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The N-terminus of yeast peptide: *N*-glycanase interacts with the DNA repair protein Rad23

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Abstract

Yeast peptide: *N*-glycanase (PNGase) is involved in the proteasomal degradation of misfolded glycoproteins where it interacts with the DNA repair protein Rad23 as first detected in a yeast two-hybrid assay and subsequently confirmed by biochemical in vivo analyses. Limited proteolysis of PNGase with trypsin led to the removal of both an N-terminal and a C-terminal stretch. Based on these truncations the N-terminal region of yeast PNGase was identified as being responsible for binding to Rad23. Secondary structure predictions of this region suggest that it is composed of a single, solvent-exposed α-helix. The interaction between PNGase and Rad23 was studied using surface plasmon resonance revealing an equilibrium binding constant of ~2.5 μM. The oligomeric nature of Rad23 was also investigated using sedimentation equilibrium analysis. Although Rad23 exists as a dimer in solution, the monomeric form of Rad23 associates with a PNGase monomer in a 1:1 stoichiometric ratio.

Keywords: Peptide: *N*-glycanase; Rad23; ERAD; Proteasome; Quality control; Analytical ultracentrifugation; Surface plasmon resonance

Newly synthesized proteins, which are destined for the secretory pathway, are subjected to a quality control system [1]. During this process proteins are sorted in the endoplasmic reticulum on the basis of their conformation. While correctly folded proteins are rerouted from the Golgi via transport vesicles to other destinations, misfolded proteins are subjected to the ER associated degradation process (ERAD) by the proteasome [2]. The characterization of this quality control system is medically important, since human genetic diseases such as autosomal dominant neurohypophyseal diabetes insipidus, α1-antitrypsin (A1Pi Z type) or polyglutamine diseases [spinobulbar muscular atrophy (SBMA)] are probably caused by toxic effects of misfolded proteins or aggregates, which are believed to accumulate due to a deficiency in the degradation machinery in the cytosol [3,4].

Previously it has been shown that the enzyme peptide: *N*-glycanase (PNGase) from *Saccharomyces cerevisiae* is involved in the efficient degradation of glycoproteins by the proteasome [5–7]. PNGase hydrolyzes the β-aspartylglycosylamine bond of asparagine linked glycopeptides and glycoproteins, releasing an intact oligosaccharide and generating at the cleavage site an aspartic acid residue in the protein/peptide. *S. cerevisiae* PNGase shares a core sequence that is highly homologous to the human enzyme (37% identity and 53% similarity). Within this core region is a transglutaminase domain, which contains a Cys-His-Asp triad and is responsible for the catalytic activity [8]. The sequence identity between yeast and human PNGase is highest

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in the transglutaminase domain, but an additional region of high similarity is present at the N-terminus (Fig. 1), suggesting a functional importance of this region. In higher eukaryotes N- and C-terminal extensions of the core region are present. Recently, the interaction between yeast PNGase and the 26S proteasome was found to be mediated via Rad23 [9], further strengthening the functional relationship between deglycosylation and proteasomal degradation. While yeast PNGase is known to interact only with Rad23, mouse PNGase has been reported to interact with several other proteins through its N-terminal extended domain that is not present in yeast [10].

Rad23 is a highly conserved protein involved in both nucleotide excision repair and degradation of proteins by association with the proteasome. The oligomeric state of Rad23 has not been studied in detail so far, although the existence of a dimeric state in solution has been suggested [11,12]. Earlier it has been established by yeast two-hybrid analysis that the C-terminal ubiquitin associating (UBA) domain of Rad23 is essential for binding to PNGase [9], while its N-terminal ubiquitin-like (Ubl) domain is required for its interaction with the 26S proteasome [13]. It has also been determined that both the internal and the C-terminal UBA domains of Rad23 participate in homodimerization of the protein [11]. Although the C-terminal domain of Rad23 is known to be responsible for its interaction with PNGase, so far no reports are available to establish the residues present in PNGase that are responsible for its interaction with Rad23.

Here, we map the site on PNGase responsible for its binding to Rad23 and investigate the interaction between Rad23 and PNGase using surface plasmon resonance (SPR). In addition, we establish the oligomeric state of Rad23 and PNGase in solution by using analytical ultracentrifugation and determine the binding stoichiometry of the two proteins in the complex.

