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Mass spectrometry-based identification of protein kinase substrates utilizing engineered kinases and thiophosphate labeling

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Abstract

Protein kinases constitute a large enzyme family with key roles in cellular signal transduction. One way to elucidate the functions of protein kinases is to systematically identify their downstream targets. We present here a simple and effective method to identify direct protein kinase substrates in native cell lysates. First, we isolate the activity of the kinase of interest by engineering the normal kinase to utilize bulky ATP analogs that cannot be used by normal cellular kinases. This allows specific labeling of substrates with thiophosphate tags by performing kinase reactions in cell lysates that also include bulky ATP- γ -S analogs. After digesting the proteins in the reaction mixture, thiophosphopeptides are isolated using a single-step capture-and-release protocol and identified by mass spectrometry. This technique is easy to use and generally applicable.

Keywords

chemical genetics; analog-sensitive kinase; thiophosphorylation; phosphopeptides

INTRODUCTION

Identifying the physiological targets of protein kinases is critical to understanding their functions, and many approaches have been developed to systematically map kinase-substrate relationships. Notably, the chemical genetics approach developed by the Kevan Shokat laboratory is an attractive way to isolate the activities of any kinase of interest from the myriad of cellular kinases. This kinase engineering scheme modifies the conserved ATP-binding pocket of a kinase, enabling it to utilize unnatural ATP analogs (Liu et al., 1998; Shah et al., 1997). A key feature of this strategy is that only the engineered (or analog-sensitive) kinase, but not cellular kinases, can use the ATP analog (Fig. 1). These analog-sensitive kinases can then be used to specifically label their substrates in kinase reactions using cell lysates (Shah and Shokat, 2003). A variety of kinase substrate identification methods using this strategy have been described (Koch and Hauf, 2010), including several recent reports (Blethrow et al., 2008; Chi et al., 2008; Holt et al., 2009).

This unit describes an *in vitro* approach to identify the direct targets of a protein kinase in native cell lysates, and it provides updated protocols based on our recent study to identify

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human CDK2 substrates (Chi et al., 2008). The basic protocols include the two key steps of the method: 1) the specific labeling of the substrates in cell lysates, and 2) the subsequent identification of the labeled proteins. For the substrate labeling, a kinase reaction is carried out in the presence of analog-sensitive kinase and an ATP- γ -S analog (Fig. 2A). Thiophosphate labeling allows stable incorporation of thiophosphate onto substrate proteins and provides an easy chemical tag for isolating the thiophosphopeptides following digestion of the protein mixture. This protocol is similar to the standard kinase assays that use unmodified kinase and ATP, but specifically label substrates with thiophosphate. In the second step (Fig. 2B), the protein mixture in the reaction is digested with trypsin, and the resulting peptide mixture is incubated with a disulfide resin, which binds both cysteine-containing peptides and thiophosphopeptides. Washing and subsequent elution of the disulfide beads with sodium hydroxide selectively releases the thiophosphopeptides (as normal phosphopeptides via hydrolysis) while leaving the cysteine-containing peptides behind. Finally, the eluted peptides are identified by mass spectrometry (MS) and database searching. This technique is broadly applicable since it is based on a simple capture-and-release chemical enrichment procedure and only requires the widely available mass spectrometry instrumentation and basic computational tools. For users' comparison, an alternative method has also been described earlier in this series (Hertz et al., 2010).

STRATEGIC PLANNING

Design, characterization and production of analog-sensitive kinases

The first step toward applying the method is to design analog-sensitive alleles of the kinase of interest. Since the ATP binding sites in protein kinases are conserved, analog-sensitive alleles of a kinase can usually be constructed by replacing a bulky or hydrophobic residue (called "gatekeeper") in the ATP binding domain to a glycine or alanine. Descriptions of the procedures and tools have been published previously (Blethrow et al., 2004; Buzko and Shokat, 2002). Since ATP- γ -S analogs are a critical feature of this method, the authors advise that the wild-type kinase of interest be tested to see if it can utilize ATP- γ -S efficiently before the design of analog-sensitive alleles. Once candidate alleles of the analog-sensitive kinases have been constructed, they need to be characterized so that at least one allele meets the expected criteria. To do this, one needs to express and purify the analog-sensitive kinase and carry out kinase assays using ATP- γ -S analogs. These test kinase assays are generally done in parallel with wild-type kinase using either a known substrate (Chi et al., 2008) or cell extracts (Blethrow et al., 2008). These assays must demonstrate that the analog-sensitive kinase, but not the wild-type version, can use the ATP- γ -S analog efficiently. Detection of the thiophosphorylation by the analog-sensitive kinase in the presence of ATP- γ -S analog can be achieved by monitoring the electrophoretic mobility shift of a known substrate or by Western blot of the protein using a phosphosite-specific antibody (Chi et al., 2008), or by autoradiography using radiolabeled ATP- γ -S analog (Blethrow et al., 2008). Once the analog-sensitive kinase has shown both activity and specificity toward the ATP- γ -S analog, it is ready to be produced in sufficient quantity to be used in the lysate experiments. Expression and purification of active analog-sensitive kinases is a preference of the user and can be produced in bacteria or human cells (Chi et al., 2008) or baculovirus-infected insect cells (Blethrow et al., 2008).

