Published in final edited form as:

Curr Protoc Chem Biol. 2012 ; 4(1): 83–100. doi:10.1002/9780470559277.ch110228.

Kinase-Catalyzed Biotinylation

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Abstract

Kinase-catalyzed protein phosphorylation plays an essential role in a variety of biological processes. Methods to detect phosphoproteins and phosphopeptides in cellular mixtures will aid in cell biological and signaling research. Our laboratory recently discovered the utility of γ -modified ATP analogues as tools for studying phosphorylation. Specifically, ATP-biotin can be used for labeling and visualizing phosphoproteins from cell lysates. Because the biotin tag is suitable for protein detection, the biotinylation reaction can be applied to multiple phosphoproteomics applications. Herein we report a general protocol for labeling phosphopeptides and phosphoproteins in biological samples using kinase-catalyzed biotinylation.

Keywords

Kinase; ATP-biotin; Biotin; Phosphoprotein; Protein labeling

Introduction

Protein phosphorylation is an important post-translational modification that occurs in cells (Hunter, 1995). Because of the role played by phosphorylation in a variety of biological process, including cell signaling, diseases, cancer and immunosuppression (Cohen, 1999; Cohen, 2002), understanding the phosphorylation process is important. Protein kinases are the enzymes responsible for protein phosphorylation (Hunter, 1995). In phosphorylation, ATP acts as a universal phosphate donor while the kinase assists in transfer of the phosphate group from the γ position of ATP (Adams, 2001). In mammalian systems, phosphorylation occurs on the hydroxyl group of serine, threonine and tyrosine. Because the neutral hydroxyl group is replaced with a negatively charged phosphate group, the activity of the protein may change (Johnson and Barford, 1993).

While a variety of methods are available to visualize phosphoproteins in complex mixtures (see Commentary), each method has its strengths and weaknesses, making the development of alternate strategies of interest. To provide an alternative, the Pflum lab has reported use of γ -modified ATP analogues as tools for studying phosphorylation (Green and Pflum, 2007; Green and Pflum, 2009; Suwal and Pflum, 2010). In particular, we demonstrated that an ATP analogue with biotin attached to the γ -phosphate (ATP-biotin, Figure 1A) acts as a cosubstrate for kinases and transfers biotin along with the phosphate group onto peptides

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and proteins (Figure 1B) (Green and Pflum, 2007). The biotinylation reaction was successful with synthetic peptides and full-length protein substrates. Importantly, kinase-catalyzed biotinylation was successfully used to label cell lysates. The availability of a facile means of biotin-labeling phosphoproteins and phosphopeptides in complex mixtures using kinases as the catalyst represents a useful method to detect phosphoproteins.

Herein we present detailed protocols to use kinase-catalyzed biotinylation to identify phosphorylated proteins. First, a protocol to test kinase-catalyzed biotinylation with recombinant kinases and peptide substrates is presented using quantitative mass spectrometry (MS). Next, we describe a protocol for biotinylation of full-length proteins for their subsequent visualization and identification using a gel analysis. Finally, the kinase-catalyzed biotinylation reaction with cellular lysates is described, which is compatible with phosphoproteomics applications.

Basic Protocol 1 Kinase-catalyzed biotinylation with peptide substrates

To initially validate the ATP-biotin cosubstrate tolerance of any recombinant kinase of interest, we provide here a protocol for kinase-catalyzed biotinylation of a peptide substrate. Establishing that a kinase of interest is compatible with ATP-biotin is necessary before visualizing phosphoproteins in a complex cellular mixture (Basic Protocol 3). The experimentalist has a choice between testing ATP-biotin compatibility using a short peptide substrate (this protocol), or a full-length protein substrate (Basic Protocol 2). Depending on the availability of known substrates, one or both of these two protocols can be utilized.

A critical aspect of protocol 1 is selection of an appropriate peptide substrate. The peptide must contain a consensus sequence that can be recognized by the kinase of interest (Adams, 2001). Phosphorylation occurs when the consensus sequence of the substrate is recognized by the appropriate protein kinase, which directs phosphorylation of the serine, threonine, or tyrosine within the consensus sequence. Generally, the amino acids immediately surrounding the phosphorylated residue are critical for kinase recognition. Consensus sequences for many kinases are known and indicate that specific kinases can recognize basic, acidic, hydrophobic or proline residues to direct phosphorylation. For example, Casein Kinase II (CK2) is a serine/threonine kinase with an acidic consensus sequence of S/T-X-X-D/E (X represents any amino acid) (Meggio et al., 1994). Abl protein kinase is a tyrosine kinase with a hydrophobic consensus sequence of I/V/L-Y-X-X-P/F (Wu et al., 2002). If the consensus sequence for the kinase of interest is known, a peptide containing that sequence can be designed and synthesized for Protocol 1. If a full length protein substrate has been identified, a short peptide containing at least four amino acids both N- and C-terminal to the phosphorylation site can be used. As an alternative, if the protein substrate is available, establishing ATP-biotin compatibility with a full-length protein substrate would also be appropriate, as described in Basic Protocol 2.

