A random mutagenesis approach to isolate dominant negative yeast sec1 mutants reveals a functional role for Domain 3a in yeast and mammalian SM proteins

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SNAP receptor (SNARE) and Sec1/Munc18 (SM) proteins are required for all intracellular membrane fusion events. SNAREs are widely believed to drive the fusion process, but the function of SM proteins remains unclear. To shed light on this, we screened for dominant negative mutants of yeast Sec1 by random mutagenesis of a GAL1-regulated SEC1 plasmid. Mutants were identified on the basis of galactose-inducible growth arrest and inhibition of invertase secretion. This effect of dominant negative sec1 was suppressed by overexpression of the v-SNAREs, Snc1 and Snc2, but not the t-SNAREs, Sec9 or Sso2. The mutations isolated in Sec1 clustered in a hotspot within Domain 3a, with F361 mutated in 4 different mutants. To test if this region was generally involved in SM protein function, the F361-equivalent residue in mammalian Munc18-1 (Y337) was mutated. Overexpression of the Munc18-1 Y337L mutant in bovine chromaffin cells inhibited the release kinetics of individual exocytosis events. The Y337L mutation impaired binding of Munc18-1 to the neuronal SNARE complex, but did not affect its binary interaction with syntaxin1a. Taken together, these data suggest that Domain 3a of SM proteins has a functionally important role in membrane fusion. Furthermore, this approach of screening for dominant negative mutants in yeast may be useful for other conserved proteins, in order to identify functionally important domains in their mammalian homologues.
INTRODUCTION

Fundamental cellular processes are controlled by similar mechanisms in all eukaryotes. An excellent example of this is intracellular membrane fusion, which is controlled by the same ubiquitous protein machinery from yeast to the human brain (FERRO-NOVICK and JAHN 1994). At the heart of this machinery are the soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors (SNAREs) (SOLLNER et al. 1993). First identified from brain as syntaxin 1, SNAP-25 and VAMP (SOLLNER et al. 1993), this family of proteins is characterised by possession of one or more signature SNARE motifs, which mediate the interaction of the individual proteins to form a 4-helical heteromeric complex (SUTTON et al. 1998). The zippering together of SNAREs localised to the vesicle (v-) and target (t-) membranes as complex formation proceeds is thought to pull the two membranes together and drive the fusion reaction (JAHN and SCHELLER 2006). This idea is supported by the demonstration that membrane fusion can be reconstituted in vitro between proteoliposomes containing the appropriate yeast or mammalian SNAREs (McNEW et al. 2000; WEBER et al. 1998).

Although much attention has focused on SNAREs, these are not the only evolutionarily conserved proteins required for intracellular membrane fusion. Genetic studies have established a universal requirement for the Sec1/Munc18 (SM) protein family in vesicle fusion in a wide variety of organisms, including yeast (SEC1, SLY1, VPS33, VPS45), plants (KEULE), nematodes (UNC18), flies (ROP) and mice (munc18-1) (HALACHI and LEV 1996; TOONEN and VERHAGE 2003). The general role of SM proteins in membrane fusion is illustrated by their requirement for ER-Golgi traffic (Sly1), transport to the vacuole (Vps33, Vps45), and exocytosis (Sec1) in yeast. SM proteins from a variety of organisms have been shown to interact with SNAREs
- particularly syntaxin homologues - suggesting that the conserved function of SM proteins in membrane fusion may be SNARE-related (Jahn 2000). However, this general requirement for SM proteins in fusion has proved difficult to reconcile with the divergent binding modes of different SM-SNARE protein interactions (Gallwitz and Jahn 2003). For example, neuronal Munc18-1 was originally shown to bind with high affinity to a closed conformation of syntaxin1a in isolation to form a complex that precludes syntaxin entering the SNARE complex (Mode 1) (Misura et al. 2000). In contrast, mammalian Munc18c, yeast Sly1 and Vps45 bind to the extreme N-terminus of their cognate syntaxins, either in isolation or as part of a SNARE complex (Mode 2) (Bracher and Weissenhorn 2002; Carpp et al. 2006; Dulubova et al. 2002; Hu et al. 2007; Peng and Gallwitz 2004). A third binding mode is evident in yeast Sec1, which is claimed not to bind efficiently to its cognate syntaxin (Sso1/2) in isolation (but see (Scott et al. 2004)), and to only interact with the ternary SNARE complex (Mode 3) (Carr et al. 1999; Togneri et al. 2006). Recently, evidence has emerged suggesting that individual SM proteins can employ multiple SNARE binding modes (Burgoyne and Morgan 2007; Toonen and Verhage 2007). For example, the binary interaction of Munc18-1 with syntaxin 1 and of Munc18c with syntaxin 4 appears to involve both Mode 1 and Mode 2 interactions (Burkhardt et al. 2008; D'Andrea-Merrins et al. 2007; Rickman et al. 2007). Similarly, the Mode 3 interaction of Munc18-1 with the SNARE complex requires Mode 2 N-terminal binding to occur (Dulubova et al. 2007; Shen et al. 2007).

The available structural information on SM-SNARE protein binding Modes 1 and 2 has enabled the design of mutations that disable these interactions. The Mode 1 interaction of Munc18-1 with the closed conformation of syntaxin can be inhibited by introduction of mutations that render syntaxin constitutively open (Dulubova et al. 1999). However, yeast and C. elegans expressing
such open mutants as the sole copy of their appropriate syntaxin are apparently normal under standard conditions (although a synthetic phenotype can seen with yeast sec9 and C. elegans unc-13 or unc-10 mutants) (Koushika et al. 2001; Munson and Hughson 2002; Richmond et al. 2001). Similarly, mutations that disable the Mode 2 interaction between Sly1 and Sed5, and between Vps45 and Tlg2, show no defects in ER-Golgi or vacuolar trafficking (Carpp et al. 2006; Peng and Gallwitz 2004). The physiological significance of these SNARE binding modes for SM function therefore remains uncertain. As both yeast Sec1 and mammalian Munc18-1 share the ability to interact with their cognate syntaxins in the assembled SNARE complex and to stimulate liposome fusion in vitro (Scott et al. 2004; Shen et al. 2007), it may be that Mode 3 binding may underlie the conserved function of SM proteins in membrane fusion. However, there are no structural data on this interaction upon which to design mutations to test this hypothesis.

To shed light on the putative conserved function of SM proteins, we undertook an unbiased screen for yeast Sec1 mutants and then tested for functional effects of conserved mutations in mammalian Munc18-1. We reasoned that dominant negative mutants might represent generally useful tools, as any mutations in conserved residues could then be introduced into other SM proteins for analysis even in the presence of the endogenous wild-type protein, which is particularly useful in mammalian systems. We therefore employed random mutagenesis using an inducible SEC1 gene and screened for dominant negative mutations active in vivo. Here we describe the isolation and characterisation of such sec1 mutants. The mutations clustered around the structurally conserved Domain 3a of SM proteins, facilitating the design of an analogous mutant in mammalian Munc18-1. This mutant inhibited exocytotic release kinetics in bovine chromaffin cells, suggesting a functional role for Domain 3a of SM proteins in the membrane fusion process.
MATERIALS AND METHODS

Materials

All materials were obtained from Sigma, unless otherwise stated. Restriction enzymes were obtained from Promega or New England Biolabs. Plasmids and yeast strains are summarized in Tables 1 and 2.

