**PNG1, a Yeast Gene Encoding a Highly Conserved Peptide: N-glycanase**

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**Abstract.** It has been proposed that cytoplasmic peptide: N-glycanase (PNGase) may be involved in the proteasome-dependent quality control machinery used to degrade newly synthesized glycoproteins that do not correctly fold in the ER. However, a lack of information about the structure of the enzyme has limited our ability to obtain insight into its precise biological function. A PNGase-defective mutant (png1-1) was identified by screening a collection of mutagenized strains for the absence of PNGase activity in cell extracts. The PNG1 gene was mapped to the left arm of chromosome XVI by genetic approaches and its open reading frame was identified. PNG1 encodes a soluble protein that, when expressed in E. scherichia coli, exhibited PNGase activity. PNG1 may be required for efficient proteasome-mediated degradation of a misfolded glycoprotein. Subcellular localization studies indicate that Png1p is present in the nucleus as well as the cytosol. Sequencing of expressed sequence tag clones revealed that Png1p is highly conserved in a wide variety of eukaryotes including mammals, suggesting that the enzyme has an important function.

**Key words:** proteasome • de-N-glycosylation • quality control

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**Introduction**

Proteins that transit through the secretory pathway are subjected to an ER quality control surveillance system that distinguishes aberrant proteins from folded proteins so that the former can be retained in the ER (Hammond and Helenius, 1995). In some cases, it has been shown that these misfolded and/or unfolded proteins are degraded by a so-called ER-associated degradation mechanism, which involves the ubiquitin-proteasome system (Kopito, 1997; Cresswell and Hughes, 1997; Suzuki et al., 1998a; Brodsky and McCracken, 1999; Plemper and Wolf, 1999; Römisch, 1999). Characterization of this process is important because the misfolding and degradation of certain glycoproteins causes a number of human genetic disorders.

In mammalian cells, there are examples when de-N-glycosylated intermediates in the overall degradation process can be detected in the presence of proteasome inhibitors (Wiertz et al., 1996a,b; Halabian et al., 1997; Hughes et al., 1997; Uppa and Ploegh, 1997; Yu et al., 1997; Bebök et al., 1998; Virgilio et al., 1998; Johnston et al., 1998; Osse et al., 1998; Yang et al., 1998). This de-N-glycosylation process is catalyzed by the action of a soluble peptide:N-glycanase (EC 3.5.1.52; PNGase), which is known to occur in mammalian cells (Suzuki et al., 1993; Kitajima et al., 1995), hen ovoviduct (Suzuki et al., 1997), as well as the budding yeast, Saccharomyces cerevisiae (Suzuki et al., 1998b). PNGase cleaves the amide bond between the proximal N-acetylglucosamine and the linker asparagine residues on glycopeptides/glycoproteins, releasing an intact oligosaccharide and generating at the site of cleavage an aspartic acid residue in the peptide/protein backbone. PNGase has been widely used as a tool in studies on N-linked glycan chains. It has been shown that soluble PNGases have in common a neutral pH optimum for activity and a requirement for –SH groups (Suzuki et al., 1994a, 1997, 1998b; Kitajima et al., 1995). Genes encoding distinct classes of PNGases have been identified in Chryseobacterium meningosepticum and Aspergillus niger (Tarentino et al., 1990; Foulquier-Paquin et al., 1997). These PNGases have no structural homology with each other; they are secreted and differ from the ubiquitous intracellular (cytoplasmic) PNGases in terms of enzymatic properties. Until now, the genes encoding the soluble PNGases involved in proteasomal degradation of proteins in eukaryotes have not been identified.

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This study describes the PNGase gene (PNG1) from S. cerevisiae. A PNGase-defective mutant (png1-1) was identified by screening a collection of temperature-sensitive mutants for the loss of PNGase activity in cell extracts. The PNG1 gene was mapped to the left arm of chromosome XVI by genetic approaches. The open reading frame (ORF) encoding Png1p was determined by performing PNGase activity assays on extracts derived from individual yeast strains that had a deletion of a particular ORF in a known position on chromosome XVI. In this way, one strain was found that was null in enzyme activity because it lacked the PNG1 gene. PNG1 was found to encode a 42.5-kD soluble protein with no apparent signal sequence. Although PNG1 is not an essential gene, it was found to be required for efficient degradation of a carboxypeptidase Y mutant protein that does not undergo folding. Subcellular localization studies indicate that the Png1p is found in the nucleus, with a lower level occurring in the cytoplasm. Comparison of the protein sequences with a variety of databases, followed by sequencing of a variety of expressed sequence tag (EST) clones, revealed highly related genes in humans, mice, plants, fruit flies, nematodes, and fungi, indicating that this class of PNGase is a highly conserved enzyme in eukaryotic cells.

### Materials and Methods

#### Yeast Strains and Media

The yeast strains used in this study are listed in Table I. For screening of the collection of temperature-sensitive strains, 440 previously isolated mutant strains (Hartwell, 1967) were assayed. Unless otherwise noted, 10 ml of cells were grown at 30°C (or 25°C for temperature-sensitive strains) in YP AD (1% bacto-yeast extract, 2% bacto-peptone [both from Difco], 2% dextrose [J.T. Baker], and 40 mg/l adenine sulfate [Sigma Chemical Co.]) in a 50-ml centrifuge tube with shaking. U nless noted, standard yeast media and genetic techniques were used (Rose et al., 1990; Sherman, 1991; E Ible, 1992).

#### PNGase Activity Assay

PNGase activity was assayed in yeast lysates using fetuin-derived asialoglycopeptide 1 ([14C]CH₃)₃Leu-A (GlcNAc₆Man₆Glc₃Gal₃-Asp-Ser-Arg) as described previously (Suzuki et al., 1994a, 1998b). Radioactivity was monitored on a PhosphorImager (Molecular Dynamics) and quantitated using ImageQuant (version 1.2). One unit was defined as the amount of enzyme that catalyzes hydrolysis of 1 μmol of fetuin-derived asialoglycopeptide 1 per minute.

