Journal of Cellular Biochemistry 94:1038–1045 (2005)

Association of Cottontail Rabbit Papillomavirus E6 Oncoproteins With the hDlg/SAP97 Tumor Suppressor

Minjie Du,[1] Xueli Fan,[1] Toshihiko Hanada,[2,4] Hua Gao,[1] Mohini Lutchman,[2] Janet L. Brandsma,[3] Athar H. Chishti,[2,4] and Jason J. Chen[1]*

[1]Department of Medicine, UMass Medical School, 364 Plantation Street, Worcester, Massachusetts 01605-2324
[2]Department of Medicine, Anatomy, and Cellular Biology, St. Elizabeth's Medical Center, Tufts University School of Medicine, Boston, Massachusetts 02135
[3]Section of Comparative Medicine, Yale University School of Medicine, New Haven, Connecticut 06520
[4]Department of Pharmacology, UIC Cancer Center, University of Illinois College of Medicine, Chicago, Illinois 60607

Abstract Papillomaviruses are small DNA viruses that infect epithelial tissues and cause warts. Human papillomavirus (HPV) infection is the primary risk factor for the development of cervical cancer. The E6 and E7 oncogenes are the only genes consistently expressed in HPV-positive cervical cancer cells. Cottontail rabbit papillomavirus (CRPV) induces papillomas and carcinomas on cottontail and domestic rabbits and provides an excellent animal model of HPV infection and vaccine development. CRPV encodes three transforming proteins; LE6, SE6, and E7. Each of these proteins is required for papilloma formation. Like HPV E7, the CRPV E7 protein binds to the tumor suppressor pRB. In contrast, unlike HPV E6, the CRPV E6 proteins do not bind the tumor suppressor p53. Although more than a dozen cellular proteins have been identified as HPV E6 interacting proteins, nothing is known about the cellular interacting proteins of CRPV E6s. Here we describe the association of CRPV E6s with hDlg/SAP97, the mammalian homolog of the *Drosophila* discs large tumor suppressor protein. HPV E6 has previously shown to bind and target hDlg/SAP97 for degradation. Our results demonstrate that both LE6 and SE6 interact with hDlg/SAP97, although their association does not lead to the degradation of hDlg/SAP97. The PDZ domains of hDlg were shown to be sufficient for interaction with CRPV E6 proteins while the C-terminus of CRPV E6 is essential for the interaction with hDlg. The association of hDlg with SE6 may be important but not sufficient for the transformation of NIH 3T3 cells by SE6. Importantly, a CRPV SE6 mutant defective for papilloma formation did not interact with hDlg. These results suggest that interaction with hDlg/SAP97 plays a role in the biological function of CRPV E6s. J. Cell. Biochem. 94: 1038–1045, 2005.

Key words: papillomavirus; E6; Dlg; SAP97; PDZ

Papillomaviruses are small DNA viruses that infect various epithelial tissues. Some Human papillomavirus (HPV) types are strongly associated with the development of cervical carcinoma (for review, see [zur Hausen, 2000]). Among animal papillomaviruses, cottontail rabbit papillomavirus (CRPV) induces papillomas that progress at a high frequency to carcinomas on domestic rabbits [Syverton, 1952]. CRPV provides an excellent animal model of HPV infection and vaccine development [Brandsma, 1994]. CRPV encodes three transforming proteins, long E6 (LE6), short E6 (SE6), and E7 [Meyers et al., 1992]. Each of these proteins can induce anchorage-independent growth of NIH 3T3 cells and transform sflEp rabbit epithelial cells [Meyers et al., 1992]. Intact CRPV LE6, SE6, and E7 genes are each

Grant sponsor: Dermatology Foundation; Grant sponsor: NIH.