Preparation of ATP- γ -S analog and cell lysates

During the characterization of the analog-sensitive kinase of interest, an appropriate ATP analog should be chosen. Two commonly used ATP analogs are the N⁶-substituted ATP analogs: N⁶-(Benzyl)-ATP and N⁶-(2-Phenylethyl)-ATP. Both the ATP and ATP- γ -S form of these ATP analogs can be chemically synthesized (Allen et al., 2007; Hertz et al., 2010),

purchased (e.g. BIOLOG Life Science Institute, Bremen, Germany), or custom synthesized (e.g. N⁶-(2-Phenylethyl)-ATP from TriLink BioTechnologies, San Diego, CA).

Kinase assays can be performed using whole cell lysates. However, the enormous complexity and dynamic range of the components within whole cell lysates limits the extent of the substrate labeling and protein identifications. It is therefore desirable to fractionate the whole cell lysates to reduce the sample complexity as well as to enrich the candidate substrates. Fractionation thus leads to more efficient and robust substrate labeling as well as more protein identifications in the mass spectrometer. The protein fractionation techniques and the extent of fractionation are flexible and depend on one's preference and instrument availability. Typically, it involves chromatography techniques, such as ion exchange (Chi et al., 2008), or precipitation techniques (Blethrow et al., 2008). As an example, a simple fractionation protocol for HEK293 cell lysate using ion exchange chromatography is provided in Support Protocol 1. Detailed descriptions on protein fractionation techniques can be found elsewhere in the literature, such as a protocol for ion exchange chromatography (Williams and Frasca, 2001).

Positive and negative controls

A positive control is desirable for monitoring the success of the protocols. Typically, a known and well-phosphorylated substrate for the kinase of interest is chosen. It is recommended that a known substrate, prior to cell lysates, is used in the kinase reaction and subsequent thiophosphopeptide purification scheme to see if these protocols can be followed successfully. An example using the GST-Rb (Glutathione S-transferase fused to the carboxyl-terminal 156 amino acids of human Retinoblastoma protein) as a standard substrate for CDK2 is provided in the Support Protocol 2. Once the method can be successfully carried out using the known substrate, this substrate can then be spiked into the kinase reactions containing cell lysates as positive control (See Basic Protocol).

Although the thiophosphate labeling is expected to be highly specific to the analog-sensitive kinase, background labeling can still occur due to the activities of cellular kinases that can use the ATP- γ -S analogs in a limited extent (Chi et al., 2008). To control for non-specific labeling, a negative control reaction without the analog-sensitive kinase should be carried out in parallel. The thiophosphopeptide identifications from this reaction can then be subtracted from the list of identifications from the reaction with the analog-sensitive kinase present.

BASIC PROTOCOL: SUBSTRATE LABELING AND PURIFICATION OF THE THIOPHOSPHOPEPTIDES

This section describes the substrate identification procedures summarized in Figure 2. Once necessary reagents described above are obtained, kinase reactions can then be carried out using the engineered kinase, ATP- γ -S analog, and cell lysates. Following tryptic digestion of the reaction mixture, the thiophosphopeptides are captured by a disulfide resin and then specifically eluted via base hydrolysis. Tandem mass spectrometry is used to identify the phosphopeptides and the substrate proteins.

Materials

- 0.6 ml and 1.7 ml Eppendorf tubes
- 1 M Tris-HCl buffer, pH 7.5
- 1 M MgCl₂
- 5 M NaCl

N^6 -(2-Phenylethyl)-ATP- γ -S (TriLink BioTechnologies)
Kinase of interest
Purified control substrate
Cell lysate (buffer composition similar to kinase assay condition)
Deionized water
5x kinase reaction buffer (see Reagents and Solutions)
Water bath
0.5 M EDTA, pH 8 (MediaTech)
Acetonitrile (VWR)
Trypsin, sequencing grade (Promega)
Thiopropyl Sepharose 6B (GE Healthcare)
Microcentrifuge
ColorpHast pH-indicator strips, pH 4.0–7.0 (Fisher Scientific)
Formic acid 99% (Fisher Scientific)
10% formic acid (store in glass bottle at room temperature indefinitely)
Mini microcentrifuge (VWR)
1ml disposable syringe (Becton Dickinson)
26G1/2 syringe needle (Becton Dickinson)
Labquake rotator (Fisher Scientific)
Iron stand and clamps (Fisher Scientific)
Dropper bulbs for Pasteur pipets, 3ml (Fisher Scientific)
Micro Bio-Spin chromatography columns, 0.8ml (Bio-rad Laboratories)
Washing solution 1 (see Reagents and Solutions)
Washing solution 2 (see Reagents and Solutions)
20 mM Sodium Hydroxide (store at room temperature up to 3 months)
1% formic acid (store in glass bottle at room temperature indefinitely)
Tandem mass spectrometer (ThermoFisher Scientific)
Database search and data filtering software

