JOURNAL OF VIROLOGY, Oct. 1993, p. 6170–6178
0022-538X/93/106170-09$02.00/0

Vol. 67, No. 10

The Human Papillomavirus Type 6 and 16 E5 Proteins Are Membrane-Associated Proteins Which Associate with the 16-Kilodalton Pore-Forming Protein

MELISSA CONRAD, VIVIEN J. BUBB,† AND RICHARD SCHLEGEL*

Department of Pathology, Georgetown University Medical School, 3900 Reservoir Road, N.W., Washington, D.C. 20007

Received 26 January 1993/Accepted 12 July 1993

The human papillomavirus (HPV) E5 proteins are predicted from DNA sequence analysis to be small hydrophobic molecules, and the HPV type 6 (HPV-6) and HPV-11 E5 proteins share several structural similarities with the bovine papillomavirus type 1 (BPV-1) E5 protein. Also similar to the BPV-1 E5 protein, the HPV-6 and HPV-16 E5 proteins exhibit transforming activity when assayed on NIH 3T3 and C127 cells. In this study, we expressed epitope-tagged E5 proteins from both the "low-risk" HPV-6 and the "high-risk" HPV-16 in order to permit their immunologic identification and biochemical characterization. While the HPV-6 and HPV-16 E5 proteins fail to form disulfide-linked dimers and oligomers, they did resemble the BPV-1 E5 protein in their intracellular localization to the Golgi apparatus, endoplasmic reticulum, and nuclear membranes. In addition, the HPV E5 proteins also bound to the 16-kDa pore-forming protein component of the vacuolar ATPase, a known characteristic of the BPV-1 E5 protein. These studies reveal a common intramembrane localization and potential cellular protein target for both the BPV and HPV E5 proteins.

Human papillomaviruses (HPVs) have been implicated in the pathogenesis of human malignancies, including cervical carcinoma (12, 42), skin carcinoma in patients with epidermodysplasia verruciformis (26), and anogenital carcinoma (41). Hybridization analyses of cervical carcinomas indicate that approximately 80 to 90% of these tumors contain HPV DNA (2, 10). In addition, these viruses are also associated with low-grade and benign lesions of the uterine cervix and are found in up to 100% of such lesions (37). The development of cervical carcinoma is strongly associated with infection by a subgroup of oncogenic ("high-risk") genital HPV types, including HPV types 16, 18, 31, 33, and 35, while "low-risk" HPV types, predominantly HPV-6 and HPV-11, are found most frequently in benign cervical lesions (12, 42). In vitro transformation assays with these malignancy-associated HPV types show that they are capable of inducing foci, although inefficiently, on NIH 3T3 cells (1, 38, 40) and can immortalize primary human keratinocytes (9, 19, 30, 32). Furthermore, genetic studies have demonstrated that the HPV E6 and E7 genes are necessary and sufficient for the immortalization transformation of primary human genital keratinocytes (32, 34).

In contrast to the HPVs, 95 to 99% of the in vitro transforming activity (focus formation) of intact bovine papillomavirus type 1 (BPV-1) DNA is due to the activity of the E5 gene. The remaining 1 to 5% of the transforming activity in BPV-1 resides in the E6 gene (31, 39). The BPV-1 E5 protein is a hydrophobic, 44-amino-acid protein which migrates on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) as a 7.0-kDa protein which is membrane associated and found within cells primarily as a 15-kDa homodimer (4, 33). The BPV-1 E5 protein has been shown to cooperate with the human epidermal growth factor (EGF) and colony-stimulating factor-1 receptors to induce transformation of NIH 3T3 cells (23) and can constitutively activate the endogenous β-subunit of the platelet-derived growth factor receptor (28). BPV-1 E5 is also known to complex with both the 16-kDa component of vacuolar ATPases (14, 16) and the activated platelet-derived growth factor receptor (27) as a trimeric complex (13).

Less is known about the role of the E5 gene in HPV-induced lesions. Amino acid sequence analysis of the HPV E5 proteins predicts that these proteins, like BPV-1 E5, are extremely hydrophobic molecules. No amino acid sequences are conserved throughout the papillomavirus E5 proteins; however, there are specific amino acid residues, notably prolines and cysteines, which are similarly located in all HPV E5s. The HPV-6 and HPV-11 E5's share structural properties with the BPV-1 E5 protein: they contain a Cys-X-Cys sequence near the carboxyl terminus and a glutamine residue within the hydrophobic domain (Fig. 1). Both of these regions have been shown to be critical for the transforming activity of BPV-1 E5 (18), with the glutamine residue apparently being important for binding to the 16-kDa vacuolar ATPase component (15). Recent reports have described a transforming activity for the HPV-16 E5 protein which is increased in the presence of EGF (21, 29, 36). Transforming activity has also been reported for the HPV-6 E5 gene in NIH 3T3 and C127 cells (6), although these activities are weak.

To evaluate the potential role of the HPV E5 genes in cellular transformation, we expressed the E5 proteins from both the malignancy-associated (high-risk) HPV-16 and the low-risk HPV-6, associated with benign lesions, to compare their biochemical properties with those of the BPV-1 E5 protein.

MATERIALS AND METHODS

Cell culture. COS cells and CMT4 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum.

Plasmid constructions. Plasmid pRNGS-40 is derived from p37S39S, a pPAVA-1 variant (35). The parent vector

* Corresponding author.
† Present address: Department of Pathology, University Medical School, University of Edinburgh, Edinburgh EH8 9AG, United Kingdom.

