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Optimizing Thiophosphorylation in the Presence of Competing Phosphorylation with MALDI-TOF–MS Detection

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Abstract

Thiophosphorylation provides a metabolically stable, chemically reactive phosphorylation analogue for analyzing the phosphoproteome in vitro and in vivo. We developed a MALDI-TOF–MS based assay for optimizing thiophosphopeptide production by a kinase even in the presence of Mg^{2+} and ATP. We found that Abl kinase thiophosphorylation rates can be 'rescued' using Mn^{2+} in the presence of Mg^{2+}. Under our ideal conditions, titration of Mn^{2+} and ATPγS in the presence of Mg^{2+} allowed relatively rapid, highly specific thiophosphorylation by Abl tyrosine kinase, both as purified enzyme and in complex cell extracts.

Keywords

thiophosphorylation; MALDI-TOF-MS; kinase activity; Bcr-Abl; phosphoproteomics

Introduction

Phosphate transfer is a major mechanism for cellular signaling, and is the subject of a wide body of research in the field of proteomics.[1] Thiophosphorylation offers a metabolically stable, chemically reactive phosphorylation analogue for investigating kinase activity in vitro and in vivo.[2–6] This technique provides a number of potential advantages for detecting phosphorylation signals in vivo. For example, the phosphatase resistance of thiophosphoproteins[7,8] may advantageously allow transient cellular signaling species to accumulate to detectable levels, facilitating analysis of low-level signaling events. Thiophosphate also provides a chemoselectively reactive handle for functionalization of thiophosphorylated peptides and proteins, aiding in pull-down of these molecules from complex solutions.[3,6] For example, recently Shokat and co-workers used a combination of a bulky ATPγS and a mutant kinase for specific thiophosphorylation and labeling of the substrates of that kinase.[5] Additionally, the ability to chemically distinguish between

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Supporting Information Available: Additional graphs from sample analysis, peptide synthesis/characterization details. This material is available free of charge via the Internet at http://www.pubs.acs.org.

existing phosphorylation patterns and recently thiophosphorylated molecules may lead to more informative time-resolution for studying the kinetics of in vivo signaling.

Thiophosphorylation is typically detected using S^{35}-labeled ATPγS or by reacting the phosphorothioate with e.g., fluorescent probes.[2–4,6,9] The pK_a of the phosphorothioate allows selective labeling at low pH even in the presence of cysteines and other nucleophilic residues in a peptide or protein.[3] One of the challenges of using this tool is the poor competition of ATPγS against ATP, with Mg^{2+} as a kinase cofactor. Unfortunately, many kinases (especially tyrosine kinases) show sluggish kinetics with ATPγS: consequently, observing 'real' kinase activity and generating enough sample for practical use can be difficult. Rate differences between phosphate and thiophosphate transfer are typically described as the 'thio-effect' by comparing the k_{cat} values for phosphate vs thiophosphate transfer.[10,11] Catalysis of thiophosphate transfer with Mg^{2+} as cofactor is often inefficient, resulting in large thio-effects. Alternative divalent metals such as Mn^{2+} and Co^{2+} can improve the efficiency of thiophosphorylation vs phosphorylation—these metals are thought to improve the activation of the terminal thiophosphate of ATPγS for transfer to the substrate via chelation with the thiol (which is poorly chelated by the 'hard' ion Mg^{2+}).[10–12] However, kinetics of thiophosphorylation are often still very slow under these conditions. Additionally, in vitro optimization of the thio-effect does not address the issue of achieving thiophosphorylation in vivo, where competition between ATP and ATPγS in the presence of Mg^{2+} remains a major challenge.

MALDI-TOF mass spectrometry has been used as a detection method for kinase assays in vitro.[13–16] These nonradioactive assays allow for the direct detection of phosphorylation of peptide substrates, and result in qualitative determination of phosphorylation. However, quantification of product yields based on the ratios of peptide to phospho-peptide peak areas must be carefully calibrated due to the inherent differences in ionization efficiency of phosphorylated, thiophosphorylated and unmodified peptides.

