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The PUB Domain Functions as a p97 Binding Module in Human Peptide *N*-Glycanase*

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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 p97 binding. 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)[3] (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

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[3] The abbreviations used are: PNGase, peptide *N*-glycanase; GST, glutathione *S*-transferase; CHES, 2-(cyclohexylamino)ethanesulfonic acid.

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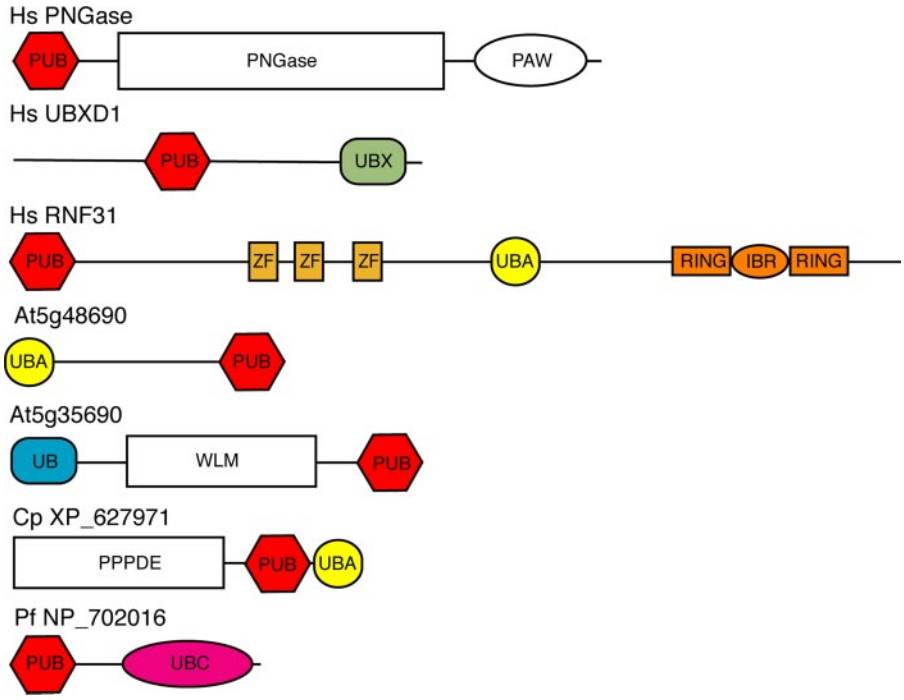

FIGURE 1. **Domain architectures of PUB domain-containing proteins.** Selected proteins from man (*Hs*), *Arabidopsis thaliana* (*At*), *Cryptosporidium parvum* (*Cp*), and *Plasmodium falciparum* (*Pf*) with links to the ubiquitin-proteasome system are shown. Domains are labeled according to the SMART data base. *WLM* and *PPDE* denote putative deubiquitinating enzyme domains (36). The proteins were either previously reported to contain the domain (15) or were identified in sequence similarity searches with the program psi-blast (www.ncbi.nlm.nih.gov/BLAST) using the amino acid sequence of the amino-terminal domain of PNGase as a query.

(Stratagene) to facilitate selenium incorporation. Seleno-methionine-substituted mutant protein was prepared exactly as above except that cells were grown in M9 minimal medium supplemented with seleno-methionine. Crystals were grown using the sitting drop vapor diffusion method at 290 K with a reservoir solution containing 0.2 M sodium acetate, 0.1 M Tris, pH 8.5, 30% polyethylene glycol 4000 using 20% glycerol as a cryoprotectant. Native and MAD data sets were collected at ID14–4, European Synchrotron Radiation Facility, Grenoble. X-ray diffraction data were indexed and integrated using the program MOSFLM and scaled with the program SCALA (20). An initial MAD density map was generated by locating four selenium sites in the data sets Peak, Inflection, High-energy remote using the program SOLVE (21), which was also used to calculate phases. RESOLVE (22) was used for solvent flattening, assuming a 40% solvent content. The structure was built using MAIN (23) and refined using CNS (24). The structure of the native protein was determined by molecular replacement using the program CNS. The PUB domain in which Asn-41, Lys-50, and Tyr-51 are replaced with alanine was crystallized in 50% polyethylene glycol 400, 0.1 M CHES, pH 9.5, 0.2 M NaCl. The structure of the mutant protein was determined by molecular replacement using the structure of the native protein.