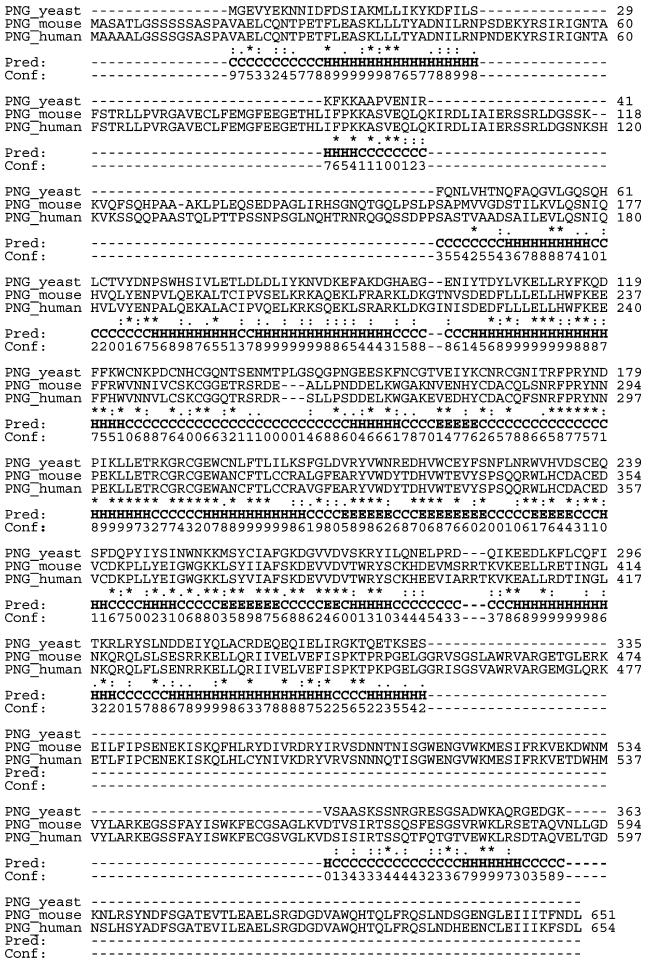

Fig. 1. Multiple sequence alignment of yeast, mouse, and human PNGase. The secondary structure of yeast PNGase was predicted using PSIPRED [19,20]. In the ΔN construct 34 residues were deleted from N-terminus. The ΔC construct had 22 residues deleted from the C-terminus. *Abbreviations:* Conf, Confidence of prediction 9-high and 0-low; Pred, prediction; C, coil; H, helix; and E, strands.

Materials and methods

Cloning of full-length, truncated forms of PNGase and full-length Rad23. The *S. cerevisiae* gene encoding PNGase was cloned into the pET 28b vector (Novagen) introducing a C-terminal His_6-tag as described elsewhere [5]. The Rad23 gene was subcloned from the pGAD424 vector in the *Nde*I/*Xho*I restriction sites of pET 28a (Novagen) introducing a N-terminal His_6-tag. Truncated forms of PNGase were constructed on the basis of limited proteolysis and mass spectrometric studies. Purified full-length PNGase was digested with trypsin for 1 h at 4 °C in a limited manner (1:50 trypsin to protein molar ratio). The digested protein was purified by size exclusion chromatography on a High Load 26/60 Superdex 200 column (Pharmacia) and subjected to mass spectrometry. N-terminal sequencing of the protein was also performed to identify the N-terminal sequence of the digested protein. The C-terminal end of the protein was estimated from the mass spectrometry data (described in detail in Results). From the secondary structure prediction and taking into consideration the limited proteolysis results, mutant constructs were prepared from the full-length protein by subcloning the gene into the pET 21b vector, which introduces a C-terminal $(His)_6$-tag. All DNA manipulations were performed according to Sambrook and Russell [14]. The PNGase deletion constructs contained the following residues of the full-length protein: (a) ΔNΔC (residues 35–341), (b) ΔN (residues 35–363), and (c) ΔC (residues 1–341).

Overexpression and purification. The three truncated constructs of PNGase, full-length PNGase, and Rad23 were transformed into BL21 DE3 (Stratagene) cells for overexpression. The cells containing full-length Rad23 and PNGase were grown at 37 °C in LB media containing 50 μg/ml kanamycin and cells of mutant PNGase in 100 μg/ml ampicillin until an A_{600} of 0.6 was reached. The cultures were induced by adding 1 mM isopropyl-1-thio-β-D-galactopyranoside at 30 °C and growth was continued for 3–4 h. Cells were harvested by centrifugation and then resuspended in lysis buffer containing 1× PBS, 1% Triton X-100, 1 mM β-mercaptoethanol, 1 mM PMSF, and 5 mM imidazole. The cells were disrupted by passing them three times through a French pressure cell followed by centrifugation for 20 min at 20,000 rpm. All proteins were purified using three different purification steps, (a) affinity chromatography, (b) anion exchange, and finally (c) size exclusion chromatography. In the first step the protein was passed through a Ni–nitrilotriacetic acid affinity column (Qiagen) at 4 °C

