E5 transforming proteins of papillomaviruses do not disturb the activity of the vacuolar H\(^+\)-ATPase

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Papillomaviruses contain a gene, E5, that encodes a short hydrophobic polypeptide that has transforming activity. E5 proteins bind to the 16 kDa subunit c (proteolipid) of the eukaryotic vacuolar H\(^+\)-ATPase (V-ATPase) and this binding is thought to disturb the V-ATPase and to be part of transformation. This link has been examined in the yeast Saccharomyces cerevisiae. The E5 proteins from human papillomavirus (HPV) type 16, bovine papillomavirus (BPV) type 1, BPV-4 E5 and various mutants of E5 and the p12\(^+\) polypeptide from human T-lymphotropic virus (HTLV) type I all bound to the S. cerevisiae subunit c (Vma3p) and could be found in vacuolar membranes. However, none affected the activity of the V-ATPase. In contrast, a dominant-negative mutant of Vma3p (E137G) inactivated the enzyme and gave the characteristic VMA phenotype. A hybrid V-ATPase containing a subunit c from Norway lobster also showed no disruption. Sedimentation showed that HPV-16 E5 was not part of the active V-ATPase. It is concluded that the binding of E5 and E5-related proteins to subunit c does not affect V-ATPase activity or function and it is proposed that the binding may be due to a chaperone function of subunit c.

Introduction

The E5 genes of papillomaviruses encode short hydrophobic polypeptides, many of which have been shown to have transforming activity. They vary in length from just over 40 (bovine) to 90 or more (human) amino acids, of which the N-terminal two-thirds are highly hydrophobic and span the membrane bilayer. E5 expression gives rise to anchorage-independent growth and, in some instances, to focus formation. It too has weak transforming properties (Franchini et al., 1993). A similar hydrophobic polypeptide is encoded by the ORF of human T-lymphotropic virus (HTLV) type I and it too has weak transforming properties (Franchini et al., 1993).

Many of the E5 proteins have been found to bind growth factor receptors (Goldstein et al., 1994; Hwang et al., 1995; Petti & DiMaio, 1994). Bovine papillomavirus (BPV) type 1 E5 binds the platelet-derived growth factor receptor, whilst human papillomavirus (HPV) type 16 E5 binds epidermal growth factor receptor and both E5 proteins activate their respective receptors (Petti et al., 1991; Pim et al., 1992; Leechanachai et al., 1992). Likewise, p12\(^+\) binds the interleukin-2 receptor (Mulloy et al., 1996). Expression of E5 proteins in cultured cells often leads to decreased receptor cycling (Martin et al., 1989). So, although there is little sequence similarity across the E5 proteins, it would seem that they have a common mode of action.

Supporting this conclusion is the finding that all E5 proteins so far examined (BPV-1 E5, BPV-4 E5, HPV-6 E5, HPV-16 E5 and HTLV-I p12\(^+\)) bind to the 16 kDa subunit c proteolipid of the vacuolar H\(^+\)-ATPase (V-ATPase) (Goldstein et al., 1991; Conrad et al., 1993; Franchini et al., 1993; Faccini et al., 1996). This multi-subunit enzyme is an essential proton pump of eukaryotes and is responsible for the low pH of many endomembrane compartments including Golgi, secretory vesicles, endosomes and lysosomes (Finbow & Harrison, 1997). The possible disturbance of V-ATPase activity has been suggested to account for the effects on growth factor receptor cycling (Finbow et al., 1991).

Subunit c is also the ‘ductin’ component of a type of gap junction, an intercellular complex that provides sites of cell-to-cell movement for low-molecular-mass solutes (Finbow et al., 1995). Connexins also form gap junction channels, and mutant forms of ductin that have transforming ability disturb connexin

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localization and down-regulate gap junction communication (Saito et al., 1998). Several E5 proteins have also been shown to down-regulate gap junction communication (Oelze et al., 1995; Facchin et al., 1996; Ashrafi et al., 2000). However, a recent mutational study on one E5 protein, BPV-4 E5 (previously designated E8), dissociates this down-regulation from anchorage-independence (Ashrafi et al., 2000).