p97 Binding Assay—Plasmids for the bacterial expression of amino-terminal glutathione *S*-transferase (GST) fusions of human PNGase (residues 11–109; GST-PUB^{PNGase}) and human UBXD1 (residues 150–264; GST-PUB^{UBXD1}) and of amino-terminal hexahistidine fusions of full-length human p97 and p47 were cloned according to standard procedures. Plasmid pProExHT-p97ΔN for the expression of hexahistidine-tagged human p97 lacking residues 1–199 (p97ΔN) was a kind gift from P. Zwickl (Martinsried, 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-PUB^{PNGase} 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 His$_6$-p97, 15 μg (0.2 nmol) of His$_6$-p97ΔN, 100 μg (2.4 nmol) of His$_6$-p47, and 150 μg (2.0 nmol) of His$_6$-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*, His$_6$-p97 and His$_6$-p47 or His$_6$-FAF1 were preincubated for 1 h at 4 °C before the preincubation mixture was added to GST-PUB^{PNGase} immobilized on glutathione-Sepharose beads. The protein concentrations in the preincubation mixture were: 2.4 μM His$_6$-p97, 28.6 μM His$_6$-p47, and 21.3 μM His$_6$-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 of the domain 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. 2*A*. The PUB domain fold consists of a bundle of five α helices that pack onto a short three-stranded anti-parallel β sheet (Fig. 2*B*). 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

TABLE 1

Crystallographic data

The first two columns refer to the Se derivative, and the third column refers to the native protein.

Data set	Peak	Inflection	High resolution peak	Native	Mutant
Symmetry	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P6_5$	$P6_5$
Wavelength (Å)	0.9797	0.9799	0.9797	0.979	1.5418
Resolution range (Å)	24.9-1.8	24.9-1.8	24.9-1.5	33.0-1.6	24.5-1.9
Unique reflections	8841	8799	15009	13734	8005
Completeness (%)[a]	98.4 (98.3)	98.0 (97.6)	98.1 (97.3)	99.5 (97.2)	99.0 (98.0)
R_{merge}[b]	0.066 (0.086)	0.072 (0.147)	0.119 (0.204)	0.070 (0.173)	0.088 (0.284)
Multiplicity[c]	5.6 (5.8)	5.5 (5.7)	5.7 (5.8)	5.9 (5.7)	3.1 (3.1)
I/sI[a]	22.2 (16.3)	19.7 (10.1)	12.2 (6.5)	18.4 (6.7)	12.4 (4.0)
No. of Se sites/monomer	4				
Figure of merit[d]	0.44				
$P2_12_12_1$ a = 29.150 Å, b = 32.779 Å, c = 95.746 Å					
$P6_5$ (native) a = 73.516 Å, c = 33.001 Å					
$P6_5$ (mutant) a = 73.987 Å, c = 32.994 Å					
Model refinement					
Resolution range (Å)			24.9-1.5	33.0-1.6	25.0-1.9
No. of reflections (working/free)			14228/762	12945/679	7588/410
No. of residues			A: 2–99	A: 1–99	A: 1–99
No. of water, ligand molecules			165	134, 3 glycerol	99, 1 PEG, 1 β-Me
R_{work}/R_{free}[e], %			0.194, 0.204	0.193, 0.206	0.185, 0.223
B average[f]			14.6 Å^2	21.9 Å^2	19.7 Å^2
Geometry bonds/angles[g]			0.014 Å, 1.253°	0.005 Å, 0.559°	0.007 Å, 1.247°
Ramachandran[h]			96.6%/0.0%	94.3%/0.0%	96.6%/0.0%
PDB ID[i]				2CCQ	2CM0

