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Mutations in the Hydrophobic Core of Ubiquitin Differentially Affect its Recognition by Receptor Proteins

Aydin Haririnia^a, Rati Verma^b, Nisha Purohit^a, Michael Z. Twarog^c, Raymond J. Deshaies^b, Dan Bolon^{c,*}, and David Fushman^{a,*}

^aDepartment of Chemistry and Biochemistry, Center for Biomolecular Structure and Organization, University of Maryland, College Park, MD 20742

^bDepartment of Biology, Howard Hughes Medical Institute, California Institute of Technology, Pasadena, CA 91125

^cUniversity of Massachusetts Medical School, Department of Biochemistry and Molecular Pharmacology, Worcester, MA 01605

Summary

Ubiquitin is one of the most highly conserved signaling proteins in eukaryotes. In carrying out its myriad functions, ubiquitin conjugated to substrate proteins interacts with dozens of different receptor proteins that link the ubiquitin signal to various biological outcomes. Here, we report mutations in conserved residues of ubiquitin's hydrophobic core that have surprisingly potent and specific effects on molecular recognition. Mutant ubiquitins bind tightly to the ubiquitin-associated (UBA) domain of the receptor proteins Rad23 and hHR23A, but fail to bind the ubiquitin-interacting motif (UIM) present in the receptors Rpn10 and S5a. Moreover, chains assembled on target substrates with mutant ubiquitins are unable to support substrate degradation by the proteasome *in vitro*, or sustain viability of yeast cells. The mutations have relatively little effect on ubiquitin's overall structure but reduce its rigidity and cause a slight displacement of the C-terminal β -sheet, thereby compromising association with UIM but not UBA domains. These studies emphasize an unexpected role for ubiquitin's core in molecular recognition, and suggest that the diversity of protein-protein interactions in which ubiquitin engages placed enormous constraints on its evolvability.

Keywords

ubiquitin; proteasomal degradation; ubiquitin receptors; molecular recognition; hydrophobic core mutation

Introduction

Ub is a small 76 a.a. protein¹ that is highly conserved amongst eukaryotes and involved in practically all aspects of eukaryotic cell biology. Ub exists within eukaryotic cells as a monomer and in the form of isopeptide-linked polymers called polyubiquitin (polyUb) chains.

* Corresponding authors Address correspondence to: David Fushman, Department of Chemistry & Biochemistry, Center for Biomolecular Structure & Organization, University of Maryland, College Park, MD 20742-3360, Phone: 301-405-3461; Fax: 301-314-0386, Email: fushman@umd.edu, Dan Bolon, University of Massachusetts Medical School, Department of Biochemistry and Molecular Pharmacology, Worcester, MA 01605, Phone: 508-856-3588; Fax: 508-856-6231, Email: Dan.Bolon@umassmed.edu.

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The enzymatic conjugation of Ub to other proteins regulates numerous biochemical pathways inside cells. This process of conjugation (termed ubiquitination) is controlled by a series of enzymes (E1, E2, and E3) and results in the formation of an isopeptide bond between Ub's C-terminal glycine residue (G76) and a lysine residue of the target protein. The existence of many E2s and E3s with different substrate specificities allows a wide variety of protein molecules to be recognized and modified with a single Ub or a polyUb chain. Outcomes of this modification range from targeted protein degradation² to altered protein trafficking³ or functional modulation⁴. A fundamental question in Ub biology is how this functional range is achieved.

A variety of protein domains/motifs that bind mono- and polyUb have been identified (reviewed in refs.^{5, 6}). Although evolutionarily distinct and structurally unrelated, most of them target the same hydrophobic-patch region on Ub's surface, comprising residues Leu8, Val70, and Ile44. The ability of Ub to interact with such a variety of receptor molecules is remarkable. A detailed understanding of the structural determinants of the binding properties of Ub is therefore required, in order to be able to predict its interaction with various downstream receptors, as well as the ability of polyUb chains to act as diverse signals. Of particular interest are proteins that act as the proteasomal receptors for polyUb chains (reviewed in ref.⁷). Two well studied examples in yeast are: Rpn10 (S5a in mammals), a subunit of the 19S regulatory complex^{8, 9}, and Rad23^{10, 11}. Unlike Rpn10, Rad23 is not an integral subunit of the proteasome. Rad23 associates with the proteasome by binding to the proteasomal subunit Rpn1^{12, 13}. Both receptors can bind Lys48-linked polyUb¹⁴, although neither of them is essential for yeast cell viability. Rpn10 binds Ub via a ubiquitin-interacting motif (UIM), while Rad23, a UBL-UBA type protein, contains two ubiquitin-associated (UBA) domains capable of binding Ub. Structurally, UIM is a single helix, while UBA domains are three-helix bundles¹⁵.

Studies in the past have focused on the identification of functionally important surface residues in Ub (e.g. ref.¹⁶). To test for essential interaction surfaces on Ub, all 63 surface side chains were individually mutated to Ala and tested as the sole Ub to support yeast growth¹⁷. Interestingly, only 16 of these mutations failed to support yeast growth. While these studies have elucidated critical interaction patches on the surface of Ub, little is known about how Ub's stability and dynamics impact its function.

Ub is extremely stable to heat denaturation; temperatures close to 100 °C are required to unfold it¹⁸. This melting temperature is unusually high for a protein from organisms that live at moderate temperature¹⁹. In addition to its extreme thermal stability, Ub is highly rigid, based on amide proton exchange measurements²⁰. These unusual physical properties may be important for Ub's function, or they may be a random byproduct of evolution.

To address the question as to how the high stability and rigidity displayed by Ub impact its function, we made point mutations (L67S and L69S) aimed at disrupting packing in the hydrophobic core of Ub. Using biophysical and structural methods we show that the mutations reduced stability and rigidity of Ub with minimal effects on its overall three-dimensional structure. *In vitro* analyses utilizing these mutant Ubs demonstrated that they unexpectedly had remarkably specific defects. For example, mutant Ubs could be conjugated to a substrate (Sic1) by the RING-based Ub ligase complex SCF^{Cdc4}, but not by the HECT domain Ub ligase complex Ubc4-Rsp5 (on PYPSic1). Although conjugates were assembled on Sic1 by Cdc34–SCF^{Cdc4}, they were not processed by purified 26S proteasomes. Remarkably, binding of mutant UbSic1 to the UIM-containing proteasomal receptor Rpn10 was abrogated, whereas binding to the UBA-domain containing Rad23 was unimpaired. Our NMR studies provide structural insight into this unprecedented discriminatory binding by the two Ub-binding domains (UBDs). Complementary to our *in vitro* analyses, we find that these mutants are unable to

support growth of budding yeast when provided as the sole source of Ub. Our studies reveal that mutations that perturb the buried core of Ub can have exquisitely specific effects on the different functions of Ub, a result unanticipated from prior mutational analysis of the surface residues of Ub.

