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# Surface hydrophobic residues of multiubiquitin chains essential for proteolytic targeting

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ABSTRACT **Ubiquitin conjugation is a signal for degradation of eukaryotic proteins by the 26S protease. Conjugation of a homopolymeric multiubiquitin chain to a substrate lysine residue results in 10-fold faster degradation than does conjugation of monoubiquitin, but the molecular basis of enhanced targeting by chains is unknown. We show that ubiquitin residues L8, I44, and V70 are critical for targeting. Mutation of pairs of these residues to alanine had little effect on attachment of ubiquitin to substrates but severely inhibited degradation of the resulting conjugates. The same mutations blocked the binding of chains to a specific subunit (S5a) of the regulatory complex of the 26S protease. The side chains implicated in this binding—L8, I44, and V70—form repeating patches on the chain surface. Thus, hydrophobic interactions between these patches and S5a apparently contribute to enhanced proteolytic targeting by multiubiquitin chains.**

A major intracellular proteolytic pathway utilizes covalent conjugation of the conserved protein ubiquitin as a signal for substrate recognition by a specific protease (1, 2). The ubiquitin-mediated proteolytic pathway is the predominant mechanism for turnover of short-lived proteins in eukaryotic cells (3). Substrates of this pathway include such critical regulatory proteins as the plant photoregulator phytochrome (4), c-Mos (5), c-Jun (6), and NF-κB (7) as well as cyclins and other regulators of cell cycle progression (8–11).

Ubiquitination occurs in three sequential enzymatic steps (1): ATP-dependent formation of a thiol ester with ubiquitin-activating enzyme (E1) at the ubiquitin C terminus (G76); ubiquitin transfer to a cysteine residue of a ubiquitin-conjugating enzyme (E2); and ubiquitin transfer to a lysine residue of the substrate, catalyzed by a ubiquitin–protein ligase (E3). Conjugated substrates are recognized and degraded by a multisubunit, ATP-dependent 26S protease that is assembled from regulatory and catalytic (20S) complexes (12, 13). The proteolytic cycle is completed upon regeneration of free ubiquitin by one or more specific isopeptidases (1, 14).

Substrates are most rapidly degraded when they are conjugated to multiple molecules of ubiquitin (15, 16). A homopolymeric multiubiquitin chain, in which successive ubiquitins are linked by K48–G76 isopeptide bonds, is an especially potent degradative signal: a substrate bearing a K48-linked chain of 8 to 12 ubiquitins is degraded ≈10 times more rapidly than a substrate bearing a single ubiquitin at the same position (17). This length-dependent differential signal is reflected in the properties of subunit 5a (S5a) of the regulatory complex of the 26S protease. S5a binds K48-linked multiubiquitin chains of $n \geq 4$ with increasing affinity as a function of chain length but has low affinity for monoubiquitin and chains of $n \leq 3$ (18).

Conjugates bearing extended multiubiquitin chains presumably undergo rapid degradation because they are efficiently targeted to the 26S protease via S5a (18). However, the interaction between multiubiquitin chains and S5a remains completely uncharacterized. We used the recently determined structure of tetraubiquitin ($Ub_4$) (19) as a starting point to address this question by site-specific mutagenesis. Here we report evidence concerning the molecular interactions responsible for enhanced proteolytic targeting by multiubiquitin chains.

## MATERIALS AND METHODS

**Ubiquitin Mutagenesis, Expression, and Purification.** Plasmids encoding mutant ubiquitins were generated by whole-plasmid (circular) PCR using pPLhUb as template (20). This procedure employs back-to-back primers, one of which encodes the desired mutation (21). The presence of each mutation was verified by determination of the complete coding sequence (fmol kit; Promega). Mutant genes under the control of the λ $P_L$ promoter in pPLhUb were expressed after heat induction of *Escherichia coli* strain AR58 (20). Briefly, 5-liter cultures were grown at 30°C to $OD_{600} \approx 1.5$ and then shifted to 42°C for 2 h. The cells were collected by centrifugation and lysed with a French press. The lysate was centrifuged at 10,000 × *g*. Perchloric acid (3.5%; vol/vol) was added to the supernatant to precipitate nonubiquitin proteins. The acid supernatant was neutralized and dialyzed, and ubiquitin was resolved by cation-exchange chromatography (22). Ubiquitin was electrophoretically homogeneous after this step.