[a] Signal to noise ratio of intensities, highest resolution bin in parentheses.
[b] R_{merge}: $S_hS_i|I(h,i) - I(h)|/S_hS_i\, I(h,i)$ where $I(h,i)$ are symmetry-related intensities and $I(h)$ is the mean intensity of the reflection with unique index h.
[c] Multiplicity for unique reflections for MAD; datasets $I(+)$ and $I(-)$ are kept separate.
[d] Value is given after density modification.
[e] 5% of reflections were randomly selected for determination of the free R-factor, prior to any refinement.
[f] Temperature factors averaged for all atoms.
[g] Root mean square deviations from ideal geometry for bond lengths and restraint angles.
[h] Percentage of residues in the "most favoured region" of the Ramachandran plot and percentage of outliers (PROCHECK).
[i] Protein Data Bank identifiers for coordinates.

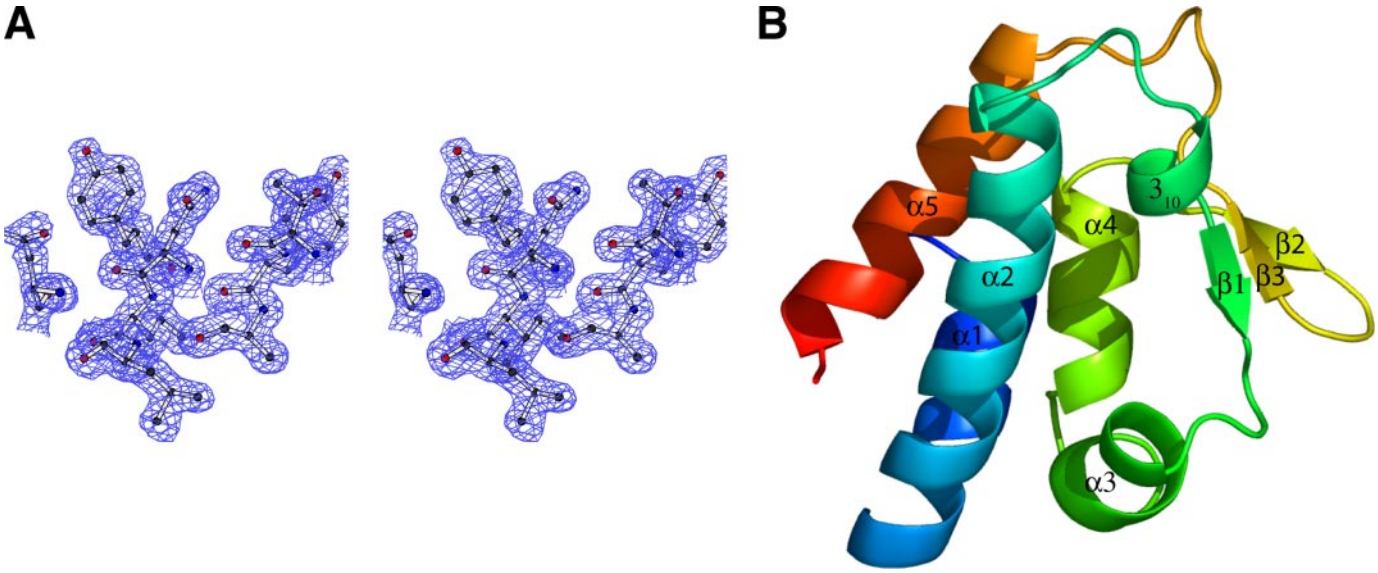

FIGURE 2. **Crystal structure of the PUB domain of human PNGase.** *A*, electron density from the 1.6 Å map. *B*, overall structure of the PUB domain. The ribbon representation is color coded from the amino terminus (*dark blue*) to the carboxyl terminus (*dark red*), and secondary structure elements are labeled (prepared using the program Pymol (www.pymol.org).

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.

