RESEARCH COMMUNICATION

Human papillomavirus type 6 E7 protein is a substrate *in vitro* of protein kinase C

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The E7 proteins of 'low-risk' and 'high-risk' human papillomaviruses (HPV) are phosphorylated by casein kinase II. In this study, we report that the 'low-risk' HPV 6 E7 protein, but not the 'high-risk' HPV 16 E7 protein, can be phosphorylated *in*

vitro on threonine at amino acid position 7 by protein kinase C. This is the first example of a qualitative biochemical difference between the HPV 6 E7 and HPV 16 E7 proteins.

INTRODUCTION

Human papillomaviruses (HPV) are divided into 'high-risk' (e.g. HPV 16) and 'low-risk' (e.g. HPV 6) types based upon their prevalence in carcinomas. An acidic, 98-residue protein, designated E7, is expressed by both viral types. The ability of the HPV 16 E7 protein to transform rodent cell lines, to transform morphologically primary baby rat kidney cells in co-operation with activated Ras, and to immortalize primary human keratinocytes *in vitro* reflects the clinical oncogenic potential of the 'high-risk' genome. The 'low-risk' 6 E7 protein lacks these activities. However, the E7 proteins of both HPV 16 and 6 share the ability to increase transcription from the adenovirus E2 promoter and to stimulate cellular DNA synthesis.

The E7 proteins of HPV 6 (6 E7) and HPV 16 (16 E7) can be phosphorylated on two serines within a consensus sequence for casein kinase II (CK II) that is adjacent to the retinoblastoma tumor suppressor protein (RB)-binding domain [1–3]. *In vitro*, HPV 16 E7 is a better substrate for phosphorylation by CK II than 6 E7 is [2].

In tissue-culture cells, the 16 E7 protein associates stably with a cellular protein kinase that phosphorylates histone H1 *in vitro* [4,5]. One study identified this kinase immunologically as the protein kinase p33^{CDK2} [5]. The 6 E7 protein associates less efficiently with the cellular complex that contains protein kinase p33^{CDK2} [6,7], a kinase that catalyses phosphorylation of serine or threonine within a consensus sequence which contains a basic residue [8–10]. Although p33^{CDK2} associates with an E7 protein *in situ*, lack of the consensus sequence X(T/S)PX(K/R), which is required for recognition by the cyclin-dependent kinases [11,12], suggests that these kinases do not phosphorylate the E7 protein.

In their context within the 6 E7 amino acid sequence, the residues between positions 4 and 9 were observed to fit the consensus pattern [(R/K₁₋₃/X₂₋₀)-S/T-(X₂₋₀/R/K₁₋₃)] for another histone H1 kinase, protein kinase C (PKC) [8], with Thr-7 being the phosphoacceptor residue. Previously, the codons for residues 4, 6 and 9 were exchanged between the 6 and 16 E7 DNAs to demonstrate that charge differences between the extreme N-termini are responsible for the aberrant migration of the 16 E7 protein in SDS/PAGE [13]. These substitutions changed two residues of 6 E7 from basic (Arg-4 and Lys-9) to acidic (Asp-4 and His-9). In this study, the codon for Thr-7

within the putative PKC site and/or the codons for Ser in the CK II site were mutated to encode Ala, in order to investigate further the phosphorylation state of 6 E7.

MATERIALS AND METHODS

Construction of plasmids and mutagenesis

Construction of fusion vector pTPC and pTPC E7 plasmids was described previously [14]. pTPC encodes a 35 kDa TrpE:Protein A:collagen (TPC) vector protein; pTPC E7 expresses E7 fused to the C-terminus of TPC. Vector pT7 and pT7 E7 plasmids have been described previously [13]; pT7 E7 expresses unfused E7. The DNAs from which E7 sequences originally were subcloned were pHPV6-W50, authentic HPV 6 DNA cloned from a benign lesion [15], and pHPV16 [16] obtained from E. M. de Villiers and H. zur Hausen (Deutsches Krebsforschungszentrum, Heidelberg, Germany). Mutations were introduced into the E7 coding regions by site-directed mutagenesis, using the Amersham oligonucleotide-directed *in vitro* mutagenesis system, following the manufacturer's instructions. The DNA sequences of the primers used to introduce the mutations were the following, with the positions of mismatched bases underlined: Ala-7: 5'-CCTTTAGGGCA-ACATGCTTCC-3' and Ala-32Ala-33: 5'-CTCATCTTCTG-CGGCGTCTACTAATTG-3'. Primers were synthesized by the Biochemistry Biotechnology Facility, Indiana University School of Medicine. The DNA sequence of the entire E7 region of all recombinant constructions was confirmed by oligo-primed double-stranded DNA sequence analysis [17].

