Tyrosine phosphorylation of VHR phosphatase by ZAP-70

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The ZAP-70 tyrosine kinase is a key component of the signaling machinery for the T cell antigen receptor (TCR). Whereas recruitment and activation of ZAP-70 are relatively well understood, the proteins phosphorylated by ZAP-70 are incompletely known. We report here that VHR, a *Vaccinia* virus VH1-related dual-specific protein phosphatase that inactivates the mitogen-activated kinases Erk2 and Jnk, is phosphorylated at Y138 by ZAP-70. Tyr138 phosphorylation was required for VHR to inhibit the Erk2–Elk-1 pathway and, conversely, the VHR^{Y138F} mutant augmented TCR-induced Erk2 kinase and activation of the gene encoding interleukin 2. These results suggest that VHR is a target for ZAP-70 and tempers activation of the Erk2 pathway in a ZAP-70–controlled manner.

One of the earliest biochemical events after generation of the T cell antigen receptor (TCR) is a transient increase in the content of phosphotyrosine (PTyr) in a number of cellular proteins[1], many of which have not yet been identified. Inhibition of this phosphorylation prevents T cell activation, as measured by both functional read-outs and biochemical assays[2,3]. The protein tyrosine kinases Lck, Fyn and ZAP-70 catalyze many of the initial phosphorylation reactions[1,4], which results in the activation of many signaling pathways, including a transient activation of the three main classes of mitogen-activated protein kinases (MAPKs): extracellular signal-regulated kinase 2 (Erk2), c-Jun N-terminal kinase (Jnk) and p38[5]. For poorly understood reasons, each MAPK follows its own distinct time-course, which can vary extensively in magnitude and duration between cell types and depending on the stimulus. The molecular mechanisms responsible for these differences likely involve protein phosphatases[5,6] that inactivate MAPKs by removing phosphate from the threonine and/or tyrosine residues in their activation loops. Resting T cells express at least two such phosphatases: the hematopoietic protein tyrosine phosphatase (HePTP[7–9]) and *Vaccinia* virus VH1-related (VHR) phosphatase[10,11]. Additional MAPK phosphatases are synthesized in T cells starting 30–60 min after TCR stimulation[5,6].

VHR is a small dual-specific phosphatase with specificity for the MAPKs Erk2 and Jnk[11–13]. VHR inactivates the kinases by dephosphorylating their activation loop phosphothreonine and PTyr, the latter more readily. The third main type of MAPK, p38, does not appear to be a substrate for VHR, at least in T cells[11]. In contrast to other MAPK phosphatases, which contain regulatory sequences and often are encoded by inducible genes[5,6,14], VHR regulation has not been elucidated. We report here that VHR accumulates at the immune synapse, contains PTyr in activated T cells and appears to be a direct substrate for the ZAP-70 kinase.

Results

VHR inhibits T cell activation

VHR is constitutively expressed in resting human T lymphocytes[11], and even modest expression of VHR can suppress TCR-induced activation of the Erk2 and Jnk kinases[11]. To extend these findings to the transactivation of *IL2*, we expressed VHR in Jurkat T cells together with a luciferase reporter driven by the entire 5′ *IL2* promoter. When cells were stimulated with monoclonal antibodies (mAbs) to the TCR + CD28 or a mAb to the TCR + phorbol ester, 23- and 110-fold increases in luciferase responses were obtained, respectively (**Fig. 1a**). In the presence of VHR, this induction was reduced by 50% and 54%, respectively (**Fig. 1a**). Compared to two other small dual-specific phosphatases expressed in T cells—VHX[15] and MKP6[16]—VHR also efficiently reduced the TCR-induced activation of an NFAT–AP-1–driven reporter[11] (**Fig. 1c**) when the three proteins were expressed at equal amounts (**Fig. 1d**). When expressed at higher concentrations, VHX also inhibited this reporter[15]; only viral VH1 was more efficient than VHR (**Fig. 1c**). Thus, VHR is a potent inhibitor of TCR signaling to *IL2*.

VHR is recruited and tyrosine-phosphorylated

To visualize VHR during antigen-driven TCR signaling, we used CD8$^+$, OVA(257–264)- K^b–specific, OT-I TCR–transgenic T cells[17] + antigen-presenting cells (APCs). With fluorescence labeling and confocal microscopy, we found that a substantial portion of cellular VHR accumulated at the immune synapse between the T cell and the APC (**Fig. 1e**). In resting T cells, VHR was diffusely distributed throughout the cytosol and did not change its location upon contact of the T cell with APCs in the absence of antigen (**Fig. 1c**, U–X). However, in the presence of antigen, VHR began to accumulate at the site of TCR enrichment after 5 min of T cell–APC contact; after 10 min, much of VHR was already at the synapse. Maximal amounts of VHR were seen after 10–20 min of contact and persisted for several hours. This time-course correlated well with the down-regulation phase of TCR-induced Erk2 activation, and these results indicated that VHR is recruited to the TCR signaling machinery under physiological conditions of T cell activation.

