Multiple modes of interaction of the deglycosylation enzyme, mouse peptide *N*-glycanase, with the proteasome

Guangtao Li*, Xiaoke Zhou*†, Gang Zhao*†, Hermann Schindelin*†, and William J. Lennarz*‡

*Department of Biochemistry and Cell Biology and †Center for Structural Biology, State University of New York, Stony Brook, NY 11794-5215

Contributed by William J. Lennarz, September 14, 2005

Peptide *N*-glycanase (PNGase) is involved in the cleavage of oligosaccharide chains from misfolded glycoproteins that are destined for degradation by the proteasome. Earlier, a number of potential binding partners of mouse PNGase (mPNGase) were detected by using the yeast two-hybrid system. In the current study, an *in vitro* system was set up to investigate direct interactions between mPNGase and these candidate proteins. Although the yeast two-hybrid system suggested an interaction of six different proteins with mPNGase, only mHR23B and the proteasome subunit mS4 were found to interact with mPNGase. In fact, mS4 competes with mHR23B for binding to mPNGase. These results suggested two possible pathways for the interaction between mPNGase and the proteasome. In one pathway, mHR23B mediates the interaction between mPNGase and the proteasome. In an alternative pathway, mPNGase directly binds to the proteasome subunit, mS4. In either case, it is clear that PNGase is located in close proximity to the proteasome and is available for deglycosylation of glycoproteins destined for degradation. Surprisingly, mPNGase also was found to mediate binding of the cytoplasmic protein, p97, to the proteasome through the formation of a ternary complex made up of mHR23B, mPNGase, and p97. Because p97 is known to bind to the endoplasmic reticulum membrane protein AMFR (gp78), an E3 ligase, we propose a model in which p97, mPNGase, and mHR23B mediate interaction of the endoplasmic reticulum with the proteasome.

mHR23B | mS4 | PNGase

The endoplasmic reticulum (ER) contains a quality control system to ensure that only properly folded proteins and glycoproteins are routed into the secretory pathway. Misfolded proteins are retrotranslocated to the cytosol, where they are degraded by the 26S proteasome. In the case of misfolded glycoproteins, the glycoprotein also is retrotranslocated from the ER, and then their N-linked glycan chains may be removed by peptide *N*-glycanase (PNGase) before proteolysis (1, 2).

PNGase is highly conserved among eukaryotes (1, 3). Although both the *Saccharomyces cerevisiae* PNG1 gene product and the mouse Png1p protein were initially believed to be catalytically active *in vitro* only on synthetic glycopeptides (3, 4), it was shown subsequently that both deglycosylate misfolded glycoproteins (2, 5, 6). In addition, both enzymes are found to have a preference for proteins that contain high-mannose oligosaccharides over those bearing complex-type oligosaccharides chains (2). Furthermore, yeast PNGase was shown to discriminate between nonnative and properly folded glycoproteins and only deglycosylated the former (2, 5, 6). The crystal structures of complexes of yPNGase and the xeroderma pigmentosum group C-binding domain of yRad23 (yRad23XBD) with or without the caspase inhibitor Z-VAD-fmk, provide a possible explanation for the specificity of yPNGase for denatured substrates (7). The active site of yPNGase was found to be located in a deep cleft generated by a domain of amino acid residues conserved in all PNGase members, and three saccharide units were found to be bound in this cleft. Although the walls of the cleft block access of folded glycoproteins to the active site of yPNGase, the cleft is sufficiently wide to accommodate denatured glycoproteins (7). Recently, it was found that in human astrocytoma cell lines treated with PNGase small-interfering RNA, glycosylated class I MHC molecules appeared in the cytosol, providing the first evidence for the appearance of an intact *N*-linked type I membrane glycoprotein in the cytosol (8). This finding also indicated that PNGase activity is not required for dislocation of these glycosylated class I MHC molecules from the ER (8). However, the biological function of PNGase is still not clear: Does PNGase only serve to remove the bulky glycans to facilitate proteasomal entry, or is there an additional regulatory or unfolding activity associated with it? To better understand the overall function of PNGase, we studied the binding partner(s) of PNGase because it seemed likely that this would provide insights into its biological role.

