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Cloning and Expression of the cDNA for E6-AP, a Protein That Mediates the Interaction of the Human Papillomavirus E6 Oncoprotein with p53

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The E6 oncoproteins of the cancer-associated or high-risk human papillomaviruses (HPVs) target the cellular p53 protein. The association of E6 with p53 leads to the specific ubiquitination and degradation of p53 in vitro, suggesting a model by which E6 deregulates cell growth control by the elimination of the p53 tumor suppressor protein. Complex formation between E6 and p53 requires an additional cellular factor, designated E6-AP (E6-associated protein), which has a native and subunit molecular mass of approximately 100 kDa. Here we report the purification of E6-AP and the cloning of its corresponding cDNA, which contains a novel open reading frame encoding 865 amino acids. E6-AP, translated in vitro, has the following properties: (i) it associates with wild-type p53 in the presence of the HPV16 E6 protein and simultaneously stimulates the association of E6 with p53, (ii) it associates with the high-risk HPV16 and HPV18 E6 proteins in the absence of p53, and (iii) it induces the E6- and ubiquitin-dependent degradation of p53 in vitro.

Many lines of evidence point to the importance of p53 in human carcinogenesis. Mutations within the p53 gene are the most frequent genetic aberration thus far associated with human cancer (44), and individuals with germ line p53 mutations have an elevated risk of developing cancer (26, 43). The mutations identified in cancers are generally point mutations which fall within evolutionally conserved domains, and most of these mutated alleles have transforming activity in various cell culture assays (reviewed in references 22 and 24). Although the p53 gene was originally classified as an oncogene, subsequent studies have shown that wild-type p53 actually has growth-suppressive and tumor-suppressive properties (9, 12). Overexpression of wild-type p53 in normal cells or in transformed cells leads to growth arrest at the G_1/S border of the cell cycle (1, 6, 27). This growth-inhibitory activity may be related to the ability of wild-type p53 to act as a modulator of transcription. p53 has been shown to have negative effects on the transcription of various genes (14) as well as to act as a DNA-binding transcriptional transactivator (11, 13, 20).

p53 was originally identified as a protein that coimmunoprecipitated with large T antigen from simian virus 40 (SV40)-transformed cells (23, 25). It was subsequently shown that the E1B 55-kDa protein of adenovirus type 5 (Ad5) and the E6 protein of human papillomavirus types 16 and 18 (HPV16 and HPV18) can also associate with wild-type p53 (37, 46). A current model is that the interaction of these viral proteins with p53 aids in releasing infected cells from a block in the cell cycle, resulting in the replication of both the cellular and viral genomes. Additional cellular proteins are involved in transformation as mediated by these viruses, the best characterized being the retinoblastoma tumor suppressor protein (pRB). SV40 large T antigen, the adenovirus E1A proteins, and the anogenital-specific HPV E7 proteins each bind to pRB (5, 8, 31, 47).

The HPVs that infect the anogenital tract can be classified as either high-risk or low-risk according to their association with cancer. HPV16 and HPV18 are the most common of the high-risk group, while HPV6 and HPV11 are among the low-risk types. Approximately 90% of cervical cancers contain HPV DNA of the high-risk types, and these same DNAs are found in the precancerous epithelial lesions (36, 49). The low-risk types are associated primarily with benign lesions such as condyloma acuminata and are only rarely found associated with cancers. 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 (7, 34, 42). Mutational analyses have shown that the E6 and E7 genes of the high-risk HPVs are necessary and sufficient for this activity in keratinocytes and fibroblasts (18, 30, 45). The specific interactions of the E6 and E7 proteins with p53 and pRB, respectively, correlate with the high- and low-risk classification. The high-risk HPV E7 proteins bind to pRB with a higher affinity than do the low-risk HPV E7 proteins (31), and only the high-risk HPV E6 proteins form detectable complexes with p53 in vitro (46). Whether this means that the E6 proteins of the low-risk viruses perform their function with regard to the viral life cycle in a different manner or simply associate with p53 with a much lower affinity is not yet clear.

A striking difference between HPV-immortalized cells and Ad5- or SV40-immortalized cells is that the p53 levels are low in HPV-containing cells (38) but greatly elevated in Ad5- and SV40-immortalized cells (33, 35). The low p53 levels in HPV-immortalized cells might be explained by the observation that complex formation between E6 and p53 in an in vitro rabbit reticulocyte system leads to the ubiquitination and proteolytic degradation of p53 (41). E1B (55 kDa) and SV40 large T, on the other hand, sequester p53 into stable complexes (33, 35). In each case, the effect of the viral oncoprotein is to functionally inactivate p53, which presumably leads to cellular proliferation.

Further support for the model that the HPV E6 and E7 proteins functionally inactivate the p53 and pRB gene prod-

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ucts comes from studies that have examined the state of the p53 and pRB genes in HPV-containing and HPV-negative cervical carcinoma cell lines (4, 38, 48). 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 RB genes. This finding implies that inactivation of the p53 and pRB gene products is an important step in cervical carcinogenesis and that this step can occur either by mutation or as a consequence of their interaction with the HPV E6 and E7 proteins.

We have previously demonstrated that complex formation between E6 and p53 is mediated by an additional cellular protein (19). The 100-kDa protein, designated E6-AP (E6-associated protein), was shown to stably interact with the high-risk HPV E6 proteins in the absence of p53 and to stably associate with p53 in the presence of either HPV16 or HPV18 E6. We report here the purification of E6-AP, the molecular cloning of a cDNA sequence encoding E6-AP, and the characterization of the in vitro-translated protein with respect to its association with p53 and the HPV E6 proteins.

MATERIALS AND METHODS

Protein purification and protein sequencing. Saos-2 cells (from the American Type Culture Collection) were maintained in Dulbecco modified Eagle medium containing 10% fetal calf serum (GIBCO). Cell extract was made from 270 176-cm^2 plates of confluent cells by lysing the cells on each plate in 2 ml of lysis buffer (0.1 M Tris-HCl [pH 7.4], 0.1 M NaCl, 1% Nonidet P-40, 1 mM dithiothreitol [DTT], 0.01% phenylmethylsulfonyl fluoride, 1 μg each of aprotinin and leupeptin per ml). The plates were scraped, and insoluble material was removed by centrifugation for 10 min at 10,000 × *g* at 4°C. The extract was split into three aliquots, and the chromatographic procedures described below were performed on each. All chromatographic procedures were performed by using a Pharmacia FPLC system. The assay of column fractions for E6-AP activity was based on the ability of E6-AP to stimulate the binding of ^{35}S-labeled wheat germ extract-translated HPV16 E6 to Sepharose beads containing glutathione *S*-transferase (GST)-p53 (19).