Stimulation of normal human T lymphocytes with mAbs to the TCR and CD28 led to the appearance of PTyr in VHR, as shown by anti-PTyr immunoblotting of anti-VHR immunoprecipitates (**Fig. 1f**). VHR also

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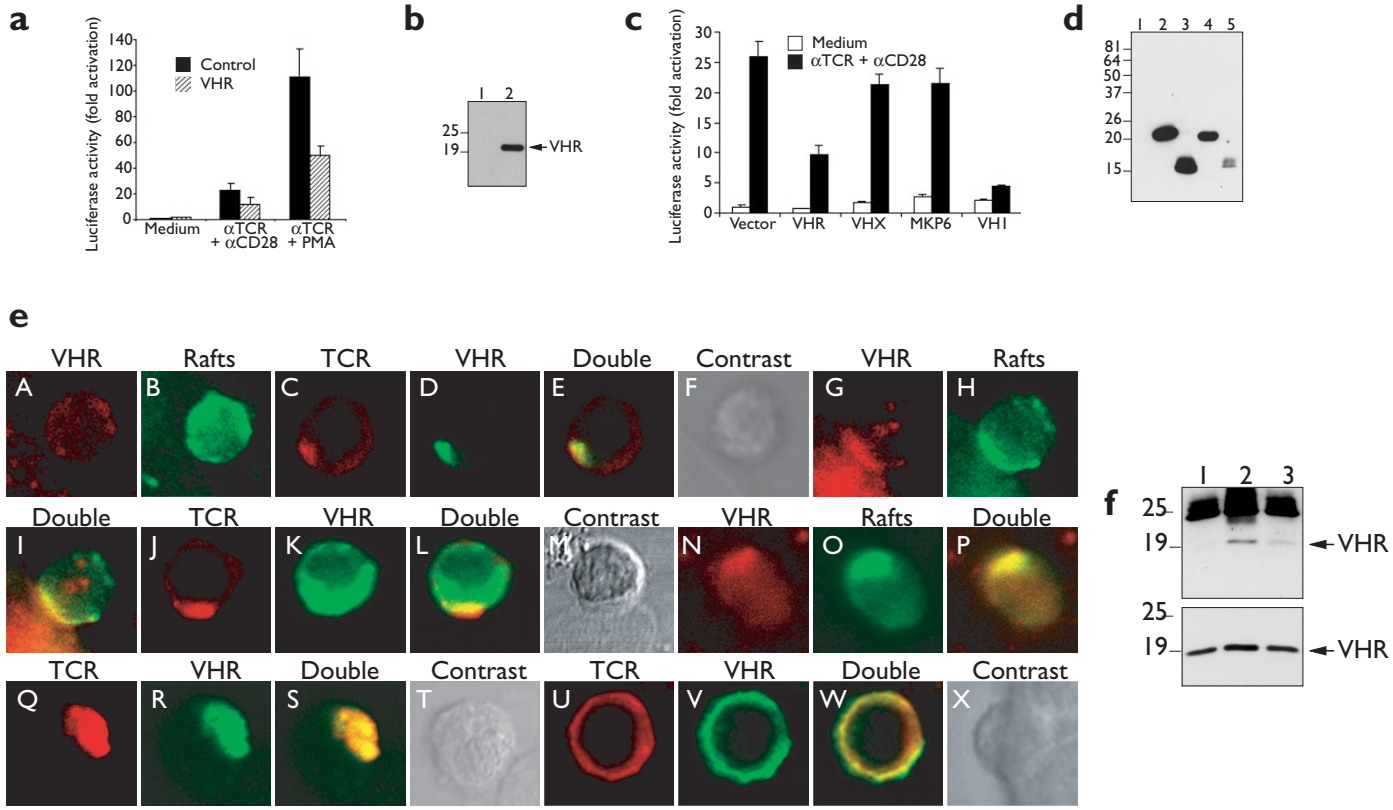

