The AAA ATPase p97/VCP Interacts with Its Alternative Co-factors, Ufd1-Npl4 and p47, through a Common Bipartite Binding Mechanism*

Roland M. Bruderer, Catherine Brasseur, and Hemmo H. Meyer‡

From the Swiss Federal School of Technology (ETH), Institute of Biochemistry, ETH Honggerberg HPM, Zurich 8093, Switzerland

The AAA ATPase p97/VCP forms complexes with different adapters to fulfill distinct cellular functions. We analyzed the structural organization of the Ufd1-Npl4 adapter complex and its interaction with p97 and compared it with another adapter, p47. We found that the binary Ufd1-Npl4 complex forms a heterodimer that cooperatively interacts with p97 via a bipartite binding mechanism. Binding site 1 (BS1) is a short hydrophobic stretch in the C-terminal domain of Ufd1. The second binding site is located at the N terminus of Npl4 and is activated upon binding of Ufd1 to Npl4. It consists of about 80 amino acids that are predicted to form a ubiquitin fold domain (UBD). Despite the lack of overall homology between Ufd1-Npl4 and p47, both adapters use identical binding mechanisms. Like the ubiquitin fold ubiquitin regulatory X (UBX)1 domain in p47, the Npl4-UBD interacts with p97 via the loop between its strands 3 and 4 and a conserved arginine in strand 1. Furthermore, we identified a region in p47 homologous to Ufd1-BS1. The UBD/UBX and the BS1 of both adapters interact with p97 independently, whereas homologous binding sites in both adapters cooperate for binding to p97. In contrast to p47, however, Ufd1-Npl4 does not regulate the ATPase activity of p97; nor does a variant of p47 that contains both binding sites but lacks the N-terminal domain. Therefore, the binding sites alone do not regulate p97 directly but rather serve as anchor points to position adapter-specific domains at critical locations to modulate p97-mediated reactions.

The essential AAA ATPase p97/VCP (valosin-containing protein) in vertebrates and its orthologues in yeast (Cdc48p) and other organisms are involved in a large variety of cellular processes. These include organelle membrane fusion, removal of soluble and membrane proteins, and regulation of gene expression, cell cycle, and apoptosis (1). p97 functions as a molecular chaperone that either extracts substrates from membranes and other structures or changes their conformation. It contains two AAA domains (D1 and D2) that are stacked and form a hexameric barrel. The N-domain, which interacts with cofactors and substrates, is positioned in a plane with D1 at the outside of the hexamer (2–4). Functional and structural work suggest that nucleotide hydrolysis and exchange in the D2 domain causes movements within the molecule, particularly of the N-domain (5–8). These movements may then be transmitted, directly or indirectly, onto substrate proteins to apply mechanical force.

For its various functions, p97 cooperates with different sets of mutually exclusive adapters and additional cofactors, two of which, p47 (9) and a binary complex of Ufd1 and Npl4 (10), have been extensively studied, and a growing number is being discovered (1). Ufd1-Npl4 and p47 form alternative complexes with the ATPase and are required for different types of reactions. The Ufd1-Npl4 adapter is needed for p97-mediated reactions during endoplasmic reticulum-associated degradation (11–14), nuclear envelope formation (15), spindle disassembly after mitosis (16), and, in yeast, the proteasome-dependent activation of transcription factors (17, 18). During endoplasmic reticulum-associated degradation, p97 and Ufd1-Npl4 mediate the transport of endoplasmic reticulum proteins into the cytosol for subsequent degradation by the ubiquitin-proteasome system. This mobilization process involves the recognition of a substrate in the membrane by the p97-Ufd1-Npl4 complex, followed by its ATP hydrolysis-driven extraction and concomitant ubiquitylation (6). The function of Ufd1-Npl4 depends on the N-terminal 200 amino acids in Ufd1 that adopt the same fold as the p97 N-domain (19) and bind lysine 48-linked polyubiquitin chains (6). The Npl4 zinc finger domain at the C terminus of metazoan Npl4 also binds ubiquitin (20–22).