Extract was loaded onto a series of four connected Bio-Rad Econo-Pac columns arranged in the following order: Bio-Rad CM, S, and two Q columns. The columns were equilibrated with 25 mM Tris-HCl (pH 7.4)–100 mM NaCl–1 mM DTT. After loading, the columns were washed thoroughly with the equilibration buffer, and then the CM and S columns were removed. Proteins bound to the Q columns were eluted with a 60-ml linear salt gradient to 500 mM NaCl. Fractions eluting at approximately 250 mM NaCl contained E6-AP activity and were pooled and dialyzed against 25 mM Tris-HCl (pH 7.4)–50 mM NaCl–1 mM DTT. This material was loaded onto a Bio-Rad heparin column equilibrated at 50 mM NaCl. A 50-ml linear gradient to 500 mM NaCl was used to elute bound protein. E6-AP activity eluted at approximately 225 mM NaCl. E6-AP-containing fractions were dialyzed against 25 mM Tris-HCl (pH 7.4)–50 mM NaCl–1 mM DTT and loaded onto a Bio-Rad DEAE Blue column equilibrated in the same buffer. E6-AP activity bound and was eluted with a linear salt gradient to 500 mM NaCl. Peak fractions were dialyzed against 25 mM Tris-HCl (pH 7.4)–125 mM NaCl–1 mM DTT and loaded onto a Pharmacia Mono Q column. E6-AP activity was eluted at approximately 325 mM NaCl with a linear salt gradient to 400 mM NaCl. Peak E6-AP fractions from the Mono Q columns were pooled and concentrated in an Amicon Centricon-10 filtration unit and loaded into a single lane of a sodium dodecyl sulfate (SDS)–7.5% polyacrylamide gel.

Following electrophoresis, the gel was soaked in Western transfer buffer (12.5 mM Tris [pH 8.3], 100 mM glycine) for 10 min and then electroblotted to nitrocellulose (0.45-μm pore size; Schleicher & Schuell) at 40 V for 2 h. The blot was stained with 0.1% Ponceau S (Sigma) in 1% acetic acid for 1 min. The blot was destained with 1% acetic acid and rinsed in water, and a 24-mm^2 section corresponding to an approximately 100-kDa protein was excised from the blot. This was sent to the Harvard Microchemistry Facility (Cambridge, Mass.), where tryptic digestion, isolation of peptides by high-pressure liquid chromatography (HPLC), and protein sequencing were performed under the supervision of William Lane. The N-terminal sequences of five peptides were determined (see Fig. 3).

cDNA cloning. Polyadenylated RNA was prepared by using an Invitrogen FastTrack mRNA isolation kit, and polymerase chain reaction (PCR) was performed with a Cetus RNA PCR kit. All PCR products were cloned into pGEM-1 (Promega) and sequenced by using a United States Biochemical Sequenase kit. Lambda clones were isolated from a random-primed cDNA library made from polyadenylated RNA isolated from normal human foreskin keratinocytes (Clontech). ^{32}P-labeled DNA probes were prepared using a Bethesda Research Laboratories random primers labeling kit and [α-^{32}P]dCTP (Amersham). Inserts from lambda clones were subcloned into pGEM-1 by first isolating the insert by PCR using primers flanking the lambda gt11 cloning site. Several positive lambda clones were isolated and sequenced. All of the sequence information shown in Fig. 3 was identified in multiple independently isolated clones. MacVector software (IBI) was used for sequence analysis, and data bases were searched with the FASTA and BLAST algorithms through GenInfo and the National Library of Medicine. Sequence alignment (Fig. 9) was done by using the LFASTA program.

The complete open reading frame (ORF) was cloned in a single fragment by taking advantage of unique restriction sites within the DNA sequence. Clones used for in vitro transcription and translation were prepared by PCR of the ORF with use of a downstream oligonucleotide spanning the translation termination codon and an upstream oligonucleotide containing an in-frame optimal translation initiation codon (21). In vitro-translated proteins were prepared by using a wheat germ extract translation system (Promega) and ^{35}S-labeled methionine (ICN or Amersham).

Protein binding and degradation assays. The 95- and 76-kDa in vitro-translated proteins were tested for association with p53 by employing essentially the same assay used for assaying column fractions for E6-AP activity. Thirty microliters of ^{35}S-labeled in vitro translation mixture was mixed with 10 μl of ^{35}S-labeled wheat germ extract-translated HPV16 or HPV11 E6 and 10 μl of glutathione-Sepharose beads containing approximately 0.2 μg of GST-p53 protein. The mixture also contained 125 μl of 25 mM Tris-HCl (pH 7.4), 50 mM NaCl, and 25 μl of lysis buffer (see above). Reaction mixtures that did not contain added E6 or E6-AP translation mixtures had an equivalent amount of a mock translation reaction mixture that was programmed with water. The mixtures were rotated at 4°C for 4 h. The beads were then collected by centrifugation, washed three times with lysis buffer, boiled in SDS-gel loading buffer, and electrophoresed on SDS–12% polyacrylamide gels. Gels were fixed, soaked in Enlightening (DuPont), dried, and exposed to Kodak XAR film. The GST-p53 and GST-mutant

