Are transforming properties of the bovine papillomavirus E5 protein shared by E5 from high-risk human papillomavirus type 16?

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Abstract

The E5 proteins of bovine papillomavirus type 1 (BPV-1) and human papillomavirus type 16 (HPV-16) are small (44–83 amino acids), hydrophobic polypeptides that localize to membranes of the Golgi apparatus and endoplasmic reticulum, respectively. While the oncogenic properties of BPV-1 E5 have been characterized in detail, less is known about HPV-16 E5 due to its low expression in mammalian cells. Using codon-optimized HPV-16 E5 DNA, we have generated stable fibroblast cell lines that express equivalent levels of epitope-tagged BPV-1 and HPV-16 E5 proteins. In contrast to BPV-1 E5, HPV-16 E5 does not activate growth factor receptors, phosphoinositide 3-kinase or c-Src, and fails to induce focus formation, although it does promote anchorage-independent growth in soft agar. These variant activities are apparently unrelated to differences in intracellular localization of the E5 proteins since retargeting HPV-16 E5 to the Golgi apparatus does not induce focus formation.

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Keywords: E5 protein; Bovine papillomavirus type 1; Human papillomavirus type 16; Golgi apparatus

Introduction

Bovine papillomavirus type 1 (BPV-1) is a double-stranded DNA tumor virus that induces benign neoplasia in cattle (Lancaster and Olson, 1982) and horses (Carr et al., 2001), and has been used as a model system to study papillomavirus-mediated transformation in vitro (Chen et al., 1982; Dvoretzky et al., 1980; Lowy et al., 1980). The major in vitro transforming protein of BPV-1 is E5 (BPV E5) (DiMaio et al., 1986; Groff and Lancaster, 1986; Schiller et al., 1986; Yang et al., 1985), a small (44 amino acids), hydrophobic polypeptide that localizes to membranes of the Golgi apparatus (Burkhardt et al., 1989; Schapiro et al., 2000). The transforming activity of BPV E5 derives in part from its ability to induce trans-phosphorylation and constitutive activation of platelet-derived growth factor receptors (PDGF-Rs) that form a complex with BPV E5 dimers (Drummond-Barbosa et al., 1995; Goldstein et al., 1994; Lai et al., 1998; Petti et al., 1991). In addition, BPV E5 binds to the 16-kDa pore-forming subunit of the vacuolar H⁺-ATPase (16K) (Goldstein et al., 1991) and inhibits normal acidification of the Golgi lumen by interfering with H⁺ transport (Schapiro et al., 2000). The interaction of BPV E5 with 16K may contribute to E5-mediated transformation, since the Q17S and L26A BPV E5 point mutants inhibit Golgi acidification and transform fibroblasts (Schapiro et al., 2000), but do not activate the PDGF-R because they are defective in PDGF-R binding or homodimer formation, respectively (Adduci and Schlegel, 1999; Sparkowski et al., 1996). These E5 mutants activate heterodimeric phosphoinositide 3-kinase (PI 3-K) and c-Src in a PDGF-R-independent manner, and c-Src activation is required for transformation by L26A E5 (Suprynowicz et al., 2000, 2002).

Epidemiological studies have shown that high-risk human papillomaviruses (HPVs) are the primary causative agent of cervical cancer worldwide, and that HPV E5, E6 and E7 genes play important roles in HPV pathogenesis (zur Hausen,
membranes (Conrad et al., 1993; Disbrow et al., 2003), and can transform fibroblasts to grow in soft agar (Leechanachai et al., 1992; Pim et al., 1992; Straight et al., 1993). HPV-16 E5 also is mitogenic in primary keratinocytes, especially in cooperation with epidermal growth factor (EGF) and HPV-16 E7 (Bouvard et al., 1994; Straight et al., 1993; Valle and Banks, 1995; Venu et al., 1998). The latter finding may be related to the ability of HPV-16 E5 to enhance HPV-16-mediated reprogramming of terminally differentiating keratinocytes to undergo DNA synthesis in order to support viral DNA amplification (Genther et al., 2003). HPV-16 E5 stably associates with the 16K V-ATPase protein (Adam et al., 2000; Ashby et al., 2001; Conrad et al., 1993; Rodriguez et al., 2000), and can bind to the EGF receptor (EGF-R), PDGF-R and colony-stimulating factor-1 receptor (Hwang et al., 1995). The association of wild-type (wt) HPV-16 E5 with 16K may interfere with vacuolar H⁺-ATPase function and inhibit endosome acidification (Adam et al., 2000; Briggs et al., 2001; Straight et al., 1995), although an inhibitory effect on endocytic trafficking also appears likely (Ashby et al., 2001; Thomsen et al., 2000). The resulting delay in endosome acidification (Straight et al., 1993, 1995), or delay in the fusion of early endocytic vesicles with acidic late endosomes (Rodriguez et al., 2000; Thomsen et al., 2000), may be responsible for enhanced EGF-R activation by EGF in cells expressing HPV-16 E5 (Crusius et al., 1998; Pim et al., 1992; Rodriguez et al., 2000; Straight et al., 1993).

