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Characterization of Human hect Domain Family Members and Their Interaction with UbcH5 and UbcH7*

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The hect domain protein family was originally identified by sequence similarity of its members to the C-terminal region of E6-AP, an E3 ubiquitin-protein ligase. Since the C terminus of E6-AP mediates thioester complex formation with ubiquitin, a necessary intermediate step in E6-AP-dependent ubiquitination, it was proposed that members of the hect domain family in general have E3 activity. The hect domain is approximately 350 amino acids in length, and we show here that the hect domain of E6-AP is necessary and sufficient for ubiquitin thioester adduct formation. Furthermore, the human genome encodes at least 20 different hect domain proteins, and in further support of the hypothesis that hect domain proteins represent a family of E3s, several of these are shown to form thioester complexes with ubiquitin. In addition, some hect domain proteins interact preferentially with UbcH5, whereas others interact with UbcH7, indicating that human hect domain proteins can be grouped into at least two classes based on their E2 specificity. Since E3s are thought to play a major role in substrate recognition, the presence of a large family of E3s should contribute to ensure the specificity and selectivity of ubiquitin-dependent proteolytic pathways.

Ubiquitin-dependent degradation operates through a two step mechanism (for recent reviews, see Refs. 1–5). Firstly, ubiquitin is covalently attached to a substrate protein. Ubiquitin itself can then serve as a substrate for ubiquitin conjugation (ubiquitination) which results in the formation of polyubiquitin chains. Finally, ubiquitinated proteins are recognized and degraded by the 26 S proteasome or, as shown for some membrane proteins, internalized and degraded via the lysosomal pathway (6–8).

Ubiquitination of proteolytic substrates requires the concerted action of ubiquitin-activating enzymes E1,[1] ubiquitin-conjugating enzymes E2, and probably in most cases, ubiquitin-protein ligases E3 (1–5). First, ubiquitin is activated at the expense of ATP and is covalently linked to E1 via a thioester bond. Ubiquitin is then transferred from the E1 to an E2, preserving the high energy thioester bond. Finally, the covalent attachment of ubiquitin to a substrate protein is catalyzed by the E2s, often in conjunction with an E3. Although the mechanisms of substrate recognition are still poorly understood, this sequential mode of ubiquitin transfer indicates that E2s and, in particular, E3s play a major role in mediating substrate recognition.

Based on their mode of action, E3s can be classified into two categories. Some E3s may function as docking proteins by binding specifically to substrate proteins and E2s, thereby allowing E2s to ubiquitinate substrate proteins. Such E3s may be represented by the recently identified SCF complexes (9, 10). SCF complexes consist of CDC53, SKP1, and one of a number of F-box proteins (*e.g.* Cdc4, Grr1) (11), which appear to determine the substrate specificity of the respective SCF complex (9, 10). Furthermore, SCF complexes have been shown to cooperate with the E2 UBC3/Cdc34 in the ubiquitination of substrate proteins. Other E3s appear to directly catalyze the attachment of ubiquitin to a substrate protein, since some E3s are loaded with ubiquitin by E2s via thioester formation. E3s with thioester-forming capacity include yeast UBR1 (5), mammalian E6-AP (12), and presumably the members of an E6-AP-related family of putative E3s termed hect domain proteins (12, 13). Members of this family have been described in all eukaryotes examined and are characterized by a C-terminal region of approximately 350 amino acids in length, the hect domain (homologous to E6-AP C terminus).

E6-AP was originally identified as a protein that is required for ubiquitination of the tumor suppressor protein p53 induced by the E6 oncoprotein of HPVs associated with cervical cancer (14, 15). Subsequent studies revealed that E6-AP has the function of an E3 (16). Furthermore, it was shown that E6-AP forms thioester complexes with ubiquitin in the presence of E1 and distinct E2s, and the position of the putative catalytic site cysteine residue was mapped to the C terminus (12). The position of this cysteine residue as well as of several surrounding residues is highly conserved among hect domain proteins, suggesting that these proteins in general share the ability to form ubiquitin thioester adducts. In support of this hypothesis, three hect domain proteins from three different organisms, *Saccharomyces cerevisiae* RSP5, *Schizosaccharomyces pombe* Pub1, and a rat 100-kDa protein have been shown to form thioester complexes with ubiquitin (13, 17).

The turnover of many cellular proteins appears to involve ubiquitin-dependent pathways, indicating that a cell contains a number of different E3s with different substrate specificities. Indeed, the genome of *S. cerevisiae* encodes for five hect domain proteins (4), of which two (RSP5, UFD4) have been shown to be involved in the degradation of natural as well as of artificial substrate proteins (18–20). Here we report that the minimal domain of E6-AP and RSP5 required for ubiquitin thioester formation coincides with the size of the hect domain. Based on this information, data base searches revealed that the human genome encodes at least 20 different hect domain proteins.

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[1] The abbreviations used are: E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin-protein ligase; SCF, SKP1, CDC53, F-box protein; GST, glutathione *S*-transferase; HPV, human papillomavirus; PCR, polymerase chain reaction; DTT, dithiothreitol.

