Solution Structure of the Ubiquitin-associated (UBA) Domain of Human Autophagy Receptor NBR1 and Its Interaction with Ubiquitin and Polyubiquitin

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Background: The autophagic receptor NBR1 is commonly found in ubiquitin-positive inclusions in neurodegenerative diseases.

Results: Molecular recognition of ubiquitin and polyubiquitin by NBR1 is described.

Conclusion: The ubiquitin-associated domain of NBR1 shows unexpectedly high affinity for monoubiquitin but lacks polyubiquitin linkage specificity.

Significance: NBR1 may be highly efficient at forming intracellular inclusions with ubiquitylated proteins via non-linkage-specific association with ubiquitin.

NBR1 (neighbor of BRCAl gene 1) is a protein commonly found in ubiquitin-positive inclusions in neurodegenerative diseases. Due to its high architectural similarity to the well studied autophagy receptor protein p62/SQSTM1, NBR1 has been thought to analogously bind to ubiquitin-marked autophagic substrates via its C-terminal ubiquitin-associated (UBA) domain and deliver them to autophagosomes for degradation. Unexpectedly, we find that NBR1 differs from p62 in its UBA structure and accordingly in its interaction with ubiquitin. Structural differences are observed on helix α-3, which is tilted farther from helix α-2 and extended by approximately one turn in NBR1. This results not only in inhibition of a p62-type self-dimerization of NBR1 UBA but also in a significantly higher affinity for monoubiquitin as compared with p62 UBA. Importantly, the NBR1 UBA-ubiquitin complex structure shows that the negative charge of the side chain in front of the conserved MGF motif in the UBA plays an integral role in the recognition of ubiquitin. In addition, NMR and isothermal titration calorimetry experiments show that NBR1 UBA binds to each monomeric unit of polyubiquitin with similar affinity and by the same surface used for binding to monoubiquitin. This indicates that NBR1 lacks polyubiquitin linkage-type specificity, in good agreement with the nonspecific linkages observed in intracellular ubiquitin-positive inclusions. Consequently, our results demonstrate that the structural differences between NBR1 UBA and p62 UBA result in a much higher affinity of NBR1 for ubiquitin, which in turn suggests that NBR1 may form intracellular inclusions with ubiquitylated autophagic substrates more efficiently than p62.

Insufficient clearance of intracellular protein aggregates has been implicated in severe human neurodegenerative disorders such as Alzheimer, Huntington, and Parkinson disease (1). Protein degradation in eukaryotic cells is mediated by two major pathways: the ubiquitin-proteasome pathway and macroautophagy (hereafter referred to as “autophagy”). The ubiquitin-proteasome pathway is limited to the degradation of proteins that, when unfolded, fit into the small (13 Å) opening of the proteasome barrel (2, 3). In stark contrast, autophagy can induce degradation of not only compact cytosolic proteins and their complexes but also large protein aggregates and even entire organelles. This evolutionarily conserved pathway involves the formation of double-membrane vesicles termed autophagosomes inside the cytosol, which thereby engulf cytosolic components. By subsequent fusion with lysosomes, the constituents of such autophagosomes are then degraded by lysosomal hydrolases under acidic conditions (4).

In addition to supplying nutrients to the cell under conditions of starvation (starvation-induced autophagy), another type of autophagy functions in a nutrient-stress-independent manner, playing an important cytoprotective role by constantly sequestering damaged organelles and protein aggregates that are potentially toxic (constitutive autophagy) (4). The vital importance of this continuous removal of cytosolic protein aggregates was recently highlighted by studies in mice, where loss of neuronal autophagy led to an accumulation of ubiquitylated protein aggregates coupled with neuronal cell death and symptoms of neurodegeneration despite normal proteasome function in those cells (5, 6).

In contrast to the ubiquitin-proteasome pathway, which specifically targets proteins marked by Lys48-linked polyubiquitin for proteasomal degradation, initial studies on autophagy concluded that autophagy chooses its substrates rather arbitrarily. However, recent evidence, in particular the discovery and characterization of autophagy receptor proteins, indicates the existence of a selective autophagy pathway in which, intriguingly,
ubiquitin is again the signal that selectively marks target substrates for autophagic degradation (4, 7).

Three mammalian autophagy receptors containing ubiquitin-binding motifs have been proposed: p62, NBR1, and NDP52. Interestingly, two of these three proteins, p62 and NBR1, share a very similar overall domain architecture consisting of an N-terminal PB1 domain, a ZZ-like zinc finger domain, a light-chain-3 interacting region and a C-terminal ubiquitin-associated (UBA) domain (Fig. 1A). This organization, reminiscent of receptor proteins in the ubiquitin-proteasome pathway such as HR23A and DSK2, has evoked the hypothesis that p62 and NBR1 may recognize autophagic substrates through their C-terminal ubiquitin binding motif (4) and deliver these substrates to autophagosomes by binding to the autophagosomal membrane protein LC-3 (microtubule-associated protein 1 light chain 3) via their light-chain-3 interacting region motif.

