Mutagenesis of human papillomavirus types 6 and 16 E7 open reading frames alters the electrophoretic mobility of the expressed proteins

Deborah J. Armstrong and Ann Roman*

Department of Microbiology and Immunology, Indiana University School of Medicine, Indianapolis, Indiana 46202-5120, U.S.A.

The E7 open reading frames of human papillomavirus type 6 (HPV-6) and HPV-16 encode proteins consisting of 98 amino acids that are quite similar in sequence yet different in electrophoretic mobility. Moreover, these proteins vary strikingly in oncogenicity. To investigate the molecular basis of the differences in structure and function, site-directed mutagenesis was used to exchange non-conserved amino acid residues between the two proteins. The mutated coding regions were expressed as fusion proteins in *Escherichia coli* and identified by Western blotting. Comparative analysis of the affinity-purified mutated E7 fusion proteins in polyacrylamide slab mini-gels in the presence of SDS and 2-mercaptoethanol revealed altered electrophoretic mobilities. This analysis suggests that the aspartic acid at residue 4 (Asp 4) contributes to the characteristic aberrant migration of the HPV-16 E7 protein in SDS–polyacrylamide gels.

Clinically, human papillomavirus type 6 (HPV-6) is associated with benign lesions of the genital tract that infrequently progress to malignancy, whereas HPV-16 is frequently identified in carcinomas (Pfister, 1987). The genomes of both HPV-6 and HPV-16 encode a protein, E7, that is phosphorylated by casein kinase II (CKII) (Firzlaff et al., 1989) and associates with RB (Dyson et al., 1989; Munger et al., 1989), the protein encoded by the retinoblastoma susceptibility gene. *In vitro* the viral E7 protein plays a major role in cellular transformation by HPVs (Matlashewski et al., 1987; Phelps et al., 1988; Storey et al., 1988; Vousden et al., 1988). However, the HPV-16 E7 protein transforms human and rodent primary cells and cell lines more efficiently, binds RB with greater affinity, and is a better substrate for phosphorylation by CKII than the E7 protein of HPV-6 (Munger et al., 1989; Barbosa et al., 1990; Gage et al., 1990; Storey et al., 1990).

At the amino acid level, approximately 50% of the residues of the two proteins are identical, and another 15% are conservative changes. The theoretical $M_r$ of the HPV-6 and -16 E7 proteins, based upon amino acid composition, is 11K, yet the apparent $M_r$ of the HPV-16 E7 protein expressed in bacteria and eukaryotic cells, or translated in vitro, is approximately 17K (Smotkin & Wettstein, 1986; Firzlaff et al., 1989; Gage et al., 1990; Tommasino et al., 1990; Munger et al., 1991). In contrast, the apparent $M_r$ of the HPV-6 E7 protein is 11K when expressed in *Escherichia coli* but approximately 14K when isolated from mammalian cells (Gage et al., 1990; Munger et al., 1991).

To compare directly the biochemical properties of the HPV-6 and -16 E7 proteins, the respective viral DNA sequences were amplified by the polymerase chain reaction (PCR) (Cetus) to contain a unique BglII site at the 5' end and a unique *Bam*HI site at the 3' end. The template DNAs were pHPV6-W50, an authentic HPV-6 DNA cloned from a benign lesion (Farr et al., 1991), and pHPV16 (Dürst et al., 1983), obtained from H. zur Hausen. The amplified sequences were cloned into pTPC, a bacterial expression vector designed to facilitate purification and analysis of the recombinant E7 proteins. Plasmid pTPC, which encodes a 35K TrpE–Protein A–collagen (TPC) vector protein, was constructed by subcloning the blunt-ended *Bcl*–EcoRI small fragment of pRIT5 (Pharmacia) containing the IgG-binding domains of Protein A into the large fragment of pATH10 (Firzlaff et al., 1987), digested with EcoRV and EcoRI. The PCR-amplified E7 coding regions of the HPV-6 and -16 genomes were inserted into pTPC at the *Bam*HI site to generate constructs pTPC6E7 and pTPC16E7, respectively. Double-stranded sequencing of the PCR products in the recombinant plasmids confirmed the prototype sequences. The plasmids were used to transform *E. coli* strain HB101.

