Calnexin and BiP Interact with Acid Phosphatase Independently of Glucose Trimming and Reglucosylation in *Schizosaccharomyces pombe*†

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ABSTRACT: The association of newly synthesized glycoproteins with the ER molecular chaperones calnexin and immunoglobulin binding protein (BiP) has been well documented in a variety of higher eukaryotes. Here we report that Cnx1p, the calnexin homologue in *Schizosaccharomyces pombe*, associates with newly synthesized molecules of the secreted glycoprotein acid phosphatase. Unlike ligand binding to mammalian calnexin, glucose trimming and reglucosylation of acid phosphatase by UDP-Glc:glycoprotein glucosyltransferase were shown to be dispensable for its binding to Cnx1p. Thus, despite the essentiality of Cnx1p for *S. pombe* viability, the glucose trimming and reglucosylation cycle does not appear to be required for protein folding in the fission yeast. The association of core-glycosylated acid phosphatase with Cnx1p after exposure of cells to heat shock or to DTT was shown to be reversible. However, Cnx1p stably associated with unglycosylated acid phosphatase after treatment with the core-glycosylation inhibitor tunicamycin. BiP was found to coprecipitate with Cnx1p, under normal and stress conditions, and following inhibition of protein synthesis by cycloheximide. We postulate that Cnx1p and BiP are part of a complex that is involved in the folding of both core-glycosylated trimmed ligands and unglycosylated proteins.

The molecular chaperone calnexin is a Ca^{2+}-binding, type I endoplasmic reticulum (ER)[1] membrane protein that assists glycoproteins during folding, and it also retains misfolded/partially folded glycoproteins (*1*−*3*). In mammalian cells, addition of the α-glucosidase inhibitors castanospermine and 1-deoxynojirimycin, as well as the glycosylation inhibitor tunicamycin, abrogates ligand binding to calnexin, demonstrating that calnexin has a lectin-like activity with a unique selectivity for monoglucosylated glycoproteins *(2, 4*−*10)*. The same α-glucosidase inhibitors also prevent dissociation of already bound transmembrane protein hemagglutinin from calnexin, suggesting that release from calnexin requires removal of the innermost glucose by glucosidase II (*9*). Therefore, the alternation in the glucosylation state of glycoproteins results in repeated binding and release of different glycoproteins to calnexin until correct folding or oligomerization is accomplished (*11*). These observations on calnexin−ligand interaction, in mammalian cells, have led to a model in which the action of calnexin and reglucosylation by the enzyme UDP-Glc:glycoprotein glucosyltransferase (GT) constitute key elements of the cellular machinery known as "quality control of protein folding in the ER" (*12, 13*). The mechanism of quality control ensures proper folding and assembly of proteins and retains misfolded and incompletely folded proteins in the ER until they are ultimately degraded (*1, 8, 9, 13, 14*). Certainly, in addition to the calnexin−GT system, other molecular chaperones such as BiP participate in this control process of protein folding (for review, see *3, 11, 15*).

Numerous publications have lent support to the aforementioned model for calnexin action. However, another set of reports has shown that calnexin also associates with unglycosylated proteins such as the T-cell receptor ϵ, an unglycosylated mutant of P-glycoprotein, the major histocompatibility complex (MHC) class I and II chains, and gp80 of MDCK cells (*16*−*20*). Further studies suggested that the initial interaction between calnexin and newly translocated MHC I, a glycoprotein, may occur via binding of the $Glc_1Man_9GlcNAc_2$ oligosaccharide. This first binding would be followed by interactions between their respective polypeptide segments, and perhaps through exposed hydrophobic patches, in a mechanism akin to that of BiP (*5, 6, 21*−*25*). This and other experiments have shown that calnexin−ligand complexes remain stable even after the *N*-linked glycans have been severed from the polypeptide chain, suggesting that even though a lectin-like interaction may be essential to

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[1] Abbreviations: BiP, immunoglobulin binding protein; Gal, galactose; GT, UDP-Glc:glycoprotein glucosyltransferase; DNJ, 1-deoxynojirimycin; ER, endoplasmic reticulum; Endo H, endoglycosidase H; Glc, glucose; IAA, iodoacetamide; Man, manose; MHC, major histocompatibility complex; GlcNac, *N*-acetylglucosamine; mAb, monoclonal antibody; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; VSV-G, vesicular stomatitis virus-glycoprotein.

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establish the binary complex, subsequent steps may involve protein−protein interactions (*6*, *20*, *25*). Furthermore, Cannon et al. (*26*) have presented evidence that unglycosylated VSV-G protein binds to calnexin in a glycan-independent fashion, in large protein aggregates. Other studies have also demonstrated the sequential interaction of BiP and calnexin during folding of thyroglobulin (*27*) and the folding of the VSV-G glycoprotein (*28*). Thus, it appears that the mechanisms of calnexin interaction with its substrates are manifold, and calnexin cooperates with other molecular chaperones of the ER in the folding of proteins.

The molecular cloning and the initial characterization of calnexin (Cnx1p), BiP, and GT (Gpt1p) from *Schizosaccharomyces pombe* have been described (*29*−*32*). Cnx1p and BiP have been shown to be essential for viability in *S. pombe*, whereas Gpt1p is dispensable for cell life (*32*). From these observations it is then possible to infer the existence in *S. pombe* of a GT-independent pathway of protein folding and quality control. The availability of the genes encoding the *S. pombe* homologues of BiP, calnexin, and GT, as well as the striking similarities of this organism to mammalian cells in terms of gene expression, protein glycosylation, and protein secretion (*30*, *33*−*36*), make this fission yeast an ideal model for the genetic study of the mechanisms involved in the quality control of protein folding in the ER. It is noteworthy that since no GT inhibitors are available as yet, the use of *S. pombe* renders possible the exploration of the cellular role(s) of this enzyme in a genetically depleted organism.

To gain a better understanding of the role of calnexin in the mechanisms of protein folding, in this paper we have addressed the characterization of the Cnx1p−ligand interaction by studying its association with newly synthesized *S. pombe* acid phosphatase, a secreted glycoprotein encoded by *pho4* (*37*). The interaction between Cnx1p and Pho4p was examined under different stress conditions that induce the misfolding of nascent proteins in the ER. As calnexin and BiP have been described to act sequentially in the mammalian ER (*27*, *28*), we therefore have also investigated the possible interaction of BiP with Cnx1p and Pho4p. Furthermore, because of the important role of reglucosylation in the binding of ligands to mammalian calnexin, we examined the interactions of Pho4p with Cnx1p in a Gpt1p-deficient background.