This suggests that the common target of E5 proteins is the V-ATPase form of subunit c. Consistent with this are the observations that HPV-16 E5 retards the acidification of early endosomes (Strait et al., 1995) and that BPV-1 E5 elevates the pH of the Golgi (Schapiro et al., 2000). Two recent studies (Adam et al., 2000; Briggs et al., 2001) also suggest a substantial impairment of the V-ATPase in Saccharomyces cerevisiae on expression of HPV-16 E5. Expression of BPV-4 E5 also results in large clusters of perinuclear vesicles, often incompletely separated, indicating endomembrane dysfunction (Ashrafi et al., 2000). In this context, a recent study (Peters et al., 2001) has shown that the V₆ domain may act in the later stages of membrane fusion and that subunit c may be part of the transient fusion-pore complex.

We have therefore investigated the effect of high levels of expression of E5 proteins on the V-ATPase. We chose S. cerevisiae for this study, as the yeast form of V-ATPase is similar in composition to higher eukaryotic forms and is not essential for viability under normal growth conditions, whereas loss of V-ATPase in higher eukaryotes is lethal. In contrast to the studies of Adam et al. (2000) and Briggs et al. (2001), we find that, although all E5 transforming proteins examined bind to Vma3p (the S. cerevisiae form of subunit c), they do not impair V-ATPase activity in any measurable way, unlike a dominant-negative form of subunit c, which inactivates the enzyme. At least one of them, HPV-16 E5, becomes dissociated from the active V-ATPase. In a compromised hybrid form of enzyme. At least one of them, HPV-16 E5, becomes dissociated in any measurable way, unlike a compatible ductin (Vma3p) construct was made in two stages. The first stage added the HA1 epitope tag and PstI and XhoI restriction sites to allow directional cloning into pCRScript (Stratagene). The second stage mutated glutamate-137 to glycine and the final product was blunt-end ligated into pPMA and sequenced to check the mutation. The primer sequences were: for HA1–BPV-1 E5; 5'-GGGGATCCGCTCCAGAGGTCTGCGCTG 3' and 3'-GGGGATCCGCCGCCACCATGTACCCATACGATGTTCCAGAGCTGC 5'. For HA1–HTLV-I p12, 5'-GGGGATCCGCCGCCACCATGTACCCATACGATGTTCCAGAGCTGC 3' and 3'-GGGGATCCGCCGCCACCATGTACCCATACGATGTTCCAGAGCTGC 5'.

Methods

**Yeast strains.** The following yeast strains were used: YPH 500 (MATα tru3 lys2 ade2 trp1 his3 leu2) (a gift from Y. Anraku, Dept of Biological Sciences, University of Tokyo, Japan) and W303-1B vat c (MATα tru3 lys2 ade2 trp1 his3 leu2 LEU2::vma3) (a gift from N. Nelson, Dept of Biochemistry, Tel Aviv University, Israel). Cultures were maintained in complete (YPD) or minimal (YNB) medium with appropriate nutrient supplements (Sherman, 1991). Media were buffered to pH 5.5 with 50 mM succinate or to pH 7.5 with MES/MOPS.

**Plasmids.** Plasmid pPMA (Jones et al., 1995) was used for expression of the E5 proteins and Vma3p E137G. The inserted gene is under the control of the plasma membrane H¹-ATPase promoter. pPMA is a 2-µ-based plasmid and contains the selectable marker URA3. A modified form of the pYES2 plasmid (Invitrogen) was used for the expression of the Norwegian lobster subunit c tagged at the C terminus with hexahistidine (Harrison et al., 1994). The URA3 gene had been replaced by the TRP1 gene (Hughes et al., 1996). Expression of the inserted gene in the pYES2 vector is under control of the GAL4 promoter.

**Antibodies.** Monoclonal antibodies (MABS) to the V-ATPase components subunit A (Vma1p) and Vph1p and to the epitopes HA1 and AU1 were purchased from Cambridge Bioscience. The rabbit polyclonal antibody to Norwegian lobster subunit c has been described previously (Leitch & Finbow, 1990).

**Oligonucleotides.** The construction of BPV-4 E5 and variant forms has been described elsewhere (O’Brien et al., 1999; Ashrafi et al., 2000). Coding sequences of BPV-1 E5 (a gift from R. Schlegel, Dept of Pathology, Georgetown University, Washington, DC, USA), HTLV-I p12 (a gift from G. Franchini, NIH, Bethesda, MD, USA) and HPV-16 E5 (a gift from A. Alonso, Deutsche Krebsforschungszentrum, Heidelberg, Germany) were amplified by PCR (PluTurbo; Stratagene) with oligonucleotides incorporating BamHI and KpnI restriction sites and an epitope tag to the HA1 antigen. These allowed ligation of the PCR product directly into the BamHI site of the pPMA vector and determination of orientation by KpnI restriction digestion. The BPV-1 E5 5' oligonucleotide was shorter because the template already contained the HA1 tag.