Because p62 accumulates in ubiquitin-positive inclusions in neurodegenerative diseases, it has been intensively studied in vivo and is now widely used as a histological marker for intracellular inclusion bodies. Moreover, both its UBA domain and its interaction with ubiquitin have been characterized by several NMR and crystallographic studies (8–10). In contrast, NBR1 was only very recently confirmed as an autophagy receptor (11). The aim of this study, therefore, was to characterize the UBA domain of NBR1 to understand why mammals require both of these two seemingly similar autophagy receptor proteins.

**EXPERIMENTAL PROCEDURES**

**Sample Preparation**—The UBA domain of human NBR1 (residues 913–959) was cloned into a pGEX-6P-1 expression vector (GE Healthcare) using the restriction sites for BamHI and XhoI. Plasmids containing point mutations of the NBR1 UBA domain were constructed by PCR. Proteins were expressed as GST fusion proteins in *Escherichia coli* in LB or M9 minimal media containing [15N]ammonium chloride and [13C]glucose and purified by glutathione-Sepharose 4FF (GE Healthcare) column chromatography. After cleavage of the GST affinity tag by the PreScission protease (GE Healthcare), proteins were further purified by size-exclusion chromatography on a HiLoad 16/60 Superdex 75pg column (GE Healthcare). Untagged ubiquitin and its point mutants were expressed and purified as described previously (12). Lys48- and Lys63-linked diubiquitin was prepared by enzymatic reaction using the enzymes UBA1, E2–25K, and Ubc13–Mms2 as described previously (12) and further purified by ion exchange on a Source-S column (GE Healthcare). The Kd value was estimated by Monte Carlo methods using 100 synthetic data (21). We further considered the uncertainty in the protein concentrations, which were determined using UV absorption and calculated extinction coefficients. A possible uncertainty in the protein concentration of the analyte protein of ±10% was incorporated into the error analysis. The Kd value for self-association of the NBR1-UBA domain was estimated by gradually diluting a concentrated sample from 1.1 mM to 8 μM.

**NMR Spectroscopy and Structure Determination of the NBR1 UBA Domain**—A [13C,15N]-labeled sample of NBR1 UBA was prepared in 20 mM potassium phosphate, pH 6.6, 5 mM potassium chloride, 1 mM EDTA, 1 mM benzamidine, 1 mM DTT, 0.02% sodium azide, and 4% D2O at a protein concentration of 1.25 mM in 5 mM Shigemi tubes. NMR spectra were acquired on a Bruker Avance 600 MHz NMR spectrometer at 298 K. Assignments for main and side-chain resonances were obtained from a series of the triple resonance experiments: HNCACB, CBCA(CO)NH, HBHA(CO)NH, H(CCO)NH, CC(CO)NH, and HCHC-TOSQSY. 4,4-Dimethyl-4-silapentane-1-sulfonic acid was used as the chemical shift reference compound. Data were processed using NMRPipe (13) and analyzed in MAGRO and CCPN (14–16). Backbone resonances were assigned by a combination of manual assignment and automatic assignment using MARS (17), whereas side-chain resonances were assigned manually. Proton-proton distances were derived from three-dimensional [13C] and [15N]-edited NOESY-HSQC spectra. NOESY cross-peaks were automatically assigned in the course of the structure calculation by the iterative CANDID algorithm implemented in CYANA (18). Backbone torsion angles were predicted by TALOS+ using chemical shift values for Hα, Cα, Cβ, and N (19). The final structure calculation was performed by CYANA 2.1 using NOE and dihedral restraints yielding a structural ensemble with the statistics given in Table 1.

**NMR Titration Experiments**—[1H,15N] HSQC spectra of 40 μM NBR1 UBA were acquired by gradually increasing the concentration of the ligand ubiquitin or diubiquitin. In the case of diubiquitin, the concentration of the ligand was defined as the concentration of monomeric ubiquitin units in the dimer. The normalized chemical shift perturbation was calculated as

\[
\Delta_{\text{obs}} = \frac{\Delta_{\text{fb}}}{2|A_0|} \left[ K_d [A_0] + [B_0] \right]
\]

in which \([A_0]\) and \([B_0]\) are the concentrations of analyte protein and titrant protein, respectively. \(\Delta_{\text{obs}}\) denotes the observed chemical change, and \(\Delta_0\) is the difference in chemical shift of the free and complexed proteins. The uncertainty in the \(K_d\) values was estimated by Monte Carlo methods using 100 synthetic data (21). We further considered the uncertainty in the protein concentrations, which were determined using UV absorption and calculated extinction coefficients. A possible uncertainty in the protein concentration of the analyte protein of ±10% was incorporated into the error analysis. The \(K_d\) value for self-association of the NBR1-UBA domain was estimated by gradually diluting a concentrated sample from 1.1 mM to 8 μM.
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A