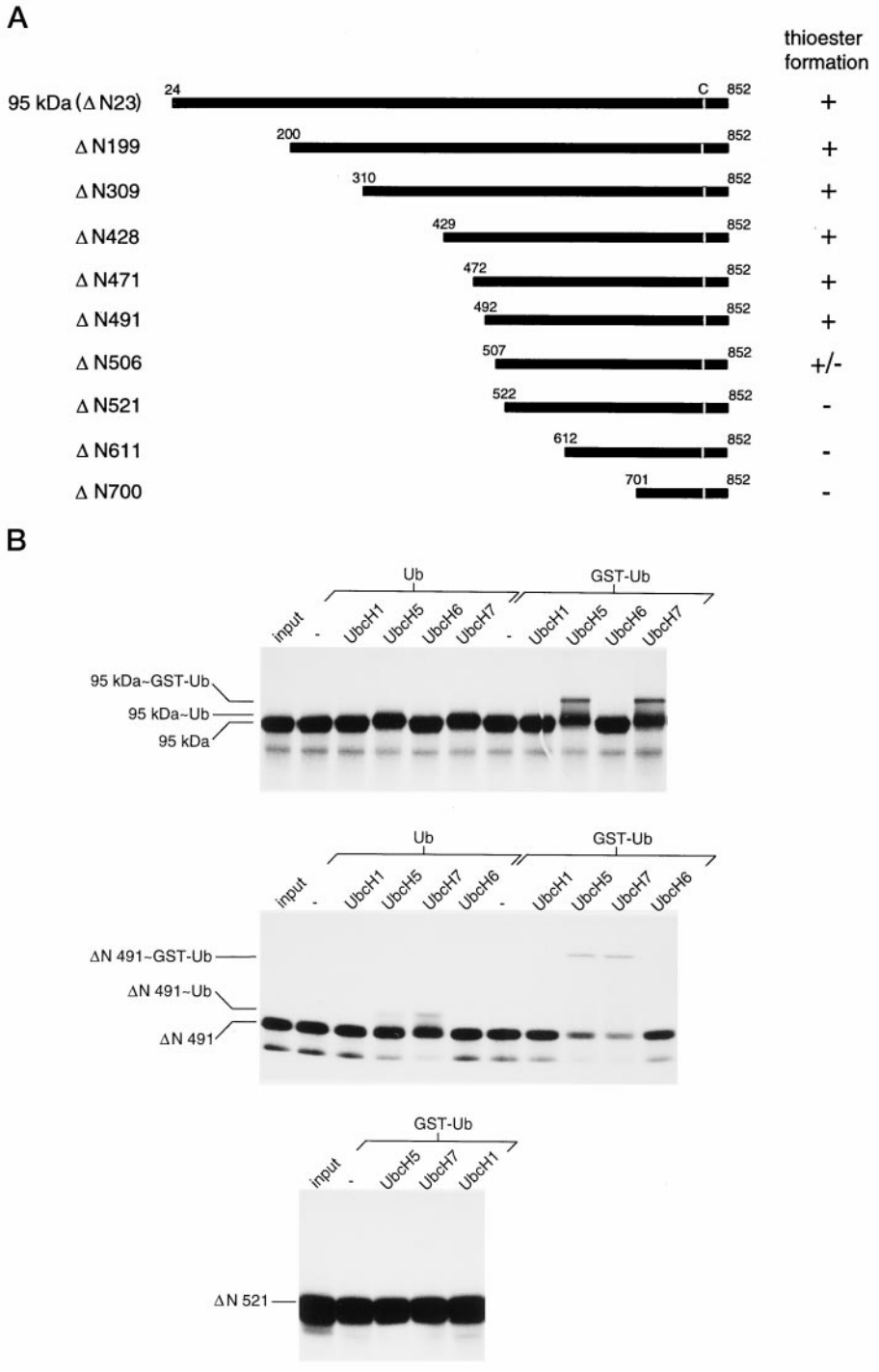


FIG. 1. **The hect domain of E6-AP is necessary and sufficient for ubiquitin thioester formation.** *A*, schematic representation of N-terminal-truncated forms of E6-AP. The various forms of E6-AP were generated in rabbit reticulocyte lysate and tested for ubiquitin thioester complex formation (see below). The ability of the different E6-AP truncation mutants to form ubiquitin thioester adducts is indicated. +, forms ubiquitin thioesters with a similar efficiency as the 95-kDa form of E6-AP; +/−, significantly less efficient in ubiquitin thioester formation than the 95-kDa E6-AP; −, thioester complex formation was not observed. The 95-kDa E6-AP represents the form commonly used in studies concerning E6-AP function. The putative catalytic site cysteine residue (*C*) at amino acid position 820 (12) is marked as a *white bar*. Numbering of the amino acid residues is according to E6-AP isoform 1 (22). *B*, the indicated E6-AP forms were generated in rabbit reticulocyte lysate in the presence of L-[^{35}S]methionine and partially purified by anion exchange chromatography (see "Experimental Procedures") to remove endogenous ubiquitin (*Ub*) and those E2s present in reticulocyte lysate that are known to interact with E6-AP (26, 27, 29, 30). The partially purified radiolabeled E6-AP forms were then incubated in the absence (input) or in the presence of ubiquitin or GST-ubiquitin, E1, and the respective recombinant E2s as indicated. After 5 min at 25 °C, the reactions were stopped in the absence or presence of a reducing agent and subjected to SDS-polyacrylamide gel electrophoresis as described previously (12). The reactions stopped in the presence of a reducing agent are not shown, but it should be noted that, under these conditions, the ubiquitin adducts of E6-AP observed in the absence of a reducing agent could not be detected. This indicates that the observed ubiquitin adducts indeed represent thioester complexes of ubiquitin with the respective E6-AP form. Furthermore, the band observed just below the ΔN491 form of E6-AP could not be detected in the presence of a reducing agent, indicating that it is a modified form of ΔN491 that is susceptible to reducing conditions. This suggests that ΔN491 may contain an intramolecular disulfide bond(s) under nonreducing conditions.

Ubiquitin thioester complex formation assays show that human hect domain proteins can be classified into two groups based on their preference for distinct E2s. Finally, generation of chimeric proteins between E6-AP and other hect domain proteins indicates that hect domains are not freely interchangeable but rather that a given hect domain has to be in a proper structural context to induce ubiquitination of associated proteins.

