The AAA ATPase, p97, achieves its versatility through binding to a wide range of cofactor proteins that adapt it to different cellular functions. The heterodimer UN (comprising Ufd1 and Npl4) is an adaptor complex that recruits p97 for numerous tasks, many of which involve the ubiquitin pathway. Insights into the structural specificity of p97 for its UN adaptor are currently negligible. Here, we present the solution structure of the Npl4 “ubiquitin-like” domain (UBD), which adopts a β-grasp fold with a 3_10 helical insert. Moreover we performed a chemical shift perturbation analysis of its binding surface with the p97 N domain. We assigned the backbone amides of the p97 N domain and probed both its reciprocal binding surface with Npl4 UBD and its interaction with the p97-binding region of Ufd1. NMR data recorded on a 400-kDa full-length UN-hexamer p97 complex reveals an identical mode of interaction. We calculated a structural model for the p97 N-Npl4 UBD complex, and a comparison with the p97-p47 adaptor complex reveals subtle differences in p97 adaptor recognition and specificity.

The AAA ATPase p97 represents ~1% of total cytosolic protein and is recruited by a myriad of adaptors to provide energy and/or mechanical force for wide-ranging cellular processes (for a recent review, see Ref. 1). The homohexameric structure of p97 has now been solved in assorted permutations of nucleotide-bound states and biophysical methods including x-ray crystallography, electron microscopy, and small-angle X-ray scattering (2–6). Each p97 monomer consists of three domains: N, D1, and D2. Six monomers associate together to form a homohexameric ring with a diameter of ~160 Å and a central hole of ~20 Å. The two ATPase domains of p97, D1 and D2, and by association the N domain, move relative to each other as a result of ATP binding/hydrolysis events, affording the hexamer important mechanical properties (for review, see Ref. 7). In most cases, the N domains form the sites of interaction between p97 and some 40 binding partners, which lend specificity to the cellular actions of p97 via intricately woven protein signaling networks.

The p97 adaptor complex comprising the heterodimer Npl4-Ufd1 (UN) (8) has biologically well characterized roles in endoplasmic reticulum-associated degradation (ERAD) (9) and regulated ubiquitin-dependent processing (10, 11) as well as other emerging functions including mitotic spindle disassembly (12). Most, if not all, of these processes involve ubiquitin or its close relations. ERAD is a quality control process whereby incorrectly folded or obsolete proteins in the ER are retrotranslocated through channels in the ER membrane, polyubiquitinated, and dismantled by the proteasome. Regulated ubiquitin-dependent processing employs the proteasome in more specific protein cleavage events, recognizing monoubiquitin signals on membrane-bound transcription factors and severing them from the membrane thereby activating transcription.

Despite extensive biochemical and genetics characterization, structural information on the UN complex is only now coming together piecemeal. Ufd1 is a 34.5-kDa protein that was discovered in a yeast screen for mutants that failed to degrade a model ubiquitinated substrate (13). The solution structure of Ufd1 residues 1–208 has been solved (14) and bears remarkable similarities to the p97 N domain structure (double β-β barrel). Ufd1 contains discrete binding sites for mono- and tetraubiquitin on different surfaces of the molecule. This has implications for the differential signaling roles of mono- and tetraubiquitin in cellular processes. There is currently no structural information for the C-terminal portion of Ufd1 (residues 209–307) and, although it encompasses binding sites for both Npl4 (residues 258–275) and p97 (residues 215–241) (15), it is unstructured when produced in isolation from these binding partners (Park et al. 14). The only structural information currently available for Npl4 is an NMR solution structure of the 28-residue zinc- and ubiquitin-binding NZF domain at its C terminus (16). Although, most of Npl4 has evaded structure determination, a ubiquitin-like domain (UBD) was identified by sequence alignment techniques and shown to bind p97 N in pull-down assays (15).

Results from biochemical data compared the mode of binding of UN to p97 with that of the other extensively character-
ized adaptor p47, which recruits p97 for homotypic membrane fusion events in a variety of different cellular processes (17). UN and p47 compete for the same binding site on p97 N domain and, despite no sequence identity, appear to use very similar binding modes (15). Both p47 and UN contain two binding sites for p97 N that permit a range of p97 hexamer:adaptor stoichiometries between 1:1 and 1:6. Cumulative evidence from electron microscopy, isothermal titration calorimetry, gel filtration, and mass spectrometry experiments now ascertains that either three p47 molecules or one UN complex bind to the p97 hexamer (18, 19). Here, we present a structural model for the Npl4 UBD in complex with the p97 N domain, the interface for the Ufd1-p97 N interaction, and discuss the implications for the UN-p97 hexamer complex.

