Although calnexin is essential in S. pombe, its highly conserved central domain is dispensable for viability

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SUMMARY

In mammalian cells, the calnexin/calreticulin chaperones play a key role in glycoprotein folding and its control within the endoplasmic reticulum (ER), by interacting with folding intermediates via their monoglucosylated glycans. This lectin activity has been mapped in mammalian calnexin/calreticulin chaperones to the central region, which is a highly conserved feature of calnexin/calreticulin molecules across species. The central domain has also been implicated in Ca²⁺ binding, and it has been proposed to be involved in the regulation of calcium homeostasis in the ER. Herein, we show that although the Schizosaccharomyces pombe calnexin is essential for viability, cells lacking its 317-amino-acid highly conserved central region are viable under normal growth conditions. However, the central region appears to be necessary for optimal growth under high ER-stress, suggesting that this region is important under extreme folding situations (such as DTT and temperature). The minimal length of calnexin required for viability spans the C-terminal 123 residues. Furthermore, cells with the central domain of the protein deleted were affected in their morphology at 37°C, probably due to a defect in cell wall synthesis, although these mutant cells exhibited the same calcium tolerance as wild-type cells at 30°C.

Key words: BiP, Chaperone, Protein folding, Yeast genetics, Schizosaccharomyces pombe

INTRODUCTION

Membrane-bound calnexin and its soluble homologue calreticulin define a new family of molecular chaperones that have been implicated in the folding of glycoproteins in the ER of mammalian cells, such as the cystic fibrosis transduction regulator (CFTR) and the T-cell receptor (reviewed in Bergeron et al., 1994; Williams, 1995; Trombetta and Helenius, 1998; Parodi, 1998). The luminal domain of calnexin shares extensive similarity with calreticulin. Both proteins contain a highly conserved central region comprising two series of tandemly repeated motifs. Motif 1 (I-DPD/EA-KPEDWDD/E) is involved in Ca²⁺ binding (Wada et al., 1991; Tjoelker et al., 1994). Ca²⁺ may be important for calnexin function since chelators disrupt its interaction with ligands in vitro (Bergeron et al., 1994; Williams, 1995; Trombetta and Helenius, 1998; Parodi, 1998). The luminal domain of calnexin shares extensive similarity with calreticulin. Both proteins contain a highly conserved central region comprising two series of tandemly repeated motifs. Motif 1 (I-DPD/EA-KPEDWDD/E) is involved in Ca²⁺ binding (Wada et al., 1991; Tjoelker et al., 1994). Ca²⁺ may be important for calnexin function since chelators disrupt its interaction with ligands in vitro (Bergeron et al., 1994; Williams, 1995; Trombetta and Helenius, 1998; Parodi, 1998). The minimal length of calnexin required for viability spans the C-terminal 123 residues. Furthermore, cells with the central domain of the protein deleted were affected in their morphology at 37°C, probably due to a defect in cell wall synthesis, although these mutant cells exhibited the same calcium tolerance as wild-type cells at 30°C.

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containing the Glc₁Man₀GlcNAc₂ glycan). Monoglucosylated glycoproteins arise from the consecutive action of the glucosidase I and II, or by reglucosylation of Glc₀Man₀GlcNAc₂-containing proteins by UDP-Glc:glycoprotein glucosyltransferase (GT; reviewed in Parodi, 1998). A distinctive feature of GT is that, in vitro, it can only reglucosylate unfolded proteins (Sousa and Parodi, 1995). These observations have led to one of the current models on quality control of glycoprotein folding in the ER, in which calnexin/calreticulin and GT constitute key elements (Trombetta and Helenius, 1998, and references therein). According to this model, the mammalian calnexin/calreticulin chaperones interact with monoglucosylated, newly synthesized proteins exclusively through their oligosaccharide moieties in a manner similar to lectins, without peptide contacts. Numerous publications have supported this model (reviewed in Trombetta and Helenius, 1998). However, peptide contacts also appear to be important for the interaction of calnexin with certain substrates. For instance, Ware et al. (1995) have shown that once the glycoprotein-calnexin complex is formed in vitro, the glycans can be removed without disrupting the interaction. Thus, these authors proposed a two-step model for calnexin-ligand interaction, where the first contact occurs through the Glc₁Man₀GlcNAc₂ oligosaccharide, while the second step is peptide-mediated, probably through exposed hydrophobic patches, in a mechanism akin to other chaperones such as BiP (Ware et al., 1995; Williams, 1995). Moreover, a number of
publications reported that calnexin/calreticulin can bind non-glycosylated proteins (reviewed in Williams, 1995; Jannatipour et al., 1998). Also, castanospermine and tunicamycin (a potent inhibitor of glycosylation) reduced but did not eliminate the binding of certain glycoproteins to calnexin/calreticulin (Pipe et al., 1998; Keller et al., 1998). Hence, the calnexin/calreticulin chaperones appear to have multiple modes of interaction with their ligands that may vary for different proteins. Calnexin and calreticulin are not the only chaperones involved in glycoprotein folding. The calnexin/calreticulin family was shown to cooperate with other chaperones such as BiP, PDI and Erp57, in the conformational maturation of folding intermediates (e.g. Kim and Arvan, 1995; Oliver et al., 1997; Elliott et al., 1997; Zapun et al., 1998; Hammond and Helenius, 1994; Tatu and Helenius, 1997).

