A Glutamine Residue in the Membrane-Associating Domain of the Bovine Papillomavirus Type 1 E5 Oncoprotein Mediates Its Binding to a Transmembrane Component of the Vacuolar H⁺-ATPase

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Received 12 September 1991/Accepted 9 October 1991

The 44-amino-acid E5 oncoprotein is the major transforming protein of bovine papillomavirus type 1. It is a highly hydrophobic polypeptide which dimerizes and localizes to the Golgi apparatus and endoplasmic reticulum membranes. Recent evidence suggests that E5 modulates the phosphorylation and internalization of the epidermal growth factor and colony-stimulating factor 1 receptors and constitutively activates platelet-derived growth factor receptors in C127 and FR3T3 cells. Although no direct interaction with these growth factor receptors has yet been identified, the E5 oncoprotein has been shown recently to interact with the hydrophobic 16-kDa component of the vacuolar H⁺-ATPase (16K protein) [D. J. Goldstein, M. E. Finbow, T. Andresson, P. McLean, K. Smith, V. Bubb, and R. Schlegel, *Nature (London)* 352:347-349, 1991]. In the current study, we have further analyzed the E5-16K protein complex by fast protein liquid chromatography and shown that each E5 dimer appears to bind two 16K proteins. In order to define the specific amino acid residues of E5 which participate in this binding, mutated E5 epitope fusion proteins were analyzed for their ability to coprecipitate 16K protein. Transformation-defective mutants containing amino acid substitutions within the short hydrophilic carboxyl-terminal domain retained the ability to associate with the 16K protein. However, E5 mutants lacking the glutamine residue in the hydrophobic domain were markedly inhibited in 16K protein binding. Most interestingly, the placement of a glutamine in several random hydrophobic sequences facilitated 16K protein binding, defining this residue as a potential binding site for the 16K protein component of the proton pump and exemplifying the critical role of hydrophilic amino acids for mediating specific interactions between transmembrane proteins.

Papillomaviruses induce the benign proliferation of epithelial cells of many vertebrate hosts (30). Some papillomaviruses, including those human papillomavirus subtypes which infect genital epithelium, are also associated with lesions that may progress to cancer (28, 41). It is becoming apparent that papillomaviruses, like other DNA tumor viruses, encode transforming proteins which interact with cellular proteins involved in the control of cell growth (32). For instance, the E6 and E7 proteins of human papillomavirus types 16 and 18 complex with the p53 (39) and RB (8, 26) proteins, respectively.

Bovine papillomavirus type 1 (BPV-1) induces the efficient transformation of murine fibroblast cell lines (7, 24) predominantly via the activity of its E5 gene (6, 18, 31, 33). The E5 oncoprotein is a highly hydrophobic protein composed of 44 amino acids that localizes to Golgi apparatus, endoplasmic reticulum, and plasma membranes (3, 4). It is composed of two distinct domains, one being a 30-amino-acid, hydrophobic, amino-terminal domain which is predicted to traverse the membrane, and the other being a 14-amino-acid, hydrophilic, carboxyl-terminal domain which is highly conserved among the fibropapillomaviruses (3, 20). Genetic analysis of the E5 oncoprotein has defined several amino acids which are required for focus-forming ability within the small hydrophilic domain (20), including two cysteine residues which mediate homodimer formation. In contrast, conservative amino acid substitutions within the hydrophobic domain do not significantly alter the transforming ability of E5 (20).

Although there does not appear to be a strict requirement for a specific hydrophobic amino acid sequence within this region, the hydrophilic glutamine residue at position 17 appears to be critical for E5 function (20, 21, 23).

Several lines of biochemical evidence suggest that growth factor activation may be an important component of E5-mediated cellular transformation. For example, E5 stimulates the phosphorylation of the epidermal growth factor receptor in a ligand-independent manner and decreases the internalization of occupied receptors (25). Similarly, the endogenous cellular β -type receptor for the platelet-derived growth factor (PDGF) is constitutively activated in C127 and FR3T3 cells stably transformed by the E5 protein (29). On the basis of amino acid sequence similarity between the carboxyl termini of the E5 protein and PDGF and the conservation of these same amino acid residues in other fibropapillomavirus E5 proteins (20), Petti et al. (29) speculated that activation of the PDGF receptor might be mediated by a direct interaction between the E5 protein and the PDGF receptor. However, direct interaction between the E5 protein and growth factor receptors has not yet been demonstrated, and it is possible that receptor activation is indirect and requires E5 interactions with other cellular proteins.

Recently, E5 epitope fusion proteins were shown to associate specifically with a 16-kDa cellular protein (16K protein). This protein has now been identified as the pore-forming constituent of vacuolar H⁺-ATPases (15) and is essential for acidification of subcellular compartments such as endosomes, lysosomes, clathrin-coated vesicles, and Golgi vesicles. Since growth factor receptors are processed

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in the Golgi apparatus and are internalized via clathrin-coated vesicles. E5 binding to the 16K protein within these components may be an intermediate step to receptor activation and signal transduction. We previously showed that a transformation-defective E5 molecule containing a glycine at position 17 failed to bind to the 16K cellular protein, suggesting that this region of the E5 protein may be a binding site for the 16K protein and that the association may be involved in E5-mediated transformation (16). To further characterize the E5-16K interaction, a number of E5 mutants were examined by coimmunoprecipitation for their ability to interact with the 16K protein. Our findings demonstrate that the glutamine at position 17 is critical for 16K protein binding, thus demonstrating that hydrophilic amino acid residues which are presumably membrane embedded can mediate specific interactions between the α -helical, trans-membrane domains of regulatory proteins.

MATERIALS AND METHODS

Cell culture. The cell lines COS-33 (a gift from J. Brady) and CMT4 (13) were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum.

Plasmid constructions. The plasmid pPava-1 consists of the *Bst*EII (nucleotide [nt] 2405)-to-*Bam*HI (nt 4450) fragment of BPV-1, which contains the entire E2-E5 open reading frames (ORFs) inserted in place of the *Hind*III (nt 5171)-to-*Bcl*I (nt 2770) large tumor (T) antigen-coding fragment of simian virus 40 (SV40) (35). These viral sequences were cloned into pBR322 at the unique *Eco*RI site in the SV40 late region (nt 1782). All plasmids containing mutated E5 ORFs (see Fig. 3B, 4B, and 5B) were derived from Pava-1 (23, 35). PL15 and mutated E5 plasmids used to analyze 16K protein binding were constructed by inserting the synthetic oligonucleotides corresponding to the HA1 epitope shown in Fig. 1 into the unique *Bst*XI sites within the E5 ORFs. These oligonucleotides contain *Bst*XI-compatible ends which, when ligated into the *Bst*XI site within the E5 gene, result in the loss of the enzyme recognition sequence.

DNA transfer and virus preparations. Recombinant virus stocks were prepared as described by Settleman and DiMaio (35). Briefly, the plasmid constructs listed in Fig. 3B, 4B, and 5B were digested with *Eco*RI, circularized by ligation at a DNA concentration of 5 μ g/ml, and used to transfect CMT4 cells by the calcium phosphate method of Graham and van der Eb (17). Following transfection, cells were allowed to incubate for 5 days at 37°C in DMEM containing 10% fetal calf serum, 1 μ M CdSO₄, and 100 μ M ZnCl₂. Primary virus stocks were prepared by scraping the cells into the medium, repeated freeze-thawing, and removal of cell debris by centrifugation. Amplification of virus stocks was achieved by infecting fresh, induced CMT4 cells with clarified supernatant and incubating the infected cells for up to 6 days. This step was repeated to ensure high-titer stocks. The final clarified supernatant was aliquoted and stored at -70°C. Virus titers were determined by the transactivation method of Settleman and DiMaio by using the NL-3D cell line (35). Typical virus stocks contained $\sim 10^8$ infectious units per ml. All subsequent infections were done at a multiplicity of infection of between 10 and 100 infectious units per cell.

Fast protein liquid chromatography (FPLC) gel filtration. Cell membranes were prepared from PL15- and Pava 17GW-infected COS cells by hypotonic lysis as described previously (3). Following final centrifugation, membrane pellets were resuspended and extracted in 0.5 ml of modified

FIG. 1. Expression plasmids for wild-type and mutated E5 epitope proteins. pPL15 and mutated E5 epitope plasmids were constructed by inserting synthetic oligonucleotides encoding the influenza virus hemagglutinin HA1 epitope into the unique *Bst*XI site within the E5 ORF of plasmid pPava-1 and of pPava-1-derived plasmids containing mutated E5 ORFs. pPava-1 expresses the E2-E5 ORFs of BPV-1 and was used for the construction of SV40-BPV-1 recombinant viruses as described in Materials and Methods (35). These viruses were used to infect COS cells and to produce high-level, transient expression of the E5 protein. The sequences encoded by the E5 ORF and the inserted epitope are indicated with single-letter amino acid abbreviations.

