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Monitoring phosphatase reactions of multiple phosphorylated substrates by reversed-phase HPLC☆

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Abstract

In an approach to gain insight into the sequence-dependent dephosphorylation of multiple phosphotyrosyl-containing peptides by the phosphatases SHP-1 and PTP1B, we applied a chromatographic technique for the analysis of the dephosphorylation products. Mono-, bi- and triphosphorylated reference peptides corresponding to positions 1999–2014 in the activation loop of the receptor tyrosine kinase Ros were first analyzed by reversed-phase HPLC and MALDI-TOF/TOF mass spectrometry. Then, the respective products from enzymatic treatment were investigated by HPLC and compared to the standard peptides. The results obtained in this study emphasize the advantage of monitoring phosphatase reactions for mono- and biphosphorylated peptides using the described procedure rather than spectrophotometric and fluorimetric methods that do not allow for a clear identification of the products formed.

Keywords: Phosphopeptide; Dephosphorylation; HPLC; MALDI-TOF/TOF

1. Introduction

The reversible phosphorylation is probably one of the most important regulatory modifications in proteins [1]. Biochemical studies of the mechanisms that regulate phosphorylation-dependent signal transduction pathways often require synthetic phosphopeptides related to natural phosphoproteins. The identification of products from a specific phosphorylation/dephosphorylation reaction of such peptides is frequently informative with respect to the relevant receptor-mediated and postreceptor-mediated reactions in intact cells. Thus, methods for a direct and micro-level analysis such as HPLC, HPCE, mass spectrometry, and protein sequencing are critical to these research areas [2–4].

Analysis of the interaction of protein tyrosine phosphatase SHP-1 with the transmembrane tyrosine kinase Ros revealed that SHP-1 strongly binds to Ros and regulates Ros signaling in a negative manner [5]. The effects of SHP-1 are mainly mediated by a direct association to the phosphorylated Ros by its N-terminal SH2-domain. In particular, phosphorylation of the tyrosine residue 2267 in the C-terminal part of Ros is responsible for the binding and activation of SHP-1, whereas the phosphorylation of residues Y2003, Y2007, and Y2008 within the activation loop of Ros is essential for full activity of the kinase. The corresponding triphosphorylated peptide is an efficient substrate of SHP-1, suggesting that SHP-1 inactivates Ros by dephosphorylation of the activation loop which comprises residues 1999–2014. Inactivation of Ros will finally

Abbreviations: HPCE, high-performance capillary electrophoresis; HPLC, high-performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; PTP, protein tyrosine phosphatase; PTP1B, protein tyrosine phosphatase 1B; N-SH2, N-terminal src homology 2 domain; SHP-1, SH2 domain protein tyrosine phosphatase-1

☆ Standard abbreviations for amino acid derivatives and peptides are according to the suggestions of the IUPAC-IUB Commission on Biochemical Nomenclature [Eur. J. Biochem. 138 (1984) 9–37]. Abbreviations for protecting groups and peptide synthesis reagents were used as recommended in the guide published in J. Peptide Sci. 9 (2003) 1–8.

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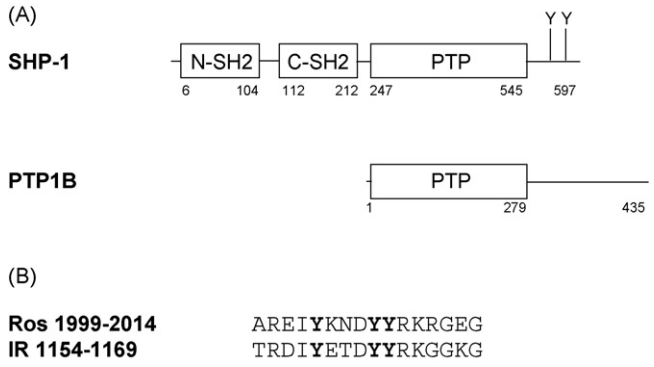


Fig. 1. Domain structure of protein tyrosine phosphatases SHP-1 and PTP1B (A), and (B) phosphorylation sites in the activation loop regions of receptor tyrosine kinase Ros [5] and the insulin receptor [7].

lead to decomposition of the SHP-1–Ros complex [5,6]. The selectivity of SHP-1 for the individual phosphotyrosines in the triple phosphorylated sequence has, however, not yet been elucidated. The case of SHP-1 and Ros may have similarities to the interaction of PTP1B, another protein tyrosine phosphatase (Fig. 1A), with the phosphorylated insulin-receptor (IR). In this case, it has been shown that the dephosphorylation of three individual phosphotyrosines in the IR activation loop (residues 1154–1169) occurs with different efficiency and is influenced by phosphorylation of neighbouring phosphotyrosines [5–8]. The activation loop sequence of Ros is highly homologous to that of the IR (Fig. 1B) and may also serve as a good substrate for PTP1B. It is therefore of interest to investigate the dephosphorylation of the Ros activation loop by SHP-1 and PTP1B, and to comparatively determine their phosphorylation-site selectivity.

In general, three types of assay have been employed for the investigation of the dephosphorylation of synthetic phosphopeptides by PTPases. One is to monitor the PTPase-catalyzed hydrolysis by measuring the production of inorganic phosphate using the malachite green colorimetric assay [14]. Another is the so-called sensitive continuous assay introduced by Zhang et al., which is based on spectroscopic differences (absorbance or fluorescence) of the phosphopeptide before and after the removal of the phosphate group [15]. A third possibility is to monitor the breakdown of peptide substrates by HPLC or HPCE, since the phosphorylated and dephosphorylated peptide species have different retention/migration times. In contrast to the above mentioned assays, this approach may allow for the analysis of the individual dephosphorylation products, in particular if coupled to mass spectrometry.

The present study aimed at the evaluation of different instrumental methods, including reversed-phase HPLC and MALDI-TOF/TOF mass spectrometry, for the analysis of different tyrosine-phosphorylated forms of Ros 1999–2014 in order to provide methodological tools for the subsequent kinetic analysis of the dephosphorylation events by SHP-1 and PTP1B. The reference peptide AREIYKNDYYRKRGEG was prepared in all possible mono-, bi- and triphosphorylated forms which were subjected to treatment with SHP-1 and PTP1B.