Isolation of mutations

Construction of pYES2-SEC1

A suitable SEC1 fragment was obtained by PCR using Pfu Turbo (Stratagene). Template DNA was p624 obtained from Dr N J Bryant (University of Glasgow, UK): this plasmid contains a genomic fragment encompassing the SEC1 gene and has been validated by sequencing. Primers used were: CGAAGGGGTCCCGGAACGATGTCTGATTTAATTGAATTAC; and CGAAGGCGATCCCGGAACGATGTCTGATTTAATTGAATTAC. The resulting fragment was purified, digested with BamHI and SphI overnight, and then ligated together with BamHI-SphI-digested pYES2 vector (Invitrogen). The resulting plasmid was named pYES-SEC1: the SEC1 region of this plasmid was confirmed by DNA sequencing.

Mutagenic PCR

pYES-SEC1 was used as a template in a set of PCR reactions set up as follows: primers used were:ACCTCTATACTTTAACGTCAAGG; and AAATAGGGACCTAGACTTCAGG. These primers amplify a fragment corresponding to the pYES-SEC1 insert fragment flanked by approx 100 bp of vector sequence on either side.
PCR conditions used included dGTP/dCTP/dTTP at 1mM (4x standard) and dATP at 0.2mM (0.8x standard). The Mg\(^{2+}\) concentration was titrated at 2, 4, 6, 8, 10 mM. All but the first of these gave detectable products and these reactions were pooled for further processing.

**Transformation of yeast with mutagenised DNA**

Yeast strain BY4741 was transformed with a mixture of *Eco*RI-*Hind*III-digested pYES2 and purified mutagenised DNA. Selection for Ura\(^+\) transformants was carried out on plates containing 2% glucose and synthetic Ura drop-out media (SD-Ura). An estimated total of 5000 transformants were washed off the plates using water, made 15% with glycerol and then frozen in aliquots at -80°C.

**Screen for Gal\(^{8}\) colonies**

An aliquot of stored transformants was thawed and cell number was estimated by cell counting. Cells were plated at approximately 120/plate on 40 plates (SD-Ura). After growth at 30°C for 2 days, colonies were replicated to SG-Ura. Allowing for colonies at the edges of plates approximately 4000 colonies were screened. Examination of the plates revealed 25 candidates for Gal-sensitive growth. These were picked from the original SD-Ura plates and propagated as patches on SD plates for retesting. Plasmid DNA was rescued from candidates by preparing DNA and selecting Ap\(^R\) transformants in XL1-Blue supercompetent cells (Stratagene).

**Shuffling**

Because dominant-negative mutant plasmids carried *SEC1* genes that had sustained several mutations it was necessary to narrow down regions of the gene carrying mutations of interest. This was accomplished by moving restriction fragments from mutant genes into a *SEC1\(^+\)* framework. In order to facilitate this, the following modifications were carried out. First of all, the single *Bsr*GI site
of pYES-SEC1 (which lies within vector sequences) was destroyed by filling-in using Klenow DNA polymerase, thus creating a SnaBI site. This plasmid, pYES-SEC1ΔA was then used as the starting point for three separate constructions each involving the destruction of a further site, within the SEC1 ORF: SexA1, AvrII and XhoI. These three plasmids were named pYES-SEC1ΔAS, pYES-SEC1ΔAA and pYES-SEC1ΔAX.

The SEC1 fragment in pYES-SEC1 is divided into three regions by internal sites for BsaB1 and EcoRI: region A, BamHI to BsaB1; region B, BsaB1 to EcoRI; region C, EcoRI to SphI. The sites for SexA1, AvrII and XhoI lie within SEC1 regions A, B and C respectively. Shuffling involved digestion of a donor plasmid (carrying mutations) and a suitable acceptor plasmid with a pair of enzymes defining a region. The acceptor plasmid was chosen to have ablated sites both for BsrG1 and for a site lying within the region being shuffled (e.g. for a region B shuffle the acceptor would be pYES-SEC1ΔAA). The resulting DNA digests were mixed, ligated and then redigested with BsrG1 to counterselect the donor plasmid. Transformed bacterial colonies were then screened for the presence of an extra SnaBI site (diagnostic of the wild-type framework of the acceptor plasmid) and for the reappearance of the region-specific restriction site (AvrII in the case of the region B example).

The D6-2 mutant, containing a single mutation, F361L, was constructed by site-directed mutagenesis using a 4-primer method, with pYES-SEC1 as a template. Separate PCR amplifications were performed using 361F (CTGCTGAGTG TCGTAGCGCA CCTGAAAGAT CTAGATGAAG AAAGAAGAAG GCTG) and pYESR (AAATAGGGAC CTAGACTTCA GG) primers; and with L361R (GGTGCGCTACGACACTCAGCAG) and pYESF (ACCTCTATAC TTTAACGTCA AGG) primers. The resultant PCR products were then mixed, amplified using pYESF and pYESR primers and then cloned as BamHI-SphI fragments into pYES2.
**Introduction of mutant alleles into the genome**

The plasmid p444wt was constructed (described below) to allow introduction of specific mutant alleles of *SEC1* into the yeast genome. The plasmid contains a *SEC1* fragment with KanMX inserted immediately downstream of the *SEC1* ORF. This SEC1-KanMX cassette is excisable from p444wt by *BamHI*-SphI digestion. The D18B and D25B alleles were introduced into p444wt by replacement of the *SexAl-XhoI* fragment.

p444wt was constructed as follows. In the first step an adapted *SEC1* PCR fragment was cloned as a *BamHI*-SphI fragment into pK19 (PRIDMORE 1987). A Taq PCR was performed using p624 as template DNA with the primers

CGAAGGGGATCCCGGAACGATGTCTGATTTAATTGAATTAC and ACTAACTGCATGC+CATTATCAGTGCCGCACACACATGGGGCGCGCCTCATTTAT CATGGTGAGATTTC. The resulting plasmid, p4, contains *BamHI*-SEC1 ORF-(AscI-NotI-SphI). In the next step a fragment was cloned into p4 to reintroduce the *SEC1* downstream region. A Pfu PCR was performed using p624 as template with the primers

GTGGTAGCGGCCGCTCCCTTAAAGAAGACAGTGATAAAAAATC and CGAAGGGCATGCGAAAGGCCACGGCGTTTGGGACGCC. Both p4 plasmid and the PCR fragment were digested with *NotI* and *SphI* and ligated together. The resulting plasmid, p44, contains *BamHI*-SEC1 ORF-(AscI-NotI)-(*SEC1* 3’ region)-SphI. Next, a fragment encompassing a KanMX gene cassette was introduced by ligating *AscI-NotI*-digested p44 together with *AscI-NotI*-digested pFA6KanMX4 (LONGTINE et al. 1998). The resulting plasmid, p444 contains *BamHI*-SEC1 ORF-AscI-KanMX-NotI-(SEC1 3’ region)-SphI.
The BamHI-SEC1 ORF-AscI portion of p444 lacks the SEC1 promoter and is derived from a Taq PCR. This region was replaced with reliable wild-type sequence from the plasmid p624XB in which the XbaI site at the 5’ end of the SEC1 fragment of p624 was converted to a BamHI site using a linker. Plasmid p624XB was used as a source of a BamHI-XhoI fragment encompassing the SEC1 promoter and most of the SEC1 ORF which was ligated together with BamHI-XhoI-digested p44, creating p444wt.

