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A protein phosphatase related to the vaccinia virus VH1 is encoded in the genomes of several orthopoxviruses and a baculovirus

(phosphorylation/tyrosine/serine/raccoonpox virus)

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ABSTRACT **The vaccinia virus VH1 gene product is a dual specificity protein phosphatase with activity against both phosphoserine- and phosphotyrosine-containing substrates. We investigated the potential presence of VH1 analogs in other viruses. Hybridization and sequence data indicated that a phosphatase related to the VH1 phosphatase is highly conserved in the genomes of smallpox variola virus and other orthopoxviruses. The open reading frames from the raccoonpox virus and the smallpox variola virus Bangladesh major strain genomes encoding the VH1 analogs were sequenced and found to be highly conserved with the vaccinia virus VH1. An open reading frame from the baculovirus *Autographa californica* has sequence similarity to the VH1 phosphatase. The viral proteins appear to be structurally related to the cell cycle control protein $p80^{cdc25}$. A recombinant phosphatase expressed from the baculovirus gene was found to share with the VH1 phosphatase the ability to hydrolyze substrates that contained both phosphoserine and phosphotyrosine.**

Tyrosine phosphorylation is tightly regulated by the balancing actions of protein-tyrosine kinases and protein-tyrosine-phosphatases (PTPases; protein-tyrosine-phosphate phosphohydrolase, EC 3.1.3.48) (1). The PTPases catalyze phosphate hydrolysis of phosphotyrosine and will not hydrolyze either phosphoserine- or phosphothreonine-containing substrates (2). Protein tyrosine phosphorylation plays a crucial role in regulation of the eukaryotic cell cycle; alterations in tyrosine phosphate levels can lead to a loss of control of cell growth (3, 4).

Bacteria are generally known to have very few tyrosine-phosphorylated proteins. Recently, however, a *Yersinia* species, which is the causative agent of the plague, was reported to encode and express a PTPase (5). Deletional analysis indicated that the *Yersinia* PTPase was necessary for virulence (6), suggesting that the PTPase functions by dephosphorylating tyrosine residues on host proteins critical for maintaining normal cellular function. Certain viruses also encode analogs of eukaryotic protein phosphatases (7). The VH1 gene of vaccinia virus (*Hin*dIII-H, first open reading frame of strain WR) has been shown to encode a phosphatase that has considerable amino acid identity with $p80^{cdc25}$, a eukaryotic protein phosphatase important for regulation of the cell cycle (8), implying structural similarity between VH1 and $p80^{cdc25}$. Site-directed mutagenesis of the cysteine residue in the putative catalytic domain of either protein results in a total loss of detectable phosphatase activity (7). The mutant $p80^{cdc25}$ also lacked biological function in an *in vivo* assay system (9). The most distinguishing feature of the 171-amino acid VH1 phosphatase was that it hydrolyzed both phosphoserine- and phosphotyrosine-containing proteins (7). Both substrates seem to be hydrolyzed by a common mechanism since mutagenesis of a critical cysteine at the active site of the phosphatase leads to a loss in hydrolytic activity toward both substrates.

Because *Yersinia* and vaccinia virus infections radically affect the host cell cycle, we speculated that phosphatases play a role in pathogenesis (5, 7). To begin exploring this concept, we report here the identification and partial characterization of other phosphatase genes in orthopoxviruses other than vaccinia virus, including smallpox variola virus, and in the baculovirus *Autographa californica*. We determined analog VH1 sequences in the raccoonpox virus, which is generally attenuated compared to vaccinia virus in smallpox vaccine relative virulence tests (10).§ Furthermore, all of the VH1-like phosphatase proteins we examined appear to have conserved active sites based on our initial characterizations and the catalytic properties of this group of viral phosphatases.

MATERIALS AND METHODS

Materials. Sequenase was purchased from United States Biochemical, casein was from Fluka, [γ-^{32}P]ATP (7000 Ci/mmol; 1 Ci = 37 GBq) was from New England Nuclear, src kinase was from Oncogene Sciences (Mineola, NY), and the random primer labeling kit was from Boehringer Mannheim. The catalytic subunit of protein kinase A was a gift from Michael Uhler (Department of Biological Chemistry, University of Michigan). *A. californica* genomic DNA was a gift from Richard Jove (Department of Microbiology, University of Michigan).