2. Experimental

2.1. Chemicals and reagents

Fmoc-amino acids, coupling reagents (HBTU, HOBt) and Rink-amide MBHA-resin were purchased from Novabiochem (Merck Biosciences AG, Darmstadt, Germany). Peptide synthesis reagents (piperidine, DIEA) and solvents (DMF, DCM) were of reagent grade from Fluka (Sigma–Aldrich–Chemie GmbH, Taufkirchen, Germany). HPLC-solvents of gradient grade were obtained from VWR International (Dresden, Germany) and Roth (Karlsruhe, Germany), respectively. The α-cyano-4-hydroxycinnamic acid was from Bruker (Leipzig, Germany). All other chemicals were of research grade from Fluka (Sigma–Aldrich–Chemie GmbH, Taufkirchen, Germany).

2.2. Peptide synthesis and purification

Peptides were synthesized by manual solid phase peptide synthesis according to the Fmoc-strategy at the 0.5 g scale using Rink-amide MBHA-resin with a loading of 0.64 mmol/g. The following side chain protecting groups were used: OtBu for Glu, Asp, Tyr; Pbf for Arg; and Fmoc-Tyr(PO(OBzl)OH) [11]. Fmoc-group deblocking was performed with 20% piperidine in DMF two times (5 and 15 min). For amino acid couplings 4 eq. of Fmoc-amino acid were activated with HBTU/HOBt (4 eq. each) in the presence of DIEA (8 eq.) for 1 h (double couplings). Fmoc-Tyr(PO(OBzl)OH) (2 eq.) was coupled with 6 eq. of DIEA. All deprotection and coupling steps were followed by intensive washings using DMF and DCM, alternately. Peptides were cleaved from the resin using a mixture of 95% TFA, 2.5% triisopropylsilane, and 2.5% water for 5–6 h at room temperature. After the precipitation in cold diethyl ether, peptides were centrifuged and washed several times with diethyl ether before lyophilisation from water. Semi-preparative purifications of the peptides were performed on a Shimadzu LC 8A HPLC-instrument equipped with a C18 column (Eurospher 100, Knauer, Berlin, Germany) using a gradient of either 10%–60% or 5%–55% eluent B in 120 min at a flow rate of 10 ml/min (eluent A: 0.1% TFA in water, eluent B: 0.1% TFA in 90% acetonitrile/water, v/v). Detection was at 220 nm.

2.3. Amino acid analysis

The amino acid composition of the peptides was confirmed by amino acid analysis. Crude samples were hydrolyzed for 24 h at 110 °C using 6 M HCl. The samples were analyzed on an amino acid analyzer LC 3000 (Eppendorf-Biotronik, Hamburg, Germany). Furthermore, amino acid analysis was used to determine the concentrations of peptides in the solutions used for the dephosphorylation assay. The procedure is described elsewhere [16].

2.4. HPLC analysis

2.4.1. Reference peptides

Purity of the fractions collected by semi-preparative HPLC was established by analytical reversed-phase HPLC on a Shi-

madzu LC-10AT chromatograph (Duisburg, Germany) with a Vydac 218TP column (5 μm particle size, 300 Å pore size, 4.6 mm × 250 mm). The column temperature was 25 °C adjusted with a Beckman 235 column heater (Krefeld, Germany). Peptides were eluted with the gradient 5–30% eluent B in 25 min at a flow rate of 1.0 ml/min, where A was 0.1% TFA in water and B 0.1% TFA in acetonitrile (detection at 220 nm).

2.4.2. Products from dephosphorylation assay

The investigation of the dephosphorylation products was performed using a gradient of either 0–30% in 30 min, 0–30% in 60 min or 0–30% in 90 min, eluent B as indicated above.

2.5. HPCE analysis

The high-performance CE instrument used for the study was a HP 3D-CE system (Hewlett Packard, Boeblingen, Germany) with a photo diode array detector (detection at 214 nm). Fused-silica capillary tube of 40 cm × 50 μm I.D. (Agilent, Waldbronn, Germany) was used. Experiments were carried out at a constant temperature of 20 °C. The sample injections were done through hydrodynamic mode with a pressure of 30 mbar for 5 s, the applied voltage was 10 kV. The buffer used for sample preparation was 50 mM Tris pH 7.4 containing 150 mM NaCl and 1 mM EDTA. The capillary was automatically purged for 4 min using HCl and the sample buffer prior to injection, respectively. In addition, after two runs the capillary was regenerated with 0.1 M NaOH, water, 0.1 M HCl, and 50 mM Tris buffer pH 7.4, each for 10 min.

2.6. Mass spectrometry

The molecular weight of the synthesized reference peptides was first confirmed by MALDI-TOF mass spectrometry on a Laser Tec Research mass spectrometer (Perseptive Biosystems, Weiterstadt, Germany) using α-cyano-4-hydroxycinnamic acid as matrix. The phosphorylation state of the reference peptides and the composition of the peptides after phosphatase treatment was then investigated by MALDI-TOF/TOF mass spectrometry on a Ultraflex TOF/TOF spectrometer (Bruker Daltonik GmbH, Leipzig, Germany) using TA30 solution (0.1% TFA/acetonitril, 2:1 (v/v)) as solvent for the samples. A sample was mixed 1:1 with saturated α-cyano-4-hydroxy-cinnamic acid in TA30 and allowed to co-crystallize with the matrix on a ground steel target. For each measurement laser position and power were adjusted to yield optimal sensitivity and resolution. The programs *flexAnalysis 2.2* and *BioTools 2.2* were used for the examination of the peptide fragments.

2.7. Phosphatase assay

Expression and purification of SHP-1 (PTP domain) and PTP1B is described elsewhere [12,13,16]. The dephosphorylation of the peptides **P2**–**P8** was investigated in a total volume of 50 μl for the assay reaction. The peptides were used in a concentration of 0.5 mM (final concentration). The reaction was initiated by the addition of 10 μl of SHP-1-PTP domain and PTP-1B (final concentration 143 nM and 100 nM, respectively). The dephosphorylation reaction was terminated by the addition of 50 μl of 10% TFA after different reaction times (1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 45, and 60 min). Afterwards, the solutions were centrifuged, the supernatants were taken off, frozen and freeze-dried. For HPLC analysis the peptides were dissolved in 100 μl of bidistilled water, and generally 20 μl of these samples were injected.

