Cell Surface Expression of the Prion Protein in Yeast Does Not Alter Copper Utilization Phenotypes

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Received for publication, March 5, 2004, and in revised form, April 15, 2004
Published, JBC Papers in Press, April 16, 2004, DOI 10.1074/jbc.M402517200

Prion diseases are fatal neurodegenerative disorders that result from conversion of a normal, cell surface glycoprotein (PrP<sup>C</sup>) into a conformationally altered isoform (PrP<sup>Sc</sup>) that is thought to be infectious. Although a great deal is known about the role of PrP<sup>Sc</sup> in the disease process, the physiological function of PrP<sup>C</sup> has remained enigmatic. In this report, we have used the yeast Saccharomyces cerevisiae to test one hypothesized function of PrP<sup>C</sup>, as a receptor for the uptake or efflux of copper ions. We first modified the PrP signal peptide by replacing its hydrophobic core with the signal sequence from the yeast protein dipeptidyl aminopeptidase B, so that the resulting protein was targeted cotranslationally to the secretory pathway when synthesized in yeast. PrP molecules with the modified signal peptide were efficiently glycosylated, glycolipid-anchored, and localized to the plasma membrane. We then tested whether PrP expression altered the growth deficiency phenotypes of yeast strains harboring deletions in genes that encode key components of copper utilization pathways, including transporters, chaperones, pumps, reductases, and cuproenzymes. We found that PrP did not rescue any of these mutant phenotypes, arguing against a direct role for the protein in copper utilization. Our results provide further clarification of the physiological function of PrP<sup>C</sup>, and lay the groundwork for using PrP-expressing yeast to study other aspects of prion biology.

Prion diseases, or transmissible spongiform encephalopathies, are fatal neurodegenerative disorders of humans and animals. These diseases are caused by conversion of an endogenous, neuronal, membrane glycoprotein (PrP<sup>C</sup>) into a conformationally altered isoform (PrP<sup>Sc</sup>) that appears to be infectious in the absence of nucleic acid (1, 2). Although a great deal has been learned about the PrP<sup>C</sup>-PrP<sup>Sc</sup> conversion event and the role of PrP<sup>Sc</sup> in the disease process, the normal function of PrP<sup>C</sup> has remained enigmatic. Identifying the function of PrP<sup>C</sup> may provide important clues to pathogenic mechanisms, because there is evidence that PrP<sup>C</sup>, in addition to serving as a precursor of PrP<sup>Sc</sup>, acts as a mediator of PrP<sup>Sc</sup>-induced neurotoxicity (3–5). A variety of functions have been proposed for PrP<sup>C</sup>, including roles in metal ion trafficking (6), protection from oxidative stress and apoptosis (7, 8), cell adhesion (9), and transmembrane signaling (10). However, the evidence in favor of each of these hypothesized functions is not definitive. In addition, attempts to deduce the function of PrP<sup>C</sup> from the phenotypes of PrP-null mice have been unrewarding. Lines of these mice, in which the gene encoding Doppel (a PrP paralog) is not artificially up-regulated, display no major anatomical or developmental deficits (11, 12), and the subtle phenotypic abnormalities that have been described in some of these lines have been controversial (13, 14).

In an effort to uncover the function of PrP<sup>C</sup>, we have been employing a novel approach, which makes use of the baker’s yeast, Saccharomyces cerevisiae. Previous studies of prion biology have generally utilized model systems involving cultured mammalian cells or whole animals. Our strategy has been to express PrP in yeast in such a way that the protein is posttranslationally processed and subcellularly localized as it is in mammalian cells. Then, we test the effect of PrP expression on the phenotype of wild-type yeast strains, or strains that harbor deletions of genes underlying a particular physiological pathway in which we hypothesize PrP is involved. Although the yeast genome does not encode a structural homologue of PrP, this organism may nevertheless express proteins that can interact physically and functionally with PrP to produce an observable phenotype. In addition, yeast contain proteins that are unrelated in primary sequence to PrP, but that display all the essential properties of prions, demonstrating strong evolutionary conservation of the cellular and molecular mechanisms underlying prion-related conformational changes (15). These considerations, combined with facile genetics and ease of experimental manipulation, make yeast a potentially attractive model system for analysis of PrP<sup>C</sup> function, and possibly the mechanism of PrP<sup>Sc</sup> formation.

