

# Noncovalent Interaction between Ubiquitin and the Human DNA Repair Protein Mms2 Is Required for Ubc13-mediated Polyubiquitination\*

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Ubiquitin-conjugating enzyme variants share significant sequence similarity with typical E2 (ubiquitin-conjugating) enzymes of the protein ubiquitination pathway but lack their characteristic active site cysteine residue. The *MMS2* gene of *Saccharomyces cerevisiae* encodes one such ubiquitin-conjugating enzyme variant that is involved in the error-free DNA postreplicative repair pathway through its association with Ubc13, an E2. The Mms2-Ubc13 heterodimer is capable of linking ubiquitin molecules to one another through an isopeptide bond between the C terminus and Lys-63. Using highly purified components, we show here that the human forms of Mms2 and Ubc13 associate into a heterodimer that is stable over a range of conditions. The ubiquitin-thiol ester form of the heterodimer can be produced by the direct activation of its Ubc13 subunit with E1 (ubiquitin-activating enzyme) or by the association of Mms2 with the Ubc13-ubiquitin thiol ester. The activated heterodimer is capable of transferring its covalently bound ubiquitin to Lys-63 of an untethered ubiquitin molecule, resulting in diubiquitin as the predominant species. In <sup>1</sup>H <sup>15</sup>N HSQC (<sup>1</sup>H <sup>15</sup>N heteronuclear single quantum coherence) NMR experiments, we have mapped the surface determinants of tethered and untethered ubiquitin that interact with Mms2 and Ubc13 in both their monomeric and dimeric forms. These results have identified a surface of untethered ubiquitin that interacts with Mms2 in the monomeric and heterodimeric form. Furthermore, the C-terminal tail of ubiquitin does not participate in this interaction. These results suggest that the role of Mms2 is to correctly orient either a target-bound or untethered ubiquitin molecule such that its Lys-63 is placed proximally to the C terminus of the ubiquitin molecule that is linked to the active site of Ubc13.

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The covalent attachment of Ub<sup>1</sup> to proteins and their subsequent degradation by the 26S proteasome represents the most commonly ascribed role for the protein ubiquitination system (1). In this respect, Ub conjugation to target substrates participates in a variety of important eukaryotic processes, such as cell cycle control (2), DNA repair (3), ribosome biogenesis (4), and the inflammatory response (5). In recent years, the role of ubiquitination has expanded to involve functions apparently unrelated to 26S-dependent proteolysis, including endocytosis of cell surface proteins (6) and nuclear factor- $\kappa$ B-dependent signal transduction (7). Therefore, the role of protein ubiquitination has broadened in scope.

Protein ubiquitination involves a cascade of enzymatic steps where Ub is passed sequentially as an activated thiol ester intermediate from a Ub-activating enzyme (E1) to a Ub-conjugating enzyme (E2) and finally to the protein target with the help of a Ub protein ligase (E3) (1, 8). In the first step, a thiol ester intermediate between the C-terminal tail of Ub (Gly-76) and the active site cysteine of the E1 is formed in an ATP-dependent manner. A subsequent transthiolesterification reaction transfers the Ub to the active site cysteine of the E2, forming another thiol ester derivative with the C terminus of Ub. In combination with an E3 enzyme, Ub is then transferred to a Lys of the target protein, forming a covalent isopeptide bond. Multi-Ub chains can then be assembled onto the mono-ubiquitinated protein, which is often portrayed as the conjugation of one Ub molecule to the next in stepwise fashion via Gly-76–Lys-48 linkages (1).

Eukaryotic organisms possess multiple E2 and E3 enzymes (1). Within the family of known E2s, all share a highly similar catalytic core domain of ~150 amino acids and are classified based on the presence or absence of C- and N-terminal extensions (9). Numerous complementary structural studies have documented that the fold of the core domain is very well conserved among family members (10–16). Furthermore, interactions between E2s and Ub within the thiol ester complex appear to be conserved among different E2 enzymes based on NMR chemical shift perturbation analysis (17–19).<sup>2</sup> Therefore, in order for E2 enzymes to effect different target substrate

<sup>1</sup> The abbreviations used are: Ub, ubiquitin; Ub<sub>2</sub>, diubiquitin; \*Ub, [<sup>35</sup>S]Ub; UEV, ubiquitin-conjugating enzyme variant; DTT, dithiothreitol; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin-protein ligase; NMR, nuclear magnetic resonance; PAGE, polyacrylamide gel electrophoresis; <sup>1</sup>H <sup>15</sup>N HSQC, <sup>1</sup>H <sup>15</sup>N heteronuclear single quantum coherence; PBS, phosphate-buffered saline; GST, glutathione S-transferase.

<sup>2</sup> Hamilton, K. S., Shaw, G. S., Williams, S., Huzil, J. T., McKenna, S., Ptak, C., Glover, M., and Ellison, M. J. (2001) *Structure*, in press.

ubiquitination, interactions with additional specificity factors are required. Target substrate specificity is thought to be dictated by different combinations of E2s and E3s in conjunction with auxiliary factors (20–23).

In addition to this core family, several atypical E2s known as ubiquitin-conjugating enzyme variants (UEVs) have been identified (24, 25). These proteins share significant sequence similarity with E2s but lack the characteristic active site cysteine residue required for thiol ester formation. Based on their similarities to E2s, it has been hypothesized that UEV proteins may function as either dominant negative (24) or positive (26) regulators of E2 function. One such UEV, encoded by the *MMS2* gene in the yeast *Saccharomyces cerevisiae*, is involved in error-free DNA postreplicative repair (25). Mms2 forms a heterodimer with Ubc13 (26), an E2 that functions in the error-free DNA postreplicative repair mechanism (27). Another UEV, encoded by the human *CROC1* gene, is involved in nuclear factor- $\kappa$ B signal transduction (28). Ubc13 is unique among known E2s in that it catalyzes the linkage of Ub molecules to one another via a Gly-76–Lys-63 isopeptide bond in an Mms2-dependent manner (26, 28). *In vivo* results demonstrated that Lys-63 chain assembly by the Ubc13/UEV heterodimer does not appear to be involved in proteasome-based degradation of target substrates (28). Furthermore, two chromatin-associated RING finger proteins, Rad5 and Rad18, are involved in the recruitment of Ubc13/Mms2 and Ubc2(Rad6) to DNA (29). A model is emerging in which the Ubc13/Mms2 heterodimer plays a central role in the recruitment and/or regulation of factors involved in the error-free tolerance to DNA damage via ubiquitination (30).