EXPERIMENTAL PROCEDURES

Protein Expression—Npl4 UBD (residues 1–96) with a C-terminal His tag was produced as described in Ref. 15. p97 N domain (residues 1–213) with an N-terminal His tag was produced as described in Ref. 20. 15N-, 15N/13C-, and 2H/15N/13C-labeled protein samples were prepared according to unlabeled protocols but in M9-based minimal media using correspondingly labeled ammonium chloride (>99% 15N), glucose (>99% U-13C), and deuterium oxide (>99.9% 2H). The UN complex and a fragment of murine p97 encompassing the N-terminal and D1 ATPase domains (p97 ND1) were produced as described previously (2, 8, 21). Uniformly deuterated samples of Npl4 or Ufd1 were prepared by expression in the appropriate Spectra9 media (Spectra Gases Ltd.).

NMR Spectroscopy and Structure Calculation for Npl4 UBD—Backbone and side-chain assignment were completed using standard double- and triple-resonance assignment methodology (22). H$_2$ and H$_{13}$ assignments were obtained using HBHA(CBCACO)NH (22). The side-chain assignments were completed using HCCH-total correlation (TOCSY) spectroscopy and (H)CC(CO)NH TOCSY (22). Three-dimensional 1H-15N/13C NOESY-heteronuclear single-quantum coherence (HSQC) (mixing time 100 ms at 500 and 800 MHz) experiments provided the distance restraints used in the final structure calculation.

A total of 42 long range NOEs, providing unambiguous three-dimensional characterization of the fold for Npl4 UBD, were manually assigned from the NOE data. The ARIA protocol (23) was used for augmentation of the NOE assignment and final structure calculation. Using the initial model as the starting structure in the first iteration, a total of 750 NOE-derived distances were assigned from 13C- and 15N-edited spectra. Dihedral angle restraints derived from TALOS were also implemented (24). The frequency window tolerance for assigning NOEs was ±0.05 and ±0.07 ppm for direct and indirect proton dimensions and ±0.7 and ±1.2 ppm for nitrogen and carbon dimensions, respectively. The ARIA parameters were set to default values with the exception of the violation tolerances, which were set to 1.0, 1.0, 0.5, 0.2, 0.1, 0.3, 0.1, 0.0, and 0.0 Å in the nine iterations.

NMR Titrations—Samples of p97 N, Npl4 UBD, UN, or Ufd1 peptide were concentrated to 50 μM in 25 mM HEPES buffer, pH 7.5, and 125 mM NaCl. Spectra were recorded in the absence and presence of the binding partner in appropriate molar ratios. For the p97 N-Npl4 UBD interaction, the molar ratios were 1:0.2, 1:0.5, 1:0.75, 1:1.3, 1:2, 1:2.4, 1:2.8, 1:3.9, and 1:6. All resolved shift changes were monitored by two-dimensional 1H-15N HSQC spectra and fitted to a single binding site saturation isotherm within Excel (Microsoft Corp.). For the other titrations, the following molar ratios were used: p97 N-Ufd1 peptide, 1:1, 1:2, 1:0.5, and 1:1; UN + p97 N, 1:0.5, 1:1, 1:2, and 1:5; UN + p97 ND1, 1:0.5, 1:1, and 1:1.5.

