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RESEARCH ARTICLE

Specific dephosphorylation of Janus Kinase 2 by protein tyrosine phosphatases

Jianzhuo Li[1,2], Xidong Liu[3,4*], Huiying Chu[5*], Xueqi Fu[1,2,4], Tianbao Li[1,2], Lianghai Hu[1,3], Shu Xing[2,4**], Guohui Li[5**], Jingkai Gu[1,3] and Zhizhuang Joe Zhao[2,6]*

[1] Key Laboratory Molecular Enzymology and Engineering, the Ministry of Education, School of Life Sciences, Jilin University, Changchun, China
[2] Edmond H. Fischer Signal Transduction Laboratory, School of Life Sciences, Jilin University, Changchun, China
[3] Research Center for Drug Metabolism, School of Life Sciences, Jilin University, Changchun, China
[4] National Engineering Laboratory of AIDS Vaccine, School of Life Sciences, Jilin University, Changchun, China
[5] State Key Laboratory of Molecular Reaction Dynamics, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian, China
[6] Department of Pathology, University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA

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Many protein kinases are activated through phosphorylation of an activation loop thereby turning on downstream signaling pathways. Activation of JAK2, a nonreceptor tyrosine kinase with an important role in growth factor and cytokine signaling, requires phosphorylation of the 1007 and 1008 tyrosyl residues. Dephosphorylation of these two sites by phosphatases presumably inactivates the enzyme, but the underlying mechanism is not known. In this study, we employed MALDI-TOF/TOF and triple quadrupole mass spectrometers to analyze qualitatively and quantitatively the dephosphorylation process by using synthetic peptides derived from the tandem autophosphorylation sites (Y1007 and Y1008) of human JAK2. We found that tyrosine phosphatases catalyzed the dephosphorylation reaction sequentially, but different enzymes exhibited different selectivity. Protein tyrosine phosphatase 1B caused rapid dephosphorylation of Y1008 followed by Y1007, while SHP1 and SHP2 selectively dephosphorylated Y1008 only, and yet HePTP randomly removed a single phosphate from either Y1007 or Y1008, leaving behind mono-phosphorylated peptides. The specificity of dephosphorylation was further confirmed by molecular modeling. The data reveal multiple modes of JAK2 regulation by tyrosine phosphatases, reflecting a complex, and intricate interplay between protein phosphorylation and dephosphorylation.

Keywords:
Cell biology / Dephosphorylation / MS / JAK2 / Tyrosine phosphatases

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1 Introduction

Protein PTM by phosphorylation plays an important role in the regulation of cellular functions, such as growth, metabolism, and differentiation [1, 2]. Among the one-thirds of total proteins phosphorylated in mammalian cells [3, 4], tyrosine phosphorylation corresponds to only 1.8% of total

Correspondence: Dr. Lianghai Hu, Life Science Building 345, College of Life Science, Jilin University, Changchun 130012, P. R. China
Fax:+86-431-85155380
E-mail: lianghaihu@jlu.edu.cn

Abbreviations: **HePTP**, hematopoietic protein tyrosine phosphatase; **JAK**, Janus kinase; **MD**, molecular dynamics; ***p*NPP**, *p*-nitrophenol phosphate; **PTK**, protein tyrosine kinase; **PTP**, protein tyrosine phosphatase; **PTP1B**, protein tyrosine phosphatase 1B; **SHP**, src homology 2 domain-containing tyrosine phosphatase; **SOCS**, suppressor of cytokine signaling

*These authors contributed equally to this work.
**Additional corresponding authors:
Dr. Shu Xing, E-mail: xingshu@jlu.edu.cn;
Dr. Guohui Li, E-mail: ghli@dicp.ac.cn.
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cellular phosphorylation [5] but usually acts in the upstream of serine/threonine phosphorylation. Tyrosine phosphorylation is involved in numerous important aspects of human cellular physiology in health and disease and is controlled by coordinate actions of protein tyrosine kinases (PTKs) and tyrosine phosphatases (PTPs) [6]. The activation of PTKs is initialized by phosphorylation of an activation loop, which subsequently activates downstream signaling pathways [7]. Abnormally increased PTK activity can have dire consequences, including developmental abnormalities [8], cancer, or fibrosis [9]. Therefore, PTPs presumably play a crucial role in the regulation of PTKs and their substrates [10].

Janus kinase 2 (JAK2) is a nonreceptor tyrosine kinase [11] and has been implicated in signaling by several receptor families. The activation of JAK2 kinase is mediated by many growth factors and cytokines [12, 13], and the JAK2 kinase activity can be downregulated by PTPs. The autophosphorylation at tyrosine 1007 (Y1007) and tyrosine 1008 (Y1008) plays a regulatory role in the function of JAK2 [14]. Phosphorylation of Y1007 and Y1008 is the initial step for activation of JAK2 and downstream signaling pathways [15]. Some mutant forms of JAK2, e.g. JAK2V617F, enhance the level of phosphorylation at Y1007 and Y1008, which causes constitutive activation of signal transduction pathways, growth factor/cytokine-independent cell growth, and finally leads to diseases including myeloprolifetative neoplasms [16, 17]. Therefore, it is of critical importance to clarify the negative regulation of JAK2 activity, especially the dephosphorylation process of pY1007 and pY1008.

