Inhibition of T Cell Antigen Receptor Signaling by VHR-related MKPX (VHX), a New Dual Specificity Phosphatase Related to VH1 Related (VHR)*

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A cDNA encoding a novel, human, dual-specific protein phosphatase was identified in the Incyte data base. The open reading frame predicted a protein of 184 amino acids related to the Vaccinia virus VH1 and human VH1-related (VHR) phosphatases. Expression VHR-related MKPX (VHX) was highest in thymus, but also detectable in monocytes and lymphocytes. A VHX-specific antiserum detected a protein with an apparent molecular mass of 19 kDa in many cells, including T lymphocytes and monocytes. VHX expression was not induced by T cell activation, but decreased somewhat at later time points. In vitro, VHX dephosphorylated the Erk2 mitogen-activated protein kinase with faster kinetics than did VHR, which is thought to be specific for Erk1 and 2. When expressed in Jurkat T cells, VHX had the capacity to suppress T cell antigen receptor-induced activation of Erk2 and of an NFAT/AP-1 luciferase reporter, but not an NF-κB reporter. Thus, VHX is a new member of the VHI/VHR group of small dual-specific phosphatases that act in mitogen-activated protein kinase signaling pathways.

Phosphate is removed from phosphoproteins by two unrelated classes of protein phosphatases, the serine/threonine-specific phosphatases (PP1, PP2, etc.) and the protein tyrosine phosphatases (PTPases; reviewed in 1,2). The latter group uses a cysteine-based catalytic mechanism (3,4) shared with a broader family of hydrolases, including phosphatases specific for phospholipids (5) and RNA (6). Phosphotyrosine specificity is generally achieved by the depth of the catalytic pocket (5). An exception from this rule is the PTPase subfamily of dual-specific phosphatases (DSPs),1 which readily dephosphorylates both phosphotyrosine and an adjacent phosphothreonine residue (7–9). Most DSPs are specific for mitogen-activated protein kinases Erk, Jnk, and p38, which are activated by dual phosphorylation of a tyrosine and threonine residue in the consensus motif Thr-X-Tyr. Accordingly, these DSPs are often referred to as the MAP kinase phosphatases or MKPs.

The first DSP to be cloned was the VH1 protein from Vaccinia virus (10). A related enzyme was subsequently found in mammalian cells and termed VHR, for VH 1-related (11). Both VH1 and VHR differ from other DSPs in that they are much smaller, only 19 and 21 kDa, respectively. VH1 has been reported to dephosphorylate both MAP kinases and Stat1 (12), while VHR appears to be specific for Erk and Jnk (13,14). However, the physiological function of VHR has remained somewhat unclear as it seems to be less efficient than many other MAP kinase-specific DSPs.

We report the identification and initial characterization of a new human gene that encodes a DSP that is much more closely related to VH1 and VHR than to other DSPs. During our work, the nucleotide sequence and predicted open reading frame of this enzyme was deposited by others in GenBank™ under the name MKPX. However, this name is already occupied (15) and cannot be used as such. Instead, we honor this name by proposing the acronym VHX for “VHR-related MKPX.”

MATERIALS AND METHODS

Antibodies—The 12CA5 anti-hemagglutinin mAb was from Roche Molecular Biochemicals (Indianapolis, IN). The anti-phosphotyrosine mAb 4G10 was from Upstate Biotechnology, Inc., (Lake Placid, NY) and the anti-phosphothreonine mAb 12G10 was from Upstate Biotechnology Inc., Lake Placid, NY) in 20 mM MOPS, pH 7.2, 25 mM MOPS, 1 mM ATP, 1 mM dithiothreitol, 5 mM EGTA at 30 °C for 30 min. The reaction was stopped by dilution into ice-cold buffer without MgCl2 and ATP, but with 5 mM EDTA. Subsequently, 200-ng aliquots of

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‡ The abbreviations used are: DSP, dual-specific protein phosphatase; MAP, mitogen-activated protein; MKP(s), MAP kinase phosphatases; VHR, VH1 related; VHX, VH1 related MKPX; Erk, extracellular signal-regulated kinase protein kinase; Jnk, c-Jun N-terminal kinase; mAb, monoclonal antibody; GST, glutathione S-transferase; MOPS, 4-morpholinepropanesulfonic acid; RT, reverse transcription; HA, hemagglutinin.