**Assays of Degradation and Determination of Steady-State Conjugate Level.** Mutant ubiquitins were assayed for their ability to support the ubiquitination and degradation of bovine lactalbumin (Sigma) in ubiquitin-depleted rabbit reticulocyte lysate or fraction II. Fraction II was prepared as described (23). The standard incubation mixture contained (25–50 μl; 37°C) 50 mM Tris·HCl (pH 7.3), 5 mM $MgCl_2$, 2 mM ATP, an ATP-regenerating system (10 mM phosphocreatine and 0.3 unit of creatine phosphokinase per ml), 0.3 unit of inorganic pyrophosphatase per ml, ≈1 mg of fraction II protein per ml, and $\approx 2 \times 10^5$ cpm of $^{125}$I-labeled lactalbumin ($10^6$ cpm/μg). Unless otherwise indicated, the concentration of ubiquitin was 9 μM. This concentration is saturating for wild type (24) and was saturating for all mutant ubiquitins that were tested in this regard (L8A, I44A, and L8A/I44A). An aliquot was removed from the incubation mixture and quenched for SDS/PAGE during the linear phase of the assay. Soluble counts were determined on a second aliquot after addition of trichloroacetic acid (25). Ubiquintinated lactalbumin was visualized by autoradiography of the dried gel; the region of the gel containing the conjugates was excised and counted in a γ-counter

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Abbreviations: E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin–protein ligase; S5a, subunit 5a of 26S protease; $Ub_4$, tetraubiquitin; $Ub_2$, diubiquitin.

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(25). Data were corrected by subtracting blanks derived from incubations lacking ubiquitin. Results are expressed relative to the control with wild-type ubiquitin.

**Inhibition of Degradation by Unanchored Ubiquitin Chains.** Degradation assays were carried out with $^{125}$I-lactalbumin as described above, except fraction II was depleted of endogenous isopeptidase(s) by passage through ubiquitin-Sepharose in buffer lacking ATP (26); the concentration of added wild-type monoubiquitin was 35 $\mu$M; and assays were further supplemented with 1.8 $\mu$M ubiquitin aldehyde, an isopeptidase inhibitor (ref. 27; gift of Keith Wilkinson, Emory University, Atlanta). Where indicated, unanchored chains (distribution as in Fig. 3*A*) were added at a total concentration of 0.8 mg of ubiquitin per ml. Without isopeptidase depletion (see above), endogenous isopeptidase T completely disassembled added wild-type ubiquitin chains within minutes, and no inhibition was seen (cf. Table 1). Isopeptidase T was purified from bovine erythrocytes by a published procedure (26).

**E3/RAD6-Dependent Ubiquitination.** Enzymes were purified as described (25, 28). $^{125}$I-ubiquitin (2 $\mu$M; 8000 cpm/pmol) was incubated with purified E1 (0.1 $\mu$M), purified yeast RAD6(UBC2) (0.1 $\mu$M), and partially purified mammalian E3 in the presence of 0.2 mg of oxidized RNase per ml (Sigma) as substrate as described (25). In this assay, the properties of yeast RAD6 are indistinguishable from those of its mammalian homolog E2-14K (28). Incubation mixtures were quenched for SDS/PAGE during the linear phase of conjugation. The portion of the dried gel containing conjugates was excised and assayed. Data were corrected by subtracting blanks derived from assays lacking E3. Initial rates of conjugation (relative to wild-type ubiquitin) were 56% (P37C), 30% (I36A), 35% (I44A), 39% (L8A), 29% (L8A/I44A), and 40% (I44A/V70A).

**Chain Synthesis.** Recombinant bovine E2-25K was purified as described (29). Chains of homogeneous ubiquitin composition ($n$ = 1–5; see Fig. 3*A*) for use in S5a binding assays were synthesized in incubation mixtures (37°C) containing purified E1 (0.4 $\mu$M), E2-25K (10 $\mu$M), and wild-type or mutant ubiquitin (≈2 mg/ml) as described (29), except that the pH was 8.0. After incubating for 45 min, the enzymes were removed by absorbing them onto anion-exchange resin (29). The chains were concentrated by ultrafiltration, labeled by radioiodination to ≈$10^6$ cpm/$\mu$g, and used at ≈0.6 $\mu$g/ml in S5a binding assays (see below).