A proposed function of PrP<sup>C</sup> that is particularly amenable to investigation in yeast is the role of the protein in copper physiology. Much of what we know about cellular utilization of copper ions in eukaryotes is derived from studies of S. cerevisiae (16–18). Key transporters, reductases, chaperones, and cuproenzymes have been identified by genetic and biochemical means, and mutant yeast strains are available that are defective in various steps of copper uptake and delivery. In addition, there is strict conservation of copper handling pathways in yeast and mammalian cells. Almost all the proteins known to be involved in copper utilization in yeast have identified counterparts in mammals, and these homologues can substitute functionally for the yeast proteins. Finally, alterations in copper metabolism produce easily detectable, non-lethal pheno-
types in yeast, making it relatively simple to assay the effects of expressed proteins.

Several lines of evidence have emerged within the past few years suggesting a connection between PrP(C) and copper ions (19, 20). Probably the most widely agreed upon fact is that PrP(C) binds copper ions. Cu2+ binds to the histidine-containing octapeptide repeats with low micromolar affinity in a pH-dependent fashion, and there is evidence for more C-terminal binding sites as well (21–24). A second important clue has emerged from studies of the cellular trafficking of PrP(C) (33). Copper ion at concentrations ≥ 100 μM rapidly and reversibly stimulate clathrin-mediated endocytosis of PrP(C) (6, 25, 26), and we have postulated that PrP(C) could function as a recycling receptor that plays a role in the cellular uptake or efflux of copper ions. A final set of observations has suggested a connection between PrP(C), copper ions, and protection of cells from oxidative stress. For example, recombinant PrP refolded in the presence of copper has been reported to exhibit a superoxide dismutase (SOD) activity (27). In addition, a number of studies indicate that the absence of PrP(C) results in increased sensitivity of neurons to several kinds of oxidative insults, a phenotype that may depend on binding of copper ions to PrP(C) (28, 29). It has also been reported that the brains of Prn-p10 mice have a reduced content of ionic copper, and decreased enzymatic activity of Cu-Zn SOD (29, 30), although these findings have not been reproduced in other laboratories (31, 32).

In the present study, we report on our use of S. cerevisiae to study the role of PrP(C) in copper metabolism. In the first step of the work, we modified the PrP molecule so that it was efficiently targeted to the secretory pathway in yeast. We then tested whether PrP expression affected the phenotypes of yeast strains deleted for various genes involved in copper utilization. Our results shed light on the role of PrP(C) in copper physiology, and they lay the groundwork for using yeast expressing PrP to study other aspects of prion biology.

EXPERIMENTAL PROCEDURES

Yeast Strains—Yeast strains were created by the Saccharomyces Deletion Project, and were obtained from Research Genetics (Huntsville, AL). The wild-type yeast strains used were BY4742 (MATa his3Δ1 leu2Δ1 lys2Δ0 ura3Δ0) and BY4743 (MATa a his3Δ1 leu2Δ1 ura3Δ0). These strains are derived from S288C, which carries a disrupted CTR3 gene (33). The following deletion strains were used: Cox17Δ (BY4743 background); SOD2 and SOD1 (BY4742 background). Yeast were grown at 30 °C on rich medium containing glucose (YPD), rich medium containing glycerol (YPG), or synthetic complete media (SC) minus uracil.

To create PrP-(1–254), wild-type mouse PrP containing the 3F4 epitope (35) was amplified by PCR using the following primers: CGGATCCATGTTGTTTAAATTTTCC (sense); GGACCGCAATTTTCA-CCACGAGAAGAA (antisense). The PCR product was cloned into the BamHI and HindIII sites of pVT102U. Yeast cells were transformed by electroporation (36).

Preparation of Yeast Microsomes and Analysis of PrP Topology—Yeast microsomes were prepared by sucrose gradient centrifugation (38). The membrane topology of PrP was analyzed by a protease K (PK) protection assay, performed as described previously for mammalian microsomes (39).

Expression of PrP in Yeast

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Endo H and α-Mannosidase Treatments—Yeast extracts prepared as described above were incubated with endo H (New England Biolabs, Beverly MA) according to the manufacturer’s directions. Some samples were then treated for 16 h at 37 °C with 50 units/ml of jack bean α-mannosidase (Glyko, Novato, CA) in the presence of 2 mM ZnSO4. Samples were subjected to anti-PrP Western blots.

