Extracellular Regulated Kinases (ERK) 1 and ERK2 Are Authentic Substrates for the Dual-specificity Protein-tyrosine Phosphatase VHR

A NOVEL ROLE IN DOWN-REGULATING THE ERK PATHWAY*

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The mammalian dual-specificity protein-tyrosine phosphatase VHR (for VH1-related) has been identified as a novel regulator of extracellular regulated kinases (ERks). To identify potential cellular substrates of VHR, covalently immobilized mutant VHR protein was employed as an affinity trap. A tyrosine-phosphorylated protein(s) of ~42 kDa was specifically adsorbed by the affinity column and identified as ERK1 and ERK2. Subsequent kinetic analyses and transfection studies demonstrated that VHR specifically dephosphorylates and inactivates ERK1 and ERK2 in vitro and in vivo. Only the native structure of phosphorylated ERK was recognized by VHR and was inactivated with a second-order rate constant of 40,000 M⁻¹ s⁻¹. VHR was found to dephosphorylate endogenous ERK, but not p38 and JNK. Immunodepletion of endogenous VHR eliminated the dephosphorylation of cellular ERK. Transfection studies in COS-1 cells demonstrated that in vivo phosphorylation of epidermal growth factor-stimulated ERK depended on VHR protein levels. Overexpression above endogenous levels of VHR led to accelerated ERK inactivation, but did not alter the normal activation of ERK. Unique among reported mitogen activated protein kinase phosphatases, VHR is constitutively expressed, localized to the nucleus, and tyrosine-specific. This study is the first to report the identification of authentic substrates of dual-specificity phosphatases utilizing affinity absorbents and is the first to identify a nuclear, constitutively expressed, and tyrosine-specific ERK phosphatase. The data strongly suggest that VHR is responsible for the rapid inactivation of ERK following stimulation and for its repression in quiescent cells.

The dual-specificity protein tyrosine phosphatases (DS-PTPs) are members of a large family of enzymes that catalyze the phosphomonoester hydrolysis of protein substrates (1). The DS-PTPs were identified as protein-tyrosine phosphatases (PTPs) that are capable of efficiently hydrolyzing phosphotyrosine as well as phosphothreonine/serine residues. Approximately 15–20 distinct DS-PTPs have been identified to date. The first identified DS-PTP (VH1, for vaccinia open reading frame H1) was discovered in the H1 open reading frame of vaccinia virus (3). More recently discovered DS-PTPs have been suggested to play a central role in the regulation of the cell cycle and signaling pathways mediated by the families of MAP (mitogen-activated protein) kinases (4). The MAP kinases facilitate intracellular signaling events triggered by mitogens, growth factors, and stress that result in cellular growth, differentiation, and death (5, 6). The MAP kinases are activated by their specific upstream dual-specificity kinases (MAP-kinase kinases) through phosphorylation on both threonine and tyrosine residues in the TXY motif. In response to extracellular stimuli, activated MAP kinases then phosphorylate an array of cellular substrates, including nuclear transcription factors. In mammals, three distinct MAP kinase families have been identified. Members of one family, ERK1 and ERK2, are primarily activated by growth and differentiation factors as well as by phorbol esters (7). The JNK family (or SAPK) and p38 MAP kinase family are activated by proinflammatory cytokines and environmental stress.

Several DS-PTPs have been implicated in ERK regulation by dephosphorylation of both Thr183 and Tyr185 (8–11). Studies have shown that such DS-PTPs, termed MAP kinase phosphatases (MKPs), harbor distinct substrate preferences for the various MAP kinase families (11–14). All of the MKPs identified to date, with the exception of Pyst1, are immediate-early genes that are induced by various mitogens, growth factors, and stresses. The induction of MKPs by distinct stimuli and their cell-specific expression suggest that there is a great diversity in the function of the various MKPs. For example, MKP-1 is induced by stress, mitogens, and phorbol esters and is localized to the nucleus (8, 15). PAC-1 is induced by mitogens and T-cell activation and is found in the nucleus of hematopoietic cells (9, 16). MKP-3/VH6 is predominantly cytosolic and is induced by nerve growth factor (17, 18). While MKP expression generally correlates with MAP kinase inactivation (8–14), numerous reports have demonstrated inconsistencies with a model in which MKPs act as the primary catalysts for dephosphorylation and inactivation of ERK1 and ERK2 (19–26). In many cells, ERK1 and ERK2 inactivation is rapid (within 15–20 min) and does not require new protein synthesis (19–26). Accordingly, MKP RNA/protein expression is normally not detectable until 30–60 min after stimulation (9, 10, 17, 18, 23,
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27, 28), and expression of MKP-1 and MKP-2 is actually induced by the ERK1 and ERK2 cascade (28). With the exception of Pyst1 (11), MKPs are not expressed in quiescent cells (29), and disruption of the mKp-1 gene does not affect mouse development and normal MAP kinase activity was observed in MKP-1-deficient fibroblasts (30). Collectively, these observations argue that a phosphatase(s) distinct from the MKPs is responsible for the rapid down-regulation of ERK.

It has been suggested that a constitutively expressed phosphatase is directly involved in ERK1 and ERK2 inactivation. In rat mesangial cells, sustained ERK2 activation by endothelin and EGF was regulated by a vanadate-sensitive protein phosphatase but not by a transcriptionally regulated protein (20). Rapid inactivation of ERK2 in 3T3-L1, PC12, and PAE cells was attributed to the serine/threonine protein phosphatase PP2A and an unknown PTP distinct from MKP-1 (22). The protein synthesis inhibitor cycloheximide failed to affect the inactivation of MAP kinase following induction with EGF in A431 and PC12 cells (21, 23), suggesting that MKPs are not involved. Others have proposed that a vanadate-sensitive and tyrosine-specific phosphatase is responsible for the repression of ERK1 and ERK2 activity in the absence of serum and that a tyrosine phosphatase regulates ERK1 and ERK2 activity in cells transformed by upstream oncogenes (19).