Figure 1. VHR inhibits T cell activation, accumulates in the immune synapse and is tyrosine-phosphorylated in normal T cells. (**a**) *IL2* activation measured with a luciferase reporter gene driven by the entire 5′ *IL2* promoter in Jurkat T cells cotransfected with VHR mutants. (**b**) A control immunoblot to show VHR expression. (**c**) Activation of a luciferase reporter gene driven by the NFAT–AP-1 element from the *IL2* promoter in Jurkat T cells cotransfected with the indicated phosphatases. (**d**) An anti-HA immunoblot to show the expression of the phosphatases, which were all cloned into the pEF vector with an N-terminal hemagglutinin (HA) epitope tag. (**e**) Phase contrast (Contrast) and confocal microscopy of CD8$^+$ T cells from OT-I TCR–transgenic mice incubated with APCs for 5 min (A–F), 10 min (G–M) or 20 min (N–T) and then either stained with fluorescein isothiocyanate (FITC)–conjugated cholera toxin to visualize cholesterol-enriched lipid rafts (Rafts) or with rhodamine-2C11 to visualize the TCR-CD3 (TCR) and with VHR mAb + either FITC- or rhodamine-conjugated anti-mouse Ig to show VHR (VHR). Cells are representative of the majority of T cells in contact with APCs. (U–X) Similarly stained T cells in contact for 20 min with APCs that do not express antigen. (**f**) (top panel) Anti-PTyr blot of VHR immunoprecipitates from untreated normal T lymphocytes (lane 1) or cells treated for 10 min with anti-CD3ε + anti-CD28 (lane 2) or 100 M pervanadate (lane 3). (bottom panel) Anti-VHR blot of the same filter.

became phosphorylated on tyrosine upon inhibition of endogenous phosphatases with pervanadate (**Fig. 1f**, lane 2). The amount of VHR was equal in each lane (**Fig. 1g**). Thus, VHR is not only recruited, but also comes in contact with TCR-associated tyrosine kinase(s) in normal T cells.

VHR tyrosine phosphorylation by ZAP-70

We next examined the requirement for ZAP-70 in VHR tyrosine phosphorylation (**Fig. 2**). Immunoprecipitation and anti-PTyr immunoblotting showed tyrosine phosphorylation after TCR-triggering of Jurkat T leukemia cells (**Fig. 2a**) and in the LAT-deficient clone JCaM2 (**Fig. 2c**), but not in the sublines JCaM1 (**Fig. 2c**) and P116 (**Fig. 2a**), which lack the Lck and ZAP-70 kinases[16], respectively. Because JCaM1 cells do not activate ZAP-70, but P116 cells contain Lck, our results implied that TCR-induced phosphorylation of VHR was ZAP-70–dependent. Re-expression of ZAP-70 in P116 cells restored VHR phosphorylation (**Fig. 2c**, lane 8). ZAP-70 and Syk also tyrosine-phosphorylated VHR in COS-1 cells (**Fig. 2d**), whereas other kinases (Csk, Lck, Fyn, Jak2, Bcr-Abl and Itk) had little effect. Finally, recombinant ZAP-70 readily phosphorylated VHR *in vitro* (**Fig. 2f**). Thus, VHR was tyrosine-phosphorylated by ZAP-70 in T cells. In addition, some ZAP-70 coimmunoprecipitated with VHR, whereas Lck was never detected in anti-VHR immunoprecipitates; this further supported the role played by ZAP-70 in phosphorylating VHR (unpublished observations).

ZAP-70 is required for VHR function

To determine whether ZAP-70 is required for the function of VHR, we assessed the ability of VHR to inhibit the Erk2–Elk-1 pathway[11] in the presence or absence of ZAP-70 (**Fig. 3**). Optimal Erk2 activation was induced by expression of a constitutively active Ras-L61 in wild-type Jurkat cells (**Fig. 3a**) or in ZAP-70–deficient P116 cells[18] (**Fig. 3c**). In the latter cells, active Ras-L61 induced a robust Elk-1 activation that was unaffected by VHR. In contrast, VHR inhibited Ras-L61–induced Elk-1 activation in wild-type Jurkat T cells, and inhibition became more prominent upon anti-CD3 stimulation. Similarly, when ZAP-70 was reintroduced into the P116 cells, VHR regained its ability to inhibit the MAPK response (**Fig. 3e**). The catalytically inactive VHR mutants VHR-C124S did not have any effect (data not shown). These results showed that VHR-mediated inhibition of the Ras-MAPK pathway is ZAP-70–dependent in T cells. Presumably, basal ZAP-70 activity was sufficient to stimulate VHR function. However, the inhibition by VHR became more pronounced upon TCR triggering.

