Distinct Functional Surface Regions on Ubiquitin*

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The characterized functions of the highly conserved polypeptide ubiquitin are to target proteins for proteasome degradation or endocytosis. The formation of a polyubiquitin chain of at least four units is required for efficient proteasome binding. By contrast, monoubiquitin serves as a signal for the endocytosis of plasma membrane proteins. We have defined surface residues that are important for ubiquitin’s vital functions in Saccharomyces cerevisiae. Surprisingly, alanine scanning mutagenesis showed that only 16 of ubiquitin’s 63 surface residues are essential for vegetative growth in yeast. Most of the essential residues localize to two hydrophobic clusters that participate in proteasome recognition and/or endocytosis. The others reside in or near the tail region, which is important for conjugation and deubiquitination. We also demonstrate that the essential residues comprise two distinct functional surfaces: residues surrounding Phe4 are required for endocytosis, whereas residues surrounding Ile44 are required for both endocytosis and proteasome degradation.

Conjugation to ubiquitin targets cellular proteins for degradation through two major pathways. Ubiquitination is required for the selective proteolysis of many intracellular proteins by the 26 S proteasome (1). In addition, a number of plasma membrane proteins require ubiquitination to signal their internalization or sorting in the endocytic pathway and subsequent degradation in the lysosome (2–4). Modification of substrates with ubiquitin proceeds through a three-enzyme cascade, resulting in the formation of a covalent isopeptide bond between the C-terminal glycine 76 of ubiquitin and a lysine residue in the target protein. Some substrates are conjugated to just one ubiquitin, whereas others are conjugated to multiple ubiquitins in the form of a polyubiquitin chain. Chains connected through three of ubiquitin’s seven lysine residues (Lys29, Lys48, and Lys63) have been identified in vivo in Saccharomyces cerevisiae (5–8). Others have been synthesized in vitro and may exist in higher eukaryotic cells (9).

Efficient recognition of ubiquitinated substrates by the 26 S proteasome requires a minimum targeting signal consisting of four ubiquitin moieties linked to each other through isopeptide bonds between Gly76 and Lys63 (5, 10). The Leu8, Ile44, and Val50 amino acids in the ubiquitin polypeptide, known collectively as the hydrophobic patch, are critical for proteasomal degradation, although mutations in these residues have little effect on the formation of ubiquitin conjugates (11). Mutations of ubiquitin tail residues that disrupt ubiquitin conjugation can also affect proteolysis (12–14).

Ubiquitination regulates the endocytic traffic of signaling receptors, ion channels, permeases, and transporters in yeast and mammalian cells (reviewed in Refs. 2, 3, 15, and 16). In mammalian cells, the down-regulation of growth factor receptors and the epithelial sodium channel by endocytosis and degradation in the lysosome is regulated by ubiquitination. In the yeast Saccharomyces cerevisiae, G protein-coupled signaling receptors (Ste2p and Ste3p) as well as permeases and transporters are internalized into the endocytic pathway by a ubiquitin signal. Ligand binding stimulates phosphorylation and ubiquitination of Ste2p and Ste3p cytoplasmic tails, and both modifications are required for rapid receptor internalization (17–19). Receptors that lack post-translational ubiquitination sites in their cytoplasmic tails can be internalized by monoubiquitin fused in-frame (20, 21). Monoubiquitin also promotes internalization of plasma membrane proteins in mammalian cells when it is fused to an N-terminal cytoplasmic domain (22). The single fused ubiquitin carries within its three-dimensional structure all of the information necessary for regulated endocytosis (21). In contrast, monoubiquitin is an inefficient signal for proteasome recognition (10).

Ubiquitin is important for other cellular functions. Monoubiquitination of histones plays a role in meiosis in yeast and the development of Drosophila embryos (23, 24), and monoubiquitination of a Fanconi anemia protein is linked to DNA repair and localization to nuclear foci (25). Monoubiquitination of the retroviral Gag protein is required for a late step in virus budding (26–28). Independent of the proteasome, the formation of polyubiquitin chains linked through Lys48 on the L28 ribosomal protein regulates ribosome activity (29), and Lys63-linked ubiquitin chains activate the Iok kinase (30). These functions of ubiquitin have recently been discovered, and little is known about how they regulate protein structure and/or activity.