radioimmunoprecipitation assay (RIPA) buffer (16) containing gel filtration standards (Bio-Rad, Richmond, Calif.). Extracts were separated by gel filtration on a Pharmacia FPLC System (Pharmacia Inc., Uppsala, Sweden), using a Superose 12 HR 10/30 column at a flow rate of 0.3 ml/min. Fifteen 1.0-ml fractions were collected over a molecular weight range of between 670,000 and 1,350 and were analyzed for the presence of E5 and the 16K protein by immunoprecipitation using the monoclonal antibody 12CA5.

Immunoprecipitation assays. Subconfluent COS cell cultures in 100-mm plates were infected at a multiplicity of infection of 100 infectious units per cell with recombinant BPV-1-SV40 viruses. Between 48 and 72 h postinfection, cells were washed with phosphate-buffered saline (PBS), incubated with methionine-free DMEM for 1.5 h, and labelled for 4 h with 750 μ Ci of a commercial mixture of ³⁵S-labelled methionine ($\sim 90\%$) and cysteine ($\sim 10\%$) (Trans-label, ICN) in 2.5 ml of DMEM at 37°C. Cells were washed twice with PBS and incubated 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, and 0.1% sodium dodecyl sulfate [SDS], pH 7.0) containing 0.1 mM protease inhibitors *N* α -*p*-tosyl-L-lysine

FIG. 2. FPLC gel filtration of wild-type- and mutant-infected COS cells. Purified cell membranes from PL15- and Pava 17G-infected COS cells were extracted in RIPA buffer containing molecular weight standards and separated on a Superose 12 column as described in Materials and Methods. Collected fractions were immunoprecipitated by using antibody 12CA5, and precipitated proteins were electrophoretically separated on SDS-15% polyacrylamide gels and visualized by fluorography. Immunoprecipitated proteins from fractions 6 through 19 are shown for both PL15- (top) and Pava 17G- (bottom) infected extracts. Major protein peaks are designated with small arrows in fraction 12 for PL15 and in fraction 14 for Pava 17G. Positions of gel filtration molecular weight standards (in thousands) are indicated at top. Positions of molecular weight markers (in thousands) used during electrophoresis are indicated to the left of the gel. Positions of the 16K and E5 epitope proteins are indicated to the right of the gel.

chloromethyl ketone (TLCK; Sigma) and 0.5 mM phenylmethylsulfonyl fluoride (Sigma). Following a 30-s vortexing, nuclei were removed by centrifugation in an Eppendorf microcentrifuge for 4 min. Five hundred microliters of each extract was incubated with 3 μ l of ascites fluid 12CA5, which recognizes the influenza virus hemagglutinin HA1 domain (a generous gift from I. Wilson) and incubated for 1 h at 4°C. Fifty microliters of protein A-Sepharose CL-4B beads (Pharmacia, Inc.) was added, and extracts were allowed to incubate for an additional 30 min. Following six washes in RIPA buffer, Sepharose beads were resuspended in 75 μ l of sample buffer with or without β -mercaptoethanol, heated at 100°C for 5 min, and separated by electrophoresis on 15% SDS-polyacrylamide gels. Gels were then fixed with glacial acetic acid-methanol, treated with Enlightening (New England Nuclear), dried, and exposed to Kodak XAR-5 film for 24 h at -70°C.

RESULTS

Construction and expression of wild-type and mutated E5 epitope fusion proteins. To identify cellular proteins that interact with the BPV-1 E5 transforming protein, we inserted two different epitopes into the amino-terminal region of the E5 protein and expressed the fusion proteins by using a recombinant SV40-BPV-1 virus in COS cells (16, 35). Fusion proteins containing insertions within this domain of E5 were shown previously to maintain biological activity and to localize normally to the Golgi apparatus. Using antibodies that recognize these epitopes, we can efficiently coimmunoprecipitate the E5 epitope fusion proteins and an associated, 16-kDa cellular protein (16) which has been recently identified as the 16K component of the vacuolar H⁺-ATPase (15).