VHR is phosphorylated at Y138

To identify the site or sites of phosphorylation, we first examined the crystal structure[19] of VHR, which showed that only tyrosine residues 38, 101 and 138 have their hydroxyl groups exposed on the surface of the protein; in contrast, tyrosines 23, 78, 85 and 128 are more or less buried in the structure. Based on this information, we generated the VHR^{Y38F}, VHR^{Y101F}

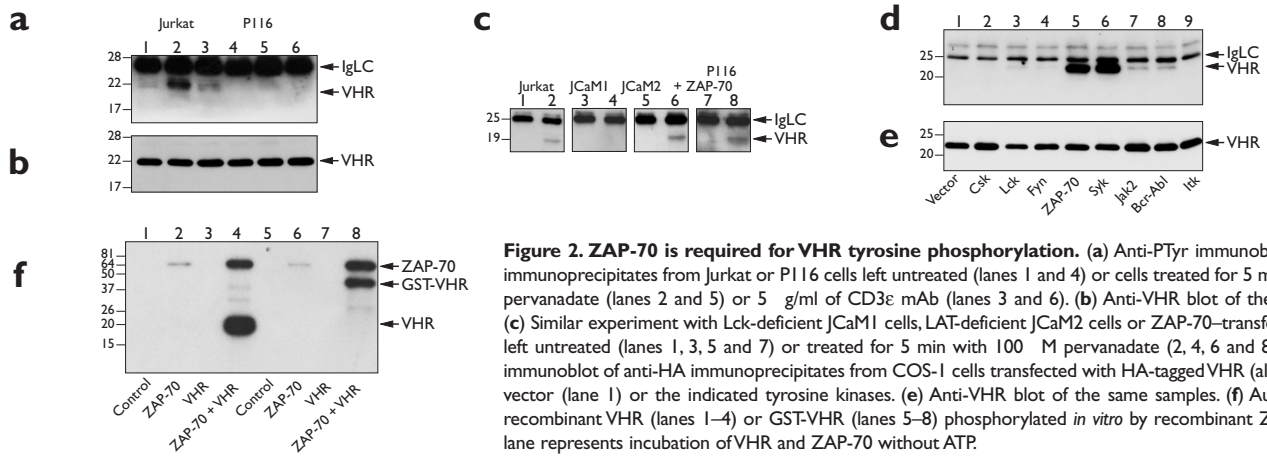

Figure 2. ZAP-70 is required for VHR tyrosine phosphorylation. (**a**) Anti-PTyr immunoblot of anti-VHR immunoprecipitates from Jurkat or P116 cells left untreated (lanes 1 and 4) or cells treated for 5 min with 100 M pervanadate (lanes 2 and 5) or 5 g/ml of CD3ε mAb (lanes 3 and 6). (**b**) Anti-VHR blot of the same samples. (**c**) Similar experiment with Lck-deficient JCaM1 cells, LAT-deficient JCaM2 cells or ZAP-70–transfected P116 cells left untreated (lanes 1, 3, 5 and 7) or treated for 5 min with 100 M pervanadate (2, 4, 6 and 8). (**d**) Anti-PTyr immunoblot of anti-HA immunoprecipitates from COS-1 cells transfected with HA-tagged VHR (all lanes) + empty vector (lane 1) or the indicated tyrosine kinases. (**e**) Anti-VHR blot of the same samples. (**f**) Autoradiogram of recombinant VHR (lanes 1–4) or GST-VHR (lanes 5–8) phosphorylated *in vitro* by recombinant ZAP-70. Control lane represents incubation of VHR and ZAP-70 without ATP.

and VHR^{Y138F} mutants, expressed them as glutathione-S-transferase (GST)-fusion proteins and incubated them with recombinant ZAP-70 in the presence of $MgCl_2$ and [γ-^{32}P]ATP. The resulting phospho-VHR was analyzed by tryptic peptide mapping, which showed that Tyr138 is a major phosphorylation site and that Tyr38 may also be phosphorylated, albeit less efficiently (**Fig. 4a**). In contrast, the tryptic peptide map of the VHR^{Y101F} mutant was indistinguishable from that of wild-type VHR. Similar results were obtained with purified Syk instead of ZAP-70 (data not shown). When the three phenylalanine mutants of VHR were expressed together with ZAP-70 in COS-1 cells and analyzed for tyrosine phosphorylation, VHR^{Y138F} was consistently less phosphorylated than wild-type VHR, VHR^{Y38F} and VHR^{Y101F} (**Fig. 4b**). The amount of VHR was equal between samples (**Fig. 4c**). Some reduction in VHR^{Y38F} phosphorylation also occurred. Thus, both Tyr38 and Tyr138 were phosphorylated by ZAP-70 in intact cells, and Tyr138 appeared to be phosphorylated more than Tyr38.

To determine whether one or both sites were also phosphorylated in intact T cells, we isolated phospho-VHR from pervanadate-treated Jurkat T cells and analyzed the protein by mass spectrometry. This detected a peptide of 1475.77 Da, which is the exact mass of the tryptic peptide containing PTyr138 (**Fig. 4d**). Peaks corresponding to other phosphopeptides were not observed. Thus, VHR is also phosphorylated at Tyr138 in normal T cells.

