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Localization of the E6-AP Regions That Direct Human Papillomavirus E6 Binding, Association with p53, and Ubiquitination of Associated Proteins

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E6-AP is a 100-kDa cellular protein that mediates the interaction of the human papillomavirus type 16 and 18 E6 proteins with p53. The association of p53 with E6 and E6-AP promotes the specific ubiquitination and subsequent proteolytic degradation of p53 in vitro. We recently isolated a cDNA encoding E6-AP and have now mapped functional domains of E6-AP involved in binding E6, association with p53, and ubiquitination of p53. The E6 binding domain consists of an 18-amino-acid region within the central portion of the molecule. Deletion of these 18 amino acids from E6-AP results in loss of both E6 and p53 binding activities. The region that directs p53 binding spans the E6 binding domain and consists of approximately 500 amino acids. E6-AP sequences in addition to those required for formation of a stable ternary complex with E6 and p53 are necessary to stimulate the ubiquitination of p53. These sequences lie within the C-terminal 84 amino acids of E6-AP. The entire region required for E6-dependent ubiquitination of p53 is also required for the ubiquitination of an artificial E6 fusion protein.

Human papillomaviruses (HPVs) that infect the anogenital tract can be classified as either high risk or low risk on the basis of their clinical associations. The high-risk HPV types, which include HPV type 16 (HPV16) and HPV18, are found in approximately 90% of cervical cancers and in a high percentage of premalignant lesions recognized to be at risk for progression to cancer (46). The low-risk types such as HPV6 and HPV11 are associated primarily with condylomata acuminata, which only rarely progress to cancer. Transfection of DNA of the high-risk HPVs results in the extended life span and immortalization of primary human keratinocytes and fibroblasts in cell culture, whereas DNA of the low-risk types does not (5, 32, 39). Mutational analyses have shown that the E6 and E7 genes are the only viral genes necessary for this activity (14, 29, 42). The E7 proteins may function in cellular transformation, at least in part, through interactions with the product of the retinoblastoma susceptibility gene, pRB (6, 30). One consequence of this interaction is disruption of the complex that pRB can form with the E2F transcription factor (2). The E7-mediated release of E2F from these complexes is thought to influence the expression of genes involved in cell cycle progression (1, 15; for a review, see reference 31).

The oncogenic activity of the E6 proteins of the high-risk HPVs has been correlated with their ability to interact with and inactivate the cellular p53 protein (43). Mutations within the p53 gene are the most frequent specific genetic alteration associated with human cancer (for a review, see reference 41), and individuals with germline p53 mutations have an elevated risk of developing cancer (26, 42). Although p53 was originally classified as an oncogene, subsequent studies have shown that wild-type p53 actually has cell growth-suppressive and tumor-suppressive properties (7, 9). This growth-inhibitory activity may be related to the ability of wild-type p53 to act as a positive as well as a negative modulator of transcription (8, 11, 12, 21). HPV16 E6 has been shown to abrogate both the transcriptional activation and the repression properties of p53 (24, 27). Additional support for the model that the HPV E6 and E7 proteins functionally inactivate the p53 and RB gene products comes from studies that have examined the state of the p53 and RB genes in HPV-containing and HPV-negative cervical carcinoma cell lines (4, 36, 44). HPV-containing cell lines were found to express wild-type p53 and pRB, whereas cell lines lacking HPV DNA contained mutations within both the p53 and the RB genes. This implies that inactivation of the p53 and RB pathways may be a common step in cervical carcinogenesis that can occur either by mutation or as a consequence of their interaction with the HPV E6 and E7 proteins.

A mechanism for how E6 might inactivate p53 was postulated on the basis of the observation that the high-risk E6 proteins stimulate the ubiquitin-dependent degradation of p53 in vitro (38). This suggested that E6 might function in immortalization by stimulating the degradation of p53. This model also accounts for the observations that p53 levels are very low in HPV-immortalized cells (36) and that the half-life of p53 in HPV16-immortalized keratinocytes is lower than in primary keratinocytes (16, 24). This mechanism differs from that postulated for how other small DNA tumor viruses inactivate p53. Both large T antigen of simian virus 40 and the 55-kDa E1B protein of adenovirus type 5 sequester p53 into complexes that inactivate p53 but result in an increase in the half-life of p53 (23, 25, 34, 45).

The enzymatic reactions involved in the ubiquitination of proteins are well characterized (for a review, see reference 10). Ubiquitin is a 76-amino-acid protein which is found in all eukaryotic organisms. The E1 ubiquitin-activating enzyme stimulates the ATP-dependent formation of a high-energy thioester between the carboxyl group of the last amino acid of ubiquitin and a thiol group of a cysteine residue of the E1 protein. The E1 protein then transfers the activated ubiquitin to a cysteine of an E2 ubiquitin-conjugating enzyme, with retention of a high-energy thioester bond. E2 proteins are a class of low-molecular-weight proteins which catalyze the

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formation of a peptide bond between the carboxyl group of the ubiquitin moiety and the ε-amino group of a lysine residue on the substrate protein. Ligated ubiquitin moieties can themselves be ubiquitinated (at lysine 48), resulting in the formation of high-molecular-weight multiubiquitinated proteins, which are then recognized and degraded by a large proteasome complex. Generally, the E2 proteins require an E3 ubiquitin ligase protein to specify the protein that is to be multiubiquitinated. E3 proteins are as yet poorly characterized and are defined broadly as an activity that is necessary for ubiquitination of a specific substrate by an E2 protein. While substrates that appear to be regulated by ubiquitin-mediated turnover (33), such as cyclin B (13), have been identified, there has not yet been a single natural target of the ubiquitin system for which the relevant E2 and E3 proteins have been identified and characterized.

The stimulation of p53 degradation by the high-risk HPV E6 proteins is a novel mechanism of action for a viral oncoprotein. How the association of E6 with p53 results in the stimulation of p53 ubiquitination is not yet fully understood. Some clues, however, have come from studies of artificial E6 fusion proteins. A fusion protein consisting of the pRB binding domain of HPV16 E7 protein and HPV16 E6 ($E7_{1-50}$-E6) can associate with pRB in vitro and stimulate the ubiquitin-dependent degradation of pRB (37), suggesting that E6 can target other proteins for degradation with which it can complex. The low-risk E6 proteins, which do not stably associate with or stimulate the degradation of p53, were also active in pRB degradation as E6-E7 fusion proteins. This implies that the high- and low-risk HPV E6 proteins share the ability to stimulate degradation of associated proteins but may differ in the cellular proteins that they target. Certain E6 fusion proteins can even stimulate their self-targeted degradation. For example, a fusion protein consisting of HPV16 E6 and the entire HPV16 E7 protein sequence (E6-$E7_{1-98}$) can be efficiently ubiquitinated in vitro (35). A possible explanation for this is that the E7 moiety is recognized by the components of the ubiquitin proteolysis system in the same way that a protein that is noncovalently bound to E6 is recognized. E6 proteins by themselves do not appear to be recognized as substrates for ubiquitination (38).