The inability of previous studies to detect expression of the HPV-16 E5 protein, unless transiently overexpressed in COS cells (Conrad et al., 1993; Hwang et al., 1995), undoubtedly has hampered characterization of its biological activity, and may account for discrepancies in its reported properties. Recently, Disbrow et al. (2003) have shown that HPV-16 E5 is expressed poorly in mammalian cells due to a high incidence (40%) of infrequently used codons, and that expression can be increased greatly by substituting codons more commonly used to encode the same amino acids. In the present study, we use codon-optimized HPV-16 E5 DNA to generate fibroblast cell lines that stably express epitope-tagged HPV-16 E5 and BPV E5 proteins at the same level. This system enables us to directly determine whether HPV-16 E5 exhibits oncogenic properties of BPV E5 in cells where the behavior of BPV E5 is well characterized.

Results

Growth factor receptor activation

To investigate whether HPV-16 E5 activates signal transduction proteins known to be activated by BPV E5, we generated NIH 3T3 cell lines that stably express the E5 proteins in equal amounts. NIH 3T3 cells were chosen for this purpose since the signaling properties of BPV E5 have been characterized extensively in these cells (Goldstein et al., 1994; Lai et al., 1998; Petti et al., 1991; Sparkowski et al., 1996; Suprynowicz et al., 2000, 2002). Normally, the HPV-16 E5 gene is expressed poorly in mammalian cells due to a high incidence (40%) of infrequently used codons, but expression can be increased more than sixfold by replacing these codons with more commonly used counterparts (Disbrow et al., 2003). As shown in Fig. 1, codon-optimized HPV-16 E5 and wt (not codon-optimized) BPV E5 proteins were present at equivalent levels when epitope-tagged and expressed in 3T3 cell lines. No E5 expression was detected in a control cell line harboring the empty JS55 expression vector. The immunoprecipitation and immunoblotting protocol used to detect E5 was not saturated by the amount of cell lysate employed in these measurements (1.5 mg), since identical results were obtained when E5 proteins were immunoprecipitated from half as much lysate (0.75 mg; Fig. 1). E5 expression remained stable in these cell lines for at least 30 passages (data not shown).

The ability of BPV E5 to induce tyrosine phosphorylation and sustained activation of the PDGF-R is well documented (Drummond-Barbosa et al., 1995; Goldstein et al., 1994; Lai et al., 1998; Petti et al., 1991). In our BPV E5-expressing cell line, the PDGF-R (immature form) was constitutively activated, especially after 24 h of serum starvation. In contrast, persistent activation of the PDGF-R was not observed in cell lines expressing HPV-16 E5 or containing the empty JS55 expression vector (Fig. 2A). Serum-starved HPV-16 E5-expressing cells and vector control cells exhibited equally substantial activation of the PDGF-R (mature form) when acutely stimulated with PDGF for 10 min (Fig. 2A), thereby demonstrating that HPV-16 E5 did not inhibit receptor activation. It is noteworthy that HPV-16 E5 also did not enhance PDGF-R activation in the presence of PDGF (Fig. 2A), given its ability to increase the recycling of ligand-bound EGF-Rs to the plasma membrane in keratinocytes (Straight et al., 1993, 1995).

![Fig. 1. Expression levels of BPV E5 and HPV-16 E5 in NIH 3T3 cell lines. Anti-AU1 Western blot of anti-AU1 immunoprecipitates from stable cell lines expressing AU1-tagged HPV-16 E5, BPV E5 or harboring the empty JS55 expression vector. Immunoprecipitates were prepared from 1.5 or 0.75 mg of cell lysate protein. Molecular mass markers (in kDa) are indicated on the left.](image-url)
HPV-16 E5 has been shown to enhance tyrosine phosphorylation and activation of the EGF-R in response to EGF in NIH 3T3 fibroblasts that overexpress exogenous EGF-Rs (Pim et al., 1992; Gottlieb et al., 1992; Rodriguez et al., 2000; Straight et al., 1993). EGF-R tyrosine phosphorylation was measured in our HPV-16 E5-expressing fibroblasts to determine whether the higher level of HPV-16 E5 expression afforded by codon optimization could induce or enhance activation of endogenous (not overexpressed) EGF-Rs. However, we found that HPV-16 E5 did not increase EGF-R activation, compared to JS55 control cells, under a variety of growth conditions: serum starvation, acute stimulation with EGF (10 min) and treatment with EGF for 24 h (Fig. 2B). The decreased level of EGF-R activation in acutely stimulated BPV E5-expressing cells (Fig. 2B) was the result of a reduction in the total number of EGF-Rs present (data not shown).

