A Single Mms2 “Key” Residue Insertion into a Ubc13 Pocket Determines the Interface Specificity of a Human Lys\(^{63}\) Ubiquitin Conjugation Complex*[^s]

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Human Ubc13 and Mms2 (or its homolog, Uev1) form a unique ubiquitin-conjugating enzyme (Ubc) complex that generates atypical Lys\(^{63}\)-linked ubiquitin conjugates. Such conjugates are attached to specific targets that modulate the activity of various cellular processes including DNA repair, mitotic progression, and nuclear factor-κB signaling. Whereas Ubc13 is a typical Ubc, Mms2 is a non-catalytic Ubc variant. Substantial biochemical evidence has revealed a mechanism whereby Mms2 properly orients ubiquitin to allow for Lys\(^{63}\) conjugation by Ubc13; however, how this specific Ubc13-Mms2 complex is formed and why Mms2 does not form a complex with other Ubes have not been reported. In order to address these questions, we used a structure-based approach to design mutations and characterize the human Ubc13-Mms2 interface. We used the yeast two-hybrid assay, glutathione S-transferase pull-downs, and surface plasmon resonance to test in vivo and in vitro binding. These experiments were paired with functional complementation and ubiquitin conjugation studies to provide in vivo and in vitro functional data. The results in this study allowed us to identify important residues of the Ubc13-Mms2 interface, determine a correlation between heterodimer formation and function, and conclude why Mms2 forms a specific complex with Ubc13 but not other Ube proteins.

In recent years, the attachment of ubiquitin (Ub)\(^1\) to its target has become one of the cornerstones of covalent post-translational modification in eukaryotes. The biochemical process, called ubiquitination, is a sequential three-step reaction whereby Ub is activated in an ATP-dependent manner by a ubiquitin-activating enzyme. The Ub C terminus then forms a thiolester intermediate with the catalytic Cys residue of a Ub-conjugating enzyme (Ubc or E2) active site. The final step involves a substrate-specific ubiquitin ligase (E3) and leads to the attachment of the Ub C terminus to an ε-amino group of a Lys residue of the target (reviewed in Ref. 1). Most often, the process is repeated, and subsequent Ub molecules are attached to one another so that poly-Ub chains are generated. Mono-Ub or poly-Ub chains are used as signals for numerous cellular processes such as cell cycle progression (2), apoptosis (3), mitochondrial inheritance (4), and transcriptional regulation (5).

The versatility of ubiquitination and its ability to function in a wide variety of cellular roles are generally attributed to three main factors. First, whereas E1s are encoded by one or few genes in the cell, E2s are a more plentiful family, with 13 such enzymes in budding yeast and many more in higher eukaryotes. Second, the most plentiful and diverse group of ubiquitination enzymes is the E3s, which function alone or as part of multi-subunit complexes. E3s are responsible for determining substrate specificity and act in either active or passive roles in ubiquitination by covalently binding Ub or acting as E2 substrate adapter proteins, respectively. A third important factor in providing versatility for ubiquitination involves the nature of the poly-Ub chains themselves.

Conventional poly-Ub chains are built via Lys\(^{48}\) and provide a characteristic signal for substrate-specific degradation by the 26S proteasome (6). Because protein turnover by the Ub proteasome system is a fundamental process, it is not surprising that Lys\(^{48}\) poly-Ub chains play such a pivotal role in the cell. However, because Ub has six other surface Lys residues, an even greater level of versatility is possible. Indeed, several studies have reported non-standard poly-Ub chains via the Lys\(^{6} (7), \text{Lys}^{29} (8), \text{Lys}^{11} (9), \text{and \text{Lys}^{63} (10)} \) residues of Ub. Furthermore, such atypical conjugates have been shown to function in roles other than proteasome degradation. Some of the best-documented examples of such poly-Ub chains involve those through Lys\(^{63}\), which have been demonstrated in a stress response (8), mitochondrial inheritance (4), plasma membrane protein endocytosis (11), ribosome function (10), and DNA postreplication repair (12).