We previously found that a 100-kDa cellular protein is required for stable interaction of high-risk HPV E6 proteins with p53. This protein was designated E6-AP (E6-associated protein) because it can form a stable complex with high-risk HPV E6 proteins in the absence of p53, but neither E6 nor E6-AP by itself stably associates with p53; only the ternary complex is stable (18, 19). The components of the ubiquitin proteolysis system that are involved in E6-mediated degradation of p53 have been characterized (35). E6-AP is necessary for E6-mediated p53 degradation, since it is required for association of E6 with p53. It is also necessary, however, for the ubiquitination of pRB with the $E7_{1-50}$-E6 fusion protein, as well as the self-targeted ubiquitination of the E6-$E7_{1-98}$ fusion protein (35). This implies that E6-AP may play an active role in ubiquitination of certain subtrates. The primary sequence of E6-AP is related to two other proteins, both of unknown function, in current data bases (19, 22, 28). In this study we have defined several functional domains of E6-AP: the E6 binding domain, the p53 binding domain, and the minimal domain necessary for the ubiquitination of p53 as well as of an artificial E6 fusion protein.

MATERIALS AND METHODS

Plasmids. p53 and HPV E6 plasmids for in vitro transcription and translation have been described previously (43). E6-AP expression plasmids were constructed by ligating polymerase chain reaction (PCR) products into pGEX-2T (Pharmacia), for expression as glutathione *S*-transferase (GST) fusion proteins in *Escherichia coli*; into pGEM1 (Promega), for in vitro transcription and translation; or into pVL1393 (Pharmingen), for expression in insect cells. Sense oligonucleotides which contained a Kozak consensus translation initiation codon (except for pGEX-2T constructs, which did not include an initiation codon) were synthesized, and antisense oligonucleotides that contained an in-frame translation stop codon were synthesized. The E6-AP cDNA described previously (19) was used as the template for PCR.

The pGEX-2TK-E6-E7 plasmid was constructed by ligation of the HPV16 E6 and HPV16 E7 open reading frames into pGEX-2TK (kindly provided by William Kaelin [20]). The protein expressed by this plasmid in *E. coli* encodes GST sequences followed by a protein kinase A site followed by the E6 and E7 sequences. A 2-amino-acid segment (Val-Asp) is encoded by the *Sal*I restriction site linking the E6 and E7 sequences. Following purification, the protein was labeled in vitro with [γ-^{32}P]ATP (Amersham) and protein kinase A (Sigma) (20).

GST fusion proteins were expressed in *E. coli* DH5α and affinity purified on glutathione-Sepharose (Pharmacia). Rabbit reticulocyte and wheat germ in vitro translation reagents were obtained from Promega. Recombinant baculoviruses were recovered by using a BaculoGold transfection kit (Pharmingen) as described by the manufacturer.

E6 and p53 binding assays. E6 binding assays were performed by combining approximately 0.1 μg of GST or GST–E6-AP fusion protein immobilized on glutathione-Sepharose with 125 μl of $T_{25}N_{50}$ (25 mM Tris-HCl [pH 7.4], 50 mM NaCl), 25 μl of lysis buffer (100 mM Tris-HCl [pH 7.4], 100 mM NaCl, 1% Nonidet P-40), and 10 μl of a ^{35}S-labeled HPV16 E6 wheat germ extract translation reaction mixture. The mixtures were rotated in microcentrifuge tubes at 4°C for 12 h. The Sepharose beads were then collected by centrifugation, washed three times with 750 μl of lysis buffer, and then boiled for 5 min in sodium dodecyl sulfate (SDS) gel loading buffer. The amount of ^{35}S-labeled E6 protein that bound to the beads was determined by SDS-polyacrylamide gel electrophoresis (PAGE; 12% acrylamide) and fluorography. A total of 1 μl of the translation reaction mixture was run on the gel to determine the fraction of the input E6 protein that bound to the GST–E6-AP proteins. Peptide competition experiments were performed as described above in the presence of peptides corresponding to amino acids 391 to 408 or 640 to 653 of E6-AP. Peptides were synthesized and high-pressure liquid chromatography purified by Peninsula Laboratories (Belmont, Calif.).

p53 binding assays were performed by mixing 0.1 μg of GST-p53 protein (18) immobilized on glutathione-Sepharose with 125 μl of $T_{25}N_{50}$, 25 μl of lysis buffer, 10 μl of a rabbit reticulocyte lysate in vitro translation reaction mixture containing ^{35}S-labeled E6-AP protein, and 10 μl of either a mock wheat germ extract translation reaction mixture or an HPV16 E6 RNA-programmed wheat germ extract translation reaction mixture. The mixtures were incubated and processed as described above for the E6 binding reactions. Aliquots of the E6-AP translation reaction mixtures were

analyzed separately by SDS-PAGE to ensure that all of the E6-AP variants were translated with similar efficiencies.

Ubiquitination assays. E6-AP variants were expressed from recombinant baculovirus in *Spodoptera frugiperda* (Sf9) insect cells in Grace's supplemented insect cell medium (Difco) containing 10% fetal bovine serum. A 15-cm plate containing Sf9 cells at 50% confluency was infected with each recombinant baculovirus at a high multiplicity of infection or with wild-type *Autographa californica* nuclear polyhedrosis virus (Ac*M*NPV; not expressing a foreign protein). Cell extract was prepared 48 h postinfection with the lysis buffer described above containing 1 mM dithiothreitol, 0.01% phenylmethylsulfonyl fluoride, and 1 μg (each) of aprotinin and leupeptin per ml. The E6-AP was partially purified by a single-batch chromatography step with DEAE-Sephacell (Pharmacia). The cell extract was combined with 75 μl of DEAE-Sephacell resin and incubated at 4°C for 10 min. The resin was then washed three times with $T_{25}N_{50}$, and bound protein was eluted with 150 μl of $T_{25}N_{500}$ (500 mM NaCl). A 25-μl aliquot of this high-salt pool was analyzed by SDS-PAGE and Coomassie blue staining. A band corresponding to each of the E6-AP variants was easily detected. The amount of the DEAE fractions used in the ubiquitination assays was adjusted to account for differences in E6-AP expression.

p53 ubiquitination assays were performed by combining 2 μl of ^{35}S-labeled wheat germ extract-translated human wild-type p53 with 10 μl of either a mock wheat germ extract translation reaction mixture or a wheat germ extract translation reaction mixture programmed with HPV16 E6 mRNA and with 10 μl of the DEAE fraction from either uninfected Sf9 cells or baculovirus-infected Sf9 cells. In addition, each reaction mixture contained 24 μl of $T_{25}N_{50}$, 2 μl of 2-mg/ml ubiquitin (Sigma), and 2 μl of 40 mM ATP-γ-S (total volume, 50 μl). The mixtures were incubated at room temperature for 4 h and then analyzed by SDS-PAGE and fluorography.

