Subcellular Localization and Ubiquitin-conjugating Enzyme (E2) Interactions of Mammalian HECT Family Ubiquitin Protein Ligases*

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In most instances, the transfer of ubiquitin to target proteins is catalyzed by the action of ubiquitin protein ligases (E3s). Full-length cDNAs encoding murine E6-associated protein (mE6-AP) as well as Nedd-4, a protein that is homologous to E6-AP in its C terminus, were cloned. Nedd-4 and mouse E6-AP are both enzymatically active E3s and function with members of the UbcH5 family of E2s. Mouse E6-AP, like its human counterpart, ubiquitinites p53 in the presence of human papilloma virus E6 protein, while Nedd-4 does not. Consistent with its role in p53 ubiquitination, mE6-AP was found both in the nucleus and cytosol, while Nedd-4 was found only in the cytosol. Binding studies implicate a 150-amino acid region that is 40% identical between mE6-AP and Nedd-4 as a binding site for the C-terminal portion of an E2 enzyme (UbcH5B). Nedd-4 was determined to have a second nonoverlapping E2 binding site that recognizes the first 67 amino acids of UbcH5B but not the more C-terminal portion of this E2. These findings provide the first demonstration of physical interactions between mammalian E2s and E3s and establish that these interactions occur independently of ubiquitin and an intact E3 catalytic domain. Furthermore, the presence of two E2 binding sites within Nedd-4 suggests models for ubiquitination involving multiple E2 enzymes associated with E3s.

The conjugation of proteins to ubiquitin (Ub) followed by their targeted degradation in the 26 S proteasome constitutes a central means of effecting regulated protein degradation in eucaryotic cells (reviewed in Refs. 1–9). Ubiquitination is implicated in the proteosomal degradation of a rapidly growing list of regulatory proteins including cell cycle regulators, transcription factors, kinases, phosphatases, and tumor suppressors. Additionally, there are recent data suggesting a role for this post-translational modification in receptor-mediated endocytosis (8–10).

Ub is a highly conserved 76-amino acid polypeptide that is found covalently bound to target proteins as monomers or chains. This occurs as the result of a multienzyme process involving sequential thiol-ester bonds with classes of enzymes termed E1—E3. This cascade culminates in the formation of isopeptide bonds between the C terminus of Ub and the e-amino group of lysines on either a substrate or the growing end of a protein-bound mult ubiquitin chain. This process is initiated by the ATP-dependent formation of high energy thiol-ester bonds between E1 (Ub-activating enzyme) and the C terminus of Ub. Ub is then transferred to E2s (UBCs or Ub-conjugating enzymes). Models based on the N-end rule E3s of yeast and mammals (1, 2) had E3s functioning primarily as docking proteins that recognize substrates and Ub-bound E2s, facilitating the transfer of Ub from E2 to substrate. However, more recent findings obtained with E6-AP (E6-associated protein) have demonstrated that, at least in some cases, Ub moieties bound to E3s through thiol-ester linkages represent the final intermediates in the ubiquitination cascade (11, 12). It remains to be determined whether the N-end rule E3s similarly form thiol intermediates with Ub.

Only one E1 enzyme has been characterized in mammals. In contrast, over 10 distinct E2s have been characterized in humans, as well as in yeast, and in the plant Arabidopsis thaliana (reviewed in Refs. 4 and 13). Since substrate specificity for ubiquitination appears to lie largely at the level of E3-substrate interactions, it is generally believed that there are a large number of E3s. This theory is supported by the partial purification of multiple distinct E3 activities from mammalian cells and other higher eucaryotes (Refs. 14–20; reviewed in Ref. 8).

cDNAs encoding three yeast E3s have been characterized (21–23). However, human E6-AP is the only characterized mammalian E3 for which the primary amino acid sequence is known (24). This E3 catalyzes p53 ubiquitination but does so only in the presence of viral E6 proteins from strains of human papilloma virus (HPV) that are high risk for the development of uterine cervical carcinomas (HPV-16 and HPV-18). E6-dependent ubiquitination is presumably responsible for the diminished levels of p53 characteristic of HPV-16- and HPV-18-infected cells (24, 25). No other cellular substrates for E6-AP have been identified.

