The Role of E6AP in the Regulation of p53 Protein Levels in Human Papillomavirus (HPV)-positive and HPV-negative Cells*

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The E6 protein encoded by the oncogenic human papillomaviruses (HPVs) targets p53 for ubiquitin-dependent proteolysis. E6-mediated p53 degradation requires the 100-kDa cellular protein E6-associated protein (E6AP). E6AP and E6 together provide the E3-ubiquitin protein ligase activity in the transfer of ubiquitin to p53. In vitro studies have shown that E6AP can form a high energy thiolester bond with ubiquitin and, in the presence of E6, transfer ubiquitin to p53. In this study we have addressed the role of E6AP in vivo in the degradation of p53. Overexpression of wild-type E6AP in HeLa cells, which are HPV18-positive and express E6, resulted in a decreased steady state level of p53 and a decrease in the half-life of p53. Mutant forms of E6AP proteins were identified that were catalytically incapable of participating in E6-dependent ubiquitination of p53 and functioned in a dominant-negative manner in that they inhibited the E6-mediated ubiquitination of p53 by the wild-type E6AP in vitro. Transient transfection of one of these dominant negative (dn) mutants resulted in an increase in both the steady state level and half-life of p53 in vivo in HeLa cells. Consistent with this observation, overexpression of the dn E6AP resulted in a marked G1 shift in the cell cycle profile. In contrast, dn E6AP had no effect on p53 levels in U2OS cells, an HPV-negative cell line that contains wild-type p53. These studies provide evidence for the involvement of E6AP in E6-mediated p53 degradation in vivo and also indicate that E6AP may not be involved in the regulation of p53 ubiquitination in the absence of E6.

There is compelling evidence associating several specific types of the human papillomaviruses (HPVs) with certain human anogenital cancers (1). These “high risk” HPV types such as HPV16 and HPV18 encode two oncoproteins, E6 and E7, which target the important cellular growth regulatory proteins p53 and pRb, respectively (2). The E6 proteins of the high risk HPV types but not of the “low risk” HPV types are able to enter into a complex with p53 (3) and interfere with the ability of p53 to transcriptionally activate p53 responsive promoters (4). The steady state levels of p53 are generally quite low in HPV-positive carcinoma cell lines and in cells immortalized by the HPV oncoproteins (5). The observation that E6 proteins of high risk HPV types 16 and 18 promote the ubiquitin-dependent degradation of the p53 protein in vitro (6) led to the hypothesis that E6-mediated ubiquitination of p53 accounted for the low steady state levels of p53. In further studies examining the mechanism of E6-mediated degradation, a 100-kDa cellular protein, E6AP (E6 Associated Protein), was found to mediate the binding of E6 to p53 (7).

E6AP functions as an E3 ubiquitin protein ligase in the ubiquitination of p53 in vitro (8–10). The functional domains of E6AP are summarized in Fig. 1 (10). The 100-kDa E6AP protein contains an 18-amino acid region (amino acids 391–408) that is sufficient for binding E6. The E6-dependent binding of p53 involves amide acids 280–781, a domain that encompasses the E6-binding region. Finally, in addition to the sequences necessary for p53 binding, an intact COOH terminus is necessary for E6-mediated p53 ubiquitination (10). The COOH-terminal 350 amino acids comprise the hect (homology to E6AP C terminus) domain, a region of homology shared by several proteins structurally and functionally related to E6AP (11).

The carboxyl-terminal segment that is required for the ubiquitination function of E6AP is highly conserved among the hect family of proteins and contains a conserved cysteine residue at position 833 (11, 12). In vitro studies have shown that an E6AP mutant with a cysteine to alanine substitution at position 833 is unable to form a thiolester bond with ubiquitin (11, 13). Another E6AP mutant with a 6-amino acid deletion from the COOH terminus retains the ability to form a thiolester bond with ubiquitin but is incapable of transferring the moiety to p53 (11).