Results

Core mutations altered the stability of ubiquitin, but preserved its overall fold

The L67S and L69S mutants and wild type Ub had similar CD spectra at 25 °C indicating that at this temperature all three proteins were folded and had similar secondary structure content (Fig. 1a). Also, both mutants and wild type Ub cooperatively unfolded when exposed to increasing concentrations of the denaturant guanidinium chloride (Fig. 1b) confirming that all three proteins were folded in the absence of denaturant. Thermodynamic fits of the data showed that compared to wild-type Ub ($\Delta G_{\text{unfold}} = 23.6$ kJ/mol), the stability of L67S (10.1 kJ/mol) and L69S (11.6 kJ/mol) were both reduced by more than 50%. The thermodynamic destabilization of L67S and L69S is likely due to loss of favorable leucine interactions in the folded state (hydrophobic and van der Waals) and the unfavorable burial of a polar hydroxyl group from the introduced serine (see also below). Despite this large thermodynamic destabilization, both L67S and L69S remained well folded at temperatures up to 55 °C as judged by circular dichroism (Fig. 1c). For comparison, wild-type Ub remained well folded at temperatures approaching 100 °C at neutral pH¹⁸. While the stability of L67S and L69S was greatly reduced relative to wild type, these mutants were well folded at physiologically relevant temperatures.

To directly verify the overall structural similarity between the wild type and mutant Ubs, we determined the three-dimensional structure of L69S in solution by NMR (see Materials and Methods). The resulting ensemble of 10 lowest-energy NMR structures was indeed very similar to that for the wild type Ub (Fig. 1d, e). Because the mutations involve only buried side chains, the exterior surface mimics wild-type Ub (Supplementary Fig. 1). According to the current understanding of the structure-function relationship in Ub the surface of Ub mediates its binding to UBDs. Therefore, based on the structural and surface similarity, one would expect the mutants to be functional.

Mutant Ubs are lethal for budding yeast when provided as the sole source of Ub

We tested the effect of expressing L67S or L69S Ub in yeast. In the SUB328 strain wild-type Ub expression is controlled from a galactose-induced promoter and can be shut off by dextrose. We introduced our mutant Ub expressed from a strong constitutive GPD promoter on a high-copy plasmid. When grown on medium containing galactose, the mutants did not prevent or slow yeast growth (Fig. 2). This result indicates that neither L67S nor L69S Ub is toxic to cells containing wild-type Ub. However, when wild-type Ub expression was shut off, neither L67S nor L69S Ub rescued growth. This result is surprising and indicates that L67S and L69S are defective for some essential process in yeast.

Expression and ubiquitination levels of L67S and L69S in yeast depleted of wild-type Ub

To gain molecular insight into the growth arrest phenotype upon shutoff of wild-type Ub we used Western Blot analysis to monitor the level of free Ub as well as high-MW ubiquitinated species (Fig. 2c). Coomassie staining of a replicate gel confirmed that similar amounts of total protein were loaded in each lane, and Western blots of purified WT, L67S, and L69S Ub confirm that the antibody detects the mutant proteins equivalent to the wild-type protein (Supplementary Fig. 2). Expression of wild-type Ub from this plasmid system was engineered to be similar to that of Ub from its endogenous promoters²¹. When wild-type Ub was present, neither L67S nor L69S perturbed the steady state level of high-MW ubiquitinated species,

suggesting that either the mutants were not incorporated into these high-MW species or they were incorporated along with wild-type Ub into chimeric chains that can be processed inside cells. After sufficient time in dextrose media to stall growth (25 hours – Fig. 2a,b), cells lacking any Ub rescue plasmid were depleted of free Ub and had low levels of high-MW ubiquitinated species (Fig. 2c,d). In contrast, cells expressing L67S or L69S had a large store of free mutant Ub that was greater than the level of free Ub in cells expressing wild-type Ub (Fig. 2c). This result indicates that yeast growth is not impeded by low expression levels of the mutants and instead is hindered by loss of subsequent interactions of the mutant Ubs with other cellular components. Cells containing L67S or L69S as the sole source of Ub had increased levels of high-MW ubiquitinated species, suggesting that the mutant Ubs were activated by E1 and assembled into chains by E2 and E3 enzymes, but that polyUb chains composed of mutant Ub subunits were not metabolized properly.

Functional assays *in vitro*: Chains assembled from mutant Ub can be recognized by UBA-containing Rad23, but not by UIM-containing Rpn10

To determine the molecular basis of the observed *in vivo* lethality of the Ub mutants, we performed functional *in vitro* assays using Sic1 as a substrate. The results (Fig. 3a) confirm the *in vivo* results by showing that single Ub mutants could be charged by E1/E2 (Cdc34) and conjugated onto a substrate (MbpSic1) by SCF to generate high molecular weight conjugates that were indistinguishable from those formed with wild-type Ub. However, these high-MW conjugates were not degraded by purified 26S proteasomes (Fig. 3b). To determine whether this was due to a failure of the proteasome to recognize polyubiquitinated MbpSic1, we assayed binding of these chains to the proteasome substrate receptors, Rpn10 and Rad23. The results (Fig. 3c) show that mutant-ubiquitinated MbpSic1 did not bind to the UIM-containing receptor protein Rpn10, but was still capable of binding to Rad23. This result by itself can explain the stability of Sic1 conjugates formed with mutant Ub, because proteasomes lacking Rpn10 are unable to degrade Sic1 *in vitro* ¹¹.

In contrast to Rpn10, Rad23 is an “extrinsic” receptor, and assays with 26S proteasome purified from *rpn10Δ* cells showed that their inability to degrade UbSic1 can be rescued by Rad23, provided the VWA domain of Rpn10 is provided *in trans* ¹¹. However, Sic1 conjugated with mutant Ub was not degraded by *rpn10Δ* 26S proteasomes supplemented with Rad23 and the VWA domain (data not shown). We speculate that despite the ability of Rad23 to bind the mutant chains, the inability of purified proteasomes to degrade Sic1 conjugated with mutant chains suggests that other events downstream from the polyUb chain recognition step are affected by the mutations. Since degradation of Ub-conjugated substrates is essential, this defect could suffice to explain the inability of the mutant Ubs to sustain life.

