Cyclin A expression and growth in suspension can be uncoupled from p27 deregulation and extracellular signal-regulated kinase activity in cells transformed by bovine papillomavirus type 4 E5

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As the biochemical detection of bovine papillomavirus type 4 E5 is problematic, a fusion form of E5 and the green fluorescent protein (GFP–E5) was constructed and its characteristics were examined. GFP–E5 was detected in cells by autofluorescence and immunoblotting. Like wild-type (wt) E5, GFP–E5 localized in the endomembranes and permitted anchorage-independent (AI) growth. However, unlike wt E5, cells expressing GFP–E5 became quiescent in low serum and failed to sustain expression of cyclins D1 and to inactivate retinoblastoma protein (pRb). The normal anchorage requirement for cyclin D1 and cyclin A expression was abolished in cells expressing wt E5 or GFP–E5, residual extracellular signal-regulated kinase (ERK 1/2) activity was not required to sustain cyclin D1 and cyclin A expression in suspension and deregulation of cyclin A–cyclin-dependent kinase (CDK) activity was sufficient to account for AI growth of cells expressing E5. Constitutive upregulation of the CDK inhibitor p27KIP1, characteristic of cells expressing wt E5, was not observed in those expressing GFP–E5; therefore, p27KIP1 deregulation is not required for E5-mediated AI growth.

INTRODUCTION

In normal cells, the decision to divide and progress through the cell cycle requires the integration of extracellular and intracellular signals. The environmental signals are the presence/absence of particular growth factors and attachment to the extracellular matrix (ECM) (Assoian, 1997). Growth factors activate a series of signal-transduction cascades, among which activation of the mitogen-activated protein kinase (MAPK) cascade, Ras–Raf–MEK–ERK, is the best-characterized. Most studies indicate that these signals eventually control G1 phase progression by regulating cyclin D1 expression (Balmanno & Cook, 1999; Pagès et al., 1993; Weber et al., 1997).

As cells progress through the cell cycle, cyclin E and cyclin A are synthesized. Cyclin E, in combination with cyclin-dependent kinase 2 (CDK2), is responsible for progression through the G1 phase, whilst the cyclin A/CDK2 complex enables S-phase progression. Finally, cyclin A and cyclin B associate with cdc2 (cell division cycle 2) to promote entry into mitosis.

In adherent cells, the presence of growth factors is required during the transition from G0 to G1; however, once beyond the restriction (R) point in G1, cells are committed to division and no longer require extracellular growth factors to complete the cell cycle. Cell-cycle control by anchorage to substrate, chiefly through integrin engagement, has been mapped to the transit through G1 into S phase (Guadagno & Assoian, 1991) and it has been shown that constitutive expression of cyclin A is necessary and sufficient for fibroblasts to proliferate in suspension (Guadagno et al., 1993).

Cell-cycle control is perturbed in transformed cells. The ability of DNA tumour viruses to stimulate unscheduled cell proliferation, overriding growth-suppressive signals by direct interaction of viral proteins with tumour suppressors or oncoproteins, has been well-documented (Lukas et al., 1994; Munger et al., 1989; Wang et al., 1993; Werness et al., 1990). However, it has emerged recently that these cellular proteins are not the only victims of viral opportunism and that both CDK inhibitors and cyclins are vulnerable to disruption by several viral gene products (Swanton & Jones, 2001). It has been shown, for example, that the E7 protein of human papillomavirus type 16 (HPV-16) and the E5 protein of bovine papillomavirus type 4 (BPV-4) both lead to constitutive expression of G1 cyclins and their associated kinase activities (O’Brien & Campo, 1998; O’Brien et al., 2001; Zerfass et al., 1995) and induce anchorage-independent (AI) growth via activation of cyclin A expression (O’Brien & Campo, 1998; Schulze et al., 1998).
BPV-4 E5, previously called E8 (Jackson et al., 1991; Morgan & Campo, 2000), belongs to a family of small, membrane-
localized peptides that are encoded by some papilloma-
viruses. BPV-4 E5 has a hydrophobic N-terminal region
(residues 1–30) with a putative α-helix conformation and a
hydrophilic C-terminal tail of 12 residues (Jackson et al.,
1991). It transforms established NIH 3T3 cells and primary
bovine fibroblasts (PalF) (which coexpress BPV-4 E7 and
an activated Ras), allowing cell growth in low serum (LS)
concentrations and in suspension (O’Brien & Campo, 1998;
O’Brien et al., 1999; Pennie et al., 1993). E5-transformed
NIH 3T3 cells have an elevated pool of cyclin D1/CDK4
complexes that retain their kinase activity, despite being
associated with a high level of the CDK inhibitor p27KIP1
(p27) under normal growth conditions or in the absence of
mitogens (O’Brien et al., 2001).