Although ubiquitin is a small protein, it is complex compared with other post-translational modifications such as phosphorylation and acetylation. In this study, we have begun to define the important functional features on ubiquitin’s surface. First, we have identified the ubiquitin amino acids that are essential for vegetative growth of a yeast cell. These residues map to the ubiquitin tail and to two distinct functional faces of the ubiquitin globular domain. Second, we demonstrate that distinct amino acids of ubiquitin mediate its two best characterized functions. Whereas Ile44 and surrounding hydrophobic residues are required for both proteasome recognition and endocytosis.
tosis (11, 21), a distinct surface region of ubiquitin containing Phe4 is required only for endocytosis.

EXPERIMENTAL PROCEDURES

Media, Reagents, Plasmids, and Strains—All yeast strains were propagated in synthetic minimal (SD) medium (31) or rich (YPUDP) medium (2% Bacto-peptone, 1% yeast extract, 2% glucose supplemented with 20 mg/liter adenine, methionine, and tryptophan). The purification of 35S-labeled /H9251-factor has previously been described (32).

Plasmid LHP585 was constructed by subcloning the CUP1-UBI-CYC1 cassette from YEp96 (33) into YEplac195 (34). Ubiquitin mutations were introduced into the sequence of plasmids encoding ubiquitin (LHP585) or Ste2p-Ub3xR (LHP587; Ref. 21) using specifically designed primers and the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA). All mutations were confirmed by automated DNA sequencing.

The plasmids containing STE2 and STE2-UBI variants were transformed into ste2/H9004 strains by single-step gene transplacement at the ura3 locus. All mutant Ste2p and Ste2p-Ub3xR proteins were able to restore mating and a-factor binding in the ste2/H9004 parental strain. The expression of Ste2p-Ub mutant proteins was assessed by immunoblotting using anti-Ste2p polyclonal antibodies. Two individual transformants of each mutant were assayed for their ability to internalize /H9251-factor, and in each case, both transformants demonstrated similar internalization kinetics.

/H9251-Factor Internalization Assays—All /H9251-factor internalization assays with strains expressing Ste2p-Ub3xR chimeras were performed as described (32, 35). Internalization half-times were calculated based on exponential curve fits performed with Kaleidagraph Software (Synergy Software, Reading, PA). Each curve was fitted to a time course assayed for 60 min (seven data points), a best fit to the data was confirmed by visual inspection, and the half-times of internalization were determined from the average rate constants for two or three independent assays. Immunoblotting with anti-Ste2p antiserum confirmed that the mutations that caused a defect in endocytosis did not lead to proteolytic clipping of the receptor-ubiquitin chimera (data not shown).

Ubiquitin Conjugation and Proteasome Assays—Ubiquitination and degradation of 125I-lactalbumin was assayed in rabbit reticulocyte fraction II (11). Lys48-linked Ub3xR (11) or Ste2p-Ub3xR (LHP587; Ref. 21) using specifically designed primers and the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA). All mutations were confirmed by automated DNA sequencing.

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α-Factor Internalization Assays—All a-factor internalization assays with strains expressing Ste2p-Ub3xR chimeras were performed as described (32, 35). Internalization half-times were calculated based on exponential curve fits performed with Kaleidagraph Software (Synergy Software, Reading, PA). Each curve was fitted to a time course assayed for 60 min (seven data points), a best fit to the data was confirmed by visual inspection, and the half-times of internalization were determined from the average rate constants for two or three independent assays. Immunoblotting with anti-Ste2p antiserum confirmed that the mutations that caused a defect in endocytosis did not lead to proteolytic clipping of the receptor-ubiquitin chimera (data not shown).

