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Thiol-ene Enabled Detection of Thiophosphorylated Kinase Substrates

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Abstract

Protein phosphorylation is a ubiquitous posttranslational modification that regulates cell signaling in both prokaryotes and eukaryotes. Although the study of phosphorylation has made great progress, several major hurdles remain, including the difficulty of the assignment of endogenous substrates to a discrete kinase and of global phosphoproteomics investigations. We have developed a novel chemical strategy for detecting phosphorylated proteins. This method utilizes adenosine 5'-O-(3-thiotriphosphate) (ATP γ S), which results in the transfer of a thiophosphate moiety by a kinase to its substrate(s). This group can subsequently be employed as a nucleophilic handle to promote protein detection. To selectively label thiophosphorylated proteins, cellular thiols (e.g. cysteine-containing proteins) must first be blocked. Most common cysteine-capping strategies rely upon the nucleophilicity of the sulfur group and would therefore also modify the thiophosphate moiety. We hypothesized that the radical-mediated thiol-ene reaction, however, would be selective for cysteine over thiophosphorylated amino acids due to the differences in the electronics and pK_a values between these groups. Here, we report rapid and specific tagging of thiophosphorylated proteins *in vitro* following chemoselective thiol capping using the thiol-ene reaction.

Protein kinases are ubiquitous in the human genome, comprising nearly 2% of open reading frames and with over 500 family members.(1) Often, these enzymes recognize multiple substrates meaning that the proportion of phosphorylated proteins is even higher, with an estimated 30% of the proteome bearing this modification.(2) The dysregulation of kinases and phosphatases has been linked to many disease processes, making these proteins important drug targets. There are two main classes of eukaryotic kinases: serine/threonine (Ser/Thr) and tyrosine (Tyr), based on the substrate amino acid that is phosphorylated. In all cases, the enzyme catalyzes the transfer of the terminal phosphate group from adenosine triphosphate (ATP) to the substrate (Figure 1A); a protein, peptide or the kinase itself (autophosphorylation). The large number of proteins involved in phosphotransfer and the ability of these proteins to interact with numerous substrates make determining the discrete role(s) of any one kinase exceptionally difficult. In addition, detection of phosphorylated substrates is challenging because phosphoproteins are frequently low abundance and substoichiometrically modified.

Current strategies for the study of kinase biology and phosphorylation in complex samples often utilize methods that rely upon nonspecific charge-, polarity-, or metal affinity-

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Supporting Information Available: Additional methods descriptions, NMR and HPLC spectra, full gel images, detection limit studies and lysozyme capping data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

mediated enrichment/visualization.(3–5) While successful, improvements are needed to enable both facile detection of newly phosphorylated proteins and detailed characterization of kinase-substrate relationships. Several research groups have employed modified ATP co-substrates as a means to address these challenges.(6, 7) Of note, strategies that facilitate crosslinking of a kinase to its substrate(s) show great promise.(8–11) Adenosine 5'-O-(3-thiotriphosphate) (ATP γ S) has also been utilized and results in the transfer of a unique group, the thiophosphate, to each substrate (Figure 1A).(12–17) ATP γ S is accepted by a wide variety of Ser/Thr and Tyr kinases.(12, 13, 18, 19) Since it is not an endogenous posttranslational modification, thiophosphorylation is a marker of a new phosphorylation event. Thiophosphorylation is also more stable than typical phosphorylation as it is resistant to phosphatase action.(20) Finally, the thiophosphate provides a chemically unique species, with reactivity similar to a thiolate, which can be exploited for detection of substrates that have been modified with this group.

Examination of thiophosphorylated proteins/peptides has been reported using several strategies. Shokat and coworkers generated an approach that utilizes an electrophilic reagent to cap both cellular thiols (e.g., cysteine-containing proteins) and thiophosphorylated amino acids. The reacted moieties are subsequently differentiated with an antibody. Importantly, labeled substrates are recognized regardless of the residue that was modified (Ser, Thr, Tyr) or the surrounding amino acid sequence, facilitating direct kinase-substrate pairing in a multitude of systems.(12, 13) Several methods have also been developed to enable proteomic profiling of thiophosphorylated proteins. Capture of thiophosphorylated species occurs through reaction of both this group and proteinacious thiols, followed by selective cleavage of the thiophosphate from solid support.(14, 15) While efficacious, this process results in loss of the thiophosphate modification and cannot facilitate detection of species that contain both a thiophosphate and cysteine(s), which become trapped on the resin. Finally, selective capture of thiophosphorylated substrates has been accomplished by performing the reaction under acidic conditions that render cysteine residues unreactive while thiophosphorylated groups remain nucleophilic.(16) This strategy has seen limited utility because the overwhelming excess of cysteine nucleophiles in complex protein mixtures makes practical application difficult.

