

A cellular protein mediates association of p53 with the E6 oncoprotein of human papillomavirus types 16 or 18

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The E6 protein of human papillomavirus types 16 and 18 (HPV-16 and HPV-18) can stably associate with the p53 protein *in vitro*. In the presence of rabbit reticulocyte lysate, this association leads to the specific degradation of p53 through the ubiquitin-dependent proteolysis system. We have examined the E6–p53 complex in more detail and have found that association of E6 with p53 is mediated by an additional cellular factor. This factor is present in rabbit reticulocyte lysate, primary human keratinocytes and in each of five human cell lines examined. The factor is designated E6-AP, for E6-associated protein, based on the observation that the E6 proteins of HPV-16 and 18 can form a stable complex with the factor in the absence of p53, whereas p53 association with the factor can be detected only in the presence of E6. Gel filtration and coprecipitation experiments indicate that E6-AP is a monomeric protein of ~100 kDa

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Introduction

A strong association exists between specific human papillomaviruses (HPVs) and some anogenital cancers, including cervical cancer (zur Hausen and Schneider, 1987). The DNA of certain HPV types, including HPV-16, 18, 31, 33 and 39, is found in ~85% of cervical carcinomas (Riou *et al.*, 1990). These virus types are considered 'high risk' because of their association with cancer and high grade intra-epithelial lesions. In contrast, the 'low risk' HPV types, such as HPV-6 and 11, have a similar tissue specificity yet are associated primarily with benign lesions that are at low risk for malignant progression. The classification into low and high risk HPV types is reflected *in vitro* by the ability of cloned DNA of only the high risk HPVs to efficiently immortalize primary human foreskin keratinocytes (Dürst *et al.*, 1987; Pirisi *et al.*, 1987; Kaur and McDougall, 1988; Schlegel *et al.*, 1988).

The HPV E6 and E7 genes are regularly expressed in HPV positive cervical cancers and derived cell lines (Baker *et al.*, 1987; Schwarz *et al.*, 1985; Smotkin and Wettstein, 1986). Genetic analyses have shown that the E6 and E7 genes are both necessary and sufficient for the efficient immortalization of primary human squamous epithelial cells (Münger

et al., 1989a; Hawley-Nelson *et al.*, 1989). Similar to other DNA tumor virus oncoproteins, the E6 and E7 proteins interact with tumor suppressor gene products. SV40 large T antigen, adenovirus E1A and the HPV E7 proteins can each associate with pRB, the retinoblastoma gene product (Whyte *et al.*, 1988; Dyson *et al.*, 1989; DeCaprio *et al.*, 1988), and the E6 proteins of HPV-16 and 18, like SV40 large T antigen and adenovirus 5 E1B protein, associate with p53 (Lane and Crawford, 1979; Linzer and Levine, 1979; Sarnow *et al.*, 1982; Werness *et al.*, 1990). The E7 proteins of the high risk HPVs bind to pRB with a higher affinity than E7 proteins of the low risk HPVs (Münger *et al.*, 1989b), and stable association of E6 with p53 *in vitro* has been observed only with the high risk HPV proteins. This suggests that these interactions may be important determinants of the oncogenic potential of the different HPV types. Further evidence for the relevance of the E7–pRB and E6–p53 interactions in cervical carcinogenesis comes from studies in which the RB and p53 genes have been examined in HPV positive and negative cervical carcinoma cell lines (Scheffner *et al.*, 1991; Crook *et al.*, 1991; Wrede *et al.*, 1991). Those cell lines containing HPV DNA were found to express wild type pRB and p53, while lines lacking HPV DNA contained mutations at both the RB and p53 genes. This suggests that inactivation of pRB and p53 is important in cervical carcinogenesis, and that inactivation can occur either through the action of the E6 and E7 proteins in the HPV positive cancers, or through mutation in the HPV negative cancers.

While the importance of p53 in cell growth control and carcinogenesis is well documented, little is known mechanistically as to how p53 exerts its effects. There have been reports that p53 can influence DNA replication (Braithwaite *et al.*, 1987; Gannon and Lane, 1987; Wang *et al.*, 1989), that p53 is a sequence specific DNA binding protein (Bargonetti *et al.*, 1991; Kern *et al.*, 1991), and that p53 possesses transcriptional transactivation properties (Fields and Jang, 1990; Raycroft *et al.*, 1990). Although the mechanism is still unknown, p53 is thought to regulate cellular proliferation negatively at the G₁/S border of the cell cycle (Diller *et al.*, 1990; Martinez *et al.*, 1991). It is present in most normal cells at very low levels and has a half-life of only 20–40 min. The levels of p53 in cells immortalized by SV40 or adenovirus 5, however, are greatly elevated, with the half-life of p53 being extended to several hours (Oren *et al.*, 1981; Reich *et al.*, 1983). Presumably these viral oncoproteins are preventing p53 from performing its normal function by sequestering it into a stable and inactive complex. In contrast, the p53 levels in most HPV-16 or 18 immortalized cells and in HPV positive cancers are very low or undetectable (Scheffner *et al.*, 1991; Wrede *et al.*, 1991). *In vitro* experiments have shown that the high risk HPV E6 proteins stimulate the degradation of p53 via the ubiquitin-dependent proteolysis system (Scheffner *et al.*, 1990). This suggests a model by which a viral oncoprotein

promotes cellular immortalization by specifically targeting a negative regulator of cell growth for proteolytic degradation.

