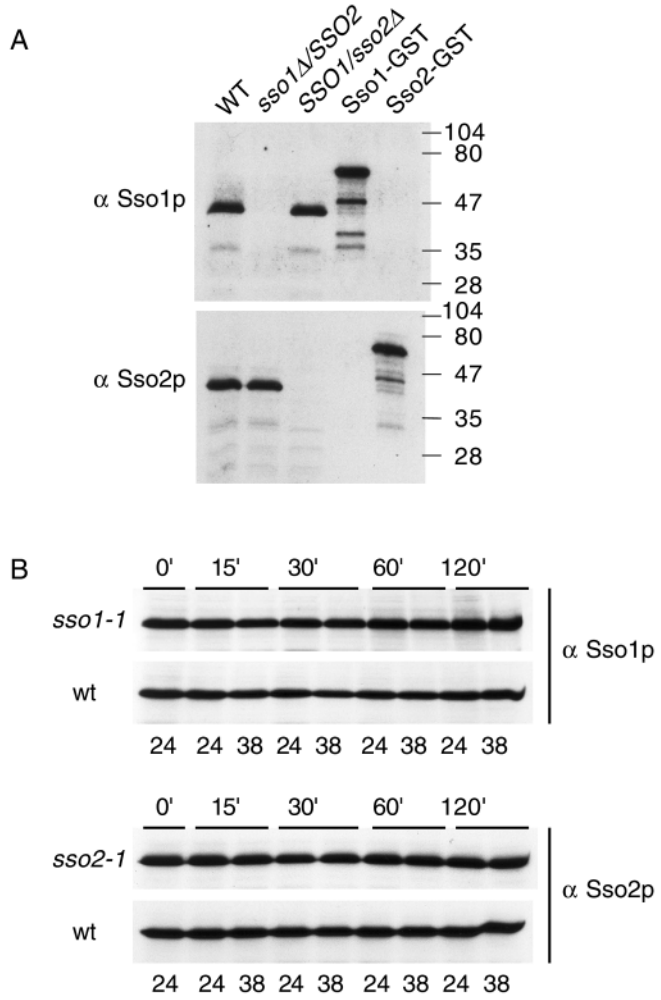

Fig. 6. (A) Characterisation of the anti-Sso1p and anti-Sso2p antisera. Cell lysates from wild-type (W303-1A), *sso1Δ* (H403) and *sso2Δ* (H404) cells (10 μg of total protein) and 15 ng of purified Sso1-GST and Sso2-GST fusion proteins were analysed by western blotting using the specific antisera against Sso1p or Sso2p. Molecular weight markers are shown on the right. (B) Expression of wild-type and mutant Sso1p and Sso2p at permissive and restrictive temperatures. Wild-type (W303-1A), *sso1-1 sso2Δ* (H1239), and *sso1Δ sso2-1* (H603) cells grown at 24°C were shifted to either 24°C or 38°C. Samples were taken at 0, 15, 30, 60 and 120 minutes after the temperature shift. Cell lysates were prepared and 20 μg of total protein was analysed by western blotting using the specific antisera against Sso1p or Sso2p.

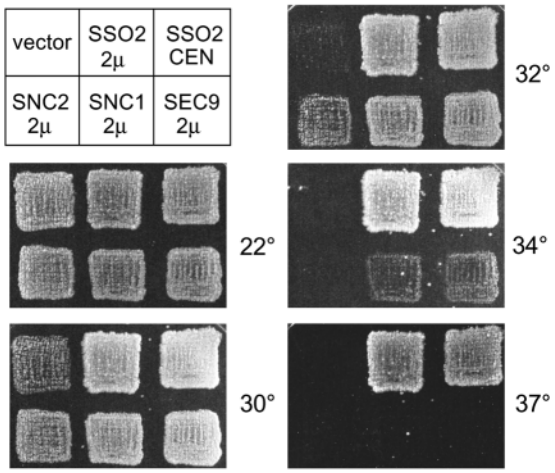

Fig. 7. Suppression of the temperature-sensitive *sso2-1* mutation by overexpression of other genes that are involved in secretion. H902 cells (*sso1Δ sso2-1*) containing different *SEC* genes on high copy number plasmids from the genomic pHR81 library were grown at 24°C, and then replicated to plates that were incubated at different temperatures, as shown in the figure. The vector control is pHR81 (Nehlin et al., 1989). Plasmid pMO9 carrying the *SSO2* gene on a single copy plasmid was included as a control (SSO2 CEN in the figure).