Ubiquitin Conjugation and Proteasome Assays—Ubiquitination and degradation of 125I-lactalbumin was assayed in rabbit reticulocyte fraction II (11). Lys48-linked Ub3xR (36) was used as an inhibitor or as a substrate for conjugation to a slightly modified form of H2O-UbDHFR (10); specifically, the C-terminal HA epitope was replaced with a consensus site for protein kinase A phosphorylation (this site was not used in the present study). Wild type, F4Y, and L8A,I44A ubiquitin were expressed and purified as previously described (11) and assembled into Lys48-linked chains using E2-25K (37) under conditions optimized to provide similar length distributions. Prolonged incubation of E2-25K
with ubiquitin leads to the accumulation of circular polyubiquitin chains that are inefficiently recognized (38). Circular chains were absent from the preparations used here, based on the finding that >85% of the Ub\(_c\) and Ub\(_g\) in each mixture was disassembled to Ub\(_b\) upon incubation with purified isopeptidase T. Proteasome assays employed 100 nM Ub\(_{b}\)DHFR and 10 nM proteasomes (10), plus 0.5 mg/ml monoubiquitin to prevent absorptive loss of Ub5DHFR. Degradation was monitored by a previously described thiol ester competition assay (39).

**Analysis of the Ability of Mutant Ubiquitins to Support Growth of S. cerevisiae**—Strains analyzed for sole source expression of mutant ubiquitin variants were derived from SUB328 (8) provided by Dan Finley (Harvard Medical School). This strain carried UBI on a LYS2-marked plasmid. SUB328 was transformed with UBI on a URA3-marked plasmid (LHP585) and mutant ubiquitin variants of this plasmid. SUB328 was transformed with UBI on a URA3-marked plasmid. These plasmids were introduced into a strain in which all endogenous ubiquitin genes were deleted and wild type ubiquitin was the sole source of ubiquitin, as assayed by growth of strains at 24 °C. Ubiquitin mutants that passed this test were further evaluated for temperature sensitivity at 37 °C and cold sensitivity at 16 °C. Strikingly, the majority of ubiquitin surface residues were not essential under our test conditions, and very few mutants exhibited conditional growth phenotypes (Table I).

**Internalization Information Carried by the Ubiquitin Polypeptide**—The majority of ubiquitin’s essential residues map to the C-terminal tail, which is involved in conjugation and deubiquitination, and to two surface patches near Phe\(^4\) and Ile\(^44\). The Leu\(^6\)/Ile\(^44\)/Val\(^70\) hydrophobic patch is required for the function of monoubiquitin to promote endocytosis (11). The key residues that are critical for the function of monoubiquitin to promote endocytosis have been identified as Ile\(^44\) and Phe\(^\) (21). To refine our information about the functionally important domains of ubiquitin, we have thoroughly defined the role of ubiquitin surface amino acids in endocytosis. To do this, we analyzed scanning alanine mutants required for ubiquitin’s known and unknown essential functions, we performed a comprehensive alanine scan of the ubiquitin surface. Plasmids encoding the mutant ubiquitin proteins were introduced into a S. cerevisiae strain in which all endogenous ubiquitin genes were deleted and wild type ubiquitin was supplied on a plasmid (8). A plasmid shuffle was then used to assess the ability of each mutant ubiquitin to sustain life as the sole source of ubiquitin, as assayed by growth of strains at 24 °C. Ubiquitin mutants that passed this test were further evaluated for temperature sensitivity at 37 °C and cold sensitivity at 16 °C. Strikingly, the majority of ubiquitin surface residues were not essential under our test conditions, and very few mutants exhibited conditional growth phenotypes (Table I).

**RESULTS**

**Ubiquitin Residues Essential for Life in Yeast**—Ubiquitin is highly conserved in eukaryotes, with only three amino acid differences between the yeast and human polypeptides at positions 19, 24, and 28. To identify surface residues (see Fig. 1A)
of all of the surface residues of yeast ubiquitin made in the context of a Ste2p-ubiquitin chimera (35). Internalization of this chimeric protein is controlled by the fused ubiquitin and not by ubiquitin that is post-translationally conjugated to the chimeric protein, because the chimeric protein (Ste2p-UB3xR) carries Lys → Arg substitutions at the known sites of polyubiquitin chain formation in vivo, Lys(48), Lys(48)′, and Lys(63) (7). Ubiquitin surface residues (see Fig. 1A) in Ste2p-UB3xR were mutated individually or in clusters of 2–4 residues, and yeast strains expressing the mutant chimeras were assayed for their ability to internalize the Ste2p ligand, α-factor. Met28 was not mutated; neither was Ser22, since this residue is an alanine in the three-dimensional structure of ubiquitin. These amino acids are Gln2, Lys6, Leu6, Thr12, Glu34, and Val67. Together with Ile44 and Phe4, these residues define two discrete surface regions in the ubiquitin globular domain that are important for endocytosis (Fig. 1C).

