Vaccinia H1-related Phosphatase Is a Phosphatase of ErbB Receptors and Is Down-regulated in Non-small Cell Lung Cancer*§

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Vaccinia H1-related phosphatase (VHR) is classified as a dual specificity phosphatase. Unlike typical dual specificity phosphatases, VHR lacks the MAPK-binding domain and shows poor activity against MAPKs. We found that EGF receptor (EGFR) was a direct substrate of VHR and that overexpression of VHR down-regulated EGFR phosphorylation, particularly at Tyr-992. Expression of VHR inhibited the activation of phosphatase of ErbB2. Consistent with these results, suppression of VHR augmented the foci formation ability of H1299 non-small cell lung cancer (NSCLC) cells, whereas overexpression of VHR suppressed cell growth in both two- and three-dimensional cultures. Expression of VHR also suppressed tumor formation in a mouse xenograft model. Furthermore, VHR expression was significantly lower in NSCLC tissues in comparison to that in normal lung tissues. Collectively, this study shows that down-regulation of VHR expression enhances the signaling of ErbB receptors and may be involved in NSCLC pathogenesis.

Among protein modifications, tyrosine phosphorylation is extensively used only in multicellular, eukaryotic organisms. Protein-tyrosine phosphorylation plays an important role in signaling transduction pathways that are involved in embryogenesis, development, and homeostasis. Disorders in protein-tyrosine phosphorylation are found in many human diseases from cancer to immune disorders. Although protein phosphorylation is a balanced action of protein kinases and phosphatases, the experimental data of protein phosphatases is proportionally much less than that of protein kinases. Dual specificity phosphatases (DUSPs)3 are structurally related to protein-tyrosine phosphatases (PTP) and are initially implicated in the down-regulation of MAPKs (1). Distinct from PTPs, which have a deep catalytic cleft; DUSPs have shallow catalytic sites, which permit the less stringent phospho-amino acid specificity of DUSPs (2–4). Several DUSPs including MAPK phosphatases (MKP)-1 to -7, M3/6 (also called VH5), and VHR have been shown to inactivate one or several MAPKs (5–12). The expression of certain DUSPs is increased by mitogenic signaling (5, 13–15). Both ERK and JNK pathways induce the expression of MKPs (15–17). The induction of MKP expression by MAPK signaling may, in turn, lead to the down-regulation of MAPK activities. Recently, many newly identified DUSPs were found to have little or no phosphate activity against MAPKs, indicating that MAPK inactivation is not the sole function of DUSPs (18–21). These novel DUSPs are smaller in size compared with MKPs and lack the MAPK-binding domain. These groups of DUSPs have been classified as atypical DUSPs (1). Others’ and our recent studies reveal that atypical DUSPs may play a critical role in the regulation of signaling triggered by protein tyrosine kinases (21–23).

VHR, a phosphatase related to the vaccinia virus phosphatase VH1, is among the first identified mammalian DUSPs (1, 24). It is initially found to be a PTP that dephosphorylates EGFR and PDGFR in vitro (24). Nevertheless, conclusive evidence supporting its role in dephosphorylating growth factor receptors in a biological context is absent. VHR has been shown to inactivate JNK and ERK pathways in different systems (25–27); however, its suppressive activity against MAPKs is weak in comparison to that of MKPs. Moreover, unlike MKPs, VHR expression is not up-regulated in response to MAPK activation (27). VHR is phosphorylated by ZAP70 at tyrosine residue 138 in response to T cell receptor signaling. This phosphorylation augments VHR activity in suppressing the ERK pathway (27). A recent report shows that expression levels of VHR are regulated during the cell cycle. Knockdown of VHR expression by RNA interference causes cell cycle arrest and senescence (28). The aim of this research was to further examine the biological function of VHR.

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3 The abbreviations used are: DUSP, dual specificity phosphatase; EGFR, EGF receptor; MKP, MAPK phosphatase; NSCLC, non-small cell lung cancer; PLC, phospholipase C; PTP, protein-tyrosine phosphatase; TR, Tet repressor; VHR, vaccinia H1-related phosphatase.
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in epithelial cells. Our results showed that VHR had very limited activity against MAPks and that VHR regulated receptor-proximal signaling through dephosphorylating ErbB receptor tyrosine kinases. Our data from cultured cells, transplanted tumors, and clinical patient samples suggested that a decrease in VHR expression could be involved in the pathogenesis of non-small cell lung cancer.