EXPERIMENTAL PROCEDURES

Plasmids—The p53 plasmid for *in vitro* transcription and translation has been described previously (21). Expression plasmids encoding the various N-terminal-truncated E6-AP forms, the N-terminal-truncated forms of RSP5, and the hect domains of seven different human hect domain proteins were constructed by ligating PCR products into pGEM-1 for *in vitro* transcription and translation. Expression plasmids encoding the hect domain of E6-AP and the E6-AP/p532 chimeric protein consisting of amino acids 200–491 of human E6-AP isoform 1 (22) fused to amino acids 4495–4861 of p532 (23, 24) were constructed by ligating PCR products into pGEX-2TK for bacterial expression. The cDNAs used as templates in PCR for E6-AP, RSP5, and p532 (hectH6) have been described (13, 15, 23). cDNAs encoding the hect domain of human hect domain proteins other than E6-AP and p532 were obtained from the following sources: hectH2 (GenBank accession number D13635) and hectH3 (D25215) were kindly provided by N. Nomura; hectH4 (D28476) and hectH5 (D42055) were obtained by RNA PCR using cytoplasmic RNA prepared from HeLa cells; hectH9 (human homolog to a rat protein termed UREB1, accession number U08214) was obtained by RNA PCR followed by screening of a HeLa cDNA library (CLONTECH); hectH7 was obtained from two EST clones (accession numbers T93069 and H19362; I.M.A.G.E. consortium). Plas-

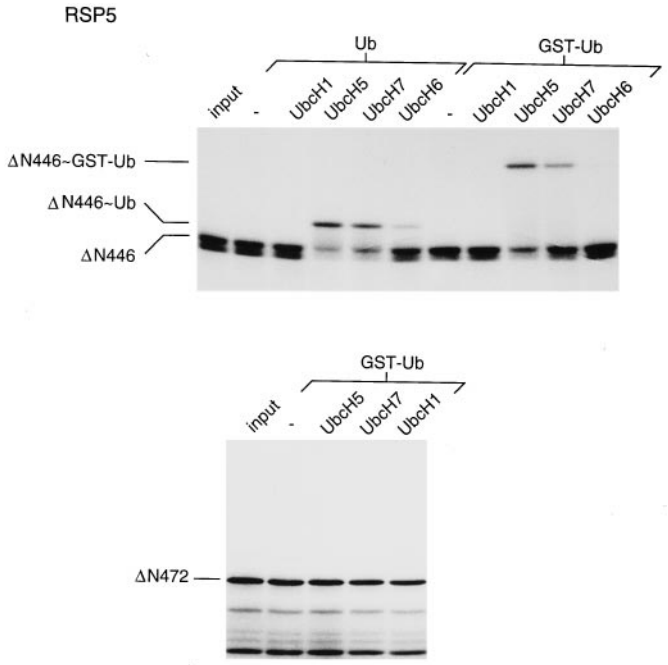


FIG. 2. **The hect domain of RSP5 is necessary and sufficient for ubiquitin thioester formation.** Two N-terminal truncation mutants of *S. cerevisiae* RSP5 comprising amino acids 447–809 (ΔN446) and 473–809 (ΔN472), respectively, were generated in rabbit reticulocyte lysate in the presence of L-[^{35}S]methionine and partially purified by anion exchange chromatography (see "Experimental Procedures"). The radiolabeled RSP5 forms were then incubated in the absence (input) or presence of ubiquitin (*Ub*) or GST-ubiquitin, E1, and the respective recombinant E2s as indicated. After 5 min at 25 °C, reaction mixtures were stopped in the absence or presence of a reducing agent and subjected to SDS-polyacrylamide gel electrophoresis as described previously (12). The reactions stopped in the presence of a reducing agent are not shown but it should be noted that, under these conditions, the ubiquitin adducts of RSP5 observed in the absence of a reducing agent could not be detected. This indicates that the observed ubiquitin adducts represent thioester complexes of ubiquitin with the respective RSP5 form.

mids encoding the GST-ubiquitin fusion protein and the 75-kDa form of E6-AP as a GST fusion protein have been described (16).

Protein Expression—E1, UbcH1, UbcH5, UbcH6, and UbcH7 were expressed in *Escherichia coli* BL21(DE3) using the pET expression system as described previously (25–27). For ubiquitination and ubiquitin thioester formation assays, E1 was partially purified by anion exchange chromatography as described (16). As a source of UbcH1, UbcH5, UbcH6, or UbcH7, crude bacterial extracts containing the respective E2s were used.

The GST fusion proteins (ubiquitin, 75-kDa form of E6-AP, hect domain of E6-AP, E6-AP/p532 chimeric protein, and E6-E7) were expressed in *E. coli* DH5α, affinity-purified on glutathione-Sepharose (Amersham Pharmacia Biotech) and eluted with 10 mM glutathione. In experiments using radiolabeled ubiquitin, the GST-ubiquitin fusion protein was radioactively labeled while bound to glutathione-Sepharose using protein kinase A (Sigma) in the presence of [γ-^{32}P]ATP (16). The radiolabeled fusion protein was eluted with 10 mM glutathione and cleaved with thrombin (Novagen) to yield free ubiquitin. Upon cleavage, thrombin was inactivated by incubation at 75 °C for 15 min.

For *in vitro* expression, the various proteins (p53, hect domains, and N-terminal truncation mutants of E6-AP and RSP5) were generated by *in vitro* transcription-translation. Transcription and translation was performed according to the manufacturer's instructions (Promega). Translation was performed in rabbit reticulocyte lysate or in wheat germ extract in the presence of L-[^{35}S]methionine as indicated in the text.

As a source of the HPV-16 E6 oncoprotein, E6 was prepared from insect cells (High Five cells; Invitrogen) infected with a recombinant baculovirus expressing E6 (kindly provided by J. M. Huibregtse). For partial purification of E6, crude extracts from infected insect cells were loaded onto a Mono S column (Amersham), the column was washed with 25 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM DTT, and bound proteins were eluted with 400 mM NaCl.