Structure Calculation for Npl4 UBD-p97 N Complex—The chemical shift perturbation studies delineated clear interaction
surface areas in the p97 N-Npl4 UBD complex, making this highly suitable for structure calculation using the HADDOCK approach (25). For the calculation, the crystal structure of p97 N and the lowest energy NMR structure from the family of Npl4 UBD structures were used. 32 amino acid residues in Npl4 UBD and 58 in p97 N were identified to have weighted chemical shift changes significantly greater than the average: 0.253 ppm in Npl4 UBD and 0.186 ppm in p97 N were chosen as suitable cut-off values. After filtering for a relative solvent accessibility greater than 50%, as calculated using the program Naccess, 12 residues in Npl4 UBD (4, 8, 16, 17, 21, 50, 51, 69, 70, 71, 74, 77) and 16 for p97 N (50, 52, 54, 107, 109, 110, 112, 113, 133, 140, 141, 142, 156, 166, 171, 179) were identified as active residues. Residues juxtaposed to these that have a relative solvent accessibility greater than 50% were termed passive residues. These included 11 further residues in Npl4 UBD (2, 3, 12, 14, 15, 23, 39, 52, 53, 68, 72) and 16 in p97 N (23, 43, 47, 48, 53, 106, 126, 131, 155, 157, 158, 169, 178, 181, 182, 191). An ambiguous distance restraint of 3.0 Å was invoked between all active residues in one partner molecule to any atom within the active and passive residues of the other protein partner. The interfacial residues that were allowed to move during the simulated annealing and water refinement were residues 12–21 and 31–43 for Npl4 UBD and 44–52 and 65–76 for p97 N. One thousand initial complex structures were generated by rigid body energy minimization, and the best 200 by total energy were selected for torsion angle dynamics and subsequent Cartesian dynamics in an explicit water solvent. Default scaling for energy terms was applied as described previously (25).

**RESULTS**

Npl4 has, thus far, evaded structure determination largely because of its intrinsic flexibility and instability in the absence of Ufd1 (NMR data not shown). Fig. 1 shows a $^1$H-$^{15}$N HSQC spectrum of the entire 100-kDa UN complex in which Npl4 is isotopically $^{15}$N labeled. Only peaks corresponding to the UBD and NZF domains have favorable relaxation...
properties and are therefore visible in HSQC spectra, indicating that these domains, at the N and C termini of Npl4, exhibit some degree of independence. The chemical shifts for amides from these domains are unchanged when compared with the individual fragments, suggesting that their structures are identical.

The predicted UBD of Npl4, comprising residues 1–96 of the full-length protein, was produced recombinantly with isotopic labeling in Escherichia coli. Although the battery of NMR spectra obtained from this protein typified a compact folded domain, a significant number of backbone amide resonances were broad or missing from the data. Varying pH, buffer, and temperature conditions failed to recover the absent peaks. Following backbone assignment (Fig. 1A), it became clear that these data corresponded to a contiguous stretch of the protein sequence (residues 60–68), implying a potentially interesting region exhibiting intermediate conformational exchange. Furthermore, residues 80–96 were completely unstructured based on their amide relaxation properties (data not shown), comparison with random coil chemical shifts, and the absence of inter-residue NOEs in NOESY spectra. Although NOE data were not available for these regions, it was possible to calculate a solution structure for the majority of the domain (Fig. 2 and Table 1). The secondary structure occupies a core βββββ topology and manual assignment of key NOEs revealed a β-grasp-like fold with similarities to ubiquitin. An initial β-grasp fold model for Npl4 UBD was used as a starting structure in an automatic NOE assignment using the ARIA protocol (23). All areas of identified secondary structure are well defined (Fig. 2A); the average pairwise root mean square deviation (r.m.s.d.) for the water-refined final family of structures is 0.88 Å for the backbone atoms of secondary structure elements. The experimentally determined secondary structure of Npl4 UBD consists of two helices (α1 between Ala-26–Phe-37 and a 3_10 (α2) between Arg-50–Glu-55) and four β-strands (β1, Ile-5–Gln-10; β2, Val-15–Ala-20; β3, Tyr-47–Ile-48; β4, Leu-74–Phe-76) assembled in a β-grasp-like fold (Fig. 2B). The β-strands form a mixed sheet in a 2-1-4-3 arrangement where 2-1 and 4-3 adopt anti-parallel pairing and 1 and 4 are parallel to each other. Fig. 2, C and D, show superimposition of Npl4 UBD with structures identified as having the most similar topology: ubiquitin (11% identity and r.m.s.d. 2.7 Å over 56 residues) and the β-grasp protein from Arabidopsis thaliana (PDB accession code: 1WF9; 20% identity, and r.m.s.d. 2.7 Å over 61 residues), respectively. These structural homologues provide hints as to the structure of the missing region from Npl4 UBD. In all UBD structures solved to date, this region corresponds to a long loop region containing a short α-helix. Fig. 2E shows a structure-based sequence alignment of Npl4 UBD, ubiquitin, p47 UBX, and the β-grasp protein from Arabidopsis (1WF9) with conserved residues highlighted.