To explore in more depth whether the mutant Ubs might have other defects, we tested their ability to be conjugated by Ubc4–Rsp5. Whereas SCF is representative of the largest class of ligases that is based on a RING domain, Rsp5 is representative of the second, mechanistically distinct branch of ubiquitin ligases whose activity is dependent upon a HECT domain ²². As shown in Fig. 3d, the Ub mutants could not be conjugated to the PYP-reporter substrate using Rsp5 and Ubc4. This is in contrast with our results for the Cdc34–SCF combination and indicates that the Ub mutations have differential effects on different Ub conjugation pathways. The inability of Ubc4–Rsp5 to conjugate mutant Ub is unlikely to account for the lethality of the L67S and L69S mutations, because the addition of unsaturated fatty acids to growth medium, which suppresses the essential requirement for Rsp5 ²³, did not allow for growth of cells in which L67S and L69S were the only source of Ub (Fig. 2a). Given the central role of Ub in many different essential biological processes, the L67S and L69S mutants could be defective in multiple pathways.

NMR studies of ligand binding to L69S Ub

The observed ability of mutant polyUb chains to discriminate between Rpn10 and Rad23 is surprising. To verify that this specificity happens at the level of the individual Ub units (as opposed to polyUb chains), we examined binding of monomeric Ub mutants to UIMs of S5a and to the UBA1 domain of hHR23A, a human homologue of Rad23.

Consistent with the binding assays presented above (Fig. 3a), no measurable perturbations were observed in L69S spectra upon titration with increasing amounts of the UIM-2 motif from S5a (residues 263-307), thus indicating a lack of binding (Supplementary Fig. 3). Also no binding was observed between L67S and an S5a construct containing both UIM-1 and UIM-2 motifs (Supplementary Fig. 3). It is worth mentioning here that while UIM-1 of S5a is highly homologous to the single UIM of Rpn10, the UIM-2 has a lesser sequence homology to Rpn10 and binds wild type Ub and chains stronger than UIM-1 does^{9, 24}.

In contrast with UIM-2, titration with the UBA1 domain of hHR23A resulted in strong perturbations in the ¹H-¹⁵N HSQC spectra of L69S (Fig. 4a,c). The largest chemical shift perturbations (CSPs) were observed in residues Thr7, Leu8, Ile13, Arg42, Leu43, Ile44, Lys48, Gln49, and Val70. In addition, several amides (most notably, Thr7, Leu8, Thr9, Lys11, and Leu71) showed strong signal attenuations indicative of an intermediate or slow exchange (on the NMR time scale) between the free and bound states of L69S. The perturbed surface is located on one side of Ub structure (Fig. 4d) and includes the same hydrophobic patch (Leu8-Ile44-Val70) involved in UBA1 binding to WT Ub (Fig. 4f)^{25, 26}. The same surface of WT Ub is also involved in its interactions with many other Ub-binding proteins (e.g. ref.¹⁵) including UIM-2 of S5a^{24, 25, 27}. Titration curves were fit to a single-site 1:1 binding model yielding an average dissociation constant of $161 \pm 29 \mu\text{M}$ (Fig. 4b, Supplementary Table 2). This K_d value is 2-3 fold smaller than reported for UBA1 binding to WT yeast Ub ($\sim 500 \mu\text{M}$ ²⁶) or to WT human Ub ($310 \pm 20 \mu\text{M}$ ²⁵), which could be due to additional interaction between UBA1 and L69S involving $\beta 5$ strand residues Val70 and His68 of Ub (see below).

Structural differences between wild type and L69S Ubs

The observed striking effect of L69S and L67S point mutations on the ligand specificity of Ub is unprecedented. Given the overall structural similarity of the proteins and identical composition of the surface residues, this effect is likely caused by subtle changes in Ub structure and/or differences in protein dynamics introduced by these core mutations.

In order to understand the structural basis of the observed effect we analyzed in detail structural differences between L69S and wild type Ub. The backbone atoms of the lowest-energy structure of L69S Ub superimpose with those of WT solution structure (PDB code 1D3Z²⁸) with the rmsd of 1.14 Å (residues 2-71) or 0.93 Å for the secondary structure elements. These differences are significantly greater than the rmsds within each ensemble of structures (Supplementary Table 1). Figure 5a shows a direct comparison of the L69S and WT Ub structures, superimposed by the α -helix and the strands $\beta 1$ and $\beta 2$ (backbone rmsd = 0.76 Å for the superimposed residues). These elements were chosen because the distance between them remains almost unchanged upon the mutation (Supplementary Table 3); in contrast, there is a significant increase in the distance between the α -helix and strands $\beta 3$ and $\beta 5$. This superimposition emphasizes an important structural difference between the two proteins, in that the strand $\beta 5$ in L69S is tilted and displaced away from the core of the protein. Distance analysis indicates that the C-terminal part of this strand (residues 68-71) is on average approximately 2 Å farther away from the α -helix (C α of Leu30) than in WT Ub. This displacement of $\beta 5$ away from the core is not unexpected, given the hydrophobic side-chain of Leu69 that “anchors” this strand to the core in WT Ub (Fig. 5a) is replaced by a shorter,

polar Ser side-chain in the mutant. Several independent lines of evidence presented below indicate that, although subtle, these differences between the wild type and L69S Ubs are real.

Spectral differences between the mutants and WT Ub—Chemical shifts reflect local electronic environment of a nucleus under observation, and amides are particularly sensitive to changes in both secondary and tertiary structure of a protein. The ^1H - ^{15}N HSQC spectra of both WT Ub and L69S Ub (Fig. 5b) show well-dispersed signals indicative of a well-defined tertiary fold with a significant β -sheet content, characteristic of Ub. Surprisingly, however, a large number of amides, not only those adjacent to the site of mutation, show significant perturbations in L69S (Fig. 5c), well above the experimental uncertainty in signal positions. This dissimilarity between the two spectra exceeds the level typically observed for a point mutation of surface residues (see e.g. Supplementary Fig. 4), where most perturbations are typically local, and suggests a possibility of a rearrangement in the core of the protein and/or in its tertiary structure.