Schizosaccharomyces pombe encodes the basic components involved in the quality control of glycoprotein folding found in the mammalian ER (Jannatipour and Rokeach, 1995; Parlati et al., 1995; Fernández et al., 1996; Fanchiotti et al., 1998). Therefore, this fission yeast represents an ideal model organism for the genetic analysis of the mechanisms of glycoprotein folding and its quality control. We and others have isolated cnx1+, the S. pombe calnexin homologue, and have shown that it encodes a protein essential for viability (Jannatipour and Rokeach, 1995; Parlati et al., 1995). Like its mammalian counterparts, Cnx1p is a type I ER-membrane protein containing the characteristic highly conserved central region including motifs 1 and 2, and a cytosolic tail (Jannatipour and Rokeach, 1995; Parlati et al., 1995). As could be expected, Cnx1p was shown to bind Ca2+ (Parlati et al., 1995; our unpublished results). Although a lectin activity for S. pombe calnexin has not been explored as yet, the presence of four copies each of motifs 1 and 2 may suggest that the central region might also be involved in glycan binding.

Like the mammalian GT enzyme, Gpt1p in S. pombe was shown to reglucosylate unfolded proteins in vitro, but its presence is dispensable for cell life under normal growth conditions (Fernández et al., 1994, 1996). From these observations it was possible to infer the existence in the fission yeast of a GT-independent mechanism for quality control of protein folding (Jannatipour et al., 1998). We have recently shown that Cnx1p associates with newly synthesized molecules of the glycoprotein acid phosphatase, independently of glucose trimming and reglucosylation by GT (Jannatipour and Rokeach, 1998). Thus, in spite of the essentiality of Cnx1p for fission yeast viability, the glucose trimming and reglucosylation cycle do not appear to be indispensable for protein folding in S. pombe. This notion is further supported by the fact that fission yeast cells genetically depleted of both Gpt1 and glucosidase II (Gls2p) are viable (Fanchiotti et al., 1998).

Based on the sequence conservation and the role of the central region in mammalian calnexin, it could be predicted that this domain might encode the essential function(s) for S. pombe viability. However, as described above, in the fission yeast the other key elements in the calnexin/calreticulin cycle do not seem to be necessary for viability under standard growth conditions. In this study, we wished to delimit the Cnx1p region required for cell viability and assess the relevance of the highly conserved calnexin/calreticulin central domain. Our results showed that the 317-amino-acid (aa) central region is dispensable under normal growth conditions but is required in situations of high-folding stress in the ER. Thus, overall our observations further suggest that the mechanism of glycoprotein folding in S. pombe might be different from that in mammalian cells.

MATERIALS AND METHODS

Strains and medium

The S. pombe strain SP6089 was used for all plasmid shuffling experiments (see below for construction and genotype). S. pombe strain SP556 (h+ ade6-M216 ura4-D18 leu1-32) was used as a wild-type control in various experiments. S. pombe transformations were performed by the lithium acetate procedure, and genomic DNA extraction was as previously described (Moreno et al., 1991). Strains were grown at 30°C (except where indicated) in EMM minimal medium supplemented with nutrient requirements (Moreno et al., 1991). The E. coli strain Top10, from TOPO XL PCR Cloning™ Kit (Invitrogen Co. CA), was used for cloning PCR products and the E. coli strain AP401 (lon::mini tetR ara- Δlac-pro naaΔ argE2::mif8 thi- Δ[F′ pro AB lacF Z M15]) was used for plasmid amplification and isolation.

Construction of the cnx1::his3 haploid strain SP6089

To construct a haploid S. pombe deleted of entire Cnx1p coding sequences, a plasmid containing a 4.5-kb PstI-PstI genomic fragment encompassing the cnx1 gene was linearized with Ncol and progressive bi-directional digestion was made with ExoIII. The his3 gene was ligated to create the plasmid pSPCA3282, in which the cnx1 coding sequences (955-2805, according to EMBL U13389) were deleted, and this was used in the subsequent steps to obtain cnx1Δ strains. Disruption of cnx1 in the haploid strain was obtained by simultaneous transformation of the haploid S. pombe strain 248 (h- his3-D1 ade6-M216 ura4-D18 leu1-32; Burke and Gould, 1994), with the PstI-PstI fragment from plasmid pSPCA3282 (containing the cnx1 deletion marked with his3) and the plasmid pSPCA3261 containing Cnx1p coding sequences in the vector pREP42 (see below). Transformants were selected for histidine prototrophy, and selected clones were analyzed by Southern blotting and PCR to ascertain correct disruptive integration. Strain SP6089 had the correct Δcnx1::his3 complemented by epissomal copies of cnx1 (plasmid pSPCA3261). The genotype of strain SP6089 is: h- his3-D1 ade6-M216 ura4-D18 leu1-32 Δcnx1::his3[pSPCA3261].

DNA manipulation and analysis

Procedures used for DNA manipulation and analysis (purification, electrophoresis, transformation, etc.) were as previously described (Sambrook et al., 1989). The DNA sequences of both strands of mutant cnx1-containing clones were determined by the dideoxy chain-terminating method using a T7 DNA Polymerase Kit™ (Amersham Pharmacia Biotech, Inc. NJ).

