Ubiquitin Binding Site of the Ubiquitin E2 Variant (UEV)
Protein Mms2 Is Required for DNA Damage Tolerance in the Yeast RAD6 Pathway*

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Colleen Tsui‡, Arani Raguraj, and Cecile M. Pickart§
From the Department of Biochemistry and Molecular Biology, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, Maryland 21205

Different ubiquitin modifications to proliferating cell nuclear antigen (PCNA) signal distinct modes of lesion bypass in the RAD6 pathway of DNA damage tolerance. The modification of PCNA with monoubiquitin signals an error-prone bypass, whereas the extension of this modification into a Lys-63-linked polyubiquitin chain promotes error-free bypass. Chain formation is catalyzed by the Mms2/Ubc13 conjugating enzyme variant/conjugating enzyme (UEV-E2) complex together with the Rad5 ubiquitin ligase. In vitro studies of this UEV-E2 complex have identified a ubiquitin binding site that is mainly localized on Mms2. However, the role of this site in DNA damage tolerance and the molecular features of the ubiquitin/Mms2 interaction are poorly understood. Here we identify two molecular determinants, the side chains of Mms2-Ile-57 and ubiquitin-Ile-44, that are required for chain assembly in vitro and error-free lesion bypass in vivo. Mutating either of these side chains to alanine elicits a severe 10–20-fold inhibition of chain synthesis that is caused by compromised binding of the acceptor ubiquitin to Mms2. These results suggest that the ubiquitin binding site of Mms2 is necessary for error-free lesion bypass in the RAD6 pathway and provide new insights into ubiquitin recognition by UEV proteins.

Ub conjugation involves the sequential action of activating (E1), conjugating (E2), and ligase (E3) enzymes (2). The E1 and E2 enzymes both form thiol ester adducts with Ub and both intermediates are strictly required for substrate ubiquitination. Most organisms possess a single E1, multiple E2s, and even more E3s. Individual E3s, many of which harbor a zinc-binding RING domain, act in conjunction with specific E2s to recognize individual substrate proteins thus imparting specificity in ubiquitination (2, 5).

Substrates can be modified with one Ub, several single Ubs, or multiple Ubs in the form of an isopeptide-linked polyUb chain. These various modifications, especially mono- versus polyubiquitination, often lead to qualitatively different outcomes (2, 3). Further variation in Ub signaling can result from the use of different lysine residues in polyUb chain assembly (6). For example, Lys-48-linked chains signal targeting to proteasomes (7, 8), whereas Lys-63-linked chains constitute a non-degradative signal in several pathways, including ribosomal protein synthesis (9), kinase activation (1), and membrane protein trafficking (3, 10). Mechanisms governing selectivity in the synthesis and recognition of these and other non-canonical polyUb signals are only beginning to be understood (6).

Besides the roles mentioned above, Lys-63-linked polyUb chains are required for a DNA damage tolerance pathway that has been most thoroughly characterized in the budding yeast *Saccharomyces cerevisiae*. The components of the RAD6 pathway are highly conserved from yeast to humans (11–14); their actions promote the bypass of DNA photo- or alkylation adducts that would otherwise block the progression of the replicative DNA polymerase. All events in this pathway depend on the Ub-conjugating activity of the Rad6 E3 complex (14–16), but the pathway subsequently divides into two branches. One branch, defined by MMS2, UBC13, and RAD5, mediates error-free bypass of DNA lesions (17–21), possibly through the recruitment of an undamaged template strand (12, 13). The other subpathway, defined by REV3 and REV7, facilitates mutagenic bypass through use of the damaged strand as a template for a translesion polymerase (12, 13). Lys-63-linked polyUb chains signal selectively in the error-free branch of the RAD6 pathway (11, 18, 22).