Recently, our laboratory reported that yPng1p interacts with the 26S proteasome via Rad23p (3). The mammalian homolog (mPng1p) differs from its yeast ortholog in that it contains extended domains at both its N and C termini (4). The N-terminal extension of mouse peptide *N*-glycanase (mPNGase) contains a PNGase/ubiquitin-associated or UBX-containing protein (PUB) domain, which may mediate protein–protein interactions (9, 10). Yeast two-hybrid library screening showed that in addition to mHR23B (a homolog of yeast Rad23p), mPNGase was found to interact with other proteins: mS4 [a subunit at the base of the 19S regulatory particle (RP) of the proteasome], mY33K (a protein of unknown function with a ubiquitin-like and ubiquitin-associated domain), ubiquitin, importin α, and mouse autocrine motility factor receptor (mAMFR, an E3 ligase located on ER membrane) (4). All these candidates are involved in or predicted to be involved in the ubiquitin-dependent degradation pathway. In this study, our objective was to determine in *in vitro* studies which of these putative protein–protein interactions actually occur. In the long term knowledge of these interactions is important because those candidates that interact would help us to understand how the function of mPNGase is regulated.

Earlier we found that among the candidates identified by the yeast two-hybrid system, only mHR23B had been shown to directly physically interact with mPNGase (4, 11). Furthermore, in COS1 cells PNGase was found to copurify with the 26S proteasome, in which mS4 is one of the subunits (4). It has been reported in yeast that yRad23p serves as a bridge between yPNGase and the 26S proteasome (3). Similar observations have been made in a mammalian system (11). Therefore, it was of interest to determine whether mPNGase directly interacts with

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Abbreviations: PNGase, peptide *N*-glycanase; mPNGase, mouse PNGase; ER, endoplasmic reticulum; RP, regulatory particle; GSH, glutathione; mAMFR, mouse autocrine motility factor receptor; PUB, PNGase/ubiquitin-associated or UBX-containing protein.

‡To whom correspondence should be addressed. E-mail: wlennarz@notes.cc.sunysb.edu.

the other candidates found by the yeast two-hybrid system. Of special interest is a possible interaction between mPNGase and mS4, which would indicate a direct interaction between mPNGase and the 26S proteasome. In addition to these above mentioned proteins, another cytoplasmic protein, p97, was also reported to interact with mPNGase (12). What are the relationships between all proteins that directly interact with mPNGase? Does mPNGase form a complex with all of these candidates? Or are there several complexes, each with one or two proteins bound to mPNGase? We addressed these issues and found that among the candidates identified in the yeast two-hybrid screening, only mS4 and mHR23B directly interact with mPNGase. The finding that both mHR23B and mS4 directly interact with mPNGase suggests there may be at least two pathways allowing mPNGase to interact with the proteasome. In addition, we identified that a ternary complex was formed between mHR23B, mPNGase, and mp97. These findings suggest a new model for the transfer of substrates to the proteasome.

Materials and Methods

Abs and Chemicals. Polyclonal antiserum against mPNGase was kindly provided by Tadashi Suzuki (University of Osaka, Osaka). mAbs against GST and poly- and monoclonal His were purchased from Santa Cruz Biotechnology. Glutathione (GSH)-agarose beads were purchased from Molecular Probes. Chelating Sepharose FastFlow was purchased from Amersham Pharmacia.

Constructs of Plasmids. The Image clone no. 4163287 purchased from Invitrogen was used as template to construct expression plasmid pGEX-5x-1-mS4. The full-length mS4 was cloned at the pGEX-5x-1 vector (Amersham Pharmacia) at the XhoI and NotI sites. Plasmid pQE9 containing the full-length of p97 was described in an earlier paper (13) and was kindly provided by Hemmo H. Meyer (Swiss Federal Institute of Technology, Zurich). pGEX-5x-1-mHR23b and a set of His-6-tagged mPNGase forms: 1–651 aa, 1–171 aa, 1–471 aa, 471–651 aa, and 171–471 aa were described (4, 11). Another set of His-6-tagged mPNGase truncated constructs: 1–111 aa, 112–450 aa, 451–651 aa, and 1–450 aa were also prepared: All fragments except 112–450 aa were cloned into the pET28a vector at the *Nde*I and *Xho*I sites. The mPNGase 112–450 aa was cloned into the pET21b vector for improved expression. The Image clone no. 3710780 purchased from Invitrogen was used as template to construct expression plasmid pGEX-5x-1-mAMFR(c, cytoplasmic domain) (309–643 aa) into the pGEX-5x-1 vector at the EcoRI and SmaI sites.

Purification of Proteins. The expression and purification of GST, GST-AMFR(c), GST-mS4, and GST-mHR23B were carried out according to the manufacturer's manual (Qiagen, Valencia, CA). In brief, the constructs were transformed into *Escherichia coli* strain BL21(DE3)pLysS, and protein expression was induced by adding 0.1 mM isopropyl-β-D-thiogalacto-pyranoside when the A_{600} reached 0.8. After 3 h of induction, cells were harvested by centrifugation and suspended in chilled lysis buffer (1× PBS/1% Triton X-100/5% glycerol/5 mM DTT/0.1% PIC I and II/4 mM PMSF, pH 7.4) and passed through a French press twice. The cell lysates were centrifuged at 14,000 × *g* for 20 min at 4°C. The supernatant was mixed with the GSH beads, which were preequilibrated with 10 vol of 1× PBS. The mixture was rocked at 4°C for 3 h, and then the beads were washed five times with washing buffer (1× PBS/1% Triton X-100/5 mM DTT/4 mM PMSF). The beads were stored at 4°C.

