Biochemical Analysis of Angelman Syndrome-associated Mutations in the E3 Ubiquitin Ligase E6-associated Protein*

Angelman syndrome is a severe neurological disorder characterized by mental retardation, absent speech, ataxia, seizures, and hyperactivity. The gene affected in this disorder is UBE3A, the gene encoding the E6-associated protein (E6AP) ubiquitin-protein ligase. Most patients have chromosomal deletions that remove the entire maternal allele of UBE3A. However, a small subset of patients have E6AP point mutations that result in single amino acid changes or short in-frame deletions that still allow translation of a full-length protein. By studying these point mutations in E6AP, we found a strong correlation between Angelman-associated mutations and a loss of E3 ubiquitin ligase activity. Interestingly, the point mutations affect E6AP activity in different ways. Some mutant proteins cannot form thiol ester intermediates with ubiquitin, others retain the thiol ester formation activity but cannot efficiently transfer ubiquitin to a substrate, and still others are unstable in cells. Our results suggest that the loss of E6AP catalytic activity and likely the improper regulation of E6AP substrate(s) is important in the development of Angelman syndrome.

Although the deletion of other genes in 15q11-13, such as the γ-aminobutyric acid neurotransmitter receptor β genes, may contribute to the severity of Angelman symptoms (7), the identification of UBE3A point mutations in numerous patients provides the most compelling evidence for E6AP being the Angelman gene (2, 3). The majority of these mutations result in frameshifts and/or premature truncations, but some are missense mutations or short in-frame deletions that still allow a translation of a full-length (or nearly full-length) protein (8, 9).

E6AP was initially identified as the cellular factor that cooperates with the human papillomavirus E6 oncoprotein to stimulate the ubiquitin-mediated degradation of the tumor suppressor p53 (10). In this process, the E6-E6AP complex comprises the E3 ubiquitin ligase activity in a ubiquitin thiol ester cascade (11). E6AP was the first identified member of a family of ubiquitin ligases called heat proteins, all of which contain a domain homologous to the E6AP carboxyl terminus (12). These are modular proteins in which the conserved heat domains catalyze ubiquitin transfer, whereas other divergent domains confer substrate specificity.

Because E6AP is a ubiquitin ligase, it is likely that the improper regulation of E6AP substrate(s) causes Angelman syndrome, although no disease-relevant E6AP targets have yet been identified. Recent structural data show that several of the Angelman-associated point mutations actually map to the E6AP catalytic cleft (13) and may affect E6AP ubiquitin ligase activity. Other Angelman-associated point mutations map to the E6AP amino terminus and are outside the catalytic domain. These mutations might affect E6AP in a manner distinct from ubiquitin transfer perhaps by compromising substrate binding, altering E6AP subcellular localization, or destabilizing the protein. To determine whether the loss of E6AP catalytic activity correlates with Angelman syndrome, we examined the activities of the non-truncating point mutations identified in Angelman patients.

MATERIALS AND METHODS

Protein Purification—His6-E6AP (wild type and mutant) baculoviruses were generated using the BaculGold system (Pharmingen) according to the manufacturer’s instructions. E6AP proteins were purified from two T175 flasks (Corning) of baculovirus-infected Hi5 cells. Forty-eight hours after infection, cells were harvested and lysed in Nonidet P-40 lysis buffer (% Nonidet P-40, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5 mM DTT, 50 mM NaF, 0.2 mM Na3VO4, and protease inhibitor mixture (Pharmingen)). Lysates were rotated for 30 min at 4 °C and cleared by centrifugation (25,000 × g for 30 min at 4 °C). Imidazole was added to a final concentration of 10 mM, and the lysates were incubated with a 200-μl bed volume of Ni2+-NTA (Qiagen) for 2 h at 4 °C. Beads were washed with 20 column volumes of 10 mM imidazole wash buffer (50 mM Tris (pH 8.0), 300 mM NaCl, 10 mM imidazole, 0.5 mM DTT) and 20 column volumes of the same buffer containing 20 mM imidazole and eluted in 0.1-M fractions of the same buffer containing 250 mM imidazole. Fractions were pooled, dialyzed against a buffer containing 25 mM Tris-HCl (pH 7.6), 50 mM NaCl, 10% glycerol, and...
Chromatography column equilibrated with 50 mM Tris (pH 7.6), 1 mM UbcH7 was further purified on a Superdex 200 fast protein liquid chromatography column equilibrated with TDE containing increasing concentrations of NaCl, and UbcH7-containing fractions were concentrated in a Centricon YM-10 device (Amicon). TDE containing increasing concentrations of NaCl, and UbcH7-containing fractions were pooled, concentrated as above, and frozen in 10% glycerol. E1 was purchased from Boston Biochem Inc. Ubiquitin was purchased from Sigma.

GST-hect proteins were expressed in *E. coli* strain BL21. Midlog phase cultures (500 ml) were induced with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside for 16 h at 15 °C. Lysates were incubated at room temperature (30 °C) for 90 min and stopped in Laemmli buffer.

