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A novel Hsp70 of the yeast ER lumen is required for the efficient translocation of a number of protein precursors

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The yeast genome sequencing project predicts an open reading frame (YKL073) that would encode a novel member of the Hsp70 family of molecular chaperones. We report that this 881 codon reading frame represents a functional gene expressing a 113–119 kDa glycoprotein localized within the lumen of the endoplasmic reticulum (ER). We therefore propose to designate this gene *LHS1* (*L*umenal *H*sp *S*eventy). Our studies indicate that *LHS1* is regulated by the unfolded protein response pathway, as evidenced by its transcriptional induction in cells treated with tunicamycin, and in various mutants defective in precursor processing (*sec11-7*, *sec53-6* and *sec59-1*). *LHS1* is not essential for viability, but an *lhs1* null mutant strain exhibits a co-ordinated induction of genes regulated by the unfolded protein response indicating a role for Lhs1p in protein folding in the ER. Furthermore, the null mutation is synthetically lethal in combination with *Δire1*, thus activation of the unfolded protein response pathway is essential for cells to tolerate loss of Lhs1p. Synthetically lethality is also seen with mutations in *KAR2*, strongly suggesting that Kar2p and Lhs1p have overlapping functions. The *lhs1* null mutant exhibits a severe constitutive defect in the translocation of several secretory preproteins. We therefore propose that Lhs1p is a molecular chaperone of the ER lumen involved in both polypeptide translocation and subsequent protein folding.

Keywords: chaperone/endoplasmic reticulum/Hsp70/translocation/UPRE

Introduction

Hsp70 is a highly conserved, ubiquitous chaperone originally identified as a stress response protein in *Drosophila* (Ashburner and Bonner, 1979). It is now clear that eukaryotes possess a number of related, but functionally distinct, Hsp70s that are involved in several aspects of protein biogenesis in a variety of subcellular compartments (Mukai *et al.*, 1993; Shirayama *et al.*, 1993; Crombie *et al.*, 1994; Cyr, 1995; see Rassow and Pfanner, 1995, and references therein). Several studies in yeast have demonstrated roles for Hsp70s in organelle biogenesis. First, cytosolic Ssa proteins are required for post-translational translocation of polypeptides into both the endoplasmic reticulum (ER) and the mitochondrion (Chirico *et al.*, 1988; Deshaies *et al.*, 1988; Murakami *et al.*, 1988). Secondly, Hsp70s located within the lumen of the ER (Kar2p), and the mitochondrial matrix (Ssc1p), are required for protein import into their respective organelles (Kang *et al.*, 1990; Vogel *et al.*, 1990; Nguyen *et al.*, 1991; Stuart *et al.*, 1994).

The role of Kar2p in ER translocation has been extensively studied both *in vivo* and *in vitro*. *KAR2* was originally identified as being involved in nuclear fusion (karyogamy; Polaina and Conde, 1982), but a number of conditional-lethal *kar2* alleles also exhibit defects in the translocation of secretory precursors under restrictive conditions (Vogel *et al.*, 1990; Nguyen *et al.*, 1991). Several lines of evidence support a direct role for Kar2p in the translocation process. First, *kar2-159* mutant cells exhibit an extremely rapid translocation defect after being shifted to their restrictive temperature (Vogel *el al.*, 1990). Secondly, functional Kar2p is required for efficient translocation of prepro-α-factor into reconstituted microsomes and into proteoliposomes (Brodsky *et al.*, 1993; Brodsky and Schekman, 1993; Panzner *et al.*, 1995). Finally, the *in vitro* analysis of *kar2* mutant phenotypes has dissected two distinct roles for Kar2p in the translocation cycle, the first being prior to the interaction of precursor with the Sec61p-containing translocase, and the second involving a direct interaction between Kar2p and the translocating chain (Sanders *et al.*, 1992; see High and Stirling, 1993, and references therein).

Whilst it is clear that Kar2p plays a direct role in post-translational translocation in yeast, the role of ER lumenal proteins in co-translational translocation remains controversial. Nicchitta and Blobel (1990, 1993) have reported that lumenal proteins are required for the net transfer of nascent chains into mammalian derived microsomes, although the identity of the lumenal factor(s) in this system is unknown. In contrast, the reconstitution of mammalian co-translational translocation into proteoliposomes has been reported in the apparent absence of lumenal proteins (Görlich and Rapoport, 1993; Oliver *et al.*, 1995). This assay requires only the Sec61 complex, SRP receptor and, for certain precursors, the TRAM protein. The observed role for lumenal proteins in co-translational import into mammalian microsomes might therefore prove to be indirect.

In addition to its role in polypeptide translocation, Kar2p/BiP also appears to play a role as a molecular chaperone in the folding of proteins within the ER lumen (see Gething and Sambrook, 1992; Simons *et al.*, 1995). As in higher eukaryotic systems (Lee, 1987; Kozutsumi *et al.*, 1988; Dorner *et al.*, 1990), Kar2p is co-ordinately regulated with other ER lumenal chaperones, including PDI, Eug1p and Fkb2p, by the unfolded protein response pathway (Tachibana and Stevens, 1992; Cox *et al.*, 1993; Kohno *et al.*, 1993; Partaledis and Berlin, 1993). The

induction of these chaperones is presumed to enhance the cell's capacity to process malfolded secretory polypeptides (see Shamu *et al.*, 1994). This transcriptional induction requires the presence of a 5′ regulatory sequence known as the unfolded protein response element (UPRE; Mori *et al.*, 1992). The identity of the *trans*-acting factor(s) acting upon the UPRE, or those involved in signal transduction from the ER to the nucleus, remains obscure, but the latter includes at least one transmembrane kinase (Ire1p/Ern1p; Cox *et al.*, 1993; Mori *et al.*, 1993).

The yeast genome sequencing project has identified an open reading frame, designated YKL073 (Rasmussen, 1994), encoding a putative Hsp70 homologue which would contain both a cleavable signal sequence (von Heijne, 1986) and an ER retention signal (-HDEL$_{COOH}$; Pelham *et al.*, 1988), suggesting an ER localization. Database comparisons reveal that the deduced amino acid sequence of the YKL073-encoded polypeptide shows significant similarity to >80 known Hsp70s from a variety of species; a typical example being Kar2p from *Saccharomyces cerevisiae* which shares 26% sequence identity with the YKL073 encoded sequence (extending to 50% similarity when conservative substitutions are included). Furthermore, Bairoch and Bucher (1994) have empirically derived a dictionary of sequence motifs (PROSITE) designed to ascribe biological function to polypeptide sequences. These include three motifs that are diagnostic for Hsp70-family members. Two of the three motifs (Hsp70-2 and Hsp70-3) are found in all known Hsp70s, whilst the third (Hsp70-1) is present in most, but not all, family members (Bairoch and Bucher, 1994). The YKL073-encoded polypeptide would contain both Hsp70-2 and Hsp70-3 motifs, and has a limited match to Hsp70-1. Given the overall sequence similarities to known Hsp70s, and the presence of these highly conserved motifs, it seems likely that YKL073 encodes a member of the Hsp70 superfamily.

We have examined the expression, localization and function of this novel Hsp70 family member. Here we report that this protein is indeed localized to the ER lumen, and that the gene is regulated by the unfolded protein response pathway. These findings are consistent with a role as an ER lumenal chaperone and we therefore propose to designate this gene *LHS1* (*L*umenal *H*sp *S*eventy). The gene is non-essential for vegetative growth, but several observations suggest that Lhs1p and Kar2p have overlapping functions. Finally, *lhs1* mutants are severely defective in the translocation of a number of secretory precursors, indicating a role for Lhs1p in protein translocation across the ER membrane.