Once the peptide substrate is generated, the next step is to perform the kinase catalyzed biotinylation reaction. Commercially available ATP-biotin is first incubated with the peptide substrate and the corresponding kinase enzyme of interest. The optimal buffer conditions for the kinase should be used for the reaction. A control reaction where the peptide is incubated

with ATP instead of ATP-biotin is necessary for the analysis. However, other control reactions where the kinase activity or ATP cosubstrate is omitted, or the biotinylation reaction includes competing ATP are recommended. We generally allow the kinase reaction to incubate for 2 hours, although the time of reaction can vary depending on the requirements of the kinase.

To determine the success of the biotinylation reaction, a quantitative mass spectrometric (MS) analysis method is performed (Figure 2). This MS-based analysis is inspired from a similar strategy used by the Aebersold lab (Tao et al., 2005). The quantitative MS experiment directly compares the efficiency of the kinase reaction with ATP-biotin to the natural kinase reaction with ATP. The requirement of a quantitative MS analysis is that the peptide products of the two reactions must be chemically identical (to ensure equivalent ionization in the MS instrument), but must be differentiated by mass to allow differentiation. To achieve chemically identical peptides differing only by mass, we use stable isotope labeling. The label is attached via a chemical esterification reaction of all carboxylic groups (the C-terminus, aspartic acid, and glutamic acid residues). Each peptide product from the kinase reactions is reacted with acidic methanol (typically, anhydrous hydrochloric acid in methanol generated with methanol in acyl chloride) to facilitate methyl esterification. To install different isotopes, either regular or deuterated methanol is employed. For the “control” ATP reaction, deuterated methanol (CD_3OD) is used to create an esterified product containing a CD_3 group (Figure 2, compound **10**). For the ATP-biotin reaction, regular methanol (CH_3OH) is used to create an esterified product containing a CH_3 group (Figure 2, compound **8**). While the ATP-biotin reaction creates a phosphobiotin peptide product (Figure 32, compound **6**), the acidic conditions of esterification result in cleavage of the phosphoramidate bond connecting the biotin to the phosphate. Therefore, in addition to isotopically labeling the peptide for the quantitative MS analysis, the esterification reaction also removes the biotin group to create a phosphopeptide product (Figure 2, compound **8**), which is chemically identical to the phosphopeptide product from the ATP reaction (Figure 2, compound **10**).

Once the two isotopically differentiated phosphopeptide products are formed via esterification, equal amounts of the two reactions are carefully mixed and analyzed using a MALDI-TOF MS instrument. The spectrum generated from the mixture of phosphopeptides contains two peaks—one containing a CD_3 ester corresponding to the ATP reaction product and the other containing CH_3 ester from the ATP-biotin reaction product (for examples, see Figure 3). Because these two mass peaks were generated from ionization of chemically identical species, their relative abundance can be directly compared. The relative conversion percentages are calculated by comparing the areas under the peaks (the shaded peaks in Figure 3), assuming reactions with ATP to be 100% complete. These initial experiments should be used to quantitatively test the cosubstrates promiscuity of a kinase.

This protocol is written generally to allow testing of any kinase and substrate. However, specific information for the biotinylation reaction with commercially available PKA and CK2 kinases is also included. A reasonable starting point for these experiments is to establish kinase-catalyzed biotinylation with either PKA or CK2 as a model system before moving on to using a new kinase and substrate.

Materials

Adenosine 5'-triphosphate (MP Biomedicals Inc, cat. no. ICN15026605: create a 10 mM stock solution in either water or Tris buffer (see recipe).

ATP-biotin (Adenosine 5'-triphosphate [γ]-biotinyl-3, 6, 9-trioxaundecanediamine, Affinity Labeling Technologies, cat. no. 864538-90-9, 10 mM stored in methanol)

Kinase enzyme stock solution: typically the stock solution is approximately 100–2500 U/ μ L, which will be diluted to a final concentration of 4–20 U/ μ L in the reaction, depending on the requirement of the given kinase. To use PKA (New England Biolabs, cat. No. P6000L), a 200 U/ μ L stock solution was prepared using deionized, purified water or kinase buffer. To use CK2 (New England Biolabs, cat. No. P6010L), a 50 U/ μ L stock solution was prepared using deionized, purified water or kinase buffer.