Because all Ubes, regardless of the poly-Ub chain synthesized, have a well-conserved core domain that houses the active site (13), part of the allure in studying atypical Ub chains lies in how they are assembled. The only example to date came as a breakthrough in the field when it was discovered that conjugation via Lys\(^{63}\) occurred through a novel mechanism requiring a heterodimeric complex between Ubc13 and a Ubc enzyme variant (Uev), Mms2 (12). Uev proteins are similar in second-

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[^1]: The abbreviations used are: Ub, ubiquitin; Ubc, ubiquitin-conjugating enzyme; E2, ubiquitin carrier protein; Uev, ubiquitin-conjugating enzyme variant; E3, ubiquitin-protein isopeptide ligase; SPR, surface plasmon resonance; MMS, methylethane sulfonate; GST, glutathione S-transferase; PBS, phosphate-buffered saline; SD, synthetic dextrose; YPD, yeast extract-peptone-dextrose; RU, response unit(s); RUeq, response unit(s) at equilibrium.

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ary and tertiary structure to E2s, but they lack the catalytic active site that forms thiolesters with Ub. Thus, it was originally proposed that the UVEs might act as dominant-negative regulators of ubiquitination (14); however, subsequent studies revealed otherwise. UBC13, MMS2, and Lys63 poly-Ub chains were shown to be indispensable for promoting postreplication repair (12).

Spurred by the uniqueness of E2-Uev-mediated catalysis of Lys63 Ub chains, the crystal structures for the human (15) and yeast (16) Ubc13-Mms2 complexes were solved; both structures were highly superimposable, and each study proposed a similar means for chain catalysis. The three-dimensional studies were followed by extensive NMR analyses that revealed the unique mechanistic basis behind Lys63 poly-Ub chain catalysis. Briefly, a T-shaped heterodimer is formed that allows Mms2 to orient an acceptor Ub through non-covalent contacts such that its Lys63 residue is presented to the donor Ub at the Ubc13 active site. An isopeptide bond can then form between the two molecules, and Lys63-di-Ub is free to exit through a channel leading from the Ubc13 active site toward a E3 binding site (17–20).

The revelation of Ubc13-Mms2-mediated catalysis suggested the necessity of heterodimer formation between the E2 and Uev; however, studies to date have not addressed in detail the significance of the Ubc13-Uev interface. Therefore, we carried out studies using structural information and a site-directed mutagenesis approach to provide a dedicated structure-function study of the human Ubc13-Mms2 interface. In our rational approach, the goals were as follows: 1) to identify key residues in Ubc13 and Mms2 that are important for complex formation; 2) to demonstrate a correlation between Ubc13-Mms2 interaction and function; and 3) to determine why Mms2 forms a specific complex with Ubc13, and not other Ubc5.

EXPERIMENTAL PROCEDURES

Strains and Cell Culture—Saccharomyces cerevisiae haploid strains used in this study include JF69-4A (MATa trpl–190 leu2–3, 112 ura3–52 his3–200 gal4a pai80a Pgal2::ADE2 LYS2::HIS3 met2::Pgal2::lacZ, a gift from Dr. P. James (University of Wisconsin, Madison, WI)). A wild-type haploid S. cerevisiae strain HK580-10D (MATa ade-1 can1–100 his3–11,15 leu2–3, 112 trpl–1 ura3–1) was received from Dr. H. Klein (New York University) and used as the recipient to delete the entire MMS2 open reading frame by a one-step gene replacement method (21) using an mms2Δ::HIS3 cassette generated through PCR amplification as previously described (22). A ubc13::HIS3 plasmid was generated as previously reported (26). The ubc13::HIS3 module was washed again three times with 500 l of PBS was loaded and incubated in the purification module for 1 h at 4 °C with gentle rocking. The module was then washed three times with 500 l of PBS. Subsequently, 0.03 mg of purified, non-fused hUbc13 or hMms2 in 1× PBS was added to the module, and the incubation was continued for another hour at 4 °C. The module was washed again three times with 500 l of PBS, and then 50 l of glutathione elution buffer was added to elute the affinity-purified proteins. Eluted samples were subjected to 12% SDS-PAGE and Western analysis.

Yeast Two-hybrid Analysis—The yeast two-hybrid strain PJ69-4A (28) was co-transformed with different combinations of Gal4AD and Gal4BD constructs. The co-transformed colonies were initially selected on SD-Trp-Leu. For each transformation, at least four independent colonies were grown in SD-Trp-Leu media, and then plated as replicates onto SD-Trp-Leu-His + 3-amino triazole to test the activation of the Pgal2::HIS3 gene.

