Human papillomavirus type 16 E7-regulated genes: regulation of S100P and ADP/ATP carrier protein genes identified by differential-display technology

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Human papillomavirus type 16 (HPV-16) is the dominant risk factor for the development of cervical cancer. The virus encodes three oncoproteins, of which the E7 oncoprotein is the major protein involved in cell immortalization and transformation. E7 is a multi-functional protein. It binds the retinoblastoma tumour-suppressor protein (pRb), which regulates progression through the G1 restriction point in the cell cycle. The E7 protein interacts with transcription-regulatory proteins such as the TATA box-binding protein and with proteins of the AP1 transcription factor family. To identify additional proteins regulated by E7, differential-display PCR was used to identify differentially expressed mRNAs in cells containing an inducible E7 protein. It is reported that E7 expression leads to regulation of the genes encoding the calcium-binding protein S100P and the mitochondrial ADP/ATP carrier protein. These data identify new functions of the E7 protein and thus expand the number of routes by which HPV-16 influences cell growth control, although the function of S100P has still to be elucidated.

Introduction

Human papillomavirus type 16 (HPV-16) is an oncogenic DNA virus that encodes six early and two late proteins, where the two early proteins E6 and E7 are major oncoproteins. They disturb the control of the cell cycle by their interaction with cellular proteins that are important for progression through the G1 restriction point. Both the E6 and E7 proteins are necessary for immortalization of human keratinocytes (Münger et al., 1989; Hawley-Nelson et al., 1989). However, both E6 and E7 alone are able to transform primary rodent cells in collaboration with activated Ras (Phelps et al., 1988; Storey & Banks, 1993).

The E6 protein binds to and degrades the p53 tumour-suppressor protein through a ubiquitin-dependent pathway and, thus, interferes with both cell cycle control and the apoptotic pathway (Schefner et al., 1990, 1993). The E7 protein exhibits multiple functions, such as protein-protein interactions with retinoblastoma protein (pRb), other pocket proteins and the cyclin-dependent kinase cdk2 (Dyson et al., 1988, 1992; Tommasino et al., 1993) and with proteins regulating transcription, such as the TATA box-binding protein (TBP) (Massimi et al., 1996; Phillips & Vousden, 1997) and the AP1 transcription factors (Antinore et al., 1996). The E7 protein decreases the transcriptional activity of p53 (Massimi et al., 1997), although p53 accumulates in E7-expressing cells (Jones et al., 1997).

E7 binding to pRb leads to the release of the transcription factor E2F (Dyson et al., 1989), which influences the expression of the genes b-myb, smooth-muscle z-actin and p14ARF (Lam et al., 1994; Morris et al., 1993; Nishida et al., 1995; Bates et al., 1998).

E7 also functions in a pRb/E2F-independent manner, as E7 mutants are defective in binding pRb but retain immortalizing potential in primary human keratinocytes (Edmonds & Vousden, 1989). Mutations in the N-terminal end, as well as in the zinc-binding cysteine motif in the C-terminal end, of the E7 protein abolished both the immortalizing and transforming properties (Jewers et al., 1992; Vousden, 1993). Although the E7 proteins from high-risk and low-risk HPV types are similar, they bind pRb with different affinities, and only the high-risk-type E7 causes immortalization and transformation of infected...
To identify genes regulated by E7 protein expression, differential-display (DD) PCR was conducted on cells where the HPV-16 E7 gene was under inducible control regulated by tetracycline (Tet). We show for the first time that the transcription of the cellular genes encoding the calcium-binding protein S100P and the ADP/ATP carrier protein are regulated in an E7 protein-dependent manner.

Methods

■ Cell culture. MCF7, a mammary adenocarcinoma cell line, was grown in Dulbecco’s modified Eagle’s medium supplemented with 10% foetal calf serum (FCS), 2 mM l-glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin (all from Gibco BRL). This medium will be referred to as DMEM. MCF7tetr cells, containing the stably integrated Tet transactivator pUHD 15-1, were grown in DMEM supplemented with 500 µg/ml geneticin (Sigma) and 2 µg/ml Tet (Sigma). MCF7E7 tet cells, which are MCF7tetr cells containing stably integrated pUHD/E7, a Tet-responsive vector containing E7 (see below), were grown in DMEM supplemented with 500 µg/ml geneticin, 100 µg/ml hygromycin B (Boehringer Mannheim) and 2 µg/ml Tet. They were divided into two cultures containing different FCS batches, designated cultures A and B.