In this work, we have developed a MALDI-TOF mass spectrometry based assay for optimizing the level of thiophosphopeptide production by a kinase even in the presence of Mg^{2+} and ATP. Mass spectrometry detection of thiophosphovs phosphopeptides revealed that for Abl kinase, ATPγS utilization efficiency could be rescued by adding relatively low levels of Mn^{2+} in the presence of Mg^{2+} and ATP. Titration of Mn^{2+} and ATPγS allowed relatively rapid, highly specific thiophosphorylation by Abl tyrosine kinases, both as purified enzymes and in complex K562 human chronic myeloid leukemia cell extracts. The assay for optimal thiophosphorylation conditions was also demonstrated for PKA, revealing Co^{2+} as the best divalent cation for ensuring high thiophosphorylation levels in the presence of ATP for this serine/threonine kinase.

Experimental Section

PKA kinase (active domain) and biotinylated PKA substrate peptide were obtained from Calbiochem. Biotinylated PKA substrate peptide was obtained from Calbiochem. The StrepII-tagged Abl substrate peptide was synthesized using Boc SPPS and purified on a Waters PrepLC 4000 system (RP–C18 column). The synthesized peptide was characterized

using LC/MS (Agilent 1100 series LC/MSD Trap). Assay analyses were performed with the Applied Biosystems Voyager 4700 MALDI-TOF/TOF mass spectrometer.

Solution Phase Assay

Assay was performed in 96-well plate format in a total volume of 30 μL. The kinase reaction buffer was composed of 50 mM Tris-HCl (pH 7.5), 30 μg/μL BSA and 1 mM DTT. Peptide concentration was 100 μM (3 nmol total). Metal cofactors and ATP and/or ATPγS were added as specified. 0.34 μg (10 pmol) of Abl (recombinantly produced kinase domain, 34 kDa) was used for each well. Reactions were performed at 30 °C for 30 min then quenched with 10 μL EDTA (0.5 M, pH 8.5). The resulting solutions were transferred to 5 μL Streptactin-sepharose beads (IBA) pre-aliquoted into a 96-well filter bottom plate (Millipore) and allowed to equilibrate at RT for 15 min to bind peptide to beads. Beads were then washed on a vacuum manifold five times with PBS buffer and 5 times with distilled water and allowed to dry partially. Bound peptides were eluted with 10 μL acetonitrile/H_2O (75/25%) and collected into a 96-well plate via centrifugation (2 min, 1500 rpm). The resulting mixture of unphosphorylated, phosphorylated and thiophosphorylated peptide was mixed with 6 μL matrix solution (75/25/0.1% acetonitrile/H_2O/TFA, 10 mg/mL CHCA) and spotted in triplicate on a stainless steel MALDI target (Applied Biosystems). Spots were analyzed in linear negative mode. Data were processed using a macro provided by Melanie Lin of Applied Biosystems to extract mass and intensity (calculated as area under the isotope cluster curve) into tab-delimited text files.

Solid-Phase Assay

Assay was performed in 96-well filter bottom plates. Streptactin-sepharose beads (6 μL) were equilibrated with kinase buffer for 1–2 min and excess buffer was removed with a vacuum manifold. Peptide (20 nmol) was added and bound to the beads for 5 min. Beads were washed and additional reagents (kinase buffer as above, ATP, ATPγS, metal as indicated in plots) and recombinant Abl (2.1 μg, ~60 pmol) were added. Reactions were performed at 30 °C for 30 min, and samples were quenched, processed and analyzed as above.

Results and Discussion

We set out to find conditions for using MALDI-TOF to quantify the efficency of thiophosphorylation in the presence of competing phosphorylation. As a model system we chose the tyrosine kinase c-Abl,[17] for which thiophosphorylation is reported to be undetectable.[12,18,19] Addition of ‘thiophilic’ divalent cations to improve Abl thiophosphorylation had not been reported. Mimicking cellular conditions (necessitating the presence of Mg^{2+} and ATP) was desirable for our overall method development. Typical thiophosphorylation assays do not address detection of competing phosphorylation. MALDI-TOF MS offered direct detection of the ratio of thiophosphorylated to phosphorylated peptide products, allowing us to determine conditions for competitive thiophosphorylation in the presence of ATP. The Abl kinase domain was used in conjunction with an ‘optimal’ substrate peptide[20] (in italics) carrying an N-terminal affinity tag (Strep-tag)[21] (WSHPQFEK)*EAIYAAPFAKKK* (**1**) to optimize the conditions for

