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High-Risk Human Papillomavirus E6 Protein Has Two Distinct Binding Sites within p53, of Which Only One Determines Degradation

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Human papillomavirus (HPV) E6 protein can inactivate tumor suppressor p53 by inducing its degradation. We now find that high-risk HPV E6 binds to p53 at two distinct sites; one is within the core structure of p53, and another is at the C terminus of p53. Binding to the core of p53 is required for E6-mediated degradation, as shown by deletion analysis and the properties of a point mutant at residue 135. Both low- and high-risk HPV E6 can bind to a C-terminal region of p53, but these interactions do not induce degradation. These results resolve previous seemingly contradictory findings that attributed the distinctive functional properties of high- and low-risk E6 proteins to either a difference in their abilities to associate with p53 or a difference in their N-terminal structures.

Tumor suppressor p53 regulates cell proliferation and inhibits its development or survival of cancer cells (29). Mutations of the p53 gene are highly associated with tumorigenesis (19). The amount of p53 protein in nontransformed cells is low because of its rapid turnover (13, 36, 41). An increase of p53 level by ectopic expression of the protein or reduction of its turnover via DNA damage leads to an arrest of cell cycle progression (9, 24). The exact mechanisms by which p53 influences cell proliferation remain undetermined, but its ability to act as a transcriptional transactivator is likely to be critical. Among the targets of p53 transcriptional induction is the p21 protein, an inhibitor of cyclin-dependent kinases (11, 14, 16, 53). p21 inhibits cyclin-dependent kinase activities and thus halts the cell cycle machine. Four functional domains have been identified within p53: a transactivation domain at its N terminus (12, 39), a DNA binding domain at the center of the molecule (3, 37, 51), a nuclear localization sequence at its C terminus (1, 47), and an oligomerization domain, also at the C terminus (22, 37, 49). In the center of the molecule is a tight structure, designated the core, that resists in vitro proteolysis (3, 37, 51). The core structure alone suffices for DNA binding. Recent X-ray crystallographic studies (4) of a complex of the core and DNA indicate that the core structure (amino acids 94 to 312) consists of a beta sandwich hydrophobic core and a loop-sheet-helix motif. Mutations in the region lead to either loss of the sites that directly contact DNA or changes of conformation that prevent DNA binding.

Inactivation of p53 suppressor function has been found in many types of human cancers. Inactivation can result from mutation of p53 or from interaction with viral oncoproteins. Most oncogenic point mutations cause conformational changes and are associated with a loss of DNA binding. p53 associates with several viral oncoproteins, such as simian virus 40 large T antigen, adenovirus 5 E1B-55K, or the E6 protein of human papillomavirus type 16 (HPV16) (26, 31, 43, 52). Although association with simian virus 40 large T antigen or with adenovirus type 5 E1B-55K increase the abundance of p53 by reducing its rate of degradation (41, 43), these interactions result in functional inactivation of p53 (36, 40). Large T antigen binds to the DNA binding domain (42) and is assumed to prevent the interactivation of p53 with its DNA substrate. E1B-55K abolishes p53 transactivation by binding to the N terminus of p53 (23). Interaction of p53 with the E6 protein of oncogenic HPVs instead decreases the effective amount of p53 protein by stimulating p53 degradation (46, 52). E6-mediated degradation requires the participation of a cellular protein, E6-associated protein (E6-AP) (20, 21). E6 and E6-AP together act as a ubiquitin ligase to promote degradation of p53 (44). The regions within p53 responsible for E6 binding and E6-mediated degradation have not been identified and are the subject of this report.

Two classes of HPVs can be distinguished according to their clinical lesions. One type, such as HPV16 or HPV18, called high risk, causes cervical cancer; a second type, such as HPV6 or HPV11, called low risk, causes benign cellular proliferation. Only the high-risk HPVs can immortalize primary human keratinocytes and fibroblasts in cell culture (10, 25, 38). The transforming activity is associated with expression of two viral proteins, E6 and E7 (17, 34). The E6 and E7 proteins target two different tumor suppressors, p53 and the retinoblastoma gene product (pRb), respectively (7, 52). The high-risk E7 proteins have higher affinity for pRb than low-risk E7 proteins (35). There is general accord that the two types of E6 proteins have dramatically different effects on p53 degradation: only high-risk HPV E6 proteins are able to induce degradation (46). However, seemingly contradictory views have been expressed on whether low-risk E6 protein can also interact with p53 (6, 52).

In this study, we show the association between E6 and p53 is more complex than previously noted and that an understanding of this complexity is needed to resolve the relationship between association and degradative activity. By using different binding assays, we were able to detect two distinct interactions of high-risk HPV16 E6 (16E6) with p53. 16E6 binds to the core structure of p53. E6-AP promotes this interaction. E6 protein alone, in the absence of E6-AP, can also bind to the C terminus of p53. Only the binding to the core structure is relevant to p53

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degradation. We found that low-risk HPV6 E6 (6E6) is also able to bind to p53 at the C terminus of p53 but that it does not interact with the core structure. These distinctive interactions provide a molecular basis for the difference between the high- and low-risk HPV E6 proteins in mediating degradation of p53.

