Mutation of chicken anemia virus VP2 differentially affects serine/threonine and tyrosine protein phosphatase activities

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Novel dual-specificity protein phosphatases (DSPs), which catalyse the removal of phosphate from both phosphotyrosine and phosphoserine/phosphothreonine substrates, have recently been identified in two viruses within the family Circoviridae. Viral protein 2 (VP2) of chicken anemia virus (CAV) and ORF2 of TT virus have been shown to possess DSP activity in vitro. CAV VP2 is unusual in possessing two vicinal cysteines within the protein phosphatase signature motif. The first cysteine residue (C95) within the motif has been identified by mutagenesis as the essential catalytic cysteine. In this study, it was shown that virus mutated at this residue displayed a marked inhibition of growth, with titres reduced 10^4-fold, and reduced cytopathogenic effect in cell culture, indicating that viral DSP activity may be significant during infection. As with virus mutated at the first cysteine residue, mutation of the second cysteine (C97) within the motif resulted in a marked reduction in viral growth and attenuation of cytopathogenicity in infected cell cultures. However, mutagenesis of this second cysteine only reduced phosphotyrosine phosphatase activity to 70% of that of wild-type VP2, but increased phosphoserine/phosphothreonine phosphatase activity by as much as 700%. The differential effect of the C97S mutation on VP2 activity does not appear to have parallels in other DSPs and suggests a unique role for the second cysteine in the function of these viral proteins, particularly in vivo.

INTRODUCTION

Novel dual-specificity protein phosphatases (DSPs) have recently been identified in two viruses within the family Circoviridae (Peters et al., 2002): TT virus (TTV), which is detected commonly in adult humans, but is of unknown significance (Takahashi et al., 2000), and chicken anemia virus (CAV), which causes severe immunosuppression in chickens (Noteborn et al., 1991). Reversible protein phosphorylation plays a crucial role in the regulation of cellular processes and the activation of immune effector cells (Chui et al., 1994; Dutz et al., 1995; Kanda & Hirai, 2001; Poovorawan et al., 2001; Safadi et al., 2001; Schievella et al., 1993; Shibayama et al., 2001; Vasconcelos et al., 2001) and thus it is possible that the DSP activity of CAV viral protein 2 (VP2) may contribute to virus-induced immunosuppression and that better understanding of the functions of these proteins may improve our understanding of the pathogenesis of both of these viruses.

The family of DSPs possesses the highly conserved signature motif CXXXXXR. Protein tyrosine phosphatases (PTPases) catalyse the removal of phosphate from phosphotyrosine via a cysteinyl phosphate intermediate formed with the active cysteine in the signature motif. The signature motif ICNCGQFRKH, from residues 94 to 103, has been identified in CAV VP2, and mutation of C95 has been shown to abrogate both PTPase and serine/threonine protein phosphatase (S/TPPase) activities (Peters et al., 2002), confirming that this residue is essential to the catalytic mechanism and that this viral DSP has features analogous to those of well-characterized PTPases.

 Numerous detailed studies of protein phosphatases have identified structural features that contribute to the activation states and substrate specificities of protein phosphatases within the cell (Chiarugi et al., 2001; Li & Dixon, 2000; Schumacher et al., 2002). In general, the structural motifs surrounding the catalytic cleft contribute to the
maintenance of the oxidative state of the catalytic cysteine and provide stabilizing interactions with the substrate within the catalytic pocket. CAV VP2 is unusual in possessing two vicinal cysteines within the signature motif. The objective of this study was to investigate further the role of this enzyme function in virus replication by examining the phenotypes of viruses containing mutations at either C95 or C97 of VP2 and to define the contribution of the second cysteine to the PTPase and S/TPPase activities in vitro.

METHODS

Mutagenesis of C95, C97 or both in CAV VP2. The Australian isolate of CAV, CAU269/7, and a cloned infectious genome of this virus, pCAU269/7, in the plasmid vector pGEX-4Z (Promega), were used in all experiments (GenBank accession no. AF227982) (Brown et al., 2000). The molecular cloning of CAV ORF1 (encoding VP2) (GenBank accession no. AAF34787.1) has been described previously.

