Identification of an α Helical Motif Sufficient for Association with Papillomavirus E6*

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We recently identified a cellular protein named E6BP or ERC-55 that binds cancer-related papillomavirus E6 proteins (Chen, J. J., Reid, C. E., Band, V., and Androphy, E. J. (1995) Science 269, 529–531). By construction of a series of deletion mutants, the region of E6BP that is necessary and sufficient for complex formation with human papillomavirus type 16 E6 has been mapped to a 25-amino acid domain. The corresponding peptide was synthesized and found by nuclear magnetic resonance spectroscopy to bind calcium and fold into a classical helix-loop-helix EF-hand conformation. Additional deletion mutagenesis showed that 13 amino acids that form the second α helix mediated E6 association. Alanine replacement mutagenesis indicated that amino acids of this helix were most important for E6 binding. Alignment of this α helical E6 binding peptide with the 18-amino acid E6 binding region of E6AP (Huibregtse, J. M., Scheffner, M., and Howley, P. M. (1993) Mol. Cell. Biol. 13, 4918–4927) and the first LD repeat of another E6-binding protein, paxillin (Tong, X., and Howley, P. M. (1997) J. Biol. Chem. 272, 33373–33376), revealed substantial similarities among these E6 binding domains. The extent of homology and the mutational data define the peptide as an E6 binding motif.

Papillomaviruses (PV)** are small DNA viruses that infect various epithelial tissues, including the epidermis and the epithelial linings of the anogenital tract. Human papillomaviruses (HPVs) that infect the anogenital tract can be classified as either high or low risk. The high risk HPV types, of which HPV type 16 (HPV-16) is the prototype, are strongly associated with the potential for development of cervical carcinoma (for review, see Ref. 4). Low risk types are also found in genital and cervical papillomavirus infections but rarely progress to cancer.

The transforming properties of HPVs reside in two genes, E6 and E7. The E6 and E7 genes are consistently expressed in HPV-positive cervical cancers and derived cell lines (5–7). They cooperate to immortalize primary human keratinocytes (8–13). HPV-16 E6 also cooperates with activated Ras in the transformation and immortalization of baby mouse kidney cells and baby rat kidney cells (14, 15). Independently of E7 or ras, HPV-16 E6 can transform NIH 3T3 cells (16), immortalize human mammary epithelial cells (17), and induce keratinocyte resistance to calcium and serum-induced differentiation (18). The activity of E6 in different biological assays implies it may influence diverse cellular pathways.

The ability of E6 protein to associate with the cellular tumor suppressor p53 has been suggested as the mechanism by which the viral protein promotes cell growth and proliferation (19). Although binding of high risk HPV E6s with p53 appears to be mediated by another cellular protein, E6AP (20), direct in vitro association of E6 with p53 has also been observed (21, 22). The complex of E6 and E6AP functions as an ubiquitin-protein ligase that results in the specific ubiquitination and subsequent degradation of p53 (23). Accumulating evidence suggests that E6 has functions independent of inactivating p53 in cellular transformation (24–36).

We have recently identified a cDNA encoding a cellular protein that binds papillomavirus E6 (E6BP or ERC-55) (1). E6BP was identified as a calcium-binding protein of the endoplasmic reticulum (37). The localization of E6BP is consistent with the localization of E6 to nonnuclear membranes (38). In vitro binding experiments demonstrated that E6BP interacted specifically with E6 proteins from cancer-related HPV types and the bovine papillomavirus type 1 (BPV-1). The transforming activity of a set of previously characterized BPV-1 E6 mutants correlated well with their E6BP binding ability. These results suggest that the E6BP interaction plays an important role for BPV-1 E6-induced transformation. More recently, it was reported that BPV-1 E6 associated with paxillin (39) as well as the trans-Golgi network-specific clathrin adaptor complex AP-1 (40) and that E6 proteins from some high risk HPVs interacts with the human homologue of the Drosophila discs large tumor suppressor protein hDlg (41, 42). These observations imply that E6 is capable of interacting with several cellular factors and participates in several pathways.

