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Expression cloning of a human dual-specificity phosphatase

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ABSTRACT **Using an expression cloning strategy, we isolated a cDNA encoding a human protein-tyrosine-phosphatase. Bacteria expressing the kinase domain of the keratinocyte growth factor receptor (bek/fibroblast growth factor receptor 2) were infected with a fibroblast cDNA library in a phagemid prokaryotic expression vector and screened with a monoclonal anti-phosphotyrosine antibody. Among several clones showing decreased anti-phosphotyrosine recognition, one displayed phosphatase activity toward the kinase *in vitro*. The 4.1-kilobase cDNA encoded a deduced protein of 185 amino acids with limited sequence similarity to the vaccinia virus phosphatase VH1. The purified recombinant protein dephosphorylated several activated growth factor receptors, as well as serine-phosphorylated casein, *in vitro*. Both serine and tyrosine phosphatase activities were completely abolished by mutagenesis of a single cysteine residue conserved in VH1 and the VH1-related (VHR) human protein. These properties suggest that VHR is capable of regulating intracellular events mediated by both tyrosine and serine phosphorylation.**

The phosphorylation of tyrosine residues in proteins plays a critical role in fundamental cell functions (1–3). Protein tyrosine phosphorylation is often transient, being regulated within the cell by protein-tyrosine kinases (PTKs) and protein-tyrosine-phosphatases (PTPs) (4, 5). Recently, increasing attention has been focused on the growing family of PTPs. Like PTKs, PTPs have been implicated in cell signaling, cell growth and proliferation, and oncogenic transformation. Moreover, some PTPs can be involved in cell cycle regulation (6–8) and embryogenesis (9, 10).

Members of the PTP gene family share a high degree of homology in their catalytic domains (11, 12). A number of PTPs have been identified by screening libraries with a known PTP cDNA probe under low-stringency hybridization conditions or by using synthetic oligonucleotide probes generated from consensus sequences of PTP catalytic domains. These cloning methods are limited, however, to the identification of PTPs which are closely related to known PTPs. We sought to develop a method for identifying new PTP gene families while simultaneously facilitating the immediate production of active enzyme for biochemical characterization and antibody production. The report that a β-galactosidase (β-gal)–bek fusion protein was autophosphorylated when expressed in bacterial cells (13) prompted us to explore the following approach to cloning modulators of phosphorylation events. We reasoned that if a phagemid expression cDNA library was used to infect bacteria expressing β-gal–bek, plaques that were not recognized by anti-phosphotyrosine antibodies might contain clones encoding active PTPs. We describe here the cDNA cloning of a human PTP by this approach.† This enzyme shows limited sequence similarity to the vaccinia virus-encoded phosphatase VH1 (14), and it is referred to herein as VH1-related (VHR) phosphatase. We also show that bacterially expressed, purified VHR has substrate specificity for both phosphotyrosine and phosphoserine.

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MATERIALS AND METHODS

Plasmid Construction of bek Kinase. The *Eco*RI–*Sal* I fragment of the mouse keratinocyte growth factor (KGF) receptor cDNA (15), which includes the kinase and carboxyl-terminal domains (residues 361–707), was ligated to the *Eco*RI–*Sal* I fragment of the expression vector pCEV-lacZ to generate the plasmid pCEV-bek (Fig. 1*A*). pCEV-lacZ is a pUC-based plasmid expression vector that produces a β-gal fusion protein under the control of the *lac* promoter (see below).

Construction of λpCEV-lacZ Prokaryotic Expression Vector. The vector designed for prokaryotic cDNA expression cloning was similar to λgt11 but incorporated several additional features. cDNAs can be inserted by the automatic directional cloning method (16), allowing construction of unidirectional libraries with high efficiency. Use of unidirectional libraries to screen negative signals excludes antisense cDNA clones, which might inhibit the expression of cDNAs in host cells (like the bek kinase used in these experiments). It also increases the possibility of cDNA expression from the promoter in the vector. Since λpCEV-lacZ is a phagemid vector, the plasmid can be rescued by a simple procedure (17).