Thioester, Ubiquitination, and p53 Binding Assays—L-[^{35}S]Methionine-labeled forms of E6-AP and RSP5 synthesized *in vitro* in rabbit reticulocyte lysate were partially purified by anion exchange chromatography as follows. 100 μl of rabbit reticulocyte lysate programmed with mRNA encoding for the respective proteins were loaded onto a Mono Q column (Amersham), the column was washed with 25 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM DTT, and bound proteins were eluted with 500 μl of the same buffer but containing 400 mM NaCl instead of 50 mM NaCl. 10 μl of the partially purified proteins were tested in ubiquitin thioester formation assays in the presence of E1, E2s as indicated (UbcH1, UbcH5, UbcH6, UbcH7), and native ubiquitin or GST-ubiquitin as described previously (12, 27). Similar amounts of the different E2s were used as assessed by their ability to form thioester complexes with ^{32}P-labeled ubiquitin in the presence of E1. To test the capacity of the hect domain of human hect domain proteins to form ubiquitin thioester complexes, the respective hect domains were translated in rabbit reticulocyte lysate, and 5 μl of the respective translate were used in standard ubiquitin thioester formation assays as described above. Thioester formation assays using GST fusion proteins (GST-hect E6-AP, GST-75 kDa E6-AP, GST-E6-AP/p532) were performed using ^{32}P-labeled ubiquitin as described for baculovirus-expressed E6-AP (12).

p53 ubiquitination assays were performed as described (16) using wheat germ extract-translated p53, partially purified HPV-16 E6 expressed in insect cells, and 1 μg of GST-75 kDa E6-AP, GST-E6-AP/p532, or GST-hect E6-AP. Ubiquitination assays using bacterially expressed GST-E6-E7 as a substrate were performed as described (16).

p53 binding assays using GST fusion proteins (GST-hect E6-AP, GST-75 kDa E6-AP, and GST-E6-AP/p532) in the presence of the HPV-16 E6 oncoprotein were performed as described (28). As a source of radiolabeled p53, p53 expressed in wheat germ extract was used.

RESULTS

The hect Domain of E6-AP or RSP5 Is Necessary and Sufficient for Ubiquitin Thioester Formation—The hect domain proteins are characterized by a conserved C-terminal region of approximately 350 amino acids (13), suggesting that this region may be necessary and sufficient to form thioester complexes with ubiquitin in the presence of distinct E2s. To test this hypothesis, a series of N-terminal-truncated forms of E6-AP (Fig. 1*A*) were generated *in vitro* using the rabbit reticulocyte lysate system. The resulting E6-AP forms were then partially purified by anion exchange chromatography to remove endogenous ubiquitin as well as E2 activities present in rabbit reticulocyte lysate that are known to interact with E6-AP (26, 27, 29, 30). Finally, the partially purified forms of E6-AP were tested for ubiquitin thioester formation in the absence or presence of distinct E2s, *i.e.* UbcH1 (the human homolog of *S. cerevisiae* UBC2/RAD6), UbcH5 (the human homolog of *S. cerevisiae* UBC4/UBC5), UbcH6, and UbcH7 (Fig. 1) (26, 27, 31). This revealed that the minimal domain of E6-AP, which is necessary and sufficient for ubiquitin thioester formation, comprises amino acid residues 492–852 (numbering is according to the sequence of E6-AP isoform 1 (22)). It should be noted that C-terminal-truncated forms were not generated, since the putative catalytic site cysteine residue of E6-AP is located at amino acid residue 820 (12), and thus, the information obtained in a deletion analysis of the C terminus would be rather limited.

As expected from previous studies (26, 27), thioester formation of all the E6-AP forms tested was observed in the presence of UbcH5 and UbcH7 but not UbcH1. Additionally, thioester formation was not observed in the presence of UbcH6. This is in contrast to the results obtained previously with the 95-kDa form of E6-AP expressed in the baculovirus system that was reported to form thioester complexes in the presence of UbcH6 although significantly less efficiently than in the presence of UbcH5 or UbcH7 (27). The reason for this apparent difference is presently unknown, but it may simply reflect different sensitivities of the respective thioester assays.