In the present study, we mapped the region of the E6BP that is critical for complex formation with HPV16 E6 and examined the structure of a peptide corresponding to this region by NMR spectroscopy. The amino acid sequence of E6BP predicts homology to EF-hand proteins. The EF-hand coordinates calcium with high specificity. It is named for a helix-loop-helix motif...
originally found in the crystal structure of parvalbumin and is usually present in two to eight copies arranged in pairs of interacting domains (for review, see Refs. 43 and 44), although there are some exceptions (45–47). The prototype calcium binding loop comprises 12 amino acids (D1\textsuperscript{X2}N3\textsuperscript{X4}D5G6\textsuperscript{X7}X8–11E12), where a single ligand is contributed to the calcium by each of the side chains of the first, third, and fifth positions in the sequence, one ligand is contributed by the backbone carbonyl of the 7th residue, and another two ligands are provided by the side chain of the 12th residue. In the NMR spectra of EF-hand-containing proteins, the NH of the invariant glycine residue at position 6 is observed to be greatly downfield-shifted (to \textsuperscript{10}ppm) on binding calcium and has been used as a reporter for the formation of a structured helix-loop-helix fragment (48). Different EF-hand motifs bind calcium with a wide range of affinities, with equilibrium dissociation constants from 10\textsuperscript{2} to 10\textsuperscript{5}M. The binding of calcium often causes a conformational change that alters the interaction with target proteins (43).

**EXPERIMENTAL PROCEDURES**

**Plasmids**—The plasmid encoding C terminus 211-amino acid fusion protein of E6BP and glutathione S-transferase (GST) (E6BP-211 or GST-E6BP) was described previously (1). Modified pGEX2T (Amersham Pharmacia Biotech) contains BamHI, XhoI, ClaI, SpeI, XbaI sites at the fusion point. Modified pGEX3X (Amersham) contains BamHI, XhoI, ClaI, SpeI, XbaI, KpnI, SpeI, and EcoRI sites at the fusion point. Plasmid pSPBPVE6 encodes BPV-1 E6 in a pSP65 vector. Plasmid pSP16E6 was obtained from Karen Vousden (49).

**Mutagenesis**—For GST-E6BP deletions E6BP-N, -N1, -N2, -M, -dlM1, and -dlM2, restriction endonuclease sites were used to delete coding sequences from GST-E6BP. E6BP-dlM was created by polymerase chain reaction amplification of GST-E6BP with the primers (CGATCGGGATCCGCTAGCATGTCCCCTATACTAGGT) and (GCGGATCCTCTTGAATGACAAATTCCG). The fragment was digested with MscI and BamHI and then used to replace the MscI-BamHI fragment in GST-E6BP. E6BP-C was constructed by insertion of a BamHI-EcoRI fragment from GST-E6BP into the BamHI and EcoRI sites of pGEX1 (Pharmacia). A HindIII-XhoI fragment of GST-E6BP was inserted into the modified pGEX2T to create E6BP-C1.
E6BP-EF4 was created by polymerase chain reaction amplification of E6BP-211 with primers (GGGGATCCCTGAGAACATTGCAGATTGCTCCACC) and (ATTCTCGAGCTACTTTTTTTTTGTAGCAGAAGCAGAGCAG) and (ATTCTCGAGCTACTTTTTTTTTGTAGCAGAAGCAGAAGCAGAGCAG). The fragments were digested with BamHI and XhoI and inserted into the modified pGEX3X. GST fusion proteins were expressed in Escherichia coli strain DH5α. One liter of LB media was inoculated with 100 ml of stationary culture and grown for 1 h before induction with 0.2 mM isopropyl-1-thio-

Protein Expression and Purification—GST fusion proteins were expressed in Escherichia coli strain DH5α. One liter of LB media was inoculated with 100 ml of stationary culture and grown for 1 h before induction with 0.2 mM isopropyl-1-thio-

Cells were harvested by centrifugation, re-suspended in 50 ml of low salt association buffer (LSAB, 100 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1% Nonidet P-40, and 1 mM phenylmethylsulfonyl fluoride) plus 0.03% SDS and 2 mM dithiothreitol, and lysed by sonication. After centrifugation at 10,000 × g for 10 min, the supernatant was collected and mixed with glutathione-Sepharose beads (Amersham). The mixture was subjected to rotary shaking for 2 h at 4 °C. The beads were then collected by centrifugation at 1000 × g for 2 min, washed three times with 20 volumes of LSAB, and stored at 4 °C. In vitro translated E6 proteins were prepared by using the rabbit reticulocyte lysate translation system (Promega) and [35S]-labeled cysteine (ICN, Irvine, California).

