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The calnexin homologue *cnx1*$^{+}$ in *Schizosaccharomyces pombe*, is an essential gene which can be complemented by its soluble ER domain

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Secretory proteins become folded by the action of a number of molecular chaperones soon after they enter the endoplasmic reticulum (ER). In mammalian cells, the ER membrane protein calnexin has been shown to be a molecular chaperone involved in the folding of secretory proteins and in the assembly of cell surface receptor complexes. We have used a PCR strategy to identify the *Schizosaccharomyces pombe* calnexin homologue, *cnx1*$^{+}$. The *cnx1*$^{+}$ encoded protein, Cnx1, was shown to be a calcium binding type I integral membrane glycoprotein. At its 5′ end, the *cnx1*$^{+}$ gene has consensus heat shock transcriptional control elements and was inducible by heat shock and by the calcium ionophore A23187. Unlike the sequence-related *Saccharomyces cerevisiae CNE1* gene, the *S.pombe cnx1*$^{+}$ gene was essential for cell viability. The full-length Cnx1 protein was able to complement the *cnx1*$^{+}$ gene disruption but the full-length mammalian calnexin could not. The ER lumenal domain of Cnx1, which was secreted from cells, was capable of complementing the *cnx1::ura4* lethal phenotype. The equivalent region of mammalian calnexin has been shown to possess molecular chaperone activity. It is possible that the lethal phenotype is caused by the absence of this chaperone activity in the *S.pombe cnx1*$^{+}$ gene disruption.

Key words: calnexin/ER/essential gene/molecular chaperone/ *Schizosaccharomyces pombe*

Introduction

In eukaryotes, the endoplasmic reticulum (ER) is the site of the folding of secretory proteins and the assembly of multimeric cell surface receptors. These processes are mediated by molecular chaperones (reviewed in Bergeron *et al.*, 1994). Some of these molecular chaperones have been identified and appear to be present in a wide variety of eukaryotic species. One such molecular chaperone, the ER membrane protein calnexin, interacts with secretory glycoproteins soon after they enter the ER (Ou *et al.*, 1993). Newly synthesized glycoproteins specifically associate with calnexin while they are monomeric, incompletely folded and their oligosaccharide modification is the $GlcNAc_2$ Man_9 Glc_1 intermediate (Ou *et al.*, 1993; Hammond *et al.*, 1994; Le *et al.*, 1994). Recently, it has been demonstrated that the $GlcNAc_2$ Man_9 Glc_1 oligosaccharide interacts directly with calnexin (Ware *et al.*, 1995). In addition, tunicamycin (Ou *et al.*, 1993) and the glucosidases I and II inhibitors, deoxynojirimycin and castanospermine, (Hammond *et al.*, 1994) inhibit the association of incompletely folded glycoproteins with calnexin, supporting the observation that the $GlcNAc_2Man_9$ Glc_1 intermediate is important for calnexin recognition.

Calnexin is associated with proteins while they are being folded by the action of other ER molecular chaperones and chaperonins. The time of association of a secretory protein with calnexin reflects the time required for its folding (Ou *et al.*, 1993). Secretory proteins that are not assembled correctly into complexes or do not fold correctly due to mutations or incorporation of amino acid analogues, are retained by calnexin in the ER (reviewed in Bergeron *et al.*, 1994). Thus calnexin also has a function as a constituent of an ER quality control apparatus.

In common with other molecular chaperones, calnexin genes have been identified in a wide variety of eukaryotes including mammals, nematodes and plants (Wada *et al.*, 1991; Sulston *et al.*, 1992; Huang *et al.*, 1993). They share sequence motifs with the ER lumenal protein calreticulin, which has similarly been found in a number of eukaryotes. Together they appear to form a gene family with a possible similarity in function (Smith and Koch, 1989; Wada *et al.*, 1991). How these proteins perform their function is a topic of considerable interest.

We have recently cloned the *CNE1* gene from *Saccharomyces cerevisiae* which shares sequence similarity with mammalian calnexin and calreticulin. The *CNE1* gene codes for an integral membrane ER glycoprotein, Cne1p. Unlike mammalian calnexin, Cne1p does not bind calcium in an *in vitro* assay, and it does not have a cytosolically directed domain at the carboxy terminus (Parlati *et al.*, 1995). We demonstrated that *CNE1* does have an effect on the retention of mutant proteins in the ER (Parlati *et al.*, 1995) but it is not essential for the viability of *S.cerevisiae*. Thus, not all of its properties correspond with those expected of a bona fide *S.cerevisiae* calnexin homologue. However, the *Schizosaccharomyces pombe cnx1*$^{+}$ gene which we characterized has several properties in common with mammalian calnexin and is most likely the *S.pombe* calnexin homologue.

Results

Cloning of an S.pombe member of the calnexin/calreticulin family, cnx1^{+}

We have previously used sequence motifs that are shared between mammalian calnexin and the ER lumenal protein calreticulin to identify a calnexin related gene in *S.cere-*

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visiae, *CNE1* (Parlati *et al.*, 1995). We used a similar strategy for cloning of an *S.pombe* member of the calnexin/calreticulin family. Degenerate oligonucleotides coding for the regions of amino acid similarity conserved between mammalian calnexin and calreticulin, YKG$^{K}/_{E}$WKP and the repeat motif KPEDWDE (Figure 1A) were used to prime a PCR using *S.pombe* genomic DNA as a template (Figure 1B). From the organization of these motifs in calnexin and calreticulin, an amplified fragment of 350 bp was expected (Figure 1A) and double-stranded (ds) DNA products of approximately this size were cloned into the plasmid pTZ19R. The DNA sequence of one of these clones and its derived amino acid sequence revealed that this PCR product shared high amino acid sequence similarity with both mammalian calnexin and calreticulin. Using this fragment as a probe, the entire gene was cloned and sequenced (Figure 1C, see Materials and methods). The *cnx1*$^+$ genomic sequence identified has consensus heat shock elements at its 5′ end (Figure 1C). The coding sequence does not contain introns and predicts a type I integral membrane protein of 560 amino acids that has a similar overall arrangement to mammalian calnexin. There is a predicted N-terminal cleavable signal sequence, an *N*-glycosylation motif at residue 418, and a membrane-spanning domain proximal to a cytoplasmic domain (Argos *et al.*, 1982; Kyte and Doolittle, 1982; von Heijne, 1986) (Figure 2). *S.pombe cnx1*$^+$ encodes four repeats related to the motif KPEDWDE, which in calnexin have been suggested to be high-affinity, low-capacity calcium binding sites (Tjoelker *et al.*, 1994). These amino acid repeats are also present in *Arabidopsis thaliana*, in mammalian calnexins and in mammalian calreticulins. Overall, *S.pombe cnx1*$^+$ is 38% identical to *A.thaliana* calnexin and 34% identical to canine calnexin and 25% identical to mouse calreticulin, but only 22% identical to *S.cerevisiae CNE1*. Thus, the *S.pombe cnx1*$^+$ gene is most likely to be a calnexin homologue since it has higher amino acid identity with calnexin than calreticulin. Additionally, it encodes a C-terminal domain after a predicted transmembrane domain, that is present only in calnexin genes. We have looked for other calnexin and calreticulin homologues in *S.pombe*. Hybridization at low stringency of a *cnx1*$^+$ probe with the entire genome, presented in the form of bacteriophage P1 clones, did not identify any other related sequences. We determined that the *cnx1*$^+$ gene maps to a region on chromosome I, between probes *57b12* and *20h4* and its location is P1 phage clone *3G10p* (data not shown) (Hoheisel *et al.*, 1993). We tentatively conclude that *cnx1*$^+$ is the only *S.pombe* calnexin gene.