thiophosphorylation, and these conditions were applied to selective thiophosphorylation of the peptide substrate **1** by cell extract. To address the inherent spot-to-spot variability in sample crystallization, all experiments were performed in duplicate and each was spotted in triplicate (i.e., six measurements per ratio). Linear mode was used for quantification of peak ratios, because reflectron mode revealed fragmentation of the thiophosphopeptide with loss of HPO_2S (see Supporting Information). Negative mode was chosen over positive mode because we find it improves the sensitivity for detection of low-level thiophosphorylation compared to positive mode.

It was important to establish calibration curves to quantify the relationship between phosphorylated and thiophosphorylated peptide. This was accomplished by combining known ratios of the two purified materials and observing the resulting signal intensity ratio in the mass spectrometer (Figure 1). Thio-to phospho-peptide signal ratios were consistently 85% of the actual ratio in the sample, thus this correction factor was applied to the ratios observed in the rest of the analyses.

We assessed the question of competition between ATPγS and ATP (1:1) in the conversion of **1** using the Abl kinase domain. Mn^{2+}, Co^{2+}, and Ni^{2+} were chosen as examples of 'thiophilic' metals previously reported in the literature. In preliminary experiments, Cu^{2+} and Hg^{2+} were also investigated but resulted in precipitation problems in the assay. Divalent metal ions Mg^{2+}, Mn^{2+}, Co^{2+}, and Ni^{2+} (5 mM) were used alone and in combination with Mg^{2+} (10 mM Mg^{2+}:1 mM other M^{2+}). The reactions were performed in solution as described above. Results are summarized in Table 1. As expected, Mg^{2+} alone greatly favored phosphorylation. Mn^{2+} alone resulted in very low overall conversion (<5%), but did improve thiophosphorylation (to approximately 1:1). Co^{2+} and Ni^{2+} alone also resulted in minimal conversion to either product. With Mg^{2+} in the reaction buffer (alone or in combination with another metal), substrate conversion to thio- and/or phospho-peptide was essentially quantitative in 30 min (with the exception of Mg^{2+}/Ni^{2+} which gave only ~30% conversion). The most positive results came from the combination of Mg^{2+}/Mn^{2+}: thiophosphorylation was improved 2-fold over Mg^{2+} alone, and importantly, conversion was quantitative. This 'rescue effect' using low levels of 'thiophilic' divalent metals in combination with Mg^{2+} is known for ribozymes containing thiophosphate linkages,[22–24] but has not previously been observed for kinases. The seemingly cooperative effect of Mg^{2+} and Mn^{2+} in rescuing thiophosphorylation may result from binary metal cofactor interactions in the kinase active site.[25] The optimized conditions using 10:1 Mg^{2+}/Mn^{2+} allowed us to achieve very high levels of thiophosphorylation in the presence of micromolar levels of ATP (Figure 2). This should be compared to the reports in the literature mentioning the failure of previous attempts to detect thiophosphorylation by Abl kinase.[10,16,17]

Additional characterization of key reactants (Mn^{2+} and ATPγS) and in depth analysis of ATP and ATPγS competition was carried out in an on-bead assay. **1** was pre-bound to the beads and subjected to the kinase reactions varying the levels of Mn^{2+} or ATPγS (up to 48 conditions in duplicate for a total of 96 reactions). The products were analyzed directly by fixing a small amount of beads to the MALDI target with matrix. The matrix solution containing acetonitrile/H_2O/TFA served to elute the peptide from the beads. These studies showed that as little as 0.5 mM Mn^{2+} in the presence of 10 mM Mg^{2+} promoted selectivity

for thiophosphorylation, and that using the optimized Mg/Mn combination buffer (10:1), a ratio of 10:1 ATPγS to ATP was sufficient to achieve 1:1 ratios of thiophosphorylated to phosphorylated product. Importantly, these reagent levels are within a practical range for use in cell extracts. We demonstrated this using the optimized conditions (described in Figure 2) with K562 chronic myeloid leukemia cell extracts (which overexpress the oncogenic fusion protein Bcr-Abl) to thiophosphorylate **1**. Specificity was shown using the Bcr-Abl inhibitor Gleevec (Figure 3). Notably, 10 mM $MgCl_2$ or 1 mM $MnCl_2$ alone did not promote thiophosphorylation in these conditions (Figure 4).

