Yeast *N*-glycanase distinguishes between native and non-native glycoproteins

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***N*-glycanase from *Saccharomyces cerevisiae* (Png1) preferentially removes *N*-glycans from misfolded proteins. The ability of Png1 to distinguish between folded and misfolded glycoproteins is reminiscent of substrate recognition by UDP-glucose glycoprotein glucosyl transferase, an enzyme that possesses this trait. The only known *in vivo* substrates of Png1 are aberrant glycoproteins that originate in the endoplasmic reticulum, and arrive in the cytoplasm for proteasomal degradation. The substrate specificity of Png1 is admirably suited for this task.**

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INTRODUCTION

The endoplasmic reticulum (ER) harbours a quality control mechanism to ensure that only properly folded proteins remain in the secretory pathway. Misfolded proteins are exported to the cytosol, where they are degraded by the proteasome. In the cytosol, aberrant glycoproteins from the ER are targeted by an activity that removes their *N*-linked glycans prior to proteolysis. The enzyme responsible is the cytosolic protein Png1 (Suzuki *et al*, 2000; Hirsch *et al*, 2003). Png1 is a member of the transglutaminase-like superfamily: three amino acids, His, Cys and Asp, constitute a catalytic triad that is part of a conserved protein domain characteristic for this superfamily (Makarova *et al*, 1999). Png1 readily deglycosylates aberrant glycoproteins such as single-chain TCR α or CD3 δ . Likewise, Png1 digests free class I major histocompatibility complex (MHC) heavy chains, whereas the assembled complex of heavy chain and β_2 -microglobulin (β_2 m) is resistant to deglycosylation by Png1 (Hirsch *et al*, 2003). Other glycosidases, such as endoglycosidase H (Endo H) or peptide:*N*-glycosidase F (Png F), act on class I MHC heavy chains regardless of their conformation. Is the ability to discriminate between folded and unfolded glycoproteins a general property of Png1? To investigate the type of substrates targeted by Png1, we examined

the interaction of yeast Png1 with two different glycoproteins and their non-native derivatives.

Bovine pancreatic ribonuclease (RNase) is a small soluble protein (Fig 1A) that occurs in two forms: RNaseA and RNaseB. The latter differs from RNaseA by the presence of a single, high-mannose-type *N*-linked glycan attached to Asn34. Reduction and alkylation of RNaseB yields a fully unfolded, inactive enzyme. Digestion of native RNaseB with subtilisin nicks the polypeptide backbone between residues Ala20 and Ser21. The first 20 amino acids of the nicked molecule can be released at low pH, yielding a nonfunctional RNaseB derivative termed RNaseBS-Prot (Fig 1B) (Richards, 1959). A catalytically active RNase termed RNaseBS can be reconstituted from RNaseBS-Prot by the addition of a synthetic form of the first 20 amino acids, the S-peptide, at neutral pH.

UDP-glucose:glycoprotein glucosyltransferase (GT) serves as a folding sensor in the ER. It glucosylates *N*-glycans attached to misfolded protein domains and targets them to the calnexin/calreticulin cycle, which assists in protein folding in the ER. Synthetic peptides bearing high-mannose glycans have also been used successfully as substrates for GT (Taylor *et al*, 2003). Deglycosylation by ER glucosidase II releases glycoproteins from this cycle. *In vitro*, GT reglucosylates only RNaseBS-Prot and fully denatured RNaseB, while active, unmodified RNaseB and the reconstituted RNaseBS are not modified (Ritter & Helenius, 2000; Trombetta & Helenius, 2000). These derivatives of RNaseB are therefore excellent substrates to monitor the substrate specificity of Png1 and compare it to that of GT.

The second substrate we examine is the serine protease inhibitor α_1 -antitrypsin (α_1 -AT), which carries three *N*-linked glycans. Several mutations in α_1 -AT interfere with the correct folding of α_1 -AT, and prevent secretion. The null Hong Kong (NHK) variant harbours a dinucleotide deletion within Leu318, and yields a premature termination codon. The resulting polypeptide fails to leave the ER and is degraded (Fabbretti *et al*, 1992).