The p47 adapter, on the other hand, contains three domains, a C-terminal ubiquitin fold (ubiquitin regulatory X; UBX)1 domain that binds p97, a SEp (shp1, eyc, p47) domain of unknown function, and a UBA (ubiquitin-associated) domain at the N terminus that binds ubiquitin (21, 23, 24). The p97-p47 complex regulates several organelle membrane fusion steps, including cisternal regrowth from mitotic Golgi fragments (9). This process involves ubiquitin, but (in contrast to endoplasmic reticulum-associated degradations) is proteasome-independent. p97-p47 is recruited to a monoubiquitylated yet unidentified substrate on mitotic Golgi membranes via the UBA domain in p47 (21). There it cooperates with another cofactor, the deubiquitinating enzyme VCIP135, to remove ubiquitin and trigger membrane fusion (25, 26).

The exact functions of the adapters and how they modulate p97-mediated reactions are not fully understood. One aspect is that they link the ATPase to different types of ubiquitin conjugates, Ufd1-Npl4 to lysine 48 linked chains and p47 to monoubiquitylated substrates (6, 21). Another aspect is the p47-mediated regulation of the ATPase activity of p97 (27).

* This work was supported by Grant 31000A0-102217 from the Swiss National Fund (to H. H. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 41-1-633-6634; Fax: 41-1-632-1298; E-mail: hemmo.meyer@bc.biol.ethz.ch.

1 The abbreviations used are: UBX, ubiquitin regulatory X; UBD, ubiquitin fold domain of Npl4; BS1, binding site 1; GST, glutathione S-transferase.
effort to cast light on the role of Ufd1-Npl4 and its relationship to p97, we have analyzed its structural organization and its interaction with the ATPase. Surprisingly, we found that it is very similar to p47.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs and Expression of Proteins**—All proteins were generated in *Escherichia coli*. Full-length His-p97, Npl4, and Ufd1-His were generated, and complexes were assembled as described (10, 15). Constructs coding for truncation mutants of Ufd1, Npl4, and p47 fused to the C terminus of GST were derived from pGEX-Ufd1, pGEX-Npl4, and pGEX-p47 (10), respectively, and generated by QuikChange mutagenesis followed by confirmation of the entire sequence. The ubiquitin fold domain of Npl4 with a C-terminal His tag was expressed using pET30 (Novagen) that contained an insert between the NdeI and XhoI sites coding for amino acids 1–96. The His-tagged p47 full-length, p47-UBX, and p47(BSI-UBX) proteins were expressed using pTrcHis-p47 (10) or derived constructs coding for amino acids 1–370, 282–370, and 244–370, respectively. His-tagged and GST-tagged fusion proteins were further purified using gel filtration. Peptides covering binding site 1 (BS1) of Ufd1 (GEVGFRAPFSQSGNKLDGKKKG) and p47 (PK-GAFAKPTGEGKQKLSTAPQ) and Ufd1-(286–306) were synthesized and biotinylated at the N terminus.

**Molecular Mass Determination**—The Ufd1-Npl4 complex was generated from recombinant proteins as described (15). 70 µg of the complex was treated to 0.22-µm filtration prior to size exclusion chromatography with a Superose-6 HR (10/30) column coupled with an in-line Dawn EOS laser light scattering apparatus (Wyatt Technology Corp.), refractometer (Wyatt Technology), and UV detector (Waters Corp.). The running buffer was 20 mM Hepes pH 7.4, 150 mM KCl, 2.5 mM MgCl₂, 2 mM 2-mercaptoethanol, 2% glycerol. The data were collected and analyzed with the ASTRA software at the HHMI Biopolymer Keck Foundation Biotechnology Resource Laboratory (Yale University).

**Binding Experiments**—For experiments using gel filtration, the pre-assembled Ufd1-Npl4 complex or individual proteins were either run alone or after 30 min of binding on ice in running buffer (150 mM KCl, 25 mM Tris, pH 8.0, 2.5 mM MgCl₂, 1 mM ATP, 5% glycerol), followed by separation on a Superose-6 HR (10/30) column (Amersham Biosciences). Co-precipitation experiments using glutathione- or streptavidin-coated beads were performed as described (10). Competition experiments were carried out with a 50-fold molar excess of soluble UBD/UBX domains or BS1 peptides over immobilized binding domains. An anti-His antibody (Amersham Biosciences) was used to detect p47 variants co-isolated with GST-p97. Far Western overlay assays were carried out as described (10).