It is notoriously difficult to detect the expression and
localization of E5 proteins. Like many non-structural viral
proteins, they are low-abundance proteins that are expres-
sed at specific stages of the viral life cycle; moreover, their
hydrophobic nature and issues of solubility in vitro have
rendered their purification extremely difficult. Antibody
reagents directed against E5 proteins are consequently
limited and not very efficient. In an attempt to obtain a
detection system for visualizing BPV-4 E5, which would
help the analysis of its functions, a form of the viral protein
fused with the green fluorescent protein (GFP) was con-
structed (GFP–E5).

Here, we analyse the transformation characteristics of
GFP–E5 and compare them with wild-type (wt) E5. GFP–
E5 induces cell growth in suspension, but not growth in LS.
However, unlike in cells expressing wt E5, we did not
observe constitutive upregulation of the CDK inhibitor
p27KIP1 and, hence, p27 deregulation is not required for
E5-mediated cyclin A deregulation and AI growth. GFP–E5
provides an excellent tool for studying the molecular
mechanisms of cell-cycle deregulation promoted by E5.

**METHODS**

**Nomenclature.** The E5 ORF, formerly called E8, is defined by nt
332–460 of BPV-4. We refer to wt E5 and GFP–E5 when comparing
the two proteins or their respective transformed cells, and to E5
when describing the general characteristics of the protein.

**Plasmid construction.** The E5 ORF, complete with a 39 bp
sequence encoding the haemagglutinin (HA) epitope at the 5’
end (O’Brien et al., 1999), was amplified by PCR and cloned into the
PstI–HindIII site of the pEGFP-C3 vector (Clontech) to generate a
fusion protein where enhanced GFP (EGFP) is expressed at the N
terminus of HA5E. For the generation of stable transfectants, GFP
and GFP–E5 were subcloned from the pEGFP-C3 vector into the
BamHI site of pZipneo (Cepko et al., 1984) by PCR.

**Cell culture and production of stable cell lines.** Cells were
cultured routinely in Dulbecco’s modified Eagle’s medium (DMEM)
containing 10 % newborn calf serum (NBCS). For transfection, 10 μg
expression plasmid (pZipneoGFP or pZipneoGFP–E5) was intro-
duced into NIH 3T3 cells by using the standard calcium phosphate
method. Cells were selected for G418 resistance (0.5 mg ml–1) and
clonal populations were derived by ring cloning. NIH 3T3 cells
expressing wt E5 or controls transfected with the empty expression
vector pZipneo (Z3T3) have been described previously (O’Brien & Campo, 1998).

**Treatment with MAP kinase kinase (MEK) inhibitors.** Cells
(3 × 105) were plated in 10 ml methocel medium containing the
following MEK inhibitors: 50 μM PD98059 (Calbiochem), 20 μM
U0126 (Promega) or an equal volume of vehicle (DMSO). Plates
were incubated at 37 °C for 48 or 72 h. At the end of the incuba-
tion time, cells were harvested, total protein was extracted and
aliquots of 30 μg were made up in lysis buffer and prepared for
immunoblotting.