MATERIALS AND METHODS

Strains and Media. YE and minimal medium (MM) were prepared as described (*34*). Nonsulfate medium (NSM) is the same as MM except that all sulfate salts in the "salt stock" and in the "mineral stock" were replaced with Cl^- salts. *S. pombe* SP556 (h^+ *ade*6-M216 *ura*4-D18 *leu*1−32) was used throughout this study, except where indicated. *S. pombe* transformations were performed by the lithium acetate procedure (*34*). *E. coli* strain XL1-Blue MRF′ was employed for all cloning procedures. The protease-deficient *E. coli* strain AP401 [*lon*::mini tet^R, *ara*$^-$, Δ*lac-pro*, *nal*A, *arg*E am, *rif*^{R}, *thi*I (F′, *pro* AB, *lac*I^q Z M15)] was used for the production of recombinant Cnx1p.

Overexpression of Cnx1p and Production of Antibodies. Recombinant Cnx1p was overproduced in *E. coli* strain AP401 as a fusion protein with a hexahistidine tag. For this purpose, two oligonucleotides were used to amplify the 1.7 kb long genomic calnexin coding sequence. The first primer (PCARPUR1 = 5′-CGG GGA TC**C** CTT GCT GAT C-3′) was used to create a *Bam*HI restriction site at position 55 bp (the restriction site is underlined with the mutagenized base shown in boldface type), deleting the predicted signal sequence of Cnx1p, and the second primer (PCARPUR2 = 5′-TCA TTA GTC TTC ATT CTT C-3′) was used to create a blunt end in the amplification product, right after the two stop codons at positions 1681 and 1684 bp (*29*). The PCR product was isolated and digested with *Bam*HI and cloned into vector pH6EX3 digested with *Bam*HI and *Sma*I (*38*). Induction and purification of histidine-tagged Cnx1p were carried out as described previously (*39*). Two rabbits were used to produce antibodies against Cnx1p. The first immunization was done with 100 μg of recombinant Cnx1p per rabbit, and three boosts of 50 μg/each were done at 2, 4, and 6 weeks, according to a published protocol (*40*). The production of rabbit anti-*S. pombe* BiP antibodies and mAb 7B4 against *S. pombe* acid phosphatase has been described elsewhere (*37*, *41*).

Plasmid Construction. For the construction of plasmid pREP-41X-Pho4-9E10, plasmid pEVP11-pho4-9E10 (*30*) was first digested with the restriction enzyme *Sac*I, blunt-ended with T4 DNA polymerase, and then digested with *Bam*HI. The vector pREP-41X (*42*, *43*) was first digested with *Xho*I, blunt-ended with T4 DNA polymerase, digested with *Bam*HI, and used to subclone the pho4-9E10 DNA fragment.

Confocal Microscopy. Confocal immunofluorescence, using anti-Cnx1p or anti-BiP antibodies diluted 1:100, was carried out exactly as previously described (*41*).

Immunoprecipitations. Haploid *S. pombe* cells (SP556) harboring either pEVP11-pho4-9E10 or pREP-41X-Pho4-9E10 were grown in minimal medium +Ade+Ura-Leu [MM-Leu; Moreno (*34*)]. Exponentially growing cells (2×10^8 cells per immunoprecipitation) were chilled on ice, collected, and washed once in ice-cold 1× PBS containing 10 mM NaN_3. The pellet was resuspended in 150 μL of immunoprecipitation buffer [IP: 50 mM HEPES, pH 7.0, 50 mM NaCl, 10 mM iodoacetamide (IAA), 1% NP-40, 1 mM PMSF, 10 μg/mL of each aprotinin (leupeptin, chymostatin, bestatin, pepstatin A)], and glass beads were added to 0.5 volume of cell suspension. Cells were disrupted by vortexing 10 times, at high speed, for 30 s followed by 30 s incubation on ice for each time. Ice-cold IP buffer was added (800 μL) to each tube, mixed, and microfuged for 5 min in the cold. The supernatant (800 μL at 2 μg/μL) was then transferred to an ice-cold fresh tube. To eliminate unspecific complexes (preclearing), the cell extracts were incubated with protein A−Sepharose (50 μL of a 10% suspension prepared in IP buffer) at 4 °C, for 30 min, and the supernatants were transferred to a fresh tube. Antibody was added (at a 1:200 dilution), and rocked for 1 h, at 4 °C. Protein A−Sepharose (50 μL of a 10% suspension prepared in IP buffer) was added and incubation continued for 1 more h. Beads were collected by centrifugation for 3 min in the cold, and washed 5 times with 1 mL each time of ice-cold IP buffer. Where indicated, RIPA buffer [50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 10 μg/mL of each aprotinin (leupeptin, chymostatin, bestatin, pepstatin A)] was used for

immunoprecipitation. The equivalent of the whole immunoprecipitation reaction was loaded and resolved by 10% SDS−PAGE (except where another acrylamide percentage is indicated). Immunoprecipitated proteins were visualized by Western blotting.

^{35}S in Vivo Labeling of Proteins. *S. pombe* cells were grown overnight in NSM containing 200 μM ammonium sulfate until the culture reached an OD_{600} of 1. From this culture, 2×10^8 cells (1 mL of culture at $OD_{600} = 2.5 \times 10^7$ cells) were spun down and washed with NSM. The cells were resuspended in 1 mL of NSM containing 200 μCi of Translabel ^{35}S labeling mix (^{35}S-Cys and ^{35}S-Met; ICN), and the cells were labeled for 3 h, following which 5 μL of cysteine and methionine (each at 5 mg/mL) was added to the medium and incubated further for 10 min. Cells were placed on ice, and NaN_3 was added to a final concentration of 10 mM, spun down, and washed with 1 mL of ice-cold PBS containing 10 mM NaN_3. Lysates were prepared by breaking the cells with glass beads as described above.

Western Blotting. Immunoblots were carried out essentially as described earlier (*44*) except that antigen bands were detected by chemiluminiscence with the Amersham ECL kit.

Characterization of Oligosaccharides. In vivo labeling of *S. pombe* cells with [^{14}C]glucose and characterization of ^{14}C-labeled, glycoprotein-associated oligosaccharides were carried out essentially as described in (*45*). Endo H-sensitive oligosaccharides were resolved by paper chromatography (Whatman1, solvent 1: 1-propanol/nitromethane/water, 5:2:4), using as standards $Glc_3Man_9GlcNac$, $Glc_2Man_9GlcNac$, $Glc_1Man_9GlcNac$, $Man_9GlcNac$, and $Man_8GlcNac$. Oligosaccharides comigrating with the standards $Glc_2Man_9GlcNac$, $Glc_1Man_9GlcNac$, and $Man_9GlcNac$ were eluted and subsequently subjected to strong acid hydrolysis, and their composition was analyzed on paper chromatography (Whatman1, solvent 2: 1-butanol/pyridine, 10:3:3), using as standards galactose, glucose, and mannose.