Traditional approaches for protein phosphorylation analyses include radioactive $^{32}P^{18}$ labeling, phospho-specific antibodies [19], and fluorescent probes [20]. However, due to the nature of all these methods, they are not reliable for detection of phosphorylation stoichiometrically at specific sites. Novel approaches need to be explored to elucidate the regulation of JAK2 tandem phosphorylation at pY1007 and pY1008 by PTPs. MS methods have been widely used in the qualitative and quantitative phosphorylation analysis due to the high mass selectivity and sensitivity [21–23]. Herein, monophosphorylated and di-phosphorylated peptides derived from the autophosphorylation sites (Y1007 and Y1008) [24] of human JAK2 were synthesized and used as substrates to monitor the dephosphorylation process by PTPs. MALDI-TOF/TOF and triple quadrupole mass spectrometers were employed for the qualitative and quantitative analysis of di-phosphorylated peptide dephosphorylation process. We found that the diphosphorylated peptides were dephosphorylated sequentially and selectively on pY1007 and pY1008 by different PTPs.

2 Materials and methods

2.1 Chemicals and reagents

HPLC-grade ACN, methanol, and water were obtained from Fisher Scientific (Fair Lawn, NJ, USA). All other chemicals were analytical grade and used with no further purification. Alkaline phosphatase and DHB was obtained from Sigma (St. Louis, MO). PQDKEpYpYKVKE, PQDKEpYYKVKE, and PQDKEYpYKVKE were synthesized with 95% purity (APeptide Co. Ltd., Shanghai, China). Recombinant proteins containing the catalytic domains of tyrosine phosphatases protein tyrosine phosphatase 1B (PTP1B), src homology 2 domain-containing tyrosine phosphatase-1 (SHP1), SHP2, and hematopoietic protein tyrosine phosphatase (HePTP) were purified as previously described [25–27].

2.2 PTP activity assays

PTP activities were measured by using *p*-nitrophenol phosphate (*p*NPP) as a substrate at 37°C as previously described [28]. The assays were performed in a 25 mM MOPS buffer (pH 7.0) containing 1.0 mM EDTA, 1.0 mM DTT, and 1 mg/mL BSA. The reactions were terminated by the addition of 0.2 M NaOH. OD_{405nm} was measured with a microplate reader. The activity of alkaline phosphatase was measured with *p*NPP in a basic buffer provided by manufacturer of the enzyme (Sigma). One unit of activity is defined as 1 nmol of phosphate released per minute.

2.3 Peptide dephosphorylation

The reaction system was a 200 μL mixture containing 160 μL working buffer (25 mM MOPS, pH 7.0, 1.0 mM EDTA, 1.0 mM DTT, 1 mg/mL BSA), 20 μL peptide (1–20 nmol), and 20 μL enzymes (0.18–5.4 U). The reaction was started with the addition of 20 μL enzymes and terminated by 10% TFA to a final pH below 3.0.

2.4 Cell culture and subcellular protein extraction

Jurkat cells were cultured at 37°C in 5% CO_2 in RPMI1640 medium (Invitrogen containing 10% FCS (Gibco). Cells were harvested by centrifugation and lysed in a whole cell extraction buffer (25 mM β-glycerolphosphate, 0.1 M NaCl, 1% Triton X-100, 10 mM EDTA, 2 mM EGTA, 10 mM 2-mercaptoethanol, and protease inhibitors). Subcellular fractionation of Jurkat cells were performed by using the ProteoExtract Subcellular Proteome Extraction Kit (EMD Biosceinces) that yielded cytosol, membrane/organelle, and nucleus fractions. As described above, total PTP activity in the cell extracts were determined by using *p*NPP, and subcellular cell extracts containing 0.54 U PTP activities were used to treat phosphopeptides.

2.5 MALDI-TOF/TOF-MS analysis

Using DHB (a concentration of 25 mg/mL dissolved in 70% ACN, 28.9% water, 0.1% TFA, and 1% phosphoric acid) as the matrix, peptides were analyzed with MALDI-TOF/TOF

5800 (Applied Biosystems, Foster City, CA) in the reflection positive-ion mode. Typically, 0.5 μL dephosphorylation reaction mixtures with 0.5 μL DHB were loaded onto sample plates for analysis. At least 1000 laser shots were typically accumulated with a laser pulse rate of 400 Hz in the MS mode, whereas in the MS/MS mode spectra, up to 2000 laser shots were acquired at the average pulse rate of 1000 Hz. Peptides were fragmented by CID with an energy of 1 kV. For CID experiments, ambient air was used as the collision gas with a medium pressure of 10^{-6} Torr.