Ubiquitination assays with ^{32}P-labeled GST–E6-E7 as the substrate were performed by combining approximately 0.02 μg of the labeled protein, 2 μl of 2-mg/ml ubiquitin, 2 μl of 40 mM ATP-γ-S, 2 μl of 100 mM dithiothreitol, 0.5 μl of 0.15 M $MgCl_2$, 10 μl of a DEAE fraction, and $T_{25}N_{50}$ to a final volume of 50 μl. The mixtures were incubated at room temperature for 4 h and then analyzed by SDS-PAGE and autoradiography.

RESULTS

The E6 binding domain of E6-AP. E6-AP can stably associate with high-risk HPV E6 proteins in the absence of p53 (18, 19). In order to map the E6-AP sequences that direct this interaction, we synthesized various portions of E6-AP in *E. coli* as GST fusion proteins. The largest segment of E6-AP fused to GST consisted of the C-terminal 653 amino acids, from amino acid 212 to 865 of the open reading frame described previously (19). This 75-kDa fragment of E6-AP contains all of the sequences necessary to direct the association of E6 with p53 and to direct the E6-dependent degradation of p53 (19). Equal amounts of GST–E6-AP fusion proteins (approximately 0.1 μg) were assayed for their abilities to associate with HPV16 or HPV11 E6 proteins by mixing the GST fusion proteins, immobilized on glutathione-Sepharose, with in vitro-translated ^{35}S-labeled E6 proteins. Wheat germ extract was used for translation of E6 proteins because it lacks endogenous E6-AP (18). Following incubation, the beads were collected and washed, and bound E6 was determined by SDS-PAGE and fluorography. The level

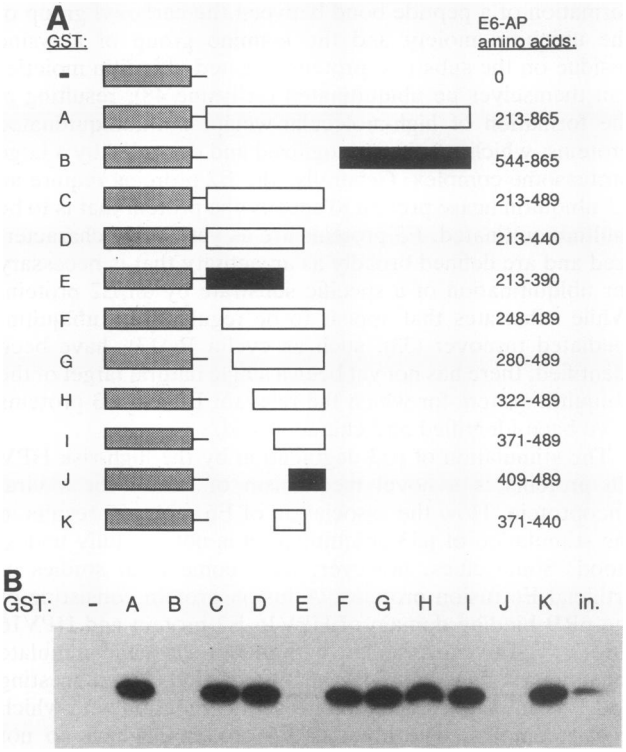

FIG. 1. (A) Schematic representation of GST–E6-AP proteins. GST proteins A through K contain the E6-AP amino acids indicated to the right. Numbering is according to the previously published sequence (19). The shaded region of each represents the GST sequence. Open boxes represent proteins that bound specifically to HPV16 E6, and blackened boxes represent those that did not. (B) Binding of HPV16 E6 to GST–E6-AP proteins A through K. ^{35}S-labeled HPV16 E6 was assayed for stable association with GST (−) or the GST–E6-AP fusion proteins A through K, as described in Materials and Methods. The last lane (in.) represents 10% of the input E6 protein used in each assay.

of binding considered nonspecific was that amount of E6 protein that bound to GST lacking E6-AP protein sequences. Figure 1A shows a schematic representation of the regions of E6-AP initially tested for E6 association, and Fig. 1B shows the corresponding data. The 75-kDa form of E6-AP (amino acids 213 to 865) bound specifically to HPV16 E6, as did the amino-terminal portion of this region (amino acids 213 to 489). The carboxy-terminal portion of E6-AP, from amino acids 544 to 865, did not bind to HPV16 E6. Additional GST fusion proteins consisting of regions from amino acid 213 to 489 localized the E6 binding domain within a 70-amino-acid region, between amino acids 371 and 440. These GST fusion proteins bound approximately 50% of the input ^{35}S-labeled HPV16 E6. None of the E6-AP fusion proteins bound HPV11 E6 above the background level (not shown).

On the basis of the above results, an additional set of fusion proteins containing regions of E6-AP from amino acid 371 to 440 was constructed and assayed for binding to HPV16 E6 (Fig. 2A and B). This set of fusion proteins localized the E6 binding domain to an 18-amino-acid region from amino acid 391 to 408. A GST fusion protein was made to the 75-kDa form of E6-AP deleted of these 18 amino acids. This protein did not bind HPV16 E6 (Fig. 3, lanes 1 and 2). Furthermore, a synthetic peptide consisting of only this 18-amino-acid region was able to compete for the binding of