**Sequence Analysis**—Primary peptide sequence alignment was performed using the ClustalW program (28). Secondary structure was calculated using Jpred (available on the World Wide Web at www.compbio.dundee.ac.uk/∼www/jpred/) (29). Protein fold recognition was done with 3D-PSSM (available on the World Wide Web at www.sbg.bio.ic.ac.uk/∼3dpsmm/) (30).

**ATPase Assay**—The assays were performed using 2 µg of recombinant p97 preincubated without or with varying amounts of the p47, p47-(244–370) or Ufd1-Npl4 for 30 min on ice. Proteins were then incubated in 100 µl of buffer (50 mM HEPES pH 7.4, 150 mM KCl, 2.5 mM MgCl₂, 5% glycerol, and 2 mM ATP) for 40 min at 37 °C. 40-µl samples were taken at the start and the end of the reaction. Formation of orthophosphate was measured using the malachite green color reagent according to the method by Lanzetta et al. (31).

**RESULTS**

**Ufd1 and Npl4 Form a Heterodimer**—In a first step toward understanding the structural organization of the Ufd1-Npl4 complex and its interaction with p97, we determined the stoichiometry of the adapter. Our previous data suggested that Ufd1 and Npl4 form a tight complex with a ratio of 1:1. The binary complex eluted at an apparent molecular mass of 200 kDa from gel filtration both when isolated from cytosol as well as when reconstituted from recombinant proteins (10). However, since migration in size exclusion columns depends on both size and shape of the proteins, we determined the molecular weight of the reconstituted Ufd1-Npl4 complex by coupling gel filtration with in-line multianalyte laser light scattering, refractive index, and UV light measurements. The Ufd1-Npl4 peak was monodisperse with an average molecular mass of 100 kDa (data not shown). This is in agreement with a heterodimer composed of one Npl4 (67-kDa) and one Ufd1 (35-kDa) peptide.

We also previously showed that Ufd1 alone binds p97. However, Npl4 stimulates complex formation, although it does not bind p97 on its own in solution (10). To reveal the relevance of this stimulation, we performed binding experiments with p97 to Ufd1 or Npl4 individually or Ufd1 and Npl4 together, separated the resulting protein complexes by gel filtration, and analyzed them in SDS-polyacrylamide gels (Fig. 1). Ufd1 bound p97, but binding was inefficient, since only a fraction of p97 was shifted to a larger molecular weight (row 2). More importantly, the p97-Ufd1 complexes were of heterogeneous size, and some eluted in the void volume, indicating that the proteins aggregated. Consistent with previous results (10, 15), Npl4 did not bind p97 (row 3), whereas Ufd1-Npl4 bound p97 efficiently and shifted it homogeneously to an apparent molecular mass of about 700 kDa (row 5). These results show that Ufd1 requires Npl4 for correct complex formation with p97.

**Ufd1 Contains Two Distinct Binding Sites for Npl4 and p97**—Ufd1 binds both p97 and Npl4 via its C-terminal ~100 amino acids, previously termed the UT6 domain (15). We decided to clarify whether p97 and Npl4 bind via the same or distinct interaction sites within this domain. Binding of the two partners to Ufd1 can be analyzed in far Western overlay assays. We generated a series of Ufd1 truncation mutants fused to GST in bacteria as depicted in Fig. 2A. Bacterial lysates were separated in SDS gels and transferred onto membranes. After partial renaturation, immobilized proteins were incubated with biotinylated p97 or Npl4, respectively. Bound probes were identified with peroxidase-coupled streptavidin and detected by chemiluminescence. The results are summarized in Fig. 2A. We found that Ufd1 contains two distinct short binding sites for p97 and Npl4, lying between residues 215 and 241 (termed...
BS1) and 258–275, respectively. To confirm the results from overlay assays, we purified critical truncation mutants and performed binding assays with p97 or Npl4 in solution. We also generated and tested GST-Ufd1-(281–307), which contains a sequence with homology to BS1. GST-Ufd1 mutant proteins were isolated using immobilized glutathione, and bound partners were analyzed in Coomassie-stained SDS gels (Fig. 2).