3. Results and discussion

3.1. Synthesis of reference peptides

The synthesis of phosphotyrosine containing peptides can be accomplished either by phosphorylation of a tyrosine-containing peptide (global phosphorylation or postassembly phosphorylation) or through incorporation of suitably N- and P-protected tyrosine residues into a growing peptide chain (pre-assembly phosphorylation) [17]. The more convenient latter technique was used successfully for phosphopeptide synthesis on the solid phase, and is generally preferred due to the availability of building blocks such as Fmoc-Tyr(PO_3H_2)-OH or Fmoc-Tyr(PO(OBzl)OH)-OH. We have previously described the preparation of linear SH2 domain ligands for SHP-1 by introducing the phosphate-unprotected building block [16]. However, for the preparation of the herein described bi- and triphosphorylated peptides we had to consider side reactions that may occur upon introduction of consecutive phosphotyrosine residues, e.g. the intramolecular pyrophosphate formation [18]. We therefore used the mono-benzyl phosphate ester of Fmoc-protected phosphotyrosine in order to avoid byproducts. Furthermore, we increased the amount of base for an efficient activation of Fmoc-Tyr(PO(OBzl)OH)-OH as recommended by White [19] to compensate for the neutralisation of 1 equivalent of base by the acidic partially protected phosphate group. We were thus able to produce the mono-, bi- and triphosphorylated peptides summarized in Table 1 in high yields (80–90% of crude isolated peptides) and good quality (70–80% HPLC purity of the crude products).

3.2. HPCE analysis of reference peptides

Numerous investigations dealing with the development of techniques for the identification of modified peptides, in particular peptides containing phosphorylated amino acids, by using instrumental micro-methods, including reversed-phase HPLC, HPCE and MALDI-TOF mass spectrometry have been described in the literature [2–4,20–24]. Therefore, in the initial phase of our investigations we intended to explore the potential of HPCE for the characterization of the different phosphopeptides. However, the studies concerned with the separation and electrophoretic behaviour of mono- and biphosphorylated peptide isomers extremely vary in the composition of the capillary and the conditions applied as well as in the running buffer used for the sample preparations [2,21,22,24]. For example, Yoo et al. investigated several pairs of peptides related to protein kinases containing Ser, Thr or Tyr in either the phosphorylated or the

Table 1
Sequences of the peptides synthesized in this study

Peptide	Sequence[a]
1 (Ros[b])	Ala-Arg-Glu-Ile-Tyr^5-Lys-Asn-Asp-Tyr^9-Tyr^{10}-Arg-Lys-Arg-Gly-Glu-Gly
2 (pY^5)	Ala-Arg-Glu-Ile-**pTyr**^{5}-Lys-Asn-Asp-Tyr^9-Tyr^{10}-Arg-Lys-Arg-Gly-Glu-Gly
3 (pY^9)	Ala-Arg-Glu-Ile-Tyr^5-Lys-Asn-Asp-**pTyr**^{9}-Tyr^{10}-Arg-Lys-Arg-Gly-Glu-Gly
4 (pY^{10})	Ala-Arg-Glu-Ile-Tyr^5-Lys-Asn-Asp-Tyr^9-**pTyr**^{10}-Arg-Lys-Arg-Gly-Glu-Gly
5 (pY^{5,9})	Ala-Arg-Glu-Ile-**pTyr**^{5}-Lys-Asn-Asp-**pTyr**^{9}-Tyr^{10}-Arg-Lys-Arg-Gly-Glu-Gly
6 (pY^{5,10})	Ala-Arg-Glu-Ile-**pTyr**^{5}-Lys-Asn-Asp-Tyr^9-**pTyr**^{10}-Arg-Lys-Arg-Gly-Glu-Gly
7 (pY^{9,10})	Ala-Arg-Glu-Ile-Tyr^5-Lys-Asn-Asp-**pTyr**^{9}-**pTyr**^{10}-Arg-Lys-Arg-Gly-Glu-Gly
8 (pY^{5,9,10})	Ala-Arg-Glu-Ile-**pTyr**^{5}-Lys-Asn-Asp-**pTyr**^{9}-**pTyr**^{10}-Arg-Lys-Arg-Gly-Glu-Gly

Positions containing a phosphotyrosine (pTyr) residue are highlighted in bold.
[a] Peptides were prepared as C-terminal amides.
[b] This sequence is derived from the activation loop of epithelial receptor tyrosine kinase Ros, representing the segment 1999–2014 [5].

non phosphorylated form. The separations were performed using extreme pH conditions of the electrolyte buffer (CAPS buffer, pH 11.0 and citric acid buffer, pH 2.5) to circumvent the problem of peptide adsorption to the capillary wall [24]. Also, the usefulness of polyacrylamide coated capillaries, SDS addition in the running buffer (phosphate buffer) and the effect of pH changes (2.5–7.5) for the resolution of mono- and biphosphorylated isomers of a peptide sequence derived from the insulin receptor has been reported [22]. However, with respect to our study of substrate dephosphorylation by SHP-1 and PTP1B we meant to abide by the buffer and pH-value used for the enzymatic reaction. In this way we wanted to enable a direct identification of the reaction products using different methods without the risk of undesired dephosphorylation/degradation evoked by additional treatment of the products by e.g. solvent and pH changes. Thus, it seemed appropriate to examine the peptides in the reaction buffer (pH 7.4). The characterization of the reference peptides **1–8** by HPCE is shown in Table 2. The peptides **1–8** were detected in the order: non-, mono-, bi, and triphosphorylated peptide, and peptides of the same phosphorylation state showed similar migration times. A comparison of the results obtained for the monophosphorylated peptides with the biphosphorylated forms revealed larger differences in migration times for the latter suggesting a possibility for isomer resolution. However, when injected as a mixture of two or three isomers, neither monophosphorylated nor biphosphorylated peptides were separated (data not shown). Except, it seemed that the peptides changed their migration times, e.g. the mixture of the biphosphorylated isomers was detected at 51.6 min, whereas peptide **5** alone had a migration time of 48.6 min. This was also found for mixtures of mono- and biphosphorylated peptides by Bonewald et al. [2]. The standard peptides were also investigated in different buffer solutions, e.g. phosphate buffer (50 mM) and at different pH values (range 2.5–7.5), but none of the changes substantially improved the electrophoretic behaviour of these peptides (data not shown). At least under the tested conditions, this method thus appeared not suitable for our intended investigations.