Particularly large resonance shifts are observed in Ser69 and Val70 (located in the strand $\beta 5$) as expected due to the mutation. Less expected are strong CSPs in Arg42 and Thr7, located in the strands $\beta 3$ and $\beta 1$ flanking $\beta 5$ on both sides and in a close proximity to the site of mutation (Fig. 5d). These CSPs likely indicate disruption in the hydrogen bond contacts (supported by the H-D exchange data, see below) between the strands, specifically, between Val70 and Arg42 (both NH and CO groups of these two residues are involved) and between NH of Leu69 and CO of Lys6 (the peptide plane that includes NH of Thr7). The strong CSP in Thr7 could also reflect the fact that in the WT Ub this residue makes side chain van der Waals contacts with Leu69. Intriguingly, significant changes in amide resonances are also observed in Ile30 in the α -helix, Ile13 in strand $\beta 2$, and Ile36 in the $\alpha/\beta 3$ loop. In WT Ub, the side chains of Ile30 and Ile13 extend into the protein core forming hydrophobic contacts with the side chains of Leu69 and Ile36 (Fig. 5e). Since the side chain of Ile36 contacts side chains of Gln41 and Leu69 in WT Ub, the large shift in the resonance of Ile36 in L69S can be attributed to the mutation as well as changes in the relative positions of $\beta 3$ and $\beta 5$. This is further corroborated by a strong CSP of the side chain amino group (NH_2) of Gln41 (Fig. 5b,c), suggesting a substantial change in the local environment of this side chain. Indeed, Gln41 side chain is oriented differently in L69S and WT Ub, although in both cases it points toward the protein core. Another strongly perturbed residue is Leu56, located proximal to the N-terminus of the α -helix, and also involved in the formation/stabilization of the core of Ub. All these spectral perturbations agree with the rearrangements in the core of Ub which involve change in the relative positions of the β -strands and the α -helix.

Interestingly, the ^1H - ^{15}N HSQC spectrum of L67S also shows significant differences from that of WT Ub (Supplementary Fig. 6). The CSP pattern in L67S is distinct from that of L69S, with generally fewer perturbed residues, but with a greater number of strong perturbations ($\Delta\delta > 1$ ppm). Strong signal shifts were observed in L67S in most of the residues in strand $\beta 5$, as well as in Phe4 and Gln2, both located in the $\beta 1$ strand and (in WT Ub) hydrogen-bonded to Leu67/Ser65 and Glu64, respectively. These CSPs likely reflect a perturbation in the hydrogen bonding between $\beta 5$ and flanking it strands $\beta 1$ and $\beta 3$ (Supplementary Fig. 6). Smaller perturbations are observed in the α -helix, consistent with the CD spectra indicating a closer correlation in the secondary structure between WT Ub and L67S, compared to L69S.

Residual dipolar coupling—The ^1H - ^{15}N residual dipolar couplings (RDCs) report directly on the orientation of the NH bonds in a protein and therefore are highly sensitive to structural changes. Our analysis shows that the structure of L69S, which is in excellent agreement with the RDCs measured for this protein (and included in its refinement), agrees less well with the experimental RDCs for WT Ub, and *vice versa* (Supplementary Fig. 7). Thus, the correlation coefficient between the experimental RDCs for L69S and those calculated for WT Ub is 0.97,

while it is 0.99 for L69S structure; the quality factors²⁹ are 0.19 and 0.09, respectively. Overall, the RDC differences for the WT structure are approximately a factor of 2 bigger than for L69S. In a reverse comparison, using the published experimental data for WT human Ub (from ref.²⁸), the corresponding correlation coefficients are 0.998 and 0.95, for WT Ub and L69S, respectively, and the quality factors are 0.042 and 0.193. All these discrepancies between the experimental RDCs for one protein and predicted for the other indicate subtle although measurable structural differences between the two proteins, resulting in a different orientation of NH bonds in some of the structural elements.

The rearrangement in the local position/orientation of the β 5 strand with respect to strands β 1, β 2, and β 3 and the α -helix would change the topography/landscape of the part of Ub's surface that includes the hydrophobic patch, and therefore could have a profound effect on its binding properties, as discussed below. The displacement of β 5 away from the protein core is also expected to weaken its hydrogen-bond contacts with the neighboring strands β 1 and β 3, which would further enhance the destabilizing effect of the mutation.

The effect of mutations on the rigidity and dynamics of Ub

H-D exchange indicates a reduced rigidity of the mutant—To verify the weakening in the inter-strand hydrogen bonding in L69S, we assayed hydrogen protection factors by monitoring H-D exchange in L69S Ub as well as in WT Ub, as a control. A relatively slow H-D exchange was observed in the β 1 and β 2 strands, consistent with the presence of hydrogen bonds between these strands. However, all β 5 residues as well as residues 40-43 in β 3 showed fast H-D exchange in L69S Ub, thus indicating that the N-terminal part of β 3 is not protected by hydrogen bonding with β 5. The fast exchange in these parts of the L69S mutant is in stark contrast with that in WT yeast (Fig. 6) and human Ub (data not shown, see also ref.²⁰). The observed 7-646 fold increase in the H-D exchange rate in L69S (Fig. 6b) clearly indicates a reduction in the hydrogen protection as a result of the structural changes introduced by the mutation. The location of the affected residues agrees with the displacement of β 5 in the L69S structure. Intriguingly, there is also an increase in the H-D exchange in amides of the α -helix residues 29-31, which also show chemical shift perturbations as a result of the mutation.

Based on the H-D exchange data, of the possible hydrogen bonds between β 5 and the neighboring strands present in WT Ub, only Glu64-Gln2, Ile44-His68, and Phe4-Ser65 (donor-acceptor) pairs were included in the structure calculation of L69S. For these pairs we also observed interstrand NOEs consistent with hydrogen bonding. It is worth mentioning here that these hydrogen bond constraints had little effect on the relative positioning of strands β 1, β 3 and β 5, which is well defined by the network of NOE constraints. Thus, calculations with no β 5- β 3 or even β 1- β 5- β 3 hydrogen bonds yielded L69S structures very similar to the one presented here, they superimpose with the backbone rmsd of 0.5 Å or 0.4 Å, respectively (residues 2-71). We also observed interstrand NOEs consistent with possible other hydrogen bond pairs (Leu69-Lys6, Arg42-Val70, His68-Ile44, Lys6-Leu67); however, given the fast H-D exchange in the amides these likely represent transient NOEs and were not included in the calculation.

L69S has a well-folded structure—As shown above, the mutations reduced the thermodynamic stability and structural rigidity of Ub. To independently verify that the L69S mutant is a well-folded protein under the conditions of our studies, we measured ^{15}N R_1 and R_2 relaxation rates and heteronuclear $^{15}\text{N}\{^1\text{H}\}$ NOEs in L69S and in WT Ub, as control. These parameters report on the overall rotational diffusion of a protein (reflecting its size and shape) as well as the backbone dynamics on a ps-ms time scale. The overall similarity between the mutant and WT Ub in their ^{15}N relaxation data (Fig. 7) clearly indicates that L69S Ub behaves as a well-folded protein. This conclusion is further supported by a detailed analysis of the

relaxation data that yielded very similar values (~5 ns) of the overall rotational correlation time for L69S and WT, thus suggesting a similar size of both proteins.