Tyr138 is required for VHR function

Next, we coexpressed VHR^{Y138F} in T cells to test its effects on TCR-dependent *IL2* activation (**Fig. 5**). Whereas wild-type VHR, VHR^{Y38F} and VHR^{Y101F} were inhibitory, VHR^{Y138F} strongly enhanced TCR-induced reporter gene activation (**Fig. 5a**). This was seen with a reporter driven by NFAT–AP-1 (**Fig. 5a**) and with a reporter driven by the entire 5′ promoter of *IL2* (data not shown). Thus, VHR requires an intact Tyr138 to exert its inhibitory function in TCR signaling. Increased gene activation by VHR^{Y138F} suggested that the mutant protein competes with endogenous VHR in a dominant-negative manner to prevent its phosphorylation.

To verify that VHR^{Y138F} augmentation of TCR-induced gene activation was causally related to the regulation of MAPKs by VHR, we generated stable transfectant clones with empty vector, VHR and VHR^{Y138F}. We measured the activation of Erk2 and Jnk in these cells by immunoblotting with antibodies that recognize the activated doubly phosphorylated form of these kinases. We found that Erk2 and Jnk activation were reduced in VHR-expressing cells but increased in VHR^{Y138F}-expressing cells compared to controls (**Fig. 5c,e**). In contrast, the TCR-induced ZAP-70 phosphorylation at Y319 was not inhibited by VHR or augmented by VHR^{Y138F} (**Fig. 5f**). The amounts of Erk2 and ZAP-70 were identical in the clones and they expressed similar amounts of TCR on their surface (**Fig. 5d,g**). These results supported the idea that VHR phosphorylation at Tyr138 is required for the function of VHR as a MAPK phosphatase in intact T cells.

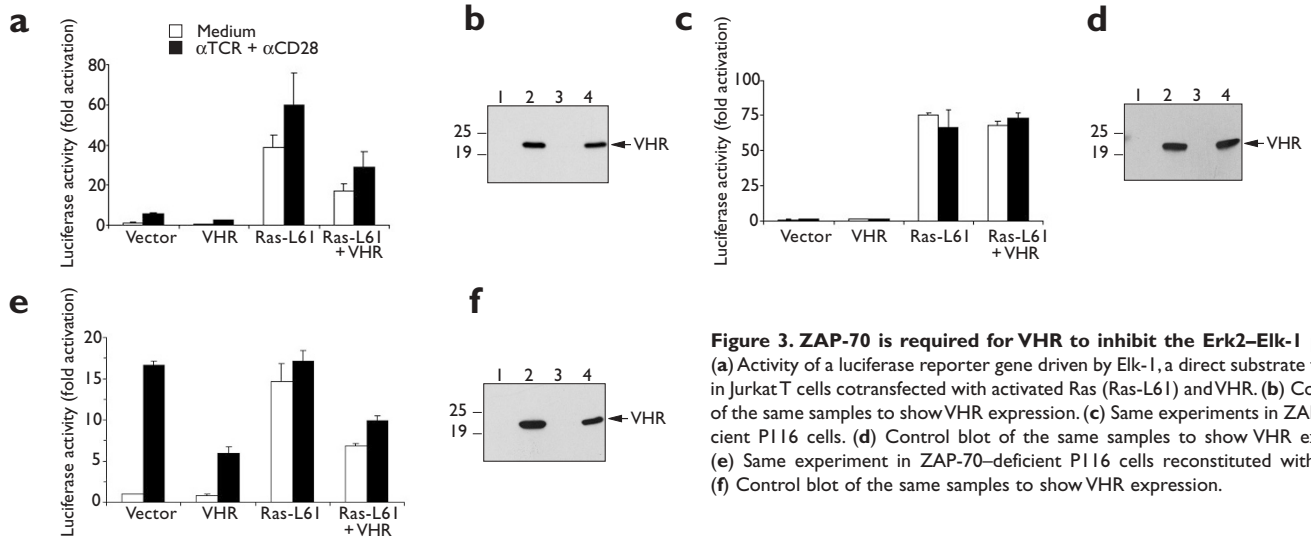

Figure 3. ZAP-70 is required for VHR to inhibit the Erk2–Elk-1 pathway. (**a**) Activity of a luciferase reporter gene driven by Elk-1, a direct substrate for Erk2[27], in Jurkat T cells cotransfected with activated Ras (Ras-L61) and VHR. (**b**) Control blot of the same samples to show VHR expression. (**c**) Same experiments in ZAP-70–deficient P116 cells. (**d**) Control blot of the same samples to show VHR expression. (**e**) Same experiment in ZAP-70–deficient P116 cells reconstituted with ZAP-70. (**f**) Control blot of the same samples to show VHR expression.

