

# A Novel Cytoplasmic Dual Specificity Protein Tyrosine Phosphatase Implicated in Muscle and Neuronal Differentiation\*

(Received for publication, October 12, 1995)

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Dual specificity protein tyrosine phosphatases (dsPTPs) are a subfamily of protein tyrosine phosphatases implicated in the regulation of mitogen-activated protein kinase (MAPK). In addition to hydrolyzing phosphotyrosine, dsPTPs can hydrolyze phosphoserine/threonine-containing substrates and have been shown to dephosphorylate activated MAPK. We have identified a novel dsPTP, rVH6, from rat hippocampus. rVH6 contains the conserved dsPTP active site sequence, VVXVHCX<sub>2</sub>GX<sub>2</sub>RSX<sub>5</sub>AY(L/I)M, and exhibits phosphatase activity against activated MAPK. In PC12 cells, rVH6 mRNA is induced during nerve growth factor-mediated differentiation but not during insulin or epidermal growth factor mitogenic stimulation. In MM14 muscle cells, rVH6 mRNA is highly expressed in proliferating cells and declines rapidly during differentiation. rVH6 expression correlates with the inability of fibroblast growth factor to stimulate MAPK activity in proliferating but not in differentiating MM14 cells. rVH6 protein localizes to the cytoplasm and is the first dsPTP to be localized outside the nucleus. This novel subcellular localization may expose rVH6 to potential substrates that differ from nuclear dsPTPs substrates.

Extracellular signals, such as mitogenic growth factors, bind to specific cell surface receptors that, in turn, initiate intracellular signaling through activation of a series of protein kinases (reviewed in Ref. 1). One such pathway involves the activation of mitogen-activated protein kinase (MAPK)<sup>1,2</sup> (2). MAPK is

regulated by an upstream kinase, MAPK/ERK kinase (MEK)<sup>3</sup> (3–5), which phosphorylates MAPK on both Tyr and Thr residues within a TXY phosphorylation motif (reviewed in Ref. 1). This diphosphorylation activates MAPK and allows it to phosphorylate nuclear transcription factors, protein kinases, cytoskeletal proteins, and other cell growth-dependent substrates (see recent reviews in Refs. 1 and 6). Recently, a family of MAPKs, including the MAPK-related proteins c-Jun N-terminal kinase/stress-activated protein kinase and p38, has been identified and implicated in kinase cascades that respond to mitogenic, differentiation, and stress-induced signals (reviewed in Ref. 7).

MAPK and MAPK-related proteins can be inactivated by dephosphorylation of either Tyr or Thr residues (reviewed in Ref. 6). Two protein phosphatases, MAP kinase phosphatase (CL100) and PAC1, have been shown to dephosphorylate and inactivate MAPK both *in vitro* (8–10) and *in vivo* (10, 11). These phosphatases belong to the protein tyrosine phosphatase (PTP) class of proteins, as defined by their conserved active site sequence, HCXAGXXR(S/T) (reviewed in Ref. 12). More specifically, MAPK phosphatase-1 and PAC1 are members of a subclass of PTPs commonly referred to as dual specificity protein tyrosine phosphatases (dsPTP) (reviewed in Ref. 13), which hydrolyze phosphate from Ser/Thr residues as well as from Tyr. Transcription of dsPTPs are induced by a variety of extracellular stimuli, where they may function in such processes as tissue regeneration (14), serum and growth factor stimulation (9, 15, 16), oxidative stress and heat shock response (15, 17), nitrogen starvation (18), and mitogen stimulation (19). dsPTPs may also regulate cell cycle events, *e.g.* controlling entry into mitosis (20) and interacting with cyclin-dependent kinases (21, 22). Dephosphorylation of MAPK and other MAPK-like proteins by dsPTPs suggests a function for dsPTPs in the regulation of cellular mitogenesis and differentiation.

In this study, we have identified a novel dsPTP from rat hippocampus, referred to as rVH6. rVH6 mRNA is detected in all tissues examined by Northern blot analysis. Recombinant rVH6 protein exhibits enzymatic activity against the artificial substrate, *p*-nitrophenyl phosphate (pNPP), and is able to dephosphorylate and inactivate MAPK *in vitro*. In COS-1 cells, expressed rVH6 protein localizes to the cytoplasm, and is thus the first dsPTP to be localized outside the nucleus. rVH6 mRNA is induced in PC12 cells following nerve growth factor (NGF)-mediated differentiation but is not induced following insulin or epidermal growth factor (EGF) mitogenic stimulation. The intermediate time course of rVH6 induction is distinct from other immediate-early gene dsPTPs and is closely correlated with the sustained activation and deactivation pattern of

but will be referred to as MAPK in this paper.

<sup>3</sup> MEK is also known as MAP kinase kinase (MAPKK) but will be referred to as MEK in this paper.

\* This work was supported by National Institutes of Health Grants 2RO1-DK18849 (to J. E. D.), 2RO1-DK4258 (to E. G. K.), 2RO1-AR18860 (to M. P. W. and S. D. H.), by funds from the Muscular Dystrophy Association (to S. D. H. and E. G. K.) and the National Kidney Foundation (to Q. C. V.), and by a grant from the Human Frontier Science Program (to J. S. C. and E. G. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U42627.

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<sup>1</sup> The abbreviations used are: MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; PTP, protein tyrosine phosphatase; dsPTP, dual specificity protein tyrosine phosphatase; pNPP, *p*-nitrophenyl phosphate; NGF, nerve growth factor; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; PCR, polymerase chain reaction; GST, glutathione *S*-transferase; HA, hemagglutinin; bp, base pair(s); kb, kilobase(s); DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; CH2 domain, cdc25 homology domain 2.

<sup>2</sup> MAPK is also known as extracellular signal-regulated kinase (ERK)

MAPK activity observed in NGF-mediated neuronal differentiation (23). Interestingly, in proliferating MM14 myoblasts, rVH6 mRNA levels are expressed at high levels and then decline rapidly following commitment to muscle differentiation. Moreover, the loss of rVH6 message correlates with the ability of basic fibroblast growth factor (bFGF) to stimulate MAPK activity in MM14 muscle cells. The widespread expression of rVH6, as well as its induction by NGF and bFGF, delineates a potential role for rVH6 in regulating proliferation and differentiation.

#### EXPERIMENTAL PROCEDURES

**Isolation of dsPTP Clones from Rat Olfactory Tissue**—Two sets of degenerate oligonucleotide primers were designed to the conserved amino acid regions within the catalytic domain of dsPTPs and used to amplify dsPTPs from rat olfactory tissue by polymerase chain reaction (PCR). The 5' primer, corresponding to amino acid sequence V(L/F)VHC(Q/L)A (5'-GT(C/G)(A/C/T)T(C/G/T)GT(C/G)CA(C/T)TG(C/T)C(A/T)GGC-3'; 288-fold degeneracy) was paired with a 3' primer corresponding to amino acids GQLL(Q/D/E)FE (5'-CT(C/T)(A/G)(A/T)(A/G)(C/G)T(C/G)C(A/C)(A/G)CAGCTG(C/G)CC-3'; 512-fold degeneracy). The template for PCR was synthesized from rat olfactory epithelial poly(A)<sup>+</sup> RNA using oligo(dT) primers and the cDNA cycle kit (Invitrogen) according to manufacturer's instructions. The PCR reactions were performed with the GeneAmp kit (Perkin-Elmer) and included 0.1 volume of the cDNA synthesis reaction and 500 ng of each primer for 35 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min. PCR products were isolated by agarose gel electrophoresis and products of expected size (approximately 177 bp) were subcloned into pCR<sup>II</sup> using the TA Cloning kit (Invitrogen). Approximately 150 independent subclones were sequenced by dideoxy chain termination using Sequenase v. 2.0 polymerase (U. S. Biochemical Corp.).