Overlap-extension PCR (Ling & Robinson, 1997) has previously been used to introduce the C95S mutation into the CAV VP2 gene sequence (Peters et al., 2002) and similar methods were used to change the C97 residue to a serine. The sequences of oligonucleotide pairs synthesized to incorporate nucleotide substitutions encoding the amino acid alterations (shown in bold) were as follows: C95S positive-sense (5'-CGTTGCTGACATCGTCTCCGTGTTGTCAAGATC-3') and negative-sense (5'-CGCGGACAATTC-3'); C97S positive-sense, (5'-ATCTGCACACGGAGAATTC-3') and negative-sense (5'-ATTGTTCCGCTGTTGCTGTTGCTGTTGTCAAGATC-3'); and C/95/97S positive-sense (5'-CGTTGCTGACATCGTCTCCGTGTTGTCAAATTC-3') and negative-sense (5'-ATTGTTCCGCTGTTGCTGTTGCTGTTGTCAAGATC-3').

Protein expression and purification. Mutated VP2 proteins were produced as a C-terminal fusion with glutathione S-transferase (GST) and the fusion proteins were expressed in Escherichia coli (Promega) as described previously (Peters et al., 2002).

Purified proteins were separated by SDS-PAGE in 12.5 % polyacrylamide gels and stained with Coomassie brilliant blue (Cleveland et al., 1977). Proteins were then electrophoresed to a PVDF membrane (Immobilon; Millipore). A Western blot of GST–VP2, GST–VP2.C95S (Peters et al., 2002), GST–VP2.C97S (Peters et al., 2002), GST–VP2.C/C95/97S/S and PPase-2B without substrate. Reactions were incubated with shaking at room temperature for 10 min and terminated by the addition of malachite green reagent. All reactions were performed in triplicate and assays were repeated on at least three occasions and the mean activity determined. Activity of the GST–VP2 fusion proteins was adjusted by a factor of 0.52 to account for the contribution to mass of the 24 kDa GST fusion tag and expressed as nmol catalysed substrate (μg protein)−1.

The release of free phosphate into solution was detected by the malachite green colorimetric assay (Geladopoulos et al., 1991). Stock malachite green solution was made by the slow addition of 60 ml concentrated sulphuric acid to 300 ml water, followed by cooling to room temperature, and 0.44 g malachite green (Fisher Scientific) was then added. Immediately before use, the colorimetric reagent was prepared from 10 ml stock malachite green, 3 % (w/v) (NH₄)₂MoO₄ (Sigma-Aldrich) and 0.15 % Tween 20 (Sigma-Aldrich). A 50 μl aliquot of the reagent was added to each of the 200 μl reactions and allowed to equilibrate for 20 min at room temperature. Acidic (pH 1) was read and phosphate release was calibrated against a standard phosphate curve, which was prepared in triplicate for each assay with phosphate at 0, 2, 5, 5, 7, 5, 10, 15, 20, 25, 30, 40 and 45 nmol in 200 μl volumes of a 1:1 ratio of AB and TB.

The kinetics of PTPhase activity of GST, GST–VP2.C97S and GST–VP2.C/C95/97S/S were determined by performing assays with 0, 2.5, 5, 7.5, 10, 15, 20, 25, 30 and 40 nmol substrate. Reactions were incubated for 1 min, with all other reaction conditions as described above. For each substrate concentration, activity was measured in at least six replicate reactions and the SEM activity was calculated for each concentration. Vmax and Km estimates were derived by linear regression analysis from a double-reciprocal plot and the SEM and P values were calculated for the constant 1/Vmax and the coefficient Kcat/Vmax.

S/TPPase assays. S/TPPase activity was assayed by using the Serine/Threonine protein phosphatase assay system (Promega) according to the manufacturer’s instructions. Assays were performed in 50 μl volumes in a microtitre plate using the general S/TPPase substrate RRA(pT)VA and an assay buffer containing 50 mM imidazole (pH 7.2; Promega), 0.2 mM EDTA (Sigma), 10 mM MgCl₂ (Sigma), 1 mM NiCl₂ (Sigma), 50 μg calmodulin (Promega) ml⁻¹ and 0.02 % 2-mercaptoethanol (Sigma). Reactions were started by the addition of 1 U protein phosphatase-2B (PPase-2B; Promega) or 5 μg GST, GST–VP2, GST–VP2.C97S, GST–VP2.C/C95/97S/S and PPase-2B without substrate, and assay buffer with neither enzyme nor substrate. Reactions were performed with RRA(pT)VA substrate concentrations of 0, 50, 100, 200, 500, 1000 or 2000 μM and incubated for 15 min at room temperature. Free phosphate was detected colorimetrically by using the malachite green system, as described for the PTPhase assays.

