The AAA ATPase p97 is a ubiquitin-selective molecular machine involved in multiple cellular processes, including protein degradation through the ubiquitin-proteasome system and homotypic membrane fusion. Specific p97 functions are mediated by a variety of cofactors, among them peptide N-glycanase, an enzyme that removes glycans from misfolded glycoproteins. Here we report the three-dimensional structure of the amino-terminal PUB domain of human peptide N-glycanase. We demonstrate that the PUB domain is a novel p97 binding module interacting with the D1 and/or D2 ATPase domains of p97 and identify an evolutionary conserved surface patch required for interaction with the D1 and/or D2 ATPase domains of p97. Furthermore, we show that the PUB and UBX domains do not bind to p97 in a mutually exclusive manner. Our results suggest that PUB domain-containing proteins constitute a widespread family of diverse p97 cofactors.

Glycoproteins of the secretory pathway that fail to fold correctly in the endoplasmic reticulum are retro-translocated to the cytosol for degradation by the ubiquitin-proteasome system in a process known as endoplasmic reticulum-associated protein degradation (1, 2). This pathway requires dedicated ubiquitin ligases and the AAA ATPase p97 (also called VCP, Cdc48) (1–3). p97 consists of an amino-terminal N domain and two AAA domains, D1 and D2. It forms a homohexameric, barrel-like structure consisting of two ring-shaped layers made of the D1 and D2 domains (4). The protein-extracting activity of p97 is believed to be the result of conformational changes that accompany nucleotide binding and hydrolysis (5, 6). The importance of functional p97 for endoplasmic reticulum-associated protein degradation is illustrated by the fact that mutations in p97 that are associated with the disorder inclusion body myopathy with Paget disease of the bone and fronto-temporal dementia cause endoplasmic reticulum-associated protein degradation defects (7, 8).

Prior to proteasomal degradation of retro-translocated, glycosylated proteins, N-linked oligosaccharide chains are removed by the enzyme peptide N-glycanase (PNGase) (9–11). The Saccharomyces cerevisiae PNGase homologue Png1 is 363 amino acids in length and contains a catalytic triad of cysteine, histidine, and aspartic acid residues typical of the transglutaminase-like superfamily of enzymes (12). It binds to the proteasomal targeting factor Rad23, thereby possibly linking glycan removal to proteasomal degradation (9).

Although the interaction between PNGase and Rad23 is evolutionary conserved in higher eukaryotes (13), differences appear to exist with respect to details of the interaction (14). Animal PNGases possess an additional amino-terminal extension that contains a PUB (also called PUG) domain, a protein module of unknown function found in many proteins linked to the ubiquitin-proteasome system on the basis of their domain architecture (15, 16) (Fig. 1). The presence of the PUB domain suggests that animal PNGases are subject to more complex regulation. Consistent with this notion, p97 and the putative retro-translocation pore component Derlin-1 have recently been shown to interact with PNGase in mammalian cells, raising the intriguing possibility that endoplasmic reticulum-associated protein degradation substrates are deglycosylated by PNGase during their p97-mediated retro-translocation and subsequently targeted to the 26 S proteasome via the Rad23 homologue, HR23B (17–19). To gain insight into the role of the PUB domain, we solved the three-dimensional structure of the PUB domain of human PNGase and found that it is a novel p97 binding module containing a conserved p97 binding site.