### Table I. Yeast Strains Used in this Study

| Strain | Genotypes | Sources |
|--------|------------|---------|
| W303-1a | MatA ade2-101 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 | Laboratory stock |
| W303-1b | MatA ade2-101 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 | Laboratory stock |
| PS593  | MatA ade2-1 leu2-3,112 trp1-289 ura3-52 | P. Sogor |
| K396-22B | MatA ade1 his1 leu2 lys7 met3 trp5 ura3 spo11 | R. Esposito |
| K393-35C | MatA his2 leu1 lys1 met4 pet8 ura3 spo11 | R. Esposito |
| K382-19D | MatA ade2 his7 hom3 tyr1 ura3 can1 cyh2 spo11 | R. Esposito |
| K381-9D | MatA ade6 arg4 ade7 asp5 lys2 met14 pet17 trp1 ura3 spo11 | R. Esposito |
| BY4742 | MatA his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 | This study |
| #2156 | MatA his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 png1Δ::KanMX4 | Research Genetics |
| #10560 | BY4742 erg6Δ::KanMX4 | Research Genetics |
| BY20941 | MatAMATα::KanMX4Δ1 his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/lys2Δ0 lys2Δ0/lys2Δ0 lys2Δ0/lys2Δ0 ura3Δ0/ura3Δ0 spo11Δ::KanMX4/SPO11 | Research Genetics |
| RSY281 | MatA sec23Δ-1 ura3-52 his4-619 | R. Schekman |
| RSY607 | MatA leu2-3,112 ura3-52 pep4Δ::URA3 | R. Schekman |
| CY629 | MatA his3Δ1 Δ0 leu2Δ1 trp1-Δ1 ade2-101 lys2 bho1Δ::HIS3 | C. Peterson |
| YEA78 | W303-1b hnr::URA3 Δ0::UbC3EU2Δ::SPO11 | R. Sternglanz |
| YWC2 | W303-1a elp3Δ::URA3 | R. Sternglanz |
| TSY5 | MatA ade2 his3 leu2 trp1 ura3 png1-1 | This study |
| TSY9 | MatA ade2 his3 leu2 trp1 ura3 png1-1 can1 | This study |
| TSY41 | MatA ade2 his3 leu2 trp1 ura3 png1-1 can1 | This study |
| TSY54 | MatA ade2 his3 leu2 trp1 ura3 png1-1 can1 | This study |
| TSY57* | MatA ade2 his3 leu2 trp1 ura3 png1-1 can1 | This study |
| TSY79 | MatA ade2 his3 leu2 trp1 ura3 gal4Δ::LEU2 | This study |
| TSY80 | MatA ade2 his3 leu2 trp1 ura3 gal4Δ::LEU2 | This study |
| TSY82 | MatA ade2 his3 leu2 trp1 ura3 gal4Δ::LEU2 | This study |
| TSY83 | MatA ade2 his3 leu2 trp1 ura3 gal4Δ::LEU2 | This study |
| TSY91 | MatA ade2 his3 leu2 trp1 ura3 gal4Δ::LEU2 | This study |
| TSY94 | MatA ade2 his3 leu2 trp1 ura3 gal4Δ::LEU2 | This study |
| TSY115 | #2156 prc1-1 | This study |
| TSY116 | W303-1a png1Δ::his5* pompbe | This study |
| TSY147 | BY4742 prc1-1 | This study |
| TSY148 | BY4742 prc1-1 erg6Δ::KanMX4 | This study |
| TSY149 | BY4742 prc1-1 png1Δ::KanMX4 | This study |
| TSY150 | BY4742 prc1-1 erg6Δ::KanMX4 png1Δ::KanMX4 | This study |

*Parentheses indicate phenotype was not determined.
Chromosomal Identification of pngl-1 Locus

To generate TSY 41, SPO 11 gene disruption of TSY 9 was carried out using SalI-PvuI digests of pEME 302 (Engerbret and Roeder, 1989). The disruption was confirmed by showing that when crossed to a spo11 tester strain and sporulated, <1% of the spores were viable. Furthermore, spores inviability was complemented by the introduction of pGB80 (provided by Dr. H. Engerbret, SUNY at Stony Brook) containing the wild-type SPO 11 gene. Chromosomal mapping of pngl-1 mutation was performed using the spo11-mapping method (K. Lapholph and Esposti, 1982). For mapping of chromosome XII, TSY 83 was prepared by sporulation of Research Genetics strain (No. 20941). CamR and CynR derivatives of TSY 41 cells (TSY 54) were isolated by selecting cells on plates containing either synthetic media–Arg 100 μg/ml canavanine or YPAD + 10 μg/ml cytoheximide. The mutants were shown to be mutant for CAN 1 and CYH 2 by complementation tests.

Genetic Mapping of pngl-1 Locus

TSY 75, which carries arco7 and sec23 as markers for the right arm of chromosome X VI, was prepared by crossing R SY 281 (a gift from Dr. Randy Schekman, University of California at Berkeley, Berkeley, CA) with K 398-4D and isolating haploid segregants of the appropriate genotype. TSY 82, which carries hho1a::HIS3 and gal4a::LEU2 as markers on the left arm of chromosome X VI, was prepared by crossing CY 629 with YEA 78 and isolating haploid segregants of the appropriate genotype. These strains were crossed to pngl-1 haploids (TSY 5 or TSY 9, respectively), and tetrad analysis of the resulting spores determined whether pngl-1 was linked to any of the markers.

To carry out mitotic mapping to determine the proximity of the mutation to known markers, TSY 94 (prepared by crossing R SY 607; a gift from Dr. Randy Schekman) with TSY 80 and isolating haploid segregants of the appropriate genotype and TSY 91 (prepared by crossing TSY 79 with YWC 2 and isolating haploid segregants of the appropriate genotype) were used. A flt mating these strains with TSY 9, diploids were selected. The diploids were grown nonselectively overnight at 30 °C in YPAD and plated to select for FOA + colonies. A mong these, LEu - cells were assayed for PNGase activity.

Bacterial Expression of Pnglp

DNA manipulations were performed according to Sambrook et al. (1989). The full-length PNG1 sequence was amplified from yeast chromosomal DNA using Vent DNA polymerase (New England Biolabs, Inc.) using 5' primer (5'-TTTGGTCGACAGCTATTTACCATCCTCCCCAC-3') and 3' primer (5'-TAAAAATTCTTGCGATATTTACCATCTCTCCCTCCAC-3'). Thus, the fragment obtained was cut by Ncol-Xhol digestion, and cloned into Ncol-Sall--digested peT-28b (Novagen, Inc.). For the (His) 6 version of Pnglp, another primers, 3' primer (5'-TTTTTTCTCTGACAGCTATTTACCATCTCCCCAC-3') and 5'- primer (5'-TTTTTTCTGACAGCTATTTACCATCTCCCCAC-3') were used. The fragment obtained was cut by Ncol-Xhol digestion and cloned into Ncol-Sall--digested peT-28b. These constructs were digested into BL2 (D E) 3 pl. yen cells, and expression of PNG1 was induced by adding 1 μm IPTG at OD 600 = 0.8/ml. A flt 3 at 37 °C, 3 ml of cells were collected and the protein was extracted by adding 400 μl of PBS/1% Triton containing 5 mM DTT and 1 mM PM SF, followed by sonication on ice using a Branson sonicator at level 3 for two 10-s intervals with a 1-min wait at 0°C between sonications. The cell extract was centrifuged at 16,000 g for 10 min at 4 °C, and the supernatant was assayed for PNGase activity.