The primers used were: for HA1–BPV-1 E5; 5'-GGGGATCCGCTCCAGAGGTCTGCGCTG 3' and 3'-GGGGATCCGCCGCCACCATGTACCCATACGATGTTCCAGAGCTGC 5'; for HA1–HTLV-I p12, 5'-GGGGATCCGCCGCCACCATGTACCCATACGATGTTCCAGAGCTGC 3' and 3'-GGGGATCCGCCGCCACCATGTACCCATACGATGTTCCAGAGCTGC 5'; for HA1–HPV-16 E5, 5'-GGGGATCCGCCGCCACCATGTACCCATACGATGTTCCAGAGCTGC 3' and 3'-GGGGATCCGCCGCCACCATGTACCCATACGATGTTCCAGAGCTGC 5'. All cloned PCR products were sequenced and found to be the same as published sequences.

The dominant-negative ductin (Vma3p) construct was made in two stages. The first stage added the HA1 epitope tag and PstI and XhoI restriction sites to allow directional cloning into pCRScript (Stratagene). The second stage mutated glutamate-137 to glycine and the final product was blunt-end ligated into pPMA and sequenced to check the mutation and the integrity of the insert. The successive pairs of oligonucleotides were as follows, with the mutated nucleotide underlined: HA1–Vma3p, 5'-GGGGATCCGCCGCCACCATGTACCCATACGATGTTCCAGAGCTGC 3' and 3'-GGGGATCCGCCGCCACCATGTACCCATACGATGTTCCAGAGCTGC 5'; Vma3p E137G, 5'-GGGGATCCGCCGCCACCATGTACCCATACGATGTTCCAGAGCTGC 3' and 3'-GGGGATCCGCCGCCACCATGTACCCATACGATGTTCCAGAGCTGC 5'; HA1–HTLV-I p12, 5'-GGGGATCCGCCGCCACCATGTACCCATACGATGTTCCAGAGCTGC 3' and 3'-GGGGATCCGCCGCCACCATGTACCCATACGATGTTCCAGAGCTGC 5'.

DNA templates for in vitro translation were synthesized by PCR using oligonucleotides that included the T7 RNA polymerase promoter, to allow T7-mediated transcription, and a ‘Kozak’ sequence to ensure efficient translation of the RNA product. The HA1–T7–KOZAK oligonucleotide primed onto the HA tag sequence, so it was also used for all of the E5 proteins except BPV-1 E5. All products were generated from pPMA constructs, allowing the universal 3' oligonucleotide PMArev to be used, which was also used for sequencing the constructs.

Primer sequences were: Vma3p E137G–T7–KOZAK, 5'-AAATA-TATACCTATATAGGGAGGAGCCACCATGCGATTTGGT-TCCTGTGCT 3'; HA1–BPV-1 E5–T7–KOZAK, 5'-AAATA-TATACCTATATAGGGAGGAGCCACCATGCGATTTGGT-TCCTGTGCT 3'; HA1–T7–KOZAK, 5'-AAATA-TATACCTATATAGGGAGGAGCCACCATGCGATTTGGT-TCCTGTGCT 3'; HA1–T7–KOZAK, 5'-AAATA-TATACCTATATAGGGAGGAGCCACCATGCGATTTGGT-TCCTGTGCT 3';
GGAGACCCACATGTACCCTACGATGTCCAG3' PMArev, 3' GGAACTGAAGGTTGAGCC5'.

■ **Vacuole isolation.** Vacuoles were isolated by a method based on that of Uchida et al. (1985). Yeast cultures (200 ml) were grown in minimal medium overnight at 30 °C to an OD$_{600}$ of 0.5–1. Sugar (glucose or galactose/raffinose) was added to restore the concentration to 2% (w/v). 2 h before the cells were harvested by centrifugation at 5000 g for 5 min at room temperature. The cells were then washed in 20 ml phosphate buffer (50 mM Na$_2$HPO$_4$/NaH$_2$PO$_4$, pH 7.5) and spheroplasts were prepared by re-suspending in 20 ml digest buffer (1 M sorbitol, phosphate buffer, pH 7.5; 2% (w/v) glucose or galactose/raffinose). 0.4% (v/v) β-mercaptoethanol, 0.5 mg/ml yeast lytic enzyme (ICN Biochemicals; 70000 units/ml) were added to the mixture, and the protein concentration was calculated from a standard curve of sodium phosphate.