To create LIVY12, the diploid strain BY4743 was transformed with a PCR fragment consisting of the URA3 gene from pYES2 flanked by SEC1 upstream and downstream sequences, creating a precise deletion of the SEC1 coding sequence. Ura+ transformants were selected and the presence of both SEC1 and sec1Δ::URA3 alleles was confirmed by PCR on genomic DNA using appropriate primers. Upon sporulation, LIVY12 asci yielded two viable spores which were Ura− and two spores which were inviable. The sec1-D18B::KanMX444; and sec1-D25B::KanMX444 fragments were excised from the corresponding p444-derived plasmids by BamHI-SphI digestion and used to transform LIVY12 and selected for resistance to geneticin, creating LIVY 18 and LIVY25. Transformant colonies were purified by restreaking on geneticin-containing medium and checked for loss of the URA3 marker as proof that the incoming fragment had transplaced at the sec1Δ::URA3 allele. Further confirmation was provided by PCR analysis using primers directed at the KanMX cassette and a chromosomal region flanking the SEC1 gene.

**PCR cloning for multicopy suppression analysis**

The following genes were amplified from yeast genomic DNA by PCR using pfu polymerase and the indicated primers:
*SSO2*: TGTGAATAAAGAAGCCAGCTAAAAG and GGGACTAATATTAAGGGCGACA.

*SNC2*: TTGGTCACATGATACGCGTG and TAAAACTGATGGCGCGAGAA.

*SEC9*: CGTAAAGATTGATCAAGACGATAAG and ACATTTCGCTGGGATACCTT.

*SEC4*: TGCGTGCCGCGTAATAAAA and CCAATCGCCTGATGAAATAC.

The PCR products were then cloned into *Sma*I-cut pK19, excised using suitable flanking polylinker sites and sub-cloned into YEpL. Double transformations were then performed with pYES-sec1-D18B and double transformants selected on –ura,-leu media.

### Invertase assay

Cells were grown overnight in selective medium containing 2% raffinose as sole carbon source.

Samples of these cultures were harvested, washed (water) and used to inoculate fresh YEP medium containing raffinose+galactose as carbon source (to induce synthesis of plasmid-encoded Sec1) to $A_{600}=0.1$. These cultures were incubated for 6h and then samples removed for invertase assay following a published method (Adamo et al. 1999). Cells were harvested washed x2 in ice-cold 10 mM sodium azide, and resuspended to 25 $A_{600}$/ml. 40 µl of cell suspension was added to 460 µl of spheroplast buffer (0.05 M Tris.HCl pH 7.5, 1.4 M sorbitol, 10 mM dithiothreitol, 100 µg/ml zymolyase 100T) and incubated for 30 min at 37º C. Spheroplasts were spun down gently and a 100 µl clean sample of supernatant removed for the assay of external invertase. The rest of the supernatant was removed, and the pellet was resuspended in 500 µl of 0.5% Triton X-100 to assay internal invertase. Samples of both fractions were assayed for invertase in a standard two-stage assay (Stage 1: sample + sucrose. Stage 2: glucose oxidase assay). Results were converted to units of invertase activity (µmol glucose liberated per minute per OD600 of cells) by comparison to a glucose calibration curve performed in parallel.
Antisera

Antisera directed against Sso1/2p, Snc1/2p and Sec9p were raised in rabbits (AbCam, Cambridge, UK), using synthetic peptide antigens, as follows:

**Sso**: VIDKNVEDAQQDVE (V234-E247).

**Snc**: RGANRVRKQMWWKD (R75-D88).

**Sec9**: TGKELDSQQKRLNN (T615-N628).

All peptides contained an additional N-terminal cysteine for conjugation. Note that the Sso and Snc antibodies were directed at a common epitope in Sso1p/Sso2p and Snc1p/Snc2p, respectively, and so do not discriminate between the two isoforms. Antibodies were affinity purified on peptide columns using a Sulfolink kit (Pierce). Anti-Sec1 antibody was purchased from Santa Cruz.

Analysis of F361 equivalent mutation in mammalian Munc18-1

**Plasmid construction**

The Y337 mutation (corresponding to F361 in Sec1) was introduced into the previously described pcDNA3.1-Munc18-1 plasmid (Ciufò et al. 2005) by site-directed mutagenesis using the four primer PCR method. The construct was fully sequenced to ensure that no additional unintended mutations were present.

**Amperometric analysis of exocytosis**

Bovine adrenal chromaffin cells were transfected by electroporation with wild type or mutant Munc18-1 plasmids and a GFP co-transfection reporter plasmid and maintained in culture for 3-5 days prior to analysis. Exocytosis was analysed by amperometry, as previously described (Ciufò et al. 2005). Briefly, cells were incubated in bath buffer (139 mM potassium glutamate, 0.2 mM EGTA, 20 mM PIPES, 2 mM ATP and 2 mM MgCl₂, pH 6.5) and a 5 μm-diameter carbon fiber
was positioned in contact with the target cell. Exocytosis was stimulated with a permeabilization/stimulation buffer (139 mM potassium glutamate, 20 mM PIPES, 5 mM EGTA, 2 mM ATP, 2 mM MgCl₂, 20 μM digitonin and 10 μM free Ca²⁺, pH 6.5) pressure-ejected from a glass pipette on the opposite side of the cell. Amperometric responses were monitored with a VA-10 amplifier (NPI Electronic, Tamm, Germany) and saved to computer using Axoscope 8 (Axon Instruments). Experiments were carried out in parallel on control (untransfected cells) and transfected cells from the same batch of cells in the same cell culture dishes. Transfected cells were identified by expression of EGFP. Amperometric data were analyzed using Origin (Microcal Software). Amperometric spikes were selected for analysis provided that the spike amplitude exceeded 40 pA, in order to remove any confounding effects of diffusion by selecting those fusion events not occurring directly beneath the carbon fiber end. Individual spikes were analyzed for total charge released (measured by the integral of the spike), amplitude (the height from baseline to peak), rise time (time from spike onset to peak) and fall time (time from spike peak to return to baseline). Spike frequency (spikes per cell) was calculated as the number of exocytotic events within the 210 seconds of recording time. Pre-spike feet were defined from foot onset (the time at which the amperometric current rose 2.5x above noise level) to spike onset (the time at which the amperometric spike began, determined by the differentiation of the amperometric trace). All data presented are shown as mean ± S.E.M. Statistical differences were assessed with nonparametric Mann-Whitney tests comparing all spikes/feet from all cells, as previously described (Ciufò et al. 2005).

**Munc18-1 binding assays**

Wild type and mutant Munc18-1 proteins were produced in ³⁵S-radiolabelled form by *in vitro* transcription/translation with the TnT T7 quick for PCR DNA system (Promega) using ³⁵S-
methionine (GE Healthcare). Binding assays between radiolabelled Munc18-1 and GST-syntaxin 1a (residues 4-266) were performed using glutathione-sepharose (GE Healthcare), as previously described (CRAIG et al. 2004). Binding of Munc18-1 to the SNARE complex was assayed via a GST-complexin pull-down method, as previously described (GRAHAM et al. 2008), but substituting radiolabelled Munc18-1. Quantification was performed by densitometry of $^{35}$S-Munc18-1 bands from 3 independent binding assays.

