Binding of bovine papillomavirus type 4 E8 to ductin (16K proteolipid), down-regulation of gap junction intercellular communication and full cell transformation are independent events

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The E8 open reading frame of bovine papillomavirus type 4 encodes a small hydrophobic polypeptide that contributes to primary cell transformation by conferring to cells the ability to form foci and to grow in low serum and in suspension. Wild-type E8 binds in vitro to ductin, a component of gap junctions, and this binding is accompanied by a loss of gap junction intercellular communication in transformed bovine fibroblasts. However, through the analysis of a panel of E8 mutants, we show here that binding of E8 to ductin is not sufficient for down-regulation of gap junction communication and that there is no absolute correlation between down-regulation of gap junction communication and the transformed phenotype.

The E8 protein of bovine papillomavirus type 4 (BPV-4) is a member of the E5 family of viral transforming proteins. These are short hydrophobic peptides with a C terminus hydrophilic domain. E8 is 42 amino acids long; its N-terminal 30 amino acids are hydrophobic and likely to form a transmembrane domain, while the 12 C-terminal amino acids make up the hydrophilic ‘tail’. E8 is expressed in the bottom layers of the mucous epithelium of early stage papillomas (Anderson et al., 1997) and is localized in the cell membranes (Pennie et al., 1993; Faccini et al., 1996).

E8 contributes to the transformed phenotype of primary cells by inducing focus formation, anchorage-independent growth and growth in low serum (Pennie et al., 1993; O’Brien et al., 1999). The last two phenotypic changes are accompanied by an E8-mediated increase in the amount of cyclin A and activation of the cyclin A–cdk2 complex (O’Brien & Campo, 1998; O’Brien et al., 1999). The presence of E8 also leads to a marked loss of cell–cell communication through gap junctions (GJIC; Faccini et al., 1996). The human papillomavirus (HPV) type 16 and BPV-1 E5 proteins also down-regulate GJIC (Oelze et al., 1995; see below).

Gap junctions are cell-surface structures that allow cell–cell communication via diffusion of small molecules (Simon & Goodenough, 1998). They contribute to the maintenance of homeostasis between cells in a tissue or in a proliferative unit (Kam & Hodgins, 1992). Gap junctions are often not functional in transformed cells and the isolation of transformed cells from their normal neighbours is thought to contribute to the neoplastic process (Budunova et al., 1995). One of the components of gap junctions is ductin (Finbow & Pitts, 1993). BPV-4 E8 and the E5 proteins of BPV-1, HPV-6 and HPV-16 bind to ductin (Goldstein et al., 1991; Conrad et al., 1993; Faccini et al., 1996). This interaction is deemed responsible for the observed down-regulation of gap junctions (Oelze et al., 1995; Faccini et al., 1996).

Ductin is also a component of vacuolar H⁺-ATPase (Finbow et al., 1995), a universal eukaryotic proton pump responsible for the acidification of cytoplasmic organelles (Finbow & Harrison, 1997). The binding between ductin and these viral oncoproteins might also be responsible for the impairment of acidification of endosomes (Straight et al., 1995) and sustained activation of growth factor receptors (Banks & Matlashewski, 1996).

We have reported that mutant forms of E8 can still induce cell growth in suspension but not in low serum (O’Brien et al., 1999), showing that these two parameters of the transformed phenotype can be dissociated. We have now examined the role of GJIC in the transformation process by E8 and find that
interaction between E8 and ductin in vitro is not necessarily accompanied by down-regulation of GJIC in vivo and that there is no absolute correlation between GJIC down-regulation and anchorage-independent growth.

The E8 mutants employed in this study, E8N17S, E8N17Y, E8N17A, E8T(1–32), E8N–E5C and E5N–E8C, have been described before (O’Brien et al., 1999). The first three mutants change residue 17 from asparagine to serine, tyrosine and alanine respectively; the fourth mutant comprises only the N terminus hydrophobic domain of E8 (aa 1–32); the fifth and sixth mutants are chimeras between the N terminus hydrophobic domain of E8 and the hydrophilic C terminus domain of...
BPV-1 E5 and vice versa, respectively. BPV-1 E5 and a mutant form with only the hydrophobic domain, E5T(1–31), were added to the study for comparison.