Polymerase chain reaction

Site-directed mutagenesis experiments were done by polymerase chain reaction (PCR) using the overlap extension method, as described (Higuchi and Krummel, 1988), with Pfu DNA polymerase, using the manufacturer’s conditions (Stratagene, La Jolla, CA). In oligonucleotide sequences (see below), the NdeI restriction site is underlined, the BamHI site is shown in bold characters, the corresponding nucleotide sequence of the ER retention signal ADEL is shown in italics, and stop codons are doubly underlined. Nucleotide numbers correspond to the position on cnx1 sequence (Jannatipour and Rokeach, 1995) as in the database (EMBL accession number U13389). Oligonucleotides used for the construction of each mutant (Fig. 1B) were: primers A, 5′-CCA CCC AAC AGC TGC ATA TGA AGT ACG GAA AG-3′ (1049-1080 nt) and B, 5′-CGG GAT CCT TAC CCA ATT TCA GGA GTC TCG ATG AGT-3′ (2532-2511 nt)
A

EMM+Ade+Ura. After 3-4 days at 30°C, colonies were replica-plated containing pREP42 plasmid, then cells were plated onto solid (see above) in pCR-XL-TOPO™ (Invitrogen Co. CA) following the marker) containing either full-length wild-type pREP42 (pSPCA7136) and the mutagenized bases are underlined) on wild-type site at position 2942 nt (the restriction site is in bold italic characters, and the internal 981-bp fragment, pSPCA7136 (pREP42-#16) of constructs #12 and #13. All proteins in the supernatant fraction were treated for 30 minutes at 4°C. The supernatant from this spin was centrifuged at 100,000 g for 1 hour at 4°C in the SW 50.1 ultracentrifuge, and the resulting pellet was resuspended in 2.5 ml of ice-cold lysis buffer.

Membrane extraction

S. pombe microsomal membranes (as described above) were treated for 15 minutes at 4°C by mixing with 1 vol. of either 1 M NaCl, 0.2% SDS, 0.2 M sodium carbonate, pH 11.5, or 3% Triton X-100. Membrane lysates were spun at 80,000 g for 1 hour at 4°C in an SW 50.1 ultracentrifuge, then the pellet from this spin was resuspended in 0.1 ml of 3 M NaCl, 0.2% leupeptine, 300 mg/ml phenanthroline). Lysates were prepared in a Potter-Elvejhem homogenizer and cleared at 1000 g for 15 minutes at 4°C. The supernatant was spun at 15,000 g for 15 minutes at 4°C in an SW 50.1 ultracentrifuge, and the resulting pellet was resuspended in 2.5 ml of ice-cold lysis buffer.

Spheroplast and membrane preparations

S. pombe wild-type, lumenal and lumenal+ADEL strains were grown at 30°C in 250 ml EMM+Ade+Ura containing 0.5% glucose, to an OD595 of 0.5. Deleted-cnx and mini-cnx strains were grown under same conditions, except that 11 of medium was used. For each strain, 109-1010 cells were harvested by centrifugation, washed in 1 ml of citrate buffer I (20 mM citrate phosphate, pH 5.6, 40 mM EDTA), and resuspended in 5 ml of citrate buffer II (50 mM citrate phosphate, pH 5.6, 1.2 M sorbitol). Cells were spheroplasted with 25 mg of NovoZym (Sigma Chemicals Co.) by a 45-minute incubation at 37°C. Spheroplasts were spun at 500 g for 5 minutes at 4°C, washed twice in citrate buffer II containing 1.2 M sorbitol. The pellet was resuspended in 2 ml of lysis buffer (0.1 M sorbitol, 20 mM Hepes, pH 7.5, 20 mM potassium acetate, pH 7.4) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, PMSF), 10 mM sodium acetamide: IAA), 300 µg/ml pepstatin A, 300 µg/ml leupeptine. 300 mg/ml phenanthroline). Lysates were prepared in a Potter-Elvejhem homogenizer and cleared at 1000 g by spinning for 5 minutes at 4°C. The supernatant was spun at 15,000 g for 15 minutes at 4°C in an SW 50.1 ultracentrifuge (Beckman Instruments, Fullerton, Ca). The supernatant from this spin was centrifuged at 100,000 g for 1 hour at 4°C in the SW 50.1 ultracentrifuge, and the resulting pellet was resuspended in 2.5 ml of ice-cold lysis buffer.

Cell-wall resistance to lytic enzymes

20 ml cultures of wild-type or mutant cnxl cells were grown in liquid EMM+Ade+Ura medium at 30°C to an OD595 of 0.2-0.5. Cells were harvested, washed in 10 ml of citrate buffer I (20 mM citrate phosphate, pH 5.6, 40 mM EDTA, pH 8.0), spun and resuspended in 5 ml of citrate buffer II (50 mM citrate phosphate, pH 5.6, 0.1 M sorbitol). 25 mg of NovoZym (Sigma Chemicals Co.) was added and cells were incubated at 37°C. Cell wall degradation was monitored by measuring cell density at OD595 and taking samples every 20 minutes. Cell viability was also followed at the same time, by plating an appropriate amount of cells on solid EMM+Ade+Ura.

Confocal microscopy

Confocal immunofluorescence using anti-Cnx1p and anti-BiP antibodies (diluted 1:100) was carried out essentially as previously described (Pidoux and Armstrong, 1993; Jannatipour et al., 1998). Briefly, cells were grown for 8 hours in EMM at 37°C before fixation and labeling with anti-Cnx1p and anti-BiP antibodies.

Calcofluor staining of cell wall

Exponentially growing cells were washed once with 1× PBS (130 mM NaCl, 2.5 mM KCl, 5 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4) and resuspended in 100 µl of the same buffer containing 20 mg/ml of Calcofluor white (000 g for 1 hour, then the cells were washed in 1× PBS and placed on a microscope slide and covered with a coverslip. Cells were observed under UV light or Nomarski interference.