Two human homologs of yeast Mms2, CROC1 (31) and human Mms2 (32), have been identified and characterized. The full-length human *MMS2* cDNA is able to complement the yeast *mms2* mutant, suggesting that human Mms2 is indeed a functional homolog of yeast Mms2. In addition, a human *UBC13* cDNA was isolated as a homolog of the *Drosophila melanogaster* *bendless* gene (33). Human *UBC13* cDNA is also able to complement the yeast Ubc13 mutant.<sup>3</sup> Therefore, the human equivalents of the yeast Ubc13 and Mms2 proteins have been identified, and their mechanism of activity remains to be determined.

Here we demonstrate that purified human Ubc13 and Mms2 proteins form a stable heterodimer, and we have uncovered several mechanistic and structural aspects that are pertinent to its function.

#### EXPERIMENTAL PROCEDURES

**Protein Expression**—Both human *UBC13* and *MMS2* open reading frames were PCR-amplified and cloned as *Bam*HI-*Sal*I fragments into the corresponding sites of a GST fusion vector pGEX6 (Amersham Pharmacia Biotech). In each case, the last codon of the N-terminal GST sequence was separated from the first codon of Mms2 and Ubc13 by intervening DNA that encoded the linker peptide, Leu-Glu-Val-Leu-Phe-Gln-Gly-Pro-Leu-Gly-Ser. The linker peptide contained the cleavage site for the PreScission Protease (Amersham Pharmacia Biotech) that cleaves between Gln and Gly. Cleavage results in the separation of GST from Mms2 or Ubc13, both of which contain Gly-Pro-Leu-Gly-Ser appended to the N terminus of the first codon. The DNA sequences were derived from plasmid-borne cloned versions for Mms2 (33) and Ubc13 (34), respectively. The DNA sequences of the human *UBC13* and *MMS2* coding regions were verified by sequencing each recombinant plasmid.

Proteins were expressed in the *Escherichia coli* strain *BL21(DE3)-RIL* (Stratagene) that contained extra copies of the *argU*, *ileY*, and *leuW* tRNA genes in addition to the pGEX6-derived plasmids as described above. 2-liter cultures were grown at 37 °C to  $A_{590} = 0.3$  in LB media containing ampicillin (50  $\mu$ g/ml) followed by induction with isopropyl  $\beta$ -D-thiogalactopyranoside (0.4 mM) for 5 h at 37 °C. Cells were harvested by centrifugation and stored at –80 °C. All subsequent steps

were performed at 4 °C. *S. cerevisiae* Ubc13 and Mms2 were produced in an identical manner to that described for the human forms. The Ubc13R92 mutant was produced by substituting the Lys-92 for Arg-92 in human Ubc13 via site-directed mutagenesis.

Other *S. cerevisiae* E2s and their derivatives (Cdc34, Cdc34 $\Delta$ 244, Ubc1 $\Delta$ 450, and Rad6 $\Delta$ 153), <sup>35</sup>S-labeled Ub (\*Ub), and Uba1 (E1) from *S. cerevisiae* were expressed and purified as described previously (36, 37, 39). It should be noted that in some of the \*Ub preparations, radiolabeled contaminant bands exist, and these are denoted in the appropriate figure legends. Ubr48 and Ubr63 were overexpressed and purified as described above with the exception that the *E. coli* strain *BL21(DE3)-RP* (Stratagene) was employed, which carries the rare Arg tRNA, AGA.

**GST Fusion Protein Purification**—Cell pellets were resuspended in 50 ml of disruption buffer (20 mM Tris/Cl, pH 7.9, 10 mM MgCl<sub>2</sub>, 1 mM EDTA, 5% glycerol, 1 mM DTT, 0.3 M ammonium sulfate, 1 mM phenylmethylsulfonyl fluoride) and lysed by two passages through a French press followed by centrifugation (40,000 rpm for 45 min). The supernatant was dialyzed against 4 liters of 1 $\times$  PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.3) overnight at 4 °C and clarified through a 0.45- $\mu$ m low protein binding filter (Millipore). The filtered lysate was applied slowly to a 5-ml glutathione-Sepharose 4B RediPack column (Amersham Pharmacia Biotech) equilibrated with 50 ml of 1 $\times$  PBS buffer. The column was washed three times with 30 ml of 1 $\times$  PBS buffer, and the retained protein was eluted with three washes of 5 ml of glutathione elution buffer (10 mM reduced glutathione, 50 mM Tris/Cl, pH 8.0). Glutathione was removed from protein samples by dialysis against 4 liters of PreScission Cleavage Buffer (50 mM Tris/Cl, pH 7.0, 150 mM NaCl, 1 mM EDTA, 1 mM DTT) for 4 h followed by the addition of PreScission Protease (Amersham Pharmacia Biotech) (40 units). Fusion proteins were completely cleaved after a 16-h incubation at 4 °C. Following cleavage, the sample was reapplied to the glutathione-Sepharose 4B column and eluted as described above. In this case, cleaved Ubc13 and Mms2 proteins appeared in the 1 $\times$  PBS flow-through, whereas the GST tag and the PreScission Protease (which is also fused to GST) remained bound to the column. The flow-through was concentrated to 2 ml using an Ultrafree Centrifugal Filter Device (Millipore, 10-kDa molecular mass cutoff) and applied to a Hi-Load 16/60 Superdex 75 column (Amersham Pharmacia Biotech) equilibrated with 200 ml of Superdex 75 buffer (50 mM HEPES, pH 7.5, 75 mM NaCl, 1 mM EDTA, 1 mM DTT). Proteins were eluted at a flow rate of 1 ml/min and collected in 1-ml fractions. Both Ubc13 and Mms2 eluted between 69 and 80 ml and were judged to be pure by SDS-PAGE. Samples were subsequently pooled and concentrated.