In the present study, we have utilized affinity trapping methods to identify potential cellular substrates of the mammalian VHR phosphatase (for VHR-related), a putative dual-specificity PTP that was previously identified by an expression cloning strategy (31) and has been the subject of detailed biochemical and structural analyses (1, 32–36). The physiological function has been uncertain since no in vivo substrates had been identified. The D92A catalytic mutant of VHR was covalently coupled to a solid matrix which then served as an affinity absorbent for specific tyrosine-phosphorylated proteins from hydrogen peroxide-stimulated COS1 cells. We selected hydrogen peroxide (H₂O₂) as a mitogen for two main reasons. We have previously demonstrated that H₂O₂ will rapidly and reversibly inactivate VHR and other PTPs by selectively oxidizing the catalytic cysteine thiolate (37). Knowing that endogenous VHR is highly expressed in COS-1 cells, we surmised that treatment with H₂O₂ would inactivate the endogenous VHR, leading to increased phosphorylation of its authentic protein substrate(s). Extracts from H₂O₂-treated COS-1 cells would then provide an enriched fraction of the physiological substrate. Moreover, reactive oxygen species such as H₂O₂ have been shown to stimulate increases in relevant cellular tyrosine phosphorylation (38–43), and intracellular generation of H₂O₂ has been shown to be required or involved in growth factor signaling pathways (44, 45), perhaps as a second messenger. Potent activation of extracellular regulated protein kinase (ERK) is observed with H₂O₂ treatment (46–49).

The phosphorylated forms of ERK1 and ERK2 were specifically retained and eluted from the VHR affinity columns. Kinetic and cellular expression studies further demonstrated that ERK1 and ERK2 are authentic in vitro and in vivo substrates for VHR. Our data suggest that VHR is a constitutively expressed and tyrosine-specific phosphatase localized to the nucleus and is responsible for the repression of ERK1 and ERK2 in quiescent cells and for their rapid inactivation following stimulation.

MATERIALS AND METHODS
Reagents and Mammalian Vectors—Full-length cDNA fragments of VHR or C124S and D92A proteins were cloned using the pdCNA3 vector (Invitrogen) to generate pdCNA3-VHR and pdCNA3-C124S, respectively. Both constructs direct the expression of all 185 amino acids, with no vector added sequences. Antisense VHR construct (pcDNA3-anti-VHR) was generated by removing the XhoI and EcoRI insert from pT7-7-VHR and inserting into the XhoI and EcoRI sites of pcDNA3. The D92A mutant was generated using the Bio-Rad Mutazyme gene method as described previously (35). Recombinant ERK2 was purified and phosphorylated by recombinant MEK1 fused to GST (50). The c-myc-ERK2 pcEXV-n plasmid was a gift from Dr. Phil Stork (14). All constructs were verified by DNA sequencing. Rabbit polyclonal anti-VHR antibodies specific to VHR were immunopurified from the serum of VHR-(full-length) immunized rabbits (Cocalico). Chicken immunopurified anti-VHR antibody was generated by immunizing chickens with the full-length VHR protein. The IgY fraction was purified from egg yolk (Aves Labs). Both VHR antibodies were affinity purified over a column containing recombinant VHR conjugated to Affi-Gel 10. Chicken antibodies were eluted with 0.1 M sodium phosphate (pH 2.5) and stored in PBS. Rabbit antibodies were eluted with 0.1 M sodium phosphate (pH 2.5) and stored in PBS.

Western Blotting—Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to Immobilon-P polyvinylidene difluoride (Millipore) membrane at 125 V for 45 min. Membranes were rinsed with TBS containing 0.05% Tween 20 (TBST) then blocked with TBST containing either 5% bovine serum albumin (BSA) (Sigma) or non-fat dry milk for 1 h at 25 °C. Blots were then incubated with primary antibodies diluted in TBST containing either 3% BSA or non-fat dry milk overnight at 4 °C. The blots were then rinsed with TBST (3 times, 15 min at 25 °C) and then incubated in the appropriate secondary antibody diluted in TBST containing either 3% BSA or 0.05% non-fat dry milk for 1 h at 25 °C. The blots were rinsed another 3 times for 5 min with TBST before detection by enhanced chemiluminescence (ECL) (NEN Life Science Products Inc.). Blots were stripped by incubating in 62.5 mM Tris-HCl, 2% SDS, 100 mM 2-mercaptoethanol (pH 6.7) for 30 min at 50 °C. Stripped blots were then rinsed extensively with TBST and reprobed as described above. The following antibodies were used: rabbit polyclonal antibody (rAb) specific to p44/42 MAPK (New England Biolabs), rAb specific to the phosphorylated forms of p44/42 MAPK (Thr202/Tyr204) and p38 MAPK (Thr180/Tyr182) (New England Biolabs), rAb specific to the phosphorylated forms of SAPK/JNK (Thr183/Tyr185) (Promega), rAb specific to VHR that was immunopurified from the serum of VHR-(full-length) immunized rabbits (Cocalico), mouse monoclonal antibody specific to anti-phosphotyrosine (4G10) (Upstate Biotechnology), monoclonal antibody-agarose conjugated and unconjugated specific to c-Myc (9E10) (Santa Cruz Biotechnology), goat anti-rabbit IgG-horseradish peroxidase conjugate (Bio-Rad), and horse anti-mouse IgG-horseradish peroxidase conjugate (New England Biolabs).

Substrate Trapping—Human VHR (C124S and D92A) proteins were both expressed and purified as described (35) and coupled to Affi-Gel 10 (Bio-Rad). Affi-Gel 10 (2 ml) was washed 5 times with ice-cold 50 mM MES (pH 7.0) and incubated with 2 ml of 0.5 mg/ml D92A or C124S VHR for 1 h at 4 °C. Unreacted activated esters were capped with 200 μl of 1 mM ethanolamine (pH 8.0) for 1 h at 4 °C and the coupled resin was washed extensively with 50 mM Tris, 50 mM NaCl (pH 6.5) (wash buffer). Alternatively, Affi-Gel 10 was washed and allowed to dry in air without protein coupling; COS-1 cells were grown to 80–90% confluence in 150-mm plates with Dulbecco’s modified Eagle’s medium containing low glucose, l-glutamine, sodium pyruvate, 10% fetal bovine serum, penicillin at 1000 units/ml, and streptomycin at 1000 μg/ml (Life Technologies, Inc.) (growth media). Cells were then treated with 200 μM H₂O₂ for 40 min at 37 °C. After treatment, cells were rinsed once with 50 mM Tris, 150 mM NaCl, 1 mM EDTA (pH 7.2), harvested, and frozen at −20 °C. Where needed, cells were lysed in 1 ml of ice-cold 50 mM Bis-Tris, 50 mM NaCl, 1% Nonidet P-40, protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 5 μg/ml aprotinin) (pH 6.5), sonicated for 30 s on ice, Dounce homogenized for 15 strokes, and centrifuged at 15,000 rpm for 10 min. Clarified extract was applied to the affinity column, attached to a Beckman Biosys 510 liquid chromatography system running at a flow rate of 1 ml/min. The column was washed with wash buffer (3 × column volumes) and retained phosphoproteins were eluted with 10 ml of 0.2 mM sodium arsenate. The eluant was collected and the level of tyrosine-phosphorylated proteins, ERK, and phosphorylated ERK were measured by Western blot analysis.

