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Solution Structure of the Ubp-M BUZ Domain, a Highly Specific Protein Module That Recognizes the C-terminal Tail of Free Ubiquitin

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Summary

The BUZ/Znf-UBP domain is a distinct ubiquitin-binding module found in the cytoplasmic deacetylase HDAC6, the E3 ubiquitin ligase BRAP2/IMP, and a subfamily of deubiquitinating enzymes. Here we report the solution structure of the BUZ domain of Ubp-M, a ubiquitin-specific protease, and its interaction with ubiquitin. Unlike the BUZ domain from isopeptidase T (isoT) that contains a single zinc finger, the Ubp-M BUZ domain features three zinc-binding sites consisted of twelve residues. These zinc ligands form a pair of cross-braced ring fingers encapsulated within a third zinc finger in the primary structure. In contrast to isoT, which can form an N-terminal loop swapped dimer in the crystal state, the formation of additional zinc fingers in the Ubp-M BUZ domain restricts its N-terminal loop to intra-domain interactions. The ubiquitin-binding site of the Ubp-M BUZ domain is mapped to the highly conserved, concave surface formed by the $\alpha 3$ helix and the central β -sheet. We further show that this site binds to the C-terminal tail of free ubiquitin, and corresponding peptides display essentially the same binding affinities as full-length ubiquitin does for the Ubp-M BUZ domain. However, modification of the G76_{Ub} carboxylate group either by a peptide- or isopeptide-bond abolishes BUZ-domain interaction. The unique ubiquitin-recognition mode of the BUZ domain family suggests that they may function as “sensors” of free ubiquitin in cells to achieve regulatory roles in many aspects of ubiquitin-dependent processes.

Keywords

BUZ domain; Znf-UBP domain; ubiquitin-binding domain; zinc finger

Introduction

Post-translational modification by ubiquitin plays an important role in many cellular processes such as transcription, translation, DNA repair, virus budding, protein re-localization, protein degradation by the 26S proteasome, and cell cycle progression^{1–7}. Ubiquitin is a highly conserved, 76 amino acid protein that can be conjugated to target proteins by the concerted

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actions of a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and a ubiquitin ligase (E3) ^{3,8}. The canonical conjugation reaction occurs between the C-terminal glycine (G76) of ubiquitin and the ϵ -amino group of a lysine sidechain of the target protein. Additionally, ubiquitin itself can be further conjugated through one of its seven conserved lysine residues, generating poly-ubiquitinated proteins. Similar to protein phosphorylation, the ubiquitination process can be readily reversed via actions of deubiquitinating enzymes (DUBs), rendering it a truly flexible signaling tag to regulate many different cellular events.

Various forms of ubiquitination have been implicated in many different pathways ^{9,10}. For example, K48 poly-ubiquitin linkage targets a substrate protein to degradation by the 26S proteasome machinery; K63 poly-ubiquitination has been associated with DNA repair; whereas mono-ubiquitination plays important roles in signaling pathways involving membrane protein trafficking, endocytosis, and transcription regulation ^{1,4}. In order for these different ubiquitin tags to signal in divergent pathways, they must be specifically recognized by distinct ubiquitin-binding domains to transmit proper signals ^{11,12}. To date, more than 16 distinct motifs have been identified as ubiquitin-binding domains ¹³. Although the sizes and topologies of these ubiquitin-binding domains vary, the majority of them interact with the hydrophobic patch centered at I44 of ubiquitin ^{14,15}. Interestingly, recent biochemical and structural studies revealed several novel ubiquitin-binding domains that associate with ubiquitin without engaging the I44 site. These include the ubiquitin-binding motif (UBM), A20-type zinc finger/Rabex-5 ubiquitin-binding zinc finger (RUZ), and ubiquitin carboxyl-terminal hydrolase-like zinc finger (Znf-UBP) domains ^{16–19}. Diversity in the binding mode makes it possible for a single ubiquitin molecule to be recognized simultaneously by multiple ubiquitin-binding domains.

The Znf-UBP domain, also known as the DAUP (deacetylase/ubiquitin-specific protease) domain, PAZ (polyubiquitin-associated zinc finger) domain or BUZ (binder of ubiquitin zinc finger) domain, is found in the cytoplasmic deacetylase HDAC6, the E3 ubiquitin ligase BRCA1-associated protein 2 (BRAP2 or Impedes Mitogenic signal Propagation, IMP), and a group of ubiquitin-specific proteases (USPs) ^{20–24}. To emphasize the fact that this domain is distributed beyond ubiquitin-specific proteases, we will refer to this domain as the BUZ domain in the present study.

The function of the BUZ domain is best characterized in HDAC6. Biochemical analysis *in vitro* demonstrated that the BUZ domain from HDAC6 interacted with mono-ubiquitin as well as poly-ubiquitin chains. It was further shown that HDAC6 bound to misfolded, ubiquitinated proteins in a BUZ-dependent manner ²⁵. This latter interaction appears to be important for the turnover of poly-ubiquitinated proteins in cells ²⁶. Recent biochemical and structural analysis of the isoT BUZ/Znf-UBP domain revealed a distinct ubiquitin-binding mode for this domain characterized by its specific recognition of the C-terminus of free ubiquitin, rather than through binding to the canonical hydrophobic surface centered around I44 of ubiquitin ¹⁹. Interestingly, these studies identified a single zinc-binding site in the BUZ/Znf-UBP domain of isoT, whereas biochemical studies of the HDAC6 BUZ domain revealed the existence of three zinc-binding sites ²⁶, suggesting that the BUZ family can be further divided into different sub-groups depending on the mode of zinc coordination.

