Cer1p, a Novel Hsp70-related Protein Required for Posttranslational Endoplasmic Reticulum Translocation in Yeast*

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Proteins enter the secretory pathway by translocation across the endoplasmic reticulum (ER) membrane. In Saccharomyces cerevisiae, import of proteins into the ER occurs both cotranslationally and posttranslationally. Presumably, the cotranslational targeting to the ER membrane is directed by the signal recognition particle, as demonstrated in other eukaryotic systems. The deletion of a gene, called CER1, inhibits the translocation of proteins that enter the ER posttranslationally, but not those that enter cotranslationally. This translocation defect is more pronounced at lower temperatures. A strain possessing a null mutation of CER1 in combination with a kar2 temperature-sensitive mutation displays synthetic growth defects, whereas overexpression of the ER DnaJ homolog Scj1p suppresses the translocation defect in cer1 strains. CER1 is predicted to encode a 100-kDa polypeptide, residing in the ER lumen that is related to the hsp70 family of molecular chaperones.

Newly synthesized proteins destined to enter the secretory pathway must first be translocated across the endoplasmic reticulum (ER) membrane. A number of secretory proteins enter the ER by virtue of a cleavable N-terminal hydrophobic signal sequence (1). Once the signal sequence has emerged from a cytosolic ribosome, the secretory protein can bind to the signal recognition particle (SRP), which helps target the protein to the translocation apparatus on the ER membrane (2, 3) and couples translation to translocation. In yeast, this coupling of translation and translocation is not absolute. Preinvertase translocates cotranslationally (4), yet does not require SRP for translocation (5). Most likely, certain imported proteins utilize cytosolic hsp70 and other factors, but not SRP, to maintain translocation competence of the precursor proteins (8, 9).

Recent evidence indicates the import pathway taken by a secretory protein depends on the hydrophathy of the signal sequence and the length of the polypeptide (7); the longer the polypeptide chain and the more hydrophobic the signal sequence, the greater the likelihood that a protein will translocate cotranslationally. Regardless of the pathway, the secretory protein is translocated through a translocation apparatus comprised of several integral membrane proteins. In addition to the translocation complex (10, 11), an ER luminal hsp70 chaperone is thought to play a critical role in the translocation process. The yeast hsp70 that resides in the ER (Kar2p) (12, 13) has been shown both genetically and biochemically to assist in both cotranslational and posttranslational translocation (14, 15), although the exact role of hsp70s in the translocation mechanism remains controversial (16).

Here we describe the partial characterization of a novel yeast protein (open reading frame YKL073) (17) that has homology to the hsp70 family. We have named the protein Cer1p, for chaperone in the ER. As shown below, Cer1p affects the import of a subset of secretory proteins, those able to translocate posttranslationally.

EXPERIMENTAL PROCEDURES

Yeast Strains—GHY1 containing the CER1 disruption was created as follows. Two oligonucleotide DNA primers were constructed containing at the 5′ ends, 40 base pairs of CER1 sequence immediately flanking the predicted CER1 open reading frame and 20 base pairs identical to the pRS306 sequence upstream and downstream of the URA3 gene. A polymerase chain reaction was performed using these primers and the pRS306 plasmid to generate a URA3 fragment with 40 base pairs of CER1 DNA flanking the gene. This fragment was used to transform SEY6210 (leu2-3, 112 ura3-52 his3Δ200 trp1Δ901 his2-801 pep4Δ40 ura3-52 his3Δ200 trp1Δ901 his2-801 pep4Δ40). The CER1 gene was disrupted as described previously in the strain MY767 (MATα ura3-52 leu2-3, 112 ade2 his3Δ200 trp1Δ901 his2-801 pep4Δ40) which was generously provided by Mark Rose, Princeton University to create GHY3, a cer1Δ kar2-1 strain. Steady State Immunoblots—Yeast strain GHY2, used in this experiment, is cer1Δ:URA3. The CER1 strain used in this experiment is LCY22. Equal aliquots of cells were harvested in log phase and total cellular protein was isolated as described previously (18). Proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Primary antibodies against α-factor, CPY, dipeptidyl aminopeptidase B (DPAP-B), and invertase were generously provided by Tom Stevens. The anti-Kar2 antibody was generously provided by Mark Rose. Detection of the primary antibody was accomplished with secondary antibodies conjugated to horseradish peroxidase and visualized using chemiluminescent reagents (Pierce). GHY1 was transformed with the 2μ-based plasmid YEpsCJ1 (19) which contains the SCJ1 open reading frame and was the generous gift of Pamela Silver, Dana Farber Cancer Institute, Harvard Medical School.

PROTEASE EXPERIMENT—Cell cultures of the GHY2 (cer1Δ pep4Δ) strain were grown to mid log phase, equal aliquots of cells were spheroplasted for 30 min at 30 °C by addition of lyticase (10 μg/ml) in supportive buffer (1.2 mM sorbitol, 50 mM Tris, pH 7.4, 2 mM MgCl2, 10 mM NaCl, 3% β-mercaptoethanol). The spheroplasts were pelleted (650 × g, 5 min) and resuspended in nonsupportive buffer (0.2 M sorbitol, 25 mM NaPO4, 0.1 M MgCl2, 10 mM NaCl, 0.1% NP-40).
**RESULTS AND DISCUSSION**

*cer1* is predicted to encode a hydrophilic 100-kDa protein (Fig. 1), possessing both an N-terminal signal sequence, for transport into the ER, and a C-terminal ER retention signal (HDEL). Therefore the Cer1 protein (Cer1p) is expected to reside in the lumen of the ER. The predicted N-terminal region of Cer1p (44 kDa) has significant homology (17% amino acid identity, 27% similarity) to the N-terminal ATPase domain of Kar2p and other hsp70s, including several 5–7 residue spans of exact identity. Amino acids predicted to contact the bound ATP (noted with an asterisk in Fig. 1) (21) are particularly well conserved. Actin and hexokinase, proteins known to share a similar three-dimensional fold with the hsp70 N-terminal domain, do not show significant homology to hsp70s or Cer1p and have no spans of homology longer than 2 residues. However, it should be noted that Cer1p is not a traditional member of the hsp70 family of molecular chaperones. In addition to the greater size, Cer1p does not display any observable homology to the highly conserved peptide binding domain of traditional hsp70s. Bacterial and eukaryotic hsp70s, for example, are over 50% identical in a 17-kDa region of the peptide binding domain (residues 385–545 of DnaK) (22).

During the preparation of this manuscript a molecular characterization of this open reading frame was published (23). The authors name the gene *LHS1*, for luminal hsp70 homolog. Our sequence analysis does not find the same degree of homology as reported by these authors, and we do not find homology extending beyond the ATP binding domain. Since homology is not found within the well conserved peptide binding domain of hsp70s, Cer1p does not share all the features of the hsp70 family and should not be considered an hsp70 homolog.

To examine the function of Cer1p we disrupted the gene in a strain, but grew poorly at lower temperatures, as compared to the parental strain, which grew normally at 30°C (Fig. 2A), the optimal growth temperature for the parent strain, but grew poorly at lower temperatures, as compared to a wild type *CER1* strain (Fig. 2B). This result suggests that either Cer1p has an important function only at lower temperatures, or other proteins can compensate for the loss of Cer1p at 30°C.

Investigation of a possible mating defect in *cer1Δ* cells of mating type a led to the discovery of decreased secretion of the mating pheromone a-factor. Since Cer1p is predicted to reside in the ER lumen, and the ER-hsp70 Kar2p is essential for translocation, we tested whether the secretory defect was due to a translocation block of the a-factor precursor (ppaf) into the ER. At the restrictive temperature (18°C), the ppaf accumulated in *cer1Δ* cells (Fig. 3A). This accumulation was more pronounced at 18°C than at 30°C.

Since increased levels of ppaf could have resulted from either an inhibition of signal sequence cleavage in the ER or from a failure of the protein to cross the ER membrane, we probed the intracellular location of ppaf by protease sensitivity (Fig. 3B). Outer membranes of yeast cells were partially disrupted by homogenization following cell wall removal. After such treatment, the ppaf that accumulated in *cer1Δ* cells could be degraded with the proteases proteinase K and trypsin under conditions in which the ER luminal protein Kar2p remained protease resistant. Only following detergent treatment did Kar2p become sensitive to proteolytic attack, indicating the ER membranes were intact following lysis of the plasma membrane. Therefore, ppaf accumulated due to a translocation block in *cer1Δ* cells.

To determine whether loss of Cer1p affected ER translocation universally, we examined the processing of other well...
characterized secretory proteins: CPY, which like ppαf translocates posttranslationally, and DPAP-B and invertase, which translocate cotranslationally (6, 7, 24). Mature CPY is a glycosylated vacuolar protein. In pep4 strains the final proteolytic processing of CPY is blocked and the vacuolar glycosylated form, which migrates at a rate corresponding to a 69-kDa protein, can be electrophoretically separated from unglycosylated forms (25). In cer1Δ pep4 cells we observed a second, faster migrating form (60 kDa) of CPY (Fig. 3A). As with ppαf, this accumulation was greater at 18°C than at 30°C. This faster migrating species is the untranslocated preproCPY (ppCPY), since endoglycosidase H treatment increased the mobility of the 69-kDa form but not the 60-kDa form (data not shown). In addition, the glycosylated ER form of CPY, termed p1 (25) could be observed in both cer1Δ and CER1 cells, but migrated more slowly than ppCPY. The conversion of p1 to the vacuolar p2 form observed in pulse-chase experiments occurred at the same rate in CER1 and cer1Δ cells, indicating that Golgi glycosyl processing and sorting to the vacuole are not affected by loss of Cer1p function (data not shown). In contrast to ppCPY and ppαf, the import of both DPAP-B and invertase were unaffected in the cer1Δ strain at all temperatures tested (Fig. 3C).

Craven et al. (23) reported a translocation defect in lsh1Δ deletion strains (cer1Δ). They also observed the accumulation of precursor proteins in cells lacking functional Cer1p. But because the secretory proteins chosen in their study could not be easily classified according to their translocation pathway, the authors were unable to conclude that Lhs1p (Cer1p) functioned only in posttranslational translocation.

Because of the sequence similarity and functional relationship between Cer1p and Kar2p, we generated kar2 cer1 double mutants to probe possible genetic interactions. As seen in Fig. 4A, a strain harboring defects in both kar2 (12) and cer1 (kar2-1 cer1Δ) grew more slowly at 30°C than cells carrying mutations in either cer1 or kar2 alone. When cer1Δ was combined with other mutations (e.g. cne1Δ; the yeast calnexin gene) such synthetic growth defects were not observed (data not shown).

In Escherichia coli, the co-chaperone hsp40 (or DnaJ) acts in concert with hsp70 (DnaK) in unfolding and replications functions (26). Similar hsp70-hsp40 pairs have been demonstrated in various compartments in yeast (27). Only one soluble hsp40 has been identified in the ER of Saccharomyces cerevisiae, termed Scj1p (28). Genetic interactions between KAR2 and SCJ1 suggest that the protein products of these two genes function as an hsp70-hsp40 pair in the ER (19). Interestingly, addition of a multicopy plasmid bearing the SCJ1 gene eliminates the ppαf translocation defect in a cer1Δ strain (Fig. 4B). Since deletion of SCJ1 has no effect on translocation, Scj1p is likely either regulating the activity of Cer1p directly, or is decreasing the effect of a cer1Δ by regulating the activity of Kar2p.

**Fig. 3. Untranslocated intermediates accumulate in cer1Δ cells.** All temperatures are in °C. A (+) indicates that cells are CER1, whereas a (−) indicates cells are cer1Δ. A, the top panel is an immunoblot of total cellular extracts probed using polyclonal antibodies raised against unglycosylated invertase. A cell lysate of a sec61-2 cer1 Δ strain that is approximately the same size as unglycosylated DAP2 (20) stain grew at the nonpermissive temperature for 3 h in the last lane to indicate the relative migration of the untranslocated preinvertase (preinv). The bottom panel shows an immunoblot analysis of Kar2p in spheroplast extracts treated as indicated with proteases and detergent. A (+) indicates the addition of proteases or detergent, whereas (−) indicates these were not added. The bottom panel shows an immunoblot analysis of α-factor from the same experiment. C, steady state forms of the SRP-dependent secretory proteins in cer1Δ strains. The top panel is an immunoblot probed using polyclonal antibody raised against unglycosylated invertase. Molecular masses indicated are based on standard molecular markers and migration of mature CPY in a PEP4 strain. B, prepro-α-factor accumulated in cer1Δ cells is exposed to the cytosolic compartment. The top panel shows an immunoblot probed using polyclonal antibodies raised against DPAP-B. The last lane contains cell extracts of CER1 cells treated with tunicamycin (tunic, 1 μg/ml final concentration) for 90 min to indicate the relative migration of the unglycosylated preDPAP-B. The faint band in cer1Δ strain that is approximately the same size as unglycosylated DPAP-B is not a DPAP-B-specific band, as it appears in extracts from strains lacking a DAP2 gene (data not shown).

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2 T. G. Hamilton, unpublished data.

3 P. Silver and T. G. Hamilton, unpublished data.
The secretory proteins that require Cer1p function (ppaf, ppCPY) have previously been shown to translocate posttranslationally in vitro and have relatively weak signal sequences whereas the proteins that are unaffected by loss of Cer1p function (preinvertase, preDPAP-B) translocate cotranslationally and possess stronger signal sequences. While it remains possible that other unknown differences between these two sets of proteins underlie the different requirements for Cer1p, the translocation pathway is the most obvious and striking difference. The correlation with SRP independence is not strong however, since translocation of preinvertase not severely affected in yeast lacking SRP. Since the translated N-terminal attachment. The ribosome could either aid directly in translocation, by generating the force required for protein movement through the membrane, or aid indirectly by linking translation, and therefore folding, to the translocation process. Either way, differences between the two import pathways extend to the lumenal side of the ER membrane.

Cer1p is the first member of a new class of hsp70 like proteins that are involved in organelle import. If Cer1p interacts directly with a translocating polypeptide, as has been shown for Kar2p, it binds through a novel peptide binding domain. It remains to be tested whether this protein displays other hsp70 chaperone functions, such as assisting in protein folding or targeting proteins for degradation.

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