

# Physical and Functional Interaction of the HECT Ubiquitin-protein Ligases E6AP and HERC2<sup>\*[5]</sup>

Received for publication, November 20, 2010, and in revised form, April 4, 2011. Published, JBC Papers in Press, April 14, 2011, DOI 10.1074/jbc.M110.205211

Simone Kühnle<sup>#1</sup>, Ulrike Kogel<sup>#1,2</sup>, Sandra Glockzin<sup>‡</sup>, Andreas Marquardt<sup>§</sup>, Aaron Ciechanover<sup>¶</sup>, Konstantin Matentzoglou<sup>#3</sup>, and Martin Scheffner<sup>#4</sup>

From the <sup>‡</sup>Department of Biology and Konstanz Research School Chemical Biology, and <sup>§</sup>Proteomics Facility, University of Konstanz, 78457 Konstanz, Germany and the <sup>¶</sup>Cancer and Vascular Biology Research Center, The Rappaport Faculty of Medicine and Research Institute, Technion-Israel Institute of Technology, Haifa 31096, Israel

Deregulation of the ubiquitin-protein ligase E6AP contributes to the development of the Angelman syndrome and to cervical carcinogenesis suggesting that the activity of E6AP needs to be under tight control. However, how E6AP activity is regulated at the post-translational level under non-pathologic conditions is poorly understood. In this study, we report that the giant protein HERC2, which is like E6AP a member of the HECT family of ubiquitin-protein ligases, binds to E6AP. The interaction is mediated by the RCC1-like domain 2 of HERC2 and a region spanning amino acid residues 150–200 of E6AP. Furthermore, we provide evidence that HERC2 stimulates the ubiquitin-protein ligase activity of E6AP *in vitro* and within cells and that this stimulatory effect does not depend on the ubiquitin-protein ligase activity of HERC2. Thus, the data obtained indicate that HERC2 acts as a regulator of E6AP.

Covalent modification of proteins by ubiquitin (“ubiquitination”) plays a prominent role in the control of many fundamental cellular processes, including cell cycle control, DNA repair, and apoptosis. Substrate recognition and, thus, specificity of the ubiquitin-conjugation system are mainly mediated by ubiquitin-protein ligases E3<sup>5</sup> (1–3). Based on structural and mechanistic considerations, E3s can be grouped into two families, E3s containing a RING or RING-like domain and E3s with a HECT domain. Both domains serve as interaction sites for E2 ubiquitin-conjugating enzymes. However, whereas in the case of RING E3s attachment of ubiquitin to substrates is catalyzed by the interacting E2, HECT E3s first accept ubiquitin from their cognate E2s by formation of a thioester complex with ubiquitin and then transfer ubiquitin to substrates (1–3).

Because many cellular processes are controlled by ubiquitination, it is not surprising that deregulation of components of the ubiquitin-conjugation system contributes to the development of human diseases (1–4). E6AP, the founding member of the HECT family of E3s, represents one of the most prominent examples for this notion. Inactivation of the E3 activity of E6AP by mutation or loss of expression results in the development of the Angelman syndrome, a neurodevelopmental disorder, whereas unscheduled activation of E6AP by interaction with the E6 oncoprotein of certain human papillomaviruses, including HPV16, contributes to cervical carcinogenesis (5–9). Thus, thorough characterization of E6AP and its substrates and regulators should provide important insights into the mechanisms underlying the pathogenesis of the aforementioned diseases.

E6AP is encoded by the *UBE3A* gene located on chromosome 15q11–13 and exists in three isoforms generated by differential splicing (7, 10, 11). The isoforms differ at their N termini, but it is currently unknown if the isoforms have different properties (e.g. protein-binding properties). Several substrates of E6AP have been reported, including HHR23A and HHR23B, Blk, AIB1, PML, alpha-Synuclein, Arc, and Ring1B (12–18). However, with the exception of Arc and potentially Ring1B (17, 18), the relevance of these interactions for development of the Angelman syndrome remains to be shown.

In this study, we report that E6AP binds to HERC2, which is a member of the HECT and RCC1-like (HERC) domain subfamily of HECT E3s (4, 19). The interaction site of E6AP on HERC2 and vice versa was mapped to RLD2 of HERC2 and a region within the N-terminal 200 amino acid residues of E6AP. Furthermore, we provide evidence that binding of the isolated RLD2 or of full-length HERC2 activates the E3 activity of E6AP *in vitro* and within cells, respectively. Thus, besides the notion that this is the first example of heteromer formation between HECT E3s, the data identify HERC2 as a potential regulator of E6AP.

## EXPERIMENTAL PROCEDURES

**Cell Lines and Plasmids**—H1299 cells, HEK293T cells, and MEFs derived from E6AP knock-out (*Ube3a*<sup>-/-</sup>) mice (20) (Charles River) or wild-type littermates were grown in DMEM supplemented with 10% (v/v) FBS.

To generate a cell line, in which HERC2 expression is stably suppressed by RNA interference, H1299 cells were transfected with pMSCVpuro-HERC2 (Clontech) expressing an shRNA directed against nucleotides 8476–8499 of the HERC2 mRNA

\* This work was supported by the Deutsche Forschungsgemeinschaft, the German-Israeli Foundation for Scientific Research and Development, and the European Union Network of Excellence RUBICON.

[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. S1–S4.

<sup>1</sup> Both authors contributed equally to this work.

<sup>2</sup> Present address: Philip Morris Research Laboratories GmbH, Köln 51149, Germany.

<sup>3</sup> Present address: Trenzyme GmbH, Konstanz 78467, Germany.

<sup>4</sup> To whom correspondence should be addressed: Dept. of Biology, Box 642, University of Konstanz, Konstanz, 78457, Germany. Tel.: 49-7531-885150; Fax: 49-7531-885162; E-mail: martin.scheffner@uni-konstanz.de.

<sup>5</sup> The abbreviations used are: E3, ubiquitin-protein ligase; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; RLD, RCC1-like domain; MEF, mouse embryo fibroblast; HPV16, human papillomavirus type 16.

