Role of ubiquitin and the HPV E6 oncoprotein in E6AP-mediated ubiquitination

Franziska Mortensen[a,b], Daniel Schneider[a,c], Tanja Barbic[a], Anna Sladewska-Marquardt[a], Simone Kühnle[a,1], Andreas Marx[b,c], and Martin Scheffner[a,b,2]

[a]Department of Biology, University of Konstanz, 78457 Konstanz, Germany; [b]Konstanz Research School Chemical Biology, University of Konstanz, 78457 Konstanz, Germany; and [c]Department of Chemistry, University of Konstanz, 78457 Konstanz, Germany

Deregulation of the ubiquitin ligase E6 associated protein (E6AP) encoded by the *UBE3A* gene has been associated with three different clinical pictures. Hijacking of E6AP by the E6 oncoprotein of distinct human papillomaviruses (HPV) contributes to the development of cervical cancer, whereas loss of E6AP expression or function is the cause of Angelman syndrome, a neurodevelopmental disorder, and increased expression of E6AP has been involved in autism spectrum disorders. Although these observations indicate that the activity of E6AP has to be tightly controlled, only little is known about how E6AP is regulated at the posttranslational level. Here, we provide evidence that the hydrophobic patch of ubiquitin comprising Leu-8 and Ile-44 is important for E6AP-mediated ubiquitination, whereas it does not affect the catalytic properties of the isolated catalytic HECT domain of E6AP. Furthermore, we show that the HPV E6 oncoprotein rescues the disability of full-length E6AP to use a respective hydrophobic patch mutant of ubiquitin for ubiquitination and that it stimulates E6AP-mediated ubiquitination of Ring1B, a known substrate of E6AP, in vitro and in cells. Based on these data, we propose that E6AP exists in at least two different states, an active and a less active or latent one, and that the activity of E6AP is controlled by noncovalent interactions with ubiquitin and allosteric activators such as the HPV E6 oncoprotein.

E6AP/UBE3A | ubiquitin | human papillomavirus | E6 oncoprotein | Angelman syndrome

In eukaryotes, posttranslational modification of proteins by ubiquitin plays a pivotal role in the regulation of many cellular processes, including cell cycle, DNA metabolism (e.g., DNA repair, transcription), and various signal transduction pathways (1 4). The specificity of the ubiquitin conjugation system is mainly ensured by E3 ubiquitin ligases, which mediate the recognition of target proteins. Based on the presence of distinct domains and their mode of action, E3 proteins can be grouped into three families, RING/RING like E3s, RING in between RING (RBR) E3s, and HECT E3s (5 7). All E3s have interaction sites for both substrate proteins and E2 ubiquitin conjugating enzymes. However, whereas in the case of RBR E3s and HECT E3s, ubiquitin is transferred from the E3 to substrates, RING/RING like E3s function as adaptors between substrates and E2s (i.e., ubiquitin is transferred from the E2 to the substrate).

E6AP, the founding member of the HECT E3 family, was originally identified as an interacting protein of the E6 oncoprotein of cancer associated human papillomaviruses (HPVs) (8, 9). The E6 E6AP complex targets the tumor suppressor p53 and other proteins which in the absence of E6 are not targeted by E6AP for ubiquitination and degradation thereby contributing to HPV induced cervical carcinogenesis (10, 11). In 1997, it was recognized that alterations in the *UBE3A* gene, which encodes E6AP, resulting in loss of E6AP expression or in the expression of E6AP variants with compromised E3 activity, are the cause of the Angelman syndrome (AS), a neurodevelopmental disorder (12 14). Recently, it was reported that amplification of the *UBE3A* gene (*i*) is found in a certain percentage of patients with autism spectrum disorders (15, 16) and (*ii*) in mice, results in increased E6AP levels and autistic phenotypes (17).

The notion that alteration of the substrate spectrum, loss of E3 function, and increased E3 function of E6AP contribute to the development of distinct disorders indicates that expression and/or E3 activity of E6AP have to be tightly controlled. Whereas some mechanisms controlling transcription of the *UBE3A* gene have been identified (e.g., the paternal allele is silenced by a *UBE3A* antisense transcript) (14, 18), only little is known about how the E3 activity of E6AP is regulated. We recently reported that E6AP binds to HERC2, a member of the HECT E3 family, and that HERC2 acts as an allosteric activator of E6AP (19). The physiological relevance of this interaction is indicated by the finding that a point mutation in the *HERC2* gene, resulting in a mutant HERC2 protein with increased turnover rate and hence decreased protein levels, underlies the development of a neurodevelopmental disorder with AS like features (20).

When analyzing the E6AP HERC2 interaction, we observed that a ubiquitin variant, in which the so called canonical hydrophobic patch of ubiquitin is mutated (Ub_hpI), is only poorly used by E6AP for ubiquitination and that HERC2 can partially rescue this disability of E6AP (19). This observation prompted us to take a closer look at the role of ubiquitin in E6AP mediated ubiquitination. We found that different surface areas of ubiquitin affect the ability of E6AP to catalyze the final transfer of ubiquitin to a substrate protein by different mechanisms, although they are not critically involved in the preceding steps (interaction

Significance

Deregulation of components of the ubiquitin–proteasome system contributes to the development of various diseases. A prominent example is the ubiquitin ligase E6AP/UBE3A, which is associated with three disorders: in complex with the E6 oncoprotein of human papillomaviruses, it contributes to cervical carcinogenesis; loss of E6AP expression results in the development of Angelman syndrome; and increased E6AP expression has been associated with autism spectrum disorders. This indicates that E6AP has to be tightly controlled; however, only little is known about how this is achieved. By analyzing the role of ubiquitin and the E6 oncoprotein in E6AP-mediated ubiquitination, we provide evidence that E6AP exists in an active state and a latent state and that its activity is controlled by allosteric effectors.

of E6AP with cognate E2 enzymes, thioester complex formation of E6AP with ubiquitin). Furthermore, we show that the HPV E6 oncoprotein rescues the disability of E6AP to use Ub_hpI, demonstrating that E6 does not only alter the substrate spectrum of E6AP but also acts as a potent allosteric activator of E6AP.

