Characterization of Two Polyubiquitin Binding Sites in the 26 S Protease Subunit 5a*

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Ubiquitylated proteins are degraded by the 26 S protease, an enzyme complex that contains 30 or more unique subunits. One of these proteins, subunit 5a (S5a), has been shown to bind ubiquitin-lysozyme conjugates and free polyubiquitin chains. Using deletional analysis, we have identified in the carboxyl-terminal half of human S5a, two independent polyubiquitin binding sites whose sequences are highly conserved among higher eukaryotic S5a homologs. The sites are approximately 30-amino acids long and are separated by 50 intervening residues. When expressed as small fragments or when present in full-length S5a molecules, the sites differ at least 10-fold in their apparent affinity for polyubiquitin chains. Each binding site contains 5 hydrophobic residues that form an alternating pattern of large and small side chains, e.g. Leu-Ala-Leu-Ala-Leu, and this pattern is essential for binding ubiquitin chains. Based on the importance of the alternating hydrophobic residues in the binding sites and previous studies showing that a hydrophobic patch on the surface of ubiquitin is essential for proteolytic targeting, we propose a model for molecular recognition of polyubiquitin chains by S5a.

Ubiquitin is a small, highly conserved eukaryotic protein that can be covalently attached to a variety of cellular proteins including itself (1). Ubiquitlayment requires an activating enzyme E1, Ub carrier proteins E2s, and substrate recognition components called E3s (2, 3). Post-translational modification of proteins by ubiquitin may serve several functions. Addition of monomers of ubiquitin to histones or insect actin appears to affect the higher order structure of chromatin or flight muscle, respectively (4, 5). More recently, ubiquilayation has been implicated in endocytosis of the Ste2p yeast mating type receptor (6), and ubiquitin has been proposed to serve a novel nonproteolytic role in the regulated activation of IkBa kinase (7). However, the best characterized function of ubiquitin is to promote the degradation of proteins to which it is attached especially when substrates are bound to a polyubiquitin chain rather than individual ubiquitin moieties (8). Polyubiquitin chains are formed by isopeptide linkages involving the C-terminal glycine (Gly76) of one ubiquitin and a lysine ε amino group on another. A β-galactosidase fusion protein covalently attached to a polyubiquitin chain consisting of 8–12 ubiquitins linked by Gly76–Lys48 isopeptide bonds is degraded approximately 10 times more rapidly than the monoubiquitlated substrate (8).

The 26 S protease, which was discovered by its ability to degrade ubiquitin-lysozyme conjugates (9), is now known to be formed from two particles, the 19 S regulatory complex (10–13) and the 20 S proteasome (14, 15). The regulatory complex confers substrate recognition and energy dependence upon the 26 S enzyme, and the proteasome provides proteolytic activities. Although the 26 S protease has been shown to degrade native unmodified ornithine decarboxylase (16), this enzyme of polyamine metabolism is the only non-ubiquitin dependent substrate so far identified. By contrast the 26 S protease has been implicated in the degradation of scores of substrates modified by ubiquitin (17, 18). It seems, therefore, that polyubiquitin chains function as a general degradation signal, and, as such, they play key roles in a variety of regulatory events. For example, polyubiquitin recognition is required for degradation of many short lived and abnormal proteins (19). Ubiquitin-mediated proteolysis is also required at many stages in the cell cycle (20) and in the elimination of specific proteins during DNA repair (21, 22). Indeed, ubiquitin-mediated proteolysis has been implicated in processes as diverse as antigen presentation on major histocompatibility complex class I molecules (23–25) and proper telomere behavior during cell division (26). Discovering how polyubiquitin chains are recognized by the 26 S protease is central to understanding a number of regulatory mechanisms.

We previously identified a subunit (S5a) of the regulatory complex that binds ubiquitylated lysozyme and free polyubiquitin chains (27). Consistent with the preference of the 26 S protease for substrates attached to polyubiquitin chains, S5a selects for longer polyubiquitin species and has little apparent affinity for ubiquitin monomers (27–29). The discovery that Saccharomyces cerevisiae S5a is not an essential protein provides clear evidence that additional ubiquitin recognition components must be present in the 26 S protease (30). However, S5a is currently the only subunit of the 19 S regulatory complex shown to bind polyubiquitin chains, and recent experiments suggest that polyubiquitin chain recognition by the 26 S protease is substantially similar to recognition by isolated S5a.2 Thus, the S5a protein provides a general model for studying recognition of polyubiquitin chains by a 26 S protease component.

