The Active Form of E6-associated protein (E6AP)/UBE3A Ubiquitin Ligase Is an Oligomer*5

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Background: E6AP/UBE3A is a Hect ligase implicated in neurodevelopment and cell transformation. Kinetic/biophysical analyses demonstrate that E6AP oligomerization is required for polyubiquitin degradation signal assembly and that changes in oligomerization regulate such activity.

Results: Employing 125I-polyubiquitin chain formation as a functional readout of ligase activity, biochemical and biophysical evidence demonstrates that catalytically active E6-associated protein (E6AP)/UBE3A is an oligomer. Based on an extant structure previously discounted as an artifact of crystal packing forces, we propose that the fully active form of E6AP is a trimer, analysis of which reveals a buried surface of 7508 Å² and radially symmetric Phe727 intercalation and trimer formation. Conversely, oncogenic human papillomavirus-16/18 E6 protein significantly enhances E6AP catalytic activity by promoting trimer formation (Kcat = 12 mM), as does a conserved α-helical peptide corresponding to residues 474–490 of E6AP isoform 1 (Kcat = 22 μM) reported to bind the hydrophobic pocket of other Hect ligases, presumably blocking Phe727 intercalation and trimer formation. Conversely, oncoprotein-deficient mouse models or those expressing higher levels of the "UBE3A" gene locus within the 15q11–13 chromosome region is associated with the neurological disorder Angelman syndrome (8–10). Patients affected with Angelman syndrome are characterized by severe intellectual and developmental disability, speech impairment, behavioral uniqueness, epilepsy, and severely abnormal electroencephalography, among other symptoms (11–14). Most of the naturally occurring mutations within the UBE3A gene introduce deletions that generate truncated forms of E6AP lacking the intact Hect domain; however, ~10% of the genetic alterations correspond to point mutations within the E6AP coding region (15, 16). Although many of the point mutants represent loss-of-function alterations and are not able to ubiquitinate their substrate, paradoxically, many retain the ability to form a thioester bond with ubiquitin (17). In contrast, duplication of the corresponding UBE3A gene is thought to result in some cases of autism spectrum disorder (18–21). These observations suggest a narrow range of E6AP activity for normal neurological development because UBE3A-deficient mouse models or those expressing higher levels of the E6AP2/UBE3A is the founding member of the Hect (homologous to E6AP C terminus) ubiquitin ligase family and is defined by a highly conserved 350-residue C-terminal catalytic domain (1, 2). The Hect domain is characterized by the presence of an active site cysteine that forms an obligatory high energy thioester bond with ubiquitin prior to transfer of the latter to specific substrate proteins, the identity of which is defined by the N-terminal targeting domain (2). The E6AP Hect domain assembles Lys48-linked polyubiquitin degradation signals that are recognized by the 26S proteasome (3, 4). Both activation and loss of E6AP function are implicated in various human diseases, as discussed elsewhere (5–7). Abrogation of E6AP function by deletion, imprinting defects, or mutation of the UBE3A gene is associated with neurodevelopment and cell transformation. These findings explain in part the etiology of specific Angelman syndrome mutations and E6-mediated cell transformation.

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2 The abbreviations used are: E6AP, E6-associated protein (gene name UBE3A); E6, human papilloma virus 16 E6 protein; Hect, homologous to E6AP C terminus; HPV, human papilloma virus; Ac-PheNH2, N-acetylphenylalanamide; E1, generic term for activating enzymes of Class 1 ubiquitin-conjugating enzymes; E2/Ubc, generic name for ubiquitin carrier protein/ubiquitin-activating enzyme; E3, generic name for ubiquitin:protein isopeptide ligase; UbcH7, human E2 carrier protein (gene name UBE2L3); Uba1, ubiquitin-activating enzyme (gene name UBE1).
ligase show phenotypes similar to Angelman syndrome or autism, respectively (18, 22–24). In general, the clinical symptoms associated with the neurological disorders and the identified targets of E6AP-catalyzed ubiquitination localize to regulatory pathways required for synaptic plasticity (25–29). The identification of Arc and Ephexin 5 as targets of E6AP, both of which function to mediate synaptic remodeling, provide a framework for reconciling the loss-of-function mutations in the E6AP maternal copy and the neurological and developmental defects present in affected individuals (25, 26, 30), although more recent evidence questions a role for E6AP in targeting Arc degradation (30).

The E6AP protein was initially identified by its interaction with the E6 viral protein encoded by human papilloma virus 16 (HPV16) (31–33). Although p53 degradation is normally mediated by the Mdm2 ubiquitin ligase (34), E6 protein is proposed to bind to E6AP and redirect its specificity to p53 (33, 35). Enhanced degradation of p53 in epithelial cells by the HPV16 and -18 viral strains induces cell transformation and development of cervical and oral cancers, depending on the site of infection (5, 36, 37). Similarly, hepatitis C virus encodes the NS5B protein that binds E6AP and induces degradation of the retinoblastoma protein tumor suppressor, increasing the risk of liver cirrhosis and hepatocellular carcinoma (38, 39). The E6AP sequence contains a leucine-rich motif (LXXXL) in the N-terminal region to which the E6 viral protein binds (40–42). Biophysical and structural analyses of the E6–E6AP interaction show that the N-terminal E6 Zn$^{2+}$-binding domain primarily interacts with E6AP, whereas the C-terminal Zn$^{2+}$-binding domain interacts with p53 (43–47). Although the interaction of E6 with E6AP is necessary for degradation of p53, the effect of such interaction on the catalytic activity of E6AP has not been adequately addressed; Kao et al. (48) have shown that ectopic E6 expression increases E6AP autoubiquitination and intracellular turnover.

Full-length E6AP is a protein of 100 kDa; however, only the structure of the truncated E6AP Hect domain in association with its cognate UbcH7 ubiquitin carrier protein has been reported (49). The Hect domain architecture displays an L-shape with distinct N-terminal and C-terminal subdomains connected by a flexible hinge region (49). The N-terminal subdomain can be further divided into large and small N-terminal subdomains also connected by flexible hinge segments (49). The active site Cys$^{820}$ to which ubiquitin forms a thioester intermediate is contained in the C-terminal subdomain (49, 50). The UbcH7 carrier protein binds to a pocket in the small N-terminal subdomain, but the geometry of the bound UbcH7–ubiquitin thioester relative to Cys$^{820}$ has been a challenge to understanding within the context of a coherent mechanism for Cys$^{820}$–ubiquitin thioester formation and subsequent polyubiquitin chain formation, discussed recently (51). However, we have used kinetic analysis of $^{125}$I-polyubiquitin chain assembly to demonstrate for the first time the presence of two functionally distinct E2–ubiquitin binding sites on the E6AP Hect domain, providing a mechanistically tractable resolution to the problem of active site thioester formation (51). Other kinetic evidence indicates that the canonical UbcH7 binding site presented in the original crystal structure (49) functions in chain elongation from the ubiquitin thioester formed at Cys$^{820}$ (51), providing an entre into reconciling a potential mechanism for polyubiquitin chain assembly.

In the present work, we use kinetic and biophysical methods to demonstrate for the first time that an E6AP oligomer is the catalytically competent form of the enzyme. Based on an earlier structure for E6AP initially discounted as an artifact of crystal packing forces (49), we propose that the fully functional form of the oligomer is a trimer, computational analysis of which allowed us to identify conserved residues located at the subunit interfaces. Using rates of $^{125}$I-polyubiquitin chain assembly as a functional readout, we have identified a subset of residues essential for stabilizing the active trimer. Furthermore, a small molecule mimic of a key stabilizing interaction is sufficient to dissociate the trimer and abrogate E6AP-catalyzed chain assembly but not Cys$^{820}$–ubiquitin thioester formation. In contrast, E6 viral protein enhances E6AP activity by promoting oligomerization as a consequence of the ability of the former to dimerize through its N-terminal Zn$^{2+}$ binding domain (51, 52). Remarkably, E6-induced oligomerization rescues synthetic and Angelman syndrome loss-of-function mutations contributing to subunit association and stabilization. The current results explain previously unresolved roles for a cohort of point mutations in the neurological pathology of Angelman syndrome, reveal new strategies for regulating E6AP function by modulating subunit assembly, and provide insights into the role of oligomerization in polyubiquitin chain formation by the Hect ligase superfamily.

**MATERIALS AND METHODS**

Bovine ubiquitin and creatine phosphokinase were purchased from Sigma. Ubiquitin was further purified to apparent homogeneity by FPLC and quantitated spectrophotometrically (53). Ubiquitin was radioiodinated by the Chloramine-T procedure to yield a specific radioactivity of $\sim$15,000 cpm/pmol using carrier-free Na$^{125}$I purchased from either GE Healthcare or PerkinElmer Life Sciences (54). Human erythrocyte Uba1 (UBA1) was purified to apparent homogeneity from outdated human blood (54). Active Uba1 was quantitated by the stoichiometric formation of $^{125}$I-ubiquitin thioester (54–56). Human recombinant UbcH7 (UBE2L3) was that described previously (57, 58), and active E2 concentration was quantitated by the Uba1-dependent stoichiometric formation of UbcH7–$^{125}$I-ubiquitin thioester (51, 59). The E2 protein was stored at $\sim$80 °C in small aliquots and was stable for more than 6 months although subject to activity loss with successive freeze-thaw cycles (59). The N-acetyl-l-phenylalanlamide (Ac-PheNH$_2$; E-1160) was obtained from Bachem Americas. The N-acetyl-NRIRMYRSERRITVLYSL peptide (purity >95%) was obtained from PEPTIDE 2.0 Inc.