Although much of the biochemistry involved in the ubiquitin-dependent proteolysis system had been characterized, little is known about how substrates are recognized and how ubiquitination might be regulated (reviewed in Hershko, 1988; Rechsteiner, 1988; Ciechanover and Schwartz, 1989). The targeted degradation of proteins is likely to be an important cell regulatory mechanism. Several examples point to the involvement of the ubiquitin-dependent proteolysis system in cell growth control. The *Saccharomyces cerevisiae CDC34* gene encodes a ubiquitin conjugating enzyme. Temperature sensitive mutations of this gene cause cell cycle arrest at the G₁/S border at the restrictive temperature, implying that the substrate(s) recognized by this enzyme must be ubiquitinated for progression of the cell cycle (Goebel *et al.*, 1988). In *Xenopus* oocytes, regulated degradation of cyclin B, which is the key step in exit from mitosis and progression into the next cell cycle, occurs through the ubiquitin pathway (Glotzer *et al.*, 1991). An *in vitro* ubiquitin degradation system has been shown to degrade several nuclear oncoproteins including p53, N-myc, c-myc and c-fos (Ciechanover *et al.*, 1991), although there is no evidence that any of these proteins are degraded *in vivo* in a regulated manner.

The stimulation of p53 degradation by the HPV E6 proteins *in vitro* has prompted a more thorough examination of the E6-p53 complex. We have found that *in vitro* association of the HPV-16 and 18 E6 protein with p53 is mediated by a 100 kDa cellular protein. Although we have not yet characterized this protein functionally, the identification of a cellular protein that participates in a stable association with E6 and p53 has implications regarding other possible targets recognized by the E6 proteins, as well as to the regulation of p53 activity in normal cells.

Results

The association of E6 with p53 requires a cellular factor

The original experiments demonstrating co-immunoprecipitation of the HPV-16 and 18 E6 protein with p53 utilized proteins translated *in vitro* in rabbit reticulocyte lysate (Werness *et al.*, 1990). Rabbit reticulocyte lysate is a rich source of the components of the ubiquitin proteolysis system, and is widely used as a system in which to study ubiquitin-dependent proteolysis *in vitro* (Hershko, 1988). The initial experiments that demonstrated E6-p53 complex formation were done at 4°C, a temperature at which the activity of the proteolysis system is sharply reduced. When the mixing experiments were performed at 25°C, complex formation of HPV-16 or 18 E6 with p53 resulted in the specific ubiquitination and degradation of p53, with E6 itself remaining stable (Scheffner *et al.*, 1990). In order to examine E6-p53 complex formation in the absence of reticulocyte lysate, E6 proteins were translated *in vitro* in wheat germ extract (Promega), and p53 was synthesized in *Escherichia coli* as a fusion protein to the carboxy terminus of glutathione S-transferase (GST). The synthesis of the GST fusion proteins is IPTG-inducible and the proteins can be rapidly affinity purified from crude lysate on glutathione Sepharose (Smith and Johnson, 1988). The GST-p53 fusion

protein immobilized on glutathione sepharose was first tested at 4°C for its ability to complex HPV E6 proteins synthesized in rabbit reticulocyte lysate. As expected, ³⁵S-labeled HPV-18 E6 protein bound to the p53 beads to a much greater extent than the low risk HPV-11 E6 protein (Figure 1A). Furthermore, complex formation was specific for wild-type p53 in that beads containing GST (no fusion) or a GST fusion to a mutated p53 did not significantly bind HPV-18 or HPV-11 E6 protein. [The mutated p53 contained a Cys to Tyr substitution at amino acid 135 and has been shown in an independent analysis of mutant p53 proteins not to bind high risk HPV E6 proteins when assayed by co-immunoprecipitation (M.Scheffner, in preparation). The gene