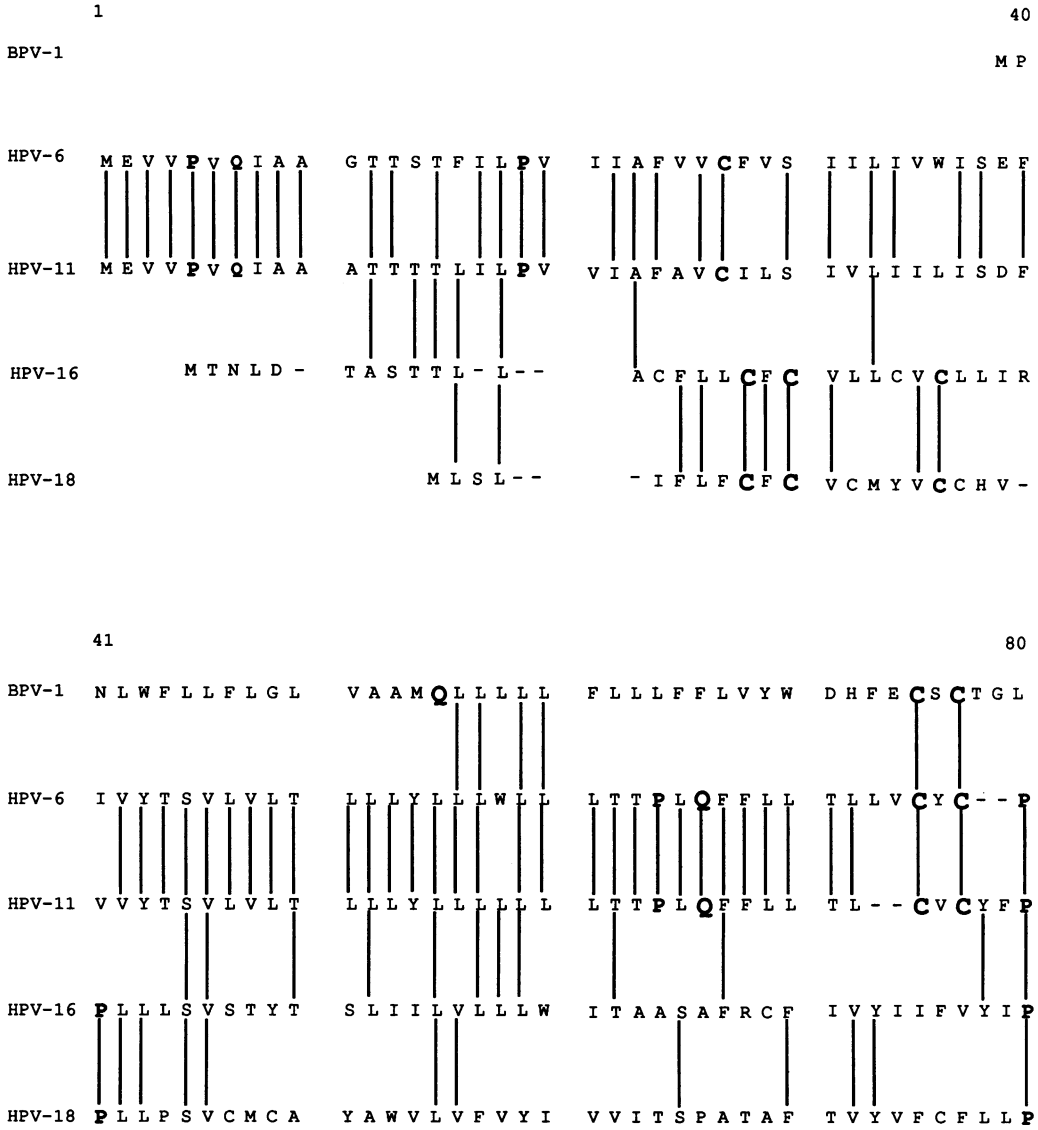

FIG. 1. Comparison of amino acid sequences of E5 proteins of BPV-1 and HPVs. Amino acid sequences have been aligned to show maximum homology. Conserved glutamine, cysteine, proline, and histidine residues are highlighted in boldface type.

pPAVA-1 contains the BPV-1 E2 to E5 region (nucleotides [nt] 2405 to 4450) in place of the simian virus 40 (SV40) early sequences (nt 2770 to 5171), so that the BPV sequences are transcribed from the SV40 early promoter. pBR322 sequences were added at the unique *Eco*RI site in SV40 (nt 1782) to facilitate propagation in bacteria. p37S39S is a variant of pPAVA-1 in which two cysteine residues at positions 37 and 39 have been converted to serine residues by site-specific mutagenesis, yielding a BPV-1 E5 protein which is no longer active in transforming assays (18). Insertion of the HPV-16 E5 gene into p37S39S was achieved by cleaving p37S39S with *Bst*XI and *Spe*I, which removes a 29-bp fragment at the 5′ end of the BPV-1 E5 open reading frame (ORF) (nt 3888 to 3916). An HPV-16 E5 ORF insert was constructed by polymerase chain reaction (PCR) technology with oligonucleotide primers flanking the HPV-16 E5 ORF (nt 3851 to 4101) and containing appropriate restriction

enzyme recognition sites on overhanging ends. The initiating methionine of the HPV-16 E5 ORF is eliminated and an in-frame fusion is created between the coding sequences of the HPV-16 E5 ORF and the codons for the first five amino acids of the BPV-1 E5 protein via the *Bst*XI cloning site. The termination codon of the HPV-16 E5 ORF is present on the 3′ oligonucleotide. Insertion of the PCR fragment into the cleaved parent plasmid resulted in a clone, pWT29, containing the HPV-16 E5 ORF. To include an antigenic epitope for expression of the HPV-16 E5 gene product as a fusion protein, plasmid pWT29 was cleaved with *Bst*XI. Oligonucleotide pairs encoding amino acids 39 to 64 of the murine lymphocyte-specific Lck protein were synthesized (termed the RNGS epitope for the first four amino acids) and annealed with oligonucleotide pairs containing *Bst*XI sites. These oligonucleotides were inserted into *Bst*XI-cleaved pWT29, yielding plasmid pRNGS-40, which encodes the HPV-16 E5 ORF with the RNGS epitope on the amino end.

Plasmid pSVL (Pharmacia, Piscataway, N.J.) contains the SV40 late promoter and the SV40 origin of replication along with the VP1 intron and late polyadenylation sequences of SV40. This vector was cleaved at the *Xho*I and *Bam*XI sites within the polylinker. HPV-6 sequences (HPV-6b E5a; nt 3887 to 4162) with an epitope tag on the 5′ oligonucleotide primer and containing appropriate restriction site ends were generated by PCR and inserted into the cleaved vector. The epitope tag included within the 5′ oligonucleotide (AU1) is a 6-amino-acid sequence from the BPV-1 L1 protein, against which a highly reactive monoclonal antibody has been raised (22). The HPV-16 insert with the RNGS epitope was generated by PCR from the previously cloned vector pRNGS-40 (described above) with oligonucleotide primer pairs containing *Xho*I and *Bam*HI sites flanking the RNGS epitope and the HPV-16 E5 ORF termination codon. The resultant clones, termed pSVL.A6E5 and pSVL.R16E5, express the HPV-6 and HPV-16 E5 proteins as fusion proteins with epitopes on their amino termini. For the truncated HPV-6 E5 clone, the 5′ oligonucleotide (including the AU1 epitope) used to generate the full-length product was used as a primer along with a 3′ oligonucleotide which inserted a termination codon after the first 45 codons of HPV-6 E5 (at nt 4023). Both primers had overlapping *Xho*I and *Bam*HI ends and were inserted into cleaved pSVL. The resultant clone is termed pSVL.TA61 and encodes the amino-terminal half of the HPV-6 E5 as an AU1 fusion protein. In all pSVL clones, the consensus Kozak sequences (20) were included on the 5′ oligonucleotide primer immediately upstream of the ATG initiation codon.