mutant protein itself is unable to compensate for the defect even if the temperature is lower than in the screen used to isolate the mutation.

Synthetic interactions between *SSO1, SSO2* and *MSO1*

The *MSO1* gene encodes a small hydrophilic protein that binds to Sec1p (Aalto et al., 1997). In wild-type cells, a disruption of *MSO1* causes a fourfold accumulation of secretory vesicles in the bud, indicating a partial block in secretion. However, the cells are otherwise healthy. By contrast, the *mso1* disruption shows strong synthetic interactions with temperature-sensitive mutations in those *SEC* genes that are involved in the terminal stage of secretion. Thus, the *mso1* disruption is lethal in the presence of *sec1*, *sec2* or *sec4* mutations, semilethal in *sec3*, *sec6*, *sec8* and *sec9* cells, and causes reduced growth at the permissive temperature in *sec5*, *sec10* and *sec15* cells (Aalto et al., 1997). We therefore wanted to examine whether the *sso1-1* or *sso2-1* mutations would show synthetic interactions with *mso1Δ*. To this end, we dissected tetrads from crosses of congenic *sso1-1 sso2Δ*, *sso1Δ sso2-1* and *mso1Δ* strains. Spores of different genotypes were isolated and tested for growth at a range of temperatures.

The results are shown in Fig. 8. Two interesting observations were made. First, we found that loss of *MSO1* has a much more pronounced effect in cells where *SSO1* is the only expressed *SSO* gene than in wild-type cells or cells expressing only *SSO2*. This effect is seen in the presence of both the wild-type *SSO1* gene and the *sso1-1* allele. Thus, *sso2Δ mso1Δ* cells, which express the wild-type Sso1 protein, are temperature sensitive at 38°C, while both single disruptions grow well at all temperatures tested. Similarly, disrupting *MSO1* in *sso1-1 sso2Δ* cells, which grow well up to 36°C, makes them more temperature sensitive with a clear effect being seen already at 30°C and no growth at all at 34°C. By contrast, disrupting *MSO1* in *sso1Δ* or *sso1Δ sso2-1* cells, which express either wild-type Sso2p or Sso2-1p, has little, if any, effect. From these observations we conclude that the Sso1 protein, whether wild type or mutant, is more dependent on Mso1p than is Sso2p. Second, we found that cells expressing *sso2-1* are somewhat more temperature sensitive than cells where the *SSO2* gene has been deleted. This effect is most clearly seen with the *sso1-1 sso2-1* double mutant, which fails to grow at 36°C, where the *sso1-1 sso2Δ* strain grows, but it is also evident from the fact that *sso2-1 mso1Δ* cells grow more poorly than *sso2Δ mso1Δ* cells at 36°C. We interpret this as a partial dominant-negative

effect of the *sso2-1* mutation with respect to temperature sensitivity. A likely explanation is that the Sso2-1 mutant protein, which functions more poorly than either wild-type or mutant Sso1p, competes with the latter for association to other SNAREs and thus reduces the number of productive SNARE complexes that are formed.

