Proteomic Identification of Proteins Interacting with a Dual Specificity Protein Phosphatase, VHZ

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Identification of Dual-specificity protein phosphatase (DSP) substrates is essential in revealing physiological roles of DSPs. We isolated VHZ-interacting proteins from extracts of 293T cells overexpressing a VHZ (C95S, D65A) mutant known to be substrate- trapping mutant. Analysis of specific proteins bound to VHZ by 2D gel electrophoresis and mass spectroscopy revealed that these proteins contained Chaperonin containing TCP1, Type II phosphatidylinositol phosphate kinase γ, Intraflagellar transport 80 homolog, and Kinesin superfamily protein 1B. VHZ-interacting proteins showed that VHZ is involved in many important cellular signal pathways such as protein folding, molecular transportation, and tumor suppression.

Key words: *dual specificity protein phosphatase, interacting protein, proteomic identification, VHZ*

A family of dual-specificity protein phosphatases (DSPs) dephosphorylates protein substrates at both the tyrosine and serine/threonine residues [Alonso *et al.*, 2004c], regulating the phosphorylation levels of several proteins that are essential for the physiological processes involved in cell survival, proliferation, and differentiation [Yu *et al.*, 2007; Rahmouni *et al.*, 2006; Sakaue *et al.*, 2004; Wu and Bunnett, 2005]. The mammalian genome encodes dozens of DSPs, and all of the catalytic domains of DSPs contain a consensus signature motif HCXXGXXR and have good alignments in the overall structures among them [Denu and Dixon, 1998]. However, each DSP has significant differences in the conformation of many residues and the surface charges, and presumably shows an individual function in the recognition of substrates and targeting to defined subcellular locations [Kim *et al.*, 2007]. Of these DSPs, the Vaccina H1-related phosphatase VHR represents a prototype of DSPs that lacks the MAP kinase-binding (MKB) domain. Without the MKB domain, VHR binds to the activated MAP kinases and downregulates the MAP kinase signaling [Alonso *et al.*, 2001].

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Recently, several interesting Vaccina H1-related phosphatases including VHX, VHY, and VHZ were cloned and characterized. VHX specifically activates c-Jun N-terminal kinase (JNK) pathway in HEK 293T cells [Chen *et al.*, 2002]. In jurkat T cells, VHX suppressed T cell antigen receptor-induced activation of extracellular signal-regulated kinase 2 (Erk2) [Alonso *et al.*, 2002]. Transient expression of VHX in COS7 cells deactivated p38 and JNK, but not Erk [Aoyama *et al.*, 2001]. Therefore, function of VHX may differ in different cell types. VHY has an N-terminal myristoylation recognition sequence and is expressed at high levels in the testis [Alonso *et al.*, 2004b].

VHZ was found to be expressed in most tissues such as heart, spleen, prostate, testis, colon, and small intestine [Alonso *et al.*, 2004c; Takagaki *et al.*, 2004]. However, to date, little is known of the physiological substrate of VHZ. VHZ was also predicted to have a different surface charge from VHR, suggesting that the substrate of VHZ may differ from that of VHR that dephosphorylates MAP kinase [Alonso *et al.*, 2004c]. In fact, overexpression of VHZ enhances the activation of JNK and p38 in COS-7 cells by activating their respective upstream kinases [Takagaki *et al.*, 2004]. To understand fully the function of VHZ, it is necessary to identify direct substrates of VHZ. In the case of protein tyrosine phosphatases (PTPs),

there are some reports that the alternation of both active Cys to Ser and/or general acid Asp to Ala resulted in an enhanced substrate-trapping mutant [Flint *et al.*, 1997; Wu *et al.*, 2006; Sun *et al.*, 1993; Shiozaki and Russell *et al.*, 1995].

Here, we constructed a trapping mutant of VHZ (C95S, D65A) and expressed the protein in 293T cells to identify substrates of VHZ *in vivo*. Proteomic approach identifies some VHZ-interacting proteins involved in the signal pathways such as protein folding, molecular transportation, and tumor suppression.

