Protein-Protein Interactions within an E2-RING Finger Complex

IMPLICATIONS FOR UBIQUITIN-DEPENDENT DNA DAMAGE REPAIR\(\textsuperscript{S}\)

Received for publication, December 2, 2002
Published, JBC Papers in Press, December 19, 2002, DOI 10.1074/jbc.M212195200

Helle D. Ulrich\‡
From the Max Planck Institute for Terrestrial Microbiology, Karl-von-Frisch-Strasse, 35043 Marburg, Germany

The RING finger protein RAD5 interacts and cooperates with the UBC13-MMS2 ubiquitin-conjugating enzyme in postreplication DNA damage repair in yeast. Previous observations implied that the function of UBC13 and MMS2 is dependent on the presence of RAD5, suggesting that the RING finger protein might act as a ubiquitin-protein ligase specific for the UBC13-MMS2 complex. In support of this notion it is shown here that the contact surfaces between the RAD5 RING domain and UBC13 correspond to those found in other pairs of ubiquitin-conjugating enzymes and ubiquitin-protein ligases. Mutations that compromise the protein-protein interactions either between the RING domain and UBC13 or within the UBC13-MMS2 dimer were found to have variable effects on repair activity \textit{in vivo} that strongly depended on the expression levels of the corresponding mutants. Quantitative analysis of the affinity and kinetics of the UBC13-MMS2 interaction suggests a highly dynamic association model in which compromised mutual interactions result in phenotypic effects only under conditions where protein levels become limiting. Finally, this study demonstrates that beyond its cooperation with the UBC13-MMS2 dimer, RAD5 must have an additional role in DNA damage repair independent of its RING finger domain.

Covalent attachment of ubiquitin to a target protein generates a signal that can function in the regulation of many biological processes, ranging from cell cycle progression and transcriptional activation to inflammatory and immune responses (1–4). Ubiquitylation of a protein is a multistep reaction involving a complex enzymatic cascade (5); in an ATP-dependent reaction, the C terminus of ubiquitin is linked via a thioester bond to a cysteine residue in the active site of ubiquitin-activating enzyme (E1).\(^1\) The ubiquitin thioester is then transferred to the active site cysteine of an E2, which mediates the attachment of the ubiquitin C terminus to a lysine residue of the target protein, resulting in an isopeptide bond. This last step is most often catalyzed by another enzyme, termed ubiquitin-protein ligase or E3. Repeated rounds of ubiquitylation result in the formation of long multiubiquitin chains in which each ubiquitin moiety is linked to an internal lysine residue, usually Lys\(^{63}\), of the preceding one (6). All of the eukaryotic genomes encode multiple E2s and an even larger number of E3s, the latter being responsible for substrate recognition (5, 7, 8). One prominent class of ubiquitin protein ligases is characterized by the presence of a RING domain, a specialized type of zinc finger in which two Zn\(^{2+}\) ions are coordinated by a group of cysteine or histidine residues in a characteristic arrangement (9–11). The RING finger has been proposed to fulfill a scaffold function and has in many cases been shown to be involved in E2 binding (12–14). The x-ray structure of the mammalian E3 c-Cbl in complex with a cognate E2, UbcH7, has given insight into the molecular details of the RING domain-E2 interaction (15). A similar contact surface was found by NMR analysis in another RING finger E3, CNOT4 (16, 17). Hundreds of RING finger proteins have been described to date; however, although a growing number of them is being identified as ubiquitin ligases (12, 13, 16, 18), by no means all of them have been shown to possess E3 activity or be involved in ubiquitylation at all, raising the question of what characteristics distinguish ubiquitin ligases from other RING finger proteins.

Modification by ubiquitin generally marks a protein for degradation by the 26 S proteasome (3). However, ubiquitylation can also convey nonproteolytic signals (2, 19–21). In particular, nonstandard multiubiquitin chains in which one ubiquitin moiety is linked to the next via Lys\(^{63}\) have been implicated in such diverse processes as DNA damage repair (22), endocytosis (23), ribosome biogenesis (24), mitochondrial inheritance (25), and NF\(\kappa\)B signaling (26). In yeast and mammals, the only enzymatic activity demonstrated so far to catalyze the assembly of Lys\(^{63}\)-linked ubiquitin chains resides in the heterodimeric complex of UBC13 and MMS2, a genuine UBC and a structurally related ubiquitin-conjugating enzyme variant (27). The function of Lys\(^{63}\)-linked ubiquitin chains in DNA damage repair has been attributed to a participation of UBC13 and MMS2 in the postreplication repair pathway (27, 28), which confers damage tolerance and ensures the completion of genome duplication in situations where lesions in the template strand cause a stalling of the replication machinery (21, 29–31). The principal mediator of postreplication repair is RAD6 (32), which encodes another UBC (33). It is believed to be targeted to sites of damage by the DNA-binding RING finger protein RAD18 (34). An error-free subpathway within the RAD6 system is mediated by another chromatin-associated RING finger protein, the SWI/SNF homolog RAD5 (35). I have previously shown that the function of UBC13 and MMS2 in the RAD6 pathway in yeast is mediated by RAD5, which contacts UBC13 through its RING
domain and recruits the E2 heterodimer to the chromatin in response to DNA damage (36). Through its association with the RAD6-RAD18 dimer, RAD5 thus coordinates the assembly of two different E2s on damaged chromatin, suggesting a cooperation of RAD6 and the UBC13-MMS2 complex with the RING finger proteins RAD18 and RAD6 in ubiquitin conjugation (36).