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1 The abbreviations used are: Ub, ubiquitin; PLuS, polyubiquitin binding site; PCR, polymerase chain reaction; NiNTA, nickel-nitrirole triacetate acid; MOPS, 4-morpholinepropanesulfonic acid; TBS, Tris-buffered saline; PAGE, polyacrylamide gel electrophoresis.

2 Beal, R. E., Toscano-Cantaffa, D., Young, P., Rechsteiner, M., and Pickart, C. (1998) Biochemistry, in press.
S5α retains the ability to bind ubiquitin chains even after it has been separated on a SDS-acrylamide gel and transferred to nitrocellulose. These properties of S5α led us to propose that readily renatured and repeated binding regions in S5α confer the ability to select for longer polyubiquitin chains (31). In support of the repeated motif model, the inferred amino acid sequences of S5α from fly, plant, and human encode conserved repeated motifs within their sequences (32−34). As a direct test for the presence of repeated polyubiquitin binding sites, we have carried out deletional mapping of the human S5α protein. These studies identify two regions of the protein able to bind polyubiquitin chains and provide evidence that the two sites bind polyubiquitin cooperatively.

**EXPERIMENTAL PROCEDURES**

**Generation of S5α Deletions**—The human S5α cDNA containing a ten histidine NH2-terminal tag (34) was cloned into a pET-26b vector (Novagen). The resulting plasmid construct expressed a protein consisting of the entire S5α protein plus an NH2-terminal 21-amino acid histidine fusion peptide. The amino acid residues are numbered using the original S5α methionine as the origin. For generation of NH2-terminal deletions, 5’ PCR primers were designed with NdeI restriction sites at selected positions within the S5α coding region. NH2-terminal deletion PCR products were generated using the NdeI 5’ primers with 3’ PCR primers containing a convenient restriction site. Restriction digestion, insertion into pET-26b, and expression in E. coli resulted in protein products fused to the NH2-terminal histidine tag sequence and lacking NH2-terminal S5α sequence.

Generation of C-terminal deletions involved a PCR reaction using a 5’ PCR primer upstream of the pET-26b promoter sequence and a 3’ primer containing a new stop codon followed by a BamHI restriction site. The C-terminal deletion PCR products were recloned into the expression pET-26b vector using the XbaI restriction site within the promoter sequence of the vector and a unique downstream BamHI site. Expression of the plasmid constructs resulted in protein products containing the entire NH2 terminal of S5α including the histidine tag but lacking the C-terminal sequence. Constructs that contained both C-terminal and NH2-terminal deletions were created by using the NH2-terminal deletion plasmids as PCR template sequences and the 5’ promoter PCR oligomer and the 3’ stop codon oligomers as primers. These were recloned back into pET-26b as described for the C-terminal deletions. All plasmids were sequenced to confirm the accuracy of the constructed fragments. Expressed deletional constructs were also confirmed by mass spectrometry. In all cases the apparent mass matched that expected for the designed recombinant protein.

**Site-directed Mutagenesis**—Site-directed mutations within the two polyubiquitin binding sites were generated using the Mutagenex phagemid in vitro mutagenesis kit (version 2) purchased from Bio-Rad (35, 36). Single-stranded DNA was generated by infection with R408 helper phage. The absence of unintended mutations in the constructs was confirmed by automated sequencing. The expressed mutant proteins were purified using Ni-NTA matrix (Qiagen).

**Expression of Constructs**—Plasmids containing S5α constructs were transformed into E. coli BL21(DE3). Cells were grown at 30 °C, and expression was induced by the addition of 300 μM to 1 mM isopropyl-β-D-thiogalactopyranoside followed by 2 h incubation at 30 °C. Approximately 300 ml of bacterial culture was harvested and lysed with a French press in 25 mM MOPS, pH 7.5, 150 mM NaCl, 50 mM sodium acetate, 1 mM dithiothreitol, and 0.025% Nonidet P-40.