Our results show that Ufd1-(215–241) was necessary and sufficient to bind p97 but did not interact with Npl4. Conversely, Ufd1-(258–275) bound Npl4, but not p97. The internal repeat Ufd1-(281–307) did not bind either of the two partners.

Sequence alignment of Ufd1-BS1 with p47 revealed a short homologous stretch in the larger linker region between the p47 SEP and UBX domains, which had previously been implicated in interaction with p97 (26). The hydrophobic residues are best conserved between the two sequences (Fig. 2C). To confirm the relevance of the homology, we used chemically synthesized peptides covering the BS1 sequences of Ufd1 and p47 as shown in Fig. 2C. Peptides were biotinylated at the N terminus and immobilized on steptavidin-beads. Incubation with p97 and subsequent co-sedimentation revealed that both peptides bound p97 specifically (Fig. 2D). In summary, we found that Ufd1 has two distinct short binding sites for p97 and Npl4. The first one, BS1, covers 21 amino acids and is homologous to a newly demarcated binding site in the other adapter, p47. An independent binding site for Npl4 resides further downstream, covering 18 amino acids.

Interaction with Ufd1 Activates Npl4 Binding to p97—The identification of the two distinct binding sites in Ufd1 allowed us to analyze Npl4-stimulated complex formation with p97.

Results are summarized on the right. Regions sufficient to bind either p97 (BS1) or Npl4 are shaded gray. B, selected GST-Ufd1 variants analyzed in A and a control covering Ufd1-(281–307) were purified and incubated with either p97 or Npl4 in solution as indicated. GST fusion proteins were then precipitated using immobilized glutathione, and bound proteins were analyzed in Coomassie-stained SDS gels. Input (10%) p97 and Npl4 are shown in the bottom panel. C, peptide sequence alignment of BS1 in Ufd1 with a homologous region in the p47 sequence. D, peptides covering the sequences of BS1 in Ufd1 and p47 as shown in C and a control peptide covering Ufd1-(286–306) were biotinylated at the N terminus and immobilized on steptavidin-coated beads. Peptide-decorated beads (as indicated) or beads alone (beads) were incubated with p97 and sedimented, and bound protein was analyzed in Coomassie-stained SDS gels.

**Fig. 2.** Ufd1 has two distinct binding sites for p97 and Npl4. A, a series of truncation mutant proteins of Ufd1 fused to GST were generated in bacteria as indicated. Binding activities were analyzed in far Western blot assays with biotinylated p97 or Npl4 in the overlay.
Again, we performed in vitro interaction studies with GST-Ufd1 truncation mutants binding to Npl4 or p97, but this time also with Npl4 and p97 together. As before, bound partners were detected in SDS gels (Fig. 3). As in the previous experiment, GST-Ufd1-(1–230) covering the N-terminal domain did not bind any of the partners (lanes 1–3), whereas GST-Ufd1-(215–307) bound both (lanes 4 and 5). Confirming our previous data, p97 recruitment to the latter was stimulated by co-incubation with Npl4 (lane 6). Ufd1-BS1-(215–241) bound p97, but not Npl4, no matter whether they were incubated individually.
or together (lanes 7–9). Conversely, the Ufd1-Npl4 binding peptide (residues 258–275) only bound Npl4 and not p97 (lanes 10 and 11). Despite the lack of direct binding activity to p97, however, this fragment co-sedimented p97, when Npl4 was present (lane 12). This result suggests that the binding of the Ufd1 fragment to Npl4 activated an otherwise inactive interaction domain in Npl4 for p97 and that Npl4 bridged between GST-Ufd1-(258–275) and p97 in this reaction.

**Npl4 Contains a Ubiquitin Fold Domain That Mediates Interaction with p97**—The notion of a hidden p97 interaction domain in Npl4 is supported by the fact that p97 binds partially denatured Npl4 on filters in overlay assays (10). We used the same technique to search for the p97-binding site in Npl4. As for Ufd1, we generated a series of Npl4 truncation mutants fused to GST as listed in Fig. 4A. We expressed the Npl4 mutants in bacteria and analyzed the lysates in far Western assays with biotinylated p97 in the overlay. As shown in Fig. 4B, we identified a fragment covering the N-terminal 89 amino acids that was sufficient and required for interaction with p97 (Fig. 4B). The loops between strands 3 and 4 of Npl4-UBD were sedimented, and bound protein was analyzed in Coomassie-stained SDS gels. 10% of input p97 is shown at the bottom of each panel. GST alone was used as a negative control. Note that each of the two ubiquitin fold domains competes with both Npl4-UBD and p47-UBX but not with the BS1 versions, and vice versa.