according to the manufacturer's instructions with the exception that the buffer used contained 1 mM β-mercaptoethanol. The proteins were eluted with a linear imidazole gradient from 50 to 500 mM in buffer containing 20 mM sodium phosphate, pH 8, 150 mM NaCl, and 1 mM β-mercaptoethanol. The protein containing fractions were concentrated, the salt concentration was reduced to 50 mM NaCl, and the protein was immediately loaded on a Source 30 Q column (Amersham–Pharmacia) equilibrated in 20 mM Tris/HCl, pH 8, 50 mM NaCl, 1 mM EDTA, and 10 mM β-mercaptoethanol. The protein was eluted with a linear NaCl gradient (50–500 mM). After concentrating the protein using Centricon, it was loaded on a 26/60 Superdex 200 size exclusion column (Amersham–Pharmacia) equilibrated in 20 mM Tris/HCl, pH 8, 250 mM NaCl, and 10 mM β-mercaptoethanol. The corresponding protein fractions were pooled, concentrated by using Centricon (Millipore, USA) and then flash-frozen in 50 μl aliquots in liquid nitrogen, and stored at −80 °C. The protein yields of full-length PNGase and Rad23 were 2 and 30 mg/L of cell culture, while the yields of the truncated forms of PNGase were as follows: ΔNΔC 5 mg, ΔN 3 mg, and ΔC 3 mg/L.

Cloning, expression, and purification of yeast ubiquitin. The coding sequence of full-length ubiquitin was amplified from yeast genomic DNA by PCR and the gene was subcloned into the pET 16b vector (Novagen), in-frame with a N-terminal His_{10}-tag. The plasmid was transformed into Rosetta DE3 (Novagen) cells and protein was overexpressed after growing in LB media containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol at 37 °C until an A_{600} of 0.6 was reached. The culture was induced by adding 0.3 mM isopropyl-1-thio-β-D-galactopyranoside and grown at 18 °C for 24 h. The cells were harvested by centrifugation and lysed by resuspending them in buffer containing 50 mM Tris/HCl, pH 8, 300 mM NaCl, and 5 mM imidazole and passing it three times through a French pressure cell. After centrifugation for 20 min at 20,000 rpm the clarified extract was applied to a Ni–NTA affinity column (Qiagen) and eluted with a linear imidazole gradient (50–500 mM). The protein was concentrated using Centricon and applied to a High Load 26/60 Superdex-200 (Amersham–Pharmacia) size exclusion column equilibrated in buffer containing 50 mM Tris, pH 7.5, and 250 mM NaCl. The corresponding protein peak fractions were pooled and concentrated to 8 mg/ml using Centricon.

Interaction analysis by analytical size exclusion chromatography. Full-length PNGase and Rad23 were mixed in a 1:1 molar ratio and incubated for 2 h at 4 °C. Similarly the three PNGase truncations were mixed and incubated at 4 °C overnight in a 2:1 Rad23 to ΔN, ΔC, and ΔNΔC PNGase molar ratio, respectively. In order to study the interaction of the PNGase–Rad23 complex with ubiquitin, the complex was mixed with ubiquitin in 1:10 molar ratio and incubated for 2 h at 4 °C. Samples were analyzed on a Superdex 200 HR 10/30 (Amersham–Pharmacia) analytical size exclusion chromatography column. The individual proteins were also analyzed on this column to determine their respective elution volumes. The column was pre-equilibrated with 50 mM Tris/HCl, pH 8, 250 mM NaCl, and 10 mM β-mercaptoethanol for PNGase–Rad23 interaction studies and with 50 mM Tris/HCl, pH 8, 150 mM NaCl, and 10 mM β-mercaptoethanol for studying interactions with ubiquitin. The corresponding peak fractions were analyzed on 12% and 15% SDS–PAGE gels.

