Association of Bovine Papillomavirus Type 1 E6 oncoprotein with the focal adhesion protein paxillin through a conserved protein interaction motif

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We have found that the E6 oncoprotein of Bovine Papillomavirus Type 1 (BE6) as well as the E6 protein of the cancer associated HPV-16 (16E6) interact with the focal adhesion protein paxillin. Mutational analysis of paxillin revealed that BE6 binds paxillin through small protein interaction motifs called LD motifs that have been previously identified as important in regulating association of paxillin with vinculin and focal adhesion kinase (FAK), and that BE6 can interact with at least two separate binding sites on paxillin. The LD motifs of paxillin that bind BE6 share homology with the E6 binding site of E6-AP, a ubiquitin ligase that together with 16E6 targets the degradation of the p53 tumor suppressor. Paxillin binding to BE6 excludes simultaneous binding to E6-AP. Mutational analysis of BE6 can distinguish the interaction of BE6 with E6-AP compared to paxillin and revealed that the interaction of BE6 with paxillin may be necessary for the induction of anchorage independent growth of cells by BE6.

Keywords: integrin; transformation; cytoskeleton; phosphotyrosine

Introduction

The papillomavirus E6 oncoproteins are small zinc-binding proteins that have been shown to function as adaptor proteins in altering the metabolism of the p53 tumor suppressor. E6 proteins from the cancer-associated human papillomaviruses (HPVs) complex with a cellular protein termed E6-AP, and together with E6-AP bind to the p53 tumor suppressor protein (Huibregtse *et al.*, 1991). Formation of the E6-E6AP-p53 complex leads to the degradation of p53 through ubiquitin-mediated proteolysis *in vitro* (Huibregtse *et al.*, 1993a). E6 interacts with a 18 amino acid sequence in E6-AP and functions as an adaptor protein in the complex with p53, inasmuch as E6-AP does not interact with p53 in the absence of E6 and the degradation of p53 requires both E6 and E6-AP (Huibregtse *et al.*, 1993b).

E6 genes from the non-cancer associated HPV types have shown surprising biochemical diversity compared to the cancer associated HPVs in that they have not been shown to bind E6-AP or degrade p53. Similarly, BPV-1 E6 (BE6), while binding E6-AP, fails to either complex with p53 or degrade other associated proteins in an *in vitro* degradation assay, implying that BE6

association with E6-AP may have functions other than the targeting of p53 (Chen *et al.*, 1995; Scheffner *et al.*, 1992).

The targeted degradation of p53 by cancer associated HPV-16 E6 is associated with the immortalization of primary breast epithelial cells by E6 (Dalal *et al.*, 1996) and the abrogation of cell cycle checkpoints by E6, but may not be related to the ability of E6 oncoproteins to induce anchorage independent growth. This is illustrated by E6 genes that do not target p53, such as BE6 and non-cancer associated HPV types that can induce anchorage independent growth in contact inhibited rodent cell lines, implying that the E6 oncoproteins have transforming functions independent of E6-AP or p53 association (Schiller *et al.*, 1984; Trujillo and Mounts, 1996; Yang *et al.*, 1985). Recently, the cancer associated HPV E6 proteins as well as BE6 have been shown to associate with a calcium-binding protein localized to the endoplasmic reticulum, called ERC-55 or E6-BP. A limited mutational analysis indicated that association of BE6 with ERC-55 correlated with transformation by BE6, yet the function of ERC-55 and the role it may play in transformation by BE6 is as yet undetermined (Chen *et al.*, 1995).

When anchorage dependent cells attach to substratum, a partly characterized series of events unfolds: cellular integrin molecules engage extracellular matrix components, resulting in tyrosine phosphorylation of proteins including pp125 focal adhesion kinase (FAK) and paxillin; this phosphorylation event is accompanied by the re-organization of actin filaments into stress fibers that terminate in discrete sites of adhesion between the membrane and the substratum, called focal adhesions (reviewed in Turner, 1994). The parallel tyrosine phosphorylation of FAK and paxillin, together with the reorganization of the actin cytoskeleton and the localization of FAK and paxillin in focal adhesions suggests that FAK and paxillin may be instrumental in the reorganization of the cytoskeleton in response to cell attachment.

How paxillin contributes to this process is unknown, but several features of paxillin suggest that it may play a regulatory rather than structural role in the re-modeling of the cytoskeleton. As would be expected for a regulatory protein, paxillin is considerably less abundant than structural cytoskeletal proteins such as talin or vinculin, yet paxillin directly associates with vinculin (Turner *et al.*, 1990; Wood *et al.*, 1994). Paxillin contains features of a regulatory protein, including SH2-binding and proline rich SH3-binding motifs (Salgia *et al.*, 1995a; Turner and Miller, 1994). Paxillin associates with or has been identified as a substrate of a number of provocative kinases and

oncogenes including the non-receptor kinases *v-src* (paxillin was first identified as a major tyrosine phosphorylated protein in *v-src* transformed cells (Glenney and Zokas, 1989)), *c-src* (Weng *et al.*, 1993), Csk (*c-terminal src* kinase) (Sabe *et al.*, 1994), FAK (focal adhesion kinase) (Bellis *et al.*, 1995; Hildebrand *et al.*, 1995; Tachibana *et al.*, 1995; Turner and Miller, 1994), the adapter protein *v-crk* (Birge *et al.*, 1993), and to BCR/ABL through its association with Crkl (Salgia *et al.*, 1995b). Phosphorylation of paxillin by FAK creates a SH2 binding motif that interacts with Crk (Schaller and Parsons, 1995). It has recently been shown that paxillin directly interacts with FAK and vinculin through short leucine rich peptide motifs termed LD motifs; there are four such motifs in paxillin, two of which can interact with FAK and vinculin (Brown *et al.*, 1996). The *c-terminal* portion of paxillin contains four LIM domains, one of which (LIM domain three) targets paxillin to focal adhesions through interactions with unidentified proteins (Brown *et al.*, 1996).