Transient Transfection of COS-1 Cells—COS-1 cells were seeded at 1.0 × 10⁶ in 100-mm plates and grown for 16–24 h. LipofectAMINE (Life Technologies, Inc.) (30 μl) and purified plasmid DNA (at 5 μg of c-myc-ERK and 1 μg all others) were combined in 500 μl of Opti-MEM I (Life Technologies, Inc.) and incubated at 25 °C for 20 min. Cells were then transfected once with Opti-MEM I and the Lipid/DNA solution was added dropwise to the cells which were then covered with Opti-MEM I (5 ml). After 6–8 h, transfected cells were placed in growth media for
16–24 h. Cells were then serum starved and left for 16–24 h before treatment with 100 nM EGF (Collaborative Biomedical Products) for 15 min at 37 °C. After treatment, cells were rinsed once with PBS, lysed with 500 μl of ice-cold 20 mM Tris, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 2 mM, 1 mM sodium vanadate, 10 mM sodium fluoride, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 M phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 2.5 μg/ml aprotinin, sonicated on ice for 30 s, and centrifuged at 15,000 rpm for 10 min. Protein concentrations were determined by the method of Bradford. Immunoprecipitation of c-myc-ERK was then performed on 500 ng of cell lysate using 10 μl of the agarose-conjugated mAb (9E10) specific to c-Myc in a total volume of 1 ml for 2 h at 4 °C. Agarose beads were washed twice with 500 μl of 50 mM Tris, 0.5 M NaCl, 5% sucrose, 0.2% Nonidet P-40 (pH 7.3) for 2 min at 4 °C and resuspended in 2 × Laemmli buffer.

Immunofluorescence—Cells were seeded at 5 × 10^5 cells per well in two-well chamber slides and grown for 24 h. The cells were then rinsed twice with PBS, fixed with 50 μl of ice-cold 20 mM Tris, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 2 mM, 1 mM sodium vanadate, 10 mM sodium fluoride, 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 2.5 μg/ml aprotinin, sonicated on ice for 30 s, and centrifuged at 15,000 rpm for 10 min. Protein concentrations were determined by the method of Bradford. Immunoprecipitation of c-myc-ERK was then performed on 500 ng of cell lysate using 10 μl of the agarose-conjugated mAb (9E10) specific to c-Myc in a total volume of 1 ml for 2 h at 4 °C. Agarose beads were washed twice with 500 μl of 50 mM Tris, 0.5 M NaCl, 5% sucrose, 0.2% Nonidet P-40 (pH 7.3) for 2 min at 4 °C and resuspended in 2 × Laemmli buffer.

**DEPHOSPHORYLATION IN COS-1 CELL EXTRACT**—Serum-starved COS-1 cells were treated with 200 μM H2O2 for 40 min at 37 °C. Cells were rinsed in PBS, harvested, and lysed as described above. Recombinant VHR, or an equal volume of buffer, was added to a final effective concentration of 0–0.16 mM and incubated for 20 min at 30 °C. The reactions were quenched by adding 5 × Laemmli sample buffer containing SDS and subjected to Western analysis.

**DEPHOSPHORYLATION OF RECOMBINANT ERK2 BY VHR**—Recombinant VHR, or an equal volume of buffer, at a final effective concentration of 0.1 μM was combined with 2.5 μM recombinant phosphorylated ERK2 in 50 mM Tris, 50 mM Bis-Tris, 100 mM sodium acetate, 1 mM dithiothreitol, 0.01% BSA (pH 7.0) and incubated for up to 40 min at 25 °C. Aliquots were withdrawn at the times indicated and the reactions were terminated by adding 5 × Laemmli sample buffer containing SDS and subjected to Western analysis.

**A**. anti-phosphotyrosine Western blot of H2O2-treated COS-1 cells (lane 1), arsente elution of the D92A VHR affinity column (lane 2), and arsente elution of control column (lane 3); C, anti-phospho-ERK Western blot from B. The anti-phosphotyrosine blot from B was stripped and reprobed with the phospho-ERK antibody (“Experimental Procedures”). D, quantitation of an elution profile. Fractions were analyzed for protein content (Bradford method) (diamonds), anti-ERK (squares), or anti-phospho-ERK (circles) immunoreactivity (densitometry of fluorescence intensity). Within each data set, the strongest signal was given a value of 1, and each point was determined relative this maximal intensity.
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**Fig. 2.** ERK is rapidly and specifically dephosphorylated by wild type VHR phosphatase. Lysates made from COS-1 cells treated with H2O2 for 40 min were incubated with 0–0.16 μM recombinant VHR for 20 min at 25 °C. A, anti-phosphoseryl phosphatase Western blot showing the rapid tyrosine dephosphorylation of the ~42-kDa protein in COS-1 cell extracts; B, Western blot from A was stripped and reprobed with the anti-phospho-ERK antibody (upper panel in B) and separately with the anti-ERK antibody (lower panel in B); C, time-dependent dephosphorylation of native ERK in COS-1 cell extracts by the anti-phospho-ERK Western blot analysis. Lysates in C were made from COS-1 cells treated with H2O2 for 40 min was incubated with 0.4 μM recombinant VHR, or an equal volume of buffer, for 0–40 min at 25 °C.