The high-throughput capabilities of this assay will in the future allow rapid, convenient determination of optimal thiophosphorylation conditions for other kinases. The key advantage is the ability to observe both phosphorylation and thiophosphorylation in the same assay. For example, we demonstrated the use of the ATP:ATPγS competition assay using the catalytic subunit of the serine/threonine kinase PKA. Thiophosphorylation of the substrate peptide LCGRTGRRNSI–NH_2 (biotinylated on the N-terminus) was carried out according to the solution phase protocol described above. Although PKA was reasonably efficient at thiophosphorylating its substrate even with only Mg^{2+} (~10–15% thiophosphopeptide produced at 1:1 ATP/ATPγS), a mild improvement in thiophosphorylation was gained (15–20% thiophosphopeptide produced) by using Co^{2+} in the reaction buffer (Table 2)—however, the reaction proceeded efficiently with Co^{2+} alone in the buffer and addition of Mg^{2+} only modestly improved the rate. We are currently screening other kinases in this assay to determine if the thiophosphorylation ‘rescue’ observed for Abl kinase with Mg^{2+}/Mn^{2+} is a general effect for tyrosine kinases or if it is specific to some kinases and not others. Our primary goal is to use these optimized conditions for Abl kinase to investigate thiophosphorylation of endogenous substrates by Bcr-Abl in K562 cell extracts, in response to changes in cellular states, with high throughput detection by MALDI-TOF mass spectrometry. The assay described here should facilitate access to thiophosphorylation as a tool for phosphoproteomics in general, but especially for systems such as ours that require optimization of not only the ‘thiophilic’ cation required for the kinase of interest, but also ATPγS competition in the presence of ATP and Mg^{2+}, a feature which will prove crucial for making thiophosphorylation practical for in vivo use.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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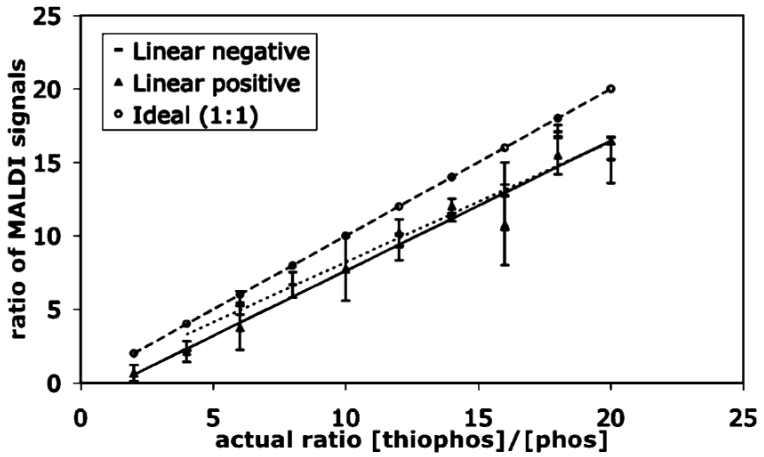

Figure 1.

Calibration curve representing signal intensity ratios of thiophosphopeptide to phosphopeptide in both linear positive and negative modes (calculated as area under the curve) compared to the ideal ratio.