**EXPERIMENTAL PROCEDURES**

*Cell Culture and Establishment of Permanently Transfected Cells*—All of the H1299-derived cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum plus penicillin and streptomycin. H1299 cells were transfected using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. For establishing the tetracycline-inducible cells, a pcDNA6/TR vector (Invitrogen), which encodes the Tet repressor (TR), was transfected into H1299 cells. The transfected cells were selected by blasticidin (40 μg/ml) for 1 week to form the H1299-TR cells. The pcDNA4/TO-Myc-VHR (or MKP-1) vector was transfected into H1299-TR cells. The transfected cells were selected by Zeocin (500 μg/ml) for 1 week to generate H1299-VHR-TR (or MKP-1 TR) cells.

*Plasmids*—To construct the human VHR expression vectors, DNA fragment encoding the VHR gene was PCR-amplified using a plasmid clone (GenBank™ number BM926103, American Type Culture Collection) as a template, 5′-GGCGGATCCACCATGTCGGGCTCGTTCGAG as the forward primer, and 5′-GTGCTCAGATGGGTTCAGAACTTCCCCTC as the reverse primer. The DNA fragment was enzyme-digested and inserted into pcDNA4/TO/Myc-His B (Invitrogen) and pGEX-GST-4T-3 (GE Healthcare) vectors between the BamHI and XhoI sites to form the Myc-VHR and GST-VHR expression vectors, respectively. The VHR-C124S phosphatase-inactive mutant (VHR-CS) constructs were generated by the side-directed mutagenesis method and sequence-verified. Mammalian and bacterial expression vector of MKP-1 were described previously (21). The RNA interference vector against human VHR was generated using the pSuper-retro vector (Oligo-Engine, Seattle, WA) as a backbone. A 19-nucleotide sequence (5′-CCTAGGGCTTACCTTGAA) corresponding to the nucleotides 291–309 of human VHR coding sequence was used for designing the inverted repeat insertion in pSuper-retro vector according to the manufacturer’s instructions. The RNA interference-resistant VHR vector was constructed by generating T300G, C303T, T306C, and A309G mutations within the coding sequence in a FLAG-VHR plasmid using the side-directed mutagenesis method.

*Reagents and Antibodies*—Protein A beads and anti-FLAG monoclonal antibody (M2) were purchased from Sigma. The anti-Myc monoclonal antibody was prepared from the culture of a hybridoma (clone 9E10). Anti-VHR, JNK, pJNK, pp38 MAPK, pERK, and anti-Tyr(P) (p-Tyr-100) antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-ERK antibody was purchased from BD Biosciences (San Diego, CA). The anti-GST (SC-138) antibody was purchased from Santa Cruz (Santa Cruz, CA). The anti-phosphotyrosine (4G10) antibody was purchased from Upstate Biotechnology (Waltham, MA). The peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies were purchased from Pierce. EGF and puromycin were purchased from Sigma-Aldrich. TNF-α was purchased from R & D System (Minneapolis, MN). Zeocin and blasticidin were purchased from Invitrogen.

*RNA Preparation from Human NSCLC Tissue and cDNA Preparation*—Fresh frozen tumor and paired nontumor lung tissue specimens from NSCLC patients receiving surgical resection at Chang-Gung Memorial Hospital were obtained from the tissue bank of Chang-Gung Memorial Hospital with signed informed consent. All of the specimens were snap frozen soon after resection and stored at −80 °C. The study protocol has been reviewed and approved by the Institutional Review Board of Chang-Gung Memorial Hospital and National Health Research Institutes. Before RNA extraction, one frozen section stained by hematoxylin and eosin stain was performed first for the histopathology examination by a pathologist (SF Huang). In this way, the tumor diagnosis and tumor percentage were determined. The benign paired lung tissue was also examined to make sure there was no tumor contamination. The RNA extraction was performed with SV total RNA isolation system (Promega) according to the instructions of the manufacturer. Reverse transcription of cDNAs were performed using the ProtoScript first strand cDNA synthesis kit (New England Biolabs, Beverly, MA) and using 1 μg of total RNA as templates. The reverse transcription mixtures were used as templates in subsequent PCRs or were stored at −20 °C.