Purification of (His) 6-tagged Pnglp (PNG1(His) 6p)

The overproduced (His) 6-tagged Pnglp (PNG1(His) 6p) in E. coli as described above was purified using a TALON metal affinity resin (CLONTECH Laboratories, Inc.) with blocked Pngl(His) 6p at 30 °C. The extract was incubated at 4°C for 2 h with 0.5 ml Talon resin that had been pre-equilibrated with 20 mM Tris-HCl/100 mM NaCl (binding buffer, pH 7.5). The entire reaction was transferred into a 3-ml gravity flow column, and the flowthrough fraction (3 ml) was collected. The resin was further washed with binding buffer with 10 mM imidazole (4 ml) and then eluted with binding buffer with 100 mM imidazole (4 ml). Finally, the column was washed with 40 ml M EsNaOH buffer, pH 5.2. Throughout the column operation, fractions of 1 ml were collected. During fractionation, DTT was immediately added to each fraction to a final concentration of 10 mM. Each fraction was analyzed by SDS-PAGE and also was assayed for PNGase activity. The pure (Pngl(His) 6p) fraction (fraction B-12) was collected, concentrated with 8.88 buffer (20 mM Hepes-KOH buffer, pH 6.8, 350 mM potassium acetate, 5 mM magnesium acetate, and 250 mM sorbitol) containing 10 mM DTT using a Microcon 10 (A-micon Inc.), and finally diluted with 8.88 with 10 mM DTT at a final concentration of 14 μg protein/ml. The protein concentration was quantitated with the Bio-Rad protein assay kit.

Product Analysis of Incubation of [14C]A siafoetuin Glycopeptide I with Purified Png1(His) 6p

Deglycosylation reaction by purified Png1(His) 6p was confirmed by product analysis using paper chromatography and paper electrophoresis as described earlier (K. Iwajima et al., 1995). [14C]A siafoetuin glycopeptide I (20,000 dpm) was digested with 12 μl of purified Png1(His) 6p, in a 10-μl reaction with 40 mM MES-NaOH buffer, pH 6.6, with 10 mM DTT at 30 °C for 15 h. The paper chromatography steps detected the removal of the glycan from glycopeptide, whereas the paper electrophoresis confirms the conversion of a glycosylated asparagine residue into an aspartic acid residue (K. Iwajima et al., 1995). Paper chromatography was carried out using 1-butanol/ethanol/H 2 O = 2:1:1 as a solvent (Suzuki et al., 1998b). Paper electrophoresis was carried out using a model EF-200 unit (A. T. O. T. O) as described earlier (Suzuki and Lennarz, 2000) and visualized using a PhosphorImager. A authentic PNGase F-deglycosylated peptide was prepared by digestion of 20,000 dpm [14C]A siafoetuin glycopeptide I with 5 units of PNGase F (Roche) in 20 μl of 50 mM Tris-HCl buffer, pH 8.0, at 37°C for 15 h.

Examination of Reactivity of Purified Png1(His) 6p toward Glycoproteins In Vitro

10 μg each of three glycoproteins, ovalbumin, ribonuclease B, and carboxypeptidase Y (Sigma Chemical Co.) was incubated in 20 μl of 40 mM MES-NaOH buffer, pH 6.6, containing 10 mM DTT with 30 μl of purified Png1(His) 6p at 30°C for 15 h. For reference, 10 μg of each glycoprotein was digested with 2 μl of PNGase F (a gift from Dr. R. O. B. K. Iwajima et al., 1995). [14C]A siafoetuin glycopeptide I with 5 units of PNGase F (Roche) in 20 μl of 50 mM Tris-HCl buffer, pH 8.0, at 37°C for 15 h. The reaction mixture was analyzed by 8% (carboxypeptidase Y), 10% (ovalbumin) or 15% (ribonuclease B) SDS-PAGE. In one experiment, glycoproteins were preincubated at 65°C for 30 min at a concentration of 1 mg/ml before incubation with Png1(His) 6p.

Construction of PNG1-containing Plasmids for Expression in Yeast

The PNG1 allele including its promoter region was amplified from yeast genomic DNA using the following primers and Vent DNA polymerase: 5'-AAAAGAAATCCTCGCTAACAACACGTAAG; and 5'-AAAGAAAATCTCGATCAACACAGCTGG-3'. PCR products were cloned into the EcoRI-XhoI site of pBluescript II S/K (Strategene) for sequencing and the EcoRI-SalI site of YEp352 for expression in yeast. After the insertion of the correct fragment into the EcoRI-XhoI site of pBluescript II S/K (Strategene), the resulting plasmid was transformed into yeast (ts5-1, the prc1-1 allele was isolated by PCR using the same primers as described above. The fragment obtained was digested with EcoRI-XhoI, and was cloned into the EcoRI-XhoI site of pBluescript II S/K (Strategene) for sequencing and the EcoRI-SalI site of YEp352 for expression of this allele in TSY 146 cells.

Construction of Carboxypeptidase Mutant (CPY*)

The prc1-1 mutation was introduced into yeast using the two-step gene replacement method (Rothstein, 1991). A n integrating plasmid containing prc1-1 was constructed using the PCR mutagenesis technique (Horton et al., 1990). In brief, the EcoRI-HindII fragment of plW1433, which contains the ORF of wild-type prc1 (Holt et al., 1996), was cloned into YEp15 (New England Biolab). plW1433 was a gift from Dr. Jacob Winther (Carlsberg Laboratory, Copenhagen Valby, Denmark). The following primers were used to amplify a fragment of prc1 using plW1433 as a template: 5'-GTGATTCTGGATGATAAAGAGC-3'; 5'-CCATCTCGATGAATTCTCTAC-3'; 5'-GATGGATCTCCAGCAGTG-3'; and 5'-AAAAAGAGATCCGTGAGGCGTG-3' (underlined letters refer to yeast genomic DNA). The amplified fragment was cloned into the EcoRI site of the expression plasmid YEp352 and the resulting plasmid was transformed into yeast strain TSY 5. The protein sequence of this mutant yeast strain was determined by the DNA sequencing method (Rothstein, 1991) and showed a change in the amino acid sequence of CPY*. The purified mutant enzyme was then assayed for PNGase activity in vitro.
fer to base change used to generate CPY*). The fragment was digested with AocI-BglII, and the equivalent fragment on Yip5-CPY was replaced by the PCR-derived pcr1-1 fragment. The sequence was confirmed using the EXCELI11 sequencing kit (Epicentre Technologies). The Yip5-CPY* was cut by AocI and integrated into strains of PRC1 locus. Transformants were grown nonselectively in YPA to allow excision of the plasmid and plated onto FOA plates. The presence of pcr1-1 was confirmed by showing disruption of the BstXI site within the gene using colony PCR as described before (Jakob et al., 1998). For PCR, the following primers were used: 5'-TTGAAACCCATCGGGAACCC-3' and 5'-AACAAGTTCCTAACAGGTGTCCT-3'; and the digests were analyzed in a 2% agarose gel.