RESULTS

Cnx1p Associates with Newly Synthesized Proteins. To examine whether Cnx1p behaves in a similar manner as mammalian calnexin, we metabolically labeled *S. pombe* cells with [^{35}S]methionine/cysteine and carried out immunoprecipitations with anti-Cnx1p antibodies. As is the case for their mammalian counterparts, numerous proteins coimmunoprecipitated with Cnx1p (Figure 1, lane 3). This interaction is specific since preimmune serum fails to precipitate any protein (Figure 1, lane 1), and no other polypeptide, besides Cnx1p, was detected when anti-Cnx1p antibodies were used under conditions disrupting protein−protein interactions (RIPA buffer; see Figure 1, lane 2). Moreover, the use of preimmune serum showed that these proteins do indeed coprecipitate with Cnx1p rather than sedimenting due to unspecific aggregation.

To investigate in detail the association of Cnx1p with glycoproteins, we used acid phosphatase (Pho4p) as a model polypeptide. Acid phosphatase is the best-characterized secreted protein in *S. pombe*, and it contains nine potential Asn-linked glycosylation sites. The unglycosylated form of acid phosphatase migrates on SDS−PAGE with an apparent mobility of 56 kDa, whereas the ER core-glycosylated form migrates as a 76 kDa protein band. Yet, the post-ER

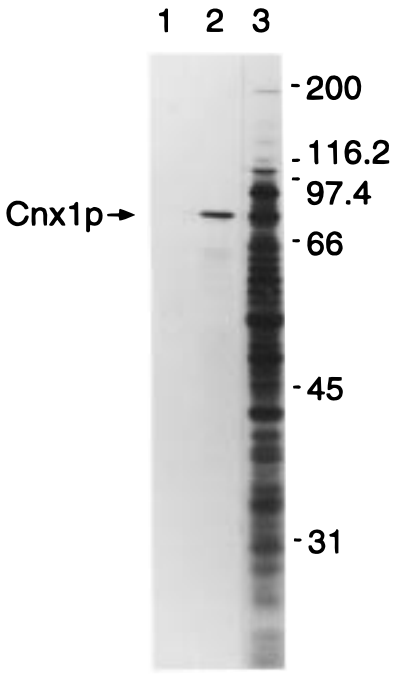

FIGURE 1: Coimmunoprecipitation of *S. pombe* cellular proteins with Cnx1p. *S. pombe* cells were metabolically labeled with ^{35}S-translabel and immunoprecipitated with preimmune rabbit serum in IP buffer (lane 1) or with anti-Cnx1p antibodies in RIPA buffer (lane 2) or in IP buffer (lane 3), as described under Materials and Methods. Immunoprecipitated material was fractionated on a 12% SDS−polyacrylamide gel, and exposed overnight at room temperature.

glycosylated species are larger and heterogeneous in size (*37*). Pho4p also contains at least one intrachain disulfide bond (Jannatipour and Rokeach, unpublished data). To facilitate its tracking, we utilized a *c-myc* epitope-tagged Pho4p. This tagged version of Pho4p has been previously shown to be correctly folded and secreted (*30*). The *c-myc* tag allowed us to immunoprecipite Pho4p and perform Western blots with the monoclonal antibody 9E10. Accordingly, for the analysis of the interaction between Cnx1p and Pho4p, cells expressing the *c-myc* Pho4p were subjected to stress conditions known to produce unfolded proteins in the ER. The corresponding cell lysates were prepared, and immunoprecipitation was carried out with anti-Cnx1p, and the presence of Pho4p in the immune complexes was detected on immunoblots with the mAb 9E10.

As shown in Figure 2 (panel A, lane 1; panel B, lane 3), core-glycosylated Pho4p (76 kDa) did bind to Cnx1p under control conditions. The amount of Pho4p precipitated with Cnx1p appeared to increase when the cells were exposed to heat shock, DTT, or β-mercaptoethanol, i.e., conditions where unfolded proteins accumulate in the ER (Figure 2A, lanes 2, 3, and 4, respectively). No post-ER forms of Pho4p bound to Cnx1p (see, for instance, Figure 8A, lane CE). It should be noted that the amount of Pho4p precipitated under control conditions is variable (compare Figures 2 and 8). Most likely, this reflects variations in Pho4p expression. In mammalian cells, the addition of glucosidase inhibitors such as castanospermine and 1-deoxynojirimycin inhibits glucose trimming and consequently binding to calnexin. Analysis of Asn-linked oligosaccharides provided evidence that 1-deoxynojirimycin indeed inhibits glucose trimming in the