**In Vitro Association Experiment**—For in vitro binding, 30 μl of glutathione-Sepharose containing approximately 4 μg of GST fusion proteins were combined with 1–10 μl of [35S]-labeled in vitro translated proteins in LSAB in a total volume of 250 μl. The mixtures were subjected to rotary shaking for 3 h at 4 °C. The mixtures were then washed six times with LSAB, boiled in SDS-gel loading buffer, and electrophoresed on SDS-polyacrylamide gels. Gels were fixed and scanned by Molecular Imager (Bio-Rad).

**Peptide Synthesis**—A 31-amino acid residue peptide was synthesized using Fmoc (N-(9-fluorenyl)methoxycarbonyl) chemistry. The crude deprotected peptide was purified by phenyl-Sepharose chromatography (50) using a linear gradient of 0–100% B (Buffer A: 10 mM CaCl₂, 50 mM Tris, 500 mM NaCl, pH 8; Buffer B: 50 mM Tris, pH 8) and followed by high performance liquid chromatography on a preparative reverse phase C₁₈ column. The peptide was greater than 98% pure by analytical HPLC and by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectral analysis (observed 3689 ± 4, theoretical 3687).

**NMR Spectroscopy**—Samples of E6bp peptide were prepared at a concentrations between 1 and 4 μM (monomer) in 10 and 100% D₂O buffers containing 20 mM NaCl, pH 6.1 ± 0.1, and 0–10% trifluoroethanol (TFE). Spectra were recorded at 30 and 35 °C on a Bruker AMX-500 spectrometer with a proton resonance frequency of 500.14 MHz. The carrier frequency was set on the water resonance, which was suppressed using presaturation. One-dimensional NMR spectra were recorded with 128 summed scans, 2048 real points, and a spectral width of approximately 7000 Hz. The spectra were processed using sinebell window functions shifted by 45°, and the residual water resonance was

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**TABLE I**

| Residue | HN | Ha | Hb | Hy | Others |
|---------|----|----|----|----|--------|
| Glu-1   | –  |    |    |    |        |
| Phe-2   | 8.10 | 4.76 | 3.08, 3.03 | 2.37, 2.37 | δ, 7.21; ε, 7.15; ζ, 7.28 |
| Val-3   | 8.11 | 4.16 | 1.95 | 0.89, 0.89 |        |
| Ile-4   | 8.11 | 3.98 | 1.79 | 1.46, 1.12 | η2, 0.86; θ1, 0.80 |
| Gln-5   | 8.35 | 4.15 | 2.10, 2.06 | 2.37, 2.37 | ε, 7.51, 6.76 |
| Glu-6   | 8.00 | 4.22 | 2.08, 2.02 | 2.29, 2.29 |        |
| Ala-7   | 7.98 | 4.35 | 1.46 |        |        |
| Leu-8   | 8.12 | 4.17 | 1.69, 1.62 | 1.69 | δ, 0.81, 0.79 |
| Glu-9   | 8.22 | 3.98 | 2.08, 2.07 | 2.36, 2.34 |        |
| Glu-10  | 7.98 | 4.11 | 1.87, 1.84 | 2.12, 2.12 |        |
| His-11  | 8.03 | 4.63 | 3.33, 3.12 | 3.14, 3.14 | δ, 7.38; ε, 8.30 |
| Asp-12  | 7.73 | 4.76 | 3.90, 3.27 | 3.18, 3.18 |        |
| Lys-13  | 7.95 | 4.15 | 1.97, 1.92 | 1.64, 1.56 | δ, 1.70, 1.70; ε, 3.09, 3.09 |
| Asn-14  | 8.17 | 4.83 | 3.31, 2.91 | 3.19, 3.19 | δ, 7.97, 6.75 |
| Gly-15  | 7.66 | 3.92, 3.94 | 3.17, 2.53 |        |        |
| Asp-16  | 8.06 | 4.56 | 3.17, 2.53 |        |        |
| Gly-17  | 9.93 | 4.10, 3.48 | 2.95, 2.88 | 0.93, 0.83 | δ, 7.12; ε, 7.26; ζ, 7.38 |
| Phe-18  | 8.05 | 4.90 | 1.96 | 1.50 | δ, 0.73, 0.71 |
| Ser-20  | 8.78 | 4.70 | 4.49, 4.05 | 4.49, 4.05 | δ, 0.73, 0.71 |
| Leu-21  | 8.77 | 3.99 | 1.67, 1.52 | 1.50 | δ, 0.73, 0.71 |
| Glu-22  | 8.85 | 3.92 | 2.07, 1.98 | 2.37, 2.29 | δ, 0.73, 0.71 |
| Glu-23  | 7.79 | 4.05 | 2.70, 2.55 | 2.62, 2.33 | δ, 0.72, 0.69 |
| Phe-24  | 8.30 | 4.08 | 3.03, 2.95 | 3.03, 2.95 | δ, 6.72; ε, 6.88; ζ, 6.89 |
| Leu-25  | 8.56 | 4.03 | 1.78, 1.50 | 1.50 | δ, 6.72; ε, 6.88; ζ, 6.89 |
| Gly-26  | 7.75 | 3.92, 3.92 | 2.70, 2.55 | 2.70, 2.55 | δ, 6.80; ε, 6.62 |
| Asp-27  | 7.71 | 4.74 | 2.78, 2.70 | 2.78, 2.70 | δ, 6.80; ε, 6.62 |
| Tyr-28  | 7.73 | 4.32 | 1.64, 1.54 | 1.38 | δ, 3.04, 3.04; ε, 7.31, η, – |
| Arg-29  | 7.67 | 4.25 | 3.23, 3.18 | 3.23, 3.18 | δ, 7.25; ε1, 10.01; ε3, 7.58 |
| Trp-30  | 7.88 | 4.60 | 6.86 | 6.86 | ζ, 7.35; ζ3, 7.08; η, 7.08 |

