Cell viability and secretion of active proteins in *Schizosaccharomyces pombe* do not require the chaperone function of calnexin

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Folding of newly synthesized proteins within the ER (endoplasmic reticulum) is a rate-limiting step in protein secretion. Thus ER molecular chaperones and foldases have a major impact in determining the rate and yield of these crucial cellular processes. Calnexin is a key ER chaperone implicated in the folding, retention and targeting for degradation of proteins that go through the secretory pathway. Calnexin molecules contain a highly conserved central domain (hcd) that has been proposed to be involved in the interaction with folding substrates and other chaperones. To gain a better understanding of the roles played by calnexin in the secretory pathway, we examined the efficiency of fission yeast (*Schizosaccharomyces pombe*) strains expressing calnexin mutants to secrete different model proteins. Remarkably, calnexin hcd-deletion mutants, although devoid of detectable

chaperone activity *in vitro*, confer viability and cause a considerable increase in the secretion of heterologous cellulase. Surprisingly the quality-control efficiency, measured as the activity/amount ratio of secreted model protein, was not severely reduced in these calnexin hcd-deletion mutant strains. Our results indicate that the essential function of calnexin does not reside in its role in the folding or in the retention of misfolded proteins. These observations suggest the existence of a highly stringent quality control mechanism in the ER of *S. pombe* that might reduce the secretion efficiency of endogenous proteins.

Key words: calnexin, endoplasmic reticulum, molecular chaperone, protein folding, secretion, yeast genetics.

INTRODUCTION

Secreted and membrane proteins enter the secretory pathway at the level of the ER (endoplasmic reticulum) [1]. Upon entering the ER, a number of newly synthesized proteins undergo covalent modifications, such as N-glycosylation and disulphide-bond formation, while they acquire their final structure. Because of the high concentration of newly synthesized and resident proteins in the ER, folding in this compartment is a major mechanistic challenge for the cell. Nevertheless, the ER constitutes a cellular compartment that is optimized for the efficient folding of secretory proteins. To this effect, the ER contains molecular chaperones and foldases that co-operate to increase the successful acquisition of the tertiary and quaternary structures of proteins (reviewed in [2,3]). In addition, a quality-control system of protein folding ensures that only correctly folded polypeptides and assembled multimeric proteins exit the ER to continue on to the secretion pathway (reviewed in [4,5]).

Membrane-bound calnexin and its soluble paralogue calreticulin are molecular chaperones that act as key components of the ER quality-control process. For instance, calnexin has been implicated in the ER-retention of misfolded mutant molecules in a vast number of hereditary protein-folding diseases (reviewed in [6]). Both calnexin [7] and calreticulin [8] were shown to interact with newly synthesized glycoproteins via their monoglucosylated glycan moieties. Nevertheless, calnexin and calreticulin were also reported to interact *in vitro* and *in vivo* via peptide–peptide contacts, as do classical chaperones such as BiP (immunoglobulin heavy-chain binding protein) and GRP94 [9,10].

The luminal portion of the calnexin molecule shares with calreticulin a highly conserved central domain (hcd) containing two tandemly repeated motifs IXDP(D/E)AXKPEDWD(D/E) and GXWXXPXIXNPXY (one-letter amino acid codes, where X indicates any amino acid). NMR [11] and crystallographic [12] studies on calreticulin and calnexin revealed that the three-dimensional structure of these chaperones is highly asymmetrical and is composed of two distinct domains [13,14]: (i) a globular core formed by the N- and C-termini; (ii) a long, extended protrusion formed by the hcd. Several reports have established *in vitro* and *in vivo* the importance of the various domains of mammalian calnexin/calreticulin for oligosaccharide recognition and binding, and peptide–peptide substrate interactions, as well as co-operation with the thiol oxidoreductase ERp57. For instance, *in vitro* experiments have shown that the lectin- and peptide-binding regions of calnexin are mainly localized in the globular domain, while the extended hairpin domain was found to be required for interaction with ERp57, both regions being required for optimal activity of the chaperone [13].

Pointing to a crucial cellular role, calnexin (Cnx1p) was shown to be essential for viability in the fission yeast *Schizosaccharomyces pombe* [14,15]. Interestingly, *S. pombe* does not possess a calreticulin-encoding gene, suggesting that the membrane-bound lectin chaperone is sufficient to ensure glycoprotein folding in this organism. In studies to assess the substrate specificity of calnexin in the fission yeast, our laboratory observed that calnexin/Cnx1p can interact *in vivo* with the glycoproteins acid phosphatase and sNEP (soluble neprilysin) in the absence of glucose trimming and reglucosylation [16,17]. These observations indicated that calnexin/Cnx1p could bind folding polypeptides

Abbreviations used: BiP, immunoglobulin heavy-chain binding protein; Cel1p, cellulase I from *Aspergillus aculeatus*; Cnx1p, calnexin from *S. pombe*; ER, endoplasmic reticulum; GST, glutathione S-transferase; HA, haemagglutinin; hcd, highly conserved central domain; HSA, human serum albumin; Ni-NTA, Ni²⁺-nitrilotriacetate; sNEP, soluble neprilysin; UPR, unfolded protein response; WT, wild-type.