*Generation and Purification of Recombinant E6AP—* Human E6AP isoform 3 (UBE3A; IMAGE clone NM00046.2) was subcloned into pGEX4T1 to yield pGEX4T1-E6AP as described previously (51). The E6APF727D, E6APY533A, E6APD543A, E6AQP563A, and E6APD583A clones were verified by DNA sequencing. The N-terminus of the recombinant protein was processed by Bovine submaxillary trypsin to yield active recombinant E6AP isoform 3.

*Measurement of E6AP-catalyzed chain assembly—* Human Uba1 (UBE1A) was purified to apparent homogeneity from outdated human blood (54). Active Uba1 was quantitated by the stoichiometric formation of $^{125}$I-ubiquitin thioester (54–56). Human recombinant UbcH7 (UBE2L3) was that described previously (57, 58), and active E2 concentration was quantitated by the Uba1-dependent stoichiometric formation of UbcH7–$^{125}$I-ubiquitin thioester (51, 59). The E2 protein was stored at $\sim$80 °C in small aliquots and was stable for more than 6 months although subject to activity loss with successive freeze-thaw cycles (59). The N-acetyl-l-phenylalanlamide (Ac-PheNH$_2$; E-1160) was obtained from Bachem Americas. The N-acetyl-NRIRMYRSERRITVLYSL peptide (purity >95%) was obtained from PEPTIDE 2.0 Inc.

*The sequence for E6AP isoform 3 differs from isoform 1, from which the original crystal structure was determined (49), by the presence of an additional 20 amino acids at the N terminus. To facilitate comparison with the crystal structure, residues for isoform 3 will be referenced to the paralogous position of isoform 1 (i.e. by subtracting 20 from the isoform 3 residue number).
Polyubiquitin Chain Assembly Requires E6AP Oligomerization

E6APR626A, and E6APK688A mutants were generated from pGEX4T1-E6 AP using the QuikChange protocol of Stratagene to yield pGEX4T1-E6APF727D, pGEX4T1-E6APY533A, pGEX4T1-E6APD543A, pGEX4T1-E6APR626A, and pGEX4T1-E6APK688A, respectively. The E6APΔ495 truncation lacking the C-terminal Hect domain was generated by inserting a STOP codon after codon 495 of pGEX4T1-E6AP to yield pGEX4T1-E6APΔ495. Residues 450–852 of full-length E6 AP were subcloned by PCR into the BamHI/NotI sites of pGEX4T1 to yield pGEX4T1-E6AP-Hect, from which was expressed the GST-E6AP-Hect domain fusion protein. The active site Cys290 was similarly mutated to alanine by the QuikChange protocol to yield GST-E6AP-HectC820A protein. The GST moiety was eluted in 50 mM Tris-HCl (pH 8.0) containing 300 mM imidazole (51). The activities of GST-E6AP and its mutants were quantitated by their stoichiometric formation of 125I-ubiquitin thioester (51, 59, 60). Unless otherwise noted, the GST moiety was not processed from the fusion proteins by thrombin digestion (51).

The full-length E6AP sequence was subsequently cloned by PCR into the BamHI/HindIII sites of pFastBac Htb (Invitrogen) for baculoviral expression of the corresponding His6-E6AP. After bacmid amplification, the complete insert was sequenced to confirm the absence of cloning errors. The bacmid was transfected into Sf9 insect cells, and then the P1 virus was isolated and amplified as a P2 stock. The P2 stock was then used to transfect Sf9 cells (3.6 and 1.6–2 mg of affinity-purified His6-E6AP protein could be isolated from 13 T150 flasks, of which 10–40% of 0.6 by the addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 0.4 mM. After 3 h at 37 °C, cells were harvested by centrifugation at 6000 × g for 30 min and then resuspended in 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl and 1 mM DTT (59). Cells were lysed by Emulsiflex (Avestin) and then centrifuged at 30,000 × g for 30 min (59). Recombinant GST-E6(HPV16) fusion proteins were purified on glutathione-Sepharose, processed with thrombin, and then passed through a second glutathione-Sepharose column to remove free GST (59). The total protein content calculated by the theoretical 280 nm extinction coefficient yielded 2.4 mg/liter of culture except for E6(HPV16)Δ91, which yielded 0.5 mg/liter of culture. The proteins were flash frozen in small aliquots and stored at −80 °C.

E6AP-catalyzed 125I-Ubiquitin Conjugation Assay—The E3 ligase activity of recombinant E6AP was quantitated in kinetic assays under initial velocity conditions (51, 58). Rates of E6AP-catalyzed 125I-polyubiquitin chain formation were measured at 37 °C in incubations of 25–μl final volume containing 50 mM Tris-HCl (pH 7.5), 1 mM ATP, 10 mM MgCl2, 1 mM DTT, 10 mM creatine phosphate, 1 IU of creatine phosphokinase, 5 μM 125I-ubiquitin (~1.5 × 104 cpm/pmol), 50 nM human Uba1, and the indicated concentrations of UbcH7 and E6AP (59, 61). Reactions were initiated by the addition of 125I-ubiquitin. After 10–30 min, the reactions were quenched by the addition of 25 μl of 2× SDS sample buffer containing 0.3% (v/v) β-mercaptoethanol, and then the samples were heated to 100 °C for 3 min. The polyubiquitin conjugates were resolved from free 125I-ubiquitin by 12% (w/v) SDS-PAGE under reducing conditions at 4 °C and visualized by autoradiography of the dried gels (59, 61). Polyubiquitin chain formation was measured by excising lanes between the top of the resolving gel and the top of the stacker gel, representing anchored and free 125I-polyubiquitin chains, respectively (62), and quantitatively associated 125I-ubiquitin by γ-counting (51, 59, 61). Absolute rates of 125I-polyubiquitin chain formation were calculated from the associated radioactivity and the corrected specific radioactivity for 125I-ubiquitin (51, 59, 61). Datum points represent single assay determinations, and complete data sets were evaluated by nonlinear regression analysis using GraFit version 5.0 (Erithicus). Active Uba1, E2, and E6AP were independently determined in parallel by their stoichiometric formation of 125I-ubiquitin thioester (59).

Static Light-scattering Measurements—His6-E6AP after the His trap affinity purification step was further purified by FPLC using a Mono Q 5/50 column (Amersham Biosciences) equilibrated in 50 mM Tris-HCl (pH 7.5) and 1 mM DTT. Recombinant His6-E6AP eluted at 300 mM NaCl in a 0–500 mM gradient (16 mM/ml, 1 ml/min). The molecular weight of His6-E6AP was determined in solution at 37 °C by static light scattering using a 235-DynaPro NanoStar laser light scattering spectrometer at 663 nm wavelength (Wyatt Technology Corp.). Spectra were collected for 18 μM His6-E6AP in 50 mM Tris-HCl (pH 7.5) containing 200 mM NaCl. The effect of Ac-PheNH2 on the molecular weight of His6-E6AP was determined in 50 mM Tris-HCl (pH 7.5) containing 200 mM NaCl, 16 μM His6-E6AP total protein, 61 mM Ac-PheNH2, and 8% (v/v) carrier methanol. The molecular weight of E6AP was also evaluated in reactions containing 0–350 μM Ac-NIRMYRSERRTVYSL in 50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 1 mM DTT, and 17 μM His6-E6AP. Data were analyzed with Dynamics® software at both default...
and maximum sensitivity to detect the presence of low concentrations of E6AP oligomeric forms.

Analysis of E6AP-Hect Interface Structure—The E6AP Hect domain structure (PDB code 1D5F) corresponding to the E6AP trimer reported by Huang et al. (49) was analyzed using PISA (PDBProtein Interactions, Surfaces, and Assemblies) (63). The Assemblies analysis predicted a strong interaction between subunits, and default conditions detected an average of 38 residues/subunit involved in surface interactions between adjacent subunits (63). All structural representations were generated with PyMOL (Schrödinger, LLC).

RESULTS

E6AP-catalyzed Polyubiquitin Chain Formation Requires Oligomerization—Early in our studies, we examined the effect of the GST moiety on E6AP activity and found that processing of GST-E6AP fusion protein with thrombin resulted in a consistent 15–30% decrease in activity that resulted from a proportionately lower $k_{\text{cat}}$ but without effect on the $K_m$ for UbcH7-125I-ubiquitin binding (not shown). Because GST protein is known to dimerize (64–66), one interpretation posits that GST promotes oligomerization of E6AP that results in the increased $k_{\text{cat}}$. Consistent with this model, the addition of free GST to disrupt the putative oligomerization of GST-E6AP showed a biphasic concentration dependence (Fig. 1A). At free GST concentrations less than 5 μM, the abrupt concentration-dependent decrease in the initial rate of polyubiquitin chain formation exhibited a $K_{1/2}$ of ~1 μM, whereas at higher concentrations, there was a linear reduction in the initial rate with [GST], (Fig. 1A). The linear phase at higher GST concentrations coincided with the appearance of a band of monoubiquitinated GST and probably results from competition of this reaction with polyubiquitin chain elongation. In contrast, the decrease in rate occurring at lower GST concentrations was consistent with disruption of GST-E6AP homodimers by free GST because the $K_f$ for GST dimerization is 0.6 μM (66).