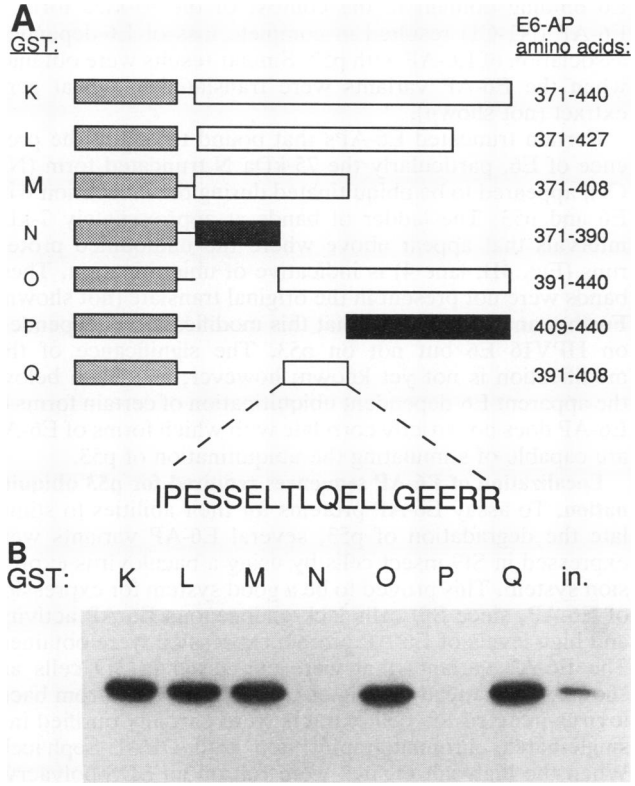

FIG. 2. (A) Schematic representation of GST proteins K through Q, containing E6-AP sequences between amino acids 371 and 440. The open boxes represent those fusion proteins that bound stably to HPV16 E6; the blackened boxes represent those that did not. The amino acid sequence of the smallest region that bound E6 (fusion protein Q) is indicated. (B) Binding of HPV16 E6 to GST–E6-AP proteins K through Q. ^{35}S-labeled HPV16 E6 was assayed for stable association with GST or GST–E6-AP fusion proteins K through Q, as described in Materials and Methods. The last lane (in.) represents 10% of the input E6 protein used in each assay.

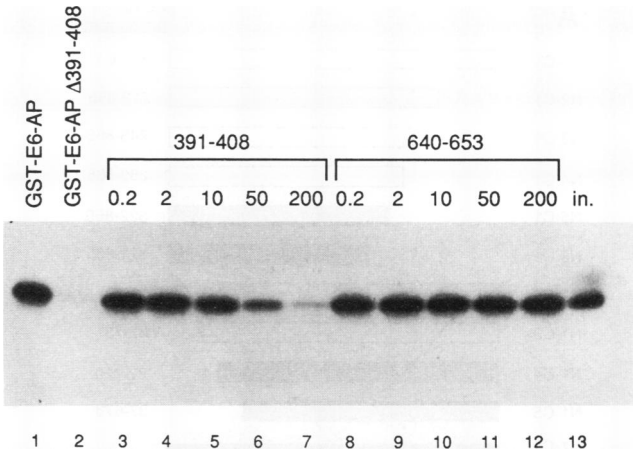

FIG. 3. Binding of HPV16 E6 to E6-AP with and without the E6 binding domain and competition for binding by synthetic peptides. The first two lanes represent HPV16 E6 binding assays to the largest GST–E6-AP fusion protein (A in Fig. 1) and to a fusion protein identical to A except that it lacks the 18-amino-acid E6 binding region (GST-E6-APΔ391-408). Lanes 3 to 12, E6 binding assays to fusion protein A in the presence of synthetic peptides corresponding to E6-AP amino acids 391 to 408 (lanes 3 to 7) or amino acids 640 to 653 (lanes 8 to 12). The amount of peptide used in each assay is indicated in micrograms. A total of 10% of the input (in.) E6 protein used in each assay is shown in lane 13.

HPV16 E6 to the GST E6-AP (75 kDa), whereas a peptide from another region of E6-AP (a 14-amino-acid peptide consisting of amino acids 640 to 653) did not (Fig. 3, lanes 3 to 12). The amount of peptide required for 50% competition (approximately 25 μg) represented an approximately 5,000-fold molar excess over the GST–E6-AP fusion protein. These results indicate that, while other regions of E6-AP might influence E6 binding, the region from amino acid 391 to 408 is necessary and sufficient for stable association with HPV16 E6.

The minimal p53 binding domain of E6-AP. Neither E6 nor E6-AP by itself stably associates with p53. Stable association of E6 with p53 requires E6-AP, and likewise, stable association of E6-AP with p53 requires E6. To localize the determinants necessary for E6-dependent association of E6-AP with p53, several amino- and carboxy-terminal deletion mutants of E6-AP were constructed in an in vitro transcription and translation vector (Fig. 4A). The largest E6-AP cDNA used in this study, N1-C1, encoding amino acids 37 to 865 of the open reading frame described previously (19), does not contain the authentic initiating methionine for E6-AP, which is as yet unknown. This cDNA encodes a protein of approximately 95 kDa that, in terms of the interaction of E6 with p53, has all of the properties of native E6-AP purified from human cells (19). In addition to N- and C-terminal deletions, an internal deletion of the 18-amino-acid E6 binding domain was constructed in the context of the 95-kDa form of E6-AP (N1-C1Δ391-408). These E6-AP variants were synthesized in rabbit reticulocyte lysate as ^{35}S-labeled proteins. Equivalent amounts of these proteins were mixed with glutathione-Sepharose beads containing GST-p53 fusion protein (wild-type p53) and either a mock wheat germ extract translation (−) or HPV16 E6 protein translated in wheat germ extract. Following incubation at 4°C, the beads were collected and washed, and bound E6-AP was determined by SDS-PAGE and fluorography. The results for the amino- and carboxy-terminal deletion mutants are shown in Fig. 4B and C, respectively. Deletion of N-terminal sequences to amino acids 213, 248, and 280 (N2-C1, N3-C1, and N4-C1, respectively) had less than a fourfold effect on the ability of E6-AP to associate with wild-type p53. Further deletion of N-terminal sequences to amino acid 322 or beyond (N5-C1 and N6-C1) led to nearly complete loss of binding. C-terminal truncation to amino acid 831 or 781 (N1-C2 and N1-C3) resulted in a slight reduction in binding to p53, with the protein deleted to amino acid 831 reproducibly binding less than that deleted to amino acid 781. Further truncation (N1-C4 and N1-C5) completely abolished binding. These deletions thus define a 502-amino-acid region, from amino acid 280 to 781, that is necessary for E6-dependent association of E6-AP with p53. As evident from the levels of p53 binding seen with some of the truncated E6-AP proteins (Fig. 4B and C), sequences flanking this minimal 502-amino-acid region do affect the efficiency of binding. Indeed, the minimal 502-amino-acid segment by itself bound to p53 at about a 20-fold-reduced efficiency compared with that of the 95-kDa (N1-C1) form of E6-AP (not shown). As anticipated, the region required for association with p53 included the E6 binding domain (amino acids 391 to 408), and as shown in Figure 4D, deletion of the