RRAP(T)VA substrate concentrations of 0, 10, 50, 70, 100, 200 or 400 μM. Reactions were incubated for 1 min, with all other reaction conditions as described above. V_max and K_m estimates were derived by linear regression analysis from a double-reciprocal plot.

**Engineering of mutated genomes.** PCR products containing the C95S and C97S mutated sequences were subcloned into the infectious cloned CAV genome in the plasmid vector pGEX-4Z (pCAU269/7). Plasmid pCAU269/7 was digested with Stsl and BsmII to remove a region of 357 bp from the VP2-coding sequence and a band of approximately 4-7 kbp, corresponding to the remainder of pCAU269/7, was purified from a 1 % agarose gel and ligated to similarly digested, mutated PCR products. The E. coli strain DH5α was electrotransformed with the ligated plasmid and grown at 37 °C on Luria–Bertani agar containing 50 μg ampicillin ml⁻¹ (Itoh et al., 2001). Plasmid was purified from selected clones with a Qiagen Midi kit according to the manufacturer’s instructions. Clones were screened for the presence of insert by PCR using the forward primer CAV.5 (5’-GGCGGCGCAGGGGCAA-3’) and reverse primer CAV.4 (5’-CTATCGAATTCGAGTGGTTACTAT-3’). The cloned DNA was sequenced by using a Taq DyeDeoxy Terminator cycle sequencing kit (Perkin Elmer) using primers CAV.2 (5’-GGCGGCGCAGGGGCAA-3’) and CAV.10 (5’-TGCTCACGTTGATGACAGTTC-3’).

**Transfection of MDCC-MSB1 cells with mutated viral genomes.** Transfection of MDCC-MSB1 cells with viral genome was used to generate mutant virus from genomic DNA that had been manipulated in vitro (Phenix et al., 1994). MDCC-MSB1 cells were grown at 37 °C in 5 % CO₂ as a suspension culture in RPMI 1640 (Sigma-Aldrich) supplemented with 2 mM glutamine (Sigma-Aldrich), 2 mM pyruvate (Sigma-Aldrich), 0.2 % NaHCO₃, 50 μg ampicillin ml⁻¹, 50 μg gentamicin (Sigma-Aldrich) ml⁻¹ and 10 % heat-inactivated fetal calf serum (FCS; CSL). The clone pCAU269/7, plasmid pEGFP-C2 (BD Biosciences Clontech) and the cloned viral constructs mut C95S and mut C97S were transfected into MDCC-MSB1 cells. Plasmid DNA for transfection was prepared by using a Qiagen Midi kit according to the manufacturer’s instructions. Purified CAV DNA was resuspended in sterile 10 mM Tris (pH 8.0).

Transfection was performed by using an adaptation of a previously described method (Noteborn et al., 1991). MDCC-MSB1 cells were passaged into fresh medium 24 h prior to transfection to synchronize growth. Cells were washed twice in FCS-free RPMI 1640, resuspended at a final concentration of 10⁶ cells ml⁻¹ and 700 μl was added to 10 μg DNA on ice. Transfection was achieved in a 0.4 cm gap-elec-troporation cuvette in a Gene Pulser apparatus (Bio-Rad) with a pulse delivered at 400 V, 900 μF, infinite resistance and extension capacitance. The time constant was typically around 3–6–4.5 ms. Cells were incubated at room temperature for 5 min and then resuspended in 5 ml warm growth medium. Transfection of pEGFP-C2, containing enhanced green fluorescent protein (EGFP) 3’ to the human cytoskeletal protein virus VP2 3’ to the human cyto-genealogivirus immediate-early promoter, was used to assess the efficiency of transfection after 48 h incubation by determining the proportion of fluorescent cells.