p62  
PB1  ZZ  UBA  440

NBR1  
PB1  ZZ  NBR1  UBA  966

NDP52  
Coil  UBZ  446

B

C

D

Q-factor: 0.16

E

16°

p62-UBA

NBR1-UBA
and following the resulting chemical shift changes. The concentration-dependent chemical shifts were fitted to a self-dimerization model using GLOVE,

$$\Delta \text{obs} = \left( \delta_{\text{dimer}} - \delta_{\text{monomer}} \right) \left[ K_0 + 2[\text{UBA}] \right] - \sqrt{\left( K_0 + 2[\text{UBA}] \right) \left( 2 - 4[\text{UBA}] \right)}$$

(Eq. 2)

Residual Dipolar Couplings—$^1$H, $^{15}$N residual dipolar coupling (RDC) constants were measured from in-phase anti-phase (IPAP) $^1$H, $^{15}$N HSQC experiments using 12.5 mg/ml Pf1 Phage (ASLA Biotech) as an alignment medium and independently analyzed in REDCAT and PALES (22–24). RDCs from α-helical residues were used to calculate the Q-factor of the CYANA-derived structure. Residual dipolar couplings for the UBA domain and ubiquitin in the complexed form were obtained from in-phase anti-phase $^1$H, $^{15}$N HSQC experiments using 0.5 mM $^{15}$N-labeled protein and 2 mM non-labeled ligand protein in the presence of 12.5 mg/ml Pf1 phage. 150 mM sodium chloride was added to alleviate strong electrostatic interactions between proteins and the negatively charged phage.

NBR1 UBA-UBiquitin Complex—Intermolecular NOEs were obtained from a filtered NOESEY experiment as previously (25) on a Bruker Avance 800-MHz NMR spectrometer using a sample consisting of 4.5 mM non-labeled ubiquitin and 12.5 mg/ml Pf1 phage. 150 mM sodium chloride and following the resulting chemical shift changes. The concentration-dependent chemical shifts were fitted to a self-dimerization model using GLOVE,

| Distance range                      | Number of NOEs (obs) | Number of NOEs (calc) | Statistics for structure Ensemble calculation |
|-------------------------------------|----------------------|-----------------------|-----------------------------------------------|
| Sequential                          | 1055                 | 1055                  | Residues in most favored regions: 80%        |
| Medium range                        | 542                  | 542                   | Residues in additional allowed regions: 15%  |
| Long range                          | 323                  | 323                   | Residues in generously allowed regions: 0%   |
|                                    | 190                  | 190                   | Residues in disallowed regions: 0%           |

The well converged residues fold into a bundle of three α-helices connected by two loops each comprising 4 and 3 residues (Fig. 1C). This three α-helical fold is the canonical fold of UBA domains (9). Accordingly, the structure of NBR1 UBA can be well superimposed on the crystal structure of the UBA domain of the autophagy receptor p62 (9) with a C$_{α}$-backbone r.m.s.d. of 0.27 ± 0.16 Å for residues 913–958 (Fig. 1B). This solution structure was validated by comparing the experimentally measured RDCs with RDC constants predicted from the averaged CYANA ensemble, yielding a Q-factor of 0.16 (Fig. 1D).

**RESULTS**

Solution Structure of the NBR1 UBA Domain—We prepared an $^{13}$C, $^{15}$N isotope-labeled sample comprising residues 913–959 of human NBR1 and conducted a series of triple resonance NMR experiments to obtain assignments for the backbone and side chain nuclei of the UBA domain. After obtaining proton-proton distance information from $^{13}$C- and $^{15}$N-edited NOESEY spectra, the solution structure of the NBR1 UBA domain was calculated from NOE and torsion angle constraints with statistics given in Table 1. The final 20 minimum energy structures converged well with a backbone r.m.s.d. of 0.27 ± 0.16 Å for residues 914–958 (Fig. 1B). This solution structure was validated by comparing the experimentally measured RDCs with RDC constants predicted from the averaged CYANA ensemble, yielding a Q-factor of 0.16 (Fig. 1D).

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Dimerization of NBR1 UBA—To examine whether the NBR1 UBA domain forms a homodimer, we investigated cross-peaks in the $^1$H, $^{15}$N HSQC spectra of NBR1 UBA at different concentrations of monomeric ubiquitin units in the dimer. The resulting data were processed using the software provided by the manufacturer (Origin 7, MicroCal Software, Inc.). Errors in the derived data represent the S.D. of three independent experiments.