**RESULTS**

**Affinity Trapping of ERK1 and ERK2 by the VHR Phosphatase**—In the current study, we used affinity chromatography to identify cellular substrates of VHR. The catalytic mutant D92A was covalently linked to a solid phase and was employed to retain authentic substrates from extracts of H2O2-stimulated COS-1 cells (Fig. 1). This method involved passing extract over the affinity column, washing away nonspecific proteins, and eluting high-affinity phosphorylated proteins with the competitive inhibitor arsenate (Kₐ of 10 μM (33)). Arsenate will only displace phosphorylated proteins that associate with VHR through the active-site. Although VHR is a putative member of the dual-specificity subfamily of PTPs, phosphopeptide substrate analysis had indicated that VHR prefers phosphotyrosine over phosphoserine/threonine by 1000-fold (33). Given this significant preference for phosphotyrosine, we postulated that authentic substrate(s) would be tyrosine-phosphorylated and could thus be detected by anti-phosphotyrosine Western blot analysis.

A major tyrosine-phosphorylated protein(s) of ~42 kDa was retained and eluted specifically from the VHR affinity columns. Fig. 1, B–D, is a representative example from three independent experiments employing D92A columns. A control column (Affi-Gel 10 resin capped with ethanolamine) was run under the identical conditions to confirm that binding of retained proteins was specific for VHR and not the resin. Panel A is an anti-phosphotyrosine Western blot demonstrating H2O2-induced tyrosine phosphorylation in COS-1 cells. Panel B is an anti-phosphotyrosine Western blot comparing COS-1 cell extract elutions from D92A and control columns. Elution from the D92A column clearly indicated the specific retention of a tyrosine-phosphorylated protein(s) of ~42 kDa. Given the size of the observed protein, we explored the possibility that this band(s) was the MAP kinase ERK. The anti-phosphotyrosine Western blot was then reprobed with an antibody specific to the active form (diphosphorylated on Thr¹⁸³ and Tyr¹⁸⁵) of ERK1 and ERK2 (51–53). The ~42-kDa protein(s) coincided exactly with the immunoreactive bands of ERK1 and ERK2 (Fig. 1C). To demonstrate that only the phosphorylated form of ERK is retained, a detailed analysis of an elution profile was performed (Fig. 1D). Fractions were analyzed for protein content (Bradford method), anti-ERK, or anti-phospho-ERK immunoreactivity (densitometry of immunofluorescence intensity). The majority of total ERK (predominantly unphosphorylated) coelutes with the general protein peak. In contrast, almost half of the total phosphorylated ERK1 and ERK2 is retained on the VHR column and rapidly eluted with arsenate. These data suggest that although the majority of ERK protein is unphosphorylated, only phosphorylated ERK can bind to VHR. A significant portion of the phosphorylated ERK pool elutes with the general protein peak, suggesting that the capacity of the column may have been exceeded or that a fraction of the phosphorylated ERK is not accessible to VHR.

ERK1 and ERK2 Are Rapidly and Specifically Dephosphorylated by the VHR Phosphatase—The VHR affinity columns demonstrated that active ERK1 and ERK2 bind specifically to immobilized VHR. Whether ERK1 and ERK2 were true substrates for the VHR phosphatase remained to be verified. We therefore examined the dephosphorylation kinetics and substrate specificity of wild type VHR for ERK1 and ERK2 from cellular extracts of COS-1 cells stimulated with H2O2. Low levels of H2O2 induce physiologically relevant tyrosine phosphorylation and transient inactivation of constitutively expressed PTPs. Purified recombinant human VHR phosphatase was added in increasing concentrations to the stimulated extract (Fig. 2, A and B). Strikingly, a protein(s) of ~42 kDa was rapidly dephosphorylated at low levels (0.08 μM) of VHR (Fig. 2A). The 42-kDa protein was almost completely dephosphorylated before any significant dephosphorylation was detected on the entire pool of tyrosine-phosphorylated proteins. Not unexpectedly, with much higher levels of VHR there was a corresponding general loss of immunoreactivity toward other phosphotyrosine proteins. To verify that this ~42-kDa protein(s) corresponded to ERK, the anti-phosphotyrosine Western blot
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Among all substrates examined to date, this represents the substrate and reflects both binding affinity and catalytic efficiency. The kinetics of ERK1 and ERK2 dephosphorylation by VHR were then investigated (Fig. 2B). VHR was capable of rapidly dephosphorylating ERK1 and ERK2 from stimulated extracts. At 0.4 μM VHR, ERK1 and ERK2 were >80% dephosphorylated in 20 min, whereas the buffer control displayed no hydrolysis even after 40 min. In the buffer control samples, the lack of ERK dephosphorylation by endogenous VHR or other ERK-specific phosphatases is consistent with the transient inactivation of PTPs from H2O2-treated cells (37, 46).

Recombinant ERK2 Is Efficiently Dephosphorylated by VHR—In order to firmly establish that VHR was responsible for the direct dephosphorylation of ERK in cellular extracts, purified recombinant ERK2 was utilized as a substrate for purified VHR. ERK2 and a constitutively active form of MEK were overexpressed separately in bacteria and purified using nickel-affinity chromatography (50, 54). ERK2 was then stoichiometrically phosphorylated and purified to homogeneity by anion exchange chromatography. The purified and phosphorylated ERK2 was then examined as a substrate of VHR. With recombinant ERK2, steady-state levels of phosphorylated ERK2 could be used for the dephosphorylation assay and accurate kinetic analyses could be performed. ERK2 (2.3 μM) was reacted with 0.1 μM VHR and the inactivation of ERK2 was assessed by the myelin basic protein kinase assay (Fig. 3). The progress curve was fitted to the integrated Michaelis-Menten equation (33) and yielded a second-order rate constant of 4.04 × 10^3 ± 4.2 × 10^3 M^-1 s^-1 (Fig. 3). Being a physiologically relevant parameter, the k_cat/K_m value is the second-order rate constant for the reaction of free enzyme with free substrate and reflects both binding affinity and catalytic efficiency. Among all substrates examined to date, this represents the largest measured value (33). For comparison, the commonly used PTP substrate p-nitrophenyl phosphate is catalyzed 80-fold less efficiently.