Comparison of the backbone dynamics in L69S and WT Ub—The values of the backbone order parameter (S^2 , Fig. 7d) in L69S are very similar to those in WT Ub and typical for a well-folded protein, thus indicating retention of structural rigidity on a ps- μ s time scale despite the lesser temperature and chemical denaturation stability of the mutant. Interestingly however, several amides in L69S show significantly elevated R_2 values indicative of conformational exchange motions on a μ s-ms time scale. Indeed, the analysis of ^{15}N relaxation data (Fig. 7e) indicates the presence of conformational exchange (on a μ s-ms time scale) in Ser28, Lys29, Ile30, Lys33, Glu34, Thr55, and Thr56 in L69S but not in WT Ub. The location of these sites, in the middle and the C-terminus of the α -helix as well as in the $\beta 4/3_{10}$ loop flanking the N-terminal end of the α -helix, combined with the fact that the side chains of most of these residues face the hydrophobic core of Ub, suggest that the observed conformational exchange could reflect some μ s-ms -time scale rearrangements/motions in the hydrophobic core of the mutant. Note that many of these residues (30, 33, 34, and 56) show strong CSPs in L69S and some of them (Lys29, Gln31) have elevated H-D exchange rates (Fig. 6b). The presence of R_{ex} terms in most of these residues was independently confirmed by the comparison of ^{15}N R_2 s with the ^{15}N CSA/dipolar cross-correlation rates (not shown). It should be pointed out that the conformational exchange motions are not unique to the L69S mutant, as some of the residues (especially Asp24) show strong R_{ex} contributions both in L69S and WT Ub (Fig. 7e).

The findings that the L69S mutation has practically no effect on the fast (ps-ns) local backbone dynamics, some effect on slow (μ s-ms) motions, and a dramatic effect on much slower motions (seconds and longer) suggest that while the secondary structure elements remain essentially intact, their contacts and packing which form the tertiary structure of Ub are affected.

Discussion

Our biochemical, biophysical, and *in vivo* assays demonstrate that the Ser mutations of Leu67 and Leu69 have a dramatic effect on Ub's function, stability, and the slow time scale dynamics. Although the effect of these mutations on the three-dimensional structure of the protein is modest, the displacement/rearrangement of the β strands that form the hydrophobic patch-containing face of Ub is the likely structural basis for its altered binding properties. In fact, this surface comprising the Leu8-Ile4-Val70 hydrophobic patch is targeted by most currently known UBDs (e.g. refs. ^{5, 6}), and the structural features of this surface should be critical for Ub's ability to recognize a vast variety of structurally different ligands. Mutations in these hydrophobic patch residues have been shown to have a strong effect on Ub function¹⁶ and cell growth¹⁷. Our results indicate that even more subtle modifications that preserve the amino acid composition of the surface but alter the dynamics of the protein and the structure/topography of its surface could have a profound effect on the binding specificity. Sequence alignment (Supplementary Table 4) shows that Leu is conserved in Ub at positions 67 and 69 in all eukaryotes, and there is also high sequence homology in both positions amongst the UBL domains. This implies that hydrophobic residues in strand $\beta 5$ at positions analogous to 67 and 69 play a significant role in stabilizing the β -sheet not only in ubiquitin but possibly in the UBLs.

Structural data provide clues to altered binding specificity of the mutant

Our structural data provide clues to altered binding specificity of the mutant. As shown in the Results section, the L69S mutation, while preserving the overall three-dimensional structure of Ub, caused perturbations in the structural arrangement and hydrogen bond contacts between

$\beta 5$ and the adjacent strands $\beta 1$ and $\beta 3$. This weakened the stability of the protein and dramatically increased its flexibility on the slow time scale, while the size of the protein and the subnanosecond backbone dynamics remain unaffected.

Why does L69S lack binding affinity for UIM?—To understand possible reasons for the lack of Rpn10 and S5a's UIM-2 binding to the mutants, we superimposed our L69S Ub structure onto the solution structure of the WT Ub/UIM-2 complex (1YX6.pdb)²⁴. When bound to WT Ub, UIM-2 lies along the $\beta 5$ strand, forming hydrophobic contacts with Val70 and His68 via its IAYAM motif, as well as electrostatic contacts with Arg72 via Glu283. The complex is further stabilized by favorable interactions of UIM-2 with several residues in the adjacent $\beta 1$ and $\beta 3$ strands of Ub, in particular, with Leu8 via Tyr289, Arg42 via Glu283, Ala46 via Leu295, and Ile44 via Ala290, Met291, and Met292. The superimposed model (Fig. 8a) suggests that the steric clashes caused by the displacement/elevation of $\beta 5$ could weaken or disrupt these interactions, e.g. by moving the UIM farther away from the rest of the hydrophobic patch. The ~ 2 Å elevation of the C-terminal part of $\beta 5$ above the β -sheet of Ub is expected to affect UIM's electrostatic interactions with Arg42, as well as the van der Waals contacts with Leu8 and possibly Ala46, thus effectively sterically hindering UIM's binding to Ub. A superimposition with the Ub/UIM-1 complex (1YX6.pdb)²⁴ (Fig. 8c) suggests that a similar conclusion holds for L69S binding to UIM-1 of S5a. Due to the sequence similarity between the UIM of Rpn10 and UIM-1 of S5a, it is natural to expect that this could also explain the lack of L69S binding to Rpn10. Moreover, the analysis of all currently available structures of Ub:UIM complexes (Supplementary Fig. 8) shows that other UIMs also bind to and are positioned along the $\beta 5$ strand; thus their binding could be affected by this mutation.

Why does L69S retain binding affinity for UBA domain?—According to the NMR-based model of the WT Ub:UBA1 complex²⁶, UBA1 contacts Lys6, Leu8, and Gly10 of the Ub's $\beta 1/\beta 2$ loop via His192, while the side chains of Met173 and Tyr175 contact Ile44 and Arg42 in $\beta 3$, respectively. Chemical shift perturbation in Val70 of WT Ub when bound to UBA1 (compared with free Ub) is below the level of significance²⁶. Moreover, no direct sidechain-to-sidechain contacts with $\beta 5$ -strand residues are present in the WT Ub:UBA1 complex²⁶. In contrast, when UBA1 is added to L69S, the largest CSP is observed in Val70 (Fig. 4c). In addition, strong signal attenuations (>70%) are present in Thr7, Leu8, Thr9, Lys11, and Leu71, indicating strong interaction of UBA1 with residues in the $\beta 1/\beta 2$ loop, as well as with the C-terminus of $\beta 5$. This is consistent with our structure of L69S. Indeed, superimposition of the L69S structure on the WT Ub:UBA1 complex (Fig. 8b) reveals that the elevation of $\beta 5$ in L69S positions Val70 properly to form a hydrophobic interaction with Met173. Also, His68 of L69S Ub is positioned properly for a hydrogen bonding interaction with Glu168. These additional interactions may be responsible for the observed increase in the binding affinity of L69S for UBA1 (see above) compared with the reported data for WT binding to UBA1^{25, 26}. A similar superimposition with the UBA2/Ub structure (Fig. 8d) suggests that L69S is expected to bind UBA2 of hHR23a as well. This is in striking contrast with the UIM:Ub complex discussed above (Fig. 8a,c), where the displacement of $\beta 5$ is expected to cause steric clashes with the UIM.

