MOLECULAR AND CELLULAR BIOLOGY, Nov. 2000, p. 8244–8253 Vol. 20, No. 21
0270-7306/00/$04.00+0
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Human Scribble (Vartul) Is Targeted for Ubiquitin-Mediated Degradation by the High-Risk Papillomavirus E6 Proteins and the E6AP Ubiquitin-Protein Ligase

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Received 17 April 2000/Returned for modification 24 May 2000/Accepted 31 July 2000

The high-risk human papillomavirus (HPV) E6 proteins stimulate the ubiquitination and degradation of p53, dependent on the E6AP ubiquitin-protein ligase. Other proteins have also been shown to be targeted for degradation by E6, including hDlg, the human homolog of the *Drosophila melanogaster* Discs large (Dlg) tumor suppressor. We show here that the human homolog of the *Drosophila* Scribble (Vartul) (hScrib) tumor suppressor protein is also targeted for ubiquitination by the E6-E6AP complex in vitro and that expression of E6 induces degradation of hScrib in vivo. Characterization of the E6AP-E6-hScrib complex indicated that hScrib binds directly to E6 and that the binding is mediated by the PDZ domains of hScrib and a carboxyl-terminal epitope conserved among the high-risk HPV E6 proteins. Green fluorescent protein-hScrib was localized to the periphery of MDCK cells, where it colocalized with ZO-1, a component of tight junctions. E6 expression resulted in loss of integrity of tight junctions, as measured by ZO-1 localization, and this effect was dependent on the PDZ binding epitope of E6. Thus, the high-risk HPV E6 proteins induce the degradation of the human homologs of two *Drosophila* PDZ domain-containing tumor suppressor proteins, hDlg and hScrib, both of which are associated with cell junction complexes. The fact that Scrib/Vart and Dlg appear to cooperate in a pathway that controls *Drosophila* epithelial cell growth suggests that the combined targeting of hScrib and hDlg is an important component of the biologic activity of high-risk HPV E6 proteins.

Human papillomaviruses (HPVs) infect basal cells of the cutaneous or mucosal epithelium, causing papillomas or warts on skin, genital tissues, and the upper respiratory tract. The viral life cycle is tightly coordinated with the differentiation program of the epithelium, and all layers of the normal epithelium are represented to various degrees in virus-producing lesions (32). Low-grade virus-producing lesions of the uterine cervix have an increased thickness of the basal cell layer, whereas in high-risk lesions the entire thickness of the epithelium consists of undifferentiated basal-cell-like cells (25). The high-grade lesions do not produce virus due to integration of the viral DNA into the host genome (44), and these lesions progress to carcinomas at a high frequency. The high-risk subgroup of HPV types detected in these lesions have been causally linked to the development of over 90% of uterine cervical carcinomas (53), the second leading cause of cancer-related deaths among women worldwide.

Two viral genes, the E6 and E7 genes, are expressed in virtually all HPV-containing cancers and are also sufficient for immortalization of cultured primary genital keratinocytes (12, 33), suggesting that E6 and E7 are cooperating viral oncoproteins. Like the oncoproteins of certain other DNA tumor viruses, the best-understood functions of E6 and E7 in promoting cellular immortalization are inactivation of the p53 and pRB tumor suppressor proteins, respectively (14). The high-risk HPV E6 proteins inactivate p53 by stimulating its ubiquitin-mediated degradation, dependent on E6AP, a cellular ubiquitin-protein ligase (16). Ubiquitin-protein ligases, or E3 activities, are the primary substrate recognition components of the protein ubiquitination machinery. E6AP serves as the last link in a cascade of thioester-linked ubiquitin transfers from the E1 ubiquitin-activating enzyme to an E2 ubiquitin-conjugating protein and finally to E6AP, which then directly catalyzes the formation of isopeptide bonds between ubiquitin and lysine residues of the substrate (41). E6AP can conjugate multiple ubiquitin molecules to substrates in the form of isopeptide-linked chains, resulting in the eventual recognition of the substrate by the 26S proteasome. Other classes of E3 proteins do not appear to function via a ubiquitin-thioester intermediate but probably function more as docking proteins for activated E2 proteins and substrates, allowing substrate ubiquitination to be catalyzed directly by the E2 protein (42).

E6AP defines a class of ubiquitin-protein ligases called HECT E3s (homologous to E6AP carboxyl terminus), each member of which has a conserved 350-amino-acid carboxyl-terminal catalytic domain, containing the active-site cysteine residue at which ubiquitin-thioester formation occurs (15). HECT E3s are large proteins, from a minimum of 92 to over 500 kDa, and characterization of E6AP and other HECT E3s suggests that the large and divergent amino-terminal domains mediate substrate recognition, while ubiquitination of bound substrates is catalyzed by the HECT domain. The HECT domain contains all of the determinants necessary for thioester formation, including the binding site for the E2 protein (13). A handful of proteins have been proposed to be natural (E6-independent) substrates of E6AP, including the human homolog of the *RAD23* protein (27), the src family kinase blk (37), and the MCM7 subunit of replication licensing factor (26). Interestingly, disruption of E6AP expression in the hippocampal and Purkinje neurons of the brain appears to be the cause of Angelman syndrome (AS), a severe mental retardation and coordination disorder, suggesting that lack of ubiq-

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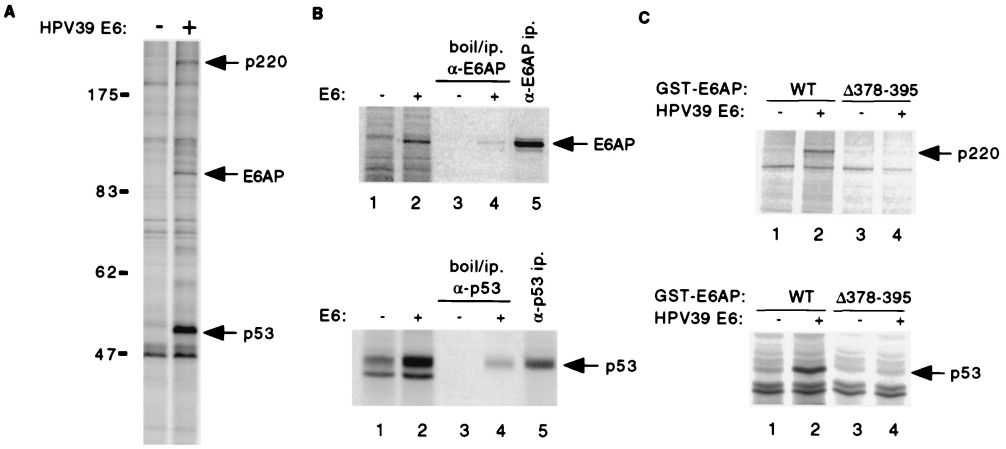

FIG. 1. Biochemical screen for E6-dependent E6AP binding proteins. (A) GST-E6AP on glutathione-Sepharose beads was incubated with ^{35}S-labeled whole-cell extract from C-33A cells, without (−) or with (+) partially purified baculovirus-expressed HPV39 E6 protein. Bound proteins were detected by SDS-PAGE and autoradiography. Molecular weight markers are indicated, as well as positions of p220 and proteins that likely correspond to p53 and E6AP. (B) The identities of the bands shown in panel A as p53 and E6AP were confirmed by performing binding experiments in duplicate, releasing the bound proteins in SDS-PAGE loading buffer, and then either running the proteins directly (lanes 1 and 2) or immunoprecipitating (ip) then with anti-p53 or anti-E6AP antibody (lanes 3 and 4). Lane 5, direct immunoprecipitation of the proteins from the labeled extract. (C) Binding experiment as in panel A with GST-E6AP (wild-type [WT]; lanes 1 and 2) or GST-E6APΔ378-395, which had the E6 binding domain deleted (lanes 3 and 4).

uitination of one or more E6AP substrates in the brain leads to the phenotypes associated with AS (6, 21, 31). A critical AS-related substrate, however, has not yet been identified.