Peptide substrate stock solution: typically the stock solution is 200 μ M, which will be diluted to a final concentration of 20 μ M in the reaction. For the PKA peptide substrate (LRRASLG, kemptide, Promega, cat. No. V5601), we created a 200 μ M stock solution using deionized, purified water or kinase buffer. For the CK2 peptide substrate (RRREEETEEE, Promega, cat. No. V5661), we generated a 200 μ M stock solution using deionized, purified water or kinase buffer.

Kinase reaction buffer: If using a commercially available recombinant kinase, the buffer is provided by the manufacturer. If using an expressed kinase, the optimal buffer should be determined. For PKA and CK2, the kinase buffer supplied by the manufacturer is provided in the Reagents and Solutions section

Acetyl chloride: Acros Organics, cat. No. 75-36-5

d₀-methanol: Acros Organic, Cat. No. 67-56-1

d₄-methanol: Cambridge Isotope Labs, cat. No. 811-98-3

Matrix solution (see recipe)

MALDI plate: Standard 384 MTP, Bruker

ThermoSavant Speedvac: Model number SPD131DDA

Kinase-catalyzed biotinylation of a peptide substrate

- 1 To setup the ATP-biotin reaction, the methanol storage solvent must be evaporated first. Dispense 1 μ L of the ATP-biotin stock solution into an eppendorf tube (to create a final concentration of 1 mM). Then, evaporate the methanol storage solvent from ATP-biotin using ThermoSavant Speedvac.

If a Speedvac concentrator is not available, evaporation can be achieved by blowing air or nitrogen gas over the sample for a few minutes until no methanol solvent is present.

- 2 To the tube containing dried ATP-biotin, add 1 μ L of the corresponding peptide substrate (which should give a final concentration of 20 μ M). Add 1 μ L of the

manufacturer's kinase buffer (using a 10X stock supplied by the manufacturer). Dilute the mixture to 9 μL using 7 μL of deionized, purified water.

- 3 To setup the ATP reaction for quantitative comparison, in a separate eppendorf tube combine 1 μL of the ATP stock solution (to give a 1 mM final concentration), 1 μL of the corresponding peptide substrate (which will give a final concentration of 20 μM), and 1 μL of the manufacturer's specified kinase buffer (using a 10X stock supplied by the manufacturer). Dilute the mixture to 9 μL using 6 μL of deionized, purified water.

While this ATP reaction constitutes a positive control reaction, inclusion of negative controls of the reaction is also strongly recommended. Relevant negative controls reactions include 1) omission of ATP-biotin, 2) omission of kinase, 3) inclusion of heat-denature kinase, 4) inclusion of ten-fold excess (10mM) ATP as a competitor.

- 4 Initiate the reactions in both tubes by adding 1 μL of the kinase enzyme stock solution to each tube.

While the volume of the kinase added should be maintained at 1 μL (to give a final 10 μL reaction volume), the concentration of the kinase stock solution and the final concentration of kinase used in the reaction can vary. Reactions containing between 4–20 U/ μL is a reasonable starting point.

- 5 Incubate the reaction mixtures at 30 °C for 2 hours.

Shaking the reaction is optional. If a shaking incubator is available, shake the reactions a 1000 rpm.

Isotopic labeling via methyl esterification

- 6 To the ATP-biotin reaction, add a solution of 2N acetyl chloride in d_0 -methanol (CH_3OH). To the ATP control reaction, add a solution of 2N acetyl chloride in d_4 -methanol (CD_3OD).

The addition of acetyl chloride should be done inside a fume hood. The acyl chloride/methanol solution can be pre-made by combining 50 μL of acyl chloride with 300 μL of methanol. Alternatively, a trick that works well is to add 300 μL of methanol into the eppendorf tube and 50 μL of acetyl chloride into the cap of the eppendorf tube. Then quickly close the cap to combine the reaction components.

Under these conditions, phosphoramidate bond cleavage occurs to remove the biotin-PEG group yielding a phosphopeptide product, which is required for quantitative mass spectrometric analysis.

- 7 Incubate the esterification reactions at 12 °C for 2 hours.

Shaking is not necessary during the esterification reaction. However, if a shaking incubator is available, shake the reactions a 1000 rpm.

- 8 After esterification, evaporate the solvent using a Speedvac concentrator and re-suspend each reaction in 5 μ L of deionized, purified water.

Complete evaporation of the acid is important as acid will corrode the MALDI plate.