(with nucleotide 1 referring to A of the start codon) by lipofection (Lipofectamine 2000, Invitrogen). Cells stably containing the expression construct were selected by resistance to puromycin (Sigma). After establishing single cell clones, protein extracts were prepared, and HERC2 levels were determined by Western blot using an anti-HERC2 mouse monoclonal antibody (BD Biosciences).

The bacterial expression construct for the GST fusion protein of HPV16 E6 was described previously (21). Bacterial expression constructs for the ubiquitin mutant ubLIA (substitution of Leu-8 and Ile-44 by Ala) and for GST fusion proteins of RLD2 and RLD3, respectively, of HERC2, and of amino acids 150–200 of E6AP (numbering according to isoform 1 (11)) (see Fig. 1) were generated by PCR-based approaches (further details will be provided upon request). Expression constructs (*in vitro* translation, transient transfection experiments) encoding HA-tagged wild-type E6AP (isoform 1), the HA-tagged catalytically inactive mutant E6AP-C820A (substitution of Cys-820 by Ala), Myc-tagged  $\Delta$ RING-Ring1B, and His-tagged ubiquitin were described previously (22–24). cDNAs encoding various deletion mutants of E6AP isoform 1 (Fig. 1) were generated by PCR-based approaches (further details will be provided upon request) and expressed as N-terminally HA-tagged forms from pcDNA3. Expression constructs encoding an N-terminally HA-tagged form of full-length HERC2 and the respective catalytically inactive mutant HERC2-C4762A (substitution of Cys-4762 by Ala) were kindly provided by Trenzyme GmbH (Konstanz, Germany).

**Transfection and Antibodies**—For transient expression, cells were transfected with the respective constructs in the presence of a reporter construct encoding  $\beta$ -galactosidase by lipofection (Lipofectamine 2000) according to the manufacturer's instructions. Protein extracts were prepared 24 h after transfection as described (22), and, when necessary (Fig. 4), transfection efficiency was determined by measuring  $\beta$ -galactosidase activity. Levels of HA-tagged E6AP, HA-tagged E6AP mutants, HA-tagged HERC2, and Myc-tagged  $\Delta$ RING-Ring1B were determined by Western blot analysis. The antibodies used for detection were the mouse monoclonal HA.11 (Hiss Diagnostics, Freiburg, Germany) for HA-tagged proteins and the mouse monoclonal 9E10 (Abcam) for Myc-tagged  $\Delta$ RING-Ring1B. Endogenous HERC2 and E6AP present in protein extracts prepared from MEFs, H1299 cells, KD1299 cells (H1299 cells with stable knockdown of HERC2 expression), and HEK293T cells were detected by an anti-HERC2 mouse monoclonal antibody (BD Bioscience) and a mouse monoclonal antibody against E6AP (25), respectively.

**Protein-Protein Interaction Assays**—*In vitro* coprecipitation experiments using GST fusion proteins were performed as described previously (21). Briefly, 10  $\mu$ l of rabbit reticulocyte lysate-translated  $^{35}$ S-labeled proteins was incubated with bacterially expressed GST or GST fusion proteins as indicated (Fig. 1, B and C). After 3 h, bound proteins were purified by glutathione affinity chromatography and electrophoresed in 8% SDS-polyacrylamide gels, and  $^{35}$ S-labeled proteins were detected by fluorography.

For coprecipitation experiments using ectopically expressed E6AP (Fig. 1E),  $\sim 5 \times 10^6$  H1299 cells were transfected with the

E6AP expression construct indicated. Protein extracts were prepared 24 h after transfection and precleared with Protein A-Sepharose. Then, 4  $\mu$ g of the mouse monoclonal antibody HA.11 was added, extracts were incubated for 4 h at 4  $^{\circ}$ C, and bound proteins were precipitated by using Protein A-Sepharose. Precipitated proteins were detected by Western blot analysis.

For coprecipitation experiments with endogenous E6AP (Fig. 2A), protein extracts were prepared from  $\sim 10^7$  E6AP null (Ube3a $^{-/-}$ ) or wild-type (Ube3a $^{+/+}$ ) MEFs and precleared with Protein A-Sepharose. 1  $\mu$ g of a mouse monoclonal anti-E6AP antibody (25) was added, extracts were incubated for 4 h at 4  $^{\circ}$ C, and bound proteins were precipitated by using Protein A-Sepharose and analyzed by Western blot.

For size-exclusion chromatography (Fig. 2C), 400  $\mu$ g of protein extract derived from H1299 cells or from KD1299 cells was applied to a Sephacryl S300 column and fractionated with 25 mM Tris-HCl, pH 7.4, 50 mM NaCl as running buffer. One half of each fraction (1 ml) was analyzed by SDS-PAGE followed by Western blot analysis using the antibodies indicated.