Functional Complementation of Yeast Mutations by Human Genes—The gradient plate assay was performed as a semiquantitative measurement of relative MMS sensitivity. The HK580-10D mms2A or ubc13Δ strains were transformed with pGAD-hMMS2 or pGBT-UBC13 two-hybrid plasmids. Colony growth was scored visually as a function of the soluble protein (analyte) at various free analyte concentrations ranging from 1 to 500 μM for Mms2 and from 2.5 to 5 μM for Ubc13. The χ² values were 5.2 and 2.3 for GST-Mms2 binding Ubc13 and GST-
Ubc13 binding Mms2, respectively. The data are deemed reliable if $x^2$ is <10. To confirm the reliability of the data, we generated plots of the response (RU) versus free analyte (nM) and calculated the $K_d$, where $K_d = [RU]_0 \times$ slope in the double reciprocal plot (1/response versus 1/free analyte).

In Vitro Ubiquitination Assay—A 0.5-mL conjugation reaction containing Uba1 (20 nm), $^{35}$S-labeled Ub (2.5 μM), and Ubc13 or Mms2 (250 nm) in an ATP mixture (10 mM HEPES, pH 7.5, 5 mM MgCl$_2$, 5 mM ATP, and 0.6 unit/ml inorganic phosphatase) was incubated at 30 °C for 60 min. The concentration of each component is noted in the figure legend. Reactions were terminated by the addition of trichloroacetic acid to a final concentration of 10% and processed for SDS-PAGE and subsequent analysis by autoradiography.

**RESULTS**

The Ubc13-Mms2 Structure—The overall structure of the human Ubc13-Mms2 heterodimer is shown in Fig. 1A. Each molecule adopts the characteristic αβ Ubc fold (for example, see Refs. 13 and 29) and exhibits a very similar three-dimensional structure, with the main exception that Mms2 lacks two C-terminal α-helices. The N terminus, α1 helix, and the Loop 1 portions of Mms2 pack against Ubc13 to form a unique end-on structure that creates a long channel which buries ~1500 Å$^2$ of solvent-accessible surface area (15, 16).

Fig. 1B is oriented similarly to Fig. 1A and reveals the residues in close proximity to the interface. Several factors were important in determining which residues to mutate. First, our early studies on heterodimer formation (17) indicate that the Ubc13-Mms2 complex is stable in salt concentrations of >1 M and therefore suggest a large portion of hydrophobic contacts. Visible inspection reveals that a significant contribution of these hydrophobic contacts likely surround a region that corresponds to a two-residue insertion (Asn$^{12}$ and Phe$^{13}$) near the amino end of the α1 helix that is unique to Uev sequences. Second, we previously identified a remarkable change in the orientation of the human Mms2 N terminus upon heterodimer formation with Ubc13 (15), implicating an importance for the Ubc13 residues in proximity to the Mms2 N terminus. Last, each of the residues that we mutated is either completely conserved or very similar when comparing protein sequence alignments across various model organisms (Fig. 1C). The panel of residues that were eventually mutated in these studies is indicated in Fig. 1, B and C.

**Design of Ubc13 Mutants and Their Interaction in the Yeast Two-hybrid Assay**—Because all evidence to date suggests a specific complex between Mms2 and a single Ubc (namely, Ubc13), we decided to initiate our mutagenesis studies with focus on Ubc13 residues. It was anticipated that this approach would narrow down the search for key residues because Ubes involved in very different cellular roles still have very conserved core domain sequences.

As a precursor to selecting mutants to test in vivo, we were first able to screen two Ali substitutions, Ubc13-Y34A and Ubc13-L63A, in vitro by using the GST pull-down approach as discussed later. These residues had been suggested previously (15) to contribute hydrophobic contacts to two separate regions of the interface (see Fig. 1B). However, neither mutation had a significant effect on binding to Mms2 as determined by GST pull-down and isothermal titration calorimetry experiments, suggesting that residues presumed to contribute hydrophobic contacts have to be changed more drastically. With this in mind, we targeted another residue of the putative hydrophobic core, Ubc13-Phe$^{57}$, and designed a non-conservative substitution to Glu to introduce a charged polar group into the region.

In another attempt to address this same hydrophobic region, we focused the role of the Ubc13-Glu$^{55}$ residue. Structure analysis implicates a dual role whereby the β- and γ-carbons of Glu$^{55}$ can provide hydrophobic contacts for Mms2-Phe$^{13}$, whereas its carboxyl group is used to H-bond with Mms2-Asn$^{12}$ (15, 16) (Fig. 1B). The only Ubc13 mutant studied to date was the corresponding Glu$^{55}$ in the yeast Ubc13 (16), for which Ala was substituted, resulting only in a small titration-dependent effect on binding and function (30). Therefore, we created a conservative E55Q mutant that was expected to disrupt the H-bonding with Mms2-Asn$^{12}$ while still retaining hydrophobic contacts and approximate size.