For assays using *in vitro* translation, E6AP constructs were excised from pcDNA4 (Invitrogen) with or without [35S]methionine and [35S]cysteine (PerkinElmer Life Sciences) using the Tnt-coupled rabbit reticulocyte lysate system (Promega). Assays were done in 50-μl volumes containing 25 mM Tris, pH 7.8, 50 mM NaCl, 8 mM ATP, 10 mM MgCl2, 0.2 mM DTT, 2 μl of [35S]cysteine for 1 h before being pulsed with the same medium supplemented with [35S]methionine and cysteine for 0, 2, 4, and 8 h. Cells were harvested in 500 μl of Nonidet P-40 lysis buffer (prepared as above), and protein concentrations were determined using the Bradford assay reagent (Bio-Rad). 500 μg of each lystate were immunoprecipitated with anti-E6AP anti-serum, resolved by 8% SDS-PAGE, and detected by autoradiography.

**Plasmid Preparations**—A plasmid for E6AP isoform III in the pGEM-1 vector has been described (17). All mutants were generated by the QuickChange method (Stratagene) using E6AP/pGEM-1 as a template. Primers used for mutagenesis are available by request. E6AP and mutants were excised from pGEM-1 by digesting with BamHI and HindIII. The fragments were ligated into BamHI/HindIII-digested pPHN (Invitrogen). The NotI/Phnl fragment was then ligated into pAC-HLT-A (Pharmingen) for baculovirus production. For mammalian expression, pPHN was digested with SpeI and in vitro translated, equilibrated with TDE. The flow-through was then loaded onto S Sepharose FF (Amersham Biosciences) equilibrated with TDE. UbcH7 was eluted stepwise with TDE containing increasing concentrations of NaCl, and UbcH7-containing fractions were concentrated as above, and frozen in 10% glycerol. E1 was purchased from Boston Biochem Inc. Ubiquitin was purchased from Sigma.

In vitro Ubiquitylation Assay—HHR23A was translated in vitro using the TNT-coupled rabbit reticulocyte lysate system (Promega). Assays were done in 50-μl volumes containing 25 mM Tris, pH 7.6, 1 mM DTT, and 125 mM NaCl. UbcH7-containing fractions were pooled, concentrated as above, and frozen in 10% glycerol. E1 was purchased from Boston Biochem Inc. Ubiquitin was purchased from Sigma.

**In Vitro Translation**—GST-hect proteins were expressed in *E. coli* strain BL21. Midlog phase cultures (500 ml) were induced with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside for 16 h at 15 °C. Lysates were incubated at room temperature (30 °C) for 90 min and stopped in Laemmli buffer.

For assays using *in vitro* translated E6AP, His6-E6AP was translated from pcDNA4 (Invitrogen) with or without [35S]methionine and [35S]cysteine (PerkinElmer Life Sciences) using the Tnt-coupled wheat germ extract system (Promega). Assays were done in 50-μl volumes containing 25 mM Tris, pH 7.8, 50 mM NaCl, 8 mM ATP, 10 mM MgCl2, 0.2 mM DTT, 2 μl of [35S]cysteine for 1 h before being pulsed with the same medium supplemented with [35S]methionine and cysteine for 0, 2, 4, and 8 h. Cells were harvested in 500 μl of Nonidet P-40 lysis buffer (prepared as above), and protein concentrations were determined using the Bradford assay reagent (Bio-Rad). 500 μg of each lystate were immunoprecipitated with anti-E6AP anti-serum, resolved by 8% SDS-PAGE, and detected by autoradiography.

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Wild type E6AP hect domain with an amino-terminal MYC or HA tag was generated from the E6AP/pGEM-1 template by PCR and encoded from residue 491 to the end (according to E6AP isoform I) of the hect domain. The amplified products were digested with BamHI/SmaI and ligated into pET23a (Novagen). The MYC-tagged CS20A and Angelman mutant fragments were amplified from the E6AP/pPhnl templates by PCR, cut with BamHI/SmaI, and ligated into the BamHI/HinII sites of pET23a (Novagen) and the BamHI/SmaI sites of pGEX 6P-1 (Amersham Biosciences) for *in vitro* translations and bacterial expression, respectively. Mutants were confirmed by sequencing.

For stable cell lines, all E6AP constructs were cloned as follows. The BamH1/NotI fragments were excised from the pDNA4 constructs described above and ligated into the BamH1/NotI sites of pBluescript (Stratagene). The Sau3A/NotI fragments were then ligated to the Xhol/NotI sites of pGEX-C, which also contains the interleukin-2 receptor gene under control of an internal ribosomal entry site. Cells were transduced with retroviruses, and stably expressing cells were sorted using magnetic beads (Dynal) coated with anti-interleukin-2 antibody.