In addition to its globular domain, ubiquitin carries a tail of four residues, Leu73, Arg74, Gly75, and Gly76, through which ubiquitin is post-translationally conjugated to substrate proteins. Gly75 and Gly76 are essential for conjugation, but their deletion had no effect on internalization (Fig. 2A, Table II). Therefore, the C-terminal glycine do not carry information needed for internalization. (This observation also indicates that internalization of the receptor-ubiquitin chimeras does not require the conjugation of the C terminus of the fused ubiquitin to a lysine residue of another protein, confirming that internalization of the mutant chimeras is mediated by the fused ubiquitin moiety alone.) When Leu73 and Arg74 were mutated individually, each mutation caused 2.3-fold inhibition (Table II). However, the deletion of the entire four-residue tail had a more modest, but still significant, effect of 1.7-fold (Fig. 2A, Table II). At present, it is unclear why deleting the tail has a smaller effect than mutation of Leu73 or Arg74 to alanine. These data suggest that the ubiquitin tail plays a minor role in post-conjugation steps of ubiquitin-mediated endocytosis. The ubiquitin residues in the two surface regions and the tail that function in endocytosis are highlighted in Fig. 1C.

Internalization is the first role to be discovered for Phe4. To further characterize the role of this amino acid in internalization we made a more conservative mutation, Phe4 → Tyr, and analyzed the ability of this mutant to direct internalization of α-factor by the corresponding chimera. The F4Y mutation had as severe an effect on internalization as F4A (Fig. 2B, Table II). Because changing Phe4 to Tyr adds a single hydroxyl group at a surface-exposed position, it is unlikely that this mutation inhibits internalization by disrupting the ubiquitin structure (this possibility was rigorously excluded; see below). Instead,
functions of ubiquitin surface domains in yeast

endocytosis. The K6A protein showed a strongly augmented level of conjugates in conjunction with a reduced degradation rate, suggesting that polyubiquitin chains assembled from this mutant are poorly recognized (11). Conjugates of high molecular weight, which are favored substrates for the proteasome (10), were underrepresented in the K6A ubiquitin assays (data not shown), suggesting that the apparent proteasome recognition defect may be an indirect consequence of a reduced efficiency of long polyubiquitin chain assembly. The competence of all the mutant ubiquitins in conjugation (Fig. 3A) indicates that they are properly folded, and consistent with this conclusion, the F4A and F4Y mutants were found to bind to the E1 ubiquitin-activating enzyme with affinities indistinguishable from wild type ubiquitin (data not shown).

To demonstrate specifically that the Phe^4 residue is not involved in the binding of polyubiquitin to proteasomes, we used an assay that measured the ability of different mutant ubiquitin chains to inhibit proteasome degradation. Unanchored chains of four wild type ubiquitin moieties (Ub^a) inhibited the degradation of a model proteasome substrate, ubiquitinated dihydrofolate reductase (Ub₅DHFR; Fig. 3B) (10). By contrast, ubiquitin chains carrying mutations that affect proteasome binding, L8A and I44A, do not efficiently inhibit Ub₅DHFR degradation. To test how UbF4Y chains behave, wild type, F4Y, and L8A,I44A ubiquitins were individually assembled into unanchored polyubiquitin chains (Fig. 3C) and the three chain mixtures were compared as inhibitors of Ub₅DHFR proteolysis. Chains assembled from wild type or F4Y ubiquitin inhibited degradation almost completely, whereas, as expected (11), L8A,I44A chains inhibited degradation more weakly (Fig. 3D). These data demonstrate that Phe^4 is not required for targeting to proteasomes mediated by polyubiquitin chains. The data also show that Phe^4 is not required for ubiquitin conjugation mediated by the enzymes used in these experiments.