MATERIALS AND METHODS

Constructs. To make fusion proteins consisting of glutathione *S*-transferase (GST) appended to the N termini of human p53, mutant p53 (Cys-135→Tyr), 16E6, and 6E6, appropriate templates were amplified by PCR and the resultant DNAs were cloned into the pGEX-3X vector at *Bam*HI and *Eco*RI restriction sites present within the oligonucleotides. GST fusion proteins were prepared and purified with glutathione-Sepharose beads as described previously (48). To make proteins by in vitro transcription and translation, p53, p53 deletion mutants, and E6 DNA templates for transcription were made by PCR. A T7 RNA polymerase recognition site was placed upstream of the translation initiation AUG codon by incorporating it into the 5′ oligonucleotide. To make the N-terminal deletions of p53, an initiation codon ATG was introduced in 5′ oligonucleotides that start at amino acid 40, 66, 101, 133, 201, or 301. To make the C-terminal deletions of p53, PCR was carried out with a 3′ oligonucleotide that incorporated a TAA stop codon immediately after amino acid 290, 318, 326, 350, 355, or 380. 16E6 and 6E6 proteins were made by the same procedures.

Degradation assay. Recombinant DNA constructs produced by PCR as described above were used as templates for in vitro transcription by T7 RNA polymerase. RNA was translated in vitro by using a rabbit reticulocyte lysate or wheat germ extract (Promega) in the presence of [^{35}S]methionine. The translated proteins were used as substrates for degradation in a rabbit reticulocyte lysate extract as described previously (30). Degradation was carried out at 30°C, and the labeled protein remaining undegraded was examined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and autoradiography. To inhibit degradation and thus allow the accumulation of polyubiquitinated forms of substrate proteins, degradation was carried out as described above except that 2 mM ATPγS was used in place of ATP and the ATP regenerating system.

Binding assays. Twenty microliters of translation lysate containing [^{35}S]methionine-labeled p53 or 12 μl of E6 protein was added to 500 μl of phosphate-buffered saline containing 0.05% Triton X-100 and 20 μl of glutathione-Sepharose 4B beads (Pharmacia) bearing the GST-16E6, GST-6E6, GST-p53, or GST-p53 (Cys-135→Tyr) fusion protein. GST–E6-AP expressed in *Escherichia coli* was purified by glutathione-Sepharose chromatography and used for determination of the p53 core binding of E6. The expression vector for GST–E6-AP was a gift of Peter Howley (20). For monoclonal antibody immunoprecipitation, 20 μl of translation lysate containing [^{35}S]methionine-labeled p53 was mixed with 1 μg of antibody and then added to 500 μl of phosphate-buffered saline containing 0.05% Triton X-100 and 20 μl of protein A-Sepharose 4B beads (Pharmacia). For E6 coprecipitation, 8 μl of translation lysate containing [^{35}S]methionine-labeled E6 was preincubated with 20 μl of p53 protein on ice for 5 min and added to the above-described mixture. All binding assays were performed at 4°C and shaken for 1 h. After shaking, the beads were washed three times with ice-cold phosphate-buffered saline. The proteins associated with the beads were made soluble in SDS sample buffer, and the radiolabeled p53 or/and E6 were analyzed by SDS-PAGE and autoradiography. Twenty percent of the amount of labeled protein used for binding assays was directly loaded on the SDS-gel as a measure of binding efficiency.

RESULTS

The core structure of p53 is needed for E6-mediated degradation. To determine the minimal structure of p53 required for E6-mediated degradation, we made a series of deletions at each end of the 393-amino-acid protein (54), as shown in Fig. 1. The [^{35}S]methionine-labeled proteins were subjected to E6-mediated ubiquitination and degradation. Intact p53 was stable in the absence of E6 protein and induced to be degraded by high-risk 16E6 (Fig. 2A, arrows). By adding ATPγS in place of ATP, ubiquitination is allowed to take place but degradation is blocked (46). High-molecular-weight polyubiquitinated forms of p53 thus accumulate and can be visualized (Fig. 2A). These assays were applied to the deleted forms of p53. The C terminus could be deleted to amino acid 350 with retention of both degradation and ubiquitination, but further deletion to amino acid 326 resulted in equivocal degradation, with retention of ubiquitination. Further truncation to amino acid 318 led to loss of degradation and reduction in ubiquitination (Fig. 2A). Deletion to amino acid 290 abolished both. Among the N-terminal deletions, removal of the first 65 amino acids had little effect, but deletion of 100 amino acids prevented both degradation and ubiquitination (Fig. 2B). A dual deletion at both ends, one producing a form of p53 containing amino acids 66 to 326, retained E6-mediated ubiquitination and degradation (result not shown). Amino acids 66 to 326 thus contain the minimal region required for E6-mediated degradation. This region barely covers the core DNA binding structure of p53.