| TABLE 1 Structural statistics for Npl4 UBD domain solution structure calculation |
|-------------------------------------------------|----------------|
| Number of experimental restraints | Npl4 UBD (PDB: 2PJH) |
| Total NOE-derived | 716 |
| Ambiguous | 630 |
| Unambiguous | 119 |
| Tails (dφ) | 511 |
| r.m.s.d. from experimental restraints | 86 |
| Distance (Å) | 0.05 ± 0.005 |
| Dihedral angle (deg.) | 0.9 ± 0.1 |
| r.m.s.d. from idealized covalent geometry | 0.0041 ± 0.0002 |
| Bonds (Å) | 0.56 ± 0.02 |
| Angles (deg.) | 73 |
| Coordinate r.m.s.d. (Å) | 22 |
| Backbone atoms in secondary structure | |
| Heavy atoms in secondary structure | 1.52 ± 0.15 |
| Ramachandran plot | |
| Residues in most favored regions (%) | 0.15 |
| Residues in allowed regions (%) | 0.16 |
| Residues in disallowed regions (%) | 0.02 |
| Residues in disallowed regions (%) | 0.005 |
| Distance (Å) | 0.02 |
| Ramachandran plot | 0.005 |

Reciprocal chemical shift perturbation studies were performed to map the interaction surface between Npl4 UBD domain and the N domain of p97. Fig. 3A shows a comparison of the 1H-15N HSQC spectra of uniformly 15N-labeled Npl4 UBD in the presence and absence of p97 N. Twenty-two significantly perturbed residues were observed, whereas most of the spectrum remained unchanged, indicating the formation of a specific complex between the two proteins. Fig. 3A highlights some of the more prominent shifts and includes multiple increments from the titration, demonstrating that binding is within the fast-exchange limits of the NMR time scale. Changes in chemical shift measured with increasing peptide concentration were fitted to a standard single binding site saturation isotherm yielding an estimated $K_d$ in the range of 10–100 μM. Mapping the perturbed residues onto the structure of Npl4 UBD delineated the binding surface, which spans the exposed face of the β-sheet and neighboring loop regions with key shifted residues congregating on β1 and β3 (Fig. 3B).

The structure of the p97 N domain is known from X-ray crystallographic studies on the larger p97 ND1 construct (2). Using a standard triple resonance NMR approach, we assigned the backbone amide resonances of the 24-kDa fragment and identified which residues participate in binding to the UBD domain of Npl4. 1H-15N HSQC spectra of uniformly 15N-labeled p97 were recorded in the presence and absence of Npl4 UBD (Fig. 4C). In this case, 35 out of a possible 213 residues were significantly perturbed, and the binding surface maps to the negatively charged groove between the two subdomains of p97 N (Nn and Nc) (Fig. 4B). Prominent sites of interaction occur in the loops between β1 and β3, β7 and β8, β12 and β13 and the long loop between β6 and β7 that connects the Nn and Nc subdomains of p97 N.