It should be pointed out here that the structural factors discussed above presumably affect the enthalpy of binding. A complete picture should account for the effect of the mutation on the entropic cost of binding. The increased structural flexibility on a slow time scale (obvious from the increased H-D exchange) suggests lower activation barriers separating various conformational states, and possibly a greater conformational ensemble available to the mutant in the native state. This would then imply a greater entropic cost associated with the rigidification of the protein upon ligand binding. Further studies are necessary to fully unveil the nature of the observed binding selectivity of the two Ub mutants studied here.

A clue to the sequence conservation of Ub

The inability of L67S and L69S to support yeast growth is surprising, given the modest changes in the structure and dynamics of these mutants relative to wild-type Ub. These results demonstrate that Ub's function is exquisitely sensitive to small perturbations in its structure and dynamics. Ub is one of the most highly conserved proteins in the eukaryotic kingdom, with yeast and human Ub differing in only 3 out of 76 residues. Our results provide a rationalization for this unusually high degree of sequence conservation. Ub must interact with a wide array of different proteins to perform its biological functions. Given Ub's small size and its large number of binding partners, we posit that there is extraordinary pressure on Ub to retain its sequence, because even relatively modest structural perturbations caused by mutations in residues that are not surface-exposed can compromise the binding of some partners (Rpn10) but not others (Rad23). Ub is a central component in many different biological pathways. Theoretical studies predict that protein hubs which intersect multiple pathways will be more sensitive to mutations than proteins that operate more independently³⁰. Perhaps this is the reason that even small perturbations to the structure and dynamics of Ub are not tolerated in the cell.

With respect to the ability of Ub to bind Rad23 but not Rpn10, the L67S and L69S mutants represent separation-of-function alleles that may be useful as tools to distinguish biological functions of Ub that depend on these two receptor proteins. If this discrimination extends to other UBA and UIM domain proteins, the L67S and L69S mutants may prove to be valuable reagents to identify effector functions of Ub that are read out by UIM domains. Indeed, analysis of all currently known complexes between Ub and UIM, UBA, and CUE domains (Supplementary Figs. 8, 9), suggests that the ability of L69S to discriminate between UIM and UBA could be a general feature of this mutant. Since a number of proteins involved in trafficking of cargo between different membrane compartments contain either a UBA or UIM domain (reviewed in ref. ³), the mutants could effectively be used to sort out steps in the endosomal pathway.

Ub is highly promiscuous with regard to its binding partners, ranging from small organic molecules like ubiquitin³¹ to protein domains⁶. The knowledge of what elements and features of Ub's surface contribute to this property of Ub is important for the understanding of what recognition signals various polyUb chains present. All binding data published to date have demonstrated that UIM and UBA domains can function interchangeably in binding polyUb, and both do not discriminate between K48- and K63-linked chains^{24, 32}. Biochemical studies presented here show, for the first time, that point mutations in its core bestow upon Ub the ability to discriminate between the two proteasomal Ub-receptor proteins, Rad23 and Rpn10.

Materials and Methods

Cell viability studies

To test for the ability of Ub mutants to support yeast viability we employed a promoter shutoff assay. SUB328²¹ cells (a kind gift of D. Finley) are deleted for all chromosomally encoded Ub. The sole Ub in these cells is supplied on a plasmid with a galactose (Gal)-regulated promoter. When Ub expression is shut off by growth in dextrose media, the cells stop growing. Into SUB328 cells, we introduced mutant Ubs (WT, L67S, L69S, or a control plasmid lacking ubiquitin) on KanMX marked 2 *micron* plasmids with a constitutive glyceraldehyde 3-phosphate dehydrogenase (GPD) promoter. Transformants were selected on yeast extract, peptone plus adenine (YPA) plates with 1% raffinose (Raf), 1% galactose (Gal) and G418 (200 µg/ml). The ability of the mutants to support yeast growth was tested by plating cells on YPA plates with 2% dextrose and G418. To monitor growth in liquid culture, cells were grown under

G418 selection in YPA Raf/Gal and transferred to YPA Dextrose. A hemocytometer was used to determine cell density as a function of time grown on dextrose.

To determine the level of mutant expression and ubiquitination, cells were grown in YPA Dextrose media for 25 hours. Cells were then collected and lysed by bead beating in 50 mM Tris, pH 7.5, 5 mM EDTA followed by the addition of SDS to 2%. After pelleting cell debris, the protein concentration in lysates was determined by the BCA assay (Pierce). Lysate containing 20 µg of protein was separated by SDS PAGE and Ub detected by Western Blotting with polyclonal αUb antibody 07-375 (Upstate Biotech). Western blots were quantified using a Fuji LAS-3000 Imager and Fuji image analysis software. Purified wild-type, L67S, and L69S Ub are all detected with similar sensitivity (Supplementary Fig. 2). For growth with unsaturated fatty acids, plates were prepared with 1 mM oleic acid (Nu-Chek Prep), or a mixture of 0.5 mM oleic acid and 0.5 mM palmitoleic acid (Nu-Chek Prep) solubilized with 1% tertigol type NP-40 (Sigma).

Preparation of ubiquitinated MbpSic1

MbpSic1 was purified from *E.coli*³³ and ubiquitinated as previously described³⁴. Briefly, MbpSic1 was phosphorylated using G1 Cdk complex expressed in insect cells and purified on glutathione beads. The supernatant containing phosphoMbpSic1 was then ubiquitinated by the Ub ligase complex SCF^{Cdc4} in the presence of E1, Cdc34, ATP, and Ub. The tetrameric SCF^{Cdc4} complex was expressed in insect cells and immunisolated on Polyoma beads (Cdc4 is tagged with the polyoma epitope).

Preparation of ubiquitinated PYPSic

Sic1 was converted from being a RING Ub ligase (SCF^{Cdc4}) substrate to a HECT Ub ligase (Rsp5) substrate by the insertion of the PY motif³⁵. It was purified from *E.coli* and ubiquitinated using E1, Ubc4, Rsp5, Ub and ATP. Both Ubc4 and Rsp5 were purified from *E.coli* as described³⁵.