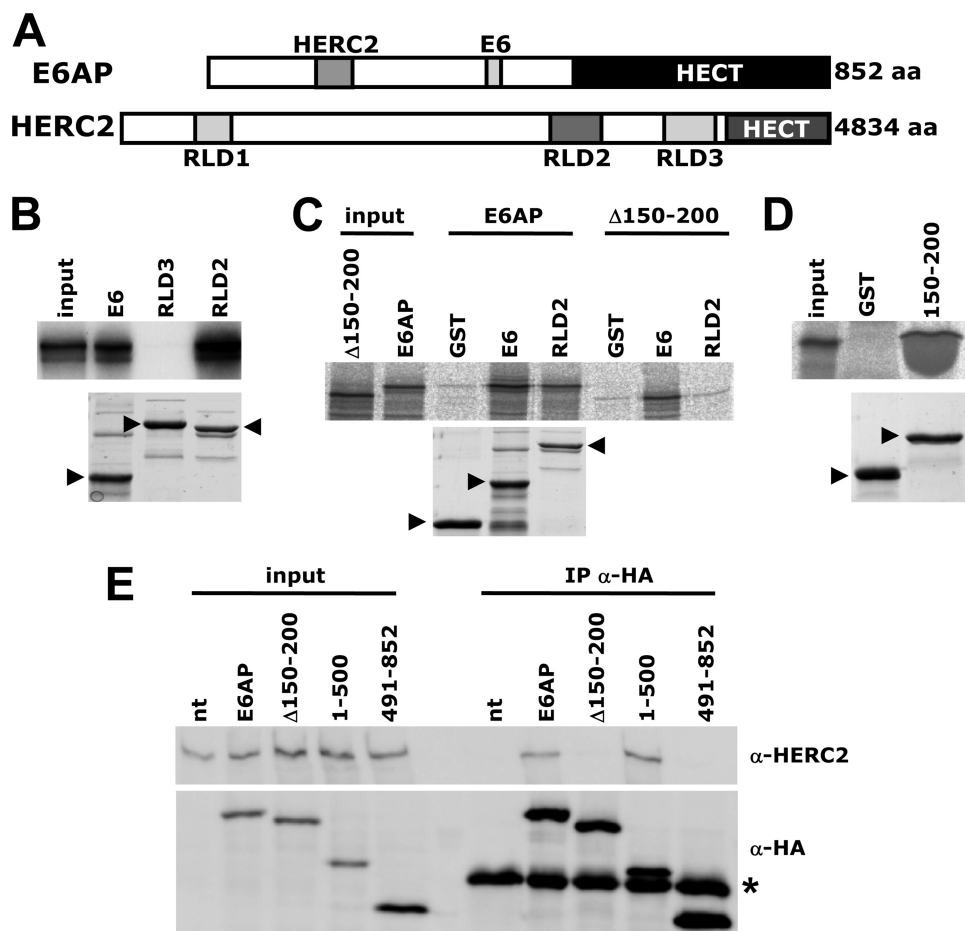
**Ubiquitination Assays**—For *in vitro* ubiquitination experiments, RLD2 was expressed as a GST fusion protein in *E. coli* BL21. The ubiquitin-activating enzyme E1 and E6AP (isoform 1) were expressed in the baculovirus system, and UbcH5b, wild-type ubiquitin, and the ubiquitin mutant ubLIA were expressed in *E. coli* BL21 by using the pET expression system as described (26). For *in vitro* ubiquitination, 1  $\mu$ l of rabbit reticulocyte lysate-translated  $^{35}$ S-labeled substrate (E6AP,  $\Delta$ RING-Ring1B) was incubated with 50 ng of E1, 50 ng of UbcH5b, 200 ng of baculovirus-expressed E6AP, and 20  $\mu$ g of ubiquitin in the absence or presence of bacterially expressed RLD2 (200 ng) in 40- $\mu$ l volumes. In addition, reactions contained 25 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM DTT, 2 mM ATP, and 4 mM MgCl<sub>2</sub>. After incubation at 25  $^{\circ}$ C for the times indicated (Fig. 3), total reaction mixtures were electrophoresed in 10% SDS-polyacrylamide gels, and  $^{35}$ S-labeled proteins were detected by fluorography. Results shown are representative of at least three different experiments with three different preparations of each protein.

For ubiquitination of E6AP and Ring1B within cells, one 6-cm plate of HEK293T cells was transfected with expression constructs encoding E6AP (2.5  $\mu$ g),  $\Delta$ RING-Ring1B (500 ng), His-tagged ubiquitin (1.5  $\mu$ g), and HERC2 or the catalytically inactive mutant HERC2-C4762A (3  $\mu$ g) as indicated (Fig. 4). 24 h after transfection, 30% of the cells were lysed under non-denaturing conditions as described (24) to determine expression levels of E6AP,  $\Delta$ RING-Ring1B, and the two forms of HERC2. The remaining cells were lysed under denaturing conditions, and ubiquitinated proteins were purified as described (24).

## RESULTS

**E6AP Interacts with HERC2**—To identify proteins that interact with E6AP, yeast two-hybrid screens and coprecipitation experiments using a bacterially expressed GST fusion protein of E6AP and cell extracts followed by mass spectrometric identification of coprecipitated proteins (27) were employed. A protein that was identified in both experimental setups is HERC2 (for details, see supplemental Fig. 1).