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in a lectin/oligosaccharide-independent fashion. Supporting this notion, we reported that *cnx1* mutants lacking the hcd and the N-terminal domain (Δhcd_cnx1 and *mini_cnx1*), including the residues implicated in direct glycan contacts by crystallography, are viable in spite of calnexin/Cnx1p being essential in the fission yeast [18]. Moreover, the hcd by itself was not sufficient to render *S. pombe* viable [18]. Consequently, these observations suggested that, at least in the ER of *S. pombe*, enough glycoproteins may fold in the absence of the calnexin lectin activity to sustain life under standard growth conditions.

Since protein folding is rate-limiting for the exit of proteins from ER, changes in the expression levels of key ER chaperones are expected to have detectable effects on secretion. Such a phenomenon has been reported for BiP, calnexin and other chaperones (reviewed in [19]). A lower level of BiP chaperone in the ER of *Saccharomyces cerevisiae* led to a diminution in the secreted quantity of three heterologous proteins [20]. In the same vein, another study reported that in *Aspergillus niger*, overexpression of calnexin resulted in a 4- to 5-fold increase in extracellular levels of *Phanerochaete chrysosporium* manganese peroxidase [21]. In contrast, a stable overexpression of BiP in CHO (Chinese-hamster ovary) cells resulted in a diminished secretion of both von Willebrand factor and a mutant form of the coagulation factor, Factor VIII [22].

In the present study, we demonstrate evidence that fission yeast calnexin/Cnx1p acts as a bona fide chaperone *in vitro*, whereas hcd-deleted mutants are not functional in the same assay; thus strongly indicating that the essential function of calnexin/Cnx1p in the ER of *S. pombe* does not reside in its chaperone activity. The relative importance of various domains of calnexin on the secretion process was directly evaluated using *cnx1* mutants. It was found that the overall secretion of proteins was substantially increased in cells devoid of the chaperone function of calnexin. Surprisingly, this did not alter significantly the ER quality-control efficiency, measured as the activity/amount of secreted model proteins. Our observations suggest that the stringency of the *S. pombe* quality-control mechanism might reduce the secretion efficiency of certain endogenous proteins.

EXPERIMENTAL

S. pombe strains and media

S. pombe (h^- , *his3-D1*, *ade6-M216*, *ura4-D18*, *leu1-32*, $\Delta cnx1::his3$) expressing full-length *cnx1*⁺, Δgly_cnx1 , *lum_cnx1*, *lumadel_cnx1*, Δhcd_cnx1 and *mini_cnx1* on pREP41 were used throughout this study [18]. *S. pombe* strains constitutively producing HA (haemagglutinin)-tagged cellulase I from *Aspergillus aculeatus* were constructed by genomic integration of the encoding gene under the control of the *adh1* promoter, both derived from the plasmid pP1Tc1 (a gift from Dr Thomas Sandal, Screen Biotechnology, Novo Nordisk A/S, Bagsvård, Denmark) at the *leu1* locus. EMM (Edinburgh minimal medium) was prepared as described [23]. *S. pombe* transformations were performed using the PEG [poly(ethylene glycol)]/lithium acetate procedure [23].

Plasmids

Plasmids pSPCA3220 [wild-type (WT) *cnx1*⁺], pSPCA3222 (Δhcd_cnx1), pSPCA7102 (*mini_cnx1*), pSPCA3221 (*lum_cnx1*), pSPCA7095 (*lumadel_cnx1*) and psNEP7019 [producing sNEP] are derivatives of the pREP41 and pREP2 vectors [24], and were constructed in our laboratory as described previously [16–18]. Plasmid pSPCA3262 (Δgly_cnx1) was constructed by site-

directed mutagenesis using the Altered Sites II *in vitro* Mutagenesis System and the pALTER-1 vector (Promega), according to the manufacturer's recommendations. The sequences encoding HSA (human serum albumin) were amplified by PCR using the plasmid pHIL-D2 HSA [25] as a template (a gift from Dr Nadhipuram Bhagavan, Department of Biochemistry and Biophysics, University of Hawaii, Honolulu, HI, U.S.A.), with the following primers: 5'-AAACTCGAGATGAAGTGGGTAACC-3' and 5'-AAAAGCGGCCGCTTAAGCCTAAGGCAGC-3'. Following amplification, this HSA expression cassette was inserted into the *XhoI* and *NotI* (underlined) sites of the *S. pombe* expression vector pSLF172, to create plasmid pSLF172 HSA. Expression from this plasmid is under the control of the thiamine-repressible promoter *nmt1*. The HSA produced with this construct contains three copies of the HA epitope at its C-terminus.