Mass spectrometry

Gel plugs from protein bands of interest were excised and the proteins were subjected to in-gel tryptic digestion and peptide extraction. After overnight digestion at 37°C, tryptic peptides were mixed with matrix ($\alpha$-cyano-4-hydroxycinnamic acid saturated solution in 50% acetonitrile, 0.5% trifluoroacetic acid) and analysed using a MALDI-ToF mass spectrometer (Voyager DE Pro, Applied Biosystems) in positive ion reflectron mode over the range of 950 to 3500 thomsons. Proteins were identified by manual searching of MSDB using MASCOT (MatrixScience, London UK). The initial search parameters allowed for a single trypsin missed cleavage, carbamidomethyl modification of cysteine residues, oxidation of methionine, acetylation of the N-terminus and an m/z error of ± 50 ppm.

Immunofluorescence

Chromaffin cells were transfected as described above and plated onto glass coverslips prior to fixation and processing for immunofluorescence, as described (CIUFO et al. 2005) At least 10 cells were imaged and quantified for each condition.
RESULTS

Identification of mutations in SEC1

We set out to find mutations in the SEC1 gene that create a dominant-negative phenotype. Since the SEC1 gene is essential, this entailed placing a copy under control of a regulated promoter on a plasmid, so that the desired mutations could be identified by their ability to inhibit growth under conditions where expression of the plasmid-borne SEC1 gene was induced. The SEC1 ORF was placed under control of the GAL1 promoter in the vector pYES2, creating the plasmid pYES-SEC1 (Table 1). Using this plasmid as template, a mutagenic PCR/gapped plasmid co-transformation procedure was used to generate a collection of yeast transformants harbouring mutagenised SEC1 genes. This collection was screened by plating on glucose-containing medium followed by replica plating onto plates containing 2% galactose as sole carbon source and maintaining selection for the plasmid. Twenty-five colonies that exhibited poor growth under inducing conditions were identified from approximately 4000 transformants. In order to confirm that the growth-inhibitory phenotype was caused by the resident plasmid, DNA was isolated from the candidate colonies and re-introduced into yeast. Twelve of these 25 candidates were chosen for further analysis because they gave the strongest galactose-sensitive (Gal⁺) growth phenotypes.

DNA sequencing of the entire SEC1 open reading frame of three of these plasmids revealed the presence of multiple substitution mutations (D9, L43P, F89S, L352P, K470E, K542R; D18, L351P, K708R, S720T; D25, I5F, I58V, Q316R, L358P, T689A), summarised in Fig 1A. Nevertheless, a possible clustering of mutations was noted, as indicated by the underlined substitutions. Partial sequencing of the remaining nine plasmids identified the following substitution mutations: D5, F361S D479G V524A; D6, A334V F361L; D7, F361P N382I Q395L I434V; D8, L332P N347I; D15, N322D F361L Q493R, D17, L439P I478T F480I; D19, I369V L370S
E463A F465L; D23, R328T T373A; D24, V354D. Strikingly, all but one (D17) contained at least one mutation (underlined) within the same region of the SEC1 gene, corresponding to amino acid residues 328-370 (Fig 1A). Mutations were especially clustered in a 20-amino-acid stretch from residues 351-370, with 9 out of 12 mutants containing a mutation in this region.

Three of the candidates (D6, D18, D25) were chosen to test the idea that the mutations in the region identified were indeed responsible for the phenotypic effect. By shuffling various restriction enzyme fragments between mutant and wild-type SEC1 sequences, the following D25 derivatives were constructed: D25A; D25BC; D25B; D25C; where letters indicate the presence of mutated regions of the gene (see Fig 1B). Of these only D25BC and D25B conferred the Gal⁰ phenotype, indicating that the changes in region B (Q316R; L358P) were responsible. Similarly it was found that the single change in region B of D18 (L351P) was responsible for the D18 phenotype. D6 also contained two mutations in region B (A334V; F361L). In this case the role of F361L was tested directly by generating this mutation in pYES-SEC1 (plasmid D6-2). The resulting plasmid was found to confer a Gal⁰ plate phenotype that was indistinguishable from that of the original plasmid, D6. These results, therefore, pinpoint mutated residues in a defined region of Domain 3a as responsible for the dominant negative phenotype. Furthermore, mutation of a single residue within this region (D6-2: F361L; D18B: L351P) is sufficient to confer the dominant negative phenotype.

Phenotypes conferred by overexpressed mutant proteins in liquid culture

Yeast cells harbouring plasmids encoding mutant Sec1 proteins D18B (L351P) and D25B (Q316R; L358P) were grown overnight in selective medium with 2% raffinose as sole carbon source. Raffinose is permissive for induction by galactose, but does not cause induction, as confirmed by the
lack of growth inhibition by the plasmids on this carbon source. Cells were then re-inoculated into fresh medium containing 2% galactose for induction of plasmid-borne SEC1 genes, together with 2% raffinose to allow for immediate growth during adaptation to the presence of galactose. As shown in Fig 2A, the presence of pYES-SEC1 had no detectable effect upon growth, whereas inhibitory effects of both pYES-sec1-D18B and pYES2-sec1-D25B were easily detected within 5-7 hours (corresponding to approximately 3 doublings of the control cells). Samples of culture were taken after 400 min of induction for analysis of levels of Sec1 protein by immunoblotting (Fig 2B). The wild-type level of endogenous Sec1 was not detectable in vector-transformed controls, but the protein was easily detected in the over-expressing cultures. The levels of the two mutant proteins were similar to the wild-type protein, indicating that the dominant negative phenotype is not simply due to Sec1 overexpression. To gain insight into the relative level of overexpression achieved, varying amounts of cell lysates from control (vector-transformed) and Sec1 overexpressing cells were immunoblotted using the Sec1 antibody. While no signal could be detected using 50 µg of control cell lysate, a signal could still be detected using 1 µg of lysate from Sec1 overexpressing cells (Fig 2C), indicating that the level of overexpression is at least 50-fold relative to endogenous Sec1 protein.

In a separate experiment the effects of mutant protein induction upon secretory traffic were investigated. The secreted enzyme invertase is induced by growth in the absence of glucose and reaches the cell exterior through the secretory pathway. Intracellular accumulation of invertase was part of the original definition of the yeast sec mutants (NOVICK and SCHEKMAN 1979) and has since been widely used to detect perturbations of secretory traffic in yeast. As shown in Fig 2D, overexpression of the Sec1-D18B and Sec1-D25B mutant proteins resulted in increased levels of intracellular invertase. Since Sec1 is required for fusion of secretory vesicles with the plasma
membrane, this accumulated intracellular invertase is likely to reflect the presence of an accumulation of secretory vesicles.