## HERC2 Stimulates E6AP-mediated Ubiquitination

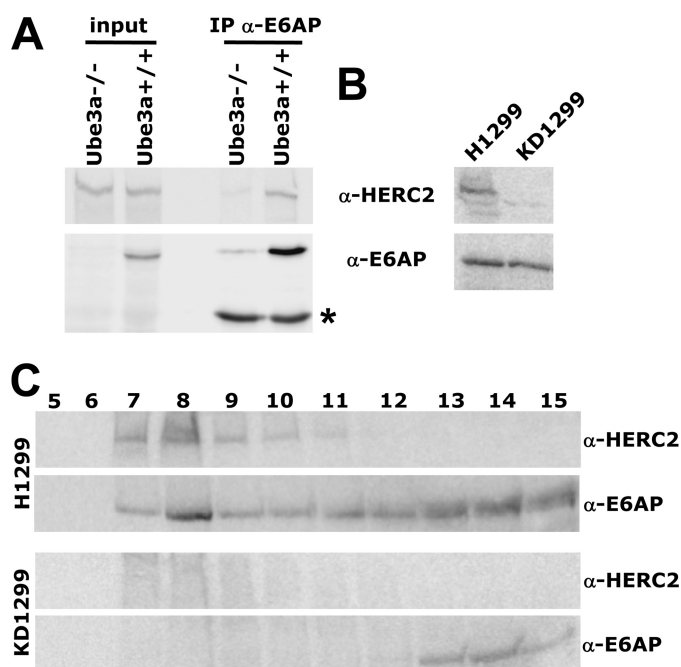


**FIGURE 1. The HECT ubiquitin ligase HERC2 binds to a region within the N terminus of E6AP.** *A*, schematic structure of human E6AP (isoform 1) and human HERC2. For E6AP, the position of the HERC2 and E6 binding domains (residues 150–200 and 378–395, respectively) and the HECT domain (residues 492–852) are indicated. For HERC2, the positions of RLD1, RLD2, and RLD3 (residues 513–778, 2958–3326, and 3951–4318, respectively) and the HECT domain (residues 4457–4834) are indicated. *B*, RLD2, RLD3, and the HPV16 E6 oncoprotein were bacterially expressed as GST fusion proteins and purified by affinity chromatography using glutathione-Sepharose. Similar amounts of the various fusion proteins (*lower panel*; running positions of the respective full-length proteins are indicated by arrowheads) were incubated with *in vitro* translated radiolabeled E6AP (isoform 1). After 3 h at 4 °C, glutathione beads were centrifuged, supernatants were removed, and beads were washed. The amount of E6AP bound to the various GST fusion proteins was determined by SDS-PAGE followed by fluorography (*upper panel*). *input*, corresponds to 20% of *in vitro* translated E6AP used for binding reactions. *C*, binding reactions and analysis of bound proteins (*upper panel*) were performed as in *B* with GST fusion proteins of RLD2 and HPV16 E6 as affinity matrix (for amounts, see the *lower panel*; running positions of the respective full-length proteins are indicated by arrowheads) and *in vitro* translated radiolabeled E6AP or E6AP $\Delta$ 150–200 (deletion of amino acid residues 150–200) as indicated. *input*, corresponds to 20% of *in vitro* translated E6AP and E6AP $\Delta$ 150–200 used for binding reactions. *D*, binding reactions and analysis of bound proteins were performed as in *B* with a GST fusion protein of amino acid residues 150–200 of E6AP (150–200) and *in vitro* translated radiolabeled RLD2 (*upper panel*). GST was used as an affinity matrix control. *input*, corresponds to 20% of *in vitro* translated RLD2 used for binding reactions. For amounts of GST and GST-E6AP150–200 used as affinity matrix, see the *lower panel*. *E*, H1299 cells were transfected with expression constructs for the HA-tagged forms of E6AP indicated. Protein extracts were prepared 24 h after transfection. 20% of the respective extracts were used to determine levels of the various forms of E6AP and endogenous HERC2 by Western blot analysis as indicated (*input*). To the remainder of the extracts, an anti-HA antibody was added, mixtures were incubated for 4 h at 4 °C, and bound proteins were precipitated by Protein A-Sepharose. Precipitated proteins were detected by Western blot analysis as indicated. *nt*, untransfected cell extracts were used as control. The band indicated with an *star* represents the heavy chain of the anti-HA antibody used.

HERC2 consists of 4834 amino acid residues, contains three RLDs, and contains a C-terminal HECT domain (Fig. 1A). It is thus a member of the HERC subfamily of HECT E3s (4, 19). Due to its size, verification of the potential interaction of full-length HERC2 with E6AP *in vitro* cannot be readily achieved. However, in the yeast two-hybrid screen several independent clones encoding distinct but overlapping regions of HERC2 were obtained. The region that is common to all clones encompasses RLD2 (amino acid residues 2958–3326; Fig. 1A and [supplemental Fig. 1A](#)) indicating that RLD2 interacts with E6AP. Indeed, GST pull-down experiments with *in vitro* translated radiolabeled E6AP revealed that RLD2 binds E6AP, whereas binding was not observed with RLD3, RLD1, or the isolated

HECT domain of HERC2 (Fig. 1B and data not shown). This indicates that RLD2 is sufficient and likely necessary for binding of HERC2 to E6AP, although the possibility that other regions of HERC2 contribute to the interaction with E6AP cannot be excluded. Furthermore, coprecipitation experiments with various deletion mutants of E6AP translated *in vitro* (Fig. 1C) or expressed in bacteria (Fig. 1D) or expressed within cells (Fig. 1E) showed that the region spanning amino acid residues 150–200 of E6AP (numbering according to isoform 1 (11)) is necessary and sufficient for E6AP to bind to HERC2.

**Endogenous E6AP Binds to Endogenous HERC2**—To provide evidence that endogenous E6AP and HERC2 are present in common complexes within cells, two approaches were used.



**FIGURE 2. Copurification of endogenous E6AP and HERC2.** *A*, extracts were prepared from MEFs derived from E6AP null embryos (*Ube3a*<sup>-/-</sup>) and matched littermates (*Ube3a*<sup>+/+</sup>). Levels of E6AP and HERC2 were either directly analyzed by Western blot analysis (*input*) or extracts were first subjected to immunoprecipitation with an anti-E6AP antibody, and the precipitates were analyzed by Western blot analysis with the antibodies indicated (*IP α-E6AP*). The band indicated with a *star* represents the heavy chain of the anti-E6AP antibody used. Note that, for preparation of E6AP null MEFs, MEFs derived from several embryos were pooled. Because the E6AP null embryos were derived from hemizygous (*i.e.* *Ube3a*<sup>+/-</sup>) animals (as female E6AP null mice rarely become pregnant and male E6AP null mice are not or only poorly fertile), embryos had to be genotyped before MEF preparation. Therefore, the weak signal corresponding to E6AP, as detected in the immunoprecipitation/Western analysis of E6AP null MEFs, is presumably due to contamination of the MEF preparation with MEFs derived from hemizygous (*i.e.* mistyped) littermates. *B*, extracts were prepared from H1299 cells and from H1299 cells, in which endogenous HERC2 expression was stably down-regulated by RNA interference (KD1299) and subjected to Western blot analysis with the antibodies indicated. *C*, extracts were prepared from H1299 and KD1299 cells, applied to a Sephacryl S300 column, and fractionated with 25 mM Tris-HCl, pH 7.4, 50 mM NaCl as running buffer. 1/2 of each fraction (1 ml) was analyzed by SDS-PAGE followed by Western blot analysis using the antibodies indicated.

