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Biochemistry

A family of proteins structurally and functionally related to the E6-AP ubiquitin–protein ligase

(papillomavirus/thioester)

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ABSTRACT **E6-AP is a 100-kDa cellular protein that interacts with the E6 protein of the cancer-associated human papillomavirus types 16 and 18. The E6/E6-AP complex binds to and targets the p53 tumor-suppressor protein for ubiquitin-mediated proteolysis. E6-AP is an E3 ubiquitin–protein ligase which accepts ubiquitin from an E2 ubiquitin-conjugating enzyme in the form of a thioester and then directly transfers the ubiquitin to targeted substrates. The amino acid sequence of E6-AP shows similarity to a number of protein sequences over an ≈350-aa region corresponding to the carboxyl termini of both E6-AP and the E6-AP-related proteins. Of particular note is a conserved cysteine residue within the last 32–34 aa, which in E6-AP is likely to be the site of ubiquitin thioester formation. Two of the E6-AP-related proteins, a rat 100-kDa protein and a yeast 95-kDa protein (RSP5), both of previously unknown function, are shown here to form thioesters with ubiquitin. Mutation of the conserved cysteine residue of these proteins destroys their ability to accept ubiquitin. These data strongly suggest that the rat 100-kDa protein and RSP5, as well as the other E6-AP-related proteins, belong to a class of functionally related E3 ubiquitin–protein ligases, defined by a domain homologous to the E6-AP carboxyl terminus (*hect* domain).**

The hallmark of the ubiquitin-mediated proteolytic pathway is the covalent attachment of the 76-aa ubiquitin polypeptide to target proteins, through isopeptide bond formation between the carboxyl terminus of ubiquitin and the ε-amino group of one or more lysine residues on the protein substrate (reviewed in refs. 1 and 2). Additional ubiquitin moieties can be ligated via lysine residues of ubiquitin itself, resulting in the formation of multiubiquitinated proteins, which are then recognized and degraded by the 26S protease complex. While much of the biochemistry of the ubiquitin proteolysis system has been elucidated, a basic question has remained largely unanswered: how are proteins specifically recognized and targeted for ubiquitination?

Protein ubiquitination involves three classes of enzymes. Ubiquitin is activated by the E1 ubiquitin-activating enzyme in an ATP-dependent reaction, resulting in thioester formation between a specific cysteine of the enzyme and the carboxyl terminus of ubiquitin. The activated ubiquitin is transferred to a cysteine residue of one of a number of low molecular weight E2 ubiquitin-conjugating enzymes. The E2 proteins have generally been thought to catalyze the final ubiquitination of the substrate protein, often in conjunction with a third group of proteins, the E3 ubiquitin–protein ligases. E3 activities have been proposed to play a major role in defining the substrate specificity of the ubiquitin system, perhaps through direct binding of substrates. In the yeast *Saccharomyces cerevisiae* at least 12 different E2 genes have been identified (1). Only two known E3 genes have been cloned, yeast *UBR1* (3) and the human E6-AP gene (4, 5).

E6-AP was discovered in the course of characterizing the mechanism by which the E6 protein of certain human papillomaviruses (HPVs) inactivates the p53 tumor-suppressor protein. The E6 proteins of HPV types 16 and 18 can complex with and promote the ubiquitin-mediated degradation of p53 (6, 7), and several lines of evidence suggest that this activity plays a role in HPV-associated carcinogenesis (8, 9). E6-AP (for E6-associated protein), a 100-kDa cellular protein, was shown to be an essential factor in mediating complex formation of E6 and p53 (10), and the identification of the components of the ubiquitin system demonstrated that E6-AP is an E3 enzyme (5).