The ubiquitination of protein substrates is carried out by a series of cellular enzymes known as E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin protein ligase) that result in polyubiquitination of proteins that are then recognized by the 26 S proteosome and degraded. In this pathway, the 76-amino acid ubiquitin moiety is activated by E1 in an ATP-dependent step, through the formation of a high energy thiolester bond between the active site cysteine on E1 and the COOH-terminal glycine of ubiquitin. This activated ubiquitin moiety is then transferred to one of a family of different E2s characterized by a highly conserved catalytic site. E2 enzymes catalyze the formation of an isopeptide bond between the COOH-terminal glycine of ubiquitin and the e-amino group on a lysine residue of the protein substrate, either directly or in conjunction with an E3. Ubiquitin protein ligases comprise the third and least well characterized group of enzymes involved in the ubiquitination of substrates destined for degradation by the ubiquitin proteolytic pathway (14).

Previously, although few E3 proteins were well character-

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¶ The abbreviations used are: HPV, human papillomavirus; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin protein ligase; dn, dominant negative; ATPγS, adenosine 5′-O-(thiotriphosphate); FAC5, fluorescence-activated cell sorting.
ized, the accepted model suggested that E3 ubiquitin ligases functioned as “docking proteins” that served to bring a specific E2 and a substrate into close proximity, enabling the E2 to transfer ubiquitin to a lysine residue on the substrate. This was based on studies of the yeast E3, Ubr-1, which was shown to bind to the E2 Rad 6 (Ubc2) as well as to the substrate (15–17). Studies with E6AP provided an alternative model in which an E3 participates directly in the ubiquitination of substrates. E6AP forms a thiolester bond with ubiquitin and in association with E6 is capable of transferring ubiquitin to the p53 target substrate (8). Thus some E3 enzymes participate directly in the “cascade” of ubiquitin thiolester transfers ultimately resulting in the ubiquitination of substrates, leading to their degradation by the 26 S proteasome (11, 13).

E6AP is a member of a family of proteins that share COOH-terminal sequence homology. Such proteins have been found in yeast, Drosophila, Caenorhabditis elegans as well as higher vertebrates. This growing family of hect proteins now has several members that have been shown to function as E3 ubiquitin ligase enzymes through their ability to form thiolester bonds with ubiquitin (11, 12). E6AP has also recently been implicated in the human genetic disorder “Angelman syndrome” (18, 19), suggesting a role for E6AP in brain development. The normal substrate(s) and regulation of E6AP in the absence of HPV E6 are currently under study in our laboratory (20). For other hect E3 enzymes, some cellular substrates have now been defined. Both Gap1 and Fur4 permeases were identified as substrates of an essential yeast E3 3ept hsc protein, Rsp5/Npi1 (21). Rpb1, the largest subunit of RNA polymerase II has also been identified as a substrate of Rsp5/Npi1 (22). Pub1, the Schizosaccharomyces pombe homologue of Rsp5/Npi1 has been demonstrated to target cdc25 for degradation (23). Further studies on E3 enzymes for which cellular substrates are defined may elucidate how the specificity of protein substrates is determined and how degradation of target substrates is regulated by the cell.

The role of E6AP in E6-mediated p53 degradation in vitro has been well characterized. The current studies were undertaken to investigate the role of E6AP in p53 degradation in vivo in HPV-positive E6 expressing cells, as well as in HPV-negative cells. A previously described in vitro p53 ubiquitination assay (8) was used to identify catalytically impaired mutant forms of E6AP, which interfered with the ability of the wild-type E6AP protein to target p53 for ubiquitination in the presence of E6. These dominant negative (dn) mutants retained the ability to enter into ternary complexes with E6 and p53 but were incapable either of forming a thiolester bond with ubiquitin (cysteine 833 to alanine substitution) or of transferring the ubiquitin moiety to p53 (a COOH-terminal 6-amino acid deletion) (11). The C833A mutant form of E6AP could be stably expressed in mammalian cells, permitting us to test the role of E6AP in mediating p53 degradation in vivo in HPV-positive cervical cancer cells as well as in human cells not expressing E6.