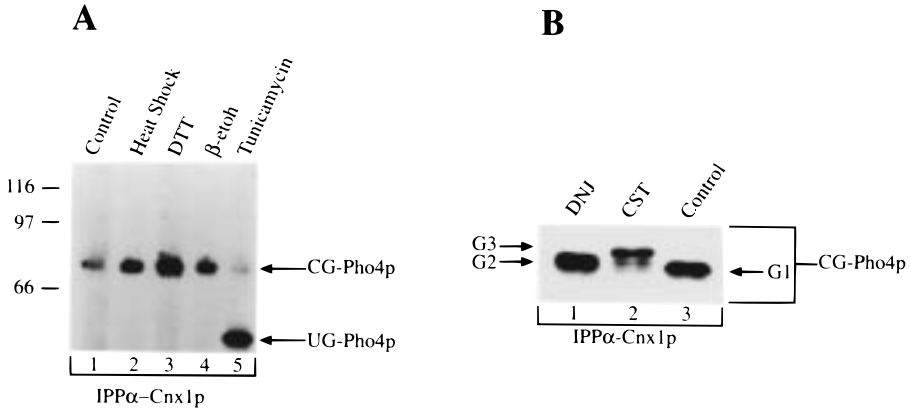

FIGURE 2: Interaction of Pho4p with Cnx1p and with BiP under various stress conditions. Panel A: Exponentially growing *S. pombe* cells expressing Pho4p tagged with the *c-myc* epitope were either incubated as control at 30 °C (lane 1) or exposed to heat shock (39 °C; lane 2), 10 mM DTT (lane 3), 15 mM β-mercaptoethanol (lane 4), or 10 μg/mL tunicamycin (lane 5), for 2 h at 30 °C. Panel B: Exponentially growing *S. pombe* cells expressing *c-myc*-tagged Pho4p were incubated for 2 h at 30 °C in the absence (lane 3) or the presence of either 1 mM castanospermine (lane 2) or 1 mM 1-deoxynojirimycin (lane1). Cell extracts were immunoprecipitated with anti-Cnx1p (2×10^8 cells in panel A, and 4×10^8 cells in panel B). Immunoprecipitated material was fractionated by SDS−PAGE (panel A, 10%; panel B, 12%), and immunoblotted with mAb 9E10 for the detection of Pho4p. UG-Pho4p and CG-Pho4p indicate the unglycosylated and core-glycosylated forms of Pho4p, respectively. G1, G2, and G3 denote the mono-, bi-, and triglucosylated forms of Pho4p, respectively. The G1 band may contain also nonglucosylated Pho4p (G0: $Man_9GlcNac$) which we cannot distinguish from G1 in this assay.

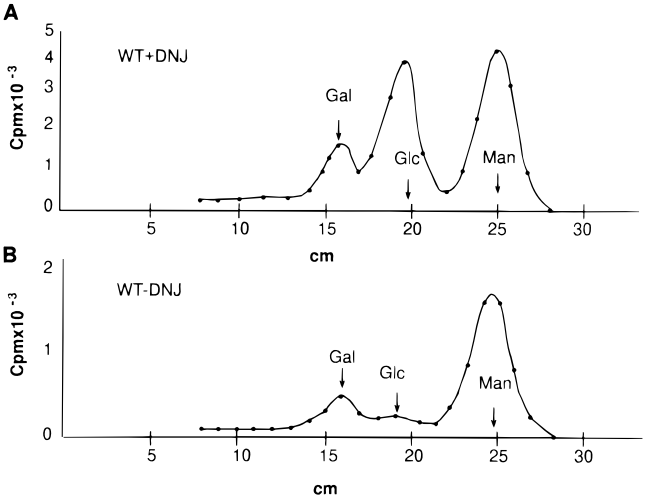

FIGURE 3: Inhibition of glucose trimming in *S. pombe* by 1-deoxynojirimycin. Wild-type *S. pombe* cells (PRY208, obtained from Dr. Phillips Robbins) were grown in the presence (+DNJ) and in the absence (−DNJ) of 6 mM 1-deoxynojirimycin, and metabolically labeled with [^{14}C]glucose, as previously described (*45*). The protein-linked oligosaccharides were liberated with endo-β-*N*-endoglycosidase H (endo H) and run on paper chromatography. The ^{14}C-labeled oligosaccharides comigrating with the standards $Glc_2Man_9GlcNac$, $Glc_1Man_9GlcNac$, and $Man_9GlcNac$ were eluted and subjected to strong acid hydrolysis. The composition of the eluted oligosaccharides was analyzed on paper chromatography using as standards glucose (Glc), mannose (Man), and galactose (Gal). Positions of monosaccharide standards are indicated with arrows.

fission yeast as shown in Figure 3. To assess the effect of castanospermine and 1-deoxynojirimycin on the Cnx1p−Pho4p interaction, *S. pombe* cells were grown in the presence of these glucosidase inhibitors. As shown in Figure 2B (lanes 1 and 2), under these conditions, Cnx1p was able to bind untrimmed forms of Pho4p. Therefore, in *S. pombe* calnexin can effectively bind glycoproteins containing two or three terminal glucose residues on their oligosaccharide moieties.

The glycosylation inhibitor tunicamycin precludes the binding of mammalian calnexin to most of its ligands (*46*). Nevertheless, in *S. pombe*, the presence of tunicamycin in the culture medium resulted in the binding of Cnx1p to both the unglycosylated form (56 kDa) and the residual amount of the core-glycosylated Pho4p species (76 kDa). Thus, the Cnx1p−Pho4p interaction can also occur in the absence of carbohydrate on the ligand protein (see also Figure 6B), as it was reported for mammalian calnexin (*16*, *17*, *26*).

BiP Coprecipitates with Cnx1p and Acid Phosphatase. Mammalian BiP has also been shown to associate transiently with many proteins in early stages of folding, retaining more tightly misfolded proteins and incompletely assembled oligomers (*13*, *47*). In addition, it has been reported that BiP and calnexin can sequentially bind to the same nascent polypeptides during their folding process (*27*, *28*).

To explore the possible interaction of Pho4p with BiP, we carried out the same experiment as described in Figure 2A, using anti-BiP antibodies in immunoprecipitation and the 9E10 mAb for the detection of Pho4p on Western blot. As depicted in Figure 4A, core-glycosylated Pho4p interacted with *S. pombe* BiP in a similar manner as it did with Cnx1p, and the amount of Pho4p associated with BiP augmented with treatments that lead to the accumulation of unfolded proteins in the ER (i.e., heat shock, DTT). Likewise, *S. pombe* BiP associates with the unglycosylated form of Pho4p (56 kDa) when cells were exposed to tunicamycin (Figure 4A, lane 5).

To test the association of Cnx1p with BiP, *S. pombe* cells were subjected to the same growth conditions as in Figure 2A, and immunoprecipitations were carried out with anti-Cnx1p, and Western blots were carried out with anti-BiP antibodies (Figure 4B). BiP was found to coprecipitate with Cnx1p in lysates from cells grown under control conditions (30 °C) as well as from cells exposed to stress.

To examine whether the coprecipitation of BiP with Cnx1p was due to their simultaneous binding to the same nascent polypeptides or to a more direct interaction between Cnx1p and BiP, we performed the immunoprecipitation experiment in the absence of translation. Accordingly, fission yeast cells were cultured in the presence of the translation inhibitor cycloheximide, and samples were analyzed at different time