Insight into the mechanism by which E6-AP functions came from the observation that E6-AP, like E1 and E2 enzymes, can form a thioester with ubiquitin (11). A cysteine residue near the carboxyl terminus of E6-AP is critical for both thioester formation and ubiquitination of p53. E3 ubiquitin thioester formation points toward a direct role of E3 proteins in the ubiquitination of substrates. Our current model for E6-AP-mediated ubiquitination is that ubiquitin is transferred sequentially in a "thioester cascade" from E1, to a specific E2, and finally to E6-AP, which then transfers ubiquitin directly to substrates in the form of a stable isopeptide bond.

There are several predicted eukaryotic protein sequences in current data bases that show similarity to E6-AP over their carboxyl-terminal regions. In this study we show that two of these *hect* (homologous to the E6-AP carboxyl terminus)-domain proteins, a rat 100-kDa protein and a yeast 95-kDa protein (RSP5), share the ability to form a thioester with ubiquitin. This strongly suggests that E6-AP is representative of a structurally and functionally related class of E3 ubiquitin–protein ligases.

MATERIALS AND METHODS

Plasmids. E6-AP expression plasmids (pGEM-1- and pVL1393-based) were identical to those described previously for *in vitro* transcription/translation and baculovirus expression (4, 11, 12). The wild-type E6-AP cDNA in these studies was that which encodes a 95-kDa protein as previously described, although this cDNA is known to be incomplete at the 5′ end (4). Amino acid numbering is according to the published sequence. The cysteine-to-alanine (C-A) mutation at amino acid 833 and the 6-aa carboxyl-terminal truncation (ΔC6) were built into the background of this cDNA. The RSP5 cDNA was subcloned by PCR from a plasmid containing the full-length open reading frame (ORF), provided by Fred Winston (Harvard Medical School; unpublished work), into pGEM-1 (Promega). The C-A and ΔC6 mutations were introduced and

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Abbreviations: DTT, dithiothreitol; GST, glutathione *S*-transferase; HPV, human papillomavirus; ORF, open reading frame.

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confirmed by standard methods. The rat p100 cDNA and the C-A mutant of rat p100 were cloned similarly from a plasmid provided by Dietmar Richter (13).

Thioester and Ubiquitination Assays. [^{35}S]Methionine-labeled proteins were synthesized *in vitro* in rabbit reticulocyte lysate with a coupled transcription/translation kit (Promega). Ubiquitin thioester formation was assayed by removing an aliquot of the translation reaction mixture (2–5 μl) into SDS/PAGE loading buffer (62 mM Tris, pH 6.8/2% SDS/10% glycerol/0.001% bromphenol blue) which either did or did not contain dithiothreitol (DTT; final concentration, 100 mM). In some cases 1 μg of glutathione *S*-transferase (GST)-ubiquitin fusion protein was added to 5 μl of translation reaction mixture and incubated for an additional 5 min at room temperature before the reaction was quenched with SDS/PAGE loading buffer. Reactions that received DTT-containing loading buffer were heated at 100°C for 5 min prior to loading onto a 9% polyacrylamide gel. Reactions that did not receive DTT were incubated at room temperature for 20 min before loading.

p53 ubiquitination assays (12) used ^{35}S-labeled wheat germ extract-translated (Promega) wild-type human p53. HPV16 E6