Purification of p97 was performed as described (11, 13). All of the mPNGase fragments were transformed into the BL21(DE3) Codon Plus-containing RIL strain. The constructs amino acids 1–111 and 451–651 were induced at 37°C for 3 h, whereas constructs 1–450 and 112–450 were induced at 15°C overnight. Purification was typically done by using a protocol involving three chromatography steps: first NiNTA affinity column, followed by anion exchange (MonoQ, Amersham Biosciences) and size exclusion chromatography (Superdex 200, Amersham Biosciences).

GST Pull-Down Experiments. All GST pull-down experiments were performed with purified proteins. If not otherwise stated, 4 μg of GST or GST fusion proteins [GST-mS4, GST-mHR23B, and GST-AMFR(c)] bound to 8 μl of GSH-agarose beads were mixed with 5 μg of full-length His-6-mPNGase or truncated forms in 0.5 ml of binding buffer (1× PBS/1% Triton X-100/5 mM DTT/5% glycerol/4 mM PMSF, pH 7.4). The binding experiments were carried out at 4°C for 3 h, and the beads were washed five times with the washing buffer. Bound proteins were eluted with sample buffer and analyzed by SDS/PAGE followed by Western blot with the appropriate Abs. For all binding experiments involving p97, 1 mM $MgCl_2$ and 1 mM ATP were added to the binding and washing buffer.

Competition experiments were carried out in the same way. GSH-agarose beads containing 0.25 μM bound GST-mS4 or GST-mHR23B were incubated with 0.25 μM His-mPNGase in the presence of a 0, 1, 2, 5, or 10 molar excess of competitors. The combinations were as follows: GST-mS4 competing with His-6-mHR23B or BSA; GST-mHR23B competing with His-6-mHR23B or His-6-p97.

SDS/PAGE and Western Blotting. Protein samples were solubilized in reducing SDS-loading sample buffer, boiled for 5 min, and analyzed by using SDS-polyacrylamide gels followed by staining with Coomassie blue R-250 or Western blotting. Western blotting was performed on nitrocellulose (Schleicher & Schuell) as described in ref. 14.

PNGase Activity Assay. PNGase activity was assayed by using fetuin-derived asialoglycopeptide I ($[^{14}C]CH_3)_2$Leu-Asn($GlcNAc_5Man_3Gal_3$)-Asp-Ser-Arg) as substrate as described in refs. 3, 15, and 16. PNGase activity was assayed by paper chromatography as reported in ref. 2. The reaction mixture (7 μl) contained 7 μg of mPNGase full-length or truncated form or 14 μg of the complex of mPNGase (1–450 aa) and mHR23B, 5 mM DTT, 70 mM Hepes-NaOH buffer (pH 7.2), and 25 μM substrate. Radioactivity was monitored by using a PhosphorImager (Molecular Dynamics).

Results

mS4 Directly Interacts with mPNGase. In an earlier report, using the yeast two-hybrid system, it was shown that several proteins, mHR23B, mS4, mImportin-α, mAMFR, mUbiquitin, and mY33k, were candidates for interaction with mPNGase (4). However, the only direct *in vitro* interaction that had been shown was between mHR23B and mPNGase (4, 11). A direct physical interaction between mY33k and mPNGase could not be detected by another group (12). Subsequently, in COS1 cells mPNGase was also found to copurify with the 26S proteasome containing subunit S4 in a process dependent on the presence of ATP (4). In the current study, the direct *in vitro* interactions among the proteins identified in the yeast two-hybrid system and mPNGase was studied. We expressed GST, GST-mHR23B, GST-mImportin α, GST-AMFR(c), GST-Ubiquitin, GST-mS4, and His-6-mPNGase in *E. coli* and purified the proteins. Subsequently, we carried out binding experiments by using GST-fusion proteins as described in *Materials and Methods*. The result showed that GST-mS4 directly associated with mPNGase (Fig. 1, lanes 2 and 3). The previously observed direct interaction between mHR23B and mPNGase was confirmed as well (Fig. 1, lane 4). However, GST itself and another GST fusion candidate

