dehydratase enzymes, this seems borne out in a convincing manner. The enzymatic *syn* addition-elimination of water with thioester substrates is not the most chemically efficient pathway but appears to depend instead on historical contingency.

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12. We have also examined the effect of solvent on these reactions. The deuteroxide-catalyzed H-D exchange of ethyl β-hydroxybutyrate in D_2O gives a 5.7:1 ratio of the 2*R**,3*R**:2*R**,3*S** diastereomers, in a pattern consistent with our other results. The 2*R**,3*R** notation refers to the absolute configurations at C-2 and C-3 and to the fact that this diastereomer is present as a racemic mixture.
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17. The extent of reaction and product diastereoselectivity were ascertained by multiple integrations of 500-MHz ^1H NMR (D_2O) and 76-MHz ^2H NMR (H_2O) spectra, respectively. At 100°C and 0.5 M KOD, ~10% H-D exchange of one α proton on malate required 30 min; the conjugate addition was one-eighth as fast under comparable conditions. All reactions were carried out to 2 to 5% addition or 2 to 15% exchange and were done in duplicate, with at least two samples from different times examined in each reaction.
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20. Because of concomitant hydrolysis, the pH of reaction mixtures dropped substantially from pH 13 to 9 over a 30-min period; in addition reactions, we added KOD to bring the pH back to 13 in order to produce sufficient adduct. Final purification of **4** was done on silica gel with a 3% methanol–97% ethyl acetate eluant. We separated the hydroxythioester and hydroxyacid from inorganic salts by extraction of the crude lyophilized product with methanol, followed by further extraction with CH_2Cl_2 to dissolve the thioester. After purification of **4** on silica gel, ^2H NMR analysis (61 MHz) was carried out in a 3:2 or 4:1 benzene-methanol mixture, which provided a baseline between the integrated peaks (δ 2.58 and δ 2.43; C_6D_6 = δ 7.15). Exchange ranged from 15 to 37%, averaging 30% of one proton (seven reactions). Addition data were averaged from five reactions.
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26. We thank T. Hoye and S. B. Philson of the University of Minnesota for help with the purification of **4** and help with 500-MHz NMR, respectively. We thank M. Mitton for examining the effect of different solvents on H-D exchange and M. McKone for helpful discussions. Supported by the Donors of the Petroleum Research Fund (administered by the American Chemical Society), the 3M Foundation, the Howard Hughes Medical Institute, and a Council on Undergraduate Research–Academic-Industrial Undergraduate Research Partnership Fellowship (E.C.P.).

20 March 1995; accepted 2 June 1995

Interaction of Papillomavirus E6 Oncoproteins with a Putative Calcium-Binding Protein

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Human papillomaviruses (HPVs) are associated with the majority of cervical cancers and encode a transforming protein, E6, that interacts with the tumor suppressor protein p53. Because E6 has p53-independent transforming activity, the yeast two-hybrid system was used to search for other E6-binding proteins. One such protein, E6BP, interacted with cancer-associated HPV E6 and with bovine papillomavirus type 1 (BPV-1) E6. The transforming activity of BPV-1 E6 mutants correlated with their E6BP-binding ability. E6BP is identical to a putative calcium-binding protein, ERC-55, that appears to be localized in the endoplasmic reticulum.

Infection with "high-risk" HPV, such as types 16, 18, and 31, can lead to malignancy, the most common of which is cervical cancer. Two viral transforming genes, *E6* and *E7*, are selectively retained and expressed in these cancers. Other HPVs such as types 6 and 11 are referred to as "low-risk" viruses because these are generally limited to benign genital and cervical papillomas that rarely progress to cancer. The high-risk HPV E6 genes induce immortalization of primary human epithelial cells either alone or in cooperation with *E7* [reviewed in (*1*)]. High-risk HPV E6 proteins bind the cellular factor E6-AP in vitro, and together these proteins bind and promote the ubiquitination and degradation of p53 (*2*, *3*). In cultured cells the introduction of HPV-16 E6 leads to increased p53 turnover (*4*, *5*), inhibits p53-regulated transcription (*6*, *7*), and blocks p53-induced G_1 growth arrest (*4*, *8*).