**Ubiquitin Chain Binding Assay**—Polypeptides were immobilized by metal chelate chromatography on Ni-NTA beads or spin columns (Qiagen) using the NH2-terminal histidine tag present on the recombinant proteins. Polyubiquitin chain mixtures consisting either of dimers to >15 ubiquitins or monomers to ≥5 ubiquitins were labeled with [125I]Lys as described previously (37). The polyubiquitin chains were homopolymeric with successive ubiquitins linked by Lys48–Gly76 isopeptide bonds. For the binding assay, 600 μl of bacterial extract was centrifuged through a Ni-NTA column and then washed with TBS, 25 mM Tris-HCl, pH 7.5, 150 mM NaCl. The labeled ubiquitin chains were added. The washed Ni-NTA beads followed by incubation were centrifuged through 600 μl of 8 μl urea through the columns. The distribution of [125I]polyubiquitin was determined by direct γ counting of both columns and wash fractions. A final wash with 300 μl imidazole displaced the S5α polypeptides from the Ni-NTA columns.

The polyubiquitin chains were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The quantity of immobilized S5α polypeptides was measured by a Coomassie protein assay (Pierce) and by relative staining after SDS-PAGE. For binding assays a BL21(DE3) extract lacking expressed S5α polypeptide was used as a negative control. For binding studies using Ni-NTA beads, the S5α polypeptides were initially incubated with the beads followed by incubation with polyubiquitin chains for 0.5−2 h and rinsed twice for 10−15 min with TBS buffer at 4 °C. The chains were released from the beads by adding SDS sample buffer. Assays for polyubiquitin chain binding to proteins immobilized on nitrocellulose filters were performed as described previously (27).

**Lysozyme Conjugate Degradation Assay**—High molecular weight polyubiquitylated [125I]lysozyme and rabbit reticulocyte lysozyme were prepared as described previously (38, 39). Ubiquitylated [125I]lysozyme conjugates were preincubated for 10 min with purified S5α or S5α fragments at room temperature and then mixed with rabbit reticulocyte lysate containing 20 mM MOPS, pH 7.8, 4 mM ATP, 5 mM MgCl2, 10 mM KC1, 0.5 mM dithiothreitol. Proteolysis was measured by the appearance of trichloroacetic acid soluble 125I (38). Protein concentrations for the small polyubiquitin binding site (PUBS) polypeptides and S5α were determined using a modified Biuret method, ESL from Boehringer Mannheim. To determine the apparent stability of S5α fragments in reticulocyte lysate, the polyubiquitin chains were added to conjugate degradation assay mixtures containing all the components just described except for rabbit reticulocyte lysate. The mixtures were incubated at 37 °C, and aliquots were blotted onto a nitrocellulose membrane at various times. The nitrocellulose filters were then incubated with 125I-polyubiquitin chains and the relative binding activity of S5α or the PUBS was determined by phosphorimaging analysis of 125I-polyubiquitin bound to the Dot-bLOTS.

**RESULTS**

**Identification of Polyubiquitin Binding Sites Within S5α**—Western blots of 26 S protease subunits separated on two-dimensional gels revealed that anti-S5α reacted with the full-length subunit as well as with smaller species. The isoelectric points of the smaller S5α immunoreactive species conformed to those expected from progressive C-terminal deletions of the intact S5α protein. As a test for polyubiquitin binding to these smaller fragments, we separated subunits of the regulatory complex by one-dimensional SDS-PAGE, transferred them to a nitrocellulose filter, and incubated the filter with 125I-polyubiquitin chains or antisera specific for S5α. Full-length S5α and two of the smaller immunoreactive polyopeptides bound polyubiquitin chains. The most abundant S5α fragment, however, did not bind polyubiquitin chains, and it did not produce amino acids upon Edman degradation indicating that it comprised the amino-terminal portion of the subunit (data not shown).