To the gradient fractions. The reactions were mixed, incubated at 30 °C for 30 min and developed using 100 µl of the double-strength phosphate detection reagent and the absorbance was measured at 630 nm against a standard curve of sodium phosphate. Kinetic analysis was performed in the same way, using 1–2 µg vacuoles per well, as described in Harrison et al. (1994). For determination of N-ethylmaleimide- (NEM; Sigma-Aldrich) sensitive ATPase activity, NEM was used to add 100 µM for 5 min before the addition of ATP.

■ **In vitro synthesis and immunoprecipitation.** In vitro synthesis in the presence of microsomal membranes and immunoprecipitation were carried out as described previously (Dunlop et al., 1995; Faccini et al., 1996; Ashrafi et al., 2000; Rodriguez et al., 2000).

■ **Protein preparation, SDS–PAGE and autoradiography.** Four vols aceton–ethanol (1:1) was added to vacuoles and the mixture was vortexed before being cooled at −20 °C for 30 min. The protein was collected by centrifugation at 20000 g for 30 min and the supernatant was removed before re-suspending the protein in SDS loading buffer [50 mM Tris–HCl, pH 6.8, 5% (w/v) glycerol, 2.5% (w/v) SDS, 0.1% (w/v) sodium azide] and 5 mM ATP and 0.02% (w/v) sodium azide was added to 5–30 µl aliquots from the gradient fractions. The reactions were mixed, incubated at 30°C for 30 min and developed using 100 µl of the double-strength phosphate detection reagent and the absorbance was measured at 630 nm against a standard curve of sodium phosphate. Kinetic analysis was performed in the same way, using 1–2 µg vacuoles per well, as described in Harrison et al. (1994). For determination of N-ethylmaleimide- (NEM; Sigma-Aldrich) sensitive ATPase activity, NEM was used to add 100 µM for 5 min before the addition of ATP.

■ **Measurement of protein content.** Protein content was measured by using the bicinchoninic acid (BCA) method. BCA reagent (Sigma-Aldrich) was prepared fresh according the manufacturer’s instructions. Samples were solubilized in 1% (w/v) SDS and the reaction mixture was incubated at 65 °C for 30 min. Absorbance at 562 nm was measured and the protein concentration was calculated from a standard curve of BSA.

■ **Purification of the V-ATPase by glycerol gradient centrifugation.** This was adapted from the method of Uchida et al. (1985) for a benchtop ultracentrifuge. Vacuoles (1 mg protein) were washed twice in TE (10 mM Tris–HCl, pH 7.5, 1 mM EDTA) by centrifugation at 400000 g$_{max}$ for 10 min at 4 °C. The pellet was re-suspended in the residual buffer, to which was added 50 µl 10% (w/v) N-dodecyl maltoside solution and 5 µl yeast protease inhibitor cocktail (Sigma-Aldrich). The solution was mixed by slow pipetting and then incubated at 4 °C for 15 min to solubilize the membrane proteins. Insoluble material was removed by centrifugation at 400000 g for 10 min at 4 °C and the supernatant was loaded onto 2 ml of a 10-step density gradient (20–50% sucrose). The gradient was incubated for 30 min at 4 °C using minimum acceleration and deceleration. Fractions (200 µl) were collected into a 96-well plate by upward displacement using fluororin (SM).

■ **Measurement of V-ATPase activity.** Reactions contained 200 µl buffer C [plus 0.02% (w/v) sodium azide] and 5 µl vacuoles (10 µg protein). Ethanol (5 µl) (with or without 5 mg/ml N,N-dicyclohexylcarbodiimide; DCCD) was added to the reactions, which were mixed brieﬂy by vortexing and then incubated at room temperature for 5 min. ATP (30 µl; 50 mM in buffer C) was added to all reactions, which were then mixed by vortexing and incubated at 30 °C for 30 min. The reaction was terminated by addition of 750 µl phosphate detection reagent (2% (v/v) sulphuric acid, 1% (w/v) SDS, 0.5% (w/v) ammonium molybdate, 0.1% (w/v) ascorbic acid). The absorbance at 830 nm was measured after 10 min and phosphate concentrations were determined from a standard curve of sodium phosphate.