Results

The Hydrophobic Patches of Ubiquitin Are Critical for E6AP-Mediated Ubiquitination. Genetic analyses have shown that three distinct surface areas of ubiquitin are essential for viability of *Saccha romyces cerevisiae* (21). Two of these "hydrophobic patches" involving Leu 8 and Ile 44 (termed patch I in the following) and Ile 36, Leu 71, and Leu 73 (termed patch II) (Fig. 1*A*) have been more closely examined for their role in ubiquitination mediated by distinct E2 ubiquitin conjugating enzymes and E3 enzymes (e.g., refs. 22 31). Notably, patch II but not patch I was shown to be required for covalent attachment of ubiquitin to substrate proteins catalyzed by the HECT E3s Rsp5 and NEDD4L (22, 30). In addition, the HECT domains of Rsp5, NEDD4L, NEDD4, and SMURF2, which are all members of the NEDD4 subfamily of HECT E3s, harbor a noncovalent binding site for a second ubiq uitin molecule (that is not in thioester complex with the HECT domain) (23, 24, 32, 33). This interaction is mediated by patch I, and although patch I is not essential for isopeptide bond formation (30), it affects the processivity of ubiquitin chain formation (23, 24, 32, 33). In contrast to Rsp5 and NEDD4L, the HECT domain of E6AP does not appear to harbor a noncovalent interaction site for free ubiquitin (24). Nonetheless, both hydrophobic patches are critically involved in E6AP mediated ubiquitination, as respective ubiquitin mutants (Ub_hpI, Leu 8 and Ile 44 replaced by Ala; Ub_hpII, Ile 36, Leu 71, and Leu 73 replaced by Ala) are not or only poorly used by E6AP for autoubiquitination as well as for ubiquitination of an inactive form of the RING E3 ligase Ring1B (Ring1B I53S), a known target of E6AP (34) (Fig. 1*B*).
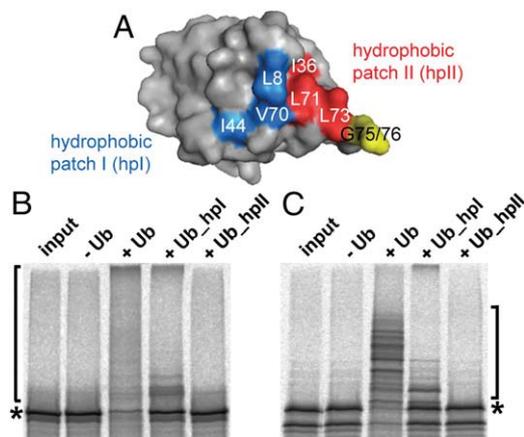

Fig. 1. Hydrophobic patches of ubiquitin are critically involved in E6AP mediated ubiquitination. (*A*) Surface model of ubiquitin (Protein Data Bank, PDB 1UBQ) with amino acid residues of the canonical hydrophobic patch I and of the noncanonical hydrophobic patch II indicated in blue and red, respectively. (*B*) For E6AP autoubiquitination, in vitro translated radiola beled E6AP was incubated with baculovirus expressed E6AP in the absence or presence of wild type ubiquitin (Ub) or the ubiquitin mutants Ub hpI (substitution of Leu 8 and Ile 44 by Ala) and Ub hpII (substitution of Ile 36, Leu 71, and Leu 73 by Ala) under standard ubiquitination conditions (*Ma terials and Methods*) as indicated. Reaction products were analyzed by SDS/ PAGE followed by fluorography. (*C*) As in *B* but E6AP mediated ubiquiti nation of in vitro translated radiolabeled Ring1B I53S (an inactive form of Ring1B that cannot ubiquitinate itself) (34) was studied. Running positions of the nonmodified form and of the ubiquitinated forms of E6AP and Ring1B I53S are indicated by asterisks and brackets, respectively.

The Hydrophobic Patches of Ubiquitin Are Not Required for Cognate E2s to Bind to E6AP. A possible explanation for the requirement of intact patches I and II for E6AP mediated ubiquitination is that E6AP interacts noncovalently with (one of) these patches, when ubiquitin is in thioester complex with E2 enzymes. If this is the case (i.e., E6AP contacts ubiquitin and E2 simultaneously), it can be predicted that the ubiquitin loading status of cognate E2s affects their affinity for E6AP. Due to both the inherent ther modynamic instability of thioester bonds and the fact that acti vated ubiquitin is rapidly transferred from E2 to E6AP, it is notoriously difficult to analyze the binding of E2 enzymes in thioester complex with ubiquitin to E6AP. It was recently shown that a UbcH5a variant, in which the catalytic Cys residue was replaced by Lys, can be efficiently loaded with ubiquitin via isopeptide bond formation and that the resulting complex is a reasonable structural mimic of the thioester complex of UbcH5a with ubiquitin (28). Thus, we adopted this strategy to study the interaction of E6AP with UbcH7 and UbcH5b (Fig. S1), which both support E6AP mediated ubiquitination in vitro (19, 35). As shown by isothermal titration calorimetry and size exclusion chromatography, the ubiquitin loading status of UbcH7 does not have a major influence on the ability of UbcH7 to interact with the isolated HECT domain of E6AP (Fig. S2), confirming pre viously published data on E6AP UbcH7 interaction (36). In contrast, whereas the HECT domain of E6AP does not detect ably interact with UbcH5b in the absence of ubiquitin, UbcH5b preloaded with ubiquitin (UbcH5b Ub) binds to the HECT domain with an efficiency similar to UbcH7 (Fig. 2 and Fig. S3). However, neither mutation of patch I nor of patch II had a significant effect on the ability of UbcH5b to bind to the HECT domain (Fig. 2), indicating that they play only a minor role in E6AP E2 interaction. This notion is further supported by the observation that like UbcH5b Ub, UbcH5b Ub_hpI and UbcH5b Ub_hpII act in a dominant negative manner in E6AP autoubiquitination [Fig. S4; note that a complex of ubiquitin with UbcH1, an E2 that does not support E6AP mediated ubiquiti nation (35), does not interfere with E6AP autoubiquitination, indicating the specificity of the dominant negative effect of UbcH5b Ub].