* Tentative assignment. –, not observable. 1 mM peptide was in a buffer containing 20 mM NaCl, 60 mM calcium chloride, pH 6.0, 8% TFE, 35 °C.
removed with a gaussian convolution function. Two-dimensional NOESY and TOCSY spectra were recorded with mixing times of 75 or 125 and 40 ms, respectively (51). Sequence-specific resonance assignments were made by identification of intraresidue spin systems using the 'H–'H through-bond connectivities found in the TOCSY spectrum followed by sequential assignment of residues on the basis of sequential 
\(d_{\alpha N}\), 
\(d_{\beta N}\), and 
\(d_{\alpha \alpha}\) NOE cross-peaks using standard methods (52).

RESULTS

The E6 Binding Domain of E6BP Is a Specific EF-hand Motif—E6BP is a 317-amino acid protein that contains six putative EF-hands (37). The original yeast two hybrid isolate of E6BP that interacts in vivo with HPV 16 E6 encoded the C-terminal 211 amino acids of E6BP (1). This protein was shown to bind high risk HPV E6 and the transforming BPV-1 E6 in vitro. To determine the region of E6BP that interacts with E6, N-terminal, C-terminal, and internal in-frame deletions were introduced into the C-terminal 211 amino acids of E6BP (Fig. 1A). These were constructed either with existing restriction sites or by employing polymerase chain reaction. Mutant proteins were synthesized in E. coli as GST fusions. Equal amounts of GST-E6BP fusion proteins were assayed for their abilities to associate with in vitro translated 35S-labeled HPV16 E6. As shown in Fig. 1B, all constructs containing amino acid residues 194–218 were capable of binding to E6, whereas truncated forms of E6BP without this region could not bind. Amino acids outside this region may contribute to the interaction, as none of the deletion mutants bound as efficiently as E6BP-211. This E6 binding domain falls within the fourth EF-hand motif in E6BP and contains all the putative loop and flanking \(\alpha\)-helical sequences.