**FIG. 5.** The two types of interaction sites bind independently, but homologous sites of both adapters compete for binding to p97. GST-tagged ubiquitin fold domains of Npl4 and p47 (UBD and UBX, respectively) or BS1 of Ufd1 and p47 were mixed with p97 as indicated. Each combination was incubated in the absence or presence of either excess His-tagged Npl4-UBD or p47-UBX proteins (A) or excess Ufd1-BS1 or p47-BS1 peptide (B) as competitors. GST fusions were sedimented, and bound protein was analyzed in Coomassie-stained SDS gels. 10% of input p97 is shown at the bottom of each panel. GST alone was used as a negative control. Note that each of the two ubiquitin fold domains competes with both Npl4-UBD and p47-UBX but not with the BS1 versions, and vice versa.

In our hands, standard computational sequence analysis of the Npl4 N terminus did not reveal significant homologies to any known proteins (other than Npl4 orthologues) or conserved domains. A fold recognition method, however, used on the 3D-PSSM server (available on the World Wide Web at www.sbg.bio.ic.ac.uk/~3dpssms/) predicted a β-grasp fold that is also found in ubiquitin and related proteins. Since p47 binds p97 via its UBX domain that also assumes a ubiquitin fold (23, 32), we aligned the peptide sequences of the two domains according to their predicted (Npl4) or confirmed (p47) secondary structure and relatively weak primary sequence similarities (Fig. 4C).

Dreveny et al. (33) showed that the p47-UBX domain interacts with p97 by inserting the loop between its strands 3 and 4 into a cleft in the p97 N-domain. Furthermore, they identified an arginine on strand 1 to be important for binding. We tested whether the Npl4 ubiquitin fold domain (UBD) binds via the same mechanism to p97. We generated a series of variants of the Npl4-UBD fused to GST with mutations in critical residues and tested binding to p97 in co-sedimentation experiments (Fig. 4D).

Mutation of the arginine residue on strand 1 that is conserved between Npl4 and p47 abolished binding of Npl4-UBD to p97 almost completely (lane 3) compared with the wild type domain (lane 2). The loops between strands 3 and 4 of Npl4-UBD and p47-UBX do not have any sequence similarities. However, mutations in the Npl4 loop (RNK to AAA, lane 5) or flanking region (INR to AAG, lane 4) again abolished binding, indicating that this region is crucial for p97 interaction with Npl4 too. In contrast, mutations in the second helix (L67A, lane 6) or S5 (L75A and F78S, lanes 7 and 8) had little or no influence on the interaction. All variants tested here were easily expressed in bacteria and soluble in large amounts, indicating correct folding. Together, Npl4 contains an interaction domain for p97 that assumes a β-grasp ubiquitin fold (UBD) but has little primary sequence homology to either ubiquitin or p47-UBX. Nevertheless, both interaction domains employ the same surfaces for binding to p97.

**Binding Sites in Ufd1 and Npl4 Are Functionally Related to Individual Sites in p47**—We identified two different binding sites in the Ufd1-Npl4 adapter that are homologous to binding sites in p47. We therefore wanted to clarify whether the different sites can interact independently and whether the homolo-
gious sites in the two adapters bind via similar mechanisms.

We incubated p97 again with GST fusions of each of the binding domains, Npl4-UBD, p47-UBX, Ufd1-BS1, or p47-BS1. We then tested the interaction in two series of competition experiments. First, we carried out the binding in the absence or presence of excess amounts of His-tagged ubiquitin fold domains, Npl4-UBD or p47-UBX (Fig. 5A). In the second series, we performed the same set of binding reactions, this time in the absence or presence of excess peptides covering the other binding site of each adapter, Ufd1-BS1 or p47-BS1 (Fig. 5B). Analysis of the amounts of p97 co-sedimented with the GST fusions under the different conditions revealed that each ubiquitin fold domain interfered with binding of both Npl4-UBD and p47-UBX (A, lanes 3 and 4 and lanes 6 and 7) but not with interaction of the BS1 of either of the two adapters (A, lanes 9 and 10 and lanes 12 and 13). Conversely, the BS1 peptides of either Ufd1 or Npl4 inhibited p97 binding to the immobilized BS1 of both adapters (B, lanes 9 and 10 and lanes 12 and 13) but did not affect interaction of the UBD/UBX domains with p97 (B, lanes 3 and 4 and lanes 6 and 7).