From these initial results we suspected that the C-terminal half of S5α contained polyubiquitin binding site(s). Hence, we used PCR to produce two fragments of S5α that divided the protein roughly in half. Recombinant human S5α containing an NH2-terminal histidine tag has polyubiquitin binding properties equivalent to the S5α subunit isolated from the 26 S protease (34) allowing us to purify histidine-tagged full-length or truncated S5α molecules on Ni-NTA beads and to test directly for 125I-polyubiquitin chain binding. The initial recombinant fragments confirmed that residues Met1−106−Lys377 of S5α bound polyubiquitin chains (Fig. 1A), whereas NH2-terminal residues Met1−Ser211 did not. Further deletional mapping identified two distinct binding sites for polyubiquitin chains within the C-terminal half of S5α. We call the two regions PUBS1 and PUBS2 (Fig. 1B). Several regions of equivalent length or longer around the two PUBS did not bind polyubiquitin chains (e.g. residues Met224−Ser278, Met236−Leu342, Met329−Lys377, or Met107−Ser211).

**Comparison of Polyubiquitin Chain Binding Properties of S5α, PUBS1, and PUBS2**—Full-length S5α protein selects for long polyubiquitin chains (27, 31, 33). The chain length selection properties of each PUBS were examined by incubating...
Two Polyubiquitin Binding Sites in Human S5a

A schematic representation of Met^{106}–Lys^{377} of S5a with deletion constructs shown below as solid bars flanked by numbers that define NH2- and C-terminal residues. A plus sign indicates that the fragment bound polyubiquitin chains; a minus sign denotes the absence of binding. B, the amino acid sequence of the C-terminal portion of human S5a is presented in the one letter amino acid code. The sequences of PUbS1 and PUbS2 are underlined. Boxed residues approximate the minimal region within each site capable of binding polyubiquitin chains.

125I-polyubiquitin chains with equal amounts of S5a, PUbS1, or PUbS2 immobilized on Ni-NTA beads. Bound chains were then eluted and resolved by SDS-PAGE (Fig. 2). All three molecules selected for longer polyubiquitin chains. Whereas PUbS2 and S5a showed appreciable apparent affinity for ubiquitin-tetramers and trimers, PUbS1 had reduced apparent affinity for these shorter species. In these experiments the recombinant proteins were present on the beads at less than 10% saturation. Moreover, varying the density of bound PUbS on the Ni-NTA beads over a 9-fold range did not alter the chain length distribution except for a very slight increase in the longest chains observed at higher peptide concentrations (data not shown). Although these results support the assumption that selection for polyubiquitin molecules does not require spanning of chains between multiple PUbS molecules bound to the beads, this possibility cannot be ruled out.

Full-length S5a inhibits ubiquitin-lysozyme conjugate degradation in rabbit reticulocyte lysates providing a way to compare the PUbS that requires the S5a fragments to bind polyubiquitin chains in solution and thereby remove concern about possible polyubiquitin chain spanning. At 2 μM, full-length S5a significantly inhibited conjugate degradation; PUbS2 and PUbS1, by contrast, had only minimal effect at this concentration (Fig. 3A). Varying the levels of the three proteins produced clear inhibition of conjugate degradation by each with a 10-fold difference in potency between S5a and PUbS2 and a 100-fold difference between S5a and PUbS1 (Fig. 3B). The NH2-terminal half of S5a (Met1–Val208) did not inhibit conjugate degradation at concentrations up to 100 μM. Upon prolonged incubation PUbS1 and PUbS2 inhibited conjugate degradation to a lesser extent. This apparently reflects their degradation or modification in reticulocyte lysate since their binding activities decreased with half-lives of −15 min. Full-length S5a, on the other hand, was stable in lysate (data not shown). Therefore, inhibition by the PUbSs might reflect both their intrinsic affinity for polyubiquitin chains and their stability in the reticulocyte lysate.

Importance of PUbS1 and PUbS2 within the Intact S5a Protein—The deletion mapping in Fig. 1 identified two polyubiquitin binding sites in subunit S5a. However, additional sites could exist that require the intact protein for proper function. To examine this possibility and to measure the relative affinities of PUbS1 and PUbS2 for polyubiquitin chains, we mutated residues within each site. As shown below, each PUbS is centered on 3 large hydrophobic residues that alternate with alanines, and mutation of the bulky aliphatic side chains to alanines eliminates polyubiquitin binding. For PUbS1, Leu^{216}, Leu^{218}, and Leu^{220} were converted to alanines; in PUbS2 Ile^{287}, Tyr^{289}, and Met^{291} were converted to alanines. In addition, a full-length S5a protein was constructed with alanine tracts at both sites. These three mutated versions of S5a and the wild-type protein were then tested for their ability to bind polyubiquitin chains. It is evident from the autoradiogram in Fig. 3C that the doubly mutated S5a did not bind polyubiquitin chains (Fig. 3C, lane 3), indicating either that additional polyubiquitin binding sites are not present within the native subunit or that such sites depend on functional PUbS1 and/or PUbS2 for activity. The autoradiogram in Fig. 3C also clearly shows that whereas inactivation of PUbS1 moderately decreased ubiquitin chain binding to full-length S5a, alanine substitutions in PUbS2 produced dramatically reduced chain binding.