Sedimentation equilibrium experiments. The analytical ultracentrifugation experiments were carried out at 4 °C in a Beckman XL-I analytical ultracentrifuge equipped with an An50 Ti rotor using six sector centerpieces. The protein was dialyzed overnight in 20 mM Tris/HCl, pH 8, 150 mM NaCl, and 1 mM β-mercaptoethanol prior to conducting the experiments. Absorbance at 280 nm was measured as a function of radial position at two different rotor speeds, 15,000 and 17,000 rpm. Equilibrium was reached after the rotors were spun at 15,000 rpm for 30 h. At that time twenty scans were averaged for each sample at each rotor speed. The sedimentation properties of both PNGase and Rad23 were analyzed by using the self-association model of the Origin software (version 6.0) provided by Beckman. The partial specific volumes were calculated based on amino acid composition and density increments of amino acids using the program SEDNTERP [15]. The protein concentration used for full-length Rad23 was 1.5 mg/ml and for PNGase it was 0.2 mg/ml. Higher concentrations of Rad23 were needed as this protein is devoid of tryptophan residues and hence has a very low extinction coefficient.

Surface plasmon resonance experiments. The binding of full-length and PNGase variants to Rad23 was determined with a Biacore-2000 instrument (Biacore, Uppsala, Sweden). Purified Rad23 (500 μg/ml) in 10 mM sodium acetate buffer, pH 5, was immobilized on a CM-5 sensor chip using standard amine coupling chemistry following the protocol recommended by the manufacturer [16]. Binding experiments were performed in a buffer containing 10 mM sodium borate buffer, pH 8, 150 mM NaCl, and 10 mM β-mercaptoethanol. The chip was primed with this buffer before performing the binding experiments. Full-length PNGase at 500 nM, 842 nM, and 1000 nM; ΔC construct at 500 nM and 1000 nM; and ΔN and ΔNΔC constructs at 1400 nM were applied to the immobilized chip at a flow rate of 10 μl/min at 20 °C to monitor binding kinetics. The sensor chip was then regenerated with 1 M NaCl. Five replicates of each concentration were taken for final analysis. The data obtained were analyzed with the BIA-evaluation software (version 4.1) provided by the manufacturer.

Results

Design of PNGase truncations

Limited proteolysis of purified full-length PNGase with trypsin yielded a truncated fragment with residues deleted from both the N- and C-termini. This protein was purified by size exclusion chromatography after trypsin digestion. From the N-terminal end 34 residues were digested which was confirmed by N-terminal sequencing of the protein. Mass spectrometry of the truncated fragment produced a mass of 34742.4 Da, while the full-length protein yielded a mass of 44001.5 Da including the His_6-tag. From these data, it was concluded that approximately 36 residues were digested by trypsin from the C-terminal end. Based on these facts and the secondary structure prediction of yeast PNGase (Fig. 1), three truncated forms were prepared by genetic engineering.

Complex formation of Rad23 and PNGase

All proteins were purified to electrophoretic homogeneity before conducting the binding experiments (Fig. 2). Full-length PNGase and Rad23, mixed at a 1:1 molar ratio, eluted as a single peak on a size exclusion chromatography column. When the corresponding fractions were analyzed on a 12% SDS gel both proteins were detected indicating the formation of a complex (Fig. 3A). The ΔNΔC as well as the ΔN constructs did not interact with Rad23 and consequently two different peaks, corresponding either to the PNGase variant or Rad23, were observed in size exclusion chromatography runs (Figs. 3B and C). For comparison, the proteins were run individually to check their elution patterns. When these

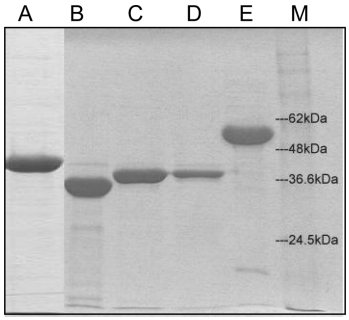

Fig. 2. SDS–PAGE (12% gel) of all proteins that were purified to electrophoretic homogeneity. Lane A, full-length PNGase; lane B, ΔNΔC PNGase; lane C, ΔN PNGase; lane D, ΔC PNGase; and lane E, full-length Rad23. Marker proteins are shown in lane M with masses as indicated.

peaks were analyzed on 12% SDS gels the presence of the respective proteins was observed in the peaks. On the other hand when the ΔC mutant of PNGase was mixed with Rad23 in a 1:2 ratio, the complex eluted as a single peak containing both proteins (Fig. 3D). In comparison to the elution of the complex, the individual proteins eluted at different elution volumes. These results clearly demonstrate that the N-terminal region of yeast PNGase is responsible for mediating the interaction with Rad23.