Firstly, coprecipitation experiments were performed using cell extracts derived from E6AP null MEFs (prepared from *Ube3a*<sup>-/-</sup> embryos) or from wild-type MEFs (prepared from littermates and *Ube3a*<sup>+/+</sup> embryos) (Fig. 2*A*). This showed that HERC2 can only be precipitated by an anti-E6AP antibody from extracts derived from wild-type MEFs demonstrating that endogenous HERC2 and E6AP can bind to each other. Secondly, H1299 cells with a stable knockdown of HERC2 expression were established by RNA interference (for details, see “Experimental Procedures”) (Fig. 2*B*, KD1299). Protein extracts derived from KD1299 cells and parental H1299 cells were applied to size-exclusion chromatography, and the fractionation behavior of HERC2 and E6AP was analyzed by Western blot analysis. As shown in Fig. 2*C*, in extracts derived from parental H1299 cells, E6AP fractionates in two peaks. One peak corresponds to a protein or protein complex with a molecular mass of >600 kDa and coincides with the presence of HERC2. The other protein peak migrates with a molecular mass of 90–120 kDa, corresponding to the mass of monomeric E6AP.

Importantly, in KD1299 extracts, E6AP is not found in the high molecular mass fractions supporting the notion that endogenous HERC2 and E6AP are present in common complexes. Due to the high molecular mass of HERC2, it is not possible to conclude if other proteins are present in the HERC2-E6AP complex or if the complexes consist of E6AP and HERC2 only. Nonetheless, the results obtained *in vitro* and in the yeast two-hybrid screen indicate that the interaction of E6AP and HERC2 does not require any additional proteins (*i.e.* E6AP and HERC2 interact directly).

**RLD2 of HERC2 Stimulates E6AP Autoubiquitination and E6AP-mediated Ubiquitination of Ring1B *In Vitro***—Because both E6AP and HERC2 are members of the HECT family of E3s, an obvious possibility is that HERC2 represents a substrate for E6AP and/or vice versa. In ubiquitination experiments *in vitro*, isolated RLD2 or an N-terminally truncated mutant of HERC2 spanning amino acid residues 2832–4834 are indeed ubiquitinated by E6AP. However, no evidence could be obtained in overexpression experiments, or in experiments in which endogenous E6AP expression was down-regulated by RNA interference (22), that endogenous or ectopically expressed full-length HERC2 represent substrates for E6AP (data not shown).

E6AP is known to ubiquitinate itself (“autoubiquitination”) *in vitro* (25). To obtain initial evidence that the interaction of E6AP with HERC2 has functional consequences, bacterially expressed RLD2 was added to an E6AP autoubiquitination reaction. This revealed that, under the conditions used, the kinetics of E6AP autoubiquitination is ~2-fold faster in the presence of RLD2 (Fig. 3*A*).

Although the effect of RLD2 on E6AP autoubiquitination is highly reproducible, different conditions were tested (*e.g.* limiting amounts of ubiquitin; see supplemental Fig. 2) to obtain more convincing evidence that binding of RLD2 stimulates the E3 activity of E6AP. In the experiment shown in Fig. 3*B*, a ubiquitin mutant termed ubLIA, in which Leu-8 and Ile-44 of wild-type ubiquitin were replaced by Ala, was employed. The mutations in ubLIA affect the hydrophobic patch of ubiquitin and, in consequence, ubLIA is compromised in its ability to interact with ubiquitin-binding proteins and is also less efficiently activated by E1 (28, 29). Indeed, in the presence of ubLIA the kinetics of E6AP autoubiquitination was significantly slowed down (Fig. 3*B*; compare with Fig. 3*A*). More importantly, addition of RLD2 resulted in a significant stimulation of E6AP autoubiquitination. Furthermore, this effect is specific for RLD2, because it was not observed with RLD3 (Fig. 3*B*) or with an E6AP mutant that does not bind to RLD2 but is active in autoubiquitination (25) (supplemental Fig. 3).

We have recently shown that the ubiquitin ligase Ring1B represents a substrate for E6AP (18). Thus, to investigate if the effect of RLD2 is restricted to E6AP autoubiquitination or if RLD2 in general acts as an activator of E6AP, E6AP-mediated ubiquitination of a Ring1B mutant, ΔRING-Ring1B, was studied (ΔRING-Ring1B was used as substrate rather than wild-type Ring1B, because, due to deletion of the RING domain, ΔRING-Ring1B does not function as an E3 ligase and in consequence cannot ubiquitinate itself. Thus, E6AP-mediated ubiquitination of ΔRING-Ring1B can be easily monitored) (23). As shown

## HERC2 Stimulates E6AP-mediated Ubiquitination

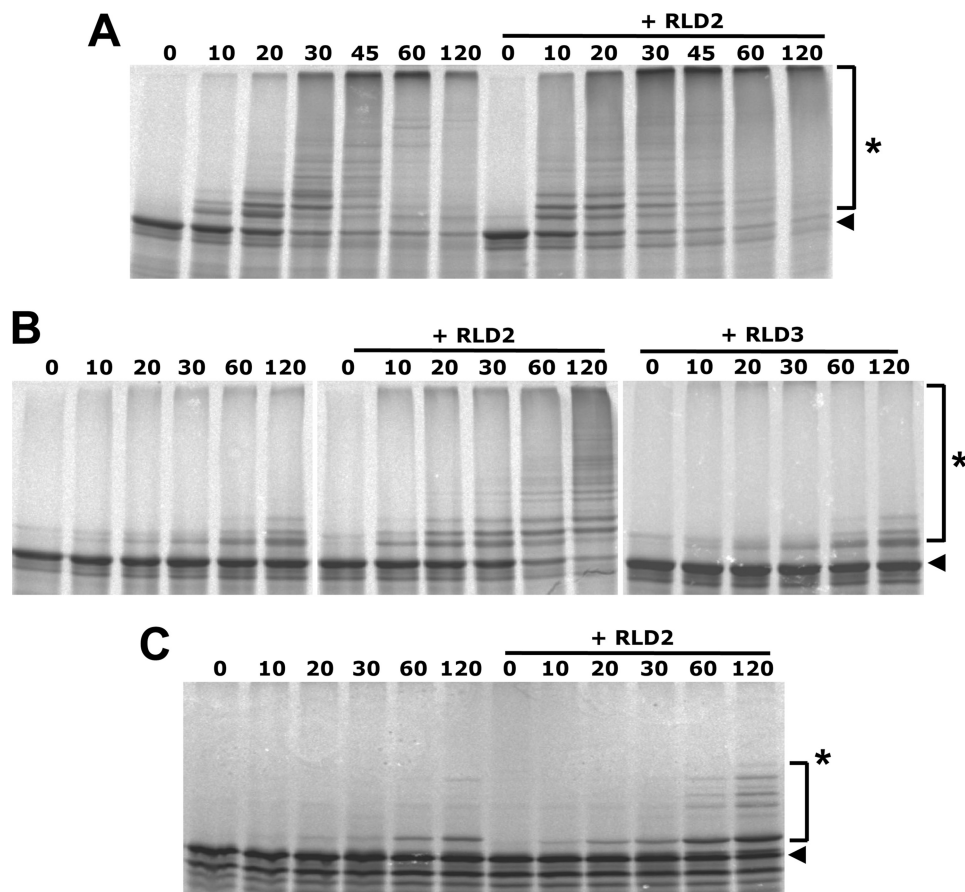


FIGURE 3. **RLD2 of HERC2 stimulates the E3 activity of E6AP *in vitro*.** A–C, *in vitro* translated radiolabeled E6AP (A and B) and  $\Delta$ RING-Ring1B (C) were incubated with baculovirus-expressed E6AP and wild-type ubiquitin (A) or the ubiquitin mutant ubL1A (B and C) under standard ubiquitination conditions in the absence or presence of bacterially expressed GST fusion protein of RLD2 or RLD3 for the times indicated (minutes). Reaction products were analyzed by SDS-PAGE followed by fluorography. Running positions of the non-modified form and of the ubiquitinated forms of E6AP and  $\Delta$ RING-Ring1B are indicated by an arrowhead and an asterisk, respectively.

in Fig. 3C, similar to E6AP autoubiquitination, E6AP-mediated ubiquitination of  $\Delta$ RING-Ring1B was significantly stimulated in the presence of RLD2.

**Full-length HERC2 Stimulates E6AP Activity within Cells**—Because we have not yet succeeded in generating bacterial or baculovirus-based expression constructs for full-length HERC2, we have not been able so far to determine *in vitro* whether the stimulatory effect of RLD2 is a peculiarity of the isolated domain or whether it can also be observed with full-length HERC2. However, we managed to ectopically express full-length HERC2 in HEK293T cells. This enabled us to ask the question as to whether HERC2 can stimulate E6AP activity within cells. To address this issue, the effect of full-length HERC2 on E6AP autoubiquitination and E6AP-mediated ubiquitination of  $\Delta$ RING-Ring1B was determined in cotransfection experiments (Fig. 4, A and B, respectively). The results obtained clearly indicate that both ubiquitination reactions are stimulated by HERC2. Furthermore, this stimulatory effect does not require a catalytically active form of HERC2, as it was observed in the presence of HERC2-C4762A, in which the catalytically active Cys residue of HERC2 was substituted by Ala. This indicates that the increase in ubiquitination observed in the presence of HERC2 was not due to HERC2-mediated ubiquitination of E6AP or Ring1B but that, similar to the *in vitro* situation, HERC2 acted as a stimulator of the E3 activity of E6AP.

## DISCUSSION

In this study, we have shown that the HECT E3 family members E6AP and HERC2 bind to each other and that HERC2 can stimulate the E3 activity of E6AP. Furthermore, the interaction of E6AP and HERC2 is not mediated via their HECT domains but rather by regions within the respective N-terminal extensions (Fig. 1A). Based on the fact that HERC2 consists of 4834 amino acids, we propose that HERC2 is a multifunctional protein and that one of its functions is to act as an allosteric activator of E6AP.

Similar to the E6AP gene (*UBE3A*), the gene encoding HERC2 is located on chromosome 15q11–13 and is frequently deleted in patients with Angelman syndrome (10). Although there is currently no evidence to indicate that loss of HERC2 activity contributes to the development of the Angelman syndrome, this does not exclude the possibility that, in neuronal cells, the activity of E6AP is in part controlled by HERC2. It was recently reported that E6AP regulates synapse development by targeting Arc, which is involved in internalization of glutamate receptors of the AMPA subtype, for ubiquitination and subsequent degradation (17). Thus, to support the notion that HERC2 is involved in controlling E6AP activity in neurons, it was of immediate interest to determine if HERC2 can also stimulate E6AP-mediated ubiquitination of Arc. However, we have