DNA transfer and virus preparations. Recombinant viral stocks from pRNGS-40 were prepared as described previously. pRNGS-40 was digested with *Eco*RI, religated at a DNA concentration of 5 μg/ml, and used to transfect CMT4 cells as described before (35). Cells were incubated for 5 days after transfection in DMEM supplemented with 10% fetal bovine serum, 1 μM $CdSO_4$, and 100 μM $ZnCl_2$. For preparation of RNGS-40 viral stocks, the cells were scraped into the medium and subjected to repeated freeze-thaw cycles. After removal of cell debris by centrifugation, amplification of virus stocks was carried out by infecting fresh CMT4 cells with the above supernatant and incubation for 5 days. A further cycle of amplification was performed to yield high-titer stocks. Virus titers were determined as described before (35), and further infections were done at a multiplicity of infection of between 10 and 100 infectious units per cell.

For transfection of pSVL constructs into COS cells, a modified calcium phosphate method, as used for the CMT4 cells above, was used. Briefly, COS cells were plated at approximately 30% confluency in 10-cm dishes. From 1 to 2 μg of circular pSVL DNA was mixed with carrier pUC DNA to a total of 10 μg of DNA and added to 500 μl of HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-buffered saline (HBS), pH 7.05. In the case of cotransfections with the 16-kDa protein-expressing pSVL construct, a 1:10 ratio of DNA (16 kDa; HPV E5) was used. Fifty microliters of 1.25 M $CaCl_2$ solution was added gradually with mixing, and the mixture was allowed to incubate for 30 min at room temperature. This mixture was added to the COS cells in 5 ml of DMEM and incubated at 37°C. After 16 to 18 h, the cells were shocked for 60 s with 15% glycerol in HBS, washed three times with phosphate-buffered saline (PBS), and further incubated in DMEM plus 10% fetal bovine serum until analysis.

Immunofluorescence assays. Subconfluent COS cell cultures grown on 18-mm coverslips were either infected with 10 to 100 infectious units of RNGS-40 virus per cell or transfected with pSVL.A6E5. Forty-eight hours after infection or transfection, the cells were washed three times with PBS and fixed for 15 min at room temperature in 3.7% formaldehyde in PBS. Coverslips were again washed three times in PBS and blocked for 20 min at room temperature with 10% nonspecific goat serum in PBS with 0.1% saponin. Staining solution with primary antibodies at appropriate dilutions (1:100 for both RNGS and AU1) was prepared in PBS with 0.1% saponin. Negative controls were either normal rabbit serum diluted 1:100 or AU1 and RNGS on uninfected COS cells. Primary antibody incubation was carried out at room temperature in a humid chamber for 1 to 2 h. Coverslips were washed extensively in PBS, and secondary-antibody solution was added. For the HPV-16 E5 staining, goat anti-rabbit immunoglobulin G (IgG) conjugated to trimethylrhodamine isothiocyanate was diluted in PBS plus 10% nonspecific goat serum and 0.1% saponin at a ratio of 1:20. For HPV-6 E5 staining, goat anti-mouse IgG conjugated to trimethylrhodamine isothiocyanate was added in a ratio of 1:50 to PBS with 0.1% saponin. Secondary-antibody incubations were carried out for 1 h at room temperature in a humid chamber. After extensive washes with PBS, coverslips were mounted on glass slides with Fluoromount and examined on a Zeiss ICM 405 UV microscope.

Immunoprecipitation and immunoblot assays. Infected and transfected COS cells were analyzed 48 h after infection or transfection by immunoprecipitation for the E5 proteins. Cells were washed with PBS, incubated with cysteine- and methionine-free DMEM for 1.5 to 2 h, and labelled for 4 h with 450 μCi of a commercial mixture of ^{35}S-labelled methionine (approx. 90%) and cysteine (10%) (Express; NEN) or a mixture of approximately 75% ^{35}S-labelled methionine and 25% cysteine (Amersham) in 2 ml of DMEM at 37°C. Cells were washed in PBS and extracted in 1 ml of a modified RIPA buffer (20 mM MOPS [morpholinepropanesulfonic acid], 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1% deoxycholate, 0.1% SDS [pH 7.0]) containing protease inhibitors (0.1 mM TLCK [*Nα-p*-tosyl-L-lysine chloromethyl ketone], 0.1 mM TPCK [*N*-tosyl-L-phenylalanine chloromethyl ketone], and 0.5 mM phenylmethylsulfonyl fluoride). After 30 s of vortexing, extracts were centrifuged for 2 min in an Eppendorf microcentrifuge. Appropriate antibody (5 μl of AU1 ascites fluid or 7 μl of RNGS antiserum) was added to the supernatant along with 50 μl of protein A-Sepharose CL-4B beads (Pharmacia). Following 1.5 h of rotation at 4°C, the Sepharose beads were pelleted, washed four times in

RIPA buffer plus protease inhibitors, and resuspended in 75 μl of sample buffer with and without β-mercaptoethanol. For double immunoprecipitations the Sepharose bead pellet was resuspended in 0.2 ml of solubilization buffer (0.4% SDS, 50 mM triethanolamine-Cl [pH 7.4], 100 mM NaCl, 2 mM EDTA, 2 mM β-mercaptoethanol) and treated for 2 min at 100°C. After cooling, 4 μl of iodoacetamide (0.5 M) and 50 μl of 10% Triton X-100 were added. The samples were pelleted, and the supernatant was removed for immunoprecipitation with the second antibody. Appropriate antibody and protein A-Sepharose beads were added, and the mixture was incubated for a further hour with rotation at 4°C. The beads were then pelleted, washed with PBS, and resuspended in sample buffer with β-mercaptoethanol for electrophoresis. Samples were heated to 100°C for 4 min and analyzed by SDS–14% PAGE. Gels were fixed with glacial acetic acid-methanol, treated with Enlightning (NEN), dried, and exposed to Kodak XAR-5 film at −70°C.

For HPV-16 E5 immunoblot assays, infected or transfected COS cells were harvested in 1 ml of RIPA buffer with protease inhibitors and immunoprecipitated as described above. Samples were electrophoresed on 14% polyacrylamide–SDS gels. The gels were blotted onto nitrocellulose, and proteins were detected by a chemiluminescence (Western-Light; Tropix) kit with RNGS antiserum at 1:50 as the primary antibody and goat anti-rabbit IgG-alkaline phosphatase conjugate (Tropix) at 1:10,000 as the secondary antibody.