To further investigate the binding of the two proteins and their role in the proteasome degradation pathway, yeast ubiquitin was mixed with PNGase–Rad23 complex in a 10:1 ratio. Size exclusion chromatography of the mixture revealed that monoubiquitin was capable of binding to the PNGase–Rad23 complex (Fig. 4). In

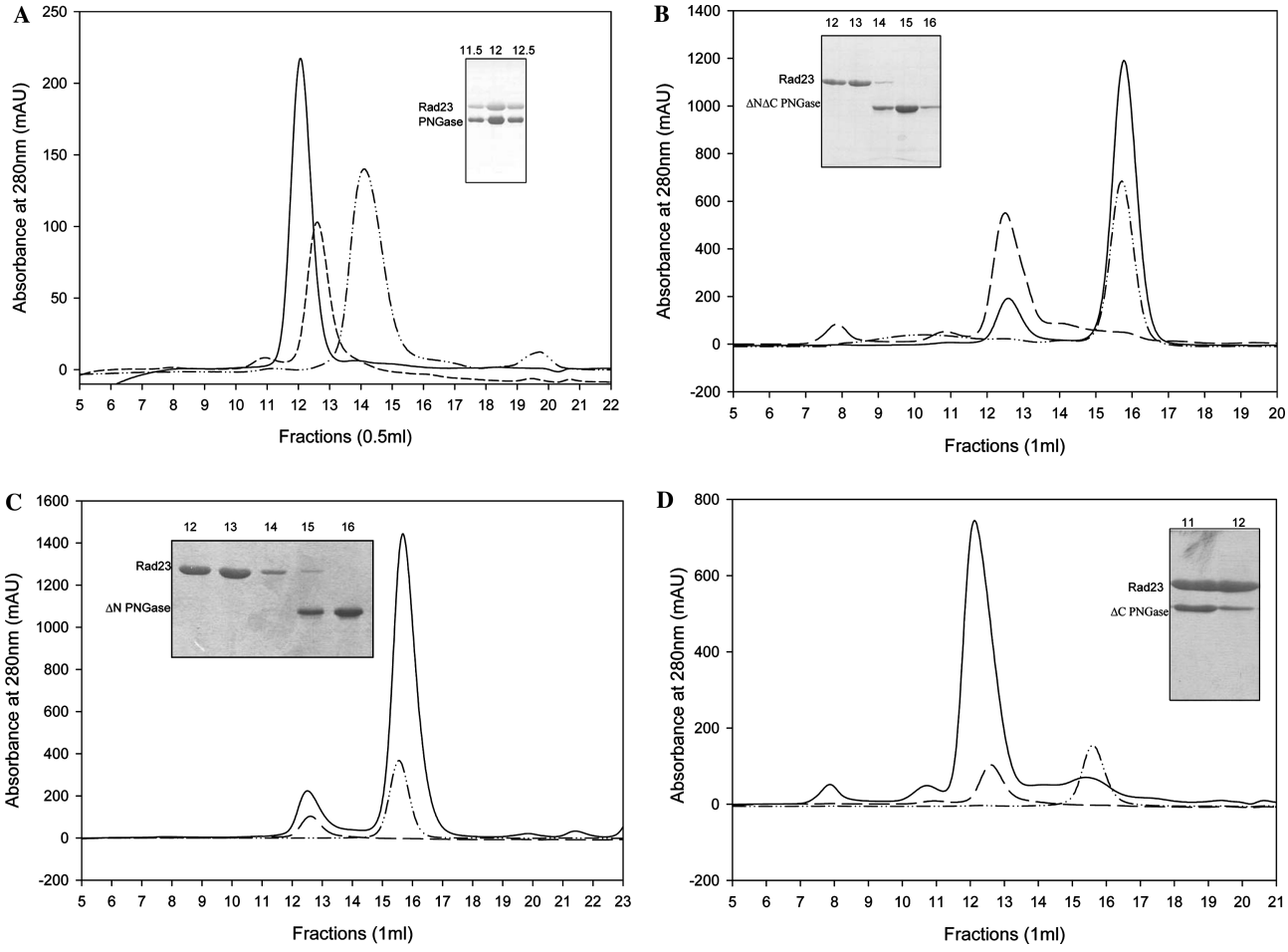

Fig. 3. Rad23–PNGase interactions. (A) Interactions between Rad23 and full-length PNGase. Analytical size exclusion chromatography profile after mixing the two proteins in a 1:1 molar ratio. The complex eluted as a single peak. The inset shows a 12% SDS–PAGE of the peak fractions. The presence of both proteins in a single peak was detected indicating the formation of a complex. The elution profiles of free Rad23 (long dash) and free PNGase (dash dot dot) are also shown. (B) Interactions between Rad23 and ΔNΔC PNGase. Analytical size exclusion chromatography profile of ΔNΔC PNGase (residues 34–341) and Rad23 mixed in a 1:2 (PNGase:RAD23) molar ratio. The inset shows a 12% SDS–PAGE gel of the fractions eluted from the two peaks. Elution profiles of free Rad23 (long dash) and ΔNΔC PNGase (dash dot dot) are also shown. (C) Interactions between Rad23 and ΔN PNGase. Analytical size exclusion chromatography profile of ΔN PNGase (residues 34–363) and Rad23 mixed in a 1:2 (PNGase:Rad23) ratio. The inset shows a 12% SDS–PAGE gel of the fractions eluted from the peaks. Individual size exclusion chromatography profiles of ΔN PNGase (dash dot dot) and Rad23 (solid line) are also indicated. (D) Interactions between Rad23 and ΔC PNGase. Analytical size exclusion chromatography profiles of ΔC PNGase (residues 1–341) and Rad23 mixed in a 1:2 ratio. A single peak is observed indicating complex formation. The inset shows a 12% SDS–PAGE gel of the fractions eluted from the peak, which contain both proteins. Individual size exclusion chromatography runs of full-length Rad23 (long dash) and ΔC PNGase (dash dot dot) are shown.

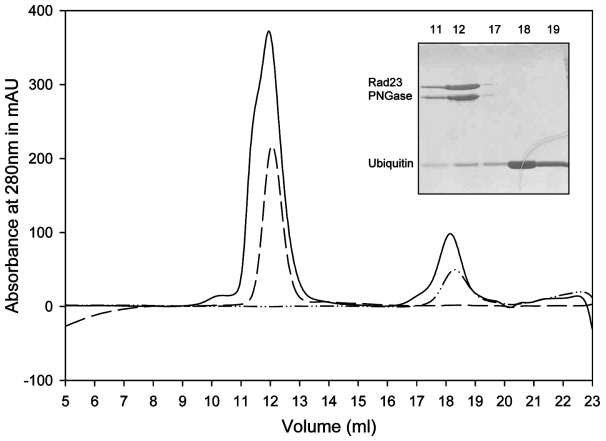