Minjie Du's present address is Department of Molecular and Medical Genetics, University of Toronto, Toronto, ON M5S 1A8, Canada

*Correspondence to: Jason J. Chen, Department of Medicine, UMass Medical School, LRB Room 323, 364 Plantation Street, Worcester, MA 01605-2324.
E-mail: Jason.Chen@UMassmed.edu

Received 22 June 2004; Accepted 9 November 2004
DOI 10.1002/jcb.20383

essential for papilloma formation [Brandsma et al., 1991; Meyers et al., 1992; Wu et al., 1994]. LE6 and SE6 differ by 97 amino acids with SE6 identical to the carboxyl-terminus of LE6. CRPV SE6 and LE6 proteins contain six and eight Cys-X-X-Cys motifs, respectively that are characteristic of zinc finger domains [Berg, 1986]. LE6 is a protein of 273 amino acids predominantly present in the nucleus and associated with the nuclear matrix. Minor portions of the LE6 are located in both cytoplasmic and the membrane fractions. SE6 is a cytoplasmic phosphoprotein of 176 amino acids.

The ability of high-risk HPV E6 and E7 proteins to associate with the cellular tumor suppressors p53 and pRB, respectively, has been suggested as one mechanism by which HPV induces tumors [Dyson et al., 1989; Munger et al., 1989; Gage et al., 1990; Werness et al., 1990]. HPV E6 and E7 also interact with multiple additional cellular proteins (reviewed in [Munger and Halpern, 1997; Rapp and Chen, 1998; Mantovani and Banks, 2001]). Notably, a group of PDZ domain-containing proteins including hDlg/SAP97, E6TP1, MUPP1, human Scribble (hScrib), and MAGIs were identified to be degradation targets for E6 [Kiyono et al., 1997; Lee et al., 1997; Gao et al., 1999; Gardiol et al., 1999; Glaunsinger et al., 2000; Lee et al., 2000; Nakagawa and Huibregtse, 2000; Thomas et al., 2002]. hDlg, a member of the SAP family, is the closest human homolog of *Drosophila* Dlg (60% amino acid identity) [Lue et al., 1994]. SAP97 is the rat homolog of *Drosophila* Dlg (54% amino acid identity) [Muller et al., 1995]. hDlg is 89% identical to SAP97. hDlg/SAP97 is a putative tumor suppressor and has been shown to be essential for the regulation of cell growth and polarity in *Drosophila* [Bilder et al., 2000].

While CRPV E7 has been shown to bind to pRB [Defeo-Jones et al., 1993; Haskell et al., 1993; Schmitt et al., 1994], CRPV E6s did not bind p53 or E6BP [Harry and Wettstein, 1996]. Therefore, the molecular mechanism of CRPV E6-mediated cellular transformation and papilloma formation remains unknown. In the present study, we describe the association of CRPV E6s with hDlg/SAP97 and correlate E6-hDlg/SAP97 interaction with cellular transformation in vitro and papilloma formation in vivo.

MATERIALS AND METHODS

Plasmids

Portions of the hDlg cDNAs were amplified by PCR, digested with restriction endonucleases, and ligated into pGEX vectors to express glutathione S-transferase (GST)-hDlg fusion proteins in *E. coli*. pdCMV-SE6 and pdCMV-E6 encoding CRPV SE6 and LE6 respectively were described previously [Sundaram et al., 1998]. The CRPV LE6 cDNA was cloned into pET-32a vector (Novagen) and expressed as a fusion with Trx/His.Tag. The CRPV E6 mutant DNAs were cloned into pSG5 as *Eco*RI-*Bgl*II fragments by PCR amplification. The *Eco*RI DNA fragment encoding rat SAP97 was subcloned from the Myc-tagged SAP97 in pGW1-CMV [Hsueh and Sheng, 1999] to EcoRI site of pSG5 to make pSG5SAP97. All constructs were verified by sequencing.