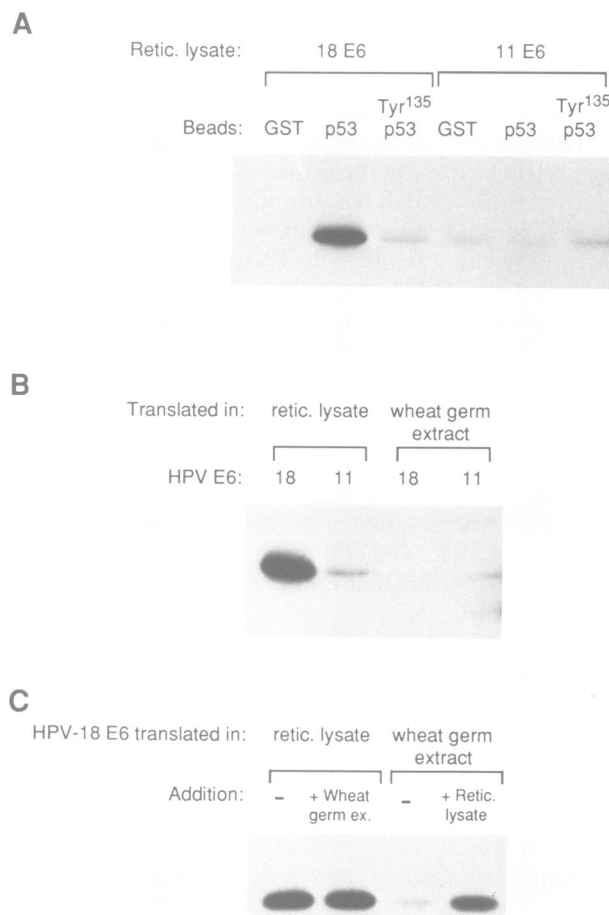


Fig. 1. Binding of *in vitro* translated E6 proteins to p53. (A) ³⁵S-labeled reticulocyte lysate-translated HPV-18 and HPV-11 E6 proteins were mixed with glutathione sepharose beads containing bound GST, GST-p53 (p53) or GST-mutant p53 (Tyr¹³⁵ p53). The beads were washed extensively and bound protein was eluted and analyzed by SDS-PAGE and fluorography, as described in Materials and methods. (B). ³⁵S-labeled HPV-18 and HPV-11 E6 proteins were synthesized either in reticulocyte lysate or wheat germ extract systems. The proteins were assayed as above for binding to GST-p53 beads. (C) ³⁵S-labeled HPV-18 E6 protein was synthesized in either reticulocyte lysate or wheat germ extract. Reticulocyte lysate-translated protein was assayed for binding to GST-p53 beads in the absence or presence of added wheat germ extract (10 μ l) and wheat germ extract-translated protein was assayed in the absence or presence of added reticulocyte lysate (10 μ l). *In vitro* protein synthesis was quantitated by determining incorporated radioactivity using an Ambis Radioanalytic Imaging System. The amount of labeled protein (c.p.m.) was the same in each mixing and ranged from 5 to 10 μ l of the translation reactions.

encoding this protein was isolated from a non-small cell lung cancer and is presumed to have played a role in carcinogenesis (Chiba *et al.*, 1990).] This indicated that, with respect to association with HPV E6 proteins, the immobilized p53 fusion protein functioned similarly to wild-type p53 present in human cell extracts or synthesized in reticulocyte lysate (Werness *et al.*, 1990).

Wheat germ extract-translated E6 proteins were next analyzed using the assay described above (Figure 1B). Neither wheat germ extract or reticulocyte lysate-translated HPV-11 E6 bound to the p53 beads to an appreciable extent, as expected. Surprisingly, however, the association of wheat germ extract-translated HPV-18 E6 with wild-type p53 was greatly reduced relative to the reticulocyte lysate translated protein. This suggested either that a factor present in wheat germ extract inhibited the high risk HPV E6 – p53 association, or that a factor present in reticulocyte lysate was necessary for the association. To distinguish between these possibilities, reticulocyte lysate-translated HPV-18 E6 protein was assayed for complex formation in the presence of added wheat germ extract, and wheat germ extract-translated proteins were assayed in the presence of added reticulocyte lysate. As shown in Figure 1C, wheat germ extract did not inhibit the ability of reticulocyte lysate-translated HPV-18 E6 protein to associate with p53, whereas reticulocyte lysate stimulated the association of wheat germ extract-translated HPV-18 E6 with p53. The same results were obtained using

HPV-16 E6 (see Figure 2). These results indicated that a factor present in rabbit reticulocyte lysate is necessary for high risk HPV E6 – p53 complex formation.

To determine if this stimulatory activity was peculiar to rabbit reticulocyte lysate, protein extracts of several cell types were analyzed for the ability to stimulate binding of wheat germ translated HPV-16 E6 protein to p53 beads. Extracts were prepared from primary human foreskin keratinocytes (HFKs), HeLa cells, C-33A cells, Saos-2 cells, and an SV40-immortalized keratinocyte cell line. The HeLa cell line is an HPV-18-containing human cervical carcinoma line, and the C-33A line is an HPV negative cervical carcinoma line that expresses only a mutated form of p53 (Scheffner *et al.*, 1991). The Saos-2 line is from a human osteosarcoma and does not express p53 (Masuda *et al.*, 1987). The SV40-immortalized human keratinocyte line was established *in vitro* (Pietenpol *et al.*, 1990). Whole cell extracts of these cell types and of rabbit reticulocyte lysate (Promega) were chromatographed on DEAE – Sephacell (Pharmacia) and bound protein was eluted with high salt. Equal amounts of eluted protein were assayed for the ability to stimulate HPV-16 E6 – p53 complex formation. As shown in Figure 2, the DEAE high salt fraction of each cell type contained comparable levels of the activity. While the chromatography step significantly increased the specific activity, the activity was also detectable in the crude lysates.