To directly evaluate the potential contribution of E6AP oligomerization to catalytic activity, we introduced a STOP codon in the GST-E6AP coding region to truncate the Hect domain from E6AP, generating GST-E6APΔ495, to test whether the truncated protein could disrupt oligomerization in a manner similar to the free GST moiety. The addition of either 54 μM GST-E6APΔ495 or 66 μM thrombin-processed E6APΔ495 to wild type enzyme decreased the initial rate of GST-E6AP-catalyzed polyubiquitin chain formation (Fig. 1B). When the experiment of Fig. 1B was repeated at different concentrations of either GST-E6APΔ495 or E6APΔ495, hyperbolic concentration-dependent decreases in the initial rates of polyubiquitin chain formation resulted (Fig. 1, C and D, respectively). At higher concentrations than shown in Fig. 1, C and D, GST-E6APΔ495 and E6APΔ495 quantitatively blocked wild type GST-E6AP-catalyzed polyubiquitin chain formation (not shown). Nonlinear hyperbolic regression analysis yielded $K_i$ values of 12 ± 3 and 19 ± 8 μM for GST-E6APΔ495 and E6APΔ495, corresponding to $\Delta G_{\text{binding}}$ values of −6.7 and −6.4 kcal/mol, respectively. Although the individual $K_i$ values are not statistically different, the corresponding $\Delta G_{\text{binding}}$ for contribution of the GST moiety to GST-E6AP dimerization predicts a $K_f$ for GST association of 1.6 μM, in good agreement with the published value of 0.6 μM (66). Results identical to those of Fig. 1, C and D, were obtained when the experiments were repeated with catalytically inactive GST-E6AP-HectC820A or E6AP-HectC820A in which the active site Cys220 of the Hect domain was mutated to alanine, yielding $K_i$ values of 31 ± 4 and 27 ± 9 μM, respectively (Fig. 1E). Inhibition by E6APΔ495 is consistent with a model in which the truncation competitively disrupts an intrinsic E6AP wild type oligomer that is required for activity, schematically illustrated in Fig. 1F. Overall, the data reveal that oligomerization of E6AP is required for catalytic competence in polyubiquitin chain assembly. The similarities in $K_i$ values between the E6APΔ495 and E6AP-HectC820A proteins suggest that the Hect domain and the targeting regions both contribute to oligomerization.

Active E6AP Is an Oligomer—Resolution of bacterially expressed protein by SDS-PAGE and visualization by Coomassie staining revealed a series of GST-associated bands in our preparations ranging from a relative mobility of ~130 kDa for full-length protein, in good agreement with the expected size of 125 kDa, to 25 kDa, representing the free GST moiety (Fig. 2A, left lane); however, only the band of highest relative molecular weight, corresponding to full-length GST-E6AP, formed a 125I-ubiquitin thioester (51). Because bacterially expressed recombinant GST-E6AP is isolated as a mixture of full-length and C-terminal truncations (Fig. 2A, left), the results of Fig. 1 suggest that our kinetic studies probably underestimate the intrinsic $k_{\text{cat}}$ for the ligase due to the presence of endogenous GST-E6AP fragments. To test this, we cloned full-length E6AP into a baculovirus expression system to yield >95% full-length ligase. The resulting N-terminal His$_6$-E6AP could be isolated from the medium without the marked fragmentation observed for bacterially expressed GST-E6AP and exhibited a relative molecular mass of 100 kDa, corresponding to the predicted value for full-length protein (Fig. 2A, right). After purification, maintaining the protein in a low concentration of NaCl was essential to prevent protein precipitation upon removal of imidazole. On a lower percentage SDS-polyacrylamide gel, the full-length affinity-purified band could be resolved into two closely migrating bands, only the slower migrating band of which could be detected on Western blots using anti-His$_6$ antibody similar with detection by anti-E6AP antibody (Fig. 2B). A trace fragment band lacking the His$_6$ tag could also be detected by anti-E6AP Western blot (Fig. 2B). Co-purification of E6AP lacking the affinity tag with His$_6$-E6AP is consistent with oligomerization of the enzyme indicated by the results of Fig. 1.

We have previously demonstrated that the recombinant GST-E6AP in preparations identical to that analyzed in Fig. 2A (left) exhibited hyperbolic kinetics for 125I-polyubiquitin chain formation with respect to [UbcH7], from which values of $K_m$ and $k_{\text{cat}}$ could be determined (51). Baculovirally expressed His$_6$-E6AP also yielded hyperbolic kinetics for 125I-polyubiquitin chain formation with respect to [UbcH7], from which $K_m$ (46 ± 7 nM) and $k_{\text{cat}}$ (0.63 ± 0.03 s$^{-1}$) could be determined by nonlinear hyperbolic regression analysis (51) (Table 1). That homogeneous full-length E6AP exhibits a $K_m$ indistinguishable from that of the bacterially expressed heterogeneous GST-E6AP preparations,
but a 20-fold higher $k_{\text{cat}}$ is consistent with our prediction based on a model for oligomerization. Similarly, GST–E6AP Hect domain protein composed of residues 450–872 is capable of modest polyubiquitin chain formation and has an affinity for UbcH7–ubiquitin thioester ($K_m = 89 \pm 11$ nm) that is slightly less than that of full-length E6AP but a $k_{\text{cat}}$ that is 2000-fold lower (Table 1). In contrast, removing the GST moiety by in situ processing with thrombin prior to assay abrogates chain formation (not shown); however, the resulting Hect domain moiety catalyzes a slow rate of monoubiquitination, as noted previously (51). The kinetics of Hect domain monoubiquitination follows hyperbolic kinetics with respect to $[\text{UbcH7}]_o$ and yields $K_m$ and $k_{\text{cat}}$ values similar to those of the unprocessed GST–Hect domain (Table 1). The results of Table 1 are not a consequence of differences in the amounts of active protein in the various preparations because in each case the ligase is quantitated by the functional assay of stoichiometric Hect domain ubiquitin thioester formation. Therefore, polyubiquitin chain formation kinetics for the various forms of E6AP (Table 1) and the effect of N-terminal truncation (Fig. 1) are consistent with the catalytically active form of the enzyme existing as an oligomer.
Polyubiquitin Chain Assembly Requires E6AP Oligomerization

A Trimer Is the Likely Fully Active Form of E6AP—To test directly the ability of E6AP to oligomerize, we determined the solution molecular weight of wild type His$_6$-E6AP by gel filtration chromatography (Fig. 3A). The relative molecular weight for E6AP, monitored by $^{125}$I-polubiquitin chain formation under E3-limiting conditions, encompasses a peak centered at 190 kDa. This molecular weight is consistent with a stable dimer of 100-kDa subunits or less stable higher oligomers subject to dissociation by dilution as the complex passes through the column. To distinguish between the latter, we analyzed a parallel sample by static light scattering, which does not involve sample dilution. Following Mono Q anion exchange chromatography to remove inactive high molecular weight aggregates, freshly prepared active His$_6$-E6AP displayed a molecular mass of 283 kDa by static light scattering that was consistent with a trimer (Fig. 3A). The low abundance higher molecular weight species of ~50-nm radius represents aggregates not completely removed by the Mono Q FPLC step. In parallel experiments, the molecular mass ranged from ~200 to 400 kDa depending on protein concentration, pH, and ionic strength, consistent with equilibrium oligomerization of the 100-kDa monomer (not shown).

Wild type E6AP is thought to exist as a monomer; however, the original publication reporting the structure of the isolated Hect domain also noted a trimeric structure that was proposed to arise from crystal packing interactions (49) (Fig. 4A). Based on the data of Fig. 3 and the requirement that E6AP-catalyzed polyubiquitin chain formation requires oligomerization, we propose that the fully active form of the ligase is a trimer. The symmetric trimer (PDB code 1D5F) reported by Huang et al. (49) buries an extensive combined surface area of 7508 Å$^2$ comprising a large fraction of apolar residues at the subunit interfaces that represents a solvation free energy of $\approx$ 4.2 kcal/mol by PISA analysis (63) (Fig. 4A), corresponding to an apparent $K_d$ of 25 μM for E6AP Hect subunit interactions without considering additional hydrogen bond or salt bridge interactions (supplemental Table 1), which are difficult mathematically to model due to uncertainties related to the actual microenvironments of the individual bonds. However, the predicted $K_d$ based on desolvation agrees well with the empirical $K_d$ obtained with GST-HectC820A (31 ± 4 μM) and HectC820A (27 ± 9 μM) (Fig. 1D).

A number of radially symmetric subunit interactions. For example, the large N-terminal subdomain of the Hect domain contains a subset of conserved apolar residues at the subunit interface (Ile$^{600}$, Tyr$^{601}$, Leu$^{723}$, Leu$^{726}$, Ile$^{732}$, and Leu$^{735}$) that forms a hydrophobic pocket into which intercalates Phe$^{727}$ of the adjacent subunit in the trimer (49) (Fig. 4C), providing a stabilization of ~2.13 kcal/mol by PISA analysis. Although there are other residues and surfaces contributing to stabilization of the trimer, intercalation of Phe$^{727}$ into the hydrophobic pocket appears to be of special importance because mutation of Phe$^{727}$ to alanine destabilizes trimeric E6AP in favor of the monomer, the latter of which retains the ability to form a ubiquitin thioester intermediate (49). Retention of the ability to form the thioester intermediate was originally interpreted as evidence that the monomer represented the biological...

TABLE 1

| Effect of E6AP length on chain formation kinetics |
|-----------------------------------------------|
| $K_m$  | $K_{cat}$ | $K_{cat}/K_m$ |
|--------|----------|--------------|
| Heterogeneous GST-E6AP                      | 58 ± 6    | 3.1 ± 0.9 × 10$^{-2}$ | 5.4 × 10$^5$ |
| E6AP Hect domain$^a$                        | 91 ± 25   | 7.0 ± 0.1 × 10$^{-4}$ | 8.5 × 10$^3$ |
| GST-E6AP Hect domain                        | 89 ± 11   | 3.2 ± 0.1 × 10$^{-4}$ | 3.6 × 10$^3$ |
| Homogeneous His$_6$-E6AP                    | 46 ± 7    | 6.3 ± 0.3 × 10$^{-1}$ | 1.3 × 10$^7$ |

$^a$ Determined by E6AP Hect domain monoubiquitination.