■ Plasmid constructs. An HPV-16 fragment from positions 505–875 of the HPV genome, including the E7 ORF covering positions 562–858, was inserted into the Tet-responsive vector pUHD 10-3 (Gossen & Bujard, 1992) to give the plasmid pUHD/E7. pUHD 15-1 contained the Tet transactivator (Gossen & Bujard, 1992). pββ hygromycin contained the hygromycin phosphotransferase gene under the control of the SV40 promoter (Morgenstern & Land, 1990).

■ Transfection. MCF7tetr cells (106) were resuspended in 50 µl PBS and mixed with 10 µl PBS containing 10 µg pUHD/E7 and 1 µg pββ hygromycin DNA. This mixture was incubated for 10 min on ice in a Gene Pulser cuvette (Bio-Rad). The cells were electroporated at 320 V and 25 µF and immediately resuspended in 4 ml pre-heated DMEM supplemented with geneticin and Tet. The medium was changed after 24 h, and the cells were transfected after a further 48 h to 10 cm dishes with DMEM supplemented with geneticin, hygromycin and Tet for the selection of resistant cell clones.

■ Purification of RNA. MCF7E7 tet cells were grown in two different batches of serum (A and B). For each batch, a series was made in which Tet was removed from the cells for 0, 24, 48 and 72 h. Total cellular RNA was purified by using Ultraspec (BioTexc) as described by the manufacturer. Contaminating DNA was removed by DNase treatment as described by Jørgensen et al. (1999) and RNA was quantified by GeneQuant.

■ Differential-display (DD) RT–PCR. Differential display (Liang & Pardee, 1992) was performed as described previously (Jørgensen et al., 1999). In brief, cDNA synthesis was performed on 1 µg total RNA with HT11V, three different primers with the general sequence AAGCTT-\text{upstream primer} \text{downstream primer}, where V is A, C or G, resulting in three cDNA populations. Each reaction was performed at 42 °C for 1 h in 20 µl of 130 mM Tris–HCl, pH 8.3, 5 mM MgCl2, 20 mM KCl, 625 µM dNTP, 10 U AMV reverse transcriptase (Stratagene) and 0.5 µg HT11V primer. The reaction was stopped by the addition of 80 µl 0.1% Triton X-100 followed by heating to 95 °C for 1 min and the cDNA samples were stored at −80 °C. An aliquot of 1 µl was used for each competitive PCR.

■ Reamplification of differentially expressed bands. Differentially expressed bands were excised from the dried gels and the DNA was recovered by shaking the sample in 50 µl TE buffer (10 mM Tris–HCl, pH 7.5, 0.1 mM EDTA) at 95 °C for 15 min. Five µl was used for PCR amplification in a total volume of 27 µl of (final concentrations) 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.8 mM MgCl2, 0.1% Triton X-100, 0.005% gelatin, 70 µM each of dATP, dCTP, dGTP and dTTP, 2.5 U AmpliTaq (Perkin-Elmer), 10 pmol upstream primer and 10 pmol extended downstream primer (7HT11V; TAATAGCAGCTAATAGGAAAGCTTTTTTTTTTTTTT). Cycle conditions were as described for competitive PCR above, except that the annealing temperature was 42 °C. Reamplified DDRT–PCR fragments were purified from 2% agarose gels. Bands were identified by sequencing the reamplified bands. Sequencing reactions were performed as cycle sequencing (Voss et al., 1997) by using the ThermoSequenase enzyme (Amersham Pharmacia Biotech) and a T7 promoter-complementary primer (TAATAGCAGCTAATAGGAAAGCTTTTTTTTTTTTTT). Cycle conditions were as described by Amersham Pharmacia Biotech. The sequencing were sequenced with the GeneBank and EBI databases by using the FASTA program (UWGGC program package).

■ Northern blotting. The hybridization probes were made from the purified differentially displayed fragments (S100P, ADP/ATP carrier protein and β-actin). Random-primer labelling was performed as described by Sambrook et al. (1989) by using 20–100 ng DNA fragment and 0.75 µg of a random 8-mer oligonucleotide. The reaction was performed in 20 µl containing 50 mM Tris–HCl, pH 7.5, 5 mM MgCl2, 10 mM β-mercaptoethanol, 250 µM of dCTP, dGTP and dTTP, 40–50 µCi [α-32P]dATP (Amersham Pharmacia Biotech) and 7.5 U Klenow enzyme (Amersham Pharmacia Biotech) at room temperature. After 1 h, 80 µl TE buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA) was added and the samples were extracted once with phenol–chloroform, precipitated and dissolved in hybridization buffer. Prehybridization, hybridization and washing were done as described by Sambrook et al. (1989). The low-stringency wash corresponded to washing at 65 °C in 1× SSC, 0.1% SDS and 0.5 mM EDTA. The high-stringency wash was in 0.1× SSC, 0.1% SDS and 0.5 mM EDTA at 65 °C. The filters were exposed to Kodak Biomax MS film.

■ Western blot analysis. Cell extracts were prepared from both MCF7tetr and MCF7E7 tet cells before induction and after 24, 48 or 72 h induction. Total cell lysates were prepared by adding Laemmli sample buffer directly to the cell culture flask and scraping off the cells (Laemmli, 1970). The samples were loaded on a 15% SDS–PAGE gel after boiling for 10 min and transferred to a PVDF membrane by semi-dry electroblotting for 1 h at 15 V. The membranes were incubated in PBS.
with 0·1% (v/v) Tween and 5% (w/v) skimmed milk for 1 h at room temperature.

Immunodetection of the E7 protein was done by incubation with a 1:50 dilution of mouse anti-E7 monoclonal antibody (MAb) (Triton) followed by a 1:100 dilution of biotinylated anti-mouse immunoglobulin (DAKO). Immunodetection of S100P was done by incubation with a 1:250 dilution of mouse anti-S100P MAb (Transduction Laboratories) followed by a 1:100 dilution of biotinylated anti-mouse immunoglobulin. Both immunoblots were developed by addition of ECL detection reagent (Amersham) according to the instructions given by the manufacturer and exposed to Hyperfilm ECL (Amersham).

**Results**

**Inducible expression of HPV-16 E7 protein**

The HPV-16 E7 gene was inserted into the pUHD 10-3 vector and transfected into the MCF7Tet cell line that was stably transfected with the Tet transactivator. To demonstrate inducible expression of the E7 protein, cells were subcloned and several clones were grown in the presence or absence of Tet. E7 protein expression was demonstrated by Western blot analysis. Clones that were leaky for expression of the E7 protein, i.e. they expressed detectable amounts of the protein in the presence of Tet, were discarded. A stable cell clone with a high level of E7 synthesized under stringent control was isolated and named MCF7E7Tet. The amount of E7 protein increased for up to 48 h after induction and then decreased. The level of E7 protein that was present at 72 h post-induction was thus similar to that measured after 24 h (Fig. 1a).

**DDRT–PCR analysis of differentially expressed genes**

DDRT–PCR was conducted on total RNA isolated from cells in which E7 expression was either uninduced or had been induced for 24, 48 and 72 h in order to follow the kinetics of mRNA accumulation or degradation. DDRT–PCR was done on two preparations of mRNA isolated from MCF7E7Tet cells grown in media containing two different FCS batches. This was done in order to reduce the risk of isolating genes regulated by serum factors. A total of approximately 200 primer combinations was used and, since each primer combination displayed about 120 bands, this corresponds to visualization of at least 24000 bands, each representing an mRNA. Since there was probably some redundancy, this may correspond to 15000–20000 different mRNAs, which is probably about 50–70% of all the mRNAs that are expressed in the MCF7 cells (Wan et al., 1996). Only mRNAs regulated in both experiments were isolated and studied further. As an internal control for the quality of the DDRT–PCR, E7 mRNA was identified with a target primer annealing to positions 606–618 in the E7 gene (Fig. 1b). Very few bands were affected by E7 expression and