In the Pi Z variant, Glu342 is changed to Lys342. This mutation interferes with folding of the protein and prevents its secretion. In some cases, the Pi Z variant is not successfully degraded from the ER, resulting in accumulation of α_1 -AT in the ER of hepatocytes. The NHK and the Pi Z variants of α_1 -AT may therefore be considered misfolded glycoproteins and were tested as substrates for Png1.

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Fig 1 | Structure of RNaseB modelled with and without S-peptide. (A) Structure of the bovine pancreatic RNaseA (Leonidas *et al*, 1997). The Asn34 that carries the *N*-linked glycan in the RNaseB form is rendered in red, and the S-peptide in white. (B) Model of the RNaseBS-Prot structure.

RESULTS

Png1 deglycosylates only denatured RNaseB

We incubated native RNaseB with several glycosidases to determine accessibility of the oligosaccharide on RNaseB to deglycosylation (Fig 2). Endo- β -*N*-acetylglucosamidase (Endo A) releases *N*-linked oligosaccharides by cleaving the di-*N*-acetylchitobiose core. As concluded from the shift in molecular weight, Endo A attacks native RNaseB (Fig 2, lane 2, compared to undigested RNaseB, lane 1). Likewise, digestion with Endo H yielded the deglycosylated form of RNaseB (Fig 2, lane 3). Incubation of native RNaseB with Png F converted the substrate into the faster migrating, deglycosylated form (Fig 2, lane 4), whereas incubation with yeast Png1 did not yield any deglycosylated material (Fig 2, lane 5). Subsequent incubation of *N*-glycanase-treated RNaseB with Endo H gave rise to the deglycosylated form of RNaseB (Fig 2, lane 6).

Denatured and alkylated RNaseB was tested in a similar set of experiments (Fig 2, lanes 7–12). All glycosidases used, including Png1, yielded the deglycosylated form of RNaseB. Subsequent incubation of Png1-treated, alkylated RNaseB with Endo H did not further reduce its apparent molecular weight, indicating that the change in mobility after Png1 digestion is due to deglycosylation

Fig 2 | (Lanes 1–6) Digestion of native RNaseB (lane 1) with the indicated glycosidases (lanes 2–6). (Lanes 7–12) Digestion of reduced and alkylated RNaseB (lane 7) with the indicated glycosidases. After enzymatic digestion, samples were analysed by SDS–PAGE followed by silver staining. The number of glycans is indicated on the right. The following amounts of enzyme were used: Endo A: 1,440 U; Endo H: 100 U; Png F: 5,000 U; Png1: 420 U. The total amount of substrate (RNaseB) for each reaction volume was 0.4 μ g in 50 μ l. Samples were incubated at 20–23 $^{\circ}$ C for 16 h.

and not proteolysis of the peptide backbone. We conclude that Png1 digests only denatured RNaseB, unlike Endo A, Endo H and Png F, which attack both native and non-native RNaseB when assayed at similar levels of enzyme activity.

RNaseBS-Prot is susceptible to deglycosylation by Png1

To test whether the truncated form of RNaseB, RNaseBS-Prot, is deglycosylated by Png1, we partially digested RNaseB with subtilisin. The complex of RNaseBS-Prot and S-peptide was then dissociated at low pH and separated from the free S-peptide by gel filtration. SDS-PAGE showed that native RNaseB and the faster migrating RNaseBS-Prot were both present in the sample (Fig 3, lane 1). Next, we tested the mixture of truncated and native RNaseB in the deglycosylation assay, using Endo H and Png1. Both native RNaseB and RNaseBS-Prot were accessible to deglycosylation by Endo H (Fig 3, lane 2), whereas Png1 deglycosylated RNaseBS-Prot only (Fig 3, lane 3). Addition of the synthetic S-peptide to RNaseBS-Prot yielded RNaseBS. While RNaseBS is sensitive to deglycosylation by Endo H (Fig 3, lane 4), inclusion of the S-peptide restored resistance to deglycosylation by Png1 (Fig 3, lane 5). It is possible that removal of the S-peptide renders RNaseBS-Prot more susceptible to reduction by DTT than native RNaseB. RNaseBS-Prot denatured by DTT would probably assume a structure comparable to that of the fully denatured RNaseB, which is deglycosylated by Png1. To exclude this possibility, we performed the deglycosylation assay in the absence of DTT. While the activity of Png1 was reduced under these

conditions, most of the RNaseBS-Prot molecules present in the sample were still deglycosylated, indicating that RNaseBS-Prot deglycosylated by Png1 is not denatured by DTT (compare Fig 3, lanes 3 and 7). Furthermore, we observed significant deglycosylation of RNaseBS-Prot even in the presence of 2 mM of the oxidizing agent GSSG (data not shown).