Label kinase substrates in a kinase reaction

- 1** Mix the following sequentially in a 1.7ml Eppendorf tube for a 200 μ l reaction:
 - a.** 5x kinase reaction buffer: 40 μ l
 - b.** Deionized water: volume needed to make up 200 μ l final reaction volume.
 - c.** cell lysate: 200–300 μ g
 - d.** control substrate: 100–200 ng

- e. N^6 -(2-Phenylethyl)-ATP- γ -S: 250 μ M final
- f. analog-sensitive kinase: 1% or less of the total protein by weight, or sufficient activity on beads.

Calculate the volume needed for each of the above reagents. If a larger reaction volume is needed, simply scale up all reagents proportionally. For reactions using a control substrate only, omit the cell lysate and reduce the reaction volume as needed (See Support Protocol 2). The analog-sensitive kinase can be added in the form of purified recombinant protein, eluted or bead-bound immunoprecipitate, or even small amount of crude cell lysate in which the analog-sensitive kinase is highly over-expressed. Ideally, the amount of kinase added should approximate the physiological amount of kinase activity in the cell lysate, but supraphysiologic kinase activity may be needed to label sufficient amounts of substrates to allow their identification. However, excessive amounts of kinase may label low affinity targets and therefore yield more false-positive identifications. Phosphatase inhibitors are not necessary for the kinase reaction because the thiophosphates are resistant to phosphatases. Avoid using reducing agents (such as Dithiothreitol, DTT) in the reaction as they may interfere with disulfide bead binding later. Low concentrations of non-ionic detergents can be included if necessary.

- 2 Incubate the kinase reaction at an appropriate temperature in a water bath for 1 hour.

Reaction temperature is typically 25 °C for yeast samples and 30 °C for mammalian samples. Reaction time can range from 30 min to several hours, but shorter incubation is preferred when possible to minimize non-specific labeling. Since kinases utilize ATP- γ -S at a much slower rate than ATP, it might be necessary to incubate reactions at longer time period to achieve sufficient labeling of proteins.

- 3 Stop the reaction by adding 0.5 M EDTA (pH 8) to a final concentration of 20 mM.
- 4 Proceed to the next step or store the reaction at -20 °C.

Digest the protein mixture

- 5 Add acetonitrile to the reaction at a final concentration of 10–15%.

Trypsin is resistant to mild denaturing conditions such as 10% of acetonitrile (Bond, 1989). Acetonitrile helps denature the lysate proteins and facilitates more complete proteolysis by trypsin. It also does not expand the sample volume appreciably. Other commonly used denaturants such as urea would require several fold dilution of sample volume before trypsin digestion. Binding to disulfide beads is a chemical reaction that is sensitive to volume expansion, and urea might also interfere with the disulfide bead binding. If urea is used as denaturant, samples should be desalted and concentrated after trypsin digestion. Avoid using reducing agents (such as DTT) at this step as they may interfere with the disulfide bead binding in the subsequent step.

- 6 Add trypsin to a final protease:protein ratio of 1:50-1:20 (w/w).
- 7 Incubate the tube at 37 °C for at least 3 hours. Allow it to go overnight if it is end of the day.

- 8 Proceed to the next step or store the sample at -20°C .

It's a common practice to treat the peptide samples with iodoacetamide to block the cysteines following the trypsin digest. This procedure should not be performed as iodoacetamide reacts readily with thiophosphates.

Bind to disulfide beads

- 9 Centrifuge the sample in a microcentrifuge at $10,000 \times g$ for 1 minute.
- This removes any precipitation that may occur during digestion. Typically, there may be minor precipitations if large amounts of protein or crude lysates are used.
- 10 Transfer the supernatant to a new Eppendorf tube.
- 11 Adjust the pH of the solution to pH ~5 using 10% formic acid. Add 1–2 μl at a time using a pipet and check the pH of the sample by spotting 1 μl onto a pH strip (pH 4.0–7.0).
- The working pH for robust binding is between 4 and 7.5, and lower pH may slightly favor thiophosphate binding. Prepare the 10% formic acid solution using 99% formic acid stock and deionized water. Handle the concentrated formic acid in a fume hood. Use glass peptides or Hamilton syringes to transfer formic acid and store the 10% formic acid stock in a glass bottle.
- 12 Prepare a 50% (v/v) slurry of disulfide beads (Thiopropyl Sepharose 6B) according to manufacture instructions.
- Bead may be prepared in advance by soaking and washing the dry beads in deionized water and storing the swollen beads at 4°C in 20% ethanol (long term) or deionized water (up to two weeks).
- 13 Mix disulfide beads stock and transfer 40 μl into a 600 μl Eppendorf tube using a pipet tip. Cut the end of the pipet tip if necessary to allow smooth transfer of the beads.
- The amount of beads used depends on the amount of sulfhydryl ($-\text{SH}$) content in the reaction mixture and should have enough capacity to theoretically capture all sulfhydryl containing molecules. Using excess beads may encourage binding of non-specific molecules to the disulfide beads.
- 14 Centrifuge the tube in a mini microcentrifuge for a few seconds to settle the beads. Remove the excess liquid using a 1 ml disposable syringe attached to a 26G1/2 needle.
- 15 Add the peptide sample to the beads. Mix by inverting the tube several times.
- The beads solution should flow smoothly upon inverting. If not, add additional 10–20% acetonitrile to help reduce the viscosity.
- 16 Incubate the tube on a rotator with continuous rotation at room temperature overnight.
- The minimal incubation time depends on the sample complexity and volume. A few hours might be sufficient if timing is important.