**DISCUSSION**

The ubiquitin molecule comprises a compact globular domain that consists of a five-stranded β-sheet and an α-helix and a flexible tail formed by four protruding residues (42, 43). Two surface regions that are important for the defined functions of ubiquitin in proteasome recognition and endocytosis have been mapped onto this structure. A hydrophobic patch including Leu^7, Ile^44, and Val^70 is required for proteasome degradation (11) and plays a critical role in endocytosis (21). Phe^4 and adjacent residues are important for the endocytic role of ubiquitin but do not function in proteasome binding and degradation. Although the C-terminal tail of ubiquitin is required for all known ubiquitin functions, the essential nature of this structural feature is due to its role in conjugation. The tail is not known to be important for downstream recognition in any ubiquitin signaling pathway.

The ubiquitin residues essential for vegetative growth of yeast lie on these same three surface features, namely the tail and two distinct clusters on the globular core domain centered around Phe^4 or the Leu^8/Ile^44/Val^70 hydrophobic patch (Fig. 1B). Many of the essential amino acids we identified are known to be involved in ubiquitination, deubiquitination, and/or proteasome-mediated degradation (see below), all essential cellular processes. Except for the C terminus, the essential surface ubiquitin residues do not correlate closely with surface residues conserved in both of the ubiquitin-like proteins Rub1p and Smt3p (Fig. 1A). Although the Leu^8/Ile^44/Val^70 hydrophobic patch is conserved in Rub1p, Rub1p does not efficiently signal endocytosis when fused to Ste2p. Our results suggest that the

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*2 J. C. Jene and L. Hicke, unpublished data.*
inability of Rub1p to signal endocytosis may be due to the absence of residues comprising the Phe4 patch. Nedd8, the mammalian Rub1p homologue, can replace one of the ubiquitins in tetraubiquitin. This observation suggests that the inability of Rub1p/Nedd8 chains (39) to recognize Rub1p/Nedd8 chains (39). internalization-defective mutant ubiquitins are not deficient in conjugation or proteasome-mediated degradation. Purified wild type or mutant ubiquitins were assayed for their ability to support the ubiquitination and degradation of a model proteasome substrate, 125I-bovine lactalbumin. Solid bars, corrected initial rate of lactalbumin degradation; gray bars, steady-state level of ubiquitin-lactalbumin conjugates. Values are expressed relative to that for wild type ubiquitin (mean ± S.D., n = 3). B, Ub4 inhibits degradation of Ub4DHFR by purified 26 S proteasomes. Assay incubations with and without 13 μM Ub4 were sampled at the indicated times and subjected to Western blotting with an antibody against an N-terminal epitope tag of the substrate. C, polyubiquitin chains used in inhibition experiments (Coomassie-stained gel): wild type chains (of various sizes) (8.5 μg), F4Y chains (8.5 μg), L8A, I44A (8.5 μg), and wild type Ub4 chains (0.25 μg). D, degradation of Ub4DHFR by purified 26 S proteasomes in the presence of wild type or mutant ubiquitins. Assay incubations were sampled at 0 or 40 min and subjected to Western blotting with an antibody against an N-terminal epitope tag of the substrate. Polyubiquitin chains (C) were added at a concentration providing ~13 μM Ub4 in each case. Stronger inhibition by wild type and F4Y chains in D relative to Ub4 in B is due to the presence of significant Ub5 and Ub6 in the chain mixtures used in D.

The ubiquitin tail consists of the essential residues Leu73, Arg74, Gly75, and Gly76. Gly75 and Gly76 are important for ubiquitin conjugation and deubiquitination (44–46). Arg74 is essential even though it is not important for E1 interaction or conjugation. These residues may be important for deubiquitination (47) and possibly for proteasome recognition as well (13). Arg74 and Leu73 play a minor role in endocytosis.