Protein Preparation

The indicated proteins were translated in vitro in the presence of [^{35}S] cysteine for E6 and [^{35}S] methionine for other proteins by using a rabbit reticulocyte lysate (RRL) transcription-translation system (Promega, WIS) according to the supplier's recommendation. GST fusion proteins were expressed in *E. coli* strain DH5α. After induction with IPTG, cells were harvested, re-suspended in 50 ml of low salt association buffer (LSAB, 100 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1% NP-40, and 1 mM phenylmethylsulfonyl fluoride) and lysed by sonication. SDS and DTT were added to the lysate immediately after sonication for a final concentration of 0.03% and 1 mM, respectively. After centrifugation at 10,000*g* for 10 min, supernatant was collected and mixed with glutathione Sepharose beads (Pharmacia). After incubation, the beads were collected by centrifugation at 1,000*g*, washed three times with 20 volumes of LSAB, and stored at 4°C. Small aliquots of fusion proteins were run on SDS–PAGE gels and stained by Coomassie blue to confirm sizes and homogeneity. To prepare Trx/His.Tag-CRPV LE6, *E. coli* BL21 (DE3) cells (Stratagene) containing pET- CRPV LE6 were induced with 0.05 mM IPTG at 25°C for 14 h. Cells were lysed in buffer A (20 mM Tris-Cl, pH 8.0, 500 mM NaCl, 5 mM Imidazole) by sonication. The cleared lysate was applied to a column of Ni-NTA His-bond Superflow (Novagen). The LE6 fusion protein with Thior-

edoxin and His-tag was eluted by 500 mM Imidazole after extensive washings with the buffer A. The eluted protein was dialyzed against PBS (pH 7.4, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl) overnight and stored at 4°C.

Protein Association Assays

For regular in vitro association, 2–20 μl [^{35}S]-labeled in vitro translated proteins were incubated with 5–30 μl glutathione-Sepharose containing 2 μg of appropriate GST fusion proteins in 250 μl LSAB. The mixtures were subjected to rotary shaking at 4°C for 3 h, and the beads were collected by centrifugation, washed four times with 1 ml LSAB buffer each wash. The bound [^{35}S]-labeled proteins were boiled in SDS sample buffer (containing fresh 200 mM DTT) for 3 min. and resolved on SDS–15% polyacrylamide gels. The gels were dried and quantified by Molecular Imager (Bio-Rad). For association assay designed to examine direct binding, two GST-fusion proteins of hDlg were used. One containing the three PDZ domains (GST-PDZ, aa 201–584) whereas the second fusion protein included three PDZ domains as well as the SH3 and GUK domains (GST-delta NT, aa 201–926) [Hanada et al., 2000]. The GST-PDZ, GST-deltaNT, and control GST proteins immobilized on the Glutathione-Sepharose 4B (Amersham) were incubated with the LE6 mentioned above in the binding buffer (PBS 0.1% Triton X-100), at 4°C overnight, and the beads were washed extensively in the binding buffer. The bound proteins were resolved as above and transferred onto the nitrocellulose membrane. The presence of LE6 was detected by Western blotting using anti-Thioredoxin antibody (Invitrogen).

In Vitro Degradation Assay

Five to ten microliters [^{35}S]-labeled in vitro translated LE6 or SE6 proteins and SAP97 proteins were incubated together for 3–5 h at 30°C in a 30 μl volume containing 10 μl additional RRL in 25 mM Tris-HCl (pH7.5), 100 mM NaCl, and 3 mM DTT. The reactions were stopped by adding 1 volume of 2× SDS sample buffer. After getting boiled for 3 min, the proteins were resolved by SDS PAGE and visualized by Molecular Imager.

RESULTS AND DISCUSSION

CRPV E6s Associate With hDlg/SAP97

To identify CRPV E6-associated proteins, we investigated the in vitro association of CRPV E6s with a group of known papillomavirus E6 interacting proteins, including Bak, c-Myc, E6AP, E6BP, hDlg, IRF-3, p300, and paxillin. We prepared ^{35}S-labeled in vitro translated CRPV LE6 and SE6 proteins and assessed their ability to bind GST fusions of human E6AP, E6BP, hDlg, IRF-3, p300, and paxillin prepared from *E. coli*. Similarly, we prepared in vitro translated ^{35}S-labeled human c-Myc and Bak and tested their ability to bind GST-CRPV E6 proteins prepared from *E. coli*. Our results demonstrated that hDlg bound both SE6 and LE6 (Fig. 1A). The affinity of CRPV E6s binding to hDlg was comparable to that of HPV-16 E6. In contrast, the bovine papillomavirus type 1