The high-risk HPV E6 proteins bind to E6AP within its amino-terminal substrate recognition domain, and formation of a stable E6-E6AP complex precedes association with p53 (17). There is no biochemical evidence indicating that p53 is a normal substrate of E6AP (40, 48), suggesting that E6 functions to redirect the substrate specificity of E6AP. Several studies have shown that high-risk HPV E6 proteins have p53-independent activities that may yet be dependent on E6AP, suggesting that E6 may direct E6AP to cellular proteins in addition to p53. E6 has been shown to induce telomerase activity (24), and a mutation in HPV type 16 (HPV16) E6 (SAT_{8-10}) that renders it unable to target p53 leaves intact the ability to induce telomerase activity and to immortalize mammary epithelial cells (22). The SAT_{8-10} mutant interacts with E6AP equivalently to wild-type HPV16 E6, consistent with the possibility that the activities of this protein are related to the targeting of other cellular proteins by the E6-E6AP complex. E6 also confers resistance to serum and calcium-induced differentiation of human genital keratinocytes (46), and analysis of E6 mutants suggested that inhibition of differentiation was not entirely accounted for by p53 inactivation (45). Transgenic mice expressing HPV16 E6 in the basal layer of the skin display cellular hyperproliferation and epidermal hyperplasia and develop malignant skin cancers. The fact that p53-null mice do not exhibit epidermal hyperplasia or cellular hyperproliferation suggests that these activities of E6 are not simply a result of p53 inactivation (47). Finally, the facts that bovine papillomavirus type 1 (BPV-1) E6 interacts with E6AP but does not inactivate or stimulate the ubiquitination p53 and that the ability of BPV-1 E6 to bind E6AP correlates with transforming activity (2, 35) further suggest that E6-E6AP complexes have targets other than p53.

HPV E6 proteins have been shown to promote the degradation of proteins other than p53, including hDlg (10), the human homolog of the *Drosophila melanogaster* tumor suppressor Discs large (Dlg). E6 expression also induces the degradation of E6TP1 (9), Bak (49, 50), and myc (11). Degradation of one or more of these proteins may be linked to the additional biologic activities of the high-risk HPV E6 proteins. We describe here the results of a biochemical screen designed to identify cellular proteins targeted for ubiquitination by the high-risk HPV E6 proteins, dependent specifically on E6AP. This has led to the identification of the human homolog of *Drosophila* Scribble (Vartul), hScrib, as a target of the E6-E6AP complex. Mutations in *Drosophila* Scrib result in a thickening of the basal cell layer of the differentiating epithelium and disorganization of the epithelium (1). P-element insertions in Scrib also result in tumors in brain hemispheres and some imaginal discs, and, furthermore, Scrib tumorigenesis can be suppressed by overexpression of Dlg (B. Mechler, personal communication). This suggests that these PDZ domain proteins, as well as lethal giant larvae (Lgl) (1a), cooperate in a pathway that controls epithelial cell polarization, growth, and differentiation in *Drosophila*.

MATERIALS AND METHODS

Plasmids and protein expression. HPV16 and 11 E6-expressing plasmids and p53-expressing plasmids for in vitro transcription have been described previously (52). Carboxyl-terminal mutations and the SAT_{8-10} mutation in HPV16 E6 were created by PCR and confirmed by DNA sequencing. The HPV39 E6 open reading frame (ORF) was isolated by PCR from cloned viral DNA (gift from G. Orth, Pasteur Institute, Paris, France) and subcloned into the pVL1393 baculovirus transfer vector for production of recombinant baculovirus. Plasmids for glutathione *S*-transferase (GST)–E6AP expression in *Escherichia coli* and baculovirus expression of E6AP have been described previously (17).

A plasmid containing cDNA KIAA0147 was provided by the Kazusa DNA Research Institute. The complete ORF or fragments were isolated by PCR and subcloned into pGEX-6p-1 (Pharmacia) for GST fusion protein expression. The ORF was also subcloned into pBluescript (with an artificially inserted ATG initiating methionine codon) for in vitro transcription, into pCDNA3 (Invitrogen) with a FLAG-encoding epitope at the 5′ end for mammalian cell transfections, and into pEGFP-C1 (Clontech) for green fluorescent protein (GFP) fusion protein expression in mammalian cells.

Recombinant baculovirus for HPV39 E6 was produced using the BaculoGold system (Pharmingen) in High5 insect cells (Invitrogen). Protein was isolated from infected cells 48 h postinfection and partially purified by cation-exchange chromatography on Bio-Rad MacroPrep S. Baculovirus E6AP was partially purified by anion-exchange chromatography as previously described (17). GST fusion proteins were expressed and purified with glutathione-Sepharose according to the manufacturer's recommendations (Pharmacia). Human E1 ubiquitin-

activating enzyme was expressed and purified from insect cells, and UbcH7 E2 protein was expressed in *E. coli* (36).

Biochemical screen and protein purification. For small-scale screening (Fig. 1), a single 10-cm-diameter plate of confluent C-33A cells was labeled with [^{35}S]methionine for 4 h, and extract was made in buffer containing 100 mM Tris (pH 8.0), 100 mM NaCl, and 1% NP-40. Associated proteins were isolated with 100 ng of GST-E6AP immobilized on glutathione-Sepharose, with or without HPV39 E6 protein (100 ng). Secondary immunoprecipitations (Fig. 1B) were done by releasing bound proteins in 20 μl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer and transferring the supernatant to a new tube and diluting it with water to 1 ml. Anti-p53 antibody (Oncogene Science) or anti-E6AP polyclonal antibody was added, and complexes were isolated with protein A-Sepharose (Pharmacia). Large-scale purification of hScrib utilized 24 15-cm-diameter plates of confluent C-33A cells as the source of protein. Whole-cell extract was precleared with 10 μg of glutathione-Sepharose-bound GST-E6AP with the E6 binding domain deleted. The supernatant was incubated at 4°C for 4 h with 10 μg of GST-E6AP and approximately 20 μg of HPV39 E6 protein. The beads were collected and washed, and bound proteins were released in SDS-PAGE loading buffer. Approximately 1 to 2 μg of the 220-kDa protein was isolated from the gel and subjected to tryptic digestion and matrix-assisted laser desorption ionization mass spectrometry protein identification (HHMI Biopolymer-Keck Foundation Biotechnology Resource Laboratory, Yale University).