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**Fig. 1.** Induction of E7 protein and mRNA in MCF7E7Tet cells in the absence of Tet. (a) Western blot analysis of cell lysates made at time 0 and 24, 48 and 72 h after induction. The proteins were separated on a 10% polyacrylamide gel and transferred to a PVDF membrane. E7 protein was visualized after incubation with E7-specific MAbs (1:50), biotinylated rabbit anti-mouse immunoglobulin (1:100), ECL detection reagent and development on Hyperfilm ECL (Amersham). (b) PAGE of α-35S-dATP-labelled RNA amplified by differential display. The RNA was prepared from cells induced for the same times as in (a). The gels were developed after autoradiography. Two independent RNA preparations are analysed in the lanes marked 1 and 2. (c) Northern blot analyses of RNA extracts prepared from cells induced for 24 and 48 h. Two independent RNA preparations were analysed, as shown in lanes 1 and 2. The filter was hybridized with a randomly [α-32P]dATP-labelled E7 probe. In the lower panel, a labelled β-actin probe was used as a control for the amount of RNA transferred to the filter.
Fig. 2. PAGE of α-35S-dATP-labelled RNA amplified by differential display. RNA was isolated from MCF7E7Tet cells induced for 24, 48 and 72 h, amplified and separated on 5% polyacrylamide gels. In the top panel, the RNA band that was reduced with time of induction is identified as S100P. In the middle panel, the RNA band that was up-regulated with time of induction is identified as the ADP/ATP carrier. In the lower panel, the β-actin and heat shock protein 27 (HSP27) RNAs are shown as bands that remained constant during E7 protein induction. Two independent RNA preparations were analysed at each time of induction and the results are shown in the lanes labelled 1 and 2. The gel was developed by autoradiography.

only six bands were excised from the gels for further analysis. Of these, only two were correctly regulated by E7.

Northern blot analysis of two independent mRNA preparations showed that E7 mRNA was induced to a maximum level after 24 h of induction and confirmed that the E7 gene was under stringent control (Fig. 1c). The higher molecular mass band that hybridized with the E7 probe under the stringent conditions used was present in both uninduced and induced MCF7E7Tet cells. It represents a cellular protein that was not characterized further. The presence of equivalent quantities of RNA loaded in each lane was controlled by hybridization with an actin probe.

E7 expression regulates the transcription of the S100P and ADP/ATP carrier protein genes

cDNAs identified by DDRT–PCR that were down- or up-regulated by E7 protein expression in MCF7E7Tet cells are shown in Fig. 2. The down-regulated band was sequenced and identified as the S100P gene. An up-regulated band was identified as the ADP/ATP carrier protein gene. Both cDNAs were amplified by PCR with specific primers (S100P, HT11C/}

Fig. 3. Northern blot analysis of RNA prepared from MCF7E7Tet cells at time 0 and 24 and 48 h after induction. Two independent RNA isolates were analysed at each time (lanes 1 and 2). The upper panel was hybridized with an S100P-specific probe, the middle panel with an ADP/ATP carrier-specific probe and the lower panel with a β-actin-specific probe. All probes were prepared by randomly primed labelling of specific DNA fragments with [α-32P]dATP. The blots were developed by autoradiography.

Fig. 4. Western blot analysis of the amount of S100P protein present in extracts made from MCF7E7Tet cells before and after induction for 24 h. Aliquots containing 40 µg total protein from each lysate were separated on 10% polyacrylamide gels and transferred to a PVDF membrane. The membrane was incubated with MAbs specific for E7 or S100P as shown, followed by biotinylated anti-mouse antibodies. The reaction was visualized by ECL detection reagent and exposed to Hyperfilm ECL (Amersham).

GH06; ADP/ATP-carrier protein, HT11C/GH12) and cloned into a pUC19 vector. The cloned cDNAs were used as probes in Northern blot analysis, which confirmed that the mRNA for the S100P protein was down-regulated after 48 h. The gene for the ADP/ATP carrier protein was up-regulated at 24 h, followed by a decrease in the mRNA after 48 h of induction (Fig. 3). Western blot analysis of 40 µg total protein from cell lysates prepared from MCF7E7Tet cells uninduced and induced for 24 h showed that S100P protein was down-regulated in the E7-expressing cells (Fig. 4).

Discussion

To study the gene-regulatory properties of HPV-16 E7, we established an MCF7 cell line in which E7 protein expression could be induced. This allowed us to investigate the immediate effect of E7 expression and its influence on the transcriptional
activity of the cells relative to uninduced MCF7E7\textsuperscript{\textit{Tet}} cells. DDRT–PCR identified differentially expressed mRNAs for S100P and the ADP/ATP carrier protein.

Expression of S100P mRNA was down-regulated by E7 protein induction. S100P belongs to the S100 super-family of calcium-binding proteins. Members of this family have been associated with cell differentiation and malignancy, when the proteins are most often up-regulated (Schafer & Heizmann, 1996; Grigorian et al., 1992), with chemokinesis (Newton & Hogg, 1998; Jinqu, et al., 1996) and with the cell cytoskeleton and motility (Donato, 1991). The genes are often clustered on chromosome 1, but S100P was mapped to chromosome 4 by Schafer et al. (1995). The best-characterized S100 gene is A4 (CAPL, mts-1), which is involved in metastasis and cell motility (Grigorian et al., 1992; Takenaga et al., 1994). In mammary carcinoma cells, the S100A2 gene (CaN19) is down-regulated (Lee et al., 1992). These authors observed that the gene was expressed abundantly not only in normal mammary keratinocytes, but also in an HPV-immortalized cell line, which is in contrast to our finding that S100P was down-regulated in cells that produced HPV-16 E7 protein. The biological function and cellular localization of S100P are not known, but the gene was first isolated and cloned from placenta by Becker et al. (1992), who expressed S100P as a homodimer. The amino acid sequence of S100P is most closely related to S100 A1 (Grigorian et al., 1992), whereas S100A2 is less related (Schafer & Heizmann, 1995; Lee et al., 1992). The only other reports on S100P describe S100P as being regulated in prostate cancer cells in an androgen-dependent fashion, which needs to be confirmed, and being up-regulated in doxorubicin-resistant colon carcinoma cell lines (Averboukh et al., 1996; Bertram et al., 1998).

The importance of the second E7-regulated gene, the ADP/ATP carrier protein gene, for control of cell growth is also unknown. Marzo et al. (1998) reported that the ADP/ATP carrier protein interacts with Bax and mediates permeabilization of the mitochondria as a first step in the apoptotic pathway. As the S100 proteins have also been described to promote apoptosis (Hu & Van Eldik, 1996; Mariggiò et al., 1994), the differential regulation of S100P and the ADP/ATP carrier protein could be important for HPV-16 E7 protein-induced cell cycle progression. However, we have not been able to show E7-induced apoptosis by FACS analysis or any changes in the levels of Bax, Bcl-2 or Bcl-x mRNA after MCF7\textsuperscript{E7\textit{Tet}} induction (data not shown). We are currently looking for biological functions of the S100P protein, and preliminary results have shown that it might be involved in the regulation of cell motility and neurite outgrowth (data not shown). It might also be involved in regulation of protein kinases, in analogy to observations on S100A1\textsubscript{4} (Heierhorst et al., 1996), or in intracellular functions related to cell cycle control, as reported for S100A2 (Lee et al., 1992) and for S100B through binding to p53 (Baudier et al., 1995).

Genes that have been described previously as regulated by HPV-16 E7 were not identified in our study. b-myb (Lam et al., 1994) and smooth-muscle \textit{\emph{α}}-actin (Nishida et al., 1995) are both regulated by E2F complexes and their expression is linked to the cell cycle (Lam et al., 1995). Cell cycle-dependent regulation can easily be overlooked by DDRT–PCR of mRNA isolated from non-synchronized cells, as used in the present study.

In conclusion, our data demonstrate for the first time that the cellular genes for S100P and the ADP/ATP carrier protein are regulated in cells expressing the HPV-16 E7 oncprotein. The contribution of these genes to the altered phenotype of transfected cells will be addressed in future studies.

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