The *cnx1*⁺ transcript is inducible by heat shock and a calcium ionophore

In eukaryotes, the consensus sequence for the heat shock element is characterized by three or more repeats of the sequence nGAAn in an alternating orientation (Pelham and Bienz, 1982; Amin *et al.*, 1988). Inspection of the upstream sequences of *cnx1*$^+$ identified two heat shock elements (see Figure 1C), which are in the correct position to act as elements controlling transcription of *cnx1*$^+$. To test this, exponentially growing *S.pombe* cells were subjected to a transient heat shock at 39°C and the RNA transcripts were analysed by Northern blots. The *cnx1*$^+$ transcript was found to be induced ~1.6-fold by a transient heat shock (Figure 3A and B). If the heat shock treatment was carried out for an extended time, the level of transcription diminished to the basal level (Figure 3A and B). Other agents that are known to cause stress were also tested for their effect on *cnx1*$^+$ transcription. Treatment with the glycosylation inhibitor tunicamycin (Figure 3C and D) or with 2-deoxyglucose (data not shown) had no effect, but treatment with the calcium ionophore A23187 gave a marked increase in *cnx1*$^+$ transcription (Figure 3C and D).

The *cnx1*⁺ gene product, Cnx1, is a calcium-binding protein

Calnexin in mammalian cells was originally described as one of the two major calcium-binding proteins of the ER membrane (Wada *et al.*, 1991). All calnexins and calreticulins tested thus far bind calcium, except for *S.cerevisiae* Cne1p (Parlati *et al.*, 1995). In order to determine if Cnx1 is a calcium-binding protein, we used a GST fusion of Cnx1 expressed in *Escherichia coli*. This fusion comprised amino acids 23–492 of Cnx1, that is, it excludes the predicted signal sequence and the C-terminal transmembrane and cytoplasmic domains of Cnx1. Tested in a calcium overlay assay, the GST::Cnx1 fusion protein bound calcium (Figure 4, lane 2) but as expected, GST alone, phosphorylase B and GST::Cne1p (*S.cerevisiae*) (Figure 4, lanes 1, 6 and 7 respectively) did not bind calcium in the same assay. The positive controls, calmodulin (Figure 4, lane 4), parvalbumin (Figure 4, lane 5) and two bands corresponding to mammalian calnexin and pgp 35 (Figure 4, lane 3) obtained from Triton X-114-extracted canine pancreatic stripped rough microsomes, bound calcium. Thus, like mammalian calnexin and calreticulin, *S.pombe* Cnx1 is a calcium-binding protein.

Cnx1 is an integral membrane glycoprotein

Polyclonal antibodies were raised to the *E.coli*-expressed GST::Cnx1 fusion protein. These antibodies recognize a protein in *S.pombe* of 91 kDa that is associated with a membrane fraction. To determine if Cnx1 is an integral membrane protein, *S.pombe* membrane preparations were treated with a regimen of sodium carbonate at pH 11.5 or 0.5 M NaCl or 2.5 M urea which did not release it from the membranes; and with 0.1% SDS and 1% Triton X-100 that led to solubilization of the 91 kDa protein (Figure 5A). Thus, the properties of *S.pombe* Cnx1 correspond to those expected of an integral membrane protein. Membrane association is expected for a calnexin homologue but not a calreticulin homologue which is a soluble ER lumenal protein.

The apparent molecular mass of 91 kDa for *S.pombe* Cnx1 is higher than predicted from the Cnx1 sequence. To confirm that the predicted site of *N*-glycosylation at residue 418 is used (see Figure 2B), *S.pombe* membrane preparations were digested with endoglycosidase H (endo-H). Immunoblotting with antibodies to Cnx1 protein revealed an increase in mobility on SDS–PAGE to ~88 kDa (Figure 5B). This mobility change corresponds to that expected if a single potential site of glycosylation (~3 kDa for each site) is modified by the addition of core sugar residues (Herscovics and Orlean, 1993). From the primary sequence, the predicted molecular mass of the non-glycosylated protein is 63 kDa (Figure 1C). This

A

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                 254
CALNEXIN         ...SREIEDPEDQKPEDWDERPKIPDPDAVKPDDWNEDAPAKIPDEEATKPDGWLDDEPEYVPDPDAEKP
CALRETICULIN     FLPPKKIKDPDAAKPEDWDERAKIDDPTDSKPEDWDK....................PEHIPDPDAKKP
                 185                                  219                 220

                                                                                    389
CALNEXIN         EDWDEDMDGEWEAPQIANPKCESAPGCGVWQRPMIDNPNYKGKWKPPMIDNPNYQGIWKPRKIPNPDFF
CALRETICULIN     EDWDEEMDGEWEPP..................VIQNPEYKGEWKPRQIDNPDYKGTWIHPEIDNPE..
                            247                 248                                 281
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B

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        PCR S                                PCR A

  K  P  E  D  W  D  E                  Y  K  G  K  W  K  P
                                                E

5· AARCCNGARGAYTGGGAYGA 3·         3· ATRTTYCCNYTYACCTTYGG 5·
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C

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                                   GtTTGATACCTGACAGTTGTTATGCGTCAGACTAAAGCATATTCTAATGAT  -451
 ***  ***  **          ***
TGAAGATTCAAAGACTACGAGTATATAggCGCTTTCAAGGCTTTATGACTAGTGATAGTTTGTAAACTTTTATTTTATCTCATTGTTTGCAT  -361
                          ***
TACTCTTTTTTTTTACTTAATGGGGnGAACAATTAAAGCCAAATACTTAGTATATTAAAATGCAAACGTTTACTGTAAACATGTTACTGC  -271
  ***          ***       ***       ***
AACCTTCATTATAGCAAGACATTCGCTCCGTAATTCACAAAGTAAGAAGTAAAATAAATATAAAGATCTAATGCAAATACAACTTACCAG  -181
                                           *** ***  ***  ***      *** ***
ATAGGTCTTTCAAATCTAATCATCTAAGTTTAATGCGATTTGTTTTTCGTTCCGGAACCTTCTCCATTATTCTTTCGATTATGTTTGCTG  -91
                                                                                     ***
ATCCATCTCATCGATCAAAGCCGTAGTACTACCAAAACACACGCAAAACCGCGCACCGTAGTTATCAACTACACCACCCAACACGTGGAA  -1

ATGAAGTACGGAAAGGTATCTTTTCTAGCGCTTTTGTGCTCTTTATATGTTCGGGGATCACTTGCTGATCCTGAATCCGAACAAGAACCT  90
 M  K  Y  G  K  V  S  F  L  A  L  L  C  S  L  Y  V  R  G  S  L  A  D  P  E  S  E  Q  E  P  30

TTGGTTTTTAACCCAACTGAAGTGAAAGCGCCTCTTGTTGAGCAGTTTCAAGGTGCATGGTCTGAACGTTGGATTCCTTCACATGCTAAA  180
 L  V  F  N  P  T  E  V  K  A  P  L  V  E  Q  F  Q  G  A  W  S  E  R  W  I  P  S  H  A  K  60

CGATTTGTAAATGGTATTGAAGAAATGAGCTATGTAGGTGAATGGACTGTTGAGGAAAGTTCTGGTCCCGGTGCCCTTAAGGGTGAAGCC  270
 R  F  V  N  G  I  E  E  M  S  Y  V  G  E  W  T  V  E  E  S  S  G  P  G  A  L  K  G  E  A  90