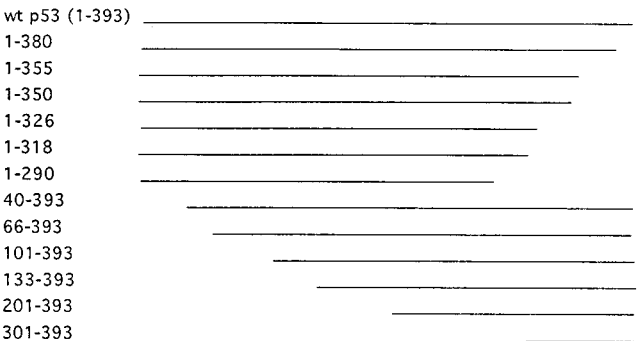

FIG. 1. Schematic representation of N- and C-terminal deletions of p53. wt, wild-type.

A binding site at the C terminus of p53 is detected by GST-E6 affinity. To determine the relationship between E6 binding and E6-mediated degradation, we examined binding more directly. Initially we used a GST-16E6 fusion protein (full-length 16E6 with GST as an N-terminal extension) to detect interaction with p53 or deleted forms of p53. p53 and its deletions were [^{35}S]methionine labeled and mixed with the GST-E6 fusion protein conjugated to glutathione-Sepharose beads, and bound labeled proteins were revealed by SDS-PAGE and autoradiography. Binding of p53 to the GST-16E6 fusion protein was efficient and the recovery was about 10 to 20% (Fig. 3A), but control GST bound no detectable p53 (see Fig. 5A). Removal of the last 13 amino acids from the C terminus of p53 (deletion 1-380) did not reduce binding, but further deletion of 25 amino acids (deletion 1-355) or more (deletions 1-350, 1-326, 1-318, and 1-290) abolished it (Fig. 3A). This assay indicated that the C terminus of p53, between amino acids 356 and 380, is required for E6 binding. All of the N-terminal deletions that we made, including the smallest remnant tested, amino acids 301 to 393, were able to bind GST-16E6 (Fig. 3B), showing that the E6 binding site is indeed within the C terminus. Furthermore PAb421, recognizing an epitope at amino acids 376 to 384 (15, 50), blocked the binding (Fig. 3C). This finding confirms that the p53 binding site detected in this assay is at or close to amino acids 376 to 384.

A second E6 binding site within the core structure of p53 is revealed by coprecipitation and correlates with degradation. Because the E6 binding site at the C terminus of p53 is not required for E6-mediated p53 degradation, we suspected that the presence of another E6 binding site not detected by the GST-16E6 binding assay. We sought evidence of the site by using coimmunoprecipitation of E6 with p53 by monoclonal antibody PAb421 or PAb1801 (2, 15). In vitro-translated p53 was mixed with in vitro-translated high-risk 16E6 and precipitated with PAb421 or PAb1801 and protein A-Sepharose. E6 was specifically coprecipitated with p53 by PAB421 (Fig. 4A) or PAb1801 (Fig. 4B) but not in control incubations without p53. The coprecipitated E6 protein was identified by its characteristic size and its absence in a control precipitation of p53 alone. Precipitation of p53 protein with PAb421 or PAb1801