**TABLE 1**

| Structure calculation of the NBR1 UBA domain |
|---------------------------------------------|
| Distance range: Sequential [$i-i'$] = 1    | 1055 |
| Medium range: [$i-i'$] ≤ 4                 | 542  |
| Long range: [$i-i'$] > 4                   | 323  |
| Statistics for structure Ensemble calculation |
| Backbone atoms r.m.s.d.                     | 0.27 ± 0.16 Å |
| All heavy atoms r.m.s.d.                    | 0.81 ± 0.17 Å |
| Number of dihedral angle violations >3°     | None |
| Number of distance violations >0.2 Å        | None |

**FIGURE 1. Solution structure of human NBR1 UBA.** A, domain architecture of mammalian autophagy receptors p62, NBR1, and NDP52 (adapted from Ref. 7). p62 and NBR1 display a similar domain architecture. The green rectangle indicates the light-chain-3 interacting region. B, ensemble of the 20 minimum energy structures of human NBR1 UBA. C, schematic representation of the averaged structure calculated from the minimum energy ensemble. D, correlation plot of RDCs calculated from the averaged solution structure and experimentally determined RDC constants. The red line indicates perfect correlation. E, overlay of the UBA structure of NBR1 (blue) and that of p62 (magenta, PDB ID 3BOF). In NBR1 UBA, helix α-3 is tilted further away from helix α-2 and is extended by about 1 turn (4 residues) as compared with p62 UBA. A flexible five-residue plasmid-derived N-terminal sequence has been omitted from all figures for clarity.
Surprisingly, amide cross-peaks exhibited concentration-dependent changes (Fig. 2A), although the total chemical shift changes were relatively small (0.12 ppm; Fig. 2B). Several residues on helix α-1 and α-3 showed above-average chemical shift perturbation implying that NBR1 UBA might form dimers. Analysis by analytical ultracentrifugation supported the idea that NBR1 UBA indeed exists in a monomer-dimer equilibrium in solution. Analysis from both NMR and analytical ultracentrifugation experiments indicated that the dimerization of NBR1 is a relatively weak interaction with a $K_d$ of 400 M (Fig. 2C) as compared with the dimer dissociation of p62 UBA ($K_d = 3$ M) (9). Notably, the surface used for dimerization differs between NBR1 UBA and p62 UBA (Fig. 2D).

Because both p62 and NBR1 are found in autophagosomes (28) and bind to each other via their N-terminal PB1 domain, the UBA domains of p62 and NBR1 may be frequently in spatial proximity. Given that both UBA domains are able to form dimers on their own, it seemed possible that an association between p62 UBA and NBR1 UBA might also occur. However, investigation by NMR spectroscopy precluded this possibility (Fig. 2E). Taken together, these results show that, although NBR1 UBA is able to dimerize, it does so by a mechanism that differs from that of p62 UBA and with much lower affinity.

Identification of the UBA-Ubiquitin Interface—To examine the interaction of NBR1 UBA with ubiquitin, we performed NMR titration experiments using $^{15}$N-labeled NBR1 UBA and non-labeled ubiquitin. On the addition of ubiquitin, most HSQC cross-peaks exhibited gradual shifts with only minor broadening (data not shown). Some peaks such as Gly928 and Leu954, however, showed significant broadening beyond recognition during the course of the titration, before reappearing at much higher concentrations of ubiquitin. This indicates that NBR1 UBA binds to ubiquitin in a fast to intermediate exchange regime on the NMR timescale.

Two regions of NBR1 UBA displayed significant (above average) chemical shift changes. The first region was centered at loop 1 at the end of helix α-1 around Gly928; the second region comprised the residues of helix α-3 with the largest chemical shift change exhibited by Leu954 (Fig. 3A). This chemical shift perturbation is mapped on the solution structure of NBR1 UBA in Fig. 3B. The residues showing the largest shifts are part of the highly conserved MGF and di-leucine motifs (Fig. 3G). This indicates that NBR1 UBA uses a canonical surface to bind to ubiquitin (29).

Next, we performed the opposite NMR titration experiment using $^{13}$N-labeled ubiquitin and unlabeled NBR1 UBA to identify residues on ubiquitin that interact with NBR1 UBA. As shown in Fig. 3C, two clusters displayed major chemical shift changes: one centered at Ile44 and another located around Val70 on ubiquitin. This indicates that NBR1 UBA binds the notori-
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ous Ile44 hydrophobic patch, consisting of Leu8, Ile44, His68, and Val70 on ubiquitin (Fig. 3D) (30). Consequently, the interaction between NBR1 UBA and ubiquitin seems to involve canonical surfaces (9, 30–32).