The cellular factor forms a stable complex with high risk HPV E6 proteins

To determine if E6 proteins can form a stable complex with the factor in the absence of p53, or if p53 can do so in the

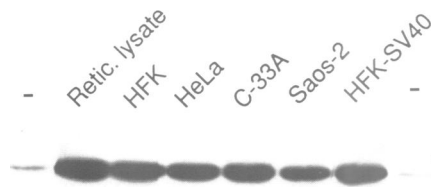


Fig. 2. Whole cell extracts of rabbit reticulocytes, primary human foreskin keratinocytes (HFK), and the HeLa, C-33A, Saos-2 and SV40-immortalized HFK cell lines were passed over DEAE – Sephacell and bound protein eluted with high salt. The high salt eluate was assayed for the ability to stimulate the binding of ³⁵S-labeled wheat germ extract-translated HPV-16 E6 to GST – p53 beads. Outside lanes (-) represent mixings with the addition of only high salt buffer.

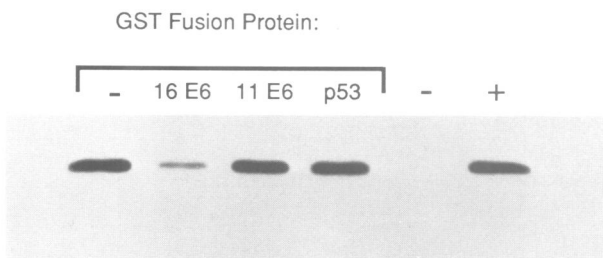


Fig. 3. Depletion of factor from reticulocyte lysate using GST fusion proteins. Reticulocyte lysate was incubated with GST fusion proteins immobilized on beads containing no fusion (-), or a fusion to HPV-16 E6, HPV-11 E6 or p53. The beads were then collected by centrifugation and the supernatant was assayed for the ability to stimulate binding of ³⁵S-labeled wheat germ extract-translated HPV-16 E6 to GST – p53 beads. Negative and positive controls were mixings without (-) and with (+) undepleted reticulocyte lysate.

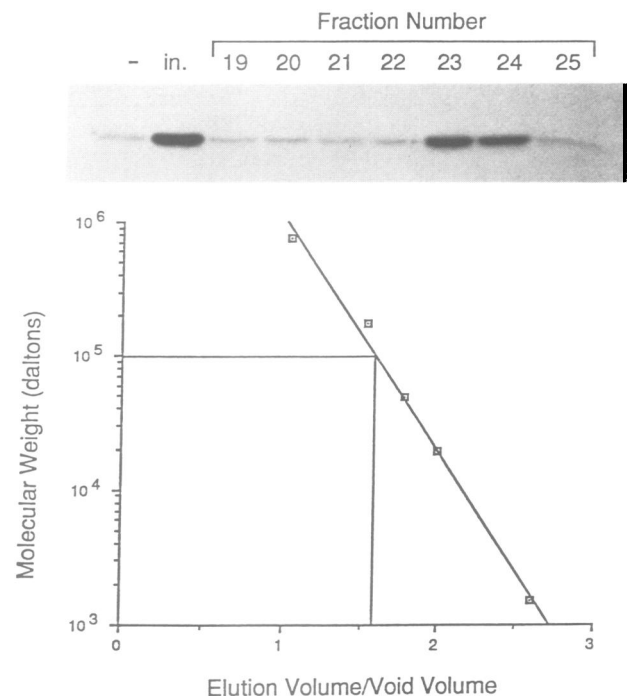


Fig. 4. Reticulocyte lysate was fractionated by gel filtration chromatography on a Superose 12 column. Fractions were assayed for the ability to stimulate binding of ³⁵S-labeled wheat germ extract-translated HPV-16 E6 to GST – p53 beads. Negative and positive controls were mixings without (-) and with (in.) the input to the column. The elution volumes of gel filtration standards relative to the void volume of the column were plotted against the native molecular weights of the standards. This plot was used to estimate the native molecular weight of the factor from the elution volume of the activity.

absence of E6, GST fusion proteins immobilized on sepharose were tested for their ability to deplete the factor from reticulocyte lysate (Figure 3). GST proteins immobilized on beads (GST, GST-HPV-16 E6, GST-HPV-11 E6, or GST-p53) were incubated for 1 h at 4°C with reticulocyte lysate. The beads were then pelleted by centrifugation, and the supernatants were assayed for the ability to stimulate E6-p53 complex formation. The HPV-16 E6 fusion protein depleted the activity from reticulocyte lysate, whereas GST, the HPV-11 E6 fusion protein, and the p53 fusion protein had little or no effect. The GST-HPV-18 E6 protein was also able to deplete reticulocyte lysate of the activity, and both the HPV-16 and 18 E6 fusion proteins could deplete the activity from HeLa cell extracts (data not shown). Therefore the cellular factor can form a stable complex with the high risk HPV E6 proteins in the absence of p53, but it does not associate detectably with HPV-11 E6 or with p53 in the absence of a high risk HPV E6 protein.