We sought to generate a novel strategy for detection of kinase-substrate pairs that avoids the use of antibodies and that can chemically differentiate thiols and thiophosphates in complex samples (Figure 1B). We wished to utilize the nucleophilicity of the thiophosphate to enable visualization of this group, thus requiring that we block cellular thiols prior to the tagging step. Most common cysteine capping reagents are electrophilic (e.g., haloacetamides, maleimide), and would also react with the thiophosphate moiety. We hypothesized that the radical-mediated thiol-ene reaction, however, might be selective for cysteine over thiophosphorylated amino acids due to the differences in the electronics and pKa values between these groups. The thiol-ene reaction is light-mediated and promotes homolytic cleavage of the S–H bond in the presence of a photoinitiator. The sulfur radical is coupled to an alkene yielding a stable thioether (Figure 2A). Following cysteine capping, thiophosphorylated proteins could be tagged with a molecule such as an iodoacetamide derivative of the fluorophore BODIPY (Figure 2B).

The thiol-ene reaction has been utilized for the generation of (bio)materials for a number of years(21) and has more recently been used in biological applications such as glycoconjugate synthesis(22) and site-specific protein modification.(23, 24) To examine the selectivity of this reaction, we subjected both a cysteine and thiophosphorylated tyrosine(25, 26) analog to thiol-ene coupling conditions. The initiator, lithium phenyl-2,4,6-trimethylbenzoylphosphine(27) (LAP), was utilized because it is suitably water soluble for use with proteins. Alkene 1-(2-methoxyphenyl)-3-buten-1-ol was used as it is both UV

active and exhibits well-resolved signals in the ^1H NMR spectrum facilitating reaction characterization by ^1H NMR and HPLC (Figure 3). As expected, the cysteine analog reacted readily to form the thioether (Figures 3A and B, Supplementary Figures 1 and 2). The ^1H NMR spectrum contained new peaks indicative of product. In addition, comparison of the relative proton integration of the phenyl protons in the alkene reagent to the internal alkene proton over the course of the reaction demonstrated that the alkene was being consumed to form the thioether (Figure 3A; cysteine starting material signals decreased, Supplementary Figure 1). HPLC analysis similarly showed formation of a new species, which was demonstrated to be the thioether by mass spectrometry (Figure 3B, Supplementary Figure 2). Gratifyingly, the thiophosphorylated Tyr analog remained unchanged even when exposed to excess alkene (4 equiv.) over several hours, indicating that it is unreactive under thiol-ene conditions (alkene to phenyl proton ratio unchanged, Figure 3C; tyrosine starting material signal unchanged, Supplementary Figures 1 and 2). In contrast, reaction of thiophosphorylated tyrosine with iodoacetamide exhibited complete conversion to the alkylated thiophosphoryl tyrosine within 15 minutes (monitored by ^{31}P NMR; Supplementary Figure 3). These data suggest that this combination of reactions may be applicable to the generation of a strategy for selective detection of thiophosphorylated moieties in proteins.

To assess this method in protein systems, three protein kinases were selected, JNK1 (serine/threonine kinase),⁽¹³⁾ p38 α MAPK (serine/threonine kinase)⁽¹⁸⁾ and Src (tyrosine kinase),⁽¹⁹⁾ all of which utilize ATP γ S as a co-substrate. These proteins were incubated with ATP γ S (5 mM) and known substrates [activating transcription factor 2 (ATF2), myelin basic protein (MBP) and self, respectively].⁽¹³⁾ Next, a cancer cell background proteome (MCF-7) was added to ensure that the sample included an ample number of cysteine-containing proteins. The thiol-ene reaction was performed on the samples to cap all cysteines. Allyl alcohol (200 mM) was selected as the “ene” due to its water solubility. Thiophosphate labeling was then accomplished by incubation with iodoacetamido-BODIPY (1 μM ; Figure 2B) and samples were separated using gel electrophoresis and visualized with in-gel fluorescence detection.