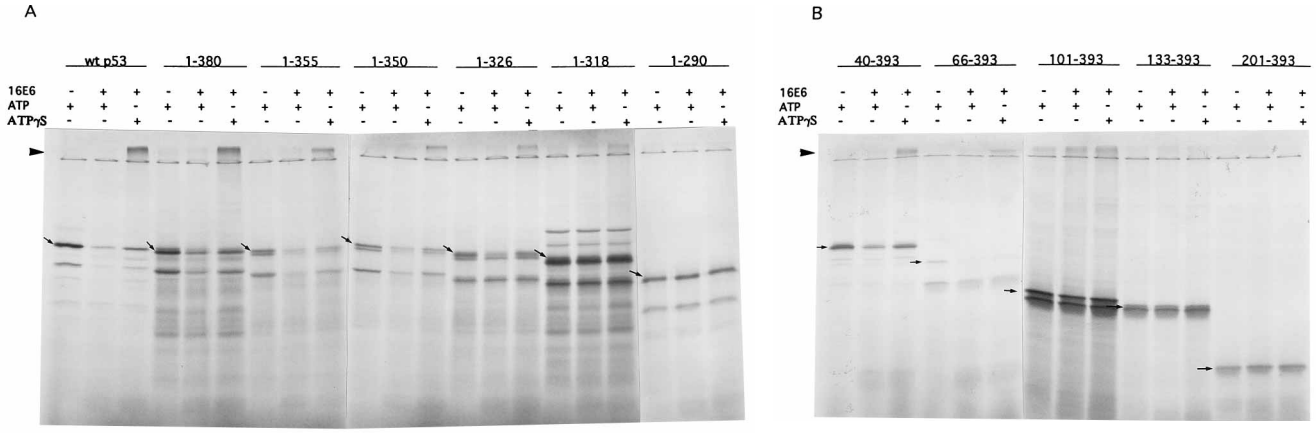

FIG. 2. 16E6-mediated degradation and ubiquitination of p53 and its deletions. Wild-type (wt) p53, p53 deletions, and 16E6 were each translated in vitro. [^{35}S]methionine-labeled p53 or its deletions were mixed with unlabeled 16E6 and subjected to either degradation in the presence of ATP or ubiquitination in the presence of ATPγS. Incubation in the presence or absence of 16E6, ATP, or ATPγS is indicated. The protein remaining undegraded or present as a high-molecular-mass ubiquitinated form was visualized by SDS-PAGE and autoradiography. (A) p53 and C-terminal deletions; (B) N-terminal deletions. Arrows indicate the positions of migration of p53 and its deletions, and arrowheads indicate ubiquitinated proteins.

was not affected by p53 (Cys-135→Tyr), which is resistant to E6-mediated degradation (45), but E6 can no longer be coprecipitated with the mutant protein (Fig. 4). Thus, the E6 binding detected by the coprecipitation assay correlates with E6-mediated degradation. To determine the binding site, we used PAb421, which recognizes the C terminus of p53, to examine E6 coprecipitation of the N-terminal deletions (Fig. 4A) and PAb1801, which recognizes the N terminus of p53, to determine coprecipitation of the C-terminal deletions (Fig. 4B). Among the C-terminal deletions, 1-350 was bound well, 1-326 was bound less strongly, and 1-318 was bound not at all. This analysis demonstrated the presence of a second E6 binding site within amino acids 66 to 326, identical to the region needed for E6-mediated degradation. Therefore, the second binding site and degradation are well correlated.

Low-risk 6E6 cannot bind to the core of p53. Low-risk 6E6, unlike high-risk 16E6, does not induce degradation of p53. The question of whether low-risk E6 proteins bind p53 and the site of their association has elicited discrepant answers. To reassess this issue, we applied the two binding assays used with 16E6. First we determined whether 6E6, like 16E6, binds the C terminus of p53, using a GST-6E6 fusion protein. As with GST-16E6, p53 associated with GST-6E6, and the binding was dependent on the presence of the C terminus of p53 (Fig. 5A). Next, we tested 6E6 binding to the p53 core structure by coprecipitation of p53 and 6E6 with the anti-p53 monoclonal antibody PAb1801. Unlike 16E6, 6E6 did not coprecipitate with p53 (Fig. 5B). There was minor precipitation of 6E6, but it was also seen in the absence of p53. We conclude that both high- and low-risk E6 proteins bind to the C-terminal site, but only the high-risk form binds to core.

E6-AP can redirect binding from C terminus to core. Next we sought to determine why two different assays of binding, GST-E6 affinity and coimmunoprecipitation, produced different results. One potential explanation lay in the very different amounts and ratios of E6 to E6-AP in the two cases: E6 in the form of a recombinant fusion protein is present in vast excess to E6-AP in the GST-E6 affinity assay. To more fully control the amount of E6 and E6-AP present, we used recombinant GST-p53 proteins as binding ligands and produced tracer amounts of labeled E6 proteins by translation in a wheat germ-derived system, which lacks E6-AP. This change makes it possible to add or withhold E6-AP, which has been shown to be needed for E6 to associate with wild-type p53 (20, 21), in amounts sufficient to saturate any E6 present. 16E6 protein was translated in a wheat germ system and tested for binding to GST-p53 or GST-p53(Cys-135→Tyr) fusion protein associated with an affinity matrix. 16E6 protein was bound equally well by an affinity matrix containing GST-p53 or GST-p53(Cys-135→Tyr) fusion protein, presumably at the C terminus of p53 (Fig. 6). Further addition of reticulocyte lysate as a source of E6-AP enhanced the binding of E6 to GST-p53 but not to the mutant GST-p53(Cys-135→Tyr). A similar result was obtained when a purified recombinant GST–E6-AP fusion protein was used in place of reticulocyte lysate (results not shown). Also required for enhanced binding is an intact core. Addition of a reticulocyte source of E6-AP actually caused GST-p53(Cys-135→Tyr) bearing a core mutation to bind less E6. This could be because E6-AP has higher affinity for E6 than for than C terminus of p53 and competes with p53 for binding of a limiting pool of E6. The 6E6 protein was similarly used to test the binding to GST-p53 and GST-p53(Cys-135→Tyr). Like 16E6, 6E6 bound similarly to both fusion proteins (Fig. 6). But strikingly different from 16E6, 6E6 binding to GST-p53 was not enhanced by adding reticulocyte lysate. We conclude that 6E6 does not bind to the core structure, regardless of the presence or absence of E6-AP. Providing E6-AP in adequate amount can redirect binding of 16E6 from the C terminus to the core. The difference in its availability likely contributes to the distinct results seen in assays using GST-E6 affinity and those using coprecipitation.