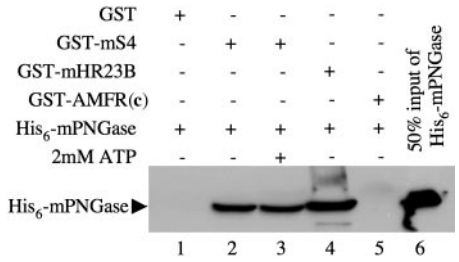

Fig. 1. mS4 directly interacts with mPNGase. Purified GST and GST fusion proteins (4 μg) bound to 8 μl of GSH-agarose beads were incubated with 5 μg of purified mPNGase in binding buffer for 3 h at 4°C and were washed five times with washing buffer. Parallel experiments were carried out with or without 2 mM ATP and 1 mM $MgCl_2$. The beads were treated with SDS-loading buffer, and the eluted proteins were subjected to SDS/PAGE, electrotransferred, and blotted with polyclonal anti-His Ab.

containing the cytoplasmic domain of AMFR [GST-AMFR(c)] did not directly interact with mPNGase under the same conditions (Fig. 1, lanes 1 and 5). All of the other candidates that were originally detected by the yeast two-hybrid system could not be shown to directly interact with mPNGase (data not shown).

In a earlier study using gel filtration in COS1 cells (4), it was reported that the proteasome–PNGase interaction required ATP. We also tested the ATP requirement in our *in vitro* binding experiments but found that mPNGase bound to GST-mS4 independent of the presence of 2 mM ATP (Fig. 1, compare lanes 2 and 3). In agreement with this result, we found that the N terminus of mS4 (1–203 aa), which lacks the ATP binding and ATPase domains, still interacted with mPNGase when tested in the yeast two-hybrid system (data not shown).

PUB Domain of mPNGase Alone Is Not Sufficient for the Interaction with mS4. To identify the region of mPNGase required for the interaction with the base subunit of the 19S RP, mS4, a set of deletion constructs of mPNGase with a His-6-tag at the N terminus (1–171, 171–471, 471–651, 1–471, and 171–651 aa), were prepared. Unfortunately, only insoluble protein was obtained by using the 171–471- and 171–651-aa constructs. Subsequently, a secondary structure analysis of mPNGase revealed a possible α-helix between 164 and 177 aa. Destruction of the helix may have caused the insolubility of these two truncated proteins. In any case, the GST-fusion protein-binding experiment was carried out by using the other three truncated forms of mPNGase. As shown in Fig. 2*A*, the truncated forms 1–471 and 1–171 aa of mPNGase were found to associate with GST-mS4 (Fig. 2*A*, lane 2). In contrast, the truncated form of the C-terminal domain 471–651 aa did not bound to GST-mS4 (Fig. 2*A*). As a positive control, it was shown that the 1–471-aa fragment of mPNGase associated with GST-mHR23B (Fig. 2*A*, lane 3). These results confirmed and extended a previous observation (11) indicating the importance of the N-terminal domain of mPNGase in its binding to other proteins.

Compared with yeast PNGase, mPNGase has additional domains at both the N and C termini. Within the extended N terminus, there is a PUB domain, which has been proposed to function in interacting with other proteins (1, 9). Because the N terminus of mPNGase(1–171 aa), encompassing the PUB domain located between residues 35 and 80, interacted with mS4, the next question we addressed was whether the PUB domain alone can mediate the interaction with mS4. For this purpose, a new set of truncated forms of mPNGase was prepared (1–111, 112–450, 1–450, and 451–651 aa). Among the four truncated forms, three had a His-6-tag at the N terminus. The 112–450 aa construct had a His-6-tag at the C terminus, but for unknown reasons, it was less reactive with the His Ab (even though the