α_1 -AT matures similarly in HepG2 and HEK-293 cells

Since HEK-293 cells do not normally synthesize α_1 -AT, we examined whether proper maturation of α_1 -AT can occur in this cell line. As a reference we used wt- α_1 -AT from HepG2 cells and compared it to wt- α_1 -AT obtained from HEK-293 cells. If both sources produce wt- α_1 -AT of an identical structure, then digestion of wt- α_1 -AT by different glycosidases should yield an identical pattern. We labelled HepG2 cells and HEK-293 cells transiently transfected with wt- α_1 -AT with 35 S-methionine for 30 min and equal aliquots of cell extracts were incubated with Endo A, Endo H, Png F or Png1 for 12 h. In the untreated control, we observed two forms of α_1 -AT: a faster migrating species that carries high-mannose-type N-glycans, and a slower migrating form with complex-type sugars (Fig 4A, lane 1) (Yilla *et al.*, 1993). Digestion of α_1 -AT from HepG2 cells with Endo A yielded two products of distinct mobility that represent polypeptides that have lost either one or two glycans (Fig 4A, lane 2). Endo H removed two N-linked glycans from wt- α_1 -AT (Fig 4A, lane 3). Digestion with Png F produced almost no deglycosylated products, although trace amounts of wt- α_1 -AT lacking one or two glycans can be observed (Fig 4A, lane 4). After digestion with Png1, a small population of α_1 -AT that had lost a single glycan could be seen (Fig 4A, lane 5). The patterns obtained after glycosidase digestion of α_1 -AT derived from HepG2 cells and HEK-293 cells are indistinguishable (compare Fig 4A,B, lanes 1–5), suggesting that both sources of α_1 -AT yield products of identical structure. Furthermore, equal amounts of α_1 -AT molecules bearing complex-type sugars are present at the end of the 30 min pulse (compare Fig 4A lane 1 to Fig 4B lane 1), indicating that α_1 -AT matures equally fast in both cell lines. Since Png1 digestion of wt- α_1 -AT produced a small population of α_1 -AT molecules that had lost a single N-linked glycan, we tested whether this material represented a population of α_1 -AT molecules that had not yet folded completely. We labelled HEK-293 cells transiently transfected with wt- α_1 -AT for 1 min and lysed the cells either at the 0 min time point or 2 min after the pulse. In untreated samples, the fully glycosylated α_1 -AT molecule was the only species that could be detected (Fig 4C, lanes 1 and 2). Digestion of the 0 min sample with Png1 produced the population of α_1 -AT lacking a single N-linked glycan that we observed in the previous digests (compare Fig 4C lane 4 to Fig 4A lane 5 and Fig 4B lane 5). Digestion of the sample from the 2 min time point did not yield any deglycosylated α_1 -AT, indicating that the α_1 -AT had acquired its mature structure 2 min after its synthesis and thus acquired resistance to deglycosylation by Png1 (Fig 4C, lane 5).

α_1 -AT Hong Kong is deglycosylated by all glycosidases

As expected, for the NHK variant, no α_1 -AT bearing complex-type oligosaccharides could be detected (Fig 5A), since the NHK variant is retained in the ER and degraded in a pre-Golgi compartment (Hosokawa *et al.*, 2003). All digests yielded the fully deglycosylated form of NHK. Only the efficiency of the digestions

Fig 3 | RNaseB was partially digested with subtilisin, yielding a preparation that contained undigested RNaseB and the faster migrating RNaseBS-Prot (lane 1). Digestion with Endo H deglycosylated both forms of the RNaseB (lane 2), whereas Png1 deglycosylated only the RNaseBS-Prot (lane 3). RNaseB reconstituted with S-peptide is susceptible to deglycosylation by Endo H (lane 4), but resistant to deglycosylation by Png1 (lane 5). The activity of Endo H is not reduced in the absence of DTT (lane 6), whereas Png1 activity shows a marked reduction in activity (lane 7). Endo H: 25 U; Png1: 42 U. Total RNaseB concentration was 5 μ g in a final volume of 30 μ l. Reactions were incubated at 20–23 $^{\circ}$ C for 16 h.