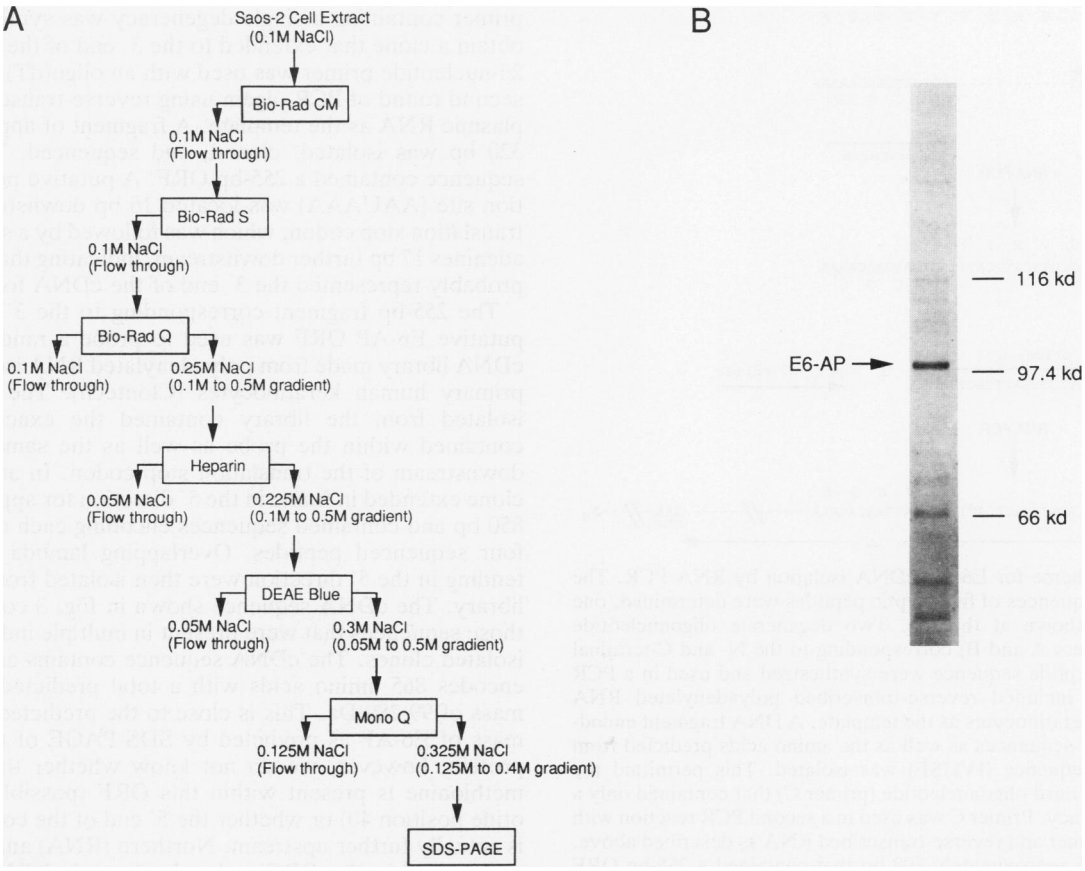

FIG. 1. (A) Chromatographic scheme for purification of E6-AP. Saos-2 cell extract was applied to Bio-Rad CM, S, and Q columns attached in series. E6-AP activity did not bind to either the CM or S column at 0.1 M NaCl but bound and was eluted from the Q column at approximately 0.25 M NaCl. E6-AP-containing fractions were subsequently chromatographed on heparin, DEAE Blue, and Mono Q columns, as indicated. Peak fractions from each column were dialyzed to lower the salt concentration before application to the next column. Mono Q fractions were pooled, concentrated by using a Centricon-10 filter (Amicon), and run in a single lane of an SDS–7.5% polyacrylamide gel. (B) A portion of the peak E6-AP-containing Mono Q fraction was run on a separate SDS–7.5% polyacrylamide gel and silver stained. Protein molecular size markers are indicated. The arrow indicates the band that corresponds to the expected size for E6-AP and that was subjected to in situ tryptic digestion and protein sequencing.

p53 (Tyr-135) plasmids and proteins have been described previously (19), and the in vitro transcription/translation plasmids used for synthesizing the E6 proteins were described by Werness et al. (46). Gels were scanned with an Ambis radioanalytic imager (Fig. 4 and 5) or an LKB densitometer (Fig. 8).

Binding of the 95- and 76-kDa proteins to GST-E6 proteins was done essentially as described above. The in vitro translation mixture was mixed with 10 μl of GST or GST-HPV16, -HPV18, -HPV11, or -HPV6 E6 immobilized on glutathione-Sepharose (approximately 0.2 μg of protein each) along with 125 μl of 25 mM Tris-HCl (pH 7.4), 50 mM NaCl, and 25 μl of lysis buffer. The mixtures were incubated for 4 h at 4°C, and bound protein was analyzed as described above. The GST-E6 plasmids and proteins have been described previously (19).

In vitro degradation assays were performed by mixing 2 μl of ^{35}S-labeled wheat germ extract-translated p53 with 10 μl of unlabeled HPV16 E6 translation mixture (or an equivalent amount of mock translation mixture), 30 μl of unlabeled 95- or 75-kDa protein translation mixture (or an equivalent amount of mock translation mixture), 40 μl of 25 mM Tris-HCl (pH 7.4), 50 mM NaCl, and 2 μl of 2-mg/ml ubiquitin (Sigma). The mixtures were incubated at room temperature for 4 h. The p53 was immunoprecipitated with a p53-specific monoclonal antibody (PAb421) as described previously (46) and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography.

Nucleotide sequence accession number. The sequence data discussed in this paper has been deposited in the GenBank data base under accession number L07557.

RESULTS

Purification of E6-AP. Saos-2 cells, which were derived from an osteosarcoma and contain a homozygous deletion of the p53 gene (6), were used as a source for purification of E6-AP. The chromatography scheme (Fig. 1A) involved a combination of anionic (CM, S, and heparin) and cationic (Bio-Rad Q, DEAE Blue, and Pharmacia Mono Q) columns. Column fractions were assayed for E6-AP activity as described previously (19), based on the ability to stimulate the binding of HPV16 E6 to p53. p53 was purified from *Escherichia coli* as a GST-p53 fusion protein, and a wheat germ extract translation system was used to synthesize [^{35}S]methionine-labeled E6 protein. The wheat germ extract trans-

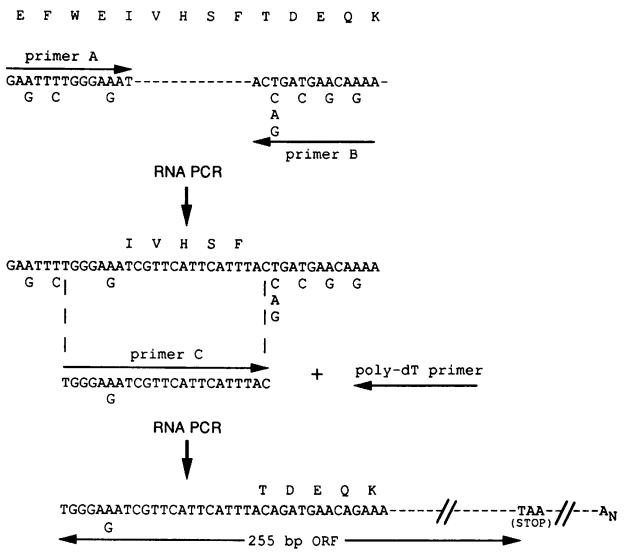

FIG. 2. Scheme for E6-AP cDNA isolation by RNA PCR. The N-terminal sequences of five tryptic peptides were determined, one of which is shown at the top. Two degenerate oligonucleotide primers (primers A and B) corresponding to the N- and C-terminal ends of the peptide sequence were synthesized and used in a PCR reaction that included reverse-transcribed polyadenylated RNA from human keratinocytes as the template. A DNA fragment encoding the primer sequences as well as the amino acids predicted from the peptide sequence (IVHSF) was isolated. This permitted the synthesis of a third oligonucleotide (primer C) that contained only a single degeneracy. Primer C was used in a second PCR reaction with a poly(dT) primer and reverse-transcribed RNA as described above. A fragment of approximately 300 bp that contained a 255-bp ORF followed by a polyadenine sequence was cloned and sequenced. The first amino acids encoded following the primer C sequence were TDEQK, as predicted from the peptide sequence. The 255-bp ORF was used as a probe for the initial screening of a cDNA library.

lation system was used for many of the experiments described below because, unlike rabbit reticulocyte lysate, it does not contain E6-AP activity. Several proteins were evident in the most purified E6-AP fractions by silver staining, with the predominant species migrating with an apparent molecular mass of approximately 100 kDa (Fig. 1B). This value was consistent with the molecular mass of E6-AP determined previously (19).