In confirmation of this model it was recently shown that proliferating cell nuclear antigen, a processivity factor for a number of DNA polymerases dedicated to replication as well as repair, is modified by Lys63-linked ubiquitin chains in response to DNA damage (37). This modification depends on the presence of RAD5, UBC13 and MMS2, whereas RAD6 and RAD18 in the absence of the former afford only mono-ubiquitylation (37). Thus, it appears that the UBC13-MMS2-RAD5 assembly indeed functions as a genuine E2-E3 complex for the assembly of Lys63-linked multiubiquitin chains. Support for this notion is presented here by a genetic and biochemical analysis of the protein-protein interactions within this E2-RING finger complex and their consequences for cooperation between RAD5 and the UBC13-MMS2 dimer in DNA damage repair.

EXPERIMENTAL PROCEDURES

**Yeast Strains and Media**—The wt yeast strain used in this study as well as the isogenic mutants ubc13::His3, rad5::His3, and rad18::HIS3 were described previously (36). Strain PJ69-4A (38) was used for two-hybrid assays. Standard protocols were followed for the preparation of yeast media and transformations (39). SC medium was a modification from that described by Guthrie and Fink (39) and contained 100 mg/liter of each amino acid except for leucine, which was present at 500 mg/liter. This corresponds to a cysteine concentration of 0.67 mM. Yeast strains harboring integrative plasmids were propagated in YPD medium following the initial selection on solid and in liquid medium after transformation; strains with centromeric plasmids were maintained in selective SC medium at all times.

**Construction of Plasmids**—Two-hybrid constructs bearing the ORFs of RAD5, UBC13, and MMS2 have been described (36). Site-directed mutants were generated by polymerase chain reaction and recloned into the two-hybrid vectors, and the amplified regions were fully sequenced. The intron was removed during construction of the UBC13 mutants; however, its presence or absence did not affect protein levels.2 For expression under the control of the native promoter, the RAD5 ORF and the RING finger mutants were recloned from the two-hybrid vectors as BamHI/PstI fragments into a derivative of the integrative vector YIp211 (40) carrying an EcoRI/BamHI fragment encompassing 245 bp of the RAD5 upstream region and a PstI/SphI fragment derived from pGBT9 (Clontech) as a transcriptional terminator (YIp211-P::RAD5::RADS). UBC13 and its mutants were expressed under the control of the native promoter and its upstream regions, which 996 bp of the UBC13 upstream regions were inserted as an EcoRI/BamHI fragment upstream of the ORF (YIp211-P::UBC13::UBC13). Regulatable expression levels were achieved by placing the UBC13 wt or mutant ORFs under the control of the MET3 promoter into the centromeric vector YCplac111 (40), again in combination with the pGBT9-derived transcriptional terminator (YIp111-P::UBC13). For the production of recombinant proteins in *Escherichia coli*, UBC13 and MMS2 as well as the respective mutants were recloned into expression vectors, allowing the production of different fusion proteins. pGEX-4T-1 (Amersham Biosciences) was used to produce N-terminal GST fusions of UBC13 and its mutants, MMS2, and GST alone. pQE-30 (Qiagen) served as a vector for expression of His,-tagged UBC13. The MMS2 ORF as well as the mutant F8A were inserted into the vector pTYB12 (New England Biolabs). This vector affords expression of MMS2 as an N-terminal fusion to a chitin-binding domain, linked by the self-cleavable VMA1 intein sequence, which allows single-step purification of MMS2 bearing three additional amino acids (AGH) at its N terminus. For simplicity, this construct will be referred to as MMS2 in the following text. Sequence maps of all the constructs used in this study are available on request.

**Two-hybrid Assays**—Analysis of the interactions between RAD5, UBC13, and MMS2 in the two-hybrid system was performed as described previously, using growth on histidine-selective medium as an indication for a positive interaction (36).
Fig. 1. Design of RAD5 and UBC13 interface mutants. The filled circles above the c-Cbl, CNOT4, and UbcH7 sequences highlight residues that were shown to be located at the RING-E2 interface based on the x-ray structure (c-Cbl and UbcH7). The open circles above the RAD5 and UBC13 sequences indicate the positions for site-directed mutagenesis. The mutants designed within the RAD5 RING finger were analyzed with respect to their interactions with the respective E2. Positions Glu943 and Tyr944 could be assigned as a position involved in interactions in both c-Cbl and CNOT4. In addition, D923A was shown to prevent the interaction with UBC13 (36). Mutation C914S, an exchange likely to disrupt the structural integrity of the entire domain, had previously been shown to prevent the interaction with UBC13 (36). Mutation D923A had no effect on association with UBC13, which was expected because this residue should occupy a position in the RING finger facing away from the putative E2 contact site. Mutations I916A, Y944A, and N959A, all predicted to reside on the surface of the RING domain facing the E2, indeed abolished interaction with UBC13 in the two-hybrid system. In contrast, mutation of Glu943, also predicted to contribute to the E2 interface, to alanine did not prevent interaction with UBC13. Thus, the interaction of the RAD5 RING finger with UBC13 involves a surface similar but not identical to that within other RING domains, including several highly conserved residues but also showing variations that are likely to affect the respective E2 specificity.

