The bovine papillomavirus type 1 (BPV-1) E6 oncoprotein can transform fibroblasts and induce anchor-age-independent growth and disassembly of the actin stress fibers. We have previously shown that the E6 protein interacts with the focal adhesion protein, paxillin, suggesting a direct role of E6 in the disruption of the actin cytoskeleton. We have now mapped the E6 binding sites on paxillin to the LD motif repeats region, which has been implicated in mediating paxillin binding to two other focal adhesion proteins, vinculin and the focal adhesion kinase. The five LD motif repeats identified in paxillin do not contribute equally to its interaction with E6. The first LD repeat is most critical for paxillin binding to E6 both in vitro and in vivo. Furthermore, the binding of recombinant wild-type E6 protein to paxillin blocked the interaction of several cellular proteins with paxillin, including vinculin and the focal adhesion kinase. A mutant E6 protein (H105S) which does not bind to paxillin had no effect on the binding of these cellular proteins to paxillin. These data suggest that E6 disruption of the actin stress fibers occurs through blocking the interaction of paxillin with its cellular effectors such as vinculin and the focal adhesion kinase.

Cellular adhesion to the extracellular matrix involves signals that are essential in many processes including cell morphology, cell division, cell motility, and tumor metastasis (for review, see Refs. 1–4). Focal adhesions are specialized structures where cells adhere to the extracellular matrix through a network of actin cytoskeleton (for review, see Refs. 5 and 6). Focal adhesions contain integrins, tensin, vinculin, the focal adhesion kinase (FAK), and paxillin. Paxillin is tyrosine phosphorylated in response to a variety of stimuli including cell adhesion, alterations in the actin cytoskeleton, and treatment with growth factors (7). Tyrosine phosphorylation of focal adhesion proteins is closely associated with changes in the structure of the actin cytoskeleton, although the precise downstream molecular consequences of such phosphorylation are currently unknown. Several lines of evidence have suggested that FAK is, at least in part, responsible for the tyrosine phosphorylation of paxillin observed in vivo (8, 9). Paxillin also binds to vinculin (10), an abundant cytoskeletal protein that is important in the assembly of the actin cytoskeleton, possibly through its interactions with other focal adhesion proteins, α-actinin, and actin (for review, see Ref. 6). The importance of a role for paxillin in cell signaling is suggested by the fact that paxillin is the target of several viral oncoproteins including v-Src (11), v-Crk (12), v-Abl (13), and the E6 oncoprotein of bovine papillomavirus type-1 (BPV-1) from our previous work (14). The interaction of paxillin with BPV-1 E6 is specific for transformation competent E6 proteins, and the expression of E6 results in the disruption of the actin stress fibers (14), suggesting that the binding of BPV-1 E6 to paxillin may be important to its oncogenic potential. Paxillin may also contribute to the tumorigenesis of human papillomaviruses (HPVs). Paxillin can bind to the E6 proteins of the cancer-associated type HPV16, but not of the nononcogenic types HPV11 and HPV6 (14). The protein levels of paxillin and FAK have also been shown to be up-regulated in cervical carcinoma cell lines and in cells immortalized by HPV18 (15).

Analysis of the primary amino acid structure of paxillin reveals several interesting motifs (13). The N-terminal half of paxillin has three potential SH2 binding domains (YXXP), the first two of which are implicated in paxillin interaction with v-Crk. Paxillin also contains one proline-rich SH3 binding domain, which is implicated in its interaction with Src. The C-terminal half of paxillin contains four LIM domains, which are cysteine-rich motifs found in two other cytoskeleton proteins, zyxin, and cysteine-rich protein, as well as in several proteins involved in development or differentiation (for review, see Ref. 16). One of the LIM domains (LIM3) has been shown to localize paxillin to focal adhesions, although the precise mechanism has not been determined (17). A structural element called the LD motif (LXXLXXL) has also recently been defined in the N-terminal half of paxillin. Paxillin contains multiple LD motif repeats, and the binding sites for vinculin and FAK on paxillin have been mapped to the regions containing the LD motifs (17).