For the microtitre assay of the purified V-ATPase, 50 µl buffer C containing 5 mM ATP and 0.02% (w/v) sodium azide was added to 5–30 µl aliquots from the gradient fractions. The reactions were mixed, incubated at 30 °C for 30 min and developed using 100 µl of the double-strength phosphate detection reagent and the absorbance was measured at 630 nm against a standard curve of sodium phosphate. Kinetic analysis was performed in the same way, using 1–2 µg vacuoles per well, as described in Harrison et al. (1994). For determination of N-ethylmaleimide- (NEM; Sigma-Aldrich) sensitive ATPase activity, NEM was used to add 100 µM for 5 min before the addition of ATP.

■ **Results**

**Binding of E5 proteins to S. cerevisiae Vma3p**

A previous study (Goldstein et al., 1992b) showed that BPV-1 E5 binds to *Schizosaccharomyces pombe* Vma3p, the subunit c proteolipid of the V-ATPase. As there is high sequence similarity both between these two and to other subunit c proteolipids (e.g. > 85% identity between fungal, human and bovine forms in the transmembrane domains), it seems likely that *S. cerevisiae* Vma3p will bind E5 proteins. We therefore examined the binding of *S. cerevisiae* Vma3p in an *in vitro* binding assay. We have shown previously, using this assay, the specificity of interaction and the requirement for interacting proteins to be located in a membrane environment (Dunlop et al., 1995). For example, BPV-1 E5 does not bind the...
Vma3p-related yeast protein Vma1p and truncated forms of HPV-16 E5 do not bind the Norway lobster subunit c, but full-length HPV-16 E5 does (Faccini et al., 1996; Rodriguez et al., 2000).

Included in this study were the following E5 proteins: BPV-1 E5A, HPV-16 E5, HTLV-I p12', BPV-4 E5 and three mutant forms, N17A, N17S and ΔCterm, in the last of which, the 12 C-terminal residues have been removed. BPV-4 E5 N17A has hypertransforming activity, while BPV-4 E5 ΔCterm has lost transformation ability (O’Brien et al., 1999). All the E5 proteins were tagged at their N termini with the HA1 epitope. Vma3p was tagged with the AU1 epitope at its C terminus. RNAs were translated in the presence of microsomes derived from pancreatic endoplasmic reticulum and pelleted microsomes were used for immunoprecipitation after solubilization.

Immunoprecipitation with MAbs to AU1 brought down Vma3p, as expected, but not any of the E5 proteins (Fig. 1a, b), when the RNAs were translated separately. However, when the RNAs for the E5 proteins were translated together with the RNA for Vma3p, E5 proteins were also precipitated. Varying amounts of the E5 proteins were seen, and we therefore repeated these experiments but precipitating with MAbs to the HA1 epitope (Fig. 1c). As expected, this antibody did not precipitate Vma3p in separate translations but, when co-translated with E5 proteins, Vma3p was present in the immunoprecipitate.

In later expression experiments (see below), we also used Norway lobster subunit c. All E5 proteins used in this study have been shown to bind this form of subunit c (Faccini et al., 1996; Ashrafi et al., 2000; Rodriguez et al., 2000), with the exception of HTLV-I p12'. We therefore carried out a similar experiment using AU1-tagged p12'. Again, immunoprecipitating with anti-AU1 antibodies or with antiserum 650 (a rabbit polyclonal antiserum to Norway lobster subunit c) resulted in both proteins being precipitated (Fig. 1 d).

Expression of E5 transforming proteins in S. cerevisiae

We used the vector pPMA, in which expression of the inserted gene is constitutive, for expression of the E5 transforming proteins. This vector was chosen because a previous study (Jones et al., 1995) had shown that expression was much higher than that from galactose-inducible promoters (e.g. GAL4), to the extent that even deleterious mutant forms of subunit c could be detected on immunoblots of vacuolar membranes. As a positive control to show that we could inactivate the V-ATPase by expression from this vector, we prepared a mutant form of Vma3p in which residue E137 had been changed to glycine (Vma3p E137G). This residue is crucial for proton pumping and we have shown previously that such a mutant can act as a dominant negative (Hughes et al., 1996). As with the viral proteins, Vma3p E137G was tagged with the HA1 epitope, but at its C terminus. The YPH500 haploid strain of S. cerevisiae was used, a strain that has been used by others to examine the V-ATPase (Umemoto et al., 1991).

YPH500 cells were transfected with the various pPMA vector constructs and RT–PCR analysis confirmed the expression of the inserted genes (data not shown). Vacuoles were isolated from transfected cells and examined for the presence of the appropriate protein by probing immunoblots or dot blots with MAbs to the HA1 epitope.