While paxillin is associated with proteins and events implicated in oncogenesis and re-modeling of the cytoskeleton, its role in these events is as yet undetermined. In *v-src* transformed fibroblasts there is loss of normal cell adhesion and cell spreading, *v-src* is localized to focal adhesions, paxillin and FAK are hyper-phosphorylated, and there is disruption of normal cytoskeletal focal adhesions. In contrast, when adherent cells bind to substratum, FAK and paxillin phosphorylation are associated with adherence to the substratum, cell spreading, and formation of focal adhesions. It is as yet unclear how to reconcile the role of paxillin in these two disparate observations.

In order to better understand the role of E6 oncoproteins in cell transformation, we sought to identify potential targets of the BE6 oncoprotein by performing a yeast two-hybrid search using a BE6 bait. We identified the focal adhesion protein paxillin as an interactor with both BE6 and the cancer-associated HPV-16 E6 (16E6). BE6 interacts with paxillin through at least 2 LD peptide motifs. The LD motifs of paxillin that interact with BE6 are homologous to the peptide motif of E6-AP that interacts with BE6. This identifies a small conserved protein interaction motif that interacts with E6 adaptor proteins, and indicates that LD domains may have a general role in mediating protein-protein interactions. An analysis of BE6 mutants is consistent with a role for the BE6-paxillin interaction in the induction of anchorage independent growth by BE6.

Results

Paxillin interacts with E6 oncoproteins

In order to identify proteins that interact with BE6 we performed a 2-hybrid interaction hunt. 1.4×10^6 human cDNAs were screened for interaction with a lexA-BE6 bait plasmid and failure to interact with lexA-BE7 (lexA fused to the BPV-1 E7 protein). BE7 was used as a non-specific bait in this assay as it contains a zinc-binding motif similar in structure to the two zinc-binding motifs of BE6. One colony that interacted with BE6 but not BE7 was determined by 5' and 3'

sequencing of the cDNA insert to contain a cDNA encoding for the first 463 amino acids of paxillin, with the cDNA extending five amino acids amino terminal of the initiating methionine. A full-length cDNA clone for chicken paxillin was expressed as a fusion to the B42 transactivation domain and tested for association with either BE6 or other non-BE6 lexA fusions in a 2-hybrid yeast interaction assay. Figure 1a shows that both paxillin and the E6-interacting protein E6-AP strongly interacted with BE6 in yeast. Paxillin did not interact with control lexA fusions including other zinc containing proteins (lexA-BE7, or lexA fused to the regulatory domain of casein kinase II). Although not visible in the Figure, longer incubation of the XGAL plate shown in Figure 1a did reveal a weak interaction between paxillin and HPV-16 E6 (16E6), that was confirmed in an *in vitro* interaction assay (Figure 1b).

The interaction between BE6 and paxillin observed in yeast was confirmed in an *in vitro* interaction assay shown in Figure 1b. The indicated proteins were expressed *in vitro* (Figure 1b upper panel) and tested for their ability to bind to a maltose-binding-protein/paxillin fusion (MBP-paxillin) immobilized on amylose-agarose beads (Figure 1b lower panel). Only BE6 and 16E6 were retained on the MBP-paxillin beads. Non-binding control proteins included the LIM-domain protein trip6 (Lee *et al.*, 1995) zinc-binding proteins (p53, E7 from BPV-1 (BE7)), the adaptor protein 14-3-3, and the E6-interacting protein E6-AP.

In order to determine if BE6 could interact with paxillin in mammalian cells, GST-BE6 or GST-BE7 was transiently overexpressed in CV-1 cells, the cells were lysed and the cell lysates incubated with glutathione agarose beads. Expression of GST-BE6 or GST-BE7 did not alter the amount or the mobility of paxillin by Western blot analysis (Figure 1c lanes 1–4). GST-BE6 retained paxillin on the glutathione-agarose beads while GST-BE7 did not (Figure 1c lanes 7 and 8). In experiments not shown here, GST-BE6 induces anchorage independent growth when expressed in mouse C127 cells (Vande Pol, S, unpublished observations). The blot shown in Figure 1c was subsequently reprobbed with a rabbit antibody to GST, confirming similar levels of expression of GST-BE6 and GST-BE7 (data not shown).

Figure 1 demonstrates that native paxillin interacts with both lexA and GST fusions of BE6 *in vitro* and *in vivo* (Figures 1a and c), and that native BE6 and 16E6 interact with paxillin fusions *in vitro* (Figure 1b). In order to determine if mammalian or yeast proteins are required to facilitate the BE6-paxillin interaction, MBP, MBP-paxillin, GST, or GST-BE6 were expressed in bacteria and the clarified but unpurified bacterial extracts were tested for the ability of BE6 and paxillin to interact *in vitro*. Figure 2 lanes 1–3 demonstrate that anti-paxillin MAbs only recognizes the MBP-paxillin fusion and its derived proteolytic breakdown products present in bacterial extracts and does not recognize either GST-BE6 or other bacterial proteins. In lanes 4–7 the indicated bacterial lysates were incubated together with glutathione agarose beads, and the washed beads were analysed for retained MBP-paxillin. GST-BE6 was able to associate with MBP-paxillin as well as with some of the MBP-paxillin degradation products, while GST alone showed no retention of MBP-paxillin (Figure 2 lane 4 compared to lane 6). This demonstrates that BE6 and