GGTCTCGTTATGAAAGACGAAGCCGCTCATCATGCTATTTCTTATGAATTTGATGAACCCATAAATGAACCAGAAAAGGATTTGGTAGTT  360
 G  L  V  M  K  D  E  A  A  H  H  A  I  S  Y  E  F  D  E  P  I  N  E  P  E  K  D  L  V  V  120

CAATATGAGGTTAACCCCGAAGAGGGTCTGAACTGTGGTGGTGCATATTTAAAGCTCTTAGCAGAACCCACTCATGGTGAAATGTCAAAC  450
 Q  Y  E  V  N  P  E  E  G  L  N  C  G  G  A  Y  L  K  L  L  A  E  P  T  H  G  E  M  S  N  150

TCCATCGATTACAGAATCATGTTTGGACCCGACAAATGTGGTGTTAATGACCGTGTTCATTTTATCTTCAAGCATAAGAATCCCCTTACG  540
 S  I  D  Y  R  I  M  F  G  P  D  K  C  G  V  N  D  R  V  H  F  I  F  K  H  K  N  P  L  T  180

GGTGAGTATTCCGAGAAGCATCTCGACTCTAGACCCGCCAGCCTTTTGAAACCTGGTATTACAAACCTTTACACTTTAATTGTGAAGCCT  630
 G  E  Y  S  E  K  H  L  D  S  R  P  A  S  L  L  K  P  G  I  T  N  L  Y  T  L  I  V  K  P  210

GACCAAACCTTCGAAGTCCGTATTAATGGCGATGTTGTCCGTCAAGGATCTTTGTTTTATGATTTTATACCTCCTGTTCTTCCTCCCGTT  720
 D  Q  T  F  E  V  R  I  N  G  D  V  V  R  Q  G  S  L  F  Y  D  F  I  P  P  V  L  P  P  V  240

GAAATTTACGATCCCGAAGATATCAAGCCTGCTGATTGGGTTGACGAACCTGAAATTCCTGACCCCAATGCGGTTAAGCCTGATGATTGG  810
 E  I  Y  D  P  E  D  I  K  P  A  D  W  V  D  E  P  E  I  P  D  P  N  A  V  K  P  D  D  W  270

GATGAAGACGCTCCCCGTATGATTCCTGATCCTGACGCAGTGAAACCTGAGGATTGGTTAGAGGATGAACCTCTTTACATTCCTGATCCT  900
 D  E  D  A  P  R  M  I  P  D  P  D  A  V  K  P  E  D  W  L  E  D  E  P  L  Y  I  P  D  P  300

GAAGCTCAGAAGCCTGAGGATTGGGATGATGAAGAAGATGGTGATTGGATTCCAAGTGAAATCATCAATCCTAAATGCATTGAAGGTGCT  990
 E  A  Q  K  P  E  D  W  D  D  E  E  D  G  D  W  I  P  S  E  I  I  N  P  K  C  I  E  G  A  330

GGCTGTGGTGAATGGAAGCCACCGATGATTCGTAACCCTAACTATCGTGGCCCTTGGTCTCCTCCTATGATCCCTAATCCTGAATTCATT  1080
 G  C  G  E  W  K  P  P  M  I  R  N  P  N  Y  R  G  P  W  S  P  P  M  I  P  N  P  E  F  I  360

GGTGAATGGTATCCTCGTAAAATTCCTAACCCTGACTATTTTGATGATGATCATCCTTCTCATTTCGGTCCACTCTACGGTGTTGGTTTC  1170
 G  E  W  Y  P  R  K  I  P  N  P  D  Y  F  D  D  D  H  P  S  H  F  G  P  L  Y  G  V  G  F  390

GAACTTTGGACTATGCAGCCCAACATCCGCTTTAGCAATATCTATGTCGGCCATTCAATTGAGGACGCTGAACGTCTTGGAAATGAAACT  1260
 E  L  W  T  M  Q  P  N  I  R  F  S  N  I  Y  V  G  H  S  I  E  D  A  E  R  L  G  N  E  T  420
                                                                                  CHO
TTCCTACCTAAATTGAAAGCTGAAAGAGAATTACTTTCTAAGCAAGAGTCCATGGAAAAGCAATCAATGCATGTTGACGAAGAGTCTAAT  1350
 F  L  P  K  L  K  A  E  R  E  L  L  S  K  Q  E  S  M  E  K  Q  S  M  H  V  D  E  E  S  N  450

CAAATTCTTGAGAAGTTTCTTGACGTATATGATATTATTAAAGCTAAACTTCCTCCAAATGTTGCCGAAAAAGTTGACTACTATGTTGAA  1440
 Q  I  L  E  K  F  L  D  V  Y  D  I  I  K  A  K  L  P  P  N  V  A  E  K  V  D  Y  Y  V  E  480

ACCATCATCGAGACTCCTGAAATTGGGATTGCAATTGTTGCCGTTCTTGGCTCTTTAACCGCTGTTATTCTTACTTGTTACTTTTATTTC  1530
 T  I  I  E  T  P  E  I  G  I  A  I  V  A  V  L  G  S  L  T  A  V  I  L  T  C  Y  F  Y  F  510

TTTGCTTCCTCTTCCCCGGCTTCTTTATCTACTGGAACTACGGAAGCTGAGAAAGAGCAGCAAGAGAAGTTTAAGCAAGAGACTGAGACT  1620
 F  A  S  S  S  P  A  S  L  S  T  G  T  T  E  A  E  K  E  Q  Q  E  K  F  K  Q  E  T  E  T  540

GAGAAGATAGACGTTTCTTACGCTCCCGAAACTGAATCACCAACTGCGAAGAATGAAGACTAATGAAACTGCTAAAATGTCAAAAGTTTT  1720
 E  K  I  D  V  S  Y  A  P  E  T  E  S  P  T  A  K  N  E  D                                560

TCTTATTTTAAGAATCGCATAGAAGTTGCAATAAACTACTTCCTGTTCCTTAAATGAATATATCAATATATATACTCACTAATACAGCAT  1810
ATTTTGTTTACAAAGTTTGATTGTGGACTTTTGACTGAAGCTCAAAGTAGCGACTCTGTTAAAGCCTTA                       1889
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Fig. 1. DNA sequence *cnx1*$^{+}$ gene and deduced amino acid sequence. (**A**) The central domains of dog calnexin and mouse calreticulin are aligned between amino acids 254–389 and 185–281 respectively (Wada *et al.*, 1991; Smith and Koch, 1989). Amino acid sequences used to design forward and reverse oligonucleotide primers are indicated in bold type. (**B**) Amino acid sequences and corresponding nucleotide sequences (PCR S, sense; PCR A, antisense) used as primers for PCR amplification of yeast (Y= C or T; R= A or G; N= A, C, G, T). (**C**) *S.pombe cnx1*$^{+}$ gene codes for a 560 amino acid protein. The predicted protein contains a signal sequence at the amino terminus (italics), a single *N*-glycosylation site at N418 (CHO), a transmembrane domain (bold type) and a 48 amino acid predicted cytosolically oriented sequence. Putative heat shock elements are indicated (underlined). Nucleotides matching the heat shock element (HSE) consensus sequences are marked by asterisks. Sequences encoding partial HSE elements are also denoted by asterisks, but are not underlined. A putative TATA box is shaded. The sequence is available as GenBank #M98799.