Cells without the calnexin/calreticulin central region are viable

Plasmids

pREP41 is an S. pombe expression multicycop vector containing the LEU2 marker and the ars1 origin of replication. Expression in this plasmid is under the control of the thiamine repressible nmt41 promoter (induction ratio 25x), flanking a polylinker site (Maundrell, 1993). In the case of pREP42, the urad marker is used for selection instead of LEU2. The full-length and the various cnxl mutants were inserted in pREP41 digested with Ndel and BamHI. The plasmids constructed with pREP41 were designated as follows: pSPCA3220 (#2; full-length cnxl), pSPCA3221 (#3), pSPCA3222 (#4), pSPCA7092 (#9), pSPCA7093 (#10), pSPCA7094 (#11), pSPCA7102 (#12), pSPCA7096 (#13), pSPCA7097 (#14), pSPCA7100 (#15) and pSPCA7095 (#16). The construction containing full-length wild-type cnxl (#2) in pREP42 was designated pSPCA3261. Constructs containing inserts #17 and #18 were cloned in pREP42 and the resulting plasmids were designated pSPCA7178 and pSPCA7180, respectively. These last plasmids were constructed by replacing the Cln-BamHI fragment pSPCA7136 (pREP42-#16) and replaced with the Cln-BamHI of constructs #12 and #13. All S. pombe cultures in this study were grown in EMM medium under induced conditions (absence of thiamine).

Plasmid shuffling experiments

S. pombe SP6089 strain containing wild-type cnxl1 inserted on pREP42 (urad4 marker) was transformed with 2 µg of pREP41 (LEU2 marker) containing either full-length wild-type cnxl1 or mutant cnxl. Transformants were grown for 6 days at 30°C in 5 ml liquid EMM supplemented with adenine (Ade) and uracil (Ura) to chase the urad4 containing pREP42 plasmid, then cells were plated onto solid EMM+Ade+Ura. After 3-4 days at 20°C, colonies were replicated onto EMM+Ade+Ura and EMM+Ade-Ura. Cells which shuffled wild-type cnxl were Leu-/Ura- and contain mutant cnxl. In the case of
Immunoprecipitation and immunoblotting

Immunoblots and immunoprecipitations were carried out essentially as previously described (Jannatipour et al., 1998).

Protein sequence alignments

Protein sequence alignments were realized by using the Block Maker multi-alignment program from BCM Search Launcher (http://brc.sourceforge.net). The sequences aligned to obtain the consensus were: A. thaliana (U08135), C. elegans (Z22181), Canis familiaris (P24643), D. melanogaster (U30466), Glycine max (P22052), H. sapiens (P27824), M. musculus (P35564), Rana rana (D78590), Helianthus tuberosus (Z35108), S. cerevisiae (U12980) and S. pombe (U13389).

RESULTS

Cells deleted of the highly conserved central region of calnexin/calreticulin are viable

Disruption of the cnxl is lethal in S. pombe (Jannatipour and Rokeach, 1995; Parlati et al., 1995). In order to identify the Cnx1p sequences required for cell viability, we undertook a deletion approach. The central region is a highly conserved feature in the calnexin/calreticulin family of chaperones (see Fig. 1A). Because in mammalian cells, this segment was shown to encompass the ligand (glycan)-binding and the Ca2+ domains (Vassilakos et al., 1996, 1998; Tjoelker et al., 1994), it could be expected that since Cnx1p contains four copies of the repeated motifs 1 and 2, the deletion of this region would be lethal. To test this assumption, we constructed mutant #4 (deleted-cnx; Fig. 1B), and assessed the importance of this region by plasmid shuffling in the strain SP6089, in which genomic cnxl was deleted and replaced with his3 (see Materials and Methods). Viability in this strain SP6089 is ensured by complementation with the plasmid pSCA3261, bearing cnxl under the control of the nmt1 promoter (see Materials and Methods). The construct encoding full-length, wild-type Cnx1p (#2) was used in this experiment as a control (see Fig. 1B), and conferred viability as expected. Surprisingly, the cells harboring construct #4 were viable in the absence of a wild-type copy of cnxl. Western blot, Southern blot and PCR analyses confirmed that the viable phenotype of strain #4 was due to gene conversion between wild-type and mutant genes, nor to integration of the wild-type sequences into the genome (not shown). From these results it is then possible to conclude that even though deletion of cnxl is lethal, the calnexin/calreticulin highly conserved region is not essential for viability in S. pombe when grown under standard laboratory conditions (minimal medium, 30°C). In order to delimit the Cnx1p essential sequences and to evaluate whether the central region by itself can confer viability, a set of cnxl deletion mutants was constructed and transformed into strain SP6089 (see Fig. 1B). For certain constructs encoding soluble proteins (#9, #10, #12, #13, #15, #17 and #18), versions with or without the ADEL ER-retention signal (Pidoux and Armstrong, 1992) were made in order to ensure that lack of viability was not due to escape of the mutant Cnx1p proteins from the ER. The functionality of the ADEL retention signal was verified by western blotting on supernatants from cultures (see Fig. 2G).
Cells without the calnexin/calreticulin central region are viable

Taken together, these results suggest that Cnx1p encodes at least two distinct domains: (1) a non-essential central domain, which in mammalian cells was demonstrated to bind folding glycoproteins (via their glycans) and Ca\(^{2+}\) (see Bergeron et al., 1994; Williams, 1995; Trombetta and Helenius, 1998; Parodi, 1994; Vassilakos et al., 1998); and (2) an essential domain for cell viability, mapping within the last 123 residues of the fission yeast molecule.

By cell fractionation studies we confirmed that wild-type Cnx1p and the mutants #4 and #11 are membrane-bound (see Fig. 2A,C,E), and that constructs #3 and #16 are soluble proteins (not shown). In addition, confocal microscopy with anti-Cnx1p antibodies showed that the mutant proteins localized to the ER (Fig. 2B,D,F). Moreover, confocal microscopy with anti-BiP antibodies presented the typical S. pombe ER staining pattern (not shown), as previously reported for cnx1\(^+\) cells (Pidoux and Armstrong, 1992; Jannatipour et al., 1998). Mutants supporting viability were studied further.

The growth rates of deleted-cnxt and mini-cnxt cells are reduced at 37°C but are similar to wild type at 30°C

As a first step to assess how the different cnxl mutants affect the physiology of S. pombe, we determined the growth rates of cells bearing the constructs luminal (#3), luminal+ADEL (#16), deleted-cnxt (#4) and mini-cnxt (#11). For comparison, we also determined the growth rates of the strain SP556
carrying an intact genomic copy of cnx1+ and the strain carrying episomal copies of full-length cnx1+ (#2). As shown in Fig. 3A, all the strains grew at about the same rate at 30°C without a major lag phase. However at 37°C, while strains #3 and #16 grew at essentially the same rate as SP556 and (not shown) strain #2, deleted-cnx (#4) and mini-cnx (#11) cells grew at considerable slower rate, reaching stationary phase after 3000 minutes and 6000 minutes, respectively (see Fig. 3B). Moreover, at 37°C, the lag phase for #2 was 100 minutes, but 500 minutes for deleted-cnx and 1500 minutes for mini-cnx cells. A prolongation of the lag phase may correspond to a reversible adaptation of the organism to the stress conditions or may represent the time required for the selection of mutant populations of cells able to grow at 37°C. To discriminate between these two possibilities, we reasoned that if the first hypothesis was correct, the cells growing at 37°C when shifted back to 30°C would grow at the same rate as wild type (#2). On the other hand, if the second possibility was true, we expected that these mutant cells would grow at an altered rate when shifted down to 30°C. As shown in Fig. 3D, the growth rates of strains #4 (deleted-cnx) and #11 (mini-cnx) after the shift-down (i.e. from 37°C to 30°C) were practically indistinguishable from those carrying wild-type cnx1+ (#2). Moreover, when these mutant strains that were shifted down to 30°C were recultured at 37°C, they exhibited the same lag phase and growth rates as in the first experiment (not shown). In addition, when these mutant cells grown at 37°C were plated on solid medium they appeared homogenous without papillae, thus excluding the possibility of selection of mutated populations.

To further explore the hypothesis of adaptation of strains #4 and #11 to the temperature stress, we measured the growth rates at intermediate temperatures. As shown in Fig. 3C, while the generation time for the wild-type strain remained practically invariant between 30°C and 37°C, that of strains #4 and #11 increased significantly with temperature. Furthermore,
Cells without the calnexin/calreticulin central region are viable.

The slow growth phenotype of cells #4 and #11 was reverted by retransformation of episomal copies of wild-type cnx1 into these strains (not shown). Taken together, these results support the notion of a gradual adaptation of the physiology of the deleted-cnx and mini-cnx cells to the temperature stress, the mini-cnx strain being more affected than the deleted-cnx strain.

52 amino acids of the lumenal region of mini-Cnx1p are sufficient to form a complex that includes BiP.

The presence of BiP was analyzed by western blotting using polyclonal anti-BiP antibodies. As shown in Fig. 4A (lanes 1-6), BiP coprecipitated with full-length and mutant Cnx1p, including deleted-cnx and mini-cnx. This interaction is specific since no BiP was precipitated from SP556 total protein extracts with or without pre-immune serum (Fig. 4A, lanes 7 and 8). Moreover, since identical results were obtained after 2 hours incubation with cycloheximide, it seems unlikely that mini-Cnx1p could bind as a misfolded substrate to BiP. The decrease in the amount of BiP coprecipitated with mini-Cnx1p (#11) could be the consequence of the lower amounts of BiP (see Fig. 4E, lane 5) and/or lesser efficiency of mini-Cnx1p precipitation due the loss of epitopes in this mutant. As shown in Fig. 4A (lower panel of lanes 1-8) the anti-Cnx1p western blotting of the immunoprecipitates detected lower amounts of mini-Cnx1p. Overall, these observations suggest that these 52 aa of the lumenal region of the mini-Cnx1p are sufficient for the formation of a complex containing both Cnx1p and BiP.
**The calnexin levels in deleted-cnxx and mini-cnxx strains drop at 37°C but BiP levels remain similar to those in cnx1+ cells**