Wash and elute the disulfide beads

- 17 Fix a 0.8 ml Micro Bio-Spin chromatography column onto an iron stand.

- 18 Centrifuge the tube containing the bead sample for 10 seconds in a mini microcentrifuge. Remove most of the supernatant containing unbound peptides.
- 19 Resuspend the beads with 200 μ l of water and load the beads mixture onto the column using a blunt-ended P200 pipet tip.
- 20 Use a rubber dropper bulb to apply air pressure to from the top of the column to squeeze out the liquid phase.
- 21 Wash the beads sequentially with 2×0.5 ml of water, 3×0.5 ml of washing solution 1, 2×0.5 ml of water, 3×0.5 ml of washing solution 2, and finally 2×0.5 ml water. At each washing step, mix the beads by pipeting up and down a few times using a blunt-ended P200 pipet tip and squeeze out the liquid phase using the rubber bulb.
- 22 Collect all beads by resuspending them in 100 μ l of water and pipeting them out of the column into a 1.7 ml Eppendorf tube. Do this several times to insure complete transfer of the beads.
- 23 Let the beads settle by standing for a few minutes or spin them down by a brief centrifugation in a mini microcentrifuge. Carefully remove most of the liquid phase with a pipet. Remove the last bit of liquid using a 1 ml syringe.
- 24 Add 20–30 μ l 20 mM NaOH. Mix and incubate on a rotator at room temperature for 30 minutes.
- The beads should be in suspension and viscosity should keep the solution from flowing around tube. The time of incubation is typically 30 minutes to 1 hour. Although the elution agent volume is flexible, a minimum of one bead bed volume is typically used. Use low elution volume if more concentrated peptide samples are desired.
- 25 Spin down the beads by a brief centrifugation in a mini microcentrifuge. Carefully collect the supernatant with a pipet tip without disturbing the beads.
- 26 (Optional) Rinse the beads by mixing it with 5 μ l of water. Spin down the beads and transfer the supernatant to the eluate above.
- 27 Acidify the eluate by adding 4–5 μ l of 1% formic acid.
- The pH should be at 3 or lower so that the sample is compatible with MS analysis. A desalting step could be included here to further concentrate or purify the peptide sample before the MS analysis, but it is not necessary.
- 28 Proceed to the next step or store the sample at -20°C .

Analyze the peptide samples by mass spectrometry

- 29 Centrifuge the sample for at $10,000 \times g$ for 1 minute in a microcentrifuge.
- This step removes any particulate material in the sample that may interfere with the HPLC system or MS instrument.
- 30 Transfer the necessary volume of the supernatant to a new vial for loading.
- The loading volume depends on user preference and the maximum loading volume allowed in the MS instrument setup.
- 31 Run the peptide sample using a tandem mass spectrometer.
- The choice of MS instruments depends on the user and their availability. Typically, a mass spectrometer that can perform tandem mass spectrometry*

(MS/MS) is required. An ion trap instrument, such the Thermo Scientific LCQ, LTQ, or LTQ Orbitrap, is suitable. Advanced instrumentation is preferred if available. As an example, the operation on the Thermo Finnigan LCQ DECA XP has been described (Yi et al., 2003).

- 32** Search the MS/MS spectra against the appropriate protein databases using a suitable search algorithm. Differential mass modification of the serine, threonine, or tyrosine residues by the phosphate group should be specified in the search parameters.

Various protein sequence databases, such as latest version of International Protein Index (IPI), and search tools, such as SEQUEST and Mascot, can be used. An overview of the current database search algorithms has been described (Kapp and Schutz, 2007).

- 33** Perform statistical analysis on the search results.

Probability-based statistical analysis software should be used to help filter the search results, especially on large datasets. Typically, peptides with high probability score (0.9 or higher) and low false discovery rate (0.1% or lower) are reported. One of these types of software tools has been described (Deutsch et al., 2010). If possible, all MS/MS spectra for the identified phosphopeptides should also be validated by manual inspection.