**Heterodimer Purification**—The Ubc13/Mms2 heterodimer was purified from its monomer components by mixing Ubc13 and Mms2 (500  $\mu$ l of each at 1 mg/ml) at 25 °C followed by Hi-Load 16/60 Superdex 75 column chromatography as described above. The heterodimer eluted between 61 and 70 ml.

**Thiol Ester Purification**—The Ubc13~[<sup>35</sup>S]Ub (Ubc13~\*Ub) thiol ester was formed as follows. A reaction (0.5 ml) containing E1 (200 nM), \*Ub (4  $\mu$ M), and Ubc13 (4  $\mu$ M) in Buffer C (10 mM HEPES, pH 7.5, 5 mM MgCl<sub>2</sub>, 5 mM ATP) supplemented with the protease inhibitors and an ATP regeneration system (37) was incubated at 30 °C for 30 min. The thiol ester was then purified by size exclusion chromatography using a Superdex 75 HR 10/30 column, as described above. Peak fractions corresponding to Ubc13~\*Ub were assayed for concentration based on the specific activity of radiolabeled Ub contained within the thiol ester.

**Thiol Ester Assays**—All thiol ester reactions were carried out in Buffer C as described above. E1 and \*Ub were incubated at 30 °C for 30 min with either Ubc13, Mms2, or the Ubc13/Mms2 heterodimer (final, 500  $\mu$ l). The concentration of each component is noted in the figure legends. DTT (10 mM) was added to one-half of the reaction, and the components of the treated and untreated samples were separated by Superdex 75 chromatography and analyzed as described above (HR 10/30 column at 0.5 ml/min).

Kinetic measurements of thiol ester formation were performed at 30 °C by removing aliquots (0.5 ml) at designated times from a mother liquor. Upon removal, aliquots were immediately quenched with EDTA (10 mM). The components of each aliquot were then separated by gel exclusion chromatography (Superdex 75 HR 10/30 column at 0.5 ml/min). Concentrations were determined from the specific activity of radiolabeled Ub contained in each fraction (0.5 ml).

**Conjugation Reactions**—All Ub conjugation reactions (0.5 ml) were performed at 30 °C for 4 to 5 h in Buffer C. The concentration of each component is noted in the figure legends. Reactions were terminated by the addition of trichloroacetic acid (final, 10%) and processed for SDS-PAGE (18%) and autoradiography as described previously (37).

<sup>3</sup> L. Pastushok and W. Xiao, unpublished observation.

**NMR Spectroscopy**—All of the NMR spectra were obtained using a Varian Unity INOVA 500 MHz spectrometer at 30 °C. The two-dimensional  $^1\text{H}$   $^{15}\text{N}$  HSQC NMR spectra were acquired using the sensitivity-enhanced gradient pulse scheme developed by Kay and co-workers (40, 41). The  $^1\text{H}$  and  $^{15}\text{N}$  sweep widths were 6000 and 1550 Hz, respectively. Spectral processing and analyses were accomplished with the programs NMRPipe (42) and PIPP (43) respectively.

All NMR samples were prepared to a final volume of 500  $\mu\text{l}$  and contained HEPES (50 mM, pH 7.5), NaCl (75 mM), EDTA (1 mM), and 2,2-dimethyl-2-silapentanesulfonic acid (1 mM) in the presence of 9:1  $\text{H}_2\text{O}:\text{D}_2\text{O}$ . In each sample,  $^{15}\text{N}$  UbK48R (300  $\mu\text{M}$ ) was employed as the NMR-detectable species. Purification of this species is described elsewhere (19). These reaction conditions represent those employed for obtaining the  $^1\text{H}$   $^{15}\text{N}$  HSQC NMR spectrum for UbK48R alone. The NMR resonance assignments for UbK48R at pH 7.5 have been described previously (19).

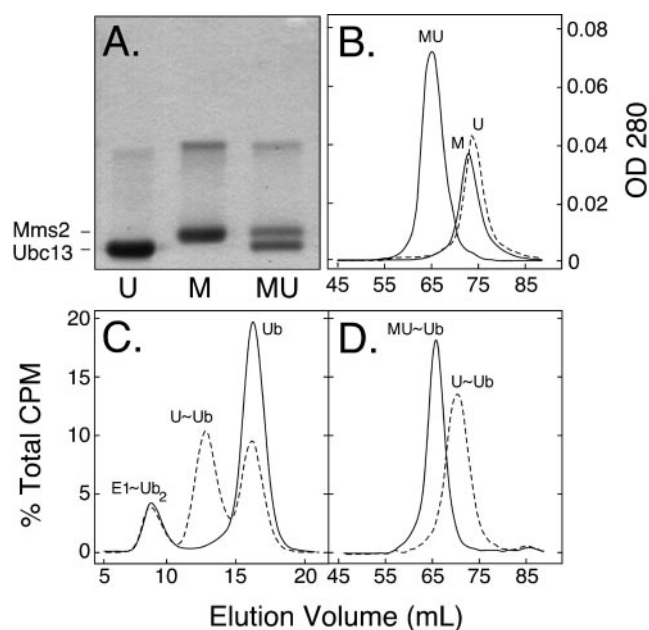
Noncovalent interactions between  $^{15}\text{N}$  UbK48R and Ubc13 were detected by including Ubc13 (310  $\mu\text{M}$ ) in addition to the reagents mentioned above. Similarly, noncovalent interactions between  $^{15}\text{N}$  UbK48R and Mms2 were detected by the inclusion of Mms2 (310  $\mu\text{M}$ ). Noncovalent interactions between  $^{15}\text{N}$  UbK48R and the Ubc13/Mms2 heterodimer were detected by adding both Ubc13 and Mms2 (310  $\mu\text{M}$ ) to the NMR sample.