Table 2
Analytical characterization of the nonphosphorylated and phosphorylated peptides derived from RTK Ros 1999–2014

Peptide	Position of pY	HPCE[a], t_M (min)	HPLC[b], t_R (min)	Molecular Weight[c]	
				Calculated	Found
1	–	24.8	17.96	2016.03	2017.16
2	pY^5	32.92	16.00	2096.01	2096.92
3	pY^9	31.74	16.25	2096.01	2096.88
4	pY^{10}	32.89	16.55	2096.01	2097.01
5	pY^{5,9}	48.63	14.34	2175.99	2176.89
6	pY^{5,10}	51.33	14.50	2175.99	2176.82
7	pY^{9,10}	52.87	14.56	2175.99	2176.83
8	pY^{5,9,10}	61.97	12.30	2255.97	2256.86

[a] Conditions: 50 mM Tris–HCl buffer pH 7.4 and an applied voltage of 10 kV.
[b] Conditions: 5–30% eluent B in 25 min. A: 0.1% TFA/water, B: 0.1% TFA/acetonitrile, flow rate 1.0 ml/min, detection: 220 nm.
[c] $[M+H]^+$.

3.3. HPLC separation of reference peptides

Analysis of the reference peptides **1–8** revealed that the different degrees of phosphorylation can clearly be distinguished by their retention times, and the elution occurred in the order: tri-, bi-, mono-, and nonphosphorylated peptide (Table 2). This was also reported by Bonewald et al. for the differentially phosphorylated forms of the amino acid sequence EDYEYTARF-NH_2, the autophosphorylation site in the activation loop of the cytosolic tyrosine kinase pp60$^{c\text{-}src}$ [2]. When injected as a mixture, peptide analogues **1–8** gave the elution pattern shown in Fig. 2. It can be seen that the three biphosphorylated isomers were not separated under the employed elution conditions (0–30% eluent B in 30 min), whereas the monophosphorylated isomers gave better results, but baseline separation was not obtained. When the same gradient was applied over an extended time range (60 or 90 min), the resolution of the monophosphorylated peptides was improved as demonstrated for the three possible mixtures of peptides **1–3** in Fig. 3. However, only the monophosphorylated peptides could be separated, whereas the mixtures of the biphosphorylated isomers always yielded only one peak even under the conditions of the more shallow gradients (data not shown). Thus, for the analysis of the products from the phosphatase assays to eluci-

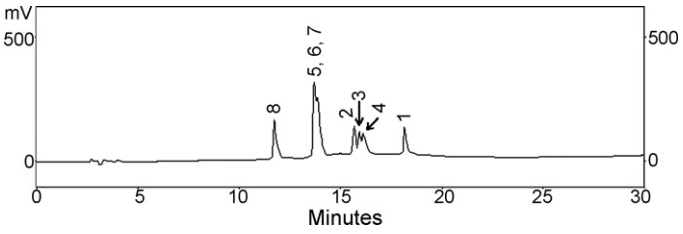


Fig. 2. Analytical HPLC elution pattern of a mixture of the reference peptides **1–8** at 220 nm. Gradient: 0–30% eluent B in 30 min with A: 0.1% TFA in water and B: 0.1% TFA in acetonitrile; flow rate at 1 ml/min.

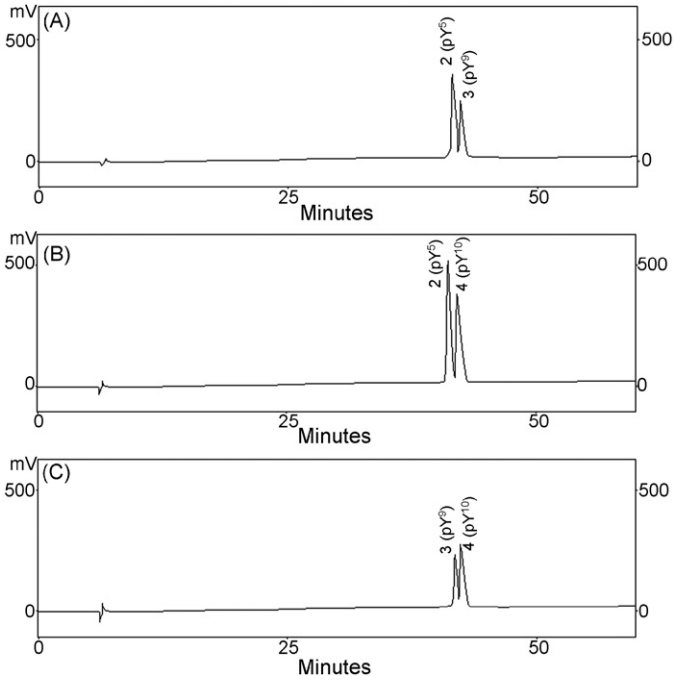


Fig. 3. Mixtures of two monophosphorylated isomers were injected and analyzed with the gradient: 0–30% eluent B in 60 min with A: 0.1% TFA in water and B: 0.1% TFA in acetonitrile; flow rate at 1 ml/min; UV detection at 220 nm. (A) Mixture of peptides **2** and **3**; (B) mixture of peptides **2** and **4**; (C) mixture of peptides **3** and **4**.

date the preferences of SHP-1 and PTP1B, a stepwise approach was required to circumvent the problem of separation of the biphosphorylated peptides. Furthermore, following HPLC analysis the peptides were examined by MALDI-TOF/TOF mass spectrometric analysis to discriminate them according to their fragmentation patterns (data not shown).

3.4. Dephosphorylation of peptides by SHP-1

We first investigated the dephosphorylation reaction by SHP-1 using the three monophosphorylated peptides. The employed enzyme concentration (5 ng/μl) was chosen according to Keilhack et al. [5]. A combinatorial phosphopeptide library screening by Pei and coworkers revealed the consensus sequence (D/E)XpY for SHP-1 substrates [25]. Only two monophosphorylated isomers (**2** and **4**) matched this consensus. In case of peptide **3** an unfavourable Asn residue is found at −2 relative to pTyr. Indeed, the analysis of the reaction products obtained for each peptide after enzyme incubation and termination of the reaction at different time points revealed that peptide **3** was dephosphorylated to a much lesser extent than peptides **2** and **4** (Fig. 4A). The dephosphorylation of isomers **2** and **4** already started after 1 or 2 min of phosphatase incubation, whereas for peptide **3** no phosphate group removal was observed over 20 min. In agreement with the results of the SHP-1-treated library [25], we can therefore conclude that the sequences EIpY (peptide **2**) and DYpY (peptide **4**) represent good substrates for SHP-1, whereas peptide **3** (NDpY) is a poor substrate of this phosphatase.