Fig. 4. Interaction of PNGase–Rad23 complex with ubiquitin mixed in a 1:10 ratio. The heterotrimeric complex eluted at a higher elution volume than the Rad23–PNGase complex. Inset shows 15% SDS–PAGE gel of the 1 ml fractions collected from the respective A_{280} peaks. Individual runs of the full-length PNGase–Rad23 complex (long dash) and free ubiquitin (dash dot dot) are shown.

addition, unbound ubiquitin was detected in the mixture from the size exclusion run. Interactions of ubiquitin with both PNGase and Rad23 were checked in control experiment and it was found that while Rad23 interacted with ubiquitin, as expected, PNGase showed no interactions with ubiquitin (data not shown). These results suggested that the internal UBA domain of Rad23 is available for ubiquitin binding, even when the C-terminal region including the second UBA domain is bound to PNGase.

Quantitating the binding interaction

Biacore experiments were performed to check the interaction of wild-type PNGase and the deletion constructs with full-length Rad23. As expected increasing concentrations of full-length PNGase gave an increase in binding signal, while two of the mutants, ΔN and ΔNΔC, did not interact with full-length Rad23 (Fig. 5). Again, the C-terminal solvent-exposed domain of PNGase was not required for its interaction with Rad23, since the ΔC variant displayed an interaction. The binding response curves obtained from the Biacore experiment were analyzed using a 1:1 Langmuir model which resulted in a fit with a χ^2 value of 0.82. The rate constants for the association and dissociation process of the wild-type PNGase–Rad23 complex were $4.05 \times 10^3\ M^{-1}\ s^{-1}$ and $0.01\ s^{-1}$, respectively, resulting in a dissociation constant of ~2.5 μM. Attempts to fit the curves to other models failed, since they did not yield reasonable χ^2 values. Thus, it was concluded that the association of the two proteins occurs with a 1:1 stoichiometry, in agreement with the size exclusion chromatography analyses.