Here we report biochemical and structural characterization of the BUZ domain from Ubp-M (hUSP16) and its interaction with ubiquitin. Ubp-M is a deubiquitinating enzyme in the USP family, and its inactivation blocks progression in cell cycle ²⁷. Ubp-M is able to deubiquitinate mono-ubiquitinated nucleosomal histone H2A at the execution phase of apoptosis ²⁸. Primary structure analysis shows that Ubp-M contains a BUZ domain at its N-terminus and a catalytic domain at its C-terminus. Mass spectrometric measurements performed on native and denatured Ubp-M BUZ domains identify three Zn^{2+} ions associated with each folded BUZ

molecule while the solution structure reveals three unique zinc-binding sites arranged in an atypical “cross-braced” ring finger configuration within a third zinc finger^{29,30}. We further show that the Ubp-M BUZ domain binds to ubiquitin or corresponding C-terminal peptides with affinities in the low micromolar range. In contrast, the Ubp-M BUZ domain does not recognize peptides with the C-terminal glycine residue blocked either by an extra residue or by a lysine sidechain, suggesting that the BUZ domain specifically recognizes the free C-terminus of ubiquitin.

Results

Defining the Minimal Sequence for the BUZ Domain in Ubp-M

Because of the functional significance of the HDAC6 BUZ domain in aggresome formation²⁵, we initially investigated whether a recombinant BUZ domain fused to glutathione transferase (GST) could bind to mono- and/or poly-ubiquitin in an *in vitro* pull-down assay (Figure 1). The wild-type HDAC6 BUZ domain, but not a C1145S or C1145S/H1164A mutant, which alters the conserved and putative zinc coordinating cysteine and histidine residues, nor GST alone, bound efficiently to both mono-ubiquitin and poly-ubiquitin chains containing a free C-terminus; similarly, a related BUZ domain from the human deubiquitinase Ubp-M²⁷ was also capable of binding to mono- and poly-ubiquitin, indicating that ubiquitin-binding is a common property of the BUZ domain family.

Despite repeated attempts, the HDAC6 BUZ domain proved to be a difficult target for NMR analysis (data not shown). We therefore focused our studies on the related BUZ domain from Ubp-M. A construct of Ubp-M containing the N-terminal 134 residues from T10 to S143 encompassing the BUZ domain was overexpressed and purified for preliminary analysis. Triple-resonance NMR experiments were used to assign the backbone resonances of the protein^{31,32}. Analysis of the ¹H-¹⁵N heteronuclear NOE spectra showed that the N-terminal residues of the Ubp-M BUZ domain, T10 to L20, were disordered (Figure S1)³³. Based on this information, a shorter fragment consisting of P22 to S143 of Ubp-M was cloned and expressed as an N-terminal His₆-tagged protein. After Ni²⁺-NTA purification and thrombin cleavage to remove the His₆-tag, the remaining fragment, containing an additional GSHM sequence at the N-terminus from the expression vector, was renumbered 1–126 for further structural and biochemical studies. Analytical ultracentrifugation analysis of this fragment revealed that the BUZ domain of Ubp-M is monomeric in solution (data not shown).

The BUZ Domain of Ubp-M Contains Three Zinc Ions

The existence of a large number of conserved cysteine and histidine residues within the BUZ domain has led to its classification as a zinc-finger-containing domain. We therefore used ESI-mass spectrometry to determine the zinc content within the BUZ domain of Ubp-M (Figure 2). The native state of the Ubp-M BUZ domain has a molecular mass of 14,649 Da. When the protein was denatured in 50% acetonitrile containing 0.1% trifluoroacetic acid, its molecular mass decreased to 14,459 Da, in agreement with the theoretical value of 14,459.3 Da. The difference of 190 Da in molecular mass between the native and denatured Ubp-M BUZ domains corresponds to a loss of three zinc ions. Although for proper protein folding the bacterial growth medium contained 100 μM ZnSO₄, no zinc was supplied during the extensive purification process that should have removed any non-specifically bound zinc ions. Based on the mass spectrometric analysis, we concluded that the BUZ domain of Ubp-M contains three zinc-binding sites.

In addition to the main peak of 14,459 Da, a smaller peak of molecular mass 14,523 Da also appeared in the spectrum of the denatured protein (Figure 2(b)). The molecular mass of this peak corresponds to that of the Ubp-M BUZ domain containing one zinc ion, suggesting that

the affinity of one particular zinc-binding site in the Ubp-M BUZ domain is significantly greater than the remaining two.

Although our studies establish three zinc ions in the Ubp-M BUZ domain, the number of zinc-binding sites appears to vary in different members of the BUZ family. For example, the recent crystal structure of the isoT BUZ/Znf-UBP domain revealed a single zinc-binding site¹⁹, whereas particle-induced X-ray emission studies suggested the existence of three zinc ions in the HDAC6 BUZ domain²⁶. Our observation of three zinc ions in the Ubp-M BUZ domain highlights its similarity to the HDAC6 BUZ domain. This assessment is further supported by the conservation of 12 potential zinc-chelating residues in the primary structures among the BUZ domains of Ubp-M, HDAC6 and a number of other ubiquitin-specific proteases (Figure 3(a)). However, the majority of these zinc ligands are not conserved in the BUZ domain of isoT, suggesting that Ubp-M and isoT employ different architectures for zinc coordination.