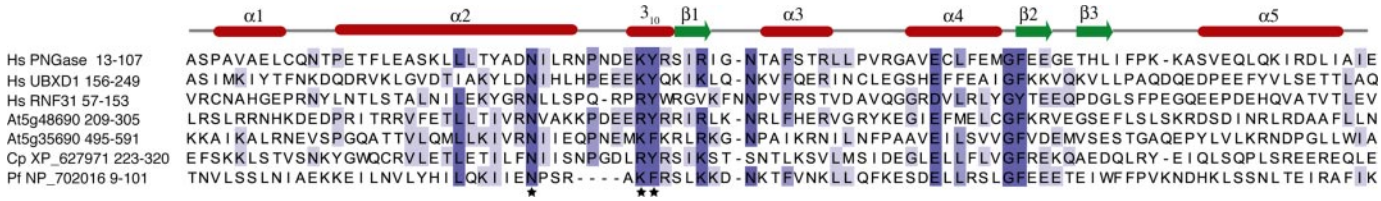

FIGURE 3. **Structure-based sequence alignment of PUB domains.** The residue numbers of the PUB domains shown in Fig. 1 are given next to the protein names. Secondary structure elements of the PNGase PUB domain are indicated at the *top*. The most highly conserved residues are *boxed*. *Asterisks* mark three highly conserved residues forming a solvent-exposed surface patch (see Fig. 4). The alignment was prepared using the program Jalview (37).

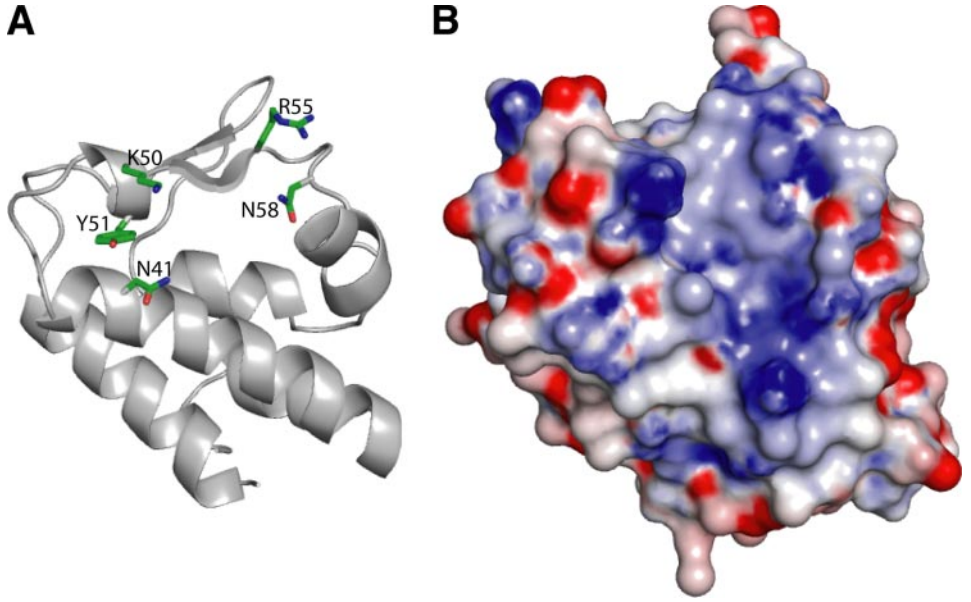

FIGURE 4. **A conserved surface patch on the PUB domain.** *A*, ribbon representation of the PNGase PUB domain with conserved, solvent-exposed residues shown as *sticks*. *B*, surface electrostatic potential shown in the same orientation.

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 3_{10} helix, Glu-73 in the third α helix, and Arg-55 in the first β 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. 5*A*). 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. 5*A*, *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 Cα atoms can be superimposed with a root mean square deviation of 0.17 Å (Fig. 5*B*). 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. 5*C*), 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