EXPERIMENTAL PROCEDURES

Structure Determination—DNA encoding the PUB domain of human PNGase (residues 11–109) was PCR amplified from a human cDNA library (Clontech) and cloned into a pRSETA (Invitrogen) derivative that expresses proteins fused to the lipoyl domain of Bacillus stearothermophilus dihydrolipoamide acetyltransferase. The resulting plasmid was transformed into Escherichia coli C41(DE3) cells. Cells were grown at 37 °C in Luria Bertani broth to mid log phase and induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside. The temperature was then reduced to 25 °C, and the cells were grown for a further 16 h. Cells were lysed by sonication, and the fusion protein was purified using a nickel-nitrilotriacetic acid Superflow affinity column (Amersham Biosciences). Following cleavage with thrombin (4 h at 30 °C), the PUB domain was further purified by ion exchange chromatography using a Source Q column (Amersham Biosciences) and subsequent gel filtration using a Superdex 75 HR column (Amersham Biosciences). A PUB domain containing L66M, L75M, and L87M mutations was created using the QuikChange II XL kit.
mid pProExHT-p97ΔN for the expression of hexahistidine-tagged human p97 lacking residues 1–199 (p97ΔN) was a kind gift from P. Zwickl (Martinried, Germany). Plasmid pQE30-FAF1 (25) for the expression of hexahistidine-tagged full-length human FAF1 was a kind gift from O. G. Issinger (Odense, Denmark). Site-directed mutagenesis of GST-PUBPNGase was performed using the QuikChange II XL kit (Stratagene) according to the manufacturer’s instructions. All proteins were purified by glutathione or nickel-nitrilotriacetic acid affinity chromatography as applicable according to standard procedures. Glutathione-Sepharose pull-downs were performed exactly as described (26), using 10 μl of beads, 40 μg (1.5 nmol) of GST, 60 μg (1.6 nmol) of GST-PUB domain fusions, 20 μg (0.22 nmol) of His6-p97, 15 μg (0.2 nmol) of His6-p97ΔN, 100 μg (2.4 nmol) of His6-p47, and 150 μg (2.0 nmol) of His6-FAF1. In the experiment described in Fig. 5,E, 40 μl of beads, 200 μg of GST (7.7 nmol), and 60 μg (1.6 nmol), 120 μg (3.2 nmol), or 300 μg (8 nmol) of GST-PUB domain fusion was used. In the experiments described in Fig. 5, D and E, His6-p97 and His6-p47 or His6-FAF1 were preincubated for 1 h at 4 °C before the preincubation mixture was added to GST-PUBPNGase immobilized on glutathione-Sepharose beads. The protein concentrations in the preincubation mixture were: 2.4 μM His6-p97, 28.6 μM His6-p47, and 21.3 μM His6-FAF1.

RESULTS

PUB Domain Structure—The amino-terminal PUB domain of human PNGase could be easily expressed in E. coli, and the purified protein produced crystals that diffracted well. As the domain contains only one methionine residue we prepared a L66M/L75M/L87M mutant protein to facilitate the incorporation of sufficient amounts of seleno-methionine to enable the structure to be solved using the multiwavelength anomalous dispersion method. The seleno-methionine-substituted mutant protein readily crystallized, and its structure was determined using multiwavelength anomalous dispersion. The structure of the native domain was then determined to 1.6 Å by molecular replacement using the mutant structure. Crystallographic data are summarized in Table 1. A representative section of the electron density is shown in Fig. 2A. The PUB domain fold consists of a bundle of five α helices that pack onto a short three-stranded anti-parallel β sheet (Fig. 2B). In addition, there is a small 3_{10} helix in the loop between the second helix and the first β strand. The structure has some resemblance to the wing helix motif, which also consists of helices packing onto a small β
sheet. The topological arrangement of the helices is, however, very different, and a search of the protein structure data base using the program DALI (27) revealed no significant similarity to other known structures. A structure-based alignment of a selection of PUB domains indicates that insertions and deletions are restricted to loops between elements of secondary structure (Fig. 3) and are unlikely to affect the overall fold. Thus, the structure of the PNGase PUB domain can serve as a good model for other PUB domains.

A number of residues are highly conserved in PUB domains (Fig. 3). Gly-79 and Phe-80 are located in the turn between the fourth α helix and the second strand of the sheet. Both residues have a structural role: a glycine residue is required for the sharp turn at the end of the helix, and the side chain of the phenylalanine residue packs into the hydrophobic core of the domain. Similarly, the highly conserved residue Leu-35 is at the center of the hydrophobic core of the domain. In contrast, several hydrophilic conserved residues are exposed at the protein surface.
Asn-41, which is at the end of the second helix, is conserved in all PUB domains, and Asn-58 in helix 3 is highly conserved. Lys-50 and Tyr-51, both of which are in the 310 helix, Glu-73 in the third helix, and Arg-55 in the first strand are also highly conserved and mostly replaced conservatively in other PUB domains. Intriguingly, the side chains of most of these residues line a basic pocket on the protein surface, and residues Asn-41, Lys-50, and Tyr-51 form a well defined surface patch (Fig. 4). The clustering of three highly conserved residues in this surface patch strongly suggests that it is a functionally important site.