Solution Structure of the BUZ Domain of Ubp-M

The structure of the BUZ domain of Ubp-M was determined by NMR on the basis of 3458 NOE, 144 dihedral angle, and 60 hydrogen bond restraints, and was further refined with 102 residual dipolar couplings. Excluding the disordered residues at the N- and C-termini (1–6 and 126) and the internally disordered loop (residues 36–53), the mean pair-wise rmsd values of backbone and heavy atoms of the NMR ensemble (Figure 3(b)) are 0.49 Å and 0.85 Å, respectively. Additional statistics are shown in Table 1.

The solution structure (Figure 3(b, c)) of the Ubp-M BUZ domain contains four α -helices and five β -strands held together by three distinct zinc-binding sites. The β -strands are arranged in an antiparallel fashion to form a twisted β -sheet located at the center. This central β -sheet is sandwiched on one side by helices $\alpha 1$ and $\alpha 4$ oriented in parallel to each other, and on the other side by helix $\alpha 3$. The edge of the β -sheet ($\beta 2$) is flanked by a short $\alpha 2$ helix, with the C-terminus of $\alpha 2$ separated from the central β -sheet by a 14-residue disordered insert loop found only in Ubp-M.

The Ubp-M BUZ domain features three well-defined zinc-binding sites formed by eight cysteine and four histidine residues. Each zinc-binding site contains four residues with their respective sidechains ideally positioned for tetrahedral zinc coordination (Figure 3(d, e, f)).

The first zinc-binding site consists of residues C7, H9, C99 and C102 (finger A, Figure 3(d)). The formation of this C₃H-type zinc finger positions the N-terminus of the BUZ domain to the vicinity of the loop connecting $\beta 4$ and $\beta 5$ of the central β -sheet. The second zinc-binding site, also belonging to the C₃H type, is formed by two cysteine residues (C31 and C34) in the CXXC motif of $\alpha 2$, C65 at the C-terminus of $\beta 2$, and H73 at the N-terminus of $\alpha 3$ (finger B, Figure 3(e)). The last zinc-binding site belongs to the C₂H₂ type and consists of two cysteine residues in the second CXXC motif (C57 at the C-terminus of $\beta 1$ and C60 in the $\beta 1$ - $\beta 2$ loop), H77 at the C-terminus of $\alpha 3$, and H86 in the loop connecting $\alpha 3$ and $\beta 3$ (finger C, Figure 3(f)). Residues of the second and third zinc-binding sites do not form typical zinc fingers, but adopt a “cross-braced” ring finger structure instead^{29,30}. These ring fingers position $\alpha 2$ to the edge and $\alpha 3$ on top of the central β -sheet to generate a concave surface encircled by $\alpha 3$ and the twisted β -sheet for ubiquitin recognition. Interestingly, these ring finger residues are located entirely between the residues of the first zinc-binding site in the primary structure, featuring an atypical topology of zinc coordination uniquely found in the BUZ domain of Ubp-M.

The BUZ Domain of Ubp-M Binds to the Free C-terminal Tail of Ubiquitin

We next investigated the binding interface between the Ubp-M BUZ domain and ubiquitin by NMR titration experiments. To map the BUZ-binding site on ubiquitin, a series of ¹H-¹⁵N

HSQC spectra of ^{15}N -labeled human ubiquitin were recorded in the presence of increasing molar ratios of the unlabeled Ubp-M BUZ domain. Surprisingly, ubiquitin residues forming the hydrophobic patch centered at I44, a common binding site for most known ubiquitin-binding domains, did not experience any noticeable perturbation in chemical shifts. In contrary, the backbone resonances of L73, R74, G75 and G76, and sidechain resonances of Q41 were severely broadened during titration. Moreover, the amide resonances of V70 and R72 were progressively perturbed by the Ubp-M BUZ domain in a concentration-dependent manner. The majority of these perturbed residues map exclusively to the C-terminal tail of ubiquitin (Figure 4(a, d)).

In order to determine the ubiquitin-binding site on the BUZ domain, a reciprocal titration was performed by adding incremental amounts of unlabeled ubiquitin to an ^{15}N -labeled Ubp-M BUZ domain sample. A number of resonances were perturbed in the resulting ^1H - ^{15}N HSQC spectra (Figures 4 and S2). Sidechain resonance of W55 and backbone resonances of L56, S69, E71, H73, A74, L75, K76, V89, L92, W98, and C99 of the Ubp-M BUZ domain were either severely perturbed or exchange-broadened during titration. These residues are located in a pocket encircled by helix $\alpha 3$ and strands $\beta 1$, $\beta 3$, and $\beta 4$ of the central β -sheet (Figure 3(a) and 4). In contrast, addition of C-terminal His₆-tagged ubiquitin to the ^{15}N -labeled BUZ domain sample did not result in any noticeable chemical shift perturbations, suggesting that the BUZ domain of Ubp-M does not recognize ubiquitin with its C-terminal glycine residue (G76) blocked. This mode of ubiquitin binding by the Ubp-M BUZ domain is reminiscent of the recognition of the ubiquitin C-terminus by the isoT BUZ/Znf-UBP domain ¹⁹.