Northern blotting

RNA was extracted from 25 ml of exponentially growing cells (D_{600} of 0.8) using TRIzol[®] reagent (Invitrogen) following the manufacturer's instructions. RNA samples (15 μ g) were analysed by Northern blot as described previously [26]. The probes were synthesized using the Prime-it[®] II Random primer labelling kit (Stratagene) following the manufacturer's instructions. The gel was stained with ethidium bromide to verify that equivalent amounts of RNA were loaded.

Purification of proteins for *in vitro* aggregation assays

The coding sequences of BiP and soluble Cnx1p (sCnx1p) were amplified by PCR and cloned in pH6EX3 [27] to create fusion proteins with His₆ tags. The primers used for BiP amplification were 5'-GGATCCAGTGGTGATGATAACTCTACAG-3' (A) and 5'-GGATCCTTAAAGTTCATCGGCCCTCATCATC-3' (B). Primers used for *cnx1* amplification were 5'-GGATCCGATCCTGATCCGAACAAGAA-3' (C) and 5'-CGGGATCCTTAAAGCTCGTCAGCCCCAATTCAGGAGTCTCGATG-3' (D). Underlined bases correspond to the *Bam*HI restriction site. To obtain GST (glutathione S-transferase) fusion proteins, the ER soluble Δhcd_Cnx1p (s Δhcd_Cnx1p) and soluble *mini_Cnx1p* (s*mini_Cnx1p*) coding sequences were cloned in pGEX-2T (Amersham Biosciences). For s Δhcd_cnx1 amplification, primers C and D, and plasmid pSPCA3222 (Δhcd_cnx1) were used. For *smini_cnx1* amplification, primers D and 5'-GGATCCATGGAAAAGCAATCAATGC-3' (E) were employed. In order to produce the recombinant proteins, the expression plasmids were transformed into the *Escherichia coli* AP401 strain (*lon::mini tet^R*, *ara⁻*, $\Delta lac-pro$, *nalA*, *argE am*, *rif^R*, *thi I*, [F⁺, *pro AB*, *lacI^q Z M15*]). Proteins were purified from a 1 litre culture of cells at a D_{554} of 0.7, induced for 3 h at 37 °C with 0.4 mM IPTG (isopropyl β -D-thiogalactoside). Cells expressing BiP were lysed by repeated freeze-thawing in buffer A (50 mM Na₂PO₄, 300 mM NaCl and 10 mM imidazole, pH 8.0) containing 0.2 mM PMSF. The lysates were then treated with 10 μ g/ml DNaseI for 15 min at 37 °C. To purify the His₆-tagged proteins, extracts were incubated overnight at 4 °C with Ni-NTA (Ni²⁺-nitrilotriacetate)-agarose (Qiagen), and the beads were washed twice with buffer B [50 mM Na₂PO₄, 500 mM NaCl, 20 mM imidazole, 10 % (v/v) glycerol and 0.1 % (v/v) Tween 20, pH 8.0], and twice with buffer C [50 mM Na₂PO₄, 1 M NaCl, 20 mM imidazole, 10 % (v/v) glycerol and 0.1 % (v/v) Tween 20, pH 8.0]. Elution of bound proteins was performed with 10 times the column's volume of buffer D (50 mM Na₂PO₄, 300 mM NaCl and 250 mM imidazole, pH 8.0). Purification of GST-s*mini_Cnx1p* using glutathione-Sepharose resin (Amersham Biosciences) was carried out following the manufacturer's recommendations. GST-s Δhcd_Cnx1p was purified

following the manufacturer's recommendations (Amersham Biosciences), followed by Q Sepharose chromatography (as described below). sCnx1p-His₆ was purified using Ni-NTA affinity-column chromatography using a different protocol. Briefly, cells expressing sCnx1p-His₆ were lysed in binding buffer [20 mM Tris/HCl pH 8.0, 500 mM NaCl, 0.5 mM PMSF, 0.75 % (v/v) Tween 20, 30 mM imidazole and 1 mM CaCl₂]. The beads were washed three times with washing buffer [20 mM Tris/HCl, pH 8.0, 500 mM NaCl, 1 mM PMSF, 0.75 % (v/v) Tween 20, 30 mM imidazole, 1 mM CaCl₂ and 20 % (v/v) glycerol] using 10 times the volume of the column. Elution was performed in the same buffer except that there was no glycerol and 200 mM imidazole were added. Fractions containing the proteins were pooled and dialysed against binding buffer for Q Sepharose which consists of 20 mM Tris/HCl, pH 8.0, 200 mM NaCl, 0.5 mM PMSF, 0.75 % (v/v) Tween 20 and 1 mM CaCl₂. Elution was made with a step gradient ranging between 200 and 1000 mM NaCl. Concentrations of proteins were determined by the method of Bradford, using the Bio-Rad Protein Assay[®] kit.