Because comparison of circular dichroism spectra between wild-type S5a and the mutant S5a with alanine substitutions at both sites revealed no detectable changes, the loss of chain binding should not be attributed to a general structured perturbation of the mutated 5a subunit. These striking differences between full-length S5a proteins with either PUbS1 or PUbS2 inactivated were also apparent when chain binding was assayed by Ni-NTA beads or when the blotted proteins were probed with ubiquitin-lysozyme conjugates rather than polyubiquitin chains (data not shown). At low concentrations, e.g., 40 ng on the blot, wild-type S5a retained 7-fold more polyubiquitin than expected from the sum of the independent sites, and this result suggests that PUbS1 and PUbS2 bind polyubiquitin chains cooperatively.

We also determined whether each full-length version of S5a could inhibit 125I-lysozyme-Ub conjugate degradation in reticulocyte lysate. Confirming the data in Fig. 3B, wild-type S5a was a potent inhibitor, and the recombinant protein bearing Ala substitutions at both PUbSs was inactive (Fig. 3D). The fact that wild-type S5a inhibited conjugate degradation 10-fold better than S5a proteins with alanine tracts at PUbS1 or PUbS2 is additional evidence for cooperative interactions between the two sites. If the sites functioned independently, wild-type S5a would, at best, only be a 2-fold better inhibitor. It is apparent in
Fig. 3. Inhibition of 125I-lysozyme-ubiquitin degradation by S5a fragments and mutated full-length S5a proteins. 

A, Ub-lysozyme degradation in the presence of S5a, PUbS1, or PUbS2. Purified 125I-lysozyme-Ub conjugates (38) were incubated at 37 °C in rabbit reticulocyte lysate containing 4 mM ATP and 2 µM test protein. Proteolysis was quantitated by the appearance of acid-soluble 125I in aliquots taken at the times indicated. 

B, concentration-dependent inhibition of Ub-lysozyme degradation. Full-length S5a, PUbS1 (Met196–Ala241), or PUbS2 (Met263–Asp307) were added to reticulocyte lysate to the concentrations shown on the abscissa. 125I-Ub-lysozyme conjugates were added, and the reactions were quenched with trichloroacetic acid after 6 min. Degradation is expressed as % acid soluble. 

C, full-length S5a molecules bearing alanine substitutions in either PUbS1, PUbS2, or both were prepared by site-directed mutagenesis. The mutant recombinant proteins were purified on Ni-NTA beads, and various amounts of the proteins were Dot blotted onto nitrocellulose as shown at the left. The filter was then incubated in a solution containing 10⁷ cpm of 125I-polyubiquitin chains, rinsed, and analyzed by phosphorimaging. 

D, Ub-lysozyme degradation in the presence of added wild-type or full-length mutant S5a molecules described in C. The assay was performed as described under B.

Fig. 3D that full-length S5a proteins with Ala tracts at PUbS1 or PUbS2 were equally good inhibitors. This result is surprising because when expressed as fragments PUbS2 is a much better inhibitor than PUbS1 (Fig. 3B), and in the context of the full-length S5a protein, PUbS2 binds polyubiquitin chains with higher apparent affinity than does PUbS1 (Fig. 3C). Possibly when S5a is present in a crude lysate, PUbS2 is partially masked, and this accounts for the unexpectedly poor inhibition by the S5a construct with PUbS1 inactivated.