**Mutant virus propagation.** Transfected cell cultures were serially passaged at a 1:10 dilution at 48 h intervals for 10 passages. Viral growth was assessed by examining the proportion of cells expressing CAV VP3 using an immunofluorescence assay (IFA) adapted from a previously published method (McNulty et al., 1988). Cells were pelleted by centrifugation at 6000 g for 5 min, washed twice in PBS to remove residual medium and resuspended in 200 μl PBS. The cell suspension was applied to a multi-well slide and dried at room temperature. Cells were fixed in ice-cold 90 % methanol for 5 min and washed briefly with 0.1 % Tween 20 (Sigma-Aldrich) in PBS containing 0.1 % BSA (0.1 % BSA/PBS). The preparation was blocked for 1 h with a solution of 5 % BSA/PBST at 37 °C in a humidified chamber. Slides were washed with 0.1 % BSA/PBST and then incubated at 37 °C in a humidified chamber with anti-VP3 mouse monoclonal antibody (mAb VP3; TropBio) diluted 1:200 in 0.1 % BSA/PBST. Slides were washed with 0.1 % BSA/PBST and then incubated for 1 h in anti-mouse sheep antibody conjugated to fluorescein iso-thiocyanate (Dako) diluted 1:100 in 0.1 % BSA/PBST. Slides were mounted in Vectashield mounting medium (Vector Laboratories) and examined by using a fluorescence microscope. The proportion of fluorescent cells was determined by reference to background fluorescence in uninfected MDCC-MSB1 cells.

The presence of infectious mutant virus was further demonstrated by reinfection of cell culture with cell-free preparations of virus. The culture was frozen and thawed three times and then clarified by centrifugation at 6000 g for 10 min. MDCC-MSB1 cells were infected with the cell-free virus preparation. Preparation of virus by this method and reinfection of cultures were repeated for at least three viral passages in each case.

Mutant viruses were titrated in microtitre plates in 200 μl volumes containing 5 × 10⁵ MDCC-MSB1 cells ml⁻¹, using an adaptation of a previously published method (Goryo et al., 1987). Six duplicate serial 10-fold dilutions of virus stocks were prepared, ranging from a final dilution of 0.05 to 0.5 × 10⁻¹⁰ and inoculated onto cells. At intervals of 48 h, infected cells were serially passed into fresh medium at a dilution of 1:4. Each well was examined for cytopathogenic effect (CPE), characterized by enlarged swollen cells, nuclear vacuolation and chromatin assemblies and cell fragmentation (McNulty, 1991), and cultures were serially passed until no difference was detected between successive passages at the end point (the lowest dilution at which CPE was observed). The observation of CPE at the end-point dilution was confirmed by IFA with mAb VP3.

**RESULTS**

**Expression and purification of mutant CAV VP2 fusion proteins**

Mutations were introduced successfully into the CAV VP2 sequence by overlap-extension PCR and the mutated sequences were expressed in the pGEX expression vector in E. coli. Fusion proteins were purified by affinity chromatography and bands of 58 kDa, corresponding to the CAV GST–VP2 fusion proteins, were identified by SDS-PAGE in the affinity-purified eluate (Fig. 1). The protein bands reacted specifically with antiserum raised against GST and also with pooled serum from chickens seropositive for CAV. Dialysed GST–VP2 was used directly in PTPase assays.

**Mutation C97S in CAV GST–VP2 changes the kinetics of PTPase and S/TPPase activities**

The GST–VP2 fusion protein containing the mutation C97S was investigated for PTPase and S/TPPase activity. The mutated fusion protein was found to have a PTPase steady-state activity that was 70 % that of the wild-type protein in a reaction with 9 μg enzyme and 10 nmol

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END(pY)INASL substrate (Table 1). In contrast, GST–VP2 containing the mutation C97S was found to have an S/TPPase steady-state activity that was 700 % of that of the wild-type protein in a reaction with 5 mM enzyme and 200 µM RRA(pT)VA substrate (Table 1).

GST–VP2 has been shown previously to have PTPase activity with an estimated \( V_{\text{max}} \) of 14 925 U mg\(^{-1}\) min\(^{-1}\) and \( K_{\text{m}} \) of 18 88 µM and S/TPPase activity with an estimated \( V_{\text{max}} \) of 28 600 U mg\(^{-1}\) min\(^{-1}\) and \( K_{\text{m}} \) of 76 µM (Peters et al., 2002). \( V_{\text{0}} \) for PTPase activity of CAV GST–VP2.C97S was measured by using 1 min reactions. From the Lineweaver–Burke plot, 1/\( V_{\text{max}} \) was found by linear regression to be 0.096 ± 0.01 (\( P < 0.001 \)) and \( K_{\text{m}}/V_{\text{max}} \) was found to be 0.634 ± 0.09 (\( P < 0.01 \)). Based on these results, \( V_{\text{max}} \) was estimated to be 10 417 U mg\(^{-1}\) min\(^{-1}\) and \( K_{\text{m}} \) to be 6.6 µM. \( V_{\text{0}} \) for S/TPPase activity of CAV GST–VP2.C97S was also measured over 1 min reactions. From the Lineweaver–Burke plot, 1/\( V_{\text{max}} \) was found by linear regression to be 0.001 ± 0.0005 (\( P = 0.1 \)) and \( K_{\text{m}}/V_{\text{max}} \) was found to be 0.839 ± 0.014 (\( P < 0.0001 \)). Based on these results, \( V_{\text{max}} \) was estimated to be 200 000 U mg\(^{-1}\) min\(^{-1}\) and \( K_{\text{m}} \) to be 839 µM (Table 2).