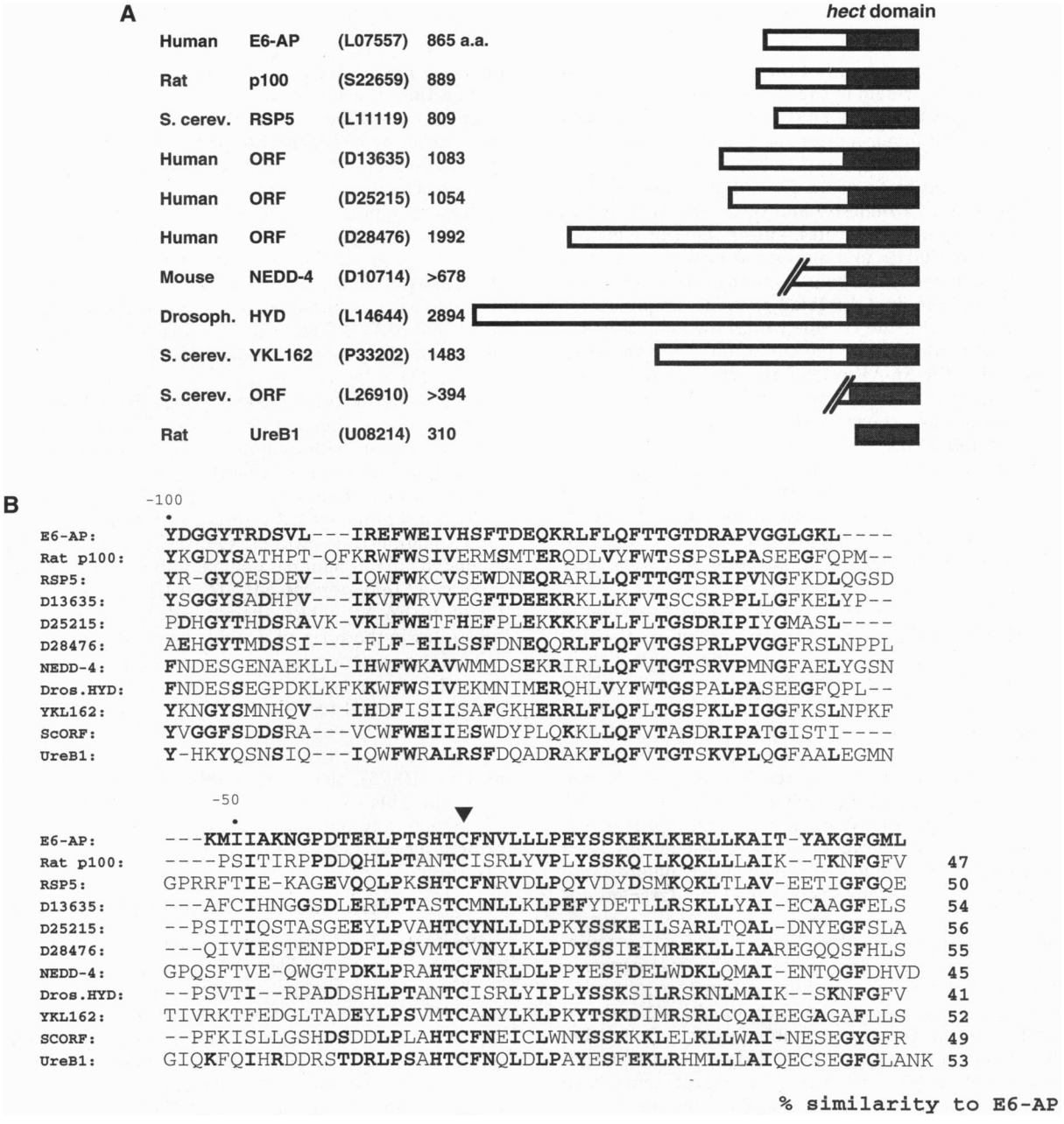

FIG. 1. (*A*) Schematic of E6-AP and 10 *hect*-domain proteins. The organism, its name designation (ORF for those without designation), GenBank accession number, and number of amino acids encoded by the cDNA are given for each. The mouse NEDD-4 and L26910 cDNAs are incomplete at the 5′ end, as is the E6-AP cDNA (4). The mouse NEDD-4 ORF as entered in GenBank terminates at the 3′ end prematurely relative to the other cDNAs. This is probably due to a sequencing error between nucleotides 1870 and 1907, since the ORF continues to encode an E6-AP-like protein in a different reading frame (see *B*). In addition to these, there are four *Caenorhabditis elegans* expressed sequence tags which encode polypeptides with similarity to the carboxyl terminus of E6-AP (GenBank accession nos. D27507, D34065, and D33580 and EMBL no. Z14494). (*B*) Alignment of the E6-AP-related proteins relative to the 100 carboxyl-terminal amino acids of E6-AP. Identical and similar amino acids are indicated in bold type. The conserved cysteine is indicated with an arrowhead, and the percent similarity of each of the proteins to E6-AP over these 100 aa is shown. The NEDD-4 sequence was determined independently over this region and as shown incorporates a correction to the database entry.