To construct λpCEV-lacZ, the *lacZ* structural gene (encoding β-gal) of pMC1871 (Pharmacia) was cloned into pUC-SVneo4ΔRI, a precursor plasmid of pCEV27 (17). Multiple cloning sites similar to those in λpCEV27 were created by inserting oligonucleotides into the *Eco*RI site of the *lacZ* gene. The resulting plasmid, pCEV-lacZ, was linearized at the *Not* I site and, after multiple excision sites were attached (17), cloned into λpCEV27 *Not* I arms to create λpCEV-lacZ.

Library Construction and Screening. An oligo(dT)-primed M426 human fibroblast cDNA library was constructed in λpCEV-lacZ. To screen the library, bacteria (*Escherichia coli* Y1091; Clontech) containing the pCEV-bek plasmid were infected with phage (1.2×10^4 plaque-forming units per 150-mm plate) and grown for 4 hr at 37°C on agar plates containing ampicillin (50 μg/ml). The plates were overlaid with nitrocellulose filters impregnated with 10 mM IPTG and were incubated for an additional 4 hr at 37°C, as described (18). The filters were removed, washed with phosphate-buffered saline containing 0.05% Tween 20 (PBST), and then blocked in PBST containing 3% dry milk for 1 hr at room temperature. Filters were probed with mouse monoclonal anti-phosphotyrosine (Upstate Biotechnology, Lake Placid, NY) overnight at room temperature, rinsed four times with PBST, incubated with ^{125}I-labeled protein A for 30 min,

Abbreviations: PTP, protein-tyrosine-phosphatase; β-gal, β-galactosidase; GST, glutathione *S*-transferase; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; KGF, keratinocyte growth factor; IPTG, isopropyl β-D-thiogalactopyranoside.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. L05147).

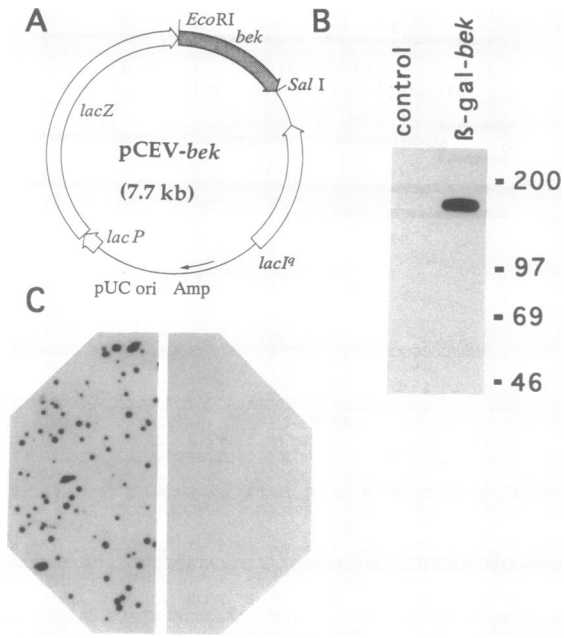


FIG. 1. Expression cloning strategy for PTPs. (*A*) Structure of pCEV-bek. The plasmid pCEV-bek encodes a *lac* repressor and the cytoplasmic domain of the mouse KGF receptor fused in frame to the *lacZ* gene under control of the isopropyl β-D-thiogalactopyranoside (IPTG)-inducible *lac* promoter. Shaded arrow, cytoplasmic domain of KGF receptor (*bek* gene product); kb, kilobases; *lacP*, *lacZ* promoter; *lacZ*, the structural gene for β-gal; *lacI^q*, *lac* repressor overproducer; Amp, ampicillin-resistance gene; ori, origin of replication. (*B*) Anti-phosphotyrosine immunoblot of control bacteria (lane 1) and bacteria expressing β-gal-bek kinase (lane 2). (*C*) Autoradiogram of two filters probed with anti-phosphotyrosine. The filters were lifted from a plate of bacteria expressing β-gal–bek and infected with control λ phage (*Left*) or with phage containing clone 27 cDNA (*Right*).

rinsed again, dried, and exposed to Kodak XAR film at −80°C overnight. Phage demonstrating decreased signals were isolated for further analysis.