Fig 4 | Deglycosylation profile of α_1 -AT from HepG2 and HEK-293 cells. (A) α_1 -AT was recovered by immunoprecipitation from 35 S-methionine-labelled HepG2 extracts that were either left untreated (lane 1) or incubated with the indicated glycosidases (lanes 2–5). Immunoprecipitates were analysed by SDS-PAGE followed by fluorography. The asterisk (*) indicates *N*-linked glycans of the complex type. (B) As in (A), the source of the α_1 -AT was transiently transfected HEK-293 cells. (C) HEK-293 cells transiently transfected with wt- α_1 -AT were pulse labelled with 35 S-methionine for 1 min and then lysed either 0 or 2 min after the chase. Samples were left untreated (lanes 1 and 2) or incubated with Png1 (lanes 4 and 5). Endo A: 2,400 U; Endo H: 1,500 U; Png F: 5,000 U; Png1: 2,800 U. Reactions were performed in a total volume of 1 ml, incubated 16 h at 4 °C.

differed, since Png F, and in particular Png1, also produced partially deglycosylated products.

α_1 -AT_{Pi Z} is susceptible to deglycosylation by Png1

The Pi Z variant of α_1 -AT comigrated with wild-type α_1 -AT, but no α_1 -AT bearing complex-type glycans is present, since the Pi Z variant is retained in the ER. Both Endo A and H yielded exclusively α_1 -AT that had lost two glycans (Fig 5B, lanes 2 and 3). From the sample treated with Png F, the fully glycosylated molecule and a form that had lost one glycan were recovered (Fig 5B, lane 4). Deglycosylation with Png1 yielded predominantly a molecule that had lost two glycans and lesser amounts of α_1 -AT with more than a single glycan (Fig 5B, lane 5).

DISCUSSION

We examined the ability of Png1 to deglycosylate RNaseB and α_1 -AT along with non-native derivatives of these two glycoproteins. Using different forms of RNaseB, we demonstrate that Png1 does not deglycosylate native RNaseB. This property is unique to Png1, since all other glycosidases tested attacked native RNaseB at similar levels of added active enzyme. By contrast, denatured

Fig 5 | Mutants of α_1 -AT are more susceptible to deglycosylation by Png1. (A) HEK-293 cells were transiently transfected with NHK-AT. NHK-AT was recovered by immunoprecipitation from 35 S-methionine-labelled lysates that were either left untreated (lane 1) or incubated with the indicated glycosidases (lanes 2–5). Immunoprecipitates were analysed by SDS-PAGE followed by fluorography. (B) Similar set of experiments as in (A), the substrate used here is Pi Z-AT. Endoglycosidase concentrations were the same as in Fig 4.

RNaseB was readily deglycosylated by Png1 and all other glycosidases as well.

To examine whether a more subtle structural change also rendered RNaseB susceptible to deglycosylation by Png1, we digested native RNaseB with subtilisin. This procedure inactivates RNaseB by removing the N-terminal 20 amino acids (S-peptide). On removal of the S-peptide, the resulting RNaseB-S-Prot molecule was deglycosylated by Png1. Generation of RNaseBS by addition of the synthetic S-peptide to RNaseB-S-Prot restored resistance of the resulting RNaseBS to digestion by Png1. Since deglycosylation of RNaseBS-Prot by Png1 occurred even under mildly oxidizing conditions and the S-peptide does not participate in disulphide bond formation, the structure of the remainder of the protein should not be significantly affected. The ability of RNaseBS-Prot to bind the S-peptide and the RNase inhibitor further supports this assumption (Neumann & Hofsteenge, 1994).