SUPPORT PROTOCOL 1: FRACTIONATION OF HEK293 CELL LYSATE

Because many kinase targets are low abundance proteins, it is necessary to enrich these proteins in order to obtain sufficient material for identification. One way to achieve this is to fractionate the whole cell lysate to reduce the complexity of the cell lysate proteins and to enrich the candidate substrates. The following protocol describes a method for HEK293 lysate preparation and subsequent fractionation using low-pressure ion exchange chromatography and ammonium sulfate precipitation.

Materials

Necessary materials from above with the following additions:

- HEK293 cells
- 15 cm cell culture dishes (Becton Dickinson)
- Phosphate buffered saline (PBS) for tissue culture
- Trypsin for tissue culture
- 15-ml and 50-ml Facon tubes (Fisher Scientific)
- Tabletop centrifuge with swinging bucket rotor
- Hypotonic lysis buffer (see Reagents and Solutions)
- DTT (Fisher Scientific)
- Triton X-100 (Sigma-Aldrich)
- Benzonase (Novagen)
- Protease inhibitors cocktail (Sigma-Aldrich)
- Branson Sonifier 250 (Branson Ultrasonics)
- Bio-rad protein assay dye reagent (Bio-rad Laboratories)

SP Sepharose Fast Flow resin (GE Healthcare)
Q Sepharose Fast Flow resin (GE Healthcare)
1.0 × 10 cm, glass chromatography columns (Bio-rad Laboratories)
Column loading buffer (see Reagents and Solutions)
Accumet AB30 conductivity meter (Fisher Scientific)
Ammonium Sulfate (Fisher Scientific)
Vortexer (VWR Scientific)
Amicon Ultra-4 Centrifugal Filter, 10,000 MWCO (Millipore)
SnakeSkin pleated dialysis tubing, 7,000 MWCO (Pierce)
Dialysis buffer (see Reagents and Solutions)

Prepare HEK293 whole cell lysate

1. Grow HEK293 cells on 4 × 15-cm plates to 80–90% confluence.
Typically, 4 to 8 15-cm plates yield enough proteins for a small scale fractionation. The starting material and the chromatography column sizes can be scaled up proportionally.
2. Wash cells with 10 ml PBS. Trypsinize cells and harvest them in a 15-ml Falcon tube by centrifugation in a tabletop centrifuge with swinging bucket rotor at 250 × g for 5 minutes. Wash cells once with 10 ml PBS and harvest them by centrifugation. Remove supernatant by aspiration.
Process the cell pellet immediately or quick freeze it in liquid nitrogen and store at −80 °C.
3. Resuspend the cell pellet in 4 ml hypotonic lysis buffer. Incubate tube at 4°C for 30 minutes.
4. Add NaCl to 150 mM and sonicate the sample using a Branson Sonifier 250. Sonicate twice for 30 seconds at 30% duty cycle and output setting “3”. Incubate sample on ice for 2 minutes between each sonication.
5. Transfer the sample to microcentrifuge tubes and pellet the cell debris by centrifugation at 20,000 × g for 10 minutes at 4 °C.
6. Collect the supernatant and store the whole cell lysate on ice. Measure the protein concentration by Bio-rad protein assay.
This protocol typically yields 20–25 mg of total protein.

Fractionate HEK293 whole cell lysate

1. Prepare a 2-ml SP Sepharose column and a 2-ml Q Sepharose column by packing each of the resins into a 1.0 × 10 cm glass column fixed on an iron stand. Equilibrate the columns by gravity flow using at least 10 ml of cold column loading buffer.
Carry out the entire fractionation procedures in a cold room. Alternatively, set up the columns at room temperature but keep all buffers and reagents on ice throughout the fractionation procedures. Instead of gravity flow, a peristaltic pump can be used to better control the flow rate.

2. Dilute the whole cell lysate ~6 fold in a 50-ml Falcon tube such that the final salt concentration is equivalent to the loading buffer. Confirm by measuring its conductivity using a conductivity meter.
3. Load the diluted whole cell lysate onto the SP column by gravity flow using a pipet. Collect the flowthrough using a 50-ml Falcon tube.
4. Wash the column with 5 x column volumes of loading buffer.
5. Elute the column sequentially with 3 x column volumes of loading buffer containing 100 mM, 200 mM, 300 mM, 400 mM, and 600 mM NaCl. Collect the eluate at each salt step.
6. Load the SP flowthrough onto the Q Sepharose column and collect the flowthrough.
7. Repeat Steps 4 & 5.
8. To the Q Sepharose flowthrough, add ammonium sulfate powder gradually to 60% (~360 mg/ml). Keep the solution on ice and mix constantly by vortexing the tube.
9. Pellet the proteins by centrifugation at $16,000 \times g$ at 4°C for 10 minutes. Remove the supernatant and resuspend the pellet in 0.5 ml of column loading buffer.
10. Concentrate all 10 column eluates by centrifugation (at $4000 \times g$ maximum) in a tabletop centrifuge with swinging bucket rotor using the Amicon Ultra-4 centrifugal filter tubes.