**Fluorescence microscopy.** Cells were seeded (3 × 104 per well)
and were fixed the next day with 3 % parafomaldehyde in PBS for
15 min at room temperature, mounted by using Vectashield and
visualized with a confocal microscope (Bio-Rad Nikon Diapht
inverted microscope). For Golgi staining, COS-7 cells were trans-
iently transfected with plasmid pEGFP–E5 by using the standard
calcium phosphate method. Live cells were incubated, 24 h post-
transfection, with 5 μM BODIPY Texas red C2 (BODIPY-
TR ceramide), Molecular Probes; Zhang et al., 1999) in 25 mM
HEPES DMEM serum-free medium (H-DMEM) for 30 min at 4 °C.
Cells were washed with H-DMEM and incubated at 37 °C for a
further 30 min. Cells were fixed, mounted and visualized with a confo-
cal microscope.

**AI growth.** Cells (5 × 105) were added to 15 ml methylcellulose
based medium (0.8 % methocel) supplemented with 20 % NBCS
and the mix was plated in two 60 mm Petri dishes. Dishes were
incubated at 37 °C for 3 weeks before scoring.

**Measurement of DNA content by flow cytometry.** One million
cells were seeded in 90 mm dishes. After 16–24 h, the medium was
replaced with LS (0.2 % serum) or normal growth medium and cells
were harvested 24 h later, fixed in ice-cold 80 % ethanol and stained
in PBS containing 20 μg propidium iodide ml–1 (Sigma) and
0.25 mg RNase A ml–1 (Qiagen). DNA content was measured on
Becton Dickinson FACScan system and cell-cycle distribution was
assessed by using MODFIT software.

**Growth curves.** Cells were seeded in triplicate in 96-well plates, at
5000 cells per well in 200 μl DMEM containing 10 % serum. One
96-well plate was set up for each day from day 0 to day 6. After 4 h
incubation, growth medium was replaced or changed to DMEM
containing 0.5 % serum in the appropriate wells.

The first plate (day 0) and subsequent plates were assayed as follows:
cells were washed once with PBS, then 100 μl 0.1 % crystal violet in
20 % methanol was added per well and plates were incubated at
room temperature overnight. The crystal violet solution was then
removed with extensive washes in water; the plate was dried at room
temperature. Finally, the dye was solubilized in 100 μl 1 % SDS and
0.2 M NaOH and A590 was determined by using an automatic micro-
well plate reader (Dynatech MR 7000).

**Antibodies.** Antibodies used were: GFP (MMS-118, Babco); cyclin
A (sc-596, Santa Cruz); cyclin D1 (sc-450, Santa Cruz); cyclin E
(sc-481, Santa Cruz); CDK2 (sc-163, Santa Cruz); total extracellular
signal-regulated kinase (ERK) 1/2 (M85670, Sigma); phosphoERK 1/2
(M8159, Sigma); and z-tubulin (T9026, Sigma). For immune com-
plex kinase assays, cyclin A (sc-751, Santa Cruz) was used.

**Immunoblotting.** Adherent cells cultured in growth medium (GM,
10 % serum) or LS (0.5 % serum) were lysed in near-boiling SDS-
PAGE sample buffer [100 mM Tris/HCl (pH 6.8), 2 % (w/v) SDS, 20 %
(v/v) glycerol], sonicated and clarified by centrifugation at
13 000 g for 5 min at 4 °C. For the assay in suspension, 5 × 10^6 cells were resuspended in 30 ml methocel medium supplied with 10 % NBCS and incubated in a humid incubator for 48 h. At the end of the incubation time, cells were pelleted and washed twice with cold PBS, resuspended in 100 μl SDS-PAGE sample buffer and processed as above. Protein concentration was determined by measurement of A_{280}. Samples containing 30–100 μg protein were made up by adding an equal volume of 2 × SDS loading buffer [4 % (w/v) SDS, 0–2 % (w/v) bromophenol blue, 20 % (v/v) glycerol, 100 mM Tris (pH 6–8), 200 mM DTT]. Samples were boiled for 5 min, loaded on an SDS gel, separated by electrophoresis and transferred onto a nitrocellulose membrane (ECL; Amersham). Antibody detection was performed by using enhanced chemiluminescence (ECL; Amersham).