We also examined different variants of α_1 -AT as substrates for Png1. Wild-type α_1 -AT derived from HepG2 cells or produced by transiently transfected HEK-293 cells yielded identical deglycosylation patterns when exposed to different glycosidases. This result is not unexpected, since no specific cofactors are known to be required for α_1 -AT to mature properly. This experiment therefore confirmed that glycosidase digestion can be used as a tool to compare the structure of two glycoproteins, especially when more than one *N*-glycan is present on the protein. Furthermore, we observed similar amounts of the α_1 -AT form that carried complex-type glycans, when comparing HepG2 and HEK-293 transfectants. Both cell lines thus supported maturation of α_1 -AT from the ER with comparable efficiency. Digestion of wt- α_1 -AT with Png1 consistently gave rise to a small population that had lost a single glycan. This material represents a fraction of α_1 -AT molecules that is targeted by Png1 presumably because it has not folded completely. After 2 min of chase, all α_1 -AT had folded properly

and acquired resistance to Png1 digestion. The ability to recognize this immature subpopulation of α_1 -AT is unique to Png1 since the other glycosidases did not allow detection of incompletely folded α_1 -AT.

The misfolded NHK variant was deglycosylated by all glycosidases tested, with Png1 being the least efficient enzyme. Therefore, the truncation of the NHK variant appears to result in a partially unfolded molecule. This result confirms the hypothesis that Png1 prefers unfolded substrates, but in this set of experiments the preference for unfolded glycoproteins does not appear to be unique for Png1. More interesting is the pattern obtained by the digestion of Pi Z, a point mutation of α_1 -AT. While digestion with Endo A and Endo H is only moderately more efficient, when compared to the wt- α_1 -AT sample, digestion with Png F yielded significantly more deglycosylated material when compared to the wild-type sample, an effect even more pronounced for the sample exposed to Png1. Here, the form that had lost two glycans predominated. The differential susceptibility of α_1 -AT variants to digestion by Png1 suggests that Png1 is indeed capable of distinguishing different glycoprotein substrates based on their conformation. The NHK or the Pi Z proteins are probably not entirely misfolded. Therefore, it cannot be expected that these mutations render all *N*-glycans on α_1 -AT equally susceptible to deglycosylation by Png1.

What structural elements are required for GT or Png1 to identify their targets? The substrate specificity of GT is not fully understood. The innermost GlcNAc unit of *N*-glycans, which can be buried inside folded proteins, is one determinant (Sousa & Parodi, 1995). Generally, it appears that GT prefers proteins in a molten-globule configuration over fully denatured proteins (Caramelo *et al.*, 2003). Png1 appears to recognize the innermost GlcNAc like GT, since *N*-glycans that are fucosylated at this position are not a substrate of Png1 (Suzuki *et al.*, 1995; Hirsch *et al.*, 2003). In addition, Png1 must also recognize structural elements within the glycoprotein itself, since class I MHC heavy chain molecules in a complex with β_2m are not deglycosylated, although the entire oligosaccharide is likely to be fully accessible based on the structure of the class I MHC molecule (Bjorkman *et al.*, 1987). The substrate specificity of Png1 reported here supports the assumption that this enzyme evolved to deglycosylate specifically the type of substrate it is likely to encounter in the cytosol: single-chain glycoproteins that are unfolded by the reducing conditions maintained in the cytosol, or glycoprotein fragments generated by proteolysis. A binding mechanism that involves recognition of both the oligosaccharide and a segment of the polypeptide backbone may increase the affinity of Png1 for its substrates. For endoglycosidases Endo H and Endo F₁, it was shown that the protein moiety of their substrates is located such that direct interactions between the polypeptide portion of the substrate and the enzyme itself do not occur (Rao *et al.*, 1995). Png F requires the *N*-linked asparagine to be part of a polypeptide, suggestive of some interaction between the protein portion of the substrate and the enzyme (Norris *et al.*, 1994). Png1 must require additional structural elements, since it does not deglycosylate native RNaseB, unlike PNGase F. Restricting access of the catalytic site to partially unfolded protein substrates may prevent binding of Png1 to other potential substrates such as the Man₅GlcAc₂-dolicholpyrophosphate assembled on the cytoplasmic face of the ER.