In this report we extend our analysis of the interaction of BPV-1 E6 with paxillin and have mapped the E6 binding sites on paxillin to the LD motif repeat regions. Although our data showed that E6 can bind to multiple LD motif repeats on paxillin, the first LD repeat (LD1) appears to be the most critical for binding E6. Furthermore, binding of E6 to paxillin can block paxillin interactions with several cellular proteins including vinculin and FAK. Finally, we propose a model of E6 interfering with paxillin function by blocking its interaction with critical cellular proteins including FAK and vinculin.
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EXPERIMENTAL PROCEDURES

GST Fusion Protein Affinity Binding—The GST fusion proteins containing various regions of paxillin have been previously described in Salgia et al. (13) or were constructed by polymerase chain reaction using standard techniques (18). E6 protein was [35S]labeled in an in vitro translation reaction (Promega) and incubated with 2 g of GST-paxillin fusion proteins as described in Tong and Howley (14). Bound E6 proteins were washed in lysis buffer and analyzed by SDS-PAGE and autoradiography. The efficiency of binding of each construct was normalized against that of full-length paxillin. 30% of the input in vitro translated material was also included.

Immunoprecipitation of Cell Lysates—The hemagglutinin (HA) epitope (BAbCO) was fused to the C terminus of full-length paxillin or of paxillin deleted of LD1 by polymerase chain reaction and cloned into the pRC/CMV vector (Invitrogen). Plasmids encoding the HA-tagged paxillin molecules were electroporated into Cos-7 cells together with the FLAG-tagged E6 plasmid (14). At 40 h after transfection, cells were lysed in lysis buffer, and immunoprecipitations were carried out either for HA-paxillin using the monoclonal antibody HA.11 (BAbCO) or for FLAG-E6 using the monoclonal antibody M2 (IBI). The immunoprecipitates were separated by SDS-PAGE followed by immunoblot analysis using HA.11 or M2 antibodies.

Generation of Recombinant E6 Protein—A baculovirus vector expressing BPV-1 E6 protein was constructed by cloning the EcoRI-BamHI fragment containing the FLAG-E6 coding sequence from pSG5FLAG-E6 (14) into pVL1392 (PharMingen). The transfection and amplification of virus stock were carried out following the manufacturer’s instructions. To produce recombinant E6 proteins, Hi5 cells infected with pVL1392-E6 were harvested at day 5 postinfection and lysed in lysis buffer. The supernatants containing E6 recombinant proteins were separated on SDS-PAGE and visualized by autoradiography. The efficiency of binding was quantitated using NIH image 1.5 software.

RESULTS

Mapping of the E6 Binding Sites on Paxillin—We have previously demonstrated an interaction between paxillin and BPV-1 E6 in vitro and in vivo (14). To extend our analysis of the paxillin/E6 association, we have mapped the E6 binding site on paxillin. For this purpose, a series of GST-paxillin fusion proteins containing various regions of paxillin were used to bind [35S]labeled, in vitro translated BPV-1 E6. As shown in Fig. 1, the N-terminal half of paxillin (aa 1–325) efficiently bound to E6, whereas the C-terminal LIM domain did not. Studies with additional truncation and deletion paxillin constructs, however, suggested the possibility of multiple E6 binding sites within the N-terminal half of paxillin. For example, both aa 100–557 and aa 1–227 could bind to E6, suggesting that the overlapping region of the two constructs (aa 100–227) was responsible for the E6 binding. However, a paxillin construct (Δ100–227) deleted of aa 100–227 still bound to E6, implying that more than one region within the N-terminal half of paxillin could bind to E6.

The N-terminal half of paxillin has recently been shown to contain multiple LD motif repeats (17). Four LD motif repeats were identified in that initial study, and our analysis has revealed a fifth repeat at aa 216–227. These repeats (LD1–5), renumbered to include the fifth LD repeat, are shown in Fig. 2. To test whether the LD motif repeats were responsible for the multiplicity of E6 binding sites on paxillin, GST fusion proteins containing or deleted of the various LD repeats were next used in the binding experiments (Fig. 3). Deletion of LD1 (aa 11–557) decreased the E6 binding efficiency to about 40% of the full-length paxillin, implicating LD1 as a major component of the E6 binding site on paxillin. A fusion protein containing LD1 (aa 1–100) bound to E6 efficiently, whereas a similar construct with LD1 deleted (aa 11–100) did not bind to E6. These data suggest that LD1 accounts for more than half of the E6 binding activity of paxillin and that the remainder of the E6 binding activity is contributed by sequences in the N-terminal half of paxillin between aa 100 and 325.