FIGURE 2. SDS-PAGE of selected E6AP preparations. A, Coomassie-stained 10% (w/v) SDS-PAGE resolution of affinity-purified recombinant GST-E6AP expressed in E. coli (left) versus His$_6$-E6AP expressed in baculovirus (right). B, Western blot of 12% (w/v) SDS-PAGE resolution of baculovirus-expressed His$_6$-E6AP stained with anti-E6AP antibody (left) and then stripped and restrained with anti-His$_6$ antibody (right). Mobility markers are shown to the left of the corresponding panels.
relevant structure (49). However, we have recently demonstrated that the E6AP catalytic cycle comprises a two-step mechanism involving rapid transthiolation of activated ubiquitin from the cognate E2/H11011 ubiquitin co-substrate to the Hect Cys820 active site residue followed by rate-limiting chain elongation to form the polyubiquitin degradation signal (51). Because of this, Cys820 thioester formation is an insensitive measure of E6AP catalytic competence (51), accounting for the reported inconsistencies between known loss-of-function mutations in Angelman syndrome and retention of the ability of the enzyme to form an active Cys820-linked thioester to ubiquitin (17). To re-examine this point, we mutated Phe727 to aspartic acid in order to more efficiently disrupt trimer formation. The E6APF727D mutation significantly ablates the ability of the enzyme to form polyubiquitin chains, as shown by the 62-fold decrease in $k_{cat}$ without significant effect on the $K_m$ for UbcH7$^\text{125I}$-ubiquitin thioester binding (Table 2) or end point Cys820 thioester formation (not shown), the latter as noted previously (49).

As additional evidence that trimeric E6AP represents the functional form of the enzyme, we examined the effect of Ac-PheNH$_2$ as a mimic of Phe727. The addition of Ac-PheNH$_2$ to His$_6$-E6AP results in quantitative dissociation of the oligomer to a 104 kDa peak in good agreement with the expected molecular weight of the monomer (Fig. 3C). This observation is consistent with substitution of Ac-PheNH$_2$ into the conserved hydrophobic pocket to disrupt the radially symmetric subunit interactions stabilizing the trimer and is accompanied by quantitative inhibition of $^{125}$I-polyubiquitin chain formation (Fig. 4D). The dependence of initial velocity on [UbcH7]$_o$ in the absence or presence of 44 mM Ac-PheNH$_2$ shows the amino acid derivative to be a non-competitive inhibitor with a $K_i$ of 12 ± 3 mM, which is in good agreement with the calculated $K_d$ of 27 mM predicted from the −2.13 kcal/mol stabilization predicted by PISA (see above). In addition, non-competitive inhibition by Ac-PheNH$_2$ is consistent with that predicted for an effect on subunit dissociation (Fig. 4E) and the observed consequence of the E6APF727D mutation (Table 2). Although Ac-PheNH$_2$ can potentially interact with other regions comprising the interfacial of full-length His$_6$-E6AP, the linearity of the double reciprocal plots for Fig. 4, D (inset) and E, are consistent with binding at a single homogeneous site, presumably the buried conserved hydrophobic pocket.

A Conserved α-Helix Blocks E6AP Trimer Formation—Examination of the extant structures for other Hect domains,
Polyubiquitin Chain Assembly Requires E6AP Oligomerization

FIGURE 4. Active E6AP is a trimer stabilized by Phe727 interactions. A, structure of the E6AP Hect domain trimer (PDB code 1D5F) reported by Huang et al. (49). Each chain is colored differently to emphasize subunit packing, whereas the forward subunit is rendered as a ribbon and colored to distinguish the large N-terminal subdomain (marine), the small N-terminal subdomain (cyan), and the C-terminal subdomain (dark blue). The active site Cys820 and N-terminal residues are rendered in CPK and colored yellow and red, respectively. B, the structure of A is rotated 90° into the plane of the page to emphasize subunit packing. Residues of the hydrophobic pocket are rendered in white for the gold subunit, and Phe727 of the blue subunit is rendered in orange. C, closer view of Phe727 intercalated into the hydrophobic pocket of the adjacent subunit. D, the concentration dependence of Ac-PheNH2 on the initial rate of 125I-polyubiquitin chain assembly conducted under E6AP-liming conditions, with the solid line representing a nonlinear inverse regression analysis fit to a hyperbolic kinetics; inset, semireciprocal plot of the resulting data. Incubations contained 400 nM Uba1, 200 nM UbcH7, 8.8 nM His6-E6AP, 5 μM 125I-ubiquitin, and the indicated concentrations of Ac-PheNH2. All incubations contained 5% (v/v) methanol as a carrier for the Ac-PheNH2.

TABLE 2
Summary of kinetic constants

|        | $K_m$ (μM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (s$^{-1}$ μM$^{-1}$) |
|--------|-----------|-----------------------|-----------------------------------|
| GST-E6AP-UbcH7$^a$ | 58 ± 8 | 3.1 ± 0.9 × 10$^{-3}$ | 5.4 × 10$^4$ |
| GST-E6APF727D-UbcH7$^a$ | 72 ± 25 | 5.0 ± 0.7 × 10$^{-3}$ | 7.6 × 10$^3$ |
| GST-E6APY533A-UbcH7 | 40 ± 12 | 3.1 ± 0.2 × 10$^{-3}$ | 7.9 × 10$^3$ |
| GST-E6APD543A-UbcH7$^a$ | 540 ± 400 | 4.0 ± 1.0 × 10$^{-3}$ | 7.5 × 10$^3$ |
| GST-E6APR626A-UbcH7$^a$ | 150 ± 50 | 2.0 ± 0.2 × 10$^{-4}$ | 1.3 × 10$^2$ |
| GST-E6APK688A-UbcH7 | 39 ± 10 | 5.2 ± 0.4 × 10$^{-2}$ | 1.3 × 10$^2$ |

$^a$ From Table I.

The observed rates of these mutants approach our limit of detection for 125I-polyubiquitin chain formation and result in a substantial increase in the S.E. of the measurements.

including WWPl (PDB code 1ND7), Smurf2 (PDB code 1ZVD), HuWE1 (PDB code 3H1D), Ned4-1 (PDB code 2XBB), Ned4-2 (PDB code 2XBF), and yeast RSP5 (PDB codes 3OLM and 4LCD), provides no other instance of trimer formation (69–73). However, the other Hect domain structures contain additional sequence N-terminal to the truncation site for E6AP at Asn397 that is not present in the E6AP structure (49). Immediately N-terminal to Asn397 is an extensive amphipathic α-helix corresponding to residues 474–490 that is relatively conserved among the Hect ligases, contributes to domain stability, and correlates with reduced autoubiquitination and target protein conjugation (71, 74). In all of the Hect domain structures containing the additional segment, the hydrophobic face of the amphipathic N-terminal α-helix binds to the hydrophobic pocket normally occupied by Phe727 in the trimeric E6AP structure (69–73). We speculate that the latter interaction of the α-helix might block oligomerization, accounting for the observed reduction in ligase activity because we here demonstrate that trimer formation is required for polyubiquitin chain assembly. To address the role of the N-terminal α-helix, the initial rate of polyubiquitin chain formation was analyzed in the absence or presence of an N-terminal blocked Ac-NRIRMYSSRRTVLVSLL peptide corresponding to residues 474–490 of E6AP isoform 1 to mimic the proposed effect of this segment (Fig. 5). With increasing concentrations of the peptide, the initial rate of E6AP-catalyzed chain formation decreased with a hyperbolic dependence (Fig. 5A), as shown by the linearity of the corresponding semireciprocal plot, yielding an apparent $K_m$ of 22 ± 2 μM (Fig. 5A, inset).
When the concentration dependence of the initial rate for polyubiquitin chain formation on [UbcH7] was examined in the absence or presence of the indicated concentrations of N-terminal peptide and evaluated by nonlinear regression fit to an inverse hyperbolic equation. Reactions contained 34 nM Uba1, 390 nM UbcH7, 1.3 nM His6-E6AP, and 4 μM 125I-ubiquitin. Inset, semireciprocal plot of the data. B, double reciprocal plot of initial rates of 125I-polyubiquitin chain assembly under E6AP-limiting conditions in the absence (closed circles) or presence (open circles) of 32 μM N-terminal peptide. Reactions contained 33 nM Uba1, 0.6 nM His6-E6AP, 4 μM 125I-ubiquitin, and the corresponding [UbcH7] concentrations. C, the static light scattering spectrum of 17 μM full-length His6-E6AP was determined as described in the legend to Fig. 3C in the absence (solid line; 10 ± 3% polydispersity) or presence (dashed line; 10 ± 2% polydispersity) of 88 μM Ac-NRIRMYSERRITVLYSL peptide.