The essential surface cluster including Leu8, Ile44, and Val70 consists of nine amino acids that extend from the base of the ubiquitin tail up to Gly77 and Lys48 (Fig. 1B). All of these residues are probably involved in ubiquitin conjugation and/or proteasome degradation, and they may also be important for deubiquitination (48). Lys48 is the major site of polyubiquitin chain formation that is necessary for proteasome degradation (5). The role of Gly77 has not been characterized, but since it bridges Lys48 and the Ile44 hydrophobic patch, it may play a role in proteasome degradation. Arg42 and Arg72 are required for efficient activation of ubiquitin by the E1 ubiquitin-activating enzyme (14, 39), and Ile44 plays a role in conjugation and/or proteasome recognition (11). The function of Leu73 has not been investigated, but since it lies adjacent to Ile44, Val70, and Arg72 it is likely to play a role in conjugation or proteasome degradation.

The second essential cluster on the globular domain surface consists of residues Gin2, Phe4, and Thr12. Phe4 is critical for endocytosis, and Gin2 and Thr12 play a minor role. The effect of the F4A and F4Y mutations is not due to structural instability, because these mutant variants are conjugated to substrates efficiently (Fig. 2A) and are expressed as well as wild type ubiquitin in yeast cells (data not shown). Moreover, the F4A and F4Y mutant proteins are recognized by proteasomes in the context of polyubiquitin chains (Fig. 3). Although most of the Phe4 patch residues involved in endocytosis are essential, endocytosis itself is not absolutely required for vegetative growth of yeast at 24 °C (49, 50). This suggests that the Phe4 patch may be involved in other, non-proteasome-dependent functions of ubiquitin. We conclude that ubiquitin carries a distinct essential surface region that is important for endocytosis, and perhaps for uncharacterized functions of ubiquitin, but not for proteasome degradation.

Our observations demonstrate that the Ile44 hydrophobic patch is critical for multiple functions of ubiquitin and suggest that Ile44 may interact with different proteins to facilitate either proteasome degradation or endocytosis. Proteasome degradation requires interaction of the Leu8/Ile44/Val70 hydrophobic patch with a regulatory subunit(s) of the proteasome. To promote endocytosis, Ile44 together with Phe4 may form an extended binding site that interacts with a component of the endocytic machinery. Alternatively, Phe4 and Ile44 may interact with different endocytic proteins. Efficient proteasome
binding requires a tetrabiubitin chain, whereas endocytosis is triggered by monoubiquitination. Therefore, the interaction of a specific binding partner with Ile44 may depend on the type of ubiquitin modification. Although monoubiquitinated receptors are efficiently recognized for endocytosis, the actual endocytic signal may be more complicated than a single ubiquitin. For example, monoubiquitinated plasma membrane proteins such as Ste2p form multimers (51, 52), potentially allowing the display of a unique multimeric binding surface composed of noncovalently linked, but closely associated, ubiquitin monomers. Alternatively, the location of the ubiquitinated proteins at the plasma membrane may influence binding partner selection.

Monoubiquitin functions as a signal for internalization not only in yeast but also in higher eukaryotes. A fused ubiquitin moiety lacking lysine residues is sufficient for the endocytosis of chimeric plasma membrane proteins expressed in mammalian cells (22). As in yeast, Ile44 is important for ubiquitin-dependent internalization in animal cells (22). The function of Phe4 in animal cell endocytosis has not been reported. Ile 44 is part of a ubiquitin sequence, DQQRL4I44, that is similar to the DKQTL4 dimerization signal that was identified in the CD3 subunit of the T cell receptor (53). Like Ile44, the mutation of the buried Leu43 to Ala severely inhibits endocytosis in animal cells (22). The function of Ile44 is independent internalization in animal cells (22). The function of Ile44 may depend on the type of binding partner with Ile44 may depend on the type of ubiquitin-dependent internalization in nonessential ubiquitin-dependent processes such as proteasomal degradation or endocytosis. Monoubiquitinated plasma membrane proteins such as Ste2p form multimers (51, 52), potentially allowing the display of a unique multimeric binding surface composed of noncovalently linked, but closely associated, ubiquitin monomers. Alternatively, the location of the ubiquitinated proteins at the plasma membrane may influence binding partner selection.

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