**Genetic properties of the sec1-D18B and sec1-D25B alleles**

We next determined whether the Sec1-D18B and Sec1-D25B proteins caused dominant-negative effects when expressed at levels equivalent to the wild-type protein. We constructed a KanMX-linked version of *SEC1* (*SEC1::KanMX444*) in a plasmid-borne cassette to allow the introduction of mutant alleles into an acceptor diploid strain of genotype *SEC1/sec1::URA3, ura3Δ/ura3Δ*. Geneticin-resistant derivatives of this strain isolated by transformation with a fragment of DNA carrying the *SEC1::KanMX444* allele (or mutant derivatives thereof) were screened for concomitant loss of the *URA3* marker by testing for resistance to FOA. This strategy allowed the easy identification of diploid transformants in which transplacement had occurred at the *sec1* allele to create a diploid strain carrying one wild-type allele of *SEC1* and one mutant allele derived from the transforming DNA. For *sec1-D18B* and *sec1-D25B*, such transformants were readily isolated, indicating that the mutant alleles do not have lethal dominant effects in heterozygous diploids.

The heterozygous diploids were sporulated (indicating that no severe dominant effects manifested during sporulation) and analysed by tetrad dissection. For the *sec1-D18B* and *sec1-D25B* alleles a 2:2 segregation of a lethal mutation was observed, and the surviving spore colonies were geneticin-sensitive, indicating that the *sec1-D18B* and *sec1-D25B* mutations are lethal in haploid cells. The spores that failed to form colonies did germinate, but only to the extent of putting out a small protrusion. No cell division occurred and so not even microcolonies were formed. Thus the *sec1-D18B* and *sec1-D25B* alleles are recessive lethal mutations; the corresponding amino acid substitutions impair an essential function of the Sec1 protein.
Suppression studies

The temperature-sensitive phenotype of the original sec mutant, sec1-1, can be rescued by overexpression of either of the t-SNARE proteins, Sec9 or Sso2 (AALTO et al. 1993; LEHMAN et al. 1999). We therefore tested various genes for the ability to suppress the galactose-sensitive growth phenotype of our dominant negative sec1 mutants. To this end, the SEC9, SSO2, SNC1, SNC2 and SEC4 genes were cloned into the YEpL vector, which carries the LEU2 marker on a multicopy plasmid. These were then transformed into a strain harbouring the D18B plasmid, and grown on SG-ura, -leu media to maintain selection for both plasmids. We observed that cells harbouring multicopy SNC1 or SNC2 plasmids were able to grow weakly and form single colonies on galactose medium. In contrast, none of the other plasmids provided any rescue, indicating a specific effect of SNC genes (Table 3). One possible explanation for the inability of SEC4, SEC9 and SSO2 to confer suppression could be that these do not actually result in the expected protein overexpression. We could not rule out this possibility for SEC4, as we did not possess an appropriate antibody. However, when extracts from cells transformed with SEC9, SSO2 and SNC2 plasmids were analysed, overexpression of all three SNARE proteins could be readily detected by immunoblotting (Fig 3), thus ruling out this trivial explanation. Therefore, the suppression of the dominant negative phenotype is specific to Snc protein overexpression, in contrast to the temperature-sensitive sec1-1 phenotype, which can be rescued by overexpression of both Sec9 and Sso2 proteins (AALTO et al. 1993; LEHMAN et al. 1999). To determine if the suppressor effect of Snc2 overexpression required its functional insertion into membranes, we truncated the SNC2 gene to create a mutant version encoding an Snc2 protein lacking the transmembrane domain (YEpL-SNC2ΔTMD). This construct was unable to rescue the dominant negative phenotype (Table 3). However, the truncated protein
was only expressed at similar levels to the endogenous full length Snc proteins (Fig 3), so the lack of suppression by this construct may simply be due to instability of the mutant protein.

**Effect of the conserved F361 mutation on mammalian Munc18-1**

The mutations identified in the sec1 mutants clustered in a 20-amino-acid stretch within Domain 3a. The tertiary structure of yeast Sec1 is not known, but the crystal structures of several SM proteins have been solved, facilitating the use of structure prediction simulations for homologous proteins. To this end, we used the I-TASSER server (Zhang 2008) to predict the structure of yeast Sec1 based on the available database of protein structures. The resulting model (Fig 4A) is generally similar to the solved crystal structures of Munc18-1 (Fig 4B) and Sly1 (Fig 4C), although some clear differences are apparent, particularly in Domain 2. It is notable that the dominant negative mutations (highlighted in green and magenta in the Sec1 structure) cluster in a region that is structurally conserved in the yeast Sly1 and rat Munc18-1 SM proteins (Fig 4). F361, which was the most common mutation, altered in four independent dominant negative alleles (Fig 1), is located at the bottom of the two finger-like alpha-helical projections at the base of Domain 3a; as are the homologous residues in Munc18-1 (Y337) and Sly1 (L390) (highlighted in magenta in Fig 4). Substitutions to amino acids with divergent chemical properties (serine, proline and leucine) were observed, suggesting that this residue is particularly sensitive to perturbation. Furthermore, a single mutation of F361 to leucine, as seen in the D6-2 mutant, is sufficient to confer the dominant negative phenotype. The corresponding residue in Munc18-1 is tyrosine 337 and so is a similar aromatic residue.

To determine whether mutation of Y337 in Munc18-1 would affect exocytosis in mammalian cells, this residue was mutated to a leucine, to mimic the mutation seen in the sec1-D6 and D15 mutants. Cultured bovine adrenal chromaffin cells were co-transfected with a mammalian
expression vector encoding Munc18-1 Y337L, along with a GFP reporter plasmid to identify transfected cells. Exocytosis from GFP-positive (co-transfected) cells were then analysed by amperometry. In this technique, carbon fibre microelectrodes are used to measure the release of oxidisable neurotransmitters (in this case, catecholamines) from individual secretory vesicle fusion events in single cells. This enables effects on the frequency and overall number of exocytotic fusion events, as well as the kinetics and quantity of transmitter release from single vesicles, to be determined. We have previously used this technique to reveal effects of Munc18-1 mutants (but not wild type Munc18-1) on both overall exocytosis and single vesicle release kinetics (BARCLAY et al. 2003; CIUFO et al. 2005; FISHER et al. 2001). No significant differences were observed between control and Munc18-1 Y337L expressing cells in terms of the frequency and overall extent of exocytotic fusion (Fig 5A, C). However, Y337L overexpression slowed and prolonged the release of catecholamine from individual vesicles, as evidenced by the increased charge (D), rise- (E) and fall-time (F) of the amperometric spikes. Representative individual spikes illustrating these changes are shown in Fig 5B. Some amperometric spikes are preceded by a pre-spike foot, thought to represent flux of catecholamine through the fusion pore prior to its dilation (CHOW et al. 1992) (see Fig 5G for example). However, no significant change in foot frequency (H), charge (I) or duration (J) was observed, suggesting that the Y337L mutation does not affect the size or stability of the initial fusion pore.

To quantify the level of overexpression of Munc18-1 Y337L in these experiments, cells were analysed by quantitative immunofluorescence microscopy using a polyclonal Munc18-1 antibody (CIUFO et al. 2005). Wild type Munc18-1 exhibited a 3.4-fold (S.E.M.=0.24, n=10 cells per condition) overexpression relative to endogenous Munc18-1, whereas Y337L was overexpressed 2.9-fold (S.E.M.=0.21, n=12 cells per condition). As overexpression of wild type Munc18-1 has no effect on exocytosis (FISHER et al. 2001), we conclude that the functional effect of Munc18 Y337L
in altering the kinetics of membrane fusion is due to a specific effect of this mutation and not simply
an effect of recombinant protein overexpression.