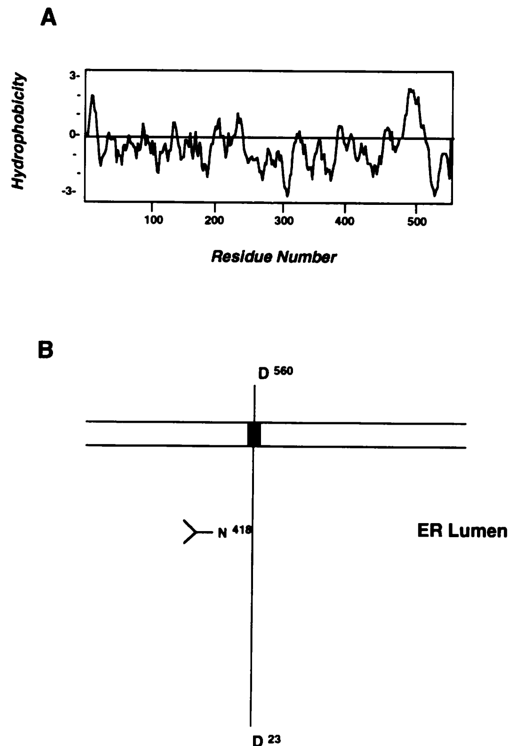

Fig. 2. Hydrophobicity plot and topology of Cnx1. (**A**) Hydrophobicity plot. (**B**) Predicted topology of Cnx1 showing the single predicted *N*-linked glycosylation site, the single transmembrane domain proximal to the carboxyl terminus and the cytoplasmic tail. The predicted signal sequence cleavage is at residue aspartate 23 (D 23).

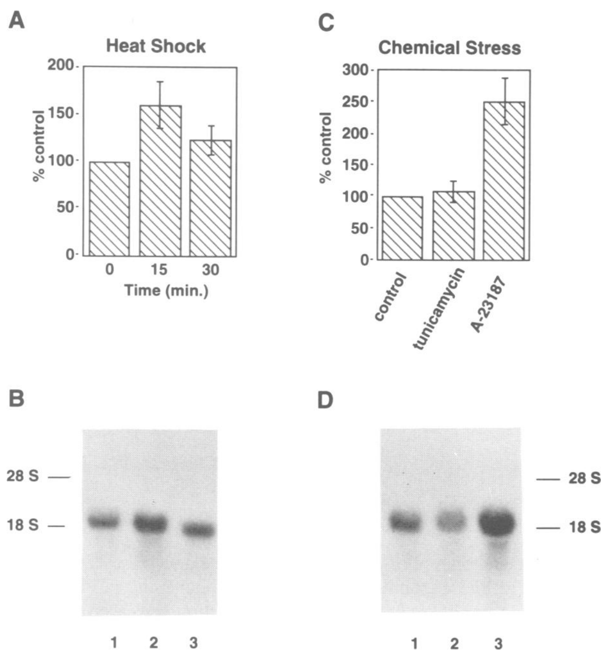

Fig. 3. Induction of *cnx1*$^+$ *by* heat shock and calcium ionophore A23187. Northern blot analysis of total *S.pombe* RNA using a probe from the complete ORF of the *cnx1*$^+$ gene *S.pombe* strain Q359 was grown at 23°C and shifted to 39°C for 0 min. (**A** and **B**, lane 1); 15 min (A and B, lane 2); and 30 min (A and B, lane 3) or grown at 30°C (**C** and **D**, lane 1) or treated with tunicamycin (C and D, lane 2) or the calcium ionophore, A23187 (C and D, lane 3). The results of three independent experiments evaluated by densitometry are illustrated in panels A and C (±SD). Equal amounts of RNA were applied to each lane and gels were stained with ethidium bromide in order to verify the quantities of 28S and 18S RNA.

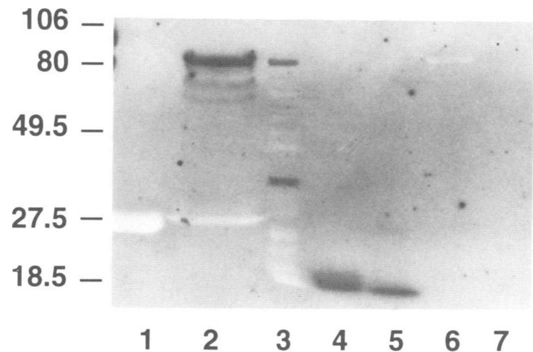

Fig. 4. Identification of *S.pombe* Cnx1 protein as a calcium binding protein. Lane 1, GST alone (20 μg protein); lane 2, GST::Cnx1 (*S.pombe)* fusion (20 μg protein); lane 3, Triton X-114 extracted canine pancreas ER (100 μg protein) showing two polypeptides of 90 kDa (calnexin) and 35 kDa (pgp 35) known to bind Ca^{2+} (Wada *et al.* 1991); lane 4, calmodulin (2 μg protein); lane 5, parvalbumin (10 μg protein); lane 6, phosphorylase B (10 μg protein); lane 7, GST::Cne1p (*S.cerevisiae*) fusion protein (20 μg protein) were electrophoresed on a 10% SDS–PAGE, transferred to nitrocellulose membrane and ^{45}Ca binding performed as described previously (Wada *et al.*, 1991). Molecular mass markers are indicated on the left.

anomalous migration on SDS–PAGE is also observed for the non-glycosylated canine calnexin that has a predicted molecular mass of 67 kDa but a mobility on SDS–PAGE corresponding to 90 kDa (Wada *et al.*, 1991). As with mammalian calnexin, the low *p*I of Cnx1 (calculated as *p*I 4.13) is likely to be responsible for this discrepancy.

The cnx1$^+$ *gene is essential*

Gene disruption was performed in order to determine if *cnx1*$^+$ is an essential gene in *S.pombe*. Approximately 80% of the coding sequence was replaced with the *S.pombe ura4*$^+$ gene. The sequences flanking *cnx1*$^+$, that is, 500 bp on the 5′ of the ORF and 1.0 kb on the 3′ side were retained in order to promote a good frequency of homologous recombination (Figure 6A). A diploid *S.pombe* strain (see Materials and methods) was transformed with this linear construct and uracil prototrophs were selected. The DNA of some transformants was analysed by PCR and Southern blots to confirm that the recombination had occurred at the *cnx1*$^+$ locus (see below). Diploid transformants heterozygous for the *cnx1*$^+$ gene disruption grew normally, were sporulated and 17 tetrads were dissected. Each tetrad gave the same segregation of two viable spores that grew to form visible colonies and two apparently non-viable spores (Figure 6C). All the spores that grew were uracil auxotrophs. Thus the *cnx1*$^+$ gene in *S.pombe* is essential for viability.