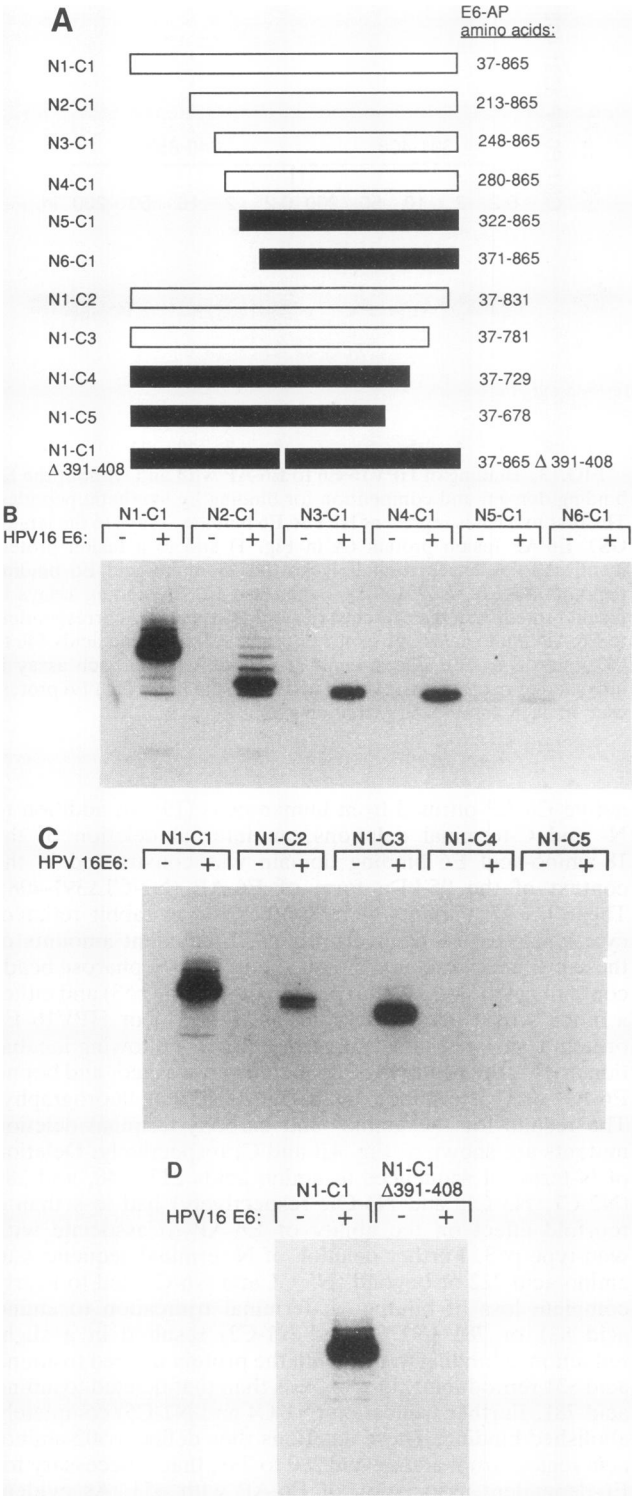

FIG. 4. (A) Schematic representation of in vitro-translated E6-AP variants. N-terminal truncations are indicated by N1-C1 through N6-C1, and C-terminal truncations are indicated by N1-C2 through N1-C5. N1-C1Δ391-408 contains an in-frame deletion of the 18-amino-acid E6 binding domain. The E6-AP amino acids encoded by each construct are indicated to the right, according to the published sequence (19). Open boxes are those variants that associated with p53 in the presence of HPV16 E6, and blackened boxes are those that did not. (B) Association of N-terminally truncated E6-AP variants with p53. ^{35}S-labeled in vitro-translated E6-AP variants were assayed for stable association with GST-p53 in the absence or presence of HPV16 E6 as described in Materials and Methods. (C) Association of C-terminally truncated E6-AP variants with p53. ^{35}S-labeled in vitro-translated E6-AP variants were assayed for stable association with GST-p53 in the absence or presence of HPV16 E6 as described in Materials and Methods. (D) The 95-kDa form of E6-AP (N1-C1) and the corresponding form lacking the E6 binding domain (N1-C1Δ391-408) were assayed for p53 association in the absence and presence of HPV16 E6 as described in Materials and Methods.

E6 binding domain in the context of the 95-kDa form of E6-AP (N1-C1) resulted in complete loss of E6-dependent association of E6-AP with p53. Similar results were obtained when the E6-AP variants were translated in wheat germ extract (not shown).

Certain truncated E6-APs that bound to p53 in the presence of E6, particularly the 75-kDa N-truncated form (N2-C1), appeared to be ubiquitinated during the incubation with E6 and p53. The ladder of bands at approximately 7-kDa intervals that appear above where the unmodified protein runs (Fig. 4B, lane 4) is indicative of ubiquitination. These bands were not present in the original translate (not shown). Further analysis showed that this modification is dependent on HPV16 E6 but not on p53. The significance of this modification is not yet known; however, as shown below, the apparent E6-dependent ubiquitination of certain forms of E6-AP does not strictly correlate with which forms of E6-AP are capable of stimulating the ubiquitination of p53.

Localization of E6-AP sequences required for p53 ubiquitination. To assay E6-AP proteins for their abilities to stimulate the degradation of p53, several E6-AP variants were expressed in Sf9 insect cells by using a baculovirus expression system. This proved to be a good system for expression of E6-AP, since Sf9 cells lack endogenous E6-AP activity, and high levels of E6-AP protein expression were obtained. The E6-AP variants that were expressed in Sf9 cells are shown schematically in Fig. 5A. E6-AP proteins from baculovirus-infected Sf9 cell extracts were partially purified in a single-batch chromatography step with DEAE-Sephacell. When the high-salt eluates were run on an SDS-polyacrylamide gel and stained with Coomassie blue, a band corresponding to the appropriate molecular weight of each of the E6-AP variants was clearly visible by comparison to the protein fraction obtained from either uninfected Sf9 cells or Sf9 cells infected with wild-type virus that did not express a foreign protein (not shown). The amounts of the DEAE fractions were adjusted according to the stained gel so that approximately equal levels of E6-AP proteins were used in each assay.

p53 degradation assays were performed by combining the E6-AP-containing DEAE fractions with ^{35}S-labeled p53 and unlabeled HPV16 E6 protein, both synthesized in the wheat germ extract translation system. A nonhydrolyzable ATP analog, ATP-γ-S, was also included in the reaction mixture, which, as previously shown, allows ubiquitination of substrates to occur while inhibiting the subsequent proteolytic degradation reaction (38). This results in the accumulation of high-molecular-weight multiubiquitinated products. As shown in Fig. 5B, p53 was not ubiquitinated in the presence of only HPV16 E6 protein or in the presence of only a DEAE fraction from uninfected Sf9 cells, from Sf9 cells infected with wild-type baculovirus, or from cells infected with virus expressing the N1-C1 form of E6-AP (lanes 2 to 5, respectively). HPV16 E6 combined with the DEAE fraction con-