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Several observations suggest that papillomavirus E6 genes encode p53-independent transformation functions. HPV-16 E6 transforms NIH 3T3 cells but trans-dominant p53 mutants did not (*9*). We have found that HPV-16 E6 induces anchorage-independent growth of p53-deficient cells (*10*). The E6 genes from HPV-5 and HPV-8, BPV-1, and cottontail rabbit PV have oncogenic properties, yet these E6 proteins do not interact with p53 (*11*). To identify additional cellular proteins that interact with HPV-16 E6, we screened (*12*) a HeLa cell complementary DNA (cDNA) library (*13*) using the yeast two-hybrid system (*14*). After screening ~10^6 colonies on X-Gal plates, we isolated a HeLa cDNA encoding a protein referred to as E6BP (E6-binding protein) that specifically interacts with HPV-16 E6 (*12*).

Sequence analysis of the E6BP cDNA revealed a 210–amino acid open reading frame encoding a protein with four potential calcium-binding motifs, the EF hand (*15*), and a putative endoplasmic reticulum (ER) retention peptide (HDEL) at the COOH-terminus. E6BP is identical in sequence to ERC-55, a protein recently isolated on the basis of its reactivity with human auto-immune antiserum (*16*). The E6BP cDNA encodes a truncated version of

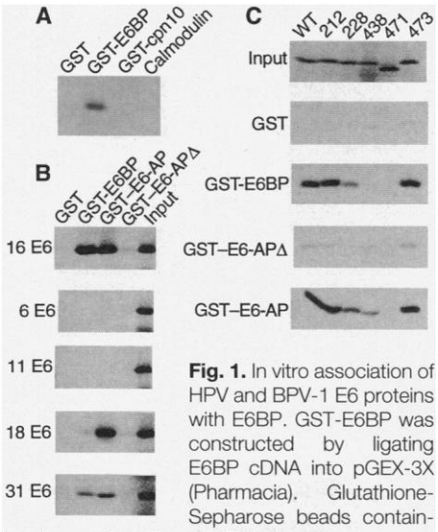

Fig. 1. In vitro association of HPV and BPV-1 E6 proteins with E6BP. GST-E6BP was constructed by ligating E6BP cDNA into pGEX-3X (Pharmacia). Glutathione-Sepharose beads containing 2 μg of GST or GST fusion proteins were mixed with ^{35}S-labeled, in vitro–translated HPV-16 E6 in LSAB [100 mM NaCl, 100 mM tris-HCl (pH 8.0), 1% NP-40, 2 mM dithiothreitol (DTT), 0.1% nonfat dry milk, and 1 mM phenylmethylsulfonyl fluoride (PMSF)] in a total volume of 250 μl. After incubation for 3 hours at 4°C and washes with LSAB, the bound products were separated by SDS–polyacrylamide gel electrophoresis. E6 binding was analyzed by autoradiography (A) or Molecular Imager (Bio-Rad) (B and C). (**A**) Binding of E6BP to HPV-16 E6. (**B**) Binding of E6BP to E6 from high-risk HPVs. (**C**) Association of E6BP with BPV-1 E6. The BPV-1 E6 mutations are indicated above each lane. Input was directly loaded into the well and represents 10% of the ^{35}S-labeled E6 used in each binding reaction. WT, wild type.

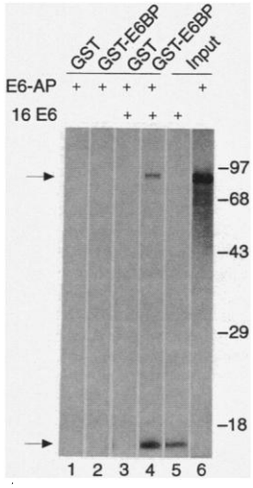

Fig. 2. Interaction of E6BP with HPV-16 E6 and E6-AP. Glutathione-Sepharose beads containing 2 μg of GST or GST fusion proteins were mixed with ^{35}S-labeled, in vitro–translated HPV-16 E6, the 95-kD form of E6-AP (*3*), or both, in LSAB as in Fig. 1. Lanes 1 (GST) and 2 (GST-E6BP), incubation with ^{35}S-labeled E6-AP; and lanes 3 (GST) and 4 (GST-E6BP), incubation with both ^{35}S-labeled HPV-16 E6 and E6-AP. Lanes 5 and 6 were loaded with 10% of the ^{35}S-labeled HPV-16 E6 and E6-AP used in each reaction. Numbers indicate the molecular size in kilodaltons. E6-AP and HPV-16 E6 are indicated by the upper and lower arrows, respectively.