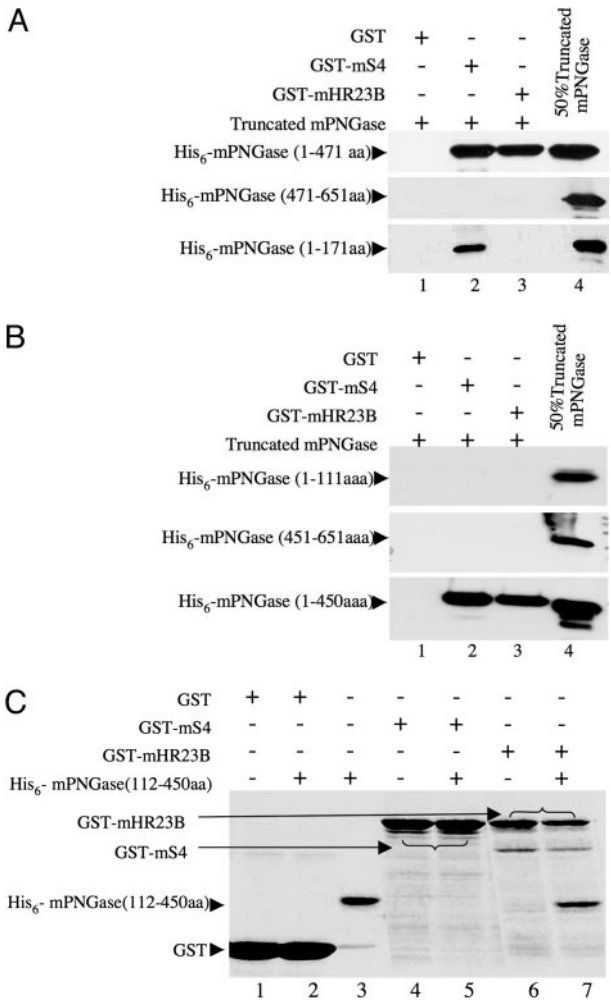

Fig. 2. Interactions of GST, GST-mS4, and GST-mHR23B with different truncated forms of mPNGase. Purified GST and GST fusion proteins (4 μg) bound to 8 μl of GSH-agarose beads were incubated with 5 μg of purified truncated mPNGase in binding buffer for 3 h at 4°C. The beads then were washed five times with washing buffer. Samples bound to the beads were eluted in 40 μl of SDS-loading buffer. (*A* and *B*) Samples (15 μl) were subjected to 12% SDS/PAGE, transferred, and blotted with polyclonal anti-His Ab. (*C*) Samples (15 μl) were subjected to 10% SDS/PAGE and stained with Coomassie blue. (*A*) Results show that the N terminus of mPNGase interacts with mS4 but not with mHR23B. (*B*) PUB domain of mPNGase is not sufficient for interaction with mS4. (*C*) The central domain of mPNGase(112–450 aa) interacts with mHR23B but not with mS4.

His-6 tag was in frame with the fragment of mPNGase). Therefore, Coomassie blue staining was used to test for the possible interaction between the 112–450-aa construct of mPNGase and GST-fusion proteins, whereas Western blot analysis was used for the other truncated form. As shown in Fig. 2 *B* and *C*, among this set of truncated forms of mPNGase, only 1–450 aa interacted with GST-mS4 (Fig. 2*B*, lane 3). Because the truncated form of 1–111 aa containing the intact PUB domain did not interact with GST-mS4 (Fig. 2*B*, lane 3), it is clear that this domain alone is not sufficient for the interaction with GST-mS4. In contrast, two truncated forms of mPNGase, 1–450 aa (Fig. 2*B*, lane 2) and 112–450 aa (Fig. 2*C*, lane 7), were found to interact with GST-mHR23B.

mPNGase Is Still Active in a Complex of mHR23B-mPNGase(1–450 aa). Because the central domain (112–450 aa) of mPNGase that contains the catalytic site of this enzyme interacts with

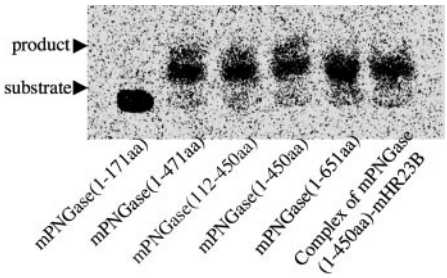

Fig. 3. mPNGase is enzymatically active when it is in a complex with mHR23B. Truncated forms of mPNGase (7 μg) or 14 μg of the complex of mPNGase(1–450 aa)–mHR23B were incubated with 1 μl of fetuin-derived asialoglycopeptide I {([^{14}C]CH_3)$_2$Leu-Asn($GlcNAc_5Man_3Gal_3$)-Asp-Ser-Arg} in a total volume of 7 μl of reaction mixture containing 70 mM Hepes-NaOH (pH 7.2) and 5 mM DTT at room temperature for 16 h. The radioactive deglycosylated peptide product was analyzed by paper chromatography and visualized by phosphorimaging.

mHR23B, we next asked whether this interaction affects the enzymatic activity of mPNGase. His-6-mHR23B and His-6-mPNGase(1–450 aa) were incubated for 1 h at 4°C, and the complex was separated from unbound proteins by using a Superdex 200 column. The complex of mPNGase(1–450 aa)-mHR23B was eluted as a single fraction and collected for mPNGase activity assays. We tested the mPNGase enzyme activity of different truncated mPNGase forms and the complex of mPNGase(1–450 aa)-mHR23B. As shown in Fig. 3, except for mPNGase(1–171 aa), all other forms of mPNGase tested and the complex of mPNGase(1–450 aa)-mHR23B had PNGase activity. These results indicated that mPNGase is active as a complex with mHR23B. This finding suggests that mPNGase could be functional during several steps in the cystosolic pathway leading to protein degradation.