The cellular factor is a monomeric protein of approximately 100 kDa

To determine the native molecular size of the E6-p53 association factor gel filtration chromatography of rabbit reticulocyte lysate, HeLa extract, and primary keratinocyte cell extract was performed using a Superose 12 column (Figure 4). The activity from each source eluted identically. The elution relative to marker proteins indicated that the factor has a native molecular mass of ~100 kDa. This, along with the heat lability of the activity (data not shown), strongly implies that the factor is a protein. The factor has therefore been designated E6-AP, for E6-associated protein.

In order to determine the subunit molecular weight of E6-AP, extracts were prepared from cultured cells (primary HFKs or Saos-2 cells) that had been metabolically labeled with [³⁵S]methionine and cysteine. E6-AP was separated from the bulk of cellular proteins by anion exchange chromatography (FPLC Mono Q, Pharmacia). This chromatography step resulted in a >100-fold purification and was necessary because of the large number of labeled proteins that absorbed non-specifically to the p53 beads when using whole cell lysate (see lane 2, Figure 5). Column fractions were assayed for the ability to stimulate binding of ³⁵S-labeled wheat germ extract-translated HPV-16 E6 protein to p53 beads. In those fractions containing E6-AP activity, a labeled protein migrating at ~100 kDa also bound to the p53 beads (fractions 28 and 29; Figure 5, lanes 8 and 9). Identical results were obtained using either primary keratinocytes or Saos-2 cells. When the E6-AP-containing Mono Q fractions were further chromatographed by gel filtration (Superose 12), the 100 kDa band continued to coprecipitate with the p53 beads from those fractions containing E6-AP activity (data not shown).

Based on the experiment shown in Figure 3, if the 100 kDa band represents E6-AP it would be predicted to bind to p53 only in the presence of a high risk HPV E6 protein, and to bind to HPV-16 E6 but not to HPV-11 E6. Figure 6 shows the results of mixing the peak ³⁵S-labeled E6-AP fraction from the Mono Q column (Fraction 28) with GST-p53 beads and GST-E6 beads. As predicted, the 100 kDa band bound to p53 beads only in the presence of a high risk HPV E6 protein (compare lane 1 with 2 and 3), it bound to HPV-16 E6 beads, and did not bind to HPV-11 E6 beads (compare lanes 4 and 5). A minor, slightly slower migrating

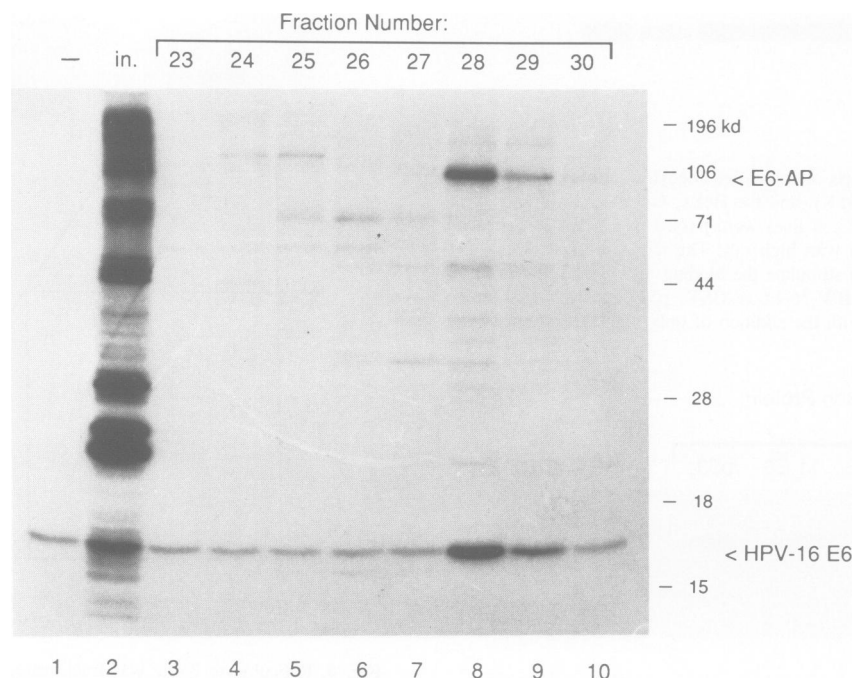


Fig. 5. Whole cell extract of metabolically labeled (³⁵S)methionine, cysteine) Saos-2 cells was prepared and chromatographed on a Mono Q column. Bound protein was eluted with a linear salt gradient. The input to the column (lane 2, in.) and fractions across the gradient were assayed for the ability to stimulate binding of ³⁵S-labeled wheat germ extract-translated HPV-16 E6 to GST-p53 beads. Total bound protein was eluted and analyzed by SDS-PAGE and fluorography. Lane 1 (-) is a negative control with no Saos-2 extract or column fractions added. The migration positions of HPV-16 E6 and the 100 kDa putative E6-AP protein are indicated. Size markers were BRL prestained high molecular weight protein markers.