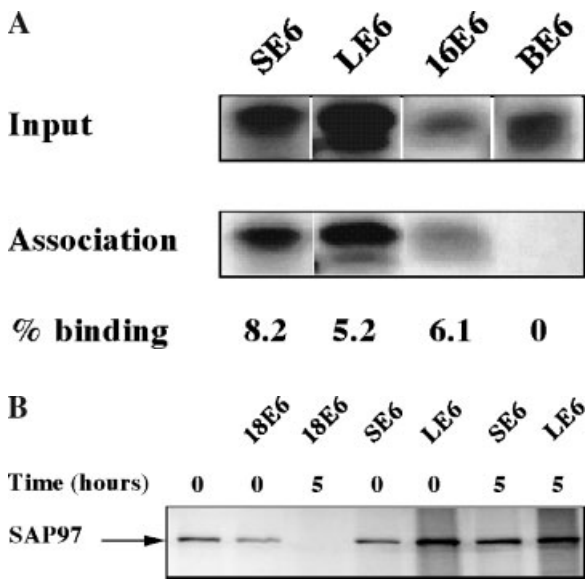

Fig. 1. Interaction of CRPV E6 with hDlg/SAP97. **A**: Glutathione Sepharose beads containing GST-full length hDlg fusion protein were combined with [^{35}S]-labeled in-vitro translated E6 proteins in LSAB buffer. The bound products were separated by SDS–PAGE and analyzed by Molecular Imager (Bio-Rad). Input was directly loaded into the well and represents 10% of the [^{35}S] cysteine-labeled E6 proteins used in each binding reaction. The average percent binding from two experiments are indicated below each lane; SE6 and LE6, CRPV SE6 and LE6; 16E6, HPV-16 E6; BE6, BPV-1 E6. **B**: Degradation assay. SAP97 and CRPV E6 were translated in vitro, [^{35}S]-labeled, and mixed in reaction mixtures containing rabbit reticulocyte lysate. Samples were incubated at 30°C for 5 h. The remaining SAP97 was separated by SDS–PAGE and quantified using Molecular Imager. The position of SAP97 is indicated by an arrow. The E6 proteins from different HPVs are indicated above each lane, 18E6, HPV-18 E6.

(BPV-1) E6 did not bind hDlg. Other cellular proteins examined did not bind or bound poorly with CRPV E6 proteins (data not shown). Thus, a specific and efficient in vitro association between hDlg and CRPV E6 proteins was demonstrated by these experiments.

As HPV E6s bind hDlg/SAP97 and target it for degradation, we investigated whether CRPV E6 proteins could degrade hDlg/SAP97. Both SAP97 and E6s were prepared and ^{35}S-labeled by in vitro translation. They were incubated in a degradation buffer in the presence of RRL. Consistent with previous observations [Gardiol et al., 1999], SAP97 was degraded by HPV-18 E6 (Fig. 1B). However, no reduction in the level of SAP97 was found when it was incubated with either CRPV LE6 or SE6 (Fig. 1B). These results suggest that hDlg/SAP97 is not a degradation target for CRPV E6, at least in vitro.

CRPV E6s Interact With the PDZ Domains of Dlg/SAP97

To further characterize the interaction of hDlg/SAP97 with CRPV E6s, we performed a mutational analysis to determine the domain(s) of hDlg/SAP97 involved in association with CRPV E6s. A set of GST-hDlg deletion mutants were prepared from *E. coli* and analyzed for their ability to bind in vitro translated and [^{35}S]-labeled CRPV E6s. As shown in Figure 2, the PDZ domains of hDlg/SAP97 are necessary and sufficient for association with CRPV E6s. In contrast, neither SH3 nor GUK bound CRPV E6s. Among the PDZ domains, either PDZ2 or PDZ3 was sufficient to bind E6s. The combination of PDZ2 and PDZ3 showed more efficient binding to SE6. Notably, HPV-16 E6 mainly bound to PDZ2 of hDlg/SAP97 [Kiyono et al.,