Identification of Additional Subunit Interface Residues Affecting E6AP Catalytic Activity—The present data and recent insights into the properties of protein subunit interfaces support the E6AP trimer as the catalytically relevant structure (67, 68). The interaction surfaces of the trimer were analyzed by PISA to define other side chain interactions contributing to stability beyond that of Phe727 (supplemental Table 1). A comprehensive sequence alignment of human Hect ligases cross-referenced to the results of PISA identified a number of conserved positions that appear potentially critical, including the hydrophobic pocket residues into which Phe727 intercalates, as discussed earlier. Interestingly, several of these residues represent or are adjacent to documented Angelman syndrome point mutations. We were particularly interested in Tyr533, which is an Angelman syndrome mutation site and which participates in a pattern of side chain interactions between subunits (75). Tyrosine 533 also exists in the region of subunit interface recently suggested by Chan et al. to be sensitive to cAbl-dependent regulation by phosphorylation of Tyr636 (76).

For two of the subunits in the trimer structure, the side chain phenolic group of Tyr533 hydrogen bonds with the amide hydrogen of Asp543 present on the same polypeptide chain (Fig. 6B), whereas for the third subunit, Tyr533 is rotated 60° and hydrogen-bonds to the amide hydrogens of Arg626 and Thr624 on the adjacent chain (Fig. 6C). More important, for all three subunits, the side chain carboxyl and amide carbonyl groups of
Asp543 hydrogen-bond to the side chain guanidinium of Arg626 (Fig. 6, B and C). Tyrosine 533 and Asp543 reside within the Hect large N-terminal subdomain, whereas Arg626 is in the small N-terminal subdomain of the adjacent subunit, so that the three residues constitute a radial symmetric pattern of subunit interactions. We independently mutated each of the residues to alanine and examined the effect on the kinetics for 125I-polyubiquitin chain formation with respect to [UbcH7]o under rigorously E3-limiting conditions (Table 2). As a control, we similarly also mutated Lys688, a residue present on the same small N-terminal subdomain interface as Arg626, which PISA analysis indicates hydrogen-bonds to the side chain of Glu535 in the adjacent subunit. All four mutants formed 125I-ubiquitin thioesters to Cys820 with kinetics qualitatively similar to that of wild type enzyme in short term incubations described previously (51).

Mutation of Lys688 did not have a significant effect on the kinetics of 125I-polyubiquitin chain assembly (Table 2). However, given the large number of predicted subunit interactions stabilizing the trimer (supplemental Table 1), abrogating a single site might not be expected a priori to have a large effect except for those interactions particularly critical for structural integrity. In contrast to the effect of mutating Tyr688, mutation of Tyr533 decreased $k_{cat}$ 10-fold but had no effect on the $K_m$ for UbcH7–ubiquitin binding (Table 2). Similarly, mutation of Arg626 decreased $k_{cat}$ 155-fold but had little effect on $K_m$ (Table 2). Both of the latter mutants are consistent with an effect on oligomerization, given their position within the trimer structure and distance from the active site Cys820 as well as the fact that neither mutation alters the ability of E6AP to bind its UbcH7–ubiquitin thioester substrate. Mutation of Asp543 similarly ablated $k_{cat}$ 8-fold but additionally increased $K_m$ significantly (Table 2). Together with the effects of Tyr636 phosphorylation (76), the consequences of these point mutants reveal complex interactions affecting the catalytic function of E6AP.

**E6 Protein Enhances E6AP Polyubiquitin Chain Synthesis**—HPV16 E6 protein is thought to promote viral replication and host epithelial cell transformation by serving as an E6AP adapter to target the p53 tumor suppressor protein for 26 S proteasome-dependent degradation (32, 77, 78). Recent structural work demonstrates that the 158-amino acid E6 viral protein contains structurally related N-terminal and C-terminal Zn$^{2+}$ binding domains connected by a linker polypeptide (46). The C-terminal domain binds p53 but also shows limited interaction with E6AP (43, 79). In contrast, the N-terminal domain exhibits significant affinity for E6AP through binding to a canonical LXXLL motif on the ligase and is additionally responsible for E6 protein self-association (40–42, 46), accounting for the observation that the complex between E6 protein and p53 consists of a p53 tetramer and an E6 dimer (45, 80). As expected, disrupting the dimerization interface by mutation inhibits E6-dependent p53 conjugation by E6AP but also enhances E6 protein solubility (46), which probably relates to earlier observations that ectopic expression of E6 protein stimulates autoubiquitination and subsequent E6AP degradation in vivo (81).

Because the trimer is the presumed catalytically fully active form of E6AP, we speculated that the effect of E6 protein on E6AP turnover (82) and the stabilization of p53 when the E6 dimerization interface is disrupted might reflect the ability of...
the viral protein to promote E6AP oligomerization. Fig. 7A shows the effect of recombinant E6 protein on the \([\text{UbcH7}]_o\) dependence of E6AP-catalyzed \(^{125}\text{I}-\text{polyubiquitin chain formation}.\) Initial rates of chain formation were significantly enhanced in the presence of E6 protein, with the \(k_{\text{cat}}\) increasing from 0.057 ± 0.029 s\(^{-1}\) in the absence of E6 protein to 0.81 ± 0.045 s\(^{-1}\) in its presence. The corresponding double reciprocal plot shows E6 protein to be a nonessential activator, exhibiting a \(k_{\text{cat}}\) effect (inset), and with a \(K_d\) for E6 binding corresponding to 1.5 nm. These results are consistent with a mechanism in which the E6 dimer promotes oligomerization of E6AP to the catalytically competent trimer through binding to a site distinct from the catalytic site, presumably the leucine-rich L\(\text{XXLL}\) motif (40–42). Although the N-terminal domain contains both the dimerization interface and the L\(\text{XXLL}\) association motif (46), the recombinant E6(HPV16)\(\Delta 91\) N-terminal domain encompassing residues 1–91 (46) inhibits E6AP-catalyzed polyubiquitin chain formation with a hyperbolic concentration dependence (apparent \(K_I = 4 \mu\text{M}\); not shown) and displays non-competitive inhibition with respect to \([\text{UbcH7}]_o\) corresponding to \(K_I = 6 \mu\text{M}\) (Fig. 7B). Similarly, the recombinant E6(HPV16)\(\Delta \text{Ct}\) C-terminal domain encompassing residues 89–158 is a non-competitive inhibitor of E6AP with respect to \([\text{UbcH7}]_o\) corresponding to \(K_I = 4 \mu\text{M}\) (not shown). Collectively, the results indicate that both E6 domains bind E6AP, presumably at different sites, to promote oligomerization of full-length E6AP. This conclusion supports earlier empirical binding evidence demonstrating interaction of each domain with E6AP (44, 46).

Because full-length E6 protein acts as a nonessential activator, presumably by promoting E6AP trimer formation, we asked whether the viral protein could rescue the loss-of-function phenotype displayed by the E6AP\(\Delta 727\) point mutant. The addition of 20 nM E6 protein significantly enhanced the initial rate of \(^{125}\text{I}-\text{polyubiquitin chain formation}\) for wild type protein (Fig. 7C, lanes 7 and 13) as seen in Fig. 7A and E6AP\(\Delta 727\)D (Fig. 7C, lanes 6 and 12). Similar rescue of \(^{125}\text{I}-\text{polyubiquitin chain formation}\) was observed in an 8-fold increase in rate for E6AP\(\Delta 533\)A (Fig. 7C, lanes 3 and 9) and a 13-fold increase in rate for E6AP\(\Delta 626\)A (Fig. 7C, lanes 4 and 10). In contrast, no enhancement in rate was observed for the E6AP\(\Delta 543\)A point mutant (Fig. 7C, lanes 5 and 11). These results are consistent with E6 rescuing the

**FIGURE 7.** E6 protein enhances E6AP ligase activity and rescues selected Angelman syndrome mutations. A, initial rates of \(^{125}\text{I}-\text{ubiquitin polyubiquitin chain formation}\) were determined under E6AP-limiting conditions in the absence (closed circles) or presence (open circles) of 20 nm E6 protein as described under “Material and Methods” and evaluated by nonlinear regression fit to the Michaelis-Menten equation. Assays contained 70 nm Uba1, 0.4 nm His6-E6AP, 4 \muM \(^{125}\text{I}-\text{ubiquitin},\) and the indicated UbcH7 concentrations. Inset, double reciprocal plot of the data. B, double reciprocal plot of the initial rates of \(^{125}\text{I}-\text{ubiquitin conjugation activity}\) determined under E6AP-limiting conditions in the absence (closed circles) or presence (open circles) of 3.3 \muM E6 (HPV16)\(\Delta 91\), as described under “Materials and Methods” and in A. Assays contained 30 nm Uba1, 1 nm His6-E6AP, 4 \muM \(^{125}\text{I}-\text{ubiquitin},\) and the indicated UbcH7 concentrations. C, conjugation reactions similar to those of B were conducted with 110 nm Uba1, 480 nm UbCH7, 5 \muM \(^{125}\text{I}-\text{ubiquitin},\) and 1 nm wild type or mutant GST-E6AP in the absence (lanes 1–7) or presence (lanes 8–13) of 20 nm E6(HPV16). D, quantitation of product formation rates from C in the absence (light gray) or presence (dark gray) of E6(HPV16). Data for wild type GST-E6AP and GST-E6AP\(\Delta 533\)A are plotted on the left axis, whereas data for GST-E6AP\(\Delta 626\)A, -\(\Delta 543\)A, and -\(\Delta 727\)D are plotted on the right axis.
loss-of-function phenotype for selected point mutants by promoting oligomerization.