The characterization of E6-AP led Huibregtse et al. (12) to identify several cDNAs in GenBankTM with deduced protein sequences homologous to the C terminus of E6-AP; they termed these regions HECT (homology to E6-AP carboxyl terminus) domains. The sine qua non of a HECT domain is a conserved cysteine residue that forms a thiol-ester with Ub. Among the HECT-encoding cDNAs was a single partial mouse cDNA termed Nedd-4, first identified by its differential expression in fetal relative to adult brain (26). Recently rat Nedd-4 was

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) U96636 (murine E6-AP) and U96635 (murine Nedd-4).

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The abbreviations used are: Ub, ubiquitin; HPV, human papilloma virus; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; GST, glutathione S-transferase; HA, hemagglutinin; MBP, maltose-binding protein; E6-AP, E6-associated protein.
E2 Interactions with Murine E6-AP and Nedd-4

cloned as a binding partner for the γ subunit of the amiloride-sensitive epithelial sodium channel using the yeast two-hybrid system (27). This association occurs through interactions of tryptophan-containing WW domains of Nedd-4 with proline-rich PY motifs within sodium channel subunits (27–29). In Liddle’s syndrome, an autosomal dominant form of hypertension characterized by increased activity and cell surface expression of sodium channels, mutations within genes encoding subunits of the sodium channel result in loss of PY motifs (30). This raises the intriguing possibility that this ion channel is regulated by Nedd-4-dependent ubiquitination. However, until now Nedd-4 has not been demonstrated to be a catalytically active E3. In the present study, we report the cloning of cDNAs encoding full-length murine Nedd-4 and murine E6-AP (mE6-AP) and determine that they are both catalytically active E3s. In addition, their subcellular localizations and their physical interactions with an E2 enzyme are investigated.

MATERIALS AND METHODS
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lated full-length cDNAs encoding mouse E6-AP and Nedd-4 from a mouse thymocyte library. mE6-AP has a predicted molecular mass of 99.4 kDa and a pI of 4.74 and exhibits 96.2 and 94.2% deduced amino acid similarity and identity, respectively, with human E6-AP (Fig. 1A). Unlike human E6-AP, an ATG in the context of a Kozak sequence (43) was identified within the murine E6-AP cDNA. In the 18-amino acid region (amino acids 396–413) implicated in the binding of HPV E6 to human E6-AP (25), a single conservative amino acid difference was found (Glu → Asp).

The full-length murine Nedd-4 (Fig. 1B) has a predicted molecular weight of 90.6 kDa and an estimated pI of 5.46. The full-length murine Nedd-4 clone exhibits 95.9% identity and 97.7% similarity with the rat Nedd-4 and shows 95.2% similarity and 90.1% identity with an unpublished GenBank™-registered human Nedd-4. The mouse, as well as the rat, Nedd-4 cDNA has a 67-amino acid gap relative to the deduced amino acid sequence of the human protein; this gap corresponds to one of the four WW domains in human Nedd-4. To determine if this difference is due to alternative splicing in the mouse, PCR amplification of first strand cDNA from multiple mouse tissues was performed across this region; however, no evidence of a longer product was found (not shown). Messenger RNA for both mE6-AP and p53 was found in all murine tissues.

Fig. 1. Protein sequences of murine E6-AP and Nedd-4. A, protein sequence alignment between mouse and human E6-AP. The putative E6 binding motif is underlined, with the crucial cysteine residue indicated by an asterisk. Dots indicate gaps in the protein sequence introduced to achieve optimum alignment. B, protein sequence alignment between mouse and human Nedd-4. WW domains (four in the human Nedd-4; three in mouse Nedd-4) are underlined, and the conserved cysteine residue is indicated by an asterisk. Potential nuclear localization sequences are located between amino acids 402 and 413 of mouse Nedd-4.
Examine mouse E6-AP mRNA was most abundant in brain, thymus, and, while Nedd-4 was found at the greatest levels in lung and kidney (not shown).