Sample preparation for MALDI analysis

- 9 Combine equal volumes (5 μ L each) of the ATP and ATP-biotin reactions into a new eppendorf tube. Thoroughly mix by gently pipetting.
- 10 Add 1 μ L of the peptide mixture to 5 μ L of matrix solution. Thoroughly mix again by gently pipetting. Spot 1 μ L of the peptide/matrix solution on a MALDI plate.

The ratio of peptide mixture to matrix solution can be varied to obtain an optimal MALDI-TOF spectrum. A 1:5 ratio of peptide to matrix is a good starting point.

- 11 Observe the presence of the phosphopeptide products using a MALDI-TOF MS instrument.

The expected masses of the two peptide products should be calculated prior to analysis. If the peptide substrate contains no aspartic acid or glutamic acid groups, then only the C-terminus will be labeled and a mass difference of 3 would be expected. If aspartic acid or glutamic acid groups are present on the peptide substrate, the masses of the two peptides will differ by an additional 3 atomic mass units (amu) for each carboxylic acid group on the peptide.

Figure 3 shows representative MALDI-TOF spectra for the PKA and CK2 model reactions. Notice that the PKA substrate peptides (which contains no aspartic acid or glutamic acid groups) differ in mass by only 3 amu, while the CK2 substrate peptides (which contains six aspartic or glutamic acid groups) differs in mass by 21 amu.

- 12 To determine the relative conversion efficiency of the ATP-biotin reaction compared to the ATP reaction, determine the area under each peptide peak. Assuming that the ATP reaction is 100 %, calculate the % conversion efficiency using the following equation:

$$\% \text{conversion efficiency} = \frac{\text{area of the ATP - biotin - reacted peptide peak}}{\text{area of the ATP - reacted peptide peak}} \times 100$$

Figure 3 displays the peptide peaks with shading to highlight the regions of the peaks that should be included in the area calculation.

Basic Protocol 2 Kinase catalyzed biotinylation with a full-length protein substrate

As an alternative to peptide reactions described in Protocol 1, the tolerance of a kinase of interest to ATP-biotin can be tested with full length protein substrates. Like with peptides, protocol 1, selection of an appropriate protein substrate is critical. The protein must be a known substrate to the kinase of interest, or must contain a consensus sequence for the kinase of interest. Dephosphorylated protein substrates are preferred because prior phosphorylation may block biotinylation. However, the substrate may be used in the phosphorylated state as long as unmodified phosphorylation sites are still available for biotinylation. For example, β -casein is a commercially available protein purified from milk that is an established substrate for the CK2 enzyme. While β -casein is already in a phosphorylated form when purified from milk, biotinylation by CK2 occurs readily because unmodified phosphorylation sites still exist. β -casein is a model protein substrate discussed in the protocol below.

The biotinylation of full-length protein substrates is analyzed using gel electrophoresis followed by visualizing the biotin tag. If a MALDI-TOF instrument is not available for protocol 1, then use of well established gel analysis may serve as an ideal alternative. A variety of biotin visualizing agents are available. Biotin has a strong binding affinity for the streptavidin protein (Green, 1963). As a result, a variety of commercially available streptavidin-fluorophore or streptavidin-enzyme conjugates can be used to detect the biotin tagged proteins after gel electrophoresis. Alternatively, biotin antibodies are also available for detection using traditional western blotting methods. In the protocol below, the streptavidin-horseradish peroxidase (SA-HRP) conjugate is used for biotinylated protein visualization. Horseradish peroxidase is an enzyme that will process substrates like ECL Plus (Amersham) or SuperSignal West Femto Plus (Thermo Scientific) to produce an observable chemiluminescent signal.

In protocol 2, kinase-catalyzed biotinylation is performed with ATP-biotin, kinase and full-length protein substrate. The biotinylation products are then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a PVDF membrane and, visualized using a SA-HRP conjugate. The biotinylation reaction is validated by the presence of biotin on the protein product band after gel analysis. As controls, reactions where ATP is used in place of ATP-biotin, the kinase is omitted, heat-denatured kinase is used, or competing ATP is included in the reaction should be tested. In addition, parallel gels can be run and stained with Coomassie Blue stain (NuSep), which will visualize all proteins to assure the presence of kinase enzyme and full-length protein in the reaction, or ProQ Diamond stain (Invitrogen), which detects all phosphorylated proteins.

Like Basic Protocol 1, protocol 2 is written to allow testing of any kinase and any full-length protein substrate. However, specific conditions for biotinylation with commercially available β -casein protein substrate and CK2 kinases are also described as a model system.

Materials

Adenosine 5'-triphosphate (MP Biomedicals Inc, cat. No. ICN15026605: create a 10 mM stock solution in either water or Tris buffer (see recipe).