Recent studies have identified the essential DNA polymerase processivity factor PCNA as a major target of Ub modifications during DNA lesion bypass (22–24). Monoubiquitination of PCNA Lys-164, catalyzed by the Rad6-Rad18 complex, signals error-prone repair (22, 23), probably by promoting the recruitment of a translesion polymerase (25, 26). This same Ub modification also serves as the substrate for extension of a Lys-63-linked polyUb chain, which promotes error-free lesion bypass (22). Chain formation requires a ternary complex composed of a RING E3, Rad5, and the heterodimeric Mms2-Ubc13 complex.

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‡ Current address: Children’s Memorial Hospital, Northwestern University, Chicago, IL 60614.
§ To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, Johns Hopkins University, 615 North Wolfe St., Baltimore, MD 21205. Tel.: 410-614-4554; Fax: 410-955-2926; E-mail: cpickart@jhmi.edu.
† The abbreviations used are: Ub, ubiquitin (subscripts denote the number of residues in certain recombinant versions); E1, ubiquitin activating enzyme; E2, ubiquitin conjugating enzyme; E3, ubiquitin-protein ligase; PCNA, proliferating cell nuclear antigen; UIM, ubiquitin-interacting motif; polyUb, polyubiquitin; UEV, ubiquitin E2 variant.
Ub synthesizes unanchored Lys-63-linked polyUb chains from free predicted. However, the finding that Rad5 is strictly required indeed binds to this site in a manner similar to that originally by Ellison and co-workers (31, 32) have confirmed that Ub defined as the molecule that donates Lys-63 to the nascent on the Mms2 modeling identified a likely binding site for the “acceptor” Ub ing site is necessary for chain assembly. These results strongly suggest that the acceptor Ub bind- ing site is necessary for chain assembly in vitro.

MATERIALS AND METHODS

Yeasts—Parental yeast strains SUB62 (MATa leu2-3,112 ura3-52 his3-200 trp1-1) and SUB280/SUB413 (MATa leu2-3 ura3-52 his3-200 trp1-1[am] ubi1-1::TRP1 ubi2-1::ura3 ubi3-Δub-2 ubi4-Δ2::LEU2[pUb39][pUb100]) were provided by D. Finley. In the latter two strains, all four Ub-encoding genes have been deleted (11), and Ub is expressed from a LYS2-marked, high copy plasmid (pUb39), whereas a HIS3-marked (pUb100) plasmid expresses the essential ribosomal protein encoded by the UBI1 gene. In SUB280, pUb39 specifies wild type Ub; in SUB413, it specifies Ub-K63R (11). In both strains the plasmid-borne Ub gene is under the control of a CUP1 promoter. In the absence of copper (the condition used here) these genes afford approximately wild type levels of Ub (33). We previously deleted the MMS2 and UBC13 genes individually from SUB62, SUB280, and SUB413 (18, 22).

Plasmids and Proteins—Existing centromeric URA3-marked yeast plasmids (18, 30) encoding His6-Mms2 or Ubc13-HA3 (the latter including the intron of UBC13) under the control of the respective endogenous promoters were mutated by standard PCR methods. All mutated open reading frames (see also below) were sequenced in their entireties. Sequences of primers used in mutagenesis and cloning are available upon request. URA3-marked 2μ plasmids encoding wild type Ub or Ub-144A were provided by L. Hicke (Northwestern University) (34).

Escherichia coli plasmids specifying His6-Mms2 (pET16b), GST- Ubc13 (pGEX4T2), and untagged Ub (pET3a) (29, 30, 35) were mutated by standard PCR-based methods. Proteins were expressed in strain BL21(DE3)pLysS (36). Ub was purified as described (35). Ubc13 was eluted from GSH beads by thrombin cleavage (30). His6-Mms2 was purified under denaturing conditions (30). Urea (4 M), glycerol (5% v/v), and NaCl (0.5 M) were added after cell lysis and DNA digestion and were maintained during nickel chelate chromatography. The eluted protein was concentrated and then renatured by successive dialysis against buffer (50 mM Tris, pH 8.0, 0.1 mM EDTA, 5% glycerol, 0.5 M NaCl) containing 1, 2, and 5 M urea, respectively. The final dialysis employed buffer without urea or salt. Dithiothreitol, 1 mM, was included in the buffers beginning with the second (1 M urea) dialysis. His6-tagged mouse E1 was produced in insect cells and purified by nickel chelate chromatography (37). C-terminally truncated (Ub74) versions of wild type and mutant Ub were generated by treatment with trypsin, which cuts after Ub-Arg-74, following by the addition of a 2-fold (w/v) excess of soybean trypsin inhibitor (Sigma) (38). Ub was radiolabeled (29) or reductively methylated (39) according to published procedures.