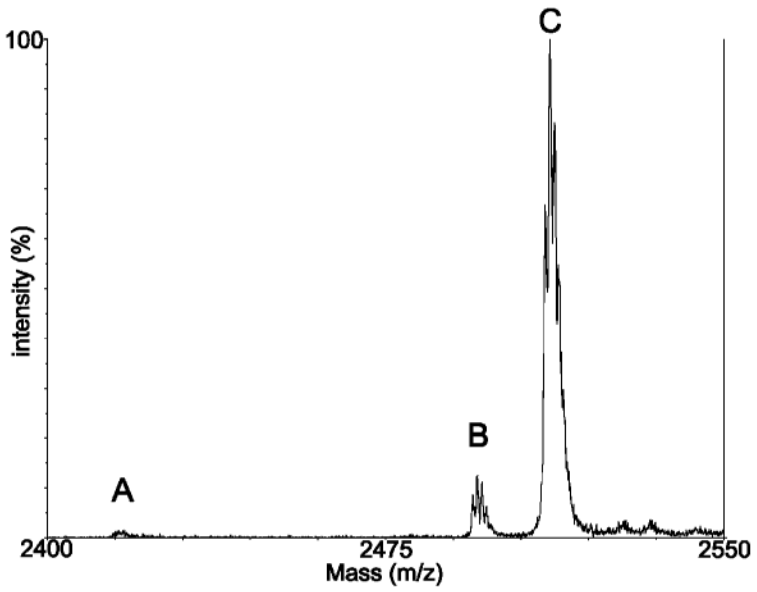

Figure 2.

Optimized thiophosphorylation conditions: ATP (2.5 μM ATPγS (1 mM), $MgCl_2$ (10 mM), $MnCl_2$ (1 mM). A: unphosphorylated; B: phosphorylated; C: thiophosphorylated.

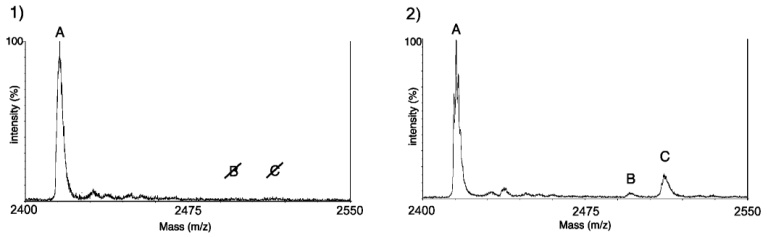

Figure 3.

Thiophosphorylation of 1 by Bcr-Abl in K562 cell extracts (1) with and (2) without inhibitor. A: unphosphorylated; B: phosphorylated; C: thiophosphorylated.

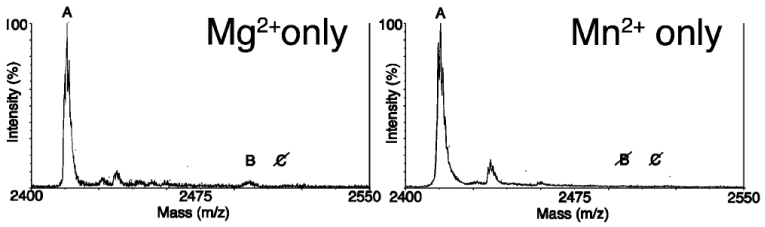

Figure 4.

Thiophosphorylation of 1 by Bcr-Abl in K562 cell extracts with Mg^{2+} and Mn^{2+} alone. No thiophosphorylation is observed with Mg^{2+} alone, and no conversion is observed for Mn^{2+} alone. A: unphosphorylated; B: phosphorylated; C: thiophosphorylated.

Table 1

Addition of Mn^{2+} with Mg^{2+} Resulted in a 2–3-Fold Improvement in Thiophosphorylation by Abl Kinase Domain Compared to Mg^{2+} Alone

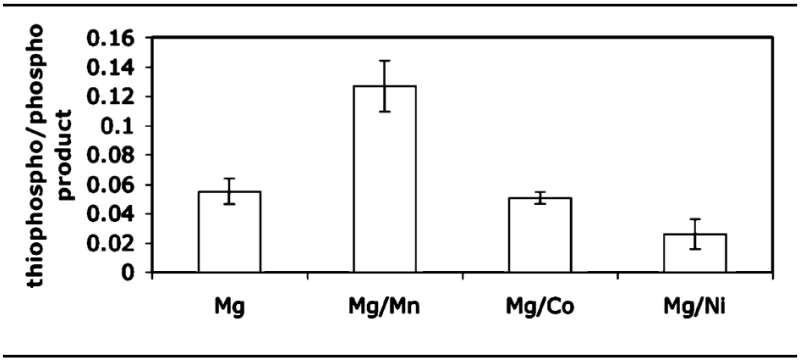

Table 2

Co^{2+} Provided a Slight Increase in Thiophosphorylation by PKA, However Conversion Was Quantitative with and without Mg^{2+}