ATP-biotin (Adenosine 5'-triphosphate [γ]-biotinyl-3, 6, 9-trioxaundecanediamine, Affinity Labeling Technologies, cat. No. 864538-90-9, 10 mM stored in methanol)

Kinase enzyme stock solution: typically the stock solution is approximately 100–2500 U/ μ L, which will be diluted to a final concentration of 4–20 U/ μ L in the reaction, depending on the efficiency of the given kinase. To use CK2 (New England Biolabs, cat. No. P6010L), a 50 U/ μ L stock solution is prepared using deionized, purified water or kinase buffer.

Protein substrate stock solution: typically the stock solution is approximately 1–10 mM, which will be diluted to a final concentration of 0.1–1 mM in the reaction. To use the β -casein protein substrate (Sigma, cat. No. 9000-71-9), we typically create a 2 mM stock solution after dilution in 10 mM ammonium bicarbonate (pH 8) buffer.

Kinase reaction buffer: If using a commercially available recombinant kinase, the buffer is provided by the manufacturer. If using an expressed kinase, the optimal buffer should be determined. For CK2, the kinase buffer supplied by the manufacturer is provided in the Reagents and Solutions section

β -mercaptoethanol: Fisher, cat. No. 75-36-5

PVDF membrane: Immobilon P^{SQ}-Millipore, cat. No. IPVH00010

Coomassie Blue stain: NuSep, cat. No. SG021

Streptavidin-horseradish peroxidase: Pierce, cat.No EN-N200

ECL Plus: Amersham Biosciences

2X SDS loading buffer (see recipe)

PBST (see recipe)

12% SDS-polyacrylamide gel

Mini-Protean electrophoretic system: BioRad

Mini-Transblot cell: BioRad

Gel imaging instrument: Molecular Dynamics Storm 8600

Kinase-catalyzed biotinylation of protein substrate

- 1 To begin the ATP-biotin reaction, the methanol storage solvent must be evaporated as described in Basic Protocol 1. Dispense 1 μ L of the ATP-biotin stock solution into an eppendorf tube (to create a final concentration of 1 mM). Then, evaporate the methanol storage solvent from ATP-biotin using a Speedvac concentrator.

If a Speedvac concentrator is not available, evaporation can be achieved by blowing air or nitrogen gas over the sample for a few minutes until no methanol solvent is present.

- 2 Add 1 μ L of the corresponding protein substrate (which should give a final concentration of 0.2 mM) and 1 μ L of the manufacturer's kinase buffer (using a 10X stock supplied by the manufacturer) to the tube containing dried ATP-biotin. Dilute the mixture to 9 μ L using 7 μ L of deionized, purified water.

Inclusion of critical reaction controls is strongly recommended. A good positive control for kinase activity is to setup an identical reaction using ATP in place of ATP-biotin. Relevant negative control reactions include 1) omission of ATP-biotin, 2) omission of kinase, 3) inclusion of heat-denature kinase, 4) inclusion of ten-fold excess (10mM) ATP as a competitor.

- 3 Initiate the reaction by adding 1 μ L of the kinase enzyme stock solution to the tube.
- 4 Incubate the reaction mixtures at 30 °C for 2 hours.

Shaking the reaction is optional. If a shaking incubator is available, shake the reactions a 1000 rpm.

Gel analysis and western blotting

- 5 Dilute the reaction with 10 μ L of SDS-PAGE loading buffer and incubate the tube to 95°C for 1 minute to heat denature all proteins.
- 6 Prepare two 12–16% SDS-polyacrylamide gels using established protocols (Sambrook and Russel, 2011) or a commercial supplier (BioRad).

The percentage of gel used is determined based on the size of the protein substrate. For β -casein, which is a 24 kDa protein, a 12% SDS-PAGE gel is appropriate.

The crude reaction(s) is loaded onto two different gels to allow separate visualization by SA-HRP and Coomassie stains. The number of gels used is dependent on the number of visualization methods desired by the user.

- 7 Load the denatured reaction(s) onto each of the gels and separate the protein bands using a constant 200 V for 1 hour. Protocols for running an SDS-PAGE gel are published elsewhere (Sambrook and Russel, 2011).
- 8 Using one of the gels, visualize the protein bands using Coomassie Blue stain according to the manufacturers' protocol.
- 9 With the second gel, transfer the separated proteins to a polyvinylidene fluoride (PVDF) membrane at 90 V for 1 hour using electrotransfer apparatus.
- 10 After protein transfer, incubate the membrane in a blocking solution of 10% (w/v) non-fat dry milk in PBST overnight. Wash the membrane with PBST and

then incubate in a 1:5000 dilution of streptavidin-horseradish peroxidase conjugate in 10% (w/v) non-fat dry milk in PBST for four hours. After washing the membrane with PBST, the bands are stained using ECL Plus reagent using the manufacturers' protocol. Finally, the membrane is visualized using a Storm 860 phosphoimaging instrument.