**Yeast Two-hybrid Assays**—Yeast two-hybrid assays were performed essentially as described previously (18). Briefly E6AP constructs were cloned into the SacI/NotI sites of pG3/7 such that they were fused in frame to the GAL4 DNA binding domain. HHR23A and UbcH7 cloned into pCB6 and fused in-frame to the GAL4 activation domain have been described previously (18). Yeast were co-transformed with two of the plasmids by the lithium acetate procedure and selected by plating on SD-Leu Trp . Single colonies were then grown in SD-Leu Trp broth to midlog phase, and aliquots in 10-fold dilutions were spotted on both SD-Leu Trp and SD-Leu Trp His .

**E2 Binding/Competion Assay**—Bovine ubiquitin (Sigma) was biotinylated using the BiotinTag microbiotination kit (Sigma) according to the manufacturer's instructions. Approximately 0.5 μg of GST-HA-hect wild type (WT) was used in ubiquitylation assays performed as described above with the following exceptions: the ubiquitin was biotinylated, and the reaction time was 10 μl at 30 °C, 90 min, and the assays were performed in the presence of increasing amounts of competitor protein, 0.3, 1, or 3 μg of bovine serum albumin or MYC-heat (WT or mutant) in which the GST was removed by cleavage with Precision protease (Amerham Pharmacia). Reactions were stopped in Laemmli buffer and resolved by SDS-PAGE, and proteins were visualized with Extravidin-peroxidase (Sigma) and anti-MYC antibody (Oncogene, Cambridge, MA).

**RESULTS**

There Is a Strong Correlation between Angelman-associated Mutations and the Loss of E6AP Ubiquitin Ligase Activity—E6AP ubiquitylation activity consists of several discrete steps. E6AP must bind to an E2 enzyme, accept ubiquitin from the E2 in the form of a thiol ester linkage, and transfer the ubiquitin to a substrate (14). We first tested whether the Angelman-associated E6AP point mutants could ubiquitylate a model substrate, reasoning that E6AP mutants that retained this activity must be able to bind to E2 enzymes and form ubiquitin thiol esters. Mutants impaired in substrate ubiquitylation were then studied further to determine the biochemical activity affected by the mutation.

To examine E6AP ubiquitin ligase activity, we performed *in vitro* ubiquitylation assays using full-length wild type and mutant E6AP proteins purified from insect cells (Fig. 1). The *in vitro* substrate was HHR23A, which had been identified previously as an E6AP-interacting protein in a yeast two-hybrid
Radiolabeled HHR23A was generated by in vitro translation in wheat germ extract, which lacks endogenous E6AP activity (20). The addition of wild type E6AP to the reaction induced the formation of a high molecular weight ladder of HHR23A (Fig. 1A, lane 2), whereas addition of the catalytically inactive (C820A) E6AP (lane 3). A, Angelman-associated mutations affect E6AP catalytic activity. Ubiquitylation reactions were performed as above, using the indicated insect cell-expressed wild type or mutant E6AP proteins. Amount of E6AP used in each reaction is indicated in the immunoblot. B, Angelman-associated mutations are impaired in E6AP-dependent ubiquitylation of HHR23A. Ubiquitylation reactions were performed as above, using the indicated insect cell-expressed wild type or mutant E6AP proteins. Amount of E6AP used in each reaction is indicated in the immunoblot. C, Non-hect domain E6AP mutation S349P is inactive in HHR23A ubiquitylation. In vitro translated His6-tagged 35S-labeled or cold E6AP proteins were purified by anion exchange followed by affinity chromatography on Ni2+-NTA. Purified E6AP was incubated with 35S-labeled HHR23A under standard ubiquitylation conditions. Lane 1, unpurified rabbit reticulocyte lysate subjected to identical purification scheme lacks an activity that ubiquitylates HHR23A. The asterisk denotes a background band. In vitro translated wild type E6AP ubiquitylates HHR23A (lanes 3–5), whereas catalytically inactive (C820A) (lanes 7–9) and Angelman-associated S349P (lanes 11–13) mutants do not. The amount of cold E6AP used was equivalent to the higher concentration of 35S-labeled E6AP. All reactions were stopped in Laemmli buffer, resolved by 10% SDS-PAGE, and visualized by autoradiography. Ub'd, ubiquitylated; Ub, ubiquitin; Unprog RRL, unprogrammed rabbit reticulocyte lysate.
point mutants to the wild type protein (Fig. 1B), we performed ubiquitylation assays using the amount of E6AP equivalent to the lowest concentration of wild type E6AP needed to catalyze the conversion of 99% of the HHR23A starting material into ubiquitylated forms. The positions of the mutants are shown schematically in Fig. 1B. Most of the Angelman-associated mutations lie within the carboxyl-terminal hect domain of the protein, whereas two lie in the non-catalytic amino-terminal portion. We predicted that the former mutations might affect E6AP enzymatic activity, whereas the amino-terminal mutations might affect substrate binding, subcellular localization, or protein stability.