Southern blot analysis of the DNA from the parental diploid strain, the *ura4*$^+$ heterozygous diploid and ura$^-$ spores were probed with the following probes: (i) the entire *ura4*$^+$ gene; (ii) a probe corresponding to nucleotides –490 to –1, 5′ to the *cnx1*$^+$ ORF; and (iii) the *NsiI*/*Eco*RV fragment of *cnx1*$^+$ (see Figure 6A). These probes were used to distinguish between the wild-type and the recombinant allele. DNA from the parental diploid strain (Figure 6B, lanes 1, 5, 9 and 13), a *ura4*$^+$ diploid transformant (Figure 6B, lanes 2, 6, 10 and 14), and ura$^-$ spores (Figure 6B, lanes 3, 4, 7, 8, 11, 12, 15 and 16) were digested with *Eco*RI (Figure 6B, lanes 1–4 and 9–12) or *Eco*RV (Figure 6B, lanes 5–8 and 13–16). Hybridization with the probe

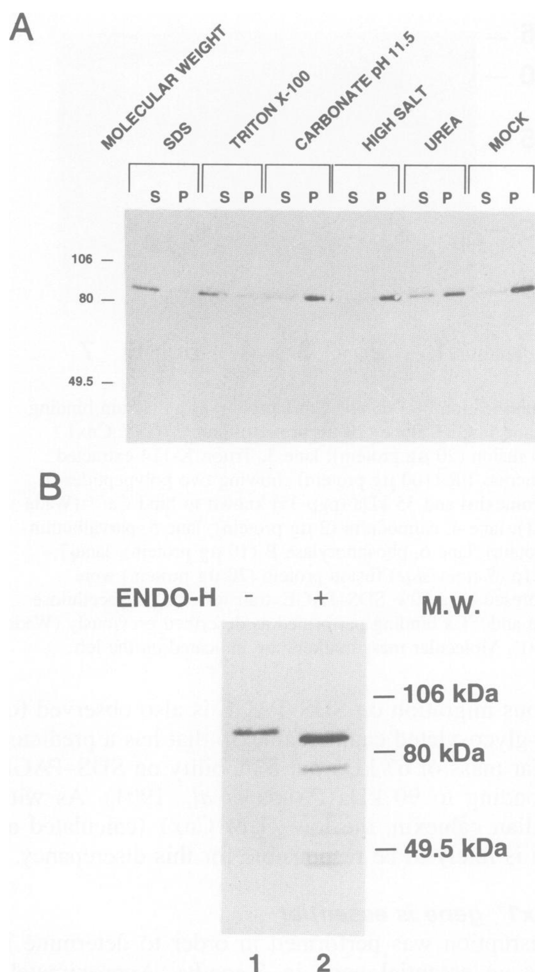

Fig. 5. Cnx1 is an integral membrane glycoprotein. (**A**) Membranes were prepared and extracted with 0.1% SDS, 1% Triton X-100, 0.1 M sodium carbonate, pH 11.5, 0.5 M NaCl (high salt), 2.5 M urea or Tris-buffered saline, pH 7.5 (mock), followed by centrifugation (30 min × 100 000 *g*) to give pellet (P) and supernatant (S) fractions that were analysed by immunoblotting with anti-Cnx1 antisera. Molecular mass markers are indicated on the left. (**B**) A total particulate fraction of homogenized spheroplasts was digested with endo-H. Analysis by immunoblotting revealed a change in mobility for Cnx1 from 91 kDa to 88 kDa. After endo-H treatment, a minor band of 74 kDa is found, most likely originating from the light 78 kDa band in lane 1. The significance of this band is unknown, although we speculate that it may represent a minor Cnx1 degradation product. Molecular mass markers are indicated on the right.

of the entire *ura4*$^+$ gene resulted in a 4.7 kb band when restricted with *Eco*RI (lane 2) and two bands of 2.3 kb and 6.0 kb when restricted with *Eco*RV (lane 6) in the heterozygous diploid. These bands were not present in the parental or ura$^-$ spores. Hybridization with the probe derived from a PCR fragment 5′ to the *cnx1*$^+$ gene (nucleotides –490 to –1) revealed a 2.9 kb band for the wild-type allele and 4.7 kb band present only in the heterozygous diploid (lane 10). Hybridization with the probe encompassing the *NsiI/Eco*RV fragment of *cnx1*$^+$ resulted in a 1.7 kb band corresponding to the wild-type allele and a 2.3 kb band only present in the heterozygous diploid (lane 14) corresponding to *cnx1*$^+$ disrupted allele. Thus we have confirmed that the *cnx1*$^+$ gene is disrupted by homologous recombination to yield a *cnx1*$^+$*/cnx1::ura4* heterozygous diploid. The spores that grew were all ura4$^-$ and contained the wild-type *cnx1*$^+$ allele. We

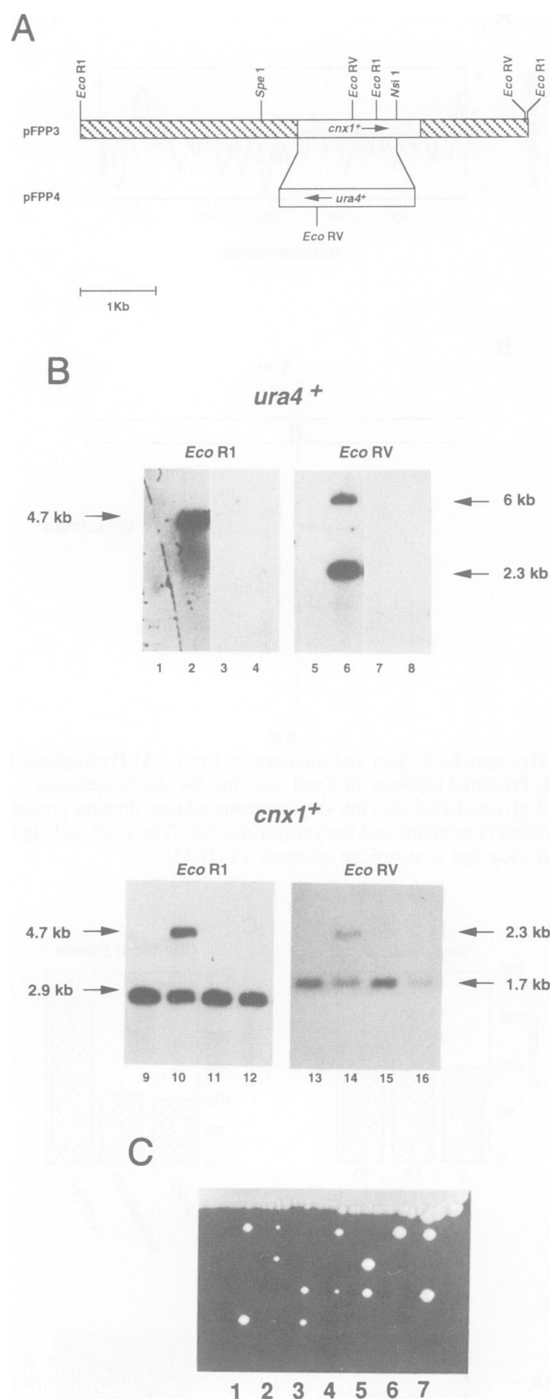

Fig. 6. Gene disruption of *cnx1*$^+$. (**A**) Schematic representation of plasmid pFPP3 containing the entire *cnx1*$^+$ gene and pFPP4 containing *cnx1::ura4*. The indicated restriction sites were used to evaluate by Southern blot analysis the presence or absence of *cnx1*$^+$ in the parental and heterozygous diploids, and in the ura$^-$ spores. (**B**) Analysis of *cnx1*$^+$ disruption by Southern blot. DNA from the parental diploid strain (lanes 1, 5, 9 and 13), heterozygous diploid strain (lanes 2, 6, 10 and 14) or ura$^-$ spores (lanes 3, 4, 7, 8, 11, 12, 15 and 16) was restricted with either *Eco*RI (lanes 1–4 and 9–12) or *Eco*RV (lanes 5–8 and 13–16) and probed with: (i) the entire *ura4*$^+$ gene (lanes 1–8); (ii) PCR fragment from nucleotides –490 to –1, 5′ of the *cnx1*$^+$ ORF (lanes 9–12); (iii) *NsiI/Eco*RV fragment of *cnx1*$^+$ (lanes 13–16). The ura$^-$ spores are from the two tetrads, i.e. lanes 3, 7, 11 and 15 from spore 1, and lanes 4, 8, 12 and 16 from spore 2. (**C**) Results of the tetrad analysis showing two viable and two non-viable spores for each tetrad.