**Radiolabeling of Cells and Immunoprecipitation**

Cells were grown to an OD600nm = 1 at 30°C in minimal medium without methionine and cysteine. Cells were concentrated to OD600nm = 10, and 1-ml aliquots were preincubated at 30°C for 30 min. During the preincubation, where indicated, MG-132 (Calbiochem) was added to a final concentration of 50 μM from a freshly made 5 mM solution in (DM SO). For control cells, the same amount of DM SO was added. Radiolabeling of the cells was initiated by the addition of Easy Tag Express-[35S] (43.48 GBq/mmol; NEN Life Science Products) to 100 μCi/OD600 of cells, and incubation was continued for 10 min for the pcr1-1 strain and 5 min for the PRC1 strain. A chase mix was added from a 100× stock solution (0.3% cysteine and 0.4% methionine), and incubation continued. At indicated periods of time, an equal volume of ice-cold 20 mM Na3HPO4/20 mM Tris-HCl, pH 7.5, was added to terminate the chase. Protein extracts were prepared and immunoprecipitated as described previously (Gillece et al., 1999) using anti-CPY antiserum (a gift from Dr. Marcus Aebi, ETH, Zürich). Proteins were analyzed on 7.5% polyacrylamide gels. When necessary, immunoprecipitated samples were divided into two aliquots; one was treated with 5 U of PNGase F (Roche) and 16 h at 37°C before gel electrophoresis. Labeled bands were visualized using the Phosphor mager and quantitated as described above. The cells used for this analysis (TSY147-150) were made by crossing TSY115 with Research Genetics strain No. 10568 and as described above. The cells used for this analysis (TSY 147-150) were made by crossing TSY115 with Research Genetics strain No. 10568 and as described above. The cells used for this analysis (TSY 147-150) were made by crossing TSY115 with Research Genetics strain No. 10568 and as described above.

**Results**

**Isolation of a PNGase-defective Mutant from a Temperature-sensitive Mutant Collection**

Earlier we reported that *S. cerevisiae* has a soluble PNGase activity that is very similar in terms of enzymatic properties to the soluble PNGases found in higher eukaryotes (Suzuki et al., 1998b). To identify the PNGase gene in yeast, mutants defective in PNGase activity were sought. From a collection of temperature-sensitive mutants, 440 strains were individually assayed for the loss of PNGase activity (Hartwell, 1967). A mong these 440 strains, 10 strains showed virtually no PNGase activity at 37°C, the nonpermissive temperature. Ten of these 10 strains were crossed to an isogenic strain (PS593). The resulting diploids were dissected and individual spore colonies were assayed for PNGase activity. One strain (No. 352) was observed to segregate 2:2 for PNGase activity in this cross (Fig. 1), demonstrating that the PNGase defect in strain No. 352 was the result of a mutation in a single gene. The PNGase defect in No. 352 did not cosegregate with the temperature-sensitive phenotype (data not shown). We named the mutation in the strain png1-1, after peptide:N-glycanase. Strains TSY 5 and TSY 9 (see Table IV for a complete strain list) carrying png1-1 were made by backcrossing strain No. 352 with wild-type strains (one cross with PS593 followed by three crosses with W303-1 strain), and used for the mapping study.

**Chromosomal Mapping of png1-1 Mutation**

Neither TSY 5 nor TSY 9 showed any phenotype or growth defect under various experimental conditions tested, making it difficult to clone by complementation. Therefore, the spo11 mapping technique (Klapholz and Esposito, 1982) was employed to localize the png1-1 mutation onto a particular chromosome. The rationale behind this approach takes advantage of the fact that there is no meiotic recombination in spo11 diploids. Therefore, if a png1-1 haploid is crossed to a spo11 strain carrying lys1 on chromosome IX, for example (thereby producing a diploid with the parental configuration (png1LY51/PNG1lys1),
recombinant spores (PNG1 L Y S1 and png1 lys1) arise primarily by independent assortment during meiosis. The presence of recombinant spores is, therefore, suggestive that png1 is not on the marked chromosome. In contrast, the absence of recombinant types suggests that png1 is located on the same chromosome as that marker. Because spo11 mutants produce predominantly inviable spores, rare viable spores were first selected on -Arg plates containing canavanine and cycloheximide (Hollingsworth and Byers, 1989). The spore colonies were patched onto YPAD plates and replica plated onto the appropriate media to score the marked chromosomes. PNGase assays were performed on the spore colonies to determine the phenotype of png1.

The data in Table I show that the cross between aro7 and png1-1 was the only one to produce a class of no recombinant types (png1 aro7). The other seven putative recombinants (Tyr+ PNGase+) were demonstrated by further genetic analysis to be due to disomy of chromosome XVI resulting from nondisjunction (data not shown). Therefore, these results suggested that png1-1 was on chromosome XVI.

Genetic Mapping of png1-1 Mutation to a Specific Site on Chromosome XVI

To localize png1-1 more precisely on chromosome XVI, png1-1 was crossed to TSY 75 and TSY 82, both of which have two markers (ARO7 and SEC23 in TSY 75 and HHO1 and GAL4 in TSY 82) on chromosome XVI (Fig. 2). Tetrad analysis of the sporulated diploids revealed that the png1-1 mutant was not linked to markers on the right arm, whereas png1-1 did exhibit linkage to the HHO1 locus, but not to GAL4, on the left arm (Table I). These results placed the png1-1 mutation approximately in the middle region of the left arm on chromosome XVI.

To further narrow down the png1-1 locus, mitotic mapping was carried out. This method uses mitotic crossing over in diploids to determine the order of genes on the same arm of a chromosome. TSY 91 contains URA3 integrated at the ELP3 locus and TSY 94 contains URA3 integrated at the PEP4 locus. These strains were crossed to the png1-1 ura3 haploid TSY 5. The diploids were grown nonselectively and plated on 5-fluoroorotic acid (FOA) to select for strains exhibiting loss of the URA3 gene. This loss of heterozygosity results from crossovers between either elp3Δ::URA3 or pep4Δ::URA3 and centromere. To ensure that FOA R was due to recombination and not gene conversion, loss of the gal4Δ::LEU2 marker on the same arm distal to elp3Δ::URA3 and pep4Δ::URA3 was also re-