To create DPAPB-PrP254, we utilized the following PCR primers: CCACGGCAATTTTCA-CCACGAGAAGAA (antisense). The PCR product was cloned into the BamH1 and XbaI sites of pVT102U. Yeast cells were transformed by electroporation.
Expression of PrP in Yeast

RESULTS

Targeting of PrP to the Yeast Secretory Pathway—In order to use yeast as a model system to study the function of PrP, it is first necessary to target the protein to the secretory pathway so that it is glycosylated, GPI-anchored, and localized to the plasma membrane, as it is in mammalian cells. The primary sequence of PrP contains two signals that play a role in its biosynthesis: an N-terminal signal peptide of 22 amino acids that is responsible for cotranslational import into the ER, and a C-terminal signal peptide of 23 amino acids that is required for attachment of the GPI anchor. We first tried to express PrP in yeast with its endogenous N- and C-terminal signal peptides (Fig. 1, PrP1–254). A single PrP band of 30 kDa was observed on SDS-PAGE, and this band was not shifted when samples were treated with endo H (Fig. 2A, lane 6 and data not shown). This result indicates that native PrP is not significantly glycosylated when expressed in yeast, possibly because it remains in the cytoplasm and does not enter the secretory pathway. PrP also remained unglycosylated when its N-terminal signal sequence was replaced with those of either yeast prepro-α-factor or Gas1p (data not shown).

In mammalian cells, almost all secretory proteins enter the ER cotranslationally via an SRP-dependent mechanism (41). In yeast, however, two pathways for ER targeting exist: a cotranslational pathway that is SRP-dependent, and a post-translational pathway that is independent of SRP (42). A major determinant of whether a protein follows the co- or post-translational pathway is the sequence of the N-terminal signal peptide (43). We hypothesized that PrP might not enter the secretory pathway in yeast because its N-terminal signal peptide is not recognized as a co-translational ER targeting signal, resulting in synthesis of the entire polypeptide chain in the cytoplasm. In this case, the polypeptide chain might misfold and fail to enter the ER.

To target PrP to the cotranslational pathway, we replaced part of its native signal sequence with the signal anchor sequence from the yeast enzyme dipeptidyl aminopeptidase B (DPAPB), a type II transmembrane protein (44) (Fig. 1, DPAPB-PrP254). It has been shown that translocation of DPAPB is strongly dependent on SRP and that transfer of its signal anchor sequence onto the hybrid signal peptide of heterologous proteins can switch their ER targeting from the post-translational to the cotranslational mode (43). We fused the DPAPB signal anchor sequence of 16 amino acids to the last 7 amino acids of the PrP signal sequence, because computer analysis using the program SignalP (45) predicted that the resulting hybrid molecule would be cleaved by signal peptidase at the normal site utilized in endogenous PrP (between cysteine 22 and lysine 23). We also appended the first two amino acids of the prepro-α-factor signal sequence to the hybrid signal peptide, because several constructs used in previous studies had incorporated these (43).

When DPAPB-PrP254 was expressed in yeast, two major bands of 27 and 35 kDa and a minor band of 33 kDa were observed on SDS-PAGE (Fig. 2A, lane 2). The two larger bands were shifted to 27 kDa when samples were treated with endo H (Fig. 2B, lane 2), indicating that the protein was glycosylated and had thus presumably entered the secretory pathway. The 33- and 35-kDa species may represent PrP molecules carrying one or two N-linked oligosaccharide chains, respectively. These larger forms are similar in size to core-glycosylated PrP synthesized in cultured cells and in translation reactions in vitro.
Expression of PrP in Yeast

(46, 47). We presume that the unglycosylated form of DPAPB-PrP254 (27 kDa; Fig. 2A, lane 2) is smaller than unglycosylated PrP (1–254) (30 kDa; Fig. 2A, lanes 3, 6, 9, and 12) because of cleavage of the N- and C-terminal signal peptides in the former molecule.

We also tried substituting the C-terminal GPI addition signal of PrP with segments of either 32, 43, or 63 amino acids derived from the C terminus of Gas1p, a GPI-anchored yeast protein (48) (Fig. 1A, DPAPB-PrP230-Gas1p32, -43, and -63). The 43 and 63 amino acid segments of Gas1p contain a serine-rich region that is normally O-glycosylated and that would be retained in the mature protein after cleavage of the C terminus and attachment of the GPI anchor. All three PrP-Gas1p constructs produced two major bands on SDS-PAGE (Fig. 2A, lanes 3–5). Lower M, bands appeared after treatment with endo H, implying that these proteins were glycosylated (Fig. 2B, lanes 5, 8, and 11). DPAPB-PrP-Gas1p43 and DPAPB-PrP-Gas1p63, but not DPAPB-PrP-Gas1p32, were shifted even further by treatment with a combination of α-mannosidase and endo H, consistent with the presence of O-linked sugars on the longer Gas1p segments (Fig. 2B, lanes 6, 9, and 12). All three PrP-Gas1p proteins displayed a less intense second band of higher M, after treatment with endo H and α-mannosidase (Fig. 2B, lanes 6, 9, and 12), which may be the result of incomplete glycosidase digestion, or may represent molecules with uncleaved N- or C-terminal signal peptides.