**DISCUSSION**

Homo- and hetero-oligomerization regulate many complex biochemical processes in the cell (83), and recent findings suggest that such protein interactions are critical for the mechanism(s) of assembling polyubiquitin signals during key events of cell regulation (52, 84–88). The present studies were prompted in part by our attempt to explicate the puzzling observation that the assembly of \(^{125}\)I-polyubiquitin chains by GST-E6AP is ablated when the affinity tag is removed following digestion with thrombin. Subsequent experiments indicated that the ability of GST to dimerize (64–66) promotes oligomerization of E6AP, confirmed by the quantitative inhibition of ligase-catalyzed chain formation by free GST, GST-E6APΔ495, and GST-E6APΔC820A (Fig. 1). The ability of thrombin-processed E6APΔ495 and E6APΔC820A to similarly inhibit wild type GST-E6AP polyubiquitin chain formation demonstrates that oligomerization is an intrinsic property of the Hect ligase, with the interaction interfaces probably spanning the N-terminal targeting and C-terminal Hect domains of the ligase (Fig. 1). In addition, co-purification of recombinant baculovirally expressed His\(_6\)-E6AP with full-length E6AP lacking the His\(_6\) affinity tag is consistent with oligomerization of the ligase (Fig. 2B). The progressive increase in \(k_{cat}\) for E6AP-catalyzed \(^{125}\)I-polyubiquitin chain assembly with the absence of competing degradative fragments is consistent with a role for oligomerization in the activity of E6AP (Table 1). Finally, static light scattering measurements are consistent with the homotrimer as the catalytically relevant form of E6AP (Fig. 3).

Trimeric E6AP was initially dismissed as an artifact of crystal packing forces, particularly because a F727A point mutant capable of disrupting the oligomer did not affect the ability of the enzyme to catalyze transthiolation from E2–ubiquitin thioester (49); however, more comprehensive functional assays were unavailable for testing additional potential effects of trimer disruption. In the present studies, we demonstrate that a paralogous F727D mutation intended to more completely disrupt trimer formation significantly ablates the ability of the enzyme to catalyze elongation of polyubiquitin chains to <2% of wild type enzyme (Table 2), concomitant with dissociation of the oligomer (Fig. 3). As noted previously for the F727A mutant (89), mutation of Phe\(^{727}\) to aspartic acid has no effect on formation of the essential Cys\(^{820}\)–ubiquitin thioester intermediate (not shown). *In silico* PISA analysis of the subunit interfaces present within the E6AP trimer identifies a cohort of conserved residues within the 7508 Å\(^2\) buried by oligomerization (supplemental Fig. 1A). Of the significant number of hydrogen bond and salt bridge interactions identified by PISA (supplemental Table 1), the conserved radially symmetric network of interactions represented by the intersubunit intercalation of Phe\(^{727}\) into the hydrophobic pocket present on the adjacent subunit appears particularly important because mutation of Phe\(^{727}\) or antagonizing the interaction by the addition of Ac-PheNH\(_2\) disrupts trimer formation and significantly ablates activity (Fig. 4). The linearity of the double reciprocal plots in the presence of Ac-PheNH\(_2\) (Fig. 4, D and E) and the good agreement between the empirical \(K_i\) of 12 mM and the value of 27 mM predicted from the calculated binding energy of Phe\(^{727}\) (−2.13 kcal/mol) are consistent with the amino acid derivative binding at the unique hydrophobic pocket present in the subunit interface.

Among the extant structures for Hect domains, that of E6AP is unique in forming a trimer (69–73). The 1DF5 E6AP trimer is also notable in being the only such Hect domain structure lacking the additional N-terminal sequence segment present in the other paralogs. In the other structures, a conserved α-helix within this sequence binds to the hydrophobic pocket otherwise occupied by Phe\(^{727}\) in the E6AP trimer (69–73). We demonstrate that E6AP residues 474–490, corresponding to the α-helical region, act as a non-competitive inhibitor of \(^{125}\)I-polyubiquitin chain formation with an affinity (\(K_i = 22 ± 2 \mu\)M) considerably greater than that of Ac-PheNH\(_2\) (\(K_i = 12 ± 3\) mM) (Fig. 5). The presence of the additional α-helical segment is reported to enhance the intrinsic stability of the Hect domain and to ablate autoubiquitination (71, 74). The latter has been interpreted as demonstrating a regulatory role for the interaction between the α-helical segment and the hydrophobic pocket in the native full-length structure. Although we cannot *a priori* rule out such a regulatory role for the α-helix, we propose that association of the α-helical segment with the hydrophobic pocket is a structural artifact of the position of the truncation for the other Hect domain constructs and predict that in the absence of this interaction, the other ligases would assume a trimeric structure analogous to that of E6AP. We note as well that in the absence of trimer formation, packing of the Hect domains within the unit cells influences the orientation of the C-terminal domain, altering their positions relative to the large N-terminal subdomains (69–73). The various positions observed for the C-terminal domains in different Hect domain structures have been previously interpreted to reflect the requirement for flexibility during the catalytic cycle of the enzyme, suggested by the ablation of activity accompanying mutations in the linker region connecting the C-terminal and large N-terminal subdomains (69, 73, 82). We suggest, instead, that these alternate orientations for the C-terminal domain may reflect artifactual crystal packing forces present in the absence of trimerization.

A second subunit interface identified as important for trimer activity localizes to interactions among Tyr\(^{533}\) and Asp\(^{543}\) within the large N-terminal subdomain, and the small N-terminal subdomain residue Arg\(^{626}\) (76) of the adjacent subunit (Fig. 6). These residues form a network of hydrogen-bonded interactions that appear to stabilize the trimer because individual mutation of each significantly affects \(k_{cat}\) but with little consequence for UbcH7–ubiquitin thioester binding (Table 2). The functional effects of these mutations collectively support the trimer as the catalytically relevant structure for E6AP. Mutation of Asp\(^{543}\) appears to be the single exception because its mutation affects both \(k_{cat}\) and binding of the UbcH7–ubiquitin thioester substrate (Table 2). The latter may reflect a structural contribution for the side chain interactions of Asp\(^{543}\) or the special role for the residue in bridging the effects of Tyr\(^{533}\) and Arg\(^{626}\) (Fig. 6), which makes its mutation equivalent to a double mutant. In contrast, disrupting the hydrogen bond between
Lys688 in the small N-terminal subdomain and Glu535 in the adjacent large N-terminal subdomain by mutation of the former has no consequence, indicating that the effects of the previous mutations are specific to those residues rather than a general feature of the interface. Interestingly, this subunit interface harbors Tyr636, which Chan et al. (76) have identified as a substrate for c-Abl phosphorylation in the regulation of E6AP function. Chan et al. (76) have speculated that the inhibition of E6AP activity observed on phosphorylation of Tyr636 might result from blocking oligomerization of the enzyme. Our empirical observations and the effect of mutation on other residues within this region support a role for Tyr636 in regulating trimer formation and E6AP activity.

The ability of E6 protein to recruit p53 to E6AP for targeted degradation is the accepted paradigm by which HPV-16 transforms infected epithelial cells (32, 77, 78). Structural studies demonstrating dimerization of E6 protein have more recently accounted for the observed stoichiometry of the resulting E6-p53 complex (45, 80). Given our observation that oligomeric E6AP is the catalytically competent form of the ligase, we asked whether the ability of E6 protein to dimerize could promote E6AP-catalyzed polyubiquitin chain formation. The data of Fig. 7 demonstrate that E6 protein is a potent nonessential activator of E6AP catalytic activity within a low nanomolar concentration range ($K_{\text{activation}} = 1.5 \text{ nm}$). The $K_{\text{activation}}$ for ligase stimulation, representing the binding of E6 to E6AP, is significantly lower than the 4 μM $K_d$ for binding of the viral protein to E6AP reported previously by surface plasmon resonance (44), probably reflecting the enhanced entropically coupled affinity associated with binding to linked sites (90). The latter indicates that E6 protein probably functions at intracellular concentrations significantly lower than suggested by the isolated in vitro equilibrium binding affinity. In the absence of substrate, E6AP catalyzes the assembly of free and anchored polyubiquitin chains, the latter attached to the ligase through autoubiquitination (51). This bifurcation of products is proposed to occur by partitioning of a Cys820—polyubiquitin intermediate between transfer to water or a lysine present on the ligase, respectively (51). The addition of E6 protein uniformly enhances the formation of both 12S1-polyubiquitin products, consistent with the partitioning model (Fig. 7C). The latter observation explains the earlier observation that ectopic expression of E6 protein stimulates the autoubiquitination and degradation of endogenous E6AP (48). More importantly, these observations anticipate that E6 protein-dependent epithelial cell transformation may arise in part through a general stimulation of ligase activity toward its normal cohort of endogenous targets rather than serving exclusively as a binding adapter to target a small subset of alternate targets, such as p53 (Fig. 7A). Such a proposal is consistent with earlier observations that an E6 SAT8–10 mutant defective in targeting E6AP-dependent p53 degradation (48) but retaining the ability to immortalize human epithelial cells, induce colony growth on soft agar, and stimulate telomerase activity (84, 86) also simulates E6AP autoubiquitination and degradation in vitro (48).

The 14-fold stimulation in E6AP activity at saturating E6 protein suggests that only 0.7% of the active ligase, determined by stoichiometric Cys820–12S1-ubiquitin thioester formation, is present as the active trimer under the conditions of Fig. 7A, although we cannot rule out an intermediate level of activity from potential dimeric species derived from the trimeric structure. Consistent with the remarkable effect of E6 in promoting oligomerization, the viral protein complements the kinetochore phenotypes resulting from the F727D, R626A, and Y533A mutations, the latter being a documented Angelman syndrome mutation site (75). Because the F727D mutation results from defective trimer formation, functional complementation of the R626A and Y533A mutations suggests that they as well represent trimer formation defects.