The Hydrophobic Patches of Ubiquitin Are Involved in E6AP-Catalyzed Isopeptide Bond Formation. Next, the possibility that patch I and/or patch II are required for efficient transfer of ubiquitin from E2 enzymes to the catalytic Cys residue of E6AP was studied. Hence, the ability of full length E6AP and of the isolated HECT domain to form thioester complexes with Ub_hpI and Ub_hpII was de termined (37). To do so, E1 and UbcH7 were used in concentra tions that are not rate limiting for the reaction to ensure that potential differences in the efficiency of the ubiquitin variants to form thioester complexes with E6AP/HECT domain are solely due to the ability of the E6AP/HECT domain to accept these. However, no significant differences were observed for ubiquitin, Ub_hpI, and Ub_hpII in their ability to be transferred from UbcH7 to the catalytic Cys residue of the HECT domain (Fig. 3*A*) or E6AP (Fig. 3*B*) (for results obtained with UbcH5b, see Fig. S5*A*).

In contrast to the reaction with full length E6AP, a band corresponding to a covalent complex of the HECT domain with Ub_hpI was observed even upon treatment with a reducing agent (DTT) (Fig. 3*A*), indicating that the isolated HECT domain can use Ub_hpI for autoubiquitination. Indeed, subsequent autou biquitination experiments showed that the isolated HECT domain uses wild type ubiquitin and Ub_hpI with similar efficiencies for autoubiquitination (Fig. S5*B*). In addition, when using UbcH5b instead of UbcH7, the HECT domain was autoubiquitined not only in the presence of ubiquitin and Ub_hpI but apparently also in the presence of Ub_hpII (Fig. S5 *A and B*). Closer examina tion of the latter result revealed that in contrast to ubiquitin and Ub_hpI, covalent attachment of Ub_hpII to E6AP was mainly
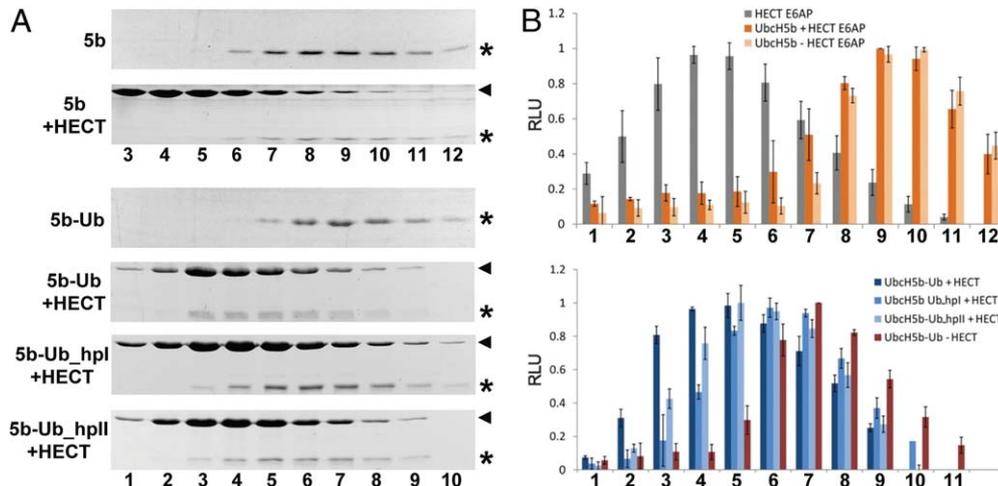
Fig. 2. The ubiquitin loading status of UbcH5b affects its ability to interact with E6AP. (*A*) UbcH5b (5b) and stable conjugates of the catalytically inactive UbcH5b C85K mutant with wild type ubiquitin (5b_Ub) or the ubiquitin mutants Ub_hpI and Ub_hpII (5b_Ub_hpI, 5b_Ub_hpII) were incubated in the absence or the presence (+HECT) of the HECT domain of E6AP. After 5 min, the mixtures were fractionated by size exclusion chromatography. Fractions were sub jected to SDS/PAGE and proteins visualized by Coomassie staining. Relative fraction numbers are indicated. Running position of the HECT domain is marked by an arrowhead; running positions of UbcH5b and the different UbcH5b_ubiquitin conjugates are marked by an asterisk. (*B*) Intensities of the bands representing the HECT domain, UbcH5b, and the different UbcH5b_ubiquitin conjugates were quantified by densitometry and are expressed in relative units (RLU). Relative fraction numbers are indicated. Error bars represent the SD from at least three independent experiments. For results obtained for UbcH7_ubiquitin conjugates, see Figs. S1 and S2.

catalyzed by UbcH5b rather than by the HECT domain itself (i.e., Ub_hpII is directly transferred from the catalytic Cys residue of UbcH5b to a Lys residue of E6AP; for further details, see legend to Fig. S5). This conclusion is based on the findings that the reaction is (*i*) independent of the catalytic Cys residue of E6AP (Fig. S5 *B* and *C*), (*ii*) only poorly catalyzed by UbcH7 (Fig. S5*B*), which is known to be weakly active in isopeptide bond formation (38), and (*iii*) only poorly catalyzed by a UbcH5b mutant (Fig. S5*B*) that can still catalyze thioester complex formation between ubiquitin and HECT E3s but is impaired in isopeptide bond for mation (39).

In conclusion, the data obtained for Ub_hpI and Ub_hpII indicate that both patch I and patch II are involved in E6AP catalyzed isopeptide bond formation, whereas their integrity is not critical for E6AP ubiquitin thioester complex formation. Furthermore, patch I and patch II affect E6AP mediated ubiq uitination by different mechanisms: Patch II appears to directly contribute to E6AP catalyzed isopeptide bond formation, be cause both the isolated HECT domain and full length E6AP cannot use Ub_hpII for ubiquitination; in contrast, patch I has an indirect effect, because the isolated HECT domain can use Ub_hpI for ubiquitination, whereas full length E6AP cannot.