The clear delineation of a binding interface using chemical shift mapping has enabled us to adopt an NMR restraint-guided docking approach (HADDOCK) (25) to generate a structural model for the complex. The stoichiometry of the UN-full-length p97 has been previously established by electron microscopy, mass spectrometry, and gel filtration in which one UN heterodimer binds one p97 hexamer (18), thus inferring a 1:1 stoichiometry for the p97 N-UBD interaction. Furthermore, chemical shift movements fit well to a single binding site isotherm. Standard ambiguous interaction restraints between Npl4 UBD and p97 N, based on residues experiencing greater than average NMR chemical shift perturbations, were used to drive the calculation (see “Experimental Procedures”). The 200 final refined structures for the p97 N-Npl4 UBD com-
plex were clustered according to a pairwise r.m.s.d. cut-off of 2.5 Å, producing two major clusters of 14 and 18 structures. The average intermolecular energy for the best cluster was $-555 \pm 55$ kcal mol$^{-1}$. Fig. 5A shows an overlay of the 10 lowest interactional energy, water-refined structures from the low energy cluster. As suggested by the chemical shift mapping data, the model shows the Npl4 UBD binding within the groove formed between the two subdomains of p97 N, in an analogous fashion to p47 UBX domain binding (Fig. 5, B and C). This interface differs from p47 in that the S3-S4 region in Npl4 UBD is elaborated with a $\beta$-sheet (Fig. 5D) that provides specific contacts to p97 N. Also highlighted in Fig. 5D are 3 critical residues in Npl4 UBD that project into the hydrophobic binding pocket with p97 N: Val-15, Leu-74, and Phe-76 (which contact Lys-109, Gly-54, and Tyr-143 in p97 N, respectively). In alignment with p47, these amino acids equate to Arg-307, Val-366, and Arg-368, region spanning residues 215–241 (15). This sequence displays some limited identity with a stretch of amino acids in p47, known to bind p97 N, which occurs in the linker region between the p47 UB and SEP domains. To localize further the interacting region within Ufd1, we synthesized a specifically labeled peptide in which a selection of $^{15}$N/$^{13}$C-labeled amino acids were incorporated throughout the sequence (i.e. labels in bold and underlined DHSGYGAGEVGRFAFGSGS-GNRKDGKKG). $^1$H/$^{15}$N and $^1$H/$^{13}$C HSQC spectra of the peptide were recorded in the presence of increasing amounts of p97 N. Gly-224, Ala-227, and Gly-230 resonances were severely broadened upon interaction with p97 N (Fig. 6A), whereas the remaining resonances were unaffected. The residues displaying broadened NMR signals delineate the p97 N-binding site, and line broadening is likely caused by the peptide exhibiting conformational exchange on the ms-μs time scale within the complex. Building on our previous

all of which make the corresponding p97 contacts and are seen to shift in the earlier NMR study.

To assess whether our structural model for Npl4 UBD-p97 N represents the likely mode of interaction within the UN heterodimer-p97 hexamer complex, we performed a TROSY- and CRINEPT-based NMR titration of 300-kDa hexameric p97 N into full-length 100-kDa UN, in which Npl4 is uniformly $^2$H/$^{15}$N-labeled and Ufd1 is unlabeled. Interestingly, the $^2$H/$^{15}$N HSQC spectrum of free UN revealed a 40–50-kDa structured core domain within Npl4, in addition to the terminal UBD and ZF domains, which folds upon interaction with Ufd1 (Fig. 3A, right). In CRINEPT and TROSY spectra of the 1:1 UN:p97 ND1 hexamer complex, a number of UBD resonances disappear, whereas the rest of the spectrum remains largely unchanged (Fig. 3A, right). These peaks correspond exactly to the UBD-p97 N interface identified in our chemical shift mapping experiments, and their absence is probably due to the faster relaxation properties exhibited when bound to the protonated 300-kDa p97 hexamer. The observation of peaks from the core domain and ZF in the TROSY spectrum of this 400-kDa complex suggests that they exhibit a degree of independence from the hexameric unit.

The portion of Ufd1 that binds to p97 N has been localized to a region spanning residues 215–241 (15). This sequence displays some limited identity with a stretch of amino acids in p47, known to bind p97 N, which occurs in the linker region between the p47 UB and SEP domains. To localize further the interacting region within Ufd1, we synthesized a specifically labeled peptide in which a selection of $^{15}$N/$^{13}$C-labeled amino acids were incorporated throughout the sequence (i.e. labels in bold and underlined DHSGYGAGEVGRFAFGSGS-GNRKDGKKG). $^1$H/$^{15}$N and $^1$H/$^{13}$C HSQC spectra of the peptide were recorded in the presence of increasing amounts of p97 N. Gly-224, Ala-227, and Gly-230 resonances were severely broadened upon interaction with p97 N (Fig. 6A), whereas the remaining resonances were unaffected. The residues displaying broadened NMR signals delineate the p97 N-binding site, and line broadening is likely caused by the peptide exhibiting conformational exchange on the ms-μs time scale within the complex. Building on our previous