**Library Screening**—A unique 177-bp PCR subclone (rVH6-pCR II), which had significant similarity to dsPTPs, was used to screen a rat hippocampus  $\lambda$ ZAPII cDNA library (Stratagene). The rVH6 insert was labeled by random priming and used as a hybridization probe to screen approximately one million library plaques by established procedures (24). Nitrocellulose filters were washed under high stringency with 0.2  $\times$  SSC (1  $\times$  SSC = 150 mM sodium chloride, 15 mM sodium citrate, pH 7.0) at 65 °C. Positive library plaques were purified and cDNA rescued into pBluescript II (pBS II) (Stratagene) according to manufacturer's instructions. Several clones of 2.1 and one 1-kb clone were isolated. The sequences of both cDNA strands were verified for all subclones.

**Bacterial Expression and Purification of Recombinant rVH6**—Recombinant rVH6 was expressed as a glutathione *S*-transferase (GST)-fusion protein containing a carboxyl-terminal polyhistidine tag. Full-length rVH6 (1.14 kb) PCR product containing 5' *Bam*HI and 3' *Eco*RI restriction sites was generated using oligonucleotides (5'-GATCG-GATCCATGATAGATACGCTCA-3') and (5'-GATCGAATTCGGTA-GATTGCAGG GAGT-3') and subcloned in-frame into *Bam*HI/*Eco*RI-digested pGEX-KT (25) modified to contain a COOH-terminal polyhistidine (6 $\times$ ) cassette. GST-rVH6-His<sub>6</sub> was produced as described previously (25) and purified on glutathione-agarose and nickel-agarose resins. Pure protein (>90%) could routinely be obtained in a single step purification using a nickel-agarose column (QIAGEN). A catalytically inactive rVH6 mutant (Cys<sup>293</sup> to Ser) generated by site-directed mutagenesis (26) was expressed and purified under conditions identical to that of wild-type rVH6.

**Enzyme Activity Assay**—rVH6 enzymatic activity was assayed against the artificial substrate, pNPP, as described previously (26). Various quantities of GST-rVH6 were incubated with 50 mM pNPP in 50 mM succinate buffer, pH 6.0, at 30 °C for 30 min. The reactions were terminated with 0.1 N sodium hydroxide, and the molar concentration of pNPP hydrolyzed was determined by absorbance at 405 nm. Analysis of the data was performed using KinetAsyst software (IntelliKinetics, State College, PA).

GST-MEK2, MAPK (p44) and the kinase-deficient mutant, MAPK K71R, were generous gifts from Dr. K. L. Guan. MAPK was activated by incubation with GST-MEK2 as described previously (27). The ability of GST-rVH6 to inactivate MAPK was determined by incubation of activated MAPK (0.2  $\mu$ g) with increasing concentrations of GST-rVH6 in Buffer A (50 mM HEPES, pH 7.5, 0.1% 2-mercaptoethanol) for 10 min at 30 °C. The reactions were terminated by the addition of sodium vanadate (final concentration, 2 mM), and the remaining MAPK activity was determined as described (28). Wild-type MAPK and mutant MAPK K71R proteins were <sup>32</sup>P-labeled by incubation with GST-MEK2 in the

presence of [ $\gamma$ -<sup>32</sup>P]ATP and purified as described (5). The ability of GST-rVH6 to dephosphorylate MAPK was determined by incubation of <sup>32</sup>P-labeled MAPK (0.2  $\mu$ g) with increasing concentrations of GST-rVH6 as described above. The reactions were stopped by the addition of 5  $\times$  Laemmli buffer, boiled, and resolved by SDS-polyacrylamide gel electrophoresis. The proteins were transferred to a polyvinylidene difluoride membrane (Schleicher & Schuell) and exposed to film. After film development, radiolabeled MAPK was excised from the membrane and subjected to phosphoamino acid analysis (5).

**Cell Culture**—All cell culture reagents were purchased from Life Technologies, Inc. PC12 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% horse serum and 5% fetal bovine serum. When cells reached 50–70% confluency, serum was withdrawn for 18 h prior to growth factor treatment. PC12 cells were then induced to proliferate with EGF (50 ng/ml) or insulin (100 nM) or to differentiate with NGF (100 ng/ml) in DMEM for the indicated times. For each time point, one 140  $\times$  20-mm tissue culture plate was harvested for poly(A)<sup>+</sup> RNA extraction.

MM14 mouse muscle cells were grown either in Ann Arbor from a cell stock generously provided by Dr. Jeffrey Chamberlain or in Seattle from the original MM14 cell stock. In both cases, the cells were grown in 0.6% gelatin-coated 140  $\times$  20-mm plates containing Ham's F-10C nutrient medium supplemented with 15% horse serum and 2 ng/ml bFGF (29). For maximal cell proliferation, MM14 cell medium was changed every 12 h, and the cells were split before reaching 400,000 cells/plate. To initiate a differentiation time course experiment, 550,000 cells were plated and allowed to proliferate for 24 h. The medium was then removed, the plates were washed with phosphate-buffered saline (PBS), and differentiation medium (Ham's F-10C containing 2% horse serum, 1  $\mu$ M insulin, without bFGF) was added. Differentiating cells were fed every 24 h. For poly(A)<sup>+</sup> RNA isolation, 20–30 plates were harvested per differentiation time point.

For detection of cellular MAPK activities, MM14 cells withdrawn from serum and bFGF for the indicated lengths of time were stimulated briefly (5 min) with either 10 ng/ml of bFGF or 1.5% horse serum in F10C medium (control). The cells were harvested and lysed, and the high speed supernatants (100,000  $\times$  g) were fractionated over DE-52 "mini columns" (Whatman) as described previously (30). MAPK activity was eluted from the columns with 0.2 M NaCl, normalized for protein, and assayed for activity using myelin basic protein (Sigma) as substrate (30).

**RNA Isolation and Northern Blot Analysis**—Rat and mouse multiple tissue Northern blots were purchased from Clontech. Northern blots of cultured PC12 and MM14 cells were prepared by extraction of total RNA with TRIzol Reagent (Life Technologies, Inc.), followed by poly(A)<sup>+</sup> RNA purification using a Poly(A)Tract kit (Promega). RNA samples were resolved in a formaldehyde-agarose gel (24) and transferred to a Nytran membrane (Schleicher & Schuell). An rVH6 riboprobe construct (rVH6A) was generated by digesting the rVH6-pBS II 1-kb partial length clone with *Acc*I, regenerating a vector construct containing an insert of 407 bp (nucleotides 1069–1476; see Fig. 1). A 440-bp [<sup>32</sup>P]UTP-labeled rVH6 riboprobe, generated by T7 RNA polymerase transcription of *Eco*RI-linearized rVH6A, was hybridized to blots overnight in 400 mM sodium phosphate, 5% SDS, 1 mM EDTA, 1 mg/ml bovine serum albumin, 50% formamide at 60 °C, 1–2 million cpm/ml. The membranes were washed at high stringency in 0.1  $\times$  SSC, 0.1% SDS, 1 mM EDTA at 70 °C and exposed to XAR 5 film (Kodak) at –80 °C with intensifying screens. A probe to mouse muscle creatine kinase was used to determine the degree of muscle differentiation, whereas rat cyclophilin was used as a control for amount of RNA loaded. RNA molecular size markers were from Life Technologies, Inc. Northern blot quantitation of mRNA was determined using a PhosphorImager (Molecular Dynamics), and RNA concentrations per lane were normalized against cyclophilin.