Southern Blot Analysis. Genomic DNA from the following orthopoxviruses was digested with *Hin*dIII, subjected to electrophoresis, and bidirectionally transferred to nitrocellulose as described (11): variola virus (Bangladesh major strain and Garcia South American minor strain), vaccinia strain NYBH virus, monkeypox virus (Copenhagen strain), cowpox virus (Brighton strain), ectromelia virus, raccoonpox virus, volepox virus, and skunkpox virus (11, 12). The blot was probed with a randomly primed ^{32}P-labeled fragment containing the coding sequence of the vaccinia VH1. The final wash of the filter was highly stringent—1× standard saline citrate at 65°C.

Sequencing of Raccoon VH1. The *Hin*dIII-E fragment (≈15 kb) of raccoonpox virus genome DNA cloned into pUC18 (13) hybridized with a probe containing the coding region of the vaccinia VH1 gene (Fig. 1). The fragment designated

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Abbreviations: PTPase, protein-tyrosine-phosphatase; pNPP, *p*-nitrophenyl phosphate; GST, glutathione *S*-transferase.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. L13165).

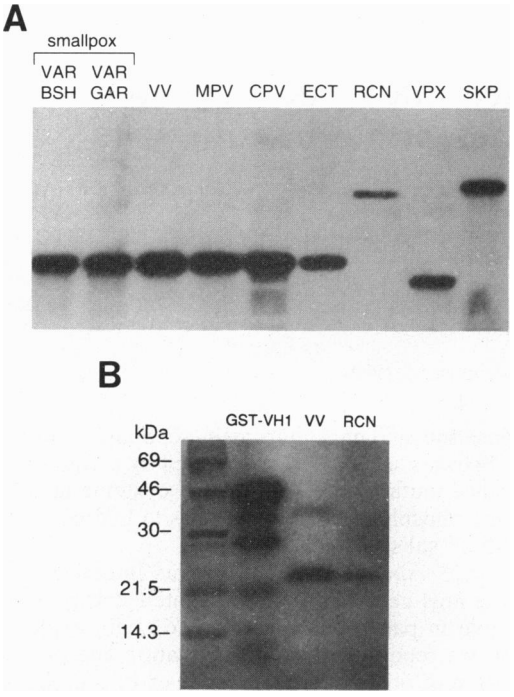

FIG. 1. The VH1 gene is conserved in many orthopoxviruses. (*A*) Genomes of certain members of the genus *Orthopoxvirus* were digested with *Hin*dIII, subjected to electrophoresis on agarose gels, transferred to nitrocellulose, and probed with the coding sequence of VH1. Genomes are labeled as follows: VAR BSH (smallpox variola virus Bangladesh major strain), VAR GAR (variola virus Garcia South American minor strain), VV (vaccinia virus), MPV (monkeypox virus Copenhagen strain), CPV (cowpox virus Brighton strain), ECT [ectromelia virus (mouse poxvirus)], RCN (raccoonpox virus), VPX (volepox virus), and SKP (skunkpox virus). (*B*) Western blot analysis of vaccinia virus VH1 (VV) and raccoonpox virus (RCN) proteins with antibody prepared against the recombinant GST–VH1. Molecular mass markers are as noted.

pUC18-RH15 was digested with *Bam*HI. Digested fragments were resolved by electrophoresis and then transferred to nitrocellulose and probed with the VH1 coding sequence. An ≈2.8-kb hybridizing fragment was then subcloned into Bluescript SK− (BS-RB2.8). BS-RB2.8 was digested with *Eco*RI and probed as described above. An ≈1.3-kb hybridizing fragment was subcloned into Bluescript SK− (BS-RE1.3). BS-RE1.3 was digested with *Eco*RV and subjected to electrophoresis. The band containing the vector and ≈950 bp of the raccoonpox virus DNA was isolated and religated. The fragment was sequenced by the Sanger dideoxynucleotide chain-termination method (14).

Sequence Analysis. DNA and protein sequence comparisons were performed with the FASTA (15, 16), PILEUP (17), and BESTFIT (18) programs.