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phospho-Erk2 were mixed with VHX (cleaved from GST-VHX by thrombin) and incubated for 1–30 min at 37 °C. Reactions were terminated by addition of SDS sample buffer, and the dephosphorylation of Erk2 analyzed by anti-phosphotyrosine immunoblotting.

RT-PCR Amplification of a 293-bp fragment of VHX cDNA was done with the 5′-upstream primer 5′-GAG CTG CCT TGT ACA CTG CCT GGC CGG GG-3′ and the 3′-downstream primer 5′-GGC CCA GAA CTT CAG AAT TCC TGG AGC GGC C-3′ from the Human MTC™ Panel II (CLONTECH, Palo Alto, CA) and Human Blood Fractions MTC™ Panel (CLONTECH) with Titanium™ Taq polymerase (CLONTECH) as recommended by the manufacturer. The manufacturer’s primers for G3PDH were used as recommended.

Plasmids and Site-directed Mutagenesis—The cDNA for VHX was also subcloned into the pEFP/HA vector (18), which adds a hemagglutinin (HA) tag to the N terminus of the insert. To generate a catalytically inactive mutant of VHX, the codon for Cys-88 was changed into a codon for serine in the pGEX-VHX plasmid using the Transformer™ site-directed mutagenesis kit as recommended by the manufacturer. The manufacturer’s primers for G3PDH were used as recommended.

Cells and Transfections—Jurkat T leukemia and murine thymoma cells were kept at logarithmic growth in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mm l-glutamine, 1 mm sodium pyruvate, nonessential amino acids, and 100 units/ml each penicillin G and streptomycin. These cells were transiently transfected with a total of 2 µg of DNA by electroporation at 950 V for 2300 µF and 240 V (14). Empty vector was added to control samples to make a constant amount of DNA in each sample. Cells were used for experiments 24 h after transfection. Peripheral blood lymphocytes (~80% T cells) were obtained from venous blood from healthy donors (Red Cross Blood Bank, San Diego, CA), by Ficoll gradient centrifugation and removal of monocytes by adherence to plastic at 37 °C for 2 h. The adherent cells were also recovered.

Immunoblotting—Proteins resolved by SDS-PAGE were transferred electrophoretically to nitrocellulose filter, which were immunoblotted as before (19–22) with optimal dilutions of mAbs, followed by anti-mouse-lg-peroxidase, and the blots developed by the enhanced chemiluminescence technique (ECL kit, Amersham Biosciences, Inc.) according to the manufacturer’s instructions.

MAP Kinase Assays and Luciferase Assays—MAP kinase assays were performed as described previously (14, 21–23). Briefly, 20 × 10⁶ cells were transfected with 2 µg of NFAT/AP-1-luc (or other reporters) together with empty pEFP/HA vector alone or VHX plasmids. After stimulation for 6 h, the cells were lysed in 100 µl of 100 mM potassium phosphate, pH 7.8, 1 mM dithiothreitol, 0.2% Triton X-100. The final assay contained 50 µl of the above cell lysate with 50 µM luciferin, 35 mM glycylglycine, pH 7.8, 20 mM MgCl₂). The activity was measured in an automatic luminometer (Monolight 2010,Analytical Luminescence Laboratory, Ann Arbor, MI). The activity of a co-transfected β-galactosidase was measured and used to normalize the luciferase activity for transfection efficiency.