FIGURE 3. Identification of the interaction surfaces on Npl4 UBD domain for p97 N. A, $^1$H/$^{15}$N HSQC spectra of the Npl4 UBD domain with increasing amounts of p97 N (left) and $^1$H/$^{15}$N TROSY spectra of $^2$H/$^{15}$N-labeled Npl4 UN in the absence and presence of hexameric p97 ND1 (right; * and † indicate resonances from the core domain and ZF, respectively). B, surface and ribbon representations of Npl4 UBD domain with residues colored white to red in 10 shaded increments of increasing normalized $^1$H/$^{15}$N chemical shift perturbations observed in the presence of p97 N. Residues greater than 80% of maximum chemical shift are colored darkest red. Between 0 and 80% is divided equally across the nine remaining colors. The maximum combined chemical shift is 1.79 ppm.
experiments, we also titrated an unlabeled peptide into $^{15}$N-labeled p97 N to observe its binding interface. Fig. 4C shows the HSQC spectra of uniformly $^{15}$N-labeled p97 N in the presence and absence of Ufd1 peptide. Surprisingly, we discovered that some of the 24 p97 N residues perturbed upon Ufd1 peptide addition fall within the binding interface.
between p97 N and Npl4 UBD (Fig. 4, A and B), implying that a degree of communication may exist in binding these two motifs. We also performed a competition experiment in which p97 N was saturated with Ufd1 peptide and then titrated with increasing amounts of Npl4 UBD. All chemical shift movements caused by the Ufd1 peptides are reversed after a saturating titer of Npl4 UBD has been added (Fig. 4C).

**DISCUSSION**

Together, Npl4 and Ufd1 recruit p97 to specific ubiquitin-related cellular processes. We provide the first structural characterization of this interaction at a molecular level. Npl4 and Ufd1 each contain a region that contacts p97 N, and these sites resemble functionally the two regions in p47, another p97 adaptor protein. The Npl4-binding site for p97 N occurs in the N-terminal UBD domain, which exhibits a novel β-grasp-like fold.

Using chemical shift perturbation studies, we delineated the region on Npl4 UBD that binds to p97 N. This surface is reminiscent of the Ile-44 face hydrophobic patch on ubiquitin that represents the binding site for many of its cohorts. Interestingly, the region of Npl4 UBD that is in severe intermediate conformational exchange (residues 60–68) lies outside the p97-binding surface identified by the HADDOCK calculation, implying that the missing data are not a consequence of the p97 N-binding site. Furthermore, resonances from this region remain conformationally broadened even in the presence of saturating amounts of p97 N. This area might represent a site of association with part of Ufd1 or another region from full-length Npl4 since the UBD represents approximately one-sixth of the whole protein. This is consistent with NMR observations suggesting that the UBD domain is not tumbling entirely independently from the main body of the UN complex.

We identified the reciprocal UBD-binding surface on p97 N domain, which is located in the cleft between the Nn and Nc subdomains. Strikingly, this Npl4 UBD-binding area encompasses the p97 N domain amino acids (Arg-93, Arg-95, and Arg-155) found missense-mutated in hereditary inclusion body myopathy with Paget disease of bone and frontotemporal dementia (IBMPFD) (27). Arg-93 is a shifted residue in our NMR titration experiments with UN and p97 N, and Arg-155 is directly adjacent to a shifted residue. Weihl et al. (27) have shown that p97 bearing the most common IBMPFD mutation, R155H, retains its usual hexameric structure and ATPase activity yet exhibits impaired ERAD function (27). Our findings suggest that this is probably a result of inhibited or modified binding to the UN complex.

Yuan et al. (28) mapped the p97 N-binding surface on p47 in a chemical shift perturbation study yielding data directly comparable with ours. Dreveny et al. (20) expanded this information by solving a crystal structure of the complex between p47 and p97 and illustrated the importance of the S3–S4 loop within p47 as a key point of interaction with the cleft of p97 N. They showed that the analogous loop from ubiquitin did not provide the necessary contacts for high affinity binding.

