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Protein & Cell

LETTER

A genetically encoded sulfotyrosine for VHR function research

Dear Editor,

Protein tyrosine phosphorylation is one of the most prevalent forms of post-translational modification and plays a significant role in the control of a variety of cellular processes, including signal transduction, and is implicated in many human diseases (Eckhart et al., 1979; Karisch et al., 2011). The human vaccinia H1-related phosphatase (VHR), the first human dual specificity protein phosphatase (DSP), hydrolyzes phosphate monoesters from phosphotyrosine, phosphothreonine, or phosphoserine residues (Aroca et al., 1995). DSPs function in signal transduction and in control of the cell cycle; more than 20 mammalian DSPs have so far been identified. Although VHR can dephosphorylate both Erk and Jnk *in vivo* (Schumacher et al., 2002), it differs from other 'typical' mitogen activated protein (MAP) kinase phosphatases (MKPs). With only 185 amino acids, VHR consists of only a catalytic domain with no recognizing or docking domain. As a member of the "atypical" DSPs, VHR has been widely used in attempts to elucidate the catalytic mechanism of DSPs. As a phosphatase, VHR has a critical role in managing the activities of the mitogen activated protein kinases (MAPKs) Erk and Jnk by dephosphorylating the phosphor-Thr and phosphor-Tyr residues in their respective active sites during the cell cycle. As a predominant nuclear enzyme, a large number of researches have been done on VHR function of phosphatase activity, but comparatively little is known about its own phosphorylation. This is largely because there is no way to site-specifically incorporate a phosphor-amino acid into a recombinant protein, so it has only been possible to conjecture about the phosphorylation site of VHR are from indirect results (Hoyt et al., 2007). Thus, the function and impact of VHR phosphorylation remain incompletely understood and hotly debated. In this manuscript, we report the first successful site-specific incorporation of sulfotyrisine into VHR through the use of expanding genetic code, confirm the site of phosphorylation, and describe the activities of the sulfation-modified VHR.

Expanding genetic code is a useful method that allows one to genetically encode unnatural amino acid (UAA) into protein during translation with high fidelity and efficiency (Wang et al., 2001). The resulting protein contains UAA at any location specified by a TAG codon, making this method significantly simpler and more versatile than competing methods such as *in vitro* enzymatic modification, chemical modification and peptide synthesis. Since this method was developed by Peter. G. Schultz about twenty-years ago (Mendel et al., 1995), more than 80 UAAs with additional functional groups have been incorporated into recombinant proteins. This methodology involves the generation of a unique codon-tRNA pair and corresponding aminoacyl-tRNA synthetase. Specifically, an orthogonal tRNA that is not a substrate for any natural aminoacyl synthetases is constructed and this tRNA with its cognate amino acid could be in response to the amber nonsense codon. An orthogonal synthetase is then selected which recognizes the unique tRNA rather than any endogenous natural tRNA, the substrate specificity of this synthetase is then evolved by changing the active sites to recognize the specific UAA instead of any endogenous natural amino acid (Xie and Schultz, 2005). Using this method, a sulfotyrosine (sulfo-tyr) specific synthetase was generated and used to catalyze the site-specific incorporation of a sulfo-tyr residue into a recombinant protein in *E. coli* (Liu and Schultz, 2006). This system has been used to both structurally and biochemically analyze the role of tyrosine sulfation in the naturally sulfated protein hirudin (Liu and Schultz, 2006; Liu et al., 2007) and to evolve 'unnatural' selectively sulfated proteins with enhanced activities through phage-based directed evolution methods (Liu et al., 2008).

We first synthetised the sulfo-tyr (Jevons, 1963) and the sulfo-tyr was identified by LC-Mass Spectrometry (Fig. 1A and 1B). The theoretical molecular weight of sulfo-tyr is 261. The result of Mass spectrometry is 262 which are the sulfotyrosine, with the addition of hydrogen. The yield of sulfo-tyr from L-tyrosine was up to 70%. Then we constructed the sulfo-tyr incorporation system (Liu and Schultz, 2006; Liu et al., 2009) which could efficiently incorporate sulfo-tyr into recombinant protein at the selected specific site. To determine that SulfotyrRS incorporated sulfotyrosine into VHR at the specific site, VHR-WT, VHR-128-sulfotyrosine and VHR-138-sulfotyrosine were over-expressed in *E. coli*. VHR-WT and its mutants were purified by His-tag affinity purification and confirmed by SDS-PAGE (Fig. 1C). The purity of the purified VHR and its mutants could reach 95%. The yield of wild type VHR was 18 mg/L. For comparison, the yield of the VHR mutant with sulfo-tyr incorporation was 8 mg/L. This difference in yield is due to the lower translation efficiency of the UAG codon compared with normal sense codon. To determine that SulfotyrRS incorporated sulfotyrosine