Concentrate until the sample volume is between 0.2–1 ml or the protein concentration is at least 2 mg/ml.
11. Dialyze all lysate fractions against 1 L of dialysis buffer for at least 3 hours at 4°C using dialysis tubing. Collect the fractions, quick freeze them in liquid nitrogen, and store at -80°C .

Avoid using lysate volumes higher than 1/5 of the total reaction volume in a kinase assay as high glycerol concentrations may inhibit the kinase reaction. If the protein concentration of a lysate fraction is low, increase the total reaction volume to compensate for higher volume of the lysate.

SUPPORT PROTOCOL 2: APPLYING THE METHOD TO A CONTROL SUBSTRATE

A simple kinase reaction using purified kinase and a known substrate is a quick way to test the method. Initial thiophosphate labeling can be achieved using wild-type kinase and ATP- γ -S, especially if the kinase is easy to produce or available commercially. The same assay can then be repeated with purified analog-sensitive kinase and an ATP- γ -S analog. The following protocol demonstrates the feasibility of the method using commercially available Cyclin A-CDK2, ATP- γ -S, and purified GST-Rb. The results can be monitored quickly by mass fingerprint using Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) analysis (Henzel and Stults, 2001). The protocol is similar to the Basic Protocol with the following modifications. For more details, refer to the Basic Protocol.

Materials

Necessary materials in above protocols with the following additions:

Cyclin A-CDK2 (New England Biolabs)

ATP- γ -S (EMD Biosciences)

GST-Rb (Expressed in *E. coli* and purified)

0.6 μ l C18 ZipTips (Millipore)

0.1% formic acid (store in glass bottle at room temperature indefinitely)

0.1% formic acid/50% acetonitrile (store in glass bottle at room temperature indefinitely)

α -cyano-4-hydroxycinnamic acid (Agilent Technologies)

4700 Proteomics Analyzer (Applied Biosystems)

1. Set up a 60 μ l reaction containing 2 μ g GST-Rb, 250 μ M ATP- γ -S, and 200 ng Cyclin A-CDK2. Incubate in 30 °C water bath for 1 hour.
2. Add 2 μ l 0.5 mM EDTA, 9 μ l acetonitrile, and then 0.5 μ g (1 μ l) trypsin. Incubate at 37 °C overnight.
3. Remove and save 1/3 (23 μ l) of the total volume as sample "A".
4. Acidify the rest sample (~47 μ l) with 1 μ l 1% formic acid (to pH ~5). Mix with 10 μ l of settled disulfide beads in a 1.7 ml Eppendorf tube. Incubate on a rotator at room temperature for 4 hour. Beads should be in suspension during the incubation. Tap the tube occasionally as needed.
5. Wash the beads and then collect them in a 1.7 ml Eppendorf tube.
6. Elute the beads by mixing with 20 μ l of 20 mM NaOH (similar to Step 4) at room temperature for 30 minutes.
7. Collect the eluate (sample "B") and acidify it with 1% formic acid (~3 μ l) to pH 3 or lower. Also, acidify sample A with 1% formic acid and then dilute the volume two-fold using 0.1% formic acid.

Samples are acidified for the C18 desalting step below. Sample A needs to be diluted to reduce the concentration of the acetonitrile present.

8. Attach a 0.6 μ l C18 ZipTip to a 20 μ l pipet and desalt samples A and B following the manufacturer's instructions. Briefly,
 - a. Wet the ZipTip by pipeting up and down several times in 100% acetonitrile.
 - b. Equilibrate the ZipTip by pipeting up and down several times in 0.1% formic acid.
 - c. Pass the ZipTip through the samples by pipeting up and down 10 times.
 - d. Wash the ZipTip by pipeting up and down several times in 0.1% formic acid.
 - e. Elute the peptides by pipeting up and down several times in 3 μ l of 0.1% formic acid/50% acetonitrile.

Passing of sample A can be achieved by pipeting the sample twice into another Eppendorf tube and then transferring back and forth between the two tubes.

9. Spot 0.5 μ l of each sample (mixed with the matrix α -cyano-4-hydroxycinnamic acid) onto a MALDI plate and perform scans in MS mode using 4700 Proteomics Analyzer. Typically, apply 1000 laser shots using the appropriate laser intensity.

10. Compare the MS spectra of sample A and sample B. Identify enrichment of phosphopeptides of GST-Rb by the correct m/z values (Fig. 3).

Phosphopeptide signal intensities depend on the degree of phosphorylation, purification yield, and their ionization efficiencies in the mass spectrometer.

REAGENTS AND SOLUTIONS

Use Milli-Q purified water or equivalent in all recipes and protocol steps.