The last of our initial Ubc13 mutagenesis focused on Ubc13-Glu$^{60}$, which is situated near the N terminus of Mms2. Although it is not resolved in the human structure, the yeast Ubc13-Mms2 heterodimer shows an H-bond formed between Ubc13-Glu$^{60}$ and Mms2-Ser$^{2}$. It was predicted that a conservative Ubc13-E60Q mutation would disrupt the bond and address the importance of the N terminus of Mms2 in binding Ubc13.

In order to determine the effect of the interface mutations on complex formation in the cell, we first employed the yeast two-hybrid assay as a qualitative measure of interaction strength. Fig. 2 shows the effects of these Ubc13 mutations. The mutation at Glu$^{60}$ (E60Q) does not affect the Ubc13 interaction with Mms2, whereas both E55Q and F57E mutations resulted in the complete disruption of heterodimer formation as visualized in the assay. The severe consequences of the Ubc13-Glu$^{55}$ and Ubc13-Phe$^{57}$ mutants prompted us to investigate another residue in this area, Ubc13-Arg$^{70}$.

The Arg$^{70}$ residue is absolutely conserved among Ubc13s (Fig. 1C). Although it has been suggested that Arg$^{70}$ can provide hydrophobic contacts in the yeast heterodimer structure (16), we note another predominant contribution to the interface as an H-bond with the carbonyl carbon of Mms2-Met$^{41}$ (Fig. 1B). To address this bond, we created a Ubc13-R70A mutant and tested its binding to Mms2 in the yeast two-hybrid assay. As seen in Fig. 2, Ubc13-R70A impaired Ubc13-Mms2 interaction.

**Design of Mms2 Mutants and Their Interaction in the Yeast Two-hybrid Assay**—The finding that all of the Ubc13 residue substitutions that affect binding lie in close proximity allowed us to narrow our search for key interface residues in Mms2. Notably, the Mms2 residues that most closely correspond to these Ubc13 mutants are Mms2-Asn$^{12}$ and Mms2-Phe$^{13}$, two amino acids unique to Uevs when aligned against Ubes (16).

Visualization of the Ubc13-Mms2 heterodimer shows an insertion of the Mms2-Phe$^{13}$ residue into a hydrophobic region, created mainly by the Ubc13 residues mutated above. As with Ubc13-F57E, we decided to exploit this area with an aggressive Mms2-F13E substitution to introduce a polar, charged group to abrogate non-polar contacts.

Unlike Phe$^{13}$, Mms2-Asn$^{12}$ does not contribute to the hydrophobic core but instead forms H-bonds with the Ubc13 residues Glu$^{55}$ and Tyr$^{34}$. However, because only the Ubc13-E55Q mutation showed an effect on binding, we sought to demonstrate a pairwise interaction between it and Mms2-Asn$^{12}$. We thus created two Asn$^{12}$ mutations, one as a conservative N12D substitution and a second mutation, N12A, in order to abolish any H-bonding possibilities.

When testing these Mms2 mutants in the two-hybrid assay, we found that neither Mms2-N12A nor Mms2-N12D had a detectable effect on inhibiting complex formation as compared with wild-type (Fig. 2). In contrast, the Mms2-F13E mutation seemed to completely disrupt the Ubc13-Mms2 interaction.

Because our mutagenesis of Ubc13 yielded mutants that resulted in severe (Ubc13-E55Q and Ubc13-F57E) and slight (Ubc13-R70A) disruption of the Ubc13-Mms2 interaction, we desired to have a complement of Mms2 mutants with similar

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2 L. Pastushok, T. F. Moraes, and J. Hu, unpublished data.
effects. Therefore, we hypothesized that because the aggressive introduction of a charged group at Mms2-Phe13 (F13E) caused a severe effect on interaction, a more conservative F13Y mutation that simply introduces a small polar hydroxyl group might cause a moderate effect. Indeed, the Mms2-F13Y mutant did not interact with Ubc13 as strongly as wild-type, but it did not completely abolish heterodimer formation either (Fig. 2).

Taken together, the yeast two-hybrid data indicate that two
mutants (Mms2-F13Y and Ubc13-R70A) result in weakened interactions between Ubc13 and Mms2. Also, three mutants (Mms2-F13E, Ubc13-E55Q, and Ubc13-F57E) led to the complete disruption of the Ubc13-Mms2 interface. Although it may be argued that some very slight binding might occur below the detectable limits of this assay, we note that the yeast two-hybrid assay is a highly sensitive tool because transient interactions are sufficient to allow transcription of the reporter gene.