2.6 LC-MS/MS analysis

The HPLC system was an Agilent 1100 series (Agilent Technologies, Palo Alto, CA) equipped with a vacuum degasser, a binary pump, a thermostatted column compartment, and an autosampler. The chromatography separation was performed on an Ascentis C18 column (5 μm, 150 mm × 4.6 mm id) maintained at 40°C. The gradient program was as follows: 0.1–4.0 min, 2% B; 4.0–4.5 min, 2%–30% B; 4.5–7.0 min, 30% B; 7.0–7.1 min, 30%–2% B; 7.1–8.0 min, 2% B, with 0.1% formic acid as solvent A, and methanol–ACN–formic acid (50:50:0.1, v/v/v) as solvent B. The flow rate was set at 1.0 mL/min; an approximately 1:1 split of the column eluant was included prior to entry into the mass spectrometer.

An API 4000 mass spectrometer (Applied Biosystem, Foster City, CA) equipped with ESI source for ion production, was employed for mass spectrometric detection. Data acquisition and integration were controlled by Analyst Software (Version 1.4.2, Applied Biosystems, Foster City, CA). Electrospray ionization was performed in the positive ion mode with nitrogen as the nebulizer, heater, and curtain gas. The mass spectrometer was operated in the MRM mode at unit resolution for both Q1 and Q3 with a dwell time of 200 ms per MRM channel. The MRM transitions from the protonated molecular ions to the product ions were controlled at m/z 503.2→666.5 for peptide PQDKEpYYKVKE and m/z 503.2→746.4 for peptide PQDKEYpYKVKE. Declustering potential and collision energy were 60 V and 30 eV for PQDKEpYYKVKE, respectively, and 60 V and 30 eV for PQDKEYpYKVKE, respectively. Optimized parameters were as follows: curtain gas, gas 1 and gas 2 were nitrogen set as 15, 55, and 50 psi, respectively, with the source temperature at 550°C and Ion Spray voltage at 5000 V.

2.7 Molecular simulation

The binding pocket was defined by the position of the peptide in the crystal structure of PTP1B (PDB code 1G1F) [29] and HePTP (PDB code 3D44) [30]. The Q-site finder was also applied on the proteins to find all the possible binding pockets [31]. The top ranked potent-binding pocket identified by Q-site finder was consistent with the defined region. The simulation was carried out by utilizing the simplest implicit solvent model to mimic solvent effect on protein conformations. Energy minimization and molecular dynamics (MD) simulations were performed using the sander module of Amber 10.0 [32] to obtain the most stable structures of PTP1B and HePTP with the peptides. Energy minimization was performed using the steepest descent minimization of 5000 steps followed by 5000-step conjugate gradient minimization. Each dynamics trajectory was run for at least 10 ns at a time step of 2 fs with all bonds involving hydrogen atoms constrained by the SHAKE algorithm and the nonbonded cutoff set at 10 Å. The particle mesh Ewald method was adopted to treat long range electrostatic interactions. The root-mean-squared deviation was calculated utilizing the ptraj module in Amber 10.0 to monitor the stability of each MD simulation. The structure was further refined by following short-time MD simulations with the generalized-born (GB) implicit solvent model. Molecular mechanics-generalized born surface area (MM-GBSA) is used to calculate the interaction energy between the two phosphotryosyl residues in the complexes of PQDKEpYpYKVKE with PTP1B and HePTP as previously described [33, 34].

3 Results

3.1 Sequential dephosphorylation of PQDKEpYpYKVKE by PTP1B

The JAK2 kinase pathway is crucial in controlling cellular activities in response to extracellular growth factor/cytokines, and over activation of the pathway results in various hematopoietic disorders. The main negative regulation of the pathway is the dephosphorylation by protein tyrosine phosphatases, such as PTP1B [35, 36] and SHP2 [37, 38]. In order to clarify the negative regulation of JAK2 kinase, a diphosphorylated PQDKEpYpYKVKE peptide derived from the autophosphorylation sites (pY1007 and pY1008) of human JAK2 were synthesized and used as a substrate to investigate its dephosphorylation process by PTPs. The amounts of PTPs used in the reactions were based on their activities measured by using *p*NPP. A typical 200 μL reaction system contained 0.18 U PTP1B and 20 nmol of PQDKEpYpYKVKE. During the reaction process, 20 μL reaction mixture was taken out and terminated by TFA at different time points and then analyzed by MALDI-TOF MS. Figure 1 illustrates the presence of di-phosphorylated PQDKEpYpYKVKE (m/z 1586), mono-phosphorylated PQDKEpYYKVKE/PQDKEYpYKVKE (m/z 1506), and nonphosphorylated PQDKEYYKVKE (m/z 1426, the final product). Interestingly, the nonphosphorylated peptide appeared only after total disappearance of the di-phosphorylated peptide and did not come out simultaneously with the mono-phosphorylated peptides. This indicates that the dephosphorylation at the two sites occurred sequentially in two steps with the peptide dissociating from the enzyme following dephosphorylation at the first site and then rebinding to the enzyme for dephosphoryation at the second site.