Selective labeling of the thiophosphorylated substrates is evident by comparison of samples in the absence (lane 1) or presence (lane 2) of treatment with the thiol-ene reagents (Figure 4). Without thiol-ene pretreatment, a large number of bands appear following addition of the electrophilic dye. Thiol-ene-facilitated thiol capping prevents this non-specific protein tagging. As expected, the labeled band was abolished when substrate was not added (lane 3). Protein labeling was determined to be both ATP γ S- (lane 4) and kinase- (lane 5) dependent. Pre-incubation with ATP also blocks band visualization, again demonstrating that protein tagging is activity dependent (lane 6). Selective labeling of all three substrates was successful indicating the generality of this method. Full-length gels and band quantification are available as supporting information (Supplementary Figure 4 and Table 1). Finally, detection limit studies were performed with the p38 α -MBP kinase-substrate pair. The protein blocking and labeling procedure was executed on samples with decreasing amounts of MBP in the presence of background proteome (MCF-7). The detection limit of this substrate was $\sim 0.5\ \mu\text{g}$, which is similar to the quantities utilized in previous studies to examine this protein pair.⁽¹³⁾ The observed signal was linear across the examined protein concentration range (0–10 μg ; Supplementary Figures 5 and 6). Quantification of the labeling of lysozyme, which contains eight cysteines, with iodoacetamido-BODIPY before and after treatment with the thiol-ene reaction indicates $>85\%$ blocking of the cysteine residues (Supplementary Figure 7).

Here, we describe a facile method for chemical differentiation of thiols and thiophosphorylated groups. Unlike most sulfhydryl capping strategies, the thiol-ene reaction

does not utilize the relatively high nucleophilicity of this group to enable selective modification. Model studies indicated that a thiophosphorylated amino acid was unaltered by the thiol-ene reaction and suggested that a two-step process could be utilized for selective visualization of thiophosphorylated proteins; thiol-ene-mediated cysteine capping followed by nucleophilic addition of the thiophosphoryl group to the tagging reagent. This strategy was readily translated into protein systems where rapid detection of the thiophosphorylated species was accomplished with three kinase-substrate pairs (capping and labeling take ~2.5 hrs). This method will find widespread utility as it takes advantage of a co-substrate, ATP γ S, which is accepted by an array of kinases. Future work will be aimed at employing alternative tagging reagents (e.g., biotin) to enable utilization of mass spectrometry-based detection to increase both the scope and sensitivity of the described approach.

METHODS

General Materials and Methods

Reagents were obtained from J.T. Baker, Mallinkrodt, Tocris Bioscience, Sigma, MP Biomedicals, EMD Biosciences, Bio-Rad, and Fisher, except where otherwise noted, and were used without further purification. ^1H nuclear magnetic resonance (NMR) spectra were recorded on Varian I500 or I400 instruments. Chemical shifts are reported relative to residual solvent peaks in parts per million. ^{31}P NMR spectra were recorded on a Varian I400 instrument. Apparent first-order multiplicities are indicated by s, singlet; d, doublet; dd, doublet of doublets; t, triplet; dt, doublet of triplets; q, quartet; m, multiplet. Compound purification was performed on an Agilent 1200 HPLC using a reverse phase column (Agilent ZORBAX C18, 5 μm , 250 $\times$ 21 mm) detected by diode array detector (200–600 nm). Gradients consisted of 5–100% B (A: H_2O , 0.1% formic acid (FA); B: CH_3CN , 0.1% FA). Mass determination was performed in the positive-ion mode using an Agilent 1200 HPLC-6130 MSD. Gradient consisted of 5–100% B (A: H_2O , 0.1% FA; B: CH_3CN , 0.1% FA). Accurate mass determination was performed in the positive-ion mode using an Agilent 1200 LC-MS-TOF equipped with a reverse phase column. Gradient consisted of 5–100% B (A: H_2O , 0.1% ammonium acetate (AA); B: CH_3CN , 0.1% AA).

Lysozyme, p38 α (active, GST-tagged, human), JNK1 (active, GST-tagged, mouse), myelin basic protein (MBP, bovine), and activating transcription factor 2 (ATF-2, maltose binding fusion protein, human) were purchased from Sigma-Aldrich. Src (active, His-tagged, human) was purchased from Millipore.