*Real Time PCR Quantification of mRNA*—Quantification of mRNA was conducted by real time PCR on the LightCycler instrument (Roche Applied Science). Specificity was further verified by checking the PCR products using agarose gel electrophoresis. Real time PCR was performed using a kit of LightCycler FastStart DNA Masterplus SYBR Green I (Roche Applied Science) in a total volume of 20 μl in the LightCycler glass capillaries. The reaction program was: initial heating to 95 °C for 10 min followed by 45 PCR cycles of heating to 95 °C for 10 s, incubation for 10 s at the annealing temperature specific for the use of primer, and incubation for 25 s at 72 °C. We conducted a melting curve analysis after every real time PCR to identify PCR product and to detect the possible presence of contaminating products. We also quantified transcripts of β-Actin gene, and each sample was normalized on the basis of its β-actin mRNA content. The relative quantification was determined using a comparative threshold cycle (CT) method.

*Immunohistochemical Staining*—Immunohistochemical staining was performed at the Pathology Core Laboratory, National Health Research Institutes. The assays were performed on 4-μm-thick formalin-fixed paraffin-embedded tissue sections. The sections were deparaffinized twice in xylene for 10 min and twice in ethanol for 2 min and then placed in 10 mM citric buffer solution (pH 6.0) and heated at 95 °C for 15 min. The samples were washed once with PBS, and the endogenous peroxidase activity was blocked by 3% H2O2 for 5 min. The slides were rinsed twice with PBS and then incubated with a VHR antibody (number AP8478a; Abgent, San Diego, CA) at 1:50 dilution for 2 h at room temperature. The slides were washed three times with PBS and then incubated with an anti-rabbit antibody-conjugated with peroxidase (1:500; Santa
The precipitates were washed three times with lysis buffer and then the cell-containing medium was mixed with 160 μl of 1% SDS, 2 mM NaCl, 0.1% Triton X-100, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, and 1 mM Na3VO4. The cell extracts were kept on ice and vigorously mixed four times at 5-min intervals. The extracts were cleared by centrifugation at 14,000 × g for 10 min, and the supernatants were collected for further analyses or stored at −80 °C.

Cell Extract Preparation—Whole cell lysate was prepared by suspending 2 × 106 cells in 200 μl of lysis buffer (50 mM Tris, pH 7.5, and 10 mM DTT). The immunocomplex was then mixed with 50 μl of phosphate reaction buffer containing indicated amounts of GST-VHR or GST-MKP-1. The phosphate reaction was performed at 37 °C for indicated times and then terminated by adding SDS sampling buffer. The reaction mixtures were heated at 95 °C for 5 min and analyzed by SDS-PAGE plus Western blot analyses.

Western Blot Analyses—Immunoprecipitated proteins or 50–100 μg of cell extracts were resolved by SDS-PAGE (8–10%) and then transferred to polyvinylidene difluoride membrane (Pall Corporation, Pensacola, FL). The membrane was first incubated in TBST buffer (20 mM Tris, pH 7.5, 135 mM NaCl, 0.1% Tween 20) containing 5% of nonfat milk powder (or 2% bovine serum albumin) for 1 h, then incubated with a primary antibody at 4 °C overnight, washed twice (10 min each) with TBST buffer, and blotted with secondary antibody conjugated with horseradish peroxidase (1:1,000 dilution) at room temperature for 1 h. The membrane was washed four times (10 min each) with TBST buffer, subjected to chemiluminescence reaction in the SuperSignal reagent (Pierce), and exposed to x-ray films.

Two-dimensional Focus Formation and Three-dimensional Matrigel Culture—H1299-VHRTR (or H1299-pSuper and VHR) cells were seeded on 60-mm dishes (100 cells/dish) and cultured with or without tetracycline (5 μg/ml) for 2 weeks. The cells were fixed and stained by the Giemsa staining method. The numbers of cell foci (bigger than 1 mm in diameter) were determined. The data are presented as the percentages of foci numbers of untreated H1299-VHRTR and that of H1299-pSuper control cells. Three-dimensional culture system was consisted of three layers. In 24-well plate, each well was firstly coated with 300 μl, 0.4% agarose in RPMI medium plus 10% serum. The second layer contained growth factor-reduced Matrigel (BD Biosciences; Bedford, MA) and H1299-VHRTR cells. One thousand cells were suspended in 320 μl of medium, and then the cell-containing medium was mixed with 160 μl of Matrigel. After seeding the total 480-μl mixture on the first gel layer, the gel culture was incubated at 37 °C for 4 h to solidify. Finally 300 μl of culture medium with or without tetracycline was added on the solidified Matrigel. The final concentration of tetracycline in the whole culture system was 5 μg/ml.