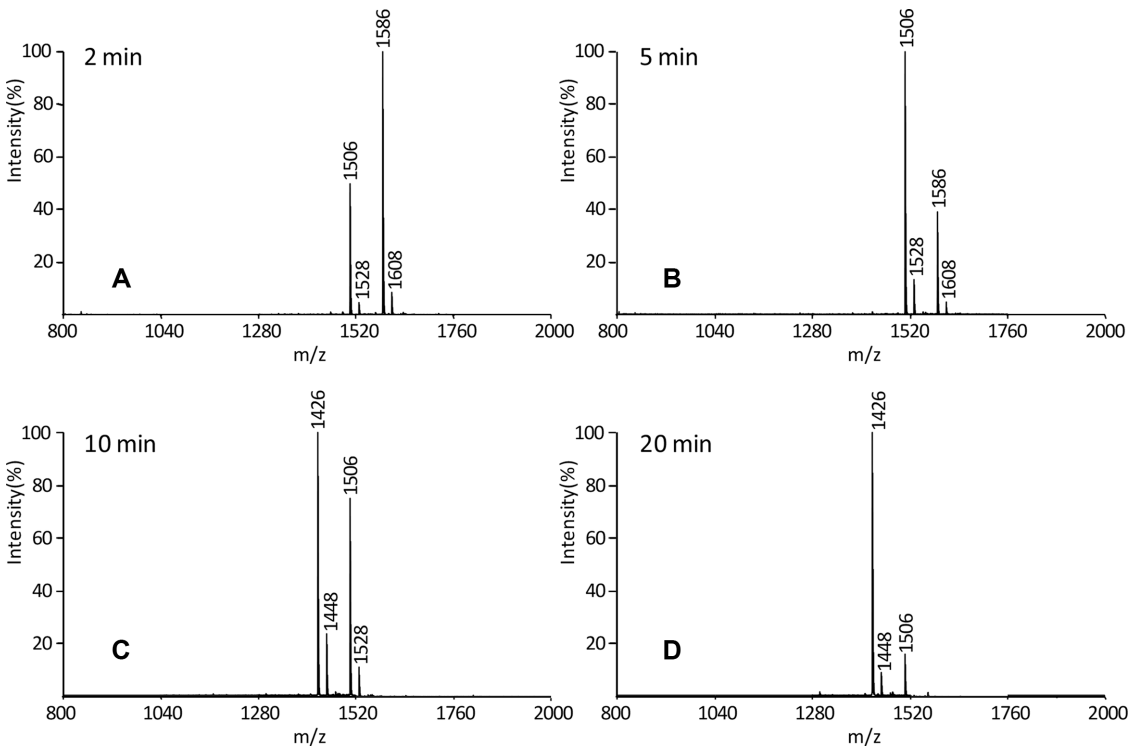

Figure 1. Time course of PQDKEpYpYKVKE dephosphorylation by PTP1B monitored by MALDI-TOF MS. The reaction systems contained 20 nmol of the peptide and 0.18 U PTP1B in a 200 μL total volume. Samples containing 15 pmol total peptides were taken at the indicated time for MALDI-TOF analysis.

Since PQDKEpYYKVKE and PQDKEYpYKVKE have the same molecular weight and charge, MALDI-TOF MS analyses cannot be used to distinguish them. For further analyses, we employed MALDI-TOF/TOF MS that breaks PQDKEpYYKVKE and PQDKEYpYKVKE into distinct as well as shared fragments as shown in Supporting Information Fig. 1. We then analyzed reaction mixtures. From the MS/MS spectra of the *m/z* 1506 ion (Fig. 2), we can clearly see the b_6 (PQDKEpY, *m/z* 841.4) and the y_5 ions (YKVKE, *m/z* 666.4) derived from PQDKEpYYKVKE but hardly any b_6 (PQDKEY, *m/z* 761.4) and y_5 ion (pYKVKE, *m/z* 764.4) derived from PQDKEYpYKVKE. The data indicate that the mono-phosphorylated peptide found in the PTP1B reaction is PQDKEpYYKVKE. Therefore, Y1008 was preferentially dephosphorylated by PTP1B. Nonetheless, PTP1B appears to be highly effective in dephosphorylating both sites.

It has been reported that PTP1B regulates leptin signaling by targeting JAK2 in vivo [39] and in vitro [35]. Myers et al. [36] reported that a substrate-trapping mutant of PTP1B formed a stable complex with JAK2 in response to interferon stimulation and that PTP1B overexpression inhibited phosphorylation of JAK2 at Y1007 and Y1008 site. Our study suggests that PTP1B can dephosphorylate JAK2 sequentially at Y1007 and Y1008 with Y1008 dephosphorylated first.