**Synthesis of Chains of Mixed Ubiquitin Composition.** $Ub_4$ molecules A–F (see Fig. 4) were assembled using E2-25K under the conditions described above, with modifications as follows. To simplify the spectrum of products obtained, all tetramers contained the K48R mutation in the distal subunit and were des-Gly-Gly in the proximal subunit (see Fig. 1 for definition of proximal and distal). Removal of the C-terminal Gly-Gly dipeptide with trypsin was carried out at the level of monoubiquitin (30). In molecules A and B, the appropriate K48R-monoubiquitin was conjugated to wild-type triubiquitin; in molecule C, K48R ubiquitin was conjugated to L8A/I44A triubiquitin; in molecule D, K48R ubiquitin was conjugated to wild-type ubiquitin ethyl ester (ref. 31; gift of Keith Wilkinson); the blocking ethyl ester group was hydrolyzed, and the dimer was conjugated to L8A/I44A diubiquitin; in molecule E, K48R ubiquitin was conjugated to wild-type diubiquitin ($Ub_2$); the resulting trimer was conjugated to L8A/I44A ubiquitin; in molecule F, K48R/L8A/I44A ubiquitin was conjugated to ubiquitin ethyl ester; the dimer was deblocked. L8A/I44A ubiquitin was conjugated to wild-type ubiquitin. The two dimers were conjugated. Intermediate chains, and the final tetramers, were purified by cation-exchange chromatography (29). Tetramers were labeled by radioiodination to ≈$10^6$ cpm/$\mu$g and used at ≈0.2 $\mu$g/ml in S5a binding assays (see below).

**Chain Binding to S5a.** Binding of radioiodinated chains and purified $Ub_4$ molecules to S5a was assayed after fractionation of the 26S regulatory complex (20 $\mu$g per lane) by SDS/PAGE (10% gel) and electrophoretic transfer of proteins to nitrocellulose (18). Chain concentration in the binding incubation mixtures ranged from ≈0.2 to ≈0.6 $\mu$g/ml (see above). Bound radioactivity was quantitated by PhosphorImager analysis. Binding data are expressed relative to a control of wild-type ubiquitin chains in the same experiment. For P37C ubiquitin chains (and the corresponding wild-type control), incubations were done in the presence of 5 mM dithiothreitol, which slightly diminished the binding of wild-type chains (40% decrease) but largely eliminated an aberrantly high signal from P37C chains. This apparently reflected dissociation of disulfide-linked aggregates of the latter chains that remained partially competent in binding. These adducts could be visualized by autoradiography after nonreducing SDS/PAGE of P37C ubiquitin chains.

## RESULTS

**Choice of Mutation Sites.** The crystal structure of K48-linked $Ub_4$ shows a compact, asymmetric dimer of dimers (Fig. 1 *Upper*) in which each of the four ubiquitin units retains the