Affinity of NBR1 UBA for Ubiquitin—To determine the dissociation constant for the binding of NBR1 UBA to ubiquitin, we fitted the chemical shift perturbation data to a classical binding model. This yielded a global $K_d$ of $3.0 \pm 0.9$ µM (see Fig. 6A), in line with the observed intermediate exchange regime, which roughly indicates dissociation constants in the range of 10–100 µM. This high affinity for ubiquitin is in stark contrast to the low affinity reported for the autophagy receptor p62 UBA-1, which roughly indicates dissociation constants in the range of about 540–750 µM (10, 33). Thus, NBR1 UBA binds ubiquitin with comparatively high affinity.

Next, we used ITC to assess the binding of NBR1 UBA to ubiquitin. We detected the binding as an exothermic reaction with a reaction enthalpy of $-4.9 \pm 0.1$ kcal mol$^{-1}$ and a dissociation constant of $4.3 \pm 0.4$ µM (Fig. 4A), in good agreement with the value obtained from NMR analysis, thereby confirming the high affinity of NBR1 UBA for ubiquitin.

Structure of the NBR1 UBA-Ubiquitin Complex—In general, both ubiquitin and UBA domains are known to undergo minimal structural rearrangements on complex formation (29, 31); therefore, we used the docking approach of HADDOCK to obtain a structural model of the NBR1 UBA-ubiquitin complex (26). A combination of ambiguous restraints from chemical shift perturbation data, intermolecular distance restraints (NOEs), and orientation restraints (RDCs) of both proteins in the complexed form were used to determine the complex structure shown in (Fig. 3E). The statistics of the docking process are given in Table 2.

The structure obtained shows that the main hydrophobic interface lies between the side chains of Leu8, Ile44, His68, Val70, on ubiquitin and the aliphatic side chains of Met927, Ile946, Leu947, Val950, Thr951, and Leu954 of NBR1 (Fig. 3F). The overall structure of the complex resembles the previously reported DSK2 UBA-ubiquitin complex (see Fig. 7A) in which the UBA helices adopt a similar geometry, and interfacial residues are involved in forming similar hydrophobic contacts.

In addition to these hydrophobic interactions, most of which occur through residues that are highly conserved among UBA domains (Fig. 3G), the structure shows the presence of polar contacts between the UBA domain and ubiquitin. The carboxyl group of Glu926 is positioned in close proximity to both the side chain ε-amino group of Lys6 and the imidazole ring of His68 on ubiquitin, permitting the formation of a salt bridge and a hydrogen bond, respectively (see Fig. 7B). Glu926 is not conserved in UBA domains; the UBA domain of DSK2, for instance, has an aspartate residue at the corresponding position, which has been shown to contribute to ubiquitin binding by similar polar contacts with both Lys6 and His68 of ubiquitin (see Fig. 7C) (31). By contrast, other UBA domains such as p62 or HR23A UBA-1, have a serine or alanine, respectively, at the equivalent site (Fig. 3G).

Validation of the UBA-Ubiquitin Surface by Site-directed Mutagenesis—To verify the calculated UBA-ubiquitin complex, we employed site-directed mutagenesis. Four single point mutants of NBR1 UBA (E926A, G928A, F929A, and L954A) and three of ubiquitin (I44A, K48A, and V70A) were generated, and binding between the wild-type and these mutant proteins was studied by ITC.

All interface mutants showed weaker binding, as compared with the respective wild-type protein. Replacement of ubiquitin Val70 by alanine resulted in an ~4-fold decrease in binding affinity (Fig. 4B), and mutation of Ile44 to alanine completely abolished binding (Fig. 4D). In the case of K48A, a small amount of heat appeared to be released; however, the binding was too weak to be detected by the ITC experiment (Fig. 4D).

In NBR1 UBA, alanine replacement of Phe929 on the first interhelix loop and alanine mutation of Leu954 on helix α-3 completely abolished binding (Fig. 4, C and D). Substitution of Glu926 for alanine reduces the ubiquitin affinity of NBR1 ~3-fold, thereby highlighting the contribution of the electrostatic contacts observed in ubiquitin binding (Fig. 4D). The G928A mutant still bound to ubiquitin, but the association was about 2-fold weaker than that of the wild-type protein (Fig. 4D). Collectively, these results confirm the UBA-ubiquitin interface determined by NMR data-driven protein-protein docking.

NBR1 UBA Does Not Discriminate between Mono- and Polyubiquitin—In living cells a large fraction of ubiquitin exists as polyubiquitin chains (34). In these chains adjacent ubiquitin units are linked via an isopeptide bond between one of seven amino groups or the N terminus of one ubiquitin molecule and the terminal carboxyl group of another ubiquitin molecule. The type of the chain linkage often serves as a specific signal, which is read by ubiquitin binding domain (UBD)-containing proteins and thereby used to regulate a large variety of cellular functions (30). In the ubiquitin-proteasome pathway, for example, the Lys48- and Lys63-linked polyubiquitin chain has been identified as a specific signal to target substrate proteins for proteasomal degradation. However, whether such a linkage-specific polyubiquitin signal is recognized in autophagy remains an open issue.