Representative gel images using the β -casein and CK2 kinase model reaction are shown in Figure 4.

Basic Protocol 3 Kinase catalyzed biotinylation using cell lysates

In addition to labeling known substrates of a specific kinase in vitro, the labeling of substrates in mammalian cell lysates provides a physiologically relevant means of monitor kinase activity. We found that ATP-biotin collaborates with cellular kinases to biotinylate endogenous substrates in Hela cell lysates (Green and Pflum, 2007). The results suggest that the kinase-catalyzed biotinylation reaction is generally successful using mammalian cell lysates to probe endogenous cellular kinases and substrates.

Kinases represent a large collection of enzymes that can accept many substrate molecules in a cell. Various cell lines are available from the American Tissue Culture Center (ATCC.org), including cancerous and noncancerous cells. With many available cell lines, the endogenous kinases and substrates in the lysates of these cell lines can be labeled using ATP-biotin, which will facilitate study of phosphoproteome. Alternatively, exogenous, purified kinases can be incubated with cell lysates to allow labeling of cellular substrates. Likewise, an exogenous, purified substrate can be included with cell lysates to allow labeling by cellular kinases. Therefore, mammalian cell lysates can provide endogenous substrates and/or kinases to probe phosphorylation in a cellular context.

Protocol 3 describes labeling of mammalian cell lysates with ATP-biotin. While the protocol specifically describes use of HeLa cell lysates, other mammalian cell lysates could also be used. Addition of recombinant kinase or substrate can be included, if desired. After incubation of the cell lysates with ATP-biotin, analysis of the biotinylation reaction is performed using SDS-PAGE, like described in Protocol #2. These experiments are useful to study the phosphoproteins in a cell, or for labeling a recombinant substrate with a recombinant kinase in the presence of cell lysates.

Materials

ATP-biotin (Adenosine 5'-triphosphate [γ]-biotinyl-3, 6, 9-trioxundecanediamine, Affinity Labeling Technologies, cat. no. 864538-90-9, 10 mM stored in methanol)

Cells lysates stock solution: typically the stock solution is approximately 10 mg/mL, which will be diluted to a final concentration of 1–5 mg/mL in the reaction, depending on the requirement of the reaction. To use Hela cells (National Cell Culture Center, www.nccc.org), 10 million cells were lysed in lysis buffer (see recipe below). A lysate stock solution of 10 mg/mL was prepared by diluting with lysis buffer.

Kinase reaction buffer: For an expressed kinase, the optimal buffer should be determined. For HeLa cell lysates, we used PKA kinase buffer supplied by the manufacturer, which is provided in the Reagents and Solutions section

Protease inhibitor cocktail: Calbiochem, cat. No. 539137

β -mercaptoethanol: Fisher, cat. No. 75-36-5

PVDF membrane: Immobilon P^{SQ}-Millipore, cat. No. IPVH00010

Coomassie Blue stain: NuSep, cat. No. SG021

Streptavidin-horseradish peroxidase: Pierce, cat. No EN-N200

Lysis buffer (see recipe)

SDS loading buffer (see recipe)

12% SDS-polyacrylamide gel

Mini-Protean electrophoretic system: BioRad

Mini-Transblot cell: BioRad

Gel imaging instrument: Molecular Dynamics Storm 8600

Create biotinylated phosphoproteins in lysates

- 1 To begin the ATP-biotin reaction, the methanol storage solvent must be evaporated as described in Basic Protocols 1 and 2. In this case, dispense 2 μ L of the ATP-biotin stock solution into an eppendorf tube (to create a final concentration of 1 mM). Then, evaporate the methanol storage solvent from ATP-biotin using Speedvac concentrator.

If a Speedvac concentrator is not available, evaporation can be achieved by blowing air or nitrogen gas over the sample for a few minutes until no methanol solvent is present.

- 2 Add 2 μ L of kinase buffer to the tube containing dried ATP-biotin. Dilute the mixture to 10 μ L using 8 μ L of deionized, purified water.

Inclusion of critical reaction controls is strongly recommended. A good positive control for kinase activity is to setup an identical reaction using ATP in place of ATP-biotin. Relevant negative control reactions include omission of ATP-biotin and use of heat-denatured lysates (produced by incubating lysates at 95 °C for 5 min). Adjust the final volume (20 μ L) by adding required amount of deionized, purified water.