The crystal structure of the hect domain reveals it to be an L-shaped molecule consisting of two lobes, the interface of which forms the catalytic cleft (13). Two Angelman-associated mutations, L502P and E550L, lie in the amino-terminal lobe of the hect domain and are entirely inactive in HHR23A ubiquitylation (Fig. 1B, lanes 6 and 7). Glu-550 forms a salt bridge with a nearby arginine residue (13); therefore, the E550L mutation may affect a critical structural element of the hect domain.

The other mutations map to the carboxyl-terminal lobe of the hect domain, two of which were also severely impaired in HHR23A ubiquitylation. They are the F782C mutation (Fig. 1B, lane 9), which removes a residue from the hydrophobic core of the hect domain (13), and an insertion at residue Lys-836 (Fig. 1B, lane 10). The latter mutation introduces a frameshift downstream of the catalytic cysteine residue (Cys-820) that inserts several amino acids and prematurely truncates much of the carboxyl-terminal α-helix of the protein.

Two other hect domain mutations consistently had partial defects in HHR23A ubiquitylation. One is the in-frame deletion of Lys-801 (Fig. 1B, lane 4), a mutation that has been identified in two unrelated patients, and the other is a missense mutation that changes Ile-804 for a lysine (I804K) (Fig. 1B, lane 8). Another patient possesses a mutation causing the insertion of an isoleucine at position 803 (I803ins). This mutation had no discernible effect on E6AP-mediated HHR23A ubiquitylation (Fig. 1B, lane 5).

The remaining two mutations reside in the non-catalytic amino terminus of E6AP. One mutant, C21Y, retained wild type activity in the HHR23A ubiquitylation assay (Fig. 1B, lane 3) whereas the other, S349P, was largely insoluble when expressed in insect cells. As an alternative source of E6AP S349P, we translated a His6-tagged version in rabbit reticulocyte lysate subjected to the same purification scheme lacked E6AP activity (Fig. 1C, lane 5). However, His6-E6AP S349P, however, was severely impaired in its ability to ubiquitylate HHR23A (Fig. 1C, lanes 11–13), identifying it as the first non-hect domain mutation to affect E6AP catalytic activity. The defect was not due to a loss of binding between E6AP S349P and HHR23A because they still interacted in a yeast two-hybrid experiment (see Fig. 3B).

Overall there was a strong correlation between the loss of substrate ubiquitylation activity and the occurrence of Angelman syndrome-associated E6AP mutations (Table I).

**TABLE I**

| Non-hect mutations | HHR23A ubiquitylation | Thiol ester formation | E2 binding | Stability in cells |
|--------------------|------------------------|-----------------------|------------|-------------------|
| C21Y               | Active                 | Yes                   | Yes        | <30 min           |
| S349P              | Inactive               | Yes                   | Yes        | 4 h               |

Hect mutations

| L502P              | Partial                | Yes                   | Yes        | >8 h              |
| E550L              | Inactive               | Yes                   | Yes        | >8 h              |
| L803ins            | Inactive               | No                    | No         | >30 min           |
| K801Δ              | Partial                | Yes                   | Yes        | >8 h              |
| I804K              | Partial                | Yes                   | Yes        | >8 h              |
| F782C              | Inactive               | No                    | No         | >30 min           |
| K836ins            | Inactive               | No                    | No         | >30 min           |

In vitro translated His8-E6AP S349P, however, was severely impaired in its ability to ubiquitylate HHR23A (Fig. 1C, lanes 11–13), identifying it as the first non-hect domain mutation to affect E6AP catalytic activity. The defect was not due to a loss of binding between E6AP S349P and HHR23A because they still interacted in a yeast two-hybrid experiment (see Fig. 3B).

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**Of the Ubiquitylation-defective Mutants, Two Are Unable to Form Thiol Esters, Whereas Others Retain the Ability to Do So—E6AP forms a covalent thiol ester intermediate between its catalytic cysteine residue and the carboxyl-terminal glycine of ubiquitin prior to transferring the ubiquitin to a substrate (14). To qualitatively determine whether the defect in the ubiquitylation-defective Angelman-associated mutants was in this activity, we tested them in an in vitro thiol ester formation assay. We used in vitro translated, radiolabeled hect domains, which are sufficient for thiol ester formation (22). The wild type and mutant hect domains were incubated under standard ubiquitylation conditions in the presence of GST-ubiquitin and an-synthesized centromeric hecat domain (13), and an insertion at residue Lys-836 (Fig. 1B, lane 10). The latter mutation introduces a frameshift downstream of the catalytic cysteine residue (Cys-820) that inserts several amino acids and prematurely truncates much of the carboxyl-terminal α-helix of the protein.

