Bovine papillomavirus oncoprotein E5 affects the arachidonic acid metabolism in cells

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Abstract

The bovine papillomavirus type 1 (BPV-1) oncoprotein encoded by the E5 ORF is a small highly hydrophobic protein, which is capable of inducing oncogenic transformation of cells. We studied the effect of the BPV-1 E5 protein expression on the arachidonic acid metabolism in monkey (COS1) and human (C33A) cells. At relatively low protein concentrations the phospholipase A2 (PLA2) activity and the arachidonic acid (AA) metabolism are activated. E5 mutant proteins, lacking cysteines responsible for the dimerisation of the protein (C37S, C37SC39S), and truncated E5, lacking the C-terminal region, are non-transforming and unable to stimulate the PLA2 activity and AA metabolism. The transformation-defective mutant D33V, which does not activate the platelet-derived growth factor receptor (PDGFR), activates AA metabolism like wt E5. Our data suggest that the BPV-1 E5 protein could stimulate the AA metabolism independently of PDGF receptor. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Viral oncogenes; Arachidonic acid metabolism; Phospholipase A2

1. Introduction

The major transforming protein of the bovine papillomavirus type-1 (BPV-1) is encoded by the E5 open reading frame (ORF) [1,2]. The E5 protein, a 44-residue highly hydrophobic onco-

Abbreviations: AA, arachidonic acid; BPV, bovine papillomavirus; EGF, epidermal growth factor; HETE, hydroxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; LO, lipoygenase; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; PLA2, phospholipase A2; TLC, thin layer chromatography.

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membranes through N-terminal hydrophobic domain. E5 co-precipitates with platelet-derived growth factor (PDGF) receptor in transformed cells [5] as well as with the 16-kilodalton subunit of the vacuolar proton-ATPase [6,7]. Several studies have shown that E5 may induce transformation through the activation of growth factor receptors by a ligand-independent mechanism [8,9]. The presence of PDGF receptor and interaction with E5 is believed to be required for transformation of the cells [10,11].

Stimulation of mitotic activity of the cells by growth factors is accompanied by marked changes in lipid turnover, including the arachidonic acid (AA) metabolism [12–15]. Signal transduction by arachidonic acid metabolism includes AA release from membranes by phospholipase A2 (PLA2) and conversion of AA to eicosanoids. The latter process includes synthesis of prostaglandins by cyclooxygenase (CO) or leukotriens by lipooxygenase (LO). AA metabolism is stimulated by numerous factors (e.g. mitogens) and is enhanced in proliferating cells [16–19]. Epidermal growth factor (EGF) and PDGF stimulate PLA2 [20]; EGF also increases 12-LO activity [21]. Mouse papillomas and squamous cell carcinomas contain 50–60-fold more 12-HETE (12-hydroxyeicosatetraenoic acid) of enzymatic origin than normal epidermis [22] and inhibition of 12-LO blocks the cell growth [23]. These data indicate that arachidonic acid metabolism is associated with cellular transformation [19,24,25].

The mechanism of activation of PLA2 is not clear yet, however, it has been shown that the activity of this enzyme could be regulated by Ca2+-level, phosphorylation of enzyme, and changes in the membrane composition [14]. In addition, it has been demonstrated that membrane-bound proteins or small transmembrane peptides could activate PLA2 in vitro [26,27]. The E5 protein compartmentalises to the cell membranes of BPV-1-transformed cells because of its hydrophobic nature [28]. In the present study we analysed the effect of the expression of the BPV-1 E5 protein and its mutants on the AA metabolism in COS and C33A cells. We used the non-transforming mutants, which do not activate the PDGFR–D33V and C37SC39S [29], or which can form complex with PDGFR–C37S and the hydrophobic amino-terminal region of E5 [7,29]. Our data show that expression of wt E5 and mutant D33V activates AA metabolism in cells, but mutants, lacking cysteines in hydrophilic region — C37SC39S, C37S and hydrophobic region of E5, are unable to stimulate the AA metabolism. It seems that E5 stimulates AA metabolism independently of PDGFR activation, and cysteines, responsible for the dimerisation of the protein, are required for the stimulation of the PLA2 activity and AA metabolism.