The information we gained from mapping both binding surfaces allowed us to generate a model for the complex of Npl4
UBD and p97 N. We also demonstrate, using NMR, that this mode of interaction exists in the full-length UN in complex with hexameric p97. Our model also reveals striking similarities to the crystal complex of p47 with p97 N, with the key p97 N-interacting residues in Npl4 UBD aligning structurally with the corresponding residues in p47. Although Npl4 UBD resembles ubiquitin rather than p47 in the length of its S3-S4 loop and overall structure, mutagenesis data confirm the importance of this region (15). Fig. 5 illustrates the key locations of these mutated residues within the protein-protein interface of the UBD-p97 N complex. Our model reveals that although these residues play a role in the interface, they lie proximal to the main region of interaction. The S3-S4 region in Npl4 is decorated with a 3_10 helix distinguishing it from both p47 and ubiquitin and suggesting a mechanism for specificity between the two p97 adaptors.

Despite dramatic variations in sequence, many of the known p97 adaptor proteins, including p37, p47, Ubx2, VCIP135, Faf1, and Saxs1, bind to the N domain via ubiquitin-like motifs (29–32). There are likely to be substantial parallels between the binding modes of these domains to p97 N. Comparing the published p47 data with the UN data presented here, we can speculate that these ubiquitin-like domains recognize the p97 N cleft using similar, yet subtly different, interaction modes. Electron microscopy and stoichiometric studies by Pye et al. (18) assign these to macroscopic differences in organization since, despite our discovery of analogous residue interactions at the binding interface, a p47 trimer appears to straddle the p97 hexamer, whereas a single UN complex projects from one side.

Upon examining the interaction between Ufd1 and p97 N, our NMR data suggest that the p97 N-binding region in Ufd1 can be localized to a short region encompassing Gly-224, Ala-227, and Gly-230. The phenylalanine, alanine, and glycine residues within this stretch are highly conserved (Fig. 6B) and expected to make key interactions with p97 N. Surprisingly, chemical shift perturbations induced in p97 N spectra in the presence of Ufd1 are reversed upon the addition the UBD. Although it may be difficult to extrapolate data on a small peptide motif to the native system, it suggests a degree of communication between the Npl4- and Ufd1-binding sites on p97. Although low resolution electron microscopy data indicate that the UN complex associates with one N domain of p97, as the authors suggest, this may represent a snapshot in a process of mechanical rearrangements associated with UN binding to the p97 hexamer during activity (18). Bearing in mind the inherent flexibility of the UN complex and potential interplay between the Ufd1- and Npl4-binding sites, it is also plausible that the UN complex spans two adjacent N domains.

Bruderer et al. (15) drew biochemical parallels between p47 binding to p97 N via two interaction points (in its UBX domain and in the linker to SEP) and UN binding through the structurally analogous regions in Npl4 UBD and Ufd1 peptide. Evidence implies that the p47 bipartite binding site can span two N domains, as p47 binds p97 as a trimer presenting two adjacent
N domains per p47 molecule (19, 20). The bipartite motif is also conserved in p37, a novel adaptor of p97 that is required for Golgi and ER biogenesis (33). The subtle differences presented in the UBD-p97 N interaction and the stark difference in their connectivities to the second binding site, i.e. covalently in the case of p47/p37 and non-covalently in UN, provide scope for varied modes of p97-mediated adaptor binding and activity.

It is conceivable that conformational changes in p97 N, induced by nucleotide binding/hydrolysis or UN binding, could facilitate a mechanism for the bipartite binding motif to alter their arrangement within the p97 hexamer and propagate signals to the substrate. Furthermore, alterations in N domain accessibility may prime them to receive new co-adaptors, for example, Ufd2 or Ufd3.