The fact that PrP molecules carrying the DPAPB-PrP hybrid signal sequence were glycosylated indicates that they had entered the secretory pathway. To prove directly that the proteins were localized in the ER lumen, we carried out protease protection experiments using isolated yeast microsomes. We found that DPAPB-PrP254 and DPAPB-PrP230-Gas1p63 were both fully protected from PK digestion, indicating that the proteins were localized entirely within the ER lumen (Fig. 3, lanes 2 and 5). We did not observe any protease-protected fragments that would indicate the presence of transmembrane forms of PrP (C<sup>TM</sup>PrP or N<sup>TM</sup>PrP) (39, 47). Permeabilization of the microsomes with Triton X-100 led to complete digestion by PK (Fig. 3, lanes 3 and 6), ruling out the possibility that the proteins were intrinsically protease-resistant. As a control for the integrity of the microsomes, the ER-resident protein calnexin (90 kDa) yielded a protease-protected fragment of 70 kDa, representing the transmembrane and luminal domains of the molecule (Fig. 3, lanes 7 and 8).

Taken together, our results indicate that the DPAPB/PrP hybrid signal sequence effectively targets PrP to the yeast secretory pathway, and that this occurs with molecules carrying the endogenous, C-terminal, GPI addition signal as well as those carrying a heterologous signal derived from Gas1p. We also found that glycosylated forms of the PrP parologue, Doppel, were synthesized when the native N-terminal signal sequence was substituted with the DPAPB/PrP hybrid signal sequence (data not shown). For Doppel, however, it was necessary to utilize a C-terminal segment from Gas1p in order to obtain glycosylated products.

PrP Targeted to the Secretory Pathway Is GPI-anchored—

Yeast synthesize a number of endogenous GPI-anchored proteins, and the steps responsible for anchor attachment in yeast are broadly similar to those in mammalian cells (49). We therefore expected that PrP targeted to the secretory pathway yeast might be GPI-anchored. We used two methods to test this possibility.

First, we assayed the partitioning of PrP into the detergent and aqueous phases of a Triton X-114 extract after treatment with phosphatidylinositol-specific phospholipase C (PIPLC), a bacterial enzyme, which cleaves GPI anchors (Fig. 4A). DPAPB-PrP254, as well as DPAPB-PrP230-Gas1p32, -43, and -63, partitioned almost entirely into the detergent phase before PIPLC treatment. After phospholipase treatment, a fraction of each of the proteins was shifted into the aqueous phase, indicating that at least some of the molecules carry a GPI anchor.
That not all the protein shifted into the aqueous phase may be caused by any of several different factors, including incomplete digestion with PIPLC, inefficient phase partitioning, inaccessibility of the anchor, or the absence of the anchor on some of the molecules.

In the second set of experiments, we tested whether the PrP constructs could be metabolically labeled with \([\text{H}]\text{inositol}\) or \([\text{S}]\text{methionine}\). All four constructs incorporated this radioactive precursor (Fig. 4B, lanes 1–4). Labeling with \([\text{S}]\text{methionine}\) confirmed synthesis of similar amounts of each of the proteins (Fig. 4B, lanes 5–8). Taken together with the Triton X-114 partitioning data, these results strongly indicate that PrP targeted to the secretory pathway in yeast is GPI-anchored.

**PrP Is Expressed on the Plasma Membrane**—We immunostained spheroplasts with anti-PrP antibody to determine whether each of the constructs was present on the plasma membrane. We found that cells expressing DPAPB-PrP254, DPAPB-PrP230-Gas1p43, and DPAPB-PrP230-Gas1p63 were brightly stained on their surface compared with control cells transformed with vector alone (Fig. 5). In contrast, cells expressing DPAPB-PrP230-Gas1p32 were not stained above background levels, suggesting that the presence of \(O\)-linked sugars facilitates delivery of the protein to the plasma membrane in the context of the Gas1p GPI addition signal. In a number of repetitions of this experiment, we observed that DPAPB-PrP-Gas1p63 consistently produced a somewhat brighter surface staining pattern than the other constructs (data not shown).