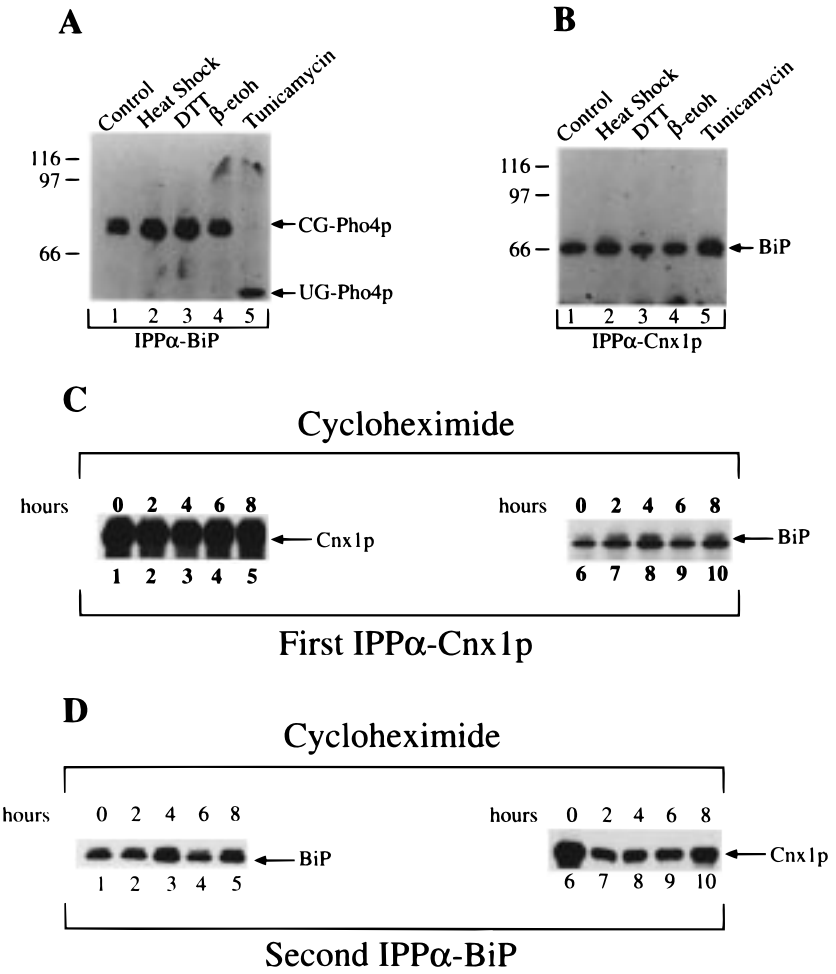

FIGURE 4: *S. pombe* BiP and Cnx1p are found in a complex. Panels A and B: Exponentially growing *S. pombe* cells expressing *c-myc*-tagged Pho4p were either incubated as control at 30 °C (lanes 1) or exposed to heat shock (39 °C; lanes 2), 10 mM DTT (lanes 3), 15 mM β-mercaptoethanol (lanes 4), or 10 μg/mL tunicamycin (lanes 5), for 2 h at 30 °C. Cell extracts were immunoprecipitated with anti-BiP antibodies (panel A) or anti-Cnx1p (panel B). The immunoprecipitated proteins were resolved on a 10% SDS−polyacrylamide gel and immunoblotted either with the 9E10 mAb to detect Pho4p (panel A) or with anti-BiP antibodies (panel B). UG-Pho4p and CG-Pho4p indicate the unglycosylated and core-glycosylated forms of Pho4p, respectively. Panel C: Exponentially growing *S. pombe* cells expressing *c-myc*-tagged Pho4p were incubated at 30 °C in the absence (lanes 1 and 6) or the presence of 2 mM cycloheximide to inhibit protein synthesis (lanes 2−5 and 7−10). Samples were taken at different time points as indicated and subjected to immunoprecipitation with anti-Cnx1p antibodies. Immunoprecipitates were resolved by SDS−PAGE and immunoblotted for the presence of Cnx1p (lanes 1−5) or BiP (lanes 6−10). Panel D: The supernatants of the immunoprecipitations in panel C were precipitated with anti-BiP antibodies. Immunoprecipitates were resolved by SDS−PAGE and immunoblotted for the presence of BiP (lanes 1−5) or Cnx1p (lanes 6−10).

points. Cnx1p was immunoprecipitated, and the presence of BiP was investigated by Western blotting. As shown in Figure 4C (lanes 6−10), BiP coprecipitated with Cnx1p even after 8 h of cycloheximide treatment. This observation would suggest that at least a certain fraction of BiP molecules are complexed with Cnx1p. To estimate the proportion of BiP complexed to Cnx1p, we performed this first immunoprecipitation of Cnx1p (shown in Figure 4C) with a 12-fold larger volume of anti-Cnx1p serum, as compared to other precipitations. However, this did not exhaust all the calnexin in the sample. Therefore, the supernatants of the immunoprecipitations depicted in Figure 4C were subsequently subjected to precipitation with anti-BiP antibodies, and the presence of bound Cnx1p was tested by Western blotting. As shown in Figure 4D (lanes 6−10), indeed Cnx1p coprecipitated with BiP which remained in the supernatants of the first immunoprecipitation.

It should be noted that since the turnover of Cnx1p and BiP is slow, even after 2 h of stress treatment the majority of the molecules of these two proteins remain native. See for instance in Figure 4B that the proportion of glycosylated BiP remains unchanged. Thus, the interactions with Pho4p we observed after the stress treatments are not the result of misfolded Cnx1p with misfolded BiP with misfolded ligand. Although stress treatments for a period of 2 h might seem excessive, the cells nevertheless remain viable. Likewise, despite Cnx1p itself being a glycoprotein, inhibition of glycosylation with tunicamycin should not affect its function, as *S. pombe* cells carrying a glycosylation mutant of Cnx1p are perfectly viable (our unpublished results).

The association of glycosylated Pho4p with Cnx1p is reversible whereas unglycosylated Pho4p forms a tight persistent complex.

To begin the characterization of the Pho4p−Cnx1p complex, we first washed the anti-Cnx1p immune complexes under stringent conditions (RIPA buffer). While the core-glycosylated Pho4p−Cnx1p complexes formed in control cells and in cells exposed to DTT or heat shock (not shown) disengaged in RIPA buffer, the association between unglycosylated Pho4p (56 kDa) with Cnx1p and with BiP persisted