To confirm these results, we engineered an additional GST fusion that incorporated only 25 amino acids (residues 194–218) from the fourth EF-hand (E6BP-M) and tested it in the in vitro binding assay (Fig. 1C). We also prepared an internal deletion of the fourth EF-hand (E6BP-d/M) of the original 211 amino acid isolate of E6BP. As shown in Fig. 1D, E6BP-M efficiently bound to HPV-16 E6, whereas E6BP-d/M did not. A GST fusion protein containing the complete 36-amino acid fourth EF-hand of (E6BP-EF4) bound HPV-16 E6. The control GST fusion protein containing the 36-amino acid fifth EF-hand (E6BP-EF5) did not bind. These results demonstrate that the

FIG. 3. The NH–NH region of the two-dimensional NOESY spectrum. Resonance assignments were accomplished by tracing connections between the NH, \(\alpha\), and \(\beta\) protons of a residue \((i)\), and the NH of the next residue \((i + 1)\). The helical nature of this peptide is illustrated by medium and strong NH to NH NOE cross-peaks between residues adjacent in sequence. Sample conditions are given in Fig. 3, and the NOE mixing time was 125 ms.

FIG. 4. Summary of the conformational data. The sequence of the peptide, corresponding to residues 289–318 of E6BP, is renumbered as residues 1–31. The residues that ligate calcium are boxed. These residues form single ligands to calcium using their side chains, except for F18, which uses its backbone carbonyl, and E23, which contributes a double ligand. NOE cross-peaks describing NH-to-NH contacts, H\(\alpha\)–NH contacts, H\(\beta\)–NH contacts, and H\(\alpha\) to H\(\beta(i + 3)\) contacts are illustrated with the thickness of the filled-in bar corresponding to NOE intensity. The absence of a bar indicates that the NOE was not observed, whereas the presence of the dotted line indicates that the NOE was likely but was obscured by resonance overlap. A hollow bar indicates that the NOE involves a glycine residue and is not applicable. A positive index indicates \(\beta\) sheet, whereas a negative index indicates \(\alpha\) helix. A summary for the secondary structure of the peptide is shown under the conformational data.
Although only amino acids 194–218 were sufficient for binding E6, comparison to other helix-loop-helix EF-hand domains (43) suggested that formation of the first helix may require residues N-terminal to the 25-amino acid E6 binding domain. A peptide was therefore synthesized as a 31-amino acid fragment (residues 188–218 of E6BP) for analysis by NMR spectroscopy. For further discussion, this peptide is renumbered as residues 1 through 31 and named E6bd peptide.

The Peptide-containing E6 Binding Domain Folds into a Classical Helix-Loop-Helix EF-hand Conformation—In the absence of metal ion, the peptide adopted a mostly random-coil conformation, as evidenced by the lack of dispersion of resonances in the spectrum, noted particularly in the aromatic and upfield methyl-group regions and by the lack of downfield-shifted amide and Hα resonances (48). Upon addition of the first three or four equivalents of calcium to the sample, very few changes were noted in the spectrum. Significant spectrum changes were observed as the calcium-to-peptide mol ratio was increased to 20:1, including the downfield migration of the amide resonance of glycine 17 to a final position around 10 ppm (Fig. 2). No significant spectrum changes were noted as the concentration of metal ion was increased to a ratio of 80:1. The weak binding of calcium to E6bp, as observed by NMR, is consistent with results obtained for a similar peptide derived from other EF-hand-containing proteins, such as troponin C (48).

In the apo form, the resonance line widths were narrow (about 3 Hz) and consistent with a monomeric form of the peptide (53). In the calcium-bound state, resonances for the residues of E6bd peptide initially were broad (about 10–20 Hz) and more so for residues 17–19 (about 20–50 Hz), indicating oligomerization (data not shown). Several EF-hand peptides have been noted before to homodimerize and mimic the heterodimer detected in full-length proteins (43, 44, 48, 54, 55). Our data were consistent with homodimer formation (see below). In addition to dimerization, the E6bd peptide visibly aggregated upon binding calcium, consistent with the exposure of a significant hydrophobic patch. As has been observed before for other EF-hand-containing peptides, the addition of a small amount of the organic solvent TFE improved solubility and sharpened the resonances without appreciably changing resonance positions (56). Subsequent samples of E6bd peptide used between 5 and 10% TFE and 60 equivalents of calcium. However, line widths still remained slightly broader (10–20 Hz) than what would be expected (4–8 Hz) for a dimer of 7000 Da (53), probably because of residual aggregation.