In vitro binding and ubiquitination assays. In vitro translations were performed in either wheat germ extract or rabbit reticulocyte lysate systems (TNT systems; Promega) in the presence of ^{35}S-labeled methionine. Five to 10 μl of translation reaction mixture was utilized per binding assay, along with 100 ng of GST fusion protein bound to glutathione-Sepharose. Binding reactions were done in buffer containing 9 parts 25 mM Tris (pH 8.0)–125 mM NaCl and 1 part cell lysis buffer (described above) in a total reaction volume of 250 μl. Reaction mixtures were rotated at 4°C for 1 h, glutathione-Sepharose beads were washed three times with cell lysis buffer, and proteins were released in SDS-PAGE loading buffer for analysis by SDS-PAGE and autoradiography.

In vitro ubiquitination assay mixtures contained 5 μl of translation reaction mixture (either wheat germ or rabbit reticulocyte lysate), 2 mM ATP, and 3 μg of ubiquitin (Sigma) in 75 μl of 25 mM Tris (pH 8.0)–125 mM NaCl–2 mM $MgCl_2$–50 μM dithiothreitol, with exogenous baculovirus human E1 and UbcH7 protein added and with or without baculovirus HPV16 or -39 E6 and/or E6AP. Reaction mixtures were incubated for 30 min at room temperature before being analyzed by SDS-PAGE and autoradiography.

Mammalian cell transfections and microscopy. For in vivo hScrib analyses, 1 μg of pCDNA3-FLAG-hScrib plasmid was transfected into 293-T cells, with or without increasing amounts of HPV16 E6 expression plasmid (from 0.5 to 2.0 μg), keeping the total plasmid amount at 3 μg by transfection with the appropriate amount of pCDNA3 vector plasmid. Cell extracts were analyzed by SDS-PAGE and immunoblotting with anti-FLAG antibody (Sigma). The relative half-lives of hScrib in the absence and presence of HPV16 E6 (0.5 μg of E6-encoding plasmid) were determined by cycloheximide chase 24 h posttransfection (final concentration, 10 μg/ml). The effect of proteasome inhibitor was determined 24 h posttransfection after 3 h of treatment with 10 μM MG132 (Calbiochem).

For fluorescence microscopy, subconfluent cultures of MDCK cells were grown on coverslips in minimal essential medium with 10% fetal bovine serum overnight. Cells were transfected with 1 μg of GFP-hScrib-expressing plasmid, pEGFP-C1 vector, and/or pCDNA-HPV16 expressing E6 using the calcium precipitation method or Lipofectamine (Gibco-BRL). For immunofluorescence visualization of ZO-1 or combined GFP–hScrib–ZO-1 visualization, cells were washed three times with phosphate-buffered saline (PBS) and then fixed for 10 min with 3.7% formaldehyde in PBS. Cells were then washed three times with PBS, rinsed with distilled water, and then permeabilized with acetone at −20°C for 10 min. Cells were washed with PBS and incubated with anti-ZO-1 antibody (Zymed) in PBS for 30 min at room temperature. Cells were then washed with PBS, incubated with rhodamine-conjugated secondary antibody (ICN), and then washed three times with PBS, mounted on a glass slide, and examined by fluorescence microscopy with an Olympus IX70 microscope. Images were captured with a cooled charge-coupled device camera (Photometric) and processed with MicroTome image deconvolution software (VeyTek). Laser-scanning confocal microscopy was performed with a Zeiss LSM 410 microscope at the Robert Wood Johnson Medical School Electronic Imaging Center.

RESULTS

Biochemical screen for E6-dependent E6AP substrates. We examined the spectrum of cellular proteins that could be isolated from cells with a GST protein fused to E6AP (GST-E6AP) in the absence or presence of HPV39 E6. HPV39 E6 is closely related to HPV18 E6, and it interacts with E6AP and targets p53 ubiquitination equivalently to the more commonly employed HPV18 or -16 E6 proteins (S. Beaudenon and J. M. Huibregtse, unpublished results) (see Fig. 3). HPV39 E6 was used because larger amounts of soluble protein could be purified from baculovirus-infected insect cells than with either HPV16 or -18 E6 baculoviruses. The C-33A cell line was used in initial screening experiments because it is a cervical carcinoma cell line that is devoid of HPV DNA and expresses elevated levels of a mutated form of p53 ($Arg_{273}Cys$) that is recognized by the E6-E6AP complex similarly to wild-type p53 (43). Therefore, p53 served as an internal control for a cellular protein that was predicted to be readily detectable in our screen for E6-dependent E6AP binding proteins.

Whole-cell extracts were prepared from C-33A cells metabolically labeled with [^{35}S]methionine and incubated with GST-E6AP immobilized on glutathione-Sepharose, in the absence or presence of HPV39 E6 protein. The Sepharose beads were collected, washed, and then boiled in SDS-PAGE loading buffer, and the released proteins were analyzed by SDS-PAGE and autoradiography. A characteristic set of proteins was detected in the absence of E6, while at least three additional proteins were detected in the presence of E6; these proteins had approximate apparent molecular masses of 50, 100, and 220 kDa (Fig. 1A). The most abundant was the 50-kDa protein, which likely represented p53. To confirm the identity of the 50-kDa protein, the binding experiment was repeated in parallel sets of reactions, and, after being boiled in SDS-PAGE loading buffer, one set of reaction mixtures was diluted 50-fold with water and an immunoprecipitation was performed with anti-p53 antibody. The approximately 50-kDa band seen in the binding assay was immunoprecipitated from the E6-containing reaction mixture, confirming that this was p53 (Fig. 1B). Likewise, we suspected that the 100-kDa band might be E6AP itself, since we have previously shown that E6 can mediate formation of an E6AP multimer (20). Immunoprecipitation with anti-E6AP antibody confirmed this (Fig. 1B). The identity of p220 was unknown. Like p53, it did not associate with GST-E6AP if the E6 binding domain was deleted (GST-E6APΔ378-395; Fig. 1C).