Results

Transcriptional regulation of LHS1

LHS1 encodes a novel member of the Hsp70 superfamily, which includes a number of proteins that are constitutively expressed and others that are induced by environmental stresses (Werner-Washburne *et al.*, 1989). The yeast *KAR2* gene encodes an Hsp70 of the ER lumen that is expressed constitutively, but which is also substantially induced by a number of stresses including heat shock (Normington *et al.*, 1989; Rose *et al.*, 1989; Werner-Washburne *et al.*, 1989). However, unlike *KAR2*, the nucleotide sequence upstream of the *LHS1* open reading frame does not contain

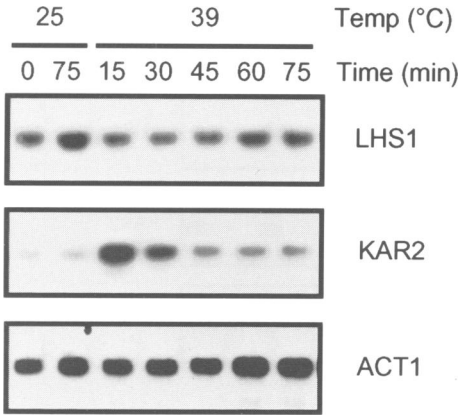

Fig. 1. *LHS1* mRNA is not regulated by heat shock. Northern blots of whole yeast RNA are shown prepared from TR2 (wild-type) cells grown in YPD at 25°C or after shifting to 39°C for the times indicated. Hybridization was carried out with radiolabelled probes for *LHS1*, *KAR2* and *ACT1* as described in Materials and methods. The results presented are from sequential hybridizations of the same filter.

KAR2	-131	G G A A C T C G A C A G C G T G T C G A A A	-110
FKB2	-121	C A T T A C T G C C A G C G C A T C T T C A	-100
EUG1	-128	T T C A A A G G C A C G C G T G T C C T T T	-107
PDI	-251	C C T G T C G G G C G G C G C C T C T T T T	-230
LHS1	-133	T A A T T A G G C G C G C G C C T C A A A T	-112

Fig. 2. The region upstream of LHS1 contains a consensus for an unfolded protein response element. Alignment of the 5′ regions from *KAR2*, *FKB2*, *EUG1* and *PDI* (see Shamu *et al.*, 1994, and references therein) with that from *LHS1*. The consensus sequence for the unfolded protein response element (UPRE) is boxed.

a recognizable heat shock element. Moreover, using Northern blot analysis we have found that *LHS1* was transcribed constitutively in cells grown in rich medium at 25°C and was largely unaffected by a heat shock at 39°C (Figure 1). In order to confirm that the heat shock response had been activated in these cells, we monitored the level of *KAR2* mRNA. This exhibited the previously reported pattern of induction, where a substantial increase in mRNA levels is rapidly attained, followed by a decrease to a new steady-state level (Werner-Washburne *et al.*, 1989; Figure 1).

The region upstream of the *LHS1* reading frame does contain a match to the consensus sequence for a UPRE (Figure 2). The presence of this putative UPRE suggests that *LHS1* might be co-ordinately regulated with a number of genes encoding ER chaperones including *KAR2*, *PDI*, *EUG1* and *FKB2* (Tachibana and Stevens, 1992; Cox *et al.*, 1993; Kohno *et al.*, 1993; Partaledis and Berlin, 1993). The transcription of such genes is induced under conditions predicted to lead to the accumulation of unfolded polypeptides within the ER, e.g. the inhibition of N-linked glycosylation by treatment with tunicamycin (see Shamu *et al.*, 1994, and references therein). Northern blot analysis of RNA prepared from wild-type cells incubated in the presence, or absence, of tunicamycin (10 μg/ml) revealed that drug treatment led to a 10-fold induction of *LHS1* mRNA (Figure 3A). This compares with a 16-fold increase in the steady-state level of *KAR2* mRNA observed in the same experiment (Figure 3A). The levels of mRNA were quantified relative to actin mRNA (*ACT1*) as a loading control (Figure 3A).

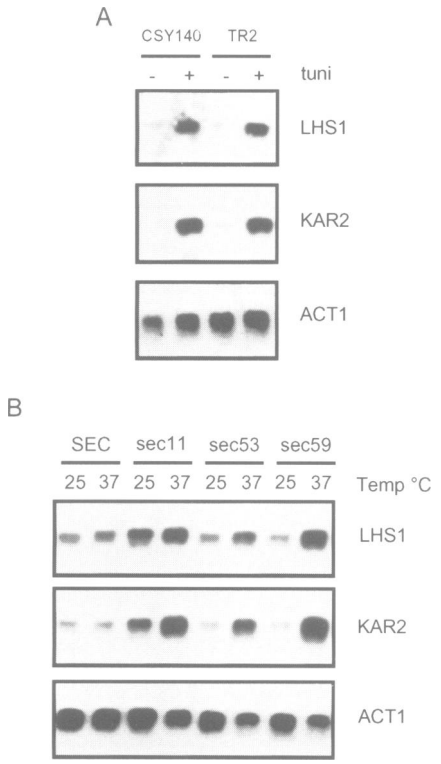

Fig. 3. *LHS1* mRNA is induced by treatments which activate the unfolded protein response pathway. Northern blots of total RNA were hybridized with radiolabelled probes specific for *LHS1*, *KAR2* or *ACT1* as indicated. (**A**) RNA prepared from two different wild-type yeast strains (CSY140 or TR2) were grown to mid-log phase in YPD at 30°C then incubated in the presence (+) or absence (–) of tunicamycin for 2.5 h prior to RNA extraction. (**B**) RNA made from strains grown at 25°C or after a 2 h shift to 37°C (*SEC*: TR2; *sec11*: PBY408A; *sec53*: RSY12; *sec59*: RSY26). The results presented are from sequential hybridizations of the same filter.

Several temperature-sensitive yeast mutations known to interfere with protein processing have also been shown to cause activation of the unfolded protein response pathway, including *sec53-6* and *sec11-7* (Normington *et al.*, 1989). *SEC53* encodes a phosphomannomutase involved in glycosylation (Képès and Schekman, 1988), and *SEC11* encodes a subunit of signal peptidase required for signal peptide cleavage (Böhni *et al.*, 1988). In order to determine the effects of these mutations on *LHS1* transcription, RNA was prepared from mutant cells grown at the permissive temperature (25°C) and from cells subjected to a 2 h shift to their restrictive temperature (37°C). Northern blot analysis indicated that, like *KAR2*, *LHS1* mRNA is substantially induced in both *sec11-7* and *sec53-6* mutant cells (Figure 3B). As previously reported, some induction is observed in *sec11-7* cells even at 24°C, indicating a sublethal defect in precursor processing at this temperature (Normington *et al.*, 1989; Kohno *et al.*, 1993). We have extended this analysis by examining mRNA levels in *sec59-1* mutant cells which are conditionally defective in dolichol kinase (Heller *et al.*, 1992). A defect in dolichol kinase will interfere with core-oligosaccharide biosynthesis and so, like tunicamycin, will inhibit N-linked glycosylation. Once again we found that both *KAR2* and *LHS1* mRNAs were induced in *sec59-1* cells at the restrictive temperature (Figure 3B). A general block in the secretory pathway produced by the translocation mutants *sec61-2* and *sec62-1* had no effect on the level of *LHS1* mRNA (R.A.Craven and C.J.Stirling, unpublished data). These results indicate that *LHS1* is regulated by the unfolded protein response pathway, from which it follows that Lhs1p plays a role in the normal cellular response to malfolded secretory polypeptides.