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**Table I. Segregation of png1-1 Mutation with Other Markers on spo11 Mapping Analysis**

| Chromosome (marker) | Parental 1 | Parental 2 | Recombinant 1 | Recombinant 2 |
|---------------------|------------|------------|---------------|---------------|
| I (ade2)            | 7          | 7          | 5             | 10            |
| II (lys2)           | 14         | 8          | 5             | 4             |
| III (leu2*)         | 21         | 23         | 12            | 13            |
| IV (trp1*)          | 11         | 20         | 9             | 11            |
| V (hom3)            | NA         | 22         | NA            | 12            |
| VI (his2)           | 6          | 7          | 2             | 7             |
| VII (cyc2)          | 2          | 14         | 8             | 11            |
| VIII (arg4)         | 11         | 6          | 7             | 7             |
| IX (lys1)           | 3          | 3          | 5             | 10            |
| X (met3)            | 4          | 3          | 4             | 3             |
| XI (met14)          | 16         | 4          | 9             | 2             |
| XII (met15)         | 2          | 1          | 2             | 3             |
| XIII (lys7)         | 9          | 9          | 11            | 6             |
| XIV (met4)          | 1          | 4          | 7             | 10            |
| XV (his3*)          | 26         | 13         | 31            | 14            |
| XVI (aro7)          | 23         | 34         | 7             | 0             |

*The results shown were obtained by cross of TSY54 to test strains (K396-22B/K381-9D/K393-35C/TSY83) except for chromosome V (hom3) and chromosome VII (cyc2), which were obtained from TSY41 × K382-19D, selecting spores only using canavanine.

†The markers indicated with asterisks were from TSY54, whereas the others were from the test strains.

‡Spores were selected using only cycloheximide.

NA, not applicable because of the selection method.

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**Table III. Genetic Mapping of the png1-1 Mutation by Tetrad Analysis**

| Cross          | Parental Ditypes | Nonparental Ditypes | Tetratypes | Linkage |
|----------------|------------------|---------------------|------------|---------|
| png1-1 × SEC23*| 3                 | 4                   | 8          | unlinked|
| png1-1 × ARO7* | 3                 | 4                   | 8          | unlinked|
| png1-1 × HHO1† | 15                | 0                   | 6          | 14.3 cM |
| png1-1 × GAL4† | 4                 | 2                   | 15         | 13 cM   |

*The results shown were obtained by crossing TSY5 with TSY75.

†The results shown were obtained by crossing TSY9 with TSY82.

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**Figure 1.** A assay of PNGase activity in four sets of tetrads from the cross of a PNGase-defective mutant (No. 352) with a wildtype strain (PS593). Protein extracts (~20 μg) from each spore colony were incubated with 25 μM of [3H]asialofetuin peptide I in 6 μl of 70 mM Hepes-NaOH buffer, pH 7.2, and 5 mM DTT at 25°C for 16 h. The reaction product was analyzed by paper chromatography and the radioactive peptides were visualized using a PhosphorImager. A paper chromatogram of four different tetrad types (1–4) are shown. P, de-N-glycosylated product ([3H]C-Leu-Asn-Ser-Arg); and S, substrate ([3H]C-Leu-Asn(GlcNA cs, Man3Gal3)-A-Ser-A rg).
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required. All of the FOA\(^+\) Leu\(^-\) colonies derived from the TSY 5/TSY 91 diploid were defective for PNGase activity, indicating that the png1-1 locus is distal to the ELP 3 locus. On the other hand, 4/16 FOA\(^+\) Leu\(^-\) strains were PNGase-positive from the TSY 5/TSY 94 diploid, indicating that the png1-1 gene is centromere proximal to the ELP 4 locus.

Given that png1-1 is located in between ELP 3 and ELP 4, the Saccharomyces Genome database (http://genome-www.stanford.edu/Saccharomyces/) was analyzed for ORFs in this interval. Among 67 ORFs, 16 candidate ORFs were chosen (YPL 103c, YPL 116w, YPL 107w, YPL 103c, YPL 101w, YPL 100w, YPL 099c, YPL 098c, YPL 096w, and YPL 095c). These ORFs were chosen as potential candidates for PNG1 based on the following criteria: (1) the ORF was uncharacterized; (2) it was at least 20 kb distance from the HHO 1 locus (based on tetrad analysis data); and (3) it encoded a protein with a molecular mass > 12 kD. Strains containing individual deletions of each ORF were obtained from Research Genetics and assayed biochemically for PNGase activity. A strain deleted in YPL 096w (No. 2156) did not exhibit any PNGase activity, thereby identifying this ORF as PNG1. To confirm that png1-1 is the same locus as YPL 096w, we crossed the Research Genetics strain (No. 2156) with the TSY 9. After tetrad dissection, 10 tetrads were assayed for PNGase activity in the TSY5/TSY94 diploid, indicating that the png1-1 gene is distal to the TSY4 locus, as expected if the two mutants are allelic. Indeed, the sequence analysis of png1-1 allele revealed that the png1-1 allele was due to a point mutation of residue 218, in which His was mutated to Tyr mutation. The catalytic inactivity of this mutant protein is consistent with the fact that this His residue is highly conserved in a variety of eukaryotic organisms (see below).

PNG1 is predicted to encode a protein (Png1p) with a molecular mass of 42.5 kD. The protein does not have either a predicted signal sequence or a membrane spanning domain. Png1p does not have any homologues in the S. cerevisiae genome nor does it exhibit any sequence similarity with known PNGases from a bacterium and a fungus (Tarentino et al., 1990; Ftoh-Paquin et al., 1997). However, a number of related proteins in other eukaryotes, including mammalian cells, were identified (see below).

**Bacterial Expression of PNG1 Shows that Png1p Is a Soluble Enzyme**

A through mutation of the PNG1 gene renders yeast cells devoid of PNGase activity, this result by itself does not distinguish whether PNG1 encodes the PNGase enzyme or a regulatory gene required for PNGase activity. Since no PNGase activity in E. coli extracts was observed under our experimental conditions, the yeast PNG1 gene was placed under the T7 expression system, introduced into E. coli, and the resulting cell extracts were monitored for PNGase activity. A similar result was observed in extracts from E. coli expressing (His\(_6\))-tagged PNG1 (data not shown). The protein was easily recovered from the soluble fractions after cell lysis with 1% Triton in the extraction buffer followed by centrifugation at 16,000 g.

For the purpose of subsequent product analysis using glycopeptide/glycoproteins as substrates, purification of Png1p was performed by using (His\(_6\))-tag and a (His\(_6\))-tagged protein-specific metal affinity resin. In Fig. 3, expression of PNG1 in BL21 (DE3)pLysS cells with the pET-28b vector resulted in very high PNGase activity. A similar result was observed in extracts from E. coli expressing (His\(_6\))-tagged PNG1 (data not shown). The protein was easily recovered from the soluble fractions after cell lysis with 1% Triton in the extraction buffer followed by centrifugation at 16,000 g.