**A Mutant PrP Is Protease-resistant and Detergent-insoluble When Expressed in Yeast**—To further document that PrP expressed in yeast has similar properties to PrP expressed in mammalian cells, we performed several biochemical analyses.

We have previously shown that PrP molecules carrying pathogenic mutations display PrP\(^{Sc}\)-like properties, including detergent insolubility and mild protease resistance, when expressed in cultured cells and transgenic mice (50, 51). We tested whether these biochemical properties were also present in a mutant PrP expressed in yeast. We found that DPAPB-PrP254 (PG14), which carries a 9-octapeptide PrP insertion associated with an inherited prion disease, was resistant to low concentrations of PK, producing a PrP-(27–30) fragment (Fig. 6A, lanes 5–8). In contrast, the corresponding wild-type PrP molecule was almost completely digested by the protease (Fig. 6A, lanes 1–4). We also found that DPAPB-PrP254 (PG14) was detergent-insoluble in a centrifugation assay: the 40-kDa glycosylated form was partially insoluble, and the 33-kDa unglycosylated form was completely insoluble (Fig. 6B, lanes 3 and 4). In contrast, the major glycosylated form of the wild protein (35 kDa) was completely soluble, and only the unglycosylated form (27 kDa) was insoluble. Previous studies have shown that unglycosylated wild-type PrP is detergent-insoluble in cultured cells (52). We conclude that PG14 PrP expressed in yeast displays two PrP\(^{Sc}\)-like biochemical properties.

**Expression of PrP Does Not Alter the Phenotypes of Yeast Strains Carrying Deletions of Copper Transporter Genes**—To determine whether PrP interacts at a genetic level with components of copper utilization pathways in yeast, we tested whether expression of PrP rescued the mutant phenotypes of strains lacking one or more of the genes encoding these components. We first investigated interactions with plasma membrane copper transporters. Yeast express two high affinity transporters, Ctr1p and Ctr3p, that are responsible for copper uptake from the extracellular medium under copper-limited conditions (18). Many laboratory strains, including the ones used here (BY4742 and BY4743), have a non-functional CTR3 gene because of insertion of a Ty2 transposon (33). However, when the CTR1 gene is also present, it provides sufficient copper transport activity for normal growth. Strains in which CTR1 has been deleted therefore display impaired copper uptake. This results in defective growth on non-fermentable carbon sources like glycerol, due to inadequate copper loading of mitochondrial cytochrome c oxidase. \(\Delta ctr1\) strains also show impaired growth under iron starvation conditions, since iron uptake is dependent on the activity of Fet3p, a ceruloplasmin-like ferroxidase that contains copper (53).

We tested the effect of PrP expression on both of these phenotypes in yeast strains deleted for CTR1. We found, as expected, that the wild-type yeast strain (BY4743) grew well on both YPD (glucose) and YPG (glycerol) media, while the \(\Delta ctr1\) mutant was unable to grow on YPG (Fig. 7A). Transformation of the \(\Delta ctr1\) strain with a plasmid encoding DPAPB-PrP-Gas1p63 did not rescue the growth deficiency on YPG medium (Fig. 7A). In a second test, yeast were spotted onto synthetic glucose-containing medium in the presence or absence of the iron chelator, ferrozine. The \(\Delta ctr1\) strain grew poorly in the presence of ferrozine, and this phenotype was also unaltered by expression of DPAPB-PrP-Gas1p63 (Fig. 7B). In both experiments, normal growth of the \(\Delta ctr1\) strains was restored by inclusion of 500 \(\mu\text{M}\) CuSO\(_4\) in the medium (Fig. 7, A and B). Similar results were obtained in both experiments when yeast expressed DPAPB-PrP254 (data not shown). The copper affinity of PrP is greatly reduced at acidic pH (22). Since unadjusted growth medium has a pH of 4.8–5.2, we therefore repeated these experiments with medium adjusted to pH 7.0. The results obtained were the same (data not shown). We also found that expression of DPAPB-PrP-Gas1p63 or DPAPB-PrP254 did not rescue the growth deficiency of a \(\Delta ctr1/\Delta ctr2\) strain on YPG media (data not shown). CTR2 encodes a low affinity copper transporter.
Fig. 5. PrP is present on the yeast plasma membrane. Spheroplasts made from yeast cells transformed with vector alone, or with plasmids encoding the indicated PrP constructs, were immunostained for PrP using 3F4 antibody. Cells were viewed using fluorescence optics (A–E) or Nomarski optics (F–J). Scale bar in J (applicable to all panels) is 5 μm.