If a recombinant source of kinase or substrate is available, it can be added to the lysate reaction at concentrations similar to those described in Protocol 1 and 2. For example, 4 U/ μ L of PKA can be added to the ATP-biotin, as was done previously (Green and Pflum, 2007).

- 3 Initiate the reaction(s) by adding 10 μ L of the lysate stock solution to each tube. For the HeLa cell lysates reaction, 10 μ L will give a final concentration of 5 mg/mL in a 20 μ L total volume.

A final reaction volume of 20 μ L is used with lysates to ensure that sufficient biotinylated substrates are present for gel analysis.

Store the lysate supernatant in single use aliquots to avoid degradation of enzymes or substrates due to freeze-thaw cycles. Remove a single tube and thaw on ice for use in kinase reactions. All pipetting must be done on ice or in a cold room, using pre-chilled eppendorf tubes.

- 4 Incubate the reaction mixture(s) at 30 °C for 2 hours.

Shaking the reaction is optional. If a shaking incubator is available, shake the reactions at 1000 rpm.

Gel analysis and western blotting

- 5 Dilute the reaction with 20 μ L of SDS-PAGE loading buffer and incubate the tube to 95°C for 1 minute to heat denature all proteins.

- 6 Follow the steps 6– 10 in Basic Protocol 2.

A representative gel image using the HeLa cell lysates as a model reaction is shown in Figure 5.

Reagents and Solutions

Use deionized, purified water in all recipes and protocol steps.

Tris buffer for ATP dilutions (1X)

25 mM Tris-HCl (pH 7.5)

PKA Kinase reaction buffer (1X)

50 mM Tris-HCl (pH 7.5) and 10 mM MgCl₂

CK2 Kinase reaction buffer (1X)

20 mM Tris-HCl (pH 7.5), 50 mM KCl, and 10 mM MgCl₂

Matrix solution

A saturated solution of alpha-cyano-4-hydroxy cinnamic acid (Acros organic, cat. No. 28166-41-8) in 70 % acetonitrile, 30 % water and 0.1 % TFA.

SDS loading buffer (2X)

100 mM Tris-HCl (pH 6.8), 4% (w/v) sodium dodecyl sulfate (SDS), 0.2% (w/v) bromophenol blue, 20 % (w/v) glycerol and 200 mM β -mercaptoethanol.

Store 2X SDS gel loading buffer at room temperature. Add 1 μ L of 14 M β -mercaptoethanol stock just before the buffer is used.

PBST buffer (1X)

137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄ and 2 mM KH₂PO₄. Adjust to pH 7.4 with HCl and/or NaOH. Add 0.1% Tween-20 from stock and store at RT.

Lysis buffer

50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10% glycerol, 0.5% Triton X-100) and 1X protease inhibitor cocktail V (Calbiochem).

Commentary**Background Information**

Protein phosphorylation is a key event in influencing protein activity. As a result, it is important to monitor protein phosphorylation to obtain a better understanding of signaling pathways. Historically, 2-D gel analysis has been used to detect phosphoproteins. In this method, proteins are separated by their isoelectric points (pI), followed by molecular weight. Differences in the pI and size of the proteins are seen between phosphorylated and unphosphorylated proteins. Due to this difference, the shifted proteins are isolated and characterized (Kawada, 2001; Marcus et al., 2000). However, 2-D analysis is challenging with proteins that are highly acidic, basic, too large, too small, highly hydrophobic or additionally modified, which can cause a non-phosphorylation dependent shift in pI and molecular weight (Kim et al., 2007; Oh et al., 2004).

Another classic method to identify protein phosphorylation involves radioactive labeling with ³²P-labeled ATP with subsequent visualizing of the ³²P-labeled phosphoprotein by autoradiography (Garcia et al., 2005; Ubersax et al., 2003). The method is not efficient since the ³²P-labeled ATP competes with the unlabeled ATP. In addition, large amounts of radioactive material are required, making the method hazardous. Phosphoprotein-specific detection using Pro-Q Diamond phosphoprotein stain (Invitrogen, (Steinberg et al., 2003) and western blotting (Mann et al., 2002) are also used widely. ProQ Diamond staining can result in nonspecific detection of unphosphorylated proteins, while western blotting requires generation of specific antibody reagents for each phosphoprotein of interest, making it less appropriate for visualizing all phosphoproteins in a complex mixture (Marcus et al., 2000; Sun et al., 2001).

Although not as widely used, phosphoproteins/phosphopeptides can be chemically modified by nucleophilic addition of a biotin group after β -elimination of the phosphate group (Meyer et al., 1986; Oda et al., 2001), although side reactions with unphosphorylated amino acids is a problem (Li et al., 2003). In addition to these methods, analog-specific kinase cosubstrates have been used for detection of kinase-specific phosphopeptides (Kraybill et al., 2002). In the analog-specific method, the kinase is mutated such that it can accept only modified ATP-analog (Blethrow Justin et al., 2008). This method was used to visualize the protein substrate for mutant kinases.