The in Vivo Function of MMS2 and UBC13 Mutants Correlates with in Vivo Interaction—MMS2 was first identified by its ability to provide yeast cells with protection from the DNA-damaging agent MMS (31). The discovery revealed the “error-free” (damage avoidance) postreplication repair pathway (reviewed in Ref. 32) that is conserved in human cells (33). Genetically speaking, error-free postreplication repair activity is dependent on UBC13, MMS2, and Lys63-conjugated poly-Ub chains (12, 20). As a result, ubc13Δ and mms2Δ are similarly sensitive to DNA damage (23).

Because mammalian MMS2 (26, 34) and UBC13 (35) genes are able to functionally complement their yeast counterparts, we decided to test the function of our human interface mutant proteins in the corresponding ubc13Δ and mms2Δ yeast strains. The approach would verify the correlation between heterodimer formation and function. As seen in Fig. 3, wild-type human MMS2 and UBC13 are able to alleviate the MMS sensitivity of their corresponding yeast deletion strains. The MMS2 mutants at Asn12 appeared to function as well as wild-type MMS2; on the other hand, the mutations at Phe13 that address the core hydrophobic region led to a slight (F13Y) or drastic (F13E) reduction in the protection of cells against MMS (Fig. 3A). As in the two-hybrid assay, the UBC13 E60Q mutant had no effect on functional complementation (Fig. 3B). However, each of the mutants in proximity to Mms2-Phe13 led to compromised Ubc13 function. Most strikingly, both Ubc13-E55Q and Ubc13-F57E mutations had no detectable ability to provide protection against MMS-induced killing when compared with the negative control. The other Ubc13 mutation, R70A, which was designed to destroy H-bonding with the Mms2 backbone, led to a reduced but incomplete loss in the ability to complement the ubc13 deletion.

The functional experiments above were repeated using UV as a DNA-damaging agent, and we observed no change in the results (data not shown). To account for the heterologous approach, functional complementation was also checked using double transformants (pGBT-hUBC13 and pGAD-hMMS2) in an mms2Δ ubc13Δ strain, and our findings were the same (data not shown).

In Vitro Binding of Interface Mutants—The yeast two-hybrid assay provides a good qualitative indication of binding strength; however, several factors such as expression levels and Gal4 fusions can hinder final conclusions. Therefore, to address and confirm the in vivo interaction data, we sought to evaluate Ubc13-Mms2 binding properties in vitro by expressing and purifying each wild-type and mutant protein as GST fusions from
formed as described in visualized by Coomassie Blue staining, and Mms2 was detected by Mms2 was then added, and after washing, the eluted GST-Ubc13 was downs were used to co-purify proteins that form stable interactions. antibodies against Ubc13 were used for Western blot. The on each figure contains purified proteins as control. bacteria and isolating the native non-fused protein when needed. Any post-translational modifications missing from bacterial expression were not a concern because previous in vitro binding and ubiquitination studies have been performed with recombinant protein from bacterial sources (17).

In addition to the wild-type proteins, two groups of mutants from each of Ubc13 and Mms2 were purified. These include those that resulted in reduced binding and functional complementation (Mms2-F13Y and Ubc13-R70A) and those that seemed to completely disrupt the interface and abolish function (Mms2-F13E and Ubc13-F57E). Despite rigorous attempts, GST-Ubc13-E55Q could not be expressed in soluble form in bacteria. To show that the lack of in vitro binding and function in this mutant was not due to insolubility in yeast cells, we tested the ability of Ubc13-E55Q and the other Ubc13 mutants to interact with TRAF6. The physical interaction between Ubc13 and TRAF6 has been demonstrated in the two-hybrid assay and is independent of Ubc13-Uev interaction (36). Therefore, the Glu55 interface mutation is not expected to affect binding to TRAF6. Indeed, we were able to show that Ubc13-E55Q and the other Ubc13 mutants interact with TRAF6 with a strength that is indifferent to wild-type Ubc13.