The coding sequences for all forms of E8 or E5 were tagged at their 5' ends with the sequences encoding the HA-1 epitope of influenza virus and cloned in pZipneo, which also carries the G418-resistance gene (Pennie et al., 1993; Faccini et al., 1996). The pZipneoHAE8 or pZipneoHAE5 plasmids were transfected into foetal primary bovine fibroblasts (PalF) (Pennie et al., 1993; O'Brien et al., 1999) together with pZipneoE7 (expressing the BPV-4 E7 ORF), pJ4Ω16-E6 (containing the HPV-16 E6 ORF; a gift from L. Crawford, Dept of Pathology, University of Cambridge, UK) and pT24 (containing the 6–6k b activated human Ha-ras oncogene). We have recently reported the transformation characteristics of the derived clones and have shown that differences in E8 expression do not correlate with degree of transformation (O'Brien et al., 1999).

Expression of E8 induces marked morphological changes. Compared to control cells (no E8) which grow as monolayers (Fig. 1), E8 cells are disorganized, criss-crossed and piled up. Moreover, these cells are larger and highly vacuolated (Fig. 1, large arrowhead), and capable of growing in the absence of serum mitogens and of substrate. The mutant E8N17S can sustain cellular growth in suspension but not in low serum; the cell morphology is different from that of E8 cells, with shorter, rounder cells, and there appear to be numerous dying cells (Fig. 1). E8N17A maintains full transformation potential and, like E8, induces disorganized growth, cell enlargement and high levels of vacuolization (Fig. 1). E8N17Y, E8T(1–32) and E8N–E5C are non-transforming and the cells look no different from control cells (not shown). The reciprocal chimera E5N–E8C has retained the ability to induce anchorage-independent growth, the culture has a criss-cross appearance and the cells are elongated but much less vacuolated than E8 cells (Fig. 1).

E8-expressing cells display similar characteristics to E8 cells: the cells are very large and highly disorganized, with numerous vacuoles and thin long pseudopods (Fig. 1). Like the C terminus truncation of E8, E5T(1–31) is transformation disabled and cells expressing this mutant display the same morphology of control cells (not shown).

Thus transformation of PalF cells by E8, E8N17A and E5 is accompanied by a very high level of vacuolization and by the formation of long pseudopodal processes. Vacuolization and pseudopods are negligible in cells expressing mutant E8N17S and chimera E5N–E8C, although these cells are still capable of anchorage-independent growth. E5 has been detected in vacuoles of transformed BALB/c cells before (Burkhardt et al., 1989). The nature of the vacuoles or the mechanisms leading to such high frequency of vacuoles and to pseudopod formation are unknown.

The mutant forms of E8 were analysed for their ability to bind to Nephrops norvegicus ductin (Faccini et al., 1996). We have previously shown (Dunlop et al., 1995; Faccini et al., 1996) that ductin is capable of forming complexes with itself and with BPV-1 E5 and BPV-4 E8 in an in vitro translation assay. This binding only occurs if the two proteins are co-translated and in the presence of membranes derived from endoplasmic reticulum (i.e. microsomes). It is also specific as ductin does not bind to other polytopic membrane proteins such as rhodopsin or to BPV-1 E5B (30% identity to BPV-1 E5), and likewise BPV-4 E8 does not bind to the yeast Vma16p, a polytopic membrane protein which has 20% identity to ductin.

When the E8 peptides and ductin are co-translated in the presence of microsomes, they all form detergent-stable com-
Fig. 3. Gap junction intercellular communication (GJIC) in transformed cells. See legend to Fig. 1 for panel labels. In all cases a single cell was injected with Lucifer yellow and the spread of dye to the surrounding cells was monitored over a 2 min period after which the cells were photographed under fluorescent light. The number of fluorescent cells is a measure of GJIC and is indicated in the panels. The corresponding phase-contrast fields are shown in Fig. 1. The left-bottom panel shows phase-
plexes that can be immunoprecipitated with either a polyclonal rabbit antiserum raised against *N. norvegicus* ductin (Fig. 2A) or the monoclonal antibody 12CA5 against the HA-1 epitope (data not shown). No complex is formed when the proteins are translated separately and then mixed together (Fig. 2B). The bands corresponding to the co-immunoprecipitated ductin and E8 proteins were quantified by scanning two independent autoradiographs using Bio-Rad’s Molecular Analyst (1.5) software. In all cases, the binding between ductin and all forms of E8 is very similar: when normalized to the level of interaction between wild-type E8 and ductin, the binding values range between 0·9 for N17S to 1·09 for E8N–E5C. The only mutant we could not test in this system was E5N–E8C, which is not efficiently translated.