Mouse Tumor Xenograft—Eight-week-old female BALB/c nude mice were purchased from BioLasco Taiwan Co., Ltd. The animals had access to food and water ad libitum. The experimental procedures using animals were approved by the Institutional Animal Care and Use Committees of the National Health Research Institutes. The mice were injected subcutaneously with H1299-VHRTR cells. Each injection contained 2 × 106 cells suspended in 150 μl of RPMI 1640 medium with 30% of growth factor-reduced Matrigel. Five days post-injection, the mice were provided with water containing 0 or 1 mg/ml of tetracycline (supplemented with 10 mg/ml sucrose for palatability). Four weeks post-injection, the tumors were harvested, weighted, and processed for immunohistochemistry or Western blot analyses.

RESULTS

VHR Has Minimal Effects on MAPK Activation—We established an inducible expression system for VHR in H1299 NSCLC cells. The expression of Myc-tagged VHR was induced by tetracycline to a level that was similar to endogenous VHR (Fig. 1A, endo VHR). Interestingly, the induction of Myc-VHR expression in H1299 cells did not decrease JNK activation by TNF-α. In contrast, expression of Myc-MKP-1, to a much lower level than that of Myc-VHR, apparently suppressed JNK activation by TNF-α (Fig. 1B). Additionally, expression of VHR did not affect ERK activation by EGF and p38-MAPK activation by TNF-α (Fig. 1, C and D).

Because our result differed from the previous reports showing that JNK was negatively regulated by VHR, we examined whether VHR could directly dephosphorylate JNK1 in vitro. We tested recombinant GST-VHR and GST-MKP-1 against JNK1 to have an equivalent comparison of these two DUSPs. Phosphorylated JNK1 was subjected to reactions with various amounts of GST phosphatases. We found that JNK1 could be efficiently dephosphorylated even by the lowest amount of GST-MKP-1. In contrast, GST-VHR failed to dephosphorylate JNK1 in the same reaction condition (Fig. 2A). This result, consistent with the data in Fig. 1, indicated that VHR was not an effective JNK phosphatase in comparison to MKP-1. Taken together, our data suggested that VHR was not a major cellular MAPK phosphatase.

VHR Is an ErbB Receptor Phosphatase—VHR is more effective in dephosphorylating phosphotyrosine residue than phosphoserine or phosphothreonine (30). Actually, the first report on VHR shows that VHR dephosphorylates multiple receptor tyrosine kinases, including EGFR, FGFR, and insulin receptor in vitro (24). However, the regulatory role of VHR on receptor signaling has not been characterized in a biological system. We hypothesized that, instead of being a dual specific MAPK phosphatase, VHR might serve as a PTP in mammalian cells. Because of the availability of various phospho motif-specific antibodies against EGFR, we selected EGFR as a candidate molecule for further examination. Immunoprecipitated EGFR was subjected to in vitro reactions with wild type or phosphatase-inactive GST-VHR; the reaction mixtures were analyzed by
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Western blotting using various phospho-EGFR antibodies. Incubation with wild type VHR, but not its mutant, apparently decreased EGFR phosphorylation at Tyr-845, Tyr-992, and Tyr-1068. The effect was weaker at Tyr-1045 and Tyr-1173 (Fig. 2B). These data supported the notion that VHR is an EGFR phosphatase. Interestingly, when we examined the EGFR phosphorylation pattern in H1299-VHRTR/EGFR in the presence of VHR induction, we detect a clear decrease of phosphorylation at only Tyr-992 but less so at other Tyr residues (Fig. 3A).

Induction of VHR expression in H1299-VHRTR/EGFR cells also decreased tyrosine phosphorylation of multiple cellular proteins in response to EGF stimulation (Fig. 3B). This result indicated that VHR did regulate EGFR signaling in a biological context. Similar experiments were performed to test whether VHR dephosphorylated ErbB2. We found that VHR decreased ErbB2 phosphorylation in vitro and in a cellular context, and the dephosphorylation of ErbB2 was more evident at Tyr-877 and Tyr-1221 than those at Tyr-1139 and Tyr-1248 (supplemental Fig. S1). Our data indicated that VHR was a cellular PTP against EGFR and ErbB2.