To obtain protein sequence information, peak fractions from the Mono Q column were concentrated by ultrafiltration and run in a single lane of an SDS-polyacrylamide gel. Proteins were electroblotted to nitrocellulose and visualized by Ponceau S staining, and the 100-kDa band was excised. After in situ tryptic digestion of the protein, peptides were isolated by HPLC. The amino-terminal sequences of five peptides were determined by automated Edman degradation chemistry at the Harvard Microchemistry Facility.

cDNA cloning. Degenerate oligonucleotide primers corresponding to the amino-terminal and carboxy-terminal ends of one of the peptide sequences as shown in Fig. 2 were synthesized. These primers were used in a PCR using random-primed reverse-transcribed cytoplasmic RNA. The RNA was isolated from normal primary human keratinocytes. A 51-bp PCR product was isolated, cloned, and sequenced. The DNA sequence between the oligonucleotide primers encoded the amino acids predicted by the peptide sequence. From this sequence information, a 26-nucleotide primer containing a single degeneracy was synthesized. To obtain a clone that extended to the 3′ end of the cDNA, the 26-nucleotide primer was used with an oligo(dT) primer in a second round of PCR, again using reverse-transcribed cytoplasmic RNA as the template. A fragment of approximately 320 bp was isolated, cloned, and sequenced. The 320-bp sequence contained a 255-bp ORF. A putative polyadenylation site (AAUAAA) was located 16 bp downstream of the translation stop codon, which was followed by a stretch of 20 adenines 17 bp further downstream, indicating that this clone probably represented the 3′ end of the cDNA for this gene.

The 255-bp fragment corresponding to the 3′ end of the putative E6-AP ORF was used to probe a random-primed cDNA library made from polyadenylated RNA isolated from primary human keratinocytes (Clontech). The first clone isolated from the library contained the exact sequence contained within the probe as well as the same sequence downstream of the translation stop codon. In addition, the clone extended in frame in the 5′ direction for approximately 850 bp and contained sequences encoding each of the other four sequenced peptides. Overlapping lambda clones extending in the 5′ direction were then isolated from the same library. The cDNA sequence shown in Fig. 3 contains only those sequences that were present in multiple independently isolated clones. The cDNA sequence contains an ORF that encodes 865 amino acids with a total predicted molecular mass of 99,289 Da. This is close to the predicted molecular mass of E6-AP as predicted by SDS-PAGE of the purified protein; however, we do not know whether the initiating methionine is present within this ORF (possibly at nucleotide position 40) or whether the 5′ end of the coding region is actually further upstream. Northern (RNA) analysis using probes within the ORF and polyadenylated RNA isolated from primary human foreskin keratinocytes showed a major hybridizing species of approximately 5 kb (not shown). The sequence shown in Fig. 3 plus the 3′ untranslated sequence constitute approximately 2,700 bp, meaning that the mRNA for this protein may have a very long 5′ untranslated region, containing perhaps as many as 2,300 nucleotides.

The association of in vitro-translated E6-AP with p53. The complete ORF shown in Fig. 3 as well as subclones that extended from amino acid 37 or 213 to the carboxy terminus were cloned into an in vitro transcription vector containing an in-frame optimal translation start codon (21). In vitro-synthesized RNA was translated in a wheat germ extract system in the presence of ^{35}S-labeled methionine. The smaller subclones were translated efficiently and gave rise to proteins of the predicted sizes of 95 and 76 kDa (Fig. 4 and 5); however, the full-length clone was translated poorly and was therefore not used in the experiments described below.

The 95-kDa in vitro-translated protein was tested for its ability to bind to wild-type or mutant p53 (Cys-to-Tyr change at position 135) in the presence or absence of HPV16 E6. Figure 4 shows that the 95 kDa E6-AP protein bound to a low degree when mixed with GST–wild-type p53 in the absence of E6 (lane 1). Similarly, [^{35}S]methionine-labeled wheat germ extract-translated HPV16 E6 bound to a low degree in the absence of the 95-kDa protein (lane 2). When the 95-kDa E6-AP and HPV16 E6 translation mixtures were combined, however, binding of the 95-kDa protein to p53 was dramatically increased (lane 3). This increase was accompanied by an increase in HPV16 E6 binding as well. In contrast, binding of the 95-kDa protein to the Tyr-135 mutant of p53 was increased only slightly when mixed with E6 (Fig. 4, lanes 4 to 6). Association of E6-AP with this mutant form of p53 has been shown to be greatly diminished relative to