Production of E7 proteins in *Escherichia coli*

Affinity-purified fusion protein

Expression and affinity purification of fusion proteins have been described previously [14]. To cleave TPC6 E7, 5 units of purified collagenase (Boehringer-Mannheim) were added to 500 µg of fusion protein in 5 ml of storage buffer, and digestion was performed for 2 h at room temperature. Cleaved E7 was isolated by passing the digestion mixture over a 1 ml IgG-Sepharose column, in order to bind the protein A moieties, and concentrated by zinc affinity chromatography by applying the flow-through from the IgG-Sepharose column to a 1 ml iminodiacetic acid-Sepharose 6B column (Sigma), equilibrated in storage buffer.

Abbreviations used: CK II, casein kinase II; HPV, human papillomavirus; PAA, phosphoamino acid; PKC, protein kinase C; PVDF, poly(vinylidene difluoride); RB, retinoblastoma tumour suppressor protein; TPC, TrpE:Protein A:collagen.

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Cleaved E7 was eluted from the column with storage buffer that contained 50 mM EDTA, and the buffer was exchanged over beaded acrylamide for storage buffer. The protein band corresponding to cleaved 6 E7 was transferred to poly(vinylidene difluoride) (PVDF; Millipore), and automated Edman degradation was performed directly by the Biochemistry Biotechnology Facility, Indiana University School of Medicine, using a Porton Instrument model PI 2090 microsequencer with on-line phenylthiohydantoin analysis and Porton Enhanced Sequencing Software [18].

Partially purified E7

To purify unfused, recombinant E7 from bacteria transformed with pT7 E7 plasmids, bacterial pellets were resuspended in 20 ml of lysis buffer [100 mM Tris/HCl (pH 8.0)/50 mM NaCl/1 mM dithiothreitol/1 mM EDTA/1 µg/ml aprotinin/10 µM Pefabloc SC (Boehringer-Mannheim)/1 µM pepstatin/1 µM leupeptin] containing 1.5 % Sarkosyl [19]. The cells were disrupted by three passages through a French pressure cell at 6210 kPa (900 lbf/in²), and the lysates were centrifuged in a Sorvall SS-34 rotor for 1 h at 4 °C and 30000 *g*. Supernatants were diluted in 130 ml of chilled lysis buffer containing 2.3 % octyl glucoside [19], re-centrifuged for 1 h, then applied to 5 ml of Bio-Rad Q-sepharose columns, equilibrated in buffer A [100 mM Tris/HCl (pH 8.0)/50 mM NaCl/1 mM EDTA/1 mM dithiothreitol], at a flow rate of 3 ml/min using a Bio-Rad Econo System. Sequentially, the columns were washed with 10 bed vol. of buffer A and 10 bed vol. of buffer B [100 mM Tris/HCl (pH 7.5)/50 mM NaCl/1 mM EDTA/1 mM dithiothreitol]. At a flow rate of 2 ml/min using a 50–850 mM NaCl gradient in buffer B, proteins were eluted from the columns and collected in 0.5 ml fractions. E7 comprised at least 80 % of the total protein content of the fractions, as assessed by SDS/PAGE [20].