To map which regions of paxillin were responsible for the residual E6 binding activity, fusion proteins containing various numbers of LD repeats were tested for their ability to bind to E6. As shown in Fig. 3, the fusion protein construct (aa 100–227) containing both LD2 and LD3 motifs could bind to E6, with an efficiency about 55% of the full-length protein. Both the LD2 and LD3 repeats appeared to be required for the binding, since each of the two fusion proteins (aa 100–227/1LD2, and 100–217) containing only a single LD motif bound E6 very poorly (less than 10% of the full-length protein). Surprisingly, unlike the deletion of LD1, deletion of LD2 and LD3 from either full-length paxillin (aa 1–557/Δ100–227) or from the N-terminal half of paxillin (aa 1–325/Δ100–227) had no effect on the efficiency of E6 binding, suggesting that the regions containing LD2 and LD3 motifs were able to bind to E6, but they were not necessary for paxillin binding to E6 when there were other LD repeats present. We next tested the role of LD4 and LD5 in mediating paxillin binding to E6. A fusion protein containing LD4 and LD5 (aa 227–325) bound to E6 with 70% of the...
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The E6 binding sites on paxillin are mapped to several regions containing the LD motif repeats. The methods are described in Fig. 1. The number of LD motifs and the efficiency of binding in each reaction are shown.

The Role of LD1 Motif in E6 Binding in Vivo—Since in vitro experiments suggested that LD1 was the most critical LD repeat in mediating E6 binding to paxillin, we next investigated the role of LD1 mediating E6 binding in mammalian cells. For these experiments, we employed a FLAG-tagged BPV-1 E6 construct which we have previously characterized and shown to be functional in cellular transformation assays (14). FLAG-tagged BPV-1 E6 was co-transfected into Cos cells with either HA-tagged full-length paxillin (1–557) or HA-tagged paxillin (11–557) deleted of LD1. Comparables levels of HA-tagged full-length paxillin and paxillin deleted of LD1 were expressed in the transfected cells as demonstrated by immunoprecipitation using the HA antibody (Fig. 4). Similarly, immunoprecipitation using the FLAG antibody (M2) showed that equal amounts of FLAG-tagged E6 were expressed in cells transfected with each of the two paxillin constructs. In agreement with our previous report, full-length paxillin coprecipitated with E6 (14). In contrast, only a small amount of paxillin (11–557) deleted of LD1 co-precipitated with E6 (Fig. 4). Quantitation of the immunoblot revealed that the amount of paxillin (11–557) complexed with E6 was about 10% of what had been observed with full-length paxillin. These data indicate that LD1 is the principal determinant for efficient binding of paxillin to E6 in vitro. Consistent with the results from the in vitro binding assays, deletion of LD1 did not completely eliminate E6 binding to paxillin, suggesting that the remaining LD motif repeats may also play a role in E6 interaction with paxillin in vitro.

The Effect of BPV-1 E6 on the Interactions between Paxillin and Cellular Proteins—There is evidence to suggest that the LD motifs also mediate paxillin binding to vinculin and FAK. Vinculin binds to a 21 aa stretch of paxillin (aa 143–164) which contains LD2, whereas maximal FAK binding requires aa 143–164 as the full-length protein. Similar to what has been observed with LD2 and LD3, deletion of LD4 and LD5 from the N-terminal half of paxillin had no effect on the efficiency of E6 binding (compare the binding result of aa 1–325 to that of aa 1–227). Taken together, the data showed that LD2–5 were responsible for part of the E6 binding activity, that LD2–3 and LD4–5 were redundant E6 binding sites, and that in the case of LD2–3, both motifs seemed to be required for E6 binding.

FIG. 3. The E6 binding sites on paxillin are mapped to several regions containing the LD motif repeats. The methods are described in Fig. 1. The number of LD motifs and the efficiency of binding in each reaction are shown.

FIG. 4. The LD1 motif is critical for the paxillin/E6 interaction in vivo. Cos cells were transiently co-transfected with FLAG-tagged E6 and HA-tagged full-length paxillin (1–557) or HA-tagged paxillin (Pax*) (11–557) deleted of LD1. Cell lysates were immunoprecipitated for E6 using the M2 antibody or for HA-paxillin using the HA.11 antibody. The immunoprecipitates were analyzed by immunoblot as indicated. The positions of paxillin, E6 and IgG heavy chain are indicated. Pax* is probably a degradation product of paxillin.