HPV E6 Acts as Allosteric Activator of E6AP. The observation that full length E6AP but not the isolated HECT domain is impaired in using Ub_hpI for autoubiquitination indicates that the N terminal region (with N terminus defined as E6AP without the HECT domain) influences the catalytic properties of the HECT domain. Thus, the N terminal region may not only represent a binding platform for substrates but also for proteins regulating E6AP activity by driving E6AP into a more active or less active conformation. This hypothesis is supported by the ability of HERC2 which binds to a region (amino acids 150 200) in the N terminus of E6AP but does not represent a substrate for E6AP to stimulate E6AP activity, and this stimulatory effect is most readily observed when Ub_hpI is used as a source of ubiquitin (19).

Similar to HERC2, the HPV E6 oncoprotein binds to a dis tinct region within the N terminus of E6AP (amino acids 378 395) (19, 40) and is not a substrate for E6AP (19, 41). To test if

binding of E6 results in activation of the catalytic properties of E6AP, the effect of E6 on the ability of E6AP to use Ub_hpI and Ub_hpII for ubiquitination was determined. Indeed, as shown in Fig. 4, E6AP mediated ubiquitination of Ring1B I53S (34) or HHR23A (42) was stimulated in the presence of E6. As for HERC2, the stimulatory effect was particularly prominent, when Ub_hpI was used as a source for ubiquitin. In contrast, E6 could not rescue the disability of E6AP to use Ub_hpII for ubiquiti nation, supporting the notion that patch I and patch II play different roles in E6AP mediated ubiquitination.

Finally, cell culture experiments were performed to determine if the stimulating effect of E6 on E6AP mediated degradation of Ring1B I53S can also be observed in cells (Fig. 4*C*). To do so, the dehydrofolate reductase (DHFR) ubiquitin fusion protein system was used (43 45). In this system, DHFR HA ubiquitin and Ring1B I53S are expressed as one protein from the same mRNA and cotranslationally cleaved by ubiquitin specific pro teases in a quantitative manner, resulting in two separate pro teins. Because upon translation and concomitant cleavage, two separate proteins are generated from a common precursor, comparison of the relative levels of Ring1B I53S and DHFR HA ubiquitin (a mutant form of ubiquitin, in which Lys 48 is replaced by Arg, is used in this system to avoid degradation of DHFR HA ubiquitin) provides a direct measure for the effect of E6AP on the turnover rate of Ring1B I53S (see also ref. 45). As shown in Fig. 4*C*, coexpression of E6 resulted in significant stimulation of E6AP mediated degradation of Ring1B I53S. Furthermore, this effect was not observed in the presence of a catalytically inactive mutant of E6AP, indicating the specificity of the E6 effect (37). Taken together, these data demonstrate that E6 does not only increase the substrate spectrum of E6AP but in addition, acts as a potent allosteric activator of E6AP.

Discussion

Although E6AP has been associated with three different disor ders and was the first HECT E3 identified, only little is known about how its activity is regulated at the posttranslational level. By analyzing the functional interaction of defined ubiquitin mutants with E6AP, we provide evidence that the N terminus of
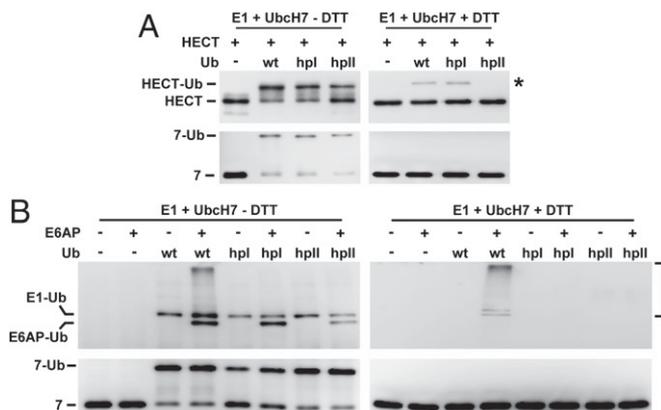
Fig. 3. Hydrophobic patches of ubiquitin do not affect thioester complex formation with E6AP. (*A*) E1, UbcH7, the HECT domain of E6AP, wild type (WT) ubiquitin (Ub), and the ubiquitin mutants hpI and hpII (Fig. 1*A*) were incubated under thioester reaction conditions (*Materials and Methods*). Reactions were stopped in the presence (+DTT) or the absence (DTT; to preserve thioester complexes) of a reducing agent. Whole reaction mixtures were subjected to SDS/PAGE followed by Western blot analysis using antibodies directed against E6AP (HECT) or His tag (UbcH7). Running positions of UbcH7 (marked as 7), the HECT domain of E6AP, and the respective ubiquitin thioester complexes are indicated. An autoubiquitinated form of the HECT domain (which is resistant to DTT treatment) is indicated by an asterisk. (*B*) As in *A* but baculovirus expressed full length E6AP and His tagged forms of wild type (WT) ubiquitin and the ubiquitin mutants hpI and hpII were used. Furthermore, Western blot analysis was performed with an anti His antibody, because due to the small size difference, the nonmodified form of E6AP is not readily distinguished from the E6AP ubiquitin thioester complex by using anti E6AP antibodies. Running positions of the various ubiquitin thioester complexes and the nonmodified form of UbcH7 (marked as 7) are indicated. Autoubiquitinated forms of E6AP (which are resistant to DTT treatment) are indicated by a bracket. For results obtained with UbcH5b, see Fig. S5.

E6AP is not only involved in substrate recognition but also has an impact on the ability of the HECT domain to catalyze the final attachment of ubiquitin to substrate proteins. Furthermore, binding of the HPV E6 oncoprotein strongly enhances the catalytic activity of E6AP, indicating that E6AP exists in at least two different states a fully active form and a catalytically less active or latent form.