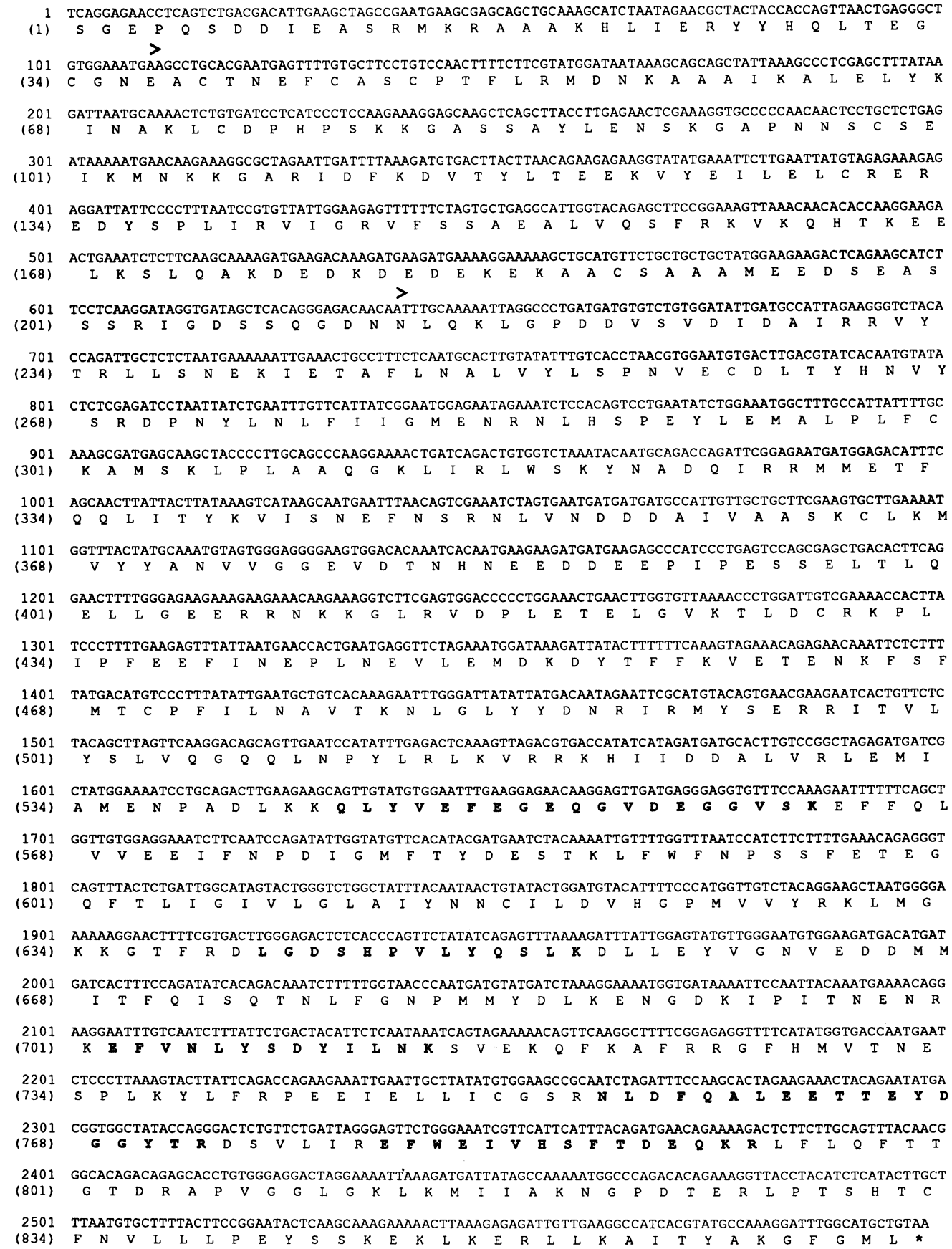

FIG. 3. Complete nucleotide and amino acid sequences of the cloned ORF. The ORF encodes 865 amino acids. Amino acid sequences in bold represent the five peptides that were sequenced. *, translation stop codon; > at amino acids 37 and 213, the 5′ ends of the cDNAs encoding the 95- and 76-kDa translation products, respectively.

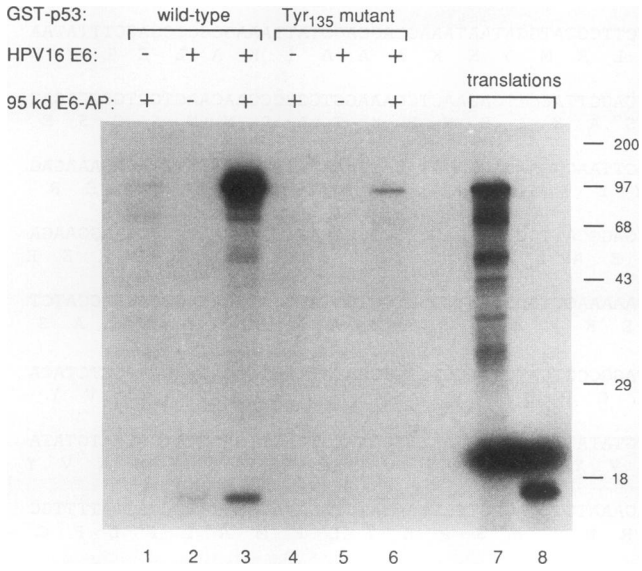

FIG. 4. In vitro translation of a 95-kDa form of the E6-AP ORF (amino acids 37 to 865) and its association with p53. The 95-kDa protein and HPV16 E6 were synthesized in wheat germ extract in the presence of [^{35}S]methionine. Each was mixed separately (lanes 1, 2, 4, and 5) or together (lanes 3 and 6) with GST-p53 fusion proteins immobilized on glutathione-Sepharose beads. Lanes 1 to 3 contained wild-type p53 fusion protein, and lanes 4 to 6 contained the Tyr-135 mutant form of p53. After incubation at 4°C, the beads were collected and washed thoroughly, and bound proteins were electrophoresed on an SDS-polyacrylamide gel. Labeled proteins were detected by autoradiography. A 1-μl aliquot of the translation reactions for the 95-kDa and HPV16 E6 proteins were run in lanes 7 and 8, respectively. Smaller translation products (lane 7) probably represent internal translation initiations, and the smear above the 18-kDa marker represents unincorporated [^{35}S]methionine that was not completely removed prior to drying the gel. Sizes are indicated in kilodaltons.

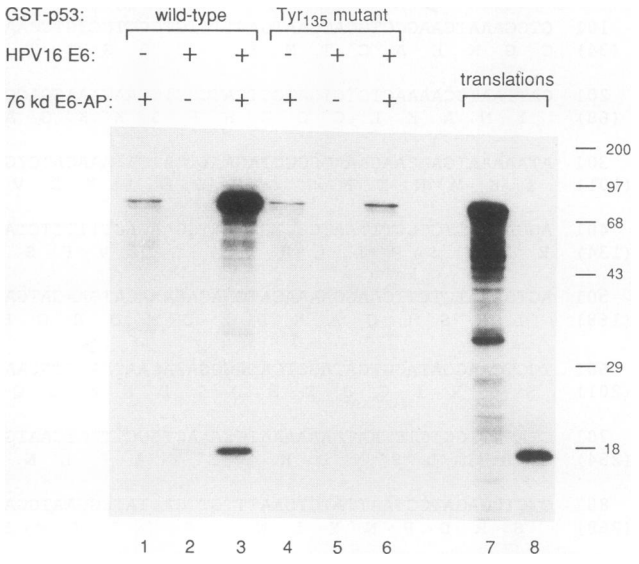

FIG. 5. In vitro translation of a 76-kDa N-terminally truncated form of the putative E6-AP ORF and its association with p53. A 76-kDa form of E6-AP (amino acids 213 to 865) and HPV16 E6 were translated in wheat germ extract in the presence of [^{35}S]methionine and assayed individually or together for the ability to associate with wild-type (lanes 1 to 3) or mutant (lanes 4 to 6) p53, as described in the legend to Fig. 4. Aliquots (1 μl) of the 76-kDa protein and HPV16 E6 translation reactions were run in lanes 7 and 8, respectively. Sizes are indicated in kilodaltons.

wild-type p53 (19), and HPV16 E6 does not bind or degrade this protein to a detectable degree in vitro (40).

The mutual stimulation of binding of E6-AP and E6 to wild-type p53 (Fig. 4, lanes 1 to 3) was quantitated. Correcting for the number of methionines in each protein (25 in the 95-kDa protein and 2 in HPV16 E6), the increase in binding relative to the background levels (lane 3 compared with lane 1 for E6-AP; lane 3 compared with lane 2 for E6) was consistent with a 1:1 ratio of the 95-kDa protein to E6 protein in complex with p53. An indication that the level of binding of the 95-kDa E6-AP protein to wild-type p53 seen in the absence of E6 (lane 1) is probably nonspecific is that a very similar level of binding to the mutant form of p53 is seen in the absence of E6 (lane 4). In addition, the level of E6 binding to p53 seen in the absence of the 95-kDa protein is also probably not specific, since a similarly low level of binding to mutant p53 in the absence of the 95-kDa E6-AP protein is seen (lanes 2 and 5).

The 76-kDa in vitro-translated form of the putative E6-AP protein was also assayed for p53 binding (Fig. 5). As with the 95-kDa form, the binding of HPV16 E6 and the 76-kDa E6-AP protein to wild-type p53 was mutually stimulatory and was not seen with the mutant form of p53. This finding further suggests that the cloned cDNA does indeed represent E6-AP and that the N-terminal 212 amino acids encoded by the ORF are not required for the p53 binding activity. Quantitation of the signals as described above again indicated roughly a 1:1 ratio of 76-kDa E6-AP to E6 protein in complex with wild-type p53. The same results were obtained when the mixing experiments were performed with wild-type p53 synthesized in wheat germ extract, followed by immunoprecipitation with a monoclonal antibody against p53 (data not shown). This finding indicates that the properties of the GST-p53 protein in our assay are the same as those of p53 synthesized as a non-fusion protein.

E6-AP association with p53 was previously observed to be dependent on the presence of high-risk as opposed to low-risk HPV E6 proteins (19). The 95- and 76-kDa in vitro-translated proteins were therefore tested for the ability to bind to GST–wild-type p53 in the presence of HPV16 versus HPV11 E6 protein. As shown in Fig. 6, stimulation of binding of both forms to p53 was only seen in the presence of HPV16 E6. Binding in the presence of HPV11 E6 was equivalent to the level seen in the absence of E6 protein.

Association of in vitro-translated E6-AP with HPV E6 proteins. Although E6-AP does not stably associate with p53 in the absence of high-risk HPV E6 proteins, it does stably associate with high-risk HPV E6 proteins in the absence of p53 (19). The 95-kDa in vitro-translated form of E6-AP was tested for its ability to bind to GST-E6 proteins (Fig. 7). Under the conditions tested, the 95-kDa protein did not bind detectably to either GST (with no fusion; lane 1) or GST fusions to HPV6 or HPV11 E6 (lanes 4 and 5). Binding was seen with both the GST-HPV16 E6 and -HPV18 E6 fusion proteins (lanes 2 and 3). Binding to the HPV16 E6 fusion protein was reproducibly greater than binding to the HPV18 E6 fusion protein. Similar results were obtained with the 76-kDa form of the translated ORF (not shown). Since HPV16 E6 associates with p53 with a higher apparent affinity than does HPV18 E6 (46), this finding suggests that the affinity of an HPV E6 protein for p53 may be directly related to its affinity for E6-AP.

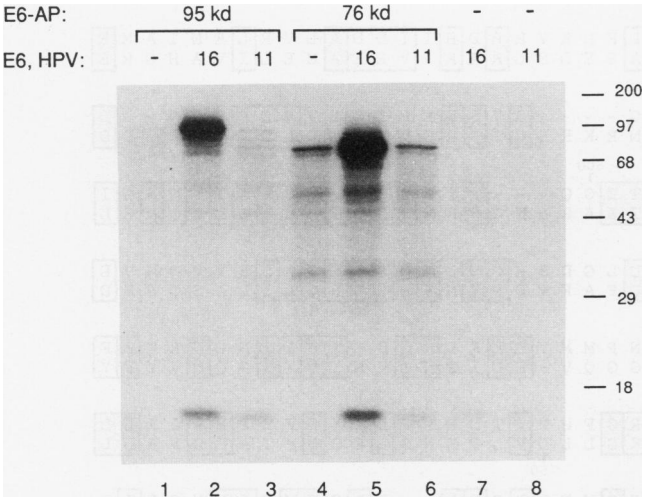

FIG. 6. Association of in vitro-translated E6-AP with wild-type p53 in the presence of HPV16 but not HPV11 E6. The 95- and 76-kDa ^{35}S-labeled in vitro-translated forms of E6-AP were mixed with GST–wild-type p53 immobilized on glutathione-Sepharose beads in the absence of E6 protein (lanes 1 and 4), in the presence of ^{35}S-labeled HPV16 E6 (lanes 2 and 5), and in the presence of ^{35}S-labeled HPV11 E6 (lanes 3 and 6). HPV16 and HPV11 E6 proteins were also mixed with the p53 beads in the absence of added E6-AP translation (lanes 7 and 8). After incubation at 4°C, the beads were collected and washed thoroughly, and bound proteins were electrophoresed on an SDS-polyacrylamide gel. Labeled proteins were detected by autoradiography. Sizes are indicated in kilodaltons.

In vitro-translated E6-AP stimulates the E6-mediated degradation of p53. It was previously shown that high-risk HPV E6 proteins target p53 for ubiquitination and proteolytic degradation when both are synthesized in rabbit reticulocyte lysate (41). The components of the ubiquitin proteolysis system are present in rabbit reticulocyte lysate and in wheat germ extract (17), yet p53 is stable in the presence of HPV16 E6 when both are synthesized in wheat germ extract (see below). Unlike rabbit reticulocyte lysate, wheat germ extract does not contain E6-AP activity, and therefore association between E6 and p53 is not detected. The addition of a highly purified E6-AP fraction to wheat germ extract-translated p53 resulted in the ubiquitination of p53 in the presence of HPV16 E6 (not shown); however, it could not be ruled out that even this highly purified E6-AP fraction might contain additional factors that are required for E6-mediated ubiquitination. To determine whether E6-AP is the only additional component necessary to stimulate E6-mediated degradation in wheat germ extract, the 95- and 76-kDa E6-AP proteins were tested for the ability to stimulate the E6-mediated degradation of p53 when all three components were translated in wheat germ extract. As shown in Fig. 8, p53 was stable in the presence of HPV16 E6 alone or either the 95- or 76-kDa form of E6-AP alone. The addition of both HPV16 E6 and either the 95- or 76-kDa form of E6-AP resulted in the degradation of p53. The 76-kDa protein was slightly more active in promoting p53 degradation than was the 95-kDa protein, possibly because the 76-kDa protein was present in a higher amount since it is translated more efficiently (not shown). This result shows that E6-AP is the only component missing in wheat germ extract that is necessary for E6-mediated degradation of p53 and that the N-terminal 212 amino acids encoded by the cloned ORF are not required for E6-mediated p53 degradation in vitro.