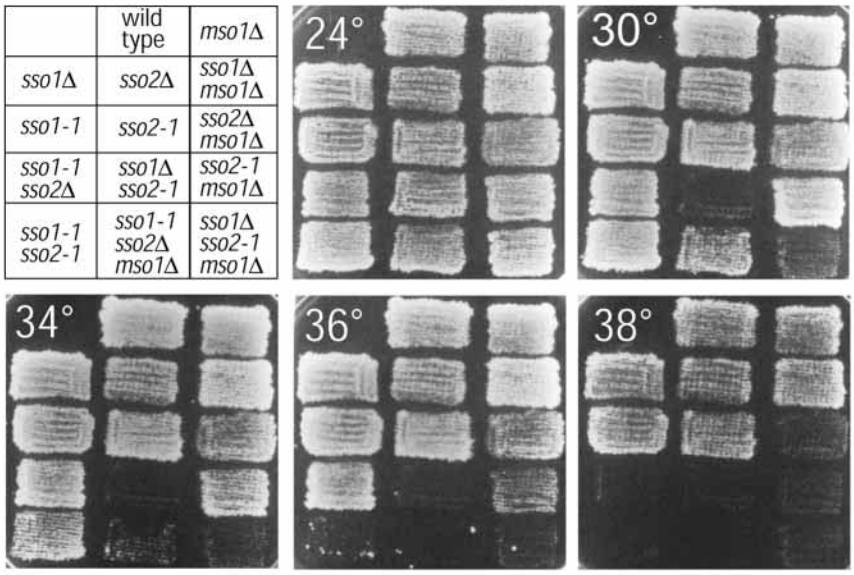

Fig. 8. Synthetic interactions between *sso1*, *sso2* and *mso1* mutations. W303 congenic strains of the indicated genotypes (Table 1) were patched onto a YPD plate and grown at room temperature. The plate was then replicated to fresh plates that were incubated at the indicated temperatures.

Sso1p and Mso1p are both required for sporulation

In a search for synthetic interactions between *sso2-1* and other temperature-sensitive *sec* mutations, we made a number of crosses in a homozygous *sso1Δ/sso1Δ* background. Surprisingly, we found that these diploids failed to sporulate. By contrast, *sso2Δ/sso2Δ* diploids sporulate normally. Further experiments confirmed that the *SSO1* gene is essential for sporulation even in the presence of the wild-type *SSO2* gene (Table 3). This is noteworthy since all experiments so far have indicated that the closely related *SSO1* and *SSO2* genes have redundant functions in vivo (Aalto et al., 1993). It should be noted that *SSO1* and *SSO2* are differentially expressed during the cell cycle (Spellman et al., 1998), and recent evidence further suggests that *SSO1* is slightly more expressed than *SSO2* during sporulation (Chu et al., 1998). This raised the possibility that the need for *SSO1* in sporulation might be due to its preferential expression rather than to a specific requirement for the Sso1 protein. We therefore tested whether overexpression of *SSO2* from a high copy number plasmid, using either its own promoter or the *ADH1* promoter, could restore the ability of *sso1Δ/sso1Δ* diploids to sporulate. We found that this was the case, but the level of sporulation was much lower than in the presence of the *SSO1* gene (Table 3). We also tested whether the *SEC9*, *SNC1* or *SNC2* genes can suppress the sporulation deficiency of *sso1Δ/sso1Δ* diploids when overexpressed (Table 3). This was not the case, indicating that the need for Sso1p cannot simply be compensated for by increased concentrations of other interacting proteins. Finally, we tested whether *SSO1* is required for pseudohyphal growth. However, this was not the case (data not shown).