To establish that inactivation of ERK2 by VHR is the direct result of dephosphorylation, the phosphorylation status of ERK2 was correlated with kinase activity. VHR was reacted with active phosphorylated ERK2, the phosphorylation state was quantitated by anti-active ERK Western analysis, and the corresponding kinase activity was determined. Quantitation of the initial velocities resulted in curves with identical slopes (data not shown), indicating that VHR inactivated ERK by direct dephosphorylation and demonstrating that immunoreactivity of the phospho-specific ERK antibody accurately reflects changes in ERK activity.

Only the Native Structure of ERK2 Is Recognized by VHR—Next, we investigated whether the native structure of ERK is required for efficient substrate recognition by VHR. It is predicted that the specificity toward an authentic substrate would be mediated through the recognition of the folded protein. If binding and catalysis are not specific, rates of hydrolysis between folded and unfolded protein should be comparable. We therefore examined the ability of VHR to recognize and dephosphorylate unfolded ERK. Active ERK2 was either maintained in its native conformation or unfolded by heat denaturation and employed as a substrate for VHR (Fig. 4A). With excess VHR (0.87 μM) and 0.5 μM phosphorylated ERK2, no dephosphorylation was observed over 40 min in the heat-denatured sample (Fig. 4A, lane 3), whereas native phosphorylated ERK (Fig. 4A, lane 2) was hydrolyzed to more than 80% of the control (Fig. 4A, lane 1). The remaining 20% of this particular phosphorylated ERK preparation was resistant to dephosphorylation by VHR (Fig. 4A, lane 2), suggesting that this 20% fraction was unfolded. Heat-denatured ERK was found to be completely inactive as a kinase, verifying loss of the native structure (data not shown). These data suggest that only native ERK is recognized by VHR.

VHR Is Specific for Phosphotyrosine 185 of ERK—Phosphoamino acid analysis of the VHR-catalyzed dephosphorylation of ERK indicated that VHR specifically hydrolyzed Tyr185 but not Thr183 (Fig. 4B). Catalytic amounts of VHR or MKP3 (0.5 μM) were reacted with active recombinant ERK (5 μM) and the specific dephosphorylation at both tyrosine and threonine was assessed by phosphoamino acid analysis. The autoradiogram clearly demonstrates that VHR rapidly catalyzes the specific dephosphorylation of tyrosine while no significant hydrolysis at phosphothreonine was observed (up to 1 h). In contrast, MKP3/VH6 (17, 55) rapidly hydrolyzed both phosphoamino acids (Fig. 4B). The buffer control demonstrated that nonenzymatic phosphoester hydrolysis over the 1-h time course was insignificant. The lower overall signal of phosphotyrosine (relative to phosphothreonine) in control samples is due to the acid-labile phosphoester of tyrosine.

To corroborate these findings, the specific tyrosine dephosphorylation was followed by anti-phosphoamino acid Western blot analysis (Fig. 4C). With both VHR (0.5 μM) and MKP3 (0.5 μM), rapid tyrosine dephosphorylation of ERK (5 μM) was observed. By 10 min, greater than 90% of the phosphotyrosine was hydrolyzed. The rates of dephosphorylation by VHR and MKP3 were comparable, with MKP3 reacting approximately 2-fold more rapidly. No hydrolysis was observed in the buffer control. The rapid dephosphorylation kinetics presented in Figs. 3 and 4, B and C, are in excellent agreement, yielding k_cat/K_m values between 20,000 and 40,000 M^-1 s^-1. The VHR dephosphorylation data presented in this study were determined by four independent approaches: 1) anti-phospho-ERK Western analysis (Fig. 2C); 2) ERK kinase activity assays (Fig. 3); 3) phosphoamino acid analysis (Fig. 4B); and 4) anti-phospho-tyrosine Western blot analysis (Fig. 4C).

VHR Does Not Significantly Dephosphorylate p38 and JNK—The ability of VHR to discriminate between the different MAP kinases was tested. Since H2O2 treatment (200 μM) of COS-1 cells leads to only modest activation either of p38 or...
Western analysis. Buffer and subjected to anti-phosphotyrosine actions were quenched by Laemmli sample at 30 °C (pH 7). Time points were taken and subjected to phosphoamino acid analysis ("Experimental Procedures"). The levels of phosphotyrosine and phosphothreonine were assessed (Fig. 6). Extracts were incubated with either capped or a heat denatured sample of 0.5 μM, VH6 (0.5 μM), or a buffer control was mixed with phosphorylated ERK2 (5.0 μM) at 30 °C (pH 7). At the times indicated, the reaction was quenched by Laemmli sample buffer and subjected to anti-phosphotyrosine Western analysis.

JNK stress MAP kinases (Fig. 5, B and C), anisomycin was used to provide robust activation of these stress kinase pathways. Anisomycin, originally identified as an antibiotic, has been extensively utilized to induce p38 and JNK MAP kinase pathways (56, 57). Interestingly, anisomycin and H2O2 are equally potent stimulators of the ERK pathway (Fig. 5A). Recombinant VHR (0.16 μM) or a buffer control were added to extracts generated from anisomycin and H2O2-treated COS-1 cells. The phosphorylation status of both p38 and JNK were then evaluated by Western blotting with antibodies specific to the phosphorylated forms of these proteins (Fig. 5, D and E). The dephosphorylation of p38 and JNK is not appreciably affected by exogenously added VHR. The dephosphorylation of p38 is rapid whether or not VHR is added, although a slight increase is noted in the presence of VHR. These data indicate the presence of a p38-specific phosphatase which rapidly dephosphorylates the kinase and is insensitive to 200 μM H2O2. Although we cannot rule out the possibility that endogenous VHR is responsible for this rapid p38 activity, it is unlikely since VHR would be rendered inactive by the H2O2 treatment. The phosphorylation state of JNK is unchanged in both the buffer control and the sample with recombinant VHR. These results are in contrast to our finding that VHR rapidly and specifically dephosphorylates ERK from H2O2 treated COS-1 cells (Fig. 2). In the above experiment, COS-1 cells were treated with both anisomycin and H2O2 to ensure that any PTPs present in the reaction will be transiently inactivated. When extracts from anisomycin-treated COS-1 cells were utilized, a significant amount of ERK dephosphorylation was observed in the buffer control (see below). These results suggested that endogenous VHR or another PTP is active in anisomycin-treated COS-1 cell lysates.