band was consistently coprecipitated, which might represent a modified form of E6-AP, or alternatively, the lower band might be a proteolyzed derivative. Most of the minor bands that bound to HPV-16 E6 beads were detected with p53 beads alone and with HPV-11 E6 beads, and are therefore considered to be non-specific. Together with the native molecular size of E6-AP from gel filtration, these results indicate that E6-AP is a monomeric protein of ~100 kDa.

Discussion

The E6 proteins of HPV-16 and 18 in conjunction with the E7 protein are necessary for efficient immortalization of primary keratinocytes (Münger *et al.*, 1989a; Hawley-Nelson *et al.*, 1989). Similar to SV40 large T antigen and the adenovirus 5 E1B 55 kDa protein, the HPV-16 and 18 E6 proteins can form stable complex with p53 *in vitro* (Lane and Crawford, 1979; Linzer and Levine, 1979; Sarnow *et al.*, 1982; Werness *et al.*, 1990). In the presence of an active ubiquitin proteolysis system E6–p53 complex formation leads to the specific degradation of p53, suggesting a novel mechanism of action for a viral transforming gene: the targeted degradation of a negative regulator of cell growth. In this study we have shown that an additional cellular factor, designated E6-AP, mediates the association of E6 with p53.

Since p53 degradation is stimulated when complexed with

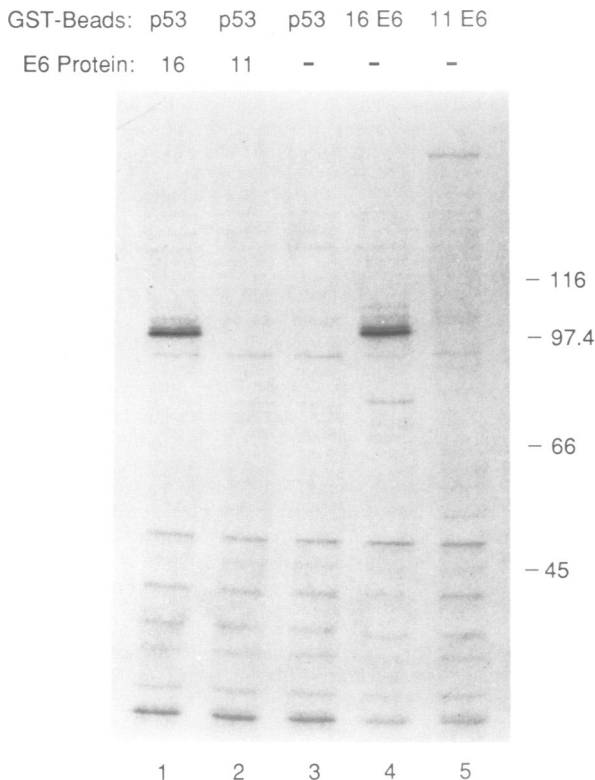


Fig. 6. Fraction 28 from the Mono Q chromatography (Figure 5) was mixed with GST–p53 beads (lanes 1–3) in the presence of unlabeled wheat germ extract-translated HPV-16 E6, HPV-11 E6, or unprogrammed wheat germ extract (–). Lanes 4 and 5 represent mixings of fraction 28 with GST-16 E6 or 11 E6 beads, also in the presence of unprogrammed wheat germ extract. Molecular weight markers were Sigma unstained protein molecular weight markers visualized by Coomassie staining of the gel.

E6 in reticulocyte lysate, it seemed possible that E6-AP might be a component of the ubiquitin-dependent proteolysis system. Because E6-AP is heat sensitive and has a molecular weight of ~100 kDa, it cannot be ubiquitin itself, which is a heat stable 76 amino acid peptide that behaves as a 9 kDa molecule in gel filtration (Ciechanover *et al.*, 1978). The initial step in ubiquitin-mediated proteolysis is the covalent attachment of multiple ubiquitin moieties to the ϵ -amino groups of lysine residues of proteins destined for degradation. Three classes of enzymes are involved in this process. All three classes of proteins can be copurified on a ubiquitin affinity column in the presence of ATP, since they all participate in readily reversible covalent or non-covalent interactions with ubiquitin (Hershko *et al.*, 1980). Our attempts to bind the E6-AP protein present in rabbit reticulocyte lysate to a ubiquitin column have been unsuccessful (data not shown), and we therefore feel that E6-AP is unlikely to represent a typical member of one of these classes of proteins. The native molecular size of E6-AP is also inconsistent with its being a characterized member of one of these classes of proteins. The other main component of the ubiquitin proteolysis system is the protease itself, which is a large (26S), multicomponent complex that rapidly recognizes and degrades fully ubiquitinated substrates in a non-selective manner (Eytan *et al.*, 1989). Because of the large size of the protease and its lack of specificity in substrate recognition, it seems unlikely that E6-AP would be a component of the protease complex. Additionally, the HPV E6 proteins themselves do not have any amino acid similarity to any of the known components of the ubiquitin proteolysis system. Therefore neither E6-AP or the E6 proteins themselves seem to be acting directly as part of the enzymatic machinery of the ubiquitin proteolysis system.