Immunoblots from vacuoles showed the presence of HPV-16 E5 and Vma3p E137G migrating at their expected sizes (Fig.
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2a). There were also aggregated forms of HPV-16 E5, which were not broken down to monomeric species by reducing agents. However, the other E5 proteins could not be detected, even though a number of different membranes and blotting conditions were tried. Presumably, the smaller size of the proteins, notably those derived from BPVs, and their high hydrophobicity makes their detection difficult. We therefore used dot blots, drying the solubilized vacuolar membranes directly on to the support membrane. By this approach, all E5 proteins were found to be present (Fig. 2b).

Measurement of the exponential growth rates of these strains in liquid minimal medium plus appropriate supplements showed no effect. Cells expressing any of the E5 proteins had doubling times similar to that of control cells (2–6 h). However, cells expressing Vma3p E137G had an increased doubling time of 5–6 h, close to the doubling time (6–4 h) of the vat c strain, in which the VMA3 gene has been disrupted.

Effects on V-ATPase-dependent growth

Growth of YPH500 under normal culture conditions (pH 5–5 and low extracellular calcium) is not dependent upon an active V-ATPase. However, at pH 7–5 or in the presence of 100 mM calcium, growth is dependent upon an active V-ATPase. We therefore examined the growth of the transfected YPH500 cells under restrictive growth conditions.

Transformed cells were grown on complete medium plates with glucose as the major nutrient source at pH 5–5 or 7–5 or with 100 mM calcium. As controls, YPH500 and VMA3-knockout cells were transfected with an empty pPMA vector. The VMA3-knockout strain was vat c, derived from W303-1B, used in previous studies (Harrison et al., 1994, 1999, 2000; Jones et al., 1995).

Cells of strains vat c and YPH500 expressing Vma3p E137G failed to grow at pH 7–5, whilst all others transfectedants grew (Fig. 3). There was weak growth of the Vma3p E137G mutant in 100 mM calcium, however. In addition, vat c cells and Vma3p E137G cells formed white colonies, consistent with the loss of V-ATPase activity. Both parental strains are ade2– and therefore give rise to red colonies in the presence of an active V-ATPase (Umemoto et al., 1991). These results showed that none of the E5 proteins, whether transforming or not, appeared to inactivate the V-ATPase and that this was unlikely to be due to poor expression from the pPMA vector. The same results were also obtained if the transfectants were grown on selective minimal medium (lacking uracil) to maintain the pPMA vector (data not shown).

The V-ATPase of S. cerevisiae is also known to be sensitive to the nutrient source. For example, switching from glucose to galactose causes the almost total loss of V-ATPase activity, and activity only recovers to approximately 40% after
galactose adaptation (Parra & Kane, 1998). We therefore examined growth on a galactose-based medium and, as expected, growth was slower. However, none of the E5 proteins affected the viability of the transfectants to grow at either pH 7.5 or in 100 mM calcium. Consistent with the poorer growth rate, the residual growth on glucose seen with Vma3p E137G on 100 mM calcium was suppressed when the cells were grown on galactose.

Effects on V-ATPase activity and ATP hydrolysis

Growth under restricted conditions occurs at low levels of V-ATPase activity or when the enzyme has been partially disabled. For example, replacing Vma3p with the Norway lobster subunit c tagged with a hexahistidine tail at its C terminus still rescues growth at pH 7.5, but elevates the $K_m$ for ATP hydrolysis tenfold relative to the native enzyme (Harrison et al., 1994). We therefore measured the V-ATPase activity in isolated vacuoles and the $K_m$ for ATP hydrolysis.

Vacuoles were isolated from exponentially growing cultures and the ATPase activity was measured in the presence of azide to remove any contamination by mitochondria. V-ATPase activity is inhibited by the fungal macrolide bafilomycin and by NEM and DCCD, in all cases giving a 70% loss of total ATPase activity. Vacuoles isolated from vat c cells have residual ATPase activity. As DCCD is known to react uniquely with the critical glutamic acid on the fourth $\alpha$-helix of subunit c (E137 of Vma3p; Finbow et al., 1992) and this residue has been identified as the site of binding of BPV-1 E5 (Andresson et al., 1995), we measured the DCCD-inhibitable ATPase activity.

The results show that none of the E5 proteins affected the level of V-ATPase activity (Fig. 4). In contrast, V-ATPase activity in the Vma3p E137G mutant was reduced by over 90% and is similar to a level of ATPase activity found in vacuoles isolated from the vat c strain. We also measured the V-ATPase activity in vacuoles isolated from cells expressing the untagged, wild-type form of HPV-16 E5 (data not shown) but, again, there was no impairment of the V-ATPase, indicating that the N-terminal tag does not interfere.