EXPERIMENTAL PROCEDURES

Plasmids—The p53 plasmid used for in vitro transcription and translation has been previously described (3), as have E6AP wild-type and mutant in vitro expression plasmids (10, 11). Baculovirus pVL1393 (Pharmingen) constructs containing either wild-type or mutant E6AP cDNAs for expression in SF9 insect cells have also previously been described (10). Mammalian E6AP expression vectors were constructed by subcloning the above cDNAs into pCMV4 (24) utilizing the EgII and HindIII sites in the polylinker. HPV16 E6 expression constructs were kindly made available by Dr. Karl Munger, with HPV16 E6 and E7 genes directed from the human CMV IE promoter. Plasmid DNA for mammalian cell transfection was prepared in DH5α and purified by standard cesium chloride gradient methods (25).

In Vitro p53 Ubiquitination Assay—In vitro assays were performed as described previously (8) using bacterially expressed wheat E1 and Arabidopsis thaliana UBC8 E2 (26, 27). p53 was translated in TNT wheat germ extract as per the manufacturer’s instructions (Promega) in the presence of radiolabeled methionine. Wild-type E6AP was translated in TNT wheat germ extract (Promega) with unlabeled amino acid components. Mutant E6AP and HPV16 E6 proteins were expressed and partially purified from SF9 insect cells as described previously (10) using DEAE-Sepharose (Pharmacia Biotech Inc.) or Bio-Rad S, respectively.

Ubiquitination reactions containing 5 μl of [35S]methionine-labeled p53 (from 100 μl of total wheat germ extract translation reaction) were incubated for 3 h at 25 °C along with 1 μl of E1, 1 μl of E2, 2 μS ATP, 0.1 mM MgCl2, 6 μg of ubiquitin, 5 μl of HPV16 E6 (a 1:100 dilution of partially purified baculovirus expressed protein) and 5 μl of E6AP (from a 1:100 dilution in translation in wheat germ extract). Competition assays for the identification of dominant mutant forms of E6AP utilized SF9 insect cell-derived mutant E6AP proteins, with wild-type E6AP protein or wild-type baculovirus SF9 cell fractions of equal volume as negative controls. These were added in 5–μl volumes containing 1 μl of baculovirus-derived protein (or corresponding wild-type baculovirus) (from 100 μl of total wheat germ extract) (from 100 μl of total wheat germ extract) (from 100 μl of total wheat germ extract) and 5 μl of E6AP (from a 1:100 dilution in translation in wheat germ extract). Competition assays for the identification of dominant mutant forms of E6AP utilized SF9 insect cell-derived mutant E6AP proteins, with wild-type E6AP protein or wild-type baculovirus SF9 cell fractions of equal volume as negative controls. These were added in 5–μl volumes containing 1 μl of baculovirus-derived protein (or corresponding wild-type baculovirus) (from 100 μl of total wheat germ extract) and 5 μl of E6AP (from a 1:100 dilution in translation in wheat germ extract). Compe-
then processed as described above for lysates, Western blotting, and quantitation by densitometric measurement.

**p21 Immunoprecipitation/Western Blotting**—p21 immunoprecipitation/Westerns were carried out using CaCl2 transfected HeLa cells at 48 h post transfection. 60-mm plates were harvested into 500 μl of RIPA buffer (20 mM Tris 7.5, 2 mM EDTA, 150 mM NaCl, 0.25% SDS, 1% Nonidet P-40, 1% deoxycholic acid) on ice. Lysates were then precleared using 20 μl of protein A and protein G-coupled agarose beads for 45 min. 0.5 μl of "15431E" anti-rabbit p21 antibody (Pharmingen) was added to clarified lysates, which were then incubated overnight at 4 °C on a rotator. Protein A- and G-coupled agarose beads were added, and lysates continued rotating for 2 h at 4 °C. Beads were pelleted at maximal speed on the Eppendorf centrifuge and were rinsed four times with RIPA buffer. After the final spin the pellet was resuspended in loading buffer and boiled for 5–7 min prior to loading on 12% polyacrylamide protein gels. These were run and transferred as described previously and stained as per Western blotting procedure using anti-p21 monoclonal antibody 15091A (Pharmingen).