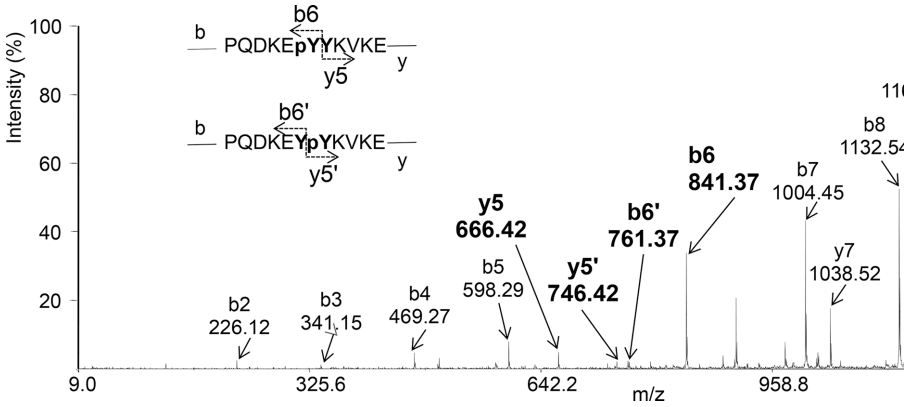

Figure 2. PTP1B-catalyzed dephosphorylation of PQDKEpYpYKVKE monitored by MALDI-TOF/TOF MS. PQDKEpYpYKVKE was treated with PTP1B for 5 min as described in Fig. 1. Data show the MS/MS spectra of the *m/z* 1506 ion.

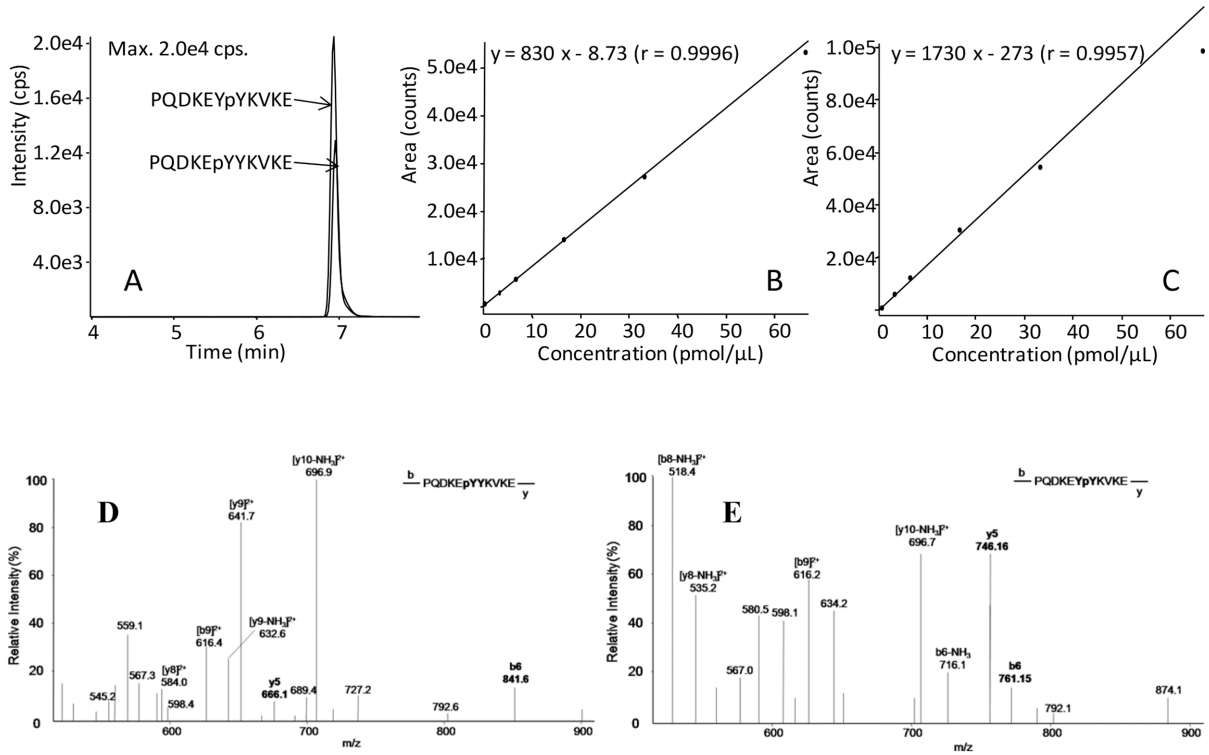

Figure 3. HPLC-MS/MS analysis of mono-phosphorylated peptides. (A) HPLC chromatograms of PQDKEpYYKVKE and PQDKEYpYKVKE peptides; standard curves obtained with given quantities of (B) PQDKEpYYKVKE and (C) PQDKEYpYKVKE; MS/MS Spectra of (D) PQDKEpYYKVKE and (E) PQDKEYpYKVKE obtained from ESI-MS/MS analyses.