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Unprogrammed rabbit reticulocyte lysate subjected to the same purification scheme lacked activity (Fig. 1C, lane 1), but His8-wild type E6AP efficiently ubiquitylated both itself and the HHR23A substrate (Fig. 1C, lanes 3–5). Ubiquitylated wild type E6AP appeared as a slightly slower migrating form, due to the addition of one ubiquitin, and a high molecular weight smear, indicative of polyubiquitylation. The appearance of both the monoubiquitylated form and the smear required that ubiquitin be supplemented in the reaction (data not shown), indicating that these are indeed ubiquitylated forms. Furthermore the high molecular smear could be collapsed to approximately five distinct species by the addition of methylated ubiquitin (data not shown). This indicates that the smear is due to polyubiquitylation (21) and that E6AP places ubiquitin on approximately five of its own lysine residues. The HHR23A substrate was modified by several ubiquitins, most clearly indicated by including a reaction in which His8-E6AP WT was translated in the presence of cold methionine so that HHR23A was the only radiolabeled protein (Fig. 1C, lane 5) in the reaction.

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As expected, mutations that caused either no or partial effects on substrate ubiquitylation (i.e. I803ins, K801Δ, and I804K) retained the ability to form thiol ester intermediates with ubiquitin (data not shown). Two of the Angelman-associated point mutants that were completely inactive in HHR23A ubiquitylation, the in-frame Phe-782 deletion (Fig. 1B, lane 9) and the carboxy-terminally truncating K836ins mutation (Fig. 1B, lane 10) did not form detectable thiol ester intermediates (Fig. 2, C and D), indicating that these mutations either impaired the ability of the hect domain to bind to the E2 enzyme or to efficiently receive ubiquitin from it.

Surprisingly two other ubiquitylation-defective mutants,
L502P and E550L, formed hect-GST-ubiquitin thiol esters as well as wild type (Fig. 2, E and F), indicating that these mutations might impair the transfer of the thiol ester-linked ubiquitin to the substrate. We next considered the fate of the ubiquitin during the ubiquitylation assay. If these mutants formed thiol esters with ubiquitin but did not transfer them to the substrate, where did the ubiquitin go? One possibility was that the ubiquitin remained “trapped” on the E6AP catalytic cysteine, causing an apparent accumulation of thiol ester-linked adducts. However, the ubiquitin-thiol ester adducts on the L502P hect domain did not accumulate any more than on the wild type hect domain (Fig. 2) even at later time points (data not shown), and those on hect E550L actually diminished slightly after 2 and 4 min (Fig. 2E). Because there was no obvious “trapping” of the thiol ester-linked ubiquitin on these mutants, the thiol ester bond must have been either hydrolyzed or the ubiquitin passed to an acceptor other than the HHR23A substrate, perhaps E6AP itself.

To address the latter possibility, we performed HHR23A ubiquitylation assays using in vitro translated 35S-labeled E6AP so we could visualize both the enzyme and substrate simultaneously. For each E6AP construct tested, one reaction was performed in which HHR23A was the only radiolabeled protein to highlight the bands which correspond to ubiquitylated HHR23A (Fig. 3A, lanes 2 and 3 and lanes 6 and 7, respectively). The L502P mutant, however, ubiquitylated itself in a hyperactive and highly processive manner (Fig. 3A, lanes 10 and 11). The hyperactivity was intrinsic to this mutant because it still occurred even when no substrate was added (data not shown). Together the data indicate that the defect caused by the E550L and L502P mutations was not in catalyzing isopeptide linkages per se because the proteins still attached ubiquitins to themselves; instead the defect was specifically in the transfer of ubiquitin to the substrate.

To rule out the possibility that E6AP E550L and L502P had simply lost the ability to bind to HHR23A, these mutants were tested in a yeast two-hybrid assay (Fig. 3B). This was the original experiment that identified this interaction (18) and remains the most reliable method to detect binding between these proteins. An interaction is denoted by growth on plates lacking leucine, tryptophan, and histidine. Both E6AP L502P and E550L scored in this assay, stimulating growth of the yeast on these plates equivalent to that of the wild type E6AP. This indicates that the mutations do not affect the interaction between E6AP and HHR23A. Moreover Fig. 3B, bottom, shows that the isolated hect domain (in which the L502P and E550L mutations reside) is not sufficient for binding to HHR23A in this assay. This suggests that the interaction with HHR23A is mediated by a non-hect domain portion of E6AP, providing additional evidence that the L502P and E550L mutations do
not affect HHR23A binding. The relative levels of GAL4 DBD-E6AP fusion proteins expressed in the yeast are shown in Fig. 3B. The levels of E6AP S349P are lower than that of the other E6AP proteins likely because the protein has a shorter half-life than wild type (see Fig. 5D) when expressed in cells; however, the mutant still scored for binding to HHR23A, indicating the sensitivity of the assay.

**Mutants That Cannot Form Thiol Ester Intermediates with Ubiquitin Do Not Functionally Interact with the E2 Enzymes**—The defect in the Angelman-associated mutants (the F782Δ and K836ins mutants) that could not form thiol ester intermediates with ubiquitin must either be in binding to the ubiquitin-conjugating enzyme or in receiving ubiquitin from it. To discriminate between these possibilities, we first tested for direct binding between bacterially purified hect domains and the ubiquitin-conjugating enzyme UbcH7. We could not reliably detect an interaction by pull-down experiments or gel filtration under the conditions we used likely because of the lower affinity of E3 ligases for uncharged ubiquitin-conjugating enzymes (23, 24).