LHS1 is not an essential gene

In order to examine the role of the Lhs1 protein *in vivo*, we have created a null mutant by targeted gene disruption. A 2520 bp *Eco*RV fragment, corresponding to codon 56 onwards of the *LHS1* open reading frame, was removed and replaced with the yeast *TRP1* selectable marker. A linear DNA fragment comprising the *TRP1* gene flanked by *LHS1* sequences was then used to transform a diploid yeast strain (TR1) to tryptophan prototrophy (Figure 4A). Southern hybridization confirmed that the transforming DNA was integrated at the *LHS1* locus, and established that a single gene copy had been replaced resulting in a *LHS1/lhs1::TRP1* heterozygous diploid (Figure 4B). Sporulation and tetrad dissection of this diploid (RCY101) resulted in four viable spores per tetrad with the expected 2:2 segregation of the *TRP1* marker (Figure 4C). Southern blotting confirmed the correct segregation of the wild-type and disrupted alleles to the progeny of a tetrad (R.A.Craven and C.J.Stirling, unpublished data). The *LHS1* gene is clearly not essential for either spore germination or for vegetative cell growth. However, *lhs1::TRP1* mutant cells grow more slowly than wild-type cells (see Figure 4C) with a doubling time at 30°C in rich medium of 2 h 15 min compared with 1 h 45 min for the wild-type haploid. The growth of the null mutant strain was neither temperature sensitive (37°C) nor cold sensitive (17°C). Similarly, no obvious defects were noted in mating of two haploid disruptants or in the sporulation of a homozygous gene disruptant. Therefore, if Lhs1p does play a role in protein folding and secretion, then that role is either non-essential or is functionally redundant.

Levels of KAR2 and PDI mRNAs are elevated in the lhs1 null mutant

The observation that *LHS1* transcription is induced in response to unfolded polypeptides suggests that the Lhs1 protein may play some role in protein folding. If this were the case, then one might expect that the absence of Lhs1p function in the *lhs1::TRP1* mutant may lead to an accumulation of unfolded polypeptides, which might in turn induce the unfolded protein response pathway. We therefore examined the levels of *PDI* and *KAR2* mRNAs as markers for the unfolded protein response. The levels of *PDI* and *KAR2* mRNAs were found to be 2.5- and 8-fold higher, respectively, in the *lhs1::TRP1* mutant versus wild-type cells (quantified relative to *ACT1* mRNA as an internal control; Figure 5, lanes 1 and 3). However, the induced mRNA levels were lower than those observed in wild-type cells treated with tunicamycin, where *PDI* and *KAR2* are induced 5- and 16-fold respectively (Figure 5, lanes 1 and 2). Significantly, the treatment of *lhs1* mutant cells with tunicamycin led to a further 2- to 2.5-fold increase of both *PDI* and *KAR2* mRNAs (Figure 5, lanes 3 and 4) to levels indistinguishable from those seen in wild-type cells treated with the drug (Figure 5, compare

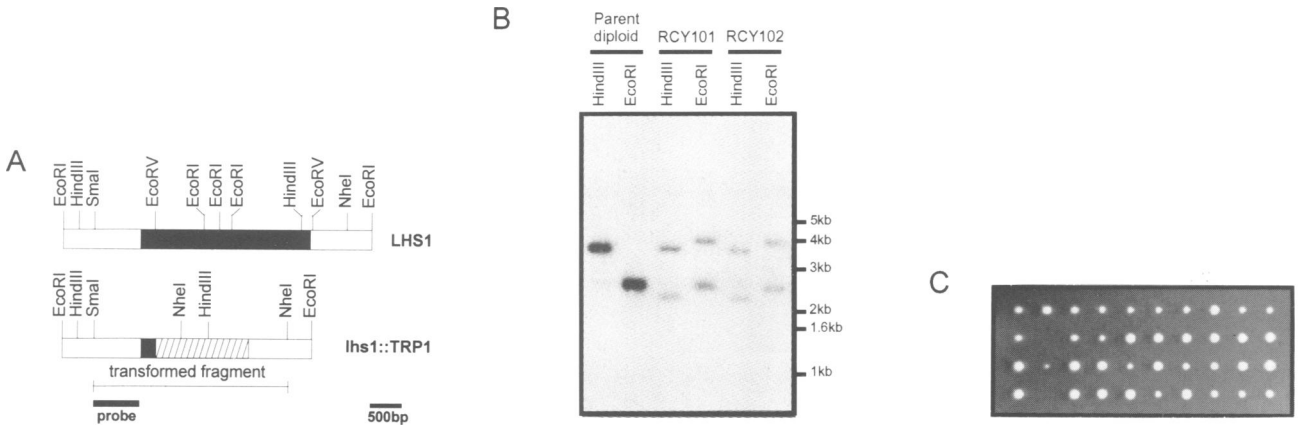

Fig. 4. *LHS1* is not an essential gene. (**A**) Restriction map of the genomic region around *LHS1* and *lhs1::TRP1* null allele constructed in pRC41 (see Materials and methods). The extent of the *LHS1* reading frame is indicated by dark shading. Lighter shading indicates the extent of *TRP1* sequences. A linear *Bam*HI/*Sac*I fragment from pRC41 fragment, corresponding to the *lhs1::TRP1* null allele, was gel purified then used to transform TR1 to tryptophan prototrophy. The fragment used to transform TR1 to tryptophan prototrophy is indicated. (**B**) Southern blot of genomic DNA from the parental diploid (TR1) and two independent trp^+ transformants (RCY101 and RCY102) digested with either *Eco*RI or *Hin*dIII. The source of the probe sequence is indicated by a black bar in (A) above. The restriction maps of the wild-type and *lhs1::TRP1* alleles predict *Eco*RI fragments of 2.4 and 4.0 kb, respectively, or *Hin*dIII fragments of 3.6 and 2.1 kb, respectively. The digestion patterns confirm that TR1 is an *LHS1/LHS1* homozygote, and that both RCY101 and RCY102 are *LHS1/lhs1::TRP1* heterozygotes. (**C**) Tetrads dissected after sporulation of a heterozygous disruptant (RCY101) then incubated at 30°C. Sporulation and spore germination were performed at 30°C. Tetrads are arranged vertically on YPD agar.

lanes 2 and 4). As expected, no *LHS1* mRNA was detected in *lhs1::TRP1* mutant cells (Figure 5). These data demonstrate a correlation between the loss of Lhs1p and the transcriptional induction of lumenal chaperones known to be regulated by the unfolded protein response pathway. The induction of the unfolded protein response requires the product of the *IRE1* gene (Cox *et al.*, 1993; Mori *et al.*, 1993). Significantly, we find that the *lhs1::TRP1* disruption is lethal in combination with an *ire1* null mutation. Sporulation of a heterozygous diploid (RCY150) produced no viable *lhs1::TRP1, Δire1* double mutants. Microscopic examination of double mutant spores indicated that they had undergone germination but had not progressed beyond one or two divisions. These results indicate that the induction of other ER lumenal chaperones is essential for cells to survive in the absence of Lhs1p.

The LHS1 gene disruption is lethal in combination with mutations in kar2

Despite the low level of overall sequence similarity between the Kar2 and Lhs1 proteins (25% sequence identity), they are nonetheless both predicted to be members of the Hsp70 superfamily. The observed induction of *KAR2* in *lhs1::TRP1* cells might therefore play some role in compensating for the loss of Lhs1p. *KAR2* is an essential gene, but several temperature-sensitive (Ts^-) alleles have been isolated, including *kar2-159* and *kar2-113* (Rose *et al.*, 1989), which are viable at 25°C but not at 37°C. We have sought to combine these mutant *kar2* alleles with the *lhs1* null allele in order to assess any synthetic interaction between these mutations. Two doubly heterozygous diploid strains were first constructed, RCY131; *lhs1::TRP1/LHS1*, *kar2-113/KAR2*: and RCY132; *lhs1::TRP1/LHS1*, *kar2-159/KAR2*. These diploids were sporulated, the resultant tetrads dissected and spores incubated at 25°C. Thirty tetrads were analysed from each cross and in both cases overall spore viability was found to be ~75%. Crucially, no viable Trp^+/Ts^- haploids were recovered,