Materials and Methods

Construction of expression plasmid. A full-length cDNA molecule encoding VHZ was obtained from human kidney Quick-Clone cDNA (Clonetech) by polymerase chin reaction (PCR). The obtained PCR product was inserted into the *Nhe*I-*Xho*I sites of the mammalian expression plasmid pcDNA3.1/Zeo (+)-FH that is constructed from pcDNA3.1/Zeo (+) (Invitrogen) to tag the expressing protein flag and Hisx6 sequence in its N-terminus. VHZ (C95S, D65A) mutant was prepared using the site-directed method and was confirmed by DNA sequence analysis.

Cell culture and cell line. 293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum and antibiotics at 37°C, in a 5% CO_2 incubator. To obtain cell lines that express VHZ stably, pcDNA3.1/Zeo (+)-F-VHZ (C95S, D65A) was introduced into 293T cells using the LipofectAMINE methods (Invitrogen). Several transfectants were selected in the complete medium containing Zeocin (0.2 mg/mL) and the protein expression was confirmed by western blotting.

Pull-down analysis. 293T cells were lysed with a buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% triton X-100, 1 mM DTT, 1 mM EDTA, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 1 mM PMSF). The His-tagged VHZ (C95S, D65A) protein was pull-downed by Ni-NTA His-Bind Resin (Qiagen). After three times washing, the pull-downed proteins were used in 2-Dimentional electrophoresis analysis.

2-Dimentional electrophoresis and protein analysis. Isoelectric focusing (IEF) was performed using a Multiphor II electrophoresis unit (Amersham Bioscience) equipped with 13cm IPG strips (pH 3-10). The protein spots in gels were stained with CBB G-250 and excised using a clean scalpel. After in-gel protein digestion with trypsin, the resulting tryptic peptides were analyzed by MALDI-TOF (Applied Biosystem). The proteins were confirmed using a peptide matching method based on the theoretical peptide masses of proteins in NCBI database.

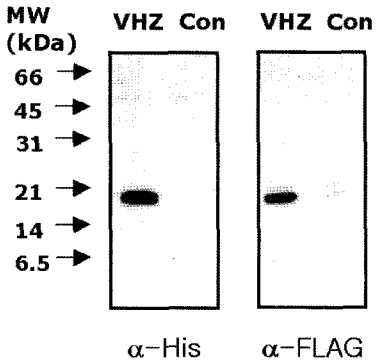

Fig. 1. Expression of VHZ in 293 T cells. HEK 293 T cells were transfected with a plasmid expressing double-tagged VHZ (C95S, D65A) and selected for stable expression of VHZ. Whole-cell lysates of the selected HEK 293 T cells and parental cells were resolved by 15% SDS/PAGE and transferred to a nitrocellulose membrane. To confirm the expression of VHZ, the membrane was immunoblotted with anti-His and anti-flag antibodies, respectively. Positions of the molecular mass markers (kDa) are shown on the left.

Results

We prepared 293T cell lines expressing flag-tagged VHZ (C95S, D65A) stably. 293T cells were transfected with a mammalian expression vector pcDNA3.1/Zeo (+)-F-VHZ (C95S, D65A), which was constructed to express VHZ (C95S, D65A) proteins. Several transfectants were selected and designated as 293T-VHR (C95S, d65A). Expression of VHZ (C95S, D65A) was confirmed by immunoblotting the cell lysates with anti-His and anti-flag antibodies, respectively. Compared to the parental 293T cells, 293T-VHZ (C95S, D65A) cells showed a protein band of predicted molecular weight (Fig. 1).

Parental and 293T-VHZ (C95S, D65A) cells were lysed, and VHZ (C95S, D65A) proteins were pull-downed with Ni-NTA bead. The pull-downed proteins were visualized by Coomassie bule staning of the 2-D gels. Several specific protein spots appeared in the both gels in similar intensities (Fig. 2), and those proteins might represent proteins interacting with His-tag or beads. We also found some specific protein spots in the lysate of 293T-VHZ (C95S, D65A), representing potential VHZ-binding proteins.