**Mutation C/C95/97S/S in CAV GST–VP2 abrogates PTPase and S/TPPase activity**

Our previous study showed that the C95S mutation abrogated both PTPase and S/TPPase activities (Peters et al., 2002).

**Table 1.** Protein phosphatase activity of mutated VP2 fusion proteins

<table>
<thead>
<tr>
<th>Protein fusion protein</th>
<th>Steady-state activity (%)†</th>
<th>END(pY)INASL §</th>
<th>RRA(pT)VA §</th>
</tr>
</thead>
<tbody>
<tr>
<td>GST–VP2</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>GST–VP2.C95S</td>
<td>0</td>
<td>0***</td>
<td></td>
</tr>
<tr>
<td>GST–VP2.C/C95/97S/S</td>
<td>0***</td>
<td>0**</td>
<td></td>
</tr>
<tr>
<td>GST–VP2.C97S</td>
<td>72***</td>
<td>700***</td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GST</td>
<td>0***</td>
<td>0***</td>
<td></td>
</tr>
<tr>
<td>PPase-2B</td>
<td>–</td>
<td>103**</td>
<td></td>
</tr>
<tr>
<td>TC-PTPase</td>
<td>97*</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>No enzyme, no substrate</td>
<td>0**</td>
<td>0***</td>
<td></td>
</tr>
<tr>
<td>GST–VP2, no substrate</td>
<td>0***</td>
<td>0***</td>
<td></td>
</tr>
<tr>
<td>GST–VP2.C95S, no substrate</td>
<td>0***</td>
<td>0**</td>
<td></td>
</tr>
<tr>
<td>GST–VP2.C/C95/97S/S, no substrate</td>
<td>0**</td>
<td>0**</td>
<td></td>
</tr>
<tr>
<td>GST–VP2.C97S, no substrate</td>
<td>0***</td>
<td>0***</td>
<td></td>
</tr>
<tr>
<td>GST, no substrate</td>
<td>0**</td>
<td>0**</td>
<td></td>
</tr>
</tbody>
</table>

* \( P < 0.05 \); ** \( P < 0.01 \); *** \( P < 0.001 \). Values of \( P < 0.05 \) were considered to be significantly different from the activity obtained with GST–VP2.

† Activity as a percentage of GST–VP2 activity.

§ General PTPase substrate.

§ General S/TPPase substrate.

Table 1. Protein phosphatase activity of mutated VP2 fusion proteins

<table>
<thead>
<tr>
<th>Protein fusion protein</th>
<th>( V_{\text{max}} ) (U mg(^{-1}) min(^{-1}))</th>
<th>( K_{\text{m}} ) (µM)</th>
<th>( V_{\text{max}} ) (U mg(^{-1}) min(^{-1}))</th>
<th>( K_{\text{m}} ) (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type GST–VP2</td>
<td>14 925</td>
<td>18 88</td>
<td>28 600</td>
<td>76</td>
</tr>
<tr>
<td>GST–VP2.C97S</td>
<td>10 417</td>
<td>6 6</td>
<td>200 000</td>
<td>839</td>
</tr>
</tbody>
</table>

**Table 2.** \( V_{\text{max}} \) and \( K_{\text{m}} \) values for PTPase and S/TPPase activity of wild-type CAV GST–VP2 and GST–VP2.C97S

**Fig. 1.** Cloning of CAV VP2 wild-type and mutated genes into the pGEX-4T-2 expression vector. Affinity-purified CAV wild-type GST–VP2 (a) and CAV GST–VP2.C95S, CAV GST–VP2.C97S and CAV GST–VP2.C/C95/97S/S (b) were separated by 12.5 % SDS-PAGE and stained with Coomassie brilliant blue. Pre-stained broad-range protein markers (New England Biolabs) were electrophoresed next to purified fusion proteins.
et al., 2002). GST–VP2.C/C95/97S/S was assayed for PTPase and S/TPPase activity. No PTPase activity was detected in reactions containing 9 µg protein and 200 µM END(pY)INASL substrate, nor was S/TPPase activity detected in reactions containing 5 µg protein with 200 µM RRA(pT)VA substrate (Table 1).