The Ubp-M BUZ Domain Binds to Ubiquitin and Ubiquitin C-terminal Peptides with Similar Affinities

The C-terminal tail of ubiquitin, consisting of residues L73 to G76, forms a disordered loop that extends beyond the globular fold of ubiquitin. Because our NMR titration data suggest that the BUZ domain of Ubp-M interacts with the C-terminus of ubiquitin, we examined whether the BUZ domain could bind to the corresponding peptide with high affinity. A peptide, YA-RLRGG, was therefore synthesized that contained the last five amino acids of ubiquitin (residues 72–76) and two additional residues, YA, at the N-terminus to facilitate concentration measurement by UV absorbance. The peptide-BUZ domain interaction was first evaluated by NMR titration. Interestingly, this peptide induced an identical HSQC chemical shift perturbation pattern in the Ubp-M BUZ domain as the full-length ubiquitin did (Figure 4(c)), suggesting that the interaction between ubiquitin and the Ubp-M BUZ domain is mediated predominantly by the C-terminal peptide of ubiquitin.

To evaluate this unique interaction between the Ubp-M BUZ domain and the ubiquitin C-terminal peptide in more quantitative terms, we measured the corresponding dissociation constant by isothermal titration calorimetry and/or by fluorescence polarization (Figure 5). As a control, we also determined the dissociation constant of the Ubp-M BUZ-ubiquitin complex by ITC and obtained a K_d of 6.53 μM . This value is similar to that of the isoT BUZ/Znf-UBP-ubiquitin complex. Importantly, the Ubp-M BUZ domain bound to the ubiquitin peptide YA-RLRGG with a K_d of 15.88 μM . Extending this peptide by three residues toward the N-terminus to include also residues L69, V70, and L71 of ubiquitin further enhanced the binding affinity of the resulting peptide (YA-LVRLRRGG). The resulting K_d of 6.77 μM is essentially identical to that of ubiquitin for the Ubp-M BUZ domain (Table 2). These data suggest that residues important for interacting with the Ubp-M BUZ domain reside exclusively in the C-terminal tail of ubiquitin, and that the BUZ domain is a protein- as well as a peptide-recognition module.

The BUZ Domain Does Not Recognize C-terminally Modified Ubiquitin Peptides

We next examined whether the Ubp-M BUZ domain could recognize the same peptides containing either an extra residue at the C-terminus (f-YA-RLRGGD, where f denotes a fluorescein moiety, see Table 2) or an isopeptide bond formed between the carboxyl group of the G76 and the ϵ -amino group of a lysine sidechain (f-YA-RLRGG-isoK or f-YA-LVLRLRGG-isoK, Table 2). The latter modification is frequently encountered in poly-ubiquitinated proteins. These C-terminally modified peptides, unlike the ubiquitin peptides containing free C-termini, completely lost the ability to interact with the Ubp-M BUZ domain (Table 2). These observations suggest that the BUZ domain of Ubp-M does not recognize a peptide bond connecting linear ubiquitin modules nor an iso-peptide bond found in poly-ubiquitinated proteins.

Discussion

Structural Comparison between the BUZ Domains of Ubp-M and IsoT

The overall fold of the BUZ/Znf-UBP domains of Ubp-M and isoT is similar and features a central, twisted β -sheet with two prominent helices packed on both sides of the β -sheet (helices $\alpha 3$ and $\alpha 4$ in Ubp-M, and αA and αB in isoT) (Figure 6). In particular, residues located on the secondary structure elements ($\alpha 3$, $\beta 1$, $\beta 3$, and $\beta 4$) that make up the ubiquitin-binding pocket are highly conserved (Figure 3(a)). Consistent with this observation, the backbone traces of $\alpha 3$ and the central β -sheet of the Ubp-M BUZ domain are essentially superimposable to those of isoT, with a backbone rmsd of ~ 0.5 Å (Figure 6, boxed regions).

There are, however, significant differences between these two domains (Figure 6). First, in the crystal structure of the isoT BUZ/Znf-UBP domain, residues N-terminal to $\beta 1$ form an extended loop devoid of any regular secondary structures that crosses the edge of the entire β -sheet. The formation of a C_3H -type zinc finger in the isoT BUZ/Znf-UBP domain restricts the C-terminal end of this loop to the tip of $\beta 2$ and αA , whereas the N-terminal portion of the loop engages the tip of $\beta 4$ and $\beta 5$ of the central β -sheet via electrostatic interactions mediated by residues R174, K178, H179, D257 and D262. This N-terminal portion of the loop appears to be flexible and contributes to the dimer interface in the crystal structures either through a disulfide-bonded bridge or as a domain-swapped loop. In contrast, the corresponding residues in the BUZ domain of Ubp-M assume a more rigid conformation and are exclusively engaged in intra-domain interactions. The very N-terminal part of the loop is restrained to the tip of $\beta 4$ and $\beta 5$ by a zinc finger (finger A). Immediately following this zinc finger, there are two helices, $\alpha 1$ and $\alpha 2$. The $\alpha 1$ helix is oriented in parallel with the $\alpha 4$ helix to cross the top of $\beta 1$ and $\beta 2$, and the $\alpha 2$ helix is held to strand $\beta 2$ and helix $\alpha 3$ by a second zinc finger (finger B). In comparison, no regular secondary structures are observed in the isoT BUZ/Znf-UBP domain in the regions corresponding to helices $\alpha 1$ and $\alpha 2$ of the Ubp-M BUZ domain.