To evaluate the E5-16K protein interaction, the HA1 epitope from the influenza virus hemagglutinin (9) was

inserted into the 5' end of E5 genes containing various substitution mutations (20, 23, 36). Oligonucleotides corresponding to the HA1 epitope were inserted into the unique *Bst*XI site of the pPava-1 plasmid (and its mutant derivatives) shown in Fig. 1 (nt 3889). SV40-BPV-1 recombinant virus particles were generated in induced CMT4 cells as described previously (see Materials and Methods) (35). Viruses expressing mutant and wild-type E5 fusion proteins were assayed for expression and binding to the 16K protein in COS cells as described previously (see Materials and Methods) (16).

FPLC analysis of the E5-16K protein complex. To further document the E5-16K protein interaction and to gain insights into the nature of the association, metabolically labelled extracts from cells expressing either wild-type HA1-E5 or mutated HA1-E5 were analyzed by gel filtration using FPLC as described in Materials and Methods. Recombinant virus PL15 expresses large amounts of the wild-type HA1-E5 fusion protein in infected COS cells (16). Virus Pava HA1/17G (previously designated p36W [16]) expresses a mutated HA1-E5 molecule containing a glycine residue in place of glutamine at position 17. This mutant was shown to be defective for focus formation, acute morphologic transformation, and induction of DNA synthesis (20, 36) and is also defective for binding to the 16K protein (16). Fractions were collected from a molecular weight range between 670,000 and 1,500 and were analyzed for the presence of E5 and the 16K protein by immunoprecipitation with the monoclonal antibody 12CA5. The autoradiograms shown in Fig. 2 demonstrate that wild-type HA1-E5 and 16K proteins coeluted from the column as a complex with a molecular weight of approximately 44,000 (lane 12).

In contrast, FPLC fractionation of extracts from Pava HA1/17G-infected cells revealed the presence of only the E5

fusion protein with virtually no detectable coprecipitated 16K protein (Fig. 2). In addition, whereas the peak of wild-type E5 eluted from the column was in fraction 12, mutant E5 protein appeared in a broad peak centered on fraction 14, a position relative to standards corresponding to a molecular weight of approximately 12,000. It appears that the reduced ability of the mutant E5 protein to bind the 16K protein resulted in a significant and reproducible alteration in the elution pattern of E5. The apparent shift corresponds to a decrease in molecular weight of about 30,000, a value which could correspond to two 16K protein molecules. Since the extractions and gradient separations were performed in nonreducing conditions, it is likely that the 12,000-molecular-weight peak observed with the mutated E5 protein represents uncomplexed E5 dimers (which run on SDS gels at 14 to 15 kDa). The peak observed in fraction 12 with the wild-type E5 represents E5 dimers, each probably complexed with two 16K protein molecules. While the resolution of FPLC for these small proteins is insufficient to definitively determine the stoichiometry of E5-16K protein binding, previous findings that monomeric E5 protein can bind the 16K protein (16) are consistent with the hypothesis that each E5 dimer can bind to two 16K protein molecules.

Transformation-defective, carboxyl-terminal mutations in E5 do not affect 16K protein binding. We previously demonstrated that the two cysteine residues at positions 37 and 39, although crucial for E5 dimer formation and transforming activity, are not required for interaction with the 16K protein (16). This suggested that the E5 hydrophilic domain may not have a direct role in 16K protein binding. To address this question, several mutants containing substitutions of conserved amino acids within the carboxyl-terminal domain of E5 were tested for 16K protein association. Included in this series of mutations were amino acid substitutions which abolish E5-mediated cellular transformation and the induction of acute DNA synthesis (20, 36). HA1 epitopes were inserted into the unique *Bst*XI site of Pava vectors with E5 ORFs containing various substitutions within this domain. A summary of the mutants tested and their abilities to induce transformation and associate with the 16K protein are shown in Fig. 3B. Levels of 16K protein binding in this and subsequent experiments (see Fig. 4 and 5) are expressed relative to that of the wild type as a ratio of 16K protein/E5 protein as measured by using both the Ambis and USB SciScan 5000 automated scanning systems.