RESULTS

Identification of a Novel Dual Specificity Phosphatase, VHX—To identify novel protein phosphatases, the Incyte data base was screened for open reading frames encoding proteins with homology to PTPases. One such cDNA encoded a 184-amino acid-residue polypeptide that had the canonical PTPase signature motif, His-Cys-X₂-Arg, at positions 87–94. In comparison with other PTPases, the new open reading frame had highest similarity to human VHR and the Vaccinia virus VHX (Fig. 1A). Very recently, the same open-reading frame was deposited in GenBank™ under the unpublished name MKPX (accession number AF165719, g0924474). However, since this name is already occupied by another unrelated DSP, MKP-X (15), also known as Pyst2 (15, 24), BS9 (25), or DUSP7 (26), we propose the name VHX for VHR-related MKPX for the new protein. This name honors the prior GenBank™ deposition by Gu and colleagues, but circumvents the confusing duplication of names. The name VHX also does not imply a function, which currently is unknown.

Expression of VHX in Tissues and Cells—To determine in which tissues and cell types VHX is expressed, we used a RT-PCR approach with gene-specific primers. An amplification product of the appropriate size (293 bp) was best obtained from Jurkat cells and thymus, while weaker bands were seen in spleen, prostate, testis, and peripheral blood lymphocytes (Fig. 1B). The band from Jurkat was sequenced to verify that the correct cDNA fragment had been obtained. As a control, we amplified glyceraldehyde-3-phosphate dehydrogenase. Because of the lymphoid bias of the result, we repeated the RT-PCR with a lymphoid cell cDNA library panel (Fig. 1B, right hand panels), which showed that monocytes, B lymphocytes, as well as CD4-positive and CD8-positive T cells express VHX. Interestingly, activated cells contained less VHX relative to the glyceraldehyde-3-phosphate dehydrogenase control than resting cells (compare lanes 3–7 with 8–11).

Detection of Endogenous VHX Protein—A GST fusion protein containing the entire open reading frame of VHX was generated, purified, and used for immunization of two rabbits. The resulting antisera reacted well with HA-tagged VHX expressed in Jurkat or COS cells (Fig. 2A). In contrast, the prebleed serum from the same rabbit did not recognize this protein. As a control, the HA-tagged VHX was readily detected by the anti-HA mAb (Fig. 2B). Immunoblotting of a number of cell lysates from human and murine leukemic cells lines detected an endogenous protein of the expected size, 19 kDa (Fig. 2C). Jurkat T cells contained a very low level of VHX, while normal T cells from peripheral blood contained a stronger band (lane 4). COS-1 cells and MyoD6 myocytes also had VHX protein, while the murine thymoma LSTRA contained very little, if any, immunoreactive VHX (lane 5). Cell lysate samples containing 70 µg of protein from Jurkat cells, isolated blood T cells, and monocytes clearly showed that Jurkat cells have a very low
three enzymes are likely to prefer substrates with acidic residues around the phosphotyrosine target residue, but VHX is likely to display a substrate preference more similar to that of VHI than VHR.

Catalytic Activity of the VHX Protein—To formally demonstrate that VHX is a catalytically active phosphatase, we first measured its capacity to dephosphorylate the general PTPase substrate p-nitrophenyl phosphate. This substrate was readily converted into the yellow p-nitrophenol by GST-VHX, but not by control GST, in a dose-dependent manner (Fig. 3A, 200 ng of VHX dephosphorylated 100 ng of Erk2 nearly completely within a few minutes at 37 °C). The same amount of VHR also dephosphorylated Erk2, but at a considerably slower rate. In contrast, the low molecular weight PTPase (LMPTP), an unrelated enzyme of similar size, did not dephosphorylate Erk2 (lanes 8 and 9). Control blots for Erk2 phosphorylation were included (Fig. 3A, lanes 2–4) and with a pH optimum of 7.0 (not shown). Thus, VHX is a catalytically active phosphatase.