Second, despite a high degree of sequence conservation of $\alpha 3$ residues involved in ubiquitin binding, the packing of $\alpha 3$ against the central β -sheet is supported by distinct mechanisms in Ubp-M when compared with isoT. In isoT, the corresponding helix (αA) interacts with the β -sheet through a salt bridge between residues D214 and H236 and van der Waals contacts between residues T213 and Y242; whereas in Ubp-M, such interactions are functionally replaced by yet another zinc finger (finger C) that mediates the packing of $\alpha 3$ against the β -sheet (Figure 6).

Third, the C-terminal helix ($\alpha 4$) in Ubp-M is significantly longer and is oriented differently from the corresponding helix (αB) of isoT. In Ubp-M, $\alpha 4$ packs parallel to $\alpha 1$ across the back of the central β -sheet, whereas the corresponding helix (αB) in isoT interacts with the N-

terminal extended loop and is buttressed by an insert loop connecting strands $\beta 3$ and $\beta 4$ (Figure 6).

Finally, the BUZ/Znf-UBP domain of isoT contains an insertion loop (L2A) between $\beta 2$ and αA , which was proposed to form a “ruler loop” to interact with ubiquitin¹⁹. Interestingly, such a loop is absent in Ubp-M. Instead, Ubp-M contains a unique insert loop between $\alpha 2$ and $\beta 1$. Compared with the “ruler loop” in isoT, the insert loop in Ubp-M is longer (14 amino acids). Surprisingly, this loop is completely disordered and its resonances are not affected by ubiquitin binding, suggesting that this insert loop is unlikely to be involved in ubiquitin binding.

Zinc Fingers Contribute to the Structural Integrity of the BUZ Domain

Our mass spectrometry and NMR studies of the BUZ domain of Ubp-M unambiguously established three zinc ions in the native protein that are coordinated by twelve residues in the form of cross-braced ring fingers encapsulated within another zinc finger in the primary structure. These zinc ligands are conserved in HDAC6, but not in isoT, suggesting that the zinc-binding modes of the BUZ domains of HDAC6 and Ubp-M are identical, but they differ from that of isoT. The existence of multiple zinc fingers in the BUZ domains of Ubp-M and HDAC6 are crucial for maintaining their structural integrity. Mutation of any of the zinc-binding residues in the BUZ domain of HDAC6 resulted in a loss of ubiquitin interaction, presumably due to impaired folding²⁶. Similarly, point-mutations of zinc-binding residues (C31S, C57S, H77S, H86S, or C99S) in the BUZ domain of Ubp-M led to protein degradation manifested by the production of proteolytic fragments during protein expression (data not shown).

Based on this current study and the difference in zinc-binding configurations, we propose to further classify the BUZ domain family into three sub-groups. The BUZ domains of Ubp-M, HDAC6, hUSP44, hUSP49, and scUSP14 form the first group since each contains 12 conserved zinc-coordinating residues and is expected to bind three zinc ions using two C_3H and one C_2H_2 fingers. In contrast, the BUZ domain of either isoT or hUSP13 contains a single C_3H zinc finger, thus belongs to a different sub-group. The functions of the two other zinc fingers found in the first group are replaced here by hydrophobic and electrostatic interactions. The hBRAP2 BUZ domain differs from the above two groups in that it harbors nine out of twelve residues involved in the formation of zinc-binding sites in the first group. Because these residues only span two of the three zinc fingers, we propose that the hBRAP2 BUZ domain belongs to a third group and likely contains cross-braced ring-fingers of the C_3H and C_2H_2 types.

Biological Implications

The finding that the BUZ domain from Ubp-M binds free glycine at the C-terminus of ubiquitin indicates that this domain should not be able to bind ubiquitin or ubiquitin chains that are conjugated to protein substrates. Such a binding mode would be consistent with the idea that this domain anchors free poly-ubiquitin chains for further processing by deubiquitinating enzymes, such as Ubp-M or isoT. This assertion, however, is not entirely consistent with the observation that HDAC6 can bind poly-ubiquitinated proteins (Figure 7(a)) and the BUZ domain is crucial for such interactions²⁵. These apparent contradictory findings might be resolved if the BUZ domain in HDAC6 does not bind directly to poly-ubiquitinated proteins, but rather serves to modulate the binding activity of HDAC6. Supporting this view, unlike HDAC6, Ubp-M does not bind poly-ubiquitinated proteins appreciably in response to proteasome inhibition (Figure 7(a)). We speculate that by virtue of its unusually high affinity for the free ubiquitin, the BUZ domain would normally be occupied by free ubiquitin or poly-ubiquitin chains, and this interaction inhibits HDAC6 binding to poly-ubiquitinated proteins. When cellular ubiquitin pool is reduced—a condition often associated with decreased

proteasome activity and the accumulation of misfolded proteins—the BUZ domain would be emptied, and the resulting conformational switch could allow HDAC6 for binding to poly-ubiquitinated proteins (Figure 7(b)), and transporting ubiquitinated proteins to aggresomes and autophagy²⁵. Although it remains possible that the BUZ domain takes part in the binding of ubiquitinated proteins by a yet to be defined mechanism, our studies suggest that the BUZ domain, at the minimum, would be required for activating the poly-ubiquitinated protein-binding activity of HDAC6 by “sensing” the levels of cellular ubiquitin pool. Such a model would suggest that the BUZ domain operates as a regulatory domain that modulates protein deacetylase, E3 ligase and deubiquitinating enzyme function in response to changes in local or cellular levels of free ubiquitin and/or poly-ubiquitin chains. If such a model is correct, proteins with the BUZ domain would act commonly as sensors for cellular ubiquitin status and likely play important roles in many aspects of ubiquitin-dependent processes.