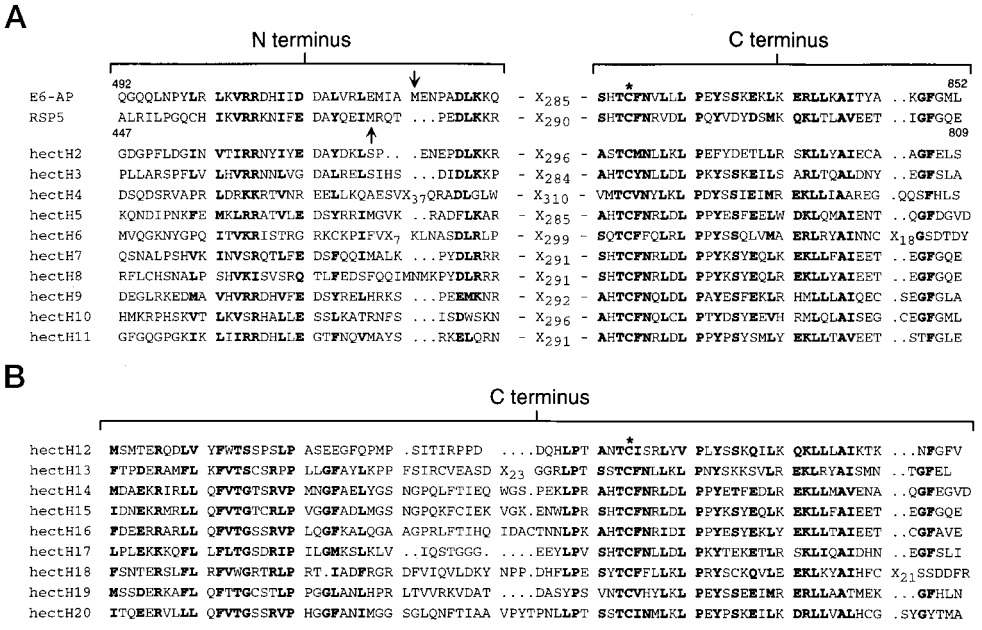


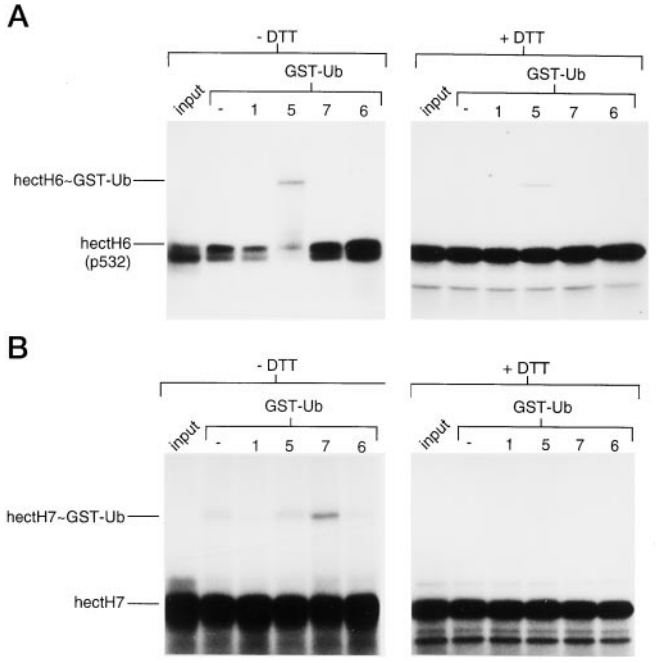
FIG. 3. **The human hect domain family.** Using amino acid residues 814–852 of E6-AP isoform 1 (22) as a query sequence, data base searches indicate that the human genome encodes at least 20 different hect domain proteins. In *A*, the amino acid sequence of the N-terminal and C-terminal ends of the hect domains is shown, for which cDNA sequences are available encoding the respective full-length hect domain protein. The putative catalytic site cysteine residue is marked with an *asterisk*. The amino acid numbers comprising the minimal hect domain of E6-AP and RSP5 as determined in the experiments shown in Fig. 1 and Fig. 2 are indicated. The start of the truncation mutants of E6-AP and RSP5, which cannot form thioester complexes with ubiquitin, are indicated by *arrows*. Amino acid residues conserved among most of the hect domain proteins are indicated in bold. In *B*, the sequence of the approximately 80 C-terminal amino acids of hect domains is shown, for which only limited sequence information is available. The sequences shown were derived by analyses of clones entered in EST data bases, and therefore sequence errors cannot be excluded. However, it should be noted that only sequences were considered, for which several EST clones are available. Accession numbers for hectH2-hectH2O (with hectH1 defined as E6-AP) are as follows. hectH2, D13635; hectH3, D25215; hectH4, D28476; hectH5, D42055; hectH6, U50078; hectH7, U96113 in combination with H19362 and T93069; hectH8, U96114; hectH9, AB002310; hectH10, AB002315; hectH11, AB002320; hectH12, W95857; hectH13, R06429 and W60127; hectH14, AA443004; hectH15, AA140512; hectH16, R23558 and AA194036; hectH17, N32787 and T62800; hectH18, H23082; hectH19, N24244 and N31202; hectH2O, N57610 and R20250.

Based on the results obtained for E6-AP, two N-terminal truncation mutants of *S. cerevisiae* RSP5 were generated (for sequence comparison, see Fig. 3*A*) and tested for ubiquitin thioester formation. Similar to E6-AP, a deletion mutant of RSP5 comprising amino acid residues 447–809 was active in ubiquitin thioester formation, whereas deletion of the N-terminal 472 amino acids resulted in an inactive form of RSP5 (Fig. 2). Thioester formation of the truncated form of RSP5 was observed in the presence of UbcH5, UbcH7, and, to a lesser extent, UbcH6. In this context it should be noted that in this experiment, as well as in all the other ubiquitin thioester experiments presented, similar amounts of UbcH1, UbcH5, UbcH6, and UbcH7 were used as determined by their ability to form thioester complexes with radioactively labeled ubiquitin in the presence of E1. Furthermore, titration experiments using decreasing amounts of the respective E2s were performed. This showed that UbcH5 and UbcH7 were similarly active in transferring ubiquitin to the truncated form of RSP5, even under conditions where the amounts of UbcH5 and UbcH7 used were rate-limiting.[2] Again, this is in contrast to results previously obtained with baculovirus expressed RSP5 (27). In this case, UbcH7 was significantly less efficient in loading RSP5 with ubiquitin than UbcH5. The reason for this discrepancy is presently unclear.

Human hect Domain Family Members Interact Preferentially with UbcH5 or UbcH7—Sequence alignment of the minimal region of E6-AP and RSP5 necessary for ubiquitin thioester formation with the amino acid sequences of other hect domain proteins present in available data bases revealed that this region coincides with the size of the region that appears to be conserved among all known members of the hect domain family (Fig. 3*A*). To obtain further evidence that a general feature of hect domain family members is the capacity to form thioester complexes with ubiquitin, the cDNAs encoding the hect domain of seven different human hect domain proteins were obtained (for further details, see "Experimental Procedures"). The respective proteins were expressed in the reticulocyte lysate system and tested for ubiquitin thioester formation without further purification. As summarized in Fig. 4, six of these proteins formed thioester complexes with ubiquitin, whereas one designated as hectH5 was inactive in the presence of the E2s used in this study (see "Discussion"). Furthermore, it appears that human hect domain proteins can be grouped into two classes based on their interaction with distinct E2s. Some hect domain proteins (*e.g.* hectH6 that represents the recently identified protein p532 (23, 24); Fig. 4*A*) form ubiquitin thioester complexes preferentially in the presence of UbcH5, whereas others (*e.g.* hectH7; Fig. 4*B*) form thioester complexes preferentially in the presence of UbcH7.

It is widely assumed that E3s play a major role in the recognition of substrates of the ubiquitin-conjugating system. Therefore, to ensure the specificity and selectivity of ubiquitin-dependent degradation, it seems likely that a cell contains a number of different hect domain proteins involved in the degradation of different proteins. This notion is supported by Northern blot analyses showing that the genes encoding the hect domain proteins tested in this study are coexpressed in several tissues, most prominently in skeletal muscle (Fig. 5), and by RNA PCR analyses demonstrating that at least six of the eight human hect domain genes tested are expressed in the cell lines HeLa (cervical carcinoma) and H1299 (non-small cell lung cancer).[2]

[2] S. E. Schwarz and M. Scheffner, unpublished data.