Two-D images of the potential VHZ-interacting proteins characterized by MALDI-TOF Mass spectroscopy are shown in Fig. 3 and listed in Table 1. Chaperonin containing TCP1, which is known to be involved in protein folding upon ATP hydrolysis [Kubota *et al.*, 1994], was found to interact with VHR. Type II phosphatidylinositol phosphate kinase (PIPKII) phosphorylates phosphatidylinositol-5-bisphosphate (Ptdins-5-P) at D-4

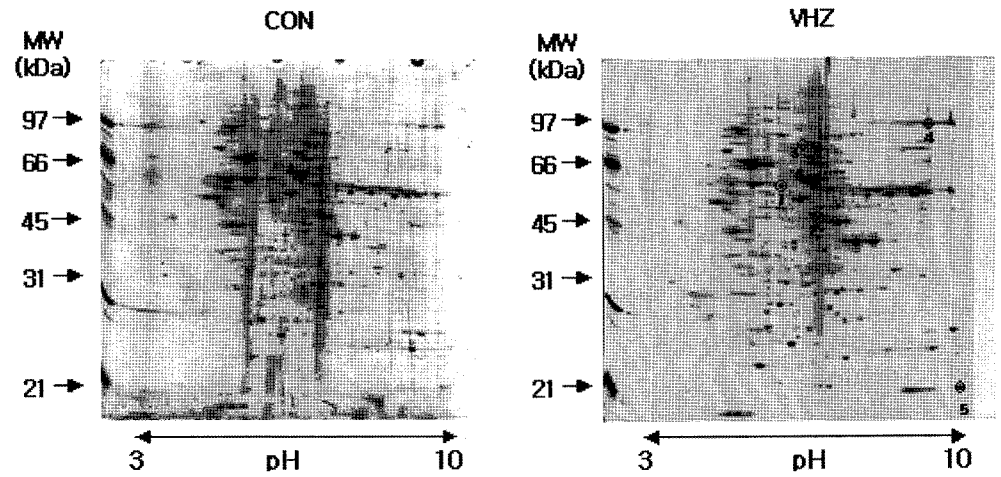

Fig. 2. Coomassie-stained 2-D SDS PAGE gels of proteins with Ni-NTA beads. Ni-NTA beads were used to pull-down proteins from whole cell lysates. The proteins bound to the beads were subjected to 13 strip, and SDS-PAGE was performed on a 12% gel. Left panel, pull-down proteins from the lysate of parental HEK 293T cells, Right panel, pull-down proteins from the lysate of HEK 293T cells expressing VHZ (C95S, D65A). The numbers represent protein spots characterized by MALDI-TOF analysis.

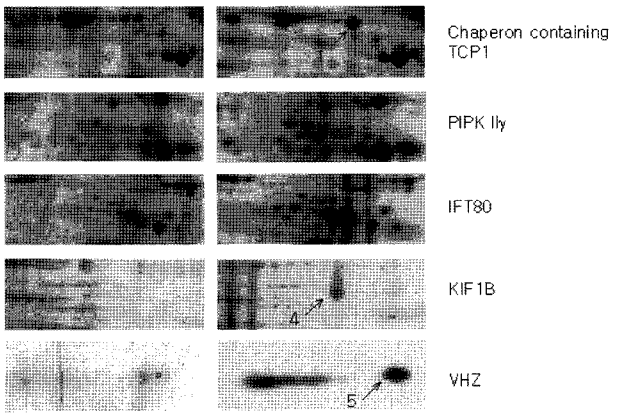

Fig. 3. Insets from Fig. 2 showing the images of altered patterns. Enlarged images of the VHZ-interacting proteins from HEK 293T cells. Arrows indicate protein spots that were selected and analyzed.