In vitro aggregation assays

Aggregation assays using luciferase as a model protein were carried out essentially as described previously [28]. Briefly, luciferase (1 μ M) was incubated alone or with smini-Cnx1p, s Δ hcd_cnx1p, sCnx1p and BiP (1 μ M) in Buffer 1 (50 mM KCl and 25 mM Tris/HCl, pH 7.8) for 5 min at room temperature (22 °C) and then at 42 °C for 10 min. Then, centrifugation at 15 000 *g* in a Sorvall 12V table-top centrifuge for 10 min at 4 °C was performed to separate aggregates (misfolded) from soluble (folded) proteins. Fractions were loaded on to SDS/10 % polyacrylamide gels (30:2.67 acrylamide/bisacrylamide) and bands were visualized by Coomassie Blue staining.

Endoglycosidase H digestions

Approx. 20 μ g of protein from total cell extracts containing cellulase, sNEP and albumin were incubated for 10 min at 100 °C in denaturing buffer [0.5 % (w/v) SDS and 1 % (w/v) 2-mercaptoethanol]. Sodium citrate was added to a final concentration of 50 mM and incubated with 1 unit of endoglycosidase H (New England Biolabs) at 37 °C for 1 h. Reaction products were separated by SDS/PAGE (10 % gel for sNEP and 12 % for the other model proteins) and subsequently detected by immunoblot analysis.

Enzymic assays

The enzymic activity assays for sNEP were carried out as described previously [17]. In the case of cellulase I from *Aspergillus aculeatus* (Cell1p), the enzymic assays were performed essentially as described in [29], except that twice the amount of substrate was used. Briefly, 100 μ l of growth supernatant from *cnx1* mutant cells containing Cell1p (*D*₅₉₅ of 1.0) were mixed in an Eppendorf tube with 100 μ l of 0.1 M citrate buffer, pH 3.0, and 100 μ l of 0.8 % AZCL-HE-cellulose (azurine cross-linked hydroxyethylcellulose) as a substrate (MegaZyme, North Rocks, N.S.W., Australia). The reactions were then incubated at 40 °C for 4 h. Water (500 μ l) was added to stop the reaction. The activity of Cell1p was expressed as the ratio *D*₆₂₀/*D*₅₉₅(cell density).

Immunoprecipitations and immunoblotting

Co-immunoprecipitations and immunoblotting were carried out as described previously [16], except that cell extracts were spun at 15 000 *g* in a Sorvall 12V table-top centrifuge for a total of

60 min. The mouse monoclonal antibody 18B5 [17] was used for the detection of sNEP, while 12CA5 mouse monoclonal antibody was used to detect HA-tagged HSA and Cell1p.

RESULTS AND DISCUSSION

Viable calnexin/Cnx1p hcd-mutants exhibit no chaperone function *in vitro*

Previously, we have shown that although calnexin is essential in the fission yeast *S. pombe*, its hcd is not required for the viability of this organism [18]. In contrast, the hcd region alone was not able to render *S. pombe* viable, since it did not rescue the calnexin knockout [18]. Therefore it was of interest to investigate whether or not calnexin mutants lacking the hcd exhibited at least a remnant of chaperone activity. To this end, we produced in *E. coli* three different forms of calnexin/Cnx1p corresponding to viable mutants of our previous studies [18]: the ER soluble domain of WT calnexin (referred to as sCnx1p; Figure 1A), the luminal portion of calnexin deleted of the 329 amino acids of the hcd (referred to as s Δ hcd.Cnx1p), and a construct consisting of the 52 C-terminal amino acids in the ER (denoted as smini-Cnx1p; Figure 1A). *S. pombe* calnexin/Cnx1p was shown to interact with glycoprotein substrates in the absence of glucose trimming and reglucosylation [16,17], indicating that this chaperone can interact in a lectin/glycan-independent manner. Therefore, to assess the potential chaperone function of these purified proteins, we measured their respective ability to prevent thermal aggregation of the non-glycosylated model protein luciferase from the firefly *Photinus pyralis* [28]. These assays were carried out in the presence or absence of ATP, ADP and MgCl₂ to analyse the possible requirement of these factors for calnexin/Cnx1p chaperone activity. As shown in Figure 1(B), sCnx1p used in a 1:1 ratio behaved as a classical chaperone preventing aggregation of about 60 % of the luciferase, while both the s Δ hcd.Cnx1p and smini-Cnx1p forms were devoid of chaperone function when the assay was carried out with or without added factors. These results indicate that the chaperone activity of calnexin/Cnx1p is dispensable for cell viability, and therefore its essentiality may reside in another function.