C

	UbcH5	UbcH7
hectH1 (E6-AP)	+	+
RSP5	+	+
hectH2	+	+/-
hectH3	+/-	+
hectH4	+/-	+
hectH6 (p532)	+	+/-
hectH7	+/-	+
hectH9	+/-	+

FIG. 4. **Preferential interaction of human hect domain proteins with UbcH5 or UbcH7.** The hect domain of the respective human hect domain proteins (hectH2-hectH7, hectH9) was generated in rabbit reticulocyte lysate in the presence of L-[^{35}S]methionine. Unlike the experiments performed with E6-AP and RSP5 (see Fig. 1 and Fig. 2), the respective radiolabeled proteins were not partially purified by anion exchange chromatography but were directly tested for ubiquitin (*Ub*) thioester complex formation in the absence (input) or presence of GST-ubiquitin and recombinant UbcH1 (1), UbcH5 (5), UbcH6 (6), and UbcH7 (7) under standard conditions (see "Experimental Procedures"). After 5 min at 25 °C, reactions were stopped in the absence (−DTT) or in the presence (+DTT) of a reducing agent and subjected to SDS-polyacrylamide gel electrophoresis followed by fluorography. Representative examples of such assays are shown for hectH6 (represents a recently identified protein termed p532 (23, 24)) (*A*) and hectH7 (*B*). The results obtained for all the hect domains tested are summarized in *C*. +, indicates that ubiquitin thioester formation was most efficient in the presence of the respective E2 (UbcH5 or UbcH7); +/−, ubiquitin thioester formation was observed in the presence of the respective E2 but with a significantly reduced efficiency compared with the other E2. Running positions of unmodified hect domains as well as of the respective thioester adducts with GST-ubiquitin are indicated. It should be noted that since the translates used were not partially purified, thioester formation was observed even in the absence of recombinant E2s, at least for some hect domains. This is explained by the fact that rabbit reticulocyte lysate contains E2 activities that can substitute for UbcH5 and UbcH7 in E6-AP-dependent ubiquitination assays (16, 26, 27, 29, 30) and thus can be considered functionally equivalent to UbcH5 or UbcH7.

The hect Domain of E6-AP Cannot Be Replaced by Other hect Domains in the Ubiquitination of p53—The studies presented above are in support of the hypothesis that hect domain proteins have a modular structure consisting of a conserved catalytic C-terminal domain (*i.e.* the hect domain) and different N-terminal extensions that determine the substrate specificity of the respective hect domain protein. If this is indeed the case, it may be possible to generate fusion proteins consisting of the hect domain fused to an unrelated protein or region of a protein that is known to mediate interaction with a protein of choice.

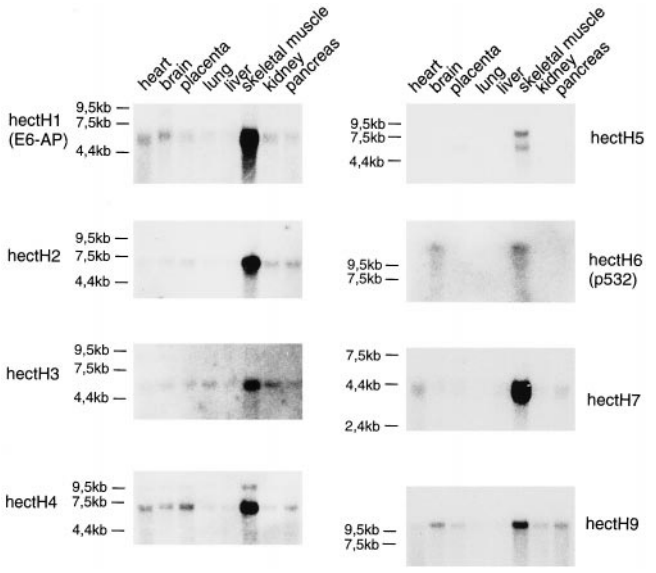


FIG. 5. **Expression of human hect domain genes.** A commercially available blot (CLONTECH) containing poly(A)$^+$ RNA derived from different human tissues was probed with cDNAs encoding the respective hect domain as indicated. Northern blot analyses using *in vitro* transcribed RNAs encoding the different hect domains, and these cDNA probes revealed that there is no cross-hybridization among the hect sequences used.[2] However, cross-hybridization with mRNAs encoding hect domain proteins not used in this study cannot be excluded. *kb*, kilobases.

Such fusion proteins could then be used to target proteins that otherwise would not be recognized by hect domain proteins selectively for ubiquitin-mediated degradation. In a first attempt to test the feasibility of this approach, a cDNA encoding a chimeric protein consisting of amino acid residues 200–491 of E6-AP and the hect domain of hectH6 (amino acid residues 4495–4861) (Fig. 6*A*) was constructed, and the chimeric protein was expressed as a GST fusion protein in *E. coli* for the following reasons. (i) The 75-kDa form of E6-AP, which starts at amino acid 200 of E6-AP isoform 1 (22), has been shown to contain the regions that are necessary and sufficient to bind to the HPV E6 oncoprotein and to p53 in the presence of the HPV E6 oncoprotein (see Fig. 6*A*) (28), and (ii) the 75-kDa form of E6-AP expressed as a GST fusion protein in *E. coli* has been shown to facilitate ubiquitination of an artificial E6-E7 fusion protein as well as of p53 in the presence of the HPV E6 oncoprotein (16). The resulting E6-AP/hectH6 chimeric protein was purified by glutathione affinity chromatography and tested for ubiquitin thioester complex formation, binding to the E6-E7 fusion protein as well as to p53, and ubiquitination of the E6-E7 fusion protein and p53 (Fig. 6, *B–D*). As controls, the 75-kDa form of E6-AP and the hect domain of E6-AP expressed as GST fusion proteins were used.

As shown in Fig. 6*B*, the 75-kDa form of E6-AP, as well as the E6-AP/hectH6 chimeric protein, were able to form thioester complexes with ubiquitin in the presence of UbcH5. In addition, both proteins bound with a similar efficiency to the E6-E7 fusion protein, and both bound to p53 in the presence of the HPV E6 oncoprotein, although the E6-AP/hectH6 chimeric protein was reproducibly less efficient in binding p53 than the 75-kDa form of E6-AP (Fig. 6*C*). In contrast to the 75-kDa form of E6-AP, however, ubiquitination of neither the E6-E7 fusion protein nor p53 was observed in the presence of the chimeric protein (Fig. 6*D*). Taken together, these results suggest that binding of a hect domain protein to a potential substrate protein is not sufficient to induce ubiquitination of the substrate protein.