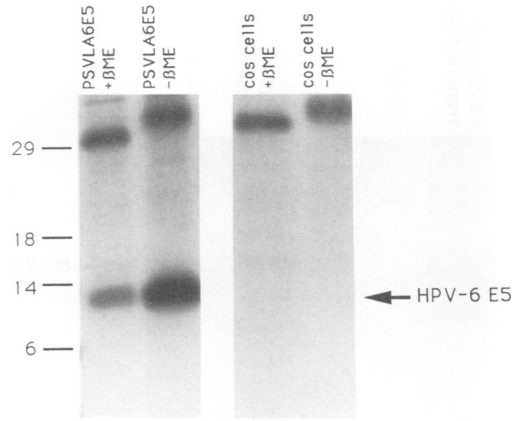

FIG. 2. Expression of the HPV-6 E5-AU1 fusion protein in COS cells. COS cells were transfected with plasmid pSVL.A6E5 and analyzed 48 h after transfection for the presence of the fusion protein. Cells were labelled with Express ^{35}S labelling mix (New England Nuclear) for 4 h, immunoprecipitated with AU1 antibody, and separated electrophoretically on a 14% acrylamide–SDS gel. The HPV-6 E5 fusion protein migrates as a 12-kDa band (arrow). Samples were run with and without the reducing agent β-mercaptoethanol (βME). Dimerization due to disulfide linkages was not detectable. Nontransfected COS cells were also immunoprecipitated with AU1 antibody and run with and without β-mercaptoethanol. Molecular sizes (in kilodaltons) are indicated at the left.

RESULTS

Expression of epitope-tagged E5 proteins of HPV-6 and HPV-16. To assist in the identification and localization of the HPV E5 proteins, the HPV E5 ORFs were cloned into expression vectors. The HPV-6 E5 ORF, with the AU1 epitope encoding the 6-amino-acid tag fused to the amino terminus, was cloned into a pSVL plasmid (containing the SV40 late promoter) for expression in COS cells. Likewise, the HPV-16 E5 ORF was inserted into the pSVL vector with the 25 amino acid RNGS epitope (lymphocyte Lck antigen) at its amino terminus. The resultant clones were termed pSVL.A6E5 and pSVL.R16E5, respectively. A recombinant SV40-BPV virus, termed RNGS-40, was also generated for the expression of the HPV-16 E5-RNGS fusion protein via infection of COS cells. Antibodies specific for the AU1 and RNGS epitopes were used to detect the respective HPV-6 E5 and HPV-16 E5 fusion proteins in COS cells transfected with pSVL constructs or infected with the recombinant virus (see Materials and Methods). Detection was done for both the HPV-6 and HPV-16 E5 proteins by immunoprecipitation of cells metabolically labelled with [^{35}S]cysteine and [^{35}S]methionine and, in the case of HPV-16 E5, by a combined technique using immunoprecipitation followed by immunoblotting.

A feature of the BPV-1 E5 protein which is critical for transforming activity is its ability to dimerize through its carboxyl terminal Cys-X-Cys sequence. Since the HPV E5 proteins contain several cysteine residues, we evaluated whether these proteins could form dimers or multimeric complexes. The HPV-6 E5-AU1 fusion protein migrated as a 12-kDa band on SDS-PAGE. When immunoprecipitates were run on acrylamide gels in the absence of the reducing agent β-mercaptoethanol, no change in band pattern was observed, indicating that the HPV-6 E5 fusion protein does not form dimers or other oligomers through disulfide linkages (Fig. 2). The HPV-16 E5-RNGS fusion protein migrated at 17 kDa on SDS-PAGE, and while it also contains multiple cysteine residues, it did not oligomerize in the absence of reducing agent (Fig. 3A and B). Both of these observations are in contrast to the case of the BPV-1 E5 protein, which is known to dimerize via disulfide linkages.

An additional 12-kDa band was seen in both radioactively labelled immunoprecipitates and immunoblots of COS cells expressing the HPV-16 E5-RNGS fusion protein (Fig. 3). This band did not appear in control experiments in which pUC or pSVL DNA had been transfected (data not shown) or in immunoprecipitates of untransfected COS cells (Fig. 3B). Although the origin of this band is presently unclear, it is detectable by immunoblotting, indicating that it contains the RNGS epitope. Studies indicate that it is not a breakdown product or the result of altered phosphorylation or glycosylation (data not shown).

Intracellular localization of the HPV-6 E5-AU1 and HPV-16 E5-RNGS fusion proteins. Immunofluorescence microscopy was used to determine the intracellular localization of the HPV-6 E5-AU1 and HPV-16 E5-RNGS fusion proteins. COS cells grown on coverslips and infected with RNGS-40 or transfected with pSVL.A6E5 were fixed in 3.7% formaldehyde in PBS at 48 h after transfection or infection. Cells were permeabilized with 0.1% saponin and blocked with 10% nonspecific goat serum, and immunofluorescence was carried out with either AU1 or RNGS as the primary antibody. The secondary antibody was a rhodamine-conjugated anti-mouse (AU1) or anti-rabbit (RNGS) IgG antibody, respectively. By this method, both the HPV-6 E5 and HPV-16 E5 fusion proteins appeared to be membrane-associated proteins. Immune staining for the HPV-6 E5-AU1 fusion protein was most pronounced in a perinuclear location, consistent with localization to the Golgi apparatus, although nuclear membrane staining and cytoplasmic staining characteristic of the endoplasmic reticulum were also seen (Fig. 4A and B). The HPV-16 E5-RNGS fusion protein showed the same three areas of staining, with slight differ-