In order to further investigate the stoichiometry of the complex we first tried to establish the molecular masses of free Rad23 and PNGase in solution by analytical ultracentrifugation. Data analysis was performed with the Origin software and the molecular mass obtained for Rad23 by using the self-association model was close to the mass calculated for a dimer. Performing a global fit with four sets of data at two different speeds and two different protein concentrations yielded a molecular mass of 86,197 Da. The theoretical monomeric mass calculated from the sequence is 43,207 Da taking into consideration the His_6-tag, or 86,414 Da for the dimer, and the observed mass therefore closely corresponds to a dimer. The data of one dataset at a rotor speed of 15,000 rpm together with the fit are shown in Fig. 6. For PNGase the data revealed a mass of 43,711 Da, while its calculated theoretical monomeric mass is 43,324 Da (Fig. 6), thus indicating that PNGase forms a monomer in solution. Analytical size exclusion

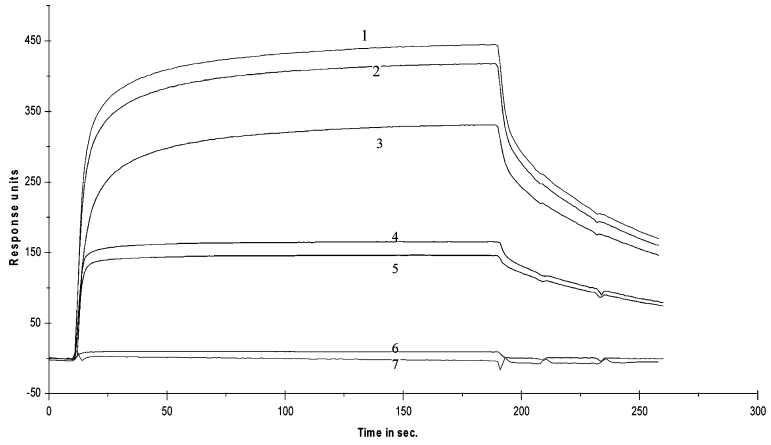

Fig. 5. Investigating the PNGase–Rad23 complex by surface plasmon resonance. Rad23 was covalently attached to CM-5 chip and full-length and PNGase variants were passed over the chip surface. Increasing concentrations of full-length PNGase [500 nM (3), (842 nM) (2), and 1000 nM (1)] together with the ΔNΔC (7) (1400 nM), ΔN (6) (1400 nM) and ΔC [1000 nM (4), and 500 nM (5)] variants of PNGase are also shown.

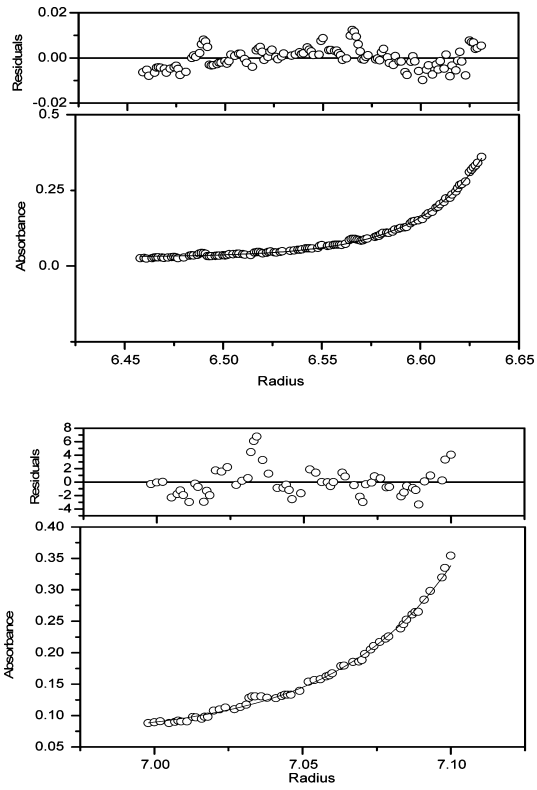

Fig. 6. Sedimentation equilibrium ultracentrifugation of full-length Rad23 (top panel) and PNGase (bottom panel) at 15,000 rpm. The absorbance at 280 nm vs. radial distance is plotted together with the residuals for the respective runs. Open circles represent the experimental data while the fits are shown as solid lines.

chromatography runs with Rad23 alone and also the complex always yielded a higher mass than that of the protein alone or the protein–protein complexes would predict from the calibration curve of the column with standard proteins. This discrepancy might be attributed to an extended shape of Rad23. The analytical size exclusion chromatography run of PNGase alone on the other hand gave a mass of approximately 46 kDa (data not shown), which is close to the value obtained by analytical ultracentrifugation.