Figure 1 Paxillin association with BPV-1 E6 (BE6) *in vivo* and *in vitro*. **(a)** Association of paxillin and BE6 in yeast. The indicated lexA fusions (baits in **(a)**) were cloned in frame with lexA in the yeast two hybrid plasmid pEG202 and introduced into the yeast reporter strain EGY48 together with a lexA responsive reporter plasmid pSH18-34 (Gyuris *et al.*, 1993). BE6 refers to BPV-1 E6, BE7 to BPV-1 E7, 16E6 refers to the E6 oncogene of HPV-16, IRF2 refers to the transcriptional repressor IRF2 (Harada *et al.*, 1994), MEKK1 is a human kinase (the generous gift of Dr Dennis Templeton), 14-3-3, (a signal transduction adapter protein) and ck2-beta (regulatory domain of casein kinase 2) are cDNAs isolated in our laboratory but are homologous to published human cDNAs (Heller-Harrison *et al.*, 1989; Leffers *et al.*, 1993; Vande Pol, unpublished observations). All of the indicated baits were shown to make lexA fusion proteins of the predicted size by Western blot analysis of yeast protein extracts using an antibody to lexA (not shown). The prey plasmids noted in the Figure express fusions of the indicated cDNAs to the B42 transcriptional activation domain in the yeast 2-hybrid prey plasmid pJG4-5 (Gyuris *et al.*, 1993). EGY48 yeast strains expressing the indicated lexA fusions were mated to YPH499 yeast strains (Sikorski and Hieter, 1989) expressing the indicated prey plasmids. Diploids were isolated on selective media and replica-plated onto galactose plates containing XGAL as described (Gyuris *et al.*, 1993). **(b)** *In vitro* association of paxillin and E6 oncogenes. In the upper panel, the indicated proteins were *in vitro* transcribed and translated in rabbit reticulocyte lysates with 35S-cysteine, and 2 μ l of the translation reactions were analysed on 15% SDS-PAGE gels followed by autoradiography. The lower panel shows the same *in vitro* translation reactions as the upper panel after *in vitro* association with MBP-

paxillin are able to interact in the absence of other eukaryotic proteins, and indicates that BE6 can associate with paxillin that is not tyrosine phosphorylated.

Paxillin interacts with a subset of E6 proteins

In order to determine if the interaction of paxillin with E6 proteins is common among different papilloma-viruses, the E6 proteins from BPV-1 (BE6), HPV16 and 18, and the rabbit cotton tail papillomavirus (CRPV) were tested *in vitro* for interaction with MBP-paxillin fusion protein. CRPV expresses two E6 proteins, a short (CRPVSE6) and long E6 (CRPVLE6) with translation of the shorter version initiated from an internal methionine (Meyers *et al.*, 1992). Figure 3 demonstrates that only BE6 and 16E6 interacted with paxillin in this assay. Thus the interaction of BE6 with paxillin is neither universal nor a non-specific feature of papillomavirus E6 proteins.

Paxillin interacts with BE6 through two separate interaction sites

To identify domain(s) of paxillin that might interact with BE6, the full-length paxillin cDNA was cloned as an amino terminal fusion to the EE monoclonal antibody epitope in the plasmid pTM1 (Templeton, 1992). The resulting plasmid was digested with restriction enzymes and the restriction products *in vitro* transcribed and translated with 35-S methionine, generating *c-terminally* truncated paxillin proteins as shown in Figure 4a lanes 1–5. Lanes 1–5 show the *in vitro* translation products for each of the restriction digests, which were tested for association with immobilized GST-BE6, as shown in lanes 6–10. The first 116 amino acids of paxillin retain the ability to bind GST-BE6 *in vitro* (Figure 4a lane 10). In order to more finely map the association of BE6 and paxillin, MBP-paxillin and GST-paxillin fusion proteins containing deletions of paxillin sequences were tested for association with wild-type BE6 (Figure 4b). While the first 79 amino acids of paxillin could interact with BE6, amino acids 12–79 could not (Figure 4b lanes 8 and 9), defining critical E6 interaction residues in the first 12 amino acids of paxillin. Surprisingly, Figure 4b revealed the presence of at least one additional BE6 interaction

paxillin immobilized on agarose beads. The expressed cDNAs are the same as those described in **(a)** with the addition of the LIM domain containing protein trip6 (Lee *et al.*, 1995), and wild-type human p53. **(c)** *In vivo* association of paxillin and BE6. GST-BE6 or GST-BE7 were transiently expressed in CV-1 cells by vaccinia/pTM1 transfection as described (Elroy-Stein *et al.*, 1989). Shown is a Western blot probed with monoclonal antibody 165 to paxillin. Samples in lanes 1–4 are 20% of the solubilized total lysate in PBB buffer (0.15 M NaCl, 50 mM Tris-Cl pH 7.4, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.5 mM sodium vanadate, 0.5 mM DTT, 0.5 mM PMSF, 1% NP40, 1 μ g/ml aprotinin, 0.5 μ g/ml leupeptin) from a 6 cm plate, while lanes 5–8 are 80% of the same lysates after association of the lysate with glutathione agarose beads, washing of the beads in PBB and elution of the glutathione agarose beads with SDS-PAGE sample buffer. Lanes 1 and 5, mock infected; lanes 2 and 6, infected and transfected with empty pTM1 plasmid; lanes 3 and 7, infected and transfected with pTM1 plasmid expressing GST-BE6 fusion; lanes 4 and 8, infected and transfected with pTM1 plasmid expressing GST-BE7 fusion

Figure 2 Association of BE6 and paxillin expressed in bacteria. The indicated glutathione-S-transferase fusions (GST) or maltose binding protein fusions (MBP) were expressed in *E. coli*, and crude bacterial lysates were prepared by sonication of bacteria in PBS 1% Triton X-100 with 0.5 mM PMSF followed by centrifugation of the extracts in a refrigerated microfuge for 10 min. Lanes 1–3 are bacterial lysate input samples. For lanes 4–7, 5 μ l of the indicated bacterial extracts were incubated in 250 μ l of PBB at 4°C for 2 h followed by the addition of glutathione agarose beads for 30 min. The beads were washed 4 $\times$ with 1.0 ml of PBB and the retained proteins solubilized in SDS sample buffer, resolved on 12% SDS–PAGE gels, transferred to PVDF membrane, and analysed by Western blot with monoclonal antibody 165 to paxillin

motif between amino acids 114–313 (Figure 4b lanes 5 and 7), distinct from the interaction motif found in amino acids 1–79. Figure 4b also reveals a low level of interaction of BE6 with the MBP-paxillin LIM domain region (lane 3), a region that mediates paxillin interactions with focal adhesions.

E6-AP and paxillin are homologous in regions implicated in the binding of E6 proteins

Previous studies of E6-AP had defined a 18 amino acid region necessary and sufficient for the interaction of E6-AP and E6 (Huibregtse *et al.*, 1993b). This region was compared to paxillin amino acids 1–79 and 114–323, with the results shown in Figure 4c. Two small regions in paxillin containing limited homology to E6-AP were in regions of paxillin implicated in association with BE6 in Figure 4a and b. The homologous regions in paxillin to E6-AP had been previously identified as sequence motifs termed LD motifs, for which a consensus sequence is shown in Figure 4c. There are 4 LD motifs in paxillin; two (amino acids 144–156 and 266–278) were shown to mediate interaction with focal adhesion kinase (FAK) and one (amino acids 144–156) was also shown to interact with vinculin (Brown *et al.*, 1996). The homology between E6-AP and paxillin is strongest for LD1 and LD2; however the data in Figure 4b does not discriminate among these motifs for interaction with BE6. LD1 sequences in amino acids 1–12 are necessary for the binding of BE6 to GST-paxillin 1–79, (Figure 4b lanes 8 and 9), and in preliminary experiments not presented here, we have observed interaction of the first 15 amino acids of paxillin with immobilized MBP-BE6 *in vitro*.

Figure 3 Paxillin association with E6 oncoproteins of differing papillomaviruses. The upper panel shows the products of *in vitro* transcription and translation reactions from rabbit reticulocyte lysates programmed to express the indicated papillomavirus products. BE6 refers to BPV-1 E6, 16E6 to HPV-16 E6, 18E6 to HPV-18E8, CRPV SE6 to the short E6 protein from cotton tail rabbit papillomavirus (CRPV) and CRPV LE6 to the long E6 protein from CRPV (Meyers *et al.*, 1992). The lower panel shows the same lysates after *in vitro* binding to and washing of MBP-paxillin-agarose beads. Protein content was quantified by beta counting of the dried gels. The numbers below the lower panel are the per cent of input counts immobilized on MBP-paxillin beads

BE6 interaction with E6-AP and paxillin is mutually exclusive

The similarity in the interaction motif in Figure 4c implies that E6-AP and paxillin may bind to the same region of BE6. In order to determine if BE6, E6-AP and paxillin can co-exist in a tri-molecular complex *in vivo*, GST-BE6 and ee-epitope-tagged paxillin and E6-AP were overexpressed in CV-1 cells, and all three proteins were readily detected on Western blots (Figure 5a, b and c). Figure 5d lanes 1 and 2 demonstrate the presence both ee-paxillin and ee-E6-AP bound to GST-BE6. When paxillin is immune-precipitated, BE6 is detected (Figure 5g), while ee-E6-AP is not detectable (Figure 5f), despite the presence of more ee-E6AP than ee-paxillin in the lysate (Figure 5c). These results indicate that GST-BE6 preferentially associated with paxillin compared to E6-AP, and that a tri-molecular complex of paxillin, E6-AP and BE6 was not detected in these assays.

Interaction of BE6 with paxillin is associated with transformation

If the interaction of paxillin and BE6 is essential for transformation, BE6 mutants that are greatly decreased for association with paxillin should be defective for transformation. A panel of BE6 point mutants were selected in yeast and characterized for transformation (Ned *et al.*, 1997). These mutants were screened for association with E6-AP and paxillin *in vivo* by yeast two hybrid association and scored for induction of anchorage independent growth as shown in Figure 6.

c

E6-AP	...IPESSELT	LQELLG	EERR	418
PAXILLIN LD1	MDDL	DALLAD	LESTT	15
PAXILLIN LD2	E	LDRLL	ELNAVQ	156
PAXILLIN LD3	E	LDEL	MASLSDFK	278
PAXILLIN LD4	QLD	TMLG	SLQSDL	313
LD CONSENSUS		LDXLL	XXL	

Figure 4 Mapping the BE6 binding domain of paxillin. (a) GST-BE6 association *in vitro* with C-terminally truncated paxillin. The full length chicken paxillin cDNA (559 amino acids) was cloned as an amino terminal fusion to the EE monoclonal antibody epitope in the pTM1 plasmid to yield pTM1-EE-paxillin. This plasmid was then digested with restriction enzymes and the resulting digested plasmids *in vitro* transcribed and translated with 35-S methionine in rabbit reticulocyte lysate to generate run-off translation products, which were released from ribosomes with 0.1 μ g of puromycin. 10 μ l of the indicated programmed reticulocyte lysates were incubated in 250 μ l of PBB buffer with 1 μ g of GST-BE6 immobilized on glutathione agarose beads for 16 h. The washed beads were re-suspended in SDS sample buffer and the products visualized by 15% SDS-PAGE and auto radiography. Lanes 1–5 show 1 μ l of the input reticulocyte lysates. Lanes 2 and 7, pTM1-EE-paxillin digested with *Eco*RI; lanes 3 and 8, digested with *Sac*I; lanes 4 and 9, digested with *Pst*I; lanes 5 and 10, digested with *Bsr*GI. (b) BE6 interaction with GST-paxillin deletion mutants. The indicated purified MBP-paxillin fusions (lanes 2 and 3) or GST-paxillin fusions (lanes 4–9) were immobilized on maltose agarose or glutathione agarose beads respectively and incubated in 250 μ l PBB for 16 h at 4°C together with 10 μ l of rabbit reticulocyte lysate programmed to express 35-S labeled BE6. The beads were washed four times and re-suspended in SDS sample buffer with the eluted proteins resolved on a 15% SDS-PAGE. The upper panel shows the coumassie-stained gel, and the lower panel the auto radiogram of the same gel. Numbers below the auto radiogram show the per cent of input counts immobilized on the beads relative to MBP-paxillin 1-559 as determined by beta counting of the dried gel with a Packard Instant Imager. (c) Homology of paxillin to E6-AP in regions implicated in the binding of E6-AP to E6 oncoproteins. The 18 amino acids of E6-AP that associate with HPV-16 (Huibregtse *et al.*, 1993b) are shown aligned to chicken paxillin sequences. The numbers refer to the last amino acid in the shown segments

Figure 5 Binding of E6-AP and paxillin to BE6 is mutually exclusive. The indicated plasmids expressing GST-BE6, and E6-AP and paxillin tagged at the amino terminus with the ee MAb epitope (ee-paxillin and ee-E6-AP respectively) were co-expressed in monkey kidney CV-1 cells by vaccinia-pTM1 transfection (Elroy-Stein *et al.*, 1989). Cells were lysed in 0.5×PBB and clarified lysates either analysed directly by Western blot with the indicated antibodies (parts A, B and C) or subjected to immune precipitation with the indicated antibodies together with bridging rabbit anti-mouse and protein A sepharose (parts E, F, G and H). Monoclonal antibodies to paxillin (Transduction Laboratories), ee epitope (Walter *et al.*, 1990), and rabbit polyclonal antibody to BE6 (Ned *et al.*, 1997) were used for precipitation and detection. GST-BE6 and associated proteins were precipitated with glutathione-agarose beads (part D). Precipitates were analysed by SDS-PAGE followed by blotting to PVDF membranes and detection with the indicated antibodies. The migration of GST-BE6, ee-paxillin, ee-E6-AP and IgG heavy chain are indicated by arrowheads left of the blots

All BE6 mutants that were greatly diminished for interaction with either E6-AP or paxillin in yeast were defective for the ability to induce anchorage independence in the contact-inhibited C127 cell line. All of the BE6 mutants that retained interaction with E6-AP also retained association with paxillin in yeast. However, several mutants showed significant interaction with paxillin while not interacting with E6-AP (F37S, Δ 135-7, P137R, L83P), demonstrating that mutant BE6 proteins can discriminate between E6-AP and paxillin. Figure 6 reveals that only BE6 mutants R42W, K114E, R129S and Δ 131-133 could induce anchorage independence at near wild-type levels, and all of these mutants retained some association with both E6-AP and paxillin. Mutants showing association with paxillin *in vivo* while losing association with E6-AP (F37S, Δ 135-7, P137R and L83P) were all defective for anchorage independence. Interaction with both E6-AP and paxillin *in vivo* was necessary but not sufficient for inducing anchorage independence as BE6 mutants K35E, and R123H were defective for

B42-fusions			lexA fusions			% lacZ Activity			Transformation	
vector	E6-AP	Paxillin				vector	E6-AP	Paxillin		
			lexA			0	0	0		0
			BE6 wt			100	100	100		++++
			BE6-C50R			6.1	0	0		0
			BE6-Δ13-15			0	0	0		0
			BE6 Δ 22-24			0	0	1.3		0
			BE6 K35E			15	96	74		0
			BE6 F37S			0	0	48		0
			BE6 R42W			0	28	70		++++
			BE6 G48S			9.1	69	122		ND
			BE6 E61G			29	84	45		ND
			BE6 E101V			0	0	5.2		0
			BE6 K102E			0	0	9.5		0
			BE6 K114E			53	73	51		++++
			BE6 R116G			0	65	25		+/-
			BE6 R121G			0	0	4.1		0
			BE6 R123H			0	52	32		0
			BE6 C128R			9	71	88		ND
			BE6 R129S			92	70	93		++++
			BE6 Δ131-133/S134A			45	61	76		+++
			BE6 Δ135-137			9	0	26		0
			BE6 P137R			8	0	23		0
			BE6 L83P			0	0	22		0
			BE6 L86P			0	0	0		0
			BE6 L58S			0	0	0		0

Figure 6 *In vivo* interaction of paxillin with BE6 and BE6 mutants, and correlation with transformation. BE6 and BE6 mutants cloned as *lexA* fusions were introduced into the yeast reporter strain EGY48 together with the *lexA*-responsive *lacZ* reporter plasmid pSH18-34 (Gyuris *et al.*, 1993). The resulting yeast strains were separately mated to YPH499 yeast strains containing plasmids that express B42-transactivation domain fusions to either E6-AP, paxillin, or the empty plasmid (vector). The resulting diploids were isolated on selective plates and individually grown in liquid glucose selective media for 4 h prior to spotting of the yeast cultures onto XGAL-containing galactose plates (the B42 fusion plasmid is induced by galactose), for a graphic indication of the 2-hybrid associations. Quantitative *lacZ* assays were as described in the Materials and methods and in (Kippert, 1995). The results shown are the *lacZ* activity relative to that obtained with wild-type *lexA*-BE6 fusion. Because BE6-*lexA* fusions have endogenous transcriptional activation properties which are altered in BE6 mutants (Lamberti *et al.*, 1990), OD405 values obtained in cultures expressing the empty B42-fusion vector alone were subtracted from values obtained in the presence of B42-E6-AP or B42-paxillin fusions, to more closely approximate the *lacZ* activity resulting from the interaction of *lexA*-BE6 with E6-AP or paxillin. Transformation results reflect the induction of anchorage independent growth in nobel agar by BE6 mutants relative to wild-type BE6. Equal numbers of pooled G418-resistant mouse C127 cells 14 days after transfection with either BE6 or BE6 mutants expressed from the retroviral expression plasmid pLXSN were seeded into agar as previously described (Vande Pol and Howley, 1995). Cultures were scored for anchorage independence 10–14 days later. Results shown are the product of at least three separate transfections for each mutant and are expressed as anchorage independence relative to wild-type BE6. + + + + refers to colonies of equivalent size and at least 50% of the frequency of wild-type BE6; + + + refers to colonies of at least 20% the frequency and similar size as wild-type BE6; + + refers to colonies distinctly smaller than wild-type BE6 that arise at less than 10% the frequency of wild-type BE6; + / - refers to small colonies that arise at 1% or less than the frequency of wild-type; 0 refers to no colonies above that seen with the empty vector pLXSN. Pooled G418-resistant colonies transfected with wild-type BE6 induced anchorage independent colonies at 55–70% efficiency in these assays. The results shown are the results from Ned *et al.* (1997)

anchorage independence yet retained association with both E6-AP and paxillin.