protein was expressed in insect cells using a recombinant baculovirus, and partially purified by single-step elution (25 mM Tris, pH 7.0/500 mM NaCl/1 mM DTT) from a Bio-Rad S column. One microgram of the E6-containing fraction was used per assay. Baculovirus-expressed E6-AP proteins were prepared as described (5).

RESULTS

***hect*-Domain Proteins.** The BLAST programs (14) were used to search data bases available through the National Center for Biotechnology Information for predicted proteins with similarity to human E6-AP. Fourteen proteins were identified with similarity to E6-AP over the ≈350 carboxyl-terminal residues of each protein. This 350-aa domain will be referred to as the *hect* domain, for homologous to E6-AP carboxyl terminus. Most of these proteins or genes are of undefined or poorly defined function (see *Discussion* for further description). Fig. 1*A* shows a schematic of the regions of similarity between E6-AP and 10 of the *hect* domain-containing protein sequences, and Fig. 1*B* shows a sequence alignment relative to the carboxyl-terminal 100 aa of E6-AP. Permitting conservative amino acid substitutions and introducing some gaps for alignment, we found that the similarity of these proteins to E6-AP was 41–56% over the last 100 aa. The cysteine residue at position 833 of E6-AP is conserved among all of the E6-AP-related proteins. This cysteine residue in E6-AP is necessary for ubiquitin thioester formation and for the ability of E6-AP to target p53 for ubiquitination. The conservation of this domain suggested that these E6-AP-related proteins might also function as E3 ubiquitin–protein ligases.

Rat p100. To test this hypothesis, cDNAs encoding the wild-type rat 100-kDa protein (hereafter referred to as rat p100) or a version containing a cysteine-to-alanine substitution (C-A) at amino acid 858, corresponding to amino acid 833 of E6-AP, were expressed in a coupled *in vitro* transcription/translation system (Fig. 2). Translation reactions were stopped with SDS/PAGE loading buffer with or without DTT (final concentration, 100 mM), and subjected to SDS/PAGE. In the absence of DTT a band ≈8 kDa larger than the expected translation product was evident with the wild-type protein. This band was not present when the translation reaction was stopped with DTT-containing buffer, nor was it evident with the C-A mutant, in either the absence or the presence of DTT. The size of this band and its sensitivity to reducing agent suggested that, like E6-AP, rat p100 can form a thioester with ubiquitin. Furthermore, when GST-ubiquitin fusion protein (molecular mass, 34 kDa) was added to the translation reaction and incubated for an additional 5 min, a band migrating with an apparent molecular mass of ≈130 kDa was evident, again only with the wild-type protein and only in the absence of DTT. This confirmed that rat p100 can form a ubiquitin adduct with characteristics of a thioester.

***S. cerevisiae* RSP5.** A second *hect* domain-containing protein, the 95-kDa *S. cerevisiae* protein encoded by *RSP5*, was similarly analyzed (Fig. 3). A cysteine-to-alanine (C-A) substitution mutant (position 777) was expressed, as well as a fortuitously isolated PCR-generated mutant encoding a protein lacking the carboxyl-terminal 6 aa (ΔC6). The ΔC6 mutant behaved similarly to wild-type rat p100 in this assay (Fig. 3, lanes 5, 6, 11, and 12). A band apparently 8 kDa larger than the expected translation product was evident in the absence of DTT. This was not present when the translation reaction was terminated with DTT-containing buffer. Also, the addition of GST-ubiquitin resulted in the production of a DTT-sensitive adduct ≈30 kDa larger than the primary translation product. The C-A mutant did not form DTT-sensitive adducts. The wild-type RSP5 protein formed an adduct with GST-ubiquitin of the expected size for the monoubiquitinated protein; however, additional adducts of higher molecular weight were also evident, and surprisingly, the adducts were largely insensitive to DTT.