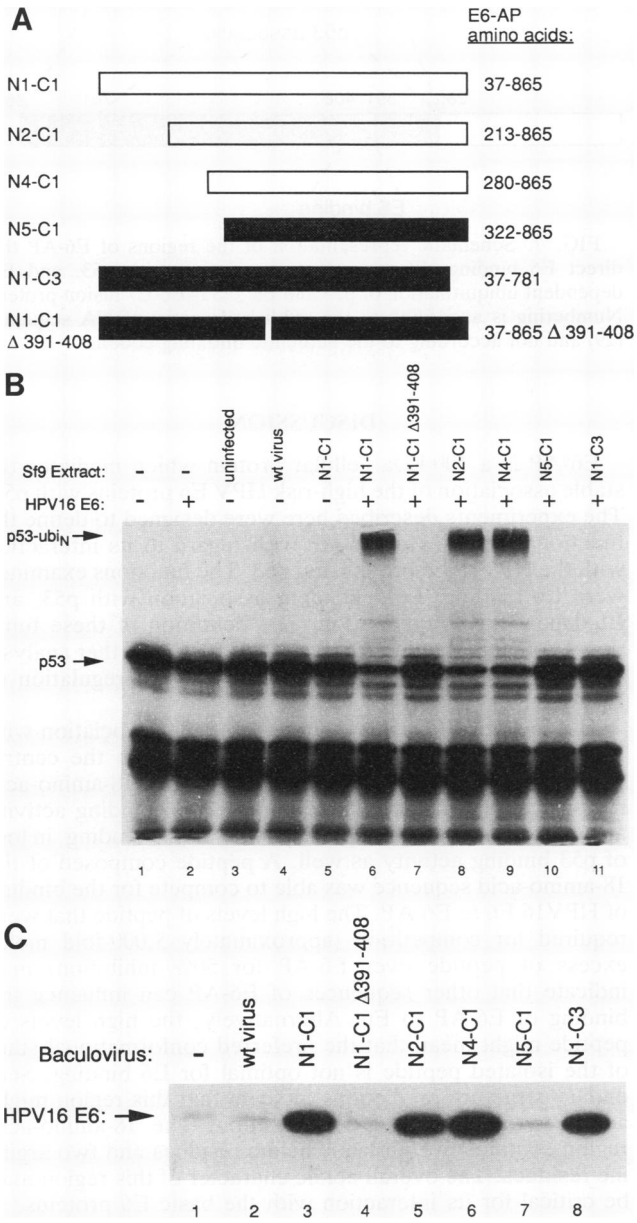

FIG. 5. (A) Schematic representation of the E6-AP variants expressed in Sf9 insect cells. Recombinant baculoviruses were obtained, and protein corresponding to each of these variants was isolated. Open boxes represent those that stimulated the E6-dependent ubiquitination of p53, and closed boxes represent those that did not. (B) p53 ubiquitination assay. ^{35}S-labeled p53 was incubated with or without HPV16 E6 protein and with or without a DEAE high-salt fraction from Sf9 cells that were either uninfected or infected with wild-type (wt) Ac*M*NPV or recombinant virus expressing the indicated E6-AP variants. The band corresponding to full-length unmodified p53 is indicated. The bands running near the top of the gel represent multiubiquitinated p53 (p53-ubi$_N$). (C) Baculovirus-expressed E6-AP variants were assayed for the ability to stimulate the association of HPV16 E6 with GST-p53. GST-p53 was incubated with ^{35}S-labeled HPV16 E6 without or with a DEAE fraction from Sf9 cells infected with wild-type (wt) Ac*M*NPV or virus expressing an E6-AP variant.

taining the N1-C1 form of E6-AP led to the accumulation of high-molecular-weight forms of p53 with a corresponding decrease in the amount of nonubiquitinated p53 (lane 6). The DEAE fraction containing the E6-AP variant lacking the 18-amino-acid E6 binding sequence (N1-C1Δ391-408) did not stimulate the ubiquitination of p53 (lane 7). The DEAE fractions containing the N-terminally truncated forms of E6-AP beginning at amino acid 213 or 280 (N2-C1 and N4-C1) stimulated the ubiquitination of p53 in the presence of HPV16 E6, while the N-terminally truncated E6-AP beginning at amino acid 322 (N5-C1) did not (lanes 8 to 10). These results are in accord with the ability of these variants to associate with p53 (Fig. 4). Interestingly, the N1-C3 C-terminally truncated form of E6-AP was unable to stimulate the ubiquitination of p53, indicating that the C-terminal 84 amino acids, while not required for stable association with p53 (Fig. 4), are necessary for the E6-induced ubiquitination of p53.

One possible explanation for why the C-terminally truncated E6-AP (N1-C3) did not stimulate the ubiquitination of p53 was that this variant, in contrast to the results shown above (Fig. 4), was unable to form a stable complex with E6 and p53 when produced in Sf9 cells. To confirm that baculovirus-expressed N1-C3 E6-AP was actually capable of forming a ternary complex with E6 and p53, the DEAE fraction was assayed for its ability to stimulate the association of ^{35}S-labeled E6 with p53. As shown previously, wheat germ-translated HPV16 E6 does not stably associate with bacterially synthesized GST-p53 because of the lack of E6-AP. DEAE fractions from uninfected Sf9 cells or Sf9 cells infected with wild-type virus did not stimulate the association of E6 with p53; however, DEAE fractions containing the N1-C1, N2-C1, and N4-C1 forms of E6-AP, as well as the N1-C3 C-terminally truncated E6-AP, could all stimulate the association of HPV16 E6 with p53 (Fig. 5C). Neither the E6-AP variant lacking the E6 binding domain (N1-C1Δ391-408) nor the N-terminally truncated form beginning at amino acid 322 (N5-C1) could stimulate the association. This showed that the ability of the E6-AP proteins produced in Sf9 cells to form ternary complexes with E6 and p53 was as predicted on the basis of the findings above (Fig. 4) and confirms that the C-terminal 84 amino acids of E6-AP, while dispensable for stable association of E6-AP with p53, are necessary for the E6- and E6-AP-dependent ubiquitination of p53.