BE6 transformed cells are altered in paxillin phosphorylation

In cells transformed by *v-src* or *bcr-abl*, tyrosine phosphorylation of paxillin is dramatically increased, contributing to speculation that paxillin may mediate some of these oncogene's effects. In order to determine if paxillin levels or phosphorylation are altered in BE6 transformed cells, mouse C127 cells were transfected wild-type BE6, mutant BE6 Δ13-15 (transformation defective and non-binding to paxillin) or vector alone. No difference was noted in paxillin content or phosphotyrosine levels as determined by Western blot (Figure 7a). In order to determine if changes in

phosphorylation of paxillin were associated with BE6 transformation of C127 cells, the same cell lines used in Figure 7a were metabolically labeled with ³²P-orthophosphate, paxillin was immune-precipitated and subjected to phospho-amino acid analysis as shown in Figure 7b. A twofold increase in phosphoserine and corresponding decrease in phosphothreonine content was observed in cells transfected with wild-type BE6 compared to the non-transforming BE6 mutant BE6 Δ13-15, but there was little difference in phosphotyrosine content.

Discussion

In this study we have found an association between the E6 oncoprotein and paxillin, a protein implicated in integrin signaling. Transformation of contact inhibited

Figure 7 (a) Levels of paxillin in BE6 transformed cells. Equal numbers of pooled G418 resistant C127 cells transfected with the retroviral expression in plasmid pLXSN expressing wild-type BE6 (pLXSN-BE6, lanes 1 and 5), a transformation defective BE6 mutant (pLXSN-BE6 Δ13-15, lanes 3 and 7) or the empty plasmid pLXSN (lanes 2 and 6) were lysed in PBB. The clarified lysates were analysed directly (lanes 1–3) or after immune precipitation with antibody to paxillin (anti-paxillin MAb, Transduction Laboratories) by SDS–PAGE followed by transfer to PVDF membranes and Western blot analysis with MAb to paxillin. In the lower panel, the same lysate samples first immune precipitated with anti-paxillin antibody were analysed for phosphotyrosine content utilizing a monoclonal anti-phosphotyrosine antibody by Western blot (MAb PY20, Transduction Laboratories). (b) Phosphorylation of paxillin in C127 cells and BE6 transformed C127 cells. Equal numbers of pooled G418 resistant C127 cells transfected with the retroviral expression plasmid pLXSN (lane 3), expressing wild-type BE6 (pLXSN-BE6, lane 1), or a transformation defective BE6 mutant (pLXSN-BE6 Δ13-15, lane 2) or the empty plasmid pLXSN (lane 3) were metabolically labeled with ^{32}P orthophosphate at 1.0 mCi/ml for 4 h prior to lysis in PBB. The lysates were pre-cleared with protein A/G sepharose and a non-specific mouse monoclonal antibody (anti-hemagglutinin MAb 12CA5) prior to immune precipitation of paxillin with mouse monoclonal antibody to paxillin. The immune precipitates were resolved on 10% SDS–PAGE gels and transferred to PVDF membranes which were analysed by autoradiography and scanned by a Packard Instant Imager to quantitate ^{32}P . The paxillin bands were excised and phospho-amino acids determined as described (Kamps and Sefton, 1989) and quantified by beta-scanning of electrophoresis plates. The relative phospho-amino acid content for each sample is indicated below the corresponding lane

cells by oncogenes is accompanied by changes in the cytoskeleton. Paxillin association with focal adhesion proteins (FAK and vinculin), its localization in focal adhesions, and its presence as a substrate of provocative oncogenes and proto-oncogenes (*v-src*, *c-src*, *v-crk*, *crkl*, *bcr-abl*) makes it plausible that paxillin exerts a regulatory role in the formation of focal adhesions, the remodeling of the cytoskeleton and transformation. We identified paxillin as an interactor with BE6 in a 2-hybrid screen in yeast. *In vitro*

association of paxillin and BE6 expressed in bacteria confirmed that the BE6-paxillin association is independent of the presence of other eukaryotic proteins (Figure 2). Like E6-AP, the association of paxillin with E6 proteins is restricted to a subset of papillomavirus types (Figure 3 and reference (Huibregtse *et al.*, 1993a)). This is another example of the surprising biochemical diversity exhibited by papillomavirus E6 proteins. Associations of E6-AP, ERC-55 and now paxillin have all demonstrated restricted interactions to only a subset of papillomavirus E6 proteins (Figure 3, and references (Chen *et al.*, 1995; Huibregtse *et al.*, 1993a)). While the role of paxillin and ERC-55 associations with E6 in the papillomavirus life cycle is as yet undetermined, E6-AP association is clearly implicated in the life cycle of papillomaviruses through the targeted degradation of p53. Yet E6-AP associates with E6 proteins from only a subset of papillomaviruses indicating that the biological importance of an association does not require that the association be universal among E6 proteins. This restriction of interactions to only a subset of E6 proteins is unlike papillomavirus E7 proteins as well as the TAg and E1a oncoproteins of papovaviruses and adenovirus respectively that interact with a common set of cellular proteins (Howley *et al.*, 1991).

There are several surprising features of the BE6-paxillin interaction. Homologous sequences between paxillin and E6-AP were implicated in the interaction of paxillin with BE6. These sequences in paxillin, previously called LD motifs, were first described as sequences in paxillin that bound to focal adhesion kinase and vinculin (Brown *et al.*, 1996). The homology between paxillin and E6-AP reveals that there is a protein interaction sequence common to E6-AP and paxillin that does not share all of the features of the LD motifs (Figure 4c). The further characterization of this sequence element may aid in the identification of additional E6 binding proteins, and determine if this motif is a general protein interaction motif or if this motif is common to proteins with shared functionality (such as the LXCXE motif found in proteins that interact with the retinoblastoma family of proteins (Harlow, 1990)).

How BE6 might interact with the LD-like motif in E6-AP and the LD motif in paxillin is unknown, but the mutational analysis performed here (Figure 6) indicates that sequences throughout the BE6 primary amino acid sequence contribute to interaction with both E6-AP and paxillin, implying that BE6 may adopt a complex folded shape to interact with paxillin, and not interact through a short peptide motif in BE6. The fact that BE6 can interact with more than one site on paxillin (Figure 4) makes the interpretation of the BE6-paxillin complex potentially complex: BE6 binding to paxillin may have different consequences depending upon the site bound, the BE6 binding sites on paxillin may vary if their affinity for BE6, and BE6 mutants may be differentially altered in their association with different sites on paxillin.