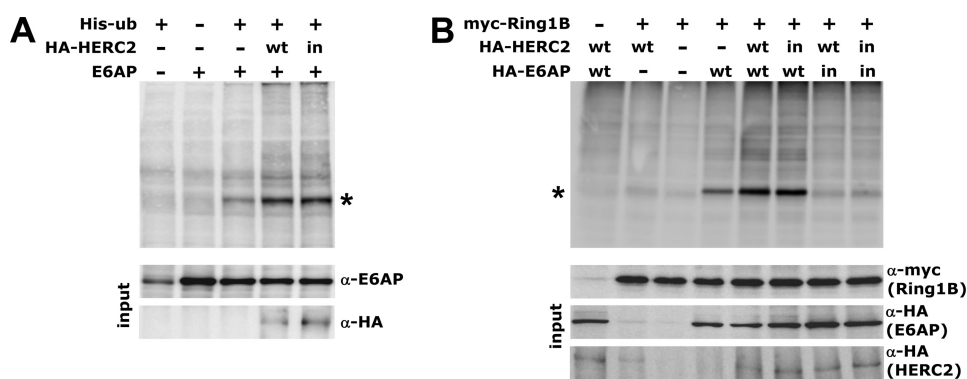


FIGURE 4. **HERC2 stimulates the E3 activity of E6AP within cells.** *A*, HEK293T cells were cotransfected with expression constructs for His-tagged ubiquitin, E6AP (isoform 1), and HA-tagged full-length HERC2 (*wt*) or the HA-tagged catalytically inactive HERC2 mutant HERC2-C4762A (*in*) as indicated. Protein extracts were prepared 24 h after transfection, and ubiquitinated proteins were isolated by  $\text{Ni}^{2+}$ -affinity chromatography. Upon affinity purification, levels of ubiquitinated E6AP were determined by Western blot analysis with an E6AP-specific antibody (*upper panel*). *input*, corresponds to 10% of the protein extracts used for affinity purification. \*, (presumably mono-)ubiquitinated E6AP. *B*, HEK293T cells were cotransfected with expression constructs for His-tagged ubiquitin, Myc-tagged  $\Delta$ RING-Ring1B, HA-tagged wild-type E6AP (*wt*) or its catalytically inactive mutant E6AP-C820A (*in*), and HA-tagged full-length HERC2 (*wt*) or the HA-tagged catalytically inactive HERC2 mutant HERC2-C4762A (*in*) as indicated. Protein extracts were prepared 24 h after transfection, and ubiquitinated proteins were isolated by  $\text{Ni}^{2+}$ -affinity chromatography. Upon affinity purification, levels of ubiquitinated  $\Delta$ RING-Ring1B were determined by Western blot analysis with an anti-myc antibody (*upper panel*). *input*, corresponds to 10% of the protein extracts used for affinity purification. \*, monoubiquitinated  $\Delta$ RING-Ring1B.

so far not been able to obtain any evidence that E6AP induces ubiquitination of Arc *in vitro* or in overexpression experiments (data not shown), neither in the absence nor in the presence of HERC2. This may indicate that additional factors (*e.g.* post-translational modification of Arc and/or E6AP) are involved in the interaction of E6AP with Arc or that Arc is not a direct target of E6AP (*i.e.* ubiquitination/degradation of Arc is mediated by another E3 enzyme that may be regulated by E6AP).

Because HERC2 affects E6AP-mediated ubiquitination of Ring1B *in vitro* and in overexpression experiments within cells (Figs. 3 and 4), we have looked into the possibility that knock-down of endogenous HERC2 expression by RNA interference affects Ring1B levels. The results obtained so far have been inconclusive. However, it should be noted that the effect of down-regulation of HERC2 expression on Ring1B levels maybe rather difficult to observe. For example, the effect of E6AP on Ring1B can be readily visualized by using Ring1B mutants devoid of E3 activity but is more difficult to observe with wild-type Ring1B (18). Similarly, E6AP may not be the only E3 enzyme involved in determining the half-life of Ring1B. Furthermore, although the efficiency of knockdown of HERC2 expression that we were able to achieve is rather good (approximately 90%), it is not complete and, thus, it is possible that the remaining level of HERC2 may suffice for stimulation of E6AP activity.

HERC2 was recently reported to be involved in DNA repair processes and to interact with RNF8, RNF168, XPA, and BRCA1 (30–32). Interestingly, although BRCA1 and XPA appear to be substrates for HERC2 (31, 32), RNF8 activity appears to be stimulated by HERC2 (30). In contrast to E6AP, however, the interaction of HERC2 with RNF8 involves the HECT domain of HERC2 suggesting that different mechanisms are involved in HERC2-mediated stimulation of E6AP and of RNF8. Furthermore, we have so far not obtained any evidence that, through its interaction with HERC2, E6AP may be involved in DNA repair processes. In this context, it should be noted that spontaneous mutations in the murine *Herc2* gene

have been associated with the phenotypes of so-called *rjs* or *jdf2* mice (33, 34). Although not contradictory, the phenotypes of these mice are not obviously linked to deficiencies in DNA repair pathways supporting the notion that HERC2 is a multifunctional protein. Furthermore, HERC2 was recently identified in a study searching for proteins that interact with the HPV16 E6 oncoprotein (35). Preliminary evidence indicates that E6 can bind to HERC2 in an E6AP-dependent as well as in an E6AP-independent manner and that E6, E6AP, and HERC2 can form ternary complexes (*supplemental Fig. 4*). Thus, it will be interesting to determine if E6 can affect HERC2 function and vice versa.

How does HERC2 stimulate E6AP activity? We have previously shown that autoubiquitination of E6AP occurs in *trans* rather than in *cis* (*i.e.* E6AP-E6AP interaction is required for autoubiquitination) (25). Thus, obvious possibilities are that HERC2 induces E6AP multimerization or that, similar to the situation in the presence of the HPV E6 oncoprotein (36), E6AP autoubiquitination occurs in *cis*. However, based on the notion that, unlike the E6 oncoprotein, the presence of RLD2 does not affect the amount of E6AP required *in vitro* to observe autoubiquitination (data not shown) both possibilities appear to be unlikely. An alternative possibility is that binding of HERC2 alters the conformation of the HECT domain of E6AP, affecting for example the kinetics of the final transfer of activated ubiquitin from the catalytic cysteine residue of E6AP to the receiving lysine residue of E6AP or of a substrate of E6AP. Unlike the HECT domain (37), the structure of full-length E6AP has not been reported yet. Because the interaction of E6AP and HERC2 does not involve the HECT domain, elucidation of the mechanism, by which HERC2 stimulates E6AP activity, will probably have to await the solution of the structure of full-length E6AP in the absence and presence of RLD2.