Approximately 1 to 2 μg of p220 was purified by scale-up of the GST-E6AP–E6 binding reaction, and a tryptic digest of the gel-isolated material was analyzed by matrix-assisted laser desorption ionization mass spectrometry (Keck Facility, Yale University). Masses were compared to those predicted by sequences in the nonredundant National Center for Biotechnology Information database using the ProFound (http://prowl.rockefeller.edu/cgi-bin/ProFound) and PeptideSearch (http://193.175.249.95:80/CGI/PPG.PeptMasses.acg) programs. To a very high degree of confidence, this analysis indicated that our sample contained a protein corresponding to the human homolog of *Drosophila* Scribble (Vartul) (1). Human (hScrib) and *Drosophila* Scrib are 35% identical and 49% similar over an alignment spanning 1,611 amino acids (BLAST2 score of 850; Fig. 2). Both proteins contain a series of leucine-rich repeats (LRRs) at their amino-terminal ends and four PDZ domains in their central regions. Both LRRs and PDZ domains have been shown to mediate protein-protein interactions. Other proteins related to Scrib include rat densin-180 and its apparent human homolog, encoded by cDNA for KIAA1225, which both contain multiple amino-terminal LRRs and a single carboxyl-terminal PDZ domain (Fig. 2). hDlg, the human homolog of *Drosophila* Discs large, which also interacts with high-risk HPV E6 proteins (29), contains three PDZ domains but does not contain LRRs.

cDNA KIAA0147, previously characterized by the Kazusa human cDNA sequencing project (34), encodes hScrib. KIAA0147 cDNA contains a 4,658-nucleotide ORF that predicts a protein of 1,551 amino acids (166-kDa calculated molecular mass). The cDNA contains a stop codon and 3′-untranslated se-

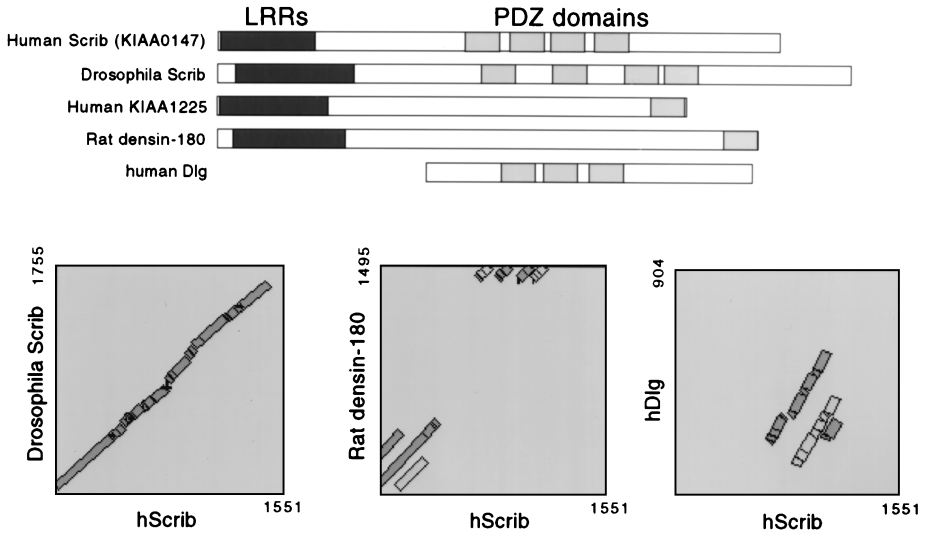

FIG. 2. Schematic of human Scrib protein relative to *Drosophila* Scrib, human KIAA1225, rat densin-180, and human Dlg (upper) and schematic representation of Blast2 comparisons of hScrib to Drosophila Scrib, densin-180, and hDlg (lower). LRRs and PDZ domains are indicated.

quences but lacks an apparent initiating methionine codon, strongly suggesting that the cDNA is incomplete at the 5′ end. Northern analysis indicates an mRNA of less than 6,000 nucleotides that is expressed in all tissue types examined (http://zearth.kazusa.or.jp/huge). By comparison with that of dScrib, KIAA0147 cDNA would be predicted to be missing coding sequences at the 5′ end specifying approximately 67 amino acids. A construct expressing the KIAA0147 ORF (with an in-frame ATG codon before the first codon of the ORF) was transcribed and translated in vitro, and the apparent molecular mass of the product, hScrib, based on gel migration was close to 220 kDa (Fig. 3). This suggests that hScrib migrates with an apparent molecular weight greater than its calculated molecular weight and further suggests that the cDNA is missing only a small amount of coding sequence at the 5′ end.

To confirm that hScrib has the characteristics of the originally isolated p220, the in vitro-translated protein was tested for binding to GST-E6AP in the absence and presence of HPV39 E6 protein. Like p53, it bound to GST-E6AP only in the presence of E6 (Fig. 3A, lanes 1 to 4). Furthermore, the addition of HPV39 E6 to rabbit reticulocyte lysate translation reaction mixtures, which contain endogenous E6AP, stimulated the multiubiquitination of both p53 and hScrib (Fig. 3A, lanes 5 to 8). Ubiquitination of wheat germ extract-translated hScrib, which lacks endogenous E6AP, was dependent on the addition of both E6AP and E6 (Fig. 3B). HPV16 E6 was used in the experiment in Fig. 3B, demonstrating that hScrib is a common target of E6 proteins of different high-risk HPV types. Thus, we conclude that the p220 detected in our screen was indeed hScrib and that hScrib is an E6-dependent substrate of E6AP.

Characterization of the interaction of E6 and E6AP with hScrib. To determine how the ternary complex of E6AP, E6, and hScrib was assembled, fusions of GST to various regions of hScrib were made, as shown in Fig. 4A. These were tested for their ability to interact with HPV16 E6, synthesized in vitro in a wheat germ extract translation system (Fig. 4B). E6 bound to the full-length GST-hScrib protein (amino acids 1 to 1551) as well as to the three fusion proteins that contained four or two PDZ domains (amino acids 655 to 1126, 655 to 932, and 933 to 1126), while it did not bind to either the amino-terminal LRR region or the carboxyl-terminal region of hScrib (amino acids 1127 to 1551). This suggests that E6 can interact directly with at least two different PDZ domains of hScrib. Consistent with the results of the initial screen, in vitro-translated E6AP only bound to GST-hScrib in the presence of E6 (Fig. 4C). The PDZ domain region of hScrib was also sufficient for E6-dependent E6AP binding. Therefore, the E6AP-hScrib interaction requires E6, yet E6 can interact independently and directly with both hScrib and E6AP (17), strongly suggesting that E6 acts as a bridge between the ubiquitin-protein ligase and substrate.

PDZ domains can mediate protein-protein interactions by recognizing a carboxyl-terminal epitope of target proteins, consisting of an X-S/T-X-V/L consensus sequence (39). All of the high-risk HPV E6 proteins contain this PDZ binding consensus site and have been previously shown to interact via this consensus sequence with the PDZ domains of hDlg (23). To determine if the carboxyl-terminal motif of E6 was necessary for hScrib interaction, the carboxyl-terminal four residues of HPV16 E6 were individually altered to those found in HPV11 E6, a low-risk HPV E6 protein (Fig. 5A). Mutation of either of the consensus residues (residues 149 or 151) significantly decreased the interaction of E6 with GST-hScrib, while mutation of the two nonconsensus residues (residues 148 and 150) had little effect on E6 binding (Fig. 5B). In addition, deletion of residue 151 (Δ151), the last amino acid of HPV16 E6, resulted in loss of binding to hScrib. Together, these results strongly suggest that the carboxyl terminus of E6 is recognized directly by the PDZ domains of hScrib.