Together, these data show that the two binding domains of each adapter independently interact with p97. However, homologous domains of the adapters compete for binding, suggesting that they bind via the same mechanism to p97.

The Binding Domains Alone Do Not Mediate Regulation of ATPase Activity—We previously reported that p47 down-regulates the ATP hydrolysis activity of p97 (27). Since results presented here revealed that Ufd1-Npl4 uses the same mechanism of interaction with p97 as p47, we asked whether Ufd1-Npl4 regulates p97 the same way. When we measured p97-adapter complexes, however, we found that, contrary to p47, Ufd1-Npl4 did not reduce the ATPase activity of p97 (Fig. 6). This differential effect could have two causes. It could be due to small variation in the binding regions between the two adapters. Alternatively, the binding domains of p47 do not regulate p97 directly but require additional adapter-specific domains for the modulation. We therefore tested whether the p47-(244–370) fragment that contains both BS1 and the UBX domain but lacks the p47-specific domains had any regulatory activity. We therefore tested whether the p47-(244–370) fragment containing both BS1 and UBX, or Ufd1-Npl4 on ice for 30 min. The ATP hydrolysis activity of the complexes was then measured at 37 °C. Shown is the mean of three experiments. B, equal binding affinities of p47 and p47-(244–370) to p97 were confirmed by co-precipitation experiments. GST or GST-p97 were incubated with either of the two His-tagged p47 variants, and bound protein was analyzed by immunoblotting using an anti-His antibody.

FIG. 6. The bipartite interaction domains do not mediate regulation of p97 directly. A, p97 complexes were formed by preincubating p97 with equal molar amounts of p47, the p47-(244–370) fragment containing both BS1 and UBX, or Ufd1-Npl4 on ice for 30 min. The ATP hydrolysis activity of the complexes was then measured at 37 °C. Shown is the mean of three experiments. B, equal binding affinities of p47 and p47-(244–370) to p97 were confirmed by co-precipitation experiments. GST or GST-p97 were incubated with either of the two His-tagged p47 variants, and bound protein was analyzed by immunoblotting using an anti-His antibody.

**DISCUSSION**

Recent work has revealed that p97/VCP mediates different types of reactions and that it requires distinct sets of cofactors to carry out these tasks. In this report, we found that despite these functional differences and lack of overall homology, the two alternative adapters Ufd1-Npl4 and p47 interact with the ATPase in a very similar manner via a bipartite binding mechanism (Fig. 7).

In the case of Ufd1-Npl4, each of the two subunits contains one binding site, and several lines of evidence show that both subunits indeed act together and bind p97 cooperatively. We have previously shown that, although Ufd1 can interact with p97 independently, the addition of Npl4 stimulates complex formation. By analyzing the various possible complexes more carefully, we now show that p97 binding to Ufd1 alone results in inefficient binding that leads to heterogeneous complex formation and aggregation. Only the preassembled Ufd1-Npl4 complex efficiently binds p97 and forms a physiological ternary complex of homogeneous size that is also found in cytosol, suggesting that all three partners act together. We also revealed how Npl4 stimulates complex formation although it is not able to bind p97 on its own in solution. Using truncation mutants, we showed that Ufd1 binding to Npl4 activates a ubiquitin fold domain (UBD) in Npl4 that then interacts with p97. The notion of an autoinhibited binding of full-length, free
Npl4 is supported by the fact that the isolated UBD of Npl4 can bind p97 in solution, and so does partially denatured Npl4 on filters. Whether this activation by Ufd1 is physiologically relevant is unclear, since we have not detected Ufd1 or Npl4 individually in cell extracts.