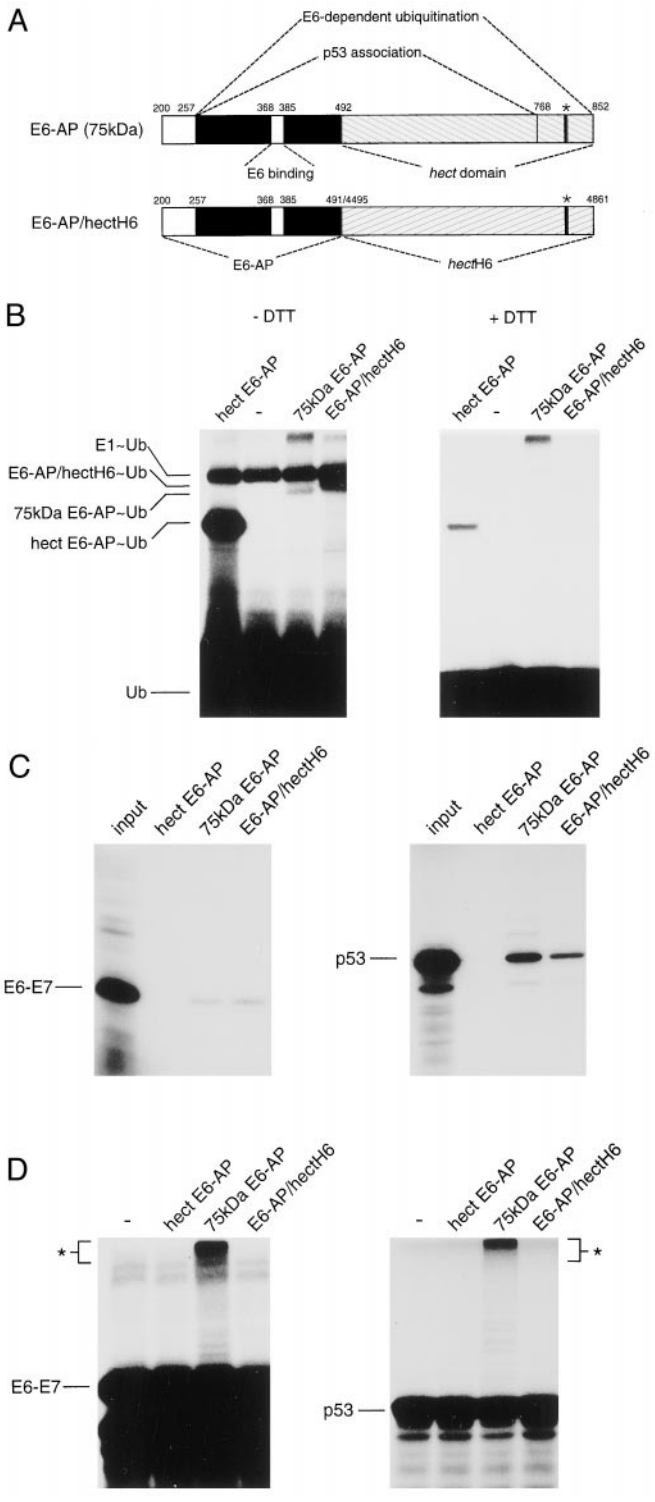


FIG. 6. **The hect domain of E6-AP cannot be replaced by other hect domains in E6-AP-dependent ubiquitination of p53.** *A*, a schematic drawing of the 75-kDa form of E6-AP (16, 28) and of a chimeric protein consisting of amino acid residues 200–491 of E6-AP fused to amino acid residues 4495–4861 of hectH6 (p532 (23, 24)) is shown. The regions of E6-AP involved in binding the HPV E6 oncoprotein and the tumor suppressor p53 in the presence of the HPV E6 oncoprotein (28) and the hect domain are indicated. The cysteine residue of E6-AP (12) and p532 (S. E. Schwarz, J. L. Rosa, and M. Scheffner, unpublished data) involved in ubiquitin thioester complex formation is marked with an *asterisk*. The 75-kDa form of E6-AP, the chimeric protein, and the hect domain of E6-AP were expressed in *E. coli* as GST fusion proteins and tested for ubiquitin thioester complex formation using ^{32}P-labeled ubiquitin (*B*), binding to the E6-E7 fusion protein and to p53 in the presence of the HPV E6 oncoprotein (*C*), and ubiquitination of the E6-E7 fusion protein and p53 (*D*) under conditions described elsewhere (for details, see "Experimental Procedures"). The E6-E7 fusion protein and p53 were generated in wheat germ extract in the presence of L-[^{35}S]methionine. The running positions of the respective thioester adducts, free ubiquitin (*Ub*), the E6-E7 fusion protein, and p53 are indicated in the respective autoradiographs. Highly ubiquitinated forms of the E6-E7 fusion protein as well as of p53 are marked with an asterisk. In *B*, thioester reactions stopped in the absence (*−DTT*) or presence (*+DTT*) of a reducing agent are indicated.

DISCUSSION

A likely possibility to account for the observed specificity and selectivity of ubiquitin-dependent degradation is the presence of a number of different E2s and E3s, which have been proposed to play a major role in substrate recognition. This hypothesis is supported by the fact that *S. cerevisiae*, for instance, encodes for 13 different E2s (4), and according to data base searches, the human genome apparently encodes at least 20 different E2s.[3] With the exception of *S. cerevisiae* UBR1 (5), however, the molecular identity of E3s remained enigmatic until recently. Similar to E2s, which are characterized by a highly conserved catalytic region termed the ubiquitin-conjugating (UBC) domain (4), recent studies suggest that eukaryotic cells encode at least two families of putative E3s. These are the hect domain proteins (12, 13) and the so-called SCF complexes (see Refs. 9 and 10 for further details). The family of hect domain proteins was originally identified based on amino acid sequence similarity of its members to the C-terminal region of E6-AP. In support of the hypothesis that hect domain proteins in general have E3 function, the present study revealed that the hect domain of several different proteins is both necessary and sufficient to form thioester complexes with ubiquitin in the presence of distinct E2s. Thus, the hect domain can be considered as being functionally equivalent to the UBC domain of E2s in that it constitutes the catalytic domain of the hect family of E3s.