Discussion

Yeast two-hybrid analyses have established that PNGase physically associates with the 26S proteasome through Rad23, a nucleotide excision repair protein [9], which also directly interacts with the Rpn1 subunit which is at the base of the regulatory particle [17]. The interaction studies of yeast PNGase with Rad23 revealed that its N-terminal solvent-exposed region is responsible for binding to Rad23, since neither the ΔN nor the ΔNΔC PNGase constructs interacted with Rad23 in Biacore and size exclusion chromatography experiments. Both truncated forms of PNGase also exhibited greater stability in solution compared to the full-length protein. Secondary structure predictions of the 34 N-terminally deleted residues reveal the presence of one longer α-helix and a sequence alignment of this region revealed that some residues including Glu-3, Phe-12, Lys-17, Leu-19, Leu-20, Phe-31, Lys-33, and Ala-35 in yeast are highly conserved among higher eukaryotes including mouse and human (Fig. 1). Hence, these conserved residues might mediate interactions between PNGase and Rad23. Higher eukaryotes are known to possess an additional PUB (peptide: *N*-glycanase/UBA or UBX containing protein) domain encompassing residues 35–80, which is inserted into the N-terminal α-helix predicted for the yeast protein. The PUB domain mediates interactions with several other proteins related to the ubiquitin–proteasome pathway including the proteasome itself [10,18]. Based on our interaction studies with yeast PNGase and Rad23, one can speculate that in mouse and human PNGase the N-terminal region prior to the PUB domain (residues 17–34) and the C-terminal region following it (residues 88–99) might also be responsible for mediating the mHR23B or hHR23B interactions and not the PUB domain itself as implicated earlier [10].

The oligomeric nature of Rad23 was investigated earlier in the absence and presence of ubiquitin. It was found that although yeast Rad23 isolated from *Escherichia coli* cells was found to be predominantly dimeric in solution, the monomeric form of the protein was associated with ubiquitin in a 1:1 ratio [12], whereas Rad23 homodimers are incapable of binding ubiquitin [11]. The UBA domains have been implicated as being responsible for dimerization, where the internal UBA domain of one Rad23 molecule associates with the C-terminal UBA domain of another Rad23 molecule [11]. In the present study it was observed that binding of ubiquitin to the PNGase–Rad23 complex was more straightforward compared to Rad23 itself. In order to form the Rad23–ubiquitin complex, Rad23 had to be diluted so that the monomeric form became available and then concentrated in the presence of ubiquitin. However, no such efforts were needed to bind ubiquitin to the Rad23–PNGase complex. This suggests that the internal UBA domain of Rad23, which is unavailable in the Rad23 dimer, becomes available in the PNGase–Rad23 complex, in which monomeric forms of both proteins are associating. As demonstrated earlier the C-terminal UBA domain of Rad23 is essential for binding to PNGase [9] and hence is not available for the interaction with ubiquitin.

The molecular mass of Rad23 from sedimentation equilibrium experiments showed that the protein, as expected, exists as a dimer in solution. However, upon association with PNGase it forms a 1:1 complex as revealed by size exclusion chromatography and Biacore experiments. The dissociation constant of the complex

was found to be ~2.5 μM indicating a tighter binding of Rad23 to PNGase compared to ubiquitin with a reported dissociation constant of 10 μM [12]. Since the binding of PNGase to Rad23 is mediated also via the C-terminal UBA domain of Rad23 the possibility of a stable complex with one molecule of Rad23 and one molecule of PNGase is favored over the dimerization of two Rad23 monomers. However, in the present investigation it was found that when PNGase is bound to Rad23 the internal UBA domain is still available for binding to monoubiquitin.

Speculating about the role of Rad23, it can be proposed that this protein is capable of acting as a carrier for both ubiquitinated proteins along with PNGase to the proteasome during the process of degradation of misfolded proteins. The data presented here further illustrate the complex set of interactions Rad23 can participate in. Besides its involvement in ubiquitin-dependent protein degradation Rad23 is also involved in nucleotide excision repair where it forms a stable complex with Rad4, which in humans corresponds to the XPC–Rad23b complex. The modular architecture of Rad23 is presumably responsible for mediating the various interactions with other proteins. This report gives the first quantitative estimate of the strength of the PNGase–Rad23 complex and also establishes the N-terminal region of yeast PNGase as the primary region responsible for this protein–protein interaction. Further mutational analysis or the crystal structure of the complex will identify the amino acids required for this interaction.

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