COS cells infected with PL15 and the carboxy-terminal substitution mutants were analyzed for expression of the HA1-E5 proteins and association with the 16K protein by coprecipitation with 12CA5 ascites fluid. The autoradiogram in Fig. 3A demonstrates that all mutants, with the exception of Pava HA1/34Q36D41A, expressed significant levels of E5 proteins and that the 16K protein was coprecipitated in each case. Previous analysis of 34Q36D41A without the HA1 epitope demonstrated that this mutant E5 protein was stable. Following overexposure of this autoradiogram, E5 and coprecipitated 16K protein could easily be detected from Pava HA1/34Q36D41A-infected COS cell extracts (data not shown). These results demonstrate that the conserved amino acids of the carboxyl-terminal domain of E5, although required for transforming activity, may not be involved directly in the association with the 16K protein. Three carboxyl-terminal mutants tested appeared to form dimers normally (Fig. 3A, lanes 8 through 14). Mutants HA1/30L35S and HA1/32S, however, consistently showed two dimer populations that migrated distinctly upon polyacrylamide gel electrophoresis analysis. This result suggests that

these mutant molecules may be aligning themselves in two different orientations (parallel and antiparallel) and therefore may not be forming properly aligned E5 dimers.

The glutamine residue in the E5 transmembrane domain regulates binding to the 16K protein. The observation that an E5 molecule containing a glutamine-to-glycine substitution at position 17 failed to induce transformation or bind the 16K protein suggested that this position within E5 may contribute to 16K protein binding and therefore modulate E5-induced transformation. To further investigate these possibilities, several substitution mutants containing HA1 epitopes were assayed for 16K protein association (Fig. 4). Pava 17L contains an E5 ORF with a leucine at position 17 and, like mutant 17G, fails to induce E5 transformation in a focus formation assay (23). Pava 15T17H contains a threonine at position 15 in place of an alanine and a histidine at position 17 in place of a glutamine. Unlike mutants 17G and 17L, this mutant induces efficient E5 transformation (20, 36). Pava 15V21F22V contains conservative substitutions at positions 15, 21, and 22 and also efficiently transforms cells (20, 36). COS cells were infected with recombinant viruses containing wild-type and mutated versions of the HA1/E5 protein and were assayed by coprecipitation of E5 and the 16K protein by using monoclonal antibody 12CA5. Like mutant HA1/17G, mutant HA1/17L was severely defective for 16K protein association (Fig. 4A, lane 2). The 16K protein was detected only when the sample was overloaded on the gel (Fig. 4A) or when the autoradiogram was overexposed. In contrast, transformation-competent mutants HA1/15T17H and HA1/15V21F22V associate efficiently with the 16K protein. These results indicate that the glutamine residue at position 17 is important for association with the 16K protein but that other polar amino acids, or perhaps charged amino acids, can substitute for glutamine. To assess the ability of mutated recombinant E5 proteins to form dimers, immunoprecipitated pellets were resuspended in sample buffer in the absence of β -mercaptoethanol. As shown in the autoradiogram in Fig. 4B, all mutants in this series appeared to form dimers normally.

Horwitz et al. (21) reported the characterization of several E5 mutants containing substitutions of the hydrophobic middle third of E5 with apparently unrelated hydrophobic sequences. In almost all cases, such drastic sequence substitutions severely inhibited E5 focus-forming ability. More recently, Kulke et al. (23) demonstrated that insertion of a glutamine residue at position 17 allowed several transformation-defective, hydrophobic substitution mutants to induce stable transformation and stimulate DNA synthesis in an acute assay using C127 cells. To determine whether there exists a correlation between the ability of these mutants to induce morphological transformation and association with the 16K protein, two mutant E5 ORFs contained within the Pava vector were analyzed for their ability to associate with the 16K protein (sequences shown in Fig. 5B). The first, Pava HR25, is a transformation-defective mutant containing a hydrophobic sequence, apparently unrelated to that of the wild type, within the middle hydrophobic domain of E5 (Fig. 5B). The second, Pava HR25Q, contains the same E5 sequence as Pava HR25, with the exception of a glutamine residue at position 17 (23). Insertion of a glutamine at this position allows this mutant to induce focus formation and DNA synthesis (23). To determine whether the generation of E5 transforming activity with the inserted glutamine was accompanied by alterations in the ability to associate with the 16K protein, the HA1 epitopes were inserted into the E5 ORF of both Pava HR25 and Pava HR25Q to generate recombinant viruses, Pava HR25W and Pava HR25QW.