3.2 Quantitative analyses of PQDKEpYpYKVKE dephosphorylation

MALDI-TOF/TOF MS analysis showed that pY1008 was more prone to dephosphorylation by PTP1B. To monitor the dephosphorylation process more quantitatively, triple quadrupole mass spectrometer was used to measure the intermediate product. For this purpose, standard PQDKEpYYKVKE and PQDKEYpYKVKE peptides were synthesized for the optimization of LC and MS conditions. As shown in Fig. 3, PQDKEpYYKVKE and PQDKEYpYKVKE showed distinct MS/MS pattern (b6 and y5). Therefore they can be distinguished by different fragment ions in MS by using the MRM mode although they were eluted from HPLC at the same retention time. Both peptides showed good linear response curves from 0.664 to 66.4 pmol/μL for PQDKEpYYKVKE ($y = 830x - 8.73$, $r = 0.9996$) and PQDKEYpYKVKE ($y = 1730x - 273$, $r = 0.9957$).

Based on the LC-MS/MS method above, dephosphorylation of PQDKEpYpYKVKE by four tyrosine phosphatases (PTP1B, SHP1, SHP2, and HePTP) and one general phosphatase (alkaline phosphatase) was analyzed. Figure 4A shows results obtained with PTP1B (see also Supporting Information Fig. 2). We can see that the concentrations of PQDKEpYYKVKE and PQDKEYpYKVKE increased sharply initially, then reached peak levels, and finally declined to zero. In the initial phase of the reaction, the accumulation rate of PQDKEpYYKVKE is about 10 times higher than that of PQDKEYpYKVKE, indicating preferential dephosphorylation at the Y1008 site by PTP1B, which is consistent with the MALDI-TOF/TOF MS results. SHP1 and SHP2 showed a similar preferential dephosphorylation at Y1008 by producing a rapidly elevated level of PQDKEpYYKVKE (Fig. 4B and C). However, the level of PQDKEpYYKVKE was sustained, suggesting its resistance to further dephosphorylation by SHP1 and SHP2. In contrast, HePTP displayed no preference to the Y1007 or Y1008 site as demonstrated by rapid and nearly equal increases in the concentrations of both PQDKEpYYKVKE and PQDKEYpYKVKE that were resistant to further dephosphorylation (Fig. 4D). This indicates that HePTP is nonselective in term of dephosphorylation at the Y1007 and Y1008 sites but has a strong preference for the di-phosphorylated peptide than either mono-phosphorylated peptide. We also used a general phosphatase, namely, alkaline phosphatase, as a nonselective enzyme. As expected, alkaline phosphatase caused total dephosphorylation of the di-phosphorylated peptide without showing clear preference to either Y1007 or Y1008 sites.

Several reports have demonstrated regulation of JAK2 by PTP1B [35, 36], SHP1 [40, 41], and SHP2 [37, 38], but there is no previous report of the involvement of HePTP in the JAK2/JAK-STAT pathway. Our study suggests that all four