The present observations significantly extend our understanding of the underlying mechanism of the E6AP catalytic cycle and that of the HECT domain superfamily. Identification of oligomeric E6AP, presumably as the trimer or multiples thereof, as the catalytically relevant form of the ligase explains a number of previous observations in the literature and reveals potential pharmacological approaches to modulate activity of the enzyme. Accordingly, Ac-PheNH$_2$ serves as an important proof of principle lead compound for potential future E6AP antagonists targeted to the disruption of oligomerization. Similarly, E6 protein suggests as yet unidentified cellular mechanisms modulating E6AP oligomerization that could serve as additional therapeutic targets. Finally, these observations emphasize the importance of quantitative biochemically defined studies unambiguously defining the mechanism of the ubiquitin ligases.

REFERENCES

1. Huibregtse, J. M., Scheffner, M., Beaudenon, S., and Howley, P. M. (1995) A family of proteins structurally and functionally related to the E6-AP ubiquitin-protein ligase. Proc. Natl. Acad. Sci. U.S.A. 92, 2563–2567
2. Rotin, D., and Kumar, S. (2009) Physiological functions of the HECT family of ubiquitin ligases. Nat. Rev. Mol. Cell Biol. 10, 398–409
3. Wang, M., and Pickart, C. M. (2005) Different HECT domain ubiquitin ligases employ distinct mechanisms of polyubiquitin chain synthesis. EMBO J. 24, 4324–4333
4. Wang, M., Cheng, D., Peng, J., and Pickart, C. M. (2006) Molecular determinants of polyubiquitin linkage selection by an HECT ubiquitin ligase. EMBO J. 25, 1710–1719
5. Beaudenon, S., and Huibregtse, J. M. (2008) HPV E6, E6AP and cervical cancer. BMC Biochem. 9, 54
6. Flashner, B. M., Russo, M. E., Boileau, J. E., Leong, D. W., and Gallicano, G. I. (2013) Epigenetic factors and autism spectrum disorders. Neuromolecular Med. 15, 339–350
7. Nicholls, R. D., and Knepper, J. L. (2001) Genome organization, function, and imprinting in Prader-Willi and Angelman syndromes. Annu. Rev. Genomics Hum. Genet. 2, 153–175
8. Matentzoglu, K., and Scheffner, M. (2008) Ubiquitin ligase E6-AP and its role in human disease. Biochem. Soc. Trans. 36, 797–801
9. Kishino, T., Lalande, M., and Wagstaff, J. (1997) UBE3A/E6-AP mutations in E6-Ap ubiquitin-protein ligase gene (UBE3A) in Angelman syndrome. Trends Neurosci. 20, 339–350
10. Sutcliffe, J. S., Jiang, Y. H., Galjaard, R. J., Matsuura, T., Fang, P., Kubota, T., Christian, S. L., Bressler, J., Cattanach, B., Ledbetter, D. H., and Beaudet, A. L. (1997) The E6-Ap ubiquitin-protein ligase (UBE3A) gene is localized within a narrowed Angelman syndrome critical region. Genome Res. 7, 368–377
Polyubiquitin Chain Assembly Requires E6AP Oligomerization

... aspects of Angelman syndrome. Mol. Syndromol. 2, 100–112

29. Philpot, B. D., Thompson, C. E., Franco, L., and Williams, C. A. (2011) Angelman syndrome. Advancing the research frontier of neurodevelopmental disorders. J. Neurodev. Disord. 3, 50–56

30. Kühnl, S., Mothes, B., Matentzoglu, K., and Scheffner, M. (2013) Role of the ubiquitin ligase E6AP/UBE3A in controlling levels of the synaptic protein. Proc. Natl. Acad. Sci. U.S.A. 110, 8888–8893

31. Scheffner, M., Huibregtse, J. M., Vierstra, R. D., and Howley, P. M. (1993) The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. Cell 75, 495–505

32. Huibregtse, J. M., Scheffner, M., and Howley, P. M. (1991) A cellular protein mediates association of p53 with the E6 oncoprotein of human papillomavirus types 16 or 18. EMBO J. 10, 4129–4135

33. Beer-Romero, P., Glass, S., and Rolfe, M. (1997) Antisense targeting of E6AP elevates p53 in HPV-infected cells but not in normal cells. Oncogene 14, 595–602

34. Haupt, Y., Maya, R., Kazaz, A., and Oren, M. (1997) Mdm2 promotes the rapid degradation of p53. Nature 387, 296–299

35. Talis, A. L., Huibregtse, J. M., and Howley, P. M. (1998) The role of E6AP in the regulation of p53 protein levels in human papillomavirus (HPV)-positive and HPV-negative cells. J. Biol. Chem. 273, 6439–6445

36. Lawson, J. S., Glenn, W. K., Heng, B. Y., Ye, Y., Tran, B., Lutz-Mann, L., and Whitaker, N. J. (2009) Koilocytes indicate a role for human papilloma virus in breast cancer. Br. J. Cancer 101, 1351–1356

37. Muench, P., Probst, S., Schuetz, J., Leiprecht, N., Busch, M., Wesseln, S., Stubenrauch, F., and Itner, T. (2010) Cutaneous papillomavirus E6 proteins must interact with p300 and block p53-mediated apoptosis for cellular immortalization and tumorigenesis. Cancer Res. 70, 6913–6924

38. Munakata, T., Liang, Y., Kim, S., McGivern, D. R., Huibregtse, J. M., Nomoto, A., and Lemon, S. M. (2007) Hepatitis C virus induces E6AP-dependent degradation of the retinoblastoma protein. PLoS Pathog. 3, 1335–1347

39. Shirakura, M., Murakami, K., Ichimura, T., Suzuki, R., Shrimjo, T., Fukuda, K., Abe, K., Sato, S., Fukasawa, M., Yamakawa, Y., Nishijima, Y., Morishita, K., Matsuura, Y., Wakti, T., Suzuki, T., Howley, P. M., Miyamura, T., and Shoji, I. (2007) E6AP ubiquitin ligase mediates ubiquitination and degradation of hepatitis C virus core protein. J. Virol. 81, 1174–1185

40. Chen, J. J., Hong, Y., Bustamadeh, E., Baleja, J. D., and Androphy, E. J. (1998) Identification of an α-helical motif sufficient for association with papillomavirus E6. J. Biol. Chem. 273, 13537–13544

41. Elston, R. C., Naphthine, S., and Doobair, J. (1998) The identification of a conserved binding motif within papillomavirus type 16 E6 binding peptides, E6AP and E6BP. J. Gen. Virol. 79, 371–374

42. Be, X., Hong, Y., Wei, J., Androphy, E. J., Chen, J. J., and Baleja, J. D. (2001) Solution structure determination and mutational analysis of the papillomavirus E6 interacting peptide of E6AP. Biochemistry 40, 1293–1299

43. Liu, Y., Cherry, J. J., Dineen, J. V., Androphy, E. J., and Baleja, J. D. (2009) Determinants of stability for the E6 protein of papillomavirus type 16. J. Mol. Biol. 386, 1123–1137

44. Zanier, K., Charbonnier, S., Baltzinger, M., Nomine, Y., Altschuh, D., and Travé, G. (2005) Genetic analysis of the interactions of human papillomavirus E6 oncoproteins with the ubiquitin ligase E6AP using surface plasmon resonance. J. Mol. Biol. 349, 401–412

45. Zanier, K., Ruhlmann, C., Melin, F., Masson, M., Ould M'hamed Ould Sidi, A., Bernard, X., Fischer, B., Brino, L., Ristriani, T., Rybin, V., Baltzinger, M., Vande Pol, S., Hellwig, P., Schultz, P., and Travé, G. (2010) E6 proteins from diverse papillomaviruses self-associate both in vitro and in vivo. J. Virol. 84, 990–1004

46. Zanier, K., Charbonnier, S., Sidi, A. O., McEwen, A. G., Ferrario, M. G., Masson, M., Poussin-Courmontagne, P., Cura, V., Brino, L., Ristriani, T., Rybin, V., Baltzinger, M., Vande Pol, S., Hellwig, P., Schultz, P., and Travé, G. (2010) E6 proteins from diverse papillomaviruses self-associate both in vitro and in vivo. J. Virol. 84, 990–1004

47. Zanier, K., Charbonnier, S., Sidi, A. O., McEwen, A. G., Ferrario, M. G., Poussin-Courmontagne, P., Cura, V., Brino, L., Ristriani, T., Rybin, V., Baltzinger, M., Vande Pol, S., Hellwig, P., Schultz, P., and Travé, G. (2010) E6 proteins from diverse papillomaviruses self-associate both in vitro and in vivo. J. Virol. 84, 990–1004

48. Zanier, K., Charbonnier, S., Sidi, A. O., McEwen, A. G., Ferrario, M. G., Poussin-Courmontagne, P., Cura, V., Brino, L., Ristriani, T., Rybin, V., Baltzinger, M., Vande Pol, S., Hellwig, P., Schultz, P., and Travé, G. (2010) E6 proteins from diverse papillomaviruses self-associate both in vitro and in vivo. J. Virol. 84, 990–1004

49. Zanier, K., Charbonnier, S., Sidi, A. O., McEwen, A. G., Ferrario, M. G., Poussin-Courmontagne, P., Cura, V., Brino, L., Ristriani, T., Rybin, V., Baltzinger, M., Vande Pol, S., Hellwig, P., Schultz, P., and Travé, G. (2010) E6 proteins from diverse papillomaviruses self-associate both in vitro and in vivo. J. Virol. 84, 990–1004
Polyubiquitin Chain Assembly Requires E6AP Oligomerization