FIG. 3. Coprecipitation of E5-16K protein complex and dimer formation in cells expressing E5 epitope proteins containing amino acid substitutions within the carboxyl-terminal domain. (A) Extracts of COS cells infected with the viruses indicated above the lanes were immunoprecipitated with 12CA5, electrophoretically separated on SDS-15% polyacrylamide gels, and visualized by fluorography. Molecular weights (in thousands) are indicated on the left, and positions of the 16K protein and E5 monomers and dimers are indicated on either side. β ME, β -mercaptoethanol. (B) Summary of mutants containing substitutions within the carboxyl terminus of E5. The expanded sequence shows the positions of substituted amino acids within the carboxyl-terminal domain. The columns on the right show phenotypes for focus formation and ability to bind the 16K protein. Data for focus formation was obtained from Horwitz et al. (20). Quantitation was performed using the Ambis scanner and the USB SciScan 5000 automated scanning system and are expressed relative to that of the wild type as a ratio of 16K protein to E5 protein. +++, >50%; ++, 20 to 50%; +, 5 to 19%; -, <5% (percentages are relative to wild-type levels). Values are averages of several immunoprecipitation experiments.

Virus preparations were generated in induced CMT4 cells and used to infect COS cells for expression of the E5 recombinant proteins. Figure 5A shows the results of immunoprecipitation experiments using Pava 25W and two independent isolates of Pava HR25QW. Whereas no 16K protein was detected in precipitations from extracts of Pava HR25W-infected cells (lane 6), significant levels of 16K protein were coprecipitated from extracts of cells infected with both Pava HR25QW isolates (lanes 7 and 8).

These results imply that the placement of a hydrophilic glutamine residue in a random hydrophobic domain of E5 is critical for cell transformation and 16K protein association. In apparent contradiction to these findings is the existence of a hydrophobic substitution mutant, Pava HR15 (Fig. 5a), which lacks glutamine and can still efficiently induce focus formation and DNA synthesis in C127 cells (21, 23). Insertion of a glutamine at position 17 (construct Pava HR15Q) enhanced focus-forming ability and acute induction of DNA

synthesis (23). To test whether these mutants associate with the 16K protein, HA1 epitopes were inserted to create Pava 15W and Pava HR15QW. Extracts from Pava HR15W- and Pava HR15QW-infected COS cells were analyzed for E5-16K protein association by immunoprecipitation with 12CA5. Figure 5A shows that little E5-associated 16K protein was detected in Pava 15W-infected extracts but that it was easily detected in extracts of cells infected with two independent isolates of Pava HR15QW.

DISCUSSION

The BPV-1 E5 oncoprotein is the smallest known protein with transforming activity for which the detailed genetic and biochemical manipulations necessary for characterization of the multiple steps of signal transduction are possible. Activation of growth factor receptors appears to be a central event in E5-mediated transformation (25, 29). The mecha-

FIG. 4. Coprecipitation of E5-16K protein complex and E5 dimer formation in cells expressing E5 epitope proteins containing amino acid substitutions within the middle hydrophobic region. (A) Extracts of COS cells infected with the indicated viruses were immunoprecipitated with 12CA5, electrophoretically separated on SDS-15% polyacrylamide gels, and visualized by fluorography. Proteins eluted from protein A-Sepharose with SDS sample buffer containing β -mercaptoethanol (+ β ME) are shown on the left (lanes 1 through 6). To analyze mutant E5 molecules for ability to form dimers, proteins were eluted in the absence of reducing agent (– β ME) and are shown in the right panel (lanes 7 through 12). Positions of E5 monomers, dimers, and 16K protein are indicated to the right and left of the gel. Positions of molecular weight markers (in thousands) are indicated to the left of the gel. (B) Summary of mutants containing substitutions within the middle hydrophobic domain of E5. The bar at the top represents the E5 protein, and the striped area indicates the substituted region. The expanded sequence shows the positions of substituted amino acids. The leftmost column contains the names of the mutant constructs. The center section shows the amino acid substitution(s) encoded by the corresponding mutants. The columns on the right show phenotypes for focus formation and ability to bind the 16K protein. Data for focus formation was obtained from Horwitz et al. (20). +++, >50%; ++, 20 to 50%; +, 5 to 19%; –, <5% (percentages are relative to wild-type levels).

nism by which E5 activates these receptors, however, remains unclear. Binding of E5 to the 16-kDa component of the vacuolar H^+ -ATPases could be one step involved in the activation of the cellular growth factor signal transduction pathway (15). The results presented in this report further verify the existence of the E5-16K protein complex, suggest that dimeric E5 binds two 16K molecules, and identify an amino acid in E5 critical for the interaction with the 16K protein.