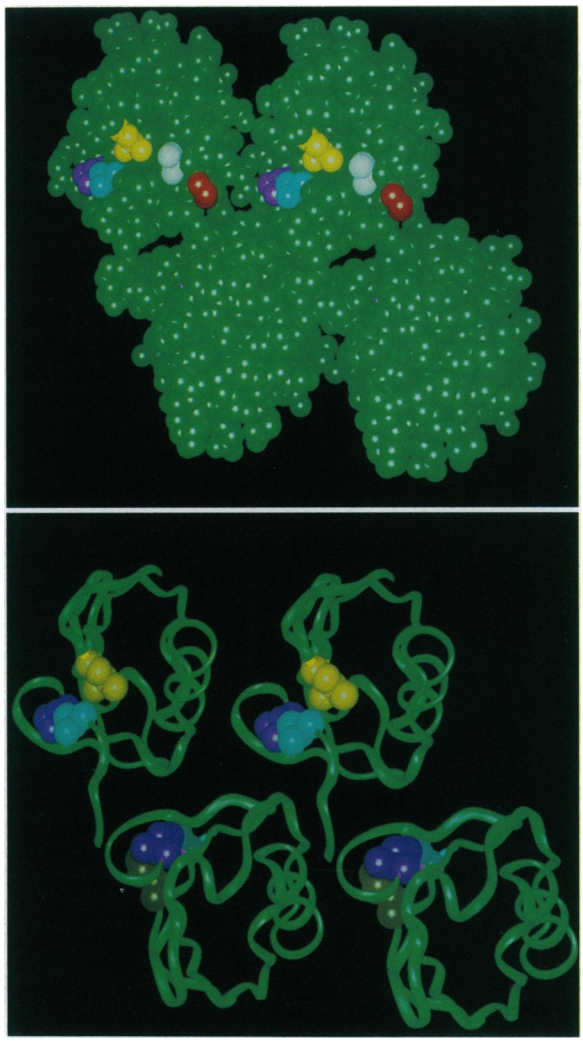

FIG. 1. $Ub_4$ structure. (*Upper*) Space-filling. (*Lower*) Ribbon. Top left, distal ubiquitin (free K48); bottom right, proximal ubiquitin (free G76). Side chains: L8, yellow; I44, purple; V70, blue; I36, white; P37, red. Images were generated with the INSIGHTII molecular visualization program (BioSym Technologies, San Diego).

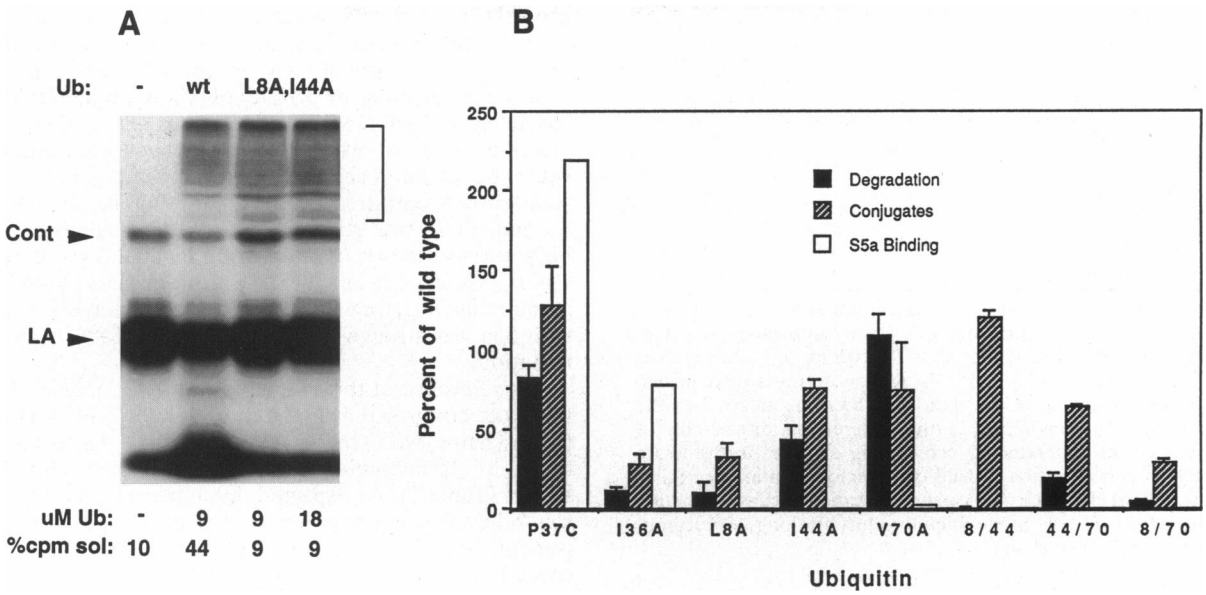


FIG. 2. Activities of mutant ubiquitins. (*A*) Bovine $^{125}$I-lactalbumin conjugation and degradation: wild-type vs. L8A/I44A ubiquitin. Aliquots of degradation assay mixtures, containing ubiquitin as indicated, were electrophoresed (autoradiograph) or assayed for acid-soluble radioactivity (bottom). LA, lactalbumin; Cont, contaminant; brackets, conjugated lactalbumin (excised and assayed for hatched bars in *B*). (*B*) Summary of mutant ubiquitin activities. Solid bars, relative rate of lactalbumin degradation; hatched bars, relative level of lactalbumin conjugates; open bars, relative binding of free chains to S5a (see Fig. 3; not determined for 8/70). The greater than wild-type binding seen with P37C ubiquitin chains probably reflects a contribution from disulfide-linked aggregates (see *Materials and Methods*).