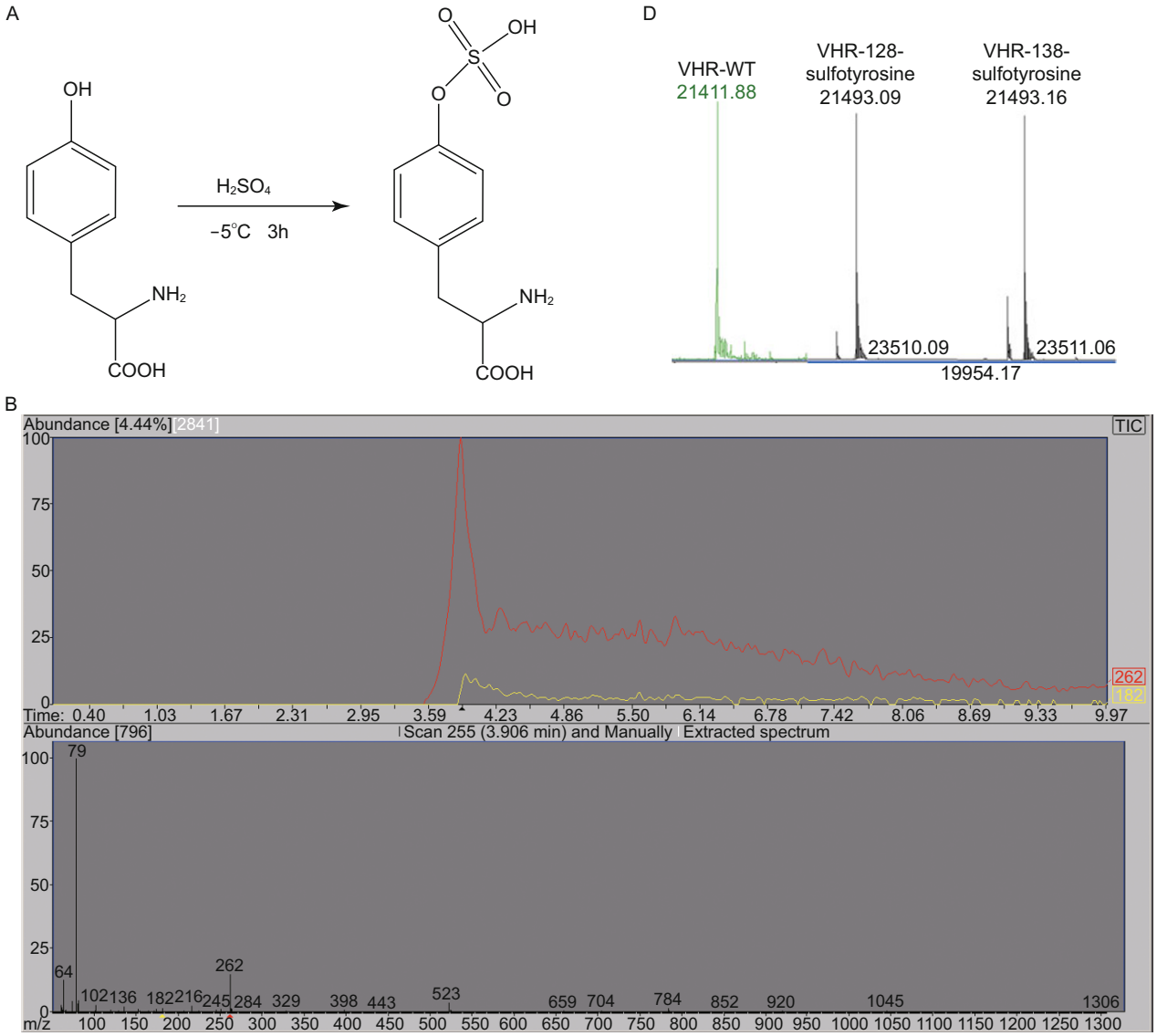

Figure 1. Sulfotyrosine synthesis and incorporation into protein. (A) The synthesis of sulfotyrosine. (B) LC-MS Single Q results of sulfotyrosine. (C) Coomassie-stained SDS-PAGE of VHR-WT, VHR-128-sulfotyrosine and VHR-138-sulfotyrosine (indicated by the arrow). Lane M, molecular standard in kDa; line 1, purified VHR-WT; line 2, purified VHR-128-sulfotyrosine; line 3, purified VHR-138-sulfotyrosine. (D) CapLC-Q-ToF micro measurement of the VHR-WT, VHR-128-sulfotyrosine and VHR-138-sulfotyrosine, respectively.