2. Materials and methods

2.1. Cell culture and transfection

The monkey cell line COS 1 (producing constitutively SV40 T antigen), and the human cell line C33A were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal calf serum (FCS) and penicillin (100 U/ml), streptomycin (0.1 mg/ml) at 37°C in a CO2 incubator. The E5-expression vectors were transfected by electroporation into COS 1 cells (1–2 × 106) at 180 V and 975 mF in 250 μl of DMEM supplemented with 10% FCS, 6 mM N,N-bis-[2-hydroxyethyl]-2-aminoethane sulfonic acid (BES) and 50 μg of denatured salmon sperm DNA (dsDNA) at room temperature [30]. The cell line C33A was electroporated at 220 V and 975 μF. In the case of mock transfection only carrier DNA (dsDNA) was used in the electroporation medium.

2.2. Plasmids

E5 protein-coding sequence was amplified by the PCR using the wt BPV-1 genome, and cloned into the eukaryotic expression vector pCG [31]. The mutant, containing only the hydrophobic amino-terminal region of E5 (30 amino acid), was amplified using 3′ primer CGGATCCCTATA-CAAGAAAAAA, (plasmid pCGnE5h). The E5-mutants 33V, 37S, and 37S39S have been described earlier [3]. Mutant 33V contains valine instead of aspartate in the position 33, 37S marks the mutant, where cysteine in the position 37, is
substituted by serine, and 37S39S contains serines instead of cysteines in positions 37 and 39. The respective coding sequences were amplified by PCR and cloned into the expression vector like the wt sequence. The sequence of all plasmids was verified by sequencing.

2.3. Assaying E5 expression

The expression of E5 protein was analysed by Western blot analysis using polyclonal serum against the E5 hydrophilic region [32]. In the case of the N-terminal hydrophobic fragment the E5 domain was tagged with a haemagglutinin HA1 epitope (16 amino acids) by cloning the N-terminal domain into the expression vector pCGN. The tag of the protein is recognised by the 12CA5 monoclonal antibody on the Western blot [33]. For visualisation of the specific E5 bands was used the enhanced chemiluminescence (ECL) (Amersham) and for quantify ImageTool software. The total cell protein was determined by the method of Bradford [34]. The expression of E5 was also determined by immunofluorescence, 24 h after transfection cells were fixed in 4% paraformaldehyde in PBS at room temperature for 30 min, permeabilized with 0.1% Triton X-100 in PBS, blocked with 1.5% fat-free milk in PBS containing 0.1% Tween-20 for 20 min at room temperature, incubated with polyclonal serum against E5 for 30 min, washed three times in PBS/Tween and incubated with FITS-labeled anti-rabbit antibody.

2.4. Assaying PLA₂ activity

Cells were grown for 24 h before transfection in the medium containing 0.5–1 μCi/ml [³H]AA or 0.1–0.5 μCi/ml [¹⁴C]AA. Cells were then washed three times with PBS, harvested and transfected with E5 or mutant E5 expression vectors and plated into 24-well flat-bottomed cluster plate at 1 × 10⁵ cells per well in DMEM, 24 h after transfection the AA and its metabolites were measured in the medium by determining the amount of ³H or ¹⁴C label by scintillation counter. The cells were then used in the assay for AA metabolites.

2.5. Assaying arachidonic acid (AA) metabolism

LO activity was determined according to Waslidge and Hayes [35]. ³H-AA or ¹⁴C-AA labelled cells were lysed 24 h after transfection using 100 μl 0.05% Triton-X 100. Same volume of ether:methanol:acetic acid (30:4:1) was added. Upper organic phase was applied to thin layer chromatography (TLC) plate and run in a mixture of hexane:ether:acetic acid (70:30:1). The plate was exposed in a PhosphoImager cassette for 3 days and quantified using software supplied on the PhosphoImager (Molecular Dynamics). In some experiments liquid scintillation counting of selected fractions on the TLC plate was used to quantify the TLC fractions. The percentage of each fraction was calculated before and after alkali treatment of the extract in 0.6 N KOH at 50°C for 1 h to liberate AA from its esters. HPETE (hydroperoxyeicosatetraenoic acid) as a marker for chromatography was produced by oxidation of AA [36]. One microgram of soybean lipoxygenase was added to a freshly prepared solution containing 6.7 mM [1-¹⁴C]AA in 30 μl of 50 mM Tris-buffer (pH 9). Reaction was carried out at 30°C for 10 min and terminated by addition of 7.5 μl of ethanol. The 12-HETE was detected by TLC using specific HETEs markers.