**mE6-AP and Nedd-4 are Ubiquitin Protein Ligases That Function with the Same Family of E2s**—To determine whether mE6-AP and Nedd-4 function as E3s, recombinant GST-E6-AP (amino acids 32–869 of mE6-AP) and GST-Nedd-4 (amino acids 52–777 of Nedd-4) were generated and assessed for their ability to catalyze the ubiquitination of bacterial cellular proteins (Fig. 2, A and B). Since multiple proteins serve as substrates in this assay, ubiquitinated species generally appear as a smear on anti-Ub immunoblots (32). In these experiments, wheat E1 (34) and a human E2 (UbcH5B) that functions with human E6-AP were utilized (32). As shown (Fig. 2A), Nedd-4 catalyzes ubiquitination in the presence of E1 and E2. Some weak E2-independent, E1-dependent Nedd-4-mediated ubiquitination is also observed. The GST fusion with mE6-AP (Fig. 2B) also exhibited a low level of E2-independent activity that was substantially enhanced by the addition of UbcH5B. We consistently observed that GST-E6-AP was less active than GST-Nedd-4. Whether this reflects differences in the activity of these two proteins, reflects issues of substrate specificity, or is a manifestation of differences in folding of these recombinant fusion proteins remains to be determined. While other studies have demonstrated thiol-ester formation involving HECT family proteins (11, 12), this provides the first evidence that a mammalian HECT family protein other than human E6-AP functions as a Ub protein ligase.

Members of the UbcH5 family of E2s, which are over 87% identical to each other, and a closely related A. thaliana E2, AtUBC8, all function with human E6-AP in protein ubiquitination, while a more distantly related E2, AtUBC1 does not (32, 44, 45). To determine whether Nedd-4 and mE6-AP exhibit specificity at the level of E2 interactions, equivalent amounts of thiol-ester-forming activity for each of these E2s were estimated as described (32) (not shown) and utilized in ubiquitination assays (Fig. 2, C and D). For both Nedd-4 and mE6-AP, UbcH5 family members and the closely related AtUBC8 all facilitated ubiquitination of bacterial cellular proteins, while AtUBC1 showed minimal or no activity. As expected, when p53 was used as a substrate in ubiquitination assays, mE6-AP catalyzed p53 ubiquitination in an E6-dependent manner, while Nedd-4 did not catalyze p53 ubiquitination either with or without added E6 (not shown).

**Differential Subcellular Localization of E6-AP and Nedd-4**—mE6-AP and Nedd-4 both manifest potential nuclear localization signals defined by the presence of clusters of basic amino acids (amino acids 413–417 of mE6-AP and amino acids 402–413 of Nedd-4) (46). To assess subcellular localization, constructs encoding full-length mE6-AP with an N-terminal Myc tag and full-length Nedd-4 with an HA tag were generated. Analysis of transfected COS-7 cells by immunofluorescence staining with antibodies specific for Myc and HA demonstrated a mixed nuclear-cytosolic distribution for mE6-AP. In contrast, Nedd-4 exhibits a pattern consistent with distribution in the cytosol and not the nucleus (Fig. 3). To substantiate these observations, nuclear and cytosolic fractions were prepared from transfected COS-7 cells and analyzed by immunoblotting (Fig. 4). In accord with the immunofluorescence results, Nedd-4 was found only in the cytosol (Fig. 4, lanes 9 and 10), while mE6-AP was detected both in the nucleus and cytosol (Fig. 4, lanes 3 and 4). A Myc-tagged cytosolic protein tyrosine kinase, ZAP-70 (47, 48), employed as a control, demonstrated a cytosolic distribution pattern. The finding of Nedd-4 in the cytosol is consistent with a putative role in regulating sodium channel activity (27), while the presence of nuclear mE6-AP is in accord with an in vivo role for this protein in catalyzing E6-mediated p53 ubiquitination (49).

**Interactions between Marine E6-AP and UbcH5B**—To evaluate the molecular basis for interactions between E2s and E3s, the binding of recombinant UbcH5B-derived proteins to GST-E3 fusion proteins was assessed (see Fig. 5 for schematic representation of constructs and Table I for summary of binding studies). GST-E6-AP was incubated with either full-length UbcH5B or an equivalent amount of a truncated UbcH5B containing only the first 67 amino acids of this E2 (nUbcH5B). Bound material was detected by immunoblotting with an antisera that recognizes the NH2 terminus of UbcH5B (Fig. 6A). UbcH5B bound specifically to GST-E6-AP; however, no specific
binding of nUbcH5B was detected. To detect associations between the carboxyl half of UbcH5B and GST-E6-AP, a chimeric protein was generated in which the maltose-binding protein (MBP) was placed upstream of amino acids 68–147 of UbcH5B (MBP-cUbcH5B). MBP-cUbcH5B bound GST-E6-AP, while MBP itself bound to neither GST alone nor to GST-E6-AP (Fig. 6B). To further characterize this association, truncated forms of mE6-AP were generated. UbcH5B and MBP-cUbcH5B bound to a GST fusion protein in which the 190 C-terminal amino acids of E6-AP, including the region implicated in catalytic activity, were deleted (GST-E6-AP679) (Fig. 6C, lane 3). More severely truncated fusions of GST with mE6-AP bound neither UbcH5B nor MBP-cUbcH5B (Fig. 6C).

In contrast to E6-AP, the amino (nUbcH5B) and carboxyl (MBP-cUbcH5B) halves of UbcH5B as well as full-length UbcH5B specifically bound GST-Nedd-4 (Fig. 7A, lanes 6 and 10, and Fig. 7B, lane 6). These associations were further eval-
uated using constructs encoding GST fusions with either amino acids 52–422 of Nedd-4 (GST-Nedd-4-N) or amino acids 423–777 of Nedd-4 (GST-Nedd-4-C). UbcH5B bound to both of these regions of Nedd-4 (Fig. 7A, lanes 7 and 8), suggesting the existence of at least two E2 binding sites within Nedd-4. Confirmation of these dual E2 binding sites comes from the findings that nUbcH5B bound to GST-Nedd-4 and GST-Nedd-4-N but not to GST-Nedd-4-C (Fig. 7A, lanes 10–12), while MBP-cUbcH5B associated specifically with GST-Nedd-4 and GST-Nedd-4-C but not GST-Nedd-4-N (Fig. 7B, lanes 6–8). Alignment of the E6-AP and Nedd-4 constructs that bind MBP-cUbcH5B implicates a region corresponding to amino acids 521–679 of mE6-AP and 423–583 of Nedd-4 as a site of interaction. Further confirmation of the ability of this region to bind the C-terminal half of UbcH5B was provided by the generation of a truncated form of GST-Nedd-4-C in which the C-terminal 116 amino acids, including the highly conserved region implicated in catalytic activity of HECT family E3s, was deleted (GST-Nedd-4-CT). As shown (Fig. 7C), both the full-length (UbcH5B) and the C-terminal (MBP-cUbcH5B) proteins bound to GST-Nedd-4-CT (amino acids 423–670 of Nedd-4), while nUbcH5B did not.

These results establish the existence of a discrete, conserved site of interaction between the C terminus of UbcH5B and at least two members of the HECT family of E3s. This binding requires neither Ub, E1, nor an intact E3 catalytic domain. Because GST-Nedd-4-C contains both an E2 binding site and the portion of Nedd-4 implicated in the catalytic activity of HECT family E3s, we evaluated whether this fusion protein has the ability to catalyze the ubiquitination of bacterial cellular proteins. Neither GST-Nedd-4-C nor GST-Nedd-4-N had detectable catalytic activity (Fig. 7D). Thus, while an E2 binding site and the conserved catalytic domain are found within the C-terminal half of Nedd-4, additional sequences, possibly including the second E2 binding site, are required for Nedd-4 to function as a ubiquitin protein ligase.