Localization of E6-AP sequences required for ubiquitination of an artificial E6 fusion protein. The region of E6-AP required for E6-dependent association with p53, as described above, spans a large region of E6-AP. While the C-terminal 84 amino acids are required for ubiquitination, we cannot further dissect the sequences involved in specifying ubiquitination, since any further deletion eliminates p53 association. A useful system for addressing this problem would be one in which the ability of E6 and E6-AP to specify ubiquitination could be assessed independently of p53 binding. A fusion protein consisting of HPV16 E6 and the entire HPV16 E7 protein sequence is unstable in the presence of E6-AP and the components of the ubiquitin proteolysis system (35). In effect, by binding E6-AP, the E6 fusion protein is targeting itself for ubiquitination. This fusion protein was used as a substrate to determine if regions required for E6-AP-dependent ubiquitination could be further separated from regions of E6-AP required for association with p53. The fusion protein, produced in *E. coli* as a GST fusion protein, contained a protein kinase A site between the GST sequence and the E6 sequence which

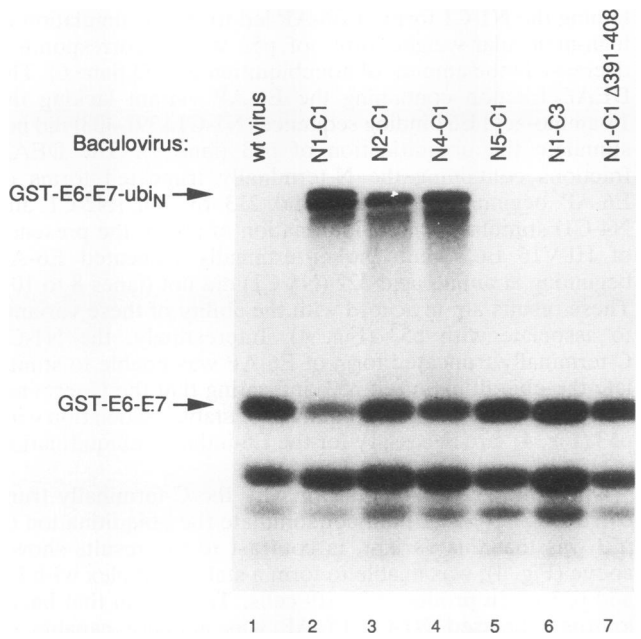

FIG. 6. Assay for ubiquitination of ^{32}P-labeled GST–E6-E7 protein. Purified labeled fusion protein was incubated with DEAE fractions from Sf9 cells infected with wild-type (wt) Ac*M*NPV or virus expressing an E6-AP variant. The band corresponding to the full-length unmodified protein is indicated (GST-E6-E7) as well as the bands corresponding to the mutiubiquitinated protein (GST-E6-E7-ubi$_N$).

allowed the protein to be labeled in vitro with ^{32}P following purification (20). Labeled GST–E6-E7 protein was mixed with unprogrammed wheat germ extract, which contains the general components of the ubiquitin proteolysis system, and the DEAE fractions from baculovirus-infected Sf9 cells as described above. The addition of wheat germ extract alone had no effect on the stability or ubiquitination of the fusion protein, nor did the addition of wheat germ extract plus DEAE fractions from uninfected Sf9 cells or Sf9 cells infected with wild-type virus (Fig. 6). DEAE fractions containing the 95-kDa E6-AP (N1-C1) and the N-terminally truncated E6-AP variants beginning at amino acid 213 or 280 (N2-C1 and N4-C1, respectively) stimulated ubiquitination of the fusion protein. The internally deleted E6-AP lacking the E6 binding site (N1-C1Δ391-408), the N-terminally truncated E6-AP beginning at amino acid 322 (N5-C1), and the C-terminally truncated E6-AP lacking the last 84 amino acids (N1-C3) did not stimulate ubiquitination of the fusion protein. This is exactly analogous to the activity of the E6-AP variants in the assay for ubiquitination of p53. In addition to showing that E6-AP is required for ubiquitination of the E6-E7 fusion protein, these results indicate again that the 84 C-terminal amino acids as well as sequences between amino acids 280 and 322 are important for E6-AP-dependent ubiquitination. Therefore, the minimal region of E6-AP that is required for ubiquitination of associated proteins spans a region that begins about 90 amino acids N-terminal to the E6 binding domain and extends to a region encompassing the last 84 amino acids. The E6 binding domain, the minimal region that can direct association with p53, and the minimal region required for ubiquitination of p53 and the E6-E7 fusion protein are shown schematically in Fig. 7.

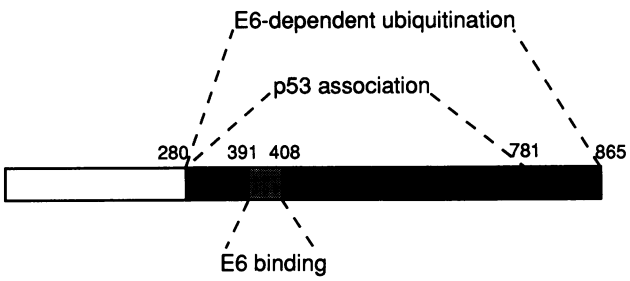

FIG. 7. Schematic representation of the regions of E6-AP that direct E6 binding, E6-dependent association with p53, and E6-dependent ubiquitination of p53 and the GST–E6-E7 fusion protein. Numbering is according to the published partial cDNA sequence (19) and not according to the authentic initiating codon.

DISCUSSION

E6-AP is a 100-kDa cellular protein which mediates the stable association of the high-risk HPV E6 proteins with p53. The experiments described here were designed to define the functional domains of E6-AP with regard to its interaction with the HPV16 E6 protein and p53. The functions examined were E6 binding, E6-dependent association with p53, and E6-dependent ubiquitination. The definition of these functional domains should provide a basis for the further analysis of the normal cellular function and possible regulation of E6-AP.

The region of E6-AP required for stable association with HPV16 E6 was mapped to 18 amino acids in the central portion of the molecule. Deletion of this 18-amino-acid region resulted in the complete loss of E6 binding activity and, since E6 binding is a prerequisite for p53 binding, in loss of p53 binding activity as well. A peptide composed of the 18-amino-acid sequence was able to compete for the binding of HPV16 E6 to E6-AP. The high levels of peptide that were required for competition (approximately 5,000-fold molar excess of peptide over E6-AP for 50% inhibition) may indicate that other sequences of E6-AP can influence the binding of E6-AP to E6. Alternatively, the high levels of peptide might mean that the preferred conformational state of the isolated peptide is not optimal for E6 binding. Secondary structure predictions indicate that this region might adopt an alpha helical conformation. The 18-amino-acid region contains five glutamic acidic residues and two arginine residues. The overall acidic character of this region may be critical for its interaction with the basic E6 proteins. A casein kinase II recognition sequence also lies within this region, suggesting that the phosphorylation state of this region might play a role in binding of the HPV E6 proteins or of cellular proteins that might interact with this region. The identification of a such a small region of E6-AP that can interact with HPV16 E6 suggests that it might be possible to develop compounds that might specifically interfere with the interaction of E6 and E6-AP. Such compounds might have application to the treatment of high-risk HPV-containing lesions or cancers.