Phosphorylation reactions *in vitro*

CK II phosphorylation reaction mixtures contained 42 mM Tris/HCl, pH 7.5, 1.4 mM ATP, 10 mM magnesium chloride, 6.4 mCi of [γ -³²P]ATP (> 5000 Ci/mmol), 150 mM 2-mercaptoethanol and approximately 1 µg of affinity-purified fusion protein, cleaved E7, partially purified E7, or phosvitin (Sigma) in a total reaction volume of 25 µl [21]. Incubations were for 1 h at 30 °C with 5 µunits of sea-star (Upstate Biotechnology, Inc.) or rabbit skeletal muscle CK II (provided by Peter Roach, Indiana University School of Medicine, Indianapolis, IN, U.S.A.) in the presence or absence of heparin (6 µg/ml), a specific inhibitor of the kinase. Substrate phosphorylation by PKC was analysed using fusion proteins, cleaved E7, partially purified E7 protein or histone H1 (Sigma) at a concentration of approximately 10 µM. Proteins were incubated with 20 µunits of recombinant rabbit brain (Gibco-BRL) or rat brain PKC- α (provided by Alexandra Newton, Indiana University, Bloomington, IN, U.S.A.) and 16 µCi of [γ -³²P]ATP (> 5000 Ci/mmol) in the presence or absence of 1.3 mM calcium chloride and lipids for 30 min at 30 °C in a total reaction volume of 80 µl [22]. Lipids (Avanti Polar Lipids) consisted of either sonicated dispersions or Triton X-100 mixed micelles of 1.4 mM bovine brain L- α -phosphatidylserine (1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine) and 38 mM dioleoylglycerol (1,2-dioleoyl-*sn*-glycerol; C_{18:1}[cis]-9) [22,23].

Phosphoamino acid (PAA) analysis

Following phosphorylation *in vitro*, electrophoresis, and transfer of proteins to PVDF, the membrane strips containing E7 protein

were incubated in 5.7 M hydrochloric acid for 2 h at 110 °C. Hydrolysates were evaporated, washed with water, and applied to cellulose plates. Thin-layer electrophoresis was conducted for 2 h at 500 V in 2.5 % formic acid/7.5 % acetic acid buffer (pH 1.9). PAA standards were 125 µg of phosphotyrosine, 50 µg of phosphoserine and 50 µg of phosphothreonine (Sigma). After electrophoresis, the standards were revealed with 0.2 % ninhydrin in acetone. ³²P-labelled phosphoamino acids were detected by autoradiography.

Antisera, immunoprecipitation, and immunoblots

Polyclonal antiserum to 6 E7 was prepared in a New Zealand White rabbit, using approximately 100 µg of purified TPC 6E7 fusion protein for the initial injection. Booster doses containing 100 µg of cleaved E7 were administered 3 and 6 weeks later. Aliquots of serum were partially purified by passage over an immobilized *Escherichia coli* lysate column (Pierce), and the IgG fraction was isolated using an Immunopure G Protein G column (Pierce). Mouse monoclonal anti-Protein A, clone SPA-27, was purchased from Sigma, and mouse monoclonal antibody to HPV 16 E7 was purchased from TRITON diagnostics. For immunoprecipitation, 10 µl of rabbit polyclonal 6 E7 antiserum or mouse monoclonal 16 E7 was added to a cell lysate or *in vitro* phosphorylation reaction. Mixtures were rocked overnight at 4 °C, 30 µl of Protein A/Protein G-agarose beads (Oncogene Science) was added, and incubation was continued for 1 h. Immunoprecipitates were collected and washed three times in 1 ml of assay buffer and analysed by SDS/PAGE [20]. Immunoblot analysis was performed using goat anti-rabbit and goat anti-mouse polyclonal antisera conjugated to alkaline phosphatase (Boehringer-Mannheim Biochemicals) as described previously [13].

RESULTS AND DISCUSSION

Differences in biological activity between 6 E7 and 16 E7 are attributable to the non-conserved amino acid residues within the N-terminal halves of the proteins [24]. Our examination of the first 10 residues (region 1) of the E7 primary amino acid sequence suggested that a consensus PKC phosphorylation site was present in 6 E7, but not in 16 E7. Experiments were designed to determine whether, indeed, there was a qualitative difference between 6 E7 and 16 E7 with respect to phosphorylation by PKC. The amino acid sequences of the N-terminal halves of 6 E7 and 16 E7, the putative PKC site and the CK II site, and the mutations analysed in this study, are depicted in Figure 1.