To obtain clues at the molecular level regarding the mechanism allowing adaptation of deleted-cnxx and mini-cnxx cells to temperature stress (see Fig. 3), we performed western blot analyses with anti-BiP antibodies with extracts of strains #2, #4 and #11 grown at the exponential phase at 30°C, 32°C, 34°C and 37°C. Fig. 4B shows the results of these experiments. In strain #2 (full-length Cnx1p), the calnexin levels remain unchanged at different temperatures since its expression is driven by an heterologous promoter (nmt1). However, in strains #4 (deleted-cnxx) and #11 (mini-cnxx) the calnexin levels dropped considerably at 37°C (see arrows in Fig. 4B). Western blotting with anti-Cnx1p antibodies using 15 μg of cell extract instead of 5 μg confirmed the presence, albeit diminished, of the deleted-Cnx1p and mini-Cnx1p in cells grown at 37°C (see Fig. 4D, lanes 3 and 4). The reduction in the levels of mutant Cnx1p at elevated temperatures could be due to instability of these proteins in vivo or to proteolytic degradation in vitro. However, the second possibility could be excluded since the levels of wild-type Cnx1p (#2) remained unchanged under these conditions (see Fig. 4B). As expected for a heat-shock protein, BiP synthesis was increased at 37°C in the cnx1+ strain (see Fig. 4B). Interestingly, at 30°C the BiP levels were higher in mutants #4 and #11 than in wild type, but remained unchanged at different temperatures. The BiP and Cnx1p levels were practically restored when the cells adapted at 37°C were shifted down and cultured at 30°C (see arrows in Fig. 4C).

To explore the possibility that an induction of BiP expression could compensate for the reduction of Cnx1p levels in strains #4 and #11 during adaptation at 37°C, we quantified by western blotting the BiP levels in the various cnx1 mutants at 30°C and 37°C. As shown in Fig. 4E, at 30°C and compared to wild type (#2), BiP accumulation is about 1.8-fold higher in the lumenal (+ADEL) (#2), 1.6-fold in the deleted-cnxx (#4) mutant and about 1.4-fold in the mini-cnxx cells (#11). Interestingly, the BiP levels did not further increase to compensate for the drop in Cnx1p levels in strains #4 and #11 cultured at 37°C (Fig. 4D, lanes 1-4; and see below). This may suggest that perhaps other chaperones compensate for the reduction in the overall chaperone efficiency in the cnx1 mutants.

**Deleted-cnxx and mini-cnxx cells exhibit temperature-dependent altered morphology**

We assessed whether the various cnx1 mutations affected the morphology of *S. pombe* cells microscopically. Lumenal and lumenal+ADEL cells presented no visible difference in their morphology (not shown). In contrast, the deleted-cnxx and mini-cnxx cells grown at 37°C had an aberrant round shape morphology and the majority contained large vesicles (Fig. 5B, see arrow). The rounded shape of these cells suggested that the synthesis of a component(s) of their cell wall could be affected. A classical way to test this hypothesis is to grow cells in a hyperosmotic medium (Fanchiotti et al., 1998). As shown in Fig. 5C, the aberrant morphology of deleted-cnxx and mini-cnxx cells grown at 37°C was suppressed in the presence of 1.2 M sorbitol (Fig. 5C) or glycerol at 1.2 M (not shown), or by transforming the plasmid encoding wild-type Cnx1p (Fig. 5D). In order to study the cell wall integrity of the cnx1 mutants, exponentially growing cells were treated by NovoZym 234, an enzyme complex able to degrade *S. pombe* cell wall polymers (Ishiguro et al., 1997). As shown in Fig. 3H, deleted-cnxx and mini-cnxx cells exhibited increased sensitivity to the lytic enzymes at 37°C when compared to cnx1+ cells. The assembly of the yeast cell wall requires the biosynthesis and transport of glycoproteins and
Cells without the calnexin/calreticulin central region are viable. β-glucans (Orlean, 1997). The antibiotic hygromycin B was reported to affect the viability of yeast cells with mutations affecting the early stages of glycoprotein biosynthesis, in particular at the ER (Dean, 1995; Silberstein et al., 1998). Hence, we tested the effect of this antibiotic on mutants #4 and #11. As it can be seen in Fig. 2H (right panel) at 30°C, the mutant strains showed increased sensitivity, while none of the strains grew at 37°C in the presence of the drug. To further study the cell wall in the cnx1 mutants, we used Calcofluor White. This fluorescent dye stains an unknown cell wall component of S. pombe cells that concentrates in the septum of dividing cells (Robinow and Hyams, 1989). The results of the microscopic observations are presented in Fig. 6. Calcofluor stained the septa of mutants #4 and #11 at 30°C and 37°C (Fig. 6A,C) in a similar manner to wild type (#2) or the mini-cnx cells also containing a plasmid expressing cnx1+. However, the large vesicles observed at 37°C in mutants #4 and #11 (see Figs 5, 6) were also labeled by the dye, thus suggesting that these vesicles might accumulate a Calcofluor-stainable cell-wall component.

Takén together, these results suggest that the conserved central domain of Cnx1p may be required, directly or indirectly, for correct cell wall synthesis and morphology at 37°C.

The slow growth phenotype of the deleted-cnx and mini-cnx strains can be rescued by glycerol and sorbitol

The growth rate of deleted-cnx and mini-cnx cells is reduced at temperatures higher than 30°C (Fig. 3B,C). We observed, however, that these mutant cells grew faster in the presence of glycerol and sorbitol at 1.2 M. Therefore, we wished to examine in more detail the effect of these polyols on the growth of cnx1 mutants. Accordingly, cells cultured exponentially were subjected to the drop test for their ability to grow at 30°C and 37°C on minimal medium (EMM) or EMM supplemented with glycerol or sorbitol.