We first used GST pull-downs (35) as a simple means to verify our in vitro interaction data. Purified wild-type or mutant GST-Ubc13 or GST-Mms2 was immobilized on glutathione-Sepharose and incubated with native Mms2 or Ubc13, respectively. Co-purified proteins were then visualized by Western blot. Whereas Ubc13-F57E was unable to interact with Mms2, Ubc13-R70A retains binding ability (Fig. 4A). Similarly, Mms2-F13E apparently lost the ability to bind Ubc13, whereas Mms2-F13Y led to dramatically reduced binding with Ubc13 (Fig. 4B). These results are in agreement with the in vitro interaction data above (Fig. 2).

Surface Plasmon Resonance—Because in vitro pull-downs lack the quantitative capabilities for measuring binding strength, we employed SPR in order to more clearly show the effects of interface mutations on heterodimer formation. SPR technology can be used to provide a sensitive real-time measure of protein-protein interactions, and the data may be used to determine kinetic and binding variables (37). In our experimental design, we used immobilized anti-GST monoclonal antibodies to capture the GST fusion protein (ligand), and the non-fused soluble binding partner (analyte) was subsequently applied to generate binding curves.

Because binding constants for the human Ubc13-Mms2 pair have been previously estimated using sedimentation (15) and NMR (18) analyses, we first sought to validate our SPR approach by determining the $K_d$ for the wild-type Ubc13-Mms2 heterodimer. Using GST-Ubc13 fusions as the ligand, we were able to generate a series of curves over varied concentrations of native Mms2 as the analyte (supplemental Fig. S1, A). These curves were used in Biacore Evaluations curve-fitting software to determine binding constants (Table I). We also generated our own double-reciprocal plots with the same data and found that our manually calculated dissociation constants were in close agreement (supplemental Fig. S2, A). To account for the possible effects of N-terminal GST fusions on binding strength, we performed the reciprocal approach using GST-Mms2 as the ligand and Ubc13 as the analyte (supplemental Figs. S1, B and S2, B). Notably, our binding constants for human Ubc13-Mms2 were found to be $50 \text{ nM}$, which is in perfect agreement with those determined in independent isothermal titration calorimetry experiments (19).

Perhaps not surprisingly, we were unable to calculate meaningful binding constants for the Ubc13 and Mms2 mutations because in each case the binding was too weak to generate reliable data at the concentrations tested. Therefore, we tested each of the mutations against wild-type as a means of comparison. While using a constant concentration of analyte, we measured the peak response during binding at equilibrium (Table I). We would like to note that in order to better visualize the relative binding of mutant proteins, we had to use a concentration of analyte that was well above that which gave a maximum response for the wild-type proteins. Fig. 5 provides a good visual representation of the binding curves as compared with wild-type. From Fig. 5A and Table I, we can see that the Ubc13-F57E mutation results in barely detectable binding ($\text{RU}_{\text{eq}} = 6$). On the other hand, the maximum response of the Ubc13-R70A mutation was almost 5-fold higher ($\text{RU}_{\text{eq}} = 27$).

Through our studies above, it became apparent that the Mms2-Phε13 residue was a critical component to the interface. Therefore, to underscore the importance of Phε13, we chose to generate a third mutant at this position, Mms2-F13A. The F13A mutation corresponds to a yeast Mms2-F8A substitution that was created by another group (16), who suggested that it eliminates interaction and function with yeast Ubc13. Fig. 5B shows that each of the mutations at Phε13 results in considerably lower binding strength. Most strikingly, the introduction
of a charged group into the core of the interface via Mms2-F13E had the greatest effect on binding (Table I). The Mms2-F13E mutation resulted in peak binding that was barely detectable (RUeq = 1), whereas the F13A mutation interaction was appreciably less weak (RUeq = 15). Again, we find that the conservative introduction of small polar hydroxyl group through Mms2-F13Y leads to a lesser disruption of the Ubc13 interaction and binds nearly 2-fold stronger than F13A. Taken together, the SPR results are consistent with our initial observations for in vivo binding in the two-hybrid assay for both the Mms2 and Ubc13 mutants.

**In Vitro Ubiquitination Assays—**Because Lys<sup>63</sup> poly-Ub chains synthesized by Ubc13-Mms2 are required for DNA repair function, the in vivo functional complementation tests in Fig. 2 are a good indicator of Ub chain catalysis. However, we wished to provide a more direct test of Ub chain formation by the mutant proteins. Therefore, we performed in vitro ubiquitination assays with purified components in which catalytic function is represented by the creation of di-Ub chains. Fig. 6 shows that wild-type Ubc13 and Mms2 form a strong band representative of di-Ub. In contrast, each of the mutations had a severe effect on di-Ub formation. In fact, neither Mms2-F13A, Mms2-F13E, Mms2-F13Y, nor Ubc13-F57E synthesized detectable Ub conjugates when paired with its wild-type partner. Only Ubc13-R70A led to observable di-Ub formation, which was much less than that observed for wild-type Ubc13. As previously reported (17), human Ubc13 has auto-Ub conjugation activity in vitro, and we observe that the interface mutations are not compromised in this regard.