**Cellular Localization of rVH6**—rVH6 protein was expressed in COS-1 cells using the eukaryotic expression vector, pCDNA1 (Invitrogen), engineered to produce a COOH-terminal triple hemagglutinin (HA) (3  $\times$  YPYDVPDYA) epitope tag fusion protein. Full-length rVH6 cDNA (wild type and C/S mutant) was subcloned by PCR using 5' (5'-GACTAAAGCTTACCGCCATGATAGATACGC-3') and 3' (5'-GAC-TAGAAATTCGATAGATGACAGGAGT-3') oligonucleotide primers. hVH3 was provided by Dr. S. Kwak and expressed as a COOH-terminal *c-myc* epitope tag-fusion protein in COS-1 cells (16). COS-1 cells were plated at 5  $\times$  10<sup>4</sup> cells/well (diameter, 3 cm) on glass coverslips (2 cm<sup>2</sup>) in DMEM + 5% fetal calf serum. Cells were transfected with the appropriate DNA (1  $\mu$ g) using lipofectamine (Life Technologies, Inc.) in serum-free DMEM for 6 h before removing transfection solution and returning the cells to DMEM + 5% serum. Cells were fixed 48 h post-transfection with 4% paraformaldehyde in PBS, followed by ac-

etone:methanol (1:1). All immunofluorescence incubations were conducted at room temperature. Primary mouse monoclonal antibodies anti-HA (Boehringer Mannheim) or anti-*myc* (9E10) (31) diluted to 1  $\mu$ g/ml in blocking buffer (PBS, 0.1% saponin, 2% bovine serum albumin) were incubated with cells for 1 h. Cells were then washed four times with PBS + 0.1% saponin and incubated for 1 h with fluorescein-conjugated goat anti-mouse secondary antibody (Vector Labs) diluted 1:500 in blocking buffer. Following secondary antibody incubation, the cells were washed four times with PBS + 0.1% saponin and twice with PBS. Coverslips were mounted with Vecta-Shield (Vector Labs), and fluorescence was viewed with a Zeiss fluorescent microscope. For negative primary antibody controls, COS-1 cells were transfected with pcDNA-1 vector alone (minus epitope tag) and treated for immunofluorescence as described above.

## RESULTS

**Identification and Cloning of rVH6 cDNA**—Degenerate oligonucleotides were used to isolate novel dsPTPs from rat olfactory epithelium cDNA by PCR. The 5' oligonucleotide was designed to partially overlap the PTP active site motif (I/V)H-CXAGXXR(S/T), whereas the 3' oligonucleotide was made against a conserved group of amino acids GQLLXFE (Fig. 1B). PCR generated a pool of cDNAs of the approximate predicted size (177 bp), which were subcloned and sequenced. Sequencing of 150 individual clones revealed that approximately 50% of the products were identical to the human dsPTP, CL100 (17). Approximately 25% of the clones contained a novel cDNA sequence, which when translated had 38–58% amino acid identity to previously identified dsPTPs. This dsPTP sequence was named rVH6 based on it being the sixth dsPTP identified in the laboratory of the senior author (J. E. D.), the first being the vaccinia H-1 gene product (VH1) (32).

Preliminary Northern blot analysis (data not shown) using the rVH6 PCR fragment indicated that rVH6 was abundantly expressed in brain. Based on the high levels of expression in brain, a rat hippocampus cDNA library was screened with the rVH6 PCR fragment to isolate full-length cDNAs. Five hybridizing clones were isolated, four of which contained identical 2104-bp inserts and one of which contained a partial length insert of 1034 bp. The 2.1-kb cDNA sequence had a 1143-nucleotide (361–1503) open reading frame (Fig. 1A) with a putative initiator methionine in a region that matched the Kozak sequence motif (nucleotides 355–363; Fig. 1A) (33). No other methionines were observed 5' to this methionine in any other reading frame. In addition, 5' to the Kozak sequence and 3' to the in-frame stop codon (nucleotides 1504–1506; Fig. 1A), stop codons were observed in all three reading frames. No polyadenylation signal or poly(A) tail was observed in any rVH6 cDNA clone isolated. Based on the 3.2-kb message size observed in Northern blot analysis (Fig. 2), up to 1.1 kb of 5' and 3' untranslated sequence was not present in the isolated cDNA clones. Northern blot analysis using the rVH6 riboprobe revealed that the mRNA is expressed as a 3.2-kb transcript in all the tissues examined (Fig. 2). Relatively higher levels of expression were seen in brain and spleen, with lower levels in heart, lung, liver, and skeletal muscle. Very low levels of rVH6 mRNA were also detected in kidney and testis. A similar size rVH6 RNA transcript was also observed in a mouse multiple-tissue Northern blot (data not shown). The rVH6 mRNA tissue distribution between rat and mouse was similar, with the exception that mouse kidney and skeletal muscle expressed higher levels of rVH6 (data not shown).

Data base searches with the deduced amino acid sequence of rVH6 (Fig. 1B) indicate that rVH6 is similar to all known dsPTPs. The rVH6 cDNA predicted open reading frame codes for a protein of 381 amino acids (~42,315 Daltons), which is similar in size and amino acid identity to CL100 (36%, (17)), Pac-1 (35%, (19)), hVH2 (35%, (27)), and hVH3 (33%, (16)). rVH6 contains the active site sequence, VXVHCXXG-

XXRSXXXXXAY(L/I)M (Fig. 1B), characteristic of virtually all dsPTPs (34). The structure of rVH6 can be divided based on its amino acid alignment with other dsPTPs (Fig. 1B) into a 181-amino acid COOH-terminal catalytic domain and a 200-residue NH<sub>2</sub>-terminal extension. The rVH6 catalytic domain possesses amino acid identities to hVH2 (45%), CL100 (44%), Pac-1 (43%) hVH5 (42%, (34)), hVH3 (38%) VHR (35%, (35)), and VH1 (27%).

The NH<sub>2</sub> terminus of rVH6 contains two regions of amino acid similarity to the cell cycle regulator phosphatase, cdc25 (36, 37). These two regions (*underlined* in Fig. 1B) are referred to as cdc25 homology domains 2 (CH2 domains) (37) and have been observed in the NH<sub>2</sub> terminus of several other dsPTPs (CL100, Pac-1, hVH2, hVH3, and hVH5). The function of these CH2 domains is unknown. Interposed between the NH<sub>2</sub>-terminal CH2-containing, and COOH-terminal catalytic domains, is a serine-rich (30%) amino acid region (amino acids 150–210; Fig. 1B) that also contains two putative proline-directed serine kinase substrate recognition motifs, XSP, (amino acids 162–164 and 200–202; Fig. 1B) (reviewed in Ref. 1). In addition, the NH<sub>2</sub>-terminal domain of rVH6 contains two putative nuclear export motifs, LXLXLXXL and LXXLXLXXL (Fig. 1A), implicated in the function of proteins that export specific proteins and RNAs from the nucleus (38, 39). No other significant amino acid similarity to other Genbank protein sequences was observed in the NH<sub>2</sub>-terminal domain of rVH6.