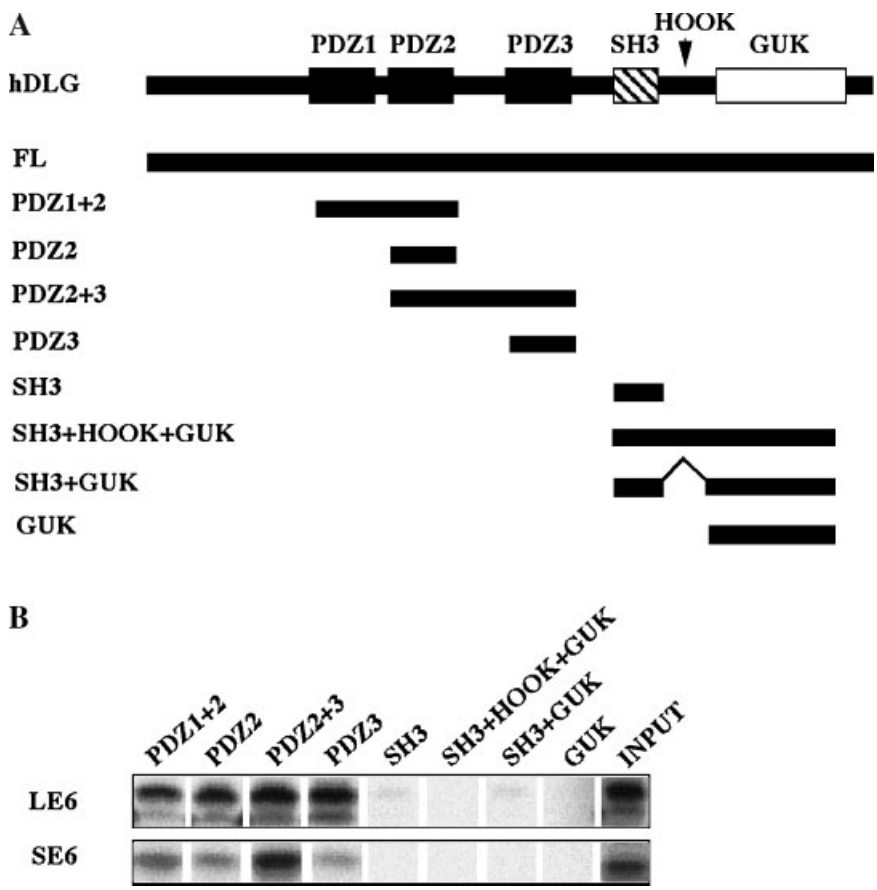

Fig. 2. PDZ domains of Dlg/SAP97 are sufficient for interaction with E6. **A**: Schematic representation of hDlg protein and its deletion mutants are used in this study. FL, full-length. **B**: Equal amount of a series of hDlg deletion mutants GST fusion proteins was combined with [^{35}S]-labeled in-vitro translated CRPV SE6 and LE6, respectively. Analyses were carried out as in Figure 1. Input was directly loaded into the well and represents 10% of the [^{35}S] cysteine-labeled E6 proteins used in each binding reaction. Domains of hDlg are indicated above each lane.

1997] while HPV-18 E6 bound equally well to all three PDZ domains [Gardiol et al., 1999]. While HPV E6 proteins from the high-risk HPV types contain a PDZ recognition consensus sequence in their carboxyl-termini, CRPV E6s do not. However, examples of PDZ-domain-mediated recognition of internal -(S/T)-x-V- sequence motifs have also been discovered, as exhibited by the PDZ–PDZ dimerization interaction between syntrophin and nNOS [Hillier et al., 1999]. The CRPV E6s do contain an internal -T-x-V- sequence (aa residues 77 through 79 of SE6 and 174 through 176 of LE6, respectively).