FPLC analysis of the E5-16K protein complex. Gel filtration by FPLC analysis has provided an alternative approach for characterizing the nature of the E5-16K protein complex. Coelution of wild-type HA1-E5 and 16K proteins supported coprecipitation data and provided independent evidence for the *in vivo* existence of the E5-16K protein complex. In addition, comparison of elution profiles of wild-type and mutant E5 molecules provided information regarding the protein composition of the multimeric complex. The elution patterns of wild-type E5-16K protein complex were consis-

tent with the existence of a complex consisting of two 16K proteins and an E5 dimer. Profiles of eluted E5 protein expressed from mutant 17G-infected cells displayed a significant and reproducible decrease in apparent molecular weight. The difference observed corresponded to the size of two 16K molecules and reflected the absence of 16K protein binding to the E5 dimer. Furthermore, these data suggest that, under the conditions of isolation utilized, no proteins other than the 16K protein are stably bound to the E5 dimer. However, cell extracts prepared with RIPA buffer do not preserve many protein-protein interactions, and it is not possible to preclude the existence of other E5-associated proteins. Because of the strongly hydrophobic nature of both E5 and the 16K protein (15), it has been difficult to extract these proteins from cells with nonionic detergents such as Nonidet P-40 and Triton X-100 (unpublished observations). However, gel filtration of extracts prepared with the zwitterionic detergents CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate} and CHAPSO {3-[(3-cholami-

FIG. 5. E5 binding to the 16K protein requires a glutamine residue within the middle hydrophobic region of E5. (A) COS cells infected with viruses containing hydrophobic substitutions within the region between amino acid positions 13 and 29 were analyzed for E5-16K protein complex formation by coprecipitation with antibody 12CA5. Immunoprecipitated proteins were separated on an SDS-15% acrylamide gel and visualized by fluorography. Positions of molecular weight markers (in thousands), 16K protein, and E5 monomers are indicated to the left of the gel. (B) Summary and amino acid sequences of hydrophobic substitution mutants. The striped and expanded area (amino acid positions 13 through 29) is the region of E5 that was mutated (23). The leftmost column shows the names of the virus constructs used in this study. The center area shows the substituted sequences. The position of the glutamine residue at position 17 is indicated by the asterisk above the wild-type sequence. The columns on the right show phenotypes for focus formation and 16K protein binding. Data for focus formation was obtained from Kulke et al. (23). +++, >50%; ++, 20 to 50%; +, 5 to 19%; —, <5% (percentages are relative to wild-type levels).

dopropyl)-dimethylammonio]-2-hydroxy-1-propanesulfonate} revealed the existence of a very high-molecular-weight complex (400,000 to 500,000), suggesting the possibility of other associated proteins (unpublished observations).

Mapping the requirements for 16K protein binding. By testing the ability of a variety of E5 mutants with single or multiple amino acid substitutions to bind the 16K protein, we identified a specific amino acid that is essential for binding the 16K protein. The primary determinant for 16K protein binding appears to reside in the hydrophobic central portion of the E5 protein. None of the missense mutations in the hydrophilic carboxyl-terminal third of the E5 protein affected 16K protein binding, including a number of mutations that severely inhibit transformation and/or block dimerization. Thus, it appears unlikely that there are many, if any, specific contacts with the 16K protein in this portion of the molecule. However, the mutants surveyed in this study each contain only one or two amino acid alterations, which may not sufficiently destabilize E5-16K protein binding, even though they interfere with transformation.

In striking contrast, there is an absolute requirement for a

hydrophilic amino acid in the middle hydrophobic domain for binding of the 16K protein as assessed by coimmunoprecipitation or by FPLC. This residue is glutamine in the case of the wild type and is an invariant amino acid among all sequenced fibropapillomavirus E5 genes, and it can be functionally replaced by histidine, another hydrophilic amino acid. In all hydrophobic sequence contexts tested, including random ones with diverse effects on transformation, there was no detectable 16K protein binding in the absence of the glutamine and readily detectable 16K protein binding in its presence. Thus, it appears that a glutamine in the hydrophobic region is sufficient for 16K protein binding, although the effect of its positioning has not been determined.