VHX belongs to the subgroup of dual-specific PTPases, many of which are specific for dually tyrosine and threonine phosphorylated MAP kinase family members. To directly test if VHX has phosphatase activity against the dual-phosphorylated Erk2 protein, we incubated purified Erk2 with GST-Mek and then treated the resulting phospho-Erk with GST-VHX. As shown in Fig. 3C, 200 ng of VHX dephosphorylated 100 ng of Erk2 nearly completely within a few minutes at 37 °C. The same amount of VHR also dephosphorylated Erk2, but at a considerably slower rate. In contrast, the low molecular weight PTPase (LMPTP), an unrelated enzyme of similar size, did not dephosphorylate Erk2 (lanes 8 and 9). Control blots for Erk2 phosphorylation were included (Fig. 3C, lower panel) and also revealed that VHX caused a rapid shift of the apparent Mr of Erk2 from 44 to 42 kDa, while VHR did not. Thus, VHX likely causes a shift of Erk2 from 44 to 42 kDa, while VHR did not. This Mr shift is caused by loss of phosphothreonine. To study this phenomenon better, we performed similar experiments that also included a shorter time point (Fig. 3, D and E), which showed that VHX caused appearance of the

**FIG. 3. Phosphatase activity of recombinant VHX.** A, GRASP representation of the substrate-interacting surface and catalytic center of VHX, VHR, and VHI. B, dephosphorylation of p-nitrophenyl phosphate by recombinant GST-VHX. The shown results represent the average of duplicate determinations, which were within 5% of each other. C, anti-phosphotyrosine blot of dual-phosphorylated Erk2 incubated for the indicated times with 200 ng of recombinant VHX (without GST), VHR, or low molecular weight PTPase (LMPTP). D, similar experiments with more time-points using 100 ng of VHX. n.a., no addition of VHX. E, same experiment with 100 ng of VHI. n.a., no addition of VHR. The same results were obtained in three independent experiments.
faster migrating Erk2 protein within 30 s and a complete conversion by 5 min. Again, VHR did not convert Erk2 to the lower $M_r$ form.

**Active VHX Inhibits MAP Kinase and Anti-CD3 Plus Anti-CD28-induced Activation of a NFAT/AP-1 Reporter Gene Taken from the Interleukin-2 Gene**—Since endogenous VHX was present in T lymphocytes, we decided to test VHX for biological activity in T cell antigen receptor signaling pathways. For these experiments, we first co-expressed VHX with a Myc-tagged Erk2 and measured its activation in response to anti-CD3 mAbs. As shown in Fig. 4A, Erk2 activation was severely reduced by VHX, but not by another PTPase (PRL-2), which was expressed at higher levels than VHX (lower panel). At the shown 5-min time point, the inhibition was 42%, and in time-course experiments the inhibition was similar (40–60%) at all time points (1, 2, 3, 5, and 10 min).

Next, we co-expressed VHX with a sensitive luciferase reporter gene, in which luciferase transcription is under the control of a tandem NFAT/AP-1 element taken from the interleukin-2 gene promoter. This reporter responds to T cell antigen receptor ligation with anti-CD3 mAbs alone or together with co-ligation of the CD28 co-stimulatory molecule (14). VHX inhibited the response in a dose-dependent manner with 50% inhibition at 0.5–1 μg of plasmid (Fig. 4B). The expression of VHX protein correlated with DNA dose (insert). In contrast to the effect of catalytically active VHX, expression of the catalytically inactive VHX-C88S mutant did not inhibit reporter gene activation (Fig. 4C), suggesting that the catalytic activity of VHX was required for inhibition. The C88S mutant protein was expressed at the same level as the wild-type VHX (insert).

**VHX Does Not Affect Anti-CD3 Plus Anti-CD28-induced Activation of a NF-kB**—We next tested VHX for effects in a parallel signaling pathway emanating from the T cell antigen receptor and the CD28 co-receptor, namely the activation of NF-κB-driven transcription. In these experiments, VHX failed to have any significant effects over the same 0.5–10 μg range as used in the NFAT/AP-1 experiments (Fig. 4D). Only at the highest concentration (10 μg) was there a slight reduction in the reporter gene response, which is unlikely of any significance. We conclude that VHX does not affect signaling pathways required for NF-κB activation in T cells.