In the reciprocal approach, the ability of the putative contact site mutants of UBC13 to interact with RAD5 was examined. Interaction with MMS2, which is independent of the UBC13-RAD5 association (36), was examined in parallel to ensure proper function of the mutants in the two-hybrid system. UBC13/E55A served as a control for a protein that should only be affected in its association with MMS2 but not with RAD5. The results of the two-hybrid analysis are shown in Fig. 2B. As expected, mutant UBC13/E55A was found to associate with RAD5, but interaction with MMS2 was abolished. In contrast, all other mutants were capable of association with MMS2 but had lost their ability to interact with RAD5. These results indicate that amino acids critical for UBC13-RAD5 contacts reside within the N-terminal helix of UBC13 (Lys^6 and Lys^10) as well as the loops L1 (Met^44) and L2 (Ser^96) (Fig. 1B). Thus, the UBC13 interface to the RAD5 RING finger closely resembles the corresponding interfaces of other E2 enzymes with their respective ubiquitin-protein ligases.

Consequences of Interface Mutations for in Vivo Function—To examine the effects of the different contact site mutations on the ability of RAD5 and UBC13 to cooperate in DNA damage repair in vivo, the mutant genes were integrated into the genome and expressed under the control of their own promoters in the respective deletion strains. Fig. 3 shows a comparison of the UV sensitivities of representative mutants. Mutations in RAD5 and UBC13 Abolish Association of the Two Proteins—The mutants designed within the RAD5 RING finger were analyzed with respect to their interactions with UBC13 in the two-hybrid system. This method has been used previously to demonstrate the interaction between the two proteins and correlates well with results obtained by co-immunoprecipitation (36). To exclude the possibility that a negative result might be due to improper folding or expression of the mutant protein in the two-hybrid system, dimerization...
Protein-Protein Interactions in an E2-RING Finger Complex

In the context of DNA repair, the expression level of the RAD5 protein is crucial. The figure below illustrates the survival curve of yeast cells after UV irradiation, with different expression levels of RAD5. The expression levels are indicated by different symbols: squares, circles, and triangles. The figure shows that the RAD5 protein's expression level significantly affects the cells' survival rate after UV exposure.

The expression of RAD5 is controlled by the UBC13 protein, which acts as an E2 enzyme in the ubiquitin-proteasome system. The interaction between RAD5 and UBC13 is essential for the proper function of the RAD5 protein. The figure also shows the survival curves of yeast cells expressing different mutants of UBC13, such as E55A, K6E, and M64A, which are involved in the E2-RING finger complex.

In summary, the expression level of RAD5 is a critical factor in determining the survival rate of yeast cells after UV irradiation, and the interaction between RAD5 and UBC13 is essential for the proper function of RAD5 in DNA repair.

DNA Repair Capacities Depend on the Expression Levels of UBC13—These results suggested that mutants in which protein-protein contacts are compromised may display defects whose magnitude depends on the genetic context, whereas those changes that directly affect the catalytic center or the structural integrity of the proteins would lead to a permanent loss of function. To examine this possibility, the UBC13 mutants were expressed from a centromeric vector under the control of the regulatable MET3 promoter. On synthetic selective medium, which contains 0.67 mM methionine, this promoter should be partially repressed (46). When UV sensitivities were compared in this context, a striking difference between wt UBC13 and the interface mutants became apparent; in fact, here all contact site mutants, including E55A, were as sensitive as the catalytic site mutant and the ubc13 deletion (Fig. 3D and supplementary Fig. 1C). Fig. 3E shows that expression from the MET3 promoter resulted in significantly lower protein levels than expression from the native UBC13 promoter even in the absence of damage-induced up-regulation. These findings imply that the UBC13 interaction mutants might only show significant defects in DNA repair when protein levels are limiting, whereas under physiological conditions, when transcription of the UBC13 promoter can be induced by DNA damage, increased expression levels can overcome the weakened interaction with RAD5 or MMS2.

To test this notion more rigorously, UV sensitivity of mutants representative for the RAD5 contact site (S96A), the MMS2 interface (E55A) as well as the catalytic center (C87S) were compared in UV gradient assays on plates of defined methionine concentration that exploited the entire range of the MET3 promoter, reaching from full induction at 0 mM methionine to full repression at 2 mM (Fig. 4A). In parallel, protein expression under both extremes was examined (Fig. 4B). In the


Fig. 3. Effect of representative mutations in RAD5 and UBC13 and expression level on UV sensitivity. A–D, survival after irradiation (254 nm) is plotted against the applied UV dosage. Expression vectors used are indicated on each panel. Those mutants mentioned in the text but not shown in this figure can be found in supplementary Fig. 1. A and B represent an analysis of RAD5 constructs in the rad5 (filled symbols) and the rad5 ubc13 background (open symbols). A, squares, RAD5; circles, C914S; triangles, I916A; diamonds, empty vector. B, squares, RAD5; circles, E943A; triangles, Y944A; diamonds, empty vector. C and D show the survival curves of UBC13 mutants in two different vectors in a ubc13 strain. Filled squares, UBC13; open squares, empty vector; filled circles, E55A; filled triangles, S96A; open triangles, C87A. E, expression levels of UBC13 and its mutants in the two different vector backgrounds. UBC13 was detected by Western blot in whole cell lysates prepared from cultures of equal densities. Shown for comparison is the endogenous UBC13 of an unmodified strain. Note that the bottom panel results from more concentrated extracts. The aberrant migration of UBC13(E55A) has been observed before (43).