BiP and calnexin/calreticulin are known to interact in a non-substrate fashion *in vivo* in fission yeast and tobacco cells [17,30]. To examine a possible synergetic co-operation between these two ER chaperones *in vitro*, we first tested the capacity of recombinant *S. pombe* BiP to prevent aggregation. As shown in Figure 1(C), BiP prevented thermal denaturation of luciferase and required ATP and Mg²⁺ for optimal activity. BiP prevented the aggregation of 40 % of the substrate when used in an equimolar fashion, reaching 60 % of anti-aggregation when present in a 4:1 molar ratio. Thus BiP required a 4 times molar excess with respect to the substrate in order to be as efficient as calnexin/Cnx1p at a 1:1 ratio (Figure 1C). Therefore, in the case of luciferase, a non-glycosylated protein, calnexin/Cnx1p is considerably more efficient than the classical chaperone BiP in preventing aggregation. This is in contrast with previous results showing that mammalian calnexin was less efficient than BiP in promoting the refolding of deglycosylated α -mannosidase [31]. However, it is in agreement with the fact that mammalian calnexin is as efficient as BiP in suppressing the thermal aggregation of the non-glycosylated substrate citrate synthase [31]. Taken together, these observations support further the notion that different substrate proteins have different chaperone requirements. The present study also provides additional proof that calnexin/Cnx1p can indeed act as a classical chaperone in the folding of non-glycosylated proteins *in vitro*, supporting further the notion that calnexin can interact with

Figure 1 Absence of chaperone function in hcd-deleted calnexin/Cnx1p mutants

(A) Schematic representation of calnexin/Cnx1p constructions used in experiments *in vitro* and *in vivo*. The hcd is indicated as the white box containing the two sets of four tandemly repeated motifs [1, IXDP(D/E)AXKPEDWD(D/E); 2, GXWXXPXIXNXPXY]. SP denotes the signal peptide, the tree symbolizes the glycosylation site and TM indicates the transmembrane domain. (B, C, D) The chaperone activities of WT and mutant calnexin/Cnx1p as well as that of BiP were assessed in a thermal denaturation assay as described previously [33]. WT and mutant calnexin/Cnx1p (1 μ M) (B), BiP (1 and 4 μ M) (C) or a combination of calnexin/Cnx1p (1 μ M) and/or BiP (1 μ M) and/or IgG (D) were incubated with *Photinus pyralis* luciferase (1 μ M) at 42 °C for 10 min to provoke thermal denaturation of the substrate. Aggregates were separated from soluble proteins by centrifugation, migrated on a SDS/12% polyacrylamide gel, stained with Coomassie Blue, and the band intensity was quantified by densitometry. Reactions were carried out in the presence or absence of the following factors: ATP or ADP (5 mM), and MgCl₂ (5 mM). The background solubility of luciferase in the absence of chaperone was subtracted in each case. Results are expressed as the percentage of soluble luciferase with respect to total input, and are the means for three independent experiments.

folding substrates both via lectin–oligosaccharide and peptide–peptide contacts [9,10,16,17].

Next, WT and mutant calnexin/Cnx1p were tested for cooperativity with BiP, using the same thermal-aggregation assay. No significant synergetic interaction between the two chaperones was observed with or without cofactors. Nevertheless, the combination of BiP with sCnx1p increased the proportion of soluble substrate to about 80%, as compared with 40% and 68% for BiP and sCnx1p alone respectively. When rabbit IgG was added to calnexin and to BiP, a 10% increase in the soluble fraction of luciferase was observed in both cases, pointing to a crowding effect caused by the higher concentration of proteins in the assay rather than actual chaperoning (Figure 1D). Consequently, the calnexin/Cnx1p mutants s Δ hcd_Cnx1p and smini_Cnx1p were not able to enhance the ability of BiP to prevent aggregation of luciferase, because the observed augmentation in the prevention of aggregation was also obtained using rabbit IgG. These results support the notion that, *in vivo*, fission yeast expressing Δ hcd_Cnx1p or mini_Cnx1p are able to survive without the chaperone function of calnexin/Cnx1p. Based on crystallographic and *in vitro* data, these mutants are also devoid of the determinants implicated in oligosaccharide binding [12,13], indicating

further that *S. pombe* is able to live without the calnexin–lectin function.

Secreted heterologous model proteins are accurately glycosylated in fission yeast calnexin mutants

To explore the role of calnexin/Cnx1p in the secretion process of fission yeast, several *cnx1* mutant strains were constructed (see Figure 1A), and the effect of these mutations was tested on three heterologous model proteins. Because WT calnexin/Cnx1p is glycosylated in *S. pombe* [14,15], whereas recombinant sCnx1p used in our *in vitro* assays is not, it was of interest to assess whether this post-translational modification had an effect on protein secretion. To this effect, the mutant Δ gly_cnx1 was constructed in which the codon for Asn⁴¹⁸ was changed to a triplet encoding the neutral residue alanine. To study a possible effect of the sub-localization of calnexin/Cnx1p within the ER, a soluble version was constructed, lum_cnx1. A second version of the latter mutant was also constructed, lumadel_cnx1, in which the encoded lumADEL_Cnx1p contains the ADEL (Ala-Asp-Glu-Leu) ER-retention signal of fission yeast at its C-terminus [32]. Mutants Δ hcd_cnx1 in which the region encoding hcd has been deleted, and

mini_cnx1 spanning the 52 luminal amino acids of the C-terminus linked to the transmembrane and cytosolic domains have been described previously [18] (see also Figure 1A). All mutants grew at rates similar to the WT *cnx1*⁺ at 30 °C in minimal medium (results not shown), indicating that they did not severely affect the physiology of *S. pombe* under these conditions.