In the crystal structure of Munc18-1 bound via Mode 1 to closed syntaxin 1a, Y337 is only
3.2 angstroms away from syntaxin and makes direct contact with N135 in the Habc domain
(MISURA et al. 2000). In addition, Y337 is within 8 angstroms of S313 in Domain 3a of Munc18-1,
and phosphorylation or mutation of S313 is known to inhibit syntaxin 1 binding (BARCLAY et al.
2003). We therefore examined if the Y337L mutation affected the Mode 1 interaction of Munc18-1
with syntaxin 1a. To this end, recombinant radiolabelled wild type and Y337L Munc18-1 proteins
were synthesized by in vitro translation and tested for their ability to bind to recombinant GST-
syntaxin 1a (cytoplasmic domain residues 4-266). Munc18-1 binding to this syntaxin construct is
strongly inhibited by mutations and treatments that affect interaction with the closed conformation,
enabling its use as an assay to selectively determine Mode 1 binding (GRAHAM et al. 2004; PALMER
et al. 2008). As can be seen in Fig 6A, equally strong binding of wild type and Y337L Munc18-1 to
GST-syntaxin 1a (4-266) was observed. This binding was specific, as little binding to GST was
evident and as a truncation mutant encoding Domain 1 of Munc18-1 (residues 1-134) showed
minimal binding to GST-syntaxin1a (4-266). Therefore, the effect of the Munc18-1 Y337 mutant
on exocytosis does not appear to be due to gross defects in Mode 1 syntaxin binding. As it has
recently been found that Munc18-1 can also bind to the SNARE complex, a binding mode shared
with yeast Sec1, we then tested the effect of the Y337L mutation on this interaction. This was
performed via a recently developed affinity purification approach (GRAHAM et al. 2008), using
GST-complexin II to pull down the SNARE complex from brain lysate (Fig 6B). The specificity of
this approach was verified by identification of major bands in the GST-complexin pull down as
Munc18-1, syntaxin 1 and VAMP2 by mass spectrometry. Quantification of these bands by
densitometry revealed a stoichiometry of 1 syntaxin:0.82 VAMP, consistent with the known 1:1
stoichiometry of the ternary SNARE complex. Substoichiometric levels of Munc18-1 (0.26 per syntaxin) were recovered, suggesting that not all SNARE complexes were associated with endogenous Munc18-1 and thus enabling binding of exogenous $^{35}$S-labelled Munc18-1 to be assessed. Again, little binding of wild type Munc18-1 to GST was seen, but strong binding was observed to GST-complexin II (Fig 6C). Interestingly, the Y337L mutant displayed clearly reduced binding to GST-complexin II. Quantification of binding over multiple assays revealed an approximately 50% reduction in Y337L binding (Fig 6D), suggesting that this impaired interaction of Munc18-1 with the SNARE complex might underlie the effect of Y337L on exocytosis.
DISCUSSION

There is a major debate about which are the conserved interactions and functions of the SM protein family. SM proteins can directly interact with their cognate SNARE binding partners in at least three distinct modes (BURGOYNE and MORGAN 2007; GALLWITZ and JAHN 2003). The functional significance of these interaction mechanisms remains unclear, however, as mutations that disrupt binding Modes 1 and 2 have very mild phenotypes in vivo (CARPP et al. 2006; PENG and GALLWITZ 2004; RICHMOND et al. 2001; TONERI et al. 2006). We therefore took a different approach: to screen for mutants that disrupt SM protein function in the cell. We chose Sec1 and Munc18-1 for this approach, as these may represent extreme examples in terms of SNARE interactions, apparently binding via Mode 3 only (TONERI et al. 2006) (but see (SCOTT et al. 2004)) or via all three modes (BURKHARDT et al. 2008; DULUBOVA et al. 2007; RICKMAN et al. 2007; SHEN et al. 2007; YANG et al. 2000), respectively. Thus, any mutations affecting both proteins are likely to reveal conserved functions of SM proteins. Interestingly, the dominant negative sec1 mutants we isolated were clustered in a 20-amino-acid stretch within Domain 3a, pinpointing this structurally conserved region in SM proteins as functionally important. Consistent with this idea, the sec1-D6-2 analogous mutation in Munc18-1, Y337L, exhibited effects on the late stages of exocytosis in mammalian neuroendocrine cells.

How might the phenotypes observed in yeast and mammalian cells be related? Clearly, the effect of the Munc18-1 mutant in slowing exocytotic release is weak compared to the inhibition of invertase secretion and growth observed in yeast. However, strong overexpression is required to observe this phenotype in yeast, as no dominant effects are seen in wt/mutant Sec1 heterozygotes. The greatly different overexpression levels between Sec1 (>50-fold) and Munc18-1 (3-fold) mutants may explain, at least in part, the observed differences in severity of the dominant phenotypes. The
effect of the Munc18-1 Y337L mutant in slowing neurotransmitter release is similar to that of the previously described Munc18-1 I133V and P242S mutants (Ciufo et al. 2005). Such reduced rates of catecholamine release detected by amperometry have been shown to be directly due to impairment of the fusion pore expansion process (Neco et al. 2008) (see Burgoyne and Barclay 2002 for review). Prolonged opening of an incompletely expanded fusion pore would retard, but not prevent, the release of catecholamine, as these low molecular mass transmitters would be relatively unimpeded by even narrow pores. However, the release of large molecules, including proteins such as invertase, would be severely restricted, as would the full collapse of the vesicle into the plasma membrane required for bud formation and hence cell growth. Thus, it is possible that an impairment of fusion pore expansion could underlie both the yeast and mammalian phenotypes. This remains speculative, however, and we cannot rule out the alternative possibility that the two mutants affect distinct SM protein functions.

The dominant negative mutations in Sec1 are localised to Domain 3a, where they cluster at the bottom of the finger-like helical projections (green and magenta coloured residues in Fig 4). This contrasts with the residues involved in SNARE binding via Mode 1 (spread across the interior groove of the arch of Munc18-1) and Mode 2 (in Domain 1 only) (Bracher and Weisshorn 2002; Burkhardt et al. 2008; Hu et al. 2007; Misura et al. 2000) (Fig 4). Only two other sec1 mutations have been previously characterised: the temperature sensitive mutants, sec1-1 and sec1-11 (Brummer et al. 2001). These mutations create G443E and R432P substitutions in Domain 3b, further supporting the importance of Domain 3 for Sec1 function. Intriguingly, a recent screen for dominant negative mutants of the yeast SM protein, Vps45, identified a single mutation, W244R, which is very close to the region we identified in Sec1, lying at the junction of Domain 3a with Domains 2 and 3b (Carpp et al. 2006). It has been suggested that the W244R mutation may cause a conformational ‘opening’ of the arch-like structure of SM proteins and so modulate protein-protein
interactions (CARPP et al. 2006). In view of the clustering of Sec1 mutations observed here, it may be that this transduced conformational change in Vps45 affects Domain 3-mediated protein interactions. As discussed above, the Y337L Munc18-1 mutation affects exocytotic release kinetics, consistent with an effect on the late stages of the membrane fusion process. Interestingly, S313 in Munc18-1, which is very close to Y337 in Domain 3a, has been shown to be regulated physiologically by PKC phosphorylation, resulting in altered exocytotic release kinetics (BARCLAY et al. 2003; CRAIG et al. 2003). Taken together, it seems likely that Domain 3a is a functionally important region in SM proteins that is involved in the membrane fusion process.