The slow growth of deleted-cnx and mini-cnx cells at 37°C was rescued in the presence of 4% (approx. 0.4 M) glycerol (see Figs 7, 3D). It should be noted that in this case, glycerol does not act as a source of carbon since EMM contains 2% glucose. The same effect was observed in the presence of 0.4 M sorbitol (not shown). Because the osmolality of the growth medium was reported to affect the levels of molecular chaperones and the stability of certain proteins (Kültz et al., 1997), we next analyzed the accumulation of Cnx1p and BiP in the presence of glycerol or sorbitol. As shown in Fig. 4D (lanes 3-6) the levels of deleted-Cnx1p and mini-Cnx1p at 37°C were restored to those at 30°C when cells were cultured in the presence of 0.4 M glycerol or sorbitol (not shown). Interestingly, the addition of these polyols resulted in the enhancement of coprecipitated BiP with Cnx1p (see Fig. 4A, lanes 9-14).

The deleted-cnx and mini-cnx strains cultured at 37°C are more sensitive to DTT, NaCl and Ca2+

The central-domain of the calnexin/calreticulin chaperones was shown to bind Ca2+, and it was proposed that membrane-bound calnexin could participate in the retention of ER-soluble proteins by anchoring a protein-calcium gel via its transmembrane domain (Wada et al., 1991; Tjoelker et al., 1994). As such, it could be expected that strains deleted of the central region of Cnx1p would be less tolerant to increased levels of Ca2+ in the medium. To assess this possibility, we performed drop tests with 10 mM Ca2+, at 30°C and 37°C. The growth of the deleted-cnx and mini-cnx strains remained unaffected in the presence of Ca2+ at 30°C (Fig. 7); however, it was severely inhibited at 37°C. This inhibition was totally rescued by the addition of 0.4 M glycerol (Fig. 7) or 0.4 M sorbitol (not shown).

Fig. 6. Calcofluor staining of cnx1 mutants. Late-log phase S. pombe cells, grown at 30°C, were diluted into fresh EMM+Ade+Ura medium and grown for 20 hours at 30°C. For cultures at 37°C, cells were grown for 20 hours for wild type and mini-cnx strain transformed with wt cnx1+, 30 hours for deleted-cnx, and 70 hours for mini-cnx. Exponentially growing cells cultured at 30°C or at 37°C were stained with Calcofluor White (see Materials and Methods) (A,C). Nomarski interference images show the same field of cells stained with Calcofluor (B,D). Arrows indicate vesicles labeled with the dye. Microscopy was done with a Zeiss microscope, at a 1500× magnification on the film. Bars, 10 μm.
DTT is a reducing agent that induces protein misfolding in the ER (Braakman et al., 1992). It was of interest to evaluate the response of the various Cnx1p mutants to a DTT challenge. As shown in Fig. 7, in the presence of 1 mM DTT the growth of deleted-cnxx and mini-cnxx cells was affected at 30°C and completely inhibited at 37°C in the case of mini-cnxx. Thus the deletion of the highly conserved central domain of Cnx1p compromised the cell’s tolerance to the combined effect of the two ER stresses (DTT and temperature; see arrows in Fig. 7).

In the presence of NaCl at a concentration as low as 50 mM in EMM, the growth of deleted-cnxx and mini-cnxx cells was drastically affected at 37°C (see Fig. 7). The inhibitory effect of NaCl suggests that these mutants cells are impaired in their regulation of NaCl homeostasis. The sensitivity to both DTT and NaCl was partially suppressed by the addition of 0.4 M glycerol (see Fig. 7); however, 0.4 M sorbitol did not rescue the growth inhibition of mini-cnxx cells at 37°C in the presence of these additives (not shown).

**DISCUSSION**

*S. pombe* cells deleted of the calnexin/calreticulin highly conserved central domain are viable

The central region is a salient feature of the calnexin/calreticulin molecules studied thus far. Its roles in mammalian cells in the tethering of folding intermediates of glycoproteins through their oligosaccharide moieties, and in the binding of Ca2+, have been demonstrated (Tjoelker et al., 1994; Vassilikos et al., 1998). Based on these observations we assumed that this 317-aa region containing motifs 1 and 2 would encode the essential function(s) of *S. pombe* calnexin. Our results showed that under normal culture conditions (minimal medium at 30°C), the fission yeast *cnx1* mutants (#4 and #11) lacking the central domain grow at the same rate as wild-type cells. Furthermore, the central region alone could not rescue the non-viability of *cnxlA* cells. These genetic observations may suggest that the interaction of monoglucosylated folding intermediates with the putative lectin determinant of *S. pombe* calnexin might not be absolutely required for the folding of glycoproteins, under normal growth conditions. Our results are consistent with four lines of evidence supporting this notion: (1) cells deleted of *gpt1*, the gene encoding glucosyl transferase, are viable at 30°C and 37°C (Fernandez et al., 1998); (2) Cnx1p interacts with acid phosphatase independently of glucose trimming and reglucosylation, i.e. the two pathways that generate monoglucosylated glycoproteins (Jannatipour et al., 1998); (3) cells genetically depleted of GT and glucosidase II (the enzyme that trims G2 glycans to G1 and G0), are viable at 30°C and 37°C (Fanchiotti et al., 1998); (4) an *alg6Δ/gpt1Δ* double mutant strain (Alg6p is the enzyme that adds glucose to the lipid-linked glycan) is viable at 30°C.

Nevertheless, strains #4 and #11, lacking the highly conserved central domain, exhibited reduced growth rates at 37°C. Because deleted-Cnx1p and mini-Cnx1p are correctly targeted to the ER (Fig. 2), the reduced growth rates of these cells at elevated temperature could be due in part to the observed decrease in Cnx1p levels (see Fig. 4). This is supported by the fact that the presence of 0.4 M glycerol or sorbitol corrected both the growth rates and the levels of mini-Cnx1p and deleted-Cnx1p. The stabilization of mutant Cnx1 proteins could be the result of general changes in chaperone levels and protein degradation activities induced by osmotic changes produced by these polyols (Kültz et al., 1997; Fernández et al., 1997). Another non-exclusive possibility could be that, in addition, glycerol might act as a chemical chaperone as previously reported in vitro (Gekko and Timasheff, 1981) and in vivo (Sato et al., 1996).