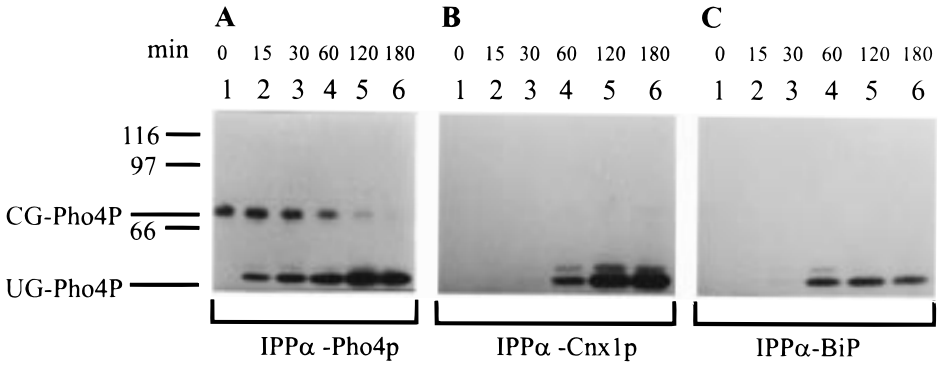

FIGURE 5: Effect of tunicamycin on the Pho4p−Cnx1p interaction. Exponentially growing *S. pombe* cells expressing Pho4p tagged with the *c-myc* epitope were grown either in the absence (lane 1) or in the presence of 10 μg/mL tunicamycin at 30 °C (lanes 2−6). Aliquots of cells exposed to tunicamycin were collected at 15, 30, 60, 120, and 180 min (lanes 2−6). Cell extracts were immunoprecipitated, using RIPA buffer, with mAb 7B4 for acid phosphatase (panel A), anti-Cnx1p antibodies (panel B), or anti-BiP antibodies (panel C). Immunoprecipitated material was fractionated on a 10% SDS−polyacrylamide gel and immunoblotted for Pho4p with the mAb 9E10.

(see Figure 5, panels B and C). Although, core-glycosylated Pho4p (76 kDa) was present in the cells at early time points after the addition of tunicamycin, the interaction with Cnx1p or BiP was disrupted when the immunoprecipitations were done in RIPA (compare panels B and C of Figure 5 with panel A). The tight complex formed between unglycosylated Pho4p (56 kDa) and Cnx1p and BiP may be due to the presence of large aggregates of unfolded polypeptides produced following tunicamycin treatment, as previously observed in mammalian cells (*26*).

To further our analysis of the Pho4p−Cnx1p interaction, we assessed the fate of the complex after the stress treatments were reversed by returning the cells to normal growth conditions. Accordingly, *S. pombe* cells producing Pho4p were exposed to heat shock, DTT, or tunicamycin for 2 h, following which the cells were washed and grown in fresh medium containing cycloheximide to inhibit de novo synthesis of proteins. The fate of the Pho4p−Cnx1p association was followed by taking samples at various time points, immunoprecipitating with anti-Cnx1p, and detecting the bound Pho4p with the 9E10 mAb. The association between core-glycosylated Pho4p (76 kDa) was reversible after removal of the stresses due to DTT (Figure 6B) or heat shock (not shown). In contrast, after removal of tunicamycin, the amount of unglycosylated Pho4p (56 kDa) bound to Cnx1p remained practically unchanged over a period of 2 h, even when the precipitation was carried out in RIPA buffer (see Figure 6A). Taken together, these observations further support the notion of the formation of large aggregates, involving Pho4p and Cnx1p, which are resilient to dissociation.

Binding of Pho4p to Cnx1p Is Independent of Reglucosylation by Gpt1p. Cells genetically depleted of UDP-Glc:glycoprotein glucosyltransferase display a viable phenotype (*32*). Moreover, confocal immunofluorescence of formaldehyde-fixed, Triton-permeabilized *S. pombe* spheroplasts prepared from both wild-type and *gpt1*Δ cells (Figure 7) showed the typical Cnx1p and BiP localization to the nuclear envelope and peripheral endoplasmic reticulum, as previously described (*41*). Thus, overall no systematic differences could be observed between wild-type and *gpt1*Δ cells. Taken together, these observations imply that reglucosylation of glycoproteins by Gpt1p may act as a spare, nonessential, mechanism for the binding of ligands to Cnx1p. To explore

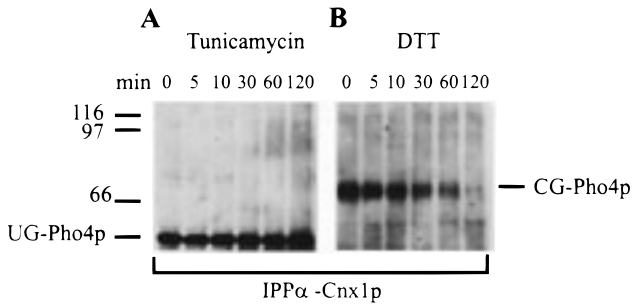

FIGURE 6: Dissociation of Pho4p from Cnx1p. Exponentially growing *S. pombe* cells expressing Pho4p tagged with the *c-myc* epitope were grown exponentially at 30 °C and then exposed for 2 h either to 10 mM DTT at 30 °C (panel A) or to heat shock at 39 °C (panel B). Subsequently, cells were collected and resuspended in fresh prewarmed media containing 2 mM cycloheximide to inhibit de novo synthesis of proteins, and incubation was continued at 30 °C. Aliquots of cells were taken at 0, 5, 10, 30, 60, and 120 min (lanes 1−6). Cell extracts were immunoprecipitated with anti-Cnx1p antibodies; immunoprecipitated material was fractionated on a 10% SDS−polyacrylamide gel and immunoblotted for Pho4p with the mAb 9E10.

this point, *c-myc*-tagged Pho4p was expressed in *gpt1*Δ cells. Both wild-type and *gpt1*$^-$ cells were grown exponentially and subjected to heat shock, DTT, tunicamycin, and 1-deoxynojirimycin treatments, as described above. Precipitation with anti-Cnx1p was followed by detection of coimmunoprecipitated Pho4p on Western blots with the anti-*c-myc* mAb 9E10. The anti-Cnx1p precipitation patterns of Pho4p in the *gpt1*-deleted background were indistinguishable from those of Pho4p expressed in *gpt1*$^+$ cells (Figure 8, panels A and B). Here again, inhibition of glucose removal by treatment with 1-deoxynojirimycin or castanospermine did not ostensibly affect the binding of Pho4p to Cnx1p (Figure 8, panel C). These results therefore show that the Cnx1p−Pho4p interactions are not dependent on reglucosylation by UDP-Glc:glycoprotein glucosyltransferase. Nevertheless, although we consider this very unlikely, the fact that the Cnx1p−Pho4p interactions are refractive to *gpt1* deletion may reflect some unique properties of Pho4p.

Further, we wished to examine whether the Cnx1p−BiP interaction was affected in cells lacking UDP-Glc:glycoprotein glucosyltransferase. To this aim, we carried out coimmunoprecipitation experiments (as described in Figure 4, panels C and D), at different time points, after the addition

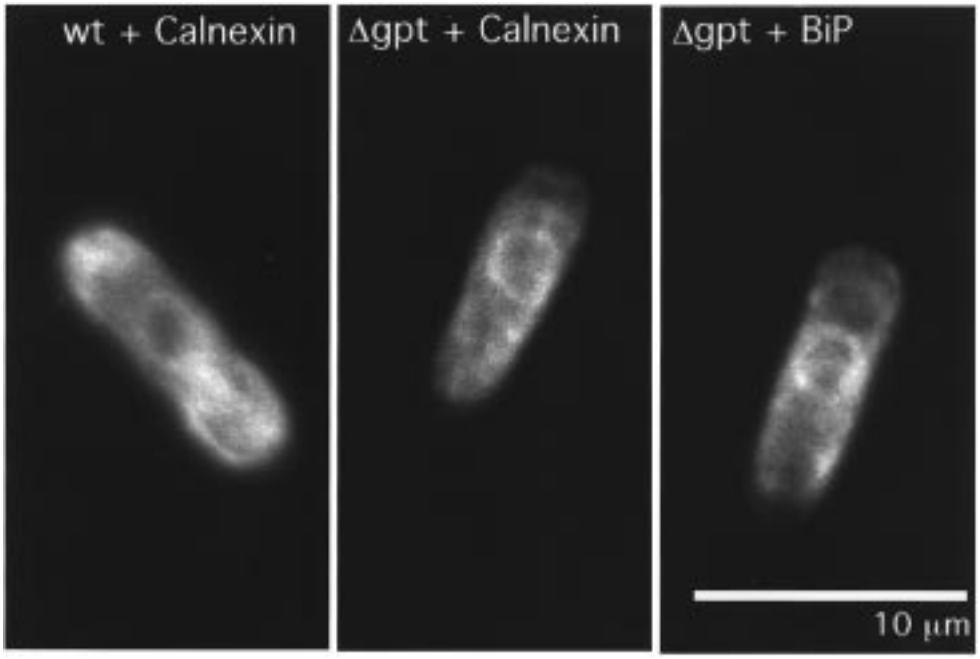

FIGURE 7: Confocal immunofluorescence of *gpt1*$^+$ and *gpt1*$^-$ *S. pombe* cells. *S. pombe* spheroplasts from wild type (wt + Calnexin) or *gpt1*$^-$ (Δ*gpt1* + Calnexin; Δ*gpt1* + BiP) were Triton-permeabilized and formaldehyde-fixed. Immunostaining was with anti-Cnx1p (+Calnexin) or anti-BiP (+BiP) antibodies, as previously described (*41*). Bar: 10 μm.

of cycloheximide to a culture of *gpt1*Δ cells. As shown in Figure 8 (panels D and E), also in the *gpt1*$^-$ background the Cnx1p−BiP complex persisted when translation was inhibited.

DISCUSSION

The striking structural similarities between *S. pombe* and mammalian calnexin (*29, 31*) suggested that Cnx1p would share with its higher eukaryotic homologue comparable ligand selectivity and mode of action. The results presented in this paper show that indeed Cnx1p displays many functional features that are characteristic of mammalian calnexin. First, akin to its mammalian homologues, Cnx1p does interact with numerous nascent polypeptides, suggesting a role of the *S. pombe* calnexin in the folding of newly synthesized proteins in the ER. Second, consistent with a possible role of Cnx1p in the quality control of protein folding, the amount of core-glycosylated acid phosphatase (Pho4p) coprecipitated with Cnx1p was shown to increase when cells were exposed to heat shock and DTT, i.e., conditions causing the accumulation of misfolded proteins in the ER. Third, although initial reports described the ligand binding to mammalian calnexin as being glycan-dependent (for reviews, see *1*−*3*), subsequent publications demonstrated that calnexin can also interact with at least certain proteins lacking their glycans as a result of mutagenesis or treatment with the glycosylation inhibitor tunicamycin (*16*−*20, 27*). In this regard, Cannon et al. (*26*) have shown that calnexin interacts with nonglycosylated VSV-G protein via the formation of high molecular weight aggregates. Based on these results, the authors suggested that this latter type of interaction could explain the binding of mammalian calnexin to nonglycosylated forms of proteins such as thyroglobulin, P-glycoprotein, and MHC I and II molecules. Similarly, our results showed that following exposure to tunicamycin Cnx1p forms a tight persistent complex with acid phosphatase, whereas the Pho4p−Cnx1p interaction was reversible following removal of the heat-shock and DTT stresses. We propose that this complex is of the same nature as the aggregates observed by Cannon et al. (*26*). Furthermore, even though the 56 kDa unglycosylated form of Pho4p was present early upon treatment with tunicamycin (Figure 5, lane 2), it only became tightly bound to Cnx1p at later times where aggregation may have occurred (Figure 5, lanes 4−6).

In contrast with mammalian calnexin, the glucosidase inhibitors 1-deoxynojirimycin or castanospermine did not disrupt the binding of core-glycosylated Pho4p to Cnx1p. In the same vein, Cnx1p did interact with core-glycosylated Pho4p in *S. pombe* cells genetically depleted of UDP-Glc:glycoprotein glucosyltransferase. Thus, from these results it seems that the biochemical behavior of Cnx1p departs from that of mammalian calnexin in its ligand selectivity, i.e., glycoproteins with monoglucosylated glycans resulting from the glucose trimming cycle. However, our observation that BiP constantly coprecipitates with Cnx1p under normal and stress conditions, even in the absence of protein synthesis, may provide an alternative explanation. Thus, the simplest interpretation to the selectivity of Cnx1p would be that nascent nonglycosylated proteins precipitate with anti-Cnx1p antibodies because these substrates are bound to BiP, and this in turn is associated with Cnx1p in a tight complex. Evidence for multichaperone complexes involving calnexin, calreticulin, BiP, and GRP94 has been presented in mammalian cells (*48*). Likewise, Crofts et al. (*49*) described, in tobacco cells, a calreticulin−BiP complex that is independent of ER stress. Thus, chaperone complexes appear to be ubiquitous in eukaryotic cells.