DISCUSSION

16E6 binds to and induces p53 degradation (43, 52). We sought to determine the sequences within p53 responsible for E6 binding and for E6-mediated degradation. Using two different assays of E6-p53 interaction, we found two distinct binding sites within p53: E6 can bind either to a region (amino acids 66 to 326) that encompasses the core (approximately amino acids 101 to 300) or to the C terminus. These interactions are distinguishable as follows. Core binding occurs with high-risk (HPV16) but not low-risk (HPV6) E6, is dependent on E6-AP, requires a wild-type core conformation, and is independent of the p53 C terminus. C-terminal binding of E6, on the other hand, requires high concentrations of E6 (or at least an excess

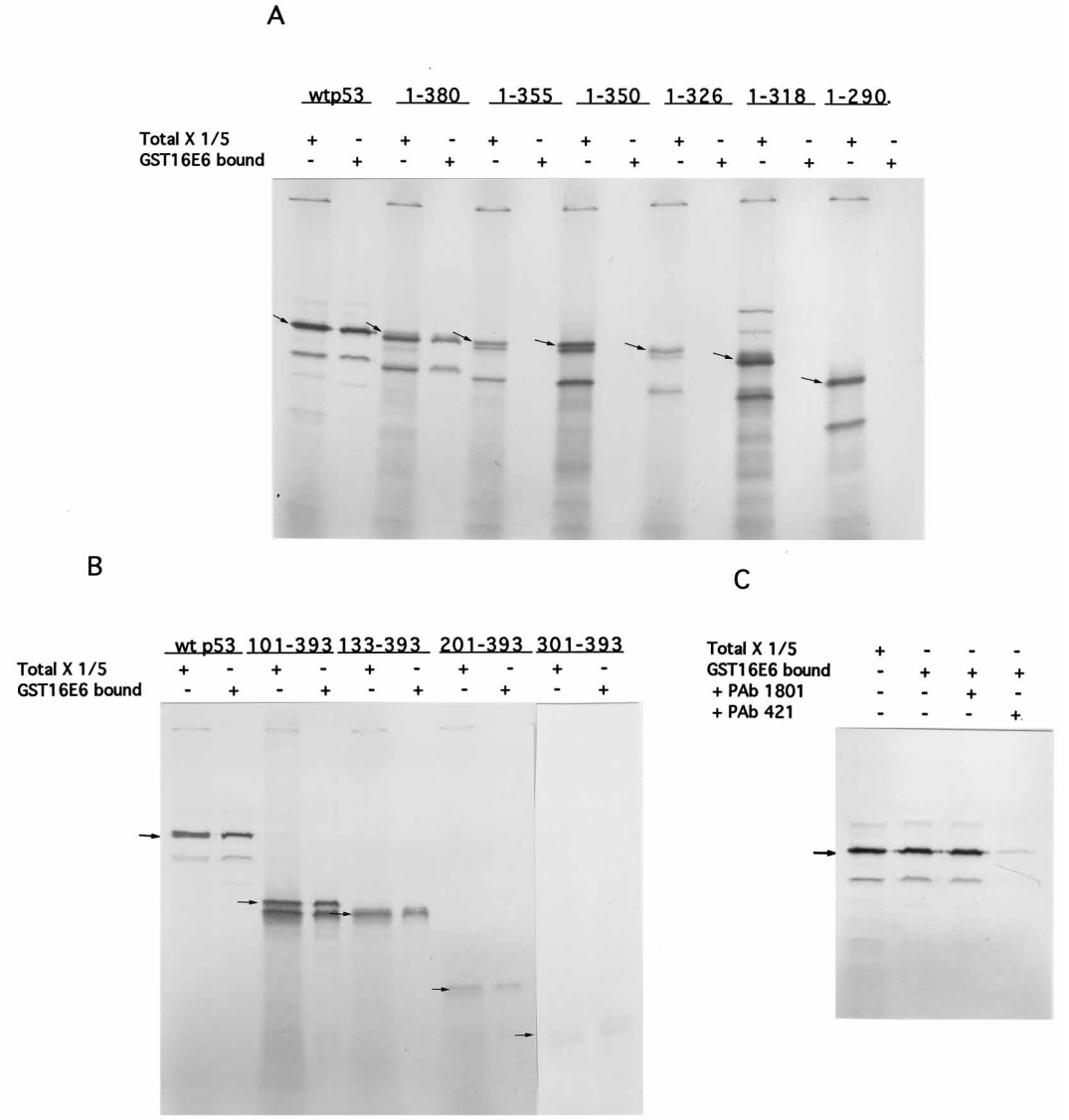

FIG. 3. GST-16E6 binding of p53 and its deletions. [^{35}S]methionine-labeled wild-type p53 (wtp53) or its deletions was mixed with the GST-16E6 fusion protein conjugated on glutathione-Sepharose 4B. The bound proteins were examined by SDS-PAGE and autoradiography. (A) N-terminal deletions; (B) C-terminal deletions. In panel C, p53 was preincubated with PAb421 or PAb1801 before mixing with GST-16E6 fusion protein, and the influence of the antibodies on binding was examined. Twenty percent of the total labeled protein used for the binding assay was directly loaded on the gel as an index of binding efficiency. Arrows indicate the positions of migration of p53 and its deletions.