We further examined the dephosphorylation reaction of the biphosphorylated isomers. In Fig. 4B the elution profiles of peptides **5** ($pY^{5,9}$), **6** ($pY^{5,10}$) and **7** ($pY^{9,10)}$ after incubation with SHP-1 for different times are shown. In contrast to peptides **5** and **7**, where only one monophosphorylated product occurred, two product peaks were generated from peptide **6**. The latter contains phosphotyrosine at positions 5 and 10. Thus, the monophosphorylated products formed are either peptide **2** or peptide **4**. We were able to identify the earlier eluting peak as peptide **2** and the second peak as peptide **4** using MALDI-TOF/TOF mass spectrometry. Thus, both phosphotyrosines (pY^5 and pY^{10}), which were recognized in the monophopshorylated sequences, were obviously also effectively dephosphorylated within the context of the biphosphorylated peptide **6**.

For both peptides, **5** and **7**, the formed product turned out to be the monophosphorylated isomer **3**. Since this peptide had been identified as a poor substrate for SHP-1 before, these results are in perfect agreement with those obtained for the monophosphorylated sequences.

Peptide **8** is most rapidly dephosphorylated among all tested phosphopeptides (Fig. 4C), which is in agreement with earlier observations [6]. The monophosphorylated peptide accumulating in the reaction mixture was identified as compound **3** by MALDI-TOF/TOF. Separation and identification of the biphosphorylated products was not possible. However, based on the previous results the following reaction scheme can tentatively be proposed for SHP-1-mediated hydrolysis of this phosphopeptide: Compound **8** becomes dephosphorylated in positions pY^5 and pY^{10} to yield the peptides **5** and **7** with position 9 remaining phosphorylated. Peptides **5** and **7** are then further converted to peptide **3** (Scheme 1A).

3.5. Dephosphorylation of peptides by PTP1B

As previously reported, PTP1B may be less specific with respect to substrate recognition compared to SHP-1 [10]. Based on the crystal structures of the PTP domains of both phosphatases this was in part explained by the different positions of the so-called WPD-loop (loop between β11 strand and α3 helix in the crystal structure) [26]. This loop is conserved among the protein tyrosine phosphatases and represents a critical component of the catalytic mechanism. The aspartic acid residue serves as general acid/base in the catalytic reaction. This loop is also supposed to contribute to substrate recognition. In case of PTP1B, a dramatic movement from an open to a closed active conformation following binding of a pTyr-substrate has been shown, whereas for SHP-1 the WPD-loop is in a half-open, half-closed conformation according to the crystal structure analysis [10,26]. Thus, it was predicted that the movement of this loop into an active closed conformation is likely to be energetically more costly for SHP-1 than for PTP1B. In conclusion, it was proposed that SHP-1 may have relatively high substrate specificity compared to PTP1B [9,10,26].

The results of the incubation of the monophosphorylated peptides with PTP1B are shown in Fig. 5A. As anticipated and in contrast to SHP-1, the three isomers **2**, **3**, and **4** were all recognized and dephosphorylated by PTP1B. However, for the

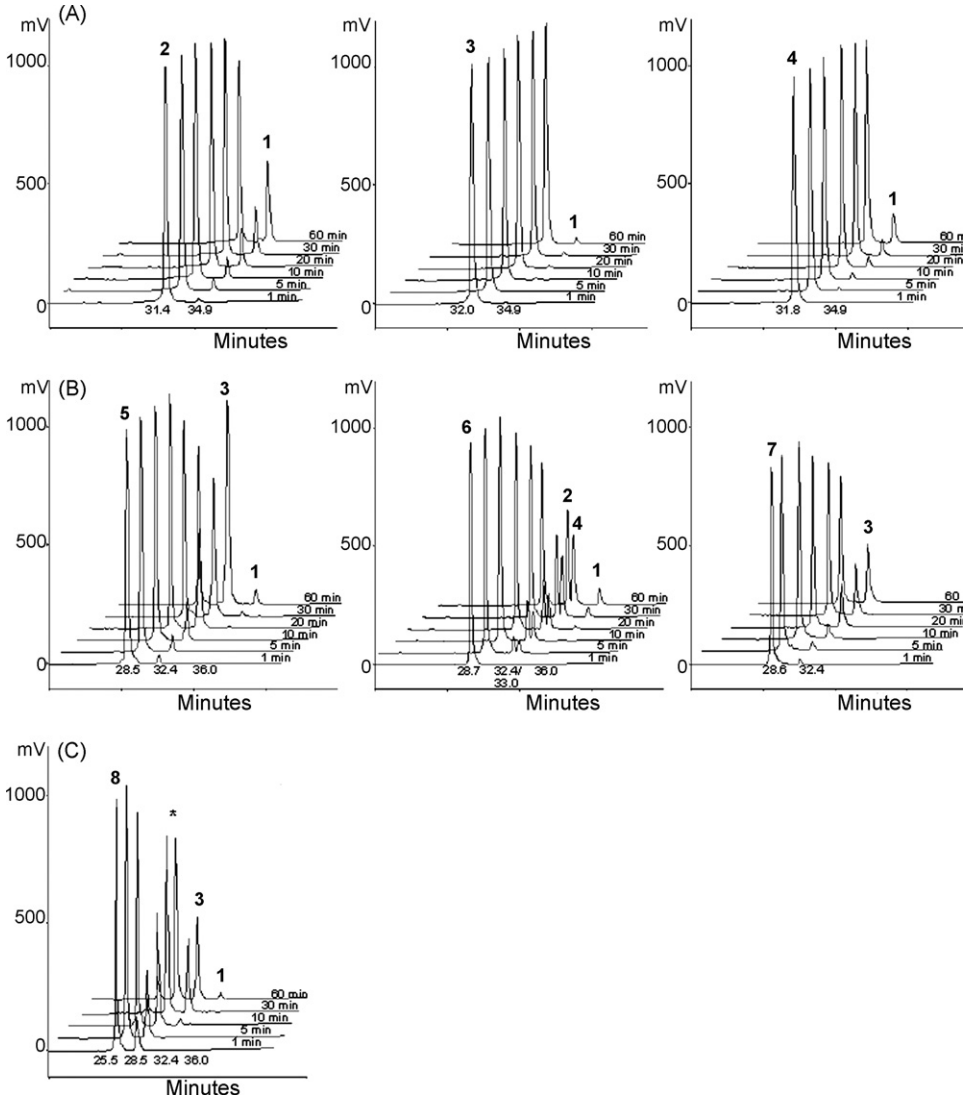


Fig. 4. HPLC analysis of the dephosphorylation reaction of peptides **1–8** by SHP-1. (A) monophosphorylated peptides **2**, **3**, and **4**; (B) biphosphorylated peptides **5**, **6**, and **7**; (C) triphosphorylated peptide **8**. Peptides were eluted with a gradient of 0–30% eluent B in either 60 min or 90 min, where B was 0.1% TFA in acetonitrile.