As an alternative to the pull-down assay, we examined the abilities of the mutant hect domains to compete with the wild type for binding to E2 enzymes in a self-ubiquitylation experiment. When wild type GST- hect was incubated with E1, UbcH7, ubiquitin, and ATP, it efficiently ubiquitylated itself (Fig. 4A, lane 1). However, when excess wild type hect protein (from which the GST moiety had been cleaved so that it would migrate distinctly from the GST- hect by SDS-PAGE) was in-
Angelman-associated Mutations Affect E6AP Catalytic Activity

Fig. 4. Two Angelman-associated mutations impair E2 binding. A, bacterially expressed GST-HA hect WT was incubated with E1, UbcH7, and biotinylated ubiquitin in the absence (lane 1) or presence (lanes 2–9) of increasing amounts of the following competitor proteins: MYC-E6AP hect WT (lanes 2 and 3), MYC-E6AP hect AF782 (lanes 4 and 5), MYC-E6AP hect K836ins (lanes 6 and 7), or bovine serum albumin (BSA) (lanes 8 and 9). The GST moieties were cleaved from the competitor hect proteins so they would migrate distinctly from the GST- hect protein. Reactions were performed for 30 min at 30 °C, stopped in Laemmli buffer, resolved by 10% SDS-PAGE, then probed with Extravidin-peroxidase to detect the ubiquitylated conjugates, and immunoblotted with anti-MYC antibody to detect the competitor hect proteins. Mono- ubiquitylated MYC-hect conjugates are detectable on the MYC-tagged wild type hect domains in lanes 2 and 3. B, yeast two-hybrid experiments performed essentially as described in Fig. 3B. The exceptions are that yeast were co-transformed with plasmids encoding UbcH7 fused to the GAL4 activation domain and one of the E6AP mutants shown fused to the GAL4 DNA binding domain. Ub, ubiquitin; CA, C820A. The asterisk denotes a cross-reactive protein that serves as a loading control.

included in the reaction, self-ubiquitylation of the GST-hect protein greatly diminished, whereas self-ubiquitylation of the smaller, untagged hect protein concomitantly increased (Fig. 4A, lanes 2 and 3). Unlike the addition of wild type hect protein, excess hect AF782A or K836ins had no effect on GST-hect self-ubiquitylation (Fig. 4A, lanes 4–7), indicating that these mutants could not compete effectively with the GST-hect protein for binding to the E2. Adding the nonspecific competitor bovine serum albumin had no inhibitory effect on the degree of self-ubiquitylation of the GST-hect protein (Fig. 4A, lanes 8 and 9). Because the F782A and K836ins mutations do not map directly to the region shown to be involved in binding to UbcH7 (14), it is possible these mutations affect the overall folding or stability of the hect domain.

Next we performed a yeast two-hybrid assay as an additional test of the binding between these two mutants and UbcH7 (Fig. 4B). This assay has been used previously to examine the E6AP/ubiquitin-conjugating enzyme interaction (19). Neither of the mutants scored in the two-hybrid assay, suggesting that they do not interact with UbcH7. The levels of E6AP AF782A and K836ins protein were lower than that of the C820A mutant in the yeast likely because they have very short half-lives when expressed in cells (around 30 min compared with >8 h, Fig. 5, G and H). However, the K836ins mutant was expressed at least as well as the E6AP S349P shown in Fig. 3B that scored for binding in the experiment. Although the levels of E6AP AF782A were also low, the complete lack of growth of the yeast suggests that this mutation also affects binding to UbcH7. Together the two-hybrid results and the competition assay are consistent with the finding that these mutations abrogate the interaction of E6AP with UbcH7.

Certain Angelman-associated Mutations Affect the Stability of E6AP in Cells—It remained possible that the Angelman-associated mutations could affect the stability of E6AP in cells. To address this, we performed pulse-chase experiments to measure the half-lives of the Angelman-associated mutant E6AP proteins. To assure that we could discriminate the mutant from the endogenous E6AP protein, we reconstituted E6AP−/− mouse embryonic fibroblasts with wild type or mutant E6AP. Wild type (Fig. 5A) E6AP was stable over the course of the 8-h chase period in agreement with previously published data (25). Note that in vitro translated E6AP was run on the same gel to indicate the position of the unmodified E6AP.

Several of the Angelman-associated mutants were degraded within 30 min after synthesis. These include the F782A and K836ins mutants (Fig. 5, G and H), respectively, which did not interact with UbcH7, the 1804K mutant (Fig. 5F), which had partial activity in the HHR23A ubiquitylation assay (Fig. 1B, lane 8), and the non-hect domain C21Y mutant (Fig. 5C), which displayed wild type activity in the in vitro ubiquitylation assay (Fig. 1B, lane 3). These results suggest that all these mutations may affect E6AP protein folding.