RESULTS

Detection and visualization of GFP–E5

GFP fusion proteins have been used extensively in biological studies to monitor gene expression, protein localization and function in a variety of cells and organisms (Kain et al., 1995; Zimmer, 2002). In an attempt to obtain a detection system for routinely visualizing BPV-4 E5, which would help the analysis of its functions, a form of the viral protein fused at its N terminus to GFP was constructed (GFP–E5) and clonal, stable cell lines of NIH 3T3 fibroblasts expressing GFP–E5 or GFP were derived. In these cells, expression of GFP–E5 was demonstrated by immunoblotting (Fig. 1a).

Both GFP cell clones showed a specific band with an apparent molecular mass of 27 kDa (Prendergast & Mann, 1992; Disbrow et al., 1993; O'Brien et al., 1995; Conrad et al., 1989; Boeke & Fields, 1984). This band, which did not appear to be an artefact of fusion protein overexpression, was suggested by colocalization studies using the Golgi marker BODIPY-TR ceramide (Fig. 1c). This does not appear to be an artefact of fusion protein overexpression, as a chiefly Golgi location was observed when the amount of GFP–E5 expression plasmid, and therefore the cellular level of fusion protein, was titrated to give weak but detectable GFP signals (results not shown).

These observations are in agreement with the previously reported localization of BPV-4, BPV-1, HPV-6 and HPV-16 E5 proteins in the GA (Burkhardt et al., 1989; Conrad et al., 1993; Disbrow et al., 2003; Pennie et al., 1993), demonstrating that, despite its much larger size, GFP does not interfere with the proper localization of E5.

NIH 3T3 fibroblasts expressing GFP–E5 grow in suspension, but not in the absence of mitogens

We next set out to determine whether the transforming abilities of wt E5 had been maintained in its fusion form with GFP. GFP–E5 cells were assayed for their growth characteristics in suspension and in reduced serum concentration (LS).

Cells transfected with an expression plasmid for GFP–E5 showed an up to 10 times higher efficiency of colony formation than the corresponding control transfectants after G418 selection, suggesting that the fusion protein confers some survival and/or growth advantage to the cells (data not shown).

We analysed three of the six GFP–E5 clones and all were capable of Al growth in semi-solid media, albeit with about 50 % reduced efficiency when compared with wt E5 (Table 1; O'Brien & Campo, 1998).

However, in contrast to wt E5 cells, GFP–E5 clones could not proliferate in LS, as assessed by flow cytometry following propidium iodide staining. Cells accumulated in G_0/G_1 and no sub-G_1 cell population was evident, suggesting that the cells did not undergo extensive apoptosis (Fig. 2a).

Growth arrest of GFP–E5 clones in LS was confirmed over a 7-day period in LS (Fig. 2b) and was due to arrested proliferation; it was not a result of a balance in cell division and cell death in the population (M. Zago & V. O'Brien, unpublished observations).

For further studies, we chose to analyse in greater detail two GFP–E5 clones (4 and 11) in suspension and GFP–E5 clone 4 in LS.

Mitogen dependence of GFP–E5 cells is enforced at the level of cyclin D1 expression and retinoblastoma protein (pRb) activation

As reported in previous studies, both cyclin D1 and cyclin A are upregulated in wt E5 cells grown in normal GM and their expression is largely mitogen-independent (O'Brien & Campo, 1998; O'Brien et al., 2001). In GM, GFP–E5 cells demonstrated a clear increase in the steady-state level of cyclin D1 compared with GFP control cells, but the expression of this cyclin remained mitogen-dependent (Fig. 3a).

We therefore expected to see inhibition of CDK4 kinase activity in GFP–E5 cells grown in LS. To confirm this, we tested the phosphorylation status of endogenous pRb by mobility shift and with antibodies directed to specific CDK4/6 phosphorylation sites (Brugarolas et al., 1999; Characteristic in suspension and in reduced serum concentration (LS).