To examine the linkage specificity of NBR1 UBA, we used NMR titration experiments to investigate the interaction of the UBA domain with polyubiquitin chains of the highest intracellular abundance: that is, Lys48- and Lys63-linked.

FIGURE 3. Structure of the NBR1 UBA-ubiquitin complex. A, normalized chemical shift perturbation values of NBR1 UBA on binding to ubiquitin plotted as a function of the amino acid residue. The evolutionarily conserved MGF and LL motifs and α-helices are indicated. B, surface representation of the chemical shift changes mapped on the solution structure of NBR1 UBA, C, ubiquitin backbone amide chemical shift changes on complex formation with NBR1 UBA, D, surface representation of panel C mapped on the structure of ubiquitin drawn from PDB ID 1UBQ. The horizontal lines in A and C and surfaces in B and D are colored as follows: magenta, $\delta_{\nu} \leq$ chemical shift perturbation (CSP) $< \delta_{\nu} + 1$ or red, CSP $> \delta_{\nu} + 1$ or $E$. F, overlay of the four lowest energy structures of NBR1 UBA in complex with ubiquitin as calculated from orientation and ambiguous and unambiguous distance restraints. F, close-up view of the UBA-ubiquitin interface. Surface residues of ubiquitin in contact with the UBA are colored green, and residues of NBR1 UBA involved in hydrophobic contacts with ubiquitin are shown as blue sticks. G, sequence conservation in UBA domains. Identical residues are colored black, whereas homologous residues are colored gray. UBA domains at least partly contain an MGF motif and a di-leucine motif, which play a crucial role in ubiquitin recognition. The red asterisk indicates a non-conserved residue with amino acid distribution shown in Fig. 7E.
polyubiquitin chains (35). NMR titration experiments with both Lys48-linked and Lys63-linked diubiquitin resulted in chemical shift changes that are essentially identical to those induced by titration with monoubiquitin (Fig. 5, A and B). Accordingly, HSQC spectra of NBR1 UBA at the end of the titration experiments using monoubiquitin, Lys48-linked diubiquitin, and Lys63-linked diubiquitin displayed essentially identical chemical shifts. This suggests that NBR1 UBA uses the same surface to bind to both Lys48- and Lys63-linked diubiquitin that it uses to bind to monoubiquitin and that it lacks a specific epitope to selectively recognize the linkage of polyubiquitin chains.

**TABLE 2**

**Structure calculation of the UBA-ubiquitin complex by HADDOCK**

| Structure Type                  | Starting structure | Ambiguous restraints (CSP) | Orientational restraints (RDC) | Unambiguous restraints (NOE) | Clusters determined by HADDOCK | RMSD from lowest-energy structure | Van der Waals energy | Electrostatic energy | Desolvation energy | Restraint violation energy | Buried surface area |
|--------------------------------|--------------------|---------------------------|-------------------------------|-------------------------------|-------------------------------|--------------------------------|----------------------|----------------------|--------------------|--------------------------|-------------------|
| Ubiquitin                     | 1D3Z               | 8                         | 27                            | 18                            | 1                             | 0.8 ± 0.5                       | -51.6 ± 2.5          | -222.2 ± 43.7        | 11.1 ± 6.7         | 8.4 ± 12.88               | 1144.6 ± 64.5      |
| NBR1 UBA                      | 2MGW               | 11                        | 29                            | 27                            | 1                             | 0.8 ± 0.5                       | -47.9 ± 1.91         | -222.7 ± 43.8        | 11.2 ± 6.2         | 6.4 ± 12.88               | 1144.6 ± 64.5      |

**FIGURE 4.** Isothermal titration calorimetry analysis of binding between NBR1 UBA and ubiquitin and their mutants. A, ITC thermogram for NBR1 UBA binding to monoubiquitin (Ub). B, binding of NBR1 UBA to the interface ubiquitin mutant V70A. C, binding of ubiquitin to the UBA mutant F929A. In all thermograms the upper panels show raw data, and the lower panels show integrated heat values. D, thermodynamic parameters for binding of wild-type and mutant proteins. N, stoichiometry; $K_d$, dissociation constant; $\Delta H$, enthalpy change. Errors represent the S.D. from three independent experiments.
This lower affinity may be due to the reduced accessibility of the proximal ubiquitin unit, which uses Lys48 for isopeptide linkage (36) because NBR1 UBA recognizes the hydrophobic patch around Ile44. The dissociation constants derived from ITC were in good agreement with those derived from NMR spectroscopy (Fig. 5, C–E), and a similar amount of heat was released in the binding of NBR1 UBA to ubiquitin dimers and to monoubiquitin. Accordingly, stoichiometry values obtained from ITC experiments further confirmed that one NBR1 UBA molecule binds simply to one ubiquitin molecule of the respective diubiquitin molecule regardless of the polymeric state or linkage type (Fig. 5E). Taken together, these results indicate that the UBA domain of NBR1 recognizes ubiquitin chains in a linkage-nonspecific manner and binds to polyubiquitin chains and ubiquitin monomers with equal strength.
DISCUSSION