Interactions between  $^{15}\text{N}$  UbK48R and Ubc13 in the Ubc13~ $^{15}\text{N}$  UbK48R thiol ester were determined by including Ubc13 (310  $\mu\text{M}$ ), ATP (5 mM),  $\text{MgCl}_2$  (5 mM), and E1 (1  $\mu\text{M}$ ) in the NMR sample. Covalent interactions between  $^{15}\text{N}$  Ubr48K and the Ubc13/Mms2 heterodimer within the Mms2/Ubc13~ $^{15}\text{N}$  Ubr48K thiol ester were delineated by the inclusion of Ubc13 (310  $\mu\text{M}$ ), MMS2 (310  $\mu\text{M}$ ), ATP (5 mM),  $\text{MgCl}_2$  (5 mM), and E1 (1  $\mu\text{M}$ ) into the NMR sample. Prior to the commencement of NMR analysis, a time course was performed to determine the kinetics of thiol ester relative to conjugate formation (data not shown). We determined that thiol ester formation is rapid (minutes), whereas the formation of conjugate is slow (hours). Furthermore, the onset of conjugate formation can be clearly identified based on the accumulation of new peaks emanating from the mixed population of Ub species. The  $^1\text{H}$   $^{15}\text{N}$  HSQC NMR experiments were therefore performed between 10 and 120 min after the addition of E1 to minimize the impact of possible side reactions.

## RESULTS

**Human Ubc13 and Mms2 Form a Heterodimer**—Our initial goal was to overexpress and purify human Ubc13 and Mms2 in high yield for the purposes of mechanistic and structural investigations. GST derivatives of Ubc13 and Mms2 were constructed and overexpressed in *E. coli*. Following elution from a glutathione column, the GST tag was proteolytically cleaved from both Ubc13 and Mms2, leaving five additional amino acids at each N terminus. The GST component of each cleaved mixture was removed by a second glutathione column step followed by size exclusion chromatography. Both Ubc13 and Mms2 eluted as a single peak at an approximate molecular mass of 18 kDa (Fig. 1B). SDS-PAGE of these peaks revealed that Ubc13 and Mms2 had been purified to virtual homogeneity (Fig. 1A). Mms2 appeared to migrate at a slightly larger molecular mass than expected, although the calculated molecular weight of Mms2 used in this study (16.8 kDa) is less than that of Ubc13 (17.6 kDa). A faint high molecular mass band was also observed, mainly associated with Mms2, which we attributed to aggregation based on the fact that no corresponding higher molecular mass species eluted from the size exclusion column.

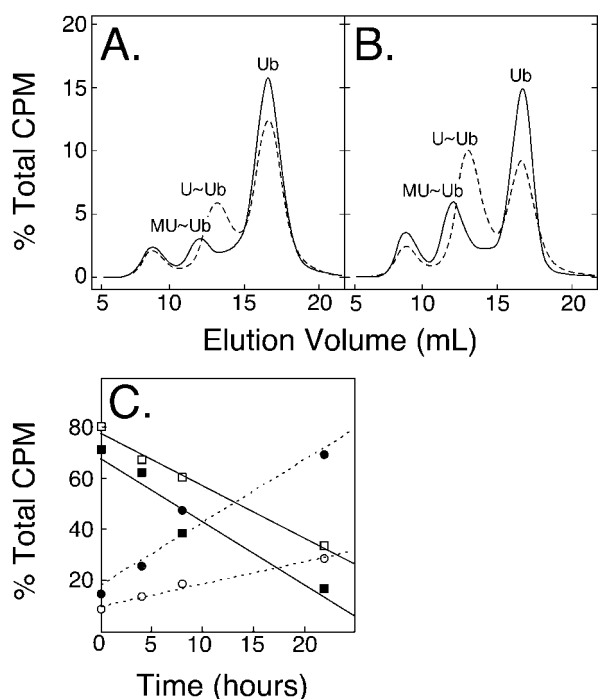
*S. cerevisiae* Ubc13 and Mms2 have been previously shown to form a heterodimer using partially purified proteins (26) or in yeast two-hybrid and coimmunoprecipitation assays (29, 30). To determine whether the purified human counterparts interact, equimolar amounts of Ubc13 and Mms2 were combined and fractionated by size-exclusion chromatography. The combined monomers were found to elute as a single peak of 40 kDa, which corresponded approximately to the expected molecular mass of a heterodimer consisting of one molecule each of Ubc13 and Mms2 (Fig. 1B). The absence of peaks of similar mass from



**FIG. 1. Ubc13 and Mms2 form a stable stoichiometric heterodimer which supports Ub thiol ester formation.** All reactions that employ Ub contain  $^{35}\text{S}$ Ub as the detectable species. A, Coomassie Blue-stained 18% SDS-PAGE demonstrating purified Ubc13 (U) (10  $\mu\text{g}$ ), Mms2 (M) (10  $\mu\text{g}$ ), and the Ubc13/Mms2 heterodimer (MU) (20  $\mu\text{g}$ ). B, size exclusion column elution profiles of purified Ubc13 (U, solid line), Mms2 (M, dashed line), and the Ubc13/Mms2 heterodimer (MU, solid line). In each case, 500  $\mu\text{g}$  of Ubc13, Mms2, or both were employed. Size exclusion column elution profiles of a 500- $\mu\text{l}$  reaction containing E1 (100 nM),  $^{35}\text{S}$ Ub (1  $\mu\text{M}$ ), and either Ubc13 (1  $\mu\text{M}$ , dashed line) or Mms2 (1  $\mu\text{M}$ , solid line) in Buffer C was reacted at 30 °C for 30 min and loaded onto a Superdex 75 HR10/30 column (C). Peaks are labeled E1~Ub<sub>2</sub> (E1 thiol ester), U~Ub (Ubc13~Ub thiol ester), and Ub (free Ub). An 800- $\mu\text{l}$  mixture containing either purified Ubc13~ $^{35}\text{S}$ Ub (0.5  $\mu\text{M}$ ) alone (dashed line) or Ubc13~ $^{35}\text{S}$ Ub (0.5  $\mu\text{M}$ ) and Mms2 (2.5  $\mu\text{M}$ ) (solid line) was reacted at 30 °C for 30 min prior to loading onto a Hi-Load Superdex 75 column (D). Peaks are labeled either MU~Ub (Mms2/Ubc13~Ub) or U~Ub (Ubc13~Ub).

control samples containing either Ubc13 or Mms2 alone eliminated the possibility of homodimer formation (data not shown). The 1:1 stoichiometry of the heterodimer was confirmed by quantitating the Ubc13 and Mms2 bands following separation by SDS-PAGE (Fig. 1A). The heterodimer was completely stable over a range of NaCl concentration (up to 1 M) suggesting a strong hydrophobic interaction between monomers (data not shown). Furthermore, Mms2 and Ubc13 failed to form heterodimers with other E2s from *S. cerevisiae* including Cdc34, Rad6, and Ubc1. Thus, the interaction between human Ubc13 and Mms2 appears to be highly specific.