Data base searches indicate that the human genome encodes at least 20 different human hect domain proteins. The rationale for choosing the particular human hect domain proteins used in this study was that when this study was initiated, cDNAs encoding the full-length protein were available for five of these (E6-AP, hectH2-hectH5). Subsequently, cDNAs encoding the full-length protein for hectH6 (p532 (23, 24)), hectH7 (32), and hectH9 (see below) became available. For hectH7, a cDNA was originally isolated encoding a C-terminal-truncated protein (32), which is now complemented to a full-length protein by this study. hectH9 represents the human homolog to a rat protein termed UREB1 (33). Initial interest in this protein was raised, since it was described to consist of approximately 300 amino acids and, thus, to represent an N-terminal-truncated hect domain. However, our own studies indicate that the human homolog of UREB1 consists of at least 800 amino acids.[2] Moreover, no evidence has been obtained to suggest that human cells contain an mRNA species encoding for a similar truncated protein as has been described in rat cells.[2] The notion that the human homolog of UREB1 is a regular hect domain protein insofar that it consists of a hect domain of approximately 350 amino acids and an N-terminal extension is further supported by a recent data base entry (accession number AB002310) indicating that human UREB1 consists of 1906 amino acids.

We previously suggested that hect domain proteins can be classified into two groups based on their preferential interaction with distinct E2s (27). This is supported by the finding that some hect domains derived from different human proteins form ubiquitin thioester complexes preferentially in the presence of UbcH5, whereas others form thioester complexes preferentially

[3] M. Scheffner, unpublished data.

in the presence of UbcH7. The only exception was hectH5, for which complex formation with ubiquitin was not observed. However, hectH5 represents the human homolog of murine Nedd4, which was recently shown to have ubiquitin-protein ligase activity in the presence of UbcH5 *in vitro* (34). This indicates that hectH5 may have the ability to form ubiquitin thioester complexes but that such complexes cannot be detected in the system used in the present study. The hect domain of E6-AP as well as of RSP5 appears to be somewhat different to the other hect domains in that the hect domain of both appears to interact *in vitro* with UbcH5 as well as with UbcH7. In contrast to these *in vitro* results, it was recently reported that in the yeast two-hybrid system, interaction of E6-AP can only be detected with UbcH7 and not with UbcH5, whereas interaction of RSP5 was observed with UbcH5 but not with UbcH7 (35). Therefore, further studies will be required to determine which if any of the interactions observed *in vitro* and in the two-hybrid system are of functional significance *in vivo*.

The finding that the hect domain is necessary and sufficient to form thioester complexes with ubiquitin indicates that hect domain proteins have a modular structure consisting of a catalytic domain (*i.e.* the hect domain) and different N-terminal extensions that determine the substrate specificity of the respective hect domain protein. This suggests the attractive possibility that, by fusion to suitable protein binding domains, a given hect domain could bind and ubiquitinate proteins that normally would not be recognized. It should be noted that a similar approach to target proteins for selective ubiquitination and degradation was suggested in previous studies using the HPV E6 oncoprotein (36) or certain E2s (37). In an attempt to test the feasibility of this approach, the N-terminal region of E6-AP was fused to the hect domain of hectH6 (p532). Although the resulting E6-AP/hectH6 fusion protein could bind to an artificial substrate protein of E6-AP with an efficiency similar to E6-AP, ubiquitination of this protein was not observed. Thus, binding of a hect domain protein to a potential target protein may not be sufficient to induce ubiquitination, suggesting that a substrate protein and a given hect domain protein have to interact in a structurally well defined manner to allow ubiquitination of the substrate protein. Furthermore, the fusion protein was able to bind to p53 in the presence of the HPV E6 oncoprotein but less efficiently than E6-AP. This suggests that, at least in some cases, the hect domain contributes toward defining the substrate specificity or, at least, in modulating the binding efficiency of the respective hect domain protein.

The fact that the similarity among human hect domain proteins is mostly limited to the hect domain indicates that different hect domain proteins are involved in the recognition and ubiquitination of different proteins. Mutations in the E6-AP gene have been reported to be the cause of a familial neurological disorder termed Angelman syndrome (38–40). However, with the exception of a recent report suggesting that the human homolog of *S. cerevisiae* RAD23 constitutes a substrate of E6-AP (35), substrate proteins of E6-AP in the absence of the HPV E6 oncoprotein have not yet been identified. p532 (hectH6) was shown to interact with the small GTP-binding protein ARF1 and, possibly, with certain Rab proteins (23, 24). However, whether ARF1 and Rab proteins are targets of p532 or regulators of p532 activity or if the observed interaction is unrelated to the proposed E3 activity of p532 is unknown at present. Nedd4, the proposed murine homolog of hectH5, was reported to bind to the epithelial Na^+ channel (41). Furthermore, it was recently shown that the stability and function of the epithelial Na^+ channel is regulated by ubiquitination and subsequent degradation (8). However, whether Nedd4 is indeed involved in this process or not remains to be determined. Similar to Nedd4, the N-terminal extension of at least two additional human hect domain proteins contains WW domains (hectH7 and hectH8) (32), and both p532 (hectH6) and hectH3 contain RCC1-like motifs (23).[2] WW domains have been implicated in the binding of peptides containing PY and PY-like domains (Ref. 32, and references therein), and it is suggested that RCC1-like domains bind small GTP-binding proteins (23). Again, however, substrates of these particular hect domain proteins have not yet been identified. In conclusion, to further understand the role of human hect domain proteins in ubiquitin-dependent degradation, it will be necessary to define both their respective cellular function and their target proteins.

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