The levels of the different calnexin/Cnx1p were evaluated by Western blotting in the mutant *S. pombe* strains (Figure 2A). While WT, Δ gly_cnx1, lum_cnx1 and lumadel_cnx1 strains contained similar amounts of calnexin, the Δ hcd_cnx1 and mini_cnx1 strains contained a much lower quantity as shown by the exposure times required to obtain equivalent signals. Because these mutants are heavily truncated forms of the WT calnexin/Cnx1p, the diminution in the signal could have been caused by a loss of available epitopes. The same ratios were obtained in strains expressing WT-Cnx1p and mini-Cnx1p fused to a *c-myc* epitope (results not shown). Thus these particular mutants appear to be more susceptible to mRNA or protein degradation. Therefore, in addition to an absent calnexin chaperone function in the Δ hcd_cnx1 and mini_cnx1 strains, these cells also contain lower amounts of calnexin/Cnx1p molecules.

To assess the impact of calnexin/Cnx1p mutations on glycoprotein secretion, two glycosylated enzymes, rabbit sNEP [17] and cellulase (Cell1p) from *Aspergillus aculeatus*, were expressed in all strains (Figure 2C). Calnexin has been shown to act efficiently as a chaperone in the folding of non-glycosylated proteins *in vitro* ([31]; and the present study), and inhibitors of glucose trimming [16,17] did not preclude the interaction between fission yeast calnexin/Cnx1p and glycoprotein substrates. Therefore it was of interest to explore a possible effect of calnexin/Cnx1p on the secretion of non-glycosylated proteins, thus HSA was also used as model protein (Figure 2C). Because of the link between glycoprotein folding and glycosylation (reviewed in [33]), and because the studied glycoproteins are heterologous to fission yeast, the N-glycosylation state of all the model proteins was first assessed by endoglycosidase H digestion. sNEP and Cell1p appeared as being correctly modified *in vivo* as demonstrated by the shift in migration of the proteins after endoglycosidase H digestion (Figure 2D). As expected, HSA, which does not contain any potential asparagine glycosylation-acceptor sites [34], was not glycosylated (Figure 2D). Hence, glycosylation of the model glycoproteins sNEP and Cell1p was not noticeably affected in the *cnx1* mutant strains.

Increased secretion of fungal cellulase in strains lacking the hcd of calnexin/Cnx1p

The secretion efficiency in the *cnx1* mutant strains was evaluated by Western blotting as the ratio of secreted/intracellular model proteins. No major variation in the secretion efficiency of sNEP and HSA could be seen in all tested strains. On the other hand, the mutants lumadel_cnx1, Δ hcd_cnx1 and mini_cnx1 secreted more cellulase than the WT, Δ gly_cnx1 and lum_cnx1 strains. This increase in secretion was greater for the Δ hcd_cnx1 strain (approx. 5-fold compared with the WT strain) than for the mini_cnx1 strain (approx. 4-fold compared with the WT strain). Thus neither the glycosylation of calnexin/Cnx1p, nor its membrane anchoring drastically affect the secretion of sNEP or HSA. However, the soluble form of calnexin/Cnx1p bearing an ER-retention signal exhibited a 2-fold increase in cellulase secretion, suggesting that, for certain proteins, ER-export may be affected by the physical accessibility of this chaperone. In addition, the overall secretion of endogenous proteins was increased substantially (approx. 5 times higher than the WT strain) in the Δ hcd_cnx1 and mini_cnx1

Figure 2 Quantification of calnexin and BiP and evaluation of the glycosylation state of heterologous proteins in mutant and WT *cnx1* *S. pombe* strains

(A) Evaluation of the intracellular amount of calnexin/Cnx1p and BiP. Cell extracts (20 μ g) were fractionated on an SDS/12% polyacrylamide gel, and analysed by immunoblotting with polyclonal antibodies against BiP and calnexin/Cnx1p. BiP level ratios compared with the WT strain (level defined as 1) were calculated by densitometry using QuantityOne (Bio-Rad), and are indicated below the anti-BiP Western blot. (B) Levels of BiP and PDI mRNA. Total RNA (15 μ g) was prepared, fractionated on a 1.5% agarose/formaldehyde gel, transferred on to a nylon membrane and probed for BiP and PDI mRNA, as described by Elagöz et al. [18]. The band corresponding to the 26 S rRNA stained with ethidium bromide is shown as a loading control. Ratios of mRNA levels compared with the WT strain (level defined as 1), normalized to the 26 S rRNA level were calculated by densitometry using QuantityOne, and are indicated below the BiP and PDI Northern blots. (C) Schematic representation of secreted model proteins. SP, signal peptide; tree, potential glycosylation sites. (D) Analysis of glycosylation of model proteins. Cell extracts (20 μ g) were either treated or not with endoglycosidase H_i as described in the Experimental section. After incubation, the material was resolved by SDS/PAGE (12% gels) and subsequently immunoblotted with the corresponding antibodies, as denoted.

strains confirming the fact that the retention capacity of calnexin is greatly reduced in these strains (results not shown).