To screen for growth factor receptor activation in a functional assay, we monitored the proliferation of E5-expressing cell lines in medium containing a low level (0.3%) of fetal bovine serum (FBS) for a period of 3 days. We previously have correlated proliferation in this assay with ligand-independent growth factor receptor activation by BPV E5 and the sis oncogene (Suprynowicz et al., 2000). Furthermore, NIH 3T3 cells have receptors for PDGF, EGF, insulin, basic fibroblast growth factor and insulin-like growth factor, since these ligands all induce proliferation in this assay (Suprynowicz et al., 2000, 2002). In the present study, the number of BPV E5-expressing cells increased by more than 200% in 3 days, due to BPV E5 activation of the PDGF-R, whereas normal NIH 3T3 cells and vector controls actually decreased in number during the same period (Fig. 2C). HPV-16 E5-expressing cells behaved in the same manner as the control cells (Fig. 2C), indicating that HPV-16 E5 did not activate a growth factor receptor capable of stimulating proliferation. Therefore, a variety of assays capable of detecting growth factor receptor activation by BPV E5 do not detect activation by HPV-16 E5, even though the two E5 proteins are present at the same level.

**Activation of PI 3-K and c-Src**

A mutational analysis of BPV E5 has shown that it can activate PI 3-K and c-Src independently of growth factor receptor activation (Suprynowicz et al., 2000, 2002). We investigated whether HPV-16 E5 can activate PI 3-K by measuring tyrosine phosphorylation of the PI 3-K 85 kDa regulatory subunit (p85) in E5-expressing and control cell lines under conditions of serum starvation and growth. p85 tyrosine phosphorylation is known to induce PI 3-K activation (Shepherd et al., 1998), and has been correlated with BPV E5-mediated PI 3-K activation in NIH 3T3 cells (Suprynowicz et al., 2000). In agreement with our previous report, p85 tyrosine phosphorylation was dramatically elevated in serum-starved cells expressing BPV E5 relative to control cells harboring the empty expression vector (Fig. 3, 0.1% FBS). Additionally, BPV E5 increased p85 tyrosine phosphorylation in exponentially growing cells (Fig. 3, 10% FBS). However, in the presence of both 0.1% FBS and 10% FBS, cells expressing HPV-16 E5 exhibited a low level of p85-associated phosphotyrosine that was indistinguishable from the vector control (Fig. 3). The similarity of p85 (and PDGF-R) tyrosine phosphorylation levels in these cells after 24 h in the presence of 0.1% and 10% FBS (Figs. 2A and 3) reflects the down-regulation of PDGF-R expression that occurs in the continuous presence of growth factors (Lih et al., 1996; Vaziri and Faller, 1995).

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**Fig. 2.** Effect of E5 proteins on acute and constitutive growth factor receptor activation. (A) PDGF-R activation. Tyrosine-phosphorylated proteins were immunoprecipitated (from equal amounts of cell protein) from NIH 3T3 cell lines expressing HPV-16 E5, BPV E5, or from control cells containing the empty JS55 vector. Prior to lysis in SDS, cells were cultured for 24 h in medium containing 0.1% FBS (−FBS), or in medium containing 10% FBS and 20 ng/ml PDGF (+FBS, +GF, 1 day). Half the cells in 0.1% FBS were acutely stimulated with 20 ng/ml PDGF for 10 min immediately preceding lysis (−FBS; +GF, 10 min). The immunoprecipitates were analyzed on anti-PDGF-R Western blots to detect tyrosine-phosphorylated PDGF-Rs. The upper and lower bands correspond to mature and immature forms of the PDGF-R, respectively. Molecular mass marker (in kDa) is shown on the left. (B) EGF-R activation. Anti-phosphotyrosine immunoprecipitates were analyzed on anti-EGF-R Western blots to detect tyrosine-phosphorylated EGF-Rs. EGF (50 ng/ml) was added to cell cultures for 24 h (+FBS; +GF, 1 day) or for 10 min (−FBS; +GF, 10 min) before lysis. (C) Proliferation assays. The proliferation of NIH 3T3 and 3T3 cell lines in medium containing 0.3% FBS was measured for 3 days as described in Materials and methods.
p85 tyrosine phosphorylation was elevated in all cell lines following treatment with 10% FBS and PDGF for 24 h, resulting from ligand-induced PDGF-R activation at the plasma membrane, and this level of phosphorylation was not further increased by HPV-16 E5 (Fig. 3, 10% FBS + PDGF). In contrast, treatment with 10% FBS and EGF did not increase p85 tyrosine phosphorylation in control cells or in cells expressing HPV-16 E5 (Fig. 3, 10% FBS + EGF). PDGF and EGF differentially modulate p85 tyrosine phosphorylation in NIH 3T3 cells because the PDGF-R and EGF-R utilize different mechanisms to activate PI 3-K. The cytoplasmic protein tyrosine kinase domain of the activated PDGF-R contains specific phosphotyrosine docking sites for p85 at Y740 and Y751. As a consequence of binding to these sites, p85 becomes tyrosine phosphorylated and the 110-kDa catalytic subunit of PI 3-K is activated (Valius and Kazlauskas, 1993). These docking sites are not present in the EGF-R, which activates PI 3-K through Ras and the Gab1 adapter protein without p85 phosphorylation (Holgado-Madruga and Wong, 2004).