Fig. 6. A mutant PrP has PrPsc-like biochemical properties when expressed in yeast. A, lysates of yeast expressing DPAPB-PrP254, either wild-type (WT) (lanes 1–4) or carrying the PG14 mutation (lanes 5–8), were digested with indicated amounts of PK. PrP was then analyzed by Western blotting using 3F4 antibody. Lanes 1 and 5 represent 200 μg of protein, and the other lanes 800 μg of protein. B, lysates of yeast expressing DPAPB-PrP254, either WT (lanes 1 and 2) or carrying the PG14 mutation (lanes 3 and 4), were centrifuged at 186,000 × g for 45 min. PrP in the supernatant fractions (lanes 1 and 3, labeled S) and pellet fractions (lanes 2 and 4, labeled P) was analyzed by Western blotting using 3F4 antibody.

did not rescue the menadione sensitivity of strains deleted for SOD1, which encodes the Cu-Zn superoxide dismutase of yeast (Fig. 9B). Thus, PrP does not significantly enhance zinc uptake or superoxide dismutase activity in yeast.

DISCUSSION

In the present study, we have used the yeast S. cerevisiae to investigate the physiological function of mammalian PrPSc. We report two major findings. First, we describe a method for efficiently targeting PrP to the secretory pathway in yeast by substituting part of the PrP signal sequence with a yeast signal sequence. Second, we use PrP-expressing yeast strains to conduct a genetic analysis of the role of PrPSc in copper and zinc metabolism, taking advantage of the fact that the pathways for utilization of these two metal ions have been extensively characterized in yeast. The results of these experiments do not support a direct role for PrPSc in cellular uptake of copper or zinc. In the future, yeast expressing PrP in the secretory pathway are likely to provide a useful experimental system to study other aspects of prion biology.

Targeting PrP to the Yeast Secretory Pathway—PrPSc normally resides on the plasma membrane of mammalian cells, and most hypotheses about the physiological function of the protein are based on this localization. It was therefore important to ensure that PrPSc was present on the plasma membrane when expressed in yeast, so as to allow us to test its functionality in this organism. We found that when PrP was expressed in yeast with its native signal sequence, or with a signal sequence from yeast prepro-α-factor or Gas1p, the protein was not glycosylated. Similar results have been reported by others using the native as well as exogenous signal sequences (62, 63).

We suspected that, in these cases, PrP had not entered the secretory pathway and instead remained in the cytoplasm in an unprocessed form. In mammalian cells, most secretory proteins, including PrP, are targeted to the ER co-translationally via binding of the signal sequence to the signal recognition particle (SRP), which temporarily arrests translation until the ribosomes docks at the ER membrane (41, 64). In contrast, yeast have two pathways for ER translocation: an SRP-dependent, cotranslational pathway and an SRP-independent, post-translational pathway (42). In the post-translational pathway, polypeptides are synthesized in the cytoplasm on membrane-free ribosomes and are imported into the ER once translation is complete. This pathway is utilized by a particular protein depends primarily on the nature of the signal sequence, in particular the hydrophobic core (h-region) of this sequence (43). Proteins with signal peptide h-regions that are highly hydrophobic (peak Kyte-Doolittle hydrophobicity index ≥ 3) are imported cotranslationally, whereas proteins with less hydrophobic h-regions (index ≤ 2) are imported post-translationally.

Since the h-region of the mouse PrP signal sequence has a peak hydrophobicity index of 1.9, it is likely that the protein would follow the SRP-independent pathway and be translated.
in the cytoplasm when expressed in yeast. This could result in misfolding of the polypeptide chain, at least in part because of failure to form the required disulfide bond in the reducing environment of the cytoplasm. The misfolded protein might then fail to be imported post-translationally into the ER. A similar phenomenon would likely occur when the signal sequence of PrP was replaced with that of prepro-/H9251-factor or Gas1p, since the latter two proteins are known to follow an SRP-independent, post-translational pathway (43).