Protein Expression and Western Blot Analysis. PCR was used to place *Bam*HI and *Eco*RI sites on the ends of the baculovirus VH1 coding region. Two synthetic oligonucleotides (5′-TTTGGATCCATGTTTCCCGCGCGTTGGCAC-3′ and 5′-TTTGAATTCTTAAAATAAATCTTGAACGTA-3′) were used in the PCR with *A. californica* viral genomic DNA as a template. The resulting fragment, coding for ORF3, located on an *Eco*RI/*Sal* I fragment of the *Hin*dIII-F region of the viral genome (19), was digested with *Bam*HI and *Eco*RI and subcloned into *Bam*HI/*Eco*RI-digested pGEX-KT (20), producing the vector pGEX-KT-BVH1. The recombinant vector was sequenced to ensure that no sequence errors had been introduced by the PCR. The glutathione *S*-transferase (GST; RX:glutathione R-transferase, EC 2.5.1.18) fusion protein was produced and purified as described (21) with the exception that induction was performed at room temperature overnight to improve the solubility of the fusion protein.

Western blot analysis of vaccinia virus and raccoonpox virus proteins in infected cells was performed as described by Auperin *et al.* (22).

Enzyme Activities. Phosphorylation of casein on serine and tyrosine was carried out by using the catalytic subunit of protein kinase A and the recombinant src kinase, respectively, as described (7). Serine or tyrosine phosphorylated casein (1 nM; 100,000 cpm) was incubated with increasing amounts of GST-BVH1 in 50 mM imidazole, pH 7.0/0.1% 2-mercaptoethanol at 37°C for 10 min. The reaction was stopped by the addition of bovine serum albumin (80 μg) and 50% trichloroacetic acid to a final concentration of 20%. The protein was precipitated on ice for 10 min. After centrifugation, the amount of ^{32}P released was determined by scintillation counting of the supernatant.

RESULTS AND DISCUSSION

Identification of VH1 Hybridizing Sequences in Members of the Genus *Orthopoxvirus*. A protein phosphatase (VH1), encoded in the *Hin*dIII-H (leftward transcription) fragment of the vaccinia virus genome DNA, was shown to have activity toward tyrosine and serine phosphate-containing substrates (7). Although the function of the phosphatase in viral replication or pathogenesis is unknown, the structural similarity between VH1 and the cell cycle gene product $p80^{cdc25}$ hints at the possibility that the viral gene may contribute to the observation that vaccinia virus rapidly shuts down the cell (23). Efforts directed at inactivation of the VH1 gene have been unsuccessful (J.E.D. and S. Broyles, unpublished observation). To gain additional insights into the importance and function of VH1, we have examined other orthopoxviruses as well as other viruses for VH1-like sequences.

A Southern blot of *Hin*dIII-digested genomic DNA of certain members of the genus *Orthopoxvirus* was probed at high stringency with the coding sequence of VH1 (Fig. 1). Strong hybridization signals were detected under stringent conditions with the genomes of two strains of smallpox variola virus (i.e., Bangladesh major strain and the Garcia South American minor strain), monkeypox virus, cowpox virus, ectromelia virus, raccoonpox virus, skunkpox virus, and volepox virus. Intense signals were observed under stringent hybridization conditions. In all of the orthopoxviruses tested, the relative genomic location of the VH1 gene cognate was conserved based on DNA maps (11, 13).

Recently, Knight *et al.* (13) showed the general genomic organization of the orthopoxviruses is highly conserved; however, the *Hin*dIII map of the raccoonpox virus is rather diverged from other orthopoxvirus DNA maps. The hybridization signal in the *Hin*dIII digest of the raccoonpox virus genomic DNA (Fig. 1*A*, lane RCN) was less intense than that of other orthopoxviruses tested. The possibility that this hybridization signal indeed corresponds to VH1-like DNA sequences was further analyzed with antibodies that recognize the vaccinia VH1 phosphatase. Infected cell lysates were prepared for vaccinia virus and raccoonpox virus (11). Antibody against bacterially expressed VH1 protein was shown to recognize proteins of ≈22 kDa in vaccinia virus- and raccoonpox virus-infected cells (Fig. 1*B*). The raccoonpox virus VH1 analog (termed RVH1) was markedly less reactive in Western blot analysis compared to the vaccinia virus VH1 protein. Addition of recombinant VH1 protein to the hybridization solution used in Western blot analysis blocks the antibody–VH1 protein interaction (data not shown). In Fig. 1*B*, products of several sizes were seen with the recombinant GST–VH1 protein. These products likely represent proteolytic breakdown products of the fusion pro-

tein. Collectively, both the Southern and Western blot analyses suggested to us that the RVH1 sequences were the least conserved among the orthopoxviruses tested. We speculated that the degree of conservation of the RVH1 protein might provide insights into residues important for catalysis and function of the VH1-like phosphatases. Thus, to determine the DNA sequence encoding the RVH1, a 15-kb genomic *Hin*dIII fragment from the raccoonpox virus was analyzed by restriction enzyme digestion. An *Eco*RI/*Eco*RV fragment that hybridized to the vaccinia VH1 gene was subcloned and sequenced (see *Materials and Methods* for details).