Several studies have reported that altering the levels of ER chaperones entails divergent effects on the secretion of heterologous proteins [19]. On one hand, overexpression of calnexin

Figure 3 Secretion and quality control efficiencies of *cnx1* mutants

(A) Secretion efficiency was calculated by dividing the amount of secreted proteins by the total amount of produced proteins (intracellular + secreted). A volume of 3 ml of spent medium from exponentially growing cells (D_{600} of 0.8) expressing the secreted model proteins was concentrated 30-fold using Microsep 10K Omega columns (Pall Life Sciences, Sydney, Australia). The concentrated supernatant and 20 μ g of cell extracts were loaded on an SDS/12% polyacrylamide gel, and subsequently quantified by Western blotting to evaluate the intracellular and extracellular levels of the model proteins. (B) Quality-control efficiency was evaluated by dividing the activity of sNEP and cellulase (see the Experimental section) by the total amount of secreted model proteins. The secretion and quality-control efficiencies of the WT strain were arbitrarily set at 1.0.

or BiP were shown to increase secretion of some heterologous proteins [21,35]. On the other hand, overexpression of BiP can also result in a reduction of the secretion efficiency of other model proteins [28]. One could speculate that, in the case of cellulase, the rate-limiting step in its secretion might be its retention by calnexin/Cnx1p and/or calnexin/Cnx1p-associated factors in the ER, whereas in the case of HSA and sNEP, removal of the chaperone function of calnexin/Cnx1p reduces their secretion slightly because folding is the determining factor in their secretion.

Quality control of protein folding is not severely affected in strains lacking the hcd of calnexin/Cnx1p

Next, the efficiency of quality control was estimated in mutants devoid of the folding/retention functions of calnexin/Cnx1p by determining the ratio of activity/quantity of secreted model enzymes cellulase and sNEP (Figure 3B). No drastic variations could be observed in the activity/quantity ratio of sNEP secreted by the *cnx1* mutants as compared with that of the WT strain. Nevertheless, in the case of cellulase, a slight reduction of the activity/quantity ratio was observed, and this roughly correlated with an increase in secretion efficiency. This hinted that the UPR (unfolded protein response) could be compensating for the lack of calnexin chaperone function in some of our strains. Indeed, we observed an increased level of BiP in the *lum_cnx1*, *lumadel_cnx1*, *Δhcd_cnx1* and *mini_cnx1* mutants compared with the WT and *Δgly_cnx1* strain (Figure 2A, lower panel). An augmentation in BiP and PDI (protein disulphide isomerase) mRNA levels confirmed that the UPR was moderately turned on in these strains

Figure 4 Interaction of calnexin/Cnx1p with glycosylated and non-glycosylated substrates

Calnexin/Cnx1p association with (A) Cel1p, (B) HSA and (C) sNEP was evaluated after treating exponentially growing cells (D_{600} of 0.8) with 10 mM DTT (dithiothreitol) for 2 h. Cells were then washed twice and resuspended in fresh medium containing 2 mM cycloheximide. Samples were removed at various time points and immunoprecipitations were performed with anti-Cnx1p antibodies, and bound model proteins were detected by immunoblotting using the appropriate antibodies as described in the Experimental section.

(Figure 2B). It is noteworthy that an active UPR was also detected in calreticulin-deficient fibroblasts [36]. Therefore an increased level of chaperones, such as BiP, combined with the functional redundancy of other factors involved in the folding, retention and targeting to degradation of proteins could be responsible for the maintenance of a functional quality-control machinery in the ER of the *cnx1* mutants studied.

The *Δhcd_cnx1* and *mini_cnx1* mutants are affected in the retention of the glycoprotein cellulase

To exert their function, chaperones interact transiently with their substrates, in binding–release cycles, freeing them once the newly synthesized proteins have acquired their mature structure. Nevertheless, it has been observed that some misfolded substrates can remain stably bound to chaperones awaiting release by other factors for their eventual correct folding or degradation [4]. To gain additional insight on the cause of the divergence of protein-secretion efficiency for the various heterologous proteins, we evaluated the length of the interaction of WT_Cnx1p, *Δhcd_Cnx1p*, and *mini_Cnx1p* with the model proteins. The mutant strains *Δgly_cnx1*, *lum_cnx1* and *lumadel_cnx1* were not analysed further because their secretion efficiency was similar to that of the WT. To perform this experiment, the cells were first treated with the reducing agent DTT (dithiothreitol) to trigger the accumulation of misfolded nascent proteins in the ER, then they were incubated with the translational inhibitor cycloheximide, and samples were collected at several time points to observe release of the model protein if the interaction is transient. The interaction of calnexin/Cnx1p with the substrates was evaluated by immunoprecipitating the chaperone and subsequently blotting against the substrates. Figure 4(A) shows that the interaction of cellulase with WT calnexin/Cnx1p was constant for 180 min following cycloheximide treatment. In contrast, no co-immunoprecipitation of cellulase could be observed with the mutants which, *in vitro*, lack chaperone activity. As shown previously, BiP interacts with the last 52 residues of the luminal domain of calnexin/Cnx1p and co-immunoprecipitates with *Δhcd_Cnx1p* and