To target PrP to the co-translational pathway, we created a chimeric signal sequence consisting of the following elements: 1) the first two amino acids of the signal sequence from yeast prepro-/H9251-factor; 2) the 16 amino acid signal anchor sequence of yeast DPAPB; and 3) the last 7 amino acids (c-region) of the native PrP signal sequence. Previous studies had shown that this region of DPAPB, which has a peak hydrophobicity index of 3.0, was sufficient to confer co-translational ER targeting on heterologous proteins (43). We found that the resulting PrP molecule (DPAPB-PrP254) was efficiently targeted to the yeast secretory pathway, as evidenced by the attachment of N-linked oligosaccharides and by protection from protease digestion in isolated microsomes.

We also created variants (DPAPB-PrP230-Gas1p32, -43, -63) in which the C-terminal, GPI attachment signal of PrP was substituted with C-terminal segments derived from Gas1p, a GPI-anchored protein of yeast. It has been shown previously that this region of Gas1p confers GPI anchoring when transferred onto heterologous proteins (65). The DPAPB-PrP230-

![Fig. 7. Expression of PrP does not rescue the growth deficiency of a Δctr1 strain on glycerol-containing or iron-depleted media.](http://www.jbc.org/)

![Fig. 8. Expression of PrP does not rescue the growth deficiency of yeast strains carrying deletions of genes encoding copper chaperones, pumps, or reductases.](http://www.jbc.org/)

![Fig. 9. Expression of PrP does not rescue the growth deficiency of yeast strains carrying deletions of zinc transporter or superoxide dismutase genes.](http://www.jbc.org/)
Gas1p constructs were also N-glycosylated and protease protected. The two longer forms, which contain a serine-rich region within the Gas1p segment, were O-glycosylated as well, indicating transit through the Golgi apparatus where elongation of O-linked mannose chains occurs in yeast (66). We showed that PrP molecules with the DPAPB chimeric signal sequence, and containing either the PrP or Gas1p C terminus, were GPI-anchored, based on Triton X-114 phase partitioning, and metabolic labeling with the anchor precursor, [3H]inositol. Finally, we demonstrated that these molecules were localized to the plasma membrane of yeast cells, based on immunofluorescence staining of spheroplasts. Taken together, our results indicate that DPAPB-PrP forms are post-translationally processed, trafficked, and localized in yeast cells similarly to native PrP in mammalian cells.

Two other studies have described expression of PrP in the secretory pathway of yeast species. Heller et al. (63) reported that PrP carrying the signal peptide from the PrP protein Kre5p was glycosylated when expressed in S. cerevisiae. Presumably, this signal sequence, which has a peak core hydrophobicity of >3, acts like DPAPB in targeting PrP to the SRP-dependent translocation pathway. However, subcellular fractionation indicated that a Kre5p-PrP chimera was localized in the vacuole, rather than on the plasma membrane. Interestingly, PrP with the Gas1p signal peptide (an SRP-independent signal) was also found to be glycosylated, but only if the PrP transmembrane domain was deleted. This result suggests that post-translational import of PrP into the ER may be inhibited by misfolding of the transmembrane region. PrP has also been expressed in the methanotropic yeast Pichia pastoris using its native signal peptide (67). In this case, the protein was glycosylated and localized to the plasma membrane. P. pastoris has a secretory apparatus that is more similar to the one in mammalian cells (68), so it is possible that the native PrP signal sequence is recognized by SRP in this species.

The Role of PrPC in Metal Ion Trafficking—A number of lines of evidence have suggested a possible role for PrPC in copper metabolism (19, 20). S. cerevisiae provides a potentially useful experimental system for studying this issue, since a great deal is known about the molecular mechanisms for copper utilization in this organism (16–18). In yeast, copper uptake begins by reduction of Cu(I) to Cu(II) by the plasma membrane reductases Fre1p and Fre2p (60). Cu(II) is then transported across the membrane by the high affinity transporters Ctr1p and Ctr3p (53), with the low affinity transporter Ctr2p contributing under conditions of high extracellular copper (54). Copper ions are subsequently transferred to one of several copper chaperones for delivery to specific target proteins in several cellular locations (55). Cox17p delivers copper to mitochondrial cytochrome c oxidase (56), Lys7p to cytoplasmic Cu-Zn SOD (57), and Atx1p to Ccc2p (58). Ccc2p is an ATP-dependent transporter that pumps copper into Golgi compartments where it is incorporated into secreted cuproproteins like Fet3p (59). Mammalian homologues of most of these copper-trafficking proteins have been identified, either by homology cloning or by functional complementation in yeast. For example, Ccc2p is homologous to the Menkes and Wilson disease proteins, and Fet3p is homologous to ceruloplasmin. These homologies imply strong conservation of copper uptake and delivery systems in yeast and mammalian cells.