It was previously shown that patch II of ubiquitin is involved in both transfer of ubiquitin from E2 to NEDD4 family members and subsequent attachment of ubiquitin to substrate proteins, whereas patch I of ubiquitin affects the processivity of ubiquitin chain formation (22 24, 30, 32, 33). In contrast, both patches contribute to E6AP mediated isopeptide bond formation, whereas their integrity is not crucial for E6AP ubiquitin thioester complex formation, supporting the notion that different HECT E3s use different catalytic strategies (46). Nonetheless, it seems likely that similar to NEDD4 family members and, for example, the RNF4 UbcH5a complex, the integrity of patch II is required to create an appropriate environment for nucleophilic attack of the E6AP ubiquitin thioester bond by the incoming amino group of a Lys residue of a target protein (28, 30, 31). In addition, we propose that in the case of E6AP, patch II serves as selectivity filter. UbcH5b catalyzes the covalent attachment of Ub_hpII to Lys 847 of E6AP, whereas this reaction is not observed with wild type ubiquitin (Fig. S5C). Thus, it appears that patch II shields the UbcH5b ubiquitin thioester complex from undesirable attack by the amino group of Lys 847, ensuring selective transthioesterification of ubiquitin from UbcH5b to the catalytic Cys residue of E6AP.

At first glance, the observation that patch I is required for efficient ubiquitination by E6AP is reminiscent of results obtained for ubiquitination mediated by RING E3s or by E2s alone (e.g., refs. 25 28). For example, in the case of the RNF4 UbcH5a

complex, Ile 44 and Leu 8 are located at the interface of ubiquitin with UbcH5a and the RNF4 RING domain contributing to the correct positioning of ubiquitin for isopeptide bond formation (28). Such a scenario seems unlikely for E6AP, because the isolated HECT domain uses wild type ubiquitin and Ub_hpI, in which Ile 44 and Leu 8 are replaced by Ala (Fig. 3 and Fig. S5), with similar efficiencies for autoubiquitination. However, in contrast to full length E6AP, the isolated HECT domain is not or only weakly active in ubiquitin chain formation (as indicated by the autoubiquitination pattern of the HECT domain, and as reported before) (46). Thus, whereas in the absence of patch I of ubiquitin, the N terminal region of E6AP interferes with the catalytic activity of the HECT domain, it is crucial for E6AP mediated ubiquitin chain formation. How can these seemingly contradictory roles of the N terminus be reconciled? An obvious possibility is that (*i*) as reported for some NEDD4 family members (summarized in ref. 7), the N terminal region of E6AP physically interacts with the HECT domain, and (*ii*) either the N terminal region or the HECT domain of E6AP harbors a noncovalent binding site for ubiquitin. However, we could observe neither a noncovalent interaction of free ubiquitin with E6AP nor a noncovalent interaction of free ubiquitin with E6AP of the isolated N terminal region with the HECT domain by size exclusion chromatography or coprecipitation experiments (Fig. S6). Thus, we propose that the affinities of the respective interactions are rather low and that the interactions occur in a functionally relevant manner only when the N terminal region and the HECT domain are present on the same polypeptide chain (i.e., intramolecular interaction) and when ubiquitin is in thioester complex with E6AP (i.e., a single ubiquitin molecule makes the covalent as well as noncovalent contact).

Although final proof of the proposed model will have to await elucidation of the structure of full length E6AP and of E6AP in covalent complex with ubiquitin, it is supported by the data obtained previously for the HERC2 E6AP interaction (19) and the data provided here for the effect of HPV E6 on the catalytic activity of E6AP. Both HERC2 and HPV E6 bind to distinct regions in the N terminus of E6AP (19, 41) and stimulate E6AP activity (note that because the structure of the N terminus has not yet been solved, we cannot exclude that the two binding sites are located within the same domain or at a similar position at the surface of E6AP). Moreover, both proteins rescue the disability of E6AP to use Ub_hpI for ubiquitination, suggesting that (*i*) in the absence of these proteins, patch I is required to partially relieve the inhibitory effect of the N terminal region, and (*ii*) at least in the presence of these proteins, patch I does not contribute or only marginally contributes to the correct positioning of ubiquitin for the final transfer to a target protein. Furthermore, it was previously reported that E6AP autoubiquitination occurs mainly via intermolecular transfer of ubiquitin (i.e., E6AP di or oligomerizes for autoubiquitination) (47), whereas in the presence of HPV E6, E6AP ubiquitinates itself preferentially in an intramolecular manner (48). Hence, all of the available evidence strongly supports the conclusion that binding of HPV E6 induces a conformational change in E6AP bringing it into a fully active state [note that it was recently reported that E6 induces E6AP oligomerization (49); however, we did not obtain any evidence that this is the case under the conditions used].