E7 was expressed initially as a fusion protein from the bacterial

Figure 1 Comparison of HPV 6 E7 and 16 E7 N-terminal sequences

Full stops within the sequences represent gaps that were introduced in order to achieve maximum amino acid identity. Region 1 was defined by the 10 N-terminal residues of the E7 proteins and contained a putative PKC site, indicated by the bar at the top of the figure. Region 2 contained the residues necessary for RB binding [25], outlined by the box. Region 3 contained the CK II site [1], indicated by underlining. Site-directed mutagenesis was used to alter the codons for the phosphoacceptor amino acids of 6 E7 (Thr-7, Ser-32, and Ser-33) to encode alanine, indicated in bold. |, Identical residues; *, conserved amino acids; P, phosphoacceptor amino acids.

Figure 2 Phosphorylation of cleaved E7 by PKC

Histone H1 and cleaved E7 were phosphorylated *in vitro* with rat brain PKC- α in the presence of calcium and lipids (+). Without calcium and lipids (—), the phosphorylation of histone was inhibited. The positions of autophosphorylated PKC (PKC), histone H1 (H1) and cleaved E7 (6E7) are indicated on the right.

expression vector pTPC. Although TPC 6 and 16 E7 were phosphorylated by CK II [14], multiple attempts to phosphorylate fusion proteins TPC 6 E7 and TPC 16 E7 using PKC were unsuccessful. Because the residues of region 1, which contained the putative PKC site, were contiguous with the TPC residues in the primary amino acid sequence of the fusion protein, it seemed likely that the phosphorylation site was inaccessible in TPC 6 E7. Indeed, cleaved 6 E7 was phosphorylated by PKC- α (Figure 2). Phosphorylation of cleaved E7 by PKC- α was observed only in the presence of calcium and lipids, congruent with a dependence on these additives for activity of this kinase isoform [22,23]. The apparent molecular mass of phosphorylated cleaved E7 was slightly less than 14 kDa.

Edman degradation analysis of the first 10 amino acid residues determined that cleaved E7 contained four additional amino acids (Gly-Pro-Asp-Leu) at the N-terminus, consistent with cleavage of TPC 6 E7 at the engineered collagenase site in the TPC portion of the fusion protein. Given the proximity of the putative PKC site, it was possible that these four additional amino acids at the N-terminus of cleaved E7 could have influenced the *in vitro* phosphorylation reaction. To exclude this possibility, preliminary phosphorylation reactions were conducted on crude lysates of *E. coli* in which non-fused 6 E7 or 16 E7 was expressed. The E7 proteins were immunoprecipitated from the reactions and analysed for phosphorylation by SDS/PAGE and autoradiography. To ensure that each E7 protein was present in the immunoprecipitation, a portion of each immunoprecipitation product was transferred to nitrocellulose after SDS/PAGE, and the blot was probed with antiserum to 6 and 16 E7. Both proteins were immunoprecipitated without evidence of proteolysis (Figure 3B). However, only the 6 E7 protein was phosphorylated by PKC, with an apparent molecular mass of approximately 14 kDa (Figure 3A). Even with overexposure of the autoradiogram or over-development of the immunoblot, no bands containing phosphorylated 16 E7 or a breakdown product were obtained (Figures 3A and 3B). Phosphorylated background bands were consistent with the migration pattern of *E. coli* proteins present in the Coomassie Brilliant Blue-stained gel.

To verify results of the experiments with crude lysates and to extend the analysis of substrates, phosphorylation reactions were

Figure 3 Phosphorylation of 6 E7 by PKC

With calcium and lipids present, phosphorylation reactions with recombinant rabbit brain PKC- α contained histone H1 or the soluble portion of *E. coli* lysates in which 6 E7 or 16 E7 was expressed. (A) The E7 proteins were immunoprecipitated from the phosphorylation reactions with rabbit polyclonal antiserum to 6 E7 (α -6E7) or mouse monoclonal antiserum to 16 E7 (α -16E7), separated by SDS/PAGE (6–20% gradient gel), and analysed by autoradiography. (B) A portion of each immunoprecipitation was transferred to nitrocellulose after electrophoresis for immunoblot analysis with α -6E7 and α -16E7. The positions of the E7 proteins (16E7, 6E7) and the polyclonal and monoclonal heavy and light immunoglobulin chains (Igs) are indicated on the right.