The SAT_{8-10} mutation of HPV16 E6 (substitution of RPR_{8-10} of HPV16 E6 for SAT, found at residues 9 to 11 of HPV11 E6) results in an E6 protein that is unable to bind to or stimulate the ubiquitination of p53, yet this protein retains certain biological activities of E6 (22, 24). We therefore wanted to determine if this mutant protein was able to target hScrib. As shown in Fig. 5C, wild-type HPV16 E6 stimulated the in vitro ubiquitination and degradation of p53, while SAT_{8-10} did not. However, both the wild-type and SAT_{8-10} mutant proteins stimulated the ubiquitination of hScrib. Figure 5D confirms that the SAT_{8-10} mutant, like wild-type HPV16 E6, retains the ability to bind specifically to GST-E6AP but not

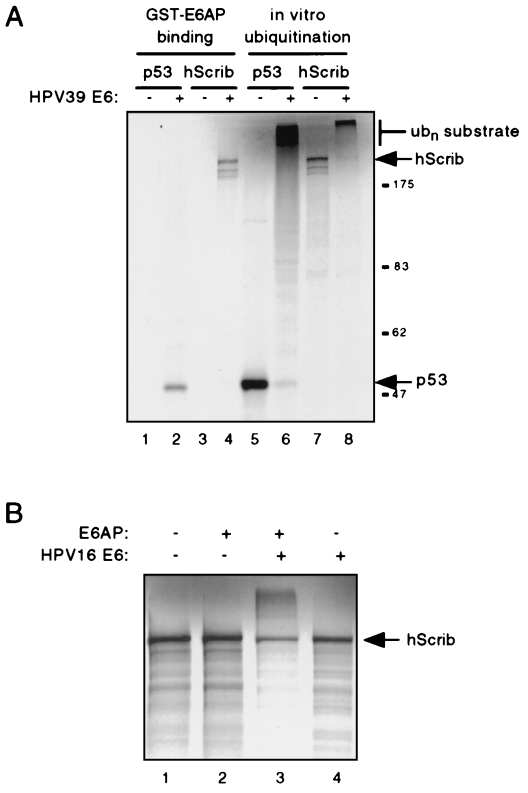

FIG. 3. hScrib has characteristics of an E6-dependent substrate of E6AP. (A) Binding of rabbit reticulocyte lysate-translated ^{35}S-labeled p53 and hScrib to GST-E6AP was performed under conditions described in the legend for Fig. 1 in the absence or presence of HPV39 E6 (lanes 1 to 4). Lanes 5 to 8, effect of the addition of HPV39 E6 to in vitro p53 and hScrib under conditions that support E6AP-dependent ubiquitination. The high-molecular-weight material (ub$_n$ substrate) represents the multiubiquitination of p53 and hScrib (lanes 6 and 8, respectively). The same amounts of translation product were used for binding (lanes 1 to 4) and ubiquitination reactions (lanes 5 to 8). (B) Wheat germ extract-translated hScrib was incubated with or without HPV16 E6 and purified E6AP, as indicated, in the presence of added E1 and E2 (UbcH7) protein, ubiquitin, and ATP. hScrib was ubiquitinated only in the presence of both E6 and E6AP (lane 3).

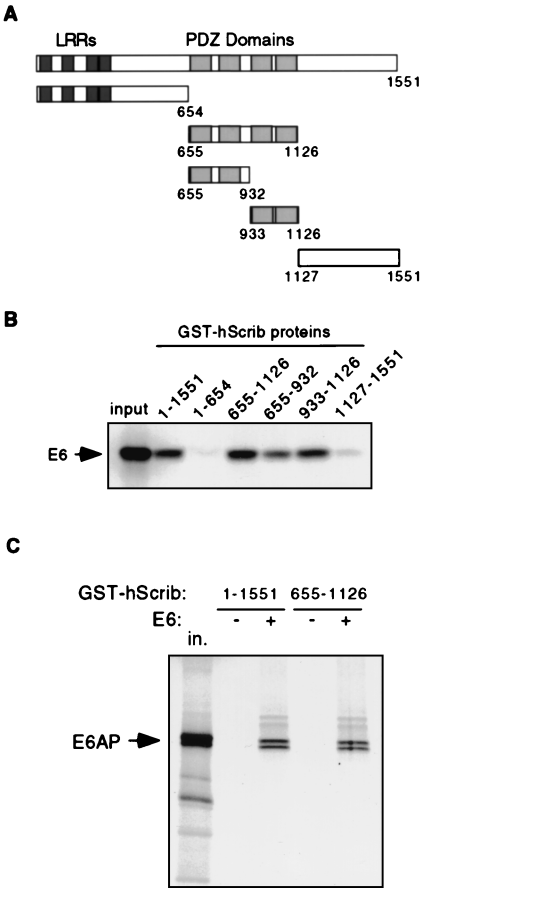

FIG. 4. (A) Schematic of hScrib regions that were expressed as GST fusion proteins. (B) GST-hScrib fusion proteins were assayed for binding to HPV16 E6 protein synthesized in a wheat germ extract translation system. (C) Full-length GST-hScrib (amino acids 1 to 1551) and the GST fusion to the PDZ domain region (amino acids 655 to 1126) were assayed for binding to E6AP in the absence and presence of HPV16 E6 protein. The amount of translation product used in the binding experiment is indicated (in.).

to GST-E6AP with the E6 binding domain deleted. The SAT_{8-10} mutation thus separates p53 targeting from hScrib targeting, and this suggests that the targeting of hScrib and/or other PDZ domain proteins such as hDlg may account for at least a subset of the biological effects mediated by the SAT_{8-10} protein.

E6-induced degradation in vivo. An epitope (FLAG)-tagged hScrib mammalian expression vector was generated and transfected into 293T cells, without or with cotransfection of an HPV16 E6-expressing plasmid. As shown in Fig. 6A, increasing amounts of E6 plasmid resulted in a corresponding decrease in the steady-state level of hScrib. To determine if E6 affects the half-life of hScrib, a FLAG-hScrib-expressing plasmid was transfected without or with an E6-expressing plasmid and cells were treated for various times with cycloheximide 24 h post-transfection. Figure 6B shows that the apparent half-life of hScrib was significantly reduced in the presence of E6 (compare lanes 1 to 4 with 5 to 8). Based on three independent experiments, the half-life in the absence of E6 was approximately 12 h, while in the presence of E6 it was less than 2 h. In addition, treatment of transfected cells with an inhibitor of the 26S proteasome partially blocked the effect of E6 on steady-state levels of FLAG-hScrib (Fig. 6C). These results, along with the corroborating in vitro results, strongly suggest that hScrib is targeted for ubiquitin-mediated degradation by the E6-E6AP complex in vivo.

hScrib localization and effect of E6 on tight junctions. *Drosophila* Scrib is localized to epithelial septate junctions, the equivalent of tight junctions in mammalian cells (1). To determine the intracellular localization of hScrib, we expressed GFP-hScrib in Madin-Darby canine kidney (MDCK) cells, normal epithelial cells in which cell junction complexes have been well characterized (4). GFP-hScrib was detected at the periphery of the cells and in the cytoplasm, and FLAG-hScrib was localized similarly by immunofluorescence (not shown). To determine if the peripheral localization corresponded to tight junctions, confocal laser microscopy was performed on GFP-hScrib-transfected cells probed with an antibody against ZO-1, a component of tight junctions (4). As shown in Fig. 7, GFP-hScrib and ZO-1 were partially colocalized at the periphery, strongly suggesting that hScrib is a component of tight junctions. GFP-hScrib was also present in membrane sections that did not contain ZO-1, suggesting that hScrib may be a component of other junctional complexes or other membrane-associated structures.