Kinase reaction buffer, 5 x

200 mM Tris-HCl, pH 7.5

50 mM MgCl₂

50 mM NaCl

Store room temperature indefinitely

Washing solution 1

0.1% Formic acid

50% Acetonitrile

Store room temperature up to 6 months

Washing solution 2

2M NaCl in water

Store room temperature indefinitely

Hypotonic lysis buffer

50 mM Tris-HCl, pH 7.5

1 mM DTT

1 mM MgCl₂

0.1% Triton X-100

25 U/ml Benzonase

protease inhibitors cocktail

Make fresh in cold water and use one time

Column loading buffer

30 mM Tris-HCl, pH 7.5

25 mM NaCl

1 mM DTT

Make fresh in cold water and use one time

Dialysis buffer

10% glycerol

30 mM Tris-HCl, pH7.5

100 mM NaCl

Make fresh in cold water and use one time

COMMENTARY

Background Information

Protein phosphorylation is perhaps the most common signaling event in the cell and plays important roles in virtually all cellular process. Protein kinases mediate cellular signal transduction events through the phosphorylation of their downstream targets. A major challenge when studying the function of kinases has been the identification of their direct targets. The large number of cellular kinases and their substrates, as well as the lack of suitable methods, have made it difficult to assign a particular protein phosphorylation to a specific kinase. Traditional and newer mass spectrometric approaches have generated ever-increasing lists of phosphorylation identifications. However, the direct kinase substrates of individual kinases remain elusive in most cases, and various strategies of substrate identification were applied with limited success.

The chemical genetic approach using analog-sensitive kinases and ATP analogs isolates the activities of a kinase of interest and allows specific and direct labeling of their targets in cell extracts. Earlier substrate identification methods using this strategy had limited number of substrate identifications or depended on tagged substrate libraries (Koch and Hauf, 2010). The method described here labels direct kinase targets with thiophosphate tags, which allow specific capture of thiophosphopeptides (as well as cysteine-containing peptides) via a sulfhydryl (-SH) reactive resin following the digestion of the labeled proteins. Elution of resin with a base specifically releases the thiophosphopeptides while leaving behind the vast majority of cysteine-containing peptides. Although cysteine-containing thiophosphopeptides are lost with this method, a large majority of the labeled peptides are recoverable. Identifying the labeled peptides directly allows more identifications to be achieved, and the sites of phosphorylation can also be mapped, providing further confidence for the identified substrates.

A similar approach has been described (Blethrow et al., 2008) and detailed protocols have been provided (Hertz et al., 2010). Users have the options to try both methods and choose the one that suits them. It is noteworthy that the method described here is based on *in vitro* reaction and therefore are subject to false-positive identifications due to various reasons, such as the lack of normal cellular context and the use of excessive amount of kinases. Nevertheless, this direct approach can identify new physiological kinase targets, and it complements the current indirect strategies (Koch and Hauf, 2010).

Critical Parameters

Effective thiophosphate labeling is an important first step towards successful substrate identification. Thus the specificity and activity of the analog-sensitive kinase toward an ATP- γ -S analog should be optimized using a known substrate protein before moving to lysate-based identifications. The ability of a kinase to utilize ATP- γ -S can sometimes be enhanced by a different divalent cation cofactor (Parker et al., 2005). Small amount of control substrate “spike-in” should be included in kinase reactions using cell lysates to monitor the sensitivity and success of the procedures. Kinase reaction efficiency can be affected by the amount of kinase, cell lysate, or ATP- γ -S analog added and the reaction time. Users can start with a reaction using more kinase, less cell lysate, and longer reaction time and then optimize the reaction conditions using less kinase and shorter incubation without compromising the results significantly.

Although small amounts of salt and non-ionic detergent are common in kinase reactions and harmless to these methods, reducing agents or sulfhydryl compounds (such as DTT), should be minimized as they will react with the disulfide resin and reduce its capacity in the subsequent step. Large amounts of reducing agent (1mM or higher) may interfere the binding of thiophosphopeptides to the resin. Reducing agents used in cell lysate preparations should be significantly diluted or removed (e.g. by dialysis) prior to kinase reaction. Similarly, sulfhydryl reacting compounds (such as iodoacetamide) should be avoided throughout the procedure as they will likely react with thiophosphopeptides.

Efficient binding of thiophosphopeptides to disulfide beads is another critical step. Since the bead binding involves covalent attachments via a chemical reaction, the ratio of sample volume to beads volume is important. It is desirable to keep the peptide sample volume as small as possible to allow efficient chemical reaction. Beads should be constantly mixed with the peptide sample and the incubation time is typically overnight for complex samples. The amount of beads used is based on the sample complexity and can be adjusted accordingly so that the bead capacity is always severalfold in excess of the total sulfhydryl content of the kinase reaction. Excess bead capacity may also increase the non-specific binding and lead to higher background in the mass spectrometry analysis.