In spite of the fact that cells without the central region are viable under standard conditions, this domain appears to be required when cells are cultured under high ER stress such as the combination of DTT and temperature. Our results are congruent with those of Fanchiotti et al. (1998), who showed that *gpt1Δ/alg6Δ* double-mutant cells do not grow at 37°C, and proposed that the interaction between monoglucosylated folding intermediates and calnexin is essential under conditions of extreme ER stress.

A possible role of the central domain in cell-wall biosynthesis

Cells deleted of the central domain (strains #4 and #11)
exhibited anomalous, round-shaped morphology at 37°C, that was suppressed by culturing the cells in the presence of 1.2 M sorbitol or glycerol. Moreover, these cnxl mutants exhibited increased sensitivity to cell-wall lytic enzymes and to hygromycin B, suggesting that these cells are defective in the biosynthesis of some cell-wall component(s), and that Cnx1p could be involved in this process. Interestingly, in Saccharomyces cerevisiae, the ER glucosidase I (CWH41), glucosidase II (GLS2) and Kar2p/BiP were shown to be involved in β-1-6 glucan synthesis (Simons et al., 1998; Shahnin et al., 1998), one of the cell wall components. Thus it is tempting to speculate that in yeasts the calnexin cycle may play a major role in the biosynthesis of cell wall components, and a secondary one in the folding of glycoproteins, whereas in mammalian cells, which obviously lack a cell wall, the calnexin cycle evolved to be specialized in the folding of glycoproteins.

The central domain is not required for Ca²⁺ homeostasis at 30°C

The highly conserved central domain of calnexin/calreticulin has been shown to bind Ca²⁺ (Wada et al., 1991; Tjoelker et al., 1994), and it was also proposed that calnexin could participate in the retention of the ER soluble proteins by anchoring a protein-calcium gel via its transmembrane domain (Wada et al., 1991). The cells deleted of the central domain were viable in the presence of 10 mM Ca²⁺ at 30°C. However, the requirement for the Cnx1p central domain was manifested under ER-stress, thus arguing that the ensemble of ER-resident proteins do not provide sufficient Ca²⁺ buffering capacity to ensure viability under these conditions.

The essential function(s) of Cnx1p is found within its 123 C-terminal residues

We have shown that the essential function(s) of Cnx1p can be delimited to the 123 C-terminal amino acids. This segment spans the last 52 aa of the luminal domain, the 23-aa transmembrane domain and the 48-aa cytosolic domain. When the mini-Cnx1p and luminal-Cnx1p molecules are compared, the overlapping region corresponds to the C-terminal 52 aa of the luminal domain. Moreover, luminal versions of Cnx1p deleted of the 52 aa failed to support viability (constructs #17 and #18). Therefore, the essential function(s) of Cnx1p is likely to be located within this 52-aa stretch of the luminal domain. Although this region displays the same basic organization as other calnexin molecules, no significant conservation at the level of aa sequence can be observed among different species. Our results may explain the observation that, in spite of the high degree of sequence conservation of the central domain and the similarities in the structural organization between mammalian and fission yeast calnexin, the canine homologue failed to rescue the lethal phenotype of cnxlΔ S. pombe cells (Parlati et al., 1995). This lack of complementation might be due to the poor similarity of sequence between these two proteins within the 52-aa segment. We have shown that the 52-aa stretch of Cnx1p is sufficient for the formation of a complex that includes BiP. Therefore, it is tempting to speculate that one of the crucial functions of Cnx1p may reside in the formation of this complex (perhaps along with other chaperones) that might be implicated in protein folding. Alternatively, this 52-aa stretch of the luminal domain could still contain enough chaperone activity by itself to support viability of S. pombe. Further experiments are required to discriminate between these possibilities, and to explore whether this region encodes a yet to be defined function.

The viability of a calnexin-less mammalian cell line has been interpreted as the result of a compensation by its soluble homologue calreticulin (Scott and Dawson, 1995). However, since glucose-trimming inhibitors do not completely obliterate the interaction of glycoproteins with calnexin/calreticulin, nor do they totally inhibit protein secretion, it may be suggested that lectin-independent glycoprotein folding pathways must exist in the ER of higher eukaryotes. Such a pathway might involve protein-protein interactions with calnexin, as it has been previously proposed, or with the assistance of other chaperones like BiP, akin to S. pombe (Williams, 1995; Jannatipour et al., 1998). In this regard, it should be noted that secretion of the fungal glycoprotein cellulase I is not diminished in S. pombe cells deleted of the calnexin central domain (our unpublished results). Complexes containing calnexin and BiP seem to exist in S. cerevisiae, since kar2-BiP mutants are synthetic with the deletion of CNE1/calnexin (Brody et al., 1999; Simons et al., 1998). Furthermore, calreticulin was shown to form a stable complex with BiP in tobacco (Crofts et al., 1998), and mammalian calnexin interacts with BiP in the absence of translation (Tatu and Helenius, 1997).

Future endeavors should focus on verifying whether the central domain of Cnx1p displays lectin activity, and the nature of a putative chaperone function encoded by the 52-aa C-terminal region of the Cnx1p luminal domain.

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