The molecular mass of the reaction product detected in control assay (Fig. 3, lane 2) was 44 K on SDS-PAGE (Fig. 4 A), which is consistent with the expected molecular mass of Png1(His\(_6\))p. This bound protein, together with the flowthrough fraction, exhibited high PNGase activity (Fig. 4 B), which was quantitated by paper chromatography. The elution pattern of PNGase correlated with the appearance of the Png1(His\(_6\))p band on the gel (Fig. 4, A and B). The purified protein exhibited 3 mU/ml of activity and the specific activity of pure protein was calculated to be 0.21 μmol of glycopeptide degraded/mg protein/min.

To rigorously prove that the reaction product detected by Png1p was formed by a de-N-glycosylation reaction, further product analysis was performed using the purified Png1p. This was particularly important because the paper chromatographic method only detects the removal of the glycan from peptide, which could be catalyzed by another type of endoglycosidase such as endo-β-N-acetyl-
glucosaminidase (Suzuki et al., 1995). For this purpose, paper electrophoresis analysis was performed since this method separates products primarily based on their charge and will detect the conversion of the glycosylated Asn into an Asp residue. This, together with the paper chromatography, has been established as the method to detect PNGase activity in cellular tissues (Kitajima et al., 1995). For a reference sample, PNGase F-deglycosylated 14C-asialofetuin glycopeptide I was used. Indeed, the reaction product from purified Png1p gave the same mobility with the authentic deglycosylated peptide formed by PNGase F using paper electrophoresis as well as paper chromatography (Fig. 4, C and D). This clearly demonstrates that Png1p possesses PNGase activity.

To determine if there was a correlation of PNG1 expression and PNGase activity, PNG1 was expressed using CEN (pRS316; single copy) or 2-micron (YEp352; multicopy) plasmids in the png1Δ strain (TSY146). As shown in Fig. 5 A, both PNG1 constructs complemented the defect of PNGase activity in png1Δ strain. A time course experiment (Fig. 5 B) showed that the PNGase activity was four- to fivefold higher in cells expressing Png1p with the 2-micron plasmid than with CEN plasmid, indicating a clear-cut correlation of PNGase activity with the level of expression of PNG1.

To assess the reactivity of Png1p toward glycoproteins in vitro, three glycoproteins (ribonuclease B, ovalbumin, and carboxypeptidase Y) were examined. In sharp contrast to bacterial PNGase F, there was no detectable de-N-glycosylated product found by SDS-PAGE analysis, even after the proteins were treated to partially denature them (data not shown).

**Deletion of PNG1 Causes a Delay in the Degradation of a Mutant Form of Carboxypeptidase Y**

A phenotypic difference between wild-type and png1Δ cells was not detected in terms of growth rate or viability (data not shown). Because soluble PNGase activity has been proposed to be involved in proteasome-dependent degradation in the cytosol in mammalian cells, the effect of deletion of PNG1 on the degradation of a mutant carboxypeptidase Y protein, CPY* (Hiller et al., 1996), was examined. CPY* is a well characterized mutant glycoprotein that does not fold correctly and, therefore, is a substrate for proteasomal degradation in yeast. We constructed strains having the prc1-1 allele, which encodes CPY*, in png1Δ and isogenic wild-type strains. The half-life of CPY* was compared in the two strains by pulse-chase experiments. As shown in Fig. 6 (A and B), the PNG1 gene is not required for CPY* degradation. However, a distinct delay in degradation was seen with the png1Δ strain, showing an increase in the half-life of the CPY* protein to 46 min compared with 26 min in the PNG1 strain. No differences were observed in the vesicular transport of CPY wild-type protein in png1Δ and isogenic wild-type strains (data not shown).

In mammalian cells, the involvement of PNGase in proteolysis of PNGase activity was measured after 16-h incubation of reaction mixture at room temperature. (C and D) Analysis of reaction products produced by incubation of [14C]asialofetuin glycopeptide I with purified Png1p using (C) paper chromatography and (D) paper electrophoresis. (Lane 1) [14C]asialofetuin glycopeptide I; (lane 2) reaction product formed by Png1p; and (lane 3) reference reaction product formed by PNGase F. The PNGase-deglycosylated product, [14C]-Leu-Ser-Asp, is indicated by asterisks.
teasomal degradation was first indicated by the appearance of de-N-glycosylated degradation intermediates in the presence of proteasome inhibitors (Wiertz et al., 1996a). In yeast, these inhibitors are ineffective because they cannot enter yeast cells unless the cells have a mutation in the ERG6 gene, which causes the cells to have increased permeability to a variety of exogenous compounds (Graham et al., 1993). Previously, it has been shown that MG-132, a specific inhibitor of the proteasome, inhibits the degradation of short-lived proteins in an erg6 mutant background (Lee and Goldberg, 1996). Because the effect of the MG-132 proteasome inhibitor on CPY* degradation had not been examined, we analyzed CPY* destruction in the presence of this inhibitor using the erg6Δ strain. As shown in Fig. 6 C, we observed a moderate, but reproducible increase in the half-life for this protein in the presence of the proteasome inhibitor in PNG1 cells (t1/2 = 27 min without MG-132 versus 47 min with MG-132). In sharp contrast to the PNG1 cells, there was no effect of the proteasome inhibitors on the degradation of CPY* in the png1Δ strain (t1/2 = 48 min without MG-132 versus 49 min with MG-132; Fig. 6 C). Thus, the lowered rate of degradation of CPY* (t1/2 = 46–48 min) was not further decreased by the addition of the proteasome inhibitor. No de-N-glycosylated intermediates were observed, even in the presence of MG-132, since there was no change in the position of migration of CPY* with and without the proteasome inhibitor and, in both cases, the CPY* was susceptible to PNGase F digestion (data not shown).

Png1-GFPp Is Found in Both the Nucleus and the Cytosol

Although in earlier studies most of the PNGase activity in a variety of tissues and cells was recovered in the soluble (cytosolic) fraction after subcellular fractionation (Suzuki et al., 1993, 1997, 1998b), the precise subcellular location of the soluble PNGase is still unclear. To localize Png1p in yeast, the green fluorescent protein (GFP) was fused to the COOH terminus of Png1p. Western blotting using anti-GFP mAbs (Fig. 7 A) revealed the absence of immunoreactivity in the png1Δ strain (TSY146), whereas a single protein of the expected size (70.5 kD) was detected when TSY146 was transformed with a plasmid containing the PNG1-GFP gene. TSY146/pPNG-GFP exhibited PNGase activity, confirming that the Png1-GFPp is enzymatically active (data not shown).

The distribution of Png1-GFPp was analyzed after fixation and staining of the nuclei with DAPI. As seen in Fig. 7 (B and C), the signal was distributed throughout the cells.
with a somewhat stronger signal in the nucleus. Much less staining was observed in large vesicles, which presumably represent vacuoles. These results imply that the protein is localized in the cytosol and nucleus, but is not present in the vesicular compartment.