The significance of the DTT-insensitive ubiquitination of wild-type RSP5 is currently unknown; however, it is an intriguing possibility that RSP5 may actually target itself for ubiquitination at one or more lysine residues in an intramolecular reaction. Since the carboxyl-terminal 6 aa of RSP5 were necessary for the apparent self-ubiquitination of RSP5 but

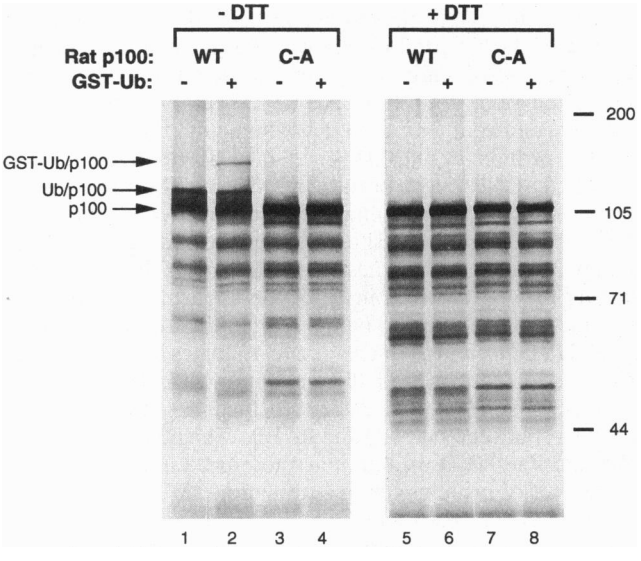

FIG. 2. Ubiquitin thioester formation of rat p100. The rat wild-type (WT) p100 cDNA and rat p100 C-A mutant were translated in rabbit reticulocyte lysate, GST-ubiquitin fusion protein (GST-Ub) was added following the translation where indicated (lanes 2, 4, 6, and 8), and the reactions were quenched with SDS/PAGE loading buffer without (lanes 1–4) or with (lanes 5–8) DTT. Reaction mixtures were electrophoresed in a 9% polyacrylamide gel and ^{35}S-labeled proteins were detected by autoradiography. Positions of molecular size (kDa) markers are indicated.

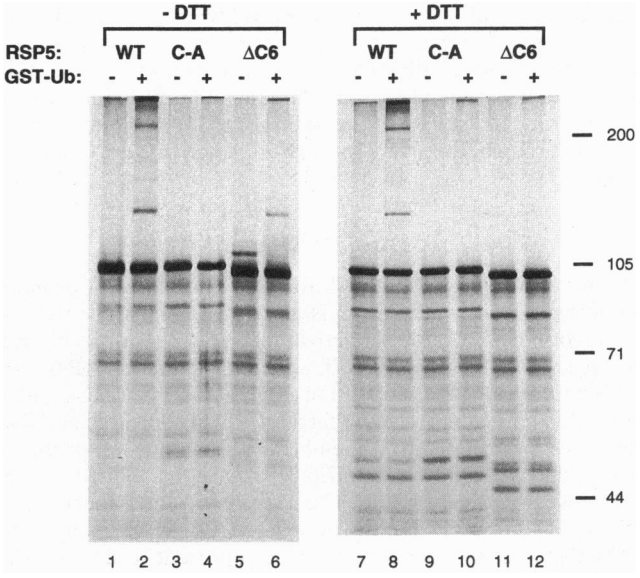

FIG. 3. Ubiquitin thioester formation of yeast RSP5. The wild-type (WT) RSP5 cDNA, the C-A RSP5 mutant, and the ΔC6 RSP5 mutant were translated in rabbit reticulocyte lysate, GST-ubiquitin was added following the translation where indicated (even-numbered lanes), and the reactions were quenched with SDS/PAGE loading buffer without (lanes 1–6) or with (lanes 7–12) DTT. Reaction products were analyzed as in Fig. 2.