The finding that Sso1p is required for sporulation and the observed genetic interactions between *SSO1* and *MSO1* prompted us to re-examine the role of Mso1p in sporulation. We found that a diploid that is homozygous for an *mso1* disruption is unable to sporulate (Table 4). However, it should be noted that this block does not appear to be as strict as that observed for *sso1Δ/sso1Δ* strains. Thus, in the W303 background, which is high-sporulating, occasional dyads and triads were formed in the *mso1Δ/mso1Δ* strain after several days on sporulation medium. In the NY179 background, which is low-sporulating, no dyads or triads were seen. We proceeded to investigate whether the *mso1Δ/mso1Δ* sporulation defect can be suppressed by overexpression of other genes involved in the terminal step of secretion. As shown in Table 4, we found that both *SEC1* and *SSO1* can restore sporulation to near wild-type levels when overexpressed from a 2μ plasmid. By contrast, overexpression of *SEC4* or *SSO2* failed to suppress the *mso1Δ/mso1Δ* sporulation defect. As expected, expression of *MSO1* itself from a centromeric plasmid could also complement the *mso1Δ* sporulation defect. The fact that overexpression of *SEC1* can suppress the *mso1Δ/mso1Δ* sporulation defect is noteworthy since *MSO1* itself was isolated as a high copy number suppressor of the *sec1-1* mutation. This cross-suppression suggests that the two encoded proteins function closely together and these proteins have in fact been

Table 3. Sporulation frequencies for *sso1Δ/sso1Δ* diploids containing different high copy number plasmids

Strain	Plasmid	Insert	Tetrads (%)
D67*	None	–	19.9
D273	YEpSSO2	*ADH1-SSO2*	0.8
D272	pSSO2	*SSO2*	0.9
D272	pSNC1	*SNC1*	0.0
D272	pSNC2	*SNC2*	0.0
D272	pSEC9	*SEC9*	0.0
D272	pHR81	–	0.0

*The congenic wild-type strain D67 was included as a positive control.

Table 4. Sporulation frequencies for *mso1Δ/mso1Δ* diploids containing different high copy number plasmids

Strain	Plasmid	Insert	Tetrads (%)
D286	YCpMSO1*	*MSO1*	12.5
D286	YEpSSO1U	*SSO1*	9.9
D286	YEpSSO2U	*SSO2*	0.0
D286	YEpSEC1aU	*SEC1*	8.5
D286	YEpSEC4	*SEC4*	0.0
D286	pRS426	–	0.0

*The *MSO1* gene on a single copy plasmid was included as a positive control.

Table 5. Sporulation frequencies for *sso1Δ/sso1Δ* diploids containing single copy plasmids expressing different *SSO1/SSO2* promoter fusions

Strain	Plasmid	Insert	Tetrads (%)
D272	pMO7	*SSO1*	23.8
D272	pMO33	*SSO1 (NdeI)**	23.2
D272	pM035	P_{SSO2}-*SSO1*	6.9
D272	pMO9	*SSO2*	0.0
D272	pMO24	*SSO2 (NdeI)**	0.0
D272	pMO37	P_{SSO1}-*SSO2*	0.0

*These constructs, which were used for the promoter switches, differ from the corresponding wild-type genes only in that they carry an *Nde*I site at the ATG.

shown to physically interact (Aalto et al., 1997). The fact that *SSO1* but not *SSO2* can suppress the *mso1Δ/mso1Δ* phenotype is also interesting. It lends further support to the notion that Sso1p has a unique function during sporulation that cannot be provided by Sso2p.

Expression of *SSO2* from the *SSO1* promoter cannot restore sporulation in an *sso1/sso1* strain

Finally, we wanted to investigate whether the inability of *SSO2* to complement the *SSO1* sporulation defect is due to the decreased expression of *SSO2* that is observed during sporulation (Chu et al., 1998), or if the Sso1 protein has a specific role in sporulation. We proceeded by introducing a restriction site at the start codon of both genes and then swapped the promotors between the genes. The fusion constructs were cloned into *CEN* plasmids that were transformed into an *sso1*Δ/*sso1*Δ strain. The transformants were plated to sporulation medium, incubated at room temperature for 5 days before determining the sporulation frequency by counting at least 1000 cells in a Bürker chamber (Table 5). As expected, we found that expression of the *SSO1* gene or its derivative with an *Nde*I site at the ATG restores sporulation to the level seen in the wild-type strain (Table 3). Expression of the *SSO2* gene from the same single copy plasmid did not restore sporulation to any detectable degree, unlike the result obtained with high copy number *SSO2* plasmids. Interestingly, nor was any sporulation seen when the *SSO2* gene was expressed from the *SSO1* promoter. By contrast, expression of the *SSO1* gene from the *SSO2* promoter did restore sporulation, but to a lower degree than in the wild-type (Table 5). From these experiments we conclude that it is the Sso1 protein itself that is specifically required during sporulation.