**Catalytic Properties of rVH6**—To determine whether the rVH6 cDNA coded for a protein possessing phosphatase activity, recombinant rVH6 was expressed, purified, and assayed for enzymatic activity. The fusion protein, GST-rVH6, hydrolyzed the tyrosine phosphatase substrate, pNPP (Fig. 3A), with kinetic parameters of  $K_m = 10$  mM and  $k_{cat} = 3.8$  min<sup>-1</sup> (Fig. 3B). These kinetic properties are considerably different from the dsPTP, VHR, which possesses a lower  $K_m$  of 1.6 mM and larger  $k_{cat}$  of 308 min<sup>-1</sup> against pNPP (40). The kinetic properties of rVH6 more closely resemble the activity of recombinant cdc25 toward pNPP, with  $K_m$  of 5.6 mM and  $k_{cat}$  of 5.3 min<sup>-1</sup> (41). Sodium vanadate, a potent inhibitor of PTPs, competitively inhibited rVH6 catalytic activity (Fig. 3B). In addition, rVH6 activity toward pNPP was dependent on the presence of reducing agents, independent of divalent cations, and optimal at pH 6.0–7.0 (data not shown), all of which are common features for the dsPTPs.

**Dephosphorylation and Inactivation of MAP Kinase**—Based on the amino acid similarity of rVH6 with MAPK phosphatase-1 and other dsPTPs, we determined whether rVH6 also recognized activated MAPK as a substrate. GST-rVH6 rapidly inactivated MAPK activity in a dose-dependent fashion, and this inactivation could be blocked by 1 mM vanadate (Fig. 4A). Approximately half of the MAPK activity was abolished in 10 min in the presence of 37 ng of GST-rVH6 (10 nM), with maximal inactivation (>95%) observed at 500 ng of GST-rVH6 under identical conditions. The ability of rVH6 to inactivate MAPK was comparable with other dsPTPs. For example, CL100 was reported to inactivate 50% of MAPK activity at 7 nM under similar buffer, incubation time, and temperature conditions (42). SDS-polyacrylamide gel electrophoresis analysis of <sup>32</sup>P-labeled MAPK indicated that the loss of biological activity correlated with the dephosphorylation of MAPK (Fig. 4B). Dephosphorylation of MAPK by GST-rVH6 was dose-dependent and closely correlated with the inactivation of MAPK activity. Catalytically inactive rVH6 (C293S) failed to dephosphorylate MAPK, as did wild-type rVH6 when it was in the presence of 1 mM sodium vanadate (Fig. 4B). rVH6 was able to completely dephosphorylate the kinase-deficient MAPK K71R mutant (27) with protein concentrations similar to that required for the

## A

1 GCACGAGACGGGGTTGGATCTGAGGTGCAGCCTCAGAGGGATTAGGAGCTGTAGACTTTTTCCTTTTTCCTTCTCCTCAGTGGCAC  
 94 GGAGTCCGAATTAATTTGATTTTCACTTGGGTAGGAAACAAACATGGGACATTTTCAATTCAGAGAGATTTCAATTCAGAGTGATCTGGT  
 187 GCAGAGGACACCGGCTTGGCTTCTGTCGCTTCCCTAACCGTAGCTTCGGCTTGGAAAGGCGCAATCAACACCGGCTCCGAGAGCCGGA  
 280 GCTTCTCACGGCTTGGCTTGGCTATGCTGCTCGAGGGGCGTCTGCTAGGCACCCCGCTTCTCTGAGCTGACCCCATGATAGATAGC  
 1 M I D T  
 373 CTCAGACCCGTGCGCTTCCGCTCGGAAATGGCGATCAGCAAGACGGTGGCTGGCTCAACGAGCAGCTGGAGCTGGGCAACGAGCAGCTGCTG  
 5 L R P V P F A S E M A I S K T V A W L N E Q L E G N E Q L E S  
 466 CTGATGAGCTCCGACCGCAGGAGCTGTACGAGTCTGCACATCGAATCTGCCATCAACGTGGCCATCCGGGCAATCATGCTGCGGCTGCTG  
 36 L M D C R P Q E L Y E S S H I E S A I N V A I P G I M L R R L  
 559 CAGAAGGGCAACCTGCGGTGCGCGCTTATTCACGCTGCGAGGACCGGACCGCTTACACGCGCTGCGGACCGGACCGCTGCTGCTGCTG  
 67 Q K G N L P V R A L F T R C E D R D R F T R R C G T D T V V L  
 652 TACGACGAGAACAGCAGCTGGAATGAGAACAGAGTGGAGTGGTCTCTGGGCTGCTGCTCAAGAACTCAAGACGAGGCTGCGG  
 98 Y D E N S S C S D W N E N T G G E S V L G L L L K K L K D E G C R  
 745 GCGTCTACCTTGAAGGTGGCTTCAGTAAGTTCCAGGCGGAGTTCGCGCTGCACTGCGAGACCAATCTAGACGGCTGTGACGAGCAGCTCC  
 129 A F Y L E G G F S K F Q A E F A L H C E T N L D G S C S S S S  
 838 CCGCGCTTGCAGTCTGGGACTCGGGGCGCTGAGGATCAGCTCCGACTTCTCTGAGACATGAGCTGACCTTGACCGAGACCCCAATAGT  
 160 P P L P V L G L G L R I S S D S S D I E S D L R D P N S  
 931 GCAACGGACTCCGATGGCAGCCCGCTTCCACAGCCAGCTTCTCTCCGCTGGAGATTTTGCCTTCTTACCTGGGCTGTGCCAAGGAC  
 191 A T D S D G S P L S N S Q P S F P V E I L P F L Y L G C A K D  
 1024 TCTACTAATTTGAGCTGTTGGAAGAGTTGGCATCAAGTACATCTTGAACGTCACCCCAATCTTTTGAAGCAATCTTTGAGAGTGGGAG  
 222 S T N L D V L E E F G I K Y I L N V T P N L P N L F E N A G E  
 1117 TTCAAGTACAAGCAATTCCTATCTCTGATCACTGAGGCAAAACCTGTCCAGTCTTTTCCCTGAGGCGCATTTCTTCATAGATGAAGCCCGA  
 253 F K Y K Q I P I S D H W S Q N L S Q F P E A I S Q F I D E A R  
 1210 GGCAGAAATGTGGTTCCTGGTGCATCTGCTGGCGGCGATCAGCGCTTCGCTGACGAGTGGCTTACCTATGCGAGAGCCCAATCTG  
 284 G K N C G V L V H C L A G I S R S V T V T V A Y L Q K L N L  
 1303 TCCATGAACGATGCTTATGACATTTGTCAAATGAAGAAGTCAACATCTCTCCCACTTCAACTTCATGGGCGAGCTGCTTGCATTTGAAGG  
 315 S M N D A Y D I V K M K K S N I S P N F N F M G Q L L D F E R  
 1396 ACCCTGGGACTCAGCAGCCCTTGCAACATCTGCTCCCGCAGCAGCTCTACTTACCGCGCCCTCAACACGAGATGCTTACCAAGTGGAC  
 346 T L G L S S P C D N R V P A Q Q L Y F T A P S N Q N V Y Q V D  
 1489 TCCCTGCAATCTAGTGAAGGACCCACCTTTCCTAGCGGGAGTGTCTCAITTCCTCAGTTTCTCTTGGGAGCATGACACAGGCTGCTTT  
 377 S L Q S T  
 1582 CTTTGTGTGTGCCCCAGGTGTCAAAAATGTCACAGCTGTCTGATTAGACAGGTTGCCAAGTGCAAAATTTGGTATTACGGAGGAGAGA  
 1675 TTTGCTCCATCAITTTTGTGAAGGACAGGGTATGCTGTCTAGATCCAGCAATAGTTTGTCTTTGTACCCAGCTACCCAGCAGG  
 1768 ACTGACCTCCATCCAGATAGAGGTAGGCAAGAGGACCGGGATAGGAGCATGTGTCTTACGGGACATATGCTGCTTCTCTGTCAT  
 1861 CTGGAACCAATATATTGCTTCAGTGAAGACTGATCAACTTTGCGTATAGTGGAGCCAAAGAGATTTTACCTCTGATTGTTGCTATCGGT  
 1954 TTTGAAACAAAGAACTGATCTTATTTGATTTATGTAATATTTGATCTTCACTTGAAGAGTGTGTTTGTGCTTGTGTTTGTGTTTAA  
 2047 TCTTTGGTTTAAACGAGATCCAAAGGAGGAGAGAGCAGTATGCTCTGTCGCGCAATTC