By affinity chromatography, the recombinant proteins were purified to near homogeneity with minimal degradation (Fig. 1). Fusion proteins were expressed by
Fig. 1. Purification of fusion protein TPC16E7. Aliquots from each stage of the purification were analysed on a 6% to 20% gradient SDS-polyacrylamide gel. Lanes 1 and 8, M, markers; lane 2, total lysate before addition of IAA; lane 3, total lysate after growth for 5 h in the presence of IAA; lane 4, supernatant applied to IgG-Sepharose; lane 5, flowthrough collected from IgG-Sepharose; lane 6, eluted TPC16E7 protein after buffer exchange; lane 7, affinity-purified TPC vector protein.

resuspending the pellet from a 75 ml stationary culture of transformed E. coli grown in NZ broth in 750 ml of M9 medium containing 2% casamino acids (Maniatis et al., 1982). The cells were grown for 1 h at 30 °C (Fig. 1, lane 2), indoleacrylic acid (IAA) was added to a final concentration of 50 μg/ml to induce expression from the trpE promoter, and incubation was continued for an additional 5 h (Fig. 1, lane 3). The cells were resuspended in 20 ml of lysis buffer (50 mM-Tris–HCl pH 7-5, 1 mM-EDTA, 100 mM-NaCl, 0-5%, NP40) and disrupted in a French press at 900 psig, and the clarified supernatant (Fig. 1, lane 4) was applied to an IgG-Sepharose column (Pharmacia). The column was washed sequentially with 10 bed volumes each of lysis buffer, 0-1 mM-glycine–HCl pH 5-0 and 0-1 mM-glycine–HCl pH 4-0. Fusion protein was eluted with 0-1 mM-glycine–HCl pH 3-0. Peak fractions were monitored by absorbance at 280 nm and pooled, and the buffer was exchanged by gel filtration over beaded polyacrylamide (Speedy desalting columns; Pierce) into buffer (50 mM-HEPES pH 7-1, 150 mM-NaCl, 10 mM-CaCl2) for storage at −20 °C (Fig. 1, lane 6). Approximately 3 mg of fusion protein was recovered from each 750 ml culture.

Western blotting was performed with a monoclonal antibody, mouse subtype IgG1, directed against Protein A (Sigma) to confirm the identity of the fusion proteins (data not shown). Relatively minor amounts of a 72K E. coli protein copurified with the recombinant proteins in nearly all the eluted fractions (Fig. 1, 2a and 4). Analysis of the mobility of the proteins by SDS–PAGE in the presence of 2-mercaptoethanol indicated that the HPV-6 E7 fusion protein (TPC6E7) migrated as predicted (M, 46K), but the HPV-16 E7 fusion protein (TPC16E7) did not (M, 52K) (Fig. 2a, lanes 5 and 7; Fig. 4, lanes 3 and 4). SDS–PAGE was performed essentially as described by Laemmli (1970) with a slight modification (DePaoli-Roach et al., 1983), using a mini-gel format (Bio-Rad).

The affinity-purified fusion proteins were used as substrates for phosphorylation by CKII. Approximately 1 µg of vector protein (TPC), TPC6E7 or TPC16E7 was incubated for 1 h at 30 °C with 5 microunits of partially purified CKII in the presence or absence of heparin (6 µg/ml), a specific inhibitor of the kinase, and [γ-32P]ATP in a total reaction volume of 25 µl. A unit of CKII activity is defined as the amount of enzyme that catalyses the incorporation of 1 µmol phosphate/min to the
substrate phosvitin (DePaoli-Roach et al., 1981). Aliquots of each reaction were analysed by SDS–PAGE and autoradiography. This analysis indicated that both E7 fusion proteins were phosphorylated by CKII, whereas TPC was not (Fig. 2b). Furthermore, phosphorylation of TPC6E7 by CKII caused an approximately 2K increase in the Mr of the fusion protein as judged by its mobility on an SDS–polyacrylamide gel, but did not alter the migration of TPC16E7 (Fig. 2a). Thus, phosphorylation by CKII can account for the apparent Mr of the HPV-6 E7 protein. This result is consistent with the data of Gage et al. (1990), who demonstrated that phosphatase treatment of E7 proteins immunoprecipitated from mammalian cells increases the mobility of the HPV-6 E7 protein in SDS–polyacrylamide gels but does not affect the electrophoretic migration of the HPV-16 E7 protein.

The amino acids that contribute to the aberrant migration of HPV-16 E7 in SDS–PAGE reside in the N-terminal half of the protein (Munger et al., 1991). Therefore, an exchange of key N-terminal amino acids between HPV-6 and HPV-16 E7 proteins may change their characteristic mobility in SDS–polyacrylamide gels. Based on amino acid sequence comparisons of the N termini (Fig. 3), single and double mutations were introduced into the HPV-6 and -16 E7 coding regions by site-directed mutagenesis using the Amersham oligonucleotide-directed in vitro mutagenesis system. The following expression plasmids were constructed: pTPC6E7D4 (Arg 4 changed to Asp), pTPC6E7D22 (Gly 22 changed to Asp), pTPC6E7D4D22 (Arg 4 and Gly 22 changed to Asp), pTPC16E7R4 (Asp 4 changed to Arg), pTPC16E7G21 (Asp 21 changed to Gly) and pTPC16E7R4G21 (Asp 4 and Asp 21 changed to Arg and Gly, respectively) (Fig. 3). Double-stranded sequencing of the entire E7 region of the recombinant expression plasmids verified that only these mutations were present. E. coli strain HB101 was transformed with each of the constructs, and the corresponding E7 fusion protein was expressed and affinity-purified as described previously.