**Interactions between Ubc13-Arg<sup>70</sup> and Mms2-Phe<sup>13</sup> Mutations—**As discussed earlier, it was originally suggested that the Ubc13-Arg<sup>70</sup> residue might have a dual role with regard to the
interface, namely, to share hydrophobic contacts with Mms2-Phe13 (Phe8 in yeast) and to form polar contacts with the Mms2 backbone via its guanidinium group. We reasoned that if the Ubc13-Arg70-Mms2 backbone H-bond plays the dominant role, we would observe an additive effect on binding by mutations that remove the H-bond (Ubc13-R70A) and interfere with the hydrophobic contacts (Mms2-F13Y). The yeast two-hybrid assay (Fig. 7A) shows that whereas the combination of each of the mutants with its corresponding wild-type partner resulted in only slightly compromised binding, cells that carry both Ubc13-R70A and Mms2-F13Y mutations completely lost the interaction. The synergistic effect was also observed in a complementation experiment (Fig. 7B), in which yeast mms2Δ ubc13Δ cells co-transformed with ubc13-R70A and mms2-F13Y were no more resistant to MMS than the control cells, in contrast to cells carrying each single mutation.

Based upon the Ubc13-Mms2 crystal structure (Fig. 1B), we originally hypothesized that the Ubc13-R70A mutation would destroy any H-bonds but retain the contribution of hydrophobic contacts to the pocket. Therefore, the effect of the Ubc13-R70A mutation (Fig. 7, A and B) is suggestive of a second important interface contact point that is independent of the hydrophobic pocket. In order to better address this question, however, we generated a Ubc13-R70L mutant that would also abolish H-bonding but would be a better candidate for retaining hydrophobic contacts with Mms2-Phe13. As is seen in Fig. 7, the combination of Ubc13-R70A and Mms2-F13Y mutations completely abolished Ubc13-Mms2 interaction and DNA repair function in yeast cells, with phenotypes indistinguishable from the negative controls. In contrast, Ubc13-R70L did not demonstrate the same additive decrease in binding when coupled with Mms2-F13Y (Fig. 7C). In fact, the Ubc13-R70L mutation may have alleviated the affect of Mms2-F13Y. In addition, the Ubc13-R70L mutation was able to protect cells from MMS treatment as well as wild-type Ubc13 in functional complementation experiments (data not shown). Taken together, these results underscore the importance of Ubc13-Arg70 in helping to create a deep Ubc13 hydrophobic pocket for Mms2-Phe13.

**DISCUSSION**

The Ubc13-Uev heterodimer is the only known E2 that creates atypical poly-Ub chains through Lys63 in vivo. The uniqueness of the Ubc-Uev complex in Ub chain synthesis led to numerous detailed biochemical studies (17–19) and the discovery of the involvement of Lys63-linked Ub chains in various cellular pathways, especially in mammalian cells (1, 38–40). Importantly, mammals have two Uev proteins, Mms2 and Uev1, which have >90% sequence identity (26) but distinct cellular functions;3 therefore, the formation of a particular Ubc13-Uev complex may serve as a means of regulation. However, whereas the mechanistic studies clearly describe how Ubc13-Uev complexes function, there is no report describing

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3 P. L. Andersen and W. Xiao, unpublished data.
why the two proteins are able to come together to form a heterodimer. Prior to this investigation, there were only two interface mutations (Ubc13-E55A and Mms2-F8A) in yeast that were reported (16, 30). In this study, we made the first attempt to systematically mutate human Ubc13 and Mms2 in order to address how the complex is formed. We employed a number of in vivo and in vitro methods, including the yeast two-hybrid assay, pull-downs, and SPR in order to assess binding strength. In addition, an in vitro ubiquitination assay and a novel heterologous complementation approach were utilized to correlate binding ability with biochemical and biological activities. Overall, an excellent correlation between these experimental results was achieved.