The DNA sequence encoding RVH1 was found to have 89% identity to the VH1 sequence (Fig. 2). At the protein level, RVH1 showed 92% identity with the VH1 phosphatase. Only conservative amino acid substitutions were noted in the VH1 and RVH1 sequences. Although DNA maps suggested that vaccinia virus and raccoonpox virus genomes are the most divergent of the orthopoxviruses (13), the coding sequences of VH1 and RVH1 are nearly identical. Residues known to be important for catalysis are invariant in the two proteins (24). One can also speculate that since the raccoonpox virus phosphatase RVH1 appears to be the most divergent of the VH1-like sequences, then the VH1-like phosphatases in the other orthopoxviruses are likely to have an even higher degree of identity to VH1. This high degree of conservation at the amino acid levels between VH1 and RVH1 and the likely possibility that VH1-like sequences in variola, cowpox, and other orthopoxviruses are even more conserved suggest a vital role(s) for these phosphatases. The smallpox variola virus Bangladesh major strain VH1 analog was sequenced and found to be 98% identical to VH1 at the amino acid level. All amino acids conserved between VH1 and RVH1 are also conserved in the variola VH1, indicating that VH1 is not a single marker of virulence (J.J.E. and R.F.M., unpublished observation).

Identification of a VH1-Like Protein in Baculovirus. To investigate whether the VH1-like phosphatases were found in other viruses, the coding region surrounding the active site residues of VH1 and RVH1 was used to search the GenBank data base. An open reading frame in baculovirus *A. californica* (19) was identified that had significant sequence identity to the VH1 phosphatase. The entire coding sequences of VH1, RVH1, and BVH1 were aligned (Fig. 2). Although the sizes of the three enzymes are very similar and the residues near the predicted active site cysteine are conserved, the BVH1 sequence shares only 20% identity with VH1 and RVH1. Taking into account conservative amino acid changes, BVH1 shares 40% similarity to VH1 and RVH1. One can anticipate that if BVH1 is indeed a phosphatase, then the invariant residues found in the three proteins are likely to play critical roles in substrate binding, structure, or catalysis.

Table 1. Relative specific activities of VH1 and BVH1

Substrate	VH1/BVH1
pNPP	40
Casein (tyrosine)	55
Casein (serine)	>2500

Purification and Expression of BVH1. The VH1 protein was previously shown to have activity toward tyrosine- and serine-phosphorylated substrates (7). The level of identity between BVH1 and VH1 raises the question of whether BVH1 was indeed a phosphatase and underscored the importance of defining its substrate specificity. To address these issues, the BVH1 coding sequence was expressed in *Escherichia coli* as a fusion protein with GST. The fusion protein GST–BVH1 was purified in a single step by glutathione agarose affinity chromatography. The results of this purification are shown in Fig. 3*A*. A crude lysate of *E. coli* cells expressing GST–BVH1 (lane 1) and purified fusion protein (lane 2) were analyzed by SDS/15% PAGE. The fusion protein was purified >95% as estimated from the single intensely staining protein band shown in Fig. 3*A* (lane 2). The purified protein effectively hydrolyzed the widely used substrate, *p*-nitrophenyl phosphate (pNPP) (Fig. 3*B*). The catalytic activity was inhibited by addition of vanadate (Fig. 3*B*). Vanadate is a general PTPase inhibitor and the vaccinia phosphatase is known to be sensitive to this reagent (GST does not show any hydrolytic activity toward pNPP). To examine the activity of BVH1 against tyrosine- and serine-phosphorylated substrates, increasing amounts of BVH1 were incubated with equal concentrations of ^{32}P-labeled serine- and tyrosine-phosphorylated casein. Fig. 3*C* shows the ^{32}P released from the tyrosine-phosphorylated and the serine-phosphorylated casein. The BVH1 phosphatase efficiently dephosphorylated the tyrosine-phosphorylated substrate but showed a low level of activity toward the serine-phosphorylated substrate. A comparison of the ratios of specific activities of BVH1 and VH1 is shown in Table 1. Using equal amounts of enzyme, VH1 has 40 times more activity against pNPP, 55 times the level of activity against tyrosine-phosphorylated casein, and >2500 the level of activity against serine-phosphorylated casein compared to BVH1. These results suggest that VH1 is more active than BVH1 toward all three substrates examined. In addition, the ratio of tyrosine to serine phosphatase activity displayed by the two proteins is radically different. VH1 shows a preference for phosphoserine-containing substrates while BVH1 hydrolyzes the same substrates poorly and shows a preference for phosphotyrosine-containing casein as opposed to phosphoserine-containing casein. These apparent differences need to be interpreted cautiously, however, since the substrates examined here are only "model proteins" and may not be accurate indicators of the specific intracellular substrate(s) used by