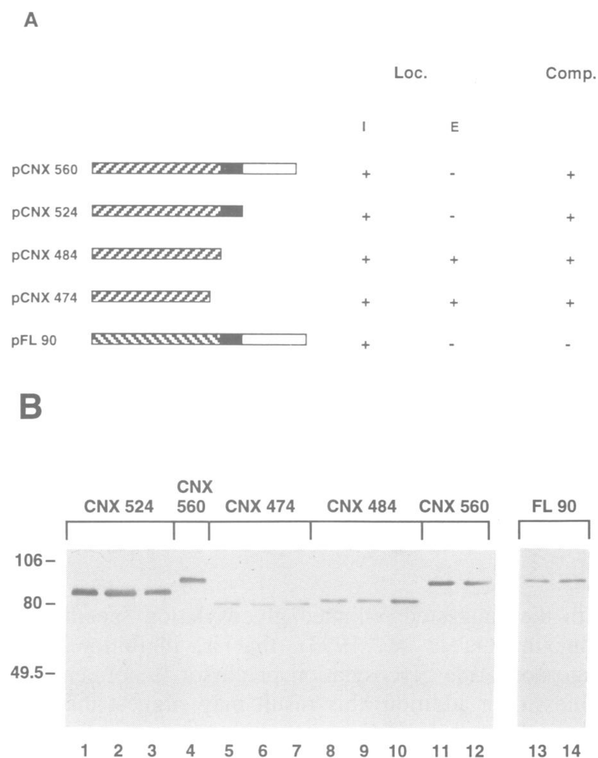

Fig. 7. Complementation of the *cnx1::ura4* disruption in haploid cells. (**A**) Constructs used to complement the *cnx1::ura4* disruption are depicted with lumenal (dashed), transmembrane (filled) and cytosolic tails (open) rectangles. Their ability to complement (Comp) the *cnx1::ura4* disruption as well as their intracellular (I) and extracellular (E) location (Loc) is noted. pCNX560 encodes the entire Cnx1 protein; pCNX 524, pCNX 484 and pCNX 474 encodes Cnx1 truncated at amino acid 524, 484 and 474 respectively. pFL90 encodes the full-length canine calnexin gene. (**B**) Immunoblot detection of Cnx1 and canine calnexin in *S.pombe* cells. Protein (10 μg) from a membrane fraction for three different *cnx1::ura4* haploids complemented with either pCNX 560 (lanes 4, 11 and 12), pCNX 524 (lanes 1, 2 and 3), pCNX 484 (lanes 8, 9 and 10) and pCNX 474 (lanes 5, 6 and 7) were electrophoresed on an 8% SDS–PAGE, transferred to nitrocellulose and blotted with anti-Cnx1 antisera. Canine calnexin was detected in a membrane fraction of diploid cells heterozygous for the *cnx1* deletion transformed with pFL90 (lanes 13 and 14). Membrane proteins (10 μg) were treated as above (lanes 1–12), but immunoblotted with anti-canine calnexin antisera. Molecular mass markers (kDa) are indicated on the left.

conclude that the *cnx1*$^{+}$ gene is essential for viability. Upon microscopic examination of the apparently non-viable spores we observed that they did germinate and divided to produce microcolonies of 20–50 cells. Therefore, *cnx1*$^{+}$ is not essential for spore germination but the results suggest that after several cell divisions, Cnx1 becomes diluted and cells eventually stop dividing.

Complementation of the cnx1::ura4 gene disruption in haploid cells

Since the *cnx1::ura4* gene disruption is lethal in haploids, we could determine which region of the molecule was essential for growth. The *cnx1::ura4* gene disruption strain was transformed with a series of deletion plasmids lacking fragments of the *cnx1*$^{+}$ C-terminus. Their ability to complement the lethal *cnx1::ura4* gene disruption, as well as the location of the Cnx1 protein was determined (Figure 7A). Plasmid pCNX 560, expressing full-length Cnx1, complemented the lethal phenotype of the *cnx1::ura4* gene disruption in haploid cells. The full-length Cnx1 protein was detectable intracellularly in membrane fractions (Figure 7B, lanes 4, 11 and 12). Plasmid pCNX 524 expressed Cnx1 lacking the cytosolic tail and produced a protein of lower molecular weight, which was also detectable intracellularly in a membrane fraction (Figure 7B, lanes 1, 2 and 3). This truncated protein was able to complement the *cnx1::ura4* gene disruption in haploids. Plasmid pCNX 484 and pCNX 474 both expressed Cnx1 protein lacking the C-terminal cytosolic domain and the putative transmembrane domain. Remarkably they both complemented the lethal phenotype of the gene disruption. For these constructs, less Cnx1 protein was detectable intracellularly (Figure 7B, lanes 5–10) and the truncated Cnx1 proteins could now be detected in the medium (not shown), thus they are not retained in the ER and are secreted (Figure 7A). We also attempted to complement the gene disruption strain with the full-length canine calnexin expressed in the same plasmid. The mammalian protein (FL 90) was detected in diploid cells heterozygous for the *cnx1* disruption (Figure 7B, lanes 13 and 14). However, we could not obtain any transformants where the mammalian calnexin complemented the lethal phenotype of the *cnx1::ura4* gene disruption in haploid cells.

Discussion

It has been established that calnexin acts as a molecular chaperone that recognizes secretory glycoproteins and unassembled multimeric cell surface receptors (see Bergeron *et al.*, 1994). In mammalian cells, its function as a molecular chaperone has been largely elucidated by *in vivo* studies showing the transient association of incompletely folded secretory proteins with calnexin. The molecular mechanism for the recognition of proteins by calnexin remains uncertain. It has been suggested that multiple mechanisms including *N*-linked glycosylation and/or direct binding to peptide motifs of incompletely folded proteins are involved (Bergeron *et al.*, 1994; Ware *et al.*, 1995). It is certain that incompletely folded proteins are recognized. For example, transferrin expressed in human HepG2 cells is released from calnexin as its disulphide bonds form (Ou *et al.*, 1993). Also, it is known that calnexin can recognize the free oligosaccharide $GlcNAc_2Man_9Glc_1$ (Ware *et al.*, 1995) but that its affinity for this oligosaccharide on a polypeptide is probably higher (Arunachalam and Cresswell, 1995). Less is known about features of calnexin that are necessary for its function. Its apparent ubiquity in mammalian cells makes experimental alteration of its function difficult, so the characterization of a yeast calnexin homologue would be an advantage. We have previously cloned *CNE1* from *S.cerevisiae* by a similar strategy to that described here. We have shown that *CNE1* has sequence similarities with the calnexin/calreticulin family and shares some of the properties of calnexins but does not bind calcium or have a C-terminal cytoplasmic domain (Parlati *et al.*, 1995). The *S.pombe cnx1*$^{+}$ gene described here also has sequence similarity and properties in common with calreticulin/calnexin, but several of these characteristics argue that *S.pombe cnx1*$^{+}$ codes for a homologue of calnexin whereas the identity of *S.cerevisiae CNE1* is less certain.