**Cell Cycle Analysis** CD20 Fluorescein Isothiocyanate (FITC) Propidium Iodide Staining—Cell cycle analysis was carried out following the methods of Van den Heuvel (28) using a CD20 expression plasmid and fluorescein isothiocyanate-conjugated anti-CD20 antibody in conjunction with propidium iodide staining. Cell cycle analysis was performed on FACS FACScan at the Core Flow Cytometry Facility at the Dana Farber Cancer Institute, Boston, Massachusetts.

**Live Cell Separation of U2OS Cells Expressing CD20 Surface Marker**—U2OS cells co-transfected with E6AP constructs and a CD20 expression plasmid (or green lantern fluorescent protein construct) were harvested live at 48 h post-transfection with 0.1% EDTA, rinsed with Dulbecco's modified Eagle's medium 10% fetal bovine serum, and stained with fluorescein isothiocyanate-conjugated anti-CD20 antibody on ice following standard procedures. Cells were sorted on either a Becton Dickinson FACScan or a Coulter EPICS 750 Series Sorter at the Core Flow Cytometry Facility at the Dana Farber Cancer Institute, Boston, MA.

**RESULTS**

**Identification of Dominant Negative E6-AP Mutants**—Dominant negative forms of E6AP were identified in an in vitro p53 ubiquitination assay using bacterially derived E1 and E2 (AtUBC8) proteins and ubiquitin as described previously (8, 26, 27). The p53 protein utilized was translated in vitro in wheat germ extract, and E6 or competing E6AP mutants were partially purified from SF9 insect cell fractions, which contain no detectable endogenous E6AP activity. In this reconstituted p53 degradation assay, p53 is efficiently ubiquitinated and degraded in the presence of ATP (8). However, in the presence of the nonhydrolyzable ATP analogue, ATPβS, p53 can be ubiquitinated but not subsequently degraded by the proteasome. Under these conditions, polyubiquitinated p53 is readily observed as a higher molecular weight, slowly migrating species by SDS-polyacrylamide gel electrophoresis (6, 8).

Two mutant forms of E6AP were tested for their ability to compete with the wild-type protein and prevent the E6-dependent ubiquitination of p53 in vitro (Fig. 1). E6AP C833A contains a cysteine to alanine substitution at amino acid 833, rendering it incapable of forming a thiolester with ubiquitin (11). E6AP C833A has the active site cysteine to alanine substitution at amino acid 833, rendering it incapable of forming a thiolester with ubiquitin but is incapable of transferring the moiety to p53 (11). wt, wild type.

**Expression of Wild-type or dn E6AP in HeLa Cells Affects Steady State Levels of p53**—Because the competition of wild-type E6AP by the two dn E6AP mutants observed in vitro occurred using protein quantities that might be attainable by transient expression in mammalian cells, we next tested the effect of the dn E6AP mutants upon E6-dependent p53 degradation in vivo. Genes encoding the wild-type and dn E6AP mutant proteins were subcloned into pCMV4 mammalian expression vectors. Transfection of plasmid DNA was performed using standard calcium phosphate methods that had been op-
p53 relative to cells transfected with vector alone (Fig. 3, B). The increased level of p53 in HeLa cells expressing E6AP C833A resulted in an induction of p21 detected by immunoprecipitation/Western blot from duplicate plates of transfected HeLa cells. Lysates were incubated with antibody 15431E overnight, precipitated, and probed using antibody 15091A by Western blot analysis.