Our working model is that Cnx1p and BiP are part of a complex and they function in a coordinated fashion. Akin to its mammalian counterpart, Cnx1p would initially bind to glycoproteins in a monoglucosylated glycan fashion and subsequently the ligand−Cnx1p association could occur

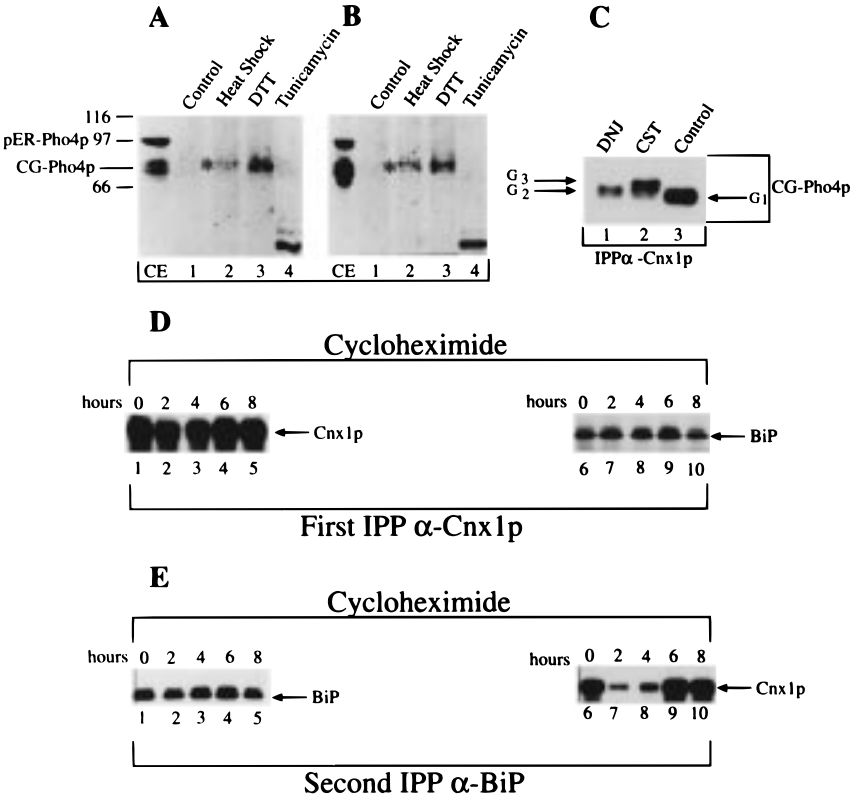

FIGURE 8: Analysis of the Cnx1p−Pho4p and Cnx1p−BiP interactions in *S. pombe* cells lacking UDP-Glc:glycoprotein glucosyltransferase (*gpt1*Δ). Exponentially growing *gpt1*$^+$ *S. pombe* cells (panel A) or *gpt1*$^-$ cells (panel B) expressing *c-myc*-tagged Pho4p were either incubated as control at 30 °C (lane 1) or exposed to heat shock (39 °C; lane 2), 10 mM DTT (lane 3), or 10 μg/mL tunicamycin (lane 4) for 2 h at 30 °C. Panel C: Exponentially growing *S. pombe* cells expressing *c-myc*-tagged Pho4p were incubated at 30 °C for 2 h in the absence (lane 3) or the presence of either 1 mM castanospermine (lane 2) or 1 mM 1-deoxynojirimycin (lane 1). Cell extracts were immunoprecipitated with anti-Cnx1p (2×10^8 cells in panels A and B, and 4×10^8 cells in panel C). Immunoprecipitated material was fractionated by SDS−PAGE (panels A and B, 10%; panel C, 12%) and immunoblotted with mAb 9E10 for the detection of Pho4p. UG-Pho4p and CG-Pho4p indicate the unglycosylated and core-glycosylated forms of Pho4p, respectively. G1, G2, and G3 denote the non/mono-, bi-, and triglucosylated forms of Pho4p, respectively. Note the larger molecular weight species corresponding to the post-ER form (pER-Pho4p) of glycosylated acid phosphatase in the total cell extract loaded in lane CE. The position of the core-glycosylated Pho4p band in the control lane is indicated with an asterisk (*). Panel D: Exponentially growing *gpt1*Δ *S. pombe* cells expressing *c-myc*-tagged Pho4p were incubated at 30 °C in the absence (lanes 1 and 6) or the presence of 2 mM cycloheximide to inhibit protein synthesis (lanes 2−5 and 7−10). Samples were taken at different time points as indicated and subjected to immunoprecipitation with anti-Cnx1p antibodies. Immunoprecipitates were resolved by SDS−PAGE and immunoblotted for the presence of Cnx1p (lanes 1−5) or BiP (lanes 6−10). Panel E: The supernatants of the immunoprecipitations in panel D were precipitated with anti-BiP antibodies. Immunoprecipitates were resolved by SDS−PAGE and immunoblotted for the presence of BiP (lanes 1−5) or Cnx1p (lanes 6−10).

through protein−protein interactions. As Cnx1p and BiP are associated in a complex, the latter would concomitantly interact with other regions of the same folding polypeptide, which would undergo numerous binding−release cycles to the Cnx1p−Bip complex until the correct tridimensional structure is achieved. In this scenario, core-glycosylated ligands would associate with Cnx1p in a glycan and glucose-trimming dependent fashion, and hence in the same manner as was observed for mammalian calnexin (reviewed in *1*−*3*). However, we cannot rule out the possibility that Cnx1p could also interact with untrimmed glycoproteins and native nonglycosylated substrates that would be first bound by BiP. In this case, the Cnx1p−ligand association would occur in a protein−protein fashion, probably through exposed hydrophobic segments on the folding polypeptide.

The Cnx1p−BiP arrangement proposed above may explain the fact that UDP-Glc:glycoprotein glucosyltransferase is not essential in *S. pombe* (*32*), while the deletion of either the BiP or the Cnx1p encoding genes entails cell death (*29*, *31*, *41*). Thus, reglucosylation of glycoproteins appears as a dispensable mechanism in the quality control of protein folding in the *S. pombe* ER. Supporting this notion is the observation that no detectable reglucosylation of proteins occurs, by alternative enzymes, in the fission yeast with a *gpt1*Δ genetic background (*32*). In the same vein, it is then possible to infer that the essentiality of calnexin for *S. pombe* viability resides in a function different than that involved in the binding of monoglucosylated ligands. Interestingly, in a recent publication Simons et al. (*50*) have elegantly demonstrated that in *S. cerevisiae* the ER glucosidases I and II, along with BiP, are involved in 1,6-β-glucan synthesis. Furthermore, these authors have shown that budding-yeast cells defective in glucose trimming are not affected in protein folding and secretion.

Finally, although whether Cnx1p is an authentic chaperone or a lectin remains to be clarified, it is possible to envisage that a complex setup such as Cnx1p−BiP could render polypeptide folding more efficient and therefore better suited for the rapid rates of the yeast's protein synthesis and secretion processes. Future studies will concentrate on testing the model proposed in this paper.

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