C-Terminal CRPV E6 Is Important for Association with Dlg

To determine the domains of CRPV E6s involved in hDlg/SAP97 association and to begin to correlate this association with known biological activities of CRPV E6s, a set of CRPV E6 deletion mutants was tested for binding with hDlg/SAP97. The mutants have been previously examined for cellular transformation and papilloma formation [Wu et al., 1994; Harry and Wettstein, 1996]. Accordingly, GST-hDlg was prepared from *E. coli* and analyzed for the ability to bind in vitro translated and [^{35}S]-labeled CRPV E6s mutants. Interestingly, the SE6 mutant with the internal -T-x-V- sequence disrupted (SE6Δ29–79) efficiently bound with hDlg (Fig. 3). This result indicates that the CRPV E6s -T-x-V- sequence is not required for association with hDlg/SAP97. The interaction of CRPV E6s with hDlg/SAP97 may be mediated by sequences that are structurally different from the PDZ binding motifs as described previously [Cuppen et al., 1998]. Interestingly, the single PDZ domain of E6TP1 also contributes little if any toward E6 binding [Gao et al., 1999]. Mutants Δ96–139 and Δ124–176 maintained partial transformation [Harry and Wettstein, 1996] and displayed reduced hDlg/SAP97 binding (Fig. 3). However, binding to hDlg/SAP97 was not in itself sufficient for transformation, as mutants Δ4–28 and Δ29–79 bound hDlg/SAP97 at wild-type levels but did not transform NIH 3T3 cells efficiently (Fig. 3). Notably, the integrity of C-terminal was essential for SE6 to bind hDlg, as the C-terminal mutant SE6Δ124–176 greatly reduced the ability of SE6 to bind hDlg while mutant SE6Δ100–176 abolished this interaction (Fig. 3). Significantly, SE6 containing the N-terminal 100 amino acid residues plus 17 additional heterogeneous amino acids was defective for the papilloma formation [Wu et al., 1994]. A recent study demonstrated that the amino acid residues in the carboxyl-terminal region of CRPV E6 influence spontaneous regression of papillomas [Hu et al., 2002]. These results suggest that E6 interaction with Dlg may play a role for CRPV to induce papillomas. However, we understand that the C-terminus truncation is likely to affect multiple functions of E6. Additional mutagenesis is required to establish the role of hDlg interaction in CRPV-induced papilloma formation.

The high-risk HPV E6 proteins contain a PDZ recognition consensus sequence in the carboxy-terminus, whereas the CRPV E6s contain an internal PDZ recognition consensus sequence. However, our data indicating that the CRPV E6s internal -T-x-V- sequence is not required for the interaction with hDlg/SAP97 suggest that the association of CRPV E6 with hDlg/SAP97 is indirect. The CRPV SE6 and LE6 proteins used for in vitro association experiments were prepared with reticulocyte lysates. The components of reticulocytes are complex and likely contain proteins that mediate the interaction between E6 and hDlg. To examine whether CRPV E6 binds Dlg directly, purified recombinant CRPV LE6 proteins expressed in bacteria were incubated with GST-hDlg fusions in the absence of reticulocyte lysates. While the GST-hDlg fusion proteins bound to the C-termini of potassium channels efficiently [Marfatia et al., 1996], they did not bind to CRPV LE6 protein (data not shown). This result suggests that the LE6 protein does not bind hDlg directly, and therefore some other cellular factors may serve as adaptors to mediate the association. Clearly, this observation does not necessarily lessen the functional importance of the observed association since many physiologically relevant associations are indirect. The best example might be the interaction between HPV E6 and p53, an association mediated by E6AP [Huibregtse et al., 1991].

In summary, we have identified hDlg/SAP97 as a CRPV E6-associated protein. This is the first CRPV E6 interacting cellular protein to be described. The PDZ domains of hDlg/SAP97 were shown to be sufficient to bind CRPV E6s. Since the interaction is likely to be indirect, it will be interesting to identify factors mediating this association. The mutational analyses presented here suggest a possible role for the