The effect of E5 proteins on c-Src tyrosine kinase activity was quantified in serum-starved cell lines using a procedure in which a selective peptide substrate (Cheng et al., 1992) is radioactively phosphorylated by anti-Src immunoprecipitates. This assay previously has been used to demonstrate activation of c-Src by BPV E5 in NIH 3T3 cells (Suprynowicz et al., 2002). Serum starvation was employed to lower the overall level of c-Src kinase activity, so that any potential activation by E5 proteins would be more apparent. As shown in Table 1, BPV E5 increased c-Src kinase activity twofold relative to JS55 control cells. The same level of c-Src activation has been observed during acute PDGF stimulation of serum-starved NIH 3T3 cells (Suprynowicz et al., 2002). In contrast, HPV-16 E5-expressing cells exhibited only slightly increased (1.2-fold) Src kinase activity (Table 1). We conclude that HPV-16 E5 does not share the ability of BPV E5 to activate PI 3-K and c-Src independently of growth factor receptor activation.

### Table 1

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Src kinase activity (% of 3T3)</th>
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<tr>
<td>3T3</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>3T3/16 E5</td>
<td>117 ± 13</td>
</tr>
<tr>
<td>3T3/JS55</td>
<td>97 ± 11</td>
</tr>
<tr>
<td>3T3/BPV E5</td>
<td>193 ± 24</td>
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*a Cells were grown in 0.1% FBS for 24 h.

**Fibroblast transformation**

The capacity of wt BPV E5 and numerous mutants that are defective for PDGF-R activation to transform fibroblasts has been demonstrated using focus formation assays, in which normal NIH 3T3 cells are transfected with BPV E5 constructs (Adduci and Schlegel, 1999; Sparkowski et al., 1994, 1996). More recently, we have shown that BPV E5-expressing fibroblast cell lines can be screened for transformation using a modified focus formation assay, in which the cell lines are co-plated with normal NIH 3T3 cells (Suprynowicz et al., 2002). When cell lines expressing equal levels of BPV E5 and HPV-16 E5 were tested in the modified focus assay, only the BPV E5 expressers formed visible foci (Fig. 4A, 10% FBS). HPV-16 E5-expressing cells also did not exhibit ligand-dependent focus formation, since they did not form foci when the assay was conducted in the continuous presence of EGF or PDGF (Fig. 4A, 10% FBS + EGF and 10% FBS + PDGF). HPV-16 E5 previously failed to transform NIH 3T3 cells in focus assays, although the level of HPV-16 E5 expression relative to BPV E5 was not shown (Disbrow et al., 2003; Straight et al., 1993). However, codon-optimized HPV-16 E5 is biologically active in NIH 3T3 cells, since it can transform the cells to undergo anchorage-independent growth in soft agar. As shown in Fig. 4B, NIH 3T3 cells expressing HPV-16 E5 formed much larger colonies in soft agar than did cells harboring the empty expression vector. Cells expressing BPV E5 also formed numerous large colonies. Consistent with our finding that neither HPV-16 E5 nor BPV E5 enhances EGF-dependent EGF-R activation in NIH 3T3 cells (Fig. 2), the addition of purified EGF to soft agar cultures did not differentially augment the growth of E5-expressing cells relative to non-E5 expressors (Fig. 4B).

**Golgi-targeted HPV-16 E5**

The localization of BPV E5 in membranes of the Golgi apparatus (Burkhardt et al., 1989; Schapiro et al., 2000) appears to be essential for its ability to transform fibroblasts. BPV E5 appended with the endoplasmic reticulum (ER) retention sequence, KDEL, remains sequestered in the ER (and cis Golgi) and is nontransforming (Sparkowski et al., 1995). Since HPV-16 E5 is localized predominantly in ER membranes (Disbrow et al., 2003), we asked whether HPV-16 E5 might acquire transforming activity in focus assays if directed to the Golgi compartment. For this analysis, we...
designed two different Golgi-targeted HPV-16 E5 constructs, termed “16 E5-SDYQRL” and “Golgi-16 E5”. In 16 E5-SDYQRL, the Golgi retrieval signal, SDYQRL (Roquemore and Banting, 1998), is appended to the C terminus of AU1-tagged, codon-optimized HPV-16 E5 (Fig. 5A). In Golgi-16 E5, AU1-tagged Q17G BPV E5 is appended to the N terminus of codon-optimized HPV-16 E5 (Fig. 5A). The Q17G BPV E5 mutant localizes to the Golgi apparatus (Schapiro et al., 2000), but does not activate the PDGF-R (Sparkowski et al., 1996), PI 3-K (Suprynowicz et al., 2000) or c-Src (Suprynowicz et al., 2002), and does not transform NIH 3T3 cells (Sparkowski et al., 1994). Anti-AU1 immunoprecipitation and immunoblot analysis showed that both Golgi-targeted HPV-16 E5 constructs were expressed in COS cells 48 h after transfection. The level of 16 E5-SDYQRL expression was identical to that of HPV-16 E5, while Golgi-16 E5 was expressed at the same level as BPV E5 (Fig. 5B).