Phosphatase Assays with Bacterial Extracts. Plasmids carrying cDNA inserts were rescued from the isolated phagemid clones as described previously (16). Cultures (50 ml) of *E. coli* Y1091 carrying either pCEV-bek, pCEV-lacZ (pCEV-lacZ vector without an insert), or pCEV clone 27 were grown and induced with 1 mM IPTG as described (19). Cells were collected by centrifugation and lysed in 1 ml of buffer containing 50 mM Hepes, pH 7.4/1% (vol/vol) Triton X-100/1 mM phenylmethylsulfonyl fluoride. Lysates were sonicated twice for 10 sec each, clarified by centrifugation, and tested for phosphatase activity as follows. Around 40 μl of *E. coli* extract from pCEV-bek was incubated with 10 μl of *E. coli* extract from pCEV-lacZ or pCEV test clone with or without 3 mM sodium vanadate at 37°C for 30 min. The reaction was stopped by boiling in SDS/PAGE sample buffer (20) for 3 min. The sample was divided into two equal aliquots, resolved by SDS/6.5% PAGE, and transferred to Immobilon (Millipore). Immunoblotting was performed with monoclonal anti-phosphotyrosine (Upstate Biotechnology, Lake Placid, NY) or monoclonal anti-β-gal (Promega) antibodies as described (21).

DNA Sequencing and Sequence Analysis. Plasmids carrying cDNA inserts were sequenced in both directions by using T7 polymerase (United States Biochemical) and the dideoxy termination method (22). Sequences were compared with the data bases by using the BLAST program (23).

Production and Purification of Bacterially Expressed VHR. A 0.8-kb *Sal* I–*Hin*dIII fragment containing the entire VHR coding region was blunt-ended with Klenow DNA polymerase, and *Bam*HI linkers were added. DNA was digested with *Bam*HI and inserted in frame into the *Bam*HI site of the pGEX-KT expression vector (24) to produce pGEX-VHR. Plasmid pGEX-VHR was used to express VHR as a fusion protein with glutathione *S*-transferase (GST). Expression and purification of GST-VHR were performed as described for a rat brain PTP (19); GST–VHR was >98% pure as determined by Coomassie blue staining of SDS/polyacrylamide gels. VHR protein obtained by thrombin cleavage of the purified GST fusion protein (19) was used for phosphatase assays.

Phosphatase Assays with Purified VHR. Dephosphorylation of *p*-nitrophenyl phosphate was performed as described (25).

Receptor-tyrosine kinases for epidermal growth factor (EGF), platelet-derived growth factor (PDGF), KGF, and insulin expressed in NIH 3T3 fibroblasts were stimulated with their respective ligands and partially purified as follows. Cells (2 mg of protein) were lysed with 1% Triton X-100 containing protease and phosphatase inhibitors at 0°C, and the lysates were clarified by centrifugation. Phosphotyrosine-containing proteins were immunoprecipitated with monoclonal anti-phosphotyrosine-agarose (Upstate Biotechnology, Lake Placid, NY) for 2 hr at 4°C, and the beads were washed three times in lysis buffer and then twice in the same buffer without phosphatase inhibitors. Proteins were eluted with 50 μl of 100 mM glycine (pH 2.5), neutralized with 2 μl of 1.5 M Tris (pH 9.0), and treated with purified GST–VHR or GST alone (1 μg) for 10 min at 25°C. Samples were then boiled in sample buffer (20), fractionated by SDS-PAGE, transferred to Immobilon membrane, immunoblotted with anti-phosphotyrosine, and detected with ^{125}I-labeled protein A. Autoradiographic imaging and quantitation were performed using a Molecular Dynamics PhosphorImager. Blots processed in parallel and probed with appropriate anti-receptor antibodies were used to identify receptor protein bands and to confirm that the amount of receptor protein was unchanged following phosphatase treatment.

Baculovirus-expressed PDGF receptor was purified to homogeneity (J. R. Beeler, R. Jensen, W. LaRochelle & S.A.A., unpublished data). Receptor (1–100 nM) was treated with VHR in 50 mM imidazole, pH 7.5/0.1% 2-mercaptoethanol for 10 min at 25°C. Reactions were stopped by addition of boiling sample buffer (20) and samples were subjected to SDS/PAGE and anti-phosphotyrosine/^{125}I-protein A immunoblotting. Dephosphorylation was quantitated with a Molecular Dynamics PhosphorImager.

Casein was phosphorylated with [^{32}P]ATP by cAMP-dependent protein kinase and enzymatically dephosphorylated as described (14). Serine-specific phosphorylation of casein was confirmed by phospho amino acid analysis (26).

Site-Directed Mutagenesis. The oligonucleotide 5′-TGCTCGTCCACTCCCGGGAAGGTTA-3′ was used to mutate VHR codon Cys-124 to Ser. A 0.8-kb *Sal* I–*Hin*dIII fragment containing an entire coding region of VHR was subcloned into our M13 vector. Site-directed mutagenesis was performed with an *in vitro* mutagenesis kit (United States Biochemical) according to the manufacturer's protocol. The mutation was confirmed by DNA sequencing. A fragment containing the mutation was subsequently cloned into pGEX-KT to produce mutant VHR protein. [Ser^{124}]VHR was purified and examined for its ability to dephosphorylate *p*-nitrophenyl phosphate and casein exactly as for wild-type VHR.

RESULTS

Expression Cloning of a PTP. Our strategy for expression cloning involved bacterial expression of an active bek kinase and screening of a phagemid cDNA expression library for clones capable of reducing the signal intensity of the autophosphorylated kinase as detected by anti-phosphotyrosine.

The bacterial expression plasmid, pCEV-bek, was designed to produce the KGF receptor cytoplasmic domain as a β-gal fusion protein (Fig. 1*A*). This construct expressed a 160-kDa protein that was readily detected by immunoblotting with anti-phosphotyrosine (Fig. 1*B*).

An M426 human lung fibroblast cDNA library in λpCEV-lacZ was used to infect bacteria containing the pCEV-bek plasmid, and 4.5×10^5 plaques were screened with anti-phosphotyrosine. Several clones displayed decreased signal intensity, and in two (clones 12 and 27), anti-phosphotyrosine recognition was completely abolished, as is shown for clone 27 (Fig. 1*C*). Dot blot hybridization under high-stringency conditions suggested that these two clones contained overlapping cDNA inserts. Clone 27, which contained the larger cDNA insert (4.1 kb), was analyzed further.

After plasmid rescue, the pCEV clone 27 insert was expressed in *E. coli* in order to investigate the biochemical basis for its activity. Lysates from *E. coli* expressing β-gal–bek were either incubated with pCEV-lacZ lysates (control) or with pCEV clone 27 lysates at 37°C and resolved by SDS/PAGE (Fig. 2). Immunoblotting with anti-β-gal revealed the 160-kDa β-gal–bek fusion protein, as well as the 137-kDa β-gal clone 27 fusion protein (Fig. 2*A*). Of note, incubation with the latter did not reduce the level of β-gal–bek (Fig. 2*A*), arguing against the possibility that clone 27 encoded a protease. Incubation with β-gal clone 27 lysates dramatically decreased the phosphotyrosine content of β-gal–bek, and this effect was inhibited by sodium vanadate, a known inhibitor of tyrosine phosphatases (Fig. 2*B*). Together, these data implied that clone 27 cDNA encoded a functional PTP.

Clones 12 and 27 had inserts of 1.8 kb and 4.1 kb, respectively. Sequence analysis confirmed that they were overlapping cDNAs of the same gene (Fig. 3). Northern analysis of M426 total RNA revealed a single transcript of about 4.1 kb recognized by the clone 27 cDNA probe, suggesting that clone 27 encompassed the entire VHR gene (data not shown). Nucleotide sequencing of the 4.1-kb insert revealed a short G+C-rich 5′ untranslated region, an open reading frame predicting translation of a 185-residue protein, and a long 3′ untranslated sequence containing a polyadenylylation signal (Fig. 3). The ATG codon at nucleotides 29–31 is the most likely start site for translation, according to Kozak criteria for authentic initiation codons (27). A stop codon, TGA, is present at nucleotides 584–586.