of E6 to E6-AP), is independent of a source of E6-AP, and is tolerant of gross structural disruption of the p53 core. Coimmunoprecipitation as used in this study provides conditions that favor core binding. The second assay, association with a matrix containing GST-E6 fusions, favors C-terminal binding. We used two alternative ligand proteins to rule out the possibility that binding to the C terminus of p53 is an artifact of the use of GST-E6. (i) By using GST-p53 as ligand, binding of 16E6 could be directed to the C terminus or core, depending, respectively, on whether a source of E6-AP was absent or present. (ii) Results similar to those reported here for GST-E6 and a glutathione matrix were found when His_6-16E6 and a nickel-chelate matrix were used in their place (data not shown). Each form of recombinant E6 associated with both wild-type p53 and the p53(Cys-135→Tyr) core mutant, a characteristic of C-terminal binding.

Medcalf and Milner have investigated the interaction with 16E6 of a single mouse p53 protein bearing a carboxyl-terminal deletion (final residue 343, corresponding to human p53 residue 349) (33). The truncated mouse protein maintained association with E6, as assessed by gel filtration. It was, however, resistant to E6-mediated degradation in vitro. We found, in apparent contrast, that truncation to (human) residue 350 had no discernible effect on degradation, while truncation to 326 was moderately inhibitory and truncation to 318 was fully stabilized. Whether the difference in outcome was due to structural differences between mouse and human p53 or other differences in methodology is not clear.

A GST-E6 affinity assay similar to that used here has been previously used to assess association between p53 and E6 proteins from a variety of HPV E6 proteins (27). Such association was not found to require a source of E6-AP. This observation and the finding that 16E6 as well as several low-risk E6 proteins bound suggests that the reported association reflected

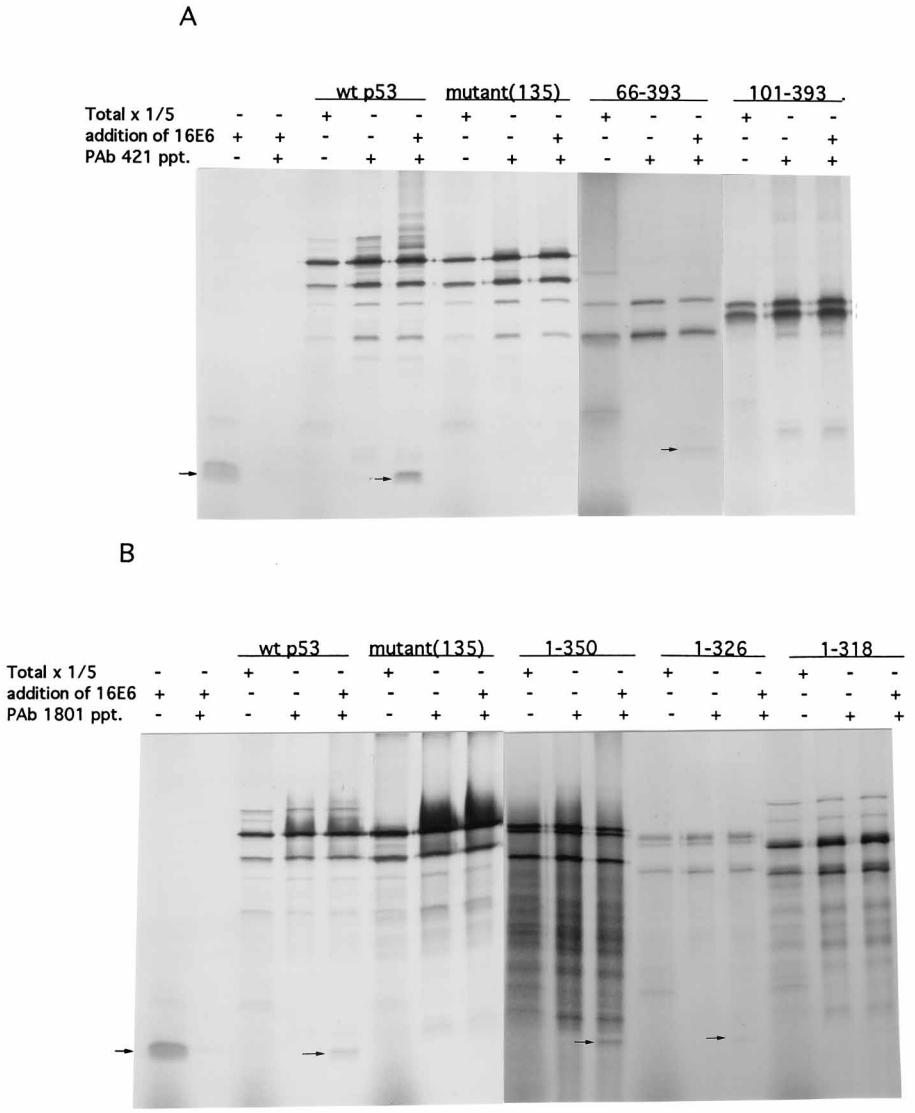

FIG. 4. Coprecipitation of 16E6 with p53 and its deletions detected by monoclonal antibody PAb421 or PAb1801. [^{35}S]methionine-labeled wild-type (wt) p53, mutant p53(Cys-135→Tyr), or p53 deletions were either directly precipitated with PAb421 or PAb1801 and protein A-Sepharose or preincubated with [^{35}S]methionine-labeled 16E6 and then precipitated. The N-terminal deletions of p53 were precipitated with PAb421 (A), and the C-terminal deletions were precipitated with PAb1801 (B). The precipitated labeled proteins were analyzed by SDS-PAGE and autoradiography. For each form of p53, 20% of the total input protein used for the precipitation assay is shown in the first lane, precipitation of p53 is shown in the second lane, and coprecipitation of 16E6 is shown in the third lane. Arrows indicate the positions of migration of 16E6.

binding to the p53 C terminus. Because p53 mutants with C-terminal alterations were not tested in that work, the presence of a second binding site outside the core could not be noted. Numerous laboratories have indicated that the conformation of p53 determines the binding of 16E6. The present report of a second site at the C terminus is novel.

The possibility that the interaction of 16E6 with the C terminus of p53 is indirectly involved in degradation, perhaps facilitating it, is excluded by three further studies (unpublished data). First, the relationship between the extent of degradation and the amount of E6 used are the same for wild-type p53 and a carboxy-truncated form (amino acids 1 to 355). Second, we observed no difference in E6-mediated p53 degradation with or without PAb421, which blocks carboxy-terminal GST-16E6 binding. Lastly, the E6 binding at the C terminus of p53 detected by GST-16E6 is independent of E6-AP, a prerequisite for E6-mediated degradation. Therefore the E6 binding site at the C terminus of p53 is unrelated to E6-mediated degradation.

Our results suggest that the regions within p53 needed for 16E6 core binding and for 16E6-mediated degradation coincide. This contrasts with the report of Mansur et al. (32), which concludes that a region within amino acids 161 to 345 is the binding domain of 16E6 but that a second region within the N terminus of p53, amino acids 1 to 160, is required for degradation. Their conclusion seems contrary to a previous report that 16E6 binding is preferential for the wild-type conformation of p53, that recognized by monoclonal antibody 1620 (33). A deletion of amino acids 1 to 160 destroys the wild-type conformation of p53 and should therefore limit 16E6 binding (4). The efficiency of binding of mutant p53 in which amino acids 1 to 160 are deleted is much less than that seen with

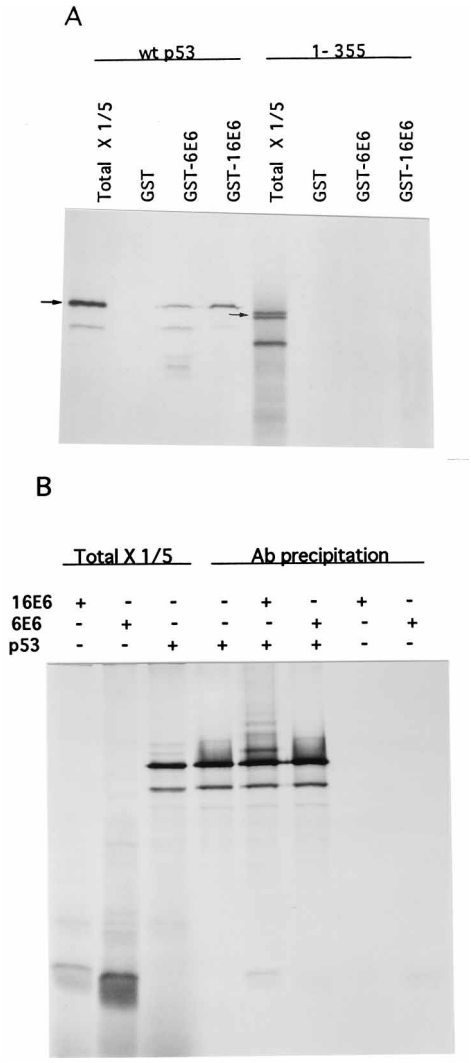

FIG. 5. Interaction of 6E6 with p53. (A) [^{35}S]methionine labeled wild-type (wt) p53 or p53 deletion 1-355 was mixed with GST alone, GST-16E6, or GST-6E6 fusion protein conjugated on beads for the binding assay. Arrows indicate the position of migration of p53. (B) In vitro translation of [^{35}S]methionine-labeled p53 was either directly precipitated with PAb1801 and protein A-Sepharose or preincubated with [^{35}S]methionine-labeled 16E6 or 6E6 and then precipitated. To provide an index of efficiency of precipitation, 20% of input 16E6 or 6E6 used for the binding assay was analyzed in parallel; the precipitation of 16E6 and 6E6 with PAb1801 in the absence of p53 was determined as a measure of nonspecific assay background.