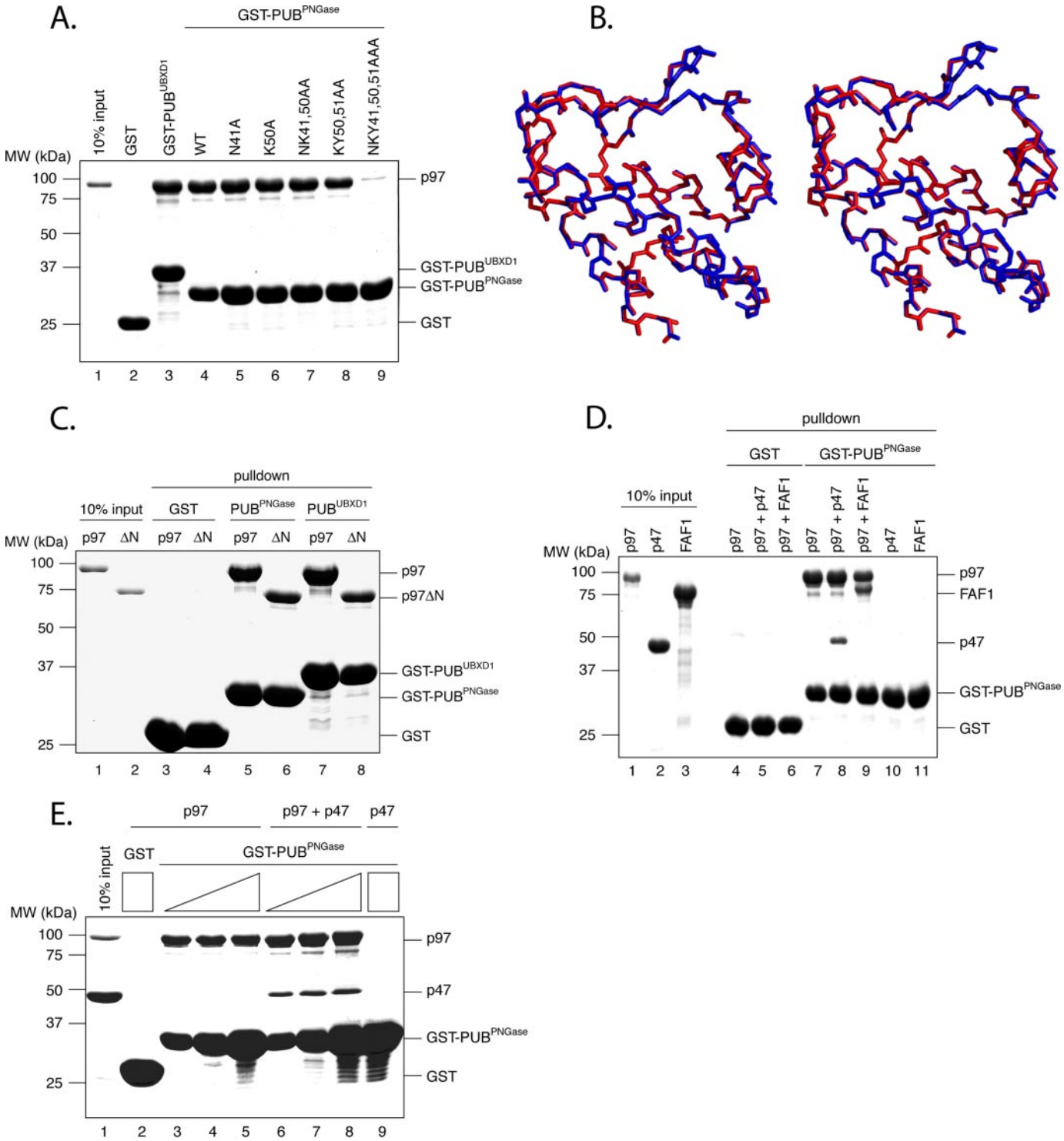

FIGURE 5. **The PUB domain is a novel p97 binding module.** *A*, p97 and the PUB domain interact directly via the conserved surface patch. GST fusion proteins of the PUB domain of human UBXD1 (*GST-PUB^{UBXD1}*; *lane 3*) or wild-type or the indicated mutant PUB domains from human PNGase (*GST-PUB^{PNGase}*; *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 p97ΔN as 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.