**Subcellular Location of VHX**—To determine where in the cell VHX is located, we first transfected Jurkat T cells with the VHX expression plasmid and stained them with a fluorescein isothiocyanate-conjugated anti-HA mAb. When these cells were viewed under a confocal microscope, it was clear that most of the fluorescence was in the cytoplasm of the cells (Fig. 5, B and C). Cells transfected with empty vector did not display any fluorescence at all (Fig. 5A). To verify that this subcellular location was also true for endogenous VHX, we stained blood T lymphocytes with preimmune serum (Fig. 5, D and E). With this staining protocol, the immunofluores-
ence was also cytoplasmic. We conclude that VHX is not nu-
clear like many DSPs, but cytoplasmic.

DISCUSSION

In this paper, we describe and begin the characterization of
a novel DSP, VHX, which is related to Vaccinia VIH1 and
the human VHR. Although, the three enzymes are quite similar in
size and share amino acid similarity, they also differ in many
significant respects. First, VHX has a longer C-terminal tail, in
which there is a possible tyrosine phosphorylation site. Second,
VHX has a different expression profile than VHR. VHX is
expressed best in the thymus, which is lowest in VHR (14).
Neither enzyme is induced, but VHX decreases at 24 and 48 h
of T cell activation, while VHR remains unchanged (14). Third,
VHX differs from VHR in that it is much more efficient toward
dually phosphorylated Erk2. In contrast to VHR, which de-
phosphorylates phosphotyrosine much faster than phos-
phothreonine (28), VHX appears to readily dephosphorylate Thr-
183 in Erk2, as well as Tyr-185. Thus, the apparent M<sub>r</sub> of Erk2,
as seen by anti-phosphotyrosine immunoblotting, shifts from
44 to 42 kDa within the first minutes of co-incubation before it
disappears. VHR, on the other hand, maintains Erk2 as a
44-kDa band that gradually weakens. Thus, VHX is a bona fide
DSP, while VHR has evolved toward a more tyrosine-selective
catalytic activity.

The group of small DSPs defined by VHR and VHX differs
from the MKPs that constitute the majority of DSPs, in that
they lack obvious non-catalytic targeting or regulatory motifs.
This explains their much smaller molecular mass, only 21 and
19 kDa, respectively, compared with over 40 kDa for most
MKPs. Nevertheless, two recent papers (14, 29) suggested that
the Erk1 and Erk2 kinases are physiological targets for VHR.
We also found that activation of Jnk in intact T cells was
diminished by co-expression of VHR, while the catalytically
inactive VHR-C124S had little effect (14). In contrast to Erk
and Jnk, activation of p38 kinase was not affected by VHR (14,
29). It remains unclear, however, if VHR dephosphorylates
MAP kinases in cells that normally express many other, and
more efficient, MKPs. In this report, we compared the new
enzyme VHX with VHR and found that VHX is considerably
more efficient as a Erk phosphatase and also removes phos-
phate from Thr-183 as well as from Tyr-185. Transfected VHX
also inhibits activation of Erk2 in Jurkat T cells. Nevertheless,
the physiological substrate(s) for VHX in intact cells remains to
be established. Our results with reporter genes suggest that
VHX can dephosphorylate a protein that is involved in T cell
antigen receptor-mediated activation of gene transcription
driven by a NFAT/AP-1 module, but not in pathways to NF-
κB driven transcription. This result is also compatible with Erk2
being a target, but since all three types of MAP kinases (Erk,
Jnk, and p38) are activated by the T cell antigen receptor and
participate in activation of components of AP-1, it is premature
to conclude that Erk2 is the only substrate. This is under
further investigation in our laboratory.