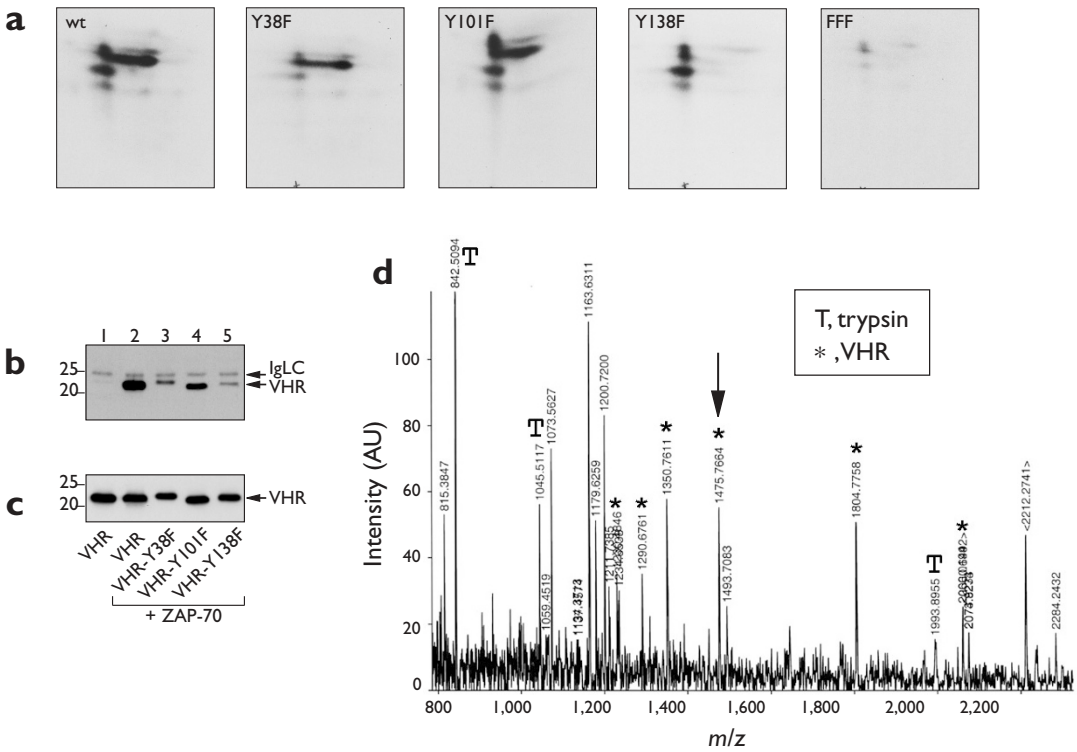

Figure 4. ZAP-70 phosphorylates VHR at Tyr38 and Tyr138. (**a**) Tryptic peptide maps of VHR phosphorylated by ZAP-70 *in vitro*. WT, wild-type. (**b**) Anti-PTyr immunoblot of VHR mutants coexpressed with ZAP-70 in COS-1 cells. (**c**) Control blot of the same samples to show VHR expression. (**d**) Mass spectrogram of a trypsin digest of VHR isolated from 500 10^6 pervanadate-treated Jurkat T cells. The arrow indicates the peptide with a 1475.77-Da mass, a precise fit for the peptide containing PTyr138. AU, arbitrary units.

Discussion

Our data show that VHR translocates from the cytosol to the pole of the T cell that faces an APC upon TCR stimulation by antigen. This redistribution brings VHR into the vicinity of the triggered TCRs, where VHR is phosphorylated at Tyr138 by ZAP-70. We found that this phosphorylation is required for the function of VHR as an inhibitor of the Erk2 and Jnk MAPKs and that a VHR^{Y138F} mutant behaves as a dominant-negative in that it augments MAPK activation and gene transactivation. Thus, VHR joins a small group of proteins that are known to be direct substrates for ZAP-70.

It is possible that phosphorylation of VHR directly activates its phosphatase activity. However, we were not able to detect such an effect. This was perhaps due to technical difficulties in preserving tyrosine phosphorylation of an active tyrosine phosphatase, which readily autodephosphorylates, and the poor activity of ZAP-70 with ATPγS as a donor for thiophosphate. In addition, the phosphorylation of VHR by ZAP-70 *in vitro* is much reduced by the ability of high concentrations of VHR to dephosphorylate and inactivate ZAP-70. This does not occur in T cells under physiological conditions, but makes the *in vitro* experiments difficult. Thus, the question of whether VHR phosphorylation directly activates its catalytic activity remains unanswered.