REFERENCES

1. Dreveny, I., Pye, V. E., Beuron, F., Briggs, L. C., Isaason, R. L., Matthews, S. J., McKeown, C., Yuan, X., Zhang, X., and Freemont, P. S. (2004) Biochem. Soc. Trans. 32, 715–720
2. Zhang, X. D., Shaw, A., Bates, P. A., Newman, R. H., Gowen, B., Orlova, E., Gorman, M. A., Kondo, H., Dokurmo, P., Lally, J., Leonard, G., Meyer, H. E., van Heel, M., and Freemont, P. S. (2000) Mol. Cell 6, 1473–1484
3. DeLaBarre, B., and Brunger, A. T. (2005) J. Mol. Biol. 347, 437–452
4. Davies, J. M., Tsuruta, H., May, A. P., and Weis, W. I. (2005) Structure (Lond.) 13, 183–195
5. McNeill, H., Knebel, A., Arthur, J. S. C., Cuenda, A., and Cohen, P. (2004) J. Cell Biol. 165, 855–866
6. Yuan, X. M., Shaw, A., Zhang, X. D., and Freemont, P. S. (2001) Cell 105, 255–263
7. Wang, B., Alam, S. L., Meyer, H. H., Payne, M., Stemmiller, T. L., Davis, D. R., and Sundquist, W. I. (2003) J. Biol. Chem. 278, 20225–20234
8. Uchiyama, K., Jokitalo, E., Kano, F., Murata, M., Zhang, X. D., and Freemont, P. S. (2007) Proc. Natl. Acad. Sci. U. S. A. 104, 467–472
9. Yuan, X. M., Shaw, A., Zhang, X. D., and Freemont, P. S. (2001) Nat. Struct. Biol. 8, 355–367
10. Pye, V. E., Beuron, F., Keetch, C. A., McKeown, C., Robinson, C. V., Meyer, H. H., Zhang, X. D., and Freemont, P. S. (2006) EMBO J. 25, 1967–1976
11. Kondo, H., Rabouille, C., Newman, R., Levine, T. P., Pappin, D., Freemont, P., and Warren, G. (1997) Nature 388, 75–78
12. McNeill, H., Knebel, A., Arthur, J. S. C., Cuenda, A., and Cohen, P. (2004) J. Biol. Chem. 279, 104, 437–452
13. Huyton, T., Pye, V. E., Briggs, L. C., Flynn, T. C., Beuron, F., Kondo, H., Zhang, X. D., and Freemont, P. S. (2003) Mol. Cell 12, 3226–3241
14. Johnson, E. S., Ma, P. C. M., Ota, I. M., and Varshavsky, A. (1995) J. Biol. Chem. 270, 17442–17456
15. Johnson, E. S., Ma, P. C. M., Ota, I. M., and Varshavsky, A. (1995) J. Biol. Chem. 270, 17442–17456
16. Weihl, C. C., Dalal, S., Pestrkonk, A., and Hanson, P. I. (2006) Hum. Mol. Genet. 15, 189–199
17. Beuron, F., Dreveny, I., Briggs, L. C., Sands, C., Beuron, F., Zhang, X. D., and Freemont, P. S. (2000) J. Struct. Biol. 131, 255–263
18. Hartmann-Petersen, R., Wallace, M., Hofmann, K., Koch, G., Johnsen, A. H., Hendil, K. B., and Gordon, C. (2004) Curr. Biol. 14, 824–828
19. Song, E. J., Yim, S. H., Kim, E., Kim, N. S., and Lee, K. J. (2005) Mol. Cell. Biol. 25, 2511–2524
20. Song, E. J., Yim, S. H., Kim, E., Kim, N. S., and Lee, K. J. (2005) Mol. Cell. Biol. 25, 2511–2524
21. Sattler, M., Schluecher, J., and Griesinger, C. (1999) Prog. NMR Spectrosc. 34, 93–158
22. Dominguez, C., Boelens, R., and Bonvin, A. (2003) J. Am. Chem. Soc. 125, 1731–1737
23. Vijaykumar, S., Bugg, C. E., Wilkinson, K. D., and Cook, W. J. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 3582–3585
24. Linge, J. P., Habeck, M., Rieping, W., and Nilges, M. (2003) Bioinformatics 19, 315–316
25. Sattler, M., Schleucher, J., and Griesinger, C. (1999) J. Mol. Biol. 289–302
26. Vijaykumar, S., Bugg, C. E., Wilkinson, K. D., and Cook, W. J. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 3582–3585
27. Kondo, H., Rabouille, C., Newman, R., Levine, T. P., Pappin, D., Freemont, P., and Warren, G. (1997) Nature 388, 75–78
28. Beuron, F., Dreveny, I., Yuan, X. M., Pye, V. E., McKeown, C., Briggs, L. C., Cliff, M. J., Kaneko, Y., Wallis, R., Isaacson, R. L., Ladbury, J. E., Matthews, S. J., Kondo, H., Zhang, X. D., and Freemont, P. S. (2006) EMBO J. 25, 1967–1976
29. Hetzer, M., Meyer, H. H., Walther, T. C., Bilbao-Cortes, D., Warren, G., and Mattaj, I. W. (2001) Nat. Cell Biol. 3, 1086–1091