folding of monomeric ubiquitin (19, 32). The structure is stabilized by electrostatic contacts between adjacent ubiquitin units (19). The $Ub_4$ structure can be extended to accommodate additional ubiquitin molecules and thus provides a good model for the longer chains ($n$ = 8–12) that efficiently target substrates to the 26S protease. We noted that a hydrophobic patch, composed of the side chains of L8, I44, and V70, is present on the surface of each ubiquitin unit in the chain (Fig. 1 *Lower*) (32, 33). With inclusion of the side chains of I36 and P37, these repeating surface patches (Fig. 1 *Lower*) coalesce to form a hydrophobic stripe on each of two chain faces (Fig. 1 *Upper*).

Recent studies have highlighted the importance of hydrophobic contacts in protein–protein interactions (e.g., see ref. 34). To test whether targeting by chains involved contacts between the protease and the stripe shown in Fig. 1 *Upper*, four of the relevant residues were mutated to alanine singly or in pairs. The fifth residue, P37, was mutated to cysteine. These mutations preserve the nonpolar character of these surface side chains and should have little effect on the stability of monoubiquitin or K48-linked multiubiquitin chains. However, the mutations should destabilize interactions that depend on hydrophobic contacts (34–36). Thus, if targeting depends on such contacts with these side chains, the mutant ubiquitins may exhibit decreased activity in degradation.

**Mutation of Specific Surface Hydrophobic Residues of Ubiquitin Inhibits Conjugate Degradation.** The mutant ubiquitins were expressed in *E. coli*, purified to homogeneity, and tested in ubiquitin-depleted reticulocyte lysate for their ability to support ubiquitination and degradation of bovine $^{125}$I-lactalbumin. The degradation of this well-characterized *in vitro* proteolytic substrate (23, 25) depends on conjugation of K48-linked multiubiquitin chains, since substitution of K48R ubiquitin for wild-type ubiquitin severely inhibits degradation (data not shown).

In the simplest case, a defect in targeting by chains would be manifested as a high substrate conjugate level but a low degradation rate. This behavior was seen with the double-mutant proteins, especially L8A/I44A ubiquitin (Fig. 2*A*; solid vs. hatched bars in Fig. 2*B*). By the same criterion, I44A ubiquitin showed evidence of a weak targeting defect, while P37C and V70A ubiquitin appeared competent in targeting. The steady-state level of conjugated lactalbumin was low for L8A and I36A ubiquitin (Fig. 2*B*), and this could explain the low degradation rates. Therefore, the targeting competence of these mutant ubiquitins was uncertain based solely on these data (see below).

All of the mutant ubiquitins supported the formation of detectable levels of high molecular weight lactalbumin conjugates (hatched bars in Fig. 2*B*). This qualitative indication that the mutant proteins were competent in conjugation was confirmed in assays with the purified enzymes responsible for lactalbumin conjugation. L8A and L8A/I44A ubiquitin were also devoid of activity in the degradation of a different *in vitro* substrate, $^{125}$I-labeled reduced/carboxymethylated serum albumin (data not shown). Thus, the effects of these mutations were independent of substrate identity. This is the expected result if the mutations blocked targeting of conjugates to the protease.

**Multiubiquitin Chains Bearing Specific Mutations Are Not Recognized by S5a of the 26S Protease.** The 26S protease is

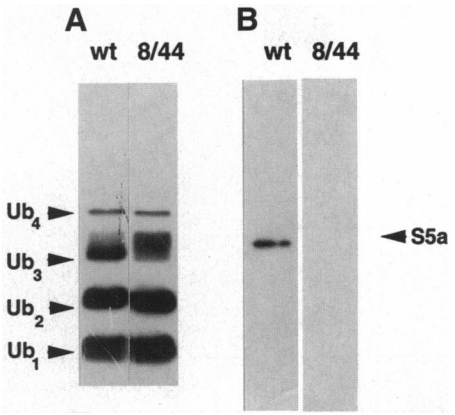


FIG. 3. Multiubiquitin chain binding to S5a (autoradiographs): wild-type vs. L8A/I44A chains. (*A*) Input chains (50 ng per lane). (*B*) Binding to immobilized S5a. Arrowhead, position of S5a as determined by Ponceau S staining of a duplicate blot strip.