VHR Suppresses EGFR Tyr-992 Phosphorylation at the Early Phase of EGF Stimulation—Because we did not detect significant endogenous ErbB2 expression in most of the NSCLC cell lines tested (data not shown), we focused our study on the effect of VHR against endogenous EGFR. We treated H1299-VHRTR cells with EGF for various times (5–60 min) in the presence or absence of VHR induction. We found that VHR expression suppressed endogenous EGFR Tyr-992 phosphorylation but mainly at the early time points of EGF treatment (0, 5, 10, and 20 min). VHR expression had minimal effects at later time points (30 and 60 min) (Fig. 4A). This result was reproducible and more evident in H1299-VHRTR/EGFR cells (Fig. 4B). The expression level of EGFR in H1299-VHRTR/EGFR was ~2.5-fold of that in control H1299 cells (supplemental Fig. S2A).

These data suggested that VHR suppressed the early phase of Tyr-992 phosphorylation during EGFR activation. Interestingly, when we examined the interaction between EGFR and VHR by co-immunoprecipitation, we found that VHR-EGFR interaction was weak but detectable in the absence of ligand stimulation. However, this interaction was decreased upon EGF stimulation (supplemental Fig. S2B). Ligand-induced dissociation between VHR and EGFR could explain the ineffectiveness of VHR on EGFR Tyr-992 phosphorylation at the later time points upon EGF stimulation.

To test whether EGFR signaling regulated the expression of VHR, we treated parental H1299 cells with EGF and collected cell samples at various time points for examining the VHR expression levels. As shown in supplemental Fig. S2C, VHR expression remained constant through 0–8 h post-EGF stimulation, indicating that VHR was not regulated by EGFR signaling.

To validate the role of endogenous VHR in EGFR signaling, we established VHR-deficient H1299 cells (H1299-VHRi) using the RNA interference method. VHR expression in H1299-VHRi cells was ~33% (an average of multiple samples) of that in control H1299-pSuper cells. In comparison to H1299-pSuper cells, H1299-VHRi cells showed a stronger phosphorylation at EGFR Tyr-992 residue (Fig. 4C) with or without EGFR stimulation. When VHR was reintroduced into H1299-VHRi cells through a RNA interference-resistant vector (H1299-VHRi/VHR), phosphorylation of EGFR Tyr-992 was reduced and less responsive to EGF (Fig. 4C). This result, consistent with the previous data, showed that VHR regulated EGFR signaling through suppressing Tyr-992 phosphorylation.

VHR Suppresses the Phospholipase Cγ (PLCγ)-PKC Pathway—Tyr-992 phosphorylation of EGFR has been shown to be
involved in the recruitment of PLCγ and Src. To test whether decreasing the early EGFR Tyr-992 phosphorylation by VHR was sufficient to affect downstream signaling, we examined the regulatory effects of VHR on the PLCγ-PKC pathway. We found that expression of VHR in H1299-VHRTR cells decreased both basal and EGF-induced Tyr-783 phosphorylation in PLCγ, suggesting that VHR did suppress PLCγ signaling (Fig. 5A). To further support this observation, we found that EGF-induced PKC activation, a downstream event of PLCγ signaling, was also suppressed in the H1299-VHRTR cell with VHR induction (Fig. 5B). These data indicated that VHR not only suppressed EGFR Tyr-992 phosphorylation, it also inhibited the downstream signaling events.

However, when we examined Src activation by detecting phosphorylation on the activating residue Tyr-416, we found that expression of VHR did not affect Src activation by EGF stimulation (supplemental Fig. S3A). Consistent with this result, VHR also did not affect the activation status of Src when both molecules were co-expressed in H1299 cells (supplemental Fig. S3B). These data indicated that VHR had a minimal effect in regulating Src activation.

VHR Suppresses Cell Proliferation and Tumor Formation—Both EGFR and ErbB2 are involved in the regulation of epithelial cell growth. Because of the suppressive effect of VHR on EGFR and ErbB2 signaling, we tested whether expression of VHR affected cell growth in H1299 cells. In regular, monolayer cell culture, we did not observe a significant difference between H1299-VHRTR cells with or without VHR induction (data not shown). However, when H1299-VHRTR cells were seeded at a low density (100 cells/60-mm dish) and allowed to grow for 2 weeks, induction of VHR significantly suppressed cell foci formation (Fig. 6A). Consistent with this observation, suppression of VHR expression by RNA interference enhanced foci formation in H1299 cells (Fig. 6A).
We also tested the growth-suppressive effect of VHR when cells were maintained in three-dimensional Matrigel cultures. In the absence of VHR induction, H1299-VHRTR cells formed colonies of significant size in 2 weeks. In the presence of tetracycline and VHR expression, the colonies formed by H1299-VHRTR cells were much less and smaller (Fig. 6B). Taken together, our data indicated that VHR expression in H1299 cells could suppress cell proliferation in both two- and three-dimensional cultures.