Our analysis of the reconstituted, binary Ufd1-Npl4 complex using multiangle light scattering combined with gel filtration revealed that the adapter is a heterodimer with only one molecule of each protein. It therefore has the same layout of binding sites as a single p47 molecule. The fact that Ufd1-Npl4 elutes at a larger apparent molecular weight from gel filtration columns points to an elongated shape of the complex. Based on these findings, any number of Ufd1-Npl4 between 1 and 6 could theoretically bind a single p97 hexamer, although a symmetric interaction of either 3 or 6 units per hexamer may be more likely. In the case of p47, recent findings indicate a binding ratio of 6 adapter molecules per hexamer p97 (33, 34), but further structural and functional work is required to confirm these results and clarify whether this is also true for Ufd1-Npl4.

The finding of a UBD in Npl4 as a binding site for p97 was unexpected to us, since primary sequence analysis did not detect any obvious homologies to ubiquitin or ubiquitin-like domains. We could, however, identify this domain using fold prediction software in Npl4 proteins as distantly related as the mammalian and the yeast form. This prediction is supported by our binding analysis using mutants of the Npl4-UBD. It revealed interaction surfaces with p97 identical to those reported for the p47-UBX domain (33). Our data, therefore, suggest that the described complex represents a general interaction mechanism of ubiquitin fold domain-containing cofactors with p97. This is not the same mechanism as was employed for the interaction of ubiquitin chains with p97, however, since cofactors and ubiquitin do not significantly compete for binding to the ATPase (33, 35). This is consistent with our findings that Ufd1-Npl4 or p47 stimulate ubiquitin binding to p97 rather than inhibiting it (6, 21). Furthermore, the absence of significant sequence homology to ubiquitin makes it unlikely that the Npl4-UBD acts in a manner similar to that of the truly ubiquitin-like domains in Rad23 and related proteins that link these factors to the proteasome (36). The sole function of Npl4-UBD may therefore be the interaction with p97.

The identification of the UBD in Npl4 as the interaction interface for p97 associates the Ufd1-Npl4 complex with a growing number of cofactors that bind p97 via this fold. Besides p47, these include VCIP135 in mammals (26), Ubx1–7p in budding yeast (37, 38), and Ubx2p and Ubx3p in fission yeast (35). The fold does not seem to be a prerequisite for interaction, though, since we did not detect this domain in the direct binding partners such as Ufd3p or Ufd2p using fold prediction.

Ufd1, on the other hand, contains a short hydrophobic binding site for p97 (BS1). We show that it is homologous and functionally related to a short stretch in p47 within the linker between the SEP and UBX domains that was previously shown to bind p97 (26). The BS1 does not seem to be as common as the ubiquitin fold, however. We noticed a homologous region in VCIP135 (amino acids 1020–1035), and it will be interesting to find out whether it has the same function. However, none of the other known p97-binding factors have segments with obvious homology to BS1. Nevertheless, BS1 is conserved in Ufd1 proteins of different species. This is also true for Caenorhabditis elegans Ufd1, which is intriguing, since Npl4 in this species lacks the UBD.

Given that the N-domain of p97 is essential for adapter binding (6), it is likely that BS1, too, directly interacts with it. Unfortunately, the structural data collected by Deverny et al. (33) using the p47-(244–370) fragment that contains both UBX and BS1 did not reveal the exact location of its binding site in p97. The orientation of the fragment in the crystal with p97, however, suggests that BS1 binds p97 on the top of the N-domain toward the center of the p97 hexamer. Based on our finding that the two binding sites interact independently with p97, one could speculate that p47-(244–370) or Ufd1-Npl4 forms a clamp around the N-domain. It was therefore important to ask whether the connected binding domains alone account for the down-regulation of p97 ATPase activity as observed for p47, by either changing the conformation of p97 or restricting the movements of the N-domains. However, since we did not detect any effect on the rate of ATP hydrolysis upon binding of either p47-(244–370) or the complete Ufd1-Npl4 complex, we conclude that bipartite adapter binding does not regulate the ATPase activity directly. Therefore, the additional adapter moieties and the way they organize the p97-adapter complexes are crucial for the differential regulation. In fact, p47 has been reported to oligomerize (24). Oligomerization of p47 units within the p97-adapter complex may be another way of restricting N-domain movements and thereby influencing ATP hydrolysis. Consistent with this, there is no evidence for oligomerization of Ufd1-Npl4. These results support a model in which the binding domains of the adapters serve as anchor points for p97 rather than directly regulating it, whereas adapter-specific domains are required for differential modulation.