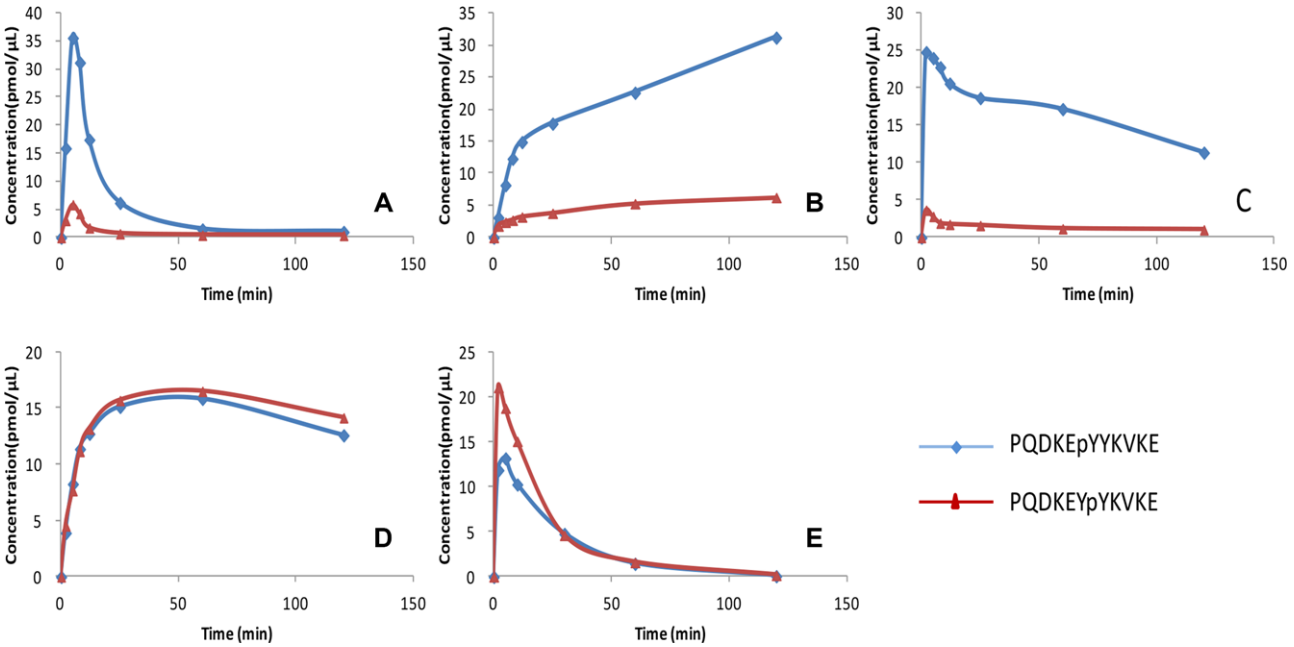

Figure 4. Time course of PQDKEpYpYKVKE dephosphorylation by different phophatases monitored with HPLC-MS/MS. Dephosphorylation reactions were carried out with 0.18 U PTP1B (A), 0.54 U SHP1 (B), 0.54 U SHP2 (C), 0.54 U HePTP (D), or 0.54 U alkaline phosphatase (E). At the indicated time, a total of 2 nmol peptides were taken for HPLC-MS/MS analyses. Data are representative of at least three independent experiments with consistent results.

PTPs effectively dephosphorylate JAK2 but with different selectivity to the Y1007 and Y1008 sites. They may regulate JAK2 through different modes.

3.3 Dephosphorylation of PQDKEpYpYKVKE by phosphatases in subcellular fractions

Protein tyrosine phophatases are ubiquitously expressed and located in different subcellular organelles. In order to reveal the dephosphorylation process in cells, we utilized different subcellular fractions of Jurkat cells. The reaction was carried out and monitored by LC-MS/MS as described above. As shown in Supporting Information Fig. 3, all cell fractions effectively dephosphorylated PQDKEpYpYKVKE with strong selectivity to the pY1008 site. However, the patterns of dephosphorylation by the cytosolic and nuclear fractions were similar to that obtained with SHP1 and SHP2, while the membrane fraction resembled PTP1B. This is somewhat expected. PTP1B is known to be a major PTP in the membrane fraction although we cannot exclude other enzymes such as CD45, a receptor tyrosine phosphatase that negatively regulates JAK2 signaling pathway. On the other hand, SHP1 and SHP2 represent major cytosolic PTPs. PTPs responsible for the dephosphorylation of JAK2 in the nuclear fraction is not known, but they must play an important role in regulating JAK2 activity because JAK2 was found in the nucleus in addition to the cytoplasm and the membrane [42].

3.4 Molecular modeling of PQDKEpYpYKVKE and PTP bindings

In order to further understand why PTPs display different selectivity to the Y1007 and Y1008 sites, molecular simulations were performed to model the complexes of PQDKEpYpYKVKE with PTP1B and HePTP. These two PTPs exhibit distinct dephosphorylation selectivity toward the di-phosphorylated peptide. The deduced structure of PTP1B-peptide complex with pY1008 in the catalytic pocket is shown in Fig. 5. Three H-bonds are formed between PTP1B and the di-phosphorylated peptide. They are Asp48 with E1012, Arg47 with D1004, and Arg24 with E1005. The Arg24 appears to extend into the pocket center, which may act as basic amino acid selector. The interaction energies of the phospho-peptide with PTP1B and HePTP are summarized in Table 1, where it shows that pY1008 has much higher interaction energy with PTP1B than pY1007 for PTP1B while almost the same for HePTP. PTP1B binds pY1007 with a vdW interaction energy (H-bond included) similar to pY1008 but a much stronger electronic interaction energy than pY1008. In the complexes of PQDKEpYpYKVKE with HePTP, no significant difference in the interaction energy is observed between pY1007 and

Table 1. The interaction energies between pY1007/pY1008 of PQDKEpYpYKVKE with PTP1B and HePTP

	PTP1B			HePTP		
	vdW interaction energy (kcal/mol)	Electronic interaction energy (kcal/mol)	Interaction energy (kca/mol)	vdW interaction energy (kcal/mol)	Electronic interaction energy (kcal/mol)	Interaction energy (kca/mol)
pY1007	3.51	–1.51	2.00	4.50	1.93	6.43
pY1008	3.66	5.63	9.29	3.72	1.65	5.37

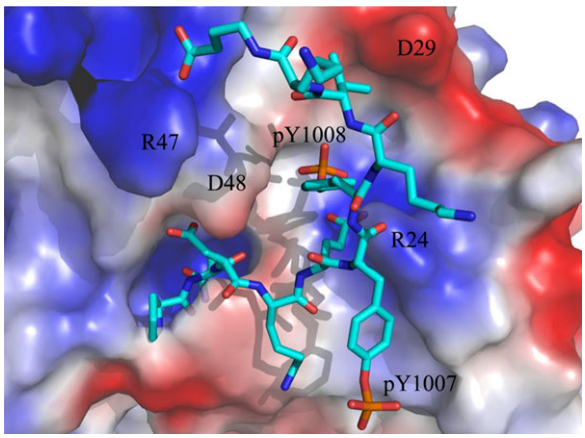

Figure 5. Molecular modeling of PTP1B-peptide binding. The PQDKEpYpYKVKE peptide is shown in the stick model, and the structure of PTP1B is represented by surface electrostatic potential.

pY1008. Together, the molecular modeling data demonstrate that PTP1B has a strong binding preference to pY1008, while HePTP exhibits no selectivity to the two sites, which is consistent with our experimental results.