Fig. 3E shows that expression from the MET3 promoter resulted in significantly lower protein levels than expression from the native UBC13 promoter even in the absence of damage-induced up-regulation. These findings imply that the UBC13 interaction mutants might only show significant defects in DNA repair when protein levels are limiting, whereas under physiological conditions, when transcription of the UBC13 promoter can be induced by DNA damage, increased expression levels can overcome the weakened interaction with RAD5 or MMS2.
absence of methionine the \textit{MET3} promoter afforded an expression level of UBC13 higher than that of the native \textit{wt} strain; under these conditions, the unmutated UBC13 as well as the interaction mutants displayed \textit{wt} UV sensitivity, and only the catalytic site mutant C87S was deficient in DNA repair. At 2 mM methionine, where no expression of UBC13 from the \textit{YCp111-P\textit{MET3}} promoter was detectable, mutants S96A and E55A showed UV sensitivities equal to that of C87S, whereas for the unmutated UBC13 under control of the \textit{MET3} promoter partial sensitivity in between that of the true \textit{wt} and the mutants was observed. Intermediate phenotypes were observed at 0.33 and 0.67 mM methionine (data not shown). Thus, these results support the notion that weakened protein-protein interactions lead to phenotypically visible repair defects only under circumstances where protein levels are limiting.

**Determination of Dissociation Constants of UBC13-MMS2 Dimers**—The variable UV sensitivities of UBC13(E55A) and the mutants unable to interact with RAD5 stand in contrast to the mutant MMS2(F8A). This mutation had previously been demonstrated to disrupt the interaction between MMS2 and UBC13 in a manner similar to UBC13(E55A), and a similar loss of \textit{in vivo} function was shown by means of UV sensitivity assays (43). Unlike the UBC13 interaction mutants, however, MMS2(F8A) proved to be a complete loss-of-function mutant in this study even when integrated into the genome under the control of its native promoter (data not shown). Considering these differences, it was of interest to determine to what degree the interactions involving UBC13 were weakened in the individual contact mutants. Thus, association between UBC13 and MMS2 was quantified for a number of combinations involving \textit{wt} or mutant proteins using surface plasmon resonance (Biacore) technology. Recombinant UBC13 was produced as a GST fusion protein for immobilization on the sensor surface, whereas MMS2 was expressed in a self-cleavable intein fusion system (see supplementary Fig. 2A). The dimer resulting from the \textit{wt} constructs was catalytically active (see below). Initial experiments showed that binding and dissociation of UBC13 and MMS2 occurred with kinetics too fast to be accurately resolved by the instrument. Therefore, dissociation constants \((K_D)\) were determined from a plot of the signal at equilibrium against the concentration of the soluble binding partner (see supplementary Fig. 2, B–F). The results for the different combinations of \textit{wt} and mutant proteins are shown in Table I. To confirm that the value of \(K_D = 3.1 \times 10^{-7} \text{ M}\) for the \textit{wt-wt} combination reflected the physiological situation as closely as possible and to exclude an effect of the GST moiety fused to UBC13, interaction was also measured for a combination of proteins where MMS2 was fused to GST and UBC13 was produced in the native form without any tag. The \(K_D\) value obtained for this pair of proteins was similar to that of the reverse combination (Table I). As expected, mutation UBC13(S96A), which should only affect binding to the RING finger of RAD5, had no effect on the affinity for MMS2. In contrast, mutation UBC13(E55A) reduced the affinity for MMS2 by \(-60\)-fold. Association of MMS2(F8A) with UBC13 was detectable only at high MMS2 concentrations, with a \(K_D\) of \(1.5 \times 10^{-3} \text{ M}\). Because of the weak signals involved, this value should be viewed as an estimation. These values imply that a moderate reduction in affinity as observed in the UBC13(E55A) mutant can be partially overcome \textit{in vivo}, whereas a more severe reduction, as exhibited by the MMS2(F8A) mutant, leads to a complete loss of function in DNA repair.

**Catalytic Activities Correlate with Affinities and \textit{in Vivo} Function**—If the repair defects observed \textit{in vivo} are really caused by the differential affinities of the proteins, then this correlation should also be reflected by the catalytic activities of the respective combinations of mutants. To test this model, RAD5-independent mult ubiquitin chain synthesis was assayed \textit{in vitro} using the same set of recombinant proteins as in the interaction analysis. Ubiquitin polymerization was monitored over a time course of 4 h at 30 °C, using two different E2 concentrations. Under these conditions the \textit{wt} combination of GST-UBC13 and MMS2 displayed detectable chain synthesis activity when present at 0.5 \(\mu\text{M}\), whereas at 5.0 \(\mu\text{M}\) E2 free ubiquitin was largely consumed at the end of the incubation period (Fig. 5A). Similar activities were observed for pairs of proteins bearing other combinations of GST and His\textsubscript{6} tags or those produced in the native form,\(^3\) confirming that N-terminal fusions to either protein influence neither mutual interaction nor catalytic activity. As expected, UBC13(S96A) showed wt levels of ubiquitin chain synthesis in combination with wt MMS2 (Fig. 5B), confirming that the repair defect observed \textit{in vivo} was not due to a reduction in catalytic activity of the protein itself but rather to a weakened interaction with RAD5. Mutants K6E, K10E, and M64A were also found catalytically active as GST or His\textsubscript{6} fusion proteins.\(^2\) In confirmation of previous results (43), MMS2(F8A) in combination with wt GST-UBC13 produced no mult ubiquitin chains except for ubiquitin dimers at high E2 concentration, (Fig. 5C). The high molecular weight species reactive to the anti-ubiquitin antibody observed at 5.0 \(\mu\text{M}\) E2 result from auto-ubiquitylation of GST-UBC13 (47), a side reaction that occurs even in the absence of MMS2.2 Interestingly, UBC13(E55A) in combination with wt MMS2 showed no detectable chain synthesis at low concentration but showed an activity comparable with the \textit{wt} combination at 5 \(\mu\text{M}\) E2 (Fig. 5D). These results indicate that with respect to catalytic activity UBC13(E55A) is able to overcome the reduced affinity for MMS2 at high protein concentration, a notion that can explain the dependence of its \textit{in vivo} repair phenotype on the expression level.