The electrophoretic mobility of the mutated proteins in SDS–polyacrylamide gels was compared to that of the wild-type fusion proteins (Fig. 4). Proteins TPC6E7D4 and TPC16E7R4 migrated at the same rate, with an apparent Mr of 49K (Fig. 4, lanes 5 and 6). Therefore, the substitution of an aspartic acid for the arginine at position 4 caused the Mr of the HPV-6 E7 fusion protein, judged from its mobility, to increase by approximately 3K, whereas the placement of an arginine at residue 4 in the HPV-16 E7 fusion produced a 3K decrease in Mr. Substitution of glycine for aspartic acid at residue 21 in the TPC16E7G21 protein had no effect upon the mobility of the HPV-16 fusion protein (Fig. 4, lane 8). The analogous exchange, aspartic acid for glycine at residue 22 in the TPC6E7D22 protein, increased the Mr of the HPV-6 fusion protein by approximately 2K (Fig. 4, lane 7), as determined from its mobility. The effect of the double amino acid substitutions upon electrophoretic mobility was additive: the apparent Mr of protein TPC16E7R4G21 was 51K (Fig. 4, lane 9), whereas protein TPC16E7R4G21 displayed the same apparent Mr as protein TPC16E7, 49K (Fig. 4, lane 10). Thus, only the amino acid exchange at residue 4 altered the mobilities of both the HPV-6 and -16 fusion proteins.

Changes in the mobility in SDS–polyacrylamide gels of proteins that differ by a single amino acid, e.g. the p21ras family, have been reported (Fasano et al., 1984). In the case of the HPV-6 E7 protein, mutation of residue 4 resulted in a protein that migrated with an apparent Mr 3K greater than that of the wild-type protein, whereas the
mutated HPV-16 E7 protein migrated with an apparent $M_r$ 3K less. This observation suggests that the amino acid that occupies residue 4 of the peptide chain is a critical determinant of the characteristic migration of the HPV-6 and -16 E7 proteins. The change in the electrophoretic behaviour of the mutated proteins may be related to the negative charge of aspartic acid and the positive charge of arginine. Inspection of the first 10 residues of each protein reveals that, in addition to a negatively charged amino acid at position 10 (Asp 10), the HPV-6 E7 protein contains two positively charged residues, Arg 4 and Lys 9. In contrast, two negatively charged residues are present in this region of the HPV-16 E7 protein, Asp 4 and Glu 10. Thus, not only is the charge of residue 4 a factor, but the local charge context in which this residue exists may contribute to the characteristic mobility of these proteins in SDS-polyacrylamide gels.

An explanation of aberrant migration based on charge may seem enigmatic, because in SDS-PAGE intrinsic electrical charges of individual amino acids presumably are nullified by the abundance of negatively charged molecules of SDS associated with the protein. However, bound SDS molecules are not necessarily distributed uniformly along the length of the denatured peptide backbone, imparting new characteristics to the protein while disrupting the native conformation (reviewed by Tanford, 1968). The binding of SDS molecules to the denatured protein is highly cooperative and results in substantial amounts of SDS bound to protein at non-polar residues. Thus, SDS-denatured proteins retain an ordered structure, but one unlike the native protein due, in part, to micelle formation at the SDS-binding sites. A change in the denatured state by an amino acid substitution that influences the formation of SDS micelles could result in a different ordered structure and different frictional properties during electrophoresis, perceived as a change in mobility in SDS-polyacrylamide gels. Similarly, shifts in electrophoretic mobility accompanying phosphorylation have also been reported (DePaoli-Roach et al., 1983) and are explicable on this basis.

The observed differences in the mobility of the HPV-16 and -6 E7 fusion proteins correlate precisely with the differences observed following immunoprecipitation of in vitro translated HPV-16 and -6 E7 protein (Gage et al., 1990; Munger et al., 1989). Therefore, it is reasonable to suggest that the change in the E7 protein, rather than a change in the interaction of E7 protein with the vector moiety, accounts for the shift in mobility seen in response to mutagenesis. In light of the difference in oncogenicity between the HPV-6 and -16 E7 proteins, it is tempting to speculate that the amino acid residues that contribute to anomalous electrophoretic mobility may be involved in activation of the transforming ability of the HPV-16 E7 protein.

We wish to thank Peter Roach for providing CKII, Mike Woody for assistance with the mutagenesis reactions, and Carol Fiol and Charles E. Wilde III for their critical reading of the manuscript. This research was supported by American Cancer Society grant MV432.

References


(Received 23 September 1991; Accepted 7 January 1992)