Discussion

Docking and fusion of secretory vesicles at the plasma membrane involves a large number of proteins (Salminen and Novick, 1987; Salminen and Novick, 1989; Aalto et al., 1992; Aalto et al., 1993; Protopopov et al., 1993; Brennwald et al., 1994; TerBush et al., 1996). Many of them were first identified by genetic screens in yeast, as temperature-sensitive mutations in the *SEC* genes that block secretion at the restrictive temperature (Novick and Schekman, 1979; Novick et al., 1981). However, the yeast homologue of syntaxin 1, a key protein in exocytosis, is encoded by duplicated genes, and was therefore not recovered in these genetic screens. The two genes, *SSO1* and *SSO2*, were instead cloned as high copy number suppressors of a temperature-sensitive mutation in the *SEC1* gene (Aalto et al., 1993). A deletion of either *SSO1* or *SSO2* is viable, but loss of both genes in vegetatively grown cells is lethal, thus proving that these t-SNAREs perform an essential function in the cell.

To gain further insights into syntaxin function, we have used in vitro mutagenesis followed by a genetic screen in yeast to isolate a temperature-sensitive mutation in the *SSO2* gene. The resulting *sso2-1* mutation is very conservative: it changes arginine-200 to a lysine. The syntaxin-like t-SNAREs comprise a large protein family (Aalto et al., 1993; Weimbs et al., 1997) which includes not only the Sso proteins and their homologs in other eukaryotes, but also more distantly related proteins such as Sed5p (Hardwick and Pelham, 1992) and Pep12p (Becherer et al., 1996), which function in transport between the ER and Golgi, and between Golgi and the vacuole, respectively. Whereas the true Sso homologs from all eukaryotes show extensive similarities to each other throughout their sequences, the similarities to Sed5p and Pep12p are limited to a highly conserved region of about 70 residues that precedes the C-terminal hydrophobic tail (Aalto et al., 1993). This domain (known as H_{core}) participates both in the formation of the closed conformation of syntaxin molecules, where it interacts with the N-terminal 3 helix bundle ($H_aH_bH_c$), and in the formation of the Ssop-Sncp-Sec9p complex (Fiebig et al., 1999). Nine residues within H_{core} are particularly strongly conserved among the syntaxin-related proteins (Aalto et al., 1993) and arginine-200 is one of them. This suggests that the arginine is functionally very important, and may thus explain why a conservative substitution to a lysine has such a pronounced effect.

Interestingly, a temperature-sensitive mutation in Sed5p, *sed5-1*, changes the same arginine, in this case to a glycine (Banfield et al., 1995). A mutation of the corresponding arginine (arginine-198) was also one of several alanine substitutions used to study how rat syntaxin 1A (a direct mammalian homologue of Sso1p and Sso2p) interacts with different proteins in vitro (Kee et al., 1995). Temperature sensitivity was not examined in that study, nor was the effect of mutating arginine-198 alone determined. However, a double mutation of arginine-198 and isoleucine-202 abolished the in vitro interaction of syntaxin 1A with VAMP/synaptobrevin (Kee et al., 1995). Furthermore, the binding of the mammalian Sec9p homolog SNAP-25 was significantly reduced in two quadruple mutants, both of which involved arginine-198, and the binding of the mammalian Sec1p homolog n-Sec1 was abolished in one of these two quadruple mutants. These results are interesting in view of the finding that *SNC1, SNC2* and *SEC9* can suppress the *sso2-1* mutation when overexpressed (Gerst, 1997) (Fig. 7). It suggests a direct interaction model for the suppression, where a reduced affinity of the Sso2-1 protein for Snc1p, Snc2p and/or Sec9p is compensated for by an increased concentration of these proteins.