into VHR mutants, CapLC-Q-ToF micro analysis of the VHR-WT, VHR-128-sulfotyrosine and VHR-138-sulfotyrosine was performed (Fig. 1D). The Mass Spectrometry results were consistent with the theoretical molecular weight of these proteins (Table 1).

VHR is a dual specificity phosphatase capable of dephosphorylating both phosphotyrosine and phosphothreonine/phosphosernine-containing substrate. Because of the similar biochemical characteristics between phosphor-Tyr and sulfo-Tyr, we tested which sites of VHR could be phosphorylated using sulfo-tyr. *Para*-Nitrophenylphosphate (pNPP) is a chromogenic substrate for acid and alkaline phosphatase. Under the influence of phosphatase, the decay to yellow *para*-nitrophenol is catalysed and this product can be measured with a 405 nm spectrophotometer. So the activities of VHR and its mutants against the substrate pNPP were measured using a spectrophotometer. As shown, the purified recombinant VHR-WT hydrolyzed the phosphate from the small molecule of

Table 1. The theoretical protein molecular weight and observed mass of VHR-WT and its mutants

VHR-WT	Theoretical Mw: 21412.21 Da
	Practical Mw: 21411.88 Da
VHR-128-sulfotyrosine	Theoretical Mw: 21492.21 Da
	Practical Mw: 21493.09 Da
VHR-138-sulfotyrosine	Theoretical Mw: 21492.21 Da
	Practical Mw: 21493.16 Da

pNPP and it showed the highest activity at pH 7.0 (Fig. 2A). Hydrolysis increased as a function of VHR concentration and the dynamic constants of VHR were determined under the optimal pH factors to pNPP (Fig. 2B and 2C).

The phosphatase activity of VHR has been extensively researched, but to date relatively little is known about the consequences of its own phosphorylation. Several investigators have speculated about the probable site of phosphorylation of VHR from some indirect results, but no direct experimental evidence has yet been published. Here we simulate phosphotyrosine with sulfotyrosin to directly study the impact of phosphorylation on VHR function. To identify the site of phosphorylation, we first checked the sequence of VHR and found that there were seven tyrosine residues. Then we examined the crystal structure of VHR (Yuvaniyama