Little is known about how substrates are specifically recognized for ubiquitination, although there is some evidence that substrates may be targeted for ubiquitination *in trans* by associated proteins (Johnson *et al.*, 1990). E6-AP or E6, while not appearing to be directly involved in the ubiquitination process, might function as a marker recognized by the ubiquitin proteolysis system, leading to the ubiquitination and degradation of p53. Our studies have not yet provided information on the stoichiometry or actual protein–protein contacts within the ternary complex involving E6, E6-AP and p53. Perhaps one of the p53-associated proteins, E6 or E6-AP, is serving to signal the ubiquitin proteolysis system while the other is serving to bind specifically to p53.

Two observations have indicated that p53 acts at the G₁/S border to control progression through the cell cycle. The introduction and overexpression of wild-type in Saos-2 cells (which lack endogenous p53) causes cell cycle arrest at the G₁/S border (Diller *et al.*, 1990), and a temperature sensitive mutant p53 causes G₁/S cell cycle arrest at the low (wild-type conformation) temperature (Martinez *et al.*, 1991). Since the steady state level of a protein is determined by the balance of its rates of synthesis and degradation, the targeted degradation of p53 at the G₁/S border might serve to regulate progression through the cell cycle. E6-AP might potentially be involved in such regulation in normal cells. Although we have not detected stable interaction of E6-AP with p53 in the absence of E6, a cellular analogue of the high risk HPV E6 proteins might exist that functions to mediate the association of E6-AP with p53. Experiments to

look at E6-AP activity and p53 levels as a function of the cell cycle are currently underway.

E6-AP activity was found in each human cell line examined, regardless of whether the cell contained normal levels of wild-type p53 (primary keratinocytes), mutant p53 (C-33A cells), no p53 (Saos-2 cells) or a high level of wild-type p53 (SV40-transformed keratinocytes). The presence of SV40 T antigen did not interfere with the ability to detect E6-AP activity, nor does T antigen interfere with E6-mediated degradation of p53 *in vitro* (Scheffner *et al.*, 1990), suggesting that E6-AP is not a part of the complex formed between p53 and SV40 T antigen. E6-AP was also detectable in HeLa cells, which express low levels of wild-type p53 along with HPV-18 E6 (Scheffner *et al.*, 1991). In addition to wheat germ extract, E6-AP activity was not detectable in extracts of the yeasts *S.cerevisiae* and *Schizosaccharomyces pombe* (data not shown). Neither plant nor yeast have been shown to have a p53 homolog, although the components of their ubiquitin proteolysis systems are in some cases well conserved with those of mammals.

E6 proteins, alone or in association with E6-AP, might be involved in targeting other cellular proteins for degradation in addition to p53. At this point we have not identified other such targets, nor have we identified any cellular targets of the low risk E6 proteins. The low risk HPV E6 proteins, which do not associate with p53 above background levels in our assays, also do not detectably interact with E6-AP, although we cannot rule out that both interactions might occur at a low level. We do, however, have preliminary indications that a chimeric protein containing the HPV-11 E6 protein can target specific protein substrates for degradation when brought into a complex (M.Scheffner, in preparation). Determination of whether E6-AP is involved in HPV-11 E6-mediated protein degradation should provide an indication as to whether the normal function of E6-AP is in providing a *trans* degradation signal for associated proteins, or in the specific recognition of p53.

Materials and methods

Plasmids and protein expression

Plasmids used for *in vitro* transcription/translation of the HPV E6 proteins have been described previously (Werness *et al.*, 1990). pGEX plasmids (Pharmacia; Smith and Johnson, 1988) were used for expression of glutathione S-transferase (GST) fusion proteins. These were constructed by ligation of PCR products into the *Bam*HI and *Eco*RI sites of pGEX-2T. The first methionine residue of the inserted open reading frame was deleted in each case. *In vitro* translations were performed using either rabbit reticulocyte lysate or wheat germ extract systems, as specified by the manufacturer (Promega). [³⁵S]cysteine (Amersham, sp. act. 1300 Ci/mmol) was used for labeling of *in vitro* translated proteins.