position, synthesizing Ptdins-4,5-P_2, a key phospholipid in the production of second messengers such as diacylglycerol and inositol 1, 4, 5-triphosphate [Itoh *et al.*, 1998]. We also detected PIPKIIγ in the precipitant of the cells expressing VHZ (C95S, D65A), suggesting VHZ is involved in the phosphoinositide signaling pathway. Intraflagellar transport 80 homolog (ITF80) was also included in the pull-downed proteins. Although its cellular functions are not clear, ITF80 has the WD domain repeat that is often found in proteins playing crucial roles in the signal transduction such as transcriptional regulation, RNA processing, and cell cycle progression [Smith *et al.*, 1999]. KIF1B is a member of the kinesin superfamily proteins that transport protein complexes and mRNAs to specific destinations within the cell [Miki *et al.*, 2001], and is shown to be a candidate for tumor suppressor gene of neuroblastoma cells [Yang *et al.*, 2001]. Compared to the parental HEK 293T cells, HEK 293T cells expressing VHZ (C95S, D65A) only showed a strong specific protein spot that is expected to VHZ at the site designated as 5 (Figs. 2 and 3). This protein was identified as VHZ by MALDI-TOF Mass spectroscopy analysis, showing that the pull-downed proteins contained VHZ and VHZ-interacting proteins.

Table 1. Identification of pull-downed proteins by MALDI-TOF analysis of HEK293T cells expressing VHZ (C95S, D65A)

Spot #	Protein name	Accession #	MW (kDa)	pI	Coverage %
1	Chaperonin containing TCP1	5453603	57448	6.0	24
2	PIPKIIγ	1251357	47328	6.4	23
3	ITF80	61217653	86446	6.9	18
4	KIF1B	33469083	130280	8.6	15
5	VHZ	12654609	16588	8.4	44

Discussion

VHZ consists of a minimal essential core of the phosphatase having only 150 amino acid residues and is a phylogenetically well-conserved enzyme with a probable ortholog from mammals to the Archea *T. kodakaraensis* [Alonso *et al.*, 2004a], suggesting that basic function of VHZ in cell physiology. In some respect, VHZ is similar to VHR, a prototype of atypical DSPs that is well characterized structurally and biologically. Although VHR is a member of the dual specificity protein phosphatases, it has a relatively deep active site pocket and prefers pY to pT in the dephosphorylation reactions [yuvaniyama *et al.*, 1996]. Similarly, recombinant VHZ was shown to dephosphorylate Tyr(P)-MBP 10^3 times rapidly than and Ser/Thr (P)-MBP [Takagaki *et al.*, 2004]. Similar to VHR, VHZ also has no MKB domain. However, VHZ is known to be different from VHR in its cellular functions. VHR shows inhibitory role in CD28-induced Erk and JNK activations, but not in p38 activation [Alonso *et al.*, 2001]. However, VHZ enhances the activation of JNK and p38, and do not affect the phosphorylation level of Erk. Both Inactive and active VHZs were similar in the activation of JNK and p38 in COS-7 cells [Takagaki *et al.*, 2004]. Therefore, the real substrate of VHZ may not be Erk, JNK, and p38 *in vivo*.

VHZ was found to interact with Chaperonin contataining TCP1, suggesting that VHZ is involved in the protein folding. However, previous studies showed that VHZ also interacts with the overexpressed proteins [Guo *et al.*, 2005; Andersen *et al.*, 2005]. Thus, it cannot be rule out that Chaperonin contataining TCP1 may help the overexpressed VHZ to be folded correctly in cells. PIPKIIγ is one of the substrate candidates for VHZ *in vivo*. Similar to VHZ, PIPKIIγ could be detected in almost all tissues including heart, brain, spleen, kidney, and testis. Upon extracellular stimuli such as EGF, PDGF, and lysophosphatidic acid, PIPKIIγ can be phosphorylated [Itoh *et al.*, 1998]. This implies that the level of phosphorylation may regulate PIPKIIγ activity, and the resulting Ptdins-4,5-P_2 can function as a second messengers in the cellular pathway leading to Map kinase. VHZ can also participate in the transcriptional regulation and RNA processing by interacting with ITF 80 and molecular transportation by KIF1B. It now remains to be determined how VHZ regulates the interacting proteins mentioned above. This is under further investigation in our laboratory.

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