FIG. 1. A, nucleotide and predicted amino acid sequence of rVH6 cDNA. Nucleotide sequence is numbered on the left, beginning with the first nucleotide of the cDNA. Amino acids are numbered in *italics* on the left, beginning at the first predicted methionine. The in-frame stop codon is denoted by an *asterisk*. The PTP conserved active site is **bold and underlined**. Two putative nuclear export signals are *underlined*. B, alignment of rVH6 with other VH1-like PTPases. The amino acid sequence of rVH6 was aligned with CL100 (17), Pac-1 (19), VHR (35), and VH1 (32) using the PILEUP program (Genetics Computing Group, Madison, WI). The *black boxes* denote conserved amino acid residues. The PTPase signature motif is *double underlined*, and the cdc25 homology domains (CH2 domains) are *single underlined*. Degenerate oligonucleotides used in PCR as described under "Experimental Procedures" are shown by *arrows*.

## B

1 MIDTLRPVFFASEMAISKTVAWNEQLELGNEQLMDCRPQELYESSHI 50  
 CL100 ... MVMEVG.TLD.AGGLRLGE...RAAQCLLDDCRSFFAFNAGHI  
 PAC-1 ... MGLEAARELECAALGTLERDP...REAERTLLDDCRPFLAFRRHV  
 51 BSAINVAIPGIMLRRLQ.KGNLPVRALFTRCEDRDRFTRRCGTDVTVLYD 100  
 CL100 AGSVNVRSTIVRRRAKG...AMGLEHIVPNAELRGRLL.AGAYHAVVLLD  
 PAC-1 RAARVPWNALLRRARGPPAAVLACLPLDRALRRLV.RGELARAVLLD  
 101 ENSSDWE...NTGGESVLGLLKKLKDEGCRAFYLDGGFSKFQAEFALH 150  
 CL100 BRSAALDGAARDGTALALAAGACRERRAA...QVFFLKGGYAFSA...  
 PAC-1 BGSASVAELRPDSPAHVLLAAHLHETRAGPTAVYFIRGGFDGFG...  
 151 CETNLDGSCSSSSPPVPLGLGLLRISDSSSDIESDRLDRPNSATDSDG 200  
 CL100 ... SCPEP...CSKQSTPTGLSLPLSTSTSPDSABS  
 PAC-1 ... CCEPD...CSEAPAP...ADP...PTGDKT  
 VHR ... MSGSFELSVQDLNLLSDGSG  
 VH1 ... MDKSLYKYLRLRSTGDMHAKS  
 201 SPLNSQPSF...PVEILFPLYLCAKDSNLDVLEFPGIKYLINVP. 250  
 CL100 GCSGCTPLDYDQGGPVEILFPLYLCAHASKRDMALGITALINVA.  
 PAC-1 SRSDSRAPVYDQGGPVEILFPLYLCAHASKRDMALGITALINVA.  
 VHR CYSLPSQPCN...VTERIVGNASVAQDIPKQKLSITHVNLNAEG  
 VH1 PTIMTR...VTNNVYLGNYKNAMDAPS.SEVKFKYVNLNLTMD  
 251 ...NLFNLFENAEFKYKQIPISDHSQNLSPFPEATSFIDEA.RG 300  
 CL100 ...NCPNHFE...GHQYKSTPVEDNHKADISSWNEADDFIDSI.KD  
 PAC-1 ...SCPNHFE...GLFRYKSTPVEDNQMVESAWQEAIGFDWV.KN  
 VHR RSFMHVNTNANFYKDSGITYLGLKANDTQEFNLSAYFERAADFIDQALAQ  
 VH1 KYTLF...NSNINIIHPLVLDTTTDSKYEDDVTAFLSKC.DQ  
 301 KNCGLVHCLAGISRSVTVTVAYLMQKLN...LSMNDAYDIVMKKSN 350  
 rVH6 AGGRVHVHQAQGISRSATICLAYLMRTNR...VKLDEAFEFVKQRNSI  
 CL100 SGGRVHVHQAQGISRSATICLAYLMQSR...VRLDEAFDFVKQRNSI  
 PAC-1 KNCGRVHCREGYSRSPTLVIAVLMRQK...MDVKSALSIIVQRNSI  
 VHR RNEPVLVHCAAGVNRSGAMILAYLMKSNKKESSPMYFLYVYHSMRDLGA  
 VH1  
 351 ISPNFNFMGQLLDFERTLCSSPCDNRVPAQQLYFT...APSNQNVYQV 400  
 rVH6 ISPNFNFMGQLLQFEISQVLAHPCSA...EAGSPAMAVLDRTSTTVFNF  
 CL100 ISPNFNFMGQLLQFEISQVLAHPCSA...EAGSPAMAVLDRTSTTVFNF  
 PAC-1 ISPNFNFMGQLLQFEISQVLAHPCSA...EAGSPAMAVLDRTSTTVFNF  
 VHR IGPNDGFLACLGLNDRLAKEGKLP\*...  
 VH1 FVENPSFKRQIIE...KYVIDKN\*...  
 401 DSLQST\*... 450  
 rVH6  
 CL100 PVSIPVHSTNSALSFLQSPITTSFSC\*

inactivation of wild-type MAPK activity (Fig. 4B). The dephosphorylation of  $^{32}$ P-labeled wild-type MAPK by rVH6 was not complete, however, presumably due to activated MAPK autophosphorylation on serine residue(s) outside of the TEY phosphorylation/activation motif (Fig. 4C). Although the serine autophosphorylation site(s) have not yet been mapped, auto-

phosphorylation of activated MAPK has been observed by others (27). The observation that rVH6 does not dephosphorylate phosphoserine on autophosphorylated MAPK suggests that rVH6 recognition of activated MAPK is specific for the diphosphorylated TEY motif.

To determine the specific amino acids dephosphorylated by

rVH6,  $^{32}\text{P}$ -labeled MAPK previously incubated with GST-rVH6 was subjected to phosphoamino acid analysis (Fig. 4C). The removal of label from tyrosine residues closely correlated with the dephosphorylation and inactivation of MAPK. Surprisingly, very little of the phosphothreonine residues were dephosphorylated by rVH6 in 10 min. However, dephosphorylation of phosphothreonine residues could be observed with rVH6 treatment greater than 1 h (data not shown). This rapid tyrosine dephosphorylation and slow threonine dephosphorylation pattern closely resembles the dephosphorylation of pTyr and pThr on p34<sup>cdc2</sup> by the dsPTP, cdc25 (20). Under similar conditions, CL100, hVH2, and hVH3 were able to rapidly dephosphorylate both tyrosine and threonine on activated  $^{32}\text{P}$ -labeled MAPK (8, 16, 27).

**Expression of rVH6 in PC12 Cells**—Several dsPTP mRNAs are induced when serum-starved PC12 cells are stimulated to proliferate or differentiate (34, 42, 43). Because rVH6 is highly expressed in brain (Fig. 2), we wanted to know whether rVH6 was also expressed in PC12 cells and whether its expression changed during conditions of proliferation or differentiation. Mitogenic stimulation of PC12 cells with either EGF or insulin failed to induce the expression of rVH6 mRNA, even after 12 h of treatment (data not shown). However, upon stimulating PC12 cells to differentiate with NGF, rVH6 mRNA was induced 18-fold over basal levels, with induction first detected at 1–2 h and maximal expression first detected at 3 h (Fig. 5). rVH6 expression was sustained up to 18 h (Fig. 5), and returned to basal levels by 24 h (data not shown).