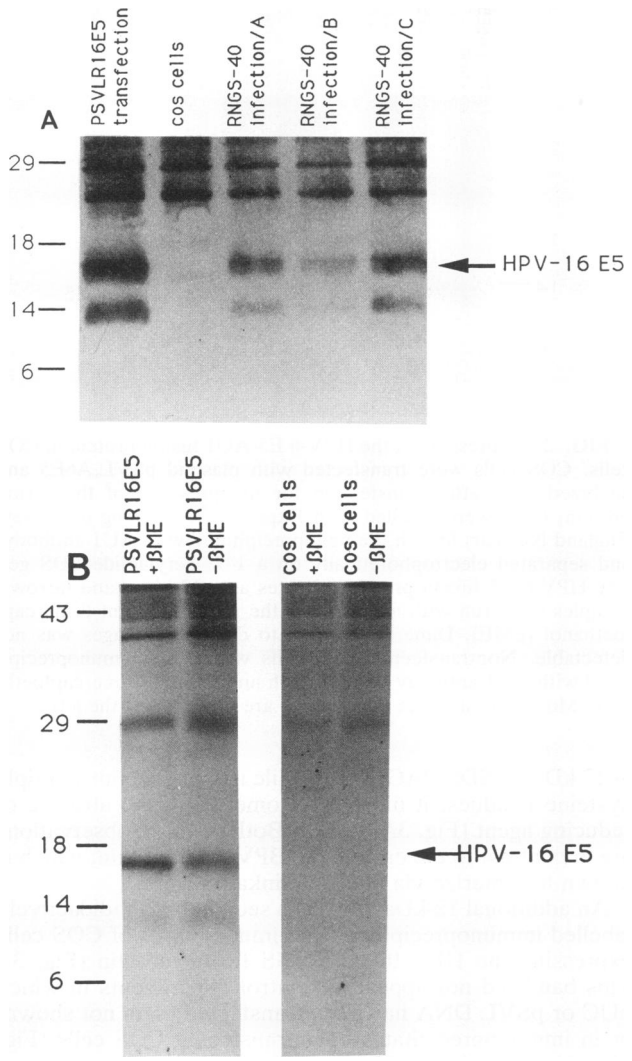

FIG. 3. Expression of HPV-16 E5 as a fusion protein with the RNGS epitope at the amino terminus. COS cells transfected with pSVL.R16E5 or infected with RNGS-40 virus were analyzed after 48 h for the presence of the HPV-16 E5 protein. (A) Infected and transfected cells were immunoprecipitated with anti-RNGS antiserum and separated by SDS-PAGE. The gel was blotted onto nitrocellulose, and immune detection was carried out with a chemiluminescent (Tropix) detection system with anti-RNGS as the primary antibody. Three different viral stocks (A, B, and C) were used for comparison. (B) Transfected cells were immunoprecipitated with anti-RNGS after 4 h of labelling with Express ^{35}S labelling mix and separated on a 14% acrylamide–SDS gel. The HPV-16 E5 fusion protein migrated as a 17-kDa band (arrow), and no dimerization was observed when samples were run in the presence and absence of β-mercaptoethanol (βME). An additional band which represents a variant form of the protein may be seen at 12 kDa (see text). Molecular sizes (in kilodaltons) are indicated at the left.

ences in distribution (Fig. 4C and D). In the case of the HPV-16 E5 fusion protein, the predominant staining was a cytoplasmic trabecular pattern consistent with endoplasmic reticulum localization, although nuclear membrane and cytoplasmic staining were also consistently observed.

Association of the HPV-6 E5-AU1 and HPV-16 E5-RNGS fusion proteins with the 16-kDa component of the vacuolar ATPase in cotransfection experiments. To determine whether the HPV E5 fusion proteins, similar to the BPV-1 E5 protein, were capable of complex formation with the 16-kDa component of the vacuolar ATPase (14), we coexpressed the HPV E5 and 16-kDa proteins in COS cells. pSVL.A6E5 and pSVL.R16E5 were cotransfected into COS cells with a pSVL construct, pTA9, which efficiently expresses the full-length bovine 16-kDa protein as a fusion protein with the influenza virus hemagglutinin HA1 epitope at its amino terminus. After metabolic labelling with a mixture of [^{35}S]methionine and [^{35}S]cysteine, immunoprecipitation with antiepitope antibodies was carried out to determine the presence of complex formation. Immunoprecipitates with RNGS (recognizing the HPV-16 E5-RNGS fusion protein) and AU1 (recognizing the HPV-6 E5-AU1 fusion protein) coprecipitated the 16-kDa protein (Fig. 5A and B). In addition, immunoprecipitation with 12CA5 (recognizing the HA1 epitope-tagged 16-kDa fusion protein) coprecipitated the HPV-6 E5 fusion protein (Fig. 5A).

Because the HPV-16 E5 fusion protein migrated at a similar position on SDS-PAGE as the 16-kDa protein, coprecipitation of HPV-16 E5 with 12CA5, recognizing the 16-kDa protein, was impossible to verify by single-immunoprecipitation techniques. For this reason, a double-immunoprecipitation experiment was performed with COS cells cotransfected with HPV-16 E5- and 16-kDa protein-expressing constructs. These cotransfected cells were first immunoprecipitated with either RNGS or 12CA5 or mock immunoprecipitated with Sepharose beads only. After washing and solubilization of the precipitated proteins, these samples were then subjected to a secondary immunoprecipitation with either RNGS or 12CA5. Figure 5C shows that the HPV-16 E5 fusion protein (which originally precipitated as a complex with the 16-kDa protein antibody 12CA5) was then directly detectable by using the RNGS antibody (Fig. 5C, lane 2). The retention of the 16-kDa protein in this secondary immunoprecipitation probably reflects incomplete dissociation or rapid reassociation of the HPV-16 E5-16-kDa protein complex during the second immunoprecipitation step and is probably due to the large amount of 16-kDa protein precipitated by 12CA5 in the first immunoprecipitation. The converse situation is also demonstrated, as 16-kDa protein (originally precipitated as a complex with HPV-16 E5 with the RNGS antibody) was then directly detected with 12CA5 in the second precipitation (Fig. 5C, lane 6). These findings illustrate that complex formation, demonstrated in both directions, between the HPV E5 fusion proteins and the 16-kDa component of the vacuolar ATPase is present for both the low-risk HPV-6 and the high-risk HPV-16 when these proteins are overexpressed in COS cells.

HPV-6 E5-AU1 fusion protein and HPV-16 E5-RNGS fusion protein do not bind to the alpha subunit of IL-2R or to the MHCI molecule. To elucidate the specificity of the interaction between the HPV E5 fusion proteins and the 16-kDa protein, control experiments were performed to determine whether cotransfection and subsequent overexpression of another membrane-associated protein (the alpha subunit of the interleukin-2 receptor [IL-2R]) would result in complex formation with HPV E5 proteins. Furthermore, association between the HPV E5 fusion proteins and an integral membrane protein (major histocompatibility complex I [MHCI]) present on COS cells was tested. Cotransfection experiments were done with the pSVL HPV E5 fusion protein-expressing constructs and an SV40 expression plasmid containing the Tac antigen (alpha chain of the IL-2R) (7). No coprecipitation of the Tac antigen with the HPV E5 fusion proteins was detected with either RNGS or AU1 antibody