Native HPV-16 E5 (Adam et al., 2000; Ashby et al., 2001; Conrad et al., 1993; Disbrow et al., 2003; Gieswein et al., 2003; Rodriguez et al., 2000) and BPV E5 (Andresson et al., 1995; Burkhardt et al., 1987; Goldstein and Schlegel, 1990; Goldstein et al., 1991) stably associates with the 16K pore-forming subunit of the vacuolar H+-ATPase and forms disulfide-linked homodimers in vivo. To demonstrate that Golgi-targeted HPV-16 E5 proteins are biologically active, we tested the ability of 16 E5-SDYQRL and Golgi-16 E5 to bind 16K and to dimerize in COS cells. In cells co-transfected with AU1-tagged E5 constructs and HA-tagged 16K, co-immunoprecipitation experiments indicated that BPV E5, HPV-16 E5 and the two Golgi-targeted HPV-16 E5 proteins stably associate with 16K (Fig. 5C). 16K was not detected in anti-AU1 immunoprecipitates if the cells were co-transfected with HA-16K and the empty E5 expression vector (Fig. 5C). In reducing SDS-polyacrylamide gels, HPV-16 E5, 16 E5-SDYQRL and Golgi-16 E5 were primarily monomeric, while BPV E5 was in the form of monomers and higher-order oligomers (Fig. 5D). In contrast, all of these E5 proteins demonstrated an ability to form disulfide-linked homodimers in nonreducing SDS gels (Fig. 5D).
The intracellular localization of 16 E5-SDYQRL and Golgi-16 E5 was evaluated by immunofluorescence microscopy in COS cells 24 h after transfection (Fig. 6). In agreement with previous findings (Disbrow et al., 2003), anti-AU1 labeling of HPV-16 E5-transfected cells yielded a reticular pattern that co-localized with the ER membrane protein, calnexin (David et al., 1993). In contrast, BPV E5 immunofluorescence was restricted to a discrete perinuclear region that co-localized with fluorescently labeled wheat germ agglutinin (WGA), a probe selective for the Golgi apparatus (Virtanen et al., 1980). The level of nonspecific background fluorescence was extremely low, since cells transfected with the empty JS55 expression vector exhibited negligible anti-AU1 immunofluorescence. 16 E5-SDYQRL localized predominantly to a perinuclear region that labeled with WGA, but also was present in the nuclear envelope in most cells. Golgi-16 E5 exclusively co-localized with WGA, and was not detected in the nuclear envelope or ER. Therefore, the SDYQRL signal was effective in directing much of HPV-16 E5 to the Golgi, while Q17G BPV E5 was highly effective in imposing Golgi localization on HPV-16 E5.

The Golgi-targeted HPV-16 E5 constructs were tested for their ability to transform NIH 3T3 cells in focus formation assays (Fig. 7). As shown, HPV-16 E5, 16 E5-SDYQRL and Golgi-16 E5 did not induce foci, which also was true for the JS55 empty vector. This result was in contrast to BPV E5, which induced many large foci.

Discussion

In this study, we generated stable fibroblast cell lines expressing epitope-tagged BPV-1 E5 and HPV-16 E5 proteins at the same level in order to determine whether any biological activities of BPV E5 are intrinsic to HPV-16 E5. The similar expression level of these proteins relied on the replacement of infrequently used codons in the HPV-16 E5 gene with commonly used synonymous codons (Disbrow et al., 2003). The six-amino-acid AU1 epitope used for immunological detection of the E5 proteins does not interfere with known biological activities of BPV E5 (Sparkowski et al., 1994) and HPV-16 E5. Like the native protein, AU1-tagged HPV-16 E5 forms homodimers, enhances EGF-R activation in primary human keratinocytes and induces anchorage-independent growth in murine fibroblasts (Disbrow et al., 2003 and this study). In addition, AU1-tagged HPV-16 E5 inhibits endosomal acidification in human keratinocytes (Disbrow et al., submitted for publication), as described for native HPV-16 E5 (Straight et al., 1995).