Transfection of GFP-hScrib alone did not affect the normal distribution of ZO-1 or the morphology of MDCK cells (Fig.

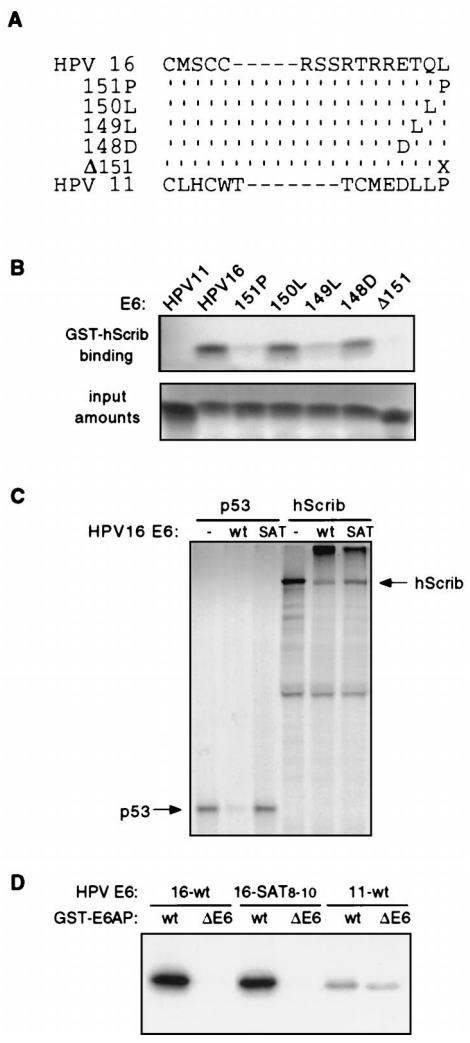

FIG. 5. (A) The carboxyl-terminal 16 residues of HPV16 E6, from the last C-x-x-C sequence to amino acid 151, are shown on the top line, and the corresponding region of HPV11 E6 is shown on the bottom line. Mutations of HPV16 E6 substituted the indicated amino acids for those present at analogous positions of HPV11 E6, relative to the carboxyl termini of the two proteins. In the HPV16 E6 Δ151 mutant the last amino acid was deleted without replacement. (B) HPV11 E6, HPV16 E6, and the mutants described for panel A were synthesized in a wheat germ extract translation system and assayed for binding to GST-hScrib (amino acids 1 to 1551). The relative input amounts are shown. (C) In vitro-translated (rabbit reticulocyte lysate) p53 and hScrib were incubated without E6 (−) or with wild-type (wt) HPV16 E6 or the HPV16 E6 SAT_{8-10} mutant (SAT). p53 was ubiquitinated in the presence of wt HPV16 E6, while hScrib was ubiquitinated in the presence of either the wt or mutant protein. (D) Binding of wt HPV16 E6, the SAT_{8-10} mutant, and HPV11 E6 to GST-E6AP (wt) or GST-E6APΔ378-395 (ΔE6), confirming that the SAT_{8-10} mutant binds to E6AP similarly to the wt protein. Amounts of input proteins were similar (not shown).

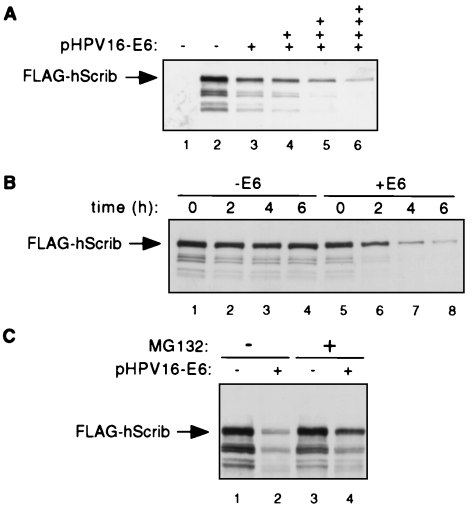

FIG. 6. HPV16 E6 expression affects steady-state level and half-life of hScrib in cells. (A) 293-T cells were transfected with a FLAG-hScrib-expressing plasmid without (lane 2) or with increasing amounts of HPV16 E6 expression plasmid (lanes 3 to 6; 0.5, 1.0, 1.5, and 2.0 μg, respectively). Cell extracts were made 48 h posttransfection and analyzed for FLAG-hScrib levels by immunoblotting with an anti-FLAG antibody. Lane 1, mock-transfected cells. (B) 293-T cells were transfected with a FLAG-hScrib-expressing plasmid without (lanes 1 to 4) or with an E6 expression plasmid (lanes 5 to 8; equivalent to lane 3 in panel A). Cycloheximide was added 24 h posttransfection, and cell extracts were made at the indicated times after addition, followed by immunoblotting analysis as for panel A. (C) Cells were transfected with a plasmid expressing FLAG-hScrib without or with an HPV16 E6-expressing plasmid, and levels of FLAG-hScrib without (lanes 1 and 2) or with incubation of MG132 for 3 h (lanes 3 and 4) were compared. Cell extracts were made and analyzed as for panel A.

8A, comparing fluorescence-positive and -negative cells). Cotransfection of GFP-hScrib and the HPV16 E6 Δ151 mutant, which does not target hScrib in vitro, resulted in a GFP fluorescence signal and a ZO-1 distribution identical to those of cells transfected with GFP-hScrib alone (Fig. 8A). However, cotransfection of GFP-hScrib with wild-type HPV16 E6 resulted in nearly complete loss of the GFP fluorescence signal (not shown), consistent with E6-induced degradation of GFP-hScrib. To determine if E6-induced degradation of hScrib affected the integrity of tight junctions, a plasmid expressing wild-type HPV16 E6 was cotransfected with a plasmid expressing GFP (pEGFP; Invitrogen) using the GFP fluorescence signal as a marker for transfected cells. Expression of GFP, by itself, did not alter ZO-1 staining relative to that of untransfected cells (Fig. 8B, top). In contrast, E6-transfected cells that were GFP positive showed an altered distribution of ZO-1, with some areas of the periphery lacking any detectable ZO-1 (Fig. 8B, bottom). Neighboring untransfected (GFP-negative) cells showed normal ZO-1 distribution. These results strongly suggest that E6 expression disrupts the integrity of tight junctions and that this is likely to occur, at least in part, through targeted degradation of hScrib.