biphosphorylated peptides (**5**–**7**) clear differences in the formation of monophosphorylated products were observed. In case of peptides **5** and **6**, the two possible monophosphorylated isomers, **2** (pY^5) and **3** (pY^9) or **2** (pY^5) and **4** (pY^{10}), respectively, occurred already after 1 or 2 min of enzyme treatment (Fig. 5B). For peptide **7**, which contains two consecutive phosphotyrosine residues only one major peak was detected. Thus, we had to find out which of the two isomers, **3** (pY^9) or **4** (pY^{10}), was formed. Unfortunately, it was not possible to clearly distinguish between peptide **3** and **4** in mass spectrometry. Slightest amounts of isomer **4** (pY^{10}) in a mixture with **3** (pY^9) prevented detection of **3**, suggesting the sole presence of isomer **4**. The retention time of the product peak, however, led us to speculate that peptide **3** rather than peptide **4** was formed. Only after 60 min of incubation, a second peak in the HPLC chromatogram was observed, which may represent peptide **4** (Fig. 5B, indicated by an arrow). We thus considered another way to identify the reaction products. A convincing experiment to solve this problem seemed the addition of either of the isomers to a reaction product mixture of peptide **7**. We found that the addition of isomer **3** to the product mixture of **7** at 20 min resulted in one increased peak of monophosphorylated peptide, whereas addition of peptide **4** yielded two peaks (data not shown). In this way we were able to determine that of the two phosphotyrosine residues occurring in peptide **7** the one at position 10 is mainly dephosphorylated and pY^9 is dephosphorylated with much lower efficiency. In case of the triphosphorylated peptide **8** we identified isomers **2** and **3** as the monophosphorylated products formed, whereas the identity of the biphosphorylated products could not be resolved. Based on the relatively low efficiency of dephosphorylation of pY^9 in the biphosphorylated sequence **7**, we propose that the primarily formed biphosphorylated isomers are the peptides **5** and **7**, as was suggested for SHP-1. These two isomers are then further converted to the nonphosphorylated peptide **1**. The tentative preferred dephosphorylation route for PTP1B is depicted in Scheme 1B.

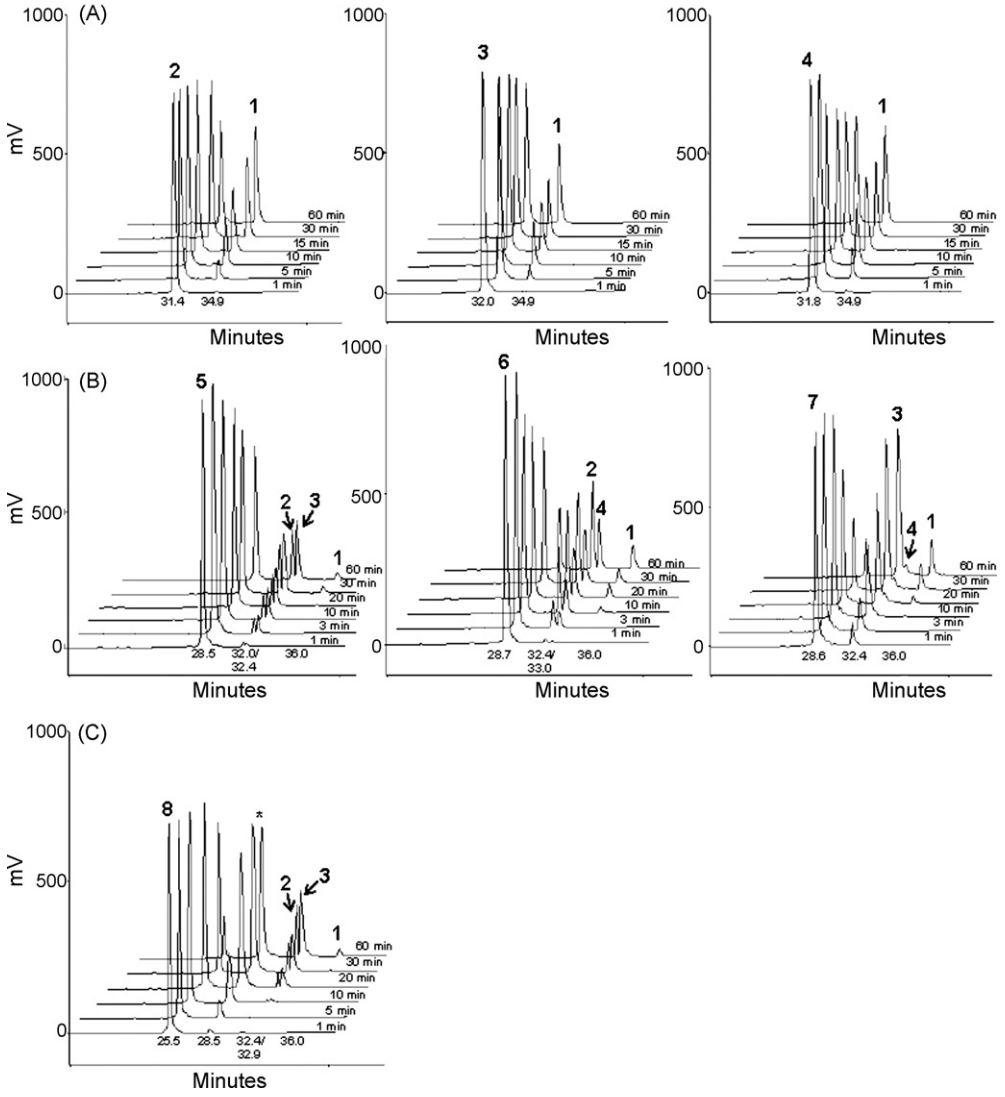


Fig. 5. HPLC analysis of the dephosphorylation reaction of peptides **1–8** by PTP1B. (A) monophosphorylated peptides **2**, **3**, and **4**; (B) biphosphorylated peptides **5**, **6**, and **7**; (C) triphosphorylated peptide **8**. Peptides were eluted with a gradient of 0–30% eluent B in either 60 min or 90 min, where B was 0.1% TFA in acetonitrile.

3.6. Kinetic analysis of dephosphorylation reactions

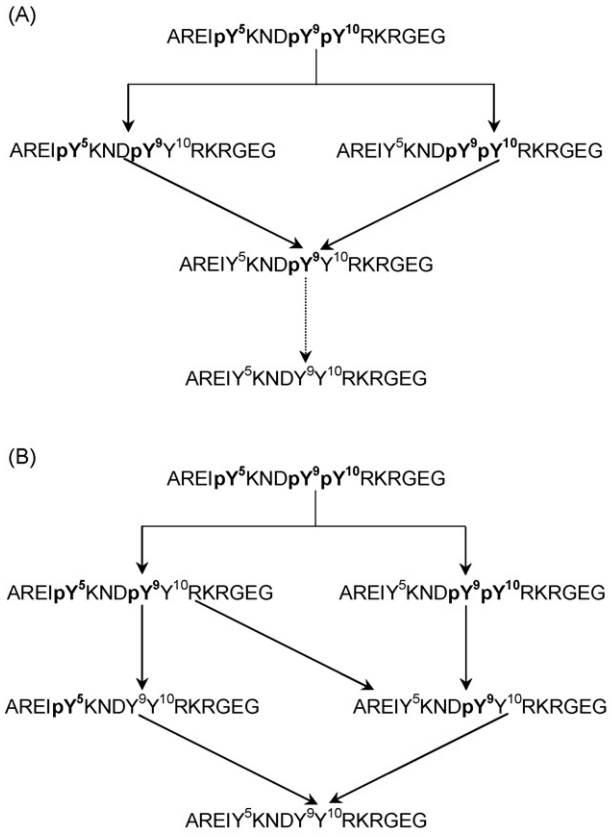
From the HPLC elution profiles we determined the reaction progress curves for the formation of the respective products, e.g. nonphosphorylated peptide **1** starting from the monophosphorylated isomers. We determined the rate constants (k) for the product formation using these progress curves in order to provide a quantitative analysis of the dephosphorylation reaction (Table 3). A representative fit is shown in Fig. 6. The rate constants indicated that the rank order for the dephosphorylation of the individual pY-residues in the monophosphorylated isomers for SHP-1 is $pY^5 \geq pY^{10} \gg pY^9$. This is slightly different to the reported preference of SHP-1 in terms of position −2 [25]. In the context of the herein studied peptides Glu ($EIpY^5$) was preferred over Asp ($DYpY^{10}$). One explanation for this finding may be the conformational aspect, especially effects of side chains other than those from the positions −1 and −2, which may influence the recognition by SHP-1, too. Besides, the sequence investigated herein is rather long (16 residues) compared to

Table 3
Rate constants (k, [10^{-2} min^{-1}]) for the dephosphorylation of substrates derived from Ros 1999–2014 by SHP-1 and PTP1B

No.	Product	SHP-1	Product	PTP1B
1	–	–	–	–
2 (pY^5)	1	2.2	1	3.9
3 (pY^9)	1	–[a]	1	3.6
4 (pY^{10})	1	1.5	1	4.5
5 ($pY^{5,9}$)	3	3.7	2	6.1
			3	6.3
6 ($pY^{5,10}$)	2	4.0	2	8.1
	4	3.8	4	7.9
7 ($pY^{9,10}$)	3	3.2	3	5.1
8 ($pY^{5,9,10}$)	bi[b]	5.4	bi[b]	8.6

[a] No analyzable progress curve for product formation over the reaction time (120 min) was detected.

[b] Data for the mixture of the biphosphorylated peptides were taken (≤20 min), afterwards the disappearance of the biphosphorylated substrates corresponds to the formation of the monophosphorylated isomer/isomer mixture.



Scheme 1. Suggestion for the dephosphorylation of the triphosphorylated substrate from Ros 1999–2014 by tyrosine phosphatases SHP-1 (A) and PTP1B (B).

the library used to determine SHP-1 recognition features (7 residues) [25]. The results of the monophosphorylated peptides were also reflected by those for the bi- and triphosphorylated substrates with pY^9 being the poorly dephosphorylated residue. A comparison of the rate constants of the three biphosphorylated isomers showed that residue pY^5 in peptide **5** was faster dephosphorylated than pY^{10} in peptide **7**. Thus, the phosphorylation at position 9 also seems to influence the hydrolysis of position 10, probably due to a sterical influence on substrate binding. In peptide **6**, both residues pY^5 and pY^{10}, were dephosphorylated with a slight preference for pY^5 over pY^{10}. Since separations of the biphosphorylated products derived from peptide **8** was not possible, we determined the rate constant for the mixture (Table 3). According to our results, the triphosphorylated peptide is the best substrate for SHP-1 followed by the biphosphorylated peptides and subsequently the monophosphorylated isomers. The following rank order can therefore be given for SHP-1:

$$\mathbf{8}\,(pY^{5,9,10}) > \mathbf{6}\,(pY^{5,10}) \geq \mathbf{5}\,(pY^{5,9}) > \mathbf{7}\,(pY^{9,10}) > \mathbf{2}\,(pY^{5}) > \mathbf{4}\,(pY^{10}) \gg \mathbf{3}\,(pY^{9}).$$

In contrast to SHP-1, the temporal analysis for PTP1B revealed that all monophosphorylated peptides were dephosphorylated though to a different extent (Fig. 5). A preference for isomer **4** (pY^{10}) over peptides **2** (pY^5) and **3** (pY^9) was observed, with the latter being the less efficiently dephosphorylated residue, i.e. regarding the residue at position −2 we found the following rank order for PTP1B: D (**4**, pY^{10}) ≥ E (**2**, pY^5) > N (**3**, pY^9). The same was found in a study of the interaction of the substrate-trapping mutant PTP1B D181A and a partially degenerate SPOT library, where pY1163 was the most efficiently dephosphorylated substrate within the sequence TRDIYETDYYRKGGKG from the IR segment 1154–1169 [27]. This sequence is very similar to the Ros-segment investigated in this study (Fig. 1B).