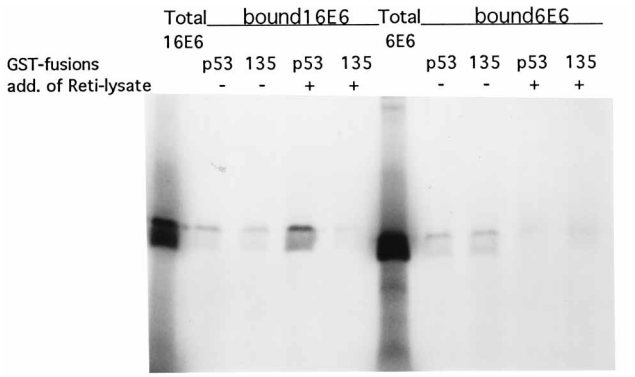

FIG. 6. E6-AP-dependent binding of 16E6 to core. 16E6 and 6E6 were separately translated in vitro in wheat germ extracts. The [^{35}S]methionine-labeled 16E6 or 6E6 was mixed with GST-p53 or GST-p53(Cys-135→Tyr) beads without or with addition of reticulocyte lysate (Reti-lysate). The bound 16E6 or 6E6 was analyzed by SDS-PAGE and autoradiography. Twenty percent of the total labeled input protein used for binding was analyzed as a measure of binding efficiency.

forms of p53 retaining that region (32). Amino acids 161 to 393 may retain some attenuated binding activity. Alternatively, the residual binding seen with the deletion mutant may result from interaction between 16E6 and the C terminus of p53, as we report here.

Low-risk 6E6 does not induce p53 degradation. Two models purport to explain why 6E6 is unable to do so. The first arises from the perception that only high-risk HPV E6 proteins, such as 16E6, are able to bind to p53 (46). A second model, provided by Crook et al., derives from the seemingly contrary observation that both high- and low-risk HPV E6 proteins are able to bind to p53 (although the latter more weakly), that their association takes place by means of a region located at the C terminus of E6 that is conserved among both high- and low-risk forms, and that an N-terminal region present only among high-risk HPV E6 proteins determines not binding but degradation (6). Our results provide another explanation that is different from these previous models in four aspects. First, we found both 16E6 and 6E6 can bind to p53, specifically to the C terminus, but that event has no perceptible effect on degradation. Second, high-risk 16E6 (but not low-risk 6E6) binds to the p53 core, and the binding is associated with degradation. Third, we found that a C-terminal E6 half molecule can bind to the C terminus of p53, but an intact 16E6 molecule is required for p53 core binding (unpublished data). Lastly, we made and examined the properties of reciprocal chimeric proteins of 16E6 and 6E6, 6/16E6 and 16/6E6, with junctions between amino acids 59 and 60; these are identical to the chimeras described by Crook et al. (6). Neither chimeric 16/6E6 protein induced p53 degradation in our hands (data not shown). Our results support similar observations for the same chimeric proteins by Foster et al. (12a).

Does E6 binding to the C terminus of p53 have any biological function? One study suggests that low-risk HPV E6 proteins can inhibit p53-mediated repression of transcription (28). p53 can act as either a transcriptional transactivator to induce gene expression or an inhibitor to repress transcription of other genes. Lechner et al. reported that both high-risk and low-risk E6 proteins can abrogate p53-mediated transcriptional repression, although low-risk HPV E6 is less active (28). Our observations suggests that low-risk HPV E6 may abrogate repression via the p53 C terminus. Alternatively, because the domain for p53 oligomerization and the signal for nuclear location are also C terminal, E6 binding may influence these functions.

Ubiquitination is an intermediate step in the degradation of many short-lived proteins, including p53 (5, 46). The cellular protein E6-AP must be present for E6 to target p53 (20, 21). E6 and E6-AP together bind to p53 and promote its degradation. The E6–E6-AP pair has recently been characterized as a ubiquitin-protein ligase that acts on p53 (44). Ubiquitination of short-lived proteins occurs by attachment of ubiquitin to lysine residue via an isopeptide bond (18), but the exact role of ubiquitination in protein degradation is not well understood. The modification has been proposed to be a direct recognition signal for the protease complex, and a specific protease subunit has recently been identified as the locus of interaction with polyubiquitin (8). Among the 20 lysine residues within the p53

protein (54), the ubiquitin modification sites have not been identified. On the basis of this work, the nine lysine residues outside of the region from amino acids 66 to 326 can be excluded as critical to degradation.

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