UBA Structure and Ubiquitin Binding—NBR1 UBA adopts a three-helix bundle structure similar to previously reported UBA domains. It shows a tendency to form homodimers in solution; the affinity ($K_d \sim 400 \mu M$) of this association may be too weak to be of physiological significance, although we note that in some cases even such weak interactions have been shown to be physiologically important (37).

The ubiquitin binding interface of NBR1 UBA was found to be largely determined by conserved hydrophobic interactions, although some important polar contacts were identified in the UBA-ubiquitin complex. Although all known UBA domains

FIGURE 6. Binding of NBR1 UBA to ubiquitin and diubiquitin as determined by NMR. $^1$H- and $^{15}$N-chemical shift changes are plotted (filled circles) against the molar ratio of ubiquitin to NBR1 UBA and separately fit (solid lines) to Equation 1 (see “Experimental Procedures”). A, binding of NBR1-UBA to monoubiquitin; $K_d = 3.0 \pm 0.9 \mu M$. B, binding of NBR1-UBA to Lys63-linked diubiquitin; $K_d = 4.5 \pm 1.2 \mu M$. C, binding of NBR1-UBA to Lys48-linked diubiquitin; $K_d = 19.0 \pm 2.0 \mu M$. In the case of diubiquitin, the data were fit assuming that one UBA domain binds to one ubiquitin unit of diubiquitin in a 1:1 stoichiometry. For comparison, data in the range of 0 – 8 mol eq are shown. Although for clarity only five example peaks are shown, the derived global $K_d$ values are the result of fitting the chemical shift difference data of all amide resonances. Yellow, Met927; red, Phe929; magenta, Gln948; blue, Glu952; green, Gln955.
display a three α-helical fold and at least partially contain the evolutionarily conserved MGF and LL motifs, their affinity for ubiquitin is highly variable.

In general, most UBA domains seem to bind to monoubiquitin with a $K_d$ of the approximate order of tens to a few hundreds of micromolar, although some UBA domains such as MARK3 UBA show extremely weak interactions with ubiquitin ($K_d > 2$ mM) (38). Therefore, NBR1 UBA achieves a comparatively strong interaction with ubiquitin ($K_d = 3–4$ μM). In particular, in the context of selective autophagy, it is puzzling that the in vitro affinity of NBR1 UBA for ubiquitin is more than 2 orders of magnitude higher than that of p62 UBA.

Possible Regulation of Ubiquitin Affinity by Phosphorylation of UBA Domains—Although homodimerization of p62 UBA inhibits ubiquitin binding and might lead to the lower $K_d$ of p62 UBA and ubiquitin, even engineered monomeric p62 UBA mutants bind to ubiquitin about an order of magnitude more weakly as compared with NBR1 UBA (9). Thus, the difference in ubiquitin affinity between NBR1 and p62 UBA domains might hint at another mechanism, such as post-translational modifications. It has recently been reported that p62 is phosphorylated at Ser403 on helix α-1 of its UBA domain, just in front of the conserved MGF motif, and that this modification significantly enhances polyubiquitin binding of p62 in vivo (39). Although no
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dissociation constant for Ser403-phosphorylated p62 UBA has been reported, a phosphorylation-mimicking S403E p62 mutant showed markedly increased binding to polyubiquitin in pulldown assays. This suggests that ubiquitin binding by p62 may be attenuated unless a double negative charge is introduced into the p62 UBA-ubiquitin interface by phosphorylation of Ser403.

Intriguingly, the residue in NBR1 UBA corresponding to p62 Ser403 is negatively charged. As our structure shows, this residue, Glu926, forms important polar contacts with the side chains of Lys46 and His68 on ubiquitin via its carboxylate group, thereby contributing to the affinity of NBR1 for ubiquitin (Fig. 7B). In the UBA domain of DSK2, the corresponding residue Asp342 forms similar interactions (Fig. 7C). The chemical shift perturbation data of p62 UBA indicate that p62 uses a surface similar to that of NBR1 and DSK2 to bind to ubiquitin (9); therefore, it is reasonable to assume that phosphorylation of p62 at Ser403 increases its affinity for ubiquitin by enabling the formation of analogous polar contacts with ubiquitin (Fig. 7, D and E).