Over its complete sequence, Cnx1 is 38% identical to

A

S.pombe Cnx1	249 KPADWVD	266 KPDDWDE	285 KPEDWLE	304 KPEDWDD
A.thaliana calnexin	230 KPEDWDE	247 KPEDWDE	266 KPEGWLD	285 KPEDWDD
Canine calnexin	284 KPEDWDE	301 KPDDWNE	320 KPDGWLD	339 KPEDWDE
S.cerevisiae Cne1p	255 KPHDWDD	272 KLSDRDE	291 EPPEWNS	310 KPSWWKE
Mouse calreticulin	215 KPEDWDE	232 KPEDWDK	-------	249 KPEDWDE

B

S.pombe Cnx1	132 CGGAYLKLL	156 IMFGPDKCG	324 PKCIEGAGCG
A.thaliana calnexin	108 CGGAYLKYL	136 IMFGPDKCG	305 PKCEAAPGCG
Canine calnexin	161 CGGAYVKLL	188 IMFGPDKCG	358 PKCESAPGCG
S.cerevisiae Cne1p	124 CGGAFIKLM	154 LVFGPDYCA	330 PLCTAERGCG
Mouse calreticulin	105 CGGGYVKLF	130 IMFGPDICG	----------

Fig. 8. Comparison of predicted high affinity calcium binding motifs (**A**) and cysteine motifs (**B**) in *S.pombe* Cnx1, *A.thaliana* calnexin, canine calnexin, *S.cerevisiae* Cne1p and mouse calreticulin proteins. Amino acid sequences were aligned using the GeneWorks program. The numbers denote the place in the original sequence of the first residue in the motif.

canine calnexin and 25% identical to mouse calreticulin, compared with an overall identity of Cne1p of 24% with canine calnexin and 21% with mouse calreticulin. As for all mammalian and the plant calnexins, *S.pombe* Cnx1 has four repeats related to the sequence KPEDWDE, but *S.cerevisiae* Cne1p has a single related motif KPHDWDD with less conserved variations of this motif at the other equivalent positions (Figure 8A). This observation is noteworthy since this repeat motif has been suggested by previous studies to represent the high-affinity calcium binding domain of calnexin as well as calreticulin (Michalak *et al.*, 1992; Tjoelker *et al.*, 1994). As shown in Figure 4, Cne1p does not bind calcium, whereas Cnx1 does. Mammalian calnexins and *S.pombe* Cnx1 possess four cysteine residues that are remarkably conserved amongst all calnexins including Cne1p, two of which are also conserved in calreticulin (Figure 8B).

The diagnostic difference between calnexins and calreticulins is that the former are type I ER membrane proteins, while calreticulins are ER lumenal proteins with carboxy terminal KDEL retrieval signals (Pelham, 1990; Michalak *et al.*, 1992). For *S.cerevisiae* we have shown that Cne1p is an integral membrane glycoprotein and is localized to the ER membrane (Parlati *et al.*, 1995). We have also shown that *S.pombe* Cnx1 is an integral membrane glycoprotein. Although we have not yet demonstrated its ER localization, the tight banding of the protein on SDS–PAGE is consistent with ER, but not Golgi glycosylation. Hence we expect Cnx1 to be localized to the ER. There is no obvious ER retention motif present in the Cnx1 cytosolic tail and the ER retention/retrieval motif for mammalian calnexin (an extreme carboxy-terminal RKPRRE motif), is not present in Cnx1 (Rajogopalan *et al.*, 1994).

S.pombe cnx1^{+} is inducible by heat shock and by treatment with the calcium ionophore A23187. This latter feature has not been described for mammalian calnexin (Bergeron *et al.*, 1994) although it is well known for other ER chaperones such as BiP, GRP 94 and PDI both in mammalian cells and yeasts (Normington *et al.*, 1989; Rose *et al.*, 1989; Mori *et al.*, 1992; Pidoux and Armstrong, 1992). The difference in mRNA abundance of *cnx1*$^{+}$ in response to A23187 but not tunicamycin may be consistent with the suggested *N*-linked glycosylation specificity of calnexin (Ou *et al.*, 1993), that is, inhibition of the oligosaccharide glycosylation precursor is not sensed by calnexin. In addition, this result may suggest that Cnx1 protein has a role in Ca^{2+} regulation or sequestration. A heat shock consensus sequence was also identified in the *S.cerevisiae CNE1* gene, but we were unable to find experimental conditions that altered its transcription (Parlati *et al.*, 1995).

The essential nature of the *S.pombe cnx1*$^{+}$ gene is in contrast to the non-essential nature of the *S.cerevisiae CNE1* gene (Parlati *et al.*, 1995). In a *S.cerevisiae cne1* deleted strain there was a small effect observed on the function of a temperature-sensitive mutant of the α-pheromone receptor (*ste2-3*) at the non-permissive temperature. Furthermore, an increase in the secretion of heterologously expressed mammalian α_1-antitrypsin was observed (Parlati *et al.*, 1995). To explain these small effects we speculated that there may be other systems for protein folding in the ER of *S.cerevisiae*. Despite a search by low stringency hybridization in both the *S.cerevisiae* and *S.pombe* genomes, we have not been able to demonstrate the presence of related calnexins or calreticulins (Parlati *et al.*, 1995).

Complementation of the lethal *cnx1::ura4* disruption strain has shown that the C-terminal part of the molecule is not essential for the viability. Indeed deletion of the transmembrane as well as the predicted cytosolic domain led to a soluble truncated form of the Cnx1 protein that was secreted but still complemented. We were able, however, to detect truncated Cnx1 protein intracellularly. Our previous studies have pointed to the lumenal domain of calnexin as important for its function as a molecular chaperone in mammalian cells (Ou *et al.*, 1993), and we speculate that this function is carried out by the ER lumenal soluble Cnx1 protein while it is in the ER. A similar result has also been observed in *S.cerevisiae* for the ER lumenal molecular chaperone BiP. When the carboxy-terminal tetrapeptide ER retrieval motif, HDEL, is deleted from the essential gene BiP, the protein is secreted. However, a small amount of BiP remains intracellular and the cells are viable (Hardwick *et al.*, 1990). By analogy, we propose that sufficient levels of

truncated soluble Cnx1 remain in the ER in order to perform the essential function. We are unable to prove definitively that the lethal phenotype of the *cnx1::ura4* disruption is due to the lack of molecular chaperone function. However, studies with the lumenal domain of the mammalian calnexin have shown that it can interact with secretory glycoproteins (W.-J.Ou *et al.*, unpublished results). Thus, it is likely that this domain has molecular chaperone properties but it may also have other functions.

The lack of complementation of the *cnx1::ura4* disruptant by mammalian (canine) calnexin is surprising. We have shown that the protein is made in *S.pombe* and is membrane-localized. The ER lumenal region of $cnx1^+$ (amino acids 1–474) that can complement the *cnx1:: ura4* disruption has 50% sequence similarity with the equivalent region in mammalian calnexin. Our observation that the lumenal domain of Cnx1 can still complement the disruption even though it is secreted, argues that if mammalian calnexin is not ER retained, enough would be present to perform its ER function. Although we expect that Cnx1 and mammalian calnexin can recognize the same secretory glycoprotein substrates, sequence differences between these proteins are of value in order to map the regions essential for molecular chaperone function.

Materials and methods

Strains and media

S.pombe strains Q358 (*h⁻ leu1-32 ura4-D18 ade6-M210*) and Q359 (*h⁺ leu1-32 ura4-D18 ade6-M216*) were used. The strains were grown at 30°C in YPD medium or EMM media supplemented with nutrient requirements as previously described (Moreno *et al.*, 1991). *E.coli* strain MC1061 (Maniatis *et al.*, 1982) was used.