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Kitagawa et al., 1996). The predominance of the growth-suppressive, active/hypophosphorylated form of pRb and accompanying decrease in Ser780 phosphorylation in GFP–E5 cells cultured in LS conditions (Fig. 3b) demonstrate that mitogen dependence of GFP–E5 cells is enforced at the level of cyclin D–CDK4/6 activity.

**Cyclin A expression and associated kinase activity are sustained in GFP–E5 cells grown in suspension**

We have shown previously that normal anchorage-dependent expression of cyclin A is lost in E5-transformed cells, which
**Fig. 1.** Detection of GFP and GFP–E5 in NIH 3T3 and COS-7 cells. (a) GFP and GFP–E5 expression was detected in NIH 3T3 clonal populations by immunoblotting using an anti-GFP mouse mAb (MMS-118, Babco). Parental NIH 3T3 cells were used as control. (b) GFP and GFP–E5 fusion protein distribution viewed 24 h after transient transfection of COS-7 cells with pEGFP–E5 plasmid (left panel) and in stable transfectants of NIH 3T3 cells (right panel), as visualized by confocal microscopy. Images were captured with a ×60 objective lens. (c) In COS-7 cells transiently expressing GFP–E5 (upper left panel), the GA was visualized by using BODIPY-TR ceramide (upper middle panel). The merge of the two images (upper right panel) shows areas of colocalization in yellow, obtained by computer-aided digital image processing. The lower panel represents a side-by-side comparison of the microscope field captured to generate the images in the top panel. The right side (BODIPY-TR ceramide) shows Golgi staining; the white arrow highlights a stained cell that also expresses GFP–E5. Non-transfected and GFP–E5-expressing cells do not show any gross differences in Golgi architecture, as revealed by BODIPY-TR ceramide staining.

**Table 1.** AI growth

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Methocel colonies (% ± SEM)</th>
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<tbody>
<tr>
<td>Z3T3*</td>
<td>3.3 ± 0.3</td>
</tr>
<tr>
<td>E5*</td>
<td>19.3 ± 0.8</td>
</tr>
<tr>
<td>GFP.3†</td>
<td>2.8 ± 0.5</td>
</tr>
<tr>
<td>GFP.10†</td>
<td>2.9 ± 0.5</td>
</tr>
<tr>
<td>GFP–E5.4†</td>
<td>12.9 ± 0.8</td>
</tr>
<tr>
<td>GFP–E5.7†</td>
<td>10.0 ± 0.8</td>
</tr>
<tr>
<td>GFP–E5.11†</td>
<td>9.5 ± 1.0</td>
</tr>
</tbody>
</table>

*Mean of two experiments.
†Mean of four experiments.

Correspondingly grow as macroscopic colonies in semisolid media (O’Brien & Campo, 1998). In wt E5 and GFP–E5 cells grown in suspension, the expression of cyclins D1 and A was sustained (Fig. 4a). We could not detect pRb kinase activity associated with cyclin D1 in control or E5 cells (results not shown) and E5 expression did not significantly alter the total levels of cyclin E or CDK2 or alter the ratio of active (Thr160-phosphorylated) to inactive enzyme, as assessed by mobility shift on SDS gels (Fig. 4b). In addition, the activity of cyclin E-associated kinase remained strictly anchorage-dependent in control and E5 cells (data not shown). So, although cyclins D1 and E are expressed in suspension cells, we found no evidence of pRb phosphorylation in E5 cells in suspension.

On the other hand, cyclin A-associated kinase activity was sustained at relatively high levels for up to 48 h in suspension in both wt E5 and GFP–E5 cells (Fig. 4c). Additionally, like wt E5 (O’Brien & Campo, 1998), GFP–E5 was able to significantly transactivate a heterologous human cyclin A promoter in cells grown in suspension (data not shown), an activity that probably underpins the sustained expression of cyclin A and cell proliferation in suspension.

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**Table 1.** AI growth

GFP, GFP–E5, Z3T3 and E5 cells (2·5 × 10⁴) were plated in duplicate on 0·8% methocel-based medium. Efficiency of colony formation was determined after 3 weeks, scoring colonies with a diameter of >0·1 mm. No. colonies is expressed as a percentage of the total no. cells seeded on day 0, and represents the mean of two or four experiments run in duplicate.