Materials and Methods

Ubiquitin Binding Assay

GST or GST-fusion proteins were purified using glutathione-sepharose resin (GE Healthcare, Piscataway, NJ). Protein levels were normalized by O.D. at 595 nM. Purified protein on beads was then incubated with 1 µg of *in vitro* synthesized mono- and poly-ubiquitin chains (Affiniti bioreagents, Golden, CO) in NETN buffer³⁴ containing 1 mM BSA to prevent the re-absorption of ubiquitin, at 4 °C for 3 hours. Samples were subjected to SDS-PAGE analysis, and then immunoblotted for ubiquitin.

For ubiquitinated protein binding *in vivo*, 293T cells were transfected with empty vector, pCDNA3-Flag-HDAC6²⁵, or pCDNA3-Flag-UbpM constructs. These transfected cells were treated with 5 µM MG132 (Sigma-Aldrich, St. Louis, MO) for 12–16 hours, and then lysed in NETN buffer in the presence of 5 mM N-ethylmaleimide (NEM, Sigma-Aldrich). Flag-tagged proteins were immunoprecipitated from 500 µg of lysate with anti-Flag antibody and Protein G Sepharose. Binding of ubiquitinated proteins was determined by immunoblotting with anti-ubiquitin and anti-Flag antibodies.

Sample Preparation

The BUZ domain constructs of Ubp-M encoding residues T10 to S143, or P22 to S143 were cloned into a pET15b vector (EMD Biosciences, Inc., Madison, WI) between NdeI and BamHI sites, and were overexpressed as N-terminal His₆-tagged proteins in *Escherichia coli* BL21 (DE3)STAR cells (Invitrogen Inc., Carlsbad, CA). Bacterial cells were initially grown in Luria broth or M9 minimal media at 37 °C. After the OD₆₀₀ reached 0.6, the cells were induced with 0.2 mM isopropyl-β-D- thiogalactopyranoside (IPTG) and 100 µM ZnSO₄ at 20 °C for 15 hrs before harvest. Isotopically labeled proteins were overexpressed in M9 media supplemented with ¹⁵N-NH₄Cl and ¹³C-glucose as the sole nitrogen and carbon sources. NMR samples were prepared with uniform ¹⁵N, ¹³C/¹⁵N, or 10% ¹³C labeling.

The N-terminal His₆-tagged fusion constructs containing T10-S143 or P22-S143 of Ubp-M were purified by a Ni²⁺-NTA column and treated with thrombin to remove the His₆-tags. The resulting fragments, containing additional four residues (GSHM) at the N-terminus and residues 10–143 or 22–143 of Ubp-M, were further purified with size-exclusion chromatography. All NMR samples were exchanged into a buffer containing 25 mM sodium phosphate, 100 mM KCl, 2 mM DTT, and 5% or 100% D₂O (pH=7.0) before experiments. Samples were degassed and sealed under argon to prevent cysteine oxidation.

Human ubiquitin with a cleavable N-terminal His₆-tag was cloned into the pET15b vector, expressed and purified similarly as described for the BUZ domain of Ubp-M except that

bacterial cells were induced with 1 mM IPTG at 37 °C for 4 hours. A second ubiquitin construct containing a non-cleavable C-terminal His₆-tag was also prepared as a negative control.

ESI Mass Spectrometry

ESI mass spectrometry was performed on the native and denatured proteins using a quadrupole time-of-flight mass spectrometer (Q-TOF 2) (Waters Corporation, Milford, MA) run in the positive ion electrospray mode. A stock solution of the Ubp-M BUZ domain was diluted into 25 mM NH₄HCO₃ to maintain the native state, whereas the denatured state was obtained by dissolving the protein sample in a solution containing 50% acetonitrile and 0.1% formic acid. Data were processed using the MaxEnt1 algorithm provided in MassLynx4.0 to yield the average mass for native and denatured proteins.

NMR Structure Determination

All NMR experiments were performed at 27°C using Varian INOVA 600 or 800 MHz spectrometers. Data were processed by NMRPIPE and analyzed with XEASY/CARA^{35,36}. Backbone resonances were collected by standard 3D triple-resonance experiments and analyzed using PACES³⁷; sidechain resonances were assigned using 3D HCCH-TOCSY and 2D homonuclear TOCSY and NOESY experiments^{31,32}. Distance constraints were derived from three-dimensional ¹⁵N- and ¹³C-separated NOESY-HSQC experiments³¹. Dihedral angles were derived from the combined input of TALOS analysis based on chemical shift information,³J_{HNH α} couplings determined from an HNHA experiment, and analysis of local NOE patterns^{38–40}. Stereo-specific assignments of valine and leucine methyl groups were obtained via a high-resolution ¹H-¹³C HSQC spectrum of a 10% ¹³C-labeled sample⁴¹. Initial structures were generated with CYANA^{42,43}. Because both the N δ 1 and N ϵ 2 atoms of histidine residues can potentially be involved in zinc binding, we evaluated the zinc-coordination geometry of these histidines individually, assuming either N δ 1 or N ϵ 2 as the zinc ligand. Only one combination (H9 N δ 1, H73 N δ 1, H77 N ϵ 2, and H86 N δ 1) satisfied all experimental constraints, which was used to calculate the final ensemble of NMR structures. Three zinc ions were included during the final stages of structure calculations.