Cloning of S.pombe cnx1⁺

To amplify the calnexin/calreticulin gene equivalent from *S.pombe*, the degenerate oligonucleotides, PCR primer **S** (5′ AARCCNGARGAYT-GGGAYGA 3′) and PCR primer **A** (3′ ATRTTYCCNYTYACCTTYGG 5′) were used to amplify genomic DNA. Amplification reactions were performed as previously described (Parlati *et al.*, 1995). The products of the PCR reaction were electrophoresed on a 2% agarose gel and stained with ethidium bromide. A band at ~350 bp was purified and cloned into the *Sma*I site of pTZ-19R (Pharmacia). The fragment was sequenced and was found to encode a peptide with high amino acid sequence similarity to canine calnexin. The PCR fragment was then ^{32}P-radiolabelled using the QuickPrime method (Pharmacia) and subsequently used as a probe to clone full-length calnexin from a *S.pombe* genomic library in plasmid pWH5 (Wright *et al.*, 1986). This gene was sequenced by standard procedures and the sequence of $cnx1^+$ was released to GenBank #M98799, December 31, 1993.

RNA extraction and transcript analysis

A 100 ml culture of strain Q359 was grown overnight at 23°C to an OD_{600} = 1 and a 25 ml aliquot was transferred to a 39°C bath for 15 or 30 min. Cells were then rapidly collected by centrifugation and frozen immediately in a dry ice–methanol bath. Overnight cultures (25 ml) grown to OD_{600} = 1 were also treated with either 1 mg/ml tunicamycin (Sigma), 10 mM A23187 (Sigma) or 10 mM 2-deoxyglucose (Sigma) for 3 h at 30°C. Cells were then harvested and quickly frozen in a dry ice–methanol bath. RNA extraction was performed by the hot phenol method (Wise, 1991) and Northern analysis was performed as previously described (Maniatis *et al.*, 1982), using a ^{32}P-probe containing the entire open reading frame of $cnx1^+$. Densitometric analyses used an LKB Ultrascan Laser densitometer.

Antibody production

Polyclonal antibodies recognizing Cnx1 were obtained by immunizing rabbits with GST::Cnx1 fusion proteins expressed in *E.coli*. This fusion protein was made by inserting a PCR fragment encoding amino acid 23–492 into pGex-2T (Smith and Johnson, 1988).

Membrane extraction and endo-H digestion

S.pombe membranes (ML fraction) were prepared and treated essentially as described (Parlati *et al.*, 1995). Membranes were mixed with 1 vol of either 1 M NaCl, 0.2 M sodium carbonate, pH 11.5, 5 M urea, 2% Triton X-100 or 0.2% SDS and were subsequently analysed as described (Feldheim *et al.*, 1992). The Cnx1 antiserum was used at a 1:4000 dilution. Endo-H digestions were performed by incubating 20 µg of ML fraction proteins in 100 mM sodium acetate, pH 4.9, 150 mM NaCl, 10 mM DTT, 1% Triton X-100, 0.1% SDS + inhibitors (1 mM PMSF, 1 mg/ml pepstatin, 1 mg/ml leupeptin and 1 mg/ml aprotinin) and incubating with 2 µg of endo-H for 16 h at 37°C.

^{45}Ca overlay

The *S.pombe* GST::Cnx1 fusion protein (residues 23–492) was expressed in *E.coli* and purified (Smith and Johnson, 1988). Samples were electrophoresed by SDS–PAGE and prepared for ^{45}Ca overlay exactly as previously described. Control experiments were carried out with canine pancreatic ER membranes obtained by extraction with Triton X-114 (Wada *et al.*, 1991), and Cne1p as previously described (Parlati *et al.*, 1995).

Gene disruption

A 4.3 kb *Pst*I DNA fragment containing the complete $cnx1^+$ gene was cloned into pTZ19R creating plasmid pFPP3. A PCR fragment containing nucleotides –490 to –1 (5′ to the coding sequence) was amplified, cut with *Spe*I and cloned into the *Spe*I/*Sma*I site of pBluescript KS+ creating plasmid pFPP3.1. The *Nsi*I/*Eco*RV fragment (3′ to the coding sequence) from pFPP3 was cut, purified and inserted into the *Eco*RV site of pFPP3.1 creating plasmid pFPP3.2. A 1.8 kb *Hin*dIII fragment containing the $ura4^+$ gene (Grimm *et al.*, 1988) was blunt-ended and inserted into the similarly treated *Eco*RI site of pFPP3.2 creating plasmid pFPP4, essentially disrupting the $cnx1^+$ gene. Plasmid pFPP4 was digested with *Xba*I, *Hin*dIII (which cleave on either side of the insert) and *Xmn*I (which cuts in the vector) and ~5 µg of the linear fragment containing the disrupted gene was isolated and purified by the GeneClean method. This fragment was used to disrupt the diploid strain *h⁻ leu1-32 ura4-D18 ade6-M210/h⁺ leu1-32 ura4-D18 ade6-M216* and uracil prototrophs were selected. Colonies were grouped into pools of 10 and chromosomal DNA was prepared (Moreno *et al.*, 1991). PCR was used to screen for homologous recombinants using a sense oligonucleotide coding for nucleotides –510 to –495 (5′ to the $cnx1^+$ ORF) and an antisense 20 bp oligonucleotide from within the $ura4^+$ gene. Among 20 pools screened, 15 were positive for the expected 550 bp PCR product. Four pools were chosen and the PCR reaction was repeated for each constituent colony. We found four colonies that were positive and chromosomal DNA was prepared from each. Southern blots were performed by digesting these DNAs with either *Eco*RI or *Eco*RV, followed by electrophoresis on 1% agarose gels and transfer to nylon membranes. The ^{32}P-labelled probes used were: (i) the entire $ura4^+$ gene hybridized to genomic *Eco*RI and *Eco*RV digests; (ii) PCR fragment from –490 to –1 hybridized to a genomic *Eco*RI digest; and (iii) *Nsi*I/*Eco*RV fragment of $cnx1^+$ hybridized to a genomic *Eco*RV digest.

Complementation of the cnx1 gene disruption

Sequences encoding full-length $cnx1^+$, and $cnx1^+$ terminated at amino acids 524, 484 and 474 were amplified using PCR (see conditions above). Oligonucleotides were designed in order to introduce a stop codon at amino acid 525, 485 and 475 respectively. These amplified sequences as well as full-length canine calnexin (Wada *et al.*, 1991) were sub-cloned into the *Sma*I site of *S.pombe* vector pREP1, under the regulation of the nmt1 promoter (Maundrell, 1993). The constructs were subsequently transformed in the diploid strain heterozygous for the $cnx1^+$ deletion, and leu^+ transformants were selected. Random spore analysis of the leu^+ strains was performed as previously described (Moreno *et al.*, 1991). Spores were plated onto phloxine B agar plates supplemented with adenine, in order to detect easily haploids that were both leu^+ (expressing the recombinant protein) and ura^+ (disrupted for $cnx1^+$). These haploids were subsequently tested for the presence of Cnx1 recombinant protein intracellularly and extracellularly by immunoblotting.

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Note added in proof

After submission of our article, Jannatipour and Rokeach published on the essential nature of the *S.pombe* calnexin homologue (*J. Biol. Chem.*, **270**, 4245–4853).