To study the terminal phenotype of the *sso2-1* mutation at the restrictive temperature we made a yeast strain that lacks the wild-type *SSO1* genes and carries the *sso2-1* allele at the *SSO2* locus. As expected, we found that *sso1Δ sso2-1* cells are temperature sensitive for growth. Furthermore, the cells

accumulate transport vesicles at the restrictive temperature (Fig. 4). This phenotype is similar to that of classical *sec* mutations (Novick et al., 1981) and consistent with our finding that secretion is impaired in these cells (Fig. 2). The same mutation was also introduced into *SSO1*, producing the *sso1-1* allele. We found that *sso1-1 sso2Δ* cells are temperature sensitive, but with a less pronounced phenotype. Thus, they fail to grow at 38°C and above (on rich media), compared with 30°C for *sso1Δ sso2-1* cells. It is noteworthy that the temperature sensitivity of both *sso1-1* and *sso2-1* is more pronounced on rich media than on synthetic media. A similar phenotype was observed for yeast cells that lack the two plasma membrane v-SNAREs, *SNC1* and *SNC2* (Protopopov et al., 1993) and for *sec3-101* strains (Haarer et al., 1996). These observations, together with our finding, suggest that cells with a partially impaired secretion have a specific problem with growth on rich media. The cause of this rich medium sensitivity remains to be determined.

We further found that *sso1Δ sso2-1* cells (but not *sso1-1 sso2Δ* cells) have a pronounced phenotype at the permissive temperature. Thus, cytokinesis and neck closure are severely affected, causing an incomplete separation of mother and daughter cells (Fig. 5). This effect is recessive and restricted to cells where Sso2-1p is the only Sso protein, so it most likely reflects a partial loss of function for the mutant protein already at the permissive temperature. Syntaxins have been shown to be required for cytokinesis in *C. elegans*, *A. thaliana* and sea urchin embryos (Lukowitz et al., 1996; Jantsch-Plunger and Glotzer, 1999; Conner and Wessel, 1999). In addition, our findings show that syntaxins are important for cytokinesis in *S. cerevisiae*. Secretion plays a key role in growth and formation of the bud, and it is therefore not surprising if a partial defect in the docking and fusion of transport vesicles can affect neck closure (e.g. by limiting new membrane formation). We further note that even when a complete septum has been formed, the cell wall is often continuous between the mother and daughter cell (Fig. 4a). It is possible that this reflects a failure in the delivery of cell wall degrading enzymes to the neck site. Finally, we note that while *sso1-1 sso2Δ* cells lack a clear morphological phenotype at 24°C, they display similar vesicle accumulation and septum formation defects at the restrictive temperature (38°C).

One reason for obtaining a temperature-sensitive *sso2-1* mutation was to study its genetic interactions with other genes involved in secretion. Several such interactions have previously been found between the late-acting *SEC* genes. Thus, the *SSO1* and *SSO2* genes can suppress temperature-sensitive mutations in the *SEC1*, *SEC3*, *SEC5*, *SEC9* and *SEC15* genes when overexpressed (Aalto et al., 1993). *SEC9* encodes the yeast homolog of SNAP-25, a subunit of the SNARE complex that interacts with the Sso proteins (Brennwald et al., 1994). The Sec1 protein is also known to interact with the syntaxins (Hata and Südhof, 1995; Carr et al., 1999). The remaining three genes that are suppressed by *SSO1* and *SSO2* encode subunits of the exocyst, another protein complex that is involved in the terminal stage of secretion (TerBush et al., 1996). We found that the *SNC1*, *SNC2* and *SEC9* genes can suppress *sso2-1* when they are overexpressed, whereas the *SEC1*, *SEC3*, *SEC4*, *SEC15* and *MSO1* genes failed to do so. As discussed above, Sso2p, Sec9p and Snc1p all interact with each other, which suggests a direct physical model for the mechanism of suppression. We further note that both *SSO2* and *SEC9* can suppress temperature-sensitive mutations in each other when overexpressed. This resembles the reciprocal cross-suppression between *SEC1* and *MSO1*.