The amino acid sequence predicted from the VHR cDNA lacks an N-terminal signal-peptide consensus sequence or a characteristic transmembrane sequence. Accordingly, VHR is likely to be a cytosolic, soluble PTP. Comparison of VHR

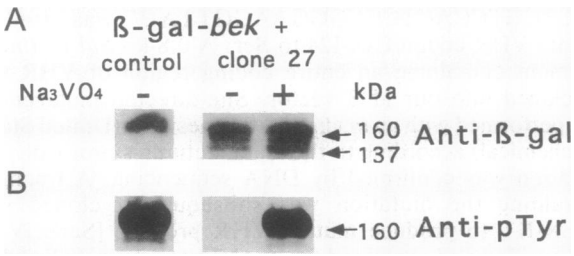
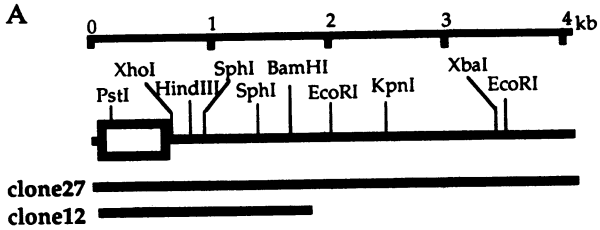


FIG. 2. PTP activity of recombinant VHR expressed in *E. coli*. Plasmid DNA rescued from a plaque showing decreased anti-phosphotyrosine staining (designated clone 27) was expressed in bacteria and a lysate was prepared. This lysate and lysate from bacteria expressing β-gal–bek were mixed and incubated at 37°C for 30 min in the presence or absence of sodium orthovanadate. A control mixture contained lysates prepared from bacteria expressing β-gal and bacteria expressing β-gal–bek. Reactions were terminated by boiling in SDS, and proteins were separated by SDS/PAGE and detected by immunoblotting with anti-β-gal (*A*) or anti-phosphotyrosine (anti-pTyr) (*B*).



B

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                                                          10
                                    M  S  G  S  F  E  L  S  V  Q  D
GCCGGGCGTGCAGGGCCCCGCCGCCGCCATGTCGGGCTCGTTCGAGCTCTCGGTGCAGGA    60
                          20                              30
  L  N  D  L  L  S  D  G  S  G  C  Y  S  L  P  S  Q  P  C  N
TCTCAACGACCTGCTCTCGGACGGCAGCGGCTGCTACAGCCTCCCGAGCCAGCCCTGCAA   120
                          40                              50
  E  V  T  P  R  I  Y  V  G  N  A  S  V  A  Q  D  I  P  K  L
CGAGGTCACCCCGCGGATCTACGTGGGCAACGCGTCTGTGGCTCAGGACATCCCCAAGCT   180
                          60                              70
  Q  K  L  G  I  T  H  V  L  N  A  A  E  G  R  S  F  M  H  V
GCAGAAACTAGGCATCACCCATGTGCTGAACGCGGCTGAGGGCAGGTCCTTCATGCACGT   240
                          80                              90
  N  T  N  A  N  F  Y  K  D  S  G  I  T  Y  L  G  I  K  A  N
CAACACCAATGCCAACTTCTACAAGGACTCCGGCATCACATACCTGGGCATCAAGGCCAA   300
                         100                             110
  D  T  Q  E  F  N  L  S  A  Y  F  E  R  A  A  D  F  I  D  Q
CGACACACAGGAGTTCAACCTCAGCGCTTACTTTGAAAGGGCTGCCGACTTCATTGACCA   360
                         120                             130
  A  L  A  Q  K  N  G  R  V  L  V  H  C  R  E  G  Y  S  R  S
GGCTTTGGCTCAAAAGAATGGCCGGGTGCTCGTCCACTGCCGGGAAGGTTATAGCCGCTC   420
                         140                             150
  P  T  L  V  I  A  Y  L  M  M  R  Q  K  M  D  V  K  S  A  L
CCCAACGCTAGTTATCGCCTACCTCATGATGCGGCAGAAGATGGACGTCAAGTCTGCCCT   480
                         160                             170
  S  I  V  R  Q  N  R  E  I  G  P  N  D  G  F  L  A  Q  L  C
GAGCATCGTGAGGCAGAACCGTGAGATCGGCCCCAACGATGGCTTCCTGGCCCAGCTCTG   540
                         180
  Q  L  N  D  R  L  A  K  E  G  K  L  K  P  *
CCAGCTCAATGACAGACTAGCCAAGGAGGGGAAGTTGAAACCCTAGGGCACCCCCACCGC   600