The transient overexpression of wild-type E6AP in HeLa cells resulted in a decrease in steady state p53 levels, whereas expression of the C833A mutant exhibited an elevated level of p53 relative to cells transfected with vector alone (Fig. 3, A and B). To determine whether higher levels of p53 corresponded to an increase in the ability of p53 to transactivate a p53-responsive promoter, we examined the levels of p21, a protein known to be induced by the p53 protein at the transcriptional level. The increased level of p53 in HeLa cells expressing E6AP C833A resulted in an induction of p21 detected by immunoprecipitation/Western analysis (Fig. 3C). We also noted that expression of wild-type E6AP, which led to a decrease in p53 level, also resulted in a lower level of p21 protein expression. These data indicate that E6AP is involved in the E6-dependent regulation of p53 protein levels.

Expression of Wild-type E6AP or E6AP C833A Alters p53 Half-Life in HeLa Cells—To determine whether the altered levels of p53 in HeLa cells induced by overexpression of wild-type or dn E6AP were due to changes in protein stability, the half-life of p53 was determined in cells transiently transfected with the above constructs. Because cycloheximide inhibits de novo protein synthesis, the half-life of p53 can be determined by Western blot analysis in cells treated with the drug (29).

Cells transfected with wild-type E6AP, the C833A mutant or the vector control were analyzed at 0, 15, 30, and 70 min following addition of cycloheximide. Differences in the steady state levels of p53 were readily apparent in cells transfected with either wild-type or E6AP C833A compared with the vector control at the zero time point of the experiment (Fig. 4A, t0, and Fig. 3). Expression of wild-type E6AP led to a slight but reproducible decrease of p53 half-life relative to vector controls, whereas expression of E6AP C833A resulted in a markedly prolonged half-life of p53. Densitometric measurement of the p53 signal from Western blots enabled the quantitation of p53 as a percentage of the total starting levels (Fig. 4B) and revealed that the half-life of p53 was between 15 and 20 min in the vector transfected cells, correlating well with the reduced p53 half-life previously demonstrated for E6-containing cells (30, 31). Expression of wild-type E6AP resulted in a shortened p53 half-life of approximately 10 min, whereas p53 half-life was extended to 70 min or longer in a dose-dependent manner following transfection of the dn E6AP C833A. From these experiments we conclude that wild-type E6AP accelerated the E6-mediated degradation of p53, whereas E6AP C833A interfered with E6-mediated p53 degradation by the endogenous E6AP protein.

Cell Cycle Effects Resulting from Altered p53 Levels in HeLa Cells—Because p53 regulates the cell cycle and can induce a G1 arrest in cells following DNA damage, we next investigated whether the effects on p53 levels observed following E6AP expression resulted in alterations in the cell cycle as determined by flow cytometric analysis. The population of HeLa cells transfected with either wild-type E6AP or E6AP C833A were selected by cotransfection with a CD20 surface marker expression vector at 48 h post transfection using fluorescein-conjugated anti-CD20 antibody. Cells expressing the CD20 surface marker as well as E6AP were isolated by FACS analysis, and propidium iodide staining provided a cell cycle profile of transfected cells. HeLa cells expressing wild-type E6AP (with a resulting reduction in p53 levels and half-life; see Figs. 3 and 4, respectively) exhibited a G2/M shift in cell cycle profile (Fig. 4B).
The G2/M population in vector controls was typically 17%, whereas cells expressing wild-type E6AP the G2/M population shifted to 40%. In contrast, cells expressing E6AP C833A (with the observed increase in steady state p53 level and half-life; see Figs. 3 and 4, respectively) exhibited a G1 shift in cell cycle profile (Fig. 5C). Although typical HeLa cell G1 profiles for vector controls were 55%, the expression of the dominant negative mutant resulted in an increase in the G1 population to 70%. Alterations in steady state p53 levels and half-life following transient expression of wild-type or dn E6AP therefore were sufficient to alter the normal cell cycle distribution of HeLa cells.

HPV-negative Cells Do Not Exhibit Altered p53 Steady State Level or Cell Cycle Profiles Following Transfection with Wild-type or dn E6AP—A critical question that remained was whether E6AP is involved in p53 ubiquitination in the absence of E6. Does E6 function to enhance the ability of E6AP to target p53 for ubiquitination, or does E6 redirect E6AP from its normal substrates to p53? To address this question, we utilized the U2OS cell line, an HPV-negative osteosarcoma cell line that contains wild-type p53. Comparable levels of transient expression of either E6AP wild-type or E6AP C833A in U2OS cells were demonstrated by Western blot (Fig. 6A, compare with Fig. 3A). In contrast to the results observed in HPV-positive cells, however, expression of either wild-type E6AP or E6AP C833A had no detectable effect on the levels of p53 (Fig. 6B). Furthermore, introduction of HPV16 E6 into U2OS cells by transfection of a plasmid expressing the E6 and E7 oncoproteins resulted in p53 degradation similar to that observed in HPV-positive cells, demonstrating that the lack of p53 degradation observed in U2OS cells was due not to a defect in E6AP function or to an intrinsic defect in the ubiquitin pathway in these cells (Fig. 6B, last lane). In addition, no effect on the cell cycle profile of U2OS cells was observed by FACS analysis following co-transfection of CD20 and propidium iodide staining with either wild-type E6AP or E6AP C833A (data not shown). These results suggest that E6AP is not involved in the regulation of p53 stability in the absence of E6.

Discussion

In this study we provide evidence that E6AP is involved in the E6-mediated degradation of p53 in vivo. Mutant forms of E6AP that prevented p53 ubiquitination in vitro also interfered with E6-mediated p53 degradation in HPV18-positive, E6 expressing HeLa cells. This conclusion is supported by the elevated p53 levels observed in HeLa cells following transient overexpression of C833A, a mutant form of E6AP that is unable to form a thiolester with ubiquitin (12). This observed increase in p53 was found to be the result of an extended half-life and to have biological consequences in terms of shifting a higher proportion of cells into the G1 phase of the cell cycle, as well as inducing cellular p21.

Our initial strategy in using dominant negative versions of E6AP was based on the hypothesis that a catalytically impaired version of E6AP that could still bind E6 might sequester E6, permitting p53 levels to rise to those normally found in HPV-negative cells. The data reported in this paper are consistent with this hypothesis. However, E6AP C833A may function in part by interfering with other cellular components of the ubiquitin machinery. This postulate is supported by other results that have shown that the COOH-terminal hect domain of E6AP can interact with specific E2 ubiquitin-conjugating enzymes (20). Therefore, overexpression of a dn E6AP containing an intact hect domain could result in the sequestration of specific E2 enzymes and a stabilization of downstream targets such as p53. However, in studies not presented here, E6AP mutants specifically incapable of binding to E6 did not function in a dominant negative manner in HPV-positive cells. The ability of only those mutants competent to bind E6 to behave in a dominant negative manner in vivo therefore implicates the sequestration of E6 mechanistically as the primary pathway for interference with p53 degradation in these studies.

Transient overexpression of wild-type E6AP in HeLa cells enhanced p53 degradation and led to an increase in the proportion of cells in the G2/M phase of the cell cycle. The de-
creased p53 levels observed in HeLa cells were the result of a shortened half-life of p53. This might suggest that in HeLa cells E6AP is limiting in E6-mediated p53 degradation. The targeted degradation of p53 by E6/E6AP may be affected by the fraction of E6 that is available to interact with E6AP to mediate ubiquitination. It may be the case that not all of the E6 protein in the cell is normally utilized to target p53 for degradation, and by increasing the levels of E6AP, additional E6 is recruited to enhance the ubiquitination of p53. The transient overexpression of wild-type E6AP also resulted in an increased proportion of cells in the G2/M phase of the cell cycle. The observed shift in G2/M cell population was cell-specific in that it was not observed in HPV-negative cells. Whether this altered G2/M profile in HeLa cells was a direct or indirect result of the observed decrease in p53 levels or resulted from E6/E6AP targeting of another as yet unidentified substrate involved in cell cycle regulation is unclear at present.