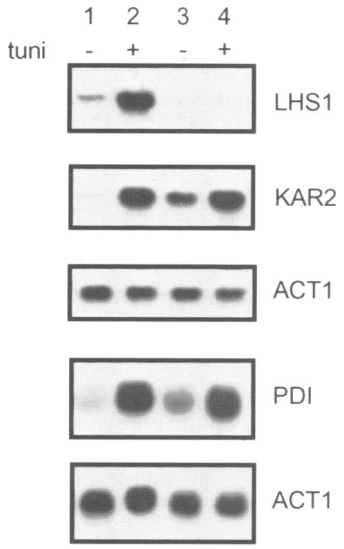

Fig. 5. Levels of *KAR2* and *PDI* mRNA are increased in the *lhs1::TRP1* mutant. Northern blots of total RNA are shown, hybridized with radiolabelled probes specific to either *LHS1*, *KAR2*, *PDI* or *ACT1* as indicated. Wild-type cells (TR2; lanes 1 and 2) and null mutant cells (RCY104; lanes 3 and 4) were grown in YPD at 30°C, and incubated in the absence (–) or presence (+) of tunicamycin for 2.5 h prior to RNA extraction. The top three panels shown are sequential hybridizations of a single filter, the bottom two panels are sequential hybridizations against a different filter.

indicating that the double mutant combination was lethal. In order to confirm this result, diploids carrying the *LHS1* gene on a *URA3*-based multicopy plasmid (pRC43) were sporulated and tetrads dissected. Viable *kar2*, *lhs1::TRP1* double mutants were now recovered, but always contained pRC43. These strains were sensitive to 5-fluoro-orotic acid (5-FOA), indicating that any cells losing plasmid through mis-segregation were inviable (Figure 6). Wild-type, and single-mutant, strains carrying pRC43 gave rise to colonies on 5-FOA medium, indicating that they

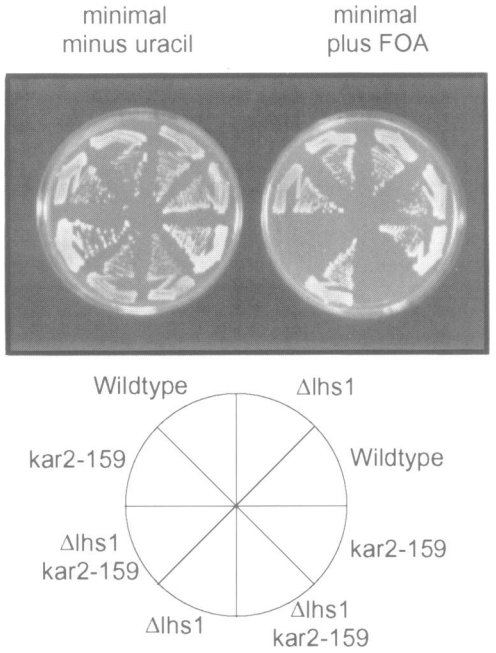

Fig. 6. *Lhs1::TRP1* is lethal in combination with *kar2* mutants. RCY132 (*LHS1/lhs1::TRP1*, *KAR2/kar2-159*) diploids carrying pRC43 (2μm, *URA3*, *LHS1*) were sporulated and tetrads dissected. Two tetrads (tetratype) where all four spores carried plasmid are shown streaked with selection for (minimal without uracil) or against (minimal plus 5-FOA) the *URA3*-containing plasmid. Spores were genotyped by scoring the ts$^-$ (*kar2*) and Trp$^+$ (*lhs::TRP1*) phenotypes. Identical results were obtained with RCY131 (*LHS1/lhs1::TRP1*, *KAR2/kar2-113*).

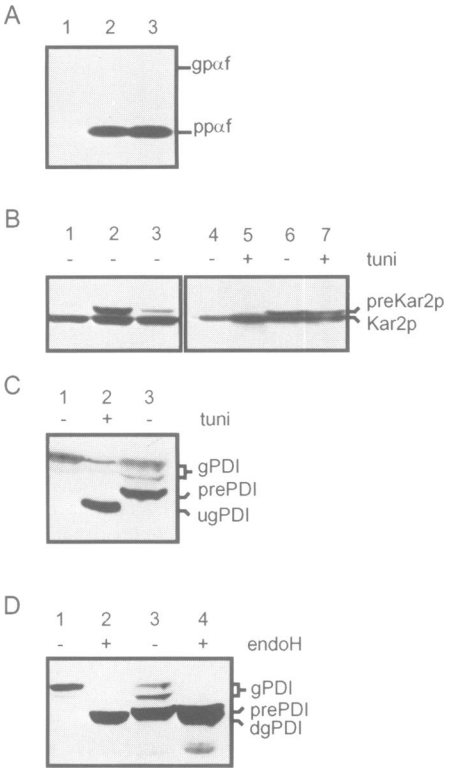

Fig. 7. The null mutant accumulates precursor forms of secretory proteins. Whole-cell extracts were separated by SDS–PAGE and analysed by immunoblotting. (**A**) Wild-type (TR3; lane 1) and null mutant (RCY110; lane 2) extracts were prepared from cells grown at 30°C. *Sec61-3* (CSY150; lane 3) was grown at 24°C and shifted to 17°C for 3 h. SDS–PAGE (12.5%) was followed by immunoblotting with anti α-factor serum. Prepro-α-factor (ppαf) and glycosylated pro-α-factor (gpαf) are indicated. (**B**) Wild-type (TR3; lanes 1, 4 and 5), null mutant (RCY110; lanes 2, 6 and 7) and sec61-3 (CSY150; lane 3) extracts were prepared from cultures grown as in (A), but where indicated were incubated in the presence (+) of tunicamycin for 2.5 h prior to cell lysis. Proteins were separated by SDS–PAGE (10%). Immunoblotting was with Kar2p specific antiserum. (**C**) Wild-type (TR3; lanes 1 and 2) and null mutant (RCY110; lane 3) extracts were prepared from cultures grown in YPD at 30°C incubated in the presence (+) or absence (–) of tunicamycin for 2.5 h prior to cell lysis. After SDS–PAGE (10%), immunoblotting was carried out with antibodies to PDI. Unglycosylated PDI (ugPDI), prePDI and glycosylated PDI (gPDI) are indicated. (**D**) Extracts from wild-type (TR3; lanes 1 and 2) and null mutant (RCY110; lanes 3 and 4) cells grown were incubated with (+) or without (–) endoH before SDS–PAGE (10%) and immunoblotting with antibodies to PDI. Deglycosylated PDI (dgPDI), prePDI and glycosylated (gPDI) are indicated.

remained viable after plasmid loss (Figure 6). These data confirm that the *lhs1* null mutation is synthetically lethal in combination with either *kar2-113* or *kar2-159*. Therefore, while *LHS1* is not an essential gene, loss of Lhs1p cannot be tolerated if Kar2p function is compromised. In itself, this observation does not demonstrate that Kar2p and Lhs1p have related functions. For example, the two mutations may attenuate quite unrelated cellular processes, either of which might be tolerated in isolation, but the combination might place an insurmountable burden on the cell. This seems unlikely in this particular case since the interaction between the *lhs1* and *kar2* mutations appears relatively specific. For example, cells carrying the *lhs1::TRP1* allele in combination with either *sec65-1*, *sec61-2* or *sec18-1* exhibit no detectable synthetic effects. Moreover, *kar2-159* has been tested against mutant alleles in a variety of essential genes with no synthetic effects other than that seen with *sec63-1* (Scidmore *et al.*, 1993). Similarly, no synthetic interactions were observed between *kar2-113* and either *sec61-2*, *sec62-1*, or indeed with *sec63-1* (Scidmore *et al.*, 1993). Given the specificity of the *lhs1/kar2* interactions, we conclude that these gene products affect the same cellular process, and may in fact have overlapping functions.