V-ATPase activity is reduced by 60% when the cells are grown with galactose/raffinose medium but, again, there was no quantitative impairment by the E5 proteins. The lack of a quantitative effect on ATPase activity attributable to the V-ATPase was mirrored by the absence of any qualitative effect on the enzyme, as the $K_m$ and $V_{\text{max}}$ for ATP hydrolysis remained unaffected (Fig. 5; $V_{\text{max}}$ data not shown). The V-ATPase activity and the $K_m$ for ATP hydrolysis are similar to values reported for the yeast enzyme (Harrison et al., 1994).

Dissociation of HPV-16 E5 from the V-ATPase

Whilst the E5 proteins are present in the yeast vacuole, it is possible that they do not remain associated with the active V-ATPase. We therefore solubilized the vacuoles in a non-ionic
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Fig. 5. E5 transforming proteins do not affect the $K_m$ for ATP hydrolysis by the V-ATPase. Measurement of the $K_m$ was made by a time-course of ATP hydrolysis at various concentrations of STP, as described in Harrison et al. (1994). Error bars show SEM.

Fig. 6. Glycerol gradient separation of the active V-ATPase from HPV-16 E5. Solubilized vacuolar membranes from YPH500 cells expressing HPV-16 E5 and control cells were centrifuged on glycerol gradients and fractions were collected. V-ATPase activity was measured (with NEM as an inhibitor) and the peak fractions of V-ATPase activity are marked with asterisks. Immunoblots were prepared and probed with antibodies to Vma1p (V1), Vph1p (V0) and HA1, to detect HPV-16 E5. Whilst Vph1p and Vma1p both co-purified with the active V-ATPase, HPV-16 E5 was present in lighter fractions.

detergent and separated the V-ATPase on glycerol gradients by ultracentrifugation. Fractions were collected and the ATPase activity was measured, in this case with NEM as the inhibitor. Immunoblots from the fractions were probed with the anti-HA1 antibody and with MAbs to Vma1p (a V1 component) and Vph1p (a V0 component).

The peak NEM-inhibitable ATPase activity coincided with the presence of V-ATPase subunits (Fig. 6). However, HPV-16 E5 was found in lighter fractions and did not overlap with the V-ATPase.

Effects of E5 proteins on a disabled enzyme in poor nutrient conditions

As mentioned already, Norway lobster subunit c tagged at the C terminus with hexahistidine (N subunit c–his$_c$) rescues growth of vat c cells at pH 7–5 but has a high $K_m$ for ATP. We transfected vat c cells with the pYES2 vector containing the cDNA for N subunit c–his$_c$, which rescued V-ATPase activity to the level reported previously (Harrison et al., 1994). However, transfecting these vat c cells with the empty pPMA vector was sufficient, in some unexplained way, perhaps due to nutrient demands, to reduce V-ATPase activity to a residual level, although there was still growth at pH 7–5. As all E5 proteins examined so far (see above) are known to bind the Norway lobster subunit c, we examined the effects of the E5 proteins in the doubly transfected vat c cells.

As expected, the cultures took longer to grow and, again, whilst Vma3p E137G blocked the rescue of growth at pH 7–5 by N subunit c–his$_c$, none of the E5 proteins did (Fig. 7). Examination of glycerol gradients showed assembled V-
ATPase present in fractions similar to those for the wild-type enzyme, but HPV-16 E5, whilst now having a broader distribution, overlapped little (Fig. 8a). We also probed for the N subunit c–his_{6}, using a polyclonal rabbit antibody and its distribution overlapped with HPV-16 E5 and Vma1p, indicating that there may still be binding between these two polypeptides. We therefore examined possible binding between HPV-16 E5 and N subunit c–his_{6}, using nickel-affinity columns to bind the hexahistidine tails. Eluates of highly stringent washes from these columns indicated the presence in the vacuole of complexes of HPV-16 E5 and N subunit c–his_{6} (Fig. 8b).

Discussion

A common property of all E5 proteins examined so far is their ability to bind the bovine and Norway lobster forms of the V-ATPase 16 kDa proteolipid subunit c. Our study extends this, showing that these E5 proteins also bind the S. cerevisiae form, Vma3p. Given the high conservation of subunit c, particularly in the transmembrane region, and the earlier observation of binding of BPV-1 E5 to S. pombe Vma3p (Goldstein et al., 1992b), this result is not unexpected. Results from other studies indicate that this binding is specific (Faccini et al., 1996; Ashrafi et al., 2000; Rodriguez et al., 2000). For example, there is no detectable binding between C-terminally truncated forms of HPV-16 E5 and subunit c, yet these forms are similar in size and hydrophobicity to BPV-1 E5 and BPV-4 E5 (Rodriguez et al., 2000; Adam et al., 2000).