HPV11 E6 did not, as previously reported (18, 19), bind to E6-AP above the level that was considered nonspecific; however, there is some evidence that the low-risk HPV E6 proteins may have the capacity to interact with E6-AP. E6-E7 fusion proteins consisting of either low- or high-risk HPV E6 proteins fused to the pRB binding region of HPV16 E7 stimulate the degradation of pRB in vitro (37), and fusion proteins consisting of either low- or high-risk HPV E6

proteins and the entire HPV16 E7 protein are capable of self-targeted ubiquitination (35). Although in both assays the low-risk HPV E6 fusion proteins are less active than the high-risk HPV E6 fusion proteins, these experiments show that the low-risk HPV E6 proteins do share the capacity to stimulate the ubiquitination of associated proteins. Since E6-AP is required for the self-targeted ubiquitination activity of the HPV16 E6-E7 fusion protein, this implies that the low-risk E6 proteins might also be able to interact with E6-AP to some extent. It is possible that the low-risk HPV E6 proteins, through this interaction, are capable of targeting cellular proteins other than p53 for ubiquitination. There may also be additional proteins that are common targets of both the low- and high-risk HPV E6 proteins. Alternatively, although we have not detected interaction of the low-risk E6 proteins with p53 in vitro, it is possible that some level of complex formation does occur in vivo but that perhaps, like binding to E6-AP, it is not detectable by in vitro mixing assays. There is one report that the low-risk E6 proteins can stably associate with p53 at significant levels; however, the same report also states that the low-risk E6 proteins lack the sequence requirements necessary to stimulate ubiquitination (3). Neither of these claims is supported by our experiments.

The minimal region of E6-AP that can direct E6-dependent association with p53 spans a large region of the protein, approximately 500 amino acids. As predicted, the E6 binding domain falls within this region. It extends approximately 90 amino acids to the amino-terminal side of the E6 binding domain and continues to between amino acids 729 and 781. The 90 amino acids amino terminal to the E6 binding site that are required for association with p53 may contribute to high-affinity binding of E6 to E6-AP rather than serving as actual contact sites for p53. Also, the region between the E6 binding domain (amino acids 391 to 408) and amino acid 781 might actually contain a much smaller region that is required for p53 contact that would not have been revealed by amino- and carboxy-terminal truncations.

The minimal region that can direct p53 association (amino acids 280 to 781) is not able to stimulate the E6-dependent ubiquitination of p53. Ubiquitination of p53 requires, in addition, a region that lies within the C-terminal 84 amino acids. As previously reported (19), E6-AP has homology to a rat 100-kDa protein of unknown function (28) over a 392-amino-acid overlap encompassing the carboxy terminus of both proteins, including the last 84 amino acids. More recently, an incomplete mouse cDNA, termed *NEDD4* (22), which is down-regulated during brain development, was found to encode a protein with similarity to the region conserved between human E6-AP and the rat 100-kDa protein, including a region within the last 84 amino acids of E6-AP. While E6-AP activity is easily detectable in both rat and mouse cell extracts, neither of these proteins is likely to encode the rat or mouse homologs of human E6-AP. A single partial cDNA from a rat library has been isolated by using the C-terminal region of human E6-AP cDNA as a probe. Over a 150-amino-acid region encoded by the rat cDNA, the human and rat E6-AP protein sequences were 100% identical (17), indicating that E6-AP is highly conserved among mammals. Therefore, it is more likely that E6-AP, the rat 100-kDa protein, and the mouse NEDD4 proteins represent a family of proteins that share a common structure or function, and given that a disruption of the region of similarity causes a loss of E6-dependent ubiquitination of p53, it may be that this common structure or function is related to ubiquitination of associated proteins.

The C-terminal truncation of 84 amino acids also eliminated the ability of E6-AP to stimulate ubiquitination of the E6-E7 fusion protein, further supporting the fact that this region is important for the ubiquitination function. An amino-terminally truncated form of E6-AP beginning at amino acid 322 abolished E6-dependent ubiquitination of p53, as expected, since this form of E6-AP did not stably associate with p53. Perhaps surprisingly, however, this form was also inactive in stimulating ubiquitination of the E6-E7 fusion protein, even though the 18-amino-acid E6 binding domain (amino acids 391 to 408) is intact. Therefore, the region between amino acids 280 and 322 must also be important for E6-mediated ubiquitination; however, as discussed above, we have not ruled out the possibility that the effect of this truncation is to indirectly influence E6 binding, which would influence both p53 association and any E6-dependent ubiquitination reaction. Additionally, we cannot rule out that this region may be required for binding of additional cellular proteins that are involved in ubiquitination.

Neither E6 nor E6-AP has homology to the highly conserved E2 ubiquitin-conjugating enzymes, which catalyze the formation of a peptide bond between ubiquitin and lysine residues of proteins destined for degradation. E3 ubiquitin ligases are proteins required for an E2 protein to specifically recognize and ubiquitinate a substrate. Since E6 and E6-AP are required for a specific E2 protein to ubiquitinate p53 (35), the combination of E6 and E6-AP is, by definition, performing the role of an E3 ubiquitin ligase. The fact that E6-AP is required for ubiquitination of the HPV16 E6-E7 fusion protein implies that the HPV16 E6 protein is not itself sufficient to signal recognition by the ubiquitin proteolysis system. It is therefore possible that the normal function of E6-AP is that of an E3 ubiquitin ligase. E6-AP may interact directly with a set of cellular proteins to regulate their recognition by the ubiquitin proteolysis system, or it may require the activity of cellular proteins that substitute for the role that E6 plays in p53 ubiquitination. In this regard, it is possible that E6-AP is involved in the turnover of p53 in normal cells, perhaps in conjunction with a cellular analog of the E6 proteins. If E6-AP does have E3-like activity in the absence of E6, it will be of interest to determine the normal targets of E6-AP-mediated ubiquitination. The definition of the E6 binding domain on E6-AP might also point to a likely site for interaction of cellular regulatory proteins.

While a minimal region of E6-AP that will stably associate with p53 in an E6-dependent manner has been defined, basic questions about the structure of the ternary complex formed among E6, p53, and E6-AP remain unanswered. For example, it is not known which protein, E6 or E6-AP, is actually contacting or binding to p53. E6 may activate E6-AP so that it binds p53, or E6-AP might activate E6 so that it binds p53, or the binding site for p53 may be composed of determinants on both molecules. The analysis of mutant E6 or E6-AP proteins may not aid in answering such questions, since, for example, an E6 mutant that associates with E6-AP but not with p53 may be defective either for actually binding to p53 or for the ability to activate E6-AP for binding to p53. Further physical and biochemical analysis of the ternary complex will be required to address this question.

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