Approximating the Minimal Length of the Polyubiquitin Binding Sites—Because PUbS1 is present and highly conserved in all known S5a sequences, it was chosen to estimate the minimal sequence required for binding polyubiquitin chains. Polypeptides containing either the N-terminal half (Met⁰⁷–Ser¹⁸⁵) or the C-terminal half (Met²⁰⁴–Ser²⁵⁷) of this region failed to associate with polyubiquitin chains (Fig. 1A), suggesting that the minimal binding site encompasses a significant portion of amino acids Met⁰⁷ through Ala²⁴¹. Two further deletions fixed the C-terminal boundary of PUbS1 to within 6 residues since a construct containing residues Met¹¹²–Gln²³¹ bound polyubiquitin chains, whereas residues Met¹¹²–Glu²⁴⁵ did not (Fig. 1A). Mapping the NH₂-terminal boundaries of PUbS1 and PUbS2 was performed using a series of synthetic peptides with Ala²⁴⁰ from S5a as the C-terminal anchor for PUbS1 and Glu³⁰⁴ as the C terminus of PUbS2. Equivalent amounts of peptides were immobilized on Ni-NTA matrix using the histidine extensions and then incubated with polyubiquitin chains. The bound 125I-polyubiquitin chains were eluted, quantitated, and displayed on an SDS-PAGE gel to determine their chain length distributions. As seen in Fig. 4, PUbS2 synthetic peptides retained more labeled chains than the PUbS1 series. The differences between PUbS1 and PUbS2 synthetic peptides were less than observed with the S5a mutants in Fig. 3C, and this is likely due to the large amounts of synthetic peptides employed in Fig. 4. For both series of peptides, the presence of amino acids NH₂-terminal to the hydrophobic region (LALAL or IAYAM) increased the amount of polyubiquitin bound. For PUbS1, the sequence of the amino acids did not appear to be of major importance since scrambling the NH₂-terminal sequence of PUbS1 did not reduce chain binding significantly. However, the scrambled version showed little selection for Ub tetramers over trimers. Both PUbS1, Met¹⁰⁶–Ala²⁴⁰, and the scrambled NH₂-terminal sequence bound ~150,000 cpm, but the ratio of bound trimer to tetramer (Ub₃/Ub₄) for the scrambled version...
was 1.4 very close to ratio of 1.9 in the starting chain mixture. By contrast the Ub$_3$/Ub$_4$ ratio was 0.4 for the wild type sequence.

The Importance of Alternating Large and Small Hydrophobic Residues in PUbs1—The combined use of internal deletions and synthetic peptides demonstrated that about 30 residues from each PUbs are required for polyubiquitin binding. Sequence alignment reveals that both sites contain a stretch of 5 residues in which large hydrophobic side chains alternate with alanines (Fig. 5). To determine the importance of the alternating pattern of hydrophobic residues we scrambled the order of alanines and large hydrophobic amino acids. We also replaced the two conserved alanines with leucines or glycines. After expression and purification, the various PUbs1 mutants were immobilized on nitrocellulose or Ni-NTA spin columns. In the nitrocellulose assay all PUbs1 variants bound less than 1% of the polyubiquitin chains relative to wild-type PUbs1 (Fig. 6). Clearly removal of the Leu residues or scrambling the order of these residues abolished polyubiquitin binding. Assaying the mutant polypeptides on Ni-NTA spin columns produced similar results, but nonspecific binding was higher. Mutation of Ile287, Tyr289, and Met291 to alanine within a 44-residue fragment of PUbs2 (Met263–Asp307) also eliminated polyubiquitin binding (data not shown).

**DISCUSSION**

Alignment of higher eukaryotic S5a sequences reveals three islands of conserved residues within the C-terminal half of the protein. The polyubiquitin binding sites are located in the two most NH$_2$-terminal of these conserved regions. Each PUbs contains 5 alternating large and small hydrophobic residues flanked by negatively charged residues (see Fig. 5). The arrangement of the 5 hydrophobic residues and a nearby serine in PUbs1 are critical for its association with polyubiquitin. After blotting 5-μg aliquots onto nitrocellulose, the filter was probed with $^{125}$I-polyubiquitin chains, rinsed, and quantitated using a phosphorimager. The majority of mutants exhibited less chain binding than a control protein, His$_6$-MAD2, a protein unlikely to bind polyubiquitin chains specifically. Relative to wild-type PUbs1 the amount of bound polyubiquitin ranged from 0.05% for Ala$_5$ to 0.6% for Leu$_5$.