Significantly, we have found that the *SSO1* and *SSO2* genes, previously thought to be functionally redundant (Aalto et al., 1993) have partially non-overlapping functions. This conclusion is supported by three observations. First and foremost, we found that Sso1p is required for sporulation, and that Sso2p cannot provide this function, even when expressed from the *SSO1* promoter (Table 5). Second, we found that overexpression of Sso1p, but not of Sso2p, can suppress the sporulation defect of an *mso1Δ/mso1Δ* diploid (Table 4). Third, we found that a disruption of *MSO1* has a much stronger effect in cells expressing only Sso1p, indicating a specific functional dependency between these two proteins (Fig. 8). It suggest that Sso1p and Mso1p function closely together, possibly within the same complex. It should be noted that while a direct interaction between Mso1p and Sso1p (or Sso2p) has not been demonstrated, Mso1p interacts with Sec1p (Aalto et al., 1997) and Sec1p in turn interacts with the Sso proteins (Carr et al., 1999).

The fact that both Sso1p and Mso1p are required for sporulation suggests that the function provided by these two proteins is particularly important during this process. In this context, it is interesting to note that Sec9p has a duplicated homologue, Spo20p, which is expressed only during meiosis, and which is required for sporulation (Neiman, 1998). It raises the question whether Sso1p and Mso1p could be part of a sporulation-specific vesicle docking complex. It should be noted that Sso1p and Mso1p are both expressed and functionally active in mitotic cells (Aalto et al., 1993; Aalto et al., 1997) so the situation is not completely analogous to that of Spo20p. Still, it is conceivable that a sporulation-specific protein such as Spo20p might interact specifically with Sso1p, but not Sso2p. This could explain why Sso1p is required during sporulation. Alternatively, a modification that is specific for Sso1p might be involved. Sso1p is phosphorylated on serine-79 and dephosphorylation of this residue is required for efficient incorporation of Sso1p into SNARE complexes (Marash and Gerst, 2001). However, this serine is conserved in Sso2p, so it is unlikely that this phosphorylation accounts for the differential behaviour of Sso1p and Sso2p during sporulation. As for the role of Mso1p, it is interesting to note that the sporulation defect of *mso1Δ/mso1Δ* cells can be suppressed by overexpression of either Sso1p or Sec1p (Table 4). A possible interpretation of this finding is that Mso1p functions by activating or potentiating Sso1p, possibly through an interaction with Sec1p, which is also known to be required during sporulation (Neiman et al., 1998). Accordingly, the need for Mso1p in meiosis would be a consequence of the specific need for Sso1p in this process. Finally, we note that a specific need of Mso1p for proper Sso1p function could explain why the *mso1* disruption has a much stronger effect in mitotic cells expressing only Sso1p than in cells expressing only Sso2p.

We thank Vesa Olkkonen for the use of animal facilities for antibody production, Markus Linder for HPLC analysis of the peptide coupling, and Dan Fredriksson, Mervi Lindman, Outi Könönen and Hanna Karmakka for skilful technical assistance, Jukka Juselius for help in the preparation of figures and Patrick Brennwald, Jeffrey

Gerst, Marja Makarow and Peter Novick for generous gifts of reagents. This work was supported by grants from the Swedish Cancer Society (to H.R.), the Academy of Finland (grants 8244, 42160, 49894, to S.K.), the Finnish Centre of Excellence Programme (2000-2005) and the Human Frontier Science Program (RG 63/95, to S.K.). M.K.A. was supported by a Fellowship from the Academy of Finland and a grant from the University of Helsinki.

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