FIG. 5. BPV-1 E6 blocks the binding of certain cellular proteins to paxillin. A, GST-paxillin fusion protein was preincubated with increasing amounts (0–250 μl) of recombinant E6 proteins expressed from a baculovirus vector. 35S-Labeled cell lysates from Cos cells were mixed with the GST-paxillin fusion protein which had been preincubated with recombinant E6 proteins (wild-type E6 or a mutant E6 H105D). The bound cellular proteins were separated on SDS-PAGE and visualized by autoradiography. The positions of cellular proteins whose binding to paxillin were blocked by wild-type E6 are indicated. B, cellular proteins bound to GST-paxillin as described in A) were further subjected to immunoblot analysis for vinculin and FAK. (+), preincubation with insect cell lysates; (−), preincubation with 200 μl of insect cell lysates expressing wild-type E6. 164 as well as a region containing LD4 and LD5 (aa 265–313). Mutational analysis indicated that the conserved glutamic acid (Asp146) contained in LD2 is required for paxillin binding to vinculin (116 kDa) (10) and FAK (125 kDa) (8). We therefore studied the potential role of BPV-1 E6 to affect paxillin interaction with LD-binding cellular proteins. Recombinant BPV-1 E6 expressed from baculovirus vector infected Hi5 cells was preincubated with full-length GST-paxillin. The GST-paxillin preincubated with recombinant BPV-1 E6 was then used to precipitate cellular proteins from 35S-labeled Cos cell lysates. As shown in Fig. 5A, GST-paxillin bound to a number of cellular proteins, some of which (p200, p180, p120, p40) were blocked by wild-type recombinant E6. Equal amounts of a mutant recombinant E6 (H105D) which cannot bind to paxillin (14) did not block the binding of these cellular proteins to paxillin, indicating that E6 must physically interact with paxillin to block its binding to certain cellular proteins.

The identity of some of these cellular proteins was next examined. p200 is similar in size to a known paxillin binding protein talin (210 kDa) (13), whereas p120 is similar in size to vinculin (116 kDa) (10) and FAK (125 kDa) (8). We therefore tested the identities of p200 and p120 by immunoblot analysis. 35S-labeled cell lysates were mixed with GST-paxillin or GST-
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Paxillin preincubated with baculovirus lysates expressing recombinant E6 protein. The bound proteins were next analyzed by autoradiography and by immunoblot analysis. Antibodies against vinculin and FAK each reacted with a protein migrating at 120 kDa whose binding to GST-paxillin was blocked by recombinant E6 (Fig. 5B). Vinculin and FAK were further shown to comigrate with p120 by matching the autoradiography to the immunoblots. Therefore p120 was identified as a doublet of vinculin and FAK. p200, on the other hand, did not correspond to talin based on immunoblot analysis (data not shown). We are currently investigating the identity of the remaining cellular polypeptides whose binding to paxillin can be blocked by E6.

***DISCUSSION***

The focal adhesion protein paxillin is tyrosine phosphorylated in response to a variety of extracellular stimuli (7). It interacts with integrin (19) and several other focal adhesion proteins including vinculin (10), talin (13) and FAK (8). These properties suggest that paxillin may be a molecular adapter for the recruitment and organization of structural and signaling molecules at focal adhesions. Paxillin is also the target of a number of retroviral oncoproteins including v-Src (11), v-Crk (12), and v-Abl (13). We have recently shown that paxillin interacts with the E6 oncoprotein of bovine papillomavirus type-1 (14). In this report we have further characterized the interaction between paxillin and BPV-1 E6. We have mapped the E6 binding sites to the LD motifs located within the N-terminal half of paxillin, and provided evidence that E6 can block the binding of several cellular proteins to paxillin. We have further identified vinculin and FAK as two of the cellular proteins whose binding to paxillin is blocked by E6.

Paxillin contains several structural motifs which have been implicated in mediating protein-protein interactions (13) including the N-terminal SH2 and SH3 binding domains, and the C-terminal LIM domains. Recently the N-terminal half of paxillin has been shown to contain tandem LD motifs (17), which may represent a new class of protein-protein interaction motifs. Four such motifs were described in the original study and we have since identified a fifth LD motif. Results from the previous work (17) as well as from our data presented here strongly suggest that the LD motifs mediate paxillin interactions with vinculin, FAK and BPV-1 E6. Several lines of evidence indicate that the individual LD motif repeats are not completely redundant. LD1 is the most critical element in paxillin’s interaction with E6, whereas it is dispensable for FAK and vinculin binding (data not shown). In contrast, the binding of FAK and vinculin to paxillin involves different LD motif repeats. LD2 is necessary and sufficient for vinculin binding to paxillin, whereas LD2 and a region containing LD4 and LD5 are required for maximal FAK binding (17). The specificity of each repeat can be conferred by the nonconserved residues in the motifs, and may thereby determine the affinity of various interactions and the outcome of signaling events in response to different stimuli.

The observation that BPV-1 E6 can block paxillin interac-

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