CTCTGCTCGAGAGGTCCGTGGGGGAGGCCGTGGGAAAGGTGTCCGAGCTGCCATGTTTAG   660
GAAACACACTGTACCCTGCTCCCAGCATCACAAGGCACTTGTCTACAAGTGTGTCCCAAC   720
ACAGTCCTGGGCCACTTTCCCCACCCTGGGGAGCACATAAAGAAGCTTGCCAAGGGGGGC   780
GTCCTTGCTCCCCAGTTGTCCTGTTTCTGTAACTTATGATGTCTTTTCCCTGAGATGGGG   840
GCTCAGAGGGGGAAGGCCTGT                                          861
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FIG. 3. VHR nucleotide sequence and predicted primary structure. (*A*) VHR cDNA is schematically shown with selected restriction sites. The open box represents coding sequence, while noncoding sequences are shown by adjoining lines. Clones 12 and 27, used for sequence analysis, are shown. (*B*) Nucleotide and predicted amino acid sequence of VHR cDNA. Amino acids are indicated in single-letter code and numbered above the sequence. Stop codon is indicated by a star.

with protein sequences in the Swiss-Prot (21.0) and GenPept (translated GenBank, Release 71.0) data bases as of May 5, 1992 revealed similarity to the recently published 3CH134 gene (28) and vaccinia virus phosphatase VH1 (14). The former is a serum-induced early response gene with no known function, and the latter is a viral enzyme which dephosphorylates both serine and tyrosine residues *in vitro*. A 30-amino acid stretch of VHR (codons 115–144) exhibited 50% identity to VH1 (Fig. 4). This domain includes a motif, HCXXXXXR, which is found in all known PTPs and is thought to include the catalytic site. The cysteine and arginine residues in this motif are essential to phosphatase activity (29, 30). Outside of this region, there was little if any sequence similarity with VH1; beyond a 15-amino acid stretch encompassing the HCXXXXXR motif, there was no detectable similarity with any other known phosphatase.

Phosphatase Activity of Purified VHR. A 0.8-kb *Sal* I–*Hin*dIII fragment containing the entire VHR-predicted coding region was inserted within the pGEX-KT vector (24). Control GST and GST–VHR fusion proteins were expressed in bacteria and purified. The ability of each protein to dephosphorylate *p*-nitrophenyl phosphate, a substrate of all known protein phosphatases, was assessed (Fig. 5). Whereas GST alone was devoid of phosphatase activity, the GST–VHR fusion protein hydrolyzed this substrate rapidly with a K_m in

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VHR      59  VLNAAEGRSFMHVNTMANFYKDSGITYLGIKANDTQEFNLSAYFERAADFIDQALAQKNG
3CH134  194  MLDALGITALINVSANCPNHFEGHYQYKSIPVEDNHKADISSWFNEAIDFID-SIKDAGG
VH1      46  PSSEVKFKYVLNLTMDKYTLPNSNINIIHIPLVDDTTTDISKYFDDVTAFLSKC-DQRNE
                                          *  *     *  *     *

VHR     119  RVLVHCREGYSRSPTLVIAYLMMRQKMDVKSALSIVRQNREIG-PNDGFLAQLCQLND
3CH134  253  RVFVHCQAGISRSATICLAYLMRTNRVKLDEAFEFVKQRRSIISPNFSFMGQLLQFES
VH1     105  PVLVHCAAGVNRSGAMILAYLMSKNKESLPMLYFLYVYHSMRD-LRGAFVENPSFKRQ
               * ** *   *  **     ****                   *
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FIG. 4. Sequence comparison of VHR with predicted 3CH134 and VH1 gene products. Amino acid sequence is shown in single-letter code and numbered at left. Boldface type highlights residues found in at least two of the sequences; identities among all three are indicated by stars.

the low micromolar range. The K_m was unchanged after cleavage of the VHR from GST with thrombin (data not shown). *p*-Nitrophenyl phosphate hydrolysis by the phosphatase was completely inhibited in the presence of 1 mM sodium vanadate; okadaic acid and sodium fluoride at similar concentrations had no inhibitory effect on the enzyme activity. All of these properties are characteristic of tyrosine phosphatases (4, 5).