Table 1. Inhibition of degradation by unanchored multiubiquitin chains

| Exp. | Chain added | Isopeptidase T | Degradation rate, % control |
|---|---|---|---|
| 1 | None (control) | − | 100 |
| 2 | None | + | 136 |
| 3 | Wild type | − | 48 |
| 4 | Wild type | + | 102 |
| 5 | L8A/I44A | − | 122 |
| 6 | L8A/I44A | + | 141 |

Assay mixtures of $^{125}$I-lactalbumin degradation containing wild-type monoubiquitin and ubiquitin aldehyde were supplemented where indicated with unanchored chains ($n$ = 1–5; 0.8 mg/ml total) and/or purified isopeptidase T (0.5 $\mu$M). Values are averages of duplicate determinations. Stimulation of degradation by isopeptidase T in the absence of added chains (Exp. 2) probably reflects disassembly of endogenous chains generated by conjugating enzymes in the lysate from the high concentration of wild-type monoubiquitin (29). Stimulation by mutant chains (Exp. 5) may reflect these chains competing as acceptors for wild-type monoubiquitin, thus blocking the accumulation of fully wild-type chains.

assembled from regulatory and catalytic (20S) complexes (12, 13). A 50-kDa subunit (S5a) of the regulatory complex has high affinity for chains of $n \geq 4$ and is likely to contribute to the specificity of the 26S (vs. 20S) protease for ubiquitin conjugates (18). To test the S5a-mediated targeting potential of the mutant ubiquitins, they were assembled into chains with a ubiquitin-conjugating enzyme, E2-25K, that synthesizes unanchored K48-linked chains from isolated ubiquitin (37). This enzyme did not discriminate kinetically against any of the mutant ubiquitins, confirming that all of them were competent in conjugation (see above). The chains were radioiodinated (Fig. 3*A*) and then assayed for binding to electrophoretically resolved S5a immobilized on nitrocellulose (18).

Wild-type chains bound strongly to S5a (Fig. 3*B*); bound chains consist mainly of $Ub_4$ and longer species (18). Chains assembled from L8A/I44A ubiquitin, which did not support proteolysis when conjugated to a substrate (Fig. 2), did not bind to immobilized S5a (Fig. 3*B*). We estimate that the affinity of these double-mutant chains for S5a is at least 100-fold lower than the affinity of wild-type ubiquitin chains based on the following considerations. First, there was no increase in signal when a 5-fold higher concentration of wild-type ubiquitin chains was used in the binding assay. The standard concentration was thus saturating (i.e., 10 times $K_{app}$). Second, there was still no detectable signal when a 5-fold higher concentration of double-mutant chains was used in the binding assay (i.e., when the concentration was at least 50 times greater than $K_{app}$ for wild type). Thus, the interaction of chains with S5a was strongly destabilized by the L8A/I44A double mutation.

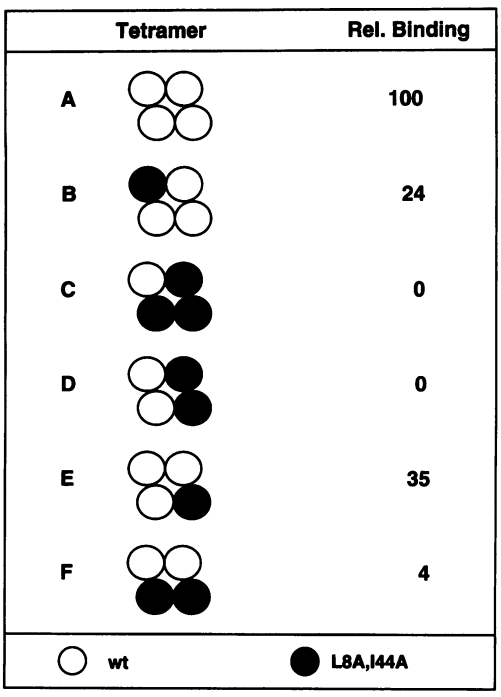


FIG. 4. Binding of mixed-composition $Ub_4$ molecules to S5a. Molecules are oriented as in Fig. 1. Values for molecules B and E are averages of duplicate determinations. All values are expressed relative to a control of wild-type ubiquitin chains in the same experiment.