**Sequence Comparison Reveals that the Png1p Is a Highly Conserved Protein in Eukaryotes**

To determine the distribution of proteins homologous to yeast Png1p in other eukaryotes, sequence searches were performed using nucleotide/protein databases. By using *Saccharomyces cerevisiae* Png1p sequence as a query, we found several Png1p homologues (Table IV). A further determining the complete sequence of a *S. cerevisiae* clone (A1019191), we used it to further carry out EST database surveys and identified several EST clones that consisted of complete or partial ORFs from human (*H. sapiens* clone ID 1316890 from Research Genetics and EST clone accession No. 97076 from ATCC), mouse (IMAGe clone ID 948982), D. melanogaster (IMAGe clone ID LD463890), and *C. elegans* (yk491.3 3; gift from Dr. Yui Kohara). Fig. 8 shows the sequence alignment of *S. cerevisiae* Png1p with *S. pombe*, *C. elegans*, D. melanogaster, mouse, and human homologues. The homologues in higher eukaryotes were found to have more extended sequences at both the NH₂ and COOH termini than either *S. cerevisiae* or *S. pombe* Png1p (Fig. 8). However, alignment of the determined sequences revealed a highly conserved core domain present in all eukaryotes examined (Fig. 8). The sequence analysis of png1-1 allele revealed that the png1-1 was due to a point mutation of a highly conserved His residue (residue 333 in Fig. 8), which was changed to Tyr, suggesting the importance of this His residue on catalytic activity of Png1p.

**Discussion**

The functional importance of de-N-glycosylation in cellular processes by PNGase was first proposed based on studies in fish oocytes and embryos (Inoue, 1990; Seko et al., 1991). Since then, interest in the biological function of PNGase in somatic eukaryotic cells has grown (Suzuki et al., 1994b; 1995; Berger et al., 1995) and the involvement of mammalian cytosolic PNGases in proteasome-mediated degradation has been proposed (Wiertz et al., 1996a,b). However, because the functional importance of the de-N-glycosylation reaction remained unclear in detail, we undertook to study this enzyme in the single cell eukaryote, *S. cerevisiae*. In our initial study (Suzuki et al., 1998b), we demonstrated the presence of PNGase in yeast and showed that it had many of the properties reported for PNGase from higher eukaryotes. In this study, we report the identification of a PNGase activity-defective yeast mutant (png1-1) strain. U sing this strain, we carried out various genetic approaches that led to the identification of the *PNG1* gene (YPL096w). PNG1 was definitively shown to encode PNGase by carrying out the analysis of reaction product of [¹⁴C]asialofetuin glycopeptide I with purified Png1p overexpressed in *E. coli*. Correlation of the expression level of PNG1 gene and PNGase activity was also confirmed through a complementation experiment using the plasmid borne PNG1 gene. PNG1 encodes a soluble protein with no apparent signal sequence. No homologues are present in the *S. cerevisiae* genome, indicating that the gene is not redundant in yeast. This observation is consistent with the finding that deletion of PNG1 abolishes PNGase activity. Sequence analysis of png1-1 revealed that the gene encoded a full-length protein corresponding to Png1p with a single mutation of His 218 to Tyr (Fig. 8; residue No. 333), indicating the importance of this His residue in catalytic activity. Indeed, this His residue was found to be conserved for all eukaryotes examined (Fig. 8).

Because the mutant strain did not have a detectable phenotype after several backcrosses to wild-type strains, the isolation of the gene by complementation was problematic. Moreover, the amount of Png1p in yeast cells seems to be very limited; for example, we can detect the PNGase activity only with a highly sensitive method using radioactive glycopeptide as a substrate, and it still requires 12-16 h to detect the activity when the yeast extract is used as an enzyme source (Suzuki et al., 1998b). Indeed, the

| Accession no. | Definition | E value |
|---------------|-----------|---------|
| AL031852      | SPBC1709; Schizosaccharomyces pombe chromosome II cosmId c1709 | 5e−30 |
| AL856765      | sb41h609.y1 Gm-c1014 Glycine max cDNA clone Genome System clone ID: Gm-c1014-282 5' | 1e−33 |
| AI019191      | ub20e10.r1 Soares 2NmMT Mus musculus cDNA clone IMAGE:1378314 | 2e−29 |
| AI491330      | 486017B11.x2 486 - leaf primordia cDNA library from Hake lab Zea mays cDNA | 5e−28 |
| AC008339      | BACR13P06; Drosophila melanogaster chromosome II BAC clone D917 | 3e−27 |
| AI117202      | CEY53H1C; Caenorhabditis elegans chromosome 1 cosmId Y53H1C | 6e−22 |
| ZZ8729        | HSB35D121 Stratagene human skeletal muscle cDNA library, cat. #936215. Homo sapiens cDNA clone 85D12 | 1e−21 |
| AI392526      | NCSC1A073 Subtracted Comidial Neurospora crassa cDNA clone SC1A9 | 2e−18 |
| AA139963      | me92e01.r1 Stratagene mouse heart (#937316) Mus musculus cDNA clone IMAGE:586200 | 7e−16 |
| AA107311      | mp066d11.r1 Life Tech mouse embryo 8 5pc 10664019 Mus musculus cDNA clone IMAGE:568743 | 1e−12 |
| AA786911      | m7603a1.r1 Aspergillus nidulans 24 h asexual developmental and vegetative cDNA lambda Zap library | 2e−12 |
| AB023033      | Arabidopsis thaliana genomic DNA, chromosome V, TAC clone: K6M13 | 5e−9 |
| Z81552        | Caenorhabditis elegans cosmId chromosId cosmId F56G4 | 6e−9 |

*Data are from tblastn search using the complete protein sequence of *S. cerevisiae* Png1p as a query. Results are from NCBI database except for AC008339, which was only found from BDGP database.*

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PNG1 turns out to have significantly low codon usage (codon bias, \(-0.032\)), indicating that it would have been extremely difficult to isolate pure Png1p. Therefore, an approach using various genetic methods, as well as application of the yeast genome sequence and the single ORF deletion strains that are commercially available was crucial in identification of the PNG1 gene.

Whereas the purified enzyme using hexahistidine tag exhibited high PNGase activity toward the glycopeptide substrate (\(~0.21\ \mu\text{mol/mg protein/min}\)), reactivity toward glycoproteins could not be detected in vitro. This is consistent with the finding that the soluble PNGase, isolated and purified from mammalian cells, also does not act on glycoproteins in vitro (Suzuki et al., 1994a). These observations are reminiscent of the fact that the 20S proteasome can only act primarily on small peptide substrates in vitro, and it has been widely supposed that the function of the 195 ATPases is to unwind protein substrates for them to be substrates for this proteolytic complex (Rubin and Finley, 1995; Wenzel and Baumeister, 1995). The result of the inability of PNGase to act on glycoproteins in vitro may indicate that additional factors are required (as is the case of 20S proteasome) for this enzyme to recognize glycoproteins as substrates in vivo. Currently, we are studying this possibility.