What is the mechanism of action of the dominant negative mutants? We hypothesise that the mutant SM proteins are defective in an essential interaction with another component of the exocytotic machinery. This may be due to an inability of the mutant to efficiently regulate SNARE zippering, or alternatively may be due to an impaired ability to bind to a different exocytotic protein. Snc2 overexpression relieved the inhibitory effect of the dominant negative mutants, and this suppression was specific to the v-SNARE, as overexpression of the t-SNAREs, Sec9 and Sso1/2 was without effect. This suggests that the defect causing the dominant negative phenotype differs from the defect in the temperature-sensitive sec1-1 mutant, as the latter can be rescued by overexpression of both Sec9 and Sso proteins (AALTO et al. 1993; LEHMAN et al. 1999). It is interesting to note that the dominant negative Vps45 mutant discussed above is similarly rescued by Snc2 overexpression (CARPP et al. 2006). Phosphorylation and mutation of S313 in Munc18-1 has been shown to inhibit Mode 1 binding to syntaxin 1a (BARCLAY et al. 2003), confirming that Domain 3 residues are important for this interaction. However, the Y337L Munc18-1 mutant described here displayed no detectable difference in Mode 1 binding to syntaxin 1a. In addition, two other Munc18-1 mutants, I133V and P242S, exhibit slowed release kinetics despite normal Mode 1 syntaxin binding (CIUFO et al. 2005). As yeast Sec1 is not thought to undergo a Mode 1 interaction with Sso1/2, it seems safe
to conclude that the observed effects of our mutants in yeast and mammalian cells are independent of closed syntaxin binding. Indeed, the observation that Munc18-1 Y337L displays impaired binding to the neuronal SNARE complex instead suggests that these effects may be due to aberrant SM protein/SNARE complex (Mode 3) interactions. Significant further work on the yeast Sec1/SNARE complex is required to test this notion rigorously, however, and to rule out the alternative possibility that the effects on exocytosis are due to alterations in interactions with other components of the fusion machinery.

Finally, the approach described here may be generally useful for identifying functionally relevant mutations in conserved proteins. We selected for dominant negative mutants, ie mutant polypeptides that disrupt the function of the wild type protein when overexpressed. Our rationale was that the ability of such mutants to confer a phenotype in a wild type genetic background should make them useful tools for probing conserved residues in SM proteins in cells/organisms where genetic null backgrounds are not available. The present manuscript provides a proof of principle for this approach, where dominant negative mutations were identified in yeast Sec1 and then applied to the analogous region in Munc18-1 and found to affect calcium-triggered exocytosis in mammalian neuroendocrine cells. As the approach used is applicable to any conserved yeast gene, it has wide potential for uncovering functionally important mutants in the appropriate mammalian orthologues.

**Acknowledgements:** We thank Dr. Nia Bryant for the gift of the SEC1 construct. This work was supported by research grants from the BBSRC and the Wellcome Trust. MR is supported by a Wellcome Trust prize studentship.
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### Tables

**Table 1: Plasmids used in this study.**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Source</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pK19</td>
<td>(PRIDMORE 1987)</td>
<td>Cloning vector</td>
</tr>
<tr>
<td>pFA6KanMX</td>
<td>(LONGTINE et al. 1998)</td>
<td>Source of Kanamycin resistance cassette</td>
</tr>
<tr>
<td>p624</td>
<td>Nia Bryant</td>
<td>SEC1 genomic fragment in pRS316</td>
</tr>
<tr>
<td>YEpl</td>
<td>(GIETZ and SUGINO 1988)</td>
<td>Expression vector (promoterless, LEU2 marker)</td>
</tr>
<tr>
<td>pYES2</td>
<td>Invitrogen</td>
<td>Expression vector (GAL1 promoter, URA3 marker)</td>
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<tr>
<td>pYES-SEC1</td>
<td>This study</td>
<td>PCR-derived SEC1 fragment from p624 cloned into pYES2</td>
</tr>
<tr>
<td>pYES-SEC1ΔA</td>
<td>This study</td>
<td>pYES-SEC1 unique BsrG1 site destroyed by filling-in: creates SnaBI</td>
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<tr>
<td>pYES-SEC1ΔAΔS</td>
<td>This study</td>
<td>pYES-SEC1ΔA, unique SexAI site destroyed by filling-in</td>
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<tr>
<td>pYES-SEC1ΔAΔA</td>
<td>This study</td>
<td>pYES-SEC1ΔA, unique AvrII site destroyed by filling-in</td>
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<tr>
<td>pYES-SEC1ΔAΔX</td>
<td>This study</td>
<td>pYES-SEC1ΔA, unique XhoI site destroyed by filling-in</td>
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<td>p4</td>
<td>This study</td>
<td>PCR-derived fragment from p624 as BamHI-SphI fragment into pK19</td>
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<tr>
<td>p44</td>
<td>This study</td>
<td>PCR-derived fragment from p624 as NotI-SphI fragment into p4</td>
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<td>p444</td>
<td>This study</td>
<td>Ascl-NotI KanMX cassette from pFA6KanMX into p44</td>
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<td>p624XB</td>
<td>This study</td>
<td>Xbal site of p624 converted to BamHI site</td>
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<td>p444wt</td>
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<td>BamHI-XhoI fragment from p624XB into p44</td>
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<td>D18, D25 etc</td>
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<td>region B+C from D18/D25 shuffled into p444wt</td>
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<td>D18B, D25B</td>
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<td>region B from D18/D25 shuffled into p444wt; used as source of cassette for construction of strain LIVY18 and LIVY25</td>
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<tr>
<td>D18C, D25C</td>
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<td>region C from D18/D25 shuffled into p444wt</td>
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<td>Truncation mutant of YEpl-SNC2 with transmembrane coding region deleted</td>
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Table 2: Yeast strains used in this study.

<table>
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<th>Source</th>
<th>Genotype</th>
<th>Selection</th>
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<tbody>
<tr>
<td>BY4741</td>
<td>Invitrogen</td>
<td>$MATa$ $his3\Delta l$ $leu2\Delta 0$ $met15\Delta 0$ $ura3\Delta 0$</td>
<td>requires His Leu Ura Met</td>
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<tr>
<td>BY4743</td>
<td>Invitrogen</td>
<td>$MATa/alpha$ $his3\Delta l/his3\Delta l$ $leu2\Delta 0/leu2\Delta 0$ $ura3\Delta 0/ura3\Delta 0$ $MET15/met15\Delta 0$ $LYS2/lys2\Delta 0$</td>
<td>Kan$^S$; requires His Leu Ura</td>
</tr>
<tr>
<td>LIVY12</td>
<td>This study</td>
<td>$BY4743$ $SEC1/sec1\Delta ;URA3$</td>
<td>Kan$^S$; requires His Leu</td>
</tr>
<tr>
<td>LIVY18</td>
<td>This study</td>
<td>$LIVY12$ $SEC1/sec1-D18B-KanMX444$</td>
<td>Kan$^R$; requires His Leu Ura</td>
</tr>
<tr>
<td>LIVY25</td>
<td>This study</td>
<td>$LIVY12$ $SEC1/sec1-D25B-KanMX444$</td>
<td>Kan$^R$; requires His Leu Ura</td>
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Table 3: Overexpression of Snc1 and 2 suppresses the dominant negative sec1 mutant phenotype.