Degradation of substrate

Ubiquitinated MbpSic1 was incubated with purified 26S proteasomes as previously described³⁶, and reactions were terminated by the addition of 5X Laemmli buffer. Degradation was monitored by running aliquots of the reaction on an 8 % gel, transferring to nitrocellulose, and visualizing Ub-conjugates with anti-Sic1 antibody.

Binding reactions

GST-Rad23 and GST-Rpn10, purified as described previously¹¹, were immobilized on glutathione beads, and incubated with ubiquitinated MbpSic1 for an hour at 4°C. Beads were washed, and bound fraction was analyzed by SDS-PAGE and immunoblotting for Sic1. Empty refers to beads that were not loaded with GST-tagged proteins.

Preparation of NMR samples

Unlabeled and uniformly isotope-labeled recombinant yeast Ub (L69S, L67S, and WT) and UBA-1 of hHR23a were expressed and purified as described in ref.³⁷. S5a constructs containing both UIM-1 and UIM-2 (residues 196-306) or only UIM-2 (residues 263-307) were expressed and purified as described in ref.⁹. NMR samples were prepared in 20 mM sodium phosphate buffer (pH 6.8), 7% D₂O, 0.02% NaN₃. S5a NMR samples contained, in addition, 150 mM NaCl.

NMR studies

All NMR measurements were performed on a Bruker DRX-600 spectrometer at 14.1 T and 25°C. The resonance assignments for L69S and L67S Ub were based on ^1H - ^{15}N HSQC, 2D TOCSY, and 3D ^{15}N -edited TOCSY spectra. $^1J_{\text{HNH}\alpha}$ couplings obtained from HMQC-J spectra³⁸ were used to confirm secondary structure assignment. Amide H-D exchange rates were measured in a series of ^1H - ^{15}N SOFAST-HMQC spectra³⁹ recorded for up to ~20 hours following resuspension of lyophilized protein in D_2O .

Binding was studied using NMR titration experiments performed as a series of ^1H - ^{15}N HSQC spectra recorded on ^{15}N -labeled L69S Ub as aliquots of a concentrated solution of the potential binding partner (UIM-2 or UBA-1) were added to solution. The starting concentration of the protein (L69S Ub) was 0.8-1.0 mM, and the titration continued until saturation in the shifts of the resonances (up to 4-fold molar excess of UBA-1). In a control titration of ^{15}N -labeled WT-Ub with UBA1, the starting concentration of Ub was 0.87 mM and the final UBA:Ub molar ratio was 4.6. Titration with UIM-2 started with 0.7 mM of ^{15}N -labeled L69S and continued until a 2:1 UIM:Ub molar ratio was reached. In another titration, L67S was added to 0.4 mM (starting concentration) of ^{15}N -labeled S5a construct (196-306) containing both UIM-1 and UIM-2 up to 2:1 Ub:S5a molar ratio. Combined amide chemical shift perturbation (CSP) was calculated as $\Delta\delta = [(\Delta\delta_{\text{H}})^2 + (\Delta\delta_{\text{N}}/5)^2]^{1/2}$, where $\Delta\delta_{\text{H}}$ and $\Delta\delta_{\text{N}}$ are shifts in ^1H and ^{15}N signals, respectively. Titration data were analyzed assuming a single binding site model as described³⁷.

The ^1H T_2 and ^{15}N relaxation measurements (T_1 , T_2 , and steady-state $^{15}\text{N}\{^1\text{H}\}$ NOE) were performed using standard methods⁴⁰. The overall rotational diffusion and the backbone dynamics of the protein were analyzed using programs ROTDIF⁴¹ and DYNAMICS⁴⁰ as detailed in ref. ⁴⁰.

Distance restraints for structure calculation were obtained from a 2D homonuclear NOESY spectrum. Residual dipolar couplings (RDCs) were determined by aligning proteins in the liquid crystal medium composed of a mixture of *n*-alkyl-poly(ethylene glycol) (C_{12}E_5) and *n*-hexanol as described⁴². The ^1H - ^{15}N couplings were measured using the IPAP ^1H - ^{15}N HSQC method⁴³ and extracted using the approximation of contour levels by ellipses⁴⁴. The RDCs were obtained from the difference in the ^{15}N - ^1H couplings observed in the oriented (25 °C) and in the isotropic phase (38 °C).

Structure calculation

Structure calculations for L69S Ub were performed using CNS⁴⁵ with ARIA⁴⁶ extensions based on the NOE-derived distance restraints, dihedral angle constraints predicted using TALOS⁴⁷, hydrogen-bonding constraints, and orientational restraints from RDCs. The statistics of the NMR constraints and of the final ensemble of 10 lowest-energy calculated structures are presented in Supplementary Table 1. Atom coordinates for L69S Ub have been deposited in the Protein Data Bank, the accession number is 2JWZ.

Protein stability studies

All CD data were acquired on a 62DS instrument (Aviv). To compare secondary structure content, Ub spectra were acquired in a 0.1 cm cuvette at a protein concentration of 50 μM in 20 mM potassium phosphate, pH 7.0. The stability of Ub variants was determined by titrating guanidinium chloride and monitoring loss of secondary structure using circular dichroism (CD). Stability measurements were made at 25 °C with a Ub concentration of 5 μM in a cuvette with a 1 cm pathlength. Samples were buffered at pH 7.0 with 20 mM potassium phosphate. Guanidinium chloride concentration was increased using an autotitrator and Ub unfolding was monitored based on the CD signal at 222 nm. Samples were equilibrated for 10 minutes

(determined to be greater than 3 times the half-life of relaxation at the midpoint of denaturation) and data were collected for 1 minute. Denaturation data were fit to a two-state transition from folded to unfolded employing linear pre and post-transition baselines as previously described⁴⁸ using the program Kaleidograph (Synergy Software).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Comparison of the structure and thermodynamic stability of purified wild type, L67S, and L69S ubiquitins. (a) Circular dichroism (CD) spectra at 25 °C indicate that all three proteins have similar secondary structure content. (b) Stability to guanidinium chloride denaturation monitored at 25 °C by loss of CD signal at 222 nm. Both L67S and L69S are greatly reduced in stability compared to wild type ubiquitin. (c) Temperature denaturation curves for L67S and L69S monitored by CD signal at 222 nm. Both mutants have $T_m \approx 72^\circ\text{C}$, and are well folded at temperatures below 55°C .