Residual dipolar couplings (¹D_{HN} and ¹D_{H α C α}) were determined from the difference of couplings between an isotropic medium and a liquid crystalline Pf1 phage medium (~12 mg/mL) in 25 mM sodium phosphate, 400 mM potassium chloride, 15% D₂O (pH=7.0). 3D HNCO-based experiments were used to measure the ¹D_{HN} and ¹D_{H α C α} couplings^{44,45}. These residual dipolar couplings were used for structure refinement using XPLOR-NIH with a water-refinement protocol^{46–48}. 20 out of 40 calculated structures were selected for presentation based the optimal geometry evaluated by MOLPROBITY^{49,50}. The statistics of the NMR ensemble are shown in Table 1. Atomic coordinates have been deposited with the Protein Data Bank (PDB entry 2I50).

Peptide Synthesis

Peptides corresponding to the C-terminal tail of ubiquitin or lysine-conjugated ubiquitin were synthesized using standard Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) chemistry on a 431A Peptide Synthesizer (Applied Biosystems, Foster City, CA). For peptides used for fluorescence polarization measurements, an appropriate amount of 5-(and-6)-carboxy-fluorescein succinimidyl ester was added to the peptide resin, and the coupling reaction was allowed to proceed for 1 hour at room temperature. The peptides were cleaved from the resin by trifluoroacetic acid treatment and purified by reverse phase HPLC using a C18 column⁵¹. The sequences of the peptides are listed in Table 2. The identity of the peptides was confirmed by mass spectrometry.

NMR Titration Studies

The binding site of ubiquitin was mapped by recording a series of ^1H - ^{15}N HSQC spectra of ^{15}N labeled ubiquitin with increasing molar ratios (from 1:0 to 1:2) of the unlabeled Ubp-M BUZ domain. Similarly, a series of ^1H - ^{15}N HSQC spectra were recorded for the ^{15}N labeled Ubp-M BUZ domain in the presence of increasing molar ratios (from 1:0 to 1:2) of unlabeled ubiquitin or synthetic peptides to map the binding surface of the BUZ domain. Chemical shift

perturbations were calculated as $\delta_{CS} = \sqrt{(\delta_{HN})^2 + (0.2\delta_N)^2}$ using resonances of ^{15}N Ubp-M or ^{15}N ubiquitin in the presence of 2:1 molar ratios of unlabeled ubiquitin or Ubp-M, respectively.

Isothermal Titration Calorimetry

The Ubp-M BUZ domain, ubiquitin, and a peptide (YA-RLRGG) containing the last five residues of ubiquitin were exchanged into a buffer containing 25 mM sodium phosphate and 100 mM KCl at pH 7.0. Isothermal titration calorimetry (ITC) measurements were performed on a MicroCal VP-ITC instrument (MicroCal, LLC, Northampton, MA). Raw data were obtained from 30 automatic injections of 10 μl aliquots of 1.5 mM ubiquitin into a solution of 50 μM Ubp-M BUZ domain at 20 $^\circ\text{C}$. Data were fit using Origin 7.0 (OriginLab Corporation, Northampton, MA) according to a 1:1 binding model.

Fluorescence Polarization Measurements

Fluorescence polarization experiments were conducted at 20 $^\circ\text{C}$ on a Beacon 2000 Polarization System (PanVera, Madison, WI). An incremental amount of the purified Ubp-M BUZ domain was titrated into a fluorescent peptide solution containing 20 mM sodium phosphate, 100 mM NaCl, pH 7.0, and the resulting polarization values were recorded. Binding data were fitted to a hyperbolic nonlinear regression model using Prism 3.0 (GraphPad Software, Inc., San Diego, CA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

DUBs	deubiquitinating enzymes
Znf-UBP domain	ubiquitin carboxyl-terminal hydrolase-like zinc finger domain
DAUP domain	deacetylase/ubiquitin-specific protease domain
PAZ domain	polyubiquitin-associated zinc finger domain
BUZ domain	binder of ubiquitin zinc finger domain
USPs	ubiquitin-specific proteases
ITC	isothermal titration calorimetry

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Figure 1.

The BUZ domain binds to mono- and poly-ubiquitin. Recombinant GST-fusion proteins containing the wild-type BUZ domain from HDAC6 (residues 1044–1273), a C1145S mutation, a C1145S/H1164A mutation, the Ubp-M BUZ domain, or GST alone were incubated with mono-ubiquitin and poly-ubiquitin chains generated *in vitro*. Bound fractions were then subjected to SDS-PAGE analysis and immunoblotted with a ubiquitin antibody (IB).

Figure 2. Mass spectrometric analysis reveals three zinc ions in the Ubp-M BUZ domain. The difference in molecular mass between the native (a) and denatured (b) proteins corresponds to the loss of three zinc ions.

Figure 3.