**rVH6 Expression in Muscle Cell Differentiation**—Following the initial observation that rVH6 was expressed in differentiating PC12 cells, we addressed the possibility that rVH6 was also modulated in other cell types during differentiation. We chose to study rVH6 expression in mouse MM14 muscle cells because the process of proliferation and differentiation has been well characterized in this cell line (29, 44, 45) and because regulation of an unknown MAPK PTP had been observed in

these cells (46). Northern blot analysis of RNA from proliferating and differentiating MM14 cells showed that rVH6 was maximally expressed in proliferating MM14 cells (Fig. 6). After 6 h of withdrawal of bFGF and serum, rVH6 mRNA levels dropped to approximately 20% maximal and by 12 h rVH6 expression was at very low levels (2–5% maximal levels). During this same time course, muscle creatine kinase, a muscle-specific protein that is indicative of the degree of differentiation, was first detected at 12 h and was maximally expressed at 24 h. Northern blot analysis of total RNA (20  $\mu\text{g}/\text{lane}$ ) showed similar results for rVH6 and muscle creatine kinase expression (data not shown). In the presence of bFGF and 2% serum (+F, Fig. 6), rVH6 mRNA expression was maintained at 55–60% of levels seen in proliferating cells. However, in high serum without bFGF (+H, Fig. 6), rVH6 expression dropped to 15% that of proliferating cells. Under both these conditions, muscle creatine kinase expression was 30–45% of that seen in cells withdrawn from bFGF and serum for 24 h. These observations suggest that bFGF is a stronger stimulus for rVH6 expression than high concentrations of serum. Both bFGF and serum, however, are required for continued cell proliferation as well as for maximal rVH6 expression.

**FGF-stimulated MAPK Activation Is Blocked in Proliferating Cells**—Weak bFGF-stimulated MAPK activity (1.2-fold stimulation) was detected in extracts from proliferating MM14 cells (Fig. 7). When the cells were switched to differentiation medium (low serum, no bFGF) for 6 and 12 h, bFGF stimulated MAPK activity 3.7- and 3.5-fold, respectively (Fig. 7). At these same time points, rVH6 mRNA levels had dropped to 80% of maximal levels by 6 h and were barely detectable by 12 h (Fig. 6). Interestingly, the decrease in rVH6 mRNA levels correlated with the ability of bFGF to stimulate MAPK activity. These results suggest that rVH6 phosphatase activity may be responsible for the previously reported lack of bFGF-stimulated MAPK activation in proliferating myoblasts (46). Western blot analysis indicated that MAPK protein levels remained constant in proliferating MM14 cells and throughout the differentiation time course (data not shown).

**rVH6 Is Localized to the Cell Cytosol**—We expressed recombinant rVH6 protein with a COOH-terminal triple HA epitope tag in COS-1 cells to determine its cellular localization (Fig. 8). Full-length expressed rVH6 protein could be detected at both 24 and 48 h by Western blotting (data not shown). Detection of expressed rVH6 by immunofluorescence (48 h post-transfection) indicated a cytoplasmic localization (Fig. 8A). The cytoplasmic localization appears to be somewhat perinuclear, avoiding the extremities of the cell body. We also detected rVH6 at 24 h post-transfection, again localized to the cytoplasm (data not shown). In a parallel experiment, expressed hVH3 (containing a COOH-terminal c-myc epitope tag) localized to

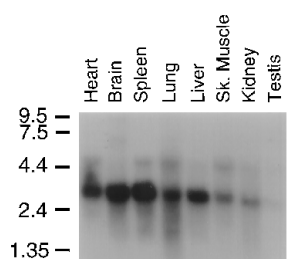


FIG. 2. **Tissue distribution of rVH6.** Rat multiple tissue Northern blot (Clontech) of poly(A)<sup>+</sup> RNA (2  $\mu\text{g}/\text{lane}$ ) was screened with a rVH6 antisense probe as described under "Experimental Procedures." RNA size markers are shown to the left.

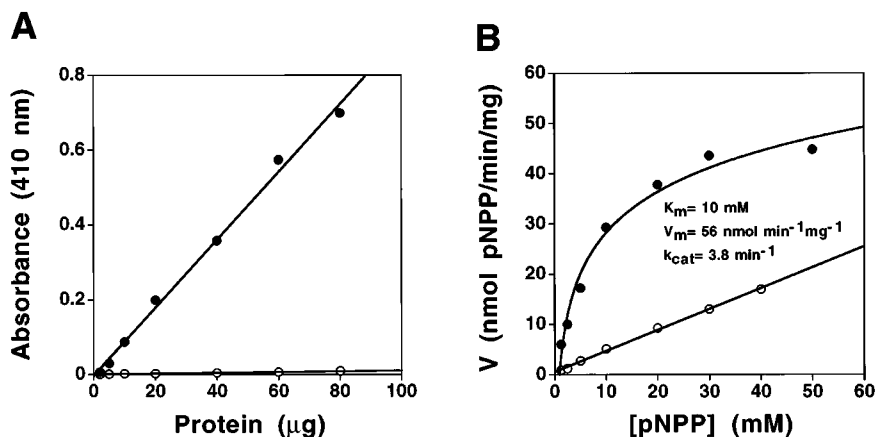


FIG. 3. **Kinetic analysis of rVH6 against pNPP.** A, hydrolysis of pNPP with increasing concentrations of recombinant GST-rVH6 (closed circles) or GST (open circles). B, hydrolysis of pNPP by GST-rVH6 (30  $\mu\text{g}$ ) in the absence (closed circles) or presence (open circles) of 1 mM sodium vanadate. Enzyme assays and determination of rVH6 kinetic properties were as described under "Experimental Procedures." Data points are the means of triplicate experiments with S.E. < 5%.

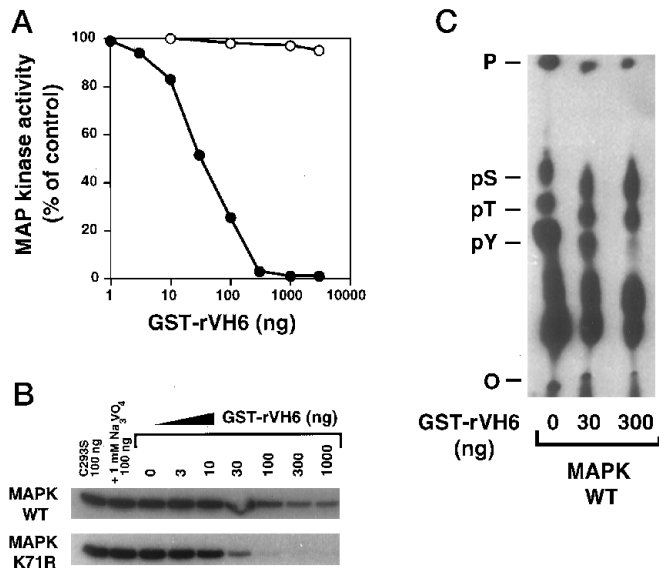


FIG. 4. **Inactivation of MAP kinase by rVH6.** A, increasing amounts of purified recombinant GST-rVH6 were assayed for their ability to inactivate p44 MAP kinase (0.2  $\mu$ g) in the absence (closed circles) or presence (open circles) of 1 mM sodium vanadate. The control MAPK activity was measured without GST-rVH6. B, autoradiograph of a SDS-polyacrylamide gel showing the dephosphorylation of  $^{32}$ P-labeled wild-type (WT) and mutant K71R MAP kinase treated with mutant C293S GST-rVH6, wild-type GST-rVH6 + 1 mM sodium vanadate, and increasing concentrations of GST-rVH6. C, phosphoamino acid analysis of  $^{32}$ P-labeled MAP kinase treated with indicated amounts of GST-rVH6. Data points in A are the mean values of triplicate experiments with S.E. < 5%.

the nucleus (Fig. 8C) as previously reported (16). Cytoplasmic localization of rVH6 was independent of enzymatic activity or epitope-tag, in that the inactive C293S rVH6 mutant or the wild-type *c-myc* epitope-tagged rVH6 constructs also localized exclusively to the cytoplasm (data not shown). Serum withdrawal or stimulation had no effect on intracellular localization (data not shown).