Thus far, studies concerning the E6 E6AP interaction have focused on the ability of E6 to increase the substrate spectrum of E6AP (i.e., identification of proteins that in the absence of E6 are not targeted by E6AP). Although several proteins have been reported to represent substrates of E6AP in the absence of E6 (summarized in ref. 7), the physiological relevance of many of these interactions remains unclear, in particular with respect to their role in the development of Angelman syndrome. The data presented here strongly indicate that E6 has a dramatic effect on the ability of E6AP to ubiquitinate its (i.e., E6AP's) regular substrate proteins. Thus, detailed analysis of the effect of E6 on
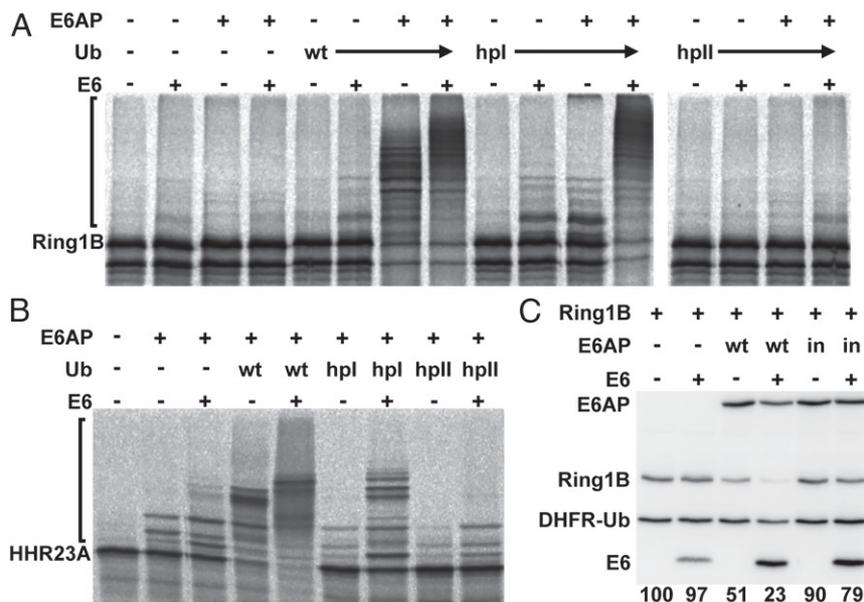
Fig. 4. The HPV E6 oncoprotein is an allosteric activator of E6AP. (*A*) In vitro translated radiolabeled Ring1B I53S was incubated with baculovirus expressed E6AP in the absence or presence of the HPV16 E6 oncoprotein and of wild type (WT) ubiquitin (Ub) or the ubiquitin mutants hpI and hpII (Fig. 1*A*) under standard ubiquitination conditions (*Materials and Methods*) as indicated. Reaction products were analyzed by SDS/PAGE followed by fluorography. Running positions of the nonmodified form and of the ubiquitinated forms of Ring1B I53S are indicated by an asterisk and a bracket, respectively. (*B*) As in *A* but in vitro translated radiolabeled HHR23A was used as substrate. (*C*) H1299 shE6AP cells, in which endogenous E6AP expression is stably down regulated by RNA interference (50), were transfected with expression constructs for HA tagged wild type E6AP (WT) or the catalytically inactive mutant E6AP C820A (in), HA tagged HPV16 E6, and a DHFR HA ubiquitin fusion protein of HA tagged Ring1B I53S (for details on the DHFR HA ubiquitin system, see text). Twenty four hours after transfection, protein extracts were prepared and levels of the various proteins were determined by Western blot analysis using an anti HA antibody and quantified. The relative ratio of HA tagged Ring1b I53S to DHFR HA ubiquitin is indicated, with the ratio of HA tagged Ring1b I53S to DHFR HA ubiquitin in the absence of E6AP set to 100%. Running positions of E6AP, Ring1B, HPV16 E6, and DHFR HA ubiquitin are indicated.

E6AP will not only contribute to further our understanding of the role of the E6 E6AP complex in cervical carcinogenesis but should also provide valuable insights into the physiological relevance of reported and yet to be identified substrate proteins of E6AP.

Materials and Methods

For plasmids, antibodies, and bacterial protein expression, see *SI Materials and Methods*.

Generation of Isopeptide Linked E2 Ubiquitin Conjugates. Synthesis of different E2 ubiquitin conjugates was carried out as described previously (28). Briefly, UbcH5b C85K, UbcH7 C86K, or UbcH1 C88K (each 200 μM) were incubated with UBA1 (1.5 μM) and either ubiquitin, Ub hpI, or Ub hpII (each 200 μM) at 37 °C for 20 22 h in a buffer containing 50 mM Tris·HCl pH 10.0, 150 mM NaCl, 5 mM MgCl₂, 3 mM ATP, and 0.8 mM TCEP. Subsequently, respective E2 Ub conjugates were purified by size exclusion chromatography. Fractions con taining E2 Ub conjugates were pooled, dialyzed against 25 mM Tris·HCl, 50 mM NaCl, 1 mM DTT, pH 7.5, concentrated by ultrafiltration, and stored at 4 °C.

Interaction Studies by Size Exclusion Chromatography. To study complex for mation of UbcH5b, UbcH7, and the different ubiquitin conjugates of UbcH5b and UbcH7 (UbcH5b Ub, UbcH5b Ub hpI, UbcH5b Ub hpII, and UbcH7 Ub) with the HECT domain of E6AP, 20 μM of the respective E2 or E2 ubiquitin conjugate were mixed with 125 μM HECT E6AP. After a 5 min incubation at room temperature, mixtures were subjected to size exclusion chromatography. As control, 20 μM of UbcH5b, UbcH7, and the various E2 ubiquitin conjugates were subjected to size exclusion chromatography in the absence of the HECT domain. Fractions were analyzed by SDS/PAGE followed by Coomassie staining and the intensities of the bands in each fraction were quantified by densi tometry. The ability of UbcH5b, UbcH7, and the various E2 ubiquitin conju gates to bind to the HECT domain of E6AP was indicated by changes in their retention times in presence and absence of the HECT domain. For each E2 variant, size exclusion experiments were done in triplicate.

In Vitro Ubiquitination and Thioester Assays. For in vitro ubiquitination, 1 μL of rabbit reticulocyte lysate translated ³⁵S labeled substrate (E6AP, Ring1B I53S,

and HHR23A) was incubated with 50 ng of baculovirus expressed E1, 50 ng of E2 enzyme (UbcH5b or UbcH7), 200 ng of baculovirus expressed E6AP, 20 μg of ubiquitin or ubiquitin mutants (Ub hpI and Ub hpII) in the absence or pres ence of GST 16 E6 (200 ng) in 40 μL volumes. In addition, reactions contained 25 mM Tris·HCl pH 7.5, 50 mM NaCl, 1 mM DTT, 2 mM ATP, and 4 mM MgCl₂. After incubation at 25 °C for 2 h, total reaction mixtures were electro phoresed in 8 15% (vol/vol) SDS polyacrylamide gels, and ³⁵S labeled proteins were detected by fluorography.

For in vitro thioester assays, 50 ng of E1, 50 ng of UbcH7 or UbcH5b, and 250 ng of E6AP or the HECT domain of E6AP were incubated with 20 μg of ubiquitin or the ubiquitin mutants (Ub hpI and Ub hpII) for 1 min at 30 °C in 40 μL volumes. In addition, reactions contained 25 mM Tris·HCl pH 7.5, 50 mM NaCl, 0.1 mM DTT, 4 mM ATP, and 10 mM MgCl₂. Reactions were terminated by incubating the mixtures for 15 min at 30 °C in 50 mM Tris·HCl pH 6.8, 2% (wt/vol) SDS, 4 M urea, 10% (vol/vol) glycerol (to preserve ubiquitin thioester complexes) or by boiling the mixtures in the same buffer containing 100 mM DTT instead of urea (37). Whole reaction mixtures were separated on 8 15% (vol/vol) SDS polyacrylamide gels and subjected to Western blot analysis using anti E6AP or anti His antibodies.