(d) The ensemble of 10 lowest-energy solution NMR structures of L69S determined here has strong similarity to that of wild type ubiquitin (PDB code 1D3Z²⁸, shown in panel e). The L69S structural ensemble is well defined with the root-mean-square deviation (rmsd) from the mean structure of 0.20 Å for the secondary structure backbone atoms and 0.79 Å for all heavy atoms over the ordered region (residues 2–71). The statistics of the experimental constraints and calculated structures are in Supplementary Table 1. The averaged (over all 10 structures) correlation coefficient between the experimental RDC data and those back-calculated for the derived ensemble of structures is 0.99, the averaged quality factor²⁹ is $Q = 0.088$. The four C-terminal residues (73–76) in both L69S and WT Ub are unstructured and highly flexible (see Fig. 7). The NMR data (NOEs, backbone dihedral angles) as well as the calculated structures

show that the same secondary structure elements are present in both L69S and wild type Ub structures.

Figure 2.

Ub mutants are incapable of sustaining yeast cell growth. (a) Comparison of SUB328 cell growth in RafGal media and under Ub expression shutoff in glucose media (Dextrose, Dextrose +unsaturated fatty acids). The type of Ub introduced on a plasmid is indicated (“none” corresponds to control plasmid lacking Ub). Also shown is growth of the *ole1* strain that is incapable of synthesizing unsaturated fatty acids. (b) Cell density as a function of time grown on dextrose. (c) Comparison of the accumulation of high-MW ubiquitinated species in SUB328 cells after 25 hours in RafGal and under cell growth arrest in dextrose media. (d) Quantitative comparison of the amounts of accumulated high-MW species for the various rescue plasmids used in this study.

Figure 3.

Functional *in vitro* assays. All assays were performed as described in the Methods section. (a) Single Ub mutants (L67S or L69S) could be charged by E1/E2 (Cdc34) and conjugated onto a substrate (MbpSic1) by SCF^{Cdc4} to generate high molecular weight conjugates that were indistinguishable from wild-type Ub conjugates. (b) The high-MW conjugates generated from Ub mutants are not degraded by the 26S proteasome. (c) The mutant high-MW conjugates bind to Rad23 but not to Rpn10. (d) Mutant Ub could not be conjugated to the PYP-reporter substrate using Rsp5 and Ubc4.

Figure 4.

NMR titration studies of UBA1 binding to L69S Ub. (a) Overlay of the ^1H - ^{15}N HSQC spectra of the free (black) and UBA1-bound (red) L69S at saturation (UBA1:Ub molar ratio is 4:1). (b) Representative titration curves for several amides (as indicated). (c) Chemical shift perturbations in L69S at the last titration point ([UBA1]/[Ub] = 4:1) as a function of residue number. Asterisks in (c) indicate residues that show >70% signal attenuation in the presence of UBA1. (d) Ribbon representation of Ub structure color-coded by the CSP values and with the side chains of Leu8, Ile44, and Val70 indicated. (e) Surface representation of Ub with the perturbed sites colored by CSPs as follows: $\Delta\delta > 0.4$ ppm (red), $0.4 > \Delta\delta > 0.3$ ppm (orange), and $0.3 > \Delta\delta > 0.16$ ppm (cyan), while residues showing significant signal attenuation (>70%) in the bound state are painted yellow.

Figure 5.

Structural and spectral differences between L69S and WT Ub. (a) A cartoon representation of the backbone superimposition of the structures of yeast L69S (green) and human WT Ub (blue). Residues 2-7 ($\beta 1$), 12-17 ($\beta 2$), and 23-34 (α) were used for the superimposition. Shown in ball-and-stick (cyan) is the side chain of Leu69 in WT Ub. The unstructured flexible C terminus (residues 73-76) is not shown. Atom coordinates of yeast WT Ub are not available in the Protein Data Bank, therefore the structure of the human variant was used for the comparison here. This is justified (1) by the comparison of the electron density maps indicating similar structures of yeast and human ubiquitin ⁴⁹, (2) by the fact the only difference in the amino acid sequence between the two WT variants is in three surface residues (19, 24, and 28) that are located away from structural changes introduced by the L69S mutation, and (3) by the observation that the HSQC spectra of yeast and human WT Ubs are very similar, with only local perturbations in the signals corresponding to mutated residues and those adjacent to them (as shown in Supplementary Fig. 5). (b) Superposition of the ^1H - ^{15}N HSQC spectra of L69S (red contours) and WT Ub (black contours). Shifted resonances are indicated. (c) Combined amide chemical shift differences between the two proteins as a function of residue number. The experimental uncertainty in $\Delta\delta$ is ≤ 0.02 ppm. The site of mutation is indicated by the arrow. (d) A cartoon representation of the 3D structure of WT Ub with the residues showing the biggest CSPs colored (from yellow to red, in increasing $\Delta\delta$). (e) Cartoon representation of the WT Ub structure, with the side chains of the perturbed residues forming or adjacent to the protein's core shown in stick representation.

Figure 6.

The results of H-D exchange assays. (a) Representative exchange kinetics for selected amides in WT (open symbols) and L69S (closed symbols). (b) The ratio of H-D exchange rates in L69S and WT ubiquitins. The inset zooms in on a smaller range of ratios. Not shown are data for several residues (marked with asterisks) where the ratio was > 1, but accurate quantification of the effect was not possible. These include Thr66 (β5) which exchanges essentially immediately in L69S (hence no reportable ratio) and Ile44 (β3); the latter amide did not exchange in WT Ub over the time course of the experiments (up to 20 h), but it did exchange although slowly in L69S. (c) A cartoon representation of the structure of Ub, with the residues showing significant increase in the exchange rate colored red (ratio > 100) and orange (ratio between 10 and 100). Overall, faster H-D exchange in L69S is localized to areas which show spectral perturbations as a result of the mutation, while the uninvolved sites retain their rigidity.

Figure 7.

Comparison of the experimental ^{15}N relaxation data and parameters of the backbone dynamics in L69S (red) and yeast WT Ub (black). Panels (a)-(c) depict longitudinal (R_1) and transverse (R_2) ^{15}N relaxation rates and the steady-state heteronuclear NOE as a function of residue number, while (d) and (e) show the values of the squared backbone order parameter (S^2) and the conformational exchange contribution term (R_{ex}). The overall rotational correlation time estimated from the ^{15}N relaxation data for L69S and WT Ub was 5.04 ± 0.4 and 4.95 ± 0.6 ns, respectively.

Figure 8. Structural superposition models show how the displacement of $\beta 5$ in L69S could affect its binding to UIMs of S5a but not to UBAs of hHR23A. Shown is a superimposition of L69S Ub structure on the known structures of WT Ub complexes: (a) Ub:UIM-2 (PDB: 1YX6) (b) Ub:UBA1, (c) Ub:UIM-1 (PDB: 1YX6), and (d) Ub:UBA2. WT Ub is colored blue, L69S Ub is green, the UIM or UBA domains are red. The Ub:UIM structures are from ref. ²⁴, the Ub:UBA docked models are from ref. ²⁶.