E5-16K protein binding and cell transformation. Our results clearly show that the ability of E5 to bind the 16K protein is not sufficient for cell transformation, because many carboxyl-terminal, transformation-defective mutants bind 16K. Among the seven amino acids in this region shown to be required for cell transformation are two cysteine residues which mediate homodimer formation. A mutant

containing amino acid substitutions at both cysteine residues fails to form dimers, suggesting that dimer formation mediated through disulfide linkage is necessary for cell transformation (20). Other transformation-defective mutations within this domain do not apparently affect dimer formation or 16K protein, indicating that these amino acids may be carrying out some other required function, such as interacting with other proteins or maintaining a critical stereochemical orientation of the hydrophobic domain.

The ability of mutant HR15 to transform cells indicates either that E5-16K protein interaction may not be absolutely required for transformation or that the hydrophobic sequence of HR15 can somehow functionally substitute for the 16K protein association. In a related study, a mutant designated HR16Q contains a glutamine within a different unrelated hydrophobic sequence and binds the 16K protein, yet it is transformation defective, indicating that not all hydrophobic sequences that bind the 16K protein can support transformation (unpublished results; 23). However, a correlation between E5 transformation and 16K protein association is supported by the finding that the transforming activity of a great majority of mutants, including HR15, is stimulated by the insertion of the glutamine which allows 16K protein binding. In addition, the transformation defect of mutant 17L, which lacks the glutamine and is defective for 16K protein binding, is more severe than that of most other mutants we have tested, including many with mutations in the carboxyl-terminal hydrophilic domain. Nevertheless, additional mutants need to be generated in order to clearly define the role of 16K protein binding in E5 transformation.

Disruption of 16K protein function as a potential mechanism of cellular transformation. The 16-kDa component of the vacuolar H^+ -ATPase has been isolated from plasma membrane and endomembrane compartments such as gap junctions (11), lysosomes (19, 34), clathrin-coated vesicles (1, 37), and Golgi membranes (14, 40). Interestingly, disruption of the gene encoding the 16-kDa component of the yeast vacuolar proton pump resulted in the failure to process newly synthesized carboxypeptidase Y, indicating that the 16K protein is required for acidification and formation of *trans*-Golgi vesicles (27). Considering the intracellular locations and activity of the 16K protein, there are several ways in which the binding of E5 to this pore-forming protein could conceivably affect growth factor receptors. For example, a local pH change induced by the E5-16K protein interaction may affect certain aspects of receptor processing, such as ligand internalization and vesicular transport. Since the E5 protein is known to accumulate in the Golgi apparatus (4, 16), it is possible that it exerts its transforming effects on growth factor receptors at this site. E5 alteration of proton-translocating ability in the Golgi network may affect the normal movement of receptors through this apparatus, perhaps resulting in receptor clustering and subsequent activation. Several lines of evidence exist which indicate that incompletely processed, Golgi-localized PDGF receptors can be activated internally before reaching the cell surface (2, 12, 22, 29). E5 is also present in the plasma membrane and may also exert an effect at this site (3). Martin et al. (25) have shown that E5 expression is associated with a greater number of epidermal growth factor receptors on the cell surface, secondary to an inhibition of receptor down-regulation. Analogous to what may be occurring in the Golgi network, E5 binding to the 16K protein in the proton channel may inhibit endosome acidification and/or endosome formation and thereby prevent receptor down-regulation.

The finding that the 16K protein may be a component of

gap junctions offers an additional regulatory site for E5 action (11). Inhibition of normal cell-cell communication via disruption of gap junction function occurs commonly during cellular transformation, and the gap junctional protein connexin is the target for the viral oncoprotein *src* (38). Identification of the physiologic responses to E5-16K protein association should help to understand the mechanism of E5-mediated transformation as well as the cellular controls of signal transduction.

Finally, the role of hydrophilic, intramembrane amino acids in mediating the interactions of transmembrane proteins such as E5 and the 16K protein may be representative of a more general biologic phenomenon. For example, recent evidence suggests that hydrophilic residues within the transmembrane domain of the α chain of the T-cell receptor induce interaction with the CD3- δ chain through an acidic residue in its transmembrane domain (5). It remains to be determined whether an analogous polar or charged amino acid in a transmembrane domain of the 16K protein induces association with the E5 protein or whether the E5 protein associates with additional cellular proteins via such interactions.

ACKNOWLEDGMENTS

This research was supported in part by grant RO1CA53371-01 from the National Cancer Institute. R.K. was supported by a fellowship from the German Cancer Research Center.

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