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**wt E5- and GFP–E5-transformed cells sustain cyclin A expression, despite pharmacological blockade of the MEK–ERK pathway**

The MEK–ERK cascade is a key signal-transduction pathway for the G1/S transition in NIH 3T3 cells under normal (monolayer) growth conditions. Substrate adhesion elicits a diverse array of mainly integrin-dependent signals that converge to regulate the induction of cyclin D1 and degradation of p27 (Rooers & Assoian, 2000). A number of studies have shown that moderate but sustained ERK activity in the nucleus is required for induction of cyclin D1 mRNA, and that ERK nuclear translocation is regulated by cell adhesion. Efficient activation of ERKs by soluble mitogens is inhibited when cells are maintained in suspension (Lin et al., 1997; Renshaw et al., 1997). Consequently, cyclin D1 expression and cell-cycle progression are inhibited (Assoian & Schwartz, 2001).

In control cells (Z3T3 and GFP), we found that ERK activity did not show such a strict requirement for cell adhesion and relatively high basal levels of ERK activity remained in cells after 48–72 h in suspension (Fig. 5a), but these cells did not proliferate (Table 1). We wanted to determine whether, in cells expressing either wt E5 or GFP–E5, the residual ERK activity and, ultimately, the expression of cyclin D1 were necessary for cell proliferation in suspension. To test this, we utilized two well-characterized inhibitors of the MAPK pathway, PD98059 and U0126 (Alessi et al., 1995; Favata et al., 1998), to block the ERK pathway through inhibition of MEK.

Treatment of cells in suspension culture with the PD98059 inhibitor (50 μm) reduced the residual level of active ERKs (p-ERK 1/2), whereas the level of total ERKs (t-ERK 1/2) was not affected. Moreover, the MAPK inhibitor reduced the expression of cyclin D1 in control Z3T3 and GFP cells, moderately decreased it in GFP–E5 cells and reduced it only slightly in wt E5 cells. However, cyclin A levels were only modestly, or not at all, affected in either wt E5 or GFP–E5 cells (Fig. 5a, b).

The independence of cyclin A expression from the MAPK pathway was confirmed in GFP–E5 cells by using the MEK inhibitor U0126 (20 μm). After 72 h U0126 treatment, p-ERK 1/2 was almost completely inhibited, but expression of...
cyclin A was maintained at high levels and that of cyclin D1 was again only moderately decreased (Fig. 5c).

We conclude that E5, whether wt or in GFP fusion form, maintains cyclin A expression chiefly or completely independently of ERK activity.

**p27 deregulation is not required for E5-mediated cyclin A deregulation and AI growth**

A significant finding in our previous studies was that p27 expression was constitutively upregulated in wt E5 cells (Fig. 5c).

We conclude that E5, whether wt or in GFP fusion form, maintains cyclin A expression chiefly or completely independently of ERK activity.
and that the ability of wt E5 (but not a truncated E5) to deregulate p27 expression correlated with cell transformation (O’Brien & Campo, 1998). Our follow-on work demonstrated that cells transformed by wt E5 display constitutively high levels of p27 and that most or all of this p27 is associated with an elevated pool of nuclear, active cyclin D1–CDK4 complexes. We have argued that wt E5 acts to uncouple cyclin D1 expression from mitogen regulation, in part through p27 deregulation, allowing for accumulation of cyclin D1–CDK4–p27 ternary complexes that act to stabilize cyclin D1 and provide a pool of enzyme activity that enforces pRb inactivation (O’Brien et al., 2001).

Given that GFP–E5 cells do not sustain cyclin D1 following mitogen withdrawal, we sought to determine whether p27 was deregulated in GFP–E5 cells. As shown in Fig. 6, p27 expression is not deregulated in GFP–E5 cells. This is a significant result that demonstrates that p27 deregulation is not required for E5-mediated transformation.