The mechanism by which BPV E5 induces sustained activation of the PDGF-R is known in considerable detail. Association of a PDGF-R molecule with each subunit of a BPV E5 dimer induces trans-phosphorylation and activation of the receptor cytoplasmic domains.
(Drummond-Barbosa et al., 1995; Goldstein et al., 1994; Lai et al., 1998; Petti et al., 1991). We observed constitutive PDGF-R tyrosine phosphorylation indicative of activation in cells expressing BPV E5 under conditions of serum-starvation and exponential growth. As an additional consequence of PDGF-R activation, BPV E5-expressing cells could proliferate in a serum-independent manner.

Like BPV E5, HPV-16 E5 can form dimers (Disbrow et al., 2003; Gieswein et al., 2003), and can bind to the PDGF-R, EGF-R and colony-stimulating factor-1 receptor (Hwang et al., 1995). Nevertheless, HPV-16 E5 did not constitu-
natively activate the PDGF-R or EGF-R, and did not promote serum-independent proliferation in our experiments. It may be that HPV-16 E5 stably associates with growth factor receptors only when both are transiently overexpressed at a high level in COS cells (Hwang et al., 1995). The inability of HPV-16 E5 to constitutively activate endogenous growth factor receptors in fibroblast cell lines may indicate that complexes of HPV-16 E5 dimers and receptors do not form when these proteins are present at lower, more physiological levels. This idea is supported by cross-linking experiments that find similar numbers of EGF-R dimers in HPV-16 E5-expressing keratinocytes and control keratinocytes in the absence of EGF (Crusius et al., 1998).

Although constitutive, ligand-independent EGF-R activation has not been demonstrated in HPV-16 E5-expressing cells (Crusius et al., 1998; Pim et al., 1992; Rodriguez et al., 2000; Straight et al., 1993), enhanced ligand-dependent EGF-R activation has been reported (Crusius et al., 1998; Pim et al., 1992; Rodriguez et al., 2000; Straight et al., 1993) in keratinocytes, which possess large numbers of endogenous EGF-Rs (Gottlieb et al., 1992), and in fibroblasts that have been engineered to express high levels of exogenous EGF-Rs (Collins et al., 1988). In contrast, NIH 3T3 fibroblasts express lower levels of EGF-Rs (and PDGF-Rs) (Collins et al., 1988; Heldin et al., 1981; Selinfreund and Wharton, 1986) and we find that HPV-16 E5 does not enhance EGF-R (or PDGF-R) activation during acute (10 min) or prolonged (24 h) treatment with EGF (or PDGF). In keratinocytes, enhanced EGF-R activation has been attributed to increased recycling of internalized EGF-Rs to the plasma membrane (Straight et al., 1993, 1995) due to an HPV-16 E5-induced defect in endosome acidification (Adam et al., 2000; Briggs et al., 2001; Straight et al., 1995) or defect in the fusion of early endosomes with acidic late endosomes (Thomsen et al., 2000). Alternatively, a different mechanism may be involved since it has been reported that enhanced EGF-R activation occurs too rapidly after EGF addition to be explained by increased receptor recycling (Crusius et al., 1998). Moreover, HPV-16 E5-expressing cells undergo ErbB2 receptor (ErbB2-R) activation during acute EGF stimulation, even though EGF is not a ligand for the ErbB2-R (Crusius et al., 1998). This finding suggests that HPV-16 E5 may induce an association of inactive ErbB2-R monomers with active EGF-R dimers in a manner that activates the ErbB2-Rs as a result of trans-phosphorylation. HPV-16 E5 may similarly enhance EGF-R activation during acute EGF stimulation by promoting an association of inactive EGF-R monomers with active dimers. Such associations of receptor molecules may require the relatively high levels of EGF-Rs, which are present in keratinocytes and fibroblasts that overexpress exogenous EGF-Rs (Collins et al., 1988; Gottlieb et al., 1992).

It previously has been reported that NIH 3T3 cells expressing low levels of HPV-16 E5 detectable only by RNA hybridization can grow in soft agar, but do not form foci in vitro (Straight et al., 1993). At the higher level of expression afforded by codon optimization, HPV-16 E5 still is unable to induce focus formation in these cells (Disbrow et al., 2003 and this study). However, codon-optimized HPV-16 E5 is biologically active in NIH 3T3 cells since we have shown that it transforms them to undergo anchorage-independent growth in soft agar. Given that HPV-16 E5 does not induce oncogenic signaling characteristic of BPV E5, its ability to promote anchorage-independent growth may be related to a unique biological activity, such as the EGF-independent activation of phospholipase C-γ (Crusius et al., 1999), or ability to confer increased resistance to apoptosis (Kabsch and Alonso, 2002).