Immunodepletion of Endogenous VHR Eliminates ERK Dephosphorylation—To explore the idea that VHR is the ERK phosphatase responsible for its rapid inactivation, endogenous VHR was immunodepleted from extracts of anisomycin-treated COS-1 cells and the time course of ERK dephosphorylation was assessed (Fig. 6). Extracts were incubated with either capped Affi-Gel 10 (mock treated) or Affi-Gel 10 coupled to an antibody (chicken polyclonal) raised against VHR. The lysates were then separated from the beads and the dephosphorylation of ERK and p38 were analyzed by Western blotting with antibodies specific to their respective phosphorylated forms (Fig. 6). Immunodepletion of endogenous VHR resulted in a marked decrease in the dephosphorylation of ERK over time (Fig. 6A), but had no effect on the dephosphorylation of p38 (Fig. 6B). After 20 min, the mock-treated sample showed nearly complete ERK dephosphorylation (Fig. 6A) while the immunodepleted sample displayed only a slight drop in ERK phosphorylation. VHR Western blot analysis indicated that >90% of the endogenous VHR was removed by immunodepletion (Fig. 6C). The slight ERK dephosphorylation observed after 20 min is consistent with the small amount of residual VHR. These results suggest that VHR may account for all the ERK phosphatase activity observed in COS-1 cell extracts.

VHR Phosphatase Is Widely Expressed in Mammalian Cells—To examine endogenous VHR expression levels, Western blotting was performed on a variety of mammalian cell lines. Using an immunopurified rabbit anti-human VHR antibody, Western blotting of whole cell extracts revealed an immunoreactive protein band of 20,600 Da corresponding to VHR in all cell lines tested (Fig. 7A). Using quantitative Western blot analysis of recombinantly expressed VHR (data not shown), we estimated that VHR constitutes as much as 0.1% of the Nonidet P-40 dissolved cellular protein. High levels of protein expression were observed in COS-1, COS-7, CV-1, and A431 cell lines. Similar high expression was also observed in human breast cancer cell lines BT474, SKBR3, and T47D and breast cell line HBL-100. VHR thus appears to be widely and highly expressed in cells, suggesting that it plays a fundamental role in cellular processes. It should be noted that VHR protein levels are not altered by serum, EGF, or other mitogenic stimulation (data not shown). This is in direct contrast to the expression of previously reported MKPs whose protein levels are only detectable after 30 min of mitogenic stimulation. Immunostaining of endogenous VHR in COS-1 cells revealed that VHR resides primarily in the nucleus, however some cytoplasmic staining was also observed (Fig. 7B).

ERK Is an in Vivo Substrate of VHR—To provide evidence for the in vivo dephosphorylation of ERK by VHR, we investigated whether ERK phosphorylation in EGF-treated COS-1 cells was dependent on VHR protein levels. To accomplish this, we cotransfected COS-1 cells with ERK2 (c-Myc tagged) and either a wild type VHR or an RNA antisense VHR construct. Serum-starved cells were stimulated with EGF for 20 min, myc-ERK2 was recovered from lysates via immunoprecipitation, and the level of ERK2 phosphorylation was determined. Overexpression of wild type VHR leads to almost complete ERK dephosphorylation after 20 min of EGF stimulation relative to the
FIG. 5. Activation of ERK, p38, and JNK pathways and analysis of VHR specificity. Activation of ERK, p38, and JNK pathways (A, B, and C). COS-1 cells were grown to 80–90% confluence and serum starved prior to treatment with 10 μg/ml anisomycin or 100 μM H₂O₂ for 30 min. Cells were then lysed and then activated in the presence of protease inhibitors. Activation of each kinase by anisomycin or H₂O₂ was determined by Western blots using antibodies specific for the phosphorylated forms of ERK (A), p38 (B), or JNK (C). Total MAP kinase levels were determined from anti-ERK, anti-p38, and anti-JNK Western analysis, shown in the lower panels of A-C. Analysis of VHR specificity (D and E). Extracts from anisomycin- and H₂O₂-treated COS-1 cells were combined with 0.16 μM recombinant VHR, or an equal volume of buffer, and incubated at 30 °C for the indicated times. D, Western blot analysis using an antibody specific to the phosphorylated form of p38 in the presence of buffer (top panels) or wild type VHR phosphatase (bottom panels). E, Western blot analysis using an antibody specific to the phosphorylated form of JNK, in the presence of buffer (top panels) or wild type VHR phosphatase (bottom panels). Total p38 and JNK levels were determined from anti-p38 and anti-JNK Western analysis and are included (D and E).

FIG. 6. Immunodepletion of endogenous VHR eliminates the in vitro dephosphorylation of ERK. COS-1 cells were grown to 80–90% confluence and serum starved prior to treatment with 10 μg/ml anisomycin for 30 min. Cells were then lysed in the presence of protease inhibitors and incubated with either capped Affi-Gel 10 (mock treated) or a specific chicken anti-VHR antibody coupled to Affi-Gel 10. The beads were removed and depleted lysates were then incubated at 30 °C for 20 min prior to Western blot analysis (A–C). Western blot analysis using antibodies specific to the phosphorylated forms of ERK (A), or p38 (B) for both mock-depleted or VHR-depleted lysates at 0 and 20 min. Total ERK and p38 levels were determined from anti-ERK and anti-p38 Western analysis and are shown in the lower panels; C, VHR Western blot analysis (immunopurified rabbit anti-VHR antibody, “Experimental Procedures”), showing that VHR protein levels are greatly diminished upon immunodepletion.

antisense vector control (Fig. 8A, left panel), but did not effect the initial activation of ERK (Fig. 8 and data not shown). To verify protein levels, Western blots of VHR (Fig. 8B), immunoprecipitated c-myc-ERK (Fig. 8C) and expressed c-myc-ERK were performed (Fig. 8D). A 6-fold decrease in ERK phosphorylation (Fig. 8A, left panel) and a >6-fold increase in VHR protein levels (Fig. 8B) were calculated in cells transfected with the wild type vector over cells transfected with the antisense control. This indicates that there is a direct correlation between the phosphorylation of ERK and the cellular levels of VHR. We were aware of the possibility that transfection of VHR antisense RNA could lower the expression of endogenous VHR in our studies (58). Direct detection of endogenous VHR levels between the pcDNA3 and pcDNA3-antisense-VHR transfected cells was difficult because of the 10–30% transfection efficiency. However, VHR Western blots of total cell lysates suggested there is a slight decrease of VHR protein levels in cells transfected with pcDNA3-antisense-VHR compared with pcDNA3. Although these small changes were within experimental error, it is quite possible that transfection with the antisense plasmid resulted in significant suppression of endogenous VHR protein. Regardless, the pcDNA3-antisense-VHR plasmid is a suitable control for comparisons with pcDNA3-VHR transfected cells.