**DISCUSSION**

**Biological characteristics of BPV-4 GFP–E5**

We have generated stable cell lines that express the BPV-4 E5-transforming protein fused to GFP. GFP–E5 could be detected easily by fluorescence and Western blotting (Fig. 1). The ease of detection of GFP–E5 contrasts with the laborious protocols that are needed to detect wt E5 (O’Brien et al., 2001) or wt E5 tagged with the HA epitope (O’Brien et al., 1999).

Despite the much larger size of GFP (27 kDa) compared with E5 (7 kDa), GFP–E5 localizes to endomembranes, predominantly to a structure identified as the GA by colocalization with the Golgi vital stain BODIPY-TR ceramide (Fig. 1c). This subcellular localization is in agreement with the observation that other E5 proteins localize mainly to the GA (Burkhardt et al., 1989; Conrad et al., 1993). The GA
is an extremely strategic location to control or regulate the transport/processing of itinerant proteins and lipids, and to influence various cellular processes either by direct interaction with targeted proteins or indirectly by modifying the organelle environment. Accordingly, a trans-Golgi location is required for BPV-1 E5-transforming abilities (Schapiro et al., 2000; Sparkowski et al., 1995) and BPV-4 E5 retains the major histocompatibility complex class I in the GA (Ashrafi et al., 2002; Marchetti et al., 2002).

Cells expressing GFP–E5, like those expressing wt E5 (O’Brien & Campo, 1998), are capable of Al growth, a phenotype believed to correlate with tumour growth in vivo. However, in contrast to wt E5 cells (O’Brien & Campo, 1998), GFP–E5 cells exit the cell cycle when deprived of serum mitogens. The segregation of these two transformation phenotypes, growth in suspension and growth in LS, has already been observed in PalF cells expressing the HA-tagged E5 mutant N17S, in which the asparagine at position 17 had been converted into serine. The inability of N17S HAE5 to induce growth in LS was not due to the addition of the HA tag, as wt HAE5 maintained all its transforming phenotypes (O’Brien et al., 1999). However, we do not know whether the cells expressing this mutant proliferate and die to the same extent in LS, hence preventing any net population growth, or become quiescent but remain viable. Full transformation

![Figure 5](image5.png)

**Fig. 5.** In E5 cells, cyclin D1 and cyclin A expression is largely independent of basal ERK 1/2 activity. Z3T3, E5, GFP and GFP–E5 cells were treated with vehicle (DMSO; DM), PD98059 inhibitor (PD) for 48 (a) or 72 (b) h or with U0126 (U0) inhibitor (c) for 72 h in suspension culture. The activation state of ERK 1/2 (p-ERK 1/2) after treatment was assessed by using a phosphospecific antibody (M8159, Sigma). Cyclin D1 and cyclin A expression levels were determined by Western blotting. Levels of total ERK 1/2 (t-ERK 1/2) or α-tubulin, detected with M5670 (Sigma) or T9026 (Sigma), respectively, served as loading controls.

![Figure 6](image6.png)

**Fig. 6.** Deregulation of p27KIP1 is not observed in GFP–E5 cells. (a) Cell lysates were prepared from representative clones of the cells indicated, grown in GM or LS for 24 h and p27 expression was detected by Western blotting with α-tubulin serving as a loading control. (b) Cells were maintained in methocel-based medium for 48 h, then harvested for Western blotting. Levels of p27 and α-tubulin (loading control) were determined by Western blotting as described above.
of PaLF cells required the addition of BPV-4 E7, HPV-16 E6 and c-Ha-Ras (Pennie et al., 1993); the presence of these additional oncogenes may confound the interpretation of results. Therefore, the GFP–E5 NIH 3T3 cells provide a model system that should be a fundamental tool for studying the molecular mechanisms responsible for the segregation of growth in suspension from growth in LS.

**Ability of E5-transformed cells to grow in suspension correlates with sustained cyclin A–CDK activity**

During the cell cycle, cyclin A gene expression is strictly regulated at the level of transcription: the protein is expressed at the G1/S transition and through the S phase (Henglein et al., 1994). E5 expression promotes the activity of the cyclin A gene promoter and a strong relationship exists between cyclin A promoter transactivation and cell growth and transformation (O’Brien & Campo, 1998; O’Brien et al., 1999). This relation is maintained in GFP–E5 cells (data not shown).