**Ubiquitin Thiol Ester Formation**—In the absence of a reducing agent, a reaction containing E1, E2, and radiolabeled Ub results in the formation of an E2~Ub thiol ester that can be separated by size exclusion chromatography and detected by autoradiography. The chromatographic profile of these reactions typically reveals three peaks that correspond to free Ub, the E2~Ub thiol ester and the E1~Ub<sub>2</sub> thiol ester. When Ubc13 was used in the thiol ester reaction, three peaks were observed corresponding to E1~Ub<sub>2</sub> (120 kDa), E2~Ub (28 kDa), and free Ub (9 kDa) (Fig. 1C). The disappearance of the Ubc13~Ub peak upon DTT treatment coupled with an increase in the free Ub peak is consistent with the lability of the thiol ester bond in the presence of reducing agents (data not shown). By comparison, Mms2 was found to be unreactive with respect to E2~Ub formation under identical conditions (Fig. 1C). This result was expected as Mms2 lacks the canonical active site cysteine required for thiol ester formation (32).



**FIG. 2. Mms2 impedes thiol ester formation while increasing its stability.** Several identical reactions (500- $\mu$ l total volume), each containing E1 (100 nM), \*Ub (2  $\mu$ M), and either Ubc13 (1  $\mu$ M, *dashed line*) or both Ubc13 (1  $\mu$ M) and Mms2 (1  $\mu$ M, *solid line*), were incubated at 30 °C for 10 (A) or 20 (B) min. Reactions were quenched by the addition of 10 mM EDTA prior to loading onto a Superdex 75 HR10/30 column. Peaks are labeled as in Fig. 1D. C, Ubc13~Ub thiol ester stability. Ubc13~\*Ub (500 nM) was purified by size exclusion chromatography and incubated at 30 °C in the presence (*open symbols*) or absence (*closed symbols*) of Mms2 (500 nM). Aliquots were removed at various times and fractionated by size exclusion chromatography to identify species containing radiolabeled Ub. The amount of thiol ester product (*solid lines*) and free Ub (*dashed lines*) are shown. *Lines* represent regression analysis of duplicate experiments and have assumed linear kinetics. Ubc13-Ub conjugate formation has not been included for clarity.

Ubc13 can also exist as a Ub thiol ester in the heterodimeric form with Mms2. The thiolated heterodimer can either be produced by combining purified Ubc13 thiol ester with Mms2 (Fig. 1D) or by thiolating the heterodimer directly (data not shown). Therefore, the heterodimer and thiol ester are structurally compatible.

The elution properties (Fig. 1D) of the heterodimer and heterodimer thiol ester are uncharacteristic of a simple molecular mass relationship. Firstly, the apparent molecular mass of the heterodimer is slightly larger than its actual mass (40 kDa compared with 35 kDa). Secondly, the addition of Ub to the heterodimer upon thiolation increases its apparent mass by only 4 kDa, less than one-half of the molecular mass of Ub.

The rate of Ubc13~Ub thiol ester formation differs in the presence or absence of Mms2 as Mms2 inhibits the rate of thiol ester formation by approximately 2-fold (Fig. 2, A and B). Furthermore, in the presence of Mms2, only the heterodimeric form of the Ubc13~Ub thiol ester is observed. These results indicate (i) that Ubc13 in the heterodimeric form is less reactive to thiol ester formation than the monomeric form and (ii) that heterodimer formation may well precede thiol ester formation. Interestingly, the Ubc13~Ub component of the heterodimer is less labile than the monomeric form of Ubc13~Ub based on the relative release of free Ub with respect to time (Fig. 2C).

**The Heterodimer Conjugates Ub Molecules via Lys-63**—A previous study has shown that yeast Ubc13/Mms2 catalyzes the linkage of Ub molecules via Lys-63 (26). We tested the

conjugation characteristics of the human heterodimer in reactions that contained E1, \*Ub, and combinations of Ubc13 and Mms2. The radiolabeled products were then separated by SDS-PAGE (Fig. 3A). A reaction containing both Ubc13 and Mms2 resulted in the formation of di- and triubiquitin (Ub<sub>2</sub> and Ub<sub>3</sub>, respectively), which were absent in either the Ubc13 or Mms2 alone reactions. The use of either wild-type Ub or UbK48R in these reactions resulted in similar yields of free Ub chains, which eliminated the possibility that the Ub moieties of the dimer were linked through Lys-48. Given that the reactions were treated with reducing agent (DTT) prior to loading onto the gel, the presence of a 26-kDa conjugate species that was unique to the Ubc13 reactions indicated that Ubc13 undergoes autoubiquitination. Human Ubc13 contains two potential sites for autoubiquitination at lysines 92 and 94 that, based on preliminary structural determinations, are in reasonable proximity to the active site. Lysine 92 was identified as the site of Ub conjugation as its mutation to arginine completely eliminated conjugate formation while not significantly affecting the amount of free chains produced (Fig. 3B).