**DISCUSSION**

The RAD5-UBC13 Interface Resembles Those of Typical E2-E3 Pairs—This study sheds light on the nature of the interfaces within the complex between the RING finger protein

\(^3\) T. K. Albert and H. D. Ulrich, unpublished observation.
RAD5 and the dimeric ubiquitin-conjugating enzyme UBC13-MMS2. Site-directed mutagenesis has demonstrated that the UBC13 subunit utilizes the same structural elements for contact with the RING finger as many monomeric E2s. In the c-Cbl-UbcH7 structure, the critical residues are Phe63 in the L1 loop as well as Pro97 and Ala98 in L2 (Ref. 15 and Fig. 1). The same positions were found to participate in binding of UbcM4 to a series of RING finger proteins (48), and even the interaction of UbcH7 with the structurally unrelated HECT-type E3, E6-AP, involves identical contacts (44). Phe63 had earlier been shown to be of particular importance for E2-E3 specificity (49).

Thus, it appears that the identities of the amino acids at the tip of the E3 outside the RING domain (14, 15, 52). Similarly, I have previously shown that the RAD5 RING finger alone is necessary but not sufficient for interaction with UBC13, suggesting that the UBC13 H1 helix may contact regions of RAD5 N-terminal of the RING domain, which might contribute to specificity.

The surface of the RING domain involved in the contact to the E2 forms a shallow groove in both c-Cbl and CNOT4 (15–17). Centrally positioned on this surface are several hydrophobic residues, Ile383 and Trp408 in c-Cbl and Leu46 and Ile45 in CNOT4, mutations of which were shown to abolish E2 binding (12, 16). Similarly, in the BRCA1 RING finger, which was recently found to possess ubiquitin ligase activity, mutation of Leu51 (corresponding to c-Cbl Trp408), to alanine resulted in a loss of E2 binding and catalytic activity (18). In RAD5, the corresponding residues, Ile914 and Tyr944, were found to fulfill similar functions, although mutations to alanine affected in vivo function of the protein to different degrees, with Ile914 having by far greater influence on the observed phenotype than Tyr944. In contrast, the neighboring Glu943, whose analog in c-Cbl is likewise involved in E2 binding, had no effect on association with UBC13. These findings demonstrate that although the E2-RING finger interface in the RAD5-UBC13 complex is structured similarly to those of several well studied E2-E3 pairs, subtle differences in the contributions of individual residues are likely to convey an exclusive specificity to the interaction.

Overall conservation of the contact surfaces, however, strongly implies a productive E2-E3 relationship for the RAD5-UBC13 interaction, thus supporting a model that attributes ubiquitin ligase activity to the RAD5 protein.

**Strong Interactions Are Disposable for Cooperation between RAD5, UBC13, and MMS2 in Vivo**—Perhaps the most intriguing result of this study is the fact that some of the contact site mutations in RAD5 and UBC13 have surprisingly small effects on the UV sensitivities of the resulting strains. If a high affinity association were necessary for cooperation between the UBC13-MMS2 dimer and RAD5, loss of this interaction by mutation of either RAD5 or UBC13 should result in the same phenotype as that of a ubc13 deletion, a condition that was observed only for the RAD5(916A) mutant. The absence of a visible phenotype would thus imply that the components could function in isolation and would not have to interact in vivo. However, this scenario seems unlikely, because all aspects of the RAD5 protein (36, 41, 53, 54), and a recruitment of the E2 to chromatin by RAD5 has been demonstrated (36). Moreover, if RAD5 indeed functions as a ubiquitin ligase, contact with its cognate E2 should be necessary for ubiquitination. Thus, it appears more likely that those mutations with minor phenotypic consequences cause only a partial weakening of the E2-RING finger interaction, which would lead to a loss of signal in the (qualitative) two-hybrid system but may not completely abolish cooperation**

The **protein-protein interactions** in an E2-RING finger complex is a key aspect of the ubiquitin pathway. The interaction is essential for the catalytic activity of ubiquitin ligases. The E2-UbcH7, for example, forms a shallow groove in both c-Cbl and CNOT4 (15–17). Centrally positioned on this surface are several hydrophobic residues, Ile383 and Trp408 in c-Cbl and Leu46 and Ile45 in CNOT4, mutations of which were shown to abolish E2 binding (12, 16). Similarly, in the BRCA1 RING finger, which was recently found to possess ubiquitin ligase activity, mutation of Leu51 (corresponding to c-Cbl Trp408), to alanine resulted in a loss of E2 binding and catalytic activity (18). In RAD5, the corresponding residues, Ile914 and Tyr944, were found to fulfill similar functions, although mutations to alanine affected in vivo function of the protein to different degrees, with Ile914 having by far greater influence on the observed phenotype than Tyr944. In contrast, the neighboring Glu943, whose analog in c-Cbl is likewise involved in E2 binding, had no effect on association with UBC13. These findings demonstrate that although the E2-RING finger interface in the RAD5-UBC13 complex is structured similarly to those of several well studied E2-E3 pairs, subtle differences in the contributions of individual residues are likely to convey an exclusive specificity to the interaction. Overall conservation of the contact surfaces, however, strongly implies a productive E2-E3 relationship for the RAD5-UBC13 interaction, thus supporting a model that attributes ubiquitin ligase activity to the RAD5 protein.

**Strong Interactions Are Disposable for Cooperation between RAD5, UBC13, and MMS2 in Vivo**—Perhaps the most intriguing result of this study is the fact that some of the contact site mutations in RAD5 and UBC13 have surprisingly small effects on the UV sensitivities of the resulting strains. If a high affinity association were necessary for cooperation between the UBC13-MMS2 dimer and RAD5, loss of this interaction by mutation of either RAD5 or UBC13 should result in the same phenotype as that of a ubc13 deletion, a condition that was observed only for the RAD5(916A) mutant. The absence of a visible phenotype would thus imply that the components could function in isolation and would not have to interact in vivo. However, this scenario seems unlikely, because all aspects of the RAD5 protein (36, 41, 53, 54), and a recruitment of the E2 to chromatin by RAD5 has been demonstrated (36). Moreover, if RAD5 indeed functions as a ubiquitin ligase, contact with its cognate E2 should be necessary for ubiquitination. Thus, it appears more likely that those mutations with minor phenotypic consequences cause only a partial weakening of the E2-RING finger interaction, which would lead to a loss of signal in the (qualitative) two-hybrid system but may not completely abolish cooperation **in vivo**. In addition, the affinity of an

**Table I** Effects of point mutations on the affinities between UBC13 and MMS2

| Ligand | Analyte | C<sup>+</sup> | RU<sub>max</sub> | K<sub>D</sub> | χ² |
|--------|---------|--------------|----------------|------------|----|
| UBC13  | MMS2    | 5 × 10<sup>-8</sup>–5 × 10<sup>-6</sup> | 209 | 3.1 × 10<sup>-7</sup> | 3.13 |
| MMS2   | UBC13   | 5 × 10<sup>-7</sup>–5 × 10<sup>-6</sup> | 187 | 4.8 × 10<sup>-7</sup> | 7.59 |
| UBC13(S96A) | MMS2 | 5 × 10<sup>-7</sup>–5 × 10<sup>-6</sup> | 219 | 3.0 × 10<sup>-7</sup> | 3.06 |
| UBC13(E55A) | MMS2 | 5 × 10<sup>-7</sup>–5 × 10<sup>-5</sup> | 183 | 1.8 × 10<sup>-5</sup> | 6.27 |
| UBC13  | MMS2(F8A) | 1 × 10<sup>-7</sup>–4 × 10<sup>-4</sup> | 215 | 1.5 × 10<sup>-3</sup> | 8.41 |

GU fusion protein immobilized on the Biacore™ sensor chip surface.

Soluble binding partner.

Range of analyte concentrations used for binding analysis.

Maximum signal in RU at infinite analyte concentration obtained by extrapolation from plots of the signal at equilibrium (RU<sub>eq</sub>) versus C.

**Fig. 5.** Catalytic activities of UBC13 and MMS2 mutants. Formation of multiubiquitin chains by in vitro polymerization reactions was analyzed by gel electrophoresis and anti-ubiquitin blots of samples taken at the indicated time points. A, GST-UBC13 and MMS2; B, GST-UBC13(S96A) and MMS2; C, GST-UBC13 and MMS2(F8A); D, GST-UBC13(E55A) and MMS2.
E2 to its cognate E3 as measured in isolation may not always correspond to the situation during the ubiquitylation reaction, because ubiquitin, substrate, or product binding may have consequences for the E2-E3 interaction. Accordingly, a number of precedents for productive low affinity interactions between E2s and RING finger E3s have been described (13), and in the case of the E3 UBR1, which contacts its cognate E2 RAD6 by means of a domain distinct from the RING finger, this interaction can be completely disrupted without significant loss of ubiquitin ligase activity (12–14). In contrast, mutation of the UBR1 RING finger abolishes E3 function, implying that an intact RING domain is required for catalytic activity rather than merely for the recruitment of the E2. The same appears to apply to the RAD5 RING finger, where perturbation of the domain structure by the C914S mutant had much more severe consequences than mutation of individual surface residues.

The concept of weak but productive interactions in the RAD5 and UBC13 contact mutants raises the question of how much the affinity in this complex can be reduced without significant loss of cooperation. I have started to approach this problem using the association within the UBC13-MMS2 dimer as a model system, because this complex is readily accessible for in vitro analysis. VanDemark et al. (43) have demonstrated that complex formation is required for ubiquitin polymerization by UBC13-MMS in vitro, because the MMS2(F8A) mutation, which abolishes interaction with UBC13, was severely compromised in a conjugation assay. Mutation of Glu55 to alanine in UBC13 similarly reduced complex formation, and both mutants were found inactive in vivo (43). I have now presented evidence that in contrast to MMS2(F8A) the defect of the UBC13(E55A) mutant can be overcome in vivo as well as in vitro by means of mass action via an increase in protein concentration. Whether the more severe phenotype of UBC13(E55A) observed by VanDemark et al. (43) is attributable to differences in strain background or details of promoter size or vector backbone remains unresolved. Consistent with the differing phenotypes observed in this study, however, the extent of interaction defects differs dramatically between UBC13(E55A) and MMS2(F8A); whereas the former causes an ~60-fold reduction in affinity, the latter mutation was found to reduce binding by more than 3 orders of magnitude. Based on the fact that the UBC13(E55A) displayed a phenotype identical to those mutants defective in their interaction with RAD5, the values obtained for interaction with MMS2 can be used as a benchmark in the analysis of the E2-RING finger contacts, which are likely to be of a similar order of magnitude, given comparable concentrations of the individual factors in the nucleus. This assumption has yet to be confirmed but appears probable based on the notion that overall RAD5 levels are significantly lower than those of UBC13 or MMS2, but the bulk of UBC13 and MMS2 resides outside the nucleus even under conditions of DNA damage (36).

Another notable property of the UBC13-MMS2 interaction is the extremely fast kinetics of association and dissociation that suggests a highly dynamic interaction rather than a permanent complex. NMR analysis of complex formation between UbcH5 and the CNOT4 RING finger implies a similar mode of interaction (16). Several interpretations can be envisioned to account for this kinetic lability. On one hand, fast dissociation rates may favor a rapid equilibrium between alternative complexes in the postreplication repair system as predicted earlier (36). On the other hand, the dynamic nature of the complex may be required for catalytic activity itself, e.g., to allow product dissociation or to facilitate activation by E1. In fact, McKenna et al. (55) argue that human UBC13 becomes less accessible to E1 when complexed to MMS2. Thus, dissociation of the UBC13-MMS2 complex might actually occur following each round of catalysis.

Additional Functions of RAD5 in DNA Repair—The fact that a rad5 deletion causes a much more severe UV sensitivity than a ubc13 or mms2 null mutant (36, 54) implies that RAD5 must have an additional role in DNA damage repair independent of its cooperation with UBC13 and MMS2. The RAD5 mutant analysis presented here allows the differentiation between RING finger-dependent and -independent aspects of RAD5 function. Mutation I916A exhibits a phenotype identical to and epistatic with the ubc13 deletion; this implies a complete loss of all activity related to cooperation with UBC13-MMS2, i.e., ubiquitin-ligase function. Yet, the C914S mutation confers a stronger phenotype than I916A, suggesting that the RING finger itself may contribute to UBC13-independent aspects of RAD5 function. Alternatively, disruption of the RING finger by removing one of the Zn2+ coordination sites may perturb the structure of the helicase-like domain into which the RING finger is embedded, which might result in a stronger phenotype than the exchange of a surface residue within the RING. Another explanation would be a decreased protein concentration of C914S; this seems unlikely, however, because preliminary experiments indicate that upon overexpression the mutant exhibits a stability similar to that of the wt protein.2

Even the C914S mutant is not fully devoid of activity, because its UV sensitivity is still less severe than that of the rad5 deletion. In light of these findings it is noteworthy that Martini et al. (56) recently identified a histone H2B mutant, htb1-3, that displayed a UV sensitivity hypertonic to RAD5 but additive with respect to a ubc13 mutation. Considering the DNA-dependent ATPase activity of RAD5 (32) and its homology to members of the SNF/SWI family, which includes helicases and chromatin remodeling factors (57, 58), it is attractive to speculate that beyond its ubiquitin ligase function RAD5 could be involved in the removal or repositioning of nucleosomes during the replication of the replication fork upon the encounter of a lesion in the template strand, thereby facilitating the strand switching process proposed to initiate postreplication repair (21, 29–31). Biochemical analyses will be necessary for a conclusive demonstration of RAD5 ubiquitin-protein ligase activity and for the investigation of additional activities independent of ubiquitylation.

Acknowledgments—I thank Margret Ludwig for excellent technical assistance, Thomas Albert for the gift of untagged UBC13, Kristine Schmidt and Thomas Albert for help with construction of UBC13 mutants, and all of the laboratory members as well as Charles Cho for helpful discussions and valuable comments on the manuscript. Regine Kahmann is acknowledged for generous support.