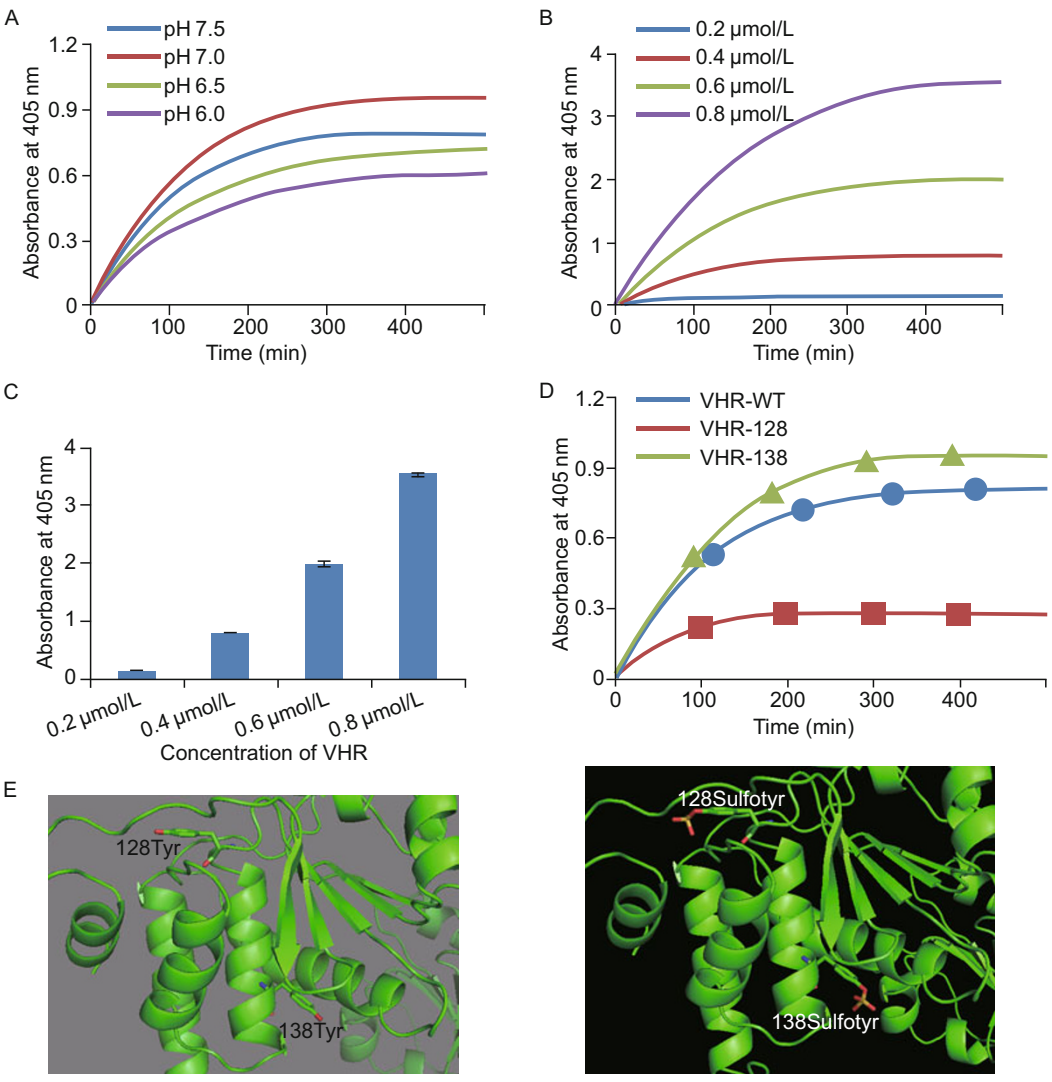

Figure 2. Phosphatase activity of purified VHR-WT and its mutants. (A) Phosphatase activity of purified VHR-WT in different pH buffer. (B) Phosphatase activity of purified VHR-WT in different concentrations. (C) The measurement of absorbance when the reaction achieved equilibrium. (D) Left: Structure of VHR (PDB code: 1VHR), bearing tyrosine at the site of 128 and 138; Right: Structure model of VHR, bearing sulfotyrosine at the site of 128 and 138. (E) Phosphatase activity of purified VHR-WT, VHR-128-sulfotyrosine and VHR-138-sulfotyrosine, respectively.

et al., 1996), which showed that only tyrosine residues of 128 and 138 are likely to be modified (Fig. 2D); in contrast, the hydroxyl groups of tyrosines 23, 78 and 85 are more or less buried internally. Tyrosine 38 may play an important role in dimerization and tyrosine 101 lies in the β-sheet. Based on this information, we generated the VHR mutants of VHR-128TAG and VHR-138ATG, then expressed and purified VHR-128-sulfotyrosine and VHR-138-sulfotyrosine in the way of VHR-WT. The enzymatic activities of all three proteins against the substrate pNPP were compared. When the Tyr 128 of VHR was replaced with sulfo-tyr, the phosphotase activity of VHR-128-sulfotyrosine declined significantly. However, when the Tyr 138 was substituted with this UAA, the enzyme activity towards pNPP increased (Fig. 2E). Collectively, our experimental evidence suggests that sulfotyrosine at the residue 138 does have direct effect on the interaction between the VHR and the small molecule substrate of pNPP. It has previously been hypothesized, based on a series of indirect results, that phosphorylation of VHR at the Tyr 138 is likely to have a functional impact on its activity; here we have been able to demonstrate that Tyr-138 phosphorylation does have direct effect on phosphatase function of VHR. Phosphorylation of tyrosine at this site is likely to modulate the activity of VHR by affecting its overall structure. Future studies will have to be done to determine how phosphorylation at this site plays a role in the function of VHR as a phosphatase *in vivo*.

FOOTNOTES

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Yueting Zheng, Xiaoxuan Lv and Jiangyun Wang declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by the any of the authors.

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