DISCUSSION

The targeting of p53 by high-risk HPV E6 proteins requires the prior formation of a complex between E6 and the E6AP ubiquitin-protein ligase, which then forms a ternary complex with p53 (17). This order of complex assembly, as well as the fact that p53 is not a natural substrate of E6AP, has suggested that E6 functions to redirect the substrate specificity of E6AP and, further, that the E6-E6AP complex might target additional cellular proteins for ubiquitination. We have demonstrated here that the human homolog of *Drosophila* Scribble, hScrib, is targeted for E6AP-dependent multiubiquitination in the presence of high-risk HPV E6 proteins. The observation that E6 expression disrupts the integrity of tight junctions and that hScrib is localized to tight junctions suggests that this activity of E6 is related, at least in part, to targeted degradation of hScrib.

hScrib is the second human homolog of a *Drosophila* tumor suppressor shown to be targeted for degradation by high-risk HPV E6 proteins. The first was hDlg, the homolog of the Discs large tumor suppressor (10, 29). Like hScrib, hDlg interacts via

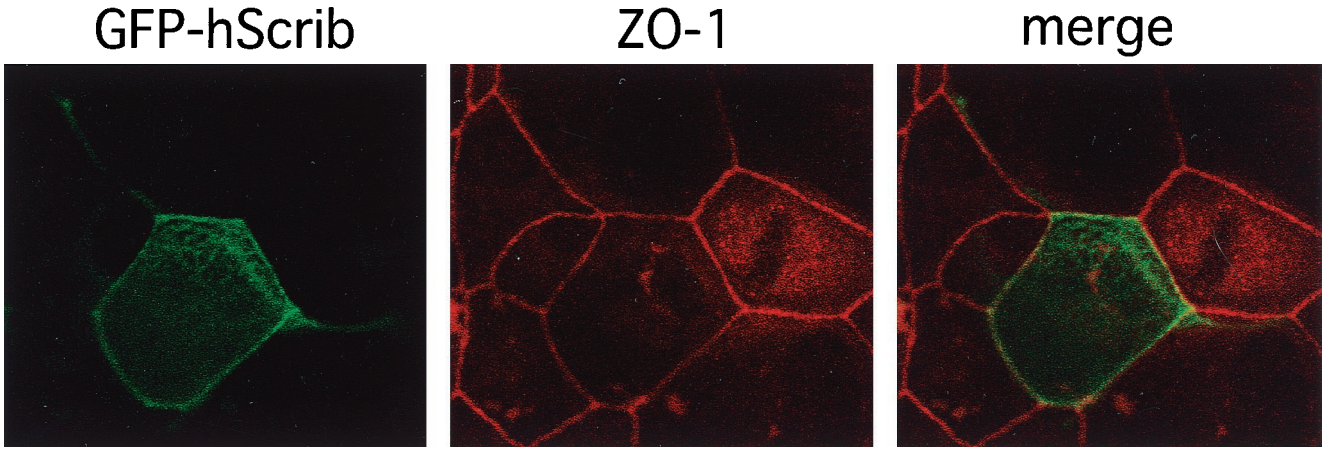

FIG. 7. GFP-hScrib-transfected MDCK cells were probed with an anti-ZO-1 antibody and rhodamine-conjugated secondary antibody, and cells were analyzed by confocal laser scanning microscopy.

PDZ domains with the carboxyl terminus of E6, and hDlg has been localized to cell junction complexes (18). hDlg is localized primarily to adherens junctions in MDCK cells, while we have shown here that hScrib is found at tight junctions. More detailed study of both hScrib and hDlg is necessary to determine if these proteins might be common components of one or more junction or other membrane complexes. Interestingly, although the phenotype of *Drosophila* Scrib mutants is not identical to that of Dlg mutants, it was found that Dlg expression can suppress tumor formation in Scrib mutants (B. Mechler, personal communication). This suggests that these proteins cooperate in a pathway that controls epithelial cell growth and/or differentiation in the fly. The fact that E6 targets both of the human homologs is consistent with the possibility that hDlg and hScrib cooperate in an analogous pathway in mammalian cells and that high-risk HPV E6 proteins must target both proteins to exert at least a subset of their biological effects.

The phenotypes of *Drosophila scrib* mutants suggest possible consequences of hScrib degradation that may be linked to the known biological effects of the high-risk HPV E6 proteins. The organization and differentiation of *Drosophila* embryonic epithelial cells are disrupted in Scrib mutants, resulting in aberrantly shaped cells and loss of monolayer organization (1). HPV16 E6 expression in the basal cells of the skin of the transgenic mice results in hyperproliferation and a disruption in their normal differentiation program (47), and these effects appear to be independent of p53 targeting. It will therefore be interesting to determine if the effects of E6 in transgenic mice are dependent on the carboxyl-terminal PDZ binding motif.

Our in vitro analyses of the E6-E6AP-dependent ubiquitination of hScrib clearly predict that E6AP is involved in mediating ubiquitination of hDlg, and preliminary data indicate that this is indeed the case (S. Nakagawa and J. M. Huibregtse, unpublished). It has been reported, however, that a different ubiquitin-protein ligase may be involved in hDlg targeting (38). E6TP1 is yet another PDZ domain-containing protein targeted for degradation by E6 proteins; however it was reported that the PDZ domains of E6TP1 do not mediate its interaction with E6 (9), and the involvement of E6AP in this reaction is as yet unclear. Bak, a proapoptotic member of the Bcl-2 family, has been shown to be targeted for E6-dependent degradation (49, 50); however it was reported that both low- and high-risk HPV E6 proteins have this activity, which would suggest that E6AP is not involved since low-risk HPV E6 proteins have not been shown to interact with E6AP (16). Thus, the induced degradation of several cellular proteins has been associated with high-risk HPV E6 expression; however, only for p53 and hScrib has E6AP been unambiguously shown to be a component of a ternary complex with E6 and substrate and to stimulate the E6-dependent multiubiquitination of the substrate in vitro. Interestingly, while HPV E7 proteins have been known for some time to associate with the retinoblastoma (pRB) tumor suppressor (5), it has more recently been found that Rb is destabilized as a result of the E7-Rb interaction (19). While the mechanism has not been elucidated, the fact E6 and E7 are structurally related proteins suggests that a second E6AP-independent degradation mechanism may underlie the ability of E6 to target some of its substrates as well as of E7 to induce destabilization of pRB.

The results described here, along with previously published results on the interaction between E6 and E6AP (17), indicate that E6 interacts directly and independently with both E6AP and hScrib, acting as a bridge between the enzyme and substrate. This may be informative with regard to the ternary complex formed with p53. While E6AP does not interact with either p53 or hScrib in the absence of E6, it has been controversial as to whether E6 interacts with p53 in the absence of E6AP (3, 17, 28, 30). For this reason it has been unclear which protein, E6 or E6AP, physically binds p53 in the ternary complex. Mutational analyses of E6AP and E6 have not resolved this issue since mutations in both E6 and E6AP that do not affect the E6-E6AP interaction yet abrogate formation of the ternary complex with p53 (the SAT_{8-10} mutant and E6AP deletion mutants) have been identified (17). If the ternary complex formed with hScrib is indicative of the ternary complex with p53, then E6 would be predicted to physically bind p53, again acting as a bridge from enzyme to substrate. Final resolution of this problem will require more detailed biophysical characterization of the ternary complex of E6-E6AP and p53.