During routine maintenance of cell lines, we observed that the growth of HPV-16 E5-expressing fibroblasts and keratinocytes was slower than control cells harboring empty expression vectors (data not shown). Previous reports linking HPV-16 E5 expression to increased cell proliferation have shown that the mitogenic effect of HPV-16 E5 is small in the absence of EGF supplementation (Bouvard et al., 1994; Straight et al., 1993; Valle and Banks, 1995). Moreover, the most profound growth-stimulatory effect of HPV-16 E5 occurred only in cooperation with the HPV-16 E7 gene (Bouvard et al., 1994; Valle and Banks, 1995). HPV-16 E7 is co-expressed with E5 and E6 during the early stages of HPV infection in vivo, where the role of E5 may be to stimulate proliferation in basal layer keratinocytes (zu Hausen, 2002), and to enhance the ability of HPV-16 to reprogram terminally differentiating keratinocytes to undergo DNA synthesis in order to support viral DNA amplification (Genther et al., 2003). Since we have shown that HPV-16 E5 does not exhibit any of the signal transduction properties of BPV E5 in fibroblasts when the two proteins are expressed at the same level, future investigations of the biological functions of HPV-16 E5 will require an analysis of its activity in keratinocytes that co-express the HPV-16 E6 and E7 proteins.

Materials and methods

Plasmid constructions

All E5 constructs used in this study were engineered to express the six-amino-acid AU1 epitope tag at their N...
terminus to facilitate immunological detection (Lim et al., 1990). The cloning of BPV E5 and codon-optimized HPV-16 E5 into the pJS55 expression vector have been described previously (Disbrow et al., 2003; Sparkowski et al., 1994). To target HPV-16 E5 to the Golgi compartment using the Q17G null mutant of BPV E5 (Sparkowski et al., 1994), a two-step PCR protocol was employed. The first step generated a DNA fragment consisting of Q17G BPV E5 appended to codons 2–7 of HPV-16 E5, as well as a second fragment comprising the last 6 codons of Q17G BPV E5 (excluding the stop codon) appended to HPV-16 E5. For the first fragment, the 5′ oligonucleotide primer (Integrated DNA Technologies) contained an upstream XhoI restriction site, Kozak sequence (Kozak, 1987), ATG translational start site and first codon of the AU1 epitope tag: 5′-CTCGAGGCCACCATGGGAC-3′ (primer A). The 3′ primer was complimentary to codons 39–44 of Q17G BPV E5 and to codons 2–7 of codon-optimized HPV-16 E5: 5′-TGCAGATATCCAGATTTGAAAGGGCA-GACCTGTACA-3′ (primer B). The 5′ primer for the second fragment corresponded to codons 39–44 of Q17G BPV E5 and codons 2–7 of codon-optimized HPV-16 E5: 5′-TGAAGGCAGTGTGGCCCTACAAACGTTGGTAGTCACTTGTAATCGGGA-TACTGCA-3′ (primer C). The 3′ primer was complimentary to the last six codons of codon-optimized HPV-16 E5 (including the stop codon) and to a downstream HindIII restriction site: 5′-AAGCTTTTATGTAATCAGAAAGCG-3′ (primer D). The two PCR products were purified from agarose gels and directly used as templates for the second PCR reaction, together with oligonucleotide primers A and D. This reaction generated full-length Q17G BPV E5 appended to full-length, codon-optimized HPV-16 E5 (Golgi-16 E5), which was ligated into pGEM-T (Promega) for amplification and sequencing, and was then cloned into the expression vector pSVL with the 11-amino-acid HA epitope tag at its N terminus (Andresson et al., 1995).

Cell culture and transfection

NIH 3T3 cell lines and COS-1 cells were maintained at 37 °C in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, 100 U/ml penicillin G and 100 µg/ml streptomycin sulfate (Invitrogen). For analysis of signaling proteins under reduced serum conditions, 15-cm tissue culture dishes of 50% confluent cells were washed twice with 25 ml Dulbecco’s phosphate-buffered saline (PBS) and cultured in 25 ml DMEM containing 0.1% FBS for 24 h. NIH 3T3 cell lines stably expressing E5 proteins were obtained following puromycin selection of cells co-transfected with E5 constructs and the puromycin resistance-conferring plasmid, pBABEpuro (Morgenstern and Land, 1990), at a ratio of 9:1 (E5 DNA: pBABEpuro) as described previously (Suprynowicz et al., 2002). At least 50 puromycin-resistant colonies were pooled for each cell line. Prior to transfection, COS-1 cells were grown to 80% confluence in 10-cm tissue culture dishes in Opti-MEM medium (Invitrogen) containing 4% FBS. For each 10-cm dish, 32 µg DNA was mixed with 4 ml serum-free Opti-MEM and 40 µl Lipofectamine 2000 transfection reagent (Invitrogen) and was added to the cells for 4 h.

To assay growth-factor-independent proliferation of NIH 3T3 cell lines, 6-cm tissue culture dishes were seeded with 1.5 × 10^5 cells in 4 ml (DMEM + 2% FBS). After 24 h, dishes were washed twice with PBS and the medium replaced with 4 ml (DMEM + 0.3% FBS) for 3 days. At the beginning and end of this 3-day period, cells were detached from duplicate dishes using trypsin/EDTA treatment, and were counted in a Beckman-Coulter particle counter. Results were calculated as the percent increase in number of cells while in 0.3% FBS.