The most interesting results, however, were obtained for the biphosphorylated peptides. The removal of the phosphate groups contained in peptide **5** corresponds to the results obtained with the monophosphorylated isomers, since pY^5 was preferentially removed compared to pY^9 (Table 3). In case of peptide **6**, it seemed that pY^{10} was better recognized than pY^5 that again is in agreement with the literature mentioned above and our findings for the monophosphorylated peptides. Peptide **7** contains two consecutive phosphotyrosine residues ($pY^{9,10}$) within the motif DYYR of the Ros segment. Several groups have reported about the interaction and hydrolysis of (D/E)pYpYR motifs by PTP1B [7,27,28]. However, there are two different interpretations of the binding modus of peptides containing adjacent pY-residues. On the one hand, Salmeen et al. discussed that the IR-derived peptide with phosphorylation at Y1162 (corresponds to pY^9 in the Ros peptide sequence) and Y1163 (pY^{10}) involves a second phosphate binding site for one of the phosphotyrosine residues [7]. Crystal structure analysis of the PTP1B-C215A mutant and the biphosphorylated peptide revealed that pY1162 bound to the catalytic site, while the second pY1163 was located within a groove on the protein surface. On the basis of this structural analysis a substrate selectivity for pY1162 relative to pY1163 was suggested. On the other hand, an analysis of the same sequence using the substrate-trapping mutant PTP1B D181A mentioned above revealed that pY1163 is the target for PTP1B rather than pY1162 in the biphosphorylated peptide [27]. Although we have not directly analyzed the IR activation loop-derived peptides, our analysis is in agreement with the results of the latter study, since peptide **3** (pY^9) was formed indicating that the adjacent pY^{10} was preferentially dephosphorylated. In addition, a comparison

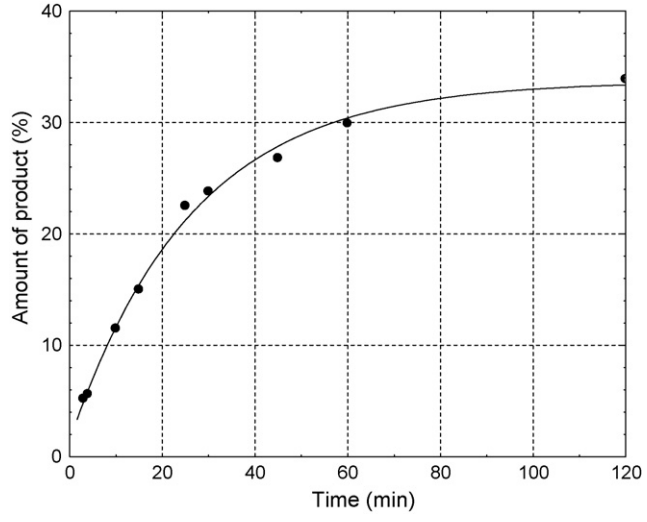


Fig. 6. Fit of the formation of peptide **4** from starting compound **6** as a function of time (*t*).

of the rate constants for the three biphosphorylated peptides revealed that $pY^{9,10}$ (peptide **7**) is the less efficiently dephosphorylated substrate (Table 3). We have currently no structural information on the binding modes of Ros-derived phosphopeptides to the catalytic site of PTP1B. Our data suggest that, in addition to the binding mode observed for the IR activation loop phosphopeptide in the PTP1B crystal structure [7], in solution additional binding modes and corresponding substrate selectivities are possible. According to our results, the rank order for substrate recogniton by PTP1B is:

$$\mathbf{8}\,(pY^{5,9,10}) \geq \mathbf{6}\,(pY^{5,10}) \geq \mathbf{5}\,(pY^{5,9}) > \mathbf{7}\,(pY^{9,10}) > \mathbf{4}\,(pY^{10}) > \mathbf{2}\,(pY^{10}) > \mathbf{3}\,(pY^{9}).$$

In addition to the HPLC analysis of the reaction progress, the kinetic analysis of the dephosphorylation of the peptides (**1–8**) revealed that the triphosphorylated compound is the preferred substrate with a k_{cat}/K_m value of $1.18 \times 10^6\,M^{-1}\,s^{-1}$ that is comparable to the value $1.51 \times 10^6\,M^{-1}\,s^{-1}$ reported for the triphosphorylated sequence from IR 1154–1169 [7]. Also, this k_{cat}/K_m was higher than the values determined for the corresponding biphosphorylated ($0.16–0.22 \times 10^6\,M^{-1}\,s^{-1}$) and monophosphorylated peptides ($0.03–0.04 \times 10^6\,M^{-1}\,s^{-1}$). Thus, the kinetic data were consistent with the rate constants determined from the reaction progress curves. In contrast to PTP1B, kinetic data for the peptides with SHP-1 could not be determined, because the peptides did not reach saturation under the conditions used. However, it is known that SHP-1 is less efficient for a number of substrates than other PTPases [29,30]. Peptide conversions by PTP1B were generally faster than by SHP-1. Due to these facts and the good correlation of the kinetic data and the rate constants for PTP1B we decided to not perform an additional kinetic analysis with a higher concentration of SHP-1.

In general, it is apparent that the trend of the effects, in particular of the number of phosphorylated sites in the sequence, for catalysis is similar between SHP-1 and PTP1B with a preference for tri- versus bi- and monophosphorylated peptides.

4. Conclusions

In the present study, we determined the utility and limits of monitoring the product formation of phosphatase reactions by reversed-phase HPLC. Substrates from the activation loop of receptor tyrosine kinase Ros (residues 1999–2014) were synthesized in all possible combinations of phosphorylation states and were subsequently used to analyze the substrate specificities of SHP-1 and PTP1B. We found that HPLC was the method of choice for the separation of the monophosphorylated peptides formed from the biphosphorylated substrates, whereas the separation of the different mixtures of biphosphorylated peptides could not be achieved. This prevented the direct analysis of phosphatase action of the triphosphorylated sequence $AREIpY^5KNDpY^9pY^{10}RKRGEG$. However, the clear identification of the monophosphorylated products formed enabled the generation of a dephosphorylation scheme for both phosphatases starting from the triphosphorylated substrate.

Compared to spectroscopic and fluorimetric enzyme assays this method has the advantage of the discrimination of different monophosphorylated products derived from biphosphorylated substrates. In particular, for the sequence containing the two adjacent phosphotyrosines ($pY^{9,10}$) we were able to clearly determine that pY^{10} rather than pY^9 is dephosphorylated in contrast to a report of the dephosphorylation of the homologous peptide TRDIYETDYYRKRGGKG from the insulin receptor (residues 1154–1169), in which the removal of the phosphate group at position 9 was suggested based on a crystal structure analysis [7]. However, our data confirm the results obtained from another study, where the same IR-motif and a partially degenerate SPOT library were used to identify the substrate specificity of PTP1B [27].

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