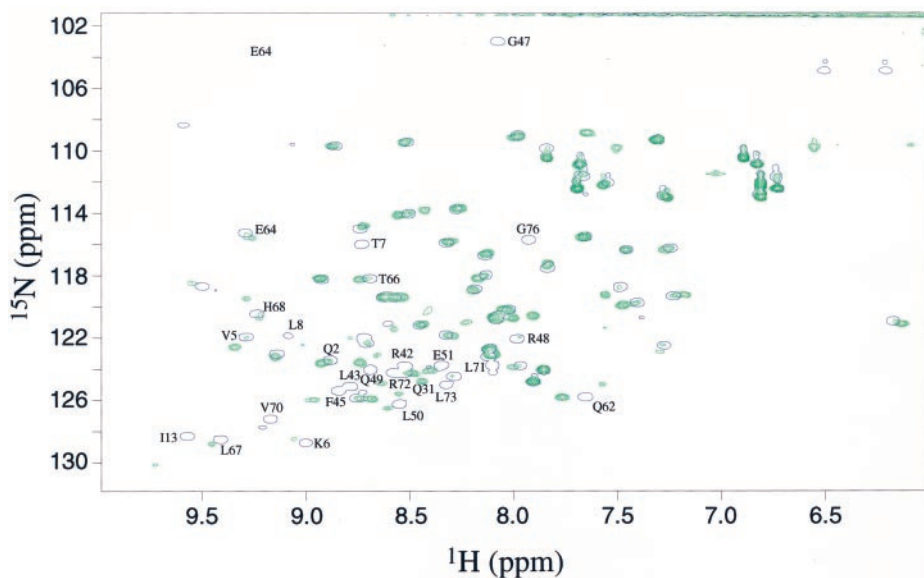
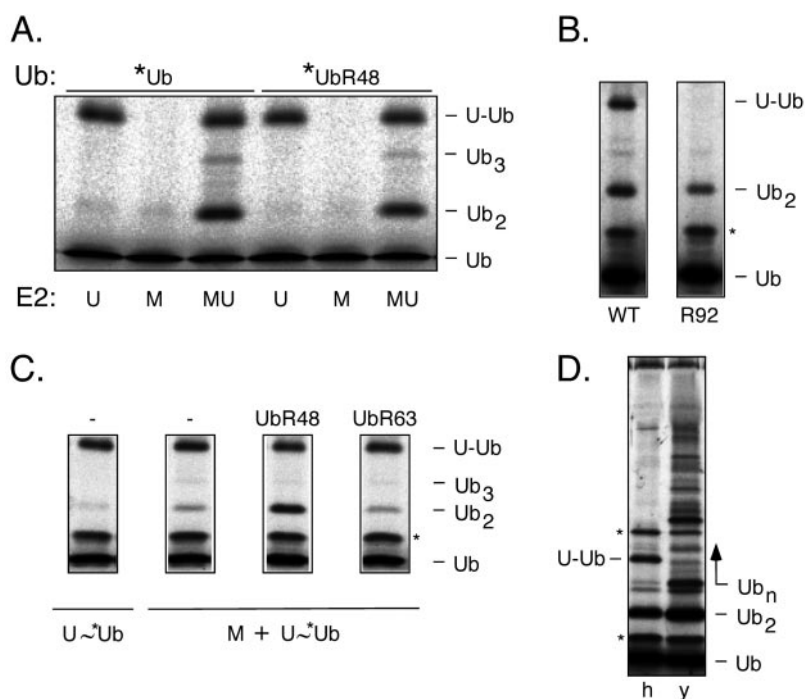
The formation of Ub<sub>2</sub>, and subsequently longer chains, by the heterodimer could occur by one of two mechanisms. Two heterodimer thiol ester molecules could, for example, interact such that one donates its Ub to the other. Alternatively, the thiol ester could target free Ub (*i.e.* not covalently attached to either Ubc13 or Mms2). To distinguish between these two possibilities, the following experiment was performed. Purified Ubc13~\*Ub thiol ester was incubated in the presence or absence of unlabeled free UbK48R, and the formation of Ub<sub>2</sub> was initiated by the addition of Mms2. As seen in Fig. 3C, the yield of Ub<sub>2</sub> increases significantly when UbK48R is added to the reaction. Thus, free Ub is the preferred substrate for Ub<sub>2</sub> formation. We attribute the presence of trace amounts of Ub<sub>2</sub> lacking an obvious source of free Ub to the Ub that arises from thiol ester hydrolysis during the time interval following thiol ester purification and prior to the addition of Mms2 (Fig. 2C). Notably, the level of Ub<sub>2</sub> in a reaction in which UbK63R is employed as the source of free Ub is comparable with the control reaction in which free Ub has been omitted. Together, these results illustrate that Ub<sub>2</sub> is formed specifically by the transfer of Ub from the thiol ester to Lys-63 of a free molecule of Ub.

**Human and *S. cerevisiae* Ubc13/Mms2 Complexes Possess Different In Vitro Activities**—Previous studies have demonstrated that the *S. cerevisiae* Ubc13/Mms2 complex appears to be more effective (26) at producing longer chains when compared with the human counterparts in the absence of any other activator proteins (28). Upon direct comparison of the *in vitro* activities of both human and *S. cerevisiae* complexes, it is clear that the *S. cerevisiae* complex does in fact synthesize much longer chains when compared with the human proteins (Fig. 3D). Furthermore, we can report that the formation of the Ubc13 conjugate is only observed with the human and not the yeast protein.

**NMR-derived Footprints of the Ub Contact Surface**—By comparing the assigned two-dimensional <sup>1</sup>H <sup>15</sup>N HSQC NMR spectra of <sup>15</sup>N UbK48R alone or in combination with unlabeled Ubc13 and/or Mms2, as shown in Fig. 4, we have been able to map the interacting surface of Ub in (i) a noncovalent complex with Mms2, (ii) a noncovalent complex with the Mms2/Ubc13 heterodimer, (iii) as a thiol ester with Ubc13, and (iv) as a thiol ester with the heterodimer.