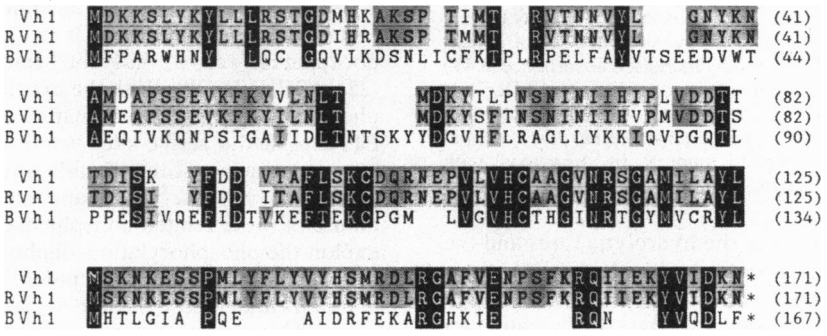

FIG. 2. Alignment of VH1, RVH1, and BVH1. Amino acid sequences of the VH1-like phosphatases from vaccinia virus, raccoonpox virus, and baculovirus were aligned. Residues that are absolutely conserved in all three proteins are indicated by solid boxes. Those residues that are conserved between two of the three phosphatases are indicated by shaded boxes. Gaps were introduced by the program to maximize areas of identity.