4 Discussion

The present study demonstrates that tyrosine phosphatases catalyze the dephosphorylation of JAK2 with distinct selectivity and efficiency. PTP1B can cause rapid dephosphorylation on Y1008 followed by Y1007. In contrast, SHP1 and SHP2 selectively dephosphorylate Y1008 only, while HePTP nonselectively dephosphorylates either Y1007 or Y1008 and yields mono-phosphorylated peptides. This suggests PTPs can regulate JAK2 activation and function through multiple modes of actions.

Activation of JAK2 is accompanied by phosphorylation on both Y1007 and Y1008 [13]. The order of phosphorylation on these two residues has been described recently [43]. By using JAK2 peptide KVLPQDKEYYKVKEPW as a substrate, Hall et al. [43] demonstrated that Y1007 was phosphorylated first and phosphorylation on Y1008 did not occur until the peptide was completely mono-phosphorylated. Therefore, phosphorylation of the two tyrosine residues follows an ordered sequence in separate catalytic reactions. The order of phosphorylation on these two tyrosine residues within the full-length JAK2 enzyme may follow the same mechanism. In this regards, the kinase reaction can only generate di-phosphorylated and Y1007-mono-phosphorylated JAK2. Our current study suggests that different PTPs dephosphorylate JAK2 with distinct specificity. While PTP1B lead to total dephosphorylation of JAK2, SHP1, and SHP2 only remove phosphate from Y1008 thereby producing Y1007-mono-phosphorylated JAK2. Importantly, dephosphorylation of di-phosphorylated JAK2 by HePTP leads to generation of Y1007- and Y1008-mono-phosphorylated JAK2. The data indicate that PTPs are able to produce diverse forms of JAK2 species. These differently phosphorylated forms of JAK2 presumably possess different enzymatic activity and confer different interactions with other signaling proteins.

The critical role of phosphorylation at Y1007 in JAK2 activation has been well accepted [15, 44–46], but recognition for the function of Y1008 phosphorylation is more recent [43]. By using purified recombinant proteins carrying the catalytic domain of JAK2, Hall et al. [43] revealed that phosphorylation at the 1007 site increased the catalytic activity by ninefold and further phosphorylation at the 1008 site resulted in an additional sixfold increase in activity. This indicates that phosphorylation at both sites are required for full activation of JAK2. Our study demonstrates that PTP1B can cause rapid dephosphorylation of JAK2 on both Y1007 and Y1008 and thus total inactivation of JAK2, while SHP1, SHP2, and HePTP can only lead to partial dephosphorylation and partial inactivation. We do not know if Y1008-mono-phsophorylated JAK2 resulted from dephosphorylation by HePTP remain active. In any case, PTPs apparently provide a more diverse control of JAK2 activity.

In addition to regulating JAK2 kinase activity, phosphorylation of Y1007 and Y1008 also modulates the interaction of JAK2 with other signaling proteins. Yasukawa et al. [47] found that phosphorylation of Y1007 is required and sufficient for initiating interactions with suppressor of cytokine signaling (SOCS1) and SOCS3 that lead to JAK2 degradation. Our study demonstrated that PTP1B causes rapid dephosphoryation of both Y1007 and 1008. This gives rise to nonphosphorylated JAK2 that is resistant to degradation and can be readily reactivated. However, SHP1 and SHP2

dephosphorylate di-phosphorylated active JAK2 on Y1008 only and produce Y1007-mono-phosphorylated JAK2 susceptible for SOCS-induced degradation that results in permanent inactivation of the JAK2 signaling pathway. On the other hand, HePTP is able to dephosphorylate di-phosphorylated JAK2 on Y1007 thereby blocking SOCS binding and preventing degradation of JAK2. It will be important to find if Y1008-mono-phosphorylated JAK2 remains partly active.

The JAK family tyrosine kinases contain four members, namely, JAK1, JAK2, JAK3, and Tyk2. All of them are regulated by phosphorylation at the conserved tandem tyrosyl residues. Importantly, amino acid sequences surrounding the tandem tyrosine phosphorylation sites are also highly conserved. We believe that the data obtained with JAK2 peptides in this study should represent other JAK family kinases. Therefore, JAK1, JAK3, and Tyk2 are likely regulated by PTPs through different modes of dephosphorylation as found with JAK2, although they may show selectivity toward specific PTPs.

In conclusion, our data demonstrate that tyrosine phosphatases catalyze the dephosphorylation reaction sequentially, but different enzymes exhibit different selectivity, providing multiple modes of JAK2 regulation by tyrosine phosphatases. Our current data also suggest MALDI-TOF/TOF and triple quadrupole mass spectrometer have enough sensitivity and accuracy to analyze the dephosphorylation of di-phosphorlyted peptides quantitatively. Therefore, this method should be applied to study dephosphorylation and phosphorylation of other proteins containing multiple phosphorylation sites.

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The authors have declared no conflict of interest.

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