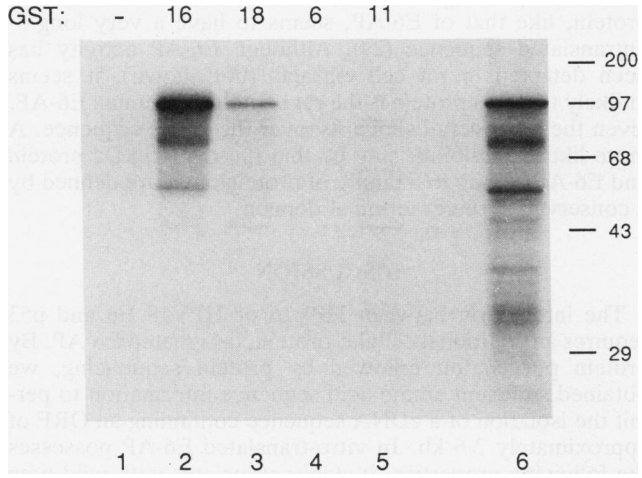

FIG. 7. Association of in vitro-translated E6-AP with GST-E6 proteins. The 95-kDa form of the E6-AP ORF was translated in wheat germ extract in the presence of [^{35}S]methionine and mixed with GST fusion proteins immobilized on glutathione-Sepharose beads. The GST proteins were either GST (no fusion; lane 1) or GST fusions to HPV16, HPV18, HPV6, and HPV11 E6 (lanes 2 to 5, respectively). After incubation at 4°C, the beads were collected and washed thoroughly, and bound proteins were electrophoresed on an SDS-polyacrylamide gel. Labeled proteins were detected by autoradiography. A 1-μl aliquot of the translation reaction was run in lane 6. Sizes are indicated in kilodaltons.

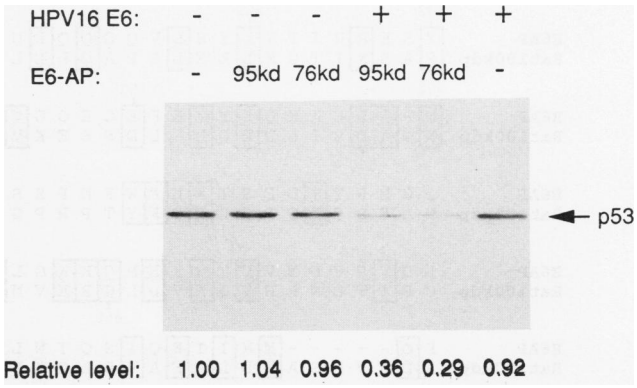

FIG. 8. Degradation of p53 in the presence of in vitro-translated HPV16 E6 and E6-AP. Wild-type human p53, HPV16 E6, and both the 76- and 95-kDa forms of the ORF were translated individually in wheat germ extract. Only the p53 translation mixture contained [^{35}S]methionine. The p53 translation mixture was mixed with the indicated combinations of E6-AP translation mixture and/or HPV16 E6 translation mixture as described in Materials and Methods. The mixtures were incubated at room temperature for 4 h, and then p53 was immunoprecipitated with PAb421. The immunoprecipitates were run on an SDS-polyacrylamide gel, and labeled p53 was detected by autoradiography. The relative amounts of immunoprecipitated p53 were quantitated with an LKB densitometer and are expressed relative to the amount immunoprecipitated when the p53 translation was incubated without E6 or E6-AP.

Analysis of the E6-AP amino acid sequence. A search for common conserved protein motifs within the E6-AP sequence revealed only several potential phosphorylation sites (casein kinase 2 and protein kinase C sites), although preliminary evidence suggests that E6-AP is not a phosphoprotein (data not shown). The encoded protein has a predicted

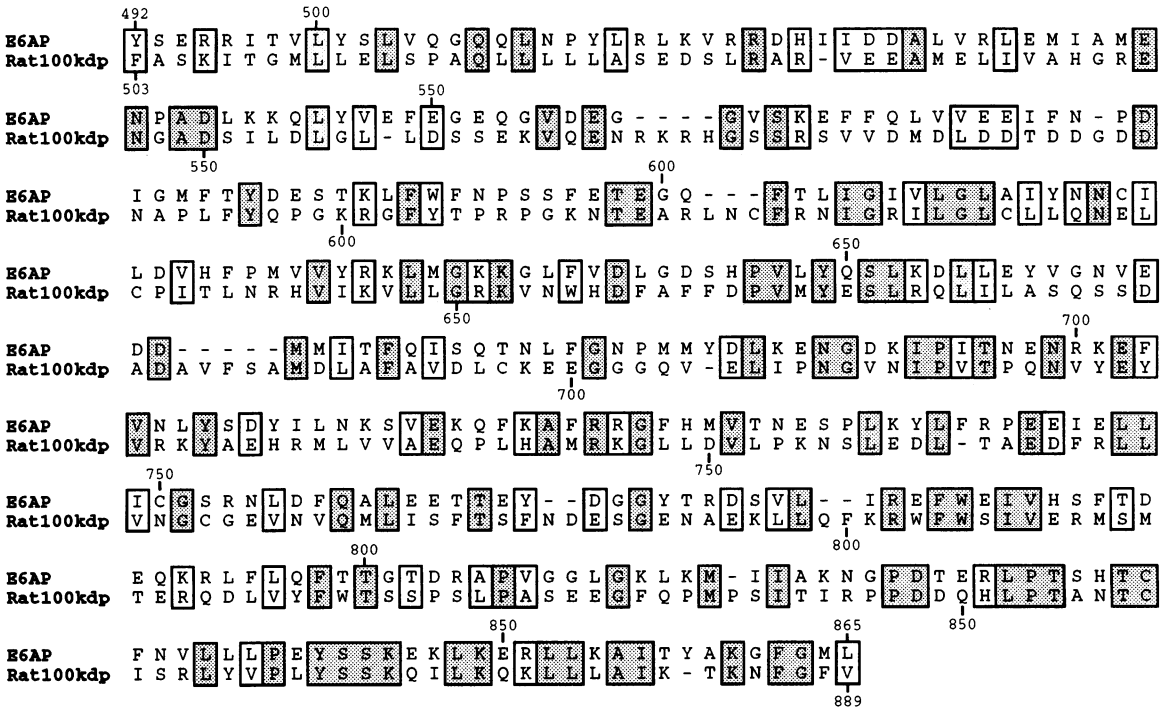

FIG. 9. Region of similarity between human E6-AP and a rat 100-kDa protein (29). A 392-amino-acid overlap that encompasses the carboxy termini of both proteins is shown. Shaded boxes represent identical amino acids, and open boxes represent conservative amino acid differences. Numbers represent amino acid positions, with amino acids 865 and 889 being the terminal amino acids of E6-AP and the rat protein, respectively. There are 101 identical amino acids and 53 similar amino acids over the 392-amino-acid overlap.

isoelectric point (pI) of 4.95, with the most acidic region being from amino acids 377 to 406, where 13 of 30 amino acids are either aspartic or glutamic acids. The most extensive reiterated amino acid sequence within the protein is NKKG-R-D--D/E, which is found beginning at amino acid positions 104 and 409. Although a definitive nuclear localization site is not present in the sequence, preliminary experiments have shown that, like E6 and p53 activities (16, 27), E6-AP activity is localized at least partially to the nucleus (not shown). There is no apparent relationship between the E6-AP cDNA and the *mdm-2* cDNA, which encodes a cellular protein that interacts with both the wild-type form and at least some mutant forms of p53 (10, 28, 32).