We also performed tumor xenograft experiments by injecting the H1299-VHRTR cells into nude mice subcutaneously. The mice were provided with water containing 0 or 1 mg/ml of tetracycline. Four weeks post-injection, the mice were sacrificed, and the tumors were harvested, weighed, and examined for VHR expression. The tumors formed in mice treated with tetracycline were significantly smaller than those formed in control mice (Fig. 6, C and D). The relative levels of endogenous and tet-induced Myc-VHR in the tumors were similar to that of cells cultured in vitro (Fig. 6E). This result was consistent with the notion that VHR suppressed cancer cell growth.

VHR Expression Is Decreased in Non-small Cell Lung Cancer Tissues—Our data strongly suggested that VHR played an important role in controlling cell proliferation, which is usually increased in cancer tissues. We hypothesized that expression of VHR might be down-regulated in cancer tissues. To test this hypothesis, we examined the mRNA expression of VHR and a closely related DUSP, MKP-6, in 36 pairs of cancer/adjacent normal tissues from NSCLC patients. We found that VHR expression was significantly lower in tumor tissues in comparison to adjacent normal lung tissues (Fig. 7). The selected sample size of 36 will yield 96.4% power for detecting a clinically meaningful difference of 0.33 between the two groups (normal versus tumor) at the 5.0% level of significance. This difference was more evident in lung adenocarcinoma than in squamous cell carcinoma (supplemental Fig. S4A) and was more significant in advanced stage III cancers than in stage I cancers (supplemental Fig. S4B). In contrast to VHR, MKP-6 expression was not significantly changed in NSCLC tissues (Fig. 7). To support the data of mRNA analyses, we performed immunohistochemical assays on tissue sections from patients with more than a 3-fold difference in VHR expression between tumor and normal tissues. As shown in supplemental Fig. S5, those tumor tissues showed weaker VHR staining intensity than the adjacent normal tissues. Taken together, these results supported our hypothesis that VHR expression is down-regulated in cancer cells.

**DISCUSSION**

VHR is among the first identified mammalian DUSPs (24). Despite the initial report showing that VHR is a protein-tyrosine phosphatase and is capable of dephosphorylating various receptor tyrosine kinases in vitro, the majority of the followed researches had focused on the ability of VHR to dephosphorylate and inactivate MAPKs (25–27). Unlike the typical MKPs, VHR displays a strong preference for dephosphorylating phosphotyrosine residue over phosphothreonine residue (30). Furthermore, although multiple reports have shown that VHR is
capable of inactivating ERKs and JNKs, its activity against MAPKs has not been compared with that of MKPs side-by-side. In this study, we first examined the ability of VHR in inactivating MAPKs using an inducible expression system in H1299 NSCLC cell line. We did not find a significant effect of VHR on ERK, p38-MAPK, and JNK (Fig. 1). In contrast, MKP-1, when expressed at a much lower level, can efficiently dephosphorylate JNK, in the same cell line. Using GST fusion recombinant proteins, we also showed that MKP-1 could dephosphorylate JNK1 much more efficiently than did VHR in the same reaction condition (Fig. 2). These data, different from the recent reports, led us to question the role of VHR in MAPK regulation and to speculate about the physiological functions of VHR.

Our experimental data were more consistent with the initial report of VHR (24). We found that VHR was an ErbB receptor phosphatase. Although VHR can dephosphorylate EGFR on multiple tyrosine residues in vitro, it preferentially reduced EGFR phosphorylation on Tyr-992 in a cellular context (Figs. 2 and 3). Consistent with this result, expression of VHR suppressed the activation of PLCy/PKC pathway (Fig. 5), which is dependent on the EGFR Tyr-992 phosphorylation (31), without affecting EGF-induced ERK activation (Fig. 1). Interestingly, Src has also been shown to bind to the phospho-Tyr-992 resi-

FIGURE 6. VHR suppresses cell proliferation. A, statistical analysis of the two-dimensional foci formation was performed by the two-tailed Student’s t test. The bar graph shows the means and standard deviations of representative quadruplicated experiments. B, colony formation in Matrigel was examined by a microscope with or without staining the cultures with Giemsa staining method. The presented result was one of three independent and consistent experiments. The magnified images in the lower panels (scale bars, 200 µm) show the morphology of the colonies. C–E, mouse tumor xenograft was performed as described under "Experimental Procedures." Four weeks post-tumor cell injection, the tumors were harvested. The tumor sizes were shown in photographs (C). The quantitative analysis of tumor weight was shown in D. The statistical analysis was performed by the nonpaired, two-tailed Student’s t test. The numbers were the means and standard deviations of samples in each group. Four tumor tissues were randomly selected from each group, extracted for protein lysate, and subjected for Western blot (WB) analysis using anti-VHR and anti-β-actin antibodies (E).