One explanation for the lack of a detectable phenotype in the png1Δ strain as well as png1-1 strain is that the enzyme is not required unless a large amount of misfolded glycoprotein is being produced by the yeast cells. We found that in png1Δ cells the rate of degradation of an incorrectly folding mutant protein, CPY* (Hiller et al., 1996) was reduced compared with wild type, suggesting that the gene is at least, to some extent, involved in ER-associated proteasomal degradation. Although our result does not...
rule out the possibility that the delay in degradation of CPY * is due to an indirect effect rising from the deletion of Png1p, it is of interest that a number of genes required for this degradation process are nonessential and do not exhibit detectable growth defects under the experimental conditions used (Plemper and Wolf, 1999). These observations imply that the ER quality control machinery seems dispensable under standard laboratory culture conditions, at least in yeast, despite the fact that this process has known to be closely associated with a number of genetic disorders in mammals (Plemper and Wolf, 1999).

When the proteasome inhibitor, MG-132, was added to cells under conditions that are known to inhibit proteasomal degradation (Lee and Goldberg, 1996; Loayza et al., 1998), partial inhibition of degradation of CPY * was observed. This degree of inhibition is consistent with the previous observations on the rate of CPY * degradation using mutated proteasome subunits; with various mutants the t1/2 was increased to 2-2.5-fold, but in no case was degradation completely blocked (Hiller et al., 1996). Partial inhibition by the proteasome inhibitor may result because this compound is not effectively taken up by yeast as compared with animal cells (Lee and Goldberg, 1998), or it may be the result of a yet uncharacterized alternative protein degradation system that is not sensitive to this inhibitor. In this connection, it is interesting to note that occurrence of a novel proteolytic complex that can compensate for the essential function of the proteasome recently has been reported in mammalian cells as well as in S. pombe (Glas et al., 1998; Geier et al., 1999; Osmulski and Gaczynska, 1999).

We were unable to detect a synergistic effect on CPY * degradation when the proteasome inhibitor was added to the PNGase deletion strain. Unlike mammalian cells, with PNGase- yeast cells to which proteasome inhibitors had been added, we were unable to detect the accumulation of de-N-glycosylated protein intermediates. This finding may mean that de-N-glycosylation is a rate-limiting step for proteasome-mediated degradation in yeast, and it may not be possible to detect the de-N-glycosylated intermediate under conditions of incomplete inhibition of proteasome activity. In addition, the lack of de-N-glycosylated protein intermediates in yeast may indicate that the level of PNGase activity in mammalian cells may be much higher than that in yeast. This hypothesis is consistent with the finding that when small glycopeptides are exported out of the ER to the cytosol, de-N-glycosylation occurs immediately in mammalian cells, but not in yeast (Römisch and A Li, 1997; Suzuki et al., 1998b). Since PNGase did not affect glycopeptide export from the ER to the cytosol (Suzuki, T., and W. J. Lennarz, unpublished observations), it is not likely that PNGase is directly involved in the translocation of misfolded proteins from the ER to the cytosol. Rather, it seems that the absence of a PNGase defect somehow slows down proteasomal degradation after the proteins are translocated from the ER to the cytosol or in the case of yeast, to the nucleus.

Although most of the soluble PNGase activity has been described as cytosolic or cytoplasmic, there still is controversy about the precise localization of PNGase in cells (Weng and Spiro, 1997). The primary structure indicates that there is no apparent signal or hydrophobic sequence that could represent a transmembrane domain. This is consistent with the hypothesis that the soluble PNGase does not enter the secretory pathway and, therefore, remains in the cytosol or a topologically equivalent compartment (i.e., the nucleus). Subcellular localization studies of Png1-GFPp in S. cerevisiae demonstrated that, although most of the protein is localized in the cytosol, a fraction of the protein is concentrated in the nucleus. At this point, it is not clear if the nucleus-associated protein represents a membrane-associated type of PNGase, which has been described earlier in animal cells (Suzuki et al., 1997; Weng and Spiro, 1997). It is also interesting to note that, unlike multicellular eukaryotes in which proteasomes are abundant in the cytosol, in S. cerevisiae, the proteasome is almost exclusively localized in the nucleus (Russell et al., 1999). Since, as discussed above, the soluble PNGase activity may be associated with the proteasomal degradation pathway, the higher concentration of Png1p in the nucleus may indicate colocalization and possible interaction between PNGase and the proteasome machinery in yeast cells.

A careful survey for Png1p homologues in various databases as well as additional sequences of EST clones from various organisms revealed homologues in mammals, plants, insects, nematodes and fungi. These findings demonstrate that this gene is highly conserved evolutionarily throughout eukaryotes. In fact, we have observed PNGase activity when the mouse homologue of Png1p is expressed in png1Δ yeast cells, establishing that the mouse homologue is indeed a functional PNGase (Suzuki, T., and W. J. Lennarz, unpublished results). Furthermore, Northern analysis using mouse PNG1 cDNA as a probe revealed the presence of mRNAs in all tissues tested, which is consistent with the biochemical findings of a wide tissue distribution of PNGase activity in mice (Kitajima et al., 1995). Although the functional role of Png1p in cellular processes still remains unclear, the ubiquitous occurrence of this protein in eukaryotes strongly suggests functional significance in certain fundamental biological processes. Finding mutants that are synthetically lethal with png1Δ might provide insight into the function of Png1p, and this line of study is currently underway.

As shown in Fig. 8, the S. cerevisiae and S. pombe PNGases have a core sequence that is highly homologous (amino acid residues 106–362 in ScPng1p) to that found in homologues in higher eukaryotes. In this core, two conserved cysteine residues form CXYC motifs (asterisks). This motif, which is found in the protein disulfide isomerase family, is known to be oxidized to form disulfide bonds. This finding is of interest in view of the fact that reducing reagents such as DTT are required for detection of the PNGase enzyme in vitro assays (Suzuki et al., 1994a, 1997, 1998b; Kitajima et al., 1995). Perhaps these conserved cysteine residues are important for its catalytic activity. It is also interesting to note that the C. elegans homologue has an apparent thioredoxin-like domain in its NH2-terminus region (66% homologous to the S. cerevisiae TRX2 gene), and the active site for thioreductase activity (WCGPC) is completely conserved. These findings suggest that the action of PNGase in the cytosol (or nucleus) may somehow be regulated by redox potential. Redox potential is known to be important for ER-associated degra-
vation in mammalian cells (Y oung et al., 1993; Tortorella et al., 1998), although, thus far, not much is known about its mechanism. Clearly, now that the primary structure of PNGase is known, future studies involving a structure-function approach should answer a number of important questions about the biological importance of this enzyme.

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