3. Results and discussion

3.1. Expression of the BPV-1 oncoprotein E5 activates PLA₂ in COS1 cells

In order to estimate the potential effect of the BPV E5 protein on PLA₂ activity we transfected COS1 cells with different amounts of the E5 protein expression vector pCGE5. The PLA₂ activity in the [³H]AA- or [¹⁴C]AA-labelled cells was measured by release of labelled AA from the transfected cells at different E5 protein concentrations. The activation of PLA₂, measured by the increased release of the AA from the membranes, was dependent on the concentration of the E5 in the cells. At 100 ng of transfected pCGE5 we identified the maximal PLA₂ activity (Fig. 1A), which was reduced to the initial level upon further
increase of the E5 concentration in the cells. The E5 expression in the transfected cells was estimated by Western blot analysis, and E5-bands were quantified. The level of E5 in the transfected cells correlated with the increase of the amount of transfected plasmid (Fig. 1B). The bell-shaped activation curve of PLA₂ was not caused by reducing amounts of E5, but might indicate that at higher concentrations of E5 inactive aggregates of E5 are formed and thereafter the reduction of the PLA₂ activity would occur (Fig. 1).

Fig. 1. BPV E5 protein activates PLA₂: (A) COS1 cells (1 x 10⁶ cells) were labelled by [³H]AA and transfected with pCGE5 plasmid, 24 h after transfection in the medium was measured radioactivity. The levels of AA released from cells indicate the activity of PLA₂; each bar represents the mean ± S.D. of measurements in four wells of microtiter plate of the same transfection; (B) cells were transfected with different amounts of pCGE5. Cell extracts containing 50 µg of total protein analysed in PAAG electrophoresis and expression of E5 was detected by Western blot using ECL kit, bands were quantified by Image Tool and are presented as integrated density (ID).
Table 1
Percentages of radioactivity in different TLC fractions before and after alkaline hydrolysis of lysate of E5-transfected COS cells

<table>
<thead>
<tr>
<th></th>
<th>Start</th>
<th>HETE</th>
<th>Arachidonic acid</th>
<th>The faster fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysate</td>
<td>39</td>
<td>9</td>
<td>8</td>
<td>43</td>
</tr>
<tr>
<td>Lysate hydrolyzed with KOH</td>
<td>5</td>
<td>7</td>
<td>80</td>
<td>6</td>
</tr>
</tbody>
</table>

We analysed released AA and its metabolites by thin layer chromatography (Fig. 2A). The alkali treatment of the extract was used for characterization of the fractions. The start-fraction and the fastest moving fraction produce AA after hydrolysis with 0.6 N potassium hydroxide (Table 1). We can therefore conclude that the fastest fractions contain apolar AA esters released from phospholipids by PLA\textsubscript{2}, while in the start-fraction of the chromatography AA is probably incorporated into the polar lipids. The release of arachidonic acid was increased three times in E5-transfected cells (Fig. 2B, line ‘−’ and ‘E5’).

3.2. E5 activates AA metabolism in COS1 cells

We also analysed other fractions of the thin layer chromatography and found that hydroxy-products of the AA metabolism contain only 12-HETE (data not shown). This indicates that upon expression of E5 12-lipoxygenase (12-LO) was also activated in the transfected cells. E5-transfected cells contained about three times more AA oxidation products (HETE) than control cells (Fig. 2B, line ‘−’ and ‘E5’). We detected maximum stimulation of HETE production at 100 ng of transfected pCGE5 (Fig. 2C).

3.3. E5 protein stimulates AA metabolism through its C-terminal domain and independently of PDGF receptor

Active E5 protein is a dimeric protein [3] and activates the PDGFR [5,8–10]. To find out whether the dimer formation of E5 and activation of the PDGFR are essential for stimulation of AA metabolism we tested the following C-terminal non-transforming mutants in the AA release and metabolism assay: 37S39S, which is unable to form dimers and to activate the PDGFR [3,29]; 37S, which has a reduced ability to form dimers [3]; 33V, which does not activate the PDGFR [29]; and truncated E5 protein containing the hydrophobic N-terminal region only (nE5h). The results are presented on the Fig. 2A,B. The production of apolar AA esters indicates the PLA\textsubscript{2} activity and changes in HETE fraction indicate the AA metabolism. We found that dimerisation defective mutants, as well as C-terminal truncated E5 proteins, are unable to activate the PLA\textsubscript{2} and the AA metabolism, while E5 mutant 33V is capable of doing this (Fig. 2B).