FIGURE 7. VHR expression is decreased in non-small cell lung cancer tissues. VHR, MKP-6, and β-actin mRNA expression levels in 36 pairs of tumor and adjacent normal tissues from NSCLC patients were determined by quantitative PCR. Every sample was measured by duplicated assays. VHR and MKP-6 expression levels were normalized by the level of β-actin within the individual sample. The means of VHR or MKP-6 expression levels of normal lung tissues were arbitrarily set as 1. The statistical analysis was performed by the paired, two-tailed Student’s t test.
due of EGFR (32), but we did not find an effect on EGF-induced Src activation by VHR expression (supplemental Fig. S3). It was likely that Src can be activated through multiple phospho-Tyr residues of EGFR and therefore was not affected by VHR. Our data also suggested that VHR could modulate EGFR signaling by dephosphorylating specific tyrosine residues. Both EGFR and ErbB2 signaling are important for the proliferation of epithelial cells. We found that expression of VHR reduced foci formation ability of H1299 cells, whereas decreasing VHR enhanced this ability. We also found that expression of VHR greatly reduced the colony forming ability of H1299 cells in three-dimensional Matrigel culture. Furthermore, expression of VHR suppressed tumor formation by H1299 cells in a mouse xenograft system (Fig. 6). These results were consistent with the suppressive activity of VHR against the EGFR and ErbB2 signaling pathway. We did not detect a significant amount of ErbB2 expression in H1299 cells; therefore, the growth-suppressive effect of VHR likely was not mediated through inhibiting ErbB2. We did not observe significant effects of VHR on Src (supplemental Fig. S3) and c-Met (data not shown). Our data also showed that VHR had a preference among different phosphotyrosine motifs, indicating that VHR is not a nonselective phosphatase. However, VHR may very likely have cellular substrates other than the ErbB receptors; therefore, we cannot conclude that VHR affected cell proliferation only through suppression of EGFR. As reported recently, VHR is involved in dephosphorylating STAT5 at Tyr-694/699 (22). Taken together, our data and that of others revealed the importance of VHR in regulating tyrosine phosphorylation-mediated signaling.

Our results also showed that VHR expression levels in NSCLC tissues are lower than those in adjacent normal tissues. The difference in VHR expression between normal lung and NSCLC tissues was statistically significant (Fig. 7). In contrast, the expression of MKP-6, a closely related DUSP, was not apparently different between normal and cancerous tissues. These data, in combination with our biochemical and functional analyses of VHR, suggested that a decrease in VHR expression and a subsequent loss of ability to suppress tyrosine phosphorylation only through suppression of EGFR. As reported recently, VHR may very well be a nonselective phosphatase. However, VHR may very likely have cellular substrates other than the ErbB receptors; therefore, we cannot conclude that VHR affected cell proliferation only through suppression of EGFR. As reported recently, VHR is involved in dephosphorylating STAT5 at Tyr-694/699 (22). Taken together, our data and that of others revealed the importance of VHR in regulating tyrosine phosphorylation-mediated signaling.