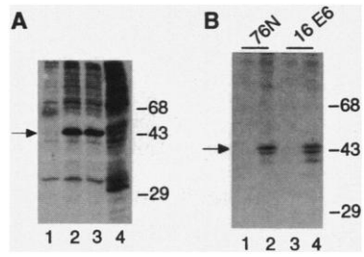

Fig. 3. Immunoprecipitation of E6BP from cultured cells. Methods are described in (*29*). The E6BP-specific antiserum was raised against the GST-E6BP fusion protein. (**A**) Immunoprecipitation of E6BP from HeLa cells. Lane 1, preimmune serum; lanes 2 to 4, E6BP antiserum. Purified GST and GST-E6BP proteins (200 ng) were added to the immunoprecipitation reactions in lanes 3 and 4, respectively. (**B**) Analysis of endogenous E6BP from primary (76N) and HPV-16 E6–immortalized (16 E6) human mammary epithelial cells (*20*). Lanes 1 and 3, preimmune serum; lanes 2 and 4, E6BP antiserum. The position of E6BP is indicated by the arrow. Numbers indicate the molecular size in kilodaltons.

ERC-55 that is missing the NH_2-terminal 107 amino acids.

To study the interaction of E6 with E6BP, we prepared glutathione-S-transferase (GST)–E6BP fusion proteins in *Escherichia coli*. GST-E6BP efficiently bound HPV-16 E6 (Fig. 1A), whereas the control GST, an irrelevant GST fusion protein, and calmodulin agarose did not bind. We next investigated the ability of E6 from other genital HPVs to bind E6BP. GST–E6-AP and GST–E6-APΔ [a mutant devoid of amino acids 391 to 408, which are necessary for E6 association (*3*)] were used as positive and negative controls. GST-E6BP and GST–E6-AP bound HPV-16 E6 equally well (Fig. 1B). GST-E6BP and GST–E6-AP bound to HPV-18 and HPV-31 E6, although binding of HPV-18 E6 was weaker with E6BP. GST-E6 fusion proteins displayed a similar hierarchy of binding affinities (HPV-16 > HPV-31 > HPV-18) in p53 binding assays (*6*). E6 from low-risk HPV-6 and HPV-11 did not bind GST-E6BP or GST–E6-AP. Thus, there is a correlation between biological risk for cervical cancer and the ability to bind E6BP.

Mutational analyses of HPV-16 E6 that compare its interactions with E6-AP and p53 to its transformation and immortalization capability have not been reported. We therefore investigated binding of E6BP to the related BPV-1 E6 transforming protein. GST-E6BP formed a complex with in vitro–synthesized BPV-1 E6 as efficiently as it did with HPV-16 E6 (Fig. 1C). The transforming activity of a series of BPV-1 E6 mutants (*17*) correlated with their ability to bind E6BP in vitro (Fig. 1C and Table 1). Mutants 212 and 473 transformed C127 cells at wild-type levels and were competent for E6BP binding, whereas mutants defective for transformation did not bind GST-E6BP. Notably, mutant 228 maintained partial transforming activity and displayed reduced E6BP binding. These results suggest that the E6BP interaction is necessary for BPV-1 E6–induced transformation. Although BPV-1 E6 does not bind (*2*, *18*) or degrade p53 in vitro (*19*), it efficiently bound E6-AP but not E6-APΔ. This indicates that binding to E6-AP is not in itself sufficient for interaction with p53 and that the 18 amino acids deleted in E6-APΔ are necessary for association with both HPV-16 and BPV-1 E6 proteins. The general correlation between BPV-1 E6 transformation and E6-AP binding suggests that this interaction may also play a role in BPV-1 E6 transformation.