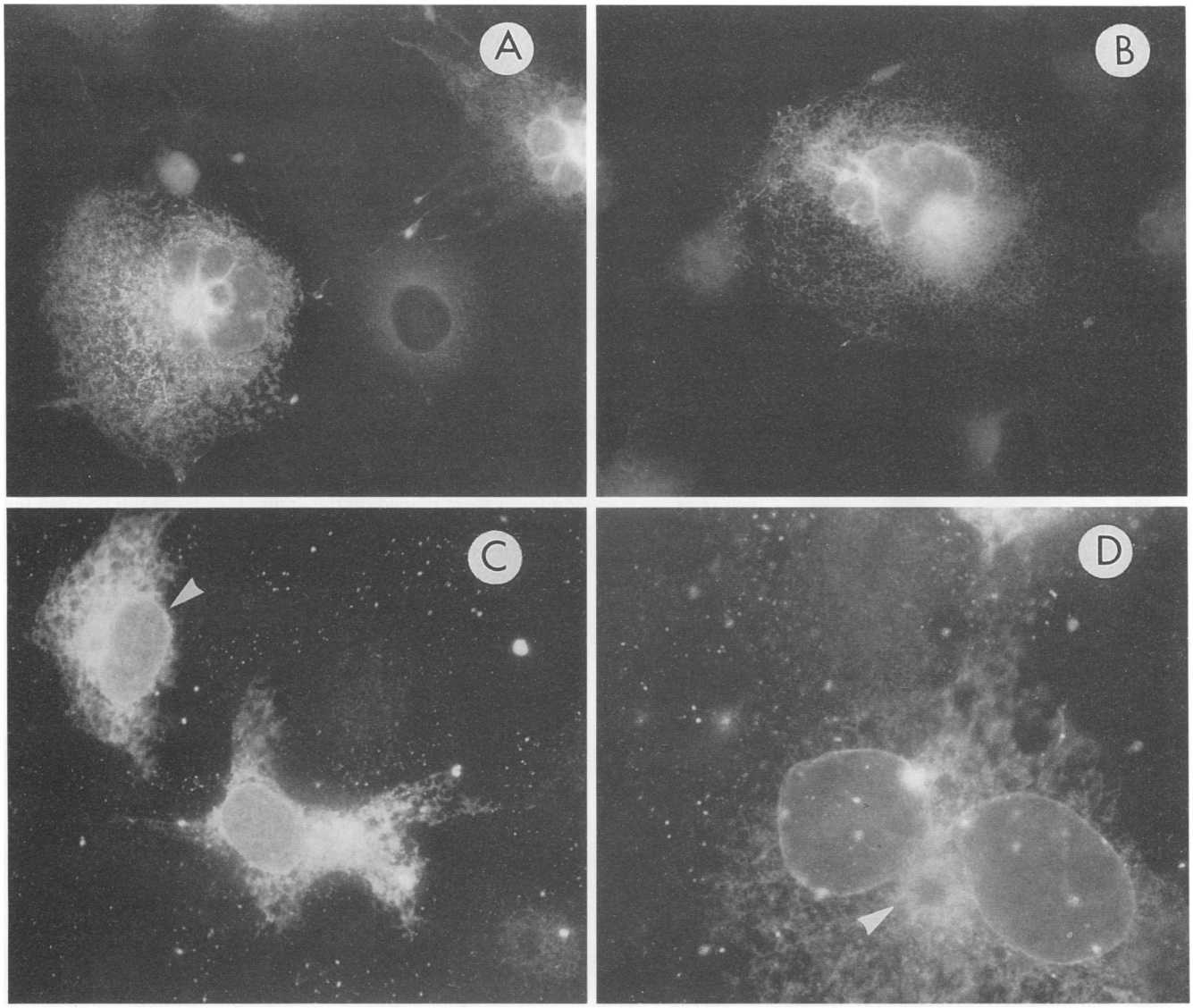

FIG. 4. Intracellular localization of the HPV-6 E5 and HPV-16 E5 fusion proteins. (A and B) Immunofluorescence staining for the HPV-6 E5-AU1 fusion protein. COS cells were transfected with pSVL.A6E5 or infected with RNGS-40 virus and analyzed 48 h after transfection or infection. Cells were fixed with formaldehyde, permeabilized with saponin, and reacted with either RNGS or AU1 antibodies and rhodamine-conjugated secondary antibodies as described in Materials and Methods. Staining is primarily within the Golgi apparatus and in small cytoplasmic vesicles. Some nuclear membrane and endoplasmic reticulum staining is also seen. (C and D) The HPV-16 E5-RNGS fusion protein is located principally within the endoplasmic reticulum, with Golgi and nuclear membrane staining also present (arrows). Nontransfected cells in panels B and C indicate that neither the AU1 nor RNGS antibody reacts significantly with nontransfected COS cells.

(data not shown). Likewise, precipitation with an anti-Tac antibody did not coprecipitate the HPV E5 fusion proteins. Although COS cells express high levels of both the alpha chain and β_2-microglobulin components of the MHCI molecule, neither component coprecipitated with the RNGS or AU1 antibody in cells transfected with HPV E5-expressing constructs (data not shown). An anti-MHCI antibody (a gift of L. Gissmann) also did not coprecipitate the HPV E5 fusion proteins.

Truncated HPV-6 E5-AU1 fusion protein associates with the 16-kDa component of the vacuolar ATPase. Attempts have been made previously to map the precise binding domain within BPV-1 E5 for the 16-kDa molecule. The glutamine residue in BPV-1 E5 appears to be important for association with the 16-kDa protein (15). However, more recent work suggests that the BPV-1 E5–16-kDa protein interaction may be quite complex, as other BPV-1 E5 mutants lacking the glutamine residue may retain the ability to complex with the 16-kDa protein (35a).

Since the HPV-6 E5 protein has certain similarities to the BPV-1 E5, including the presence of a glutamine residue within a hydrophobic domain, we evaluated whether this residue was important for HPV-6 E5 binding to the 16-kDa protein. A construct, pSVL.TA61, was generated, which expresses the first 45 amino acids of the HPV-6 E5 as a fusion protein with the AU1 epitope and lacks the glutamine residue which resides in the carboxyl terminus of the HPV-6 E5 molecule. When the truncated HPV-6 E5 and 16-kDa