Interaction of mS4 and mPNGase Occurs at a Molar Ratio of 1:1. Next we investigated the stoichiometry of the complex of GST-mS4 and mPNGase. Because the M_r of GST-mS4 and His-6-PNGase are similar, ≈75 kDa, it was not possible to distinguish between the two proteins by SDS/PAGE. Therefore, the truncated form of mPNGase, 1–450 aa, was used. GST-mS4 (0.1 μM) was mixed with truncated His-6-PNGase (0.5 μM), 1–450 aa, in the binding buffer, and the complex was bound to GSH beads, washed, and eluted with sample buffer. The sample was then subjected to SDS/PAGE followed by Coomassie blue staining. Because we already knew that mPNGase interacts with mHR23B in a molar ratio of 1:1 (G.Z. and H.S., unpublished data), GST-mHR23B (0.1 μM) mixed with His-6-PNGase(1–450 aa) (0.5 μM) was used as control. After SDS/PAGE and Coomassie blue staining, we quantified the amount of GST-mS4, GST-mHR23B, and His-6-mPNGase(1–450 aa) by densitometry and converted the values to molar equivalents. We found that GST-mS4 interacts with His-6-mPNGase(1–450 aa) with an approximate molar ratio of 1:1, as does GST-mHR23B (data not shown).

mS4 Competes with mHR23B to Bind to mPNGase. Because both mS4 and mHR23B bind mPNGase, we considered two possibilities for the interaction among the three proteins: In one, mS4 competes with mHR23B to bind mPNGase, and two complexes are formed, one containing mS4 and mPNGase and the other mHR23B and mPNGase. In the second possibility, mS4 and mHR23B both bind to mPNGase and form one ternary complex, which is composed of the three proteins, mS4-mPNGase-mHR23B.

To distinguish between the two possibilities, a competition experiment was carried out. First, we standardized positive experimental conditions to detect the competition between

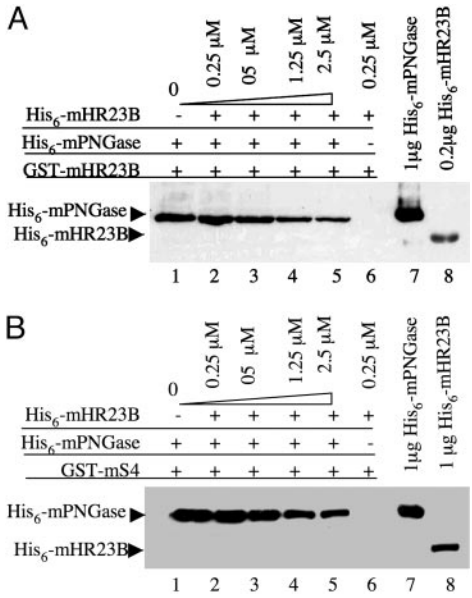

Fig. 4. mS4 competes with mHR23B to bind to mPNGase. GSH-agarose beads (8 μl) containing 0.25 μM bound GST-mS4 or GST-mHR23B were incubated with 0.25 μM His-6-mPNGase in the presence of a 0, 1, 2, 5, or 10 molar excess of the specified competitors. The samples were eluted in 45 μl of SDS-loading buffer and subjected to SDS/PAGE, electrotransferred, and blotted with polyclonal Ab against His. (*A*) His-6-mHR23B competes with GST-mHR23B for binding to mPNGase. (*B*) His-6-mHR23B competes with GST-mS4 for binding to mPNGase.

GST-mHR23B and His-6-mHR23B. Initially, the same molar amounts of GST-mHR23B bound to GSH beads and His-6-mPNGase (0.25 μM) were present in all reactions. Then the amount of His-6-mHR23B was increased from 0- to 10-fold the molar amount of His-6-mPNGase. The results in Fig. 4 demonstrate that the amount of His-6-mPNGase bound to GST-mHR23B decreases when the amount of His-6-mHR23B increases (Fig. 4*A*, lanes 1–5). This result clearly indicated that His-6-mHR23B competes with GST-mHR23B for binding to mPNGase. This finding indicates that this system can be used to carry out a competition study between GST-mS4 and His-6-mHR23B. As shown in the control (Fig. 4*A*, lane 6), GST-mHR23B did not bind to His-6-mHR23B, demonstrating that GST-mHR23B did not form a heterodimer with His-6-mHR23B under this condition.

As in the competition experiment with mHR23B, the same molar amount of GST-mS4 bound to GSH beads and His-6-mPNGase (0.25 μM) were present in all reactions. The molar amount of His-6-mHR23B was increased from 0- to 10-fold the molar amount of GST-mS4. The results demonstrate that the amount of His-6-PNGase bound to GST-mS4 decreases in proportion to the amount of His-6-mHR23B added (Fig. 4*B*, lanes 1–5). However, His-6-mHR23B was not detected as a component of this GST-mS4–mPNGase complex (Fig. 4*B*, lanes 1–5). This result indicates that a ternary complex containing of mS4-mPNGase-mHR23B is not formed. Furthermore, it is clear that there is no direct interaction between mHR23B and mS4 (Fig. 4*B*, lane 6).