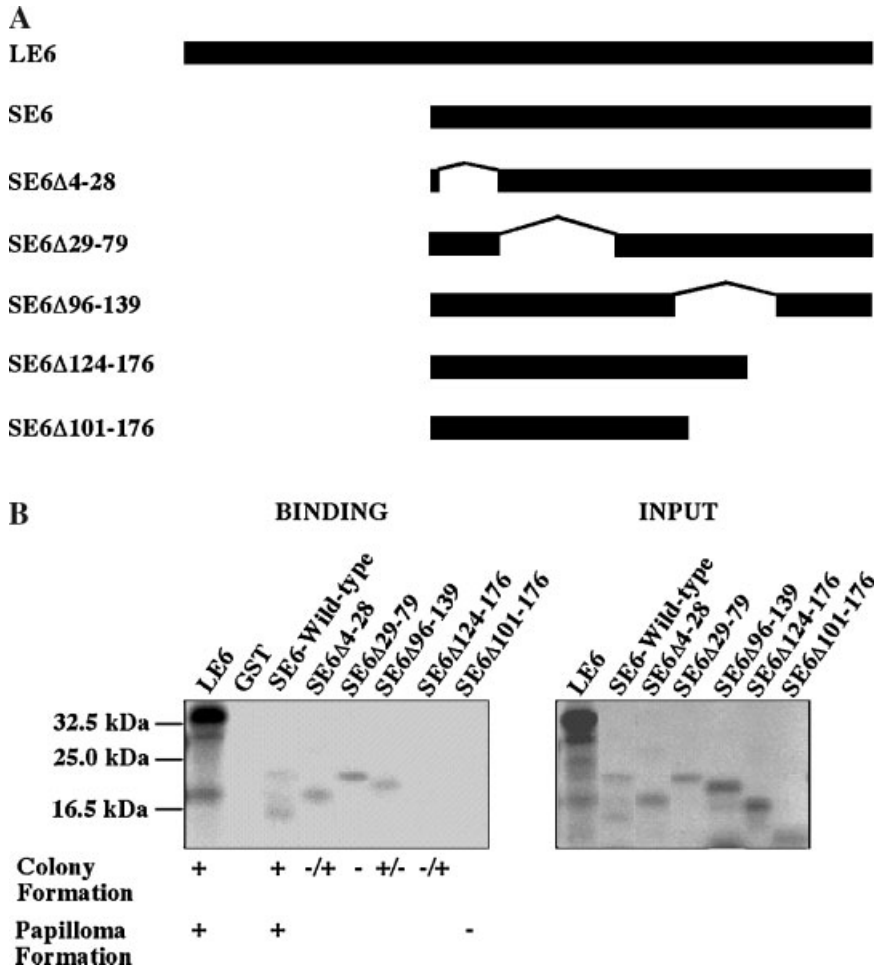

Fig. 3. The integrity of C-terminal CRPV E6 is important for Dlg interaction. **A**: Schematic representation of CRPV SE6 mutants used in this study. **B**: Association of CRPV SE6 and LE6 with hDlg. In vitro association of full-length hDlg-GST fusion protein with [^{35}S]-labeled in vitro translated E6 protein was similar to what was described in Figure 1 legend. The E6 mutant proteins are indicated above each lane. Results from a representative of three experiments are shown. GST represents a control association of wild-type SE6 with GST. Colony formation data and papilloma formation data are from Harry and Wettstein [1996] and Wu et al. [1994], respectively. Data are summarized as follows: +, >90% (wild-type E6 activity = 100%); +/−, 63–67%; −/+, 19–38%; −, <7%.

E6-hDlg/SAP97 interaction in CRPV-induced cellular transformation in vitro and papilloma formation in vivo. More extensive studies will be able to establish the physiological relevance of this interaction in transformation and other viral functions.

ACKNOWLEDGMENTS

We thank Dr. Elliot J. Androphy for advice throughout the course of this work and critical reading of the manuscript; members of our laboratory for helpful discussions; Dr. Morgan Sheng and Dr. Daniel Pak for plasmids. M.D. was supported in part by the Dermatology Foundation Postdoctoral Fellowship. A.H.C. was supported by the NIH grant CA94414. J.J.C. was supported in part by the Dermatology Foundation Dermik Laboratories, Inc. Research Grant and Career Development Award.

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