Troubleshooting

Table 1 describes some of the potential problems that may occur during the procedure.

Anticipated results

Generally, when the method is applied to simple reactions, such as the control kinase reaction described in Support Protocol 1, it is expected to be more efficient than reactions using complex cell lysates. For a known substrate, multiple phosphopeptides (if possible) should be readily recovered and easily detectable by MALDI or electrospray mass spectrometers. For complex reactions in lysates, the phosphopeptide yield may be lower due to lower labeling efficiency, more competitive binding to the disulfide beads, and lower elution efficiency. Also, the sensitivity of method may be lower due to higher background in the mass spectrometry analysis. When a control substrate is included in the lysate reactions, recovery of phosphopeptide(s) from the control substrate is expected. The more distinct peptides recovered from the control substrate, the better the overall reaction efficiency and yield of the phosphopeptides. Failure to recover any control phosphopeptides indicates problems during the procedure.

Time Considerations

The most time-consuming parts are the preparatory procedures described in the Strategic Planning. Once all necessary reagents are ready, and the activity and specificity of the engineered kinase has been optimized, an experiment using the standard substrate can be completed within 2 days, and an experiment involving multiple lysate reaction samples can also be processed in 2–3 days.

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Figure 1. **Kinase** engineering strategy. Mutation of a conserved gate-keeper residue in the ATP binding domain of a kinase allows the binding of ATP analogs. Although most analog-sensitive kinases can still use ATP, only they, but not the wild-type kinases can use the ATP analogs.

Figure 2.

General scheme of the substrate identification method. **(A)** Labeling of candidate substrates via kinase reactions using cell lysate, analog-sensitive kinase and ATP- γ -S analog. **(B)** Identification of the labeled substrates. Proteins in the kinase reaction are digested with trypsin. The resulting peptides are incubated with disulfide beads, which capture both thiophosphopeptides and cysteine-containing peptides. The beads are then treated with basic solution to selectively release only the thiophosphopeptides, which are converted to normal phosphopeptides via hydrolysis. The peptide sample is subjected to liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) to yield the identification of the candidate substrates.

Figure 3.

MALDI-TOF analysis of purified phosphopeptides from thiophosphorylated GST-Rb. MALDI MS spectra with mass (m/z) shown on x-axis and % intensity shown on y-axis. **(A)** Peptide sample before thiophosphopeptide isolation. All expected Rb thiophosphopeptides are near background signal intensity. **(B)** After thiophosphopeptide isolation, three expected Rb phosphopeptides are enriched and clearly detectable: singly phosphorylated ISEGLPTPTK, $m/z = 1122.46$; singly phosphorylated SPYKFPSSPLR, $m/z = 1358.56$; and doubly phosphorylated ISEGLPTPTKMTPR, $m/z = 1687.64$.

Table 1

Troubleshooting Guide

Problem	Possible Cause	Solution
No thiophosphate labeling of a known substrate using ATP- γ -S	Not enough active kinase, or kinase uses ATP- γ -S poorly.	Use more kinase. Increase the kinase reaction time. Try adding other divalent cations, such as Mn^{2+} .
No phosphopeptide identifications after the purification procedure on a known substrate.	Insufficient labeling	See above.
	Inefficient bead binding	Try smaller sample volume and longer incubation time (e.g. overnight).
	Expected tryptic phosphopeptides are too large or small	Try a different substrate or protease.
No thiophosphate labeling using cell lysates and ATP- γ -S analog.	Not enough active kinase, or kinase uses ATP- γ -S analog poorly.	See above
	Insufficient ATP- γ -S analog	Increase ATP- γ -S analog concentration (up to 0.5 mM)
	Inhibitory effect by the cell lysate.	Use less cell lysate. Use fractionated cell lysates.
No phosphopeptide identifications after the purification procedure on a lysate reaction.	Insufficient labeling	Include a known substrate as control. Add more kinase and increase reaction time.
	Insufficient digestion	Use more trypsin. Digest for longer time. Use denaturant during digestion followed by desalting of the peptide sample.
	Inefficient bead binding	Reduce sample volume and increase incubation time with beads. Avoid high concentration of reducing agents and denaturants (e.g. urea). Desalt the peptide sample prior to bead binding.
	Low stoichiometry of substrates	Enrich the concentration of substrates by fractionating the cell lysates or cell compartments.
Phosphopeptides identified in "no-kinase" control	Background labeling due to other kinases using the ATP- γ -S analog	Subtract out these peptide identifications from the list of "+ kinase" reaction.
		Include background kinase specific inhibitors in the reaction (e.g. casein kinase II inhibitors).
High background signals in mass spectrometry analysis	Small molecules from the ATP- γ -S analog reagent that co-purify with phosphopeptides	Use purer ATP- γ -S analog. Desalt the final peptide sample using mixed cation exchange (MCX) prior to mass spectrometry.