The sequential assignment of the 1H NMR spectrum was carried out using the conventional two-dimensional NMR approach. TOCSY spectra were used to identify through-bond connectivities associated with spin systems of residues, and NOESY spectra were used to establish interresidue connectivities (52). The chemical shift assignments for the calcium-bound peptide are presented in Table I. Fig. 3 shows a contour plot of the NH to NH region of a NOESY spectrum and illustrates the connectivities associated with spin systems of residues, and the chemical shift data, from a qualitative analysis of this sort and can only be defined more precisely from a complete structure determination to be presented elsewhere. Our preliminary analysis of the NOE data showed contacts between residues and is consistent with an antiparallel dimer similar to the previously solved structures of EF-hand-containing proteins. A model of the E6bd peptide was prepared using the calcium-bound EF-hand pair observed in the 2.1-Å resolution structure of turkey troponin C (57). The EF-hands formed dimer contacts nearly throughout the entire length of the peptide sequence and was centered about a small β sheet formed using residues 18 to 20 of each monomer (Fig. 5).

The E6 Binding Domain Is a Short α Helical Peptide That Is Homologous to E6 Binding Regions Found in Other E6-binding Proteins—To further map the domain that is important for E6 binding, additional deletion mutants from the E6 binding domain (E6BP-M) were constructed and tested for E6 binding (Fig. 6A). Because BPV-1 E6 binds this domain with higher efficiency than HPV-16 E6, subsequent quantitative experiments were performed with BPV-1 E6. As shown in Fig. 6B, deletion of the first α helix (E6bd-20) did not affect binding nor did deletion of two additional amino acids from C-terminal end of E6bd (E6bd-18). More importantly, a peptide of 13 amino acids encompassing the second α helix (E6bd-13) retained the ability to bind E6, although at reduced efficiency. Notably, the majority of the loop region of the EF-hand motif was not present in this 13-amino acid peptide. The ability of the second α helix to bind E6 demonstrates that the interaction of E6BP with E6 is independent of calcium binding, as the first α helix and loop region from the EF-hand motif are both required for coordination of calcium.

Next, alanine replacement mutations in the E6 binding domain (E6bd) were constructed to define amino acids important
for E6 interaction within this α helix (Fig. 6A). Some mutations were also made in the surrounding regions. As expected, mutants E6bd-V19A, -S20A, -Y28A, -R29A, and -W30A, which are adjacent to the α helix, bound E6 at wild-type levels or with modest changes (Fig. 6B). The mutant E6bd-F18A showed some reduced binding (about 3-fold), probably reflecting the role of hydrophobic residue interactions between E6 and E6bd. Although some mutations within the α helix (E6bd-E22A and -E23A) showed modest changes of E6 binding activity, all other mutants within the α helical structure demonstrated substantial decreases. Consistent with the notion that hydrophobic residue interactions play important roles in E6-E6bd interaction, E6bd-L21A, E6bd-F24A, and E6bd-L25A were severely impaired for their ability to bind E6. Notably, a change of leucine to alanine at amino acid 25 (E6bd-L25A) totally abolished binding. Maintenance of E6 association by the mutant E6bd-E23A substantiated the prediction that the interaction of E6BP with E6 is independent of calcium binding, as glutamic acid 23 contributes two ligands to calcium. Finally, we created a leucine to proline change at amino acid 25, which was expected to disrupt the α helical structure of E6bd. The L25P mutation totally abolished E6 binding, indicating importance of the α helix. In summary, the sequence of amino acids in E6BP that bind E6 is FVSLEELGDLG, with the amino acids in bold most critical.

Previous studies of E6AP defined a span of 18 amino acids that is necessary for interaction with E6 (2). Comparison of the amino acid sequence of the α helical E6 binding domain of E6BP with this 18-amino acid E6 binding region revealed a striking degree of homology with a common motif, L(E/Q)E(F/L)LG(D/E) (Fig. 7A). Moreover, this motif has similarity to the LD1 motif in paxillin, which has been reported to be critical for BPV-1 E6 binding (3). Using a secondary structure analysis program (58), the E6 binding motif from E6AP and the paxillin LD motifs are each strongly predicted to form an α helix. We have thus identified an α helical E6 binding domain that is conserved among these E6-binding proteins. The consensus sequence is LhxXoxLx, where h denotes an amino acid that can make multiple hydrogen bonding interactions with its side chain, x is an amino acid with a small side chain, and X is any amino acid.