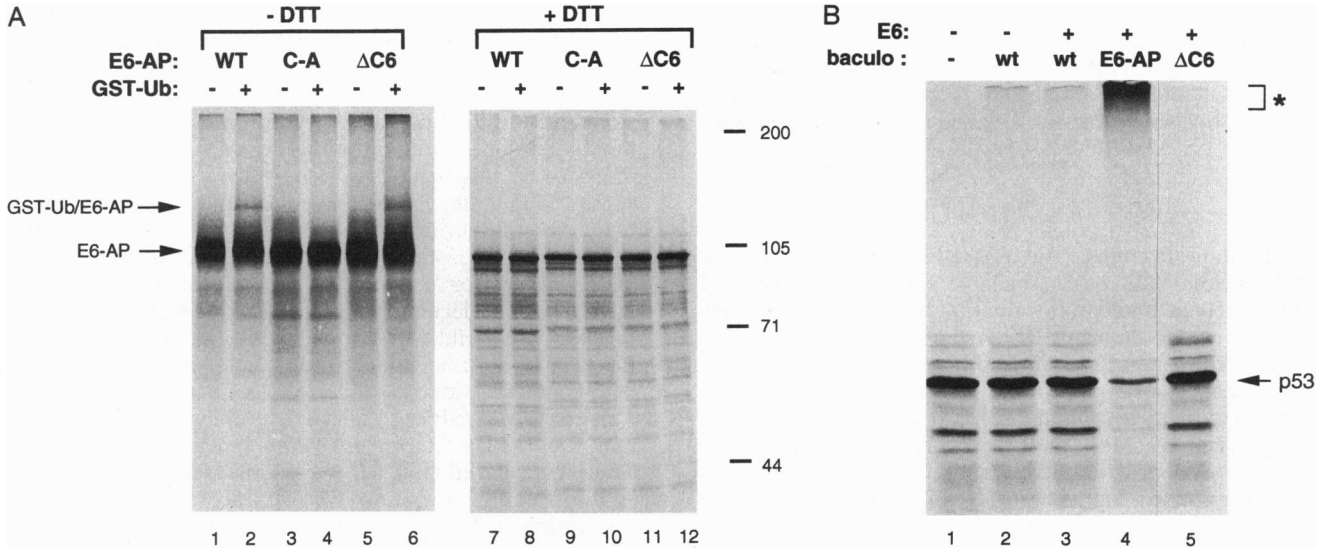

FIG. 4. (*A*) Ubiquitin thioester formation of human E6-AP. The wild-type (WT) E6-AP cDNA, the C-A E6-AP mutant, and the ΔC6 E6-AP mutant were translated in rabbit reticulocyte lysate, GST-ubiquitin was added following the translation where indicated (even-numbered lanes), and the reactions were quenched with SDS/PAGE loading buffer without (lanes 1–6) or with (lanes 7–12) DTT. Reaction products were analyzed as in Fig. 2. Molecular size markers are indicated, as well as running positions of E6-AP and the GST-ubiquitin/E6-AP conjugate. (*B*) p53 ubiquitination assay. Baculovirus-expressed E6-AP and the ΔC6 E6-AP mutant, along with an equivalent fraction from wild-type (WT) virus-infected cells, were assayed for their ability to ubiquitinate ^{35}S-labeled wheat germ extract-translated wild-type human p53. Lane 1, the p53 translation product without E6 or a baculovirus DEAE high-salt fraction; lane 2, with a DEAE high-salt fraction from wild-type baculovirus-infected cells; lane 3, as in lane 2, with a baculovirus HPV16 E6-containing fraction; lanes 4 and 5, as in lane 3, but with E6-AP-containing DEAE high-salt fractions. Equivalent amounts of the E6-AP and the ΔC6 mutant were used in this assay as determined by Western immunoblotting (data not shown). The HPV16 E6 protein was expressed in baculovirus-infected insect cells and partially purified by cation-exchange chromatography. Bracket with asterisk indicates high molecular weight multiubiquitinated p53.