Degradation Assay in Cells. H1299 shE6AP cells (stable knockdown of E6AP expression) (50) were grown in DMEM supplemented with 10% (vol/vol) FBS. For degradation assays, one 6 cm plate of cells was transfected with ex pression constructs encoding HA tagged E6AP or the catalytically inactive mutant E6AP C820A (2.5 μg), HA tagged HPV16 E6 (1.5 μg), and DHFR HA ubiquitin HA Ring1B I53S (1 μg) as indicated (Fig. 4*C*). Twenty four hours after transfection, cells were lysed and levels of E6AP, DHFR HA ubiquitin, HA Ring1B I53S, and HA E6 were determined by SDS/PAGE followed by Western blot analysis using an anti HA antibody. Quantification of the intensity of the signals was performed with the Aida 4.08 software package (Raytest).

1. Kerscher O, Felberbaum R, Hochstrasser M (2006) Modification of proteins by ubiquitin and ubiquitin-like proteins. *Annu Rev Cell Dev Biol* 22:159–180.

2. Schwartz AL, Ciechanover A (2009) Targeting proteins for destruction by the ubiquitin system: Implications for human pathobiology. *Annu Rev Pharmacol Toxicol* 49:73–96.

3. Varshavsky A (2012) The ubiquitin system, an immense realm. *Annu Rev Biochem* 81:167–176.

4. Popovic D, Vucic D, Dikic I (2014) Ubiquitination in disease pathogenesis and treatment. *Nat Med* 20(11):1242–1253.

5. Metzger MB, Hristova VA, Weissman AM (2012) HECT and RING finger families of E3 ubiquitin ligases at a glance. *J Cell Sci* 125(Pt 3):531–537.

6. Dove KK, Klevit RE (2012) RING-between-RINGs–keeping the safety on loaded guns. *EMBO J* 31(19):3792–3794.

7. Scheffner M, Kumar S (2014) Mammalian HECT ubiquitin-protein ligases: Biological and pathophysiological aspects. *Biochim Biophys Acta* 1843(1):61–74.

8. Huibregtse JM, Scheffner M, Howley PM (1991) A cellular protein mediates association of p53 with the E6 oncoprotein of human papillomavirus types 16 or 18. *EMBO J* 10(13):4129–4135.

9. Huibregtse JM, Scheffner M, Howley PM (1993) Cloning and expression of the cDNA for E6-AP, a protein that mediates the interaction of the human papillomavirus E6 oncoprotein with p53. *Mol Cell Biol* 13(2):775–784.

10. Scheffner M, Huibregtse JM, Vierstra RD, Howley PM (1993) The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. *Cell* 75(3):495–505.

11. Beaudenon S, Huibregtse JM (2008) HPV E6, E6AP and cervical cancer. *BMC Biochem* 9(Suppl 1):S4.

12. Kishino T, Lalande M, Wagstaff J (1997) UBE3A/E6-AP mutations cause Angelman syndrome. *Nat Genet* 15(1):70–73.

13. Matsuura T, et al. (1997) De novo truncating mutations in E6-AP ubiquitin-protein ligase gene (UBE3A) in Angelman syndrome. *Nat Genet* 15(1):74–77.

14. Dagli A, Buiting K, Williams CA (2012) Molecular and clinical aspects of Angelman syndrome. *Mol Syndromol* 2(3-5):100–112.

15. Glessner JT, et al. (2009) Autism genome-wide copy number variation reveals ubiquitin and neuronal genes. *Nature* 459(7246):569–573.

16. Hogart A, Wu D, LaSalle JM, Schanen NC (2010) The comorbidity of autism with the genomic disorders of chromosome 15q11.2-q13. *Neurobiol Dis* 38(2):181–191.

17. Smith SE, et al. (2011) Increased gene dosage of Ube3a results in autism traits and decreased glutamate synaptic transmission in mice. *Sci Transl Med* 3(103):103ra97.

18. Meng L, et al. (2015) Towards a therapy for Angelman syndrome by targeting a long non-coding RNA. *Nature* 518(7539):409–412.

19. Kühnle S, et al. (2011) Physical and functional interaction of the HECT ubiquitin-protein ligases E6AP and HERC2. *J Biol Chem* 286(22):19410–19416.

20. Harlalka GV, et al. (2013) Mutation of HERC2 causes developmental delay with Angelman-like features. *J Med Genet* 50(2):65–73.

21. Sloper-Mould KE, Jemc JC, Pickart CM, Hicke L (2001) Distinct functional surface regions on ubiquitin. *J Biol Chem* 276(32):30483–30489.

22. Kamadurai HB, et al. (2009) Insights into ubiquitin transfer cascades from a structure of a UbcH5B approximately ubiquitin-HECT(NEDD4L) complex. *Mol Cell* 36(6):1095–1102.

23. Kim HC, Steffen AM, Oldham ML, Chen J, Huibregtse JM (2011) Structure and function of a HECT domain ubiquitin-binding site. *EMBO Rep* 12(4):334–341.

24. Maspero E, et al. (2011) Structure of the HECT:ubiquitin complex and its role in ubiquitin chain elongation. *EMBO Rep* 12(4):342–349.

25. Saha A, Lewis S, Kleiger G, Kuhlman B, Deshaies RJ (2011) Essential role for ubiquitin-ubiquitin-conjugating enzyme interaction in ubiquitin discharge from Cdc34 to substrate. *Mol Cell* 42(1):75–83.

26. Wickliffe KE, Lorenz S, Wemmer DE, Kuriyan J, Rape M (2011) The mechanism of linkage-specific ubiquitin chain elongation by a single-subunit E2. *Cell* 144(5):769–781.