To confirm that the competition is specific between mHR23B and mS4, we replaced His-6-mHR23B with an unrelated protein, BSA, to detect any possible competition with GST-mS4 bound to GSH beads. As expected, the amount of His-6-PNGase bound to the GST-mS4 remained constant when the molar amount of BSA was increased to 10-fold over GST-mS4 (data not shown).

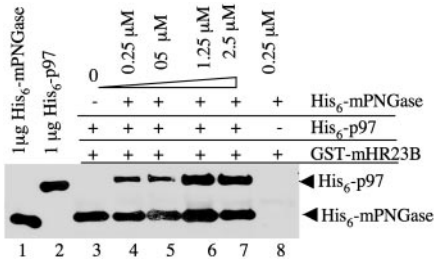

Fig. 5. mHR23B, mPNGase, and p97 form a complex. GSH-agarose beads (8 μl) containing 0.25 μM bound GST-mHR23B were incubated with 0.25 μM His-6-mPNGase in the presence of 0, 1, 2, 5, or 10 molar excess of His-6-p97. The samples were eluted in 45 μl of SDS-loading buffer and subjected to SDS/PAGE, electrotransferred, and then blotted with anti-His mAb.

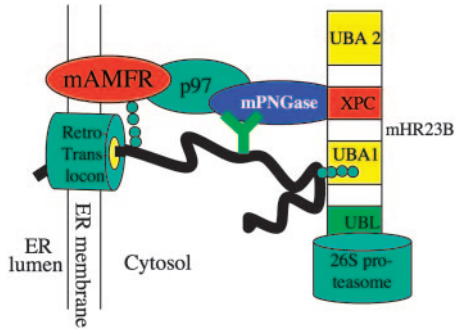

Fig. 6. Model of a mAMFR/p97/mPNGase dependent escort pathway. A protein substrate (black line) is being retrotranslocated (through the retrotranslocon) from the ER lumen to cytosol and recognized by the mAMFR-p97-mPNGase-mHR23B-proteasome complex in the cytosol. Polyubiquitin chain (green dot) is being added by mAMFR, an E3 ligase. The glycan moiety (green Y) is recognized by mPNGase, and a polyubiquitin chain is bound to the ubiquitin-associated (UBA) domain (yellow square) of mHR23B.

mPNGase Mediates the Interaction Between mHR23B and p97. The direct *in vitro* interaction between mPNGase and p97 was reported recently (12). This result was confirmed by using a coimmunoprecipitation experiment (data not shown). We also tested whether mPNGase, mHR23B, and p97 form a complex. The competition experiment between His-6-p97 and GST-mHR23B was carried out as described above by using His-6-p97 as a competitor. As shown in Fig. 5, the amount of His-6-mPNGase bound to GST-mHR23B remained the same when the molar amount of His-6-p97 was increased to 10-fold over GST-mHR23B (Fig. 5, lanes 3–7). At the same time, when His-6-p97 was added to the system, His-6-p97 bound to the GST-mHR23B–mPNGase complex (Fig. 5, lanes 4–7), and the amount of His-6-p97 bound increased with the input until it was saturated (Fig. 5, lanes 6 and 7). When His-6-mPNGase was absent from the system, GST-mHR23B did not bind to His-6-p97 (Fig. 5, lane 8). This result indicates that a ternary complex of mHR23B-mPNGase-p97 is formed and that mPNGase mediates the formation of this complex.

Discussion

Protein degradation was once thought to be a nonspecific scavenger process. It is now clear that this process is highly complex and tightly regulated. A cascade of enzymes and binding proteins with a high degree of substrate specificity keeps the cell from cannibalizing itself. In the case of N-linked glycoproteins, it is clear that PNGase is an important component among the enzymes involved in the ER-associated degradation pathway. mPNGase removes the glycans from misfolded glycoproteins to promote their degradation by the 26S proteasome (5, 8, 10). However, it is not clear how the function of mPNGase is regulated. Does it act more efficiently when it is coupled to other components of the ER-associated degradation pathway? Does it function strictly at only one point in the degradation chain, or is it active during sequential steps, starting when the misfolded glycoproteins are being retrotranslocated through the ER and up to the point of where they are degraded?