Because Tyr138 is located on the opposite side of the VHR catalytic center, it appears more likely that tyrosine phosphorylation of VHR affects other aspects of its function, such as protein-protein interactions, subcellular location or substrate targeting. These possibilities require further study, but would readily explain the dominant-negative effects of the VHR^{Y138F}. For example, if VHR was complexed to an inhibitor and only released upon tyrosine phosphorylation, then VHR^{Y138F} would remain bound and reduce the phosphorylation of endogenous VHR by competing for recognition by ZAP-70. This model also predicts that VHR overexpression will mimic VHR phosphorylation if the expressed amount of VHR exceeds the capacity of the inhibitor. Both these predictions are

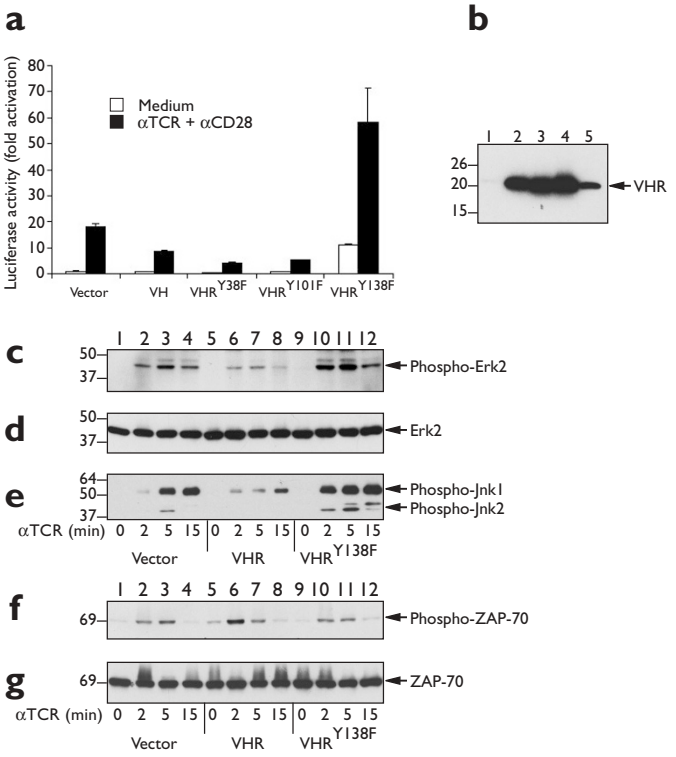

Figure 5. VHR^{Y138F} augments T cell activation. (**a**) Activation of a luciferase reporter gene driven by the NFAT–AP-1 element from the 5′ *IL2* promoter in Jurkat T cells cotransfected with various VHR constructs. (**b**) An anti-HA immunoblot of the same transfectants. (**c**–**g**) Immunoblots with antibodies specific for dually phosphorylated Erk2 (**c**), total Erk2 (**d**), phospho-Jnk (**e**), phospho-Y319 ZAP-70 (**f**) or total ZAP-70 (**g**) of lysates of Jurkat clones stably transfected with empty vector, wild-type VHR or VHR^{Y138F}. The blots represent time-courses after stimulation with the anti-TCR mAb C305.

compatible with our experimental findings. The location of Tyr138 also reveals that VHR can autodephosphorylate only *in trans*. Thus, if VHR is inactivated by autodephosphorylation, this reaction is highly dependent on its concentration. This may represent a negative feedback regulation that ensures that only low concentrations of tyrosine phosphorylated VHR exist at any time in the T cell. It is also possible that VHR is dephosphorylated by another phosphatase.

The accumulation of VHR at the immune synapse not only places VHR within reach of activated ZAP-70, but also raises the question of whether this population of VHR is the functionally active one. If so, MAPKs activated as a result of TCR signaling could be dephosphorylated by VHR before leaving the vicinity of the immune synapse. It may be relevant that VHR does not accumulate in the immune synapse until 10 min after antigen-presentation, a time point that coincides with the beginning of MAPK inactivation.

Unlike the *Vaccinia* virus VH1 phosphatase[20], which likely represents a 'stolen' cellular gene subverted to serve viral purposes and is a potent inhibitor of T cell activation, the cellular VHR does not completely block MAPK activation. Instead, VHR tempers and shortens Erk2 activation, as would be expected from a physiologically negative regulator. Thus, VHR phosphorylation by ZAP-70 may give the TCR signaling machinery more control over the duration of the Erk2 and Jnk MAPK responses. Because ZAP-70 plays a differential role in T cells differentiated along the T helper type 1 or 2 lines[21], in T cell anergy[22] and during thymic development[23], this may, in part, explain marked differences in the relative activation profiles of the Erk2 and Jnk MAPKs in these cells.