Model Compound Synthesis and Assessment

Synthesis of N-acetyl-L-thiophosphotyrosine—*N*-acetyl-L-tyrosine (Sigma-Aldrich, 30 mg, 0.13 mmol) was dissolved in D_2O , and 6M KOH was added dropwise until the pH reached 10. This solution was added dropwise to a solution of D_2O (100 μL), 6 M KOH (60 μL), and PSCl_3 (125 μL , 0.54 mmol) over two min. KOH was added as necessary to maintain the solution pH at roughly 10. After three hour, when the reaction was largely complete as judged by ^{31}P and ^1H NMR, the reaction mixture was purified by HPLC. ^1H NMR (400 MHz, D_2O) δ 8.15 (s, formic acid salt), 7.31–7.22 (m, 4H, phenyl), 4.50–4.46 (m, 1H, ABX), 3.22–2.97 (m, 2H, ABX), 2.02 (s, 3H, Me); ^{31}P NMR (162 MHz, D_2O , proton coupled) δ 84.27 (s); $[\text{M}+\text{H}]^+ \text{C}_{11}\text{H}_{15}\text{NO}_6\text{PS}^+$, expected = 320.0235, observed = 320.0351.

Thiol-ene Reaction with Model Amino Acid Cysteine—*N*-acetyl-L-cysteine-OH (8.2 mg, 0.05 mmol) and lithium phenyl-2,4,6-trimethylbenzoylphosphinate(27) (LAP, 1.5 mg, 0.005 mmol) were dissolved in 300 μL D_2O . 1-(2-methoxyphenyl)-3-buten-1-ol (8.6 μL , 0.05 mmol [1:1 ratio of Cys:ene]; 1:4 ratio of Cys:ene used in HPLC experiments) was

dissolved in 300 μL methanol- d_4 , which was required for solubility of the alkene and the resulting alkylated Cys product, and the two solutions were combined and irradiated at 365 nm for one hour. Reaction progress was monitored by ^1H NMR and HPLC. The product was purified by HPLC. $[\text{M}+\text{H}-\text{H}_2\text{O}]^+ \text{C}_{16}\text{H}_{22}\text{NO}_4\text{S}^+$, expected = 324.1, observed = 324.2.

Thiol-ene Reaction with Model Amino Acid Thiophosphotyrosine—*N*-acetyl-L-thiophosphotyrosine (0.004 mmol) and lithium phenyl-2,4,6-trimethylbenzoylphosphinate(27) (0.12 mg, 0.0004 mmol) were dissolved in 400 μL D_2O . 1-(2-methoxyphenyl)-3-buten-1-ol (2.8 μL , 0.016 mmol; 1:4 ratio of tpTyr:ene used in both experiments) was dissolved in 300 μL methanol- d_4 , which was required for the solubility of the alkene, and the two solutions were combined and irradiated at 365 nm for one hour. Reaction progress was monitored by ^1H NMR and HPLC; no change was observed.

Protein Labeling and Detection

Buffers for protein experiments—The reaction buffer used was 50 mM Tris-HCl, 0.2 M KCl, 5 mM MgCl_2 , final pH 7.8. The 2X SDS-PAGE loading buffer contained 125 mM Tris, pH 6.8, 20% glycerol, 4% SDS (w/v), 5% BME (v/v), and 0.2% bromophenol blue (w/v).

Protein thiophosphorylation reactions—Reaction mixtures (5 μL) were prepared with 0.1–3 μg purified kinase, 5–10 μg substrate, and 1 μL ATP S (5 mM final concentration) in reaction buffer. The mixtures were incubated at 37 $^\circ\text{C}$ for 12–24 hours.

Protein thiol-ene reactions—Following completion of the thiophosphorylation reaction, background proteome (BGP, 20 μg) and urea (6 M final concentration) were added to the reaction mixture (30 μL final volume), and the reaction was incubated at room temperature for 10 minutes. LAP, allyl alcohol (water-soluble ene; 200 mM final concentration), and glutathione (50 mM final concentration) were added and the mixture was irradiated at 365 nm for 20 min. To quantify the ability of the thiol-ene to cap a specific thiol-containing protein, lysozyme, the reaction was set-up as above with the addition of 1 μg of this protein.

Protein precipitation protocol—Protein precipitation using trichloroacetic acid (TCA) was carried out prior to labeling to remove unreacted ATP S, thiophosphate, and glutathione. Reaction mixtures were incubated on ice for 5 min, TCA solution (5 μL , 100% w/v) was added, and the resulting mixture was incubated on ice for 10 min. The samples were centrifuged for 10 min at $20,000 \times g$ at 4 $^\circ\text{C}$. The supernatant was discarded and the pellet was washed with cold methanol (40 μL) and cold acetone (350 μL), and the pellets were dried at room temperature for 30 min. The pellets were then resuspended in reaction buffer (14 μL).