Glutathione S-transferase fusion proteins were expressed in *E.coli* strain DH5 α or HB101. One liter cultures were inoculated with 100 ml of stationary culture and grown to an OD₆₀₀ of 0.6. Fusion protein synthesis was then induced with 0.1 mM IPTG for 3 h. Cells were harvested by centrifugation, resuspended in 10 ml of phosphate buffered saline (PBS) containing 1% Triton X-100, and lysed by sonication. Cellular debris was removed by centrifugation at 10 000 g for 10 min. Glutathione sepharose (Pharmacia) was added to the supernatant and the mixture rotated at room temperature for 10 min. The beads were collected by centrifugation at 1000 g for 3 min and were washed four times with 20 vol of PBS, 1% Triton-X100 and stored in this buffer. The amount of glutathione sepharose used per liter of *E.coli* culture was adjusted for each fusion protein in order to give an approximately equal amount of fusion protein per ml of sepharose beads (~1–2 mg protein/ml of beads).

E6–p53 association assays

E6–p53 association assays were done as follows. 10 μ l of GST–p53 beads were combined with 5–10 μ l of ³⁵S-labeled *in vitro* translated E6 protein

and 250 μ l of solution containing 25 mM Tris–HCl (pH 7.2) 50 mM NaCl and 0.2% NP-40. The volume of protein extracts or column fractions assayed was generally 25–100 μ l. The mixtures were rotated at 4°C for 2 h. The beads were collected by centrifugation and washed three times with 750 μ l of cold lysis buffer (100 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1% NP-40). SDS–PAGE sample buffer was added to the beads, the beads were heated at 100°C for 4 min, and the supernatants loaded onto a 12% SDS–polyacrylamide gel. Gels were fixed and soaked in DuPont Enlightening prior to exposure to Kodak XAR5 film. Prestained protein molecular weight standards were obtained from BRL and unstained molecular weight markers from Sigma.

E6-AP depletion experiments (Figure 4) were performed by mixing 50 μ l of reticulocyte lysate with 200 μ l of 25 mM Tris–HCl (pH 7.2), 50 mM NaCl, 0.2% NP-40, and 50 μ l of glutathione sepharose loaded with either GST or GST fusion protein. The mixture was rotated at 4°C for 1 h. The beads were then collected by centrifugation and the supernatant removed to a tube containing 25 μ l of p53 beads and 10 ml of ³⁵S-labeled wheat germ extract-translated HPV-18 E6. These mixtures were then rotated at 4°C for 2 h, after which the beads were washed and bound ³⁵S-labeled E6 protein detected as described above.

Preparation of cell extracts and chromatography

Cell lysates were prepared from subconfluent 75 cm² plates. Primary human foreskin keratinocytes were prepared as described (Schlegel *et al.*, 1988) and maintained in keratinocyte growth medium (Gibco). HeLa, C-33A and Saos-2 cell lines (obtained from American Type Culture Collection) were maintained in DMEM containing 10% fetal calf serum (Gibco), and SV40 immortalized human keratinocytes (Pietenpol *et al.*, 1990) were maintained in 3 parts keratinocyte growth medium and 1 part DMEM containing 10% fetal calf serum. Cells were washed twice with cold PBS prior to lysis on the plates with 0.5 ml lysis buffer (above) containing 1 mM DTT, 0.01% PMSF and 1 μ g/ml aprotinin and leupeptin. After 30 min at 0°C, the plates were scraped and the lysates collected. Debris was removed by centrifugation for 5 min at 14 000 g. Protein concentrations were determined using Pierce Coomassie protein determination reagent.

Metabolic labeling of cultured cells was done using NEN Express protein labeling mixture containing [³⁵S]methionine and cysteine (sp. act. 1100 Ci/mmol). Cells were starved for 1 h in DMEM lacking methionine and cysteine (supplemented with 125 U/ml penicillin, 125 μ g/ml streptomycin and 150 μ g/ml glutamine) prior to addition of 1 mCi Express labeling mixture per 75 cm² plate. Cells were labeled for 4 h at 37°C and lysates prepared as described above.

Batch chromatography of cell lysates was performed by adding 100 μ l DEAE–Sephacell (Pharmacia) to 0.5 ml of cell lysate in a 1.5 ml microfuge tube. The mixture was rotated at 4°C for 15 min, and the gel was washed several times with 25 mM Tris–HCl (pH 7.2), 125 mM NaCl. Bound protein was then eluted with two 200 μ l washes of 25 mM Tris–HCl (pH 7.2), 500 mM NaCl.

Gel filtration chromatography was performed using a Pharmacia FPLC system and a Superose 12 HR 10/30 column. The column was equilibrated and run in buffer containing 25 mM Tris–HCl (pH 7.2), 125 mM NaCl and 1 mM DTT. 0.5 ml fractions were collected at a flow rate of 0.5 ml/min. Gel filtration standards were obtained from Bio-Rad. Mono Q chromatography was performed using a Pharmacia Mono Q HR 5/5 column. The column was equilibrated with 125 mM NaCl in the above buffer. Bound protein was eluted with a 15 ml linear gradient to 500 mM NaCl. One ml fractions were collected at a flow rate of 0.5 ml/min.

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