What might be the consequences of the BE6-paxillin association? In the case of E6-AP and p53, E6 acts as an adaptor protein bringing p53 into contact with the E6-AP ubiquitin ligase. Based upon this model, one might predict that BE6 is bringing a modifying function such as a kinase to paxillin or perhaps using

paxillin to gain access to an as yet identified protein. The alteration in paxillin phosphoserine content in BE6 transformed cells is suggestive of this possibility. Serine phosphorylation of paxillin has recently been described as a result of integrin signaling, indicating that phosphorylation of paxillin associated with the formation of focal adhesions can in some circumstances involve serine and not tyrosine residues (Bellis *et al.*, 1997; De Nichilo and Yamada, 1996). The change in paxillin phosphoserine content in BE6 transformed cells was significant but not dramatic, indicating that potential BE6 regulation of paxillin function through altered phosphorylation may be subtle, and/or restricted to particular sites of paxillin. We did not observe dramatic differences in focal adhesions or stress fibers in BE6 transformed C127 cells compared to untransformed C127 cells (unpublished observations). BE6 may compete with other cell regulatory proteins for binding to paxillin since the LD motifs of paxillin have recently been shown to mediate the interaction of paxillin with FAK and vinculin (Brown *et al.*, 1996). The BE6-binding LD1 at the extreme amino terminus of paxillin (amino acids 1–15) does not yet have an ascribed function, but it is close to tyrosine residues phosphorylated by FAK (amino acids 31 and 118) and *c-src* *in vitro* that create SH2-binding sites for the Crk adaptor protein (Bellis *et al.*, 1997; Schaller and Parsons, 1995).

The evidence that the association of BE6 and paxillin may play a role in induction of anchorage independence by BE6 is biologically plausible, given the association of paxillin with focal adhesions and the postulated role of paxillin in integrin signaling events. BE6 mutants that induce anchorage independence all associate with paxillin, and BE6 mutants that failed to associate with paxillin do not induce anchorage independence (Figure 6). The interaction of E6-AP and paxillin with BE6 is mutually exclusive *in vitro* (Figure 7), raising the possibility that the interaction of BE6 with paxillin may repress the interaction of BE6 with E6-AP, or *vice versa*. Alternatively, the interaction of BE6 with paxillin and E6-AP may be separate and complementing effects in the induction of anchorage independence by BE6.

Recently, another group has independently demonstrated the association of BE6 and HPV-16 E6 with paxillin after immune precipitation of BE6 and microsequencing of associated proteins (Tong and Howley, 1997).

Materials and methods

Plasmids

Paxillin clones: The full-length chicken paxillin cDNA clone (paxillin 11–559) (Turner and Miller, 1994) were per amplified and cloned into the *EcoRI* and *XhoI* sites of pJG4-5 to create a paxillin fusion to the B42 transactivation domain for expression in a yeast two hybrid system (Gyuris *et al.*, 1993). Maltose binding protein (MBP) paxillin fusion for expression in *E. coli* was created by per amplification of the full length paxillin cDNA and was cloned as a *EcoRI* to *BamHI* fragment into pMAL-cR1 (New England Biolabs). MBP-paxillin fusion amino acids 3231–559 was per amplified and cloned as a *EcoRI* to *BamHI* fragment into pMAL-cR1. GST-paxillin fusions

have been previously described (Brown *et al.*, 1996). The full-length paxillin cDNA from pJG-paxillin was cloned as a *EcoRI* to *XhoI* fragment into a modified pTM1-5' ee plasmid (Elroy-Stein *et al.*, 1989; Yan and Templeton, 1994), where paxillin is fused to the ee monoclonal antibody epitope (Walter *et al.*, 1990). GST fusions to BE6 and to BPV-1 E7 (BE7) were cloned into pTM1 for expression in mammalian cells by T7-vaccinia virus expression (Elroy-Stein *et al.*, 1989), and into pGEX-4T1 (Pharmacia) for expression in *E. coli*. BE6 point and deletion mutants are from (Ned *et al.*, 1997). For transformation assays, BE6 and BE6 mutants were expressed from the retroviral expression plasmid pLXSN (Miller and Rosman, 1989). BE6, BE7, and the E6 genes from HPV-16, 18, 11, and cotton tail rabbit papillomavirus (CRPV) were per amplified with primers containing 5' *EcoRI* and 3' *XhoI* sites and cloned into pTM1 for *in vitro* transcription and translation, and into pEG202 for expression as *lexA* fusions in yeast (Gyuris *et al.*, 1993). The cDNA for E6-AP was the generous gift of Dr John Huibregtse (Rutgers University), and was cloned into pTM1-5' ee for *in vitro* transcription and translation. A yeast 2 micron expression plasmid expressing *lexA* fusions from the ADH promoter with URA3 selection was created by per amplification of the 1301 nt *SphI* fragment containing the ADH promoter, *lexA* open reading frame and ADH1 terminator with 5' *SacI* and 3' *KpnI* cloning sites from the plasmid pEG202; the resulting per fragment was cloned into the *SacI* and *KpnI* sites of the yeast 2 micron URA3 plasmid pRS426 (Sikorski and Hieter, 1989), to create p1704A.

Cell lines, transfections and culture conditions

Monkey kidney CV-1 cells and mouse C127 cell lines were maintained in DMEM media supplemented with 10% newborn calf serum, glutamine and antibiotics. Vaccinia virus pTM1 transfections were performed as described (Rose *et al.*, 1991).

Yeast expression systems

BE6 and BE6 mutants were cloned as *lexA* fusions into the *lexA* DNA binding domain yeast expression plasmid pEG202 (Gyuris *et al.*, 1993). pEG202 and the *lexA* responsive LacZ reporter plasmid pSH18-34 were the generous gift of Roger Brent (Harvard University). Human E6-AP (provided by John Huibregtse, Rutgers University) was cloned as a fusion to the B42 transactivation domain in the yeast 2-hybrid prey plasmid pJG4-5 (Gyuris *et al.*, 1993). All yeast media, culture conditions and transfections were done with standard techniques (Ausubel *et al.*, 1995).