P. M. (2000) Human papillomavirus type 16 E6 induces self-ubiquitination of the E6AP ubiquitin-protein ligase. J. Biol. Chem. 74, 6408–6417

Huang, L., Kinnucan, E., Wang, G., Beaudenon, S., Howley, P. M., Huibregtse, J. M., and Pavletich, N. P. (1999) Structure of an E6AP-UbchH7 complex. Insights into ubiquitination by the E2-E3 enzyme cascade. Science 286, 1321–1326

Scheffner, M., Huibregtse, J. M., and Howley, P. M. (1994) Identification of a human ubiquitin-conjugating enzyme that mediates the E6-AP-dependent ubiquitination of p53. Proc. Natl. Acad. Sci. U.S.A. 91, 8797–8801

Ronchi, V. P., Klein, J. M., and Haas, A. L. (2013) E6AP/UBE3A ubiquitin ligase harbors two E2–ubiquitin binding sites. J. Biol. Chem. 288, 10349–10360

Heer, A., Alonso, L. G., and de Prat-Gay, G. (2011) E6*, the 50 amino acid product of the most abundant splice transcript of the e6 oncoprotein in high-risk human papillomavirus, is a promiscuous folder and binder. Biochemistry 50, 1376–1383

Baboshina, O. V., and Haas, A. L. (1996) Novel multiubiquitin chain linkages catalyzed by the conjugating enzymes E2ap and Rads are recognized by the 26S proteasome subunit 5. J. Biol. Chem. 271, 2823–2831

Haas, A. L. (2005) Purification of E1 and E1-like enzymes. Methods Mol. Biol. 301, 23–35

Haas, A. L., and Rose, I. A. (1982) The mechanism of ubiquitin activating enzyme. A kinetic and equilibrium analysis. J. Biol. Chem. 257, 10329–10337

Ronchi, V. P., and Haas, A. L. (2012) Measuring rates of ubiquitin chain formation as a functional readout of ligase activity. Methods Mol. Biol. 832, 197–218

Siepmann, T. J., Bohnsack, R. N., Tokgoz, Z., Baboshina, O. V., and Haas, A. L. (2003) Protein interactions within the N-end rule ubiquitin ligation pathway. J. Biol. Chem. 278, 311–321

Tokgoz, Z., Siepmann, T. J., Striech, F., Jr., Kumar, B., Klein, J. M., and Haas, A. L. (2012) E1-E2 interactions in ubiquitin and Nedd8 ligation pathways. J. Biol. Chem. 287, 311–321

Ronchi, V. P., and Haas, A. L. (2012) Measuring rates of ubiquitin chain formation as a functional readout of ligase activity. Methods Mol. Biol. 832, 197–218

Klingelhutz, A. J. (1998) Both Rb/p16INK4a inactivation and telomerase expression, leading to cellular transformation and full malignant phenotype. Cancer Res. 58, 1027–1035

Medcalf, E. A., and Milner, J. (1993) Targeting and degradation of p53 by 53BP1. Nature 364, 829–835

Thomas, M., Tomaic, V., Pim, D., Myers, M. P., Tommasino, M., and Banks, L. (2013) Interactions between E6AP and E6 proteins from α and β HPV types. Virology 435, 357–362

Streathfild, P. J., Almeida, L., Mitchell, P. J., and Miller, D. J. (2010) Structural, evolutionary, and assembly principles of protein oligomerization. Prog. Biol. Transl. Sci. 117, 25–51

Perica, T., Chothia, C., and Teichmann, S. A. (2012) Evolution of oligomeric state through geometric coupling of protein interfaces. Proc. Natl. Acad. Sci. U.S.A. 109, 8127–8132

Verdecia, M. A., Joaozeiro, C. A., Wells, N. J., Ferrer, J. L., Bowman, M. E., Hunter, T., and Noel, J. P. (2003) Conformational flexibility underlies ubiquitin ligation mediated by the WWP1 HECT domain E3 ligase. Mol. Cell 11, 249–259

Ogunjimi, A. A., Wiesner, S., Briant, D. J., Varelas, X., Sicheri, F., Forman-Kay, J., and Wrana, J. L. (2010) The ubiquitin binding region of the Smurf HECT domain facilitates polyubiquitylation and binding of ubiquitylated substrates. J. Biol. Chem. 285, 6308–6315

Pandya, R. K., Partridge, J. R., Love, K. T., Schwartz, T. U., and Ploegh, H. L. (2010) A structural element within the HuWHE1 HECT domain modulates self-ubiquitination and substrate ubiquitination activities. J. Biol. Chem. 285, 5566–5574

Maspero, E., Mari, S., Valentini, E., Musacchio, A., Fish, A., Pasqualato, S., and Polo, S. (2011) Structure of the HECT-ubiquitin complex and its role in ubiquitin chain elongation. EMBO Rep. 12, 342–349

Kamadurai, H. B., Soupourn, J., Scott, D. C., Duda, D. M., Miller, D. J., Stringer, D., Piper, R. C., and Schulman, B. A. (2009) Insights into ubiquitin transfer cascades from a structure of a UbcH5A ubiquitin–HECT(Nedd4L) complex. Mol. Cell 36, 1095–1102

Ogunjimi, A. A., Briant, D. J., Perez-Barbara, N., Le Roy, C., Di Guglielmo, G. M., Kavask, P., Rasmussen, R. K., Seet, B. T., Sicheri, F., and Wrana, J. L. (2005) Regulation of Smurf2 ubiquitin ligase activity by anchoring the E2 to the HECT domain. Mol. Cell 19, 297–308

Fang, P., Lev-Lehman, E., Tsai, T. F., Matsuura, T., Benton, C. S., Sutcliffe, J. S., Christian, S. L., Kubota, T., Halley, D. J., Meijers-Heijboer, H., Langlois, S., Graham, J. M., Jr., Beuten, J., Willems, P. J., Ledbetter, D. H., and Beaudet, A. L. (1998) The spectrum of mutations in UBE3A causing Angelman syndrome. Hum. Mol. Genet. 8, 129–135

Chan, A. L., Grossman, T., Zuckerman, V., Campigli Di Giammartino, M., Mosher, O., Scheffner, M., Monahan, B., Pillng, P., Jiang, Y. H., Haupt, S., Schueler-Furman, O., and Haupt, Y. (2013) C-Ab1 phosphorylates E6AP and regulates its E3 ubiquitin ligase activity. Biochemistry 52, 3119–3129

Scheffner, M., Wernsen, B. A., Huibregtse, J. M., Levine, A. I., and Howley, P. M. (1990) The E6 oncprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell 63, 1129–1136

Huibregtse, J. M., Scheffner, M., and Howley, P. M. (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, 4918–4927

Largrange, M., Charbonnier, S., Orfanoudakis, G., Robinson, P., Zanier, K., Masson, M., Lutz, Y., Trave, G., Weiss, E., and Deryckere, F. (2005) Binding of human papillomavirus 16 E6 to p53 and E6AP is impaired by monclonal antibodies directed against the second zinc-binding domain of E6. J. Gen. Virol. 86, 1001–1007

Medcalf, E. A., and Milner, J. (1993) Targeting and degradation of p53 by E6 of human papillomavirus type 16 is preferential for the 1620+ p53 conformation. Oncogene 8, 2847–2851

Thomas, M., Tomaic, V., Pim, D., Myers, M. P., Tommasino, M., and Banks, L. (2013) Interactions between E6AP and E6 proteins from α and β HPV types. Virology 435, 357–362

Dai, B., Pieper, R. O., Li, D., Wei, P., Liu, M., Yang, S., Aldea, K. D., Sawaya, R. B., Xie, K., and Huang, S. (2010) FoxM1B regulates NEDD4–1 expression, leading to cellular transformation and full malignant phenotype in immortalized human astrocytes. Cancer Res. 70, 2951–2961

Marianayagam, N. J., Sunde, M., and Matthews, J. M. (2004) The power of two. Protein dimerization in biology. Trends Biochem. Sci. 29, 618–625

Spikovsky, D., Aengeneyndt, F., Braspenning, J., and von Knobel Döberitz, M. (1996) p53-independent growth regulation of cervical cancer cells by the papillomavirus E6 oncogene. Oncogene 13, 1027–1035

Kiyono, T., Foster, S. A., Koop, J. L., McDougall, I. K., Galloway, D. A., and Klingelfuss, A. J. (1998) Both Rb/p16INK4a inactivation and telomerase activity are required to immortalize human epithelial cells. Nature 396, 84–88

Kentis, A., Gordon, R. E., and Borden, K. L. (2002) Control of biochemical
reactions through supramolecular RING domain self-assembly. Proc. Natl. Acad. Sci. U.S.A. 99, 15404–15409
88. Sun, L., Deng, L., Ea, C. K., Xia, Z. P., and Chen, Z. J. (2004) The TRAF6 ubiquitin ligase and TAK1 kinase mediate IKK activation by BCL10 and MALT1 in T lymphocytes. Mol. Cell 14, 289–301
89. Wang, X., Herr, R. A., Chua, W. J., Lybarger, L., Wiertz, E. J., and Hansen, T. H. (2007) Ubiquitination of serine, threonine, or lysine residues on the cytoplasmic tail can induce ERAD of MHC-I by viral E3 ligase mK3. J. Cell Biol. 177, 613–624
90. Page, M. I., and Jencks, W. P. (1971) Entropic contributions to rate accelerations in enzymic and intramolecular reactions and the chelate effect. Proc. Natl. Acad. Sci. U.S.A. 68, 1678–1683