DISCUSSION

Using a variety of approaches including affinity chromatography, enzyme kinetics, and cellular transfection studies, we demonstrated that ERK1 and ERK2 are authentic in vitro and in vivo substrates of VHR. Our data suggest a novel role for VHR in down-regulating the ERK pathway, distinct from that proposed for the MKPs. Identifying physiological substrates of DS-PTPs and PTPs is a fundamental step toward defining their roles in regulating signaling pathways.

Affinity Chromatography—A substrate trapping approach has been recently employed using the protein-tyrosine phosphatases PTP1B (59), YopH (60), and TCPTP (61). However, instead of identifying substrates from immunoprecipitated complexes (59–61), we have used affinity chromatography to absorb substrates to catalytically inactive mutants of VHR. With this approach, phosphoproteins only interact with the
coupled phosphatase and the use of immune complexes is avoided. Utilizing this technique, we have identified ERK1 and ERK2 as authentic substrates of the dual-specificity phosphatase VHR. With the D92A VHR affinity absorbent, the phosphorylated forms of ERK1 and ERK2 were specifically retained, and eluted with the strong competitive inhibitor arsenate (Fig. 1). This method should have wide utility for determining the physiological substrates of other dual-specificity phosphatases and PTPs.

**In Vitro Dephosphorylation of ERK1 and ERK2 by VHR—**

Detailed biochemical and kinetic experiments were then performed to validate ERK1 and ERK2 as physiological substrates of VHR. Our study demonstrated that VHR is highly specific for ERK1 and ERK2 (Fig. 2), that VHR recognizes the native structure (Fig. 4A), and that VHR dephosphorylates ERK with a second-order rate constant of 40,000 $\text{M}^{-1}\text{s}^{-1}$ (Fig. 3). VHR was shown to have no effect on the dephosphorylation of JNK and only slight effect on p38 dephosphorylation (Fig. 5, D and E). In addition, immunodepletion of endogenous VHR nearly eliminated dephosphorylation of cellular ERK but not of p38, suggesting that VHR accounts for nearly all of the ERK phosphatase activity in COS-1 cells. VHR catalyzes the specific hydrolysis of phospho-Tyr185 from activated ERK (Fig. 4B) from activated ERK (Fig. 4B). With those considerations, the rapid dephosphorylation (40,000 $\text{s}^{-1}\text{s}^{-1}$) of ERK by low catalytic levels of VHR supports the proposal that ERK1 and ERK2 are relevant substrates. We believe that a stringent kinetic analysis is critical to any investigation in which the validation of physiological substrates of DS-PTPs and PTPs is required.

**In Vivo Dephosphorylation of ERK by VHR—** We demonstrated that the in vivo phosphorylation of ERK depends on VHR protein levels in EGF-stimulated COS-1 cells (Fig. 8). These transfection studies provide convincing evidence that ERK1 and ERK2 are in vivo targets of the VHR phosphatase. These studies complement the detailed biochemical and kinetic investigations which provide direct proof for the specific binding and efficient catalysis of ERK by VHR. Overexpression of VHR resulted in a 6-fold decrease in the total level of phosphorylated ERK following 20 min of stimulation, as compared with a VHR antisense control vector (Fig. 8A). In addition, the kinetics of VHR-dependent dephosphorylation indicate that overexpression of VHR leads to rapid ERK inactivation following normal activation (Fig. 8A and data not shown).

Western blot analysis demonstrated that total VHR protein levels do not change upon EGF, H$_2$O$_2$, and serum stimulation, consistent with a previous report of unaltered RNA transcription upon mitogenic stimulation (10). It should be again noted that significant levels of endogenous VHR protein were observed in COS-1 cells. As a consequence, transient overexpression of VHR in COS-1 cells simply results in elevating the concentration of a normally expressed protein. We estimate that overexpression of VHR results in a 6-fold or greater increase above normal levels (Fig. 8B). It would be predicted that only the rate of VHR's normal function would be accelerated by increasing the enzyme level. As predicted, the dephosphorylation rate of ERK is enhanced by higher in vivo concentrations of VHR.

**Role of VHR in ERK Signaling—** Constitutive expression and nuclear localization of VHR is consistent with the proposal that nuclear VHR is involved in the rapid inactivation of ERK by dephosphorylating Tyr185. Our data would also suggest that VHR may function in quiescent cells to maintain ERK in the inactive state. In this capacity, VHR would ensure that spurious or below threshold activation is maintained until appropriate stimulation is communicated to the cell. As discussed earlier, a constitutively expressed and tyrosine-specific phosphatase has been implicated in direct inactivation of ERKs. In rat mesangial cells, sustained ERK2 activation by endothelin and EGF was regulated by a vanadate-sensitive protein phosphatase but not by a transcriptionally regulated protein (20). Rapid inactivation of ERK2 in a variety of cell lines was attributed to the serine/threonine protein phosphatase PP2A and an unknown PTP (22). The
protein synthesis inhibitor cycloheximide failed to affect the inactivation of MAP kinase following induction with EGF in A431 and PC12 cells (21, 23). Gopalbhai et al. (19) have proposed that a tyrosine-specific phosphatase represses ERK1 and ERK2 activity in the absence of serum, and that a tyrosine phosphatase regulates ERK1 and ERK2 activity in cells transformed by upstream oncogenes. The data presented in the current study strongly suggests that VHR is the candidate phosphatase responsible for these previously described phenomena.