The relevance of this binding defect to conjugate degradation was confirmed by inhibition studies with unanchored (free) chains; wild-type chains markedly reduced the degradation of lactalbumin, whereas L8A/I44A chains did not inhibit (Table 1). As expected, inhibition by wild-type chains was relieved when assays were supplemented with purified isopeptidase T, an enzyme that rapidly disassembles unanchored K48-linked chains (26, 37). Thus, the failure of the mutant chains to bind to S5a (Fig. 3) can explain the failure of L8A/I44A ubiquitin to support degradation (Fig. 2). We expect that the inhibition observed with wild-type ubiquitin chains in Table 1 underestimates what could be achieved at this chain concentration, since Western blot analysis with anti-ubiquitin antibodies indicated that the added chains were partially disassembled by endogenous isopeptidases (data not shown) despite the presence of an isopeptidase inhibitor in the assay.

For most of the mutant ubiquitins, binding of chains to S5a correlated with activity in degradation (cf. P37C, L8A, and double-mutant ubiquitins; open bars vs. solid bars in Fig. 2*B*). For I36A ubiquitin chains, the greatly reduced size of the conjugate pool may have led to slow degradation, since these chains bound well to S5a (Fig. 2*B*). The binding and degradation data indicate that combinations of mutations at positions 8, 44, and 70 inhibited targeting strongly. In contrast, mutation of I36 and P37 was benign. Thus, a series of hydrophobic patches involving L8, I44, and V70, rather than a generalized hydrophobic stripe that also includes I36 and P37 (Fig. 1), is apparently critical for targeting mediated by multiubiquitin chains.

Single mutation of I44 or V70 had a stronger inhibitory effect on binding to S5a than on degradation (open vs. solid bars in Fig. 2*B*). Here the high cooperativity of the chain–S5a interaction (18) and the moderate length of the chains used in the binding assay (Fig. 3*A*) may have prevented detection of an impaired, but partially productive, interaction. We note that many lactalbumin conjugates apparently bear chains of 10 or more ubiquitins, since they migrate with molecular masses > 100 kDa (e.g., see Fig. 2*A*). Thus, chains present in the degradation assays were longer than those used in the binding assays (Fig. 3*A*). However, it is also possible that an additional 26S protease subunit(s), not detected in the binding assay, assists in chain binding and retains the ability to bind chains assembled from I44A or V70A ubiquitin.

**Effects on Chain Binding to S5a Are Independent of Mutant Ubiquitin Position.** Degradation was not inhibited when assays were simultaneously supplemented with wild-type and L8A/I44A (mono)ubiquitin under conditions estimated to give a 1:1 ratio of incorporation into conjugates (data not shown). This finding suggested that mixed wild-type/mutant chains are functional and raised the possibility that the position of a specific ubiquitin within the chain could be significant.

To address this possibility, we synthesized $Ub_4$ molecules bearing mutant subunits at defined positions and tested them for binding to S5a. The results (Fig. 4) showed that the fractional content of wild-type ubiquitins, rather than their

specific positions, determined the strength of interaction with S5a (Fig. 4). We noted previously that the top and bottom faces of the $Ub_4$ molecule (as shown in Fig. 1) are identical and speculated that this degeneracy might reduce geometric constraints in binding to the protease (19). However, the results in Fig. 4 (A vs. F) exclude a simple model in which an intact set of hydrophobic patches on one face of the chain is sufficient for binding to S5a.

## DISCUSSION

We have shown that mutation of specific hydrophobic residues on the surface of K48-linked multiubiquitin chains has effects that are consistent with these residues functioning in targeting conjugates to the 26S protease. None of the mutations strongly affected the ability of ubiquitin to be conjugated to proteolytic substrates. However, certain mutations severely inhibited the ability of the resulting conjugates to be degraded. Chains bearing these same mutations were specifically defective in binding to S5a of the regulatory complex of the 26S protease. An important role for S5a in targeting had already been suggested by its properties in chain binding as well as its presence in the regulatory complex that confers conjugate recognition on the 20S catalytic core of the protease (18). The present results provide additional strong support for the hypothesis that S5a plays a critical role in targeting conjugates to the 26S protease.