The Ubc13-Mms2 interface is long and narrow and buries a large solvent-accessible surface area of ~1500 Å. Our study began with the systematic mutagenesis of a number of residues along the Loop 1, α1 helix, and the N terminus of Mms2, all of which contain portions that visibly contact Ubc13 in the human (15) (Fig. 1, A and B) and yeast (16) crystal structures. We ended up with the identification of a rather unexpected pocket of interactions close to the α1 helix of Mms2, which appears to be critical for both complex formation and specificity. This pocket consists of Phe57 and Glu55 of Ubc13 residing in close proximity to the N-terminal end of the Mms2 α1 helix believed to be the core of the Ubc13-Mms2 interface. Accordingly, both the Ubc13-F57E and Ubc13-E55Q mutations have extreme effects on disrupting the Ubc13-Mms2 interaction. In addition, Arg70 can be viewed as a bridge between two Mms2 contact points because it putatively contributes hydrophobic contacts to the α1 helix of Mms2 (via Mms2-Phe13) and an H-bond with the Loop 1 backbone (via Mms2-Met154). Underlining its importance as another key interface residue, the Ubc13-R70A mutation compromised binding and function. Among residues in the Mms2 complement of the core interface region, the Mms2-N12D and Mms2-N12A mutations that disrupt the H-bond with Ubc13-Glu55 did not have the same effect as the corresponding Ubc13-E55Q mutation. On the other hand, mutating Mms2-Phe13 had profound effects on binding and in vivo functions. Based on the Ubc13-Mms2 crystal structures, Mms2-Phe13 is seen to insert itself deep within a hydrophobic pocket formed by the Ubc13 backbone and several side chain carbons, reminiscent of a key in a keyhole. To fully explore the significance of this critical interaction, we created three very different mutations at this same Phe13 residue. An aggressive F13E mutation resulted in the complete loss of detectable binding in all of our in vivo and in vitro studies. To explore the importance of these hydrophobic contacts further and more precisely, we created and tested a more conservative mutation, F13Y. We found that the introduction of this small hydroxyl group was sufficient to severely disrupt the Ubc13-Mms2 interface, and this underscores the importance of the deep hydrophobic contacts required for the Phe13 insertion. Similarly, the Ubc13-Mms2 heterodimer is abrogated with an Mms2-F13A substitution that maintains the hydrophobic characteristic but cannot breach the Ubc13 surface to create deep hydrophobic contacts.

Whereas our studies have revealed a number of crucial Ubc13 interface residues, Mms2-Phe13 appears to be the only interface residue identified with a role in binding to Ubc13. A model based on crystal structures and this study suggests that Mms2-Phe13 is inserted between Ubc13-Glu55 and Ubc13-Phe57 and is flanked closely by Ubc13-Arg70, which together form a critical hydrophobic interface pocket. This model is in agreement with the demonstrated stability of Ubc13-Mms2 salt concentrations as high as 2.5 M.4 It is thus likely that polar contacts, such as those observed in the crystal structures via the Ubc13-Arg70 and Ubc13-Glu55 residues, do not significantly contribute to binding strength and might instead play more subtle roles such as aiding Mms2-Ubc13 recognition.

Because of their functional interchangeability, the human (15) and yeast (16) crystal structures of the Ubc13-Mms2 heterodimers are very similar (26, 35). In particular, the corresponding interface residues that we mutated in the human proteins are conserved and oriented identically in yeast. A small caveat is the human Ubc13-Phe57 residue, which corresponds to a Tyr27 in yeast; however, Tyr27 has its hydroxyl group pointed away from the critical hydrophobic pocket (supplemental Fig. S9). The above information allows us to use sequence alignments to determine the residues in other yeast Ubc structures (Fig. S8) that correspond to human Ubc13-Glu55, Ubc13-Phe57, and Ubc13-Arg70. The positions of these corresponding residues for each of Ubc2/Rad6 (29), Ubc4 (13), and Ubc7 (41) are physically oriented such that the necessary hydrophobic pocket for Mms2-Phe13 is not permitted (Fig. 8, B–E). Although Ubc1 (42) might spatially allow an Mms2-Phe13 insertion, the stoutness of its Asp and Gln residues at the Ubc13-Glu55 and Ubc13-Arg70 positions, respectively, would not allow sufficient hydrophobic contact from their side chains (Fig. 8F). It would be of great interest to see in future studies whether these critical Ubc13 residues are not only required but also sufficient to interact with Mms2.

4 L. Pastushok, unpublished results.
A Structural and Functional Basis for Ubc13-Mms2 Interaction

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A Single Mms2 "Key" Residue Insertion into a Ubc13 Pocket Determines the Interface Specificity of a Human Lys65 Ubiquitin Conjugation Complex

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