**FIG. 6.** Polyubiquitin binding to PUbs1 with mutated hydrophobic cores. Various recombinant versions of PUbs1 were generated by site-directed mutagenesis of the LALAL region (Leu$^{216}$–Leu$^{220}$) in the S5a fragment encompassing Met$^{107}$–Ala$^{241}$. The expressed proteins were purified and confirmed by mass spectrometry. In all cases the apparent mass matched that expected for the designed recombinant protein. After blotting 5-μg aliquots onto nitrocellulose, the filter was probed with $^{125}$I-polyubiquitin chains, rinsed, and quantitated using a phosphorimager. The majority of mutants exhibited less chain binding than a control protein, His$_6$-MAD2, a protein unlikely to bind polyubiquitin chains specifically. Relative to wild-type PUbs1 the amount of bound polyubiquitin ranged from 0.05% for Ala$_5$ to 0.6% for Leu$_5$. The two PUbs are approximately 30 amino acids in length. Although the absolute minimal lengths of PUbs1 and PUbs2 needed to bind polyubiquitin have not been defined precisely, we do know that the Phe$^{204}$–Glu$^{232}$ in PUbs1 binds polyubiquitin chains, and it

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2 A. M. Christensen, P. Young, M. Rechsteiner, and W. I. Sundquist, manuscript in preparation.
is close to the minimal peptide able to do so.

The NH₂-terminal half of S5a does not contribute to polyubiquitin chain binding (Fig. 3). Nonetheless, it is highly conserved among all known S5a sequences. This portion of the protein may be involved in association of S5a with other components in the 19 S regulatory complex. Alternatively, the NH₂-terminus of S5a has been reported to bind and regulate the function of the helix-loop-helix protein Id1 (40). Thus, it is possible that both the NH₂-terminal portion of S5a and the two PUBs interact with 26 S protease substrates. If this is true, then the third conserved region in the C-terminal half of S5a, residues Phe³³⁴–Lys³⁷⁷, may mediate association of S5a with the 19 S regulatory complex. It should be noted that S5a binds the 26 S protease weaker than most other subunits since a significant portion of Drosophila S5a binds almost 10-fold more polyubiquitin chains than expected from the sum of the two sites, and it is at least a 10-fold better inhibitor than full-length molecules with either PUBS1 or PUBS2 inactivated (Fig. 3, C and D). This raises the possibility that PUBS2 is partially masked under normal physiological conditions becoming fully active only under special circumstances, such as heat shock.

We began these studies with the hypothesis that multiple binding sites in S5a would explain the ability of this 26 S protease subunit to bind long polyubiquitin chains selectively. Molecular dissection of S5a has, indeed, identified two binding sites in the protein (Fig. 1), and there is evidence that the two sites may cooperate in binding polyubiquitin chains. This follows from the fact that at low concentration wild-type S5a binds almost 10-fold more polyubiquitin chains than expected from the sum of the two sites, and it is at least a 10-fold better inhibitor than full-length molecules with either PUBS1 or PUBS2 inactivated (Fig. 3, C and D). The evidence for cooperativity is not conclusive, however, since one must assume that alanine substitutions in PUBS1 do not alter the structure and/or accessibility of PUBS2 or vice versa. Several considerations support this assumption. First, the CD spectrum of S5a with penta-alanine tracts at both sites is indistinguishable from the CD spectrum of wild-type S5a (data not shown). Second, 2D-NMR studies demonstrate that the LALAL region of PUBS1 is α-helical, and both alanine and leucine are strong helix-forming residues (42). Although it seems unlikely that substituting alanines for leucine would disrupt the α-helix in this region of the protein, we cannot eliminate the possibility that such substitutions perturb the structure of S5a.