It has become evident that proteins that contain both LRRs and PDZ domains constitute a new family of proteins, known collectively as LAP proteins (LRRs and PDZ domain proteins [1b]). These include Scribble (Vartul), rat and human densin-180 and erbin, and *Caenorhabditis elegans* LET-413. A common feature of the LAP proteins is their polarized localization in membranes. *Drosophila* Scrib has been shown to restrict

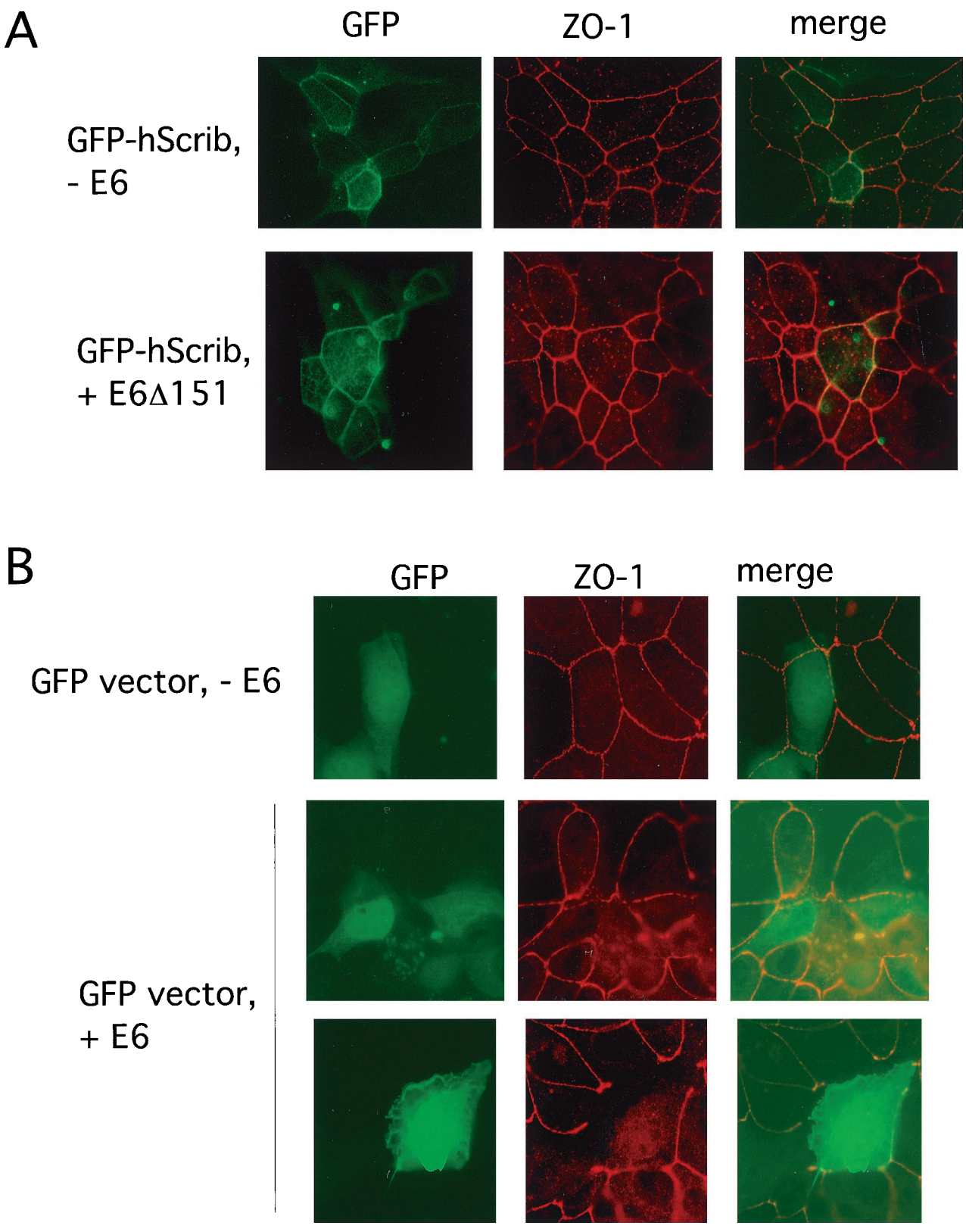

FIG. 8. (A) MDCK cells were transfected with a GFP-hScrib-expressing plasmid alone (top) or with a plasmid expressing the HPV16 E6 Δ151 mutant (bottom), and the fixed cells were probed with anti-ZO-1 antibody and rhodamine-conjugated secondary antibody. Cells were observed by fluorescence microscopy. (B) MDCK cells were transfected with pEGFP-C1 vector alone (top) or pEGFP-C1 vector with pCDNA-HPV16 E6 plasmid (bottom). The GFP signal served as a marker for transfected cells, and the ZO-1 antibody was detected with a rhodamine-conjugated secondary antibody.

localization of apical proteins in epithelial cells, including Crumbs (Crb) and Discs lost (Dlt) (1). Both of these proteins are normally found apically but are found both apically and basolaterally in *scrib* mutants. It has been suggested that Scrib may serve as a diffusion barrier between apical and basolateral membrane surfaces or, alternatively, that Scrib plays a role in the polarized targeting of transport vesicles delivering apical proteins (1). By analogy to results for Scrib mutant flies, the targeted degradation of hScrib by the HPV E6-E6AP complex may result in loss of polarized localization of membrane-associated proteins in HPV-infected keratinocytes.

PDZ domain proteins, in general, have been implicated in many aspects of cytoskeletal organization, signal transduction, and protein-trafficking pathways (7, 8). The finding that at least three PDZ domain proteins (hScrib, hDlg, and E6TP1) are targets of E6 suggests that E6-E6AP targets multiple proteins involved in maintenance of the cell structure and that this may be related to the effect of E6 on the differentiation program of keratinocytes (45). BPV-1 E6 and HPV16 E6 also interact with paxillin (51), a component of focal adhesions, resulting in a disruption of the actin fiber network. While the E6-paxillin interaction apparently does not lead to degradation of paxillin, this nevertheless supports the role of E6 in affecting several aspects of cell structure and intracellular communication. Finally, the adenovirus 9ORF1 and human T-cell leukemia virus type 1 Tax oncoproteins have also been shown to interact with hDlg (29). It will therefore be of interest to determine if other viral oncoproteins share with the high-risk HPV E6 proteins the ability to target and inactivate hScrib.

ACKNOWLEDGMENTS

This work was supported by a grant to J.M.H. from the National Institutes of Health (CA72943), and S.N. was supported by a fellowship from the University of Tokyo, Faculty of Medicine, and the Kanzawa Medical Research Foundation.

We thank Bernard Mechler for communication of unpublished results, members of our laboratory for helpful discussions, Sylvie Beaudenon for generation and characterization of HPV39 E6 baculovirus, the Kazusa DNA Research Institute, Chiba, Japan, for providing cDNA for KIAA0147, and Go Totsukawa, Fumio Matsumura, and Herb Geller and the Robert Wood Johnson Medical School Confocal and Electronic Imaging Center for assistance with microscopy and image analysis.

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