**Viruses with the VP2 mutations C95S or C97S are replication-competent, but grow to low titre**

Viruses with mutant genotypes were recovered by transfection of MDCC-MSB1 cells with mutated genomic constructs. Transfection with the wild-type CAV genome in pCAU269/7 resulted in an initial phase of transient expression of CAV VP3, as observed by immunofluorescence. Four to five serial passages were required at 10-fold dilutions before an exponential increase in cells expressing VP3 was seen, suggesting active virus replication and infection, rather than simply maintenance of transfected DNA constructs. CAV VP2.C95S and CAV VP2.C97S mutant genome constructs were found to be infectious and to be able to replicate in vitro, when assessed in parallel with pCAU269/7- and mock-transfected cells. Mutant virus was harvested from the earliest passage at which an exponential increase in VP3 expression was observed. For each construct, cell-free virus was prepared by lysis and clarification of the transfected culture and used to reinfect fresh cultures. Infection with the mutant viruses was confirmed by Western blotting to detect CAV VP3, by Southern blotting using a CAV-specific probe and by CAV-specific PCR. CAV PCR was performed on extracted DNA after digestion with DpnI to remove any residual transfected DNA that might otherwise have served as a target.

Although replication-competent virus was generated from both mutant genomes, the mutated viruses produced maximal titres of only $10^{5.5–7}$ TCID$_{50}$ ml$^{-1}$, despite repeated attempts to optimise culture conditions (Table 3).

Virus-induced CPE was investigated by using phase-contrast microscopy and immunofluorescent staining of fixed cells. CPE was identified readily within 24–72 h after inoculation of MDCC-MSB1 cells with wild-type CAU269/7 virus (Fig. 3). Viruses containing the C95S and C97S mutations in VP2 induced only mild CPE. Mild cellular swelling, mild cytoplasmic vacuolation and mild nuclear pyknosis were evident. MDCC-MSB1 cells infected with each of the mutant viruses were examined by immunostaining and fluorescence microscopy. Nuclei of cells infected with these mutant viruses stained uniformly with Hoechst stain, as in uninfected cells, and there was no evidence of nuclear fragmentation or chromatin clumping. VP3 immunofluorescent staining identified intensely stained, crescent-shaped aggregates of VP3 in the cytoplasm.

**DISCUSSION**

Our previous studies have shown that the first of the two cysteines in the catalytic motif is the essential catalytic cysteine for both PTPase and S/TPPase activities of the VP2 protein of the circovirus CAV. The circovirus protein phosphatases are unusual in possessing two vicinal cysteines within the catalytic cleft. In the current study, mutagenesis of both cysteine residues (C95 and C97) to serines eliminated both PTPase and S/TPPase activities. However, mutagenesis of C97S alone differentially affected the protein phosphatase activity for phosphoserine/phosphothreonine as opposed to phosphotyrosine substrates, slightly decreasing PTPase activity, but greatly increasing S/TPPase activity. The partial reduction in PTPase activity and the enhanced S/TPPase activity suggested that this residue is important in the catalytic mechanism of the enzyme. Such a differential effect of specific mutagenesis on S/TPPase and PTPase activities of a DSP has not been reported previously.

A second viral DSP, human vaccinia H1-related phosphatase (VHR), like CAV VP2, shows greater activity for phosphotyrosine than for phosphothreonine substrates (Schumacher et al., 2002). The catalytic cysteine in VHR is C124 and simultaneous mutagenesis of both residues, E126A and Y128I, has been shown to increase the activity of VHR for phosphothreonine ninefold (Schumacher et al., 2002). Structural studies of VHR suggest that the reason for the selectivity of VHR for phosphotyrosine is that the relatively bulky tyrosine and glutamate residues lie at the lip of the catalytic cleft and reduce the width of the entrance into the cleft, reducing the access of phosphotyrosine. The substitutions would be expected to increase the width of the entrance to the catalytic cleft, as the residues blocking access are replaced with the smaller residues found in these positions in most DSPs (Schumacher et al., 2002). This appears to be analogous to the effect of the C97S mutation in VP2, as C97 is in a position homologous to that of E126 in VHR. In Fig. 2, the effect of the substitution is shown on the width of the entrance to the cleft. The VP2 structure has been modelled by the threading of the VP2 sequence on to the solved crystallographic structure of muPTPase (unpublished data). In this model, the catalytic fold is embedded within a deep cleft formed by five β-strands and three α-helices. The cleft is narrowest between the side chains of residues C97 to R89 and is only 3·04 Å (0·304 nm) at this point. The distance is increased to 8·39 Å (0·839 nm) when the substitution C97S is made. The wider entrance to the deep pocket of the cleft may accommodate bulkier phosphoamino acid side-chain moieties. The differential effect of the C97S mutation on activity in CAV VP2 implies...
that the mechanism of catalysis for phosphoserine/ phosphothreonine may differ from that for phosphotyrosine, even though the C95S mutation showed that the same cysteine residue is essential for both activities.

