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

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Deregulation of the ubiquitin ligase E6 associated protein (E6AP) encoded by the \textit{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 E6 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.

\textit{E6AP/UBE3A} \mid \text{ubiquitin} \mid \text{human papillomavirus} \mid \text{E6 oncoprotein} \mid \text{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 \textit{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 \textit{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 \textit{UBE3A} gene have been identified \((e.g., the paternal allele is silenced by a \textit{UBE3A} antisense transcript)\) \((14, 18)\), 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 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 \textit{HERC2} gene, resulting in a mutant HERC2 protein with increased turnover rate and hence decreased protein levels, underlies the development of a neuredevelopmental 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.


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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 Saccharomyces 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. 1A) 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 ubiquitin 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. 1B).

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 determined (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. 3A) or E6AP (Fig. 3B) (for results obtained with Ubch5B, see Fig. 5A).

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. 3A), indicating that the isolated HECT domain can use Ub_hpI for autoubiquitination. Indeed, subsequent autoubiquitination experiments showed that the isolated HECT domain uses wild type ubiquitin and Ub_hpI with similar efficiencies for autoubiquitination (Fig. S5B). In addition, when using Ubch5B instead of Ubch7, the HECT domain was autoubiquitinated 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 examination of the latter result revealed that in contrast to ubiquitin and Ub_hpI, covalent attachment of Ub_hpII to E6AP was mainly
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. S5B), which is known to be weakly active in isopeptide bond formation (38), and (iii) only poorly catalyzed by a UbcH5b mutant (Fig. S5B) that can still catalyze thioester complex formation between ubiquitin and HECT E3s but is impaired in isopeptide bond formation (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 ubiquitination by different mechanisms: Patch II appears to directly contribute to E6AP catalyzed isopeptide bond formation, because 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 distinct 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 HHRI23A (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 ubiquitination, 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. 4C). 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 proteases in a quantitative manner, resulting in two separate proteins. 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. 4C, expression 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 disorders 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
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 meclophilic 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 absence of these proteins, patch I does not contribute to the correct positioning of ubiquitin for the final transfer to a target protein. Further more, 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 absence of HPV E6, E6AP ubiquitinates itself preferentially in a 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 to the correct positioning of ubiquitin for the final transfer to a target protein. Furthermore, it was previously reported that E6AP autoubiquitination occurs via 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

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**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 hpl and hpII (Fig. 1A) 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 anti-WT ubiquitin (Ub), and the ubiquitin mutants hpI and hpII (Fig. 1B). A si n B

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**Table 1.** Summary of results obtained with Ubch5b, see Fig. S5.

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**Fig. S5.**
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 hpl, or Ub hplII (each 200 μM) at 37 °C for 20 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 containing 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 formation of UbcH5b, UbcH7, and the different ubiquitin conjugates of UbcH5b and UbcH7 (UbcH5b Ub, UbcH5b Ub hpl, UbcH5b Ub hplII, 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 densitometry. The ability of UbcH5b, UbcH7, and the various E2 ubiquitin conjugates 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 hpl and Ub hplII) in the absence or presence 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 electrophoresed 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 230 ng of E6AP or the HECT domain of E6AP were incubated with 20 μg of ubiquitin or the ubiquitin mutants (Ub hpl and Ub hplII) 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% (vol/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 expression 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 tagged Ring1B I53S (1 μg) as indicated (Fig. 4C). 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).

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