#### DISCUSSION

Tissue regeneration and serum stimulation have been shown to induce the expression of dsPTPs (9, 14–16). Certain dsPTPs also appear to play a role in proliferation and differentiation by regulating the activities of MAPKs (reviewed in Ref. 13). In olfactory epithelium, olfactory neurons undergo continuous regeneration, proliferation, and differentiation to replace damaged olfactory receptors (47). We therefore considered rat olfactory tissue as a likely source of novel dsPTPs. rVH6 was identified from rat olfactory cDNA by degenerate oligonucleotide PCR and subsequently cloned from rat brain cDNA. The cDNA codes for a protein that shares amino acid identity with other dsPTPs, including the extended PTP active site consensus sequence, VXVHCXXGXXRSXXXXXAY(L/I)M (34). All of the amino acids previously shown to be important for dsPTP catalytic activity are conserved in rVH6, including Cys<sup>293</sup>, the catalytic cysteine that functions as the active site nucleophile, and Ser<sup>300</sup> and Asp<sup>262</sup>, both involved in hydrolysis of the thiol-phosphate intermediate (48, 49). Similar to other dsPTPs, rVH6 contains two CH2 domains in an NH<sub>2</sub>-terminal extension. Although the functions of the CH2 domains are unknown, it has been suggested that they may confer substrate specificity or function via interactions with other regulatory proteins (36). The rVH6 protein also contains a serine-rich region between the NH<sub>2</sub>-terminal CH2 and COOH-terminal catalytic domains. This amino acid region containing two putative proline-directed serine kinase substrate recognition motifs may be ame-

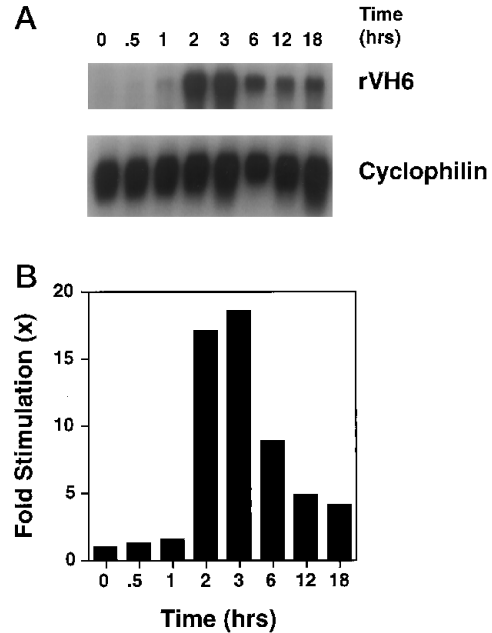


FIG. 5. **Induction of rVH6 expression in NGF-mediated neuronal differentiation.** Autoradiograph (A) and quantitation (B) of a PC12 cell poly(A)<sup>+</sup> RNA (2  $\mu$ g/lane) Northern blot hybridized with rVH6 and cyclophilin riboprobes. PC12 cells were serum-starved for 18 h and induced to differentiate with NGF (100 ng/ml) treatment for the indicated times. rVH6 message levels were quantitated and normalized against endogenous cyclophilin mRNA as described under "Experimental Procedures." Fold stimulation of rVH6 mRNA levels was calculated relative to levels at time 0 in the absence of NGF. The data shown are representative of a typical experiment repeated three times.

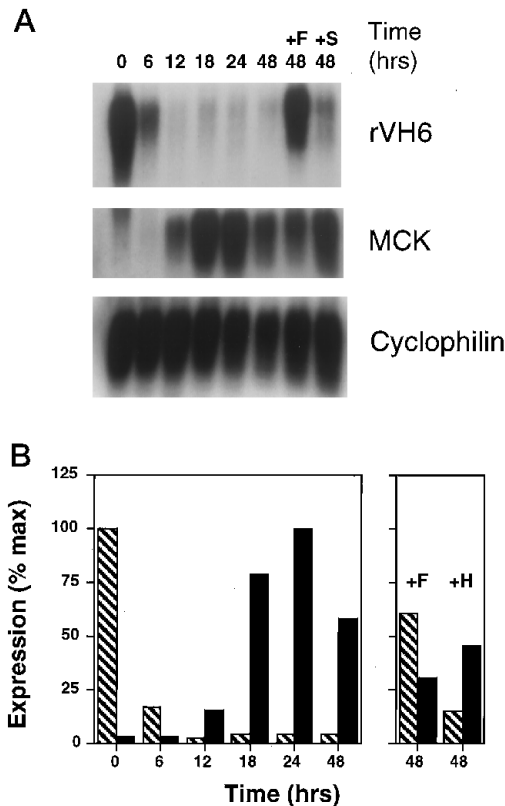
nable to phosphorylation, thereby potentially contributing to the regulation of the protein.

Bacterially expressed GST-rVH6 protein possesses PTP activity and hydrolyzes the artificial substrate, pNPP. The affinity of rVH6 for pNPP is 10- and 2-fold lower than that of VHR and cdc25, respectively (40, 41). The  $k_{cat}$  for pNPP hydrolysis is also 80-fold slower than VHR and more closely resembles cdc25 with only a 1.4-fold lower turnover rate. The differences in kinetic parameters between rVH6 and VHR may demonstrate a higher selectivity of rVH6 for its endogenous substrate. In contrast, rVH6 appears to have activity similar to CL100 in dephosphorylating and inactivating MAPK *in vitro*. CL100 inactivates MAPK both *in vitro* and *in vivo* and has therefore been suggested to be the physiological MAPK phosphatase-1 (8, 11). rVH6 inactivates MAPK at similar protein concentrations as CL100, 10 nM *versus* 7 nM, respectively (42), suggesting that rVH6 may also be able to physiologically recognize MAPK as a substrate and regulate its activity *in vivo*.

Several differences between rVH6 and other dsPTPs are noted. Although rVH6 will rapidly dephosphorylate phosphotyrosine in  $^{32}$ P-labeled MAPK, the rate of hydrolysis for phosphothreonine is much slower than with other dsPTPs (8, 16, 27, 40) and more closely resembles the dephosphorylation of p34<sup>cdc25</sup> by the dsPTP, cdc25 (41). Although dephosphorylation of phosphotyrosine is sufficient by itself to inactivate MAPK, the differences in rate of phosphothreonine hydrolysis may reflect differences in substrate specificity.