VP2 PTPase and S/TPPase activities have been demonstrated in vitro, but physiological activity in vivo has not been established, nor has a cellular substrate been identified (Peters et al., 2002). The effects of the C95S and C97S VP2 mutations on viral function were therefore of considerable interest to the investigation of in vivo activity. The identification of the C95 and C97 residues as critical to protein phosphatase activity and the concurrent altered phenotypes observed for viruses mutated at these positions indicates that viral protein phosphatase activity is of physiological significance in vivo. The viruses mut C95S and mut C97S grew in cell culture, but were only able to grow to low titres and had a markedly reduced CPE. This suggests that lack of phosphatase activity may disrupt viral functions that are not essential, but are required for efficient replication.
It was notable that both the C95S and C97S mutations had similar effects on virus replication, even though only the C95S mutation was able to eliminate PTPase and S/TPPase activities in vitro. It might be expected that the increased S/TPPase activity induced by the C97S mutation would enhance virus replication if this activity was significant in vivo. This apparently incongruous observation might be explained by recent studies on the eukaryotic cellular low-molecular-mass PTPase (LMM-PTPase) (Chiarugi et al., 2001). LMM-PTPase is unusual within the PTPase family in possessing vicinal cysteines within the catalytic pocket at residue positions 12 and 17 (Chiarugi et al., 2001). Within the cell, the catalytic C12 is activated by reduction to a sulphenic derivative by the glutaredoxin/glutathione/glutathione reductase/NADPH system. The C17 residue forms a reversible disulphide bond with C12 under oxidizing conditions within the cell, protecting the catalytic cysteine from further oxidation to the second and third oxidized states, sulphinic and sulphonic acid, and thus irreversible loss of enzyme activity. In the oxidized state, the enzyme is inactive, but is in a state conducive to rapid activation by reduction by the glutaredoxin/glutathione/glutathione reductase/NADPH system. A C17A mutation in LMM-PTPase was found only to reduce activity to 70% in vitro, but to result in severe impairment of the capacity of the enzyme to recover function in vivo after oxidative stress. The impairment of in vitro PTPase activity of VP2 by the C97S mutation was of a similar magnitude, and it is possible that the significant effect of the VP2 C97S mutation on virus replication might result from impaired protection of the PTPase activity from oxidative stress. The vicinal cysteines in LMM-PTPase facilitate redox regulation of activity in the cell, with inactivation occurring in times of oxidative stress, but activity being regenerated rapidly under reducing conditions. Thus, the viral protein phosphatase activity could similarly be under reversible redox regulation, which may be associated intimately with the host-cell activation state.

An alternative explanation for the unexpected effect of the C97S mutation in vivo might be that it plays a role in dephosphorylation of specific substrates and that this activity is not reflected in in vitro studies using generalized phosphopeptide substrates. Differentiation of these two possible roles of the C97 residue will require identification of the substrate for VP2 in infected cells.

In conclusion, this study demonstrated a differential role for the two cysteines adjacent to the catalytic site of the CAV DSP. Only one of the cysteines, C95, was critical for enzymic function in vitro, and mutagenesis of C97 was shown to increase activity significantly for a phosphothreo- nine substrate. However, both residues were shown to be equally significant for the function of VP2 in virus replication, suggesting further roles for the C97 residue aside from its effect on S/TPPase activity. The differential effects of the two mutations introduced into the virus in this study will help in further studies to identify the cellular substrates for the viral phosphatase and its effects on signalling pathways in infected cells and will enhance our understanding of the molecular pathogenesis of CAV and TTV infection.

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