**DISCUSSION**

We have cloned and characterized two widely expressed murine ubiquitin protein ligases that interact with a family of core E2 enzymes. Consistent with an in vivo role in p53 ubiquitination, mE6-AP is found in the nucleus as well as the cytosol. We have determined that Nedd-4, first identified as a partial cDNA differentially expressed in developing brain (26), also functions as a Ub protein ligase. Nedd-4 shows no activity toward p53, and, in contrast to mE6-AP, Nedd-4 was not detected in the nucleus.

The only previous demonstration of a physical interaction between an E2 and E3 came from work on UBR1, the N-end rule E3 of S. cerevisiae (50). In that study, UBR1, which is structurally unrelated to Nedd-4 and E6-AP, co-purified with ScUBC2 when yeast lysates expressing each of these enzymes were mixed. We have established the existence of two E2 binding sites within Nedd-4. One of the two sites within Nedd-4 binds the C-terminal half of UbcH5B (cUbcH5B), while the other binds the N-terminal half of this E2 (nUbcH5B). A cUbcH5B binding site was also found within mE6-AP, and while no association between nUbcH5B and mE6-AP was found, this does not preclude a physiologically relevant interaction not detectable by in vitro binding. Since all of the proteins used in these studies were expressed in E. coli, these E2-E3 interactions do not require Ub, E1, or other known
When mE6-AP and Nedd-4 are aligned, the region common to all of the recombinant proteins that bind cUbcH5B (amino acids 522–680 of mE6-AP; 423–582 of Nedd-4) exhibits 40% identity (Fig. 8). Notably, this region does not encompass the C-terminal 100 amino acids of these E3s, which includes the catalytic cysteine. Three other HECT family proteins, a rat 100-kDa protein, S. cerevisiae Rsp5, and Schizosaccharomyces pombe Pub1 have been demonstrated to form thiol-ester bonds with Ub (11, 12, 22). Two of these, Rsp5 and Pub1, have been shown to be enzymatically active E3s (12, 22). When mE6-AP, Nedd-4, Rsp5, and Pub1 are aligned (Fig. 8), the region implicated in binding cUbcH5B demonstrates 27% identity, not substantially different from the C-terminal 100 amino acids that surround the catalytic cysteine (30% identity). By comparison, the more NH2 regions of these four E3s exhibit no significant identity. While the rat 100-kDa protein also exhibits substantial homology with Nedd-4 and mE6-AP in the region implicated in E2 binding, this rat protein also has insertions of 9, 32, and 8 amino acids in this part of the protein (not shown). Since there is little information available on this rat protein other than the fact that it forms E2-dependent thiol-esters with Ub, it is premature to speculate on the significance of these differences.

As already alluded to, Nedd-4 also manifests a second E2 binding site. This binding site for cUbcH5B is located between amino acids 55 and 422 of Nedd-4. While it is possible that in vitro one E2 molecule binds Nedd-4 through two distinct contact points, the fact that the interactions of GST-Nedd-4 fusion proteins with cUbcH5B and nUbcH5B are each of sufficient strength to be detected in an in vitro binding assay makes it likely that Nedd-4 has the capacity to simultaneously bind two E2 molecules. The existence of these two binding sites within an E3 is complemented by genetic studies in S. cerevisiae, where multiple E2s participate in the ubiquitination of the MATa2 repressor (52), by findings from the yeast two-hybrid system indicating hetero- and homotypic interactions of E2 molecules (52, 53), and by cross-linking studies (54). Taken together with our observations, these findings indicate that E2-E2 interactions may occur in the context of E3-containing complexes and open for consideration models in which multiple E2s cooperate in the generation of protein-bound chains of Ub.

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