In vitro association of HPV-16 E6 with p53 requires E6-AP (*3*). In contrast, HPV-16 E6 bound E6BP in the absence of E6-AP (*19*). Although GST-E6BP did not directly bind E6-AP, addition of HPV-16 E6 allowed coprecipitation of E6-AP (Fig. 2). Thus, HPV-16 E6, E6-AP, and E6BP can simultaneously be present in a complex, implying that the region of E6 that binds E6-AP is distinguishable from the E6BP interaction domain. Although HPV-16 E6 and E6-AP assemble with GST-E6BP, we have not been able to demonstrate entry of p53 into this complex.

Rabbit polyclonal antiserum against GST-E6BP immunoprecipitated a 50-kD protein from HeLa cells (Fig. 3A), which is probably identical to the 55-kD product ERC-55 (*16*). In primary human keratinocytes, the target cell of HPVs, and in human mammary epithelial cells that are susceptible to HPV and BPV-1 E6 immortalization (*20*), the antiserum recognized 50- and 48-kD proteins. The amount of the E6BP was similar in normal and immortalized cells (Fig. 3B). The antiserum immunoprecipitated proteins of similar molecular mass from murine cell lines, and the amount of E6BP in BPV-1 E6–transformed cells did not differ from that in the parental C127 cell line (*19*). Although these data imply that HPV-16 and BPV-1 E6 do not induce E6BP degradation, it remains possible that this occurs under specific conditions. It is also possible that E6BP attracts and stimulates degradation of other cellular proteins by mediating their entry into the E6–E6-AP–E6BP complex.

HPV-16 E6 protein has not been identified in complex with E6-AP and p53 in vivo, and similar experiments with E6BP have been unsuccessful. This is probably due to the low amount of E6 in a trans-

Table 1. Binding of E6BP to BPV-1 E6 mutants. E6BP binding and E6-AP binding are expressed as percent of wild-type BPV-1 E6, after subtraction of the background binding of each E6 mutant to GST or GST–E6-APΔ. Data represent two to four independent experiments.

E6 mutant	Amino acid change	Class*	Number of foci†	Growth in agar†	E6BP binding	E6-AP binding
Wild type			68	+++	100	100
212	$Ile^{41} \rightarrow Thr$	N	64	+++	128	90
473	$Cys^{128} \rightarrow Ser$	N	26	+++	105	70
228	$Arg^{46} \rightarrow Ser$ $Tyr^{47} \rightarrow His$	N	20	+	14	23
238	$Cys^{50} \rightarrow Gly$	Zn	14	+	12	10
139	$Cys^{17} \rightarrow Pro$	Zn	0	–	0	0
149	$Cys^{20} \rightarrow Ser$	Zn	0	–	0	0
247	$Cys^{53} \rightarrow Arg$	Zn	0	–	0	15
359	$Cys^{90} \rightarrow Ser$	Zn	0	–	0	0
367	$Cys^{93} \rightarrow His$	Zn	0	–	0	0
368	$Cys^{93} \rightarrow Ser$	Zn	0	–	0	11
403	$His^{105} \rightarrow Asp$	C	0	–	0	0
438	$Arg^{116} \rightarrow Ser$	C	0	–	0	17
460	$Cys^{124} \rightarrow Val$	Zn	0	–	0	4
471	$Cys^{127} \rightarrow Stop$	P	0	–	0	0
491	$Ser^{134} \rightarrow Stop$	P	0	–	0	16

*N, nonconserved amino acid mutations; Zn, zinc finger mutations; C, conserved amino acid mutations not in the zinc finger; P, premature termination. †Transformation data are from (*17*).

formed cell. By immunoelectron microscopy, ERC-55 was localized to the ER (*16*). By cell fractionation, both BPV-1 and HPV E6 proteins were distributed in nuclear and membranous compartments (*21*, *22*), the latter fraction consistent with their presence in the ER. Immunofluorescence studies with the monoclonal antibody C1P5, which is specific for both HPV-16 and HPV-18 E6, localized E6 to the cytoplasm (*23*). In preliminary experiments with C1P5 and affinity-purified rabbit antisera to E6BP, we observed colocalization with granular cytoplasmic fluorescence and perinuclear accentuation in HPV-16 E6–expressing Caski cervical carcinoma cells and HPV-16–immortalized mammary epithelial cells (*19*). A monoclonal antibody to the ER protein BiP produced an identical pattern. These results suggest that the association between E6 and E6BP is physiologically relevant.