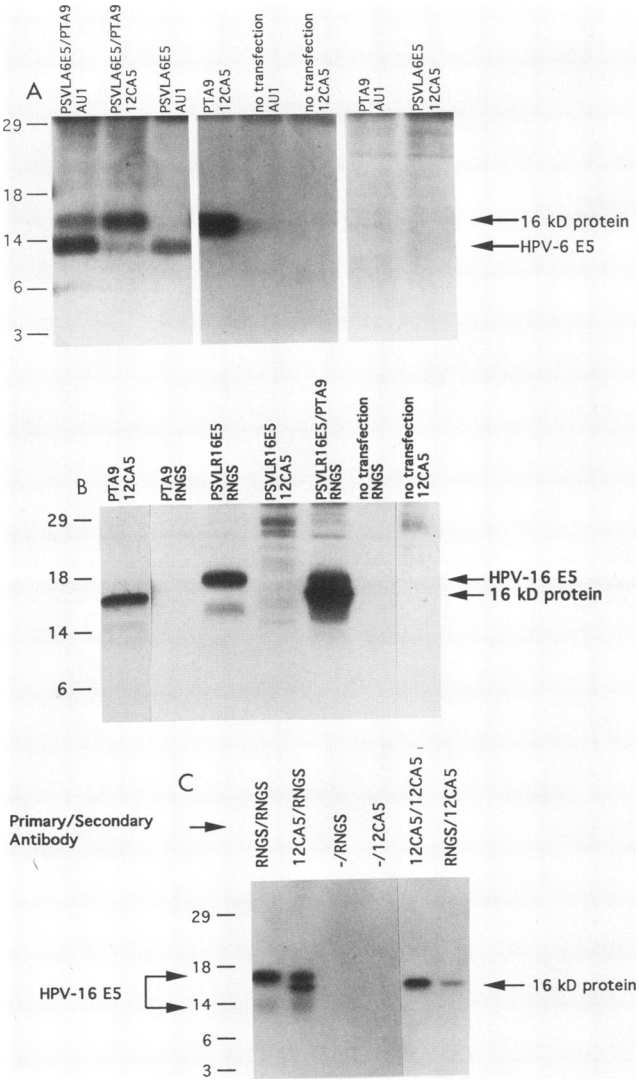

FIG. 5. Association of the HPV-6 E5 and HPV-16 E5 fusion proteins with the 16-kDa component of the vacuolar ATPase. (A) COS cells were transfected with plasmid pSVL.A6E5 (expressing the HPV-6 E5-AU1 fusion protein) or pTA9 (expressing the 16-kDa protein with an HA1 epitope tag) and labelled 48 h posttransfection with Express ^{35}S labelling mix. Samples were immunoprecipitated with AU1 or 12CA5, a monoclonal antibody reactive against the HA1 epitope, and separated on an SDS–14% PAGE gel. The first line above each lane represents the transfected DNA plasmid, and the second line represents the antibody used for immunoprecipitation. In cotransfection experiments with both plasmids (first two lanes), both the 16-kDa band and the 12-kDa HPV-6 E5 band (arrows) were precipitated by both antibodies. Controls included cells transfected only with pSVL.A6E5 and immunoprecipitated with either AU1 or 12CA5 antibodies, cells transfected with pTA9 and immunoprecipitated with 12CA5 or AU1, and untransfected COS cells precipitated with both antibodies. Molecular sizes (in kilodaltons) are indicated at the left. (B) COS cells were transfected with plasmids expressing the HPV-16 E5 (pSVL.R16E5) or the 16-kDa protein with the HA1 epitope tag (pTA9). After 48 h, cells were metabolically labelled with Express ^{35}S label mix and immunoprecipitated with RNGS antiserum or 12CA5, a monoclonal antibody against the HA1 epitope. Samples were run on a 14% acrylamide–SDS gel. In cotransfection experiments with HPV-16 E5- and 16-kDa protein-expressing constructs, precipitation with the RNGS antibody showed a band corresponding to the 16-kDa protein located between the two HPV-16 E5 species. Association in the reverse direction (HPV-16 E5 coprecipitation with 12CA5) is difficult because of the strong signal of the 16-kDa protein and the similar gel locations of HPV-16 E5 and the 16-kDa protein. Controls included cells transfected only with pSVL.R16E5 and immunoprecipitated with either RNGS or 12CA5, cells transfected with pTA9 and immunoprecipitated with 12CA5 or RNGS, and untransfected COS cells precipitated with both antibodies. Molecular sizes are indicated at the left. (C) COS cells were cotransfected with pSVL.R16E5 and pTA9 and labelled 48 h after transfection with Express ^{35}S label mix. Samples were then double immunoprecipitated with RNGS (recognizing HPV-16 E5) and 12CA5 (recognizing the 16-kDa protein) as primary and secondary antibodies as indicated above the lanes. Mock primary immunoprecipitated cells (—) were used as negative controls. In secondary immunoprecipitations, 12CA5 was able to precipitate the 16-kDa protein previously coprecipitated with RNGS (last lane). Likewise, RNGS precipitates HPV-16 E5 protein first coprecipitated with 12CA5 (second lane). Molecular sizes are indicated at the left. The 12CA5 secondary immunoprecipitations in panel C represent 10-fold reductions in the radiographic exposure times used for panels A and B.

proteins were coexpressed in COS cells, it was possible to coprecipitate the 16-kDa protein readily with antibodies directed against E5 (Fig. 6, lane 2). On the other hand, coprecipitation of the truncated E5 protein with antibodies directed against the 16-kDa protein was much less efficient (Fig. 6, lane 3) and required longer exposures for adequate visualization (data not shown). Evidently, neither the Cys-X-Cys nor the glutamine residues in the HPV-6 E5 protein are required for HPV E5–16-kDa protein binding.

DISCUSSION

In this study, the E5 proteins of HPV-6 and HPV-16 were expressed, localized to membranes, and shown to interact with a common cellular target of the BPV-1 E5 protein, the 16-kDa pore-forming component of the vacuolar ATPase.

Epitope addition. Two HPV E5 proteins were tagged with antigenic epitopes at their amino termini in order to facilitate analysis of their biochemical properties and intracellular localization. Similar studies have been done previously with the BPV-1 E5 protein (14, 16), and in these previous studies, epitopes were placed on the amino termini of the E5 proteins (16). HPV-6 E5 shows some homology to BPV-1 E5, and the amino terminus of BPV-1 E5 is known to withstand significant insertion, deletion, and substitution mutations without effects on its transforming ability (8, 17, 18). The AU1 epitope was chosen because of its small size (6 amino acids) and the availability of a high-affinity monoclonal antibody against this epitope. When it was added to the amino terminus of HPV-6 E5, the resultant fusion protein was readily detectable in COS cells. Addition of the AU1 epitope to the HPV-16 E5 protein was more problematic and did not result in a detectable fusion protein, perhaps because of alterations in protein conformation or masking of the epitope within the E5 molecule. The HPV-16 E5-RNGS fusion protein, however, was detectable. The RNGS epitope is significantly larger (25 amino acids) than AU1 and presumably is not masked by protein conformation.