Protein thiophosphate tagging reactions—Labeling was achieved by adding 1 μL BODIPY-iodoacetamide (1 μM final concentration) in reaction buffer. Individual mixtures were incubated at room temperature for 1 h in the dark to prevent fluorophore photobleaching. After incubation, reactions were quenched with 2X SDS-PAGE loading buffer (5 μL) and heated at 100 $^\circ\text{C}$ for 5 min. Each well was loaded with 16 μL of the reaction sample, corresponding to 80 ng of kinase (2.4 μg in the case of Src) and 8 μg of substrate per well. Detection limit experiments utilized the indicated amount of protein in each sample.

Gel separation and in-gel fluorescence detection—Polyacrylamide gels were composed of 12.5% resolving gel (2.3 mL of 1.5 M Tris-HCl buffer pH 8.8, 2.8 mL of bis-acrylamide 37.5:1 (40% solution), 3.9 mL of H_2O , 31.5 μL of 10% ammonium persulfate

(APS), 15.8 μ L of tetramethylethylenediamine (TEMED)) and 4.5% stacking gel (0.6 mL of 0.5 M Tris-HCl buffer pH 6.8, 0.3 mL of bis-acrylamide 37.5:1 (40% solution), 1.6 mL of H₂O, 15 μ L of 10% APS, 7.5 μ L of TEMED). Running parameters were 180 V, 400 mA, and 60 W for 1 h. After SDS-PAGE, gels were washed three times with distilled water and scanned on a Typhoon Variable Mode Imager 9210 (Amersham Biosciences) using 526-nm (short-pass filter) detection for BODIPY (λ_{ex} : 504 nm, λ_{em} : 514 nm). For comprehensive protein visualization, gels were coomassie stained following fluorescence scanning.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Use of ATP γ S as an alternative kinase co-substrate provides a functional group that can be used for protein visualization. a) Kinases catalyze the transfer to the substrate protein of a phosphate from ATP or a thiophosphate from ATP γ S. b) General strategy for detection of phosphorylated proteins in complex mixtures. Substrate(s) are thiophosphorylated by kinase partner and ATP γ S. Proteinacious thiols must be capped to facilitate subsequent chemoselective detection of the thiophosphorylated species.

Figure 2. Methods and reagents utilized to facilitate selective detection of thiophosphorylated proteins. a) Cysteine residues are capped by formation of the stable thioether. b) Thiophosphate groups are subsequently labeled by reaction with an iodoacetamido-BODIPY reagent.

Figure 3.

The thiol-ene reaction is specific for thiols. a) ^1H NMR spectra of the treatment of cysteine with 1-(2-methoxyphenyl)-3-buten-1-ol (1:1 ratio) in the presence of LAP and 365 nm light at $t = 0$ and 1 h. The ratio of the phenyl protons to internal alkene protons is reduced, indicating that a reaction is occurring. b) Cysteine capping under thiol-ene conditions was monitored by HPLC (alkene $t_R = 22.2$ min; A_{280}). Formation of the expected thioether was readily observed ($t_R = 18.4$ min). c) ^1H NMR spectra of the treatment of thiophosphotyrosine yielded a spectrum in which the ratio of the phenyl protons to internal alkene protons is unchanged, indicating no reaction (thiophosphotyrosine:ene of 1:4).

Figure 4.

Specific fluorescent labeling of JNK1 (a), p38 α MAPK (b), and Src (c) substrates. The serine/threonine kinases p38 α and JNK1 were incubated with ATP γ S (5 mM) and known substrates, MBP and ATF2, respectively (80 ng kinase, 8 μ g substrate in 5 μ L). In-gel fluorescence detection of the BODIPY fluorophore in the absence (lane 1) or presence (lane 2) of treatment with the thiol-ene reagents. Substrate labeling is abolished in the absence of ATP γ S (lane 4) or kinase (lane 5) or pre-incubation with ATP (lane 6; 100 mM). Lane 3 in part a (no substrate; ATF2) contains lysozyme (14 kDa), which was required to facilitate effective protein precipitation in the absence of ATF2. The tyrosine kinase, Src, was also examined (2.4 μ g). This kinase autophosphorylates. Coomassie staining showed even protein loading.