This binding of E8 mutants to ductin indicates that the asparagine residue at position 17 of E8 is not critical for interaction. This contrasts with earlier reports (Goldstein et al., 1992; Kulke et al., 1992; Sparkowski et al., 1994, 1996) for BPV-1 E5 in which the homologous residue (Q17) is important both for ductin binding and for the maintenance of the transformation activity. However, as with BPV-1 E5 (Goldstein et al., 1992), a ductin-binding domain is located within the N-terminal hydrophobic core of E8. The C-terminal domain is dispensable in this respect and can be substituted by the tail of E5. The main difference between the C termini of E8 and E5 is the presence in the latter of a C-X-C motif, responsible for E5 dimerization (Horwitz et al., 1988), which is lacking in the former. This difference clearly does not affect the interaction with ductin.

The transformed cells were assayed for their ability to communicate through gap junctions by measuring the spread of the fluorescent dye Lucifer yellow injected into a single cell (Faccini et al., 1996). Control cells not expressing E8 communicate well (Fig. 3) as measured by the spread of the dye to the surrounding cells. Cells expressing wild-type E8 or E8N17A fail to communicate (Fig. 3). In contrast, none of the other E8 mutants, including the chimeras, retain the ability of down-regulating gap junctions (Fig. 3, shown only for N17S), and the spread of the dye is very similar to that of control cells despite their ability to bind ductin in *vivo* (Fig. 2). PaFL cells expressing wild-type E5 fail to communicate (Fig. 3), demonstrating that E5 inhibits GJIC also, while cells expressing E5T display functional gap junctions (not shown).

In the above experiments Lucifer yellow present in the cytoplasm of the injected cell did not enter the vacuoles, suggesting they are impermeable to the dye over the time-course of the experiment (Fig. 3, bottom right). The large size of these vacuoles in E8-transformed cells allowed injection of Lucifer yellow directly into an individual vacuole. The dye spread very rapidly from the injected vacuole to a group of adjacent vacuoles but not to all vacuoles in a cell (Fig. 3, bottom left). Slowly, over a period of a minute or two, the dye spread to the cytoplasm. These observations indicate that vacuoles are connected in groups and that not all the groups are connected to each other. How E8 induces vacuole formation remains to be elucidated and the structures that mediate the connection between vacuoles are not known.

The above results allow the following conclusions: (1) binding to ductin may be necessary but not sufficient for down-regulation of GJIC; (2) the nature of residue 17 in E8 is critical for gap junction functionality but not for ductin binding; (3) the hydrophilic tail is necessary both in E8 and in E5 for effective inhibition of GJIC, and (4) the overall integrity of the protein is required, as neither chimera can down-regulate junctional communication. The differences in the ability of E8 and its mutant versions to down-regulate GJIC do not appear to be due to different protein levels as we have shown that in these cells there is no relationship between level of E8 expression and degree of transformation (O’Brien et al., 1999). There may be other causes for the inability to inhibit GJIC, including lack of or incorrect interaction *in vivo* or differential compartmentalization of E8 mutants. Whatever the reason, it is clear that GJIC down-regulation can be dissociated from growth in suspension. As anchorage-independent growth is one of the strongest indicators of transformation, we conclude that down-regulation of GJIC is not a pre-requisite for cell transformation. However, no mutation of E8 has dissociated GJIC down-regulation from vacuolization and growth in low serum, leaving open the possibility that these phenotypes co-segregate.

Overall, these results indicate that inhibition of GJIC is not necessary for transformation of primary cells, although it may help the establishment of a transformed clone. This may be of particular importance *in vivo*: the expression of E8 in the deep layers of the papilloma would lead to temporary closure of the gap junction channels and isolation of the infected cells from the homeostatic control exerted by their uninfected neighbours. Once a transformed clone is established, E8 expression would be extinguished and the gap junction channels would regain their functionality. This is consistent with observations that the tumour promoter TPA has no effect on GJIC when applied to mouse epidermis (Kam & Pitts, 1988).

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contrast and fluorescent photographs of an E8 cell injected into a single vacuole with Lucifer yellow; the microinjector needle is visible to the left of the picture; the spread of dye to the surrounding vacuoles was monitored over a 2 min period. The right-bottom panel shows phase-contrast and fluorescent photographs of ‘unconnected’ vacuoles, with no Lucifer yellow, showing black against the fluorescent cytoplasm, in an E8 cell. Bottom panels, magnification 800 ×; all other panels, magnification 400 ×.
References


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