As an alternative to these methods, we reported a kinase-catalyzed biotinylation reaction to allow biotin labeling of kinase substrates for subsequent detection using either MS or gel analysis. In the case of MS-based detection, the biotin group is reported to reduce the

ionization of the attached peptide (Zhou et al., 2001), which makes direct MS analysis challenging. To avoid the ion suppression caused by biotin, we utilized a quantitative MS analysis that requires removal of biotin prior to MS detection. By using isotopic labels and comparing the ATP-biotin reaction to the natural ATP reaction, a percentage conversion value is obtained. As a result, the approach can assess the efficiency of the ATP-biotin reaction with any kinase of interest.

If MS instrumentation is unavailable or a qualitative analysis is desirable, an alternative is use of gel analysis. In this case, visualizing the biotinylated protein products is performed similar to traditional western blotting techniques, although in this case streptavidin conjugates can be used in place of an antibody reagent. Gel analysis is also useful when biotinylation is performed with kinases in cell lysates. The choice of detection methods (MS or gel analysis) will depend on the instrumentation, substrates, and kinases available. In addition, the need for qualitative or quantitative analysis should be also be considered.

Critical Parameters

Selection of the appropriate peptide or protein substrates—Selection of peptide and/or protein substrate is an important factor for Protocols 1 and 2. The peptide or protein must be recognized and phosphorylated by the kinase of interest. In addition, the peptide substrates should be designed to contain only one phosphorylation site per sequence to simplify subsequent analysis. Ideally, the protein substrate should be used in the unphosphorylated state. However, we have had success using protein substrates that were purified in a phosphorylated state, as long as there are additional site(s) available for kinase-catalyzed biotinylation (for example, β -casein).

Storage conditions and handling—The quality of the ATP-biotin is very important for this reaction. Like any triphosphate, ATP-biotin is prone to hydrolyze to ATP or ADP. Degraded ATP will compete with ATP-biotin in the kinase reaction. For protocol 1, the presence of contaminating ATP will affect the quantitative analysis and possibly skew the results. Therefore, the ATP-biotin should be stored at -20°C to minimize degradation during storage. In addition, it is critical that freeze thaw cycles be avoided with ATP-biotin. The methanol storage solvent will not freeze at -20°C , which will reduce degradation due to thaws. Finally, ATP-biotin should be kept as cold as possible during manipulations. All transfers must be done on ice or in a cold room, using pre-chilled eppendorf tubes. Even with these precautions, ATP-biotin will degrade over time and should be used within 6 months.

The storage conditions of the kinase enzymes are also very important to maintain the activity of the compounds. The enzymes or lysates should be store at -80°C . In addition, we recommend storing the enzymes/lysates in single use aliquots, which will avoid inactivation due to freeze/thaw cycles. Remove a single tube and thaw on ice for use in kinase reactions. After this one freeze/thaw, any unused portion remaining in the tube should be discarded. It is not advisable to refreeze. Creating these single use aliquots will ensure that the enzymes (or lysates) do not lose activity due to freeze/thaw cycles. The enzymes should also be manipulated in a cold room or on ice.

Troubleshooting

Appropriate troubleshooting is described in following table.

Anticipated results

Basic Protocol 1—The anticipated result is the generation of a quantitative MS spectrum, like that produced in Figure 3. The percentage conversion efficiency of the ATP-biotin reaction can be determined by comparing to the ATP reaction. For the model reactions with PKA and CK2 displayed in Figure 3, the spectra indicated that the PKA peptide was converted to the corresponding biotin phosphopeptide with efficiency of 82%, (Figure 3A) while the CK2 peptide was converted with an efficiency of 53% relative to ATP phosphorylation (Figure 3B).

Basic Protocol 2—The anticipated result is generation of a gel image (see Figure 4 for an example) where a biotinylated substrate protein is observed using the streptavidin detection reagent. In the model reaction with β -casein, CK2 kinase, and ATP-biotin (Figure 4, lane 4), a protein band at ~24 kDa was visualized with SA-HRP due to successful biotinylation.

Basic Protocol 3—The anticipated result is generation of a gel image (see Figure 5 for an example) where biotinylated protein substrates are visualized using the streptavidin detection reagent. In the model reaction with HeLa cell lysates and ATP-biotin (Figure 5, lane 7), a variety of biotinylated proteins visualized by the SA-HRP conjugate are observed.

Time considerations

Kinase-catalyzed biