Physical Interaction between Specific E2 and Hect E3 Enzymes Determines Functional Cooperativity*

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The cellular protein E6AP functions as an E3 ubiquitin protein ligase in the E6-dependent ubiquitination of p53. E6AP is a member of a family of functionally related E3 proteins that share a conserved carboxy-terminal region called the Hect domain. Although several different E2 ubiquitin-conjugating enzymes have been shown to function with E6AP in the E6-dependent ubiquitination of p53 in vitro, the E2s that cooperate with E6AP in the ubiquitination of its normal substrates are presently unknown. Moreover, the basis of functional cooperativity between specific E2 and Hect E3 proteins has not yet been determined.

Here we report the cloning of a new human E2, designated UbcH8, that was identified in a two-hybrid screen through specific interaction with E6AP. We demonstrate that UbcH7, an E2 closely related to UbcH8, can also bind to E6AP. The region of E6AP involved in complex formation with UbcH8 and UbcH7 was mapped to its Hect domain. Furthermore, we show that UbcH5 and UbcH6, two highly homologous E2s that were deficient for interaction with E6AP, could associate efficiently with another Hect-E3 protein, RSP5. Finally, only the E6AP-interacting E2s could function in conjunction with E6AP in the ubiquitination of an E6 independent substrate of E6AP, whereas the noninteracting E2s could not. Taken together, these studies demonstrate for the first time complex formation between specific human E2s and the Hect domain family of E3 proteins and suggest that selective physical interaction between E2 and E3 enzymes forms the basis of specificity for functionally distinct E2:E3 combinations.

Ubiquitin-dependent proteolysis constitutes a major pathway in the cell for selective protein degradation (1–3). The covalent attachment of multiple ubiquitin molecules to lysine residues of a target protein serves to signal its recognition and rapid degradation by the 26 S proteasome. Ubiquitin conjugation can also result in nonproteolytic modification of target proteins (4–7). Ubiquitination of a protein substrate requires the concerted action of three classes of enzymes; the ubiquitin activating enzyme E1 initially activates ubiquitin in an ATP-dependent reaction through the formation of a thiol ester bond between the carboxyl terminus of ubiquitin and the thiol group of a specific cysteine residue of E1. Ubiquitin is then transferred to a specific cysteine residue on one of several ubiquitin-conjugating enzymes (Ubc or E2s). E2 enzymes in turn may transfer the ubiquitin either directly to a substrate or to the final class of enzymes known as ubiquitin protein ligases (E3s). The E3 enzymes catalyze the formation of an isopeptide bond between the carboxyl terminus of ubiquitin and the ε-amino group of lysine residues on a target protein (3, 8, 9). A substrate may be multiply ubiquitinated through the attachment of additional ubiquitin molecules to specific lysine residues (lysine 48 or 63) of ubiquitin itself, although the progressive nature of a mult ubiquitination reaction is presently unclear (3, 4, 7). In order for this process to be efficient, it is likely that the E1, E2, and E3 enzymes involved form multi-protein complexes to allow rapid thiol ester transfer of ubiquitin molecules (4, 7). Whereas mult ubiquitination of some proteins leads to their rapid degradation by the proteasome, in other instances ubiquitination may serve as a modification resulting in functional regulation (5, 6). At present it is unclear how specific proteins are recognized as substrates for the ubiquitin system and what precise roles the E2 and E3 enzymes play in the recognition as well as in the ubiquitination of a substrate.

While only one functional E1 ubiquitin activating enzyme has been identified thus far, over 30 different E2 ubiquitin-conjugating enzymes have been isolated from various organisms (10). All E2s contain a conserved domain of approximately 14 kDa (~130 amino acids) and an active site cysteine residue that is required for thiol ester formation with ubiquitin. E2 enzymes that consist almost exclusively of the conserved Ubc domain (class I E2s) are unable to transfer ubiquitin to protein substrates in vitro, suggesting that this class of E2s may require E3 ubiquitin protein ligases for substrate recognition. A second group of E2 enzymes (class II) contain unique carboxy-terminal extensions (e.g. cdc34, Rad6, Ubc6) (10) that may contribute to substrate specificity and cellular localization (10).

The E3 ubiquitin protein ligases may be the key enzymes that provide substrate specificity for the ubiquitin conjugation system. Although two E3 enzymes have been previously identified from rabbit reticulocytes (E3α and E3β) and one from yeast (UBR1), it was not until the cloning and characterization of E6AP that the structural and functional features of a new class of E3 enzymes was revealed (10–13). E6AP was initially identified as a 100-kDa cellular protein that in conjunction with the E6 oncprotein of the human papillomavirus type 16 (HPV) constituted the E3 activity in the ubiquitination of p53 (8, 14–16). E6AP can also promote the ubiquitination of cellular proteins in the absence of E6, indicating that E6AP can...
function as an E3 independent of E6 (8). Sequence analysis of E6AP revealed a region of approximately 350 amino acids in the carboxyl terminus that was highly conserved among a number of proteins from various organisms (17). This region, termed the Hect domain (homologous to E6AP carboxyl terminus), also contains a conserved cysteine residue that serves as the active site for thioester formation with ubiquitin (17). In addition to E6AP, two other Hect domain proteins, RSP5 and rat p100, have been shown to form thioester complexes with ubiquitin (9, 17). A total of six genes encoding Hect proteins have been identified in Saccharomyces cerevisiae and several in Drosophila melanogaster, Caenorhabditis elegans, and mammals (17).

The question of substrate specificity is key to understanding how the ubiquitin system is regulated. Although the recognition of specific proteins as substrates appears to involve protein-protein interactions with specific E3s, the relative contribution of E2 enzymes in substrate recognition and ubiquitination has not yet been established. The existence of multiple E2s and Hect E3 proteins, however, suggests that specific combinations of these proteins are likely to function together in the ubiquitination of a substrate. At least 12 different E2s have been isolated from S. cerevisiae and shown to be involved in a variety of cellular functions, including DNA repair, cell cycle control, protein translocation, stress response, and chromosomal organization (3, 10). Moreover, gene inactivation experiments in mice have indicated very specific roles for certain E2s, suggesting that, despite belonging to a large multigene family, their functions are not redundant. For example, proviral integration and inactivation of UbcM4 (mouse homologue of UbcH7) results in placental defects and embryonic lethality (18). In another recent study, it was demonstrated that inactivation of mHR6B (one of the mouse homologues of yeast Rad6/Ubch2) in mice causes male sterility associated with chromatin modification (19). This is an intriguing result in terms of functional specificity of E2 enzymes since another mouse homologue of Rad6, mHR6A, which shares over 90% sequence identity with mHR6B and is expressed in all the same organs and tissues as mHR6B, was intact in these mice (19). In addition, both human homologues of Rad6, hHR6A and hHR6B, were shown to complement Rad6 function in DNA repair but not in sporulation in yeast (20). These data strongly indicate that individual E2 enzymes, despite having closely related homologues, carry out very specific functions in the cell.

The critical question with regard to different E2s is how functional specificity for each enzyme is achieved. Although the expression profiles, tissue distribution and subcellular localization of individual E2s may contribute to functional specificity, an equally important aspect is the ability of E2s to cooperate with specific E3 proteins in substrate ubiquitination. However, the basis underlying the specificity of why certain E2 enzymes and not others function with particular E3s is not yet known.

To identify potential substrates and regulators of E6AP, we have carried out a yeast two-hybrid screen using E6AP as bait. In this study, we report the cloning of a new human E2 (designated UbcH8) that was isolated as an E6AP-interacting protein and demonstrate that UbcH8 can transfer ubiquitin to E6AP. We have extended this analysis to additional E2s and Hect domain E3s. Our results demonstrate that only a subset of structurally related E2s physically interact with E6AP and function in the ubiquitination of an E6AP substrate (22). Furthermore, we show that a different subfamily of structurally related E2s bind to the S. cerevisiae Hect protein RSP5 (17, 20).

Taken together, these studies demonstrate specific complex formation between E2 enzymes and the Hect domain family of E3 proteins, and suggest that the ability of E2s to physically associate with specific Hect E3s constitutes the basis of specificity for functionally distinct E2:E3 combinations.

**EXPERIMENTAL PROCEDURES**

**Isolation of UbcH8 cDNA—**A modified version of the yeast two-hybrid screen was carried out to identify E6AP-interacting proteins (21, 24–26). The bait vector was constructed by inserting a catalytically inactive mutant of E6AP (C833A) into pPC97, in-frame with the Gal4 DNA binding domain (amino acids 1–147). The prey cDNA library contained the Gal4 activation domain fused to cDNAs derived from activated human T cells in pPC86 vector. The bait and prey constructs were transformed into yeast strain MaV103 (MATa ura3-52 leu2-3, 112 trpl-901 his3200 ade2-101 gal434080 A GAL1::HIS3 @ lys2 2FL10::URA3) and transformants plated in the absence of histidine. Since increased HIS3 expression resulting from an interacting clone will render the yeast resistant to 3-amino triazole (3-AT), selection of interacting clones was performed in the presence of 25 mM 3-AT. pPC86-derived interaction positive cDNAs were rescued by transformation of competent HB101 with total yeast DNA. The DNA sequence of isolated cDNAs was determined by dideoxynucleotide sequencing using appropriate oligonucleotide primers. The pPC86-derived human T cell cDNA library and the yeast host strain MaV103 were kindly provided by Dr. Marc Vidal (Massachusetts General Hospital, Charlestown, MA).

**Plasmid Constructs—**The ubcH8, ubch7, ubch5, and ubch9 (17) genes were amplified by polymerase chain reaction (PCR) using appropriate oligonucleotide primers and inserted into pET23a vector (Novagen) for bacterial expression. For expression in MaV103 yeast strain, PCR-derived E2 cDNAs (UbcH8, UbcH7, UbcH6, UbcH5, and UbcH9) were cloned in-frame with the Gal4 activation domain in the pPC86 plasmid. E6AP, RSP5, and rat p100 cDNAs harboring a substitution of the active site cysteine residue with alanine were amplified by PCR and cloned into the pPC97 vector in-frame with the Gal4 DNA binding domain. E6AP deletion mutants were generated by PCR using appropriate oligonucleotide primers and inserted in-frame with the Gal4 DNA binding domain in pPC97 (see Fig. 3B).

**Protein Expression—**E1, UbcH8, UbcH7, UbcH5, and UbcH9 were expressed in Escherichia coli BL21 using the pET expression system (Novagen) (23, 28, 29). The relative amounts of expression of various E2 proteins was determined by Coomassie Blue staining. Construction, purification, and radioactive labeling of GST-ubiquitin have been described previously (8). Preparation of E6AP from H15 cells infected with recombinant baculovirus expressing WT or mutant E6AP proteins has been described (8, 17, 23). In vitro expression of HHHR23A was performed in TNT-coupled wheat germ extracts (WGE) in the presence of [35S]methionine as per manufacturer’s instructions (Promega). The expression of fusion proteins in yeast strain MaV103 was confirmed by Western blotting with anti-Gal4 DNA binding domain and anti-Gal4 activation domain antibodies from Santa Cruz Biotechnology.

**Thiol Ester Assay—**Ubiquitin thiol ester formation on UbcH8 and E6AP was determined as described previously (9). Reaction mixtures contained approximately 5–10 ng of E1, 100 ng of UbcH8, 200 ng of WT-E6AP or E6AP (C833A), and 500 ng of [35S]-labeled GST-ubiquitin in 20 mM Tris-HCl, pH 7.6, 50 mM NaCl, 4 mM ATP, 10 mM MgCl2, and 0.2 mM dithiothreitol for 3 min at 25 °C. Reactions were terminated by incubating the mixtures for 15 min at 30 °C in SDS-sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 0.2% bromphenol blue) in the absence of reducing agents and resolved by SDS-PAGE. Radioactively labeled proteins were visualized by autoradiography.

**Protein Interaction Assay—**The yeast strain MaV103 was co-transformed with pC97-derived Hect protein constructs and pPC86-derived E2 constructs using standard lithium acetate procedures and plated in the absence of leucine and tryptophan (21). Individual colonies were picked and equivalent number of cells, as determined by OD reading at 600 nm, were spotted on histidine drop-out plates containing 25 mM 3-AT. Interactions were scored based on growth in the presence of 3-AT after incubation at 30 °C for 3 days.

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A nucleotide and deduced amino acid sequence of UbcH8. Amino acids are indicated in single letter code. The stop codon is denoted by an asterisk. B, comparison of amino acid sequences of UbcH8 with other human E2s. Identical amino acids are denoted in gray. The conserved active site cysteine residue is marked by an arrowhead and the carboxyl-terminal ends by an asterisk. Percent identity between the different E2s was obtained using the DNA sis program and is shown above the sequence alignment.

UbcH8 is 45.7% identical to UbcH7 and UbcH6 is 53.7% identical to UbcH5. UbcH8 and UbcH5 are 22.4% identical; UbcH7 and UbcH5 share 17.6% identity.
RESULTS

Isolation of UbcH8—To identify cDNA clones that encode E6AP-interacting proteins, a modified version of the yeast two-hybrid screen was performed using E6AP as bait (21). A catalytically inactive form of E6AP in which the active site cysteine residue has been substituted with alanine (C833A) was used to avoid potential degradation of interacting proteins. The yeast reporter strain MaV103 expressing E6AP (C833A) as a fusion protein with the Gal4 DNA-binding domain was transformed with the prey cDNA library derived from human T cells (21). A total of 12 independent interacting clones were isolated by plating the transformants on histidine drop-out plates containing 25 mM 3-AT. Whereas both E6AP and various E2s, and interactions were scored by growth on histidine-minus plates containing 25 mM Tris-HCl, pH 7.6, 50 mM NaCl, 4 mM ATP, 10 mM MgCl2, and 0.2 mM dithiothreitol. Reactions were terminated after 2 h. at 30 °C by the addition of SDS-sample buffer. Samples were boiled for 5 min, resolved by SDS-PAGE, and visualized by autoradiography.

Ubiquitination Assay—HHR23A protein was synthesized in vitro in the presence of [35S]methionine for 90 min at 30 °C using TNT-coupled wheat germ extracts (22) (Promega). 5-μl aliquots of in vitro translated HHR23A were incubated with 5–10 ng of E1, approximately 100 ng of various E2s, 200 ng of E6AP, and 4 μg of ubiquitin (Sigma) in 20 mM Tris-HCl, pH 7.6, 50 mM NaCl, 4 mM ATP, 10 mM MgCl2, and 0.2 mM dithiothreitol. Reactions were terminated after 2 h. at 30 °C by the addition of SDS-sample buffer. Samples were boiled for 5 min, resolved by SDS-PAGE, and visualized by autoradiography.

Ubiquitin Thiol Ester Formation and Transfer—The finding that UbcH8 can form thiol ester complexes with ubiquitin and transfer the ubiquitin to the active site cysteine residue of E6AP, the UbcH8 cDNA was inserted into pET23a vector and translated in E. coli (23, 28, 29). Bacterially expressed UbcH8 could efficiently form thiol ester adducts on UbcH8 and E6AP, suggested by the high degree of homology to UbcH8 (45.7% identity, Fig. 1B) (30).

Ubiquitin Thiol Ester Formation and Transfer—To ascertain whether UbcH8 can form thiol ester complexes with ubiquitin and transfer the ubiquitin to the active site cysteine residue of E6AP, the UbcH8 cDNA was inserted into pET23a vector and expressed in E. coli (23, 28, 29). Bacterially expressed UbcH8 was incubated in the presence of ATP with E1, wild type or catalytically inactive E6AP (C833A) and radiolabeled ubiquitin. As shown in Fig. 2 (lanes 2 and 3), UbcH8 efficiently form thiol ester complexes with ubiquitin. Furthermore, UbcH8 could transfer ubiquitin to wild type E6AP and not to the E6AP mutant (C833A) containing a substitution of the active site cysteine residue with alanine (Fig. 2, lanes 2 and 3). The complex of E6AP and ubiquitin could be disrupted by the addition of dithiothreitol, indicating the labile nature of a reduction sensitive thiol ester bond (data not shown). No thiol ester adduct was detected on E6AP in the absence of UbcH8 (lane 1). It should be noted that in the presence of mutant E6AP (C833A), the amount of ubiquitin thiol ester complexes remaining on UbcH8 was greater than in the presence of wild type E6AP indicating the specificity of ubiquitin transfer from UbcH8 to E6AP. These results demonstrate that like other E2s that have been characterized thus far, UbcH8 can form thiol ester complexes with ubiquitin. In addition, UbcH8 has the ability to transfer ubiquitin to the active site cysteine residue of E6AP.

Interaction of E6AP with UbcH8 and UbcH7—The finding that UbcH8 can not only transfer ubiquitin to E6AP but also physically interact with E6AP, suggested that the binding of E2 enzymes to Hect E3 proteins might be a general property important for functional cooperation between E2 and E3 enzymes. We therefore examined the ability of other human E2s to associate with E6AP using the yeast two-hybrid assay. The yeast strain MaV103 was co-transformed with E6AP (C833A) and various E2s, and interactions were scored by growth on histidine-minus plates containing 25 mM 3-AT. Whereas both UbcH8 and the closely related UbcH7 (30) interacted efficiently with E6AP, UbcH5 and UbcH9 (23, 27) did not show significant levels of interaction (Fig. 3A). Thus E6AP can associate with only a subset of E2s, namely UbcH8 and UbcH7. In addition, as suggested by the high degree of homology between UbcH8 and UbcH7 (shown in Fig. 1B), these E2s may represent a structurally and functionally related subfamily.

To map the region of E6AP that is required for its interaction with UbcH8 and UbcH7, several deletion constructs of E6AP were generated and assayed for their ability to interact with the different E2s. As summarized in Fig. 3B, the Hect domain of E6AP was found to be required and sufficient for interaction with UbcH8 and UbcH7, while the amino-terminal sequences of E6AP were dispensable. Each of the deletions within the Hect region resulted in a loss of binding, further indicating the requirement of this region for interaction with the specific E2s (Fig. 3B). Piner deletions within the Hect domain will be required to determine whether the entire region is involved in the interaction with E2s or whether specific segments mediate the binding. Nonetheless, these results suggest that the conserved Hect domains of this family of proteins may enable them to associate with specific E2 enzymes and as such provide the specificity for functional cooperation in substrate ubiquitination.

Interaction of RSP5 with UbcH6 and UbcH5—Since the conserved Hect domain defines a family of proteins believed to
function as E3s, the Hect domains of two other proteins, RSP5 and the rat p100 protein (17), were tested for their ability to interact with different E2s using the two-hybrid assay. Whereas the E6AP Hect domain interacted selectively with UbcH8 and UbcH7, the RSP5 Hect region was found to interact with UbcH6 and UbcH5, and not with other E2s (Fig. 4). It should be noted that just as UbcH8 and UbcH7 are closely related to each other (45.7% identity; Fig. 1B), UbcH6 and UbcH5 share strong homology with each other (53.7% identity; Fig. 1B). The rat p100 protein was unable to interact with any of the five E2s that were tested in these experiments. On the basis of these results, it seems that different Hect containing proteins interact with specific subsets of closely related E2s and that the selective association of E2 and E3 proteins may constitute the basis of specificity for distinct E2:E3 combinations.

**DISCUSSION**

The ubiquitin system plays a major role in selective protein degradation and may also be an important pathway for protein modification (4–7). The specificity of substrate recognition by the ubiquitin system may be achieved by different E3 enzymes capable of interacting with specific substrates. However, specificity within the enzymatic components of the pathway, i.e. different E2:E3 combinations, may also influence substrate recognition and ubiquitination. As noted earlier, gene inactivation experiments in mice have indicated very specific roles for E2 enzymes (18, 19), although the way functional specificity for different E2s is achieved is not yet known. The existence of multiple E3 proteins and their likely role in substrate recognition strongly suggests that cooperation between specific E2 and E3 enzymes may play an important role in defining the specificity involved in substrate ubiquitination. Accordingly, ubiquitination of a substrate may depend upon the transfer of ubiquitin from E1 to a given E2, the ability of that E2 to cooperate with a specific E3, and finally the interaction between the E3 and the substrate.

To date, however, the only physical interaction demonstrated between E2 and E3 proteins involves the *S. cerevisiae* Rad6 (Ubc2) protein and UBR1, a non-Hect domain E3 involved in the N-end rule pathway for protein degradation (13,
Hect-domain protein from S. cerevisiae and UbcH5, were found to interact selectively with RSP5, a Interestingly, two other E2s closely related to each other, UbcH6 and UbcH5, were found to interact selectively with E6AP, suggesting that other Hect family E3 proteins may also have the ability to interact with specific E2s. This interaction was mapped to the Hect domain of E6AP, suggesting that other Hect family E3s specifically with E6AP. This interaction was mapped to the Hect domain of E6AP, suggesting that other Hect family E3s form the basis for functional cooperativity between E2 and E3 enzymes in substrate ubiquitination as they can form high energy thiol ester bonds with ubiquitin prior to the transfer and covalent attachment of ubiquitin to a substrate (9, 17). Therefore, the Hect domain family of E3s do not function simply as adaptors or docking protein by bringing in close proximity the relevant E2 and the substrate. The Hect domain family of E3 proteins, as established for E6AP, play a more direct role in substrate ubiquitination as they can form high energy thiol ester bonds with ubiquitin prior to the transfer and covalent attachment of ubiquitin to a substrate (9, 17). However, the basis of specificity for ubiquitin thiol ester transfer from E2s to E3s has not been addressed, and prior to this study, physical association of specific E2 and Hect E3 proteins had not been demonstrated.

The studies presented here demonstrate for the first time complex formation between specific E2s and Hect E3 proteins in vitro, and suggest that the ability of E2s to interact selectively with Hect domain E3s forms the basis for functional cooperativity between E2 and E3 enzymes in substrate ubiquitination. UbcH8 and the closely related UbcH7 interacted specifically with E6AP. This interaction was mapped to the Hect domain of E6AP, suggesting that other Hect family E3 proteins may also have the ability to interact with specific E2s. Interestingly, two other E2s closely related to each other, UbcH6 and UbcH5, were found to interact selectively with RSP5, a Hect-domain protein from S. cerevisiae (17, 23, 30). None of the five E2s examined in this study interacted with the rat p100 protein. Finally, we demonstrate that the two E6AP-interacting E2s also functioned in conjunction with E6AP in the ubiquitination of HHR23A, an E6AP substrate (22). The noninteracting E2s were unable to enhance the ubiquitination of HHR23A above background levels observed in wheat germ extracts.

31–33). UBR1 has not been shown to form thiol ester complexes with ubiquitin, suggesting that it may function more like an adaptor or docking protein by bringing in close proximity the relevant E2 and the substrate. The Hect domain family of E3 proteins, as established for E6AP, play a more direct role in substrate ubiquitination as they can form high energy thiol ester bonds with ubiquitin prior to the transfer and covalent attachment of ubiquitin to a substrate (9, 17). Therefore, the Hect domain family of E3s do not function simply as adaptors or docking protein by bringing in close proximity the relevant E2 and the substrate. The Hect domain family of E3 proteins, as established for E6AP, play a more direct role in substrate ubiquitination as they can form high energy thiol ester bonds with ubiquitin prior to the transfer and covalent attachment of ubiquitin to a substrate (9, 17). However, the basis of specificity for ubiquitin thiol ester transfer from E2s to E3s has not been addressed, and prior to this study, physical association of specific E2 and Hect E3 proteins had not been demonstrated.

Prior to this report, the only assay used to define the specificity of ubiquitin thiol ester transfer from E2s to Hect E3s was based upon in vitro reactions in which relatively high amounts of partially purified components were used to detect the transfer of a radiolabeled GST-ubiquitin fusion protein to E6AP (9, 30). Using these in vitro thiol ester assays, E6AP was shown to accept ubiquitin from A. thaliana Ubc8, UbcH5, and UbcH7 (23, 30); and, RSP5 from UbcH5 (30). However, as shown in this study, UbcH5 fails to interact efficiently with E6AP in the yeast two-hybrid system whereas it can interact strongly with RSP5. The most probable explanation for a lack of efficient interaction between E6AP and UbcH5 may be that the two proteins do not have a high affinity for each other. Accordingly, it is possible that unlike the assay conditions for thiol ester transfer in vitro (9), the comparatively low levels of protein expression from centromeric plasmids in yeast cells (21) may preclude the detection of relatively low affinity interactions. Consistent with this possibility, UbcH5 was unable to enhance the ubiquitination of HHR23A under conditions where E6AP-interacting UbcH8 and UbcH7 did so efficiently.

In this regard, it is also important to note that UbcH5, which is very similar to Arabidopsis thaliana Ubc8, was cloned by reverse transcription-PCR using two degenerate primers corresponding to conserved sequences in A. thaliana Ubc8 and other similar E2s (23). As such, the isolation of UbcH5 was not based upon any functional criteria that link it directly to E6AP. On the other hand, UbcH7 (initially called E2-F1) was isolated as an E2 activity from reticulocyte lysates and shown to function with E6 and E6AP in the ubiquitination of p53 (34, 35). The cDNA for E2-F1 was subsequently cloned by Nuber et al. (30) based upon peptide sequences derived from direct protein sequencing (34) and designated as UbcH7 (30). UbcH7 was shown to be capable of transferring ubiquitin to E6AP in vitro (30). As described in this report, we isolated UbcH8 as a specific E6AP-interacting protein, and show that it also can transfer ubiquitin to E6AP. Sequence comparison revealed that UbcH8

FIG. 5. Ubiquitination of HHR23A using E6AP and various E2s. The HHR23A protein was synthesized in WGE with [35S]methionine and incubated with ATP, E1, and E6AP either in the absence (lane 2) or in the presence of bacterially expressed E2s as indicated. Reactions were terminated after 90 min at 30 °C and analyzed by SDS-PAGE and autoradiography. Equivalent amounts of the respective E2s were used as determined by Coomassie staining. The position of unmodified input HHR23A is indicated by arrows and slower migrating ubiquitinated forms are marked by a bracket on the right.
was most closely related to UbcH7, and both E2s could interact specifically with E6AP. As noted above, UbcH5 and UbcH6 share a high degree of homology with each other. Both of these E2s failed to bind E6AP efficiently but interacted strongly with RSP5. Consistent with this observation, UbcH6 and to a lesser extent UbcH5, were also able to bind Pub-1, the *Schizosaccharomyces pombe* homologue of RSP5.3

Based on the interaction and functional data presented here, UbcH6 and UbcH7 represent a structurally related subfamily of E2s that may function physiologically with E6AP in the ubiquitination of its substrates, whereas UbcH6 and UbcH5 may belong to a subfamily that functions with the mammalian homologues of RSP5. Further studies are currently underway to extend the analysis of E2:E3 interactions to additional E2s and Hect E3s. As more substrates of the E3 enzymes become known, different E2:E3 combinations can be tested for their ability to function in the ubiquitination of specific substrates, thereby allowing the identification of E2-E3 pairs that cooperate in substrate ubiquitination.

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