prevent its binding to the PUB domain in a GST pulldown experiment (Fig. 5*D*, *lanes 7–9*), suggesting that binding of UBX and PUB domains is not mutually exclusive. Consistent with this interpretation, significant binding of p47 and FAF1 to the GST-PUB domain fusion was observed in the presence, but not the absence, of p97 (compare *lanes 8* and *9* with *lanes 10* and *11*). To ensure that the amount of PUB domain used in this experiment was saturating with respect to p97, we performed a titration experiment with increasing amounts of GST-PUB domain fusion protein (Fig. 5*E*). We found that the lowest PUB domain concentration, which had been used in the previous experiments, was already saturating, because no additional p97 bound to a 5-fold higher concentration (*lanes 3–5*). Consistently, the amount of p47 bound to p97 remained constant even at the highest concentration of PUB domain used (*lanes 6–8*), whereas no p47 bound to the PUB domain in the absence of p97 (*lane 9*). Taken together, these data demonstrate that binding of the PUB domain and of UBX domain proteins to p97 is not mutually exclusive.

DISCUSSION

p97 is a chaperone-like molecular machine involved in a variety of cellular functions, including protein degradation, membrane fusion, and cell cycle regulation (31, 33). This remarkable versatility of p97 depends critically on its ability to form complexes with a number of different cofactors mediating distinct activities (31). Substrate-recruiting cofactors including the heterodimer Ufd1/Npl4 and members of the large UBX protein family regulate the substrate specificity of p97 (26, 34), while substrate-processing cofactors like the ubiquitin chain elongation enzyme Ufd2 or the deubiquitinylating enzymes VCIP135 and Otu1 determine the fate of p97 substrates (31, 35). In addition, a significant number of p97-binding proteins exist whose mechanism of action on p97 is still poorly characterized, among them PNGase (17).

In a structural approach to investigate p97 cofactor functions, we solved the three-dimensional structure of the PUB domain of human PNGase. Although the structure did not possess significant similarities to known proteins, it revealed a conserved surface patch suggestive of a functionally important site (Fig. 4). Indeed, our biochemical analysis showed that the PUB domain is a novel p97 binding module and that the conserved surface patch is a major p97 binding site (Fig. 5*A*). Interestingly, the PUB domain does not bind to the N domain of p97, unlike the well established substrate-recruiting cofactors Ufd1/Npl4 and p47, but rather to the D1 and/or D2 ATPase domains. Consequently, binding of the PUB domain is not mutually exclusive with binding of substrate-recruiting UBX proteins (Figs. 5, *D* and *E*). The use of a different binding site on p97 by the PUB domain could simply allow the assembly of larger complexes on p97. Alternatively, the interaction with the D1 and/or D2 domains may enable the PUB domain to coordinate the ATP hydrolysis-dependent substrate-extracting activity of p97 with PNGase-catalyzed deglycosylation and substrate-processing activities of other cofactors, perhaps including the proteasomal targeting factor HR23B (9, 17).

Our finding that the two distantly related PUB domains of human PNGase and human UBXD1 both bind to p97 (Fig. 5*A*) suggests strongly that PUB domain-containing proteins constitute a novel family of p97 cofactors. PUB domain proteins are found in the plant and animal kingdoms and are particularly common in trypanosomes, which contain several unique families of PUB domain-containing proteins (36). The PUB domain is often found in proteins that also contain domains associated with ubiquitin conjugation and removal (Fig. 1). It is tempting to speculate that these proteins coordinate assembly and/or processing of ubiquitin chains with p97 function.

UBXD1-like proteins containing both PUB and UBX domains together are evolutionary conserved and represent the most widespread group of PUB domain proteins (28). Although none of these proteins has been characterized in any detail, the simultaneous presence of two p97 binding domains suggests that UBXD1-like proteins may be important regulators of p97 function. Intriguingly, the major p97 binding motif found in most UBX domains (residues FPR in the turn between strands 3 and 4 of the UBX domain) (29, 32) is significantly altered in UBXD1-like proteins (28, 29), thus potentially decreasing the affinity for p97. We speculate that the presence of a second p97 binding module (the PUB domain) within UBXD1-like proteins allowed for the modulation of the binding properties of the UBX domain during evolution. It will be interesting to study the details of such a dual p97 binding mechanism. The identification of PUB domain proteins as an entire new family of potential p97 cofactors further emphasizes the key role of substrate-recruiting and substrate-processing proteins for the regulation of the p97 molecular machine and opens a new direction for the investigation of p97 functions in various organisms.

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