In this study, we tested the effect of PrP expression on the growth phenotypes of yeast strains harboring deletions in genes that encode key components of copper utilization pathways, including transporters, chaperones, pumps, reductases, and cuproenzymes. We chose to use deletion strains for our analysis, since wild-type yeast are already very efficient at copper uptake, and thus an effect of PrP may be apparent only under circumstances where utilization of the metal is partially compromised. How the growth of these strains would be altered by PrP expression would depend on exactly how the protein is predicted to function. In one possible model, we have hypothesized that PrPC enhances the efficiency of copper uptake by acting as a recycling receptor that concentrates the metal in endocytic compartments, from which it is then transferred into the cytoplasm by transmembrane transporters (6). In this case, PrP expression would be predicted to ameliorate the mutant phenotype of strains deleted for individual CTR genes, assuming that the remaining CTR genes could still transport copper delivered by PrPC, or that other, non-CTR-dependent pathways exist. In an alternative model, we have proposed that PrP functions in copper efflux by binding metal ions that are pumped into the Golgi, and delivering them to secreted cuproproteins like ceruloplasmin (25). In this case, PrP might rescue or ameliorate the iron deficiency phenotype of Δact1 and/or Δcce2 strains, assuming that alternate pathways existed for transporting copper into secretory compartments where it could interact with PrP.

Several previous experimental results also led us to test the effect of PrP expression on the growth phenotype of yeast strains carrying deletions of ZRT1, which encodes the major high affinity zinc transporter (61), or SOD1, which encodes cytoplasmic, Cu-Zn superoxide dismutase. Although the affinity of recombinant PrP for copper is much higher than for zinc, we found that both metals are equally effective in stimulating endocytosis of PrPC (6). In addition, it has been reported that recombinant PrP exhibits a superoxide dismutase activity in the presence of copper (27), and that SOD1 activity is diminished in the brains of Prn-o/0 mice (29).

We failed to find an effect of PrP expression on the growth deficiency of any of the yeast strains we tested. Thus, our results do not support a direct role for PrPC in trafficking of either copper or zinc ions. Of course, it is possible that PrP performs some function in mammalian cells related to copper or zinc that is not detectable in yeast, perhaps because yeast lack interacting partners for PrP. Many of the mammalian proteins involved in copper and zinc metabolism are able to functionally complement mutations or deletions of the corresponding yeast proteins. However, the yeast genome does not encode a structural homologue of PrP, making it difficult to carry out a supplementation test of PrP activity in this organism. PrP homologues have been described in several non-mammalian species, including birds (69), frogs (70), turtles (71), and fish (72). Interestingly, the homologues from Xenopus laevis and Salmo salar lack the histidine-containing repeats that are postulated to bind copper in mammalian PrP (72), arguing against a universal role for copper in PrP function.

Further Studies using PrP-expressing Yeast—S. cerevisiae have been utilized for studying the molecular and cellular mechanisms underlying other neurodegenerative disorders, including Alzheimer’s, Huntington’s, and Parkinson’s diseases (73–75). Yeast expressing PrPC in the secretory pathway may prove useful for addressing several other issues in prion biology. These include the role of the proteasomal and lysosomal pathways in PrP degradation, the involvement of chaperones in PrP maturation, and the activation of stress response pathways as a result of PrP misfolding. With respect to the last point, we report here that mutant PrP molecules acquire protease-resistance when expressed in yeast, and another group has observed that abnormal forms of PrP have a toxic effect on the growth of yeast cells (63). It may also be possible to identify...
previously unknown activities and interacting partners of PrP using yeast genetic screens. Finally, it may be feasible to infect PrP-expressing yeast cells with prions, and to use them for analyzing the processes involved in PrP\textsuperscript{Sc} formation. Because yeast are capable of propagating their own, endogenous prions, they may also possess the mechanisms for catalyzing conversion of PrP\textsuperscript{Sc} to PrP\textsuperscript{Sc}.

Acknowledgments—We thank Ken Blumer for helpful advice at all stages of this project and Heather True-Krob for reading the manuscript. We also acknowledge Suzanne Wharle for performing the PK protection experiment in Fig. 3.

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J. Biol. Chem. 2004, 279:29469-29477.
doi: 10.1074/jbc.M402517200 originally published online April 16, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M402517200

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