Cells arrested by lack of substrate adhesion fail to produce cyclin A; however, ectopic expression of cyclin A enables the cells to bypass the adhesion requirement, implicating cyclin A as the major target of cell-cycle control by anchorage-signalling pathways (Guadagno & Asoian, 1991). From the results presented here, we conclude that in E5- and GFP–E5-transformed cells, sustained cyclin A expression and associated kinase activity is sufficient to mediate the observed AI growth (Figs 4 and 5, Table 1).

**E5-mediated upregulation of cyclins D1 and A is largely independent of the MAPK cascade**

The Ras–Raf–MEK–ERK pathway is a well-characterized response to both receptor tyrosine kinase (activated by growth factors) and integrin activation (by ECM) (Danen & Yamada, 2001) and several studies have established a link between sustained ERK activation and cell proliferation (Balmanno & Cook, 1999; Pagès et al., 1993; Weber et al., 1997). However, inhibition of the residual ERK activity in wt E5 and GFP–E5 cells in suspension demonstrates the independence of cyclin D1 expression from ERK activity, as the cyclin D1 expression level is almost unaltered or only slightly reduced following prolonged exposure to MEK inhibitors (Fig. 5). Thus, in E5 cells, the normal sequential activation of G1 cyclins is disrupted and the ERK signalling cascade that links growth factor signals to cyclin D1 is dispensable, at least in suspension culture.

**p27 deregulation is not required for E5-mediated transformation**

The present report defines a form of E5 that does not deregulate p27 expression, yet transforms cells so that p27 deregulation is not required for E5-mediated AI growth. In turn, this suggests that the observed increase in cyclin D–CDK4/6 and constitutive hyperphosphorylation of pRb/inactivation in wt E5 cells (O’Brien et al., 2001) may not be required for cyclin A expression and AI growth. Cyclin A is a crucial downstream target of pRb and ectopic expression of cyclin A is sufficient to overcome pRb-mediated growth suppression (Knudsen et al., 1999). It remains to be determined whether pRb inactivation is strictly required for E5-mediated transformation; the GFP–E5 cells will allow us to investigate this important question.

It has been shown recently that another viral protein, BPV-1 E5, transforms NIH 3T3 cells with minimal or abolished requirement for the ERK pathway (Suprynowicz et al., 2000). Instead, a central role for PI-3 kinase (PI-3K) activity has been demonstrated for this oncoprotein. wt BPV-1 E5 and transforming mutants that are defective for activating the well-established target of BPV-1 E5, the platelet-derived growth factor (PDGFβ) receptor, constitutively activate PI-3K (Suprynowicz et al., 2000). Sustained PI-3K activation can induce AI growth, but not proliferation, in LS (Chang et al., 1997; Klippel et al., 1998). The PI-3K pathway is therefore a potential key mediator of the observed phenotypes of BPV-4 E5 cells (Jirmanova et al., 2002).

**Why is GFP–E5 compromised in its transforming functions?**

Unlike wt E5, GFP–E5 cannot provide the conditions required for growth in LS, but does provide the full complement of signals required for AI growth. Whether this is due to GFP–E5 activating only a subset of pathways that can be activated by wt E5, or instead is a manifestation of differences in the efficiency, intensity or duration of a common set of signalling mechanisms, awaits elucidation. It is also possible that the observed differences in activity of GFP–E5 and wt E5 might result from subtle differences in the subcellular distribution of the proteins within the endomembrane system. The molecular mechanism(s) underlying the compromised activity of GFP–E5 might then arise as a result of the lack of (or improper) association with crucial cellular targets or with other GFP–E5 monomers as a result of steric hindrance of the relatively large GFP moiety. Whatever the precise details, GFP–E5 cells provide an important model system to facilitate the study of E5-mediated AI growth at the molecular level.

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