Whereas DSK2 UBA contains an aspartate residue at the respective position and is also a relatively strong binder of ubiquitin ($K_d = 15 \mu M$ (31)), the weak binder HR23A UBA-1 ($K_d = 540 \mu M$, (33)) possesses an alanine residue in front of the MGF motif. Therefore, the charge on the side chain in front of the MGF motif in UBA domains may play a critical role in the interaction with ubiquitin; in the case of p62, this charge may be adjusted by phosphorylation and dephosphorylation.

Among UBA domains contained in the SMART database, there is a clear preference for a negatively charged residue (42%) at the position just in front of the MGF motif; thus, this residue may contribute to the interaction of UBA domains with ubiquitin in the same way as in NBR1 (Fig. 7F). In stark contrast, positively charged amino acids are virtually absent at this site. Intriguingly, among the 1725 UBA domains, $\sim 300$ (17%) have a serine residue at this position, suggesting the possibility that the regulation of ubiquitin binding affinity by phosphorylation might be a general mechanism in UBA domains.

Binding of NBR1 UBA to Lys48- and Lys63-linked Polyubiquitin Chains—The interaction of UBA domains with ubiquitin and polyubiquitin is often investigated by surface plasmon resonance studies using GST fusion proteins of UBA domains. Nevertheless, a recent study found that dimerization of the fusion partner GST significantly alters the dissociation constants determined for the binding of a UBA domain to various kinds of ubiquitin (32). In fact, both $K_d$ values and ubiquitin-linkage preferences were found to differ between the GST-free samples and the corresponding GST fusion proteins (32).

In the case of NBR1 UBA, a previous study reported that GST-fused NBR1 UBA binds to Lys63-linked diubiquitin about 60 times more strongly than to monoubiquitin (11). On the basis of this result and similar experiments with GST-fused p62 UBA, it was proposed that the Lys63-linked polyubiquitin chain may act as a signal for selective autophagy (40). In this study we deliberately studied the association of NBR1 UBA with ubiquitin by experiments in the solution state, namely ITC and NMR, to eliminate the artifact of GST dimerization. The $K_d$ values obtained for the NBR1 UBA domain differed from those previously reported for GST-fused NBR1 UBA and showed no preference for Lys48-, or Lys63-linked diubiquitin over monoubiquitin. Furthermore, the values obtained from ITC suggested that a given NBR1 UBA molecule binds one monomeric unit of diubiquitin in a 1:1 stoichiometry (Fig. 5E). In good agreement, NMR titration experiments showed that NBR1 UBA binds diubiquitin via the same surface as monoubiquitin. Collectively, these results indicate that there is no specificity for Lys48- or Lys63-linked ubiquitin chains encoded in the isolated NBR1 UBA domain.

Possibility of the Specificity of NBR1 UBA for Other Ubiquitin Linkages—Ubiquitin chains other than Lys48- and Lys63-linked diubiquitin were not used in our experiments; however, Lys6-, Lys11-, Lys29-, and Lys33-linked polyubiquitin chains account for $<1\%$ of the total concentration of cellular polyubiquitin (35). The linear (M1-linked) ubiquitin chain shows an extended conformation, similar to Lys63-linked diubiquitin (41), making it unlikely to be selectively recognized by the small and compact NBR1 UBA domain; however, we cannot rule out the possibility that NBR1 UBA may show a higher affinity for the compact K11-linked polyubiquitin chain (42).

Whereas the isolated UBA domain appears to have no specificity regarding ubiquitin linkage, such a polyubiquitin preference may arise in the context of full-length NBR1, which is reported to bind to p62 via its N-terminal PB1 domain and dimerize via a coiled-coiled region (4, 32). By such a mechanism, two C-terminal UBA domains may be spatially arranged in a way to specifically recognize a particular ubiquitin linkage.

No Linkage Specificity and Ubiquitin-positive Inclusion Bodies—On the other hand, the absence of a particular ubiquitin-linkage type preference in NBR1 UBA is intriguing in the presence of recent cell biological studies, which highlighted that ubiquitin chains in cellular ubiquitin-positive inclusion bodies do not show any linkage specificity either (35). Because such ubiquitin-positive inclusions are substrates of autophagy (43), it is reasonable that an autophagy receptor protein such as NBR1 also displays no chain-linkage selectivity.

Conclusion—Our study presents the first characterization of the UBA domain of the human autophagy receptor NBR1 and its interaction with various types of ubiquitin. We revealed structural differences between NBR1 UBA and the previously studied autophagy receptor p62, which give rise to a much higher affinity of NBR1 UBA for ubiquitin. Moreover, NBR1 was found to have a non-selective UBA domain, in good agreement with recent cell biological data indicating that ubiquitin-positive aggregates, which serve as substrates for autophagy, similarly show no ubiquitin-linkage specificity.

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