Given the specificity of binding, it would be expected that the E5 transforming proteins would affect the functions of subunit c complexes such as the V-ATPase. Several studies indicate that this might be the case. For example, HPV-16 E5 appears to delay the acidification of the early endosome (Straight et al., 1995), whilst BPV-1 E5 and BPV-4 E5 both appear to elevate Golgi pH (Schapiro et al., 2000; M. S. Campo and R. Schlegel, unpublished results). Also, HPV-16 E5 and various mutant forms decrease the exponential growth rate of S. cerevisiae at pH 7.5 when present at high levels (Adam et al., 2000), indicating impairment of the V-ATPase.

Our study shows that none of the E5 proteins, whether capable of transformation or not, perturbs V-ATPase activity in any detectable way, even when a compromised subunit c is used under poor growth conditions. In addition, at least one of these proteins, HPV-16 E5, is not part of the active V-ATPase, although it can remain bound to subunit c complexes within the vacuolar membrane. It is not clear why we found no disturbance of the V-ATPase in S. cerevisiae whilst Adam et al. (2000) and Briggs et al. (2001) found that HPV-16 E5 and many mutant forms impaired growth at pH 7.5 and appeared to destabilize the V-ATPase. However, not all mutants of HPV-16 E5 that bind subunit c impaired growth at the restrictive pH, indicating that other factors may have come into play in these two studies.

If E5 proteins bind specifically to subunit c, why do they not remain attached to the active V-ATPase? There are two possibilities. Firstly, Vma12p and Vma22p of S. cerevisiae are not dissimilar to the E5 transforming proteins, being short, highly hydrophobic polypeptides (Graham et al., 1998). They are essential assembly factors of Vh_{1} holoenzyme and are presumably lost during maturation or targeting of the enzyme. A similar situation might also occur for E5 proteins. Secondly, the proposed rotation (Harrison et al., 1997) of the subunit c core driven by ATP hydrolysis past the Vph1p ‘stator’ may physically dislodge E5 proteins. Recent structural data (Páli et al., 1999; Harrison et al., 1999, 2000) on the arrangement of subunit c shows helix 4 to be outermost, facing the bilayer, and this helix forms part of the binding region with BPV-1 E5 and HPV-16 E5 (Goldstein et al., 1992a; Adam et al., 2000), notably the key glutamic acid residue. Thus, E5 transforming proteins are likely to be attached peripherally to V_{0} rather than being integrated within the complex. In addition, recent work on the related F_v subunit c (Rastogi & Girvin, 1999) predicts that much of the BPV-1 E5 binding domain on the V_{0} subunit c may undergo a substantial
conformational change during proton translocation and be transiently re-located within the interior of V$_p$.

A number of recent mutational studies (Adam et al., 2000; Ashrafi et al., 2000; Rodriguez et al., 2000) show that the ability of E5 proteins to bind subunit c does not correlate with their transforming activity. The biological significance of this interaction is therefore unclear. One possibility is a chaperone function. Tagging BPV-1 E5 with an endoplasmic reticulum retention signal results in the inability to transform, indicating that the site of action of this transforming polypeptide lies outside the endoplasmic reticulum (Sparkowski et al., 1995). Binding to subunit c may therefore provide an export mechanism for E5 transforming proteins to the Golgi and post-Golgi compartments. Once in the Golgi, when the V+ATPase is first activated, E5 proteins may become dissociated to bind to other targets, e.g. growth factor receptors.

Another explanation for binding of E5 proteins to subunit c relates to the recent finding that apposing V$_p$ sectors form inter-membrane complexes during late stages of vesicle fusion (Peters et al., 2001), possibly being the ‘fusion pore’ observed a number of years ago in exocytosis (Breckenridge & Almers, 1987; Finbow et al., 1991). Such a possibility might account for the profusion of vesicles in fibroblasts expressing E5 proteins. Coincidentally, these vesicles are ‘coupled’ to one another, as shown by the transfer of the gap junction-permeant tracer dye Lucifer yellow, indicating stalled fusion events.

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References


in Saccharomyces cells expressing the Nephrops 16-kDa proteolipid. European Journal of Biochemistry 221, 111–120.


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