Expression of HPV E5 fusion proteins. The HPV-16 E5-RNGS fusion protein was initially detected in COS cells by infection with recombinant SV40-BPV viruses which express the fusion protein. This system has several advantages in cells expressing the SV40 large T antigen, including activation of the SV40 late promoter and driving of the SV40 origin of replication, resulting in enhanced viral copy num-

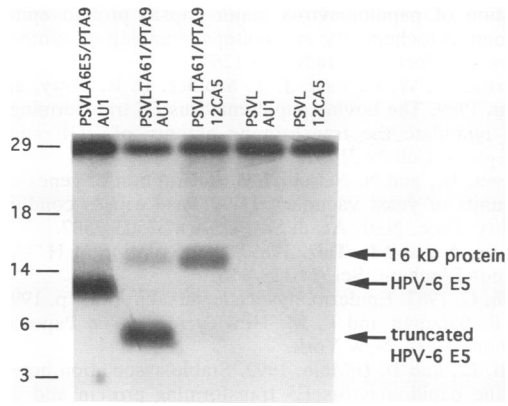

FIG. 6. Binding of truncated HPV-6 E5-AU1 fusion protein to the 16-kDa component of the vacuolar ATPase. Plasmids expressing the full-length HPV-6 E5 fusion protein (pSVL.A6E5) and truncated forms of this protein (pSVL.TA61) were cotransfected in COS cells with pTA9, which expresses the 16-kDa protein as a fusion protein with the HA1 epitope. The cells were labelled at 48 h after transfection with Express ^{35}S label mix, immunoprecipitated, and analyzed by SDS-PAGE. The first line above each lane represents the transfected plasmids, and the second line represents the antibody used for immunoprecipitation. Immunoprecipitation with AU1 antibody, recognizing both the truncated and full-length HPV-6 E5 fusion proteins, shows that both proteins coprecipitate with the 16-kDa protein (first two lanes from left). Additionally, cotransfection of the truncated HPV-6 E5 with the 16-kDa protein and immunoprecipitation with 12CA5 (which recognizes the epitope-tagged 16-kDa protein) shows a faint band revealing coprecipitation of the truncated protein. Molecular sizes (in kilodaltons) are indicated at the left.

ber and enhanced production of the fusion protein. The HPV-6 E5 was also efficiently expressed in the PAVA system as a fusion protein with the influenza virus hemagglutinin HA1 epitope (data not shown). The disadvantages of this system include the need to generate and purify viral stocks for infection and the presence of BPV segments within the construct (such as the E2 ORF). However, no truncated fragments of BPV E5 were detectable within COS cells infected with PAVA viruses (data not shown). The pSVL vector has the advantage of expressing the isolated HPV E5 proteins while retaining the advantages of the large-T-antigen-driven SV40 late promoter and origin of replication.

The HPV-6 E5-AU1 fusion protein and the HPV-16 E5-RNGS fusion protein both migrate more slowly on SDS-PAGE than would be predicted by their molecular weights. The retarded migration of the E5's in acrylamide gels is most likely attributable to SDS-resistant protein structure. Experiments with polyclonal antisera raised against portions of the HPV-6 and HPV-16 wild-type E5 proteins can also detect these proteins and verify their abnormal migration properties (data not shown), indicating that epitope addition alone is not responsible for their altered mobility.

Localization of the HPV-6 E5 and HPV-16 E5 fusion proteins. The HPV-6 E5-AU1 and HPV-16 E5-RNGS fusion proteins are membrane proteins localized to the Golgi apparatus, endoplasmic reticulum, and nuclear membrane. This intramembrane location was anticipated from the extreme hydrophobicity of the molecules and is consistent with the intracellular localization of the BPV-1 E5. This location is also consistent with the finding that the E5 proteins complex with the 16-kDa molecule, which is known to reside in Golgi and vesicle membranes. Although the BPV-1 E5 protein has been described to be oriented asymmetrically in the Golgi, with its carboxyl terminus oriented intraluminally (5), the precise orientation of the HPV E5 proteins within membranes remains unknown. However, both the HPV-6 and HPV-16 E5 proteins do contain three potential transmembrane domains which would orient their amino and carboxyl termini on opposite membrane surfaces (3).

Association of the HPV-6 E5-AU1 and HPV-16 E5-RNGS fusion proteins with the 16-kDa pore-forming component of the vacuolar ATPase. The 16-kDa protein is a membrane component of the vacuolar proton-ATPase, which is also found in clathrin-coated vesicles, lysosomes, endosomes, and Golgi vesicles (11). The proton ATPase is essential for the acidification of these intracellular compartments and may be important in protein processing, sorting, and endocytosis (24, 25). The 16-kDa pore-forming protein is known to associate with the BPV-1 E5 protein (14) and also to form a complex with the BPV-1 E5 protein and the platelet-derived growth factor receptor (13). In this study, we showed that HPV E5's from both a low-risk and a high-risk HPV type also complex with the 16-kDa molecule when these proteins are over-expressed in COS cells. This association with the 16-kDa protein may interfere with proton pump function, possibly resulting in altered growth factor receptor recycling and degradation (13). Thus, the reported oncogenic activity of HPV-16 E5 could reflect an altered processing or degradation of the EGF receptor, since EGF appears to be critical for the observed HPV-16 E5-mediated transforming activity (21, 29, 36). Indeed, it has recently been shown that expression of HPV-16 E5 leads to a delay in EGF receptor internalization and degradation in human keratinocytes and to an increased recycling of these receptors to the cell surface (36).

Recent data showing that some mutants of BPV-1 E5 lacking the glutamine residue can complex with the 16-kDa protein (35a) suggest that the E5–16-kDa protein interaction may involve multiple domains. The fact that an HPV-6 E5 construct lacking the carboxyl terminus and glutamine residue binds as well as the full-length HPV-6 E5 to the 16-kDa pore-forming molecule supports the concept that a binding domain other than the glutamine residue is present in the HPV-6 E5 molecule. The HPV E5's, while extremely hydrophobic, contain additional polar amino acid residues which might mediate binding to the 16-kDa protein. Further studies of both HPV-6 E5 and HPV-16 E5 will be necessary to further characterize their 16-kDa binding domains as well as to determine whether they interact with other cellular targets.

ACKNOWLEDGMENTS

We thank David Goldstein for suggestions and advice regarding the 16-kDa protein binding studies and D.G. and Hubert Stöppler for critical review of the manuscript.

This work was supported by Public Health Service grants CA-01626 (M.C.) and RO1 CA-53371 (R.S.) from the National Cancer Institute.

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