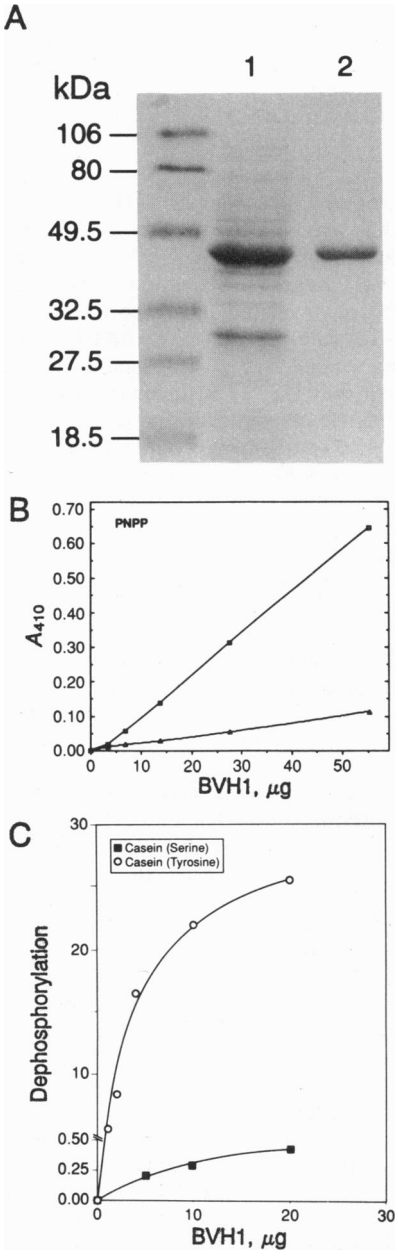

FIG. 3. (*A*) Purification of pGEX-KT-BVH1. The GST–BVH1 fusion protein was expressed in *E. coli* and isolated by affinity chromatography using a glutathione agarose resin. Crude cell lysate (lane 1) and affinity-purified GST–BVH1 fusion protein (lane 2) were subjected to electrophoresis on a SDS/15% polyacrylamide gel. Molecular mass markers were run as standards (sizes are indicated on the left). (*B*) Hydrolysis of *p*-nitrophenyl phosphate (pNPP) by BVH1. Hydrolysis of pNPP in the absence (■) and presence (▲) of 10 μM sodium orthovanadate was monitored by increased absorbance at 410 nm (*y* axis) with increasing amounts of protein (*x* axis). (*C*) Dephosphorylation of phosphoserine- and phosphotyrosine-containing casein. Release of ^{32}P from serine-phosphorylated (■) and tyrosine-phosphorylated (○) casein is plotted (cpm × 10^{-3}) (*y* axis) with increasing amounts of BVH1 (*x* axis).

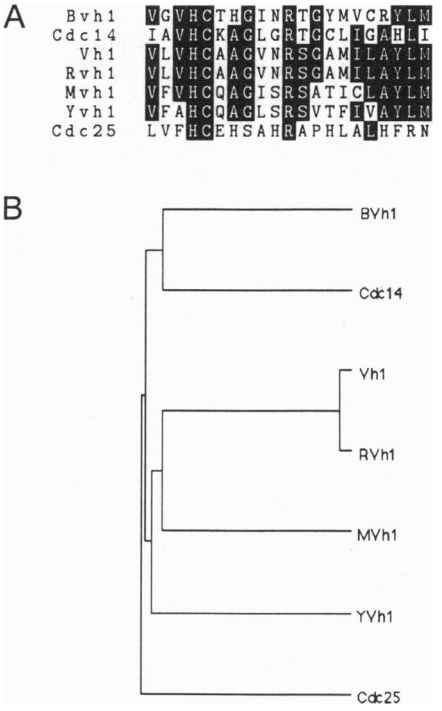

FIG. 4. (*A*) Active site signature sequences in the VH1 phosphatase family. Amino acid sequences surrounding the active sites of the VH1-like phosphatases from a baculovirus (BVH1), vaccinia virus (VH1), raccoonpox virus (RVH1), mice (MVH1), and yeast (YVH1) as well as the cell cycle regulatory proteins cdc14 and cdc25 from *Schizosaccharomyces pombe* were aligned by using the PILEUP program. Residues that are conserved among four of the seven proteins are indicated by solid boxes. (*B*) Family tree of VH1-like phosphatases. A representative family tree based on the degree of amino acid identities was constructed by the PILEUP program.

these enzymes. Comparisons of the hydrolytic rates and the substrate specificity need to be readdressed when the naturally occurring substrates for the two phosphatases are identified. Nevertheless, it appears that the baculovirus phosphatase is similar to the vaccinia phosphatase in being able to hydrolyze both phosphoserine- and phosphotyrosine-containing proteins. The dual specificity of these phosphatases is in contrast to the specificity noted with all of the mammalian and bacterial PTPases whose substrate specificities are restricted to the hydrolysis of tyrosine phosphate-containing protein.

VH1-Like Proteins Comprise a Subfamily of Phosphatases. When the VH1 and BVH1 phosphatase domains were used to search for related eukaryotic proteins, several were identified. These include the cell cycle proteins cdc25 (8) and cdc14 (25) in fission yeast; an open reading frame in budding yeast genome adjacent to the *DAL1* locus (26) known as YVH1 [studies on YVH1 are presented elsewhere (27)]; and a serum-stimulatable immediate-early gene, 3CH134, in the mouse (28) (termed MVH1, for mouse VH1-like protein, in this paper). A comparison of the residues surrounding the predicted active site cysteine residue in each enzyme is shown in Fig. 4*A*. All of the enzymes contain the conserved His-Cys sequence as well as an arginine residue 6 amino acids C-terminal to the cysteine. It is important to point out that phosphatase activity has not been demonstrated for cdc14 (25) or 3CH134 (28). With the exception of cdc14, all of these phosphatases are more similar to one another than to other PTPases at the active site. We therefore suggest that these enzymes may constitute a subfamily of phosphatases that are present in viruses, yeast, and mammals. The widespread nature of these related phosphatases, their ability to alter or exploit the phosphorylation–dephosphorylation signal transduction pathway, and their potential importance as disease cofactors underscore the importance of understanding events associated with their interactions in attenuated and pathogenic organisms.

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