27. Dou H, Buetow L, Sibbet GJ, Cameron K, Huang DT (2012) BIRC7-E2 ubiquitin conjugate structure reveals the mechanism of ubiquitin transfer by a RING dimer. *Nat Struct Mol Biol* 19(9):876–883.

28. Plechanovová A, Jaffray EG, Tatham MH, Naismith JH, Hay RT (2012) Structure of a RING E3 ligase and ubiquitin-loaded E2 primed for catalysis. *Nature* 489(7414):115–120.

29. Pruneda JN, et al. (2012) Structure of an E3:E2~Ub complex reveals an allosteric mechanism shared among RING/U-box ligases. *Mol Cell* 47(6):933–942.

30. Kamadurai HB, et al. (2013) Mechanism of ubiquitin ligation and lysine prioritization by a HECT E3. *eLife* 2:e00828.

31. Maspero E, et al. (2013) Structure of a ubiquitin-loaded HECT ligase reveals the molecular basis for catalytic priming. *Nat Struct Mol Biol* 20(6):696–701.

32. French ME, Kretzmann BR, Hicke L (2009) Regulation of the RSP5 ubiquitin ligase by an intrinsic ubiquitin-binding site. *J Biol Chem* 284(18):12071–12079.

33. Ogunjimi AA, et al. (2010) The ubiquitin binding region of the Smurf HECT domain facilitates polyubiquitylation and binding of ubiquitylated substrates. *J Biol Chem* 285(9):6308–6315.

34. Zaaroor-Regev D, et al. (2010) Regulation of the polycomb protein Ring1B by self-ubiquitination or by E6-AP may have implications to the pathogenesis of Angelman syndrome. *Proc Natl Acad Sci USA* 107(15):6788–6793.

35. Nuber U, Scheffner M (1999) Identification of determinants in E2 ubiquitin-conjugating enzymes required for hect E3 ubiquitin-protein ligase interaction. *J Biol Chem* 274(11):7576–7582.

36. Purbeck C, Eletr ZM, Kuhlman B (2010) Kinetics of the transfer of ubiquitin from UbcH7 to E6AP. *Biochemistry* 49(7):1361–1363.

37. Scheffner M, Nuber U, Huibregtse JM (1995) Protein ubiquitination involving an E1-E2-E3 enzyme ubiquitin thioester cascade. *Nature* 373(6509):81–83.

38. Wenzel DM, Lissounov A, Brzovic PS, Klevit RE (2011) UBCH7 reactivity profile reveals parkin and HHARI to be RING/HECT hybrids. *Nature* 474(7349):105–108.

39. Wu PY, et al. (2003) A conserved catalytic residue in the ubiquitin-conjugating enzyme family. *EMBO J* 22(19):5241–5250.

40. Huibregtse JM, Scheffner M, Howley PM (1993) Localization of the E6-AP regions that direct human papillomavirus E6 binding, association with p53, and ubiquitination of associated proteins. *Mol Cell Biol* 13(8):4918–4927.

41. Tomaić V, Pim D, Banks L (2009) The stability of the human papillomavirus E6 oncoprotein is E6AP dependent. *Virology* 393(1):7–10.

42. Kumar S, Talis AL, Howley PM (1999) Identification of HHR23A as a substrate for E6-associated protein-mediated ubiquitination. *J Biol Chem* 274(26):18785–18792.

43. Bachmair A, Finley D, Varshavsky A (1986) In vivo half-life of a protein is a function of its amino-terminal residue. *Science* 234(4773):179–186.

44. Varshavsky A (2005) Ubiquitin fusion technique and related methods. *Methods Enzymol* 399:777–799.

45. Kühnle S, Mothes B, Matentzoglu K, Scheffner M (2013) Role of the ubiquitin ligase E6AP/UBE3A in controlling levels of the synaptic protein Arc. *Proc Natl Acad Sci USA* 110(22):8888–8893.

46. Wang M, Pickart CM (2005) Different HECT domain ubiquitin ligases employ distinct mechanisms of polyubiquitin chain synthesis. *EMBO J* 24(24):4324–4333.

47. Nuber U, Schwarz SE, Scheffner M (1998) The ubiquitin-protein ligase E6-associated protein (E6-AP) serves as its own substrate. *Eur J Biochem* 254(3):643–649.

48. Kao WH, Beaudenon SL, Talis AL, Huibregtse JM, Howley PM (2000) Human papillomavirus type 16 E6 induces self-ubiquitination of the E6AP ubiquitin-protein ligase. *J Virol* 74(14):6408–6417.

49. Ronchi VP, Klein JM, Edwards DJ, Haas AL (2014) The active form of E6-associated protein (E6AP)/UBE3A ubiquitin ligase is an oligomer. *J Biol Chem* 289(2):1033–1048.

50. Kuballa P, Matentzoglu K, Scheffner M (2007) The role of the ubiquitin ligase E6-AP in human papillomavirus E6-mediated degradation of PDZ domain-containing proteins. *J Biol Chem* 282(1):65–71.

51. Berndsen CE, Wolberger C (2011) A spectrophotometric assay for conjugation of ubiquitin and ubiquitin-like proteins. *Anal Biochem* 418(1):102–110.

52. Yamamoto Y, Huibregtse JM, Howley PM (1997) The human E6-AP gene (UBE3A) encodes three potential protein isoforms generated by differential splicing. *Genomics* 41(2):263–266.

53. Glockzin S, Ogi FX, Hengstermann A, Scheffner M, Blattner C (2003) Involvement of the DNA repair protein hHR23 in p53 degradation. *Mol Cell Biol* 23(24):8960–8969.

54. Hengstermann A, et al. (2005) Growth suppression induced by downregulation of E6-AP expression in human papillomavirus-positive cancer cell lines depends on p53. *J Virol* 79(14):9296–9300.

55. Huang L, et al. (1999) Structure of an E6AP-UbcH7 complex: Insights into ubiquitination by the E2-E3 enzyme cascade. *Science* 286(5443):1321–1326.