A comparison of the E6-AP amino acid sequence with those present in current data bases revealed only a single cDNA with seemingly significant similarity. The cDNA, which encodes a 100-kDa rat protein of unknown function, was isolated from a library by using a probe that had been used to isolate rat insulin-degrading enzyme (IDE) (29). The encoded protein itself, however, is apparently not related to IDE (29). The protein contains a region with homology to poly(A)-binding proteins (67% identity over a 30-amino-acid region) and a region of alternating acidic and basic residues similar to those found in the 70-kDa protein of the U1 small nuclear ribonucleoprotein particle. Neither of these regions, however, is contained within human E6-AP. The region of homology between E6-AP and the rat protein extends over a 392-amino-acid overlap comprising the carboxy termini of both proteins (Fig. 9). Allowing for several small alignment corrections, the sequences are approximately 25% identical over this region, and considering conservative amino acid substitutions, they are 40% similar. Within this region, a 40-amino-acid segment, comprising amino acids 817 to 856 of E6-AP and amino acids 837 to 876 of the rat protein, is 50% identical. The rat protein, in addition to having a molecular mass similar to that of E6-AP, has a similar overall acidic isoelectric point (pI of 5.7), and the mRNA for the rat protein, like that of E6-AP, seems to have a very long 5′ untranslated sequence (29). Although E6-AP activity has been detected in rat cell extracts (not shown), it seems unlikely that this protein is the rat homolog of human E6-AP, given the low overall similarity over the entire sequence. A more likely possibility may be that the rat 100-kDa protein and E6-AP belong to a family of proteins that are defined by a conserved carboxy-terminal domain.

DISCUSSION

The interaction between HPV16 or HPV18 E6 and p53 requires an additional cellular protein, designated E6-AP. By protein purification followed by protein sequencing, we obtained sufficient amino acid sequence information to permit the isolation of a cDNA sequence containing an ORF of approximately 2.6 kb. In vitro-translated E6-AP possesses the following properties: it stably associates with wild-type p53 in the presence of HPV16 E6, it simultaneously stimulates the association of HPV16 E6 with p53, and it can stably associate with the high-risk HPV E6 proteins in the absence of p53. These are characteristics previously described for E6-AP activity (19). These data suggest that the association of E6-AP with the high-risk HPV E6 oncoproteins occurs prior to the simultaneous association of both proteins with p53. In addition, in vitro-translated E6-AP is able to induce the degradation of p53 in the presence of HPV16 E6 when all three proteins are translated and mixed in wheat germ

extract. This finding indicates that E6-AP is the only protein missing from wheat germ extract that is required for both the association of E6 with p53 and the E6-mediated degradation of p53. From the experiments using the 76-kDa N-terminally truncated form of the protein, we conclude that the N-terminal 212 amino acids of the ORF are not required for complex formation with p53 or for HPV16 E6-mediated degradation of p53. Further experiments will be directed toward identifying the protein domains that are required for these activities.

The foremost question concerning E6-AP is what its function is in normal cells. One hypothesis is that E6-AP is a protein that normally targets a specific protein or group of proteins for ubiquitination in a regulated manner. Its association with the HPV E6 proteins might draw it into complexes with proteins that it normally does not target, such as p53, thereby leading to their degradation. If such a model is correct, it would be of interest to determine the normal cellular targets of E6-AP, as well as any additional targets of the E6/E6-AP complex. Although E6-AP does not appear to belong to any of the known classes of proteins involved in the ubiquitin proteolysis system, little is known about how substrates are recognized and targeted for ubiquitination (3). A useful system for determining the role of E6 and E6-AP in the ubiquitination of p53 may be the previously described degradation of pRB by an HPV16 E7-E6 fusion protein (39). In the E6-p53 system, it is not possible to directly assess the role of E6 or E6-AP in ubiquitination of p53 because neither associates with p53 without the other. E6-AP is not required for binding of the E7-E6 fusion protein to pRB, since binding is mediated by the E7 portion of the molecule. The question will then be whether E6-AP is required for the ubiquitination of pRB or, alternatively, the tethering of the E6 portion of the fusion protein to the E7-pRB complex is sufficient to target pRB for ubiquitination.

An alternative hypothesis is that E6-AP is a protein that is normally involved in regulating p53 activities. Although E6-AP does not stably or detectably interact with p53 in vitro in the absence of E6, it is possible that the interaction does take place in cells or that there is an E6 analog in cells that can stimulate this interaction. The cloning of the cDNA for E6-AP should aid in the search for a possible cellular analog to the high-risk E6 oncoproteins. There is some evidence that wild-type p53 might normally be degraded by the ubiquitin proteolysis system in the absence of HPV E6 proteins (2, 15), which might be the basis for the short half-life of wild-type p53 (33). E6-AP might therefore be considered a likely candidate to be involved in the normal ubiquitin-mediated degradation of p53. Another possibility is that E6-AP is normally involved in regulating p53 function but in a manner not related to its degradation, such as by regulating the growth-suppressive or transcriptional repression or activation properties of p53. The degradation of p53 when in complex with E6 and E6-AP, as discussed above, may be a result of its association with E6 rather than E6-AP.

The primary sequence of E6-AP yields little information as to the normal function of the protein, since a significant similarity was found to only a single known translated cDNA, the product of which is also of unknown function. This cDNA is from rat and encodes a protein that, like E6-AP, has a molecular mass of approximately 100 kDa (29). The two proteins are 25% identical over a 392-amino-acid overlap that encompasses the carboxy termini of both proteins. This finding implies that E6-AP and the rat 100-kDa protein may be members of a family of proteins defined by a conserved carboxy-terminal domain. As discussed above, such a family might be postulated to play a role in the ubiquitin proteolysis system or in the regulation of p53 activities.

Given the high frequency of p53 mutations in human cancers, it will be of interest to determine whether any genes whose products are involved in p53-related pathways might also be altered at a high frequency in cancers. This is apparently the case for the human *mdm-2* gene. *mdm-2* was originally identified as a dominant transforming oncogene in mice (10), and the proteins encoded by the rat and human *mdm-2* homologs have been shown to interact with both wild-type and mutant forms of p53 (28, 32). The *mdm-2* gene was found to be amplified in 17 of 47 human sarcomas (32), which implies that overexpression of *mdm-2* might be a mechanism of overriding p53-regulated growth control. Chromosomal mapping studies are currently under way to determine whether the gene for E6-AP is localized to a region that is associated with alterations in any human cancers.

While much work has been done in establishing the importance of p53 in human carcinogenesis, very little is known about how the growth-suppressive effects of wild-type p53 or the oncogenic effects of mutant forms of p53 are mediated at the biochemical level. The cDNA cloning of the gene for E6-AP may aid in our understanding of cellular immortalization as mediated by the HPV E6 proteins and provide further insight into the mechanisms by which p53 functions.

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