Correct anchoring, however, may be crucial. Whereas the UBD could serve as a general recruitment domain, the BS1 may provide higher affinity and may position the adapter in the right orientation with respect to p97 and its movements. The exact role of the adapters in p97-mediated reactions is still a matter of speculation. If they are involved in transmitting mechanical force from p97 to a substrate, correct positioning is essential. However, this is also true, if they are involved in further processing of substrates and regulating recruitment of additional cofactors, a deubiquitinating enzyme in the case of p47, and possibly ubiquitin conjugation machinery in the case of Ufd1-Npl4. Future analysis of yet uncharacterized domains of the adapters will shed light on their roles in p97-mediated reactions.

Acknowledgments—We thank Graham Warren, in whose laboratory this project was initiated. We also thank Martin Lowe and Lars Ellgaard for critical reading of the manuscript.

REFERENCES
1. Woodman, P. G. (2003) J. Cell. Sci. 116, 4283–4290
2. Zhang, X., Shaw, A., Bates, P. A., Newman, R. H., Gonen, B., Orlova, E., Gorman, M. A., Kondo, H., Dokurno, P., Lally, J., Leonard, G., Meyer, H., van Heel, M., and Freemont, P. S. (2000) Mol. Cell 6, 1475–1484
3. Huyton, T., Pye, V. E., Briggs, L. C., Flynn, T. C., Beurun, F., Kondo, H., Ma, J., Zhang, X., and Freemont, P. S. (2003) J. Struct. Biol. 144, 337–348
4. Delabarre, B., and Brunger, A. T. (2003) Nat. Struct. Biol. 10, 856–863
5. Song, C., Wang, Q., and Li, U. C. (2003) J. Biol. Chem. 278, 3648–3655
6. Ye, Y., Meyer, H. H., and Rapoport, T. A. (2000) J. Cell Biol. 162, 71–84
7. Rouiller, I., Delabarre, B., May, A. P., Weis, W. I., Brunger, A. T., Milligan, R. A., and Wilson-Kubalek, E. M. (2002) Nat. Struct. Biol. 9, 850–857
8. Beurun, F., Flynn, T. C., Ma, J., Kondo, H., Zhang, X., and Freemont, P. S. (2003) J. Mol. Biol. 327, 619–629
9. Kondo, H., Rabouille, C., Newman, R., Levine, T. P., Pappin, D., Freemont, P., and Warren, G. (1997) Nature 388, 75–78
10. Meyer, H. H., Shorter, J. G., Seemann, J., Pappin, D., and Warren, G. (2000) EMBO J. 19, 2181–2192
11. Bay, N. W., Wilhovsky, S. K., Goradia, A., Hodgkiss-Harlow, K., and Hampton, R. Y. (2001) Mol. Biol. Cell 12, 4114–4128
12. Braun, S., Matuschewski, K., Rape, M., Thomas, S., and Jentsch, S. (2002) EMBO J. 21, 615–621
13. Jarnosch, R., Texis, C., Volkwein, C., Bordaloo, J., Finley, D., Wolf, D. H., and Sommer, T. (2002) Nat. Cell Biol. 4, 134–139
14. Ye, Y., Meyer, H. H., and Rapoport, T. A. (2001) Nature 414, 652–656
15. Hetzer, M., Meyer, H. H., Walthier, T. C., Bilbae-Cortes, D., Warren, G., and Mattaj, I. W. (2001) Nat. Cell Biol. 3, 1086–1091
16. Cao, K., Nakajima, R., Meyer, H. H., and Zheng, Y. (2003) Cell 115, 355–367
17. Rape, M., Hoppe, T., Gorr, I., Kalocay, M., Richly, H., and Jentsch, S. (2001) Cell 107, 667–677
18. Hitchcock, A. L., Krebber, H., Fritze, S., Lin, A., Lutterich, M., and Silver, P. A. (2001) Mol. Biol. Cell 12, 3226–3241
19. Coles, M., Dererks, T., Liermann, J., Grager, A., Rockel, B., Baumeister, W.,