The expression of E5 and mutants was analysed by Western blot analysis. All the mutant proteins were expressed at approximately the same level in COS cells (Fig. 2D).

All these mutants were also tested in the human cell lines C33A and the effect of the wild type and mutant E5 proteins on PLA\textsubscript{2} activity and AA metabolism were measured. Wild type and mutant E5 proteins were expressed less efficiently from the pCG plasmid in these cells and achieved the effective level of expression at 2000 ng of the input plasmid (Fig. 3A). We found that PLA\textsubscript{2} activity and AA metabolism were influenced by
Fig. 3.
E5 and its mutants in the same way they were in COS1 cells, but the changes were smaller (Fig. 3A,B). The latter is due to low level of E5 in C33A cells. As the containing of E5 in C33A cell extracts is hardly visible by Western blot (Fig. 3C), the expression of E5 and mutant proteins was detected by immunofluorescence (Fig. 3D). About 5–10% of C33A cells contained detectable level of E5, at the same time E5 was detectable in 30–40% of COS cells.

Since the transfection of pCGE5 into the COS cells and C33A cells stimulates PLA₂ activity and AA metabolism in cells (Figs. 2 and 3), we conclude that BPV E5 can affect the AA metabolism in various cell lines.

All tested E5 mutant proteins contain an intact hydrophobic domain, which might integrate into the membrane, but the ability to activate AA metabolism was different. E5 has previously been shown to bind to PDGFR and 16 k, the integral-membrane subunit of the proton-ATPase, through its hydrophobic membrane-associating domain, an E5 mutant lacking the hydrophilic domain did so also [7]. Our results demonstrate that the truncated E5 protein, which contains the hydrophobic region only, does not stimulate AA metabolism. Consequently, the binding of E5 to membrane-associated proteins is not sufficient for activation of AA metabolism. Evidently there are more reasons for the activation of AA metabolism than the incorporation of a hydrophobic protein into the membrane. All the mutants that could not stimulate the AA metabolism — 37S, 37S39S, and nE5h — are defective in forming dimers. The mutants 37S39S and nE5h do not contain cysteines for formation of dimers. The transformation-defective mutant with a single cysteine residue 37S has been shown earlier to form dimer 50% less effective than wild type E5 in C127 cells [3]. It is possible that the ability to form dimers through two cysteines is essential for E5 protein to express its activity. The 33V mutant, containing C-terminal cysteines for dimerisation and has previously been shown to form dimers in C127 cells [3], does not transform cells [37] but can stimulate the AA metabolism. This suggests that the ability to activate AA metabolism is not sufficient for transformation of cells, but might be involved in this process.

The classical target of E5 is PDGFR [5,7]. It has been shown that COS cells do not contain PDGFR in detectable level [7] and besides, the 33V mutant does not activate this receptor [29]. Since 33V mutant stimulates the AA metabolism in COS cells and C33A cells to the same extent as wt E5 (Figs. 2 and 3), we conclude that BPV E5 can activate the AA metabolism independently of PDGF receptor. It is noteworthy that several BPV E5 proteins with mutations in the transmembrane domain have been demonstrated to transform cells without activating the PDGFR [38]. Consequently, there was no absolute correspondence between the ability of E5 to bind PDGFR, to transform cells and to stimulate the AA metabolism.

COS and C33A are transformed cell lines and may respond to oncoprotein E5 expression differently compared to normal cells. Nevertheless, our results allow to conclude that BPV-1 transforming protein E5 affects many processes in the cell and E5 has more target proteins in the cell than PDGF receptor. We show a new activity of BPV-1 protein E5, ability to modulate the AA metabolism. Recently have been shown that the human papillomavirus type 16 E5 (HPV E5) protein activates PLCγ-1 and modulates membrane signaling phospholipids in an EGF-independent manner [39]. HPV-16 E5 is 83 residue
protein strongly hydrophobic and associated with cellular membranes like the BPV-1 E5. It is unclear whether the activated metabolism of membrane phospholipids is involved in transformation process or in the cellular response to virus infection.

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