Solution structure of the Ubp-M BUZ domain. (a) Sequence alignment of representative BUZ domains from hUbp-M (NP_006438), hHDAC6 (NP_006035), hUSP44 (AAH30704), hUSP49 (NP_061031), scUBP14 (NP_009614), hBRAP2 (NP_006759), hUSP13 (NP_003931), and hIsoT (NP_003472). Conserved residues are highlighted, with hydrophobic and glycine residues colored in yellow, basic residues in blue, and residues involved in zinc binding in pale green, brown, and purple according to the formation of individual zinc fingers, respectively. The majority of these conserved residues are located in the central β -sheet and $\alpha 3$ that comprise the ubiquitin-binding interface. Secondary structures are labeled. Residues that experience chemical shift perturbation upon ubiquitin binding are denoted by green dots above the sequence. The NMR ensemble and the ribbon diagram of the Ubp-M BUZ domain are shown in panels (b) and (c), respectively. Secondary structures are highlighted, with helices in red and strands in blue. The zinc ions (brown) are shown in sphere. Details of the three zinc-binding sites are shown in panels (d), (e), and (f), with sidechains of zinc ligands shown in stick model. The topology of the three zinc fingers in the primary structure is shown in panel (g).

Figure 4.

NMR titration maps the binding interface to the concaved surface of the BUZ domain of Ubp-M and the C-terminal tail of ubiquitin. (a) ^1H - ^{15}N HSQC spectra of ubiquitin in the absence (black) and presence (red) of the Ubp-M BUZ domain. ^1H - ^{15}N spectra of Ubp-M in the absence (black) and presence (red) of ubiquitin or the ubiquitin peptide YA-RLRGG are shown in panels (b) and (c), respectively. (d) A surface representation of ubiquitin with significantly perturbed residues labeled in black and colored in orange. (e, f) Surface representations of the Ubp-M BUZ domain with significantly perturbed residues labeled in black and colored in orange.

Figure 5.

The BUZ domain of Ubp-M binds to ubiquitin and a ubiquitin C-terminal peptide YA-RLRGG with similar affinities. ITC analyses of the BUZ domain interactions with ubiquitin and the ubiquitin peptide YA-RLRGG are shown in panels (a) and (b), respectively.

Figure 6.

Structural comparison of the BUZ domains of Ubp-M and isoT. Ribbon diagrams of Ubp-M and isoT are colored in pale blue and pale green, respectively. Secondary structures and zinc fingers are denoted. Boxed regions indicate the highly conserved BUZ domain interface for ubiquitin binding, which consists of a central β -sheet and an α -helix ($\alpha 3$ in Ubp-M or αA in isoT). Residues of isoT that replace the structural role of the two structural zinc fingers A and C of Ubp-M are shown in stick model and labeled, with hydrophobic residues colored in brown, basic residues in blue, and acidic residues in red, respectively.

Figure 7.

Proteasome inhibitors induce specific association of poly-ubiquitinated proteins with HDAC6, but not with Ubp-M. (a) FLAG-HDAC6 or FLAG-UbpM expression plasmids were transfected into 394T cells and treated with MG132 as indicated. HDAC6 and UbpM were immuno-precipitated by anti-FLAG (M2) antibody followed by immuno-blotting with a ubiquitin antibody. (b) A potential model for the association of HDAC6 with poly-ubiquitinated proteins. In this model, depletion of free ubiquitin enables binding of HDAC6 to poly-ubiquitinated proteins.

Table 1Structural statistics for the Ubp-M BUZ domain (20 structures) ^a

NOE distance restraints	3458
Intra-residue	1578
Sequential ($ i - j = 1$)	673
Medium-range ($2 \leq i - j \leq 4$)	530
Long-range ($ i - j \geq 5$)	677
Hydrogen bonds ^b	60
Dihedral angle constraints	144
Residual dipolar couplings ($^1D_{NH}$)	51
Residual dipolar couplings ($^1D_{CH}$)	51
Dipolar coupling <i>R</i> factor of $^1D_{NH}$, ^c %	14.3 ± 0.2
Dipolar coupling <i>R</i> factor of $^1D_{CH}$, ^c %	15.9 ± 0.6
Ramachandran plot ^d	
Favored region	92.0%
Allowed region	98.4%
Deviations from idealized geometry	
Bonds, Å	0.015 ± 0.000
Angles, °	1.724 ± 0.031
Improper, °	1.799 ± 0.083
Mean pairwise rmsd	
Backbone (residues 7–35, 54–125), Å	0.49
Heavy atoms (residues 7–35, 54–125), Å	0.85

^aNone of these structures exhibit distance violations greater than 0.5 Å or dihedral angle violations greater than 5°.

^bTwo constraints per hydrogen bond ($d_{HN-O} \leq 2.0$ Å and $d_{N-O} \leq 3.0$ Å) are implemented for amide protons protected from solvent-exchange.

^c*R*-factor for residual dipolar coupling is defined as the ratio of the r.m.s deviation between observed and calculated values to the expected r.m.s deviation if the vectors were randomly distributed ⁵².

^dMOLPROBITY was used to assess the quality of the structures ^{49,50}.

Table 2

Binding affinities of the Ubp-M BUZ domain for ubiquitin or ubiquitin-derived peptides

Name	Sequences	K _d (μM)	Method
Ubiquitin	WT ubiquitin	6.5	ITC
penta-peptide	YA-RLRGG-COOH	15.9	ITC
f-penta-peptide	f-YA-RLRGG-COOH	15.9	FP
f-octa-peptide	f-YA-LVLRLRGG-COOH	6.8	FP
f-penta-D	f-YA-RLRGGD	TLQ	FP
f-penta-isoK	f-YA-RLRGG-isoK	TLQ	FP
f-octa-isoK	f-YA-LVLRLRGG-isoK	TLQ	FP

YA: residues added to aid concentration determination

f: fluorescein

TLQ: affinity too low for accurate quantification

ITC: isothermal titration calorimetry

FP: fluorescence polarization