Figure 4 PKC substrate and PAA analysis *in vitro*

Phosphorylation reactions with recombinant rabbit brain PKC- α contained the substrates indicated at the top of the figure. (A) Aliquots of the phosphorylation reactions were separated on a SDS/17.5% polyacrylamide gel and were analysed by autoradiography. (B) After electrophoresis, proteins were transferred to nitrocellulose and stained with Ponceau S. The bands corresponding to each substrate were excised, each substrate was hydrolysed, and PAA analysis was performed. To the left of the autoradiogram, the positions of PAA standards, sample origin, and hydrolysed phosphate are indicated.

conducted on partially purified 6 E7, 16 E7, and phosphoacceptor site mutants 6 E7A7, 6 E7A32A33, and 6 E7A7A32A33. The E7 proteins were purified by anion exchange from the soluble fraction of the respective *E. coli* lysate.

Figure 5 CK II substrate and PAA analysis *in vitro*

Phosphorylation reactions contained the substrates indicated at the top of the Figure. (A) Aliquots of the phosphorylation reactions were separated on an SDS/17.5% polyacrylamide gel and were analysed by autoradiography. (B) After electrophoresis, proteins were transferred to nitrocellulose and stained with Ponceau S. The bands corresponding to each substrate were excised, each substrate was hydrolysed, and PAA analysis was performed. To the left of the autoradiogram, the positions of PAA standards, sample origin, and hydrolysed phosphate are indicated.

Substrates 6 E7 and 6 E7A32A33 were phosphorylated by PKC, with apparent molecular masses of 14 kDa, whereas 16 E7, 6 E7A7, and 6 E7A7A32A33 were not (Figure 4A). Thus, residue Thr-7 in region 1 of 6 E7 was the phosphoacceptor. Phosphoamino acid analysis confirmed the results, indicating that phosphothreonine was released only from the hydrolysis of 6 E7 and 6 E7A32A33 (Figure 4B). Phosphorylation and hydrolysis of histone H1 were included for comparison. As a control, the same panel of E7 proteins was subjected to phosphorylation reactions with CK II, to ensure that the proteins were acceptable reagents. In these experiments, the CK II substrate phosvitin was the positive control for phosphorylation and hydrolysis. Proteins 16 E7, 6 E7 and 6 E7A7 were phosphorylated by CK II, with apparent molecular masses of 19 kDa, 14 kDa and 14 kDa respectively (Figure 5A); phosphoamino acid analysis indicated that phosphoserine was present (Figure 5B).

Whereas previous reports have demonstrated quantitative differences between the affinity of 6 E7 and 16 E7 for the RB family of proteins or cyclin A [4,6,25,26] and have indicated that 16 E7 is phosphorylated more efficiently by CK II [2], this is the first report of a qualitative biochemical difference between the two proteins. It is noteworthy that adenovirus E1A protein and simian-virus-40 large T antigen, viral oncoproteins with domains of limited amino acid similarity to the E7 N-termini and biological activities similar to HPV 16 E7, lack the PKC consensus sequence. In contrast, we observed no apparent correlation between viral type, tissue tropism, or oncogenic potential and the presence of an optimal PKC consensus sequence in comparisons of the N-termini of the more than 50 published E7 amino acid sequences.

To date, the role that differential protein phosphorylation may

play in the natural history of papillomavirus infections remains unclear. Such investigations have been hindered by the lack of a keratinocyte culture system that will support viral replication and gene expression. In other experimental systems, serine/threonine protein kinases such as CK II and PKC are involved in the regulation of cell growth, transcription, replication and differentiation [27–35]. The clear demonstration of 6 E7 as a PKC substrate *in vitro* and the specificity of the reaction are compelling arguments for further analyses of the interactions of PKC with the HPV life cycle, ideally in an experimental system that permits both cell growth and differentiation.

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