2-hybrid assay

The L40 strain of yeast (Hollenberg *et al.*, 1995) expressing a *lexA*-VP16 fusion was EMS mutagenized (Lawrence, 1991) and a blue colony *ura3* mutant was isolated on a selective plate containing uracil, FOA (Boeke *et al.*, 1987) and XGAL. The resulting strain of yeast was cured of the *lexA*-VP16 plasmid by growth in the presence of tryptophan, and a white colony was selected on a plate containing tryptophan, leucine, adenine, histidine, uracil, FOA and XGAL, resulting in the strain L40A5. L40A5 has the same genotype as L40 but is *ura3* from the EMS mutagenesis. A yeast cDNA expression library was created in the plasmid pJG 4-5 as follows: 5 µg of polyA-selected mRNA from a HPV-18 immortalized human keratinocyte cell line (Romanczuk *et al.*, 1991) was reverse transcribed utilizing both a random primer with a 3' *Xho* cloning site (GAGAGAGAGACTCGAGNNNNNNNN) and an

oligo dT primer with a 3' *Xho* cloning site, converted to double stranded cDNA with *Eco*RI adapters utilizing a commercial cDNA synthesis kit (Gibco-BRL), 5-methyl-dCTP, and ligated into *Eco*RI and *Xho*I cut pJG4-5 according to the manufacturer's directions and (Gubler and Hoffman, 1983). 1.4×10^6 primary colonies resulted from electroporation of the ligated cDNA. A lex-BE6 fusion in the yeast 2 micron URA3 plasmid p1704A was created by cloning BE6 in frame to lexA in the *Eco*RI and *Xho*I sites of p1704A, resulting in the plasmid p1704A-BE6. This was introduced into yeast strain L40A5. The human keratinocyte cDNA library in pJG4-5 was transfected into the resulting strain and colonies selected on CM plates minus histidine, uracil, tryptophan, and containing 1% galactose and 2% raffinose as the carbon source, and 50 mM 3-aminotriazole. Colonies were lifted on nitrocellulose filters from galactose-raffinose CM plates minus histidine and transferred to galactose plates containing XGAL. Blue colonies were picked and transferred to glucose CM plates containing histidine, uracil, FOA, and leucine to select for loss of the lexA-BE6 bait plasmid. The resulting colonies were mated to YPH500 yeast containing a lexA-BE7 on a URA3 plasmid, diploids were selected and screened on galactose plates containing XGAL. White colonies were selected, containing potential prey cDNA expressing plasmids that interacted with lexA-BE6 but not lexA-BE7.

Yeast beta-galactosidase assays

These were performed in 96 well microtiter plates as follows: Yeast were inoculated into 0.1 ml of selective media with raffinose as the carbon source diluted 1:10 with sterile water and grown to saturation overnight. (Raffinose is used instead of glucose to avoid repression of the prey plasmid *Gall* promoter by glucose). In the morning 0.05 ml of $3 \times$ selective media with raffinose as a carbon source was added to each well and the plates grown at 30°C for 3 h. 0.02 ml of 20% galactose was added per well to induce the galactose-inducible promoter on the prey plasmids and the cells were incubated at 30°C for another 6 h. The plates were then centrifuged, the media discarded, and the yeast re-suspended with 0.2 ml of sterile water. The plates were then read with a 96 well spectrophotometer at 650 nm to determine the yeast concentration in each well, and the plates subsequently re-centrifuged and the water discarded. The yeast were then re-suspended in 0.1 ml per well of beta galactosidase assay buffer (Z buffer) containing ONPG and 0.2% sarkosyl to permeabilize the yeast (Kippert, 1995). After the development of yellow color, the reaction was stopped with 0.1 ml of 0.1 M sodium carbonate, the plates were re-centrifuged, and 0.1 ml per well removed to a new microtiter plate and the plate optical density read at 410 nm.

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Immune precipitations and Western blots

Cells were lysed in 1.0 ml of PBB buffer (0.15 M NaCl, 0.05 M TrisCl pH 7.5, 50 mM NaF, 5 mM Na Pyrophosphate, 0.5 mM sodium vanadate, 0.5 mM DTT, 0.5 mM PMSF, 1% NP40, 1 µg/ml aprotinin, 0.5 µg/ml leupeptin) per 10^7 cells on ice for 30 min and the lysates clarified by centrifugation in a microfuge at 4°C for 10 min. Immune precipitation with monoclonal antibodies to paxillin (MAb 165 (Turner et al., 1990) and MAb to paxillin from Transduction Laboratories, Inc.) was performed with 0.5 µg of monoclonal antibody, bridging rabbit anti-mouse antibody and protein A sepharose beads. SDS-PAGE gels were transferred to PVDF membranes, probed with monoclonal antibodies to paxillin or phosphotyrosine (a mix of PY20, Transduction Laboratories, Inc., and Ab-2 from Oncogene Science); detection of bound antibody was with goat anti-mouse coupled to alkaline phosphatase and BCIP/NBT colorimetric detection.

In vitro translation and in vitro binding assays

In vitro coupled transcriptions and translations were performed utilizing standard nuclease treated reticulocyte lysate (Promega) according to the manufacturer's recommendations, supplemented with 1.5 mM $MgCl_2$, 2.5 mM nucleotide triphosphates and 25 units T7 RNA polymerase (Gibco-BRL) per 50 µl translation reaction. For *in vitro* binding assays approximately 1–5 µg of MPB or GST fusions immobilized on agarose beads were suspended in 150 µl PBB buffer (for MBP-paxillin fusions) or $0.5 \times$ PBB buffer (for GST-E6-AP fusions) and 10 µl reticulocyte lysate programmed to express the indicated proteins was added. The samples were incubated for 16 h at 4°C, and the beads washed three times with 1.0 ml of the appropriate binding buffer. Retained proteins were eluted with SDS sample buffer, resolved by 15% SDS-PAGE, fluorographed with salicylate (Chamberlain, 1979), and subjected to autoradiography. Where indicated, radioactive proteins were quantitated by gel scanning with a Packard Instant Imager.

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