We next examined the interaction of this enzyme with several tyrosine-phosphorylated growth factor receptors. Autophosphorylated receptors for PDGF, EGF, insulin, and KGF were immunoprecipitated with anti-phosphotyrosine-agarose, eluted with low pH buffer, neutralized, and treated with purified VHR or GST. After incubation, samples were subjected to SDS/PAGE and immunoblotted with anti-phosphotyrosine. Incubation with VHR decreased the anti-phosphotyrosine staining of all four receptors, as well as other phosphotyrosine-containing proteins in the same samples (Fig. 6*A*). Immunoblotting of parallel samples with the appropriate receptor antibodies showed that the amounts of receptor in GST- and VHR-treated samples were identical (data not shown). Further analysis of VHR phosphatase activity on highly purified PDGF receptor indicated that the K_m of this interaction was <100 nM.

Although VHR showed only limited similarity to VH1, we sought to determine whether the enzyme possessed serine phosphatase activity. In fact, VHR effectively dephosphorylated serine-phosphorylated casein (Fig. 6*B*). All of these findings indicated that VHR had dual specificity for phosphotyrosine and phosphoserine residues. The limited region of sequence similarity between VHR and VH1 contains a cysteine residue at position 110 that is essential for VH1 catalytic activity (14). Thus, we analyzed the effects of substitution of serine for the corresponding cysteine residue at position 124 in VHR (Fig. 4). This substitution resulted in complete loss of activity toward *p*-nitrophenyl phosphate (data not shown) and phosphorylated casein (Fig. 6*B*).

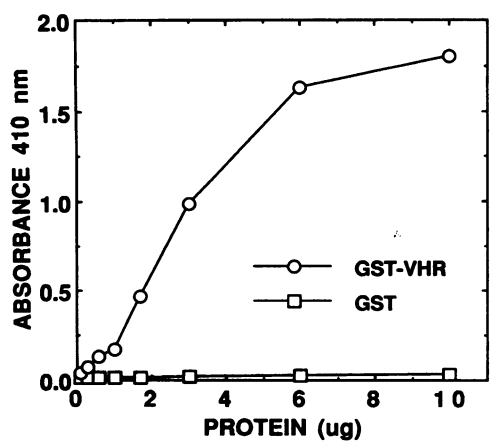

FIG. 5. Phosphatase activity of purified VHR. GST–VHR fusion protein and GST were expressed in bacteria and purified in parallel by using glutathione-agarose. Dephosphorylation of *p*-nitrophenyl phosphatase, expressed as increased absorbance at 410 nm, was measured as a function of protein concentration. The results shown are representative of three independent experiments.

DISCUSSION

In this report, we describe an expression cloning approach that led to the isolation of a human PTP. Because this