The relative decreases in cross-peak intensities in the <sup>1</sup>H <sup>15</sup>N HSQC NMR spectrum that occur upon complex formation were determined for each amino acid residue in <sup>15</sup>N UbK48R. Normally, cross-peak intensity decreases in proportion to the size

**FIG. 3. The Ubc13/Mms2 heterodimer synthesizes free Ub chains.** *In vitro* ubiquitination reactions were performed using  $^3\text{H}$ -Ub in combination with 18% SDS-PAGE and autoradiography. All reactions were precipitated with trichloroacetic acid and solubilized in SDS-PAGE load buffer. Contaminants present in some of the  $^3\text{H}$ -Ub preparations are denoted (\*). Either Ubc13 (U, 250 nM), Mms2 (M, 250 nM), or both were included in reactions (500- $\mu\text{l}$  total volume) containing E1 (25 nM) and either wild-type Ub (1250 nM) or UbR48K (1250 nM) (A). Reactions proceeded for 5 h at 30 °C. Prominent bands include U-Ub (Ub conjugated onto Ubc13), Ub<sub>2</sub>, Ub<sub>3</sub>, and free Ub. Either wild type (WT) or Ubc13R92 (R92) (250 nM) was included in reactions containing E1 (25 nM),  $^3\text{H}$ -Ub (1  $\mu\text{M}$ ), and Mms2 (250 nM) (B). Reactions proceeded for 4 h at 30 °C. A reaction containing purified Ubc13~ $^3\text{H}$ -Ub (250 nM), Mms2 (250 nM), and either (i) no free Ub, (ii) free unlabeled UbK48R (2  $\mu\text{M}$ ), or (iii) free unlabeled UbK63R (2  $\mu\text{M}$ ) was reacted for 4 h at 30 °C (C). Either human (*h*) or *S. cerevisiae* (*y*) Ubc13 (200 nM) and Mms2 (200 nM) was included in reactions containing E1 (15 nM) and  $^3\text{H}$ -Ub (2  $\mu\text{M}$ ) (D). Reactions proceeded for 5 h at 30 °C.



**FIG. 4. Superposition of  $^1\text{H}$ - $^{15}\text{N}$ -HSQC NMR spectra of  $^{15}\text{N}$ -labeled Ub, free and in complex with Ubc13.** 500  $\mu\text{l}$  of NMR samples including either  $^{15}\text{N}$  UbK48R (300 nM) (blue) or  $^{15}\text{N}$  UbK48R (300 nM), E1 (1  $\mu\text{M}$ ), and Ubc13 (310 nM) (green, 20 contours) were studied. Samples were studied at 30 °C, pH 7.5, and included 1% 2,2-dimethyl-2-silapentanesulfonic acid and a mixture of 9:1  $\text{H}_2\text{O}:\text{D}_2\text{O}$  as the solvent. Only selected backbone cross-peaks that were affected by complex formation are labeled.

of the protein or protein-protein complex due to peak broadening as a result of increased rotational tumbling time. A decrease in peak intensity beyond this effect likely reflects changes in chemical environment that occur because of protein-protein interactions (19).

$^1\text{H}$   $^{15}\text{N}$  HSQC NMR spectroscopy was used to determine whether Ub associates with Ubc13 and/or Mms2 in a noncovalent fashion (that is, in the absence of E1 and  $\text{ATP}/\text{Mg}^{2+}$ ). The  $^1\text{H}$   $^{15}\text{N}$  HSQC NMR spectrum of  $^{15}\text{N}$  UbK48R alone is indistinguishable from the spectrum acquired in the presence of Ubc13. This observation indicates the lack of an interaction between Ub and Ubc13 at a concentration of 300  $\mu\text{M}$  (data not shown). In the presence of either Mms2 or the Mms2/Ubc13 heterodimer, the  $^1\text{H}$   $^{15}\text{N}$  HSQC NMR spectrum of  $^{15}\text{N}$  UbK48R undergoes two types of changes. First, a global reduction in peak intensities is observed when compared with  $^{15}\text{N}$  UbK48R alone. A 57% average reduction in peak intensity is observed in the presence of Mms2, and a 70% average reduction in peak intensity is observed in the presence of the heterodimer. Each

of these results appears reasonable based on the predicted increase in molecular mass of the noncovalent protein complexes. Second, a marked decrease of peak intensity was observed over and above these average decreases corresponding to specific residues that cluster to one face of Ub (Fig. 5). It can be concluded from these findings that Ub interacts with Mms2 and the Mms2/Ubc13 heterodimer through noncovalent contacts and that a similar contact surface is used by Ub in each case.

The interactions of  $^{15}\text{N}$  UbK48R with the Ubc13 thiol ester or within the heterodimeric thiol ester complex were also examined using  $^1\text{H}$   $^{15}\text{N}$  HSQC NMR spectroscopy. The incorporation of  $^{15}\text{N}$  UbK48R into each of the two thiol ester forms resulted in an average reduction of  $^1\text{H}$   $^{15}\text{N}$  HSQC NMR cross-peak intensity (relative to  $^{15}\text{N}$  UbK48R alone) of 88% for Ubc13~Ub and 91% in the case of the heterodimer thiol ester. Upon comparison of the two thiol ester forms, residues corresponding to the largest reductions in cross-peak intensity are clustered on similar surfaces of Ub (Fig. 5). The most notable difference exhib-