The LHS1 null mutant is defective in protein translocation

The regulation of *LHS1*, the induction of ER chaperones in the *lhs1::TRP1* mutant, and its synthetic lethality with *kar2* alleles, indicate some role for the Lhs1p in precursor processing. Western blotting of whole yeast extracts was used to investigate the biogenesis of a variety of pre-proteins. Prepro-α-factor is processed by core glycosylation and signal peptide cleavage in the ER. Further glycosylation and proteolytic maturation in the Golgi produces the mature form, which is secreted (Julius *et al.*, 1984). Processing is rapid such that only a faint band, corresponding to an ER-glycosylated intermediate, is detected in Western blots of wild-type cell extract (Figure 7A, lane 1). In contrast, cell extract prepared from the *lhs1::TRP1* null mutant contained an immunoreactive species which co-migrates with the untranslocated prepro-α-factor accumulated in *sec61-3* cells (Figure 7A, lanes 2 and 3; Stirling *et al.*, 1992). Similarly, the null mutant also contains a form of Kar2p, not observed in wild-type cells (Figure 7B, lanes 1 and 2), which co-migrates with

the preKar2p accumulated in the *sec61-3* mutant (Figure 7B, lane 3). These findings would be consistent with the *lhs1* null mutant being severely defective in the translocation of both prepro-α-factor and preKar2. It has been reported that overexpression of Kar2p can lead to the accumulation of preKar2, perhaps via the saturation of some component of the targeting/translocation machinery (Rose *et al.*, 1989). Clearly, the levels of *KAR2* mRNA are elevated in the *lhs1* mutant, raising the possibility that the observed accumulation of preKar2 is a simple consequence of its overexpression. However, tunicamycin treatment of either wild-type or *lhs1::TRP1* mutant cells led to a far greater increase in the level of *KAR2* mRNA with little or no concomitant accumulation of preKar2 (Rose *et al.*, 1989; Figure 7B, lanes 4–7). Overexpression alone therefore cannot account for the preKar2 accumulation observed in the *lhs1* mutant.

Finally, we examined the processing of protein disulfide isomerase (PDI) in the *lhs1* disruptant. Yeast PDI is an ER-resident glycoprotein predicted to have a cleavable signal sequence (Mizunaga *et al.*, 1990; Farquhar *et al.*, 1991; LaMantia *et al.*, 1991; Tachikawa *et al.*, 1991). Wild-type cell extracts probed with anti-PDI antiserum contained two immunoreactive bands, of which the slower migrating form was most abundant (Figure 7C, lane 1). The *lhs1* null mutant extract contained three forms of PDI. Two of these co-migrated with those observed in wild-type cells, but their relative levels were significantly altered, with a greater proportion of the higher mobility form being apparent in the mutant (Figure 7C, lanes 1 and 3). These two forms clearly appear to be glycosylation variants since both collapse to a single band upon digestion with endoglycosidase H (Figure 7D, lanes 1 and 2).

The third PDI species observed in the mutant (labelled 'prePDI') migrated more slowly than the signal processed forms observed either after tunicamycin treatment of wild-type cells (Figure 7C, lanes 2 and 3) or EndoH digestion of wild-type extracts (Figure 7D, lanes 2 and 3). EndoH digestion of the mutant extract resulted in two closely migrating bands (Figure 7D, lane 4). The larger of these co-migrates with the 'prePDI' observed in mock-digested extracts (Figure 7D, lane 3), whilst the smaller of the two bands precisely co-migrates with the deglycosylated PDI seen after EndoH treatment of wild-type extract (Figure 7D, lane 2). From this we conclude that the mutant accumulates a form of PDI whose gel mobility is unaffected by EndoH digestion, indicating that it is unmodified by N-linked oligosaccharides. These data would be consistent with this novel form corresponding to prePDI, lacking both *N*-glycosylation and signal processing.

In order to determine whether the *lhs1* defect corresponded to a genuine translocation phenotype, or was due to a defect in the processing of translocated precursors, the membrane association and protease accessibility of the accumulated form of PDI were examined. The accumulated prePDI was found to be membrane associated, in a form which was resistant to salt washing, but which could be released into the soluble fraction by low levels of detergent (0.5% NP40; Figure 8A). The membrane-associated prePDI was found to be sensitive to exogenously added protease, whilst the glycosylated forms were completely resistant to protease unless membranes were first solubilized with detergent (Figure 8B). The protease accessibility

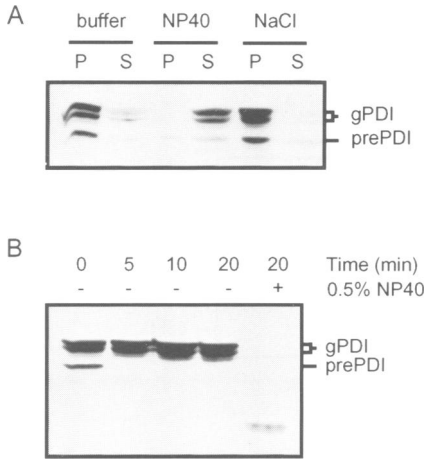

Fig. 8. The accumulated form of PDI is membrane associated, but accessible to exogenous protease. RCY110 cells were grown in YPD at 30°C and fractionated producing a 660 *g* supernatant as described in Materials and methods. (**A**) This extract was treated with lysis buffer or with lysis buffer containing either 0.5% NP40 or 500 mM NaCl, then incubated on ice for 30 min before spinning at 100 000 *g* to give pellet (P) and supernatant (S) fractions. Samples were analysed by immunoblotting with anti-PDI antiserum. PrePDI and glycosylated PDI (gPDI) are indicated. (**B**) Proteinase K was added to the low-speed supernatant to a final concentration of 300 μg/ml in the presence (+) or absence (–) of 0.5% NP40 and aliquots were removed at the times indicated. Again samples were separated by SDS–PAGE (10%) and analysed by immunoblotting with antibodies to PDI.

of the prePDI accumulated in the *lhs1* mutant demonstrates that it is predominantly associated with the cytosolic surface of the ER membrane, and that it is therefore largely untranslocated. The membrane association of prePDI would be consistent with effective targeting, but a subsequent failure to either initiate, or propagate, the translocation reaction. The lack of signal peptide cleavage would suggest that little or no translocation of the accumulated precursor had occurred. Whilst *lhs1* mutant cells are clearly defective in the translocation of prePDI, prepro-α-factor and preKar2, no accumulation of precursor forms of either invertase or dipeptidylaminopeptidase B was detectable in Western blots (R.A.Craven and C.J.Stirling, unpublished data). We therefore conclude that Lhs1p is required for the efficient translocation of a subset of precursors across the yeast ER membrane.

Finally, we noted an increased abundance in the *lhs1* mutant of a glycosylation variant of PDI located within the ER lumen. This may indicate either that PDI folding is perturbed, resulting in a restricted availability of some glycosylation site(s) within the protein or, alternatively, may reflect some general defect in the process of *N*-glycosylation in the mutant strain.

LHS1 encodes an ER glycoprotein

The nature of the regulation of *LHS1*, and the phenotypes of the null mutant, are consistent with the ER localization predicted from the deduced amino acid sequence of the protein. In order to empirically determine the cellular localization of Lhs1p, we tagged the protein with an epitope from c-*myc* against which there is a well-characterized monoclonal antiserum (9E10; Evan *et al.*, 1985). A 117 bp DNA sequence, encoding two tandem 9E10-specific epitopes, was inserted at a unique *Hin*dIII site