The interaction between papillomavirus E6 and E6BP provides several leads for investigation. Calcium ions and calcium-binding proteins are involved in signaling cell growth and cell differentiation. Through interaction with E6BP, E6 may inhibit terminal differentiation of epithelial cells, establishing the necessary environment for viral DNA replication. E6 dramatically alters differentiation of keratinocytes (*24*), and keratinocytes immortalized by HPV-16 E6 and E7 are resistant to calcium-induced differentiation (*25*). Calcium is required for entry into mitosis, and E6 may play a role in this stage of cell growth. Transgenic expression of HPV-16 E7 in the murine ocular lens induces apoptosis, which is reduced in E6-E7 double transgenic mice (*26*). Adenovirus 19K E1b and Bcl-2 inhibit apoptosis, and these proteins bind to a putative calcium-binding protein that is localized to the nuclear envelope and ER (*27*). The E6-E6BP association may similarly prevent apoptosis of HPV-infected cells. Characterization of the E6-E6BP interaction should provide better understanding of p53-independent E6 activities.

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12. The DNA binding hybrid plasmid 16E6:E2R encodes HPV-16 E6 fused in-frame with the DNA binding domain of BPV-1 E2 (E2R). The *Saccharomyces cerevisiae* strain DL72 contains a 2-μm plasmid carrying the CYC-1 promoter with four E2 binding sites and a *lacZ* reporter. 16E6:E2R did not stimulate *lacZ* expression in DL72. DL72 cells containing the 16E6:E2R hybrid gene were transformed with a library of randomly primed HeLa cell cDNA fused to the VP16 transcription activation domain and a nuclear localization signal (*13*). Activation of the *lacZ* reporter required both 16E6:E2R and VP16:E6BP. As negative controls, plasmids encoding the BPV-1 E2 sequence used in the E6 chimera or a full-length transactivation-defective mutation in the BPV-1 E2 gene (E2-145R) did not stimulate *lacZ* expression when coexpressed with VP16:E6BP. E6Δ111-115:E2R encodes a p53-binding and degradation-defective HPV-16 E6 mutant (*28*) fused with E2R which, after cotransformation with VP16-E6BP into DL72, did not stimulate *lacZ* expression. These results indicate the specificity of the interaction between HPV-16 E6 and E6BP.
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29. Cells were metabolically labeled for 12 hours with 1 mCi of ^{35}S Translabel (ICN Biomedicals, Irvine, CA) per 10-cm dish in methionine and cysteine-free Dulbecco's minimum essential medium containing 5% dialyzed fetal calf serum. Cells were lysed at 4°C in 1 ml of radio immunoprecipitation assay (RIPA) buffer [150 mM NaCl, 50 mM tris-HCl (pH 8.0), 1% NP-40, 0.1% SDS, and 0.5% sodium deoxycholate] plus 2 mM DTT, 0.1% nonfat dry milk, and 1 mM PMSF. Insoluble debris was pelleted at 10,000*g* for 10 min, and the supernatant was incubated with appropriate antiserum and protein A–Sepharose beads. After they were washed with RIPA buffer, the beads were boiled in SDS sample buffer and loaded onto SDS-polyacrylamide gels.

30. We thank D. Lowy, C. Mansur, J. T. Schiller, and B. Schaffhausen for comments on the manuscript; D. McNealy and X. Lu for technical assistance; D. Birk for advice on immunofluorescence; R. Treisman and S. Dalton for the HeLa hybrid library; S. Reedy and X. Yang for advice on the two-hybrid system; and P. Howley, J. Huibregtse, K. Vousden, and D. Breiding for plasmids. Supported by National Cancer Institute grants RO1CA44174 (E.J.A.) and R29CA56803 (V.B.), American Cancer Society Faculty Research Award FRA-393 (E.J.A.), and Dermatology Foundation Marion Merrell Dow Pharmaceuticals Fellowship (J.J.C).

7 December 1994; accepted 17 May 1995