<table>
<thead>
<tr>
<th>Multicopy plasmid</th>
<th>Growth of sec1-D18B on glucose</th>
<th>Growth of sec1-D18B on galactose</th>
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<tbody>
<tr>
<td>YEpL (empty vector)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>YEpL-SEC4</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>YEpL-SEC9</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>YEpL-SSO2</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>YEpL-SNC1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>YEpL-SNC2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>YEpL-SNC2ΔTMD</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Wild type cells were co-transformed with pYES2-sec1-D18B and high-copy YEpL plasmids encoding the indicated genes. Double transformants were selected on –ura,-leu media and tested for ability to grow on galactose-containing media.
Figure legends

Figure 1: Mapping of dominant negative mutations in SEC1.

A. The open reading frame of three strong dominant negative mutants (D9, D18 and D25) was fully sequenced, revealing a clustering of mutations (represented by filled circles) in a central region of the SEC1 gene. Further sequencing showed that all but one of the remaining mutants contained a mutation in this region, corresponding to amino acids 328-370 (enclosed by rectangle). The amino acid sequence of this region is shown, with the various substitution mutations emboldened.

B. To determine which of the multiple mutations in D18 and D25 were responsible for the dominant negative phenotype, the SEC1 gene was divided into three restriction fragment cassettes: A, B and C. These cassettes were then used to create domain-swap constructs carrying hybrid wild type and mutant cassettes. The ability of the hybrids to confer the dominant negative phenotype was then assessed by scoring growth inhibition on galactose media.

Figure 2: Effects upon growth and secretion of high level expression of mutant sec1 genes.

Wild type cells were transformed with plasmids encoding wild type (SEC1+) or dominant negative (sec1-D18B+, sec1-D25B+) SEC1 constructs, or with empty vector. Raffinose-grown transformants were re-inoculated into fresh raffinose medium and pre-grown before addition of galactose (2%: time = 0). Subsequent growth of the four strains was followed. After 360-400 min, samples were withdrawn for protein extraction and for assay of internal and external levels of invertase.

A. Growth of the four strains was followed by measurement of culture absorbance at 600 nm.

B. Detergent-soluble protein extracts of each strain were prepared, then analysed by SDS-PAGE with subsequent immunoblotting using an anti-Sec1 antiserum. Note that the antibody fails to detect endogenous levels of Sec1 protein in control (vector-transformed) wild type cells.
C. Different amounts of extracts from control (vector-transformed) and Sec1-overexpressing cells were immunoblotted with anti-Sec1 antiserum to calibrate the relative level of Sec1 protein overexpression.

D. Samples of cells and of culture medium from the various strains were assayed for the level of invertase activity. Open bars indicate intracellular invertase activity, closed bars represent extracellular (secreted) invertase activity.

**Figure 3: Overexpression of Sec9p, Sso2p and Snc2p by high-copy plasmids.**

Wild type cells were transformed with empty vector or plasmids carrying *SEC9*, *SSO2* and *SNC2* genes. These were then grown up in liquid culture and detergent-solubilised protein extracts were prepared. The resulting samples were analysed by SDS-PAGE and immunoblotting.

**Figure 4: Dominant negative sec1 mutations cluster in a structurally conserved domain in SM proteins.**

A. The structure of Sec1 as predicted by I-TASSER computer simulations is shown with the dominant negative mutations isolated in Domain 3a highlighted (*green* = found in a single mutant, *magenta* = F361, mutated in 4 separate mutants).

B. The crystal structure of Munc18-1 bound to syntaxin 1a (pdb entry: 1dn1) is shown with the F361 equivalent residue, Y337, highlighted in *magenta*.

C. The crystal structure of Sly1 bound to Sed5 (pdb entry: 1mq5) is shown with the F361 equivalent residue, L390, highlighted in *magenta*.

Structures were rendered using Chimera software.
**Figure 5:** The sec1 dominant negative mutation equivalent in Munc18-1 (Y337L) slows exocytosis release kinetics.

A. Typical amperometric responses from untransfected cells (left panel) or cells transfected with the Munc18-1 Y337L mutant (right panel) following addition of digitonin and Ca\(^{2+}\) to elicit exocytosis.

B. Representative amperometric spikes from untransfected (left panel) or Y337L-transfected cells.

C. Analysis of the frequency of exocytotic fusion events reveals no significant difference between control and transfected cells.

D-F. Expression of Munc18-1 Y337L increases charge (C) and slows the kinetics of release by increasing both the rise-time (D) and fall-time (E) of individual amperometric spikes.

G. Representative example of a pre-spike foot (marked by arrow).

H-J. Analysis of the frequency (H), charge (I) and duration (J) of pre-spike feet reveals no significant difference between control and transfected cells.

Data were analysed from 240 spikes and 34 feet from 18 cells (control) and 366 spikes and 100 feet from 26 cells (Y337L) and statistical significance assessed using Mann-Whitney tests.

**Figure 6:** The Y337L mutation impairs binding of Munc18-1 to the neuronal SNARE complex, but not to syntaxin 1a.

A. \(^{35}\)S-radiolabelled, in vitro translated Munc18-1 wild type, Y337L and a truncation mutant comprising domain I (residues 1-136) were incubated with GST or GST-syntaxin1a (4-266) immobilised on glutathione-sepharose beads. Bound radiolabelled protein remaining after washing and the corresponding inputs were analysed by SDS-PAGE followed by exposure to \(^{35}\)S-sensitive film. GST and GST-syntaxin1a (4-266) bait proteins were visualised by Ponceau S staining.

B. Detergent-solubilized brain extract was incubated with GST or GST-complexin II immobilised on glutathione-sepharose beads. Bound proteins remaining after washing were visualised by
colloidal Coomassie blue staining, excised from the gel and analysed by MALDI mass spectrometry. The indicated bands were identified as Munc18-1, syntaxin 1 and VAMP2.

C. $^{35}$S-radiolabelled, in vitro translated Munc18-1 wild type and Y337L were incubated with GST or GST-complexin II that had been pre-incubated with detergent-solubilized brain extract and immobilised on glutathione-sepharose beads. Bound radiolabelled protein remaining after washing and the corresponding inputs were analysed by SDS-PAGE followed by exposure to $^{35}$S-sensitive film. GST and GST-complexin II bait proteins were visualised by Ponceau S staining.

D. Quantitative densitometry of Munc18-1 wild type and Y337L binding to the GST-complexin-affinity-purified neuronal SNARE complex.
Boyd et al Fig 2

A

Invertase activity (μmol glucose/min/OD_{600} cells)

B

Blot: Sec1

C

Blot: Sec1

D

Invertase activity (μmol glucose/min/OD_{600} cells)
Boyd et al Fig 5

A

Control

Y337L

B

Control

Y337L

C

D

E

P<0.001

F

P<0.001

G

H

I

J

Control

Y337L

Control

Y337L

Control

Y337L