The other non-hect domain Angelman-associated mutant, S349P, had a half-life of approximately 3 h (Fig. 5D), which is shorter than that of the wild type. This mutant does not ubiquitylate HHR23A in vitro, so both its loss of catalytic activity and decreased half-life may contribute to the E6AP enzyme defects in the Angelman syndrome patient with this mutation.
Because the L502P mutant self-ubiquitylated so efficiently in vitro, we expected it to be less stable than wild type E6AP when expressed in cells. Consistent with the in vitro data, E6AP L502P was self-ubiquitylated much more than wild type (note the multiple polypeptides in Fig. 5F as opposed to in Fig. 5A); however, E6AP L502P was stable over the course of the chase (Fig. 5F). Since the E6AP L502P protein was highly self-ubiquitylated, why wasn’t its half-life decreased? One possibility was that the E6AP L502P protein was monoubiquitylated on multiple sites rather than polyubiquitylated. This would not constitute a degradation signal since a chain of at least four ubiquitins is required for targeting proteins to the proteasome (26). Alternatively the self-ubiquitylation may have occurred after the cells were lysed. Indeed we could prevent self-ubiquitylation of E6AP L502P (and E6AP WT) by lysing the cells under denaturing conditions (data not shown). Therefore, the L502P protein was stable in cells but became readily self-ubiquitylated after cell lysis, likely during the immunoprecipitation. The other mutant that formed thiol ester adducts with ubiquitin was E6AP E550L, but this mutation had no effect on the half-life of the protein (Fig. 5E).

The half-life of the other partially active hect domain mutant, K801A (Fig. 5I), was also shorter than wild type, around 4 h. It is unclear whether or not this difference in half-life would be of physiologic significance. Additional Angelman-relevant substrates will need to be identified to test this further. Finally the I803ins (Fig. 5K) mutation had no effect on the stability of the protein. This mutant had no defects in any of the assays tested in our analysis.

**DISCUSSION**

There is a strong correlation between the occurrence of Angelman syndrome and the loss of E6AP catalytic activity, suggesting that the dysregulation of E6AP substrates is an important factor in this disease. Of the E6AP point mutations examined here, seven map to the catalytic hect domain, and they affected all steps in the ubiquitylation process. Four of the hect domain mutations severely impaired the ability of E6AP to ubiquitylate the model substrate HHR23A. Three of these, the F782Δ, K836ins, and I804K mutations, were likely misfolded. The F782Δ and K836ins mutants did not form detectable thiol ester intermediates with ubiquitin (Fig. 2, C and D), were unable to functionally interact with the ubiquitin-conjugating enzyme UbcH7 (Fig. 4A, lanes 4–7, and h), and were unstable when expressed in cells (Fig. 5, G and H). The hect domain crystal structure predicts that these two residues would make important contributions to the structural integrity of the protein. Phe-782 resides in the hydrophobic core, and its side chain comprises the hect domain. The K836ins mutation, on the other hand, deletes the carboxyl-terminal α-helix of the hect domain, which makes numerous contacts with residues in the other lobes of the domain (13). The extreme carboxyl terminus is also necessary for hect domain function (in addition to structure) because deleting the six carboxyl-terminal amino acids slows (or stops) the transfer of the thiol ester-linked ubiquitin from the catalytic cysteine residue of the hect domain, causing the accumulation of E6AP-ubiquitin thiol ester adducts (12).

I804K retained partial activity in HHR23A ubiquitylation but was also very unstable when expressed in cells. Qualitatively this mutant still formed thiol ester intermediates with ubiquitin (data not shown) but did not compete as effectively as wild type E6AP for E2 binding. The rapid turnover of this mutant in cells suggests that it may be misfolded, although it still possessed partial activity in vitro.

Two other hect domain mutants, E550L and L502P, retained the ability to form thiol ester adducts with ubiquitin (Fig. 2, E and F), although they were impaired in transferring the ubiquitin to the substrate (Fig. 1B, lanes 6 and 7). Their defect was not in catalyzing isopeptide linkages because the proteins still
affixed ubiquitin to themselves (Fig. 3, lanes 6 and 7 and lanes 10 and 11, respectively) with the L502P mutant doing so hyperactively. Moreover the mutations did not abrogate E6AP-substrate binding (Fig. 3B). These phenotypes may indicate that the E550L and L502P mutations either impair or sterically limit access of a substrate lysine to the hect domain catalytic cleft. The hyperactivity of the L502P mutant may reflect an increase in the efficiency with which it catalyzes the transfer of thiol ester-linked ubiquitin to itself.