were not required for thioester formation, we were interested in the effect of a similar mutation in the context of E6-AP. Fig. 4*A* shows a thioester assay using *in vitro* translated E6-AP [95-kDa form (4)], the C-A mutant (position 833), and the ΔC6 mutant. As with RSP5, the ΔC6 mutant did not significantly affect the ability of E6-AP to form a thioester, whereas, as previously shown (11), the C-A mutation completely abolished this activity. To determine whether the ΔC6 E6-AP mutant could catalyze the transfer of ubiquitin to a substrate, equivalent amounts of baculovirus-expressed wild-type E6-AP and the ΔC6 mutant were assayed for their ability to ubiquitinate p53 in the presence of HPV16 E6. As shown in Fig. 4*B*, the ΔC6 mutant was unable to stimulate the ubiquitination of p53. Neither rat p100 nor RSP5 could target p53 for ubiquitination, regardless of whether HPV16 E6 protein was present (data not shown).

DISCUSSION

We have proposed a model for E6-AP-mediated protein ubiquitination in which there is an ordered transfer of ubiquitin from the E1 ubiquitin-activating enzyme to a specific E2 enzyme, from the E2 to E6-AP, and finally from E6-AP to the substrate as a stable isopeptide conjugate (11). The results presented here demonstrate that a rat 100 kDa protein and the yeast RSP5 protein, two members of a family of proteins structurally related to human E6-AP, share the ability to form a thioester with ubiquitin. The structural and biochemical similarities between these proteins and E6-AP strongly suggests that the *hect*-domain proteins represent a class of E3 ubiquitin–protein ligases.

The regions of the E6-AP-related proteins amino-terminal to the *hect* domain are generally divergent. That the domain of E6-AP that is involved in binding to HPV16 E6 is located amino-terminal to the conserved carboxyl-terminal domain suggests that the *hect* domain may represent a modular E3 domain which targets specific substrates for ubiquitination based on protein–protein interactions directed by the amino-terminal region. Additionally, it is possible that the substrate specificity of the *hect* E3 proteins is controlled by their interaction with cellular E6-like proteins.

Thus far, since most of the E6-AP-related cDNAs were isolated by nonfunctional screens, we have few clues as to what the normal substrates of their putative E3 activities might be. The cDNA encoding rat p100 was cloned in a screen for an apparently unrelated cDNA (13). Initially, the most interesting feature of the rat p100 cDNA was that it encoded a protein with a region of similarity (amino acids 485–514) to a conserved element found in poly(A) RNA-binding proteins (PABPs) and another region that is similar to that found in the 70-kDa protein of the U1 small nuclear ribonucleoprotein particle (snRNP). Both of these regions are N-terminal to the conserved carboxyl-terminal domain, and while neither of these domains has been shown to be involved in direct RNA binding, one might hypothesize that targets of the rat p100 E3 activity may be involved in RNA metabolism. Another *hect*-domain protein, encoded by the *Drosophila* hyperplastic discs gene (HYD protein), which appears to play a role in imaginal disc growth control and differentiation (15, 16), has large regions of similarity to rat p100, including the region of similarity between rat p100 and PABPs. The E3 activities of rat p100 and the HYD protein may therefore target a similar or related set of proteins for ubiquitination.

S. cerevisiae RSP5 is an essential gene that was isolated in a screen for extragenic suppressors of *spt3* mutations (Barbara Berg, Anne Happel, and Fred Winston, personal communication). *SPT3* encodes a protein that interacts with the *SPT15* gene product (17), which encodes the TATA box-binding protein (TBP) of TFIID. Genetic analyses suggest that SPT3 itself is not a target of RSP5, since an *rsp5* allele has been isolated which suppresses an *spt3* null mutation. Further genetic analyses may prove useful in identification of the proteins or pathways affected by RSP5 activity. In addition to the

conserved carboxyl-terminal domain, RSP5 shares sequence similarity with another E6-AP-related protein, mouse NEDD-4 (18).