## REFERENCES

1. Kerscher, O., Felberbaum, R., and Hochstrasser, M. (2006) *Annu. Rev. Cell Dev. Biol.* **22**, 159–180

## HERC2 Stimulates E6AP-mediated Ubiquitination

2. Deshaies, R. J., and Joazeiro, C. A. (2009) *Annu. Rev. Biochem.* **78**, 399–434
3. Rotin, D., and Kumar, S. (2009) *Nat. Rev. Mol. Cell. Biol.* **10**, 398–409
4. Scheffner, M., and Staub, O. (2007) *BMC Biochem.* **8**, Suppl. 1, S6
5. Matsuura, T., Sutcliffe, J. S., Fang, P., Galjaard, R. J., Jiang, Y. H., Benton, C. S., Rommens, J. M., and Beaudet, A. L. (1997) *Nat. Genet.* **15**, 74–77
6. Kishino, T., Lalonde, M., and Wagstaff, J. (1997) *Nat. Genet.* **15**, 70–73
7. Clayton-Smith, J., and Laan, L. (2003) *J. Med. Genet.* **40**, 87–95
8. Beaudenon, S., and Huijbregtse, J. M. (2008) *BMC Biochem.* **9**, Suppl. 1, S4
9. Shai, A., Pitot, H. C., and Lambert, P. F. (2010) *Cancer Res.* **70**, 5064–5073
10. Nicholls, R. D., and Knepper, J. L. (2001) *Annu. Rev. Genomics Hum. Genet.* **2**, 153–175
11. Yamamoto, Y., Huijbregtse, J. M., and Howley, P. M. (1997) *Genomics* **41**, 263–266
12. Kumar, S., Talis, A. L., and Howley, P. M. (1999) *J. Biol. Chem.* **274**, 18785–18792
13. Oda, H., Kumar, S., and Howley, P. M. (1999) *Proc. Natl. Acad. Sci. U.S.A.* **96**, 9557–9562
14. Mani, A., Oh, A. S., Bowden, E. T., Lahusen, T., Lorick, K. L., Weissman, A. M., Schlegel, R., Wellstein, A., and Riegel, A. T. (2006) *Cancer Res.* **66**, 8680–8686
15. Louria-Hayon, I., Alsheich-Bartok, O., Levav-Cohen, Y., Silberman, I., Berger, M., Grossman, T., Matentzoglou, K., Jiang, Y. H., Muller, S., Scheffner, M., Haupt, S., and Haupt, Y. (2009) *Cell Death Differ.* **16**, 1156–1166
16. Mulherkar, S. A., Sharma, J., and Jana, N. R. (2009) *J. Neurochem.* **110**, 1955–1964
17. Greer, P. L., Hanayama, R., Bloodgood, B. L., Mardinly, A. R., Lipton, D. M., Flavell, S. W., Kim, T. K., Griffith, E. C., Waldon, Z., Maehr, R., Ploegh, H. L., Chowdhury, S., Worley, P. F., Steen, J., and Greenberg, M. E. (2010) *Cell* **140**, 704–716
18. Zaaroor-Regev, D., de Bie, P., Scheffner, M., Noy, T., Shemer, R., Heled, M., Stein, I., Pikarsky, E., and Ciechanover, A. (2010) *Proc. Natl. Acad. Sci. U.S.A.* **107**, 6788–6793
19. Garcia-Gonzalo, F. R., and Rosa, J. L. (2005) *Cell Mol. Life Sci.* **62**, 1826–1838
20. Jiang, Y. H., Armstrong, D., Albrecht, U., Atkins, C. M., Noebels, J. L., Eichele, G., Sweatt, J. D., and Beaudet, A. L. (1998) *Neuron* **21**, 799–811
21. Huijbregtse, J. M., Scheffner, M., and Howley, P. M. (1993) *Mol. Cell. Biol.* **13**, 775–784
22. Kuballa, P., Matentzoglou, K., and Scheffner, M. (2007) *J. Biol. Chem.* **282**, 65–71
23. Ben-Saadon, R., Zaaroor, D., Ziv, T., and Ciechanover, A. (2006) *Mol. Cell* **24**, 701–711
24. Glockzin, S., Ogi, F. X., Hengstermann, A., Scheffner, M., and Blattner, C. (2003) *Mol. Cell. Biol.* **23**, 8960–8969
25. Nuber, U., Schwarz, S. E., and Scheffner, M. (1998) *Eur. J. Biochem.* **254**, 643–649
26. Nuber, U., Schwarz, S., Kaiser, P., Schneider, R., and Scheffner, M. (1996) *J. Biol. Chem.* **271**, 2795–2800
27. Matentzoglou, K., and Scheffner, M. (2008) *Biochem. Soc. Trans.* **36**, 797–801
28. Beal, R., Deveraux, Q., Xia, G., Rechsteiner, M., and Pickart, C. (1996) *Proc. Natl. Acad. Sci. U.S.A.* **93**, 861–866
29. Dikic, I., Wakatsuki, S., and Walters, K. J. (2009) *Nat. Rev. Mol. Cell Biol.* **10**, 659–671
30. Bekker-Jensen, S., Rendtlew Danielsen, J., Fugger, K., Gromova, I., Nerstedt, A., Lukas, C., Bartek, J., Lukas, J., and Mailand, N. (2010) *Nat. Cell Biol.* **12**, 80–86, suppl. pp. 1–12
31. Kang, T. H., Lindsey-Boltz, L. A., Reardon, J. T., and Sancar, A. (2010) *Proc. Natl. Acad. Sci. U.S.A.* **107**, 4890–4895
32. Wu, W., Sato, K., Koike, A., Nishikawa, H., Koizumi, H., Venkitaraman, A. R., and Ohta, T. (2010) *Cancer Res.* **70**, 6384–6392
33. Lehman, A. L., Nakatsu, Y., Ching, A., Bronson, R. T., Oakey, R. J., Keiper-Hrynko, N., Finger, J. N., Durham-Pierre, D., Horton, D. B., Newton, J. M., Lyon, M. F., and Brilliant, M. H. (1998) *Proc. Natl. Acad. Sci. U.S.A.* **95**, 9436–9441
34. Walkowicz, M., Ji, Y., Ren, X., Horsthemke, B., Russell, L. B., Johnson, D., Rinchik, E. M., Nicholls, R. D., and Stubbs, L. (1999) *Mamm. Genome* **10**, 870–878
35. Vos, R. M., Altreuter, J., White, E. A., and Howley, P. M. (2009) *J. Virol.* **83**, 8885–8892
36. Kao, W. H., Beaudenon, S. L., Talis, A. L., Huijbregtse, J. M., and Howley, P. M. (2000) *J. Virol.* **74**, 6408–6417
37. Huang, L., Kinnucan, E., Wang, G., Beaudenon, S., Howley, P. M., Huijbregtse, J. M., and Pavletich, N. P. (1999) *Science* **286**, 1321–1326