Yeast Extracts and Western Blotting—Cells (2 optical density units) from log-phase cultures of SUB413 (with or without the Ub-144A-expressing plasmid) were pelleted, washed, and then sonicated in 90 µl of alkaline extraction buffer as previously described (40). Aliquots were analyzed by Western blotting with affinity-purified polyclonal antibodies directed against Ub (produced by these authors). Blots were devel- oped colorimetrically by means of an alkaline phosphatase-conjugated secondary antibody (Bio-Rad).

Assays—UV sensitivity was assayed in triplicate as previously described (19). Chain assembly was assayed at pH 7.6 and 37 °C and monitored by SDS-PAGE (29, 30). The standard steady-state assay (detection by Coomassie staining) contained 0.1 µM E1, 2 µM each Ub and Mms2, and 117 µM Ub. To determine initial rates of Ub2 synthesis, Ub14 or Ub-Asp-77 (117 µM) was used as the acceptor in conjunction with full-length Ub (2–6 µM). Reactions were termi- nated during the linear phase of 125I-Ub2 synthesis, and this product was quantitated (following autoradiography) by band excision/counting or phosphorimage analysis (18). In some cases enzyme concentrations were adjusted, and/or modified forms of Ub were used, as described in the figure legends. Published procedures were used to produce Lys-63- linked Ub carrying the K63R mutation in its distal Ub and an extra residue (Asp-77) in its proximal Ub (29); this chain is chemically inert in the Mms2/Ubc13-catalyzed reaction. The integrity of the Mms2/ Ubc13 interaction was assessed by monitoring the binding of untagged Ub13 to H10-Mms2 immobilized on nickel beads as in our previous work (30). Expression levels of His6-Mms2 and Ubc13-HA3 in yeast cell extracts were monitored by Western blotting with antibodies (Santa Cruz) directed against the His6 tag (Sc-803) and the hemagglutinin tag (Sc-805), respectively.

RESULTS

A Model for Ub Interaction with the Mms2-Ubc13 Complex—Structural and modeling studies have identified two Ub binding sites on the Mms2/Ubc13 heterodimer (30, 32). One of these sites (Fig. 1) interacts with the Ub molecule bound to the active site cysteine residue of Ubc13. We define this site, which is entirely located on Ubc13, as the “donor” site. The other site mainly encompasses a concave lower surface of Mms2 (as orien- ted in Fig. 1). This site binds the Ub that provides Lys-63 to the isopeptide bond. In the discussion below we refer to this as the “acceptor” site. We proposed the existence of donor and acceptor sites based on the results of modeling and site-specific mutagenesis (30). The donor site is a universal feature of E2 enzymes (32, 41), but so far, the acceptor site is unique to the Mms2-containing heterodimer. Solution structural studies of Ellison and co-workers (32) have shown that Ub indeed binds non-covalently to the acceptor site, which is primarily located on Mms2.
An Mms2-interacting Residue of the Acceptor Ub (Ile-44) Is Important for Chain Synthesis In Vitro and DNA Damage Tolerance in Vivo—Our goal in the present work was to test the involvement of the acceptor Ub site in the biochemical and biological activities of the Mms2-Ubc13 complex. To identify residues of Ub that are important for its binding to this site, we began with three residues that comprise a hydrophobic patch on the surface of Ub. The side chains of Leu-8, Ile-44, and Val-70 are important for the recognition of Lys-48-linked polyUb chains by proteasomes and for several other Ub-dependent processes (34, 42, 43). In addition, NMR results indicate that the surface encompassing these residues contacts the acceptor Ub binding site of the human Mms2-Ubc13 complex (31). To test the functional significance of such contacts, each of the three residues was individually mutated to alanine, and the purified mutant proteins were screened in steady-state chain assembly assays with the yeast Mms2-Ubc13 complex. As shown in Fig. 2A, chain synthesis with the L8A and V70A mutants proceeded at a similar rate to the reaction with wild type Ub (lanes 7–14 versus 1–3; the anomalous migration of Ub-L8A seen in lanes 7–10 is frequently observed). In contrast, the Ub-I44A mutation caused a profound defect in chain synthesis (Fig. 2A, lanes 4–6).

Because the Ub-I44A mutation is lethal (34), we used an indirect approach to ask whether the in vitro defect conferred by this mutation correlated with a deficiency in error-free DNA lesion bypass. A plasmid specifying wild type Ub or Ub-I44A was transformed into a yeast strain engineered to express Ub-K63R as the sole form of Ub (11). The parent strain, called SUB413, is UV light sensitive because of a specific defect in error-free lesion bypass that is due to the inability of Ub-K63R to be assembled into Lys-63-linked chains (Fig. 2B, open circles) (11, 18). As expected, expression of wild type Ub from a control plasmid complemented this phenotype (filled circles). Note that Ub-K63R does not act as a dominant chain terminator, presumably due to rapid disassembly and re-synthesis of chains within cells (8). In contrast, expression of Ub-I44A in SUB413 failed to complement the UV light-sensitive phenotype of this strain (Fig. 2B, filled squares), despite robust expression as evidenced by increased levels of free and conjugated Ub (Fig. 2C). We postulate that the inability of Ub-I44A to promote DNA damage tolerance reflects the inability of this mutant to be used as a substrate for Mms2-Ubc13-catalyzed synthesis of Lys-63-linked chains (Fig. 2A).

Polar Effect of Ub-I44A Mutation—we next sought to determine whether Ub-I44A was defective in binding to one or to both of the Ub binding sites in the Mms2-Ubc13 complex. To address this question we created two versions of Ub-I44A, each of which could perform only one role (i.e. donor or acceptor). Blocking the C terminus of Ub via biosynthetic introduction of an extra residue (Asp-77) prevents activation of Gly-76; this blocks the donor site. (Although we cited this result in an earlier publication (30), these data have not been presented previously.) Quantitative reactions with a radiolabeled donor Ub were performed as described under “Materials and Methods” with 117 µM Ub and 4 µM Mms2-Ubc13 complex. Assays were quenched at the indicated times. Only the region of the gel containing Ub and Ub$_n$ is shown. Lanes 1–3, wild type (wt) Ub; lanes 4–6, Ub-I44A; lanes 7–10, Ub-L8A; lanes 11–14, Ub-V70A. No Ub$_n$ product was present in the zero time samples from the wild type Ub and Ub-I44A reactions (data not shown, but see Figs. 3 and 4). B, I44A mutation in Ub compromises error-free lesion bypass. S. cerevisiae strain SUB413, which expresses Ub-K63R as the sole form of Ub, is UV light sensitive because of the inability to synthesize Lys-63-linked polyUb chains (open circles; SUB413 was transformed with empty vector). Wild type Ub (filled circles) or Ub-I44A (filled squares) was expressed in this strain from a 2 µm plasmid under the control of a ÇUP1 promoter, in the absence of added copper. Each point is the mean $\pm$ S.D. of triplicate determinations. Error bars that cannot be seen are smaller than the diameter of the symbol. C, Ub-I44A is strongly expressed (anti-Ub Western blot). Extracts from log-phase SUB413 cells transformed with empty vector (lanes 1 and 3) or the Ub-I44A-expressing plasmid (lanes 2 and 4) were analyzed (lanes 1 and 2, 2 µl of extract; lanes 3 and 4, 4 µl of extract). Migration of molecular mass standards is shown at the left. Coomassie staining of duplicate lanes confirmed equal loading of the sample pairs (not shown). Ub$_1$ and Ub$_n$ refer to mono-Ub and Ub-conjugated proteins (the latter including free polyUb chains), respectively.
As competitors. Incubations were sampled at a constant time during the site. The value of plex) were sampled at the indicated times. Previously estimated subsaturating Ub-Asp-77 (25\(\text{RM}\)) donor Ub (see the text). The two Ubs were combined at 117\(\text{D77}\) mutation) or the reductively methylated (yeast) gel). The Ub-I44A mutation was introduced selectively into the acceptor Ub functionality (Coomassie-stained I44A acceptor; filled circles, wild type acceptor; open circles, Ub-I44A mutation selectively compromises the interaction of Ub with the acceptor site of the Mms2-Ubc13 complex. These results reinforce the notion that the inability of Ub-I44A to support error-free bypass \textit{in vivo} (Fig. 2) reflects compromised binding to this site. Our standard catalytic assays were done at a Ub concentration (117 \(\mu\text{M}\)) that is below saturation for the acceptor site. Thus, the ability of Ub-L8A and Ub-V70A to support chain synthesis (Fig. 2A) suggests that unlike Ile-44, the Leu-8 and Val-70 side chains do not make important contributions to the interaction of Ub with the acceptor site of yeast Mms2. (Note that the donor site is saturated in this assay, because its occupancy is mainly determined by the upstream interaction of Ub with E1, which follows \(K_m \approx 1–2 \mu\text{M}\) for wild type Ub (46, 47)).

Role of Mms2-Ile-57 in Acceptor Ub Binding—Based on our model for acceptor Ub binding to the Mms2-Ubc13 complex, it appeared possible that Ub-Ile-44 could contact Mms2 near the side chain of Mms2-Ile-57 (Fig. 1). As a first test of this model, we introduced the I57A mutation into Mms2 and compared the purified mutant protein to wild type Mms2 in a steady-state chain synthesis assay. The I57A mutant displayed strongly reduced activity (Fig. 4A). Quantitative assays with \(^{125}\text{I}\)-labeled donor Ub showed that the initial rate of Ub\(_2\) synthesis was reduced by 15-fold (data not shown). Control experiments showed that wild type Mms2 and the I57A mutant bound with similar efficiencies to wild type Ubc13 (data not shown). Therefore the defect in chain synthesis seen with Mms2-I57A cannot be attributed to a weakened interaction with Ubc13, consistent with the large distance between Mms2-Ile-57 and the heterodimer interface (Fig. 1 (30, 48)). To test whether the heterodimer containing Mms2-Ile-57 was impaired in its ability to bind the acceptor Ub, we again turned to competition assays. Here we used catalytically inert Lys-63-Ub\(_1\) as the competitor (see “Materials and Methods”). The weak competition seen in assays involving Mms2-I57A (Fig. 4B, open circles), in comparison to the result with wild type Mms2 (filled circles), indicates that Mms2-Ile-57 plays an important role in acceptor Ub binding. The I57A mutation in Mms2 led to a \(\approx 6\)-fold increase in the value of \(K_{\text{app}}\).

Mms2-Ile-57 Is Important for DNA Damage Tolerance—We next tested whether the \textit{in vitro} defect conferred by the Mms2-I57A mutation (Fig. 4, A and B) translates into compromised DNA damage tolerance \textit{in vivo}. A low copy yeast plasmid specifying Mms2-I57A under the control of the endogenous MMS2 promoter was transformed into an mms2\(A\) strain of \textit{S. cerevisiae}. As shown in Fig. 4C, expression of Mms2-I57A afforded minimal rescue of the UV-light sensitive phenotype of the mms2\(A\) strain (filled squares versus open circles) in comparison to results obtained for wild type Mms2 in the same vector backbone (filled circles). Blotting experiments confirmed that the mutant protein was expressed at a comparable level to wild type Mms2 (data not shown). Thus, Mms2-Ile-57 is important for chain synthesis \textit{in vivo}.

Mutation of Ubc13-Asp-81 Inhibits DNA Damage Tolerance—Ubc13-Asp-81 is predicted to lie near the boundary of the acceptor site in the Mms2-Ubc13 complex (Fig. 1) (30). We found in earlier work that mutating Ubc13-Asp-81 to arginine or alanine inhibited \textit{in vitro} chain synthesis completely or severely, respectively (30). To address the importance of Ubc13-Asp-81 \textit{in vivo}, we expressed each of these mutant Ubc13 proteins in a \textit{ubc13A} strain. As shown in Fig. 4D, the phenotype of the ubc13-Asp-81 strain (filled squares) was indistinguishable from that of the null strain (open circles), whereas expression of the D81A mutant (filled triangles) provided weak rescue. Both proteins were expressed comparably to wild type Ubc13 expressed in the same vector backbone (data not shown). These phenotypes correlate well with the biochemical effects of the corresponding mutations.
FIG. 4. Mms2-I57A mutation impedes acceptor Ub binding. A, steady-state Ub2 synthesis assays (Coomassie-stained gel). Assays contained Mms2/Ubc13 complex at 2 μM (assembled from wild type [wt] Mms2 or Mms2-I57A as indicated), together with Ub-Asp77 and Ub-K63R at 117 μM each. Incubations were sampled at the indicated times. B, competition assays. Quantitative assays employed a subsaturating concentration of Ub-Asp77 acceptor (25 μM) with [125I]-Ub (5 μM) as donor. The Mms2/Ubc13 complex (2 μM) was formed from wild type Mms2 (filled circles) or Mms2-I57A (open circles); chain synthesis reactions were sampled during the linear phase of Ub2 formation. Reactions contained the indicated concentration of chemically inert Lys-63-Ub4 as competitor (see “Materials and Methods”). Rates are expressed relative to the control-lacking competitor. Because the control rate with Mms2-I57A was slow (A), incubations containing this mutant were sampled at a 4-fold later time than incubations containing wild type Mms2. C, Mms2-I57A is defective in error-free lesion bypass. The SUB62-derived mms2Δ yeast strain (30) was transformed with empty centromeric vector (open circles), a version of this plasmid expressing H10-Mms2/I57A (filled squares), or a version expressing wild type H10-Mms2 (filled circles), in both cases under the control of the MMS2 promoter. Assays at each UV light dose were performed in triplicate. D, mutation of Ubc13-Asp81 inhibits error-free lesion bypass. Experiments were performed as in C, except that plasmids were transformed into the SUB62-derived ubc13Δ strain. Triple hemagglutinin-tagged versions of wild type Ubc13 (filled circles), Ubc13-D81A (filled triangles), or Ubc13-D81R (filled squares) were expressed under the control of the endogenous UBC13 promoter in a centromeric plasmid. Open circles denote strain transformed with empty vector.

DISCUSSION

The ability of chemically distinct polyUb chains to act as functionally distinct signals can be appreciated by comparing the properties of Lys-48-linked with Lys-63-linked chains. Lys-48-linked chains are the principal signal for targeting to proteasomes (7, 8), whereas Lys-63-linked chains are non-proteolytic signals in several different pathways. For example, the modification of TRAF6 with Lys-63-linked chains activates a specific protein kinase upstream of IκBα kinase, ultimately leading to phosphorylation and degradiation of IκBα, translocation of NFκB into the nucleus, and the induction of inflammatory responses (1). In the Ub-dependent DNA damage tolerance pathway studied here, the modification of PCNA with a Lys-63-linked chain promotes an error-free mode of DNA lesion bypass (11, 22). Because TRAF6 and PCNA are metabolically stable proteins, Lys-63-linked chains do not elicit target protein degradation in these signaling pathways. Instead of being recognized by proteasomes, the non-canonical TRAF6-linked chains bind to the kinase adaptor proteins TAB2 and TAB3 (49); the receptor of the PCNA-bound chain signal is not yet known. Although the target protein that is modified by the non-canonical chain differs between the two signaling pathways, there are notable similarities in the biochemistry of signal generation. In both cases, a heterodimeric UEV-Ubc13 complex collaborates with a RING domain E3 to generate the chain. Here we have identified specific molecular determinants of the UEV-Ub interaction in the DNA damage tolerance pathway.

The Ub-based determinant, the Ile-44 side chain, is essential for viability in budding yeast (34). This side chain is important for the recognition of mono-Ub signals in the non-essential process of endocytosis (34) and for the recognition of Lys-48-linked chains by proteasomes, which is an essential process (8, 43). Ub-Ile-44 is known to make an important contribution to the affinity of mono-Ub for representative members of two families of Ub-interacting elements, the ubiquitin-interacting motif (50, 51) and CUE (52–54) domains. The present results add the UEV protein Mms2 to this list. This conclusion is based on the profound inhibition of Mms2/Ubc13-catalyzed chain synthesis caused by the Ub-I44A mutation (Fig. 2A), an effect that is specifically because of compromised interaction of this mutant Ub with the acceptor site of the heterodimeric UEV-E2 complex (Fig. 3, A and B). Although the weak affinity of wild type mono-Ub for this site of the yeast Mms2/Ubc13 complex precluded a direct determination of the affinity of Ub-I44A, the results of competition studies (Fig. 3C) indicate that reduced binding to this site is the principal cause of the catalytic defect. The other major constituents of the hydrophobic patch of Ub do not seem to contribute importantly to the Mms2/Ub interaction, because the individual Ub-L8A and Ub-V70A mutations have no detectable effect on the kinetics of chain synthesis at a subsaturating Ub concentration (Fig. 2A).

NMR chemical shift perturbation studies indicate that the hydrophobic patch of Ub contacts Mms2 in the human Ub-Mms2 complex (31). Our finding that the Ile-44 side chain plays a unique role in mediating the interaction is consistent with these results but represents a significant refinement. Further studies will be necessary to determine whether Ub-Ile-44 also plays a predominant role in the interaction of Ub with human Mms2. The higher affinity of the latter interaction, $K_d \approx 30 \mu M$ (45) versus $K_d \approx 100–400 \mu M$ in the yeast case (Fig. 3C and Ref. 29), suggests that additional interactions are available in the human Mms2-Ub complex, which could involve contacts with other residues in the hydrophobic patch of Ub. However, the presence of Leu-8 and Val-70 at the interface of the human complex does not necessarily translate into an
important energetic contribution of these side chains to binding, as seen in recent studies of ubiquitin-associated domains (55). Indeed, we found that Ub-I44A is fully functional when covalently bound at the donor site in Ubc13 (Fig. 4A) even though NMR data suggest that the Ub-Ile-44 side chain contacts human Ubc13 and yeast Ubc1 in their respective Ub thiol ester complexes (32, 41).Ub-Ile-44 is also located at the interface in complexes of mono-Ub with representative NZF and ubiquitin-associated domains (55–58). To our knowledge, an important energetic contribution of the Ile-44 side chain to binding has not been established in these cases.

Although we have not systematically addressed how affinity depends on chain length, data obtained in the present study suggest that Lys-63-Ub_4 binds only ~2-fold more tightly than mono-Ub to the acceptor site (Fig. 3C versus 4B). This is not surprising given the limited area of this site (Fig. 1). Because Ub-Ile-44 remains exposed in the extended, open conformation of Lys-63-linked Ub (59), each Ub in Lys-63-Ub_4 is likely to be accessible for interaction; this will enhance affinity through a simple concentration effect. These considerations, as well as the positions of the donor/acceptor sites, argue for a distributive (versus processive) mechanism of chain extension by the isolated Mms2/E2 complex (32). Earlier kinetic data are largely in agreement with this model (29). It remains possible that additional relevant conjugation factors (Rad5 and the Rad6-Rad18 complex) increase processivity in vivo.

Based on our model for acceptor Ub binding (30), we postulated that Mms2-Ile-57 might be part of the acceptor Ub binding site. The current results confirm this hypothesis. The L57A mutation has no effect on the Mms2/Ubc13 interaction, but it caused a severe inhibition of chain assembly (Fig. 4A) that could be assigned, through competition studies, to reduced affinity for PCNA. The current results confirm this hypothesis. The I57A mutation (see Fig. 1) had no detectable effect on chain synthesis in vitro or UV sensitivity in vivo (data not shown). These results exclude an important contribution of the Ser-33-hydroxyl to acceptor Ub affinity. It remains possible that this side chain helps to generate shape complementarity.

We proposed previously that Ubc13-Asp-81 resides at the border of the acceptor site (Fig. 1), because mutating this residue inhibited catalytic activity severely without impeding Ub occupancy of the donor site (30). Although competition studies revealed detectably weakened binding of the acceptor Ub to the Mms2-Ubc13-D81A complex (see Fig. 5C of Ref. 30), this effect was modest relative to the effects of the Mms2-I57A and Ub-I44A mutations in the present study (Figs. 3C and 4B). The new results presented here suggest that Ubc13-Asp-81 does not play a major role in determining the affinity of the acceptor Ub. Further work is needed to explain why mutating this residue inhibits catalysis so strongly. Perhaps Ubc13-Asp-81 helps to position Ub-Lys-63 correctly in the Ubc13 active site.

We observed a perfect correlation between in vitro and in vivo effects of the mutations studied here (Figs. 2–4), suggesting that the Mms2/Ubc13-catalyzed reaction contributes to rate limitation in vivo in our experimental system. (This may not be true in all yeast strains (60).) This outcome is most significant in the case of the Mms2-I57A mutation, which has no known effect besides reducing the affinity of the acceptor Ub. Thus, the UV light sensitivity conferred by this mutation (Fig. 4C) strongly argues that the Ub binding site of Mms2 remains relevant when Rad5 participates in chain synthesis. The location of the Rad5 binding site at the top of Ubc13 (as oriented in Fig. 1 (30, 48, 60)), distant from the acceptor site of Mms2, is consistent with this model. The precise role of Rad5 is uncertain, but it is likely that its interaction with the Rad18-Rad6 complex (19) helps to guide the Mms2/Ubc13 heterodimer to monoubiquitinated PCNA. Rad18 possesses autonomous affinity for PCNA, and the two proteins associate in mammalian cells (25, 26). Such a bivalent interaction with PCNA could help to overcome the weak intrinsic affinity of Ub for the acceptor site of Mms2.

Understanding the principles that enforce linkage specificity in polyUb chain synthesis, recognition, and disassembly is an important challenge, because these principles underlie signaling specificity and in some cases, may guide pharmacologic intervention (61). Here we have identified molecular determinants that are important for binding of the acceptor Ub to the UEV protein Mms2. Our finding that Ub-Ile-44 is an important determinant reinforces the notion that linkage specificity in this particular reaction arises through an indirect mechanism (30). The Ub-Ile-44 determinant is distant from the site of chemical reaction, but contact with this side chain acts to exclude lysine residues other than Lys-63 from the active site of Ubc13. In marked contrast, the small ubiquitin-like modifier-specific conjugating enzyme Ubc9 directly binds side chains immediately adjacent to the target lysine, generating specificity for lysines within a particular consensus sequence (62). Therefore, target lysine specificity in the ligation of Ub family proteins can arise through at least two different mechanisms (63).

Interestingly, the other well characterized UEV protein, Tsg101/Vps23, interacts with mono-Ub through a different UEV surface that is mainly contributed by a unique insertion in the primary structure of Tsg101 (64, 65). (Although Ub-Ile-44 is centrally located in the Tsg101/Ub interface, its quantitative contribution to affinity has not been determined.) The outcome of the Ub/UEV interaction is also different between the two proteins. Tsg101 promotes the entry of monoubiquitinated cargo proteins into multivesicular bodies, whereas Mms2 acts in conjunction with Ubc13 to catalyze a specific chemical reaction (63). Diverse modes of Ub recognition by these and other Ub/polyUb receptors will contribute to the selectivity of Ub-dependent signaling.

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