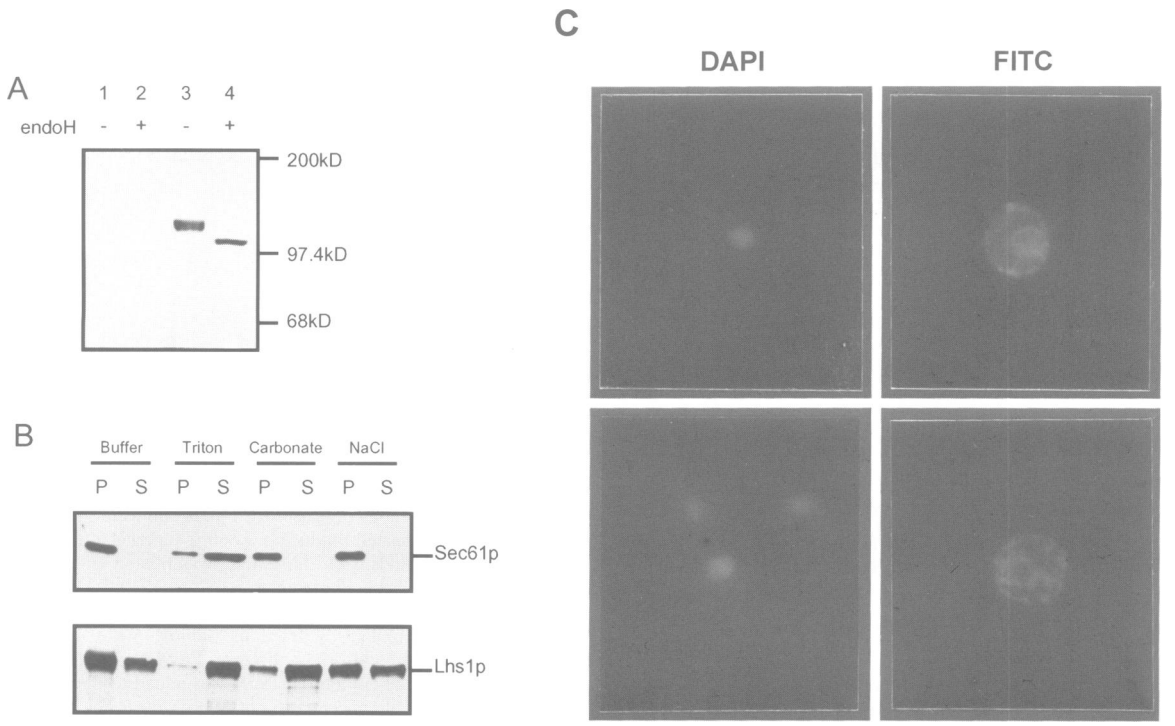

Fig. 9. Lhs1p is an ER-localized glycoprotein. (**A**) Extracts from RCY104 containing pRC44 (vector control; lanes 1 and 2) and pRC45 (myc tag; lanes 3 and 4) were made from cells grown in minimal medium at 30°C and incubated with (+) or without (–) endo H, separated by SDS–PAGE (7.5%) and immunoblotted with 9E10 tissue culture supernatant. (**B**) RCY104 containing pRC45 was grown in minimal medium at 30°C and fractionated as described in Materials and methods. Pellet (P) and supernatant (S) fractions were collected after treatments with the reagents indicated and analysed by SDS–PAGE and immunoblotting. (**C**) RCY104 containing pRC45 was grown in minimal medium. Cells were fixed and processed for immunofluorescence as described in Materials and methods, and probed with purified anti-c-*myc* antibodies followed by secondary decoration with FITC-conjugated rabbit anti-mouse IgG antibodies. DAPI and FITC fluorescence are shown in separate panels. The upper and lower panels illustrate two different fields of the same stained cells. Cells carrying vector plasmid alone showed no detectable fluorescein staining (not shown).

situated 46 codons upstream of the *LHS1* stop codon. The resultant construct would be predicted to encode a fusion protein some 4.5 kDa larger than native Lhs1p. This epitope-tagged version of Lhs1p was expressed from a multicopy vector under the control of its own promoter (pRC45). The tagged protein was functional as determined by the ability of pRC45 to rescue the translocation phenotype of the *lhs1::TRP1* mutant (R.A.Craven and C.J.Stirling, unpublished data). Western blotting of whole-cell extracts with 9E10 monoclonal antibodies detected a diffuse band with a relative mol. wt of ~117–123 kDa which was not present in control extracts (Figure 9A). This diffuse band shifts to a single discrete band of ~103 kDa after digestion with EndoH (Figure 9A). These results indicate, first, that the tagged Lhs1p contained N-linked oligosaccharide and, secondly, that the observed diffuse nature of the band is due to heterogeneous glycosylation. On some gels, the diffuse band appeared to be resolved into a doublet, suggesting that the heterogeneous glycosylation may arise from addition of a variable number of core oligosaccharides. Given that N-linked glycosylation occurs only within the ER, then these observations conclusively demonstrate that Lhs1p is targeted to the ER. The primary sequence of Lhs1p contains seven potential sites for N-linked glycosylation (Rasmussen, 1994). The observed shift in relative molecular weight would be consistent with six or seven of these sites being core glycosylated, but this interpretation awaits future verification. Fractionation studies indicate that the tagged Lhs1p is associated with the microsomal fraction, and can be released into the supernatant by permeabilizing membranes with low levels of detergent, or by carbonate extraction, but not by salt washing (Figure 9B). In these experiments Sec61p, an integral membrane protein, serves as a control (Stirling *et al.*, 1992). We therefore conclude that Lhs1p is a soluble protein localized within the lumen of a membrane-bound compartment. In order to determine the identity of this compartment, the tagged Lhs1 protein was localized by indirect immunofluorescence microscopy. Cells carrying pRC45 exhibited strong perinuclear staining with further staining at, or near, the cell periphery. Frequently, strands were observed which appeared to connect the perinuclear and peripheral staining (Figure 9C). This pattern is reminiscent of those seen for Kar2p (Rose *et al.*, 1989), and the ER membrane proteins Sec62p and Sss1p (Deshaies and Schekman, 1990; Esnault *et al.*, 1993), and is consistent with yeast ER. There was substantial variation observed in the intensity of staining in different cells within the population which we attribute to likely copy number variation of the 2μm-based plasmid. No staining was observed in control cells lacking the *myc*-tagged Lhs1p (R.A.Craven and C.J.Stirling, unpublished data). Taken together, these data demonstrate that Lhs1p is a soluble glycoprotein located within the lumen of the ER.

Discussion

Our data indicate that the *LHS1* gene encodes a glycoprotein localized within the lumen of the ER. An epitope-

tagged version of Lhs1p has been expressed in RCY104 cells where it was found to complement an *lhs1* null mutation, indicating that it retains Lhs1p function. The tagged protein was localized to the lumen of the ER, where it was extensively modified with N-linked oligosaccharide. Some variation in the extent of N-glycosylation was suggested by the appearance of a diffuse band, possibly a doublet, with a relative mol. wt of 117–123 kDa. Taking into account the predicted increase in molecular weight conferred by the tag, these data predict a gel mobility for native Lhs1p of between 113 and 119 kDa.

The Lhs1 protein shares sequence similarities with members of the Hsp70 superfamily of molecular chaperones, but like many Hsp70s Lhs1p is not inducible by heat shock. The *LHS1* gene is regulated by the unfolded protein response pathway, suggesting a role for Lhs1p in the processing of malfolded polypeptides. Moreover, in the absence of Lhs1p, we observed a substantial increase in the levels of both *KAR2* and *PDI* mRNA. The only known mechanism for the co-ordinated regulation of these two genes is through the unfolded protein response (UPR). The simplest interpretation of our data would therefore be to propose that the UPR pathway is induced in *lhs1* mutant cells, indicative of the accumulation of unfolded polypeptides. This would in turn suggest a role for Lhs1p in protein folding within the ER under normal circumstances. The observed increase in mRNA levels corresponds to 50% of the level attained when the UPR was fully induced by tunicamycin treatment. This would therefore require either that the UPR can be induced in a graded fashion or, alternatively, that the pathway is induced in only 50% of cells. In the latter case, the UPR may be cycling on and off, with maximal induction resulting in suppression of the *lhs1* folding defect and a consequent downregulation of UPRE-containing genes.

Our data demonstrate that *LHS1* is not essential for viability. However, the required induction of the unfolded protein response complicates interpretation of the null mutant phenotype since increased levels of other ER chaperones may compensate for the loss of Lhs1p. More specific evidence for such functional redundancy arose from the study of genetic interactions between mutant alleles of *kar2* and *lhs1*. The *lhs1* null mutation is synthetically lethal in combination with either *kar2-113* or *kar2-159*, indicating that cells cannot tolerate the loss of Lhs1p when Kar2p function is impaired. Previous studies have demonstrated that *kar2-159* is synthetically lethal when combined with *sec63-1*, which has been interpreted as the consequence of two partially debilitated proteins being unable to interact productively (Scidmore *et al.*, 1993). Subsequent biochemical studies have confirmed the predicted interaction between the Kar2 and Sec63 proteins (Brodsky and Schekman, 1993; Brodsky *et al.*, 1993). Obviously, in the case of the *lhs1* null allele, no direct physical interaction with Kar2p is implied. However, the specific nature of the genetic interactions between *kar2* and *lhs1* indicates that these gene products are involved in some common pathway that is essential for viability. Given that both proteins are members of the Hsp70 superfamily and, furthermore, that our data indicate a role for Lhs1p in both protein translocation and folding, then it is tempting to speculate that Lhs1p and Kar2p share some common activity. This activity can be provided by Kar2p alone, but the *kar2-113* and *kar2-159* mutations are constitutively defective, resulting in a dependence upon Lhs1p. Clearly, since *KAR2* is an essential gene, we must conclude that Lhs1p cannot compensate for all the essential activities of Kar2p.