Transformation assays

The focus forming activities of E5-expressing cell lines were assayed by plating 500 E5-expressing cells with 5 × 10^5 normal NIH 3T3 cells as detailed previously (Suprynowicz et al., 2002). The focus forming activities of Golgi-targeted HPV-16 E5 constructs were analyzed by transfecting 10-cm tissue culture dishes of 80% confluent NIH 3T3 cells using the Lipofectamine 2000 procedure outlined above. Following transfection, the medium was replaced with (DMEM + 10% FBS). After 3 days, the cells were replated in 175-cm² tissue culture flasks, which were maintained for 2–3 weeks with additions of fresh medium every 3 days. Foci were stained with 1% methylene blue (Sigma) as described (Suprynowicz et al., 2002). Where indicated, purified PDGF or EGF (Invitrogen) also were added to cultures every 3 days.

Anchorage-independent growth was assayed in 3.5-cm tissue culture dishes by layering 1.7 × 10^5 cells in 1 ml DMEM containing 10% FBS and 0.3% (w/v) agarose over 1 ml DMEM containing 10% FBS and 0.6% (w/v) agarose (Suprynowicz et al., 2000).

Immunofluorescence microscopy

COS-1 cells were grown to 80% confluence on 22 × 22 mm glass cover slips in six-well cluster plates containing Opti-MEM + 4% FBS. For transfection, 4 µg DNA was
mixed with 0.5 ml serum-free Opti-MEM and 5 μl Lipofectamine 2000 and was added to each well for a period of 4 h. Twenty-four hours later, cells were fixed in 4% paraformaldehyde (25 min), permeabilized in PBS containing 0.1% (w/v) saponin (10 min) and blocked in Pgel-S (PBS + 0.2% (w/v) gelatin + 0.1% saponin) containing 10% normal donkey serum (20 min). Cells were labeled for 30 min with primary antibodies diluted in Pgel-S (PBS + 0.2% (w/v) gelatin + 0.1% saponin) containing 10% normal donkey serum (20 min). Cells were washed twice with PBS containing 0.2% gelatin and twice with PBS before mounting in 9:1 glycerol/PBS containing 5% (w/v) n-propyl gallate. The Golgi compartment was labeled using Alexa Fluor 488-conjugated wheat germ agglutinin (Molecular Probes) at 1 μg/ml in Pgel-S. DNA was stained using Hoechst dye 33258 (Sigma) at 0.5 μg/ml in PBS.

**Metabolic labeling**

Ten-centimeter dishes of 80% confluent cells were washed with PBS (15 ml per dish) and incubated for 2 h in DMEM without cysteine and methionine (4 ml per dish; BioSource International). [35S] methionine/[35S] cysteine protein labeling mix (Perkin-Elmer “Easy Tag”) was added (200 μCi per dish) for a period of 3 h, and the cells subsequently lysed in modified RIPA buffer as described below.

**Immunoprecipitation and immunoblotting**

Phosphotyrosine-containing proteins were immunoprecipitated from SDS lysates of 80–90% confluent 15-cm tissue culture dishes as detailed previously (Suprynowicz et al., 2000). AU1-tagged E5 proteins were immunoprecipitated using the same protocol (10 μl anti-AU1 ascites per 0.75–1.5 mg cell lysate), except that cells were lysed in a modified RIPA buffer (150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1% deoxycholate, 0.1% SDS, 20 mM MOPS-NaOH, pH 7.0, 0.5 mM phenylmethylsulfonil fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin and 2 μg/ml pepstatin). Immunoprecipitates were fractionated on 1.5-mm SDS polyacrylamide mini-gels (Invitrogen), transferred to Immobilon-P membranes (Millipore) and labeled with polyclonal antibodies (Upstate Biotechnology, Inc.) recognizing the PDGF-R, EGFR, or 85 kDa regulatory subunit of PI 3-K as previously described (Suprynowicz et al., 2000, 2002). The same method was used to label anti-AU1 immunoblots, except that three labeling steps were employed: anti-AU1 ascites diluted 1:2000, unconjugated rabbit anti-mouse IgG (Pierce) at 1 μg/ml and alkaline phosphatase-conjugated goat anti-rabbit IgG (Applied Biosystems) diluted 1:5000. Anti-HA immunoblots were blocked with 2% (w/v) bovine serum albumin in PBS containing 0.05% (w/v) Tween-20, and were labeled with 1:5000-diluted anti-HA ascites (Covance) and alkaline phosphatase-conjugated goat anti-mouse IgG.

**Src kinase assay**

c-Src immunoprecipitates were incubated in the presence of [γ-32P]ATP and the selective substrate peptide KVEK1GEGTYGVVYK (Cheng et al., 1992) as described previously (Suprynowicz et al., 2002).

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