REFERENCES
1. Ben-Neriah, Y. (2002) Nat. Immunol. 3, 20–26
2. Conaway, R. C., Brower, C. S., and Conaway, J. W. (2002) Science 296, 1254–1258
3. Hershko, A., and Ciechanover, A. (1998) Annu. Rev. Biochem. 67, 425–479
4. Jorgensen, P., and Tyers, M. (1999) Curr. Opin. Microbiol. 2, 610–617
5. Pickart, C. M. (2001) Annu. Rev. Biochem. 70, 503–533
6. Chau, V., Tobias, J. W., Bachmair, A., Marriott, D., Ecker, D. J., Gonda, D. K., and Varshavsky, A. (1989) Science 243, 1576–1583
7. Jackson, P. K., Eldridge, A. G., Freed, E., Furstenthal, L., Heu, J. Y., Kaiser, B. K., and Reimann, J. D. (2000) Trends Cell Biol. 10, 429–438
8. Ulrich, H. D. (2002) Curr. Top. Microbiol. Immunol. 269, 137–174
9. Borden, K. L., Freemont, P. S., Huibregtse, J. M., Scheffner, M., Beaudenon, S., and Howley, P. M. (1996) Curr. Opin. Struct. Biol. 6, 395–401
10. Freemont, P. S. (2000) Curr. Biol. 10, R54–R57
11. Joazeiro, C. A., and Weissman, A. M. (2000) Cell 102, 549–552
12. Joazeiro, C. A., Wing, S. S., Huang, H., Leveryson, J. D., Hunter, T., and Liu, Y. C. (1999) Science 286, 309–312
13. Lorick, K. L., Jensen, J. P., Fang, S., Ong, A. M., Hatakeyama, S., and Howley, P. M. (1996) Science 279, 309
14. Xie, Y., and Varshavsky, A. (1999) EMBO J. 18, 6832–6840
15. Zheng, N., Wang, P., Jeffrey, P. D., and Pavletich, N. P. (2000) Cell 102, 533–539
16. Albert, T. K., Hanzawa, H., Legenthenberg, Y. I., de Ruwe, M. J., van den Heuvel,
F. A. Collart, M. A., Boelens, R., and Timmers, H. T. (2002) EMBO J. 21, 355–364
17. Hanzawa, H., de Ruwe, M. J., Albert, T. K., van Der Vliet, P. C., Timmers, H. T., and Boelens, R. (2001) J. Biol. Chem. 276, 10145–10150
18. Ruffner, H., Joazeiro, C. A., Hemmati, D., Hunter, T., and Verma, I. M. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 5134–5139
19. Hicke, L. (1999) Trends Cell Biol. 9, 107–112
20. Pickart, C. M. (2001) Mol. Cell 8, 499–504
21. Ulrich, H. D. (2002) Embryonic Cell 1, 1–10
22. Spence, J., Sadis, S., Haas, A. L., and Finley, D. (1995) Mol. Cell. Biol. 15, 1265–1273
23. Galan, J., and Haguenauer-Tsapis, R. (1997) EMBO J. 16, 5847–5854
24. Spence, J., Gali, R. R., Dittmar, G., Sherman, F., Karin, M., and Finley, D. (2000) Cell 102, 67–76
25. Fisk, H. A., and Yaffe, M. P. (1999) J. Cell Biol. 145, 1199–1208
26. Deng, L., Wang, C., Spencer, E., Yang, L., Braun, A., You, J., Slaughter, C., Pickart, C., and Chen, Z. J. (2000) Cell 103, 351–361
27. Hofmann, R. M., and Pickart, C. M. (1999) Cell 96, 645–653
28. Broomfield, S., Chow, B. L., and Xiao, W. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 5678–5683
29. Broomefield, S., Hryciw, T., and Xiao, W. (2001) Mutat. Res. 486, 167–184
30. Friedberg, E. C., Walker, G. C., and Siede, W. (1995) DNA Repair and Mutagenesis, American Society for Microbiology, Washington, D.C.
31. Lawrence, C. (1994) BioEssays 16, 253–258
32. Reynolds, P., Weber, S., and Prakash, L. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 168–172
33. Jentsch, S., McGrath, J. P., and Varshavsky, A. (1987) Nature 329, 131–134
34. Bally, V., Lauder, S., Prakash, S., and Prakash, L. (1997) J. Biol. Chem. 272, 23360–23365
35. Johnson, R. E., Prakash, S., and Prakash, L. (1994) J. Biol. Chem. 269, 28359–28362
36. Ulrich, H. D., and Jentsch, S. (2000) EMBO J. 19, 3388–3397
37. Hoege, C., Pfander, B., Moldovan, G. L., Pyrowelakis, G., and Jentsch, S. (2002) Nature 419, 135–141
38. James, P., Halliday, J., and Craig, E. A. (1996) Genetics 144, 1425–1436
39. Guthrie, C., and Fink, G. R. (1991) Guide to Yeast Genetics and Molecular Biology, Academic Press, San Diego, CA
40. Gietz, R. D., and Sugino, A. (1988) Gene (Amst.) 74, 527–534
41. Ulrich, H. D. (2001) Nucleic Acids Res. 29, 3487–3494
42. Silver, P. A., Chiang, A., and Sadler, I. (1988) Genes Dev. 2, 707–717
43. VanDemark, A. P., Hofmann, R. M., Tou, C., Pickart, C. M., and Wolberger, C. (2001) Cell 105, 711–720
44. Huang, L., Kinnucan, E., Wang, G., Beaudenon, S., Howley, P. M., Hubrengtse, J. M., and Pavletich, N. P. (1999) Science 286, 1321–1326
45. Moraes, T. F., Edwards, R. A., McKenna, S., Pastushok, L., Xiao, W., Glover, J. N., and Ellison, M. J. (2001) Nat. Struct. Biol. 8, 669–673
46. Cherest, H., Nguyen, N. T., and Surdin-Kerjan, Y. (1985) Gene (Amst.) 34, 269–281
47. Hofmann, R. M., and Pickart, C. M. (2001) J. Biol. Chem. 276, 27936–27943
48. Martinez-Noel, G., Muller, U., and Harbers, K. (2001) Eur. J. Biochem. 288, 5912–5919
49. Nuber, U., and Scheffner, M. (1999) J. Biol. Chem. 274, 7576–7582
50. Madura, K., Dohmen, R. J., and Varshavsky, A. (1993) J. Biol. Chem. 268, 13046–13054
51. Watkins, J. F., Sung, P., Prakash, S., and Prakash, L. (1993) Genes Dev. 7, 250–261
52. Bally, V., Prakash, S., and Prakash, L. (1997) Mol. Cell. Biol. 17, 4536–4543
53. Cejka, P., Vondrejs, V., and Storchova, Z. (2001) Genetics 159, 953–963
54. Torres-Ramos, C. A., Prakash, S., and Prakash, L. (2002) Mol. Cell. Biol. 22, 2419–2426
55. McKenna, S., Spyracopoulos, I., Moraes, T., Pastushok, L., Ptak, C., Xiao, W., and Ellison, M. J. (2001) J. Biol. Chem. 276, 40120–40126
56. Martini, E. M., Keeney, S., and Osley, M. A. (2002) Trends Biochem. Sci. 27, 1375–1387
57. Pazin, M. J., and Kadonaga, J. T. (1999) Cell 96, 737–740
58. Peterson, C. L., and Tamkun, J. W. (1995) Trends Biochem. Sci. 20, 143–147