mini-Cnx1p mutants [18]. Hence, whereas WT calnexin/Cnx1p retained at least a fraction of cellulase in the ER for the time evaluated, the hcd-deleted calnexin/Cnx1p mutants, which *in vitro* lack chaperone activity, were unable to do so. These observations would correlate the increase in secretion of cellulase with an abrogated interaction of this model protein with the truncated mutants of calnexin/Cnx1p.

HSA did co-precipitate with WT calnexin, indicating that this chaperone can interact, directly or indirectly, with non-glycosylated folding proteins. The amount of HSA co-precipitated with WT calnexin/Cnx1p lessened with time (Figure 4B). The amount of BiP found in complex with WT remained constant, indicating that the reduction in HSA levels observed was not due to a diminution in calnexin content. However, the amount of this model protein precipitated with the hcd-deleted calnexin/Cnx1p mutants remained apparently constant during 180 min (Figure 4B). Thus, whereas HSA appeared to bind and release from WT calnexin/Cnx1p, this model protein seemed to be retained by Δ hcd-Cnx1p and mini-Cnx1p. Interestingly, the secretion of HSA was slightly diminished in *cnx1* mutant strains (Figure 3A), hinting at a possible correlation between binding of this model protein to calnexin/Cnx1p mutants and reduced protein secretion. In the case of HSA, a functional calnexin appears to be required for optimal secretion efficiency. In contrast, there is a diminution in the interaction between sNEP and the hcd-truncated calnexin/Cnx1p mutants (Figure 4C), but no detectable increase in secretion was observed (Figure 3A). This could be due to the interaction of sNEP with other components of the quality-control machinery after release from the hcd-deleted calnexin/Cnx1p mutants, since there are higher levels of ER chaperones in the *cnx1* mutant strains due to UPR induction. Moreover, since Δ hcd-Cnx1p and mini-Cnx1p are devoid of lectin activity, it is possible that the residual productive interaction observed between truncated mutants of calnexin/Cnx1p and HSA or sNEP, occurs via BiP which is found in a stable association with calnexin/Cnx1p (right-hand panels of Figure 4). Such co-operative interactions among ER chaperones have been well documented [16,30,37,38].

Concluding remarks

In the present study, we have established that *S. pombe* calnexin acts as a classical chaperone *in vitro*, whereas mutants lacking the hcd are devoid of this activity. Since hcd-deleted *cnx1* mutants are viable, these observations indicate that the essentiality of calnexin/Cnx1p for viability is not in its chaperone activity (peptide interactions), nor in its lectin role, but in another yet to be determined function. We have shown previously that calnexin/Cnx1p interacts with BiP independently of translation [16]. Δ hcd-Cnx1p and mini-Cnx1p are able to form stable complexes with BiP (Figure 4), thus mapping the essential function of calnexin/Cnx1p and the site of interaction with BiP to the last 52 residues of the luminal domain of calnexin/Cnx1p. Therefore it is tempting to speculate that the essential function of calnexin/Cnx1p in the fission yeast could be in the binding of BiP and/or the scaffolding of other ER folding/quality-control components. Interestingly, the equivalent of the 52-residue region of the *Yarrowia lipolytica* calnexin homologue was reported to interact with the β subunit of the Sec61 translocon [39], stressing further the importance of this calnexin region.

Although the chaperone function and substrate specificity of calnexin/calreticulin have been investigated intensively in the past, the specific role of these chaperones in the secretion process remains poorly understood. Recent papers studied the effects of calnexin/calreticulin overexpression on the secretion efficiency of various heterologous proteins in different organisms and ob-

tained contradictory results [21,22,40]. Similarly, in the present study with three model proteins, we observed that strains devoid of the calnexin/Cnx1p chaperone function exhibited an important increase in the secretion of fungal cellulase and in overall endogenous proteins, whereas secretion of rabbit sNEP and HSA was not affected or slightly reduced. It is interesting to consider that among our model proteins studied here, cellulase is the only fungal protein and is also the only one showing an increase in its secretion. On the other hand, mammalian proteins for which folding in the ER of *S. pombe* is rate-limiting might require additional chaperone content in order to improve their secretion. Our studies may provide hints for future attempts at secreting large quantities of heterologous proteins in fungal hosts.

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