Alternatively the E550L and L502P mutants may not properly undergo conformational changes required for normal E6AP activity. Such changes are predicted from the two known hect domain crystal structures. The structure of the E6AP hect domain bound to the ubiquitin-conjugating enzyme UbcH7 depicted a 40-Å distance between the catalytic cysteine residues of the two enzymes (13). This distance is too great to enable the ubiquitin to be transferred from one enzyme to the other, making a conformational change necessary. In addition, the WWP1 hect domain structure depicts a dynamic protein that swivels about a flexible linker, thereby facilitating ubiquitin transfer from the ubiquitin-conjugating enzyme to itself. Mutations predicted to constrain the flexibility of the linker reduced hect domain activity (27). This study used self-ubiquitination to assay enzymatic activity, but such a change is likely also required for substrate ubiquitylation. The E550L and L502P mutations might alter the ability of the hect domain to undergo such conformational switches, but the thiol ester-linked ubiquitin would still be passed to the nearest eligible lysine residue, which would be on E6AP itself.

Although the L502P mutant self-ubiquitylated efficiently in vitro (Fig. 3A, lanes 10 and 11) and appeared as a series of slower migrating forms when immunoprecipitated from cells, it was stable over the course of the 8-h chase period (Fig. 5F). We believe that the L502P mutant self-ubiquitylated after the cells were lysed because L502P migrated as a single polypeptide when we lysed the cells under denaturing conditions. We hypothesize that immunoprecipitating the protein stimulated self-ubiquitylation likely by forcing the dimerization of two E6AP molecules similar to the way in which dimerization of kinases induces them to phosphorylate each other (28).

The I803ins mutation had no defects in any of the assays performed here. This mutation may cause only a subtle defect that cannot be detected under the in vitro conditions tested here. Alternatively this mutation could affect ubiquitylation of a specific E6AP substrate relevant to Angelman syndrome or affect an E6AP activity other than ubiquitylation. E6AP has been reported to act as a coactivator in progesterone-dependent transcription and does not require its catalytic activity to do so (29). Interestingly the F782Δ and I804K mutants retained the coactivator function in that report, although these mutations destabilized the proteins in the pulse-chase experiments performed here (Fig. 5, G and J). This suggests that coactivator activity does not require an intact hect domain and that the E6AP protein must be utilized for coactivator function rapidly after protein synthesis.

A small percentage of the wild type E6AP migrated slightly slower than the unmodified protein on the SDS-polyacrylamide gel (Fig. 5A, open arrowhead). This modification required E6AP catalytic activity because it was not present on the inactive E6AP C820A mutant (Fig. 5, compare A and B) and likely corresponds to E6AP modified by a single ubiquitin. Strikingly a more robust, even slower migrating form of E6AP appeared in the stable cell lines likely due to the addition of an unknown modification to E6AP. This was not a cross-reactive polypeptide for two reasons: it was not present in non-transduced cells, and it reacted with both anti-E6AP antiserum and an epitope tag-specific probe when a His6 tag was appended to E6AP (data not shown). This modification must have been attached to E6AP during or shortly after E6AP synthesis since it was present at the end of the “pulse” period. Also this modified form had a much shorter half-life than the unmodified E6AP (Fig. 5A), and its disappearance during the chase required its own catalytic activity since it persisted on all the ubiquitylation-defective mutants (Fig. 5, compare A with B and D–F). The nature of this modification is currently under investigation.

The non-hect domain C21Y mutant displayed wild type activity in the in vitro ubiquitylation assay (Fig. 1B, lane 3) but had a half-life of less than 30 min (Fig. 5C) in cells, which is significantly shorter than that of wild type E6AP. Also its rapid degradation was proteasome-dependent (data not shown). Cys-21 is one of four conserved cysteine residues in a 60-amino acid region that is highly conserved among E6AP orthologs in humans, fruit flies, and the soft-shell clam (30). Although the spacing of the cysteines does not conform to any known consensus, the instability of the C21Y mutant in cells suggests that it may be misfolded and that Cys-21 makes important structural contributions to the protein.

S349P, the other non-hect domain mutant, did not ubiquitylate HHR23A although it possesses a wild type hect domain and retained the ability to bind to HHR23A (Fig. 3B). The loss of the activity of this mutant may be due to several possibilities. First Ser-349 may stimulate hect domain activity perhaps via an intramolecular interaction. Alternatively the S349P mutation may disrupt the structure of the protein, which would explain its insolubility and its slightly decreased half-life in cells (Fig. 5D).

In conclusion, we undertook a thorough biochemical analysis of E6AP mutations identified in patients with Angelman syndrome. None of the examined mutations have been found in unrelated control individuals, although normal individuals occasionally have benign polymorphisms in UBE3A. We show here that there is a strong correlation between the occurrence of Angelman syndrome-associated E6AP mutations and the loss of E6AP ubiquitylation activity, indicating that the improper regulation of E6AP substrate(s) is likely to be an important determinant in this disease. It will be important to identify additional E6AP substrates and examine their relevance to the pathiology of Angelman syndrome. Finally, 15–20% of people with Angelman syndrome have no large deletion, no uniparental disomy, no imprinting defect, and no UBE3A mutation in the exons or intron-exon boundaries. This suggests either that UBE3A is not expressed in or active in the brains of these patients or that additional gene(s) may be involved in causing Angelman syndrome.

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