Preferential binding of longer polyubiquitin chains by S5a cannot be attributed solely to the presence of two binding sites since each site alone selectively binds longer polyubiquitin chains (Fig. 2). It is also clear from the data in Fig. 4 that wild-type PUBS1 bound tetramers tighter than trimers, whereas the opposite was observed when the sequence NH₂-terminal to the LALAL region was scrambled. This result indicates that Leu²¹⁶–Glu²³² in PUBS1 have substantial affinity for trimers and that the wild-type sequence NH₂-terminal to Leu²¹⁶ confers additional binding energy for association with ubiquitin tetramers. Some portion of the ubiquitin tetramer may directly contact Met¹⁹⁶–Leu²¹⁶ in PUBS1. Alternatively, the increased apparent affinity for tetramers could result from conformational constraints imposed by the wild-type sequence on Leu²¹⁶–Glu²³².

The central region of PUBS1 has been shown to be an α-helix in aqueous solution. Taking into account this structural information and previous site-directed mutational studies on ubiquitin’s hydrophobic surface residues (29), we offer a model for molecular interaction between PUBS1 and a polyubiquitin chain. We imagine that 10 residues of α-helix within PUBS1 (Glu²¹⁵–Met²²⁴) are critical for its association with polyubiquitin chains. We propose that the side chains of the first and last Leu residues in the LALAL sequence interdigitate between Leu⁶, Ile⁴⁴, and Val⁷⁰ on the surface of a ubiquitin monomer. Molecular modeling reveals that the Leu side chains in PUBS1 can make reasonable van der Waals contacts with the 3 hydrophobic residues (43). Moreover, Leu⁶, Ile⁴⁴, and Val⁷⁰ of ubiquitin have been shown to be important for interaction of polyubiquitin chains with S5a and required for ubiquitin-mediated proteolysis (29). The presence of Ser²²³ in PUBS1 and Ser²⁸⁴ in PUBS2 are also very important since their conversion to alanine eliminates or greatly reduces polyubiquitin binding (See Fig. 5 legend). These two serines may be involved in the formation of hydrogen bonds to His⁵⁸ or Lys⁶, which are adjacent to the hydrophobic patch on ubiquitin. Alternatively, they may be necessary for maintaining structure within each PUBS.

The described hydrophobic interaction is also accessible to mono-ubiquitin, which does not bind S5a with appreciable affinity. Therefore, the proposed hydrophobic interactions cannot be sufficient to explain polyubiquitin selection. We propose that polyubiquitin selection arises from two additional interactions. The first involves association of the PUBS helix with the opposing ubiquitin present at an interface formed between two ubiquitins. Although Drosophila S5a has detectable affinity for di-ubiquitin (41), human S5a has little apparent affinity for ubiquitin dimers. Hence, we propose that selection of longer polyubiquitin chains requires portions of PUBS1 on either side of the LALAL region that associate with additional ubiquitins. The loss of tetramer selection after scrambling the residues NH₂-terminal of the LALAL region within PUBS1 supports this idea (Fig. 4). In general, the ability of short S5a peptides to bind polyubiquitin selectively indicates that the interactions do not require long through space distances. However, too little is known to speculate on the molecular details of such interactions. So in essence, we are suggesting that the LALAL regions in S5a and the hydrophobic patch on the surface of ubiquitin generated by Leu⁶, Ile⁴⁴, and Val⁷⁰ nucleate association between the 26 S protease subunit and polyubiquitin. Additional site-directed mutagenesis studies and co-crystallization of PUBS with polyubiquitin should provide rigorous tests of this model and help in identifying additional interactions between S5a and polyubiquitin.

The S. cerevisiae 26 S protease recognizes polyubiquitylated
proteins in the absence of S5a. This finding demonstrates that other recognition factors for polyubiquitin must be present in the 26 S protease. Current evidence indicates that the polyubiquitin binding properties of these factors are very similar to the binding properties of S5a. Thus, it of interest that the two largest proteins of the 19 S regulatory complex, subunits 1 and 2, contain a number of repeated sequences that resemble the LALAL or IAYAM found within the PUBS1 and PUBS2 regions of S5a, e.g., L154ALGLVMLGS252 within human subunit 1 and L689ALALISVS700 in human subunit 2. Moreover, like S5a, these two subunits contain KEKE motifs that have been postulated to promote protein-protein associations (44). Hence, these two subunits contain KEKE motifs that have been postulated to promote protein-protein associations (44). Hence, these two subunits contain KEKE motifs that have been postulated to promote protein-protein associations (44). Hence, these two subunits contain KEKE motifs that have been postulated to promote protein-protein associations (44).

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