Our studies provide in vivo evidence for a role of E6AP in E6-mediated degradation of p53 in mammalian cells. Our conclusions are in agreement with other published studies, which have demonstrated an increase in nuclear p53 levels measured by immunofluorescence following microinjection of antisense oligonucleotides complimentary to E2 (human Ubc 4) and to E6AP into HeLa cells (32). Originally identified by Huibregtse et al. as a required factor for E6 binding to p53, E6AP has been subsequently characterized biochemically as an E3 (9). The studies presented in this paper indicate that E6AP may not be involved in targeting the ubiquitination of p53 in the absence of E6. This may suggest that E6 redirects E6AP from other substrates to p53. It is not yet clear what determines the normal regulation of E6AP and whether under some circumstances E6AP could target p53 for ubiquitination in the absence of E6. Recent studies have reported that Mdm2 promotes the rapid degradation of p53 through the ubiquitin proteolytic pathway (33, 34). It may therefore be the case that one or more downstream effectors mediate p53 degradation in the cell, either in conjunction with E6AP or through other E3 enzymes. Maki et al. (29) have demonstrated that p53 is degraded by the ubiquitin-dependent proteolytic pathway in HPV-negative cells; however, the specific E2 and E3 enzymes involved in p53 regulation have not yet been identified. It also remains to be established whether the interaction of HPV E6 with E6AP has additional cellular consequences. For instance, there may be specific cellular proteins in addition to p53 targeted by E6 and E6AP for ubiquitination. In redirecting E6AP to p53 as a target, E6 may actually stabilize some cellular proteins that are normally substrates of E6AP.

Our data suggest that E6AP is not involved in the regulation of p53 levels in cells that do not contain E6. No effect on the levels of p53 was observed in U2OS cells by either wild-type or dn E6AP proteins, even though comparable levels of expression to those achieved in HeLa cells were demonstrated. Furthermore, the ability to induce p53 degradation in U2OS cells transfected with E6 demonstrated that the E6AP ubiquitin pathway components required for ubiquitination and degradation of p53 are present and functional in these cells but only mediate p53 degradation in the presence of E6. Our conclusion that E6AP does not have a role in regulating the ubiquitination of p53 in the absence of E6 is also in agreement with a recent study demonstrating that microinjection of E6AP antisense oligonucleotides into HPV-negative cells fails to affect intracellular p53 levels (35). We believe that there is an advantage to the use of the dn E6AP protein as opposed to an antisense oligonucleotide to address the identification of potential substrates of E6AP. Dominant negative methodology provides specificity that is based on our mechanistic understanding of the role of E6AP in the ubiquitin pathway. Using the dn E6AP, we have recently identified additional E6-independent substrates of E6AP.2

There is clearly a high degree of specificity involved in the regulated transfer of ubiquitin to individual target substrates. However, few of the molecular determinants of this specificity have been identified, and little is known about the regulation of ubiquitin pathway components. The three-dimensional molecular structure of two E2 enzymes have now been determined (36, 37), and conserved surface “patches” are postulated to interact with ubiquitin, presumably in the form of an E1-mediated thiolester. The nonconserved regions of the E2 peptides presumably mediate interactions with the E3 enzymes and possibly with specific substrates. For the subset of E3 proteins that contain het domains, the interaction with E2 enzymes appears to be mediated through this COOH-terminal domain, whereas the nonconserved NH2-terminal regions of these proteins appear to specify substrate interactions (20). It may also be the case for the het proteins that additional cellular “specificity factors” normally mediate substrate recognition, with the E6 protein serving as a virally encoded example of one such factor. Clearly the regulation of any of the individual components of this pathway, including the deubiquitinating components comprising the counterbalancing set of reactions, may be relevant to the kinetics of substrate degradation.

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