REFERENCES

1. Tonks, N. K., and Neel, B. G. (1996) Cell 87, 365–368
2. Mustelin, T., Brockdorff, J., Gjorolf-Wingren, A., Tailor, P., Han, S., Wang, X.,
   and Saxena, M. (1998) Front. Bionics. 3, 1069–1096
3. Barford, D., Plett, A. J., and Tonks, N. K. (1994) Science 263, 1379–1404
4. Zhou, G., Denu, J. M., Wu, L., and Dixon, J. E. (1994) J. Biol. Chem. 269,
   28084–28090
5. Maehama, T., and Dixon, J. E. (1998) J. Biol. Chem. 273, 13375–13378
6. Takagi, T., Moore, C. R., Dief, F., and Buratowski, S. (1997) Cell 89, 867–873
7. Keyse, S. M. (2000) Curr. Opin. Cell Biol. 12, 186–192
8. Saxena, M., and Mustelin, T. (2000) Sem. Immunol. 12, 387–396
9. Camps, M., Nicholas, A., and Arkin, J. S. (1999) EMBO J. 14, 6–16
10. Guan, K. L., Bryoles, S. S., and Dixon, J. E. (1991) Nature 350, 359–362
11. Ishibashi, T., Bottaro, D. P., Chan, A., Kiki, T., and Aaronson, S. A (1992)
    Proc. Natl. Acad. Sci. U. S. A. 89, 12170–12174
12. Najarro, P., Traktman, P., and Lewis, J. A. (2001) J. Virol. 75, 3185–3196
13. Denu, J. M., and Dixon, J. E. (1996) Proc. Natl. Acad. Sci. U. S. A. 92,
    5910–5914
14. Almoao, A., Saxena, S., Williams, S., and Mustelin, T. (2001) J. Biol. Chem.
    276, 4766–4771
15. Groom, I. A., Sneddon, A. A., Alessi, D. R., Dowd, S., and Keyse, S. M. (1996)
    EMBO J. 15, 3621–3632
16. Tailor, P., Williams, S., Gilman, J., Couture, C., and Mustelin, T. (1997) J.
    Biol. Chem. 272, 5371–5376
17. Khodad, N., and Mustelin, T. (2001) BioTechniques 31, 322–328
18. von Willebrand, M., Jascu, T., Bennefray-Berard, N., Yano, H., Altman, A.,
    Matsuda, Y., and Mustelin, T. (1996) Eur. J. Biochem. 235, 828–835
19. Couture, C., Deckert, M., Williams, S., Otero Russo, F., Altman, A., and
    Mustelin, T. (1996) J. Biol. Chem. 271, 24284–24289
20. Couture, C., Songyang, Z., Jascu, T., Williams, S., Tailor, P., Cantley, L. C.,
    and Mustelin, T. (1996) J. Biol. Chem. 271, 24880–24884
21. Saxena, M., Williams, S., Brockdorff, J., Gilman, J., and Mustelin, T. (1999)
    J. Biol. Chem. 274, 11693–11700
22. Saxena, M., Williams, S., Tasken, K., and Mustelin, T. (1999) Nat. Cell. Bio.
    1, 305–311
23. Jascu, T., Gilman, J., and Mustelin, T. (1997) J. Biol. Chem. 272, 14483–14488
24. Dowd, S., Sneddon, A. A., and Keyse, S. M. (1998) J. Cell Sci. 111, 3389–3399
25. Shin, D. Y., Ishibashi, T., Choi, S. S., Chung, E., Chung, I. Y., Aaronson, S. A,
   and Bottaro, D. P. (1997) Oncogene 14, 2633–2639
26. Smith, A., Price, C., Cullen, M., Mada, M., Kling, A., Ozone, B., Arrnkin, A.,
   and Ashworth, A. (1997) Genomics 42, 524–527
27. Yuvaniyama, J., Denu, J. M., Dixon, J. E., and Saper, M. A. (1996) Science
    272, 1328–1331
28. Denu, J. M., Zhou, G., Wu, L., Zhou, R., Yuvaniyama, J., Saper, M. A., and
    Dixon, J. E. (1995) J. Biol. Chem. 270, 3796–3803
29. Todd, J., Tanner, K., and Dent, J. (1999) J. Biol. Chem. 19, 13271–13280