The intracellular localization of rVH6 is novel. Previously, expressed epitope-tagged dsPTPs (Pac-1, MAPK phosphatase-1, hVH2, and hVH3) have been immunolocalized to the nucleus (16, 19, 27, 50). rVH6 is the first dsPTP to be localized outside the nucleus. Interestingly, rVH6 contains two putative nuclear export signals (38, 39) (Fig. 1A), one of which falls within the serine-rich amino acid extension found between the

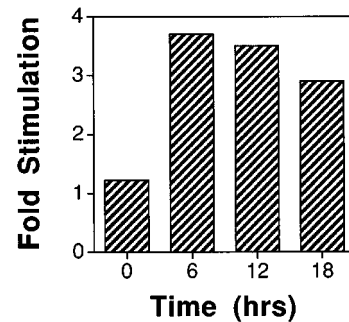




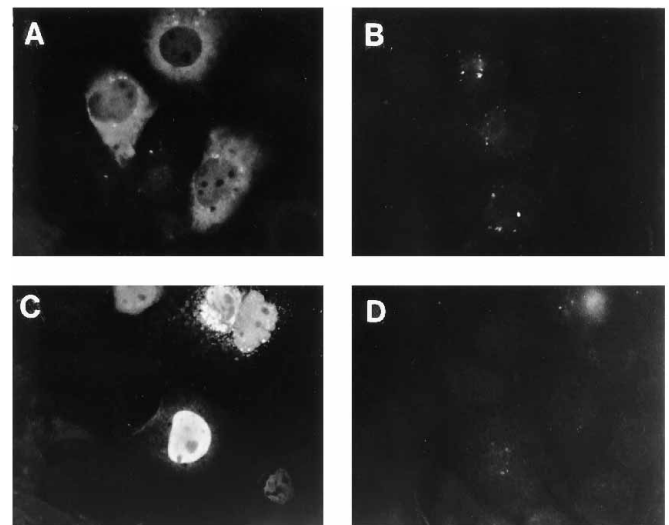
**FIG. 6. rVH6 expression in MM14 muscle cell differentiation.** Autoradiograph (A) and quantitation (B) of a MM14 cell poly(A)<sup>+</sup> RNA (1  $\mu$ g/lane) Northern blot hybridized with rVH6 (hashed bars), muscle creatine kinase (black bars), and cyclophilin riboprobes. Proliferating cells (0 h) were withdrawn from bFGF and high concentrations of serum. Isolation and analysis of the RNA at the indicated time points was performed as described under "Experimental Procedures." Lane +F was treated for 48 h in the presence of 2 ng/ml bFGF and 2% serum, whereas lane +S was treated with 15% serum and no bFGF. RNA levels were normalized against endogenous cyclophilin mRNA. The data shown are representative of a typical experiment repeated twice with similar results.

NH<sub>2</sub>-terminal CH2-containing domain and the COOH-terminal catalytic domain of rVH6 (Fig. 1B). We were unable to find these motifs in the NH<sub>2</sub>-terminal regions of any other mammalian dsPPT, suggesting that these nuclear export signals may be responsible for the novel cytoplasmic localization of rVH6. This intracellular localization could enable rVH6 to interact with potential substrates different from the nuclear dsPPTs, including the inactivation of MAPK prior to its nuclear translocation. In addition, 30–50% of MAPK activity has been shown to be associated with the microtubule cytoskeleton (51). This cytoskeletal MAPK could, therefore, be a potential substrate for a cytoplasmic dsPPT. Recently, other diphosphorylated MAPK family members have been identified (52–54). One of these members, p38, has been expressed and immunolocalized to both the cytoplasm and nucleus in COS-1 cells (55). Interestingly, following environmental stress (ultraviolet radiation), p38 shows an increased localization to the perinuclear region of the cytoplasm, an area rich in expressed rVH6. As more potential substrates are identified and examined, the question of rVH6 substrate specificity may be addressed.

Based on the abundant expression of rVH6 mRNA in brain, we wished to determine whether rVH6 was also expressed in PC12 cells and whether it played a potential role in cell proliferation or differentiation. MAPK can be activated in PC12 cells by both mitogenic (EGF or insulin) or differentiation (NGF) stimuli. The duration of MAPK activation has been suggested



**FIG. 7. MAP kinase activity during MM14 muscle cell differentiation.** MM14 cells were withdrawn from bFGF and high serum and harvested at the indicated times. Following a 5-min stimulation by bFGF (10 ng/ml), cell extracts were prepared, and MAPK activity was isolated and measured as described under "Experimental Procedures." The data are presented as fold stimulation of MAPK activity (bFGF-stimulated MAPK activity divided by non-bFGF-stimulated MAPK activity). Similar results were seen in two separate experiments.



**FIG. 8. rVH6 localizes to the cell cytoplasm.** The localization of expressed epitope-tagged rVH6 or hVH3 in COS-1 cells was visualized by immunofluorescence. HA-tagged rVH6 (A), anti-HA antibody control (B), c-myc-tagged hVH3 (C), and anti-myc antibody control (D) were detected with a fluorescein-conjugated anti-mouse secondary antibody as described under "Experimental Procedures." COS-1 cell immunofluorescence was photographed under a 40 $\times$  microscope objective.

as the controlling factor between proliferation (transient activation) or differentiation (sustained activation) (23). Some evidence suggests that the Ser/Thr phosphatase, PP2A, is involved in the rapid deactivation of MAPK activity in PC12 cells (43). dsPPTs, CL100, and hVH5 are transcriptionally induced as immediate early genes in response to EGF, insulin, or NGF (34, 42, 43). rVH6, however, is not expressed under mitogenic conditions following EGF or insulin stimulation of serum-starved PC12 cells. Rather, stimulation to differentiate with NGF results in sustained rVH6 mRNA expression 1–2 h later than the dsPPT immediate early genes. Moreover, the time course of rVH6 mRNA expression parallels the sustained activation and delayed inactivation of MAPK observed in NGF-treated PC12 cells (23). The inability of EGF or insulin to induce rVH6 expression, as well as the time course of rVH6 expression induced by NGF, suggests that rVH6 may play a role in PC12 neuronal differentiation.

In the mouse skeletal muscle cell line, MM14, FGF represses muscle differentiation and together with serum maintains cell proliferation (45). It has recently been shown that bFGF activates MEK but not MAPK in MM14 myoblasts that have been

withdrawn from mitogens for 3 h (46). However, both MEK and MAPK are activated by bFGF after 10 h of mitogen withdrawal. The inability of bFGF to activate MAPK in myoblasts appears to be due to a PTP activity that decreases with time following mitogen withdrawal (46). Interestingly, rVH6 mRNA is highly expressed in proliferating myoblasts but rapidly declines following mitogen withdrawal. After 6 h in differentiation medium, a time at which bFGF is able to stimulate MAPK activity (Fig. 7), the levels of rVH6 message have dropped by 80% (Fig. 6). Thus, expression of rVH6 is inversely correlated with the ability of bFGF to stimulate MAPK activity in MM14 cells. In contrast to PC12 cells, rVH6 expression in MM14 cells is highest in proliferating cells and lowest in differentiated cells.

In this study, we have identified a novel dual specificity phosphatase that is expressed in many tissues and appears to have a unique intracellular localization. rVH6 dephosphorylates and inactivates MAPK *in vitro*. The novel cytoplasmic localization of rVH6 may allow it to interact with substrates that are distinct from the substrates of nuclear dsPTP. Our results also suggest that rVH6 may play a role in promoting proliferation and repressing differentiation in MM14 muscle cells while promoting differentiation in PC12 neuronal-like cells.

**Acknowledgments**—We thank Drs. John M. Denu and Gaochao Zhou for helpful advice in the characterization of rVH6 and Lauri D. Aicher for assistance with the mouse multiple-tissue Northern blot analysis. We also acknowledge the generous gifts of Dr. Jeffrey Chamberlain for MM14 cells and muscle creatine kinase probe, Laura Mauro for the cyclophilin probe, Kunliang Guan and Elizabeth Butch for MAPK and MEK2, Seung Kwak for the hVH3-pcDNA expression construct, and Brian Largent for rat olfactory epithelium cDNA.

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