Fig 6 | Kinetics of Png1. (A) Rate of deglycosylation of RNaseB by Png1. Png1 (0.56 nM) was incubated with RNaseB (0.122 mM) at 30 °C. Samples were taken at the indicated time points and subjected to SDS-PAGE followed by Coomassie staining. The zero time point was taken prior to addition of Png1. (B) Quantitation of RNaseB deglycosylation by densitometry. The glycosylated form of RNaseB (+ CHO; substrate, black diamonds) and the deglycosylated form of RNaseB (–CHO; product, grey squares) are indicated. (C) Png1 does not attack native RNaseB, while PNGase F recognizes and deglycosylates native RNaseB. Native RNaseB was incubated with the indicated units of Png1 or PNGase F at 20–23 °C for 16 h, and fractions were subjected to SDS-PAGE, followed by Coomassie staining. Even at high enzyme concentrations, Png1 fails to deglycosylate native RNaseB.

Since Png1 recognizes the same RNaseB derivatives as GT, could the two enzymes use a similar mechanism of recognition to target their substrates? Although there is an analogy between Png1 and GT in terms of substrate recognition, we cannot conclude that their actual mechanism of recognition is the same in the absence of further structural data. A crystal structure of Png1 will be

required to understand fully the mechanism by which substrate recognition occurs.

METHODS

Cell culture, transient transfections, pulse-chase analysis, immunoprecipitations and SDS-PAGE were performed as described (Hirsch et al, 2003). α_1 -AT antibodies and subtilisin were purchased from ICN. RNaseB was purchased from Sigma. Glycosidase digestions of RNaseB were performed overnight at 20–23 °C and glycosidase digestions of antitrypsin were carried out at 4 °C in NP-40 lysis buffer (0.5% NP-40, 50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM MgCl₂ and 1 mM DTT). Digestion of RNaseB with subtilisin was performed as described before (Richards, 1959). The S-peptide was synthesized by standard Fmoc solid-phase peptide synthesis and used at 1 mM final concentration. Molecular modelling of the images was carried out using Pymol available at <http://www.pymol.org>.

Plasmid constructs. His-tagged yeast Png1 was amplified using the primers described below and cloned into the *NcoI* and *XhoI* sites of the pET-28a(+) vector (Novagen). Sense: CATGCCATGG GAGAGGTATACGAAAAA; antisense: CCGCTCGAGTTTACCA TCCTCCCCACGCTG.

Png1 expression and purification. BL21/DE3 *Escherichia coli* bacteria transformed with PNG-6His plasmid were grown at 37 °C (30 mg l⁻¹ kanamycin). After 3 h of induction with IPTG (1 mM), cells were harvested and lysed using a french press followed by sonication, in buffer A (20 mM Tris (pH 8), 150 mM NaCl, 5% glycerol) containing 5 mM imidazole and 1% Triton X-100. The sample was loaded on a Ni²⁺-NTA column (QIAGEN), equilibrated with buffer A. The column was washed with five column-volumes (CV) of buffer A plus 10 mM imidazole and 0.1% Triton X-100, followed by 10 CV of buffer A containing 15 mM imidazole. Png1-6His was eluted with 5 CV of elution buffer (200 mM imidazole (pH 8.0), 5% glycerol, 1 mM DTT). The eluted Png1-6His was further purified on a superdex-200-prep-grade 16/600 sizing column (Pharmacia), equilibrated with elution buffer. Png1 was concentrated to 6 mg ml⁻¹, and its activity was calculated to be approximately 140 U μ l⁻¹. Endo A was cloned from *Arthrobacter protophormiae*. It was purified (Takegawa et al, 1997) and concentrated to 1.3 mg ml⁻¹, and its activity was determined to be 480 U μ l⁻¹. Endo H and PNGase F activities were 500 U μ l⁻¹ (NEB). One unit (U) is defined as the amount of enzyme required to remove more than 95% of glycans from 10 μ g of denatured RNaseB in 1 h.

Png1 kinetics. The kinetic parameters of purified N-glycanase were assessed for RNaseB and quantitated by densitometry of Coomassie-stained gels (Fig 6).

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