The regulation of *LHS1* by the unfolded response pathway, and the observed phenotypes of the *lhs1* null mutant, are consistent with a role for Lhs1p as a novel molecular chaperone of the ER lumen. Despite its size, Lhs1p shares no significant sequence similarity with the Hsp90 class of chaperones, but rather appears more closely related to the Hsp70 family. Many members of the Hsp70 family are very highly conserved whilst others, such as yeast Sse1p/Msi3p and Sse2p, appear more divergent (Mukai *et al.*, 1993; Shirayama *et al.*, 1993). Like Lhs1p, both Sse1p/Msi3p and Sse2p share ~26–29% identities with other family members. However, whilst Sse1p/Msi3p and Sse2p are closely related to one another, they appear no more closely related to Lhs1p than are other family members. Whether or not Lhs1p corresponds to a functionally distinct subclass of the Hsp70 family remains to be determined.

The *lhs1* null mutant is defective in the translocation of a number of protein precursors, including prepro-α-factor, preKar2 and prePDI. The accumulated prePDI is membrane associated in a form which is resistant to salt washing, but which remains accessible to exogenous protease. These results suggest that prePDI is efficiently targeted to the ER, where its membrane association may be mediated by hydrophobic interactions, perhaps involving its partial membrane translocation. However, the majority of the prePDI polypeptide chain remains on the cytosolic side of the ER membrane. In mitochondrial protein import, a model for the role of the matrix Hsp70, Ssc1p, has been proposed in which Ssc1p binds precursor as it emerges into the matrix, then acts to 'pull' the remainder of the polypeptide through the bilayer (Kang *et al.*, 1990). A similar role has been proposed for Kar2p in ER translocation (see Schekman, 1994). The nature of the accumulation of prePDI in the *lhs1* mutant would be entirely consistent with an identical role for lhs1p in the translocation of prePDI.

Our data clearly demonstrate a severe translocation defect in the *lhs1* mutant, but we cannot exclude the possibility that this translocation defect is indirect, perhaps via the recruitment of Kar2p to some other activity, thereby reducing its availability to participate in polypeptide translocation. This would appear unlikely given that conditional *kar2* mutants have been shown to accumulate a broad range of precursors, including prepro-α-factor and invertase (Vogel *et al.*, 1990; Nguyen *et al.*, 1991; Brodsky *et al.*, 1995). If the translocation defect observed in *lhs1* mutant cells was a result of an indirect effect upon Kar2p, then one would expect a similar profile of precursors to be affected. This is not the case. The *lhs1* null mutation affects prepro-α-factor, preKar2 and prePDI, but no accumulation of preInvertase was detected in Western blots. The subcellular localization of Lhs1p is clearly consistent with the proposed role for this protein in both ER protein folding and in polypeptide translocation across the ER membrane. Further studies are under way to determine the nature of the role played by Lhs1p in these events.

Table I. Bacterial and yeast strains

Strain	Genotype	Source or reference
Saccharomyces cerevisiae		
TR1	*trp1/trp1 ura3/ura3 his3/his3 ade2/ade2 lys2/lys2 MAT***a**/*MAT*α	Parker *et al.*, 1988
TR2	*trp1 ura3 his3 ade2 lys2 MAT***a**	Parker *et al.*, 1988
TR3	*trp1 ura3 his3 ade2 lys2 MAT*α	Parker *et al.*, 1988
RCY101	as TR1 but *lhs1::TRP1/LHS1*	this study
RCY102	as RCY101, independent isolate	this study
RCY104	as TR2 but *lhs1::TRP1*	this study
RCY110	as TR3 but *lhs1::TRP1*	this study
CSY140	*leu2 ura3 MAT*α	this study
RSY12	*ura3 leu2 sec53-6 MAT*α	F.Képès
RSY26	*ura3 suc2 lys2 sec59-1 MAT***a**	F.Képès
PBY408A	*ura3 leu2 his4 sec11-7 MAT*α	Böhni *et al.*, 1988
CSY150	*leu2 trp1 ura3 sec61-3 MAT*α	Stirling *et al.*, 1992
RCY 115	*ura3 ade2 trp1 lys2 kar2-113 MAT*α	this study
RCY131	RCY104×RCY115 diploid	this study
RCY117	*ura 3 ade2 leu2 trp1 kar2-159 MAT*α	this study
RCY132	RCY104×RCY117 diploid	this study
CS165	*ire1::URA3 leu2 his3 trp1 ura3 ade2 can1 met his3::HIS*$^+$ *UPRE-lacZ leu2::LEU+UPRE-lacZ MAT***a**	Cox *et al.*, 1993
RCY150	RCY110×CS165 diploid	this study
Escherichia coli		
DH5α	*supE44* Δ*lac*U169 (*F80 diac*ZDM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Hanahan, 1983

Materials and methods

Materials

Restriction enzymes, modifying enzymes, dNTPs, ampicillin, endoglycosidase H and dNTPs were from Boehringer Mannheim. Tunicamycin, proteinase K and DAPI were from Sigma. Yeast lytic enzyme and [α-^{32}P]dCTP were from ICN. Culture media were obtained from Difco.

Strains and growth conditions

Escherichia coli and yeast strains are listed in Table I. *Escherichia coli* cells were grown in LB (1% tryptone, 0.5% yeast extract, 1% NaCl). Where appropriate, ampicillin was used at a final concentration of 100 μg/ml. Yeast strains were routinely grown in YPD (2% peptone, 1% yeast extract, 2% glucose) or in YNB (0.675% yeast nitrogen base) with 2% glucose and appropriate amino acid supplements. Tunicamycin was used at a final concentration of 10 μg/ml. URA3$^+$ cells were counterselected using 5-FOA-containing medium (Sikorski and Boeke, 1991). Solid media were supplemented with 2% Bacto agar. Diploids were sporulated on 1% KOAc, 0.1% yeast extract, 0.05% glucose, plus appropriate amino acid supplements, at 25°C. Tetrad dissection was as described by Sherman and Hicks (1991).

Plasmids and nucleic acid manipulations

Nucleic acid manipulations were carried out essentially as described by Sambrook *et al.* (1989). *LHS1* was subcloned from cosmid clone pEKG080 (Rasmussen, 1994) as a 4 kb *Sma*I/*Nhe*I fragment filled in with Klenow and dNTPs, and ligated into the *Sma*I site of pUC118 to generate pRC40. An *lhs1* null allele was constructed by digesting pRC40 with *Eco*RV to remove the coding sequence of *LHS1*, and replacing this with a 1.45 kb *Bam*HI/*Sac*I fragment (filled in with Klenow and dNTPs) corresponding to the yeast *TRP1* gene, thus generating pRC41. The 1.45 kb *Bam*HI/*Sac*I *TRP1* fragment was derived from pMR38 which was constructed by ligating the *TRP1*-containing *Eco*RI fragment from YRp7 (Parent *et al.*, 1985) filled in with Klenow and dNTPs into the *Sma*I site of pUC118 (M.Régnacq, personal communication). To allow expression of *LHS1* in yeast, a *Bam*HI/*Sac*I fragment from pRC40 was cloned into the *Bam*HI/*Sac*I sites of YEp352 (Hill *et al.*, 1986) creating pRC43. An epitope tag was inserted into the *LHS1* coding sequence as follows. First, the polylinker *Hin*dIII site in pRC43 was deleted by digesting with *Nar*I and *Sal*I, filling in with Klenow and dNTPs, then recircularizing to produce pRC44. A 113 bp *Nde*I fragment, encoding the epitope tag, from pGEMMH (K.S.Sheldrick, D.J.E.Griffiths, A.M.Carr and I.M.Hagan, in preparation) was filled in with Klenow and dNTPs, then ligated into the unique *Hin*dIII site in pRC44 similarly filled in with Klenow and dNTPs. This created pRC45 which contains a net insertion of 117 bp, encoding two tandem 9E10 epitopes, in frame within the *LHS1* coding sequence. The sequence of the *Nde1* fragment from pGEMMH is as follows: CATATGGGTAGCAGCCACCATCATCACCATCATGCTGAGGAGCAAAAGTTAATTTCTGAAGAAGATTTGTCCATGGCTGAAGAACAAAAATTGATCAGCGAGGAGGACTTACATATG). Plasmids were transformed into yeast using the lithium acetate method (Ito *et al.*, 1983).