Direct and Indirect Interactions Between mPNGase and the Proteasome. In this study, we have identified another protein, mS4, which directly interacts with mPNGase in addition to mHR23B and p97 (4, 11, 12). Interestingly, both of them have a close relationship to the proteasome. mHR23B is a binding partner of the 19S RP base and mS4 is one of the six AAA ATPases of the 19S RP base. In yeast, Rad23p mediates the interaction between yPNGase and the proteasome (3). mHR23B exhibits high homology to yRad23p, and a similar mediator function between mPNGase and the mammalian proteasome was demonstrated (11). The 19S RP ATPases function by unfolding protein substrates before their degradation by the 20S catalytic domain of the proteasome (17, 18). In addition, mS4 is an essential component for the entry of substrates into the 20S proteasome via its ATPase domain (19). Moreover it has been reported that the base of the 19S proteasome regulatory particle exhibits chaperone-like activity (20). We found that the interaction of mS4 with mPNGase is ATP independent. This result is consistent with our results with the yeast two-hybrid system analysis, indicating that the N terminus of mS4 lacking the ATP-binding domain was still functional in the interaction with mPNGase (data not shown).

Our competition experiments demonstrate that mS4 competes with mHR23B to bind to mPNGase. Because the proteins were found to bind overlapping but not identical mPNGase fragments (residues 1–171 and 112–450, respectively), our results suggest the binding sites share some structural determinants but that additional residues are required for specific interactions of each protein with mPNGase. We found that a ternary complex of mS4-mPNGase-mHR23b does not exist. Instead, two binary complexes are formed: mS4-mPNGase and mHR23B-mPNGase. This result may indicate that there are at least two pathways, which bring mPNGase into close spatial proximity of the proteasome: (*i*) mPNGase is indirectly connected to the proteasome by the mediator mHR23B, and (*ii*) mPNGase directly connects to the 19S RP subunit, mS4. In the case of the latter interaction, it seems likely that deglycosylation immediately precedes entry of the glycoprotein into the core of the proteasome.

The 26S proteasome not only degrades proteins earmarked with polyubiquitin chains, but also degrades some proteins in a ubiquitin-independent manner (21). There are two possible hypotheses to explain the competition between mS4 and mHR23B in binding to mPNGase. One possibility is that different substrates require different pathways to be routed to the 26S proteasome: The polyubiquitylated proteins are transferred to the proteasome through mHR23B, whereas the proteins destined for degradation by the proteasome in an ubiquitin-independent fashion may be transferred to the ATPases of the 19S RP base directly. Another possibility is that sequential steps are involved in the transfer of a substrate glycoprotein to mPNGase and from there to mHR23B and then to mS4: mHR23B recruits both the glycoprotein and mPNGase from the cytosol, and, after deglycosylation, the unfolded, deglycosylated protein is routed via mHR23B to mS4 for delivery to the catalytic core for degradation.

mPNGase and mHR23B Interact with mAMFR and p97 to Form a Bridge Between the ER and the Proteasome. Recently it was reported that another cytosolic AAA ATPase, p97, (a homolog of yeast Cdc48) directly interacts with mPNGase (12). P97 is a multifunctional adaptor protein that has been implicated in a number

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of membrane-related cellular processes including extraction of ER-associated degradation substrates from the ER (22–24). In this study, we detected mPNGase-mediated formation of a ternary complex containing mHR23B-mPNGase-p97. Because the xeroderma pigmentosum group C domain of mHR23B interacts with mPNGase (G.Z. and H.S., unpublished data) and the ubiquitin-like domain on the N terminus of yRad23 (and its mammalian homolog, mHR23B), interact with the proteasome (25), the interaction between mHR23B and mPNGase does not interrupt the interaction between mHR23B and the proteasome. Interestingly, in yeast, another protein, UFD2, mediates interaction of Rad23p and Cdc48 (the yeast homolog of p97) by forming a ternary complex containing Cdc48-UFD2-Rad23p (26). However, Rad23p binds UFD2 via its ubiquitin-like domain, and binding to the proteasome and to UFD2 are competing events (27). The ternary complex of Cdc48-UFD2–Rad23p complex functions in the generation and transfer of the substrates containing short oligo-ubiquitinated chains to the proteasome (26). Our finding indicates that another model for the transfer of substrates to the proteasome exists in mammalian cells (Fig. 6). In this model, the E3 enzyme mAMFR is shown to ubiquitinate the misfolded substrate while it is being retrotranslated, and p97, mPNGase, and mHR23B form a complex bridging the ER and the proteasome. The substrate is deglycosylated by mPNGase and the misfolded ubiquitinated and deglycosylated protein is routed via mHR23B to the proteasome. In fact, it has been shown that p97 directly interacts with AMFR (28), and we have found that p97 is an adaptor that mediates mPNGase binding to mAMFR through the formation of a mAMFR–p97–mPNGase complex (G.L. and W.J.L., unpublished data). This model is different from the system in which the proteasome is directly coupled to the retrotranslocon (29). Further experiments should be carried out to test the validity of our alternative model.

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