Methods

Antibodies and reagents. The mAb to VHR was from Transduction Laboratories (Los Angeles, CA). mAb 12CA5 to hemagglutinin was from Boehringer Mannheim (Indianapolis, IN). The OKT3 hybridoma that produced the CD3ε mAb was from American Type Culture Collection (Bethesda, MD). Both mAbs were used as ascites. Anti-CD28 was from PharMingen (San Diego, CA). The polyclonal anti-Erk2 was from Santa Cruz Biotechnology (Santa Cruz, CA).

Plasmids and site-directed mutagenesis. The VHR expression plasmids were as described[11]. GAL4-Elk was from J. Tian and G. Hauser (Burnham Institute, La Jolla, CA). Expression plasmids for Lck, Fyn, ZAP-70, Syk, Bcr-Abl, Jak2 and Csk were as described[24,25]. Codons for tyrosines were changed to codons for phenylalanine with the Transformer site-directed mutagenesis kit, as recommended by the manufacturer (Clontech, Palo Alto, CA). All mutations were verified by nucleotide sequencing.

Cells and transfections. Normal T lymphocytes were isolated from venous blood of healthy volunteers by Ficoll gradient centrifugation. Blood from anonymous donors was purchased from the San Diego Red Cross Blood Service. Institutional Human Subjects Assurance No. M-1383. Monocytes-macrophages were eliminated by adherence to plastic for 1 h at 37 °C. Jurkat T leukemia cells and the variants P116, JCaM1 and JCaM2 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 U/ml each of penicillin G and streptomycin. Transfection of Jurkat T cells and assays for luciferase activity were done as described[11,15]. The APCs used were K^b-expressing MEC cells stably transfected with B7.1 and a signal-peptide-OVA peptide fusion construct that directs the antigen into the endoplasmic reticulum for efficient MHC loading. MEC cells without the fusion construct were used as controls.

Immunoprecipitation, SDS-PAGE and immunoblotting. These procedures were done as described[11]. Briefly, cells were lysed in 20 mM Tris-HCl (at pH 7.5), 150 mM NaCl and 5 mM EDTA containing 1% NP-40, 1 mM Na_3VO_4, 10 g/ml of aprotinin and leupeptin, 100 g/ml soybean trypsin inhibitor and 1 mM phenylmethylsulphonyl fluoride and clarified by centrifugation at 15,000 rpm for 20 min. The clarified lysates were preabsorbed on protein G Sepharose and then incubated with antibody for 2 h, followed by protein G Sepharose beads. Immune complexes were washed three times in lysis buffer, once in lysis buffer with 0.5 M NaCl, again in lysis buffer and either suspended in SDS sample buffer or used for *in vitro* kinase assays. Proteins resolved by SDS-PAGE were transferred electrophoretically to nitrocellulose filters, which were immunoblotted with optimal dilutions of mAbs, followed by horseradish peroxidase–anti-mouse Ig, and the blots were developed by the enhanced chemiluminescence technique (ECL kit, Amersham) according to the manufacturer's instructions.

***In vitro* phosphorylation and tryptic peptide mapping.** The phosphorylation reaction contained 2 g of GST-VHR, 100 ng recombinant ZAP-70 (Upstate Biotechnology Inc., Lake Placid, NY) in 25 l of 50 mM HEPES (at pH 7.5), 150 mM NaCl, 10 mM $MnCl_2$, 1 mM Na_3VO_4 and 10 Ci of [γ-^{32}P]ATP and 10 M ATP. After 30 min at 30 °C, the proteins were resolved on SDS gels and transferred onto nitrocellulose filters. Phospho-VHR was localized by autoradiography, excised and digested with trypsin as described[26]. The resulting peptides were separated in two dimensions by thin layer electrophoresis at pH 1.9 followed by ascending chromatography.

Mass spectrometry. VHR was immunoprecipitated from 500 10^6 pervanadate-treated Jurkat cells, resolved by SDS-PAGE and visualized by Coomassie blue staining. The band was excised and digested with trypsin. The resulting peptides were analyzed in a Voyager 2000 matrix-assisted laser desorption ionization-time-of-flight mass spectrometer. The spectra were further fine-calibrated with characteristic trypsin autolysis peptides, and the monoisotopic masses of all nontrypsin peptides used in a database search with a ± 0.05-Da tolerance. This search gave VHR as a hit with a probability of 1 and a sequence coverage of 37% and a mass accuracy of ± < 0.038 Da.

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Competing interests statement
The authors declare that they have no competing financial interests.

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