Our results provide information about the nature of the interaction between chains and S5a and about structural features of the chain that are important for this interaction. A cavity-forming Leu-to-Ala mutation can decrease the stability of the related side chain interaction by 2–6 kcal/mol (1 cal = 4.184 J) (34–36). Mutation of ubiquitin residue 8, 44, or 70 to alanine decreased the affinity of chains for S5a by at least 3 kcal/mol; mutation of I36 and P37 was benign. Thus, a series of hydrophobic patches involving L8, I44, and V70, rather than a generalized hydrophobic stripe including I36 and P37 (Fig. 1), is critical for proteolytic targeting mediated by S5a. Single mutation of residue 8, 44, or 70 to alanine strongly inhibited the binding of chains to S5a, suggesting that these side chains are normally in close van der Waals contact with a nonpolar surface and that all of them contribute to the interaction of a given patch with a site on S5a.

Single mutations generally impacted more negatively on chain binding to S5a than on degradation (Fig. 2*B*). Differences in the conditions of the two assays probably contributed to this discrepancy, but we cannot exclude the possibility that the regulatory complex contains another targeting component with binding properties that differ slightly from those of S5a. However, the strong degradative defect seen with the double-mutant ubiquitins (Fig. 2*B*) indicates a high likelihood that any other targeting components also engage in hydrophobic contacts with the surface residues of ubiquitin that we have identified. In preliminary studies, we have found that the degradative activity of ubiquitin increases in proportion to the size of the side chain at position 8 (Ala < Val < Ile ≈ Leu; D. Toscano-Cantaffa and C.P., unpublished experiments), further supporting the hypothesis that a hydrophobic contact involving L8 is important for targeting. Moreover, the L8A mutation is lethal in *Saccharomyces cerevisiae* (R. Seet, C.P., and D. Finley, unpublished data).

Besides $Ub_4$, the structure of K48-linked $Ub_2$ has also been determined (38). The dimeric units of $Ub_4$ and $Ub_2$ are distinct and are related by a 120° rotation about the isopeptide bond (19). Unlike $Ub_4$, which is stabilized entirely by electrostatic interactions (19), $Ub_2$ is stabilized in part by sequestration of the side chains of L8, I44, and V70 of each ubiquitin into a pocket between the two ubiquitins (38). $Ub_2$ was considered to be a poor model for higher-order chains because its twofold symmetry will not allow stabilizing interactions to develop with the next ubiquitin in the chain unless there is structural reorganization (38). Such reorganization, which permits infinite extension of the chain, was indeed apparent in the structure of $Ub_4$ (19).

Modeling studies suggest that the distal ubiquitin unit in the $Ub_4$ chain can easily rotate 120° about the isopeptide bond, causing the two distal ubiquitins in the chain to adopt the $Ub_2$ conformation (data not shown). If this rotation occurred, and if the resulting $Ub_2$ cap was important for recognition of the chain by S5a, the hydrophobic contacts suggested by our mutagenesis results (see above) could be ubiquitin–ubiquitin, rather than ubiquitin–S5a, contacts. However, a specific prediction of this model, that a single mutant ubiquitin in an otherwise wild-type chain should destabilize binding to S5a most when located at the distal chain end, was not borne out in our studies with mixed-composition $Ub_4$ molecules (Fig. 4, molecule A vs. B and E).

Since monoubiquitin has little affinity for S5a (18), S5a must interact with multiple patches in the chain. This could lead to the observed cooperative increase in affinity with chain length (18) through several mechanisms: the presence of multiple S5a molecules in each molecule of the 26S protease, chain–chain interactions in the bound state, or the presence in each S5a molecule of binding sites for multiple patches. The sequence of an *Arabidopsis* S5a homolog shows a series of hydrophobic repeats in the C-terminal region (39) that may contribute to the structure–function relationship revealed by our work.

K48-linked multiubiquitin chains play a well-documented role in degradative targeting (17, 40, 41). However, isopeptide multiubiquitin chains linked through other lysine residues of ubiquitin have also been described (42, 43). Although in most cases the function of these chains remain unknown, K11-linked chains are able to target proteins for degradation and are recognized by S5a (A. Haas, personal communication). The specific structure of the K48-linked chain (Fig. 1) allows the hydrophobic patches composed of L8, I44, and V70 to be present on the surface of the chain and thus available for interaction with S5a. It remains to be determined whether the structures of other isopeptide chains permit this same feature to be present on the chain surface.

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