Three human E6-AP-related cDNAs, listed in GenBank but not described in the literature (Fig. 1), were identified through the cloning of randomly sampled cDNAs. One of these (GenBank accession no. D28476) has some limited regions of homology to E6-AP over the more amino-terminal sequences of E6-AP. The *S. cerevisiae* gene YKL162 was identified in the course of the chromosome XI sequencing project (19). Preliminary genetic analyses indicate that, unlike *RSP5*, YKL162 is a nonessential gene (Alex Bortvin and Fred Winston, personal communication). An E6-AP-related protein that is clearly unique compared with the others is the rat UreB1 protein, in that it consists of only a portion of the *hect* domain (310 aa), without any amino-terminal extension. UreB1 has been proposed to be a DNA-binding transcriptional regulator (20). In addition to the 10 predicted E6-AP-related proteins shown in Fig. 1, there are 4 *C. elegans* expressed sequence tags which have homology to regions of the *hect* domain.

A cysteine residue is located 32–36 aa from the carboxyl end of each of the E6-AP-related proteins. This cysteine is required for ubiquitin thioester formation in E6-AP, rat p100, and RSP5 and, as the only cysteine residue conserved among the E6-AP-related proteins, is likely to represent the actual site of thioester formation. We have also defined a determinant within the last 6 aa of E6-AP which, while not required for thioester formation, is required for transfer of ubiquitin to p53. This determinant is also necessary for the self-ubiquitination of RSP5. As shown in Fig. 1*B*, there is a single highly conserved amino acid within this region (phenylalanine or tyrosine).

The ubiquitination of RSP5 appears to be the result of an intramolecular transfer of ubiquitin, since mixing of full-length RSP5 with the cysteine-to-alanine mutant did not result in the ubiquitination of the mutant protein (data not shown). It is an intriguing possibility that the autoubiquitination represents a mechanism for regulating the E3 activity of RSP5. This phenomenon was not observed with rat p100 or E6-AP under the same reaction conditions, but preliminary results suggest that E6-AP also undergoes self-ubiquitination specifically in the presence of HPV16 E6 protein (S.B., J.M.H., and P.M.H., unpublished work). Interestingly, an E2 ubiquitin-conjugating enzyme, UBC3 (*S. cerevisiae CDC34* gene product) has also been shown to ubiquitinate itself (21).

In experiments not shown, baculovirus-expressed and partially purified rat p100 and RSP5 were shown to accept ubiquitin from *Arabidopsis thaliana* UBC8 or human UbcH5, but not *A. thaliana* UBC1. This is similar to the E2 specificity of E6-AP (22), suggesting that the *hect* E3 proteins share a common E2 specificity. *A. thaliana* UBC8 and human UbcH5 belong to a subgroup of highly related E2 proteins that includes *S. cerevisiae* UBC4 and UBC5, *Drosophila* UbcD1, and *C. elegans* ubc-2 (23–26).

While many of the enzymes that are involved in protein ubiquitination have been identified and characterized, the least characterized have been those that are thought to play a key role in substrate recognition, the E3 ubiquitin–protein ligases. Besides E6-AP and the putative E3 genes described here, only one E3 gene has been cloned, *S. cerevisiae UBR1* (3). UBR1 does not share sequence similarity with the E6-AP-related proteins and is therefore likely to be representative of a different class of E3 proteins, which might function through a mechanism distinct from that of E6-AP. The identification of a class of structurally and functionally related E6-AP-like E3 enzymes, particularly in genetically tractable organisms, should contribute toward our understanding of how substrate specificity of the ubiquitin proteolysis system is controlled.

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