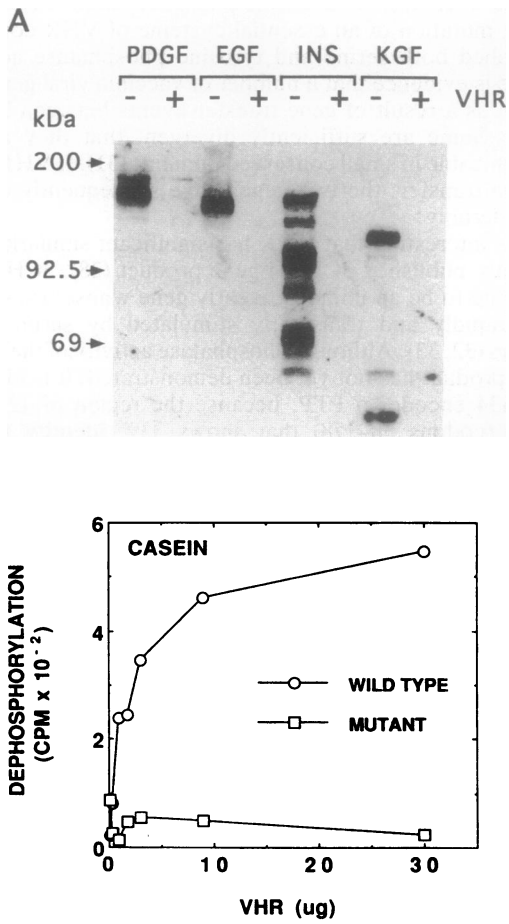

FIG. 6. Hydrolysis of phosphotyrosine and phosphoserine by VHR. (*A*) After stimulation with the mitogen indicated, phosphotyrosine-containing proteins were purified from NIH 3T3 cells overexpressing receptors for either PDGF, EGF, insulin (INS), or KGF. Purified GST (−) or VHR (+) was incubated with the purified phosphoproteins as indicated, and the reaction mixtures were subjected to SDS/PAGE and immunoblotting with anti-phosphotyrosine. Samples processed in parallel and blotted with appropriate receptor antibodies showed that the amounts of receptor in GST- and VHR-treated samples were identical (data not shown). (*B*) [^{32}P]Phosphocasein was prepared using cAMP-dependent protein kinase. Phosphorylation exclusively on serine was confirmed by phospho amino acid analysis (data not shown). Substrate was incubated with purified VHR (wild type) or [Ser^{124}]VHR (mutant), and dephosphorylation (expressed as [^{32}P]phosphate release) was measured as a function of enzyme concentration. Results are representative of several experiments. Mutation of Cys-124 to Ser also abolished *p*-nitrophenyl phosphate activity (data not shown).

approach is based upon function rather than on sequence similarity, it offers the possibility of identifying PTPs which may be only distantly related to known PTPs. The phosphatase isolated bears very little sequence similarity to known PTPs, and conventional methods, such as low-stringency hybridization, would not have allowed its detection. The use of prokaryotic expression cloning also affords the opportunity to produce large quantities of the enzyme. This makes it possible to characterize PTP activity rapidly and efficiently prior to extensive sequence analysis of the cloned cDNA.

Among the PTPs from bacteria to human, VHR was found to be most similar biochemically to a phosphatase encoded by the vaccinia virus late gene H1 (14). VH1 is a 20-kDa PTP that is only distantly related to the eukaryotic PTPs and that efficiently dephosphorylates both phosphotyrosine- and phosphoserine-containing substrates *in vitro* (14). We found that VHR displayed phosphatase activity *in vitro* toward *p*-nitrophenyl phosphate, as well as a number of tyrosine kinase receptors and serine-phosphorylated casein. Like VH1, mutation of an essential cysteine of VHR completely abolished both serine and tyrosine phosphatase activities. There is evidence that a number of vaccinia viral genes have arisen as a result of gene transfer events between host and virus. Some are sufficiently divergent that they are only recognizable in small conserved domains (31). If VH1 reflects such a transfer, the two genes have subsequently diverged considerably.

It is interesting that VHR has significant similarity to the recently published 3CH134 gene product (28). 3CH134 was reported to be an immediate early gene whose transcription was rapidly and transiently stimulated by serum growth factors (32, 33). Although phosphatase activity of the 3CH134 gene product has not yet been demonstrated, it is likely that 3CH134 encodes a PTP, because the region of 121 amino acids (codons 58–178) that shows 33% identity to VHR includes the putative active site within the HCXXXXXR motif.

CDC25 of *Schizosaccharomyces pombe* is the only previously identified eukaryotic phosphatase with substrate specificity for both tyrosine and serine/threonine phosphate residues. This enzyme is thought to dephosphorylate Tyr-15 and Thr-14 of p34/CDC2 kinase in a complex with cyclin B (reviewed in ref. 34). The catalytic activity of CDC25 is stimulated 4- to 5-fold in the presence of cyclin B, and it is otherwise rather inefficient against substrates such as *p*-nitrophenyl phosphate and reduced, carboxamidomethylated, and maleylated lysozyme *in vitro* (35, 36). In contrast, we have shown that VHR exhibits broad substrate specificity *in vitro*. These differences and their lack of significant sequence similarity outside of the conserved HCXXXXXR motif suggest that CDC25 and VHR define two distinct subfamilies of tyrosine/serine/threonine phosphatases.

There is accumulating evidence for a class of protein kinases with dual specificity for tyrosine and serine/threonine residues (37). Members include *S. pombe* wee1 and mik1, a redundant homolog of wee1, which act in opposition to CDC25 with respect to the regulation of p34/CDC2 kinase (34). Other dual specificity kinases include MAP kinase activator (38, 39), which has been implicated in mitogenic signaling in mammalian cells. If wee1 kinase were to phosphorylate both of the residues that regulate p34/CDC2 kinase, it would be tempting to speculate that VHR may act in opposition to some other dual-specificity protein kinase on substrates involved in cellular growth regulation.

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