**Single and Dual-specificity Protein Phosphatases—** Upon dual-phosphorylation by MEK, activated ERK is translocated to the nucleus where it regulates expression of immediate-early genes (63–66). Subsequent dephosphorylation of ERK is then thought to mediate its exit from the nucleus. Our results are consistent with a model in which the dephosphorylation of nuclear ERK by VHR may lead to its removal from the nucleus. Because dual-phosphorylation is required to maintain high activity (1000-fold activation) (65), the actions of single-specificity phosphatases are sufficient to inactivate the MAP kinases. The constitutive and serine/threonine-specific phosphatase PP2A may act in concert with VHR by dephosphorylating Thr183 of ERK (21, 22, 67). The cell’s use of single-specificity phosphatases may reflect an additional level of regulation for ERK activation, not yet fully realized. This type of regulatory mechanism prompts the consideration of several interesting areas for future study, including the regulation of ERK by single-specificity phosphatases, the mechanism of subsequent reactivation of monophosphorylated ERK by MEK or single specificity kinases, the relevance of ERK auto-phosphorylation, and the subcellular distribution of the various phosphorylated forms of ERK. Although dephosphorylation of ERK promotes its nuclear translocation and retention, the consequent trafficking of the monophosphorylated ERKs has not been described. The fission yeast phosphatase Pyp1 inactivates the stress-activated MAP kinase Spc1/Sty1 by specific dephosphorylation of Tyr173 within the activation motif (68), suggesting that MAP kinase regulation by single-specificity phosphatases may be conserved throughout eukaryotic evolution.

Feedback regulation of the MAP kinase pathways is thought to be mediated by the inducible MKPs which act to turn off the pathway by dephosphorylating both threonine and tyrosine. The tyrosine specificity of VHR distinguishes this phosphatase from these truly dual-specific MKPs. The physical basis for the two distinct specificities has not been established. However, the major structural differences between VHR and the MKPs may hold the answer. VHR is a single domain structure consisting of the catalytic core that is conserved among all DS-PTPs (36). The MKPs harbor a catalytic domain, but also contain an amino-terminal domain. In the case of MKP3, this amino-terminal domain binds ERKs independently of phosphorylation and causes a 34-fold activation of MKP3 phosphatase activity (69). In the absence of this activation MKP3 is a rather inefficient enzyme. The amino-terminal domain is thus thought to provide high affinity binding to ERK. Using small diphasphorylated peptides corresponding to the activation lip of the MAP kinases, it has been demonstrated that both VHR and MKP3 prefer hydrolysis at phosphotyrosine relative to phosphothreonine in the context of these synthetic peptides (33, 55). For VHR, the preference for phosphotyrosine hydrolysis is 1000-fold higher than for phosphothreonine. The phosphothreonine hydrolysis by MKP3 could not be detected. The 1000-fold lower activity was concluded to reflect the lower affinity for phosphotyrosine. For the MKPs, like MKP3, the amino terminus may provide the basis for the efficient phosphothreonine hydrolysis observed against diphasphorylated ERK protein. Independent of phosphorylation, the high affinity binding of the MKP amino-terminal domain to ERK provides an efficient means to increase the “effective concentration” of both phosphomonoesters, leading to efficient catalytic rate enhancement. On the other hand, since VHR appears to bind to ERK in classical enzyme/substrate fashion, there is no added energetic basis to compensate for the 3–4 orders of magnitude difference in the intrinsic reactivity between phosphotyrosine and phosphothreonine. We are currently exploring this model as a mechanism for dual-specificity of the MKPs.

**Regulation of VHR and PTPs—** The proposed function of VHR raises an interesting question regarding constitutively expressed PTPs. That is, how is a growth signal maintained if the PTPs that function to down-regulate the pathway are present during mitogenic signaling? It is likely that a negative regulatory mechanism exists to turn off the PTPs. Support for negative regulatory mechanisms has come from several investigations (37, 71–74).

Numerous reports have now indicated that cellular redox status plays an important role in the mechanisms which regulate the function of growth factors and tyrosine phosphorylation-dependent signal transduction pathways (38–43). Hydrogen peroxide has been shown to stimulate a complete program of mitogenic signal transduction. Hydrogen peroxide-treated cells mimic the phosphorylation cascades that are induced by several growth factors such as EGF (44) and platelet-derived growth factor (45). Potent activation of ERK is observed after H2O2 treatment (Refs. 46–49 and see “Results”). Recent reports have indicated that the cellular production of H2O2 is involved in normal receptor-mediated signal transduction. In vascular smooth muscle cells, platelet-derived growth factor transiently increased the intracellular concentration of H2O2 (45). When growth factor stimulation of intracellular H2O2 production was blocked, tyrosine phosphorylation, MAP kinase activation, DNA synthesis, and chemotaxis were inhibited. Similarly, EGF treatment induced the transient intracellular generation of H2O2 in human epidermoid carcinoma cells (75).
and the tyrosine kinase activity of EGF receptor was required for the production of H$_2$O$_2$. There is now significant evidence that H$_2$O$_2$ is generated by receptor-mediated events and thereby may play a critical role in signal transduction. We (37) and others (74) have proposed that PTPs are a logical target of H$_2$O$_2$, leading to transient and reversible inactivation of phosphatase activity by oxidizing the catalytic cysteine to a sulfenic acid (37).

In support of this proposal, we have observed robust activation of ERK by low levels (200 µM) of H$_2$O$_2$ in COS-1 cells. Curiously, only slight activation of the stress MAP kinase p38 was observed (Fig. 5, B and C). Treatment of COS-1 cells with H$_2$O$_2$ eliminates the dephosphorylation of ERK by endogenous VHR (Fig. 2). Addition of reduced thiols recovers the ERK-specific phosphatase activity (data not shown). In anisomycin-treated cells, VHR rapidly dephosphorylates ERK (Fig. 6A), indicating that VHR is not inactivated during anisomycin stimulation. Collectively, the results suggest that VHR is transiently inactivated in H$_2$O$_2$-treated cells and are consistent with a model of PTP inactivation in which the catalytic cysteine is reversibly oxidized. These observations support a mechanism of ERK regulation whereby H$_2$O$_2$ negatively regulates the activity of VHR, allowing for full ERK activation during mitogenic stimulation.

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