**p97 Binding**—Recently, p97 has been shown to interact with human PNGase in vivo and in vitro (17, 19). To test whether the PUB domain is involved in p97 binding, we performed an in vitro GST pulldown experiment using the isolated PUB domain of human PNGase (Fig. 5A). Indeed, the isolated PUB domain bound p97 very efficiently (compare lane 4 to the input shown in lane 1), whereas no background binding of p97 to GST alone was detectable (lane 2). Similar results were obtained with the PUB domain of human UBXD1 protein (lane 3) (28). These data show for the first time the direct binding of PUB domains to p97. Furthermore, they suggest that the PUB domain is a novel p97 binding module.

We next analyzed the importance of the conserved surface patch consisting of residues Asn-41, Lys-50, and Tyr-51 for p97 binding, using a site-directed mutagenesis approach. Altering residues Asn-41 and Lys-50 of the PUB domain of human PNGase to alanine, either alone or in combination, did not result in a significant decrease in p97 binding as compared with the wild-type PUB domain (Fig. 5A, lanes 4–7). The combined mutation of residues Lys-50 and Tyr-51 led only to a slight reduction in p97 binding (lane 8). In contrast, mutation of all three conserved residues almost completely abolished binding of p97 to the mutant PUB domain (lane 9). To verify that the three amino acid exchanges did not affect the overall protein structure, we determined the crystal structure of the NKY41,50,51AAA mutant PUB domain. The structure of the triple mutant PUB domain is identical to that of the native protein, and the Ca atoms can be superimposed with a root mean square deviation of 0.17 Å (Fig. 5B). The lack of binding activity of the mutated domain is therefore solely the result of the removal of functionally important side chains, indicating that the conserved surface patch comprising residues Asn-41, Lys-50, and Tyr-51 forms part of the major p97 binding site on the PUB domain.

Many p97 cofactors, including the heterodimer Ufd1/Npl4 and members of the large family of UBX domain-containing proteins, bind to the amino-terminal N domain of p97 (29–31). We therefore tested in NMR chemical shift-mapping experiments whether the PUB domain of human PNGase similarly binds to the N domain of p97. However, unlike the p47 UBX domain (32), the PUB domain did not show detectable binding to the isolated N domain of p97 (data not shown). To test whether the PUB domain binds to the D1 and/or D2 ATPase domains of p97, we repeated the pulldown experiment with a truncated p97 variant lacking the N domain, p97ΔN. p97ΔN bound efficiently and specifically to the PUB domains of PNGase and UBXD1 (Fig. 5C), showing that the PUB domain binding site resides within the D1 and/or D2 ATPase domains of p97.

The distinct binding regions of p97 for the PUB versus UBX domains raised the possibility that both domains can bind simultaneously to p97. Indeed, incubation of p97 with a 10-fold molar excess of the UBX domain proteins p47 or FAF1 did not...
FIGURE 5. The PUB domain is a novel p97 binding module. A, p97 and the PUB domain interact directly via the conserved surface patch. GST fusion proteins of the PUB domain of human UBXD1 (GST-PUBUBXD1; lane 3) or wild-type or the indicated mutant PUB domains from human PNGase (GST-PUBPNGase; lanes 4–9) bound to glutathione beads were incubated with p97. After repeated washing steps, binding of p97 was analyzed by SDS-PAGE followed by Coomassie staining of the gel. GST served as negative control (lane 2). For comparison, 10% of the p97 input is shown (lane 1). B, the NKY41,50,51AAA triple mutant PUB domain has the same structure as the wild-type domain. Stereo view of the superimposed backbone traces of the wild-type (red) and mutant (blue) PUB domains. C, the PUB domain binds to the D1 and/or D2 ATPase domains of p97. Binding of p97 or p97/H9004 indicated to the PUB domains of human PNGase or human UBXD1 was tested as in panel A. D, binding of the PUB domain and UBX domain proteins is not mutually exclusive. Binding of p97 to the PUB domain of human PNGase was tested as in panel A after preincubating p97 without (lane 7) or with a 10-fold molar excess of the UBX domain proteins p47 (lane 8) or FAF1 (lane 9). E, p47 binds to p97 in the presence of saturating amounts of PUB domain. Binding of p97 and p47 to the PUB domain of human PNGase was tested as in panel D in the presence of increasing amounts of PUB domain. In lanes 3 and 6, the same amount of PUB domain as in panels A, C, and D was used. In lanes 4 and 7, twice the amount of PUB domain was; in lanes 2, 5, 8, and 9, the 5-fold amount of GST and PUB domain, respectively, was used.
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