### DISCUSSION

The high risk HPV E6 proteins are consistently expressed in all HPV-positive cervical carcinomas. E6 is a small protein and is not known to possess intrinsic enzymatic activity. Similar to the oncoproteins of other tumor viruses, E6 is thought to exert multiple functions through interaction with cellular factors. Five proteins have been reported to associate directly with E6: E6AP (1, 20), E6BP (1), paxillin (39), hDlg (41, 42), and AP-1 (40). Although association with E6AP is believed to be necessary for E6 to target p53 for degradation, interaction with the...
other proteins, including E6BP, represents a p53-independent property of E6.

In the present study, we first mapped the region of the E6BP that is necessary and sufficient for complex formation with HPV16 E6 to a 25-amino acid residue domain. A peptide containing this E6 binding domain was synthesized and shown by NMR spectroscopy to bind calcium and fold into a classical dimeric helix-loop-helix conformation. Subsequently we determined that a smaller peptide of 13 amino acids containing the second α helix of the EF-hand retained the ability to bind E6. Smaller peptides have not been tested yet, so this may not be the minimal interaction domain.

Comparison of the α helical E6 binding domain identified in this study with the E6 binding region of E6AP and paxillin revealed strong homology. Alanine replacement mutagenesis studies in E6BP clearly demonstrated the importance of these common amino acids. The greatest effects on E6 binding resulted from substitutions of the hydrophobic amino acids and the negatively charged amino acid on one side of the α helix (Fig. 7B). Thus E6 can bind proteins bearing the sequence LhXdLs− (see above). Proteins that do not contain this sequence or ones that are not α helical do not bind E6 (Figs. 1D, and 7B). For example, in EF-hand 5 there is a proline at the s position of the consensus sequence, which is likely to disrupt the helical structure. In addition, a tryptophan occupies the negatively charged amino acid position of the consensus sequence.

EF-hand-containing proteins interact with their target proteins in different ways (43). In all cases, association is predominantly mediated through hydrophobic residues and is complemented by acidic side chains from the EF-hands interacting with basic amino acid residues of the target molecule. The consensus binding motif contains several hydrophobic residues and one negatively charged residue. We showed that the residues Leu-21, Phe-24, Leu-25, and Asp-27 were critical for E6 binding. The hydrophobic Phe-18 residue, although not essential, may enhance the interaction between E6 and E6 binding domain. Alternatively, it may contribute to dimerization of the domain. We observed that the E6bd domain peptide dimerizes on binding calcium, but the role of this dimerization on interaction with E6 is not understood. Since GST is dimeric, it is likely that the GST fusions to E6BP derivatives used in the binding experiments are also dimeric. The amino acid sequence of E6 itself predicts a pseudodimeric protein (59), and it could be that two E6 binding motifs simultaneously interact with E6. Our future experiments are aimed at examining the conformation of E6bd peptides while bound to E6.

Despite bearing EF-hand motifs, the mutational data show that E6BP does not require calcium for binding E6. In addition, we find that the extent of binding is insensitive to the addition of calcium (data not shown). Other EF-hand-containing proteins, such as calmodulin and calcykin, have been observed to interact with some target proteins in the calcium-free state (reviewed in Ref. 43). The other proteins that bind E6, E6AP, paxillin, and AP-1 have no known calcium binding motifs and are unlikely to bind calcium. We hypothesize that the E6/E6BP complex targets a regulatory protein in a calcium-dependent manner, although that protein has not yet been discovered.

The fourth protein that binds E6, hDlg, appears to operate by using a different mechanism (41). The hDlg protein does not contain the consensus motif. E6 binding has been mapped to interaction between its third PDZ domain and the C-terminal four-amino acid residues of HPV-16 E6. A PDZ domain is a protein recognition surface that recognizes peptides bearing the sequence Xaa-Thr/Ser-Xaa-Val-COO−. Although the C terminus of HPV-16 E6 contains the XTXL motif, BPV-1 E6 does not. Crystallographic analysis of a PDZ domain-peptide complex shows that the target peptide is bound in an extended conformation (60). The most recently identified E6-binding protein, AP-1, does not contain all elements of the consensus sequence, although the exact region required for interaction has not been defined (40). In contrast, based on our observa-
tions, we predict that E6AP, E6BP, and paxillin use an α-helical motif to bind E6. Identification of a conserved E6 binding motif provides a basis for structure-based drug design to block HPV-associated malignant transformation.

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