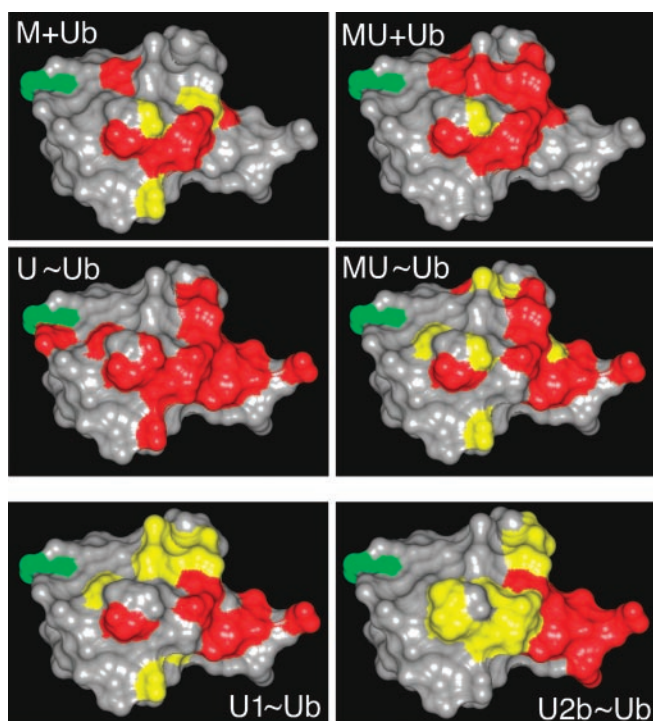


FIG. 5. **Connolly surfaces of the binding interfaces on Ub.** The surface of Ub is presented, and residues whose  $^1\text{H}$   $^{15}\text{N}$  HSQC NMR peak height intensities are affected by complex formation moderately (yellow, corresponding to  $\geq 20\%$  reduction when compared with average decreases in peak intensity upon complex formation) and more significantly (red, corresponding to the disappearance of intensity beyond detection limits) are colored. Lys-63 is colored in green as a point of reference. *M+Ub*, noncovalent interaction between Ub and Mms2. *MU+Ub*, noncovalent interaction between Ub and the Ubc13/Mms2 heterodimer. *U~Ub*, interactions between Ub and Ubc13 in the Ubc13~Ub thiol ester. *MU~Ub*, interactions between Ub and the Ubc13/Mms2 heterodimer within the Mms2/Ubc13~Ub thiol ester. *U1~Ub*, interactions between Ub and yeast Ubc1 within the Ubc1~Ub thiol ester.<sup>2</sup> *U2b~Ub*, interactions between Ub and human Ubc2b within the hUbc2b~Ub thiol ester (18).

ited by the thiol ester-linked forms of Ub, when compared with the unlinked forms, is that the C-terminal tail makes extensive contacts in the former but not in the latter. Excluding the C-terminal tail as well as obvious differences in detail, Ub utilizes an analogous contact surface in both the thiol ester and untethered complexes described above.

#### DISCUSSION

The present work illustrates that, as is true of its yeast counterpart, the human form of the Mms2/Ubc13 heterodimer links Ub molecules together via Lys-63. This work also extends previous studies on these proteins (26) with a more comprehensive understanding of how their structures relate to their functions.

The human heterodimer is formed rapidly and quantitatively by combining Mms2 and Ubc13. Furthermore the association of these subunits is significant ( $K_d = 2 \mu\text{M}$ ) (45) and is stable over a wide range of salt concentration. The Ub thiol ester form of the heterodimer can be created in either of two ways: 1) by direct activation of the Ubc13 subunit by E1 or 2) by assembly of the heterodimer from Mms2 and Ubc13~Ub thiol ester. The rate of dimer assembly *in vitro* exceeds the rate of thiol ester formation. Thus, the first pathway may be the preferred route of thiol ester formation *in vivo*.

Although the Ubc13 component of the heterodimer is less reactive to activation than its monomeric counterpart, it is more stable with respect to hydrolysis. One explanation for these observations is that the active site cysteine and the C

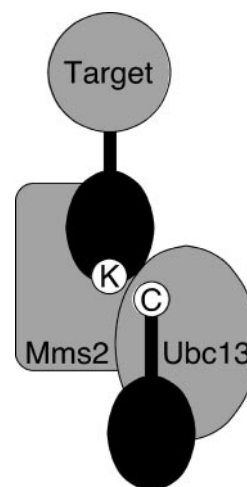


FIG. 6. **Model of Ubc13/Mms2 catalyzed ubiquitination of target substrates.** Heterodimer formation between Ubc13 (dark gray) and Mms2 (light gray) proceeds rapidly followed by the formation of thiol ester between the active site cysteine of Ubc13 and the C-terminal tail of Ub (C). Mms2 may serve to correctly position a second Ub via a noncovalent binding site such that transfer of thiol ester linked Ub to Lys-63 (K) of the second may proceed. Target substrates may be bound to the noncovalently bound Ub or alternatively may become ubiquitinated via a downstream mechanism.

terminus of Ub become less accessible to E1 and solvent when incorporated into the heterodimer.

The human and *S. cerevisiae* Ubc13/Mms2 complexes demonstrate markedly different *in vitro* activities with respect to both chain formation and autoubiquitination. Lys-92 of human Ubc13 becomes autoubiquitinated, a phenomenon that occurs with other ubiquitin-conjugating enzymes, namely *S. cerevisiae* Ubc1, which undergoes conjugate formation on an analogous Lys at position 93 (36). In contrast, no conjugate formation was observed upon yeast Ubc13, but chains of significantly greater length were formed in the presence of Mms2.

The Ub conjugation studies taken together with the results of the NMR spectroscopy experiments are consistent with a mechanistic model that can account for the linkage between Ub molecules via Lys-63 (Fig. 6). The model portrays one Ub molecule linked to the active site cysteine of the Ubc13 subunit making noncovalent contacts primarily with Ubc13. The other Ub molecule is not covalently tethered but makes its principal noncovalent contacts with the Mms2 subunit. The variation in the Ub contact surfaces that are observed when either the Mms2 or Ubc13 monomers assume their heterodimeric forms may reflect adjustments of Ub on the E2 surface and/or additional contacts that are formed with the second protein. The Ub molecule bound to Mms2 is oriented such that Lys-63 is close to the active site of Ubc13, thereby facilitating linkage with the C terminus of the thiol ester-linked Ub molecule of Ubc13. Unlike the Ubc13-bound Ub molecule, the C-terminal region of the Mms2-bound Ub molecule is not sequestered. From the model presented herein, we speculate that the C-terminal region of Mms2-bound Ub is sterically free, thereby allowing for coupling to a suitable target without interference from the heterodimer. This raises the possibility that subsequent Ub molecules are added to the Ub molecule of a monoubiquitinated substrate.

Recently, the high-resolution x-ray crystal structures of both the human (45) and *S. cerevisiae* (38) Ubc13/Mms2 heterodimer have been solved, and both structures propose models that could accommodate the active site tethered and noncovalently bound Ub molecules. In combination with the data presented in this manuscript, these structures establish a foundation for the assembly of Lys-63 chains by the Ubc13/Mms2 heterodimer.

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