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REFERENCES
1. Alonso, A., Sasin, J., Bottini, N., Friedberg, I., Friedberg, I., Osterman, A., Godzik, A., Hunter, T., Dixon, J., and Mustelin, T. (2004) Cell 117, 699–711
2. Camps, M., Nichols, A., and Arkinstall, S. (2000) FASEB J. 14, 6–16
3. Yuwaniyama, J., Denu, J. M., Dixon, J. E., and Saper, M. A. (1996) Science 272, 1328–1331
4. Stewart, A. E., Dowd, S., Keyse, S. M., and McDonald, N. Q. (1999) Nat. Struct. Biol. 6, 174–181
5. Sun, H., Charles, C. H., Lau, L. F., and Tonks, N. K. (1993) Cell 75, 487–493
6. Misra-Press, A., Rim, C. S., Yao, H., Roberson, M. S., and Stork, P. J. (1995) J. Biol. Chem. 270, 14587–14596
7. Mourey, R. J., Vega, Q. C., Campbell, J. S., Wenderoth, M. P., Hauschka, S. D., Krebs, E. G., and Dixon, J. E. (1996) J. Biol. Chem. 271, 3792–3802
8. Muda, M., Boschert, U., Smith, A., Antonsson, B., Gillieron, C., Chabert, C., Camps, M., Martinou, I., Ashworth, A., and Arkinstall, S. (1997) J. Biol. Chem. 272, 5141–5151
9. Tanoue, T., Moriguchi, T., and Nishida, E. (1999) J. Biol. Chem. 274, 19949–19956
10. Marti, F., Krause, A., Post, N. H., Lyddane, C., Dupont, B., Sadelain, M., and King, P. D. (2001) J. Immunol. 166, 197–206
11. Tanoue, T., Yamamoto, T., Maeda, R., and Nishida, E. (2001) J. Biol. Chem. 276, 26629–26639
12. Smith, A., Ramos-Morales, F., Ashworth, A., and Collins, M. (1997) Curr. Biol. 7, 893–896
13. Ward, Y., Gupta, S., Jensen, P., Wartmann, M., Davis, R. J., and Kelly, K. (1994) Nature 367, 651–654
14. Rohan, P. J., Davis, P., Moskaluk, C. A., Kearns, M., Krutzsch, H., Siebenlist, U., and Kelly, K. (1993) Science 259, 1763–1766
15. Grumont, R. J., Rasko, J. E., Strasser, A., and Gerondakis, S. (1996) Mol. Cell. Biol. 16, 2913–2921
16. Bokemeyer, D., Sorokin, A., Yan, M., Aah, N. G., Templeton, D. J., and Dunn, M. J. (1996) J. Biol. Chem. 271, 639–642
17. Brondello, J. M., Brunet, A., Pouysségur, J., and McKenzie, F. R. (1997) J. Biol. Chem. 272, 1368–1376
18. Chen, A. J., Zhou, G., Juan, T., Colicos, S. M., Cannon, J. P., Cabrera-Hansen, M., Meyer, C. F., Jurecic, R., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Fletcher, F., Tan, T. H., and Belmont, J. W. (2002) J. Biol. Chem. 277, 36592–36601
19. Hood, K. L., Tobin, J. F., and Yoon, C. (2002) Biochem. Biophys. Res. Commun. 298, 545–551
20. Niwa, R., Nagata-Ohashi, K., Takeichi, M., Mizuno, K., and Uemura, T. (2002) Cell 108, 233–246
21. Wang, J. Y., Lin, C. H., Yang, C. H., Tan, T. H., and Chen, Y. R. (2006) J. Neurochem. 98, 89–101
22. Hoyt, R., Zhu, W., cerignoli, F., Alonso, A., Mustelin, T., and David, M. (2007) J. Immunol. 179, 3402–3406
23. Li, J. P., Fu, Y. N., Chen, Y. R., and Tan, T. H. (2010) J. Biol. Chem. 285, 5472–5478
24. Ishibashi, T., Bottaro, D. P., Chan, A., Miki, T., and Aaronson, S. A. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 12170–12174
25. Todd, J. L., Rigas, J. D., Raffy, L. A., and Denu, J. M. (2002) Oncogene 21, 2573–2583
26. Todd, J. L., Tanner, K. G., and Denu, J. M. (1999) J. Biol. Chem. 274, 13271–13280
27. Alonso, A., Saxena, M., Williams, S., and Mustelin, T. (2001) J. Biol. Chem. 276, 4766–4771
28. Rahmouni, S., Cerignoli, F., Alonso, A., Tsutji, T., Henkens, R., Zhu, C., Louis-dit-Sully, C., Moutschen, M., Jiang, W., and Mustelin, T. (2006) Nat. Cell Biol. 8, 524–531
29. Chen, Y. R., Han, J., Kori, R., and Tan, T. H. (2002) J. Biol. Chem. 277, 39334–39342
30. Schumacher, M. A., Todd, J. L., Rice, A. E., Tanner, K. G., and Denu, J. M. (2002) Biochemistry 41, 3009–3017
31. Rotin, D., Margolis, B., Mohammad, M., Daly, R. J., Daum, G., Li, N., Fischer, E. H., Burgess, W. H., Ullrich, A., and Schlessinger, J. (1992) EMBO J. 11, 559–567
32. Sierke, S. L., Longo, G. M., and Koland, J. G. (1993) Biochem. Biophys. Res. Commun. 191, 45–54