Southern blotting

Genomic DNA was prepared essentially as described by Philippsen *et al.* (1991). Three micrograms were digested and resolved on a 0.8% agarose gel. Southern blotting to nylon membranes (Hybond N, Amersham) was carried out as described by Sambrook *et al.* (1989) using 0.4 M NaOH as the transfer buffer. Pre-hybridization and hybridization were carried out at 65°C in Church buffer (Church and Gilmore, 1984; 0.5 M Na_2HPO_4, 7% SDS, 1 mM EDTA) and washes in Church wash buffer (Church and Gilmore, 1984; 40 mM Na_2HPO_4, 1% SDS) at the same temperature. A *Sac*I/*Pst*I digest of pRC40 was used to generate a 756 bp fragment corresponding to the region immediately 5′ of the coding sequence. This fragment was radiolabelled using a random priming kit supplied by Boehringer Mannheim.

Northern blotting

Total yeast RNA was prepared from cultures grown to mid-log phase (Schmitt *et al.*, 1990), and 15 μg were loaded and separated on a 1.2% agarose gel. Northern blotting to nylon membranes (Hybond N; Amersham) was as described by Sambrook *et al.* (1989). Pre-hybridizations and hybridizations were carried out in Church buffer at 65°C, and washes with 2× SSC, 1% SDS and 0.2× SSC, 0.1% SDS at the same temperature. Northern blots were stripped with 0.1% SDS as described in the manufacturer's instructions. The 2520 bp *Eco*RV fragment from pRC40 was used as a probe for *LHS1*. *KAR2* and *PDI* specific probes were generated by polymerase chain reaction (PCR) from genomic DNA. For *KAR2*, a 560 bp fragment corresponding to positions 1991–2559 was amplified (Rose *et al.*, 1989). The 766 bp PDI probe corresponded to positions 351–1116 (LaMantia *et al.*, 1991). A 1.6 kb *Eco*RI/*Hin*dIII fragment corresponding to the 3′ end of the gene and untranslated regions was used as a probe for actin (Gallwitz and Sures, 1980; Ng and Abelson, 1980). All probes were again radiolabelled by random priming. Samples were visualized by autoradiography and, where appropriate, samples were quantitated after detection using a Fujix BAS2000 Bioimager.

Immunoblotting

Whole yeast extracts were prepared by glass bead lysis in SDS sample buffer (Laemmli, 1970) from cultures grown to mid-log phase, resolved by SDS–PAGE, transferred to nitrocellulose membranes (HybondC,

Amersham) and probed with reagent antisera essentially as described previously (Stirling *et al.*, 1992). The following antisera were used at the dilutions indicated in parentheses: α-factor (1:5000; from R.Schekman), Kar2p (1:5000; from M.Rose), PDI (1:1000 of affinity-purified antibodies; from M.Tuite), Sec61p (1:5000; Stirling *et al.*, 1992), peroxidase-conjugated goat anti-rabbit IgG (Sigma; 1:5000), 9E10-containing tissue culture supernatant (1:2; from K.Sheldrick) and peroxidase-conjugated rabbit anti-mouse IgG (1:5000; Dakopatts, Denmark). Peroxidase-conjugated secondary antibodies were detected by enhanced chemiluminesence (ECL, Amersham).

Endo H digestion was performed using 20 μl whole-cell extract (prepared as above and corresponding to 1 OD_{600} equivalent of cells) added to 400 μl of 150 mM sodium citrate (pH 5.5), 1 mM phenylmethylsulfonyl fluoride (PMSF). One unit of Endo H was added and the sample incubated at 37°C for 4 h. Mock digests were incubated without enzyme. Trichloroacetic acid (TCA) was added to a final concentration of 10% and after precipitation protein pellets were washed with acetone and resuspended in SDS sample buffer. Samples were analysed by SDS–PAGE and immunoblotting.

Cell fractionation

Cultures grown to mid-log phase were harvested and cells resuspended at 50 OD_{600}/ml in 0.1 M Tris–sulfate (pH 9.4), 10 mM dithiothreitol (DTT). After 10 min at room temperature, cells were collected and resuspended at 100 OD_{600}/ml in 0.75× YP, 0.7 M sorbitol, 0.5% glucose, 10 mM Tris–HCl (pH 7.4). One unit/OD of yeast lytic enzyme was added and cells incubated at 30°C for 30 min. Spheroplasts were harvested and resuspended at 100 OD_{600}/ml in lysis buffer [20 mM HEPES (pH 7.4), 50 mM KOAc, 2 mM EDTA, 250 mM sorbitol] and lysed by 15 manual strokes of a Potter–Elvejem homogenizer (Wheaton, USA). The homogenate was spun at 660 *g* for 5 min to remove unlysed cells and the supernatant removed. The 660 *g* supernatant was diluted 2.5-fold into lysis buffer alone or lysis buffer containing either 0.4% Triton X-100, 0.5% NP40, 0.1 M Na_2CO_3 (pH 11.5) or 0.5 M NaCl, then incubated on ice for 30 min before being centrifuged at 100 000 *g* for 30 min and the pellet and supernatant fractions collected. Samples were resuspended in SDS loading buffer and analysed by SDS–PAGE and immunoblotting. Protease protection experiments were performed by adding proteinase K to the 660 *g* supernatant to a final concentration of 300 μg/ml in the presence or absence of 0.5% NP40 and samples incubated on ice. Aliquots were removed at specified time points and added to an equal volume of 40% TCA, precipitated proteins collected by centrifugation, washed with acetone and resuspended in SDS sample buffer. Samples were analysed by SDS–PAGE and immunoblotting.

Immunofluorescence

Immunofluorescence microscopy was carried out essentially as described by Pringle *et al.* (1991). Cultures were grown to mid-log phase and formaldehyde added to a final concentration of 5%, incubated for 5 min with shaking, followed by a further 2 h standing at room temperature. Formaldehyde-fixed cells were resuspended in 1.2 M sorbitol, 100 mM HEPES (pH 7.4), 10 mM DTT at 5 OD_{600}/ml and washed twice in the same buffer. Two units of yeast lytic enzyme were added per OD_{600} equivalent of cells and incubated for 10 min incubation at 30°C, resulting in 50% spharoplasting. Cells were then washed twice in the same buffer minus DTT and finally in phosphate-buffered saline (PBS) before being resuspended in PBS at 25 OD/ml. PBS containing 0.05% NP40 and 1% BSA was used for blocking. Antibody bindings were carried out in PBS for 2 h and washes were carried out with PBS. Affinity-purified anti-c-*myc* antibodies (0.5 mg/ml; a generous gift from Greg Steele) were used at a 1:40 dilution, and decorated with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG antibodies (1:100; Sigma). Slides were incubated for 5 min with 1 μg/ml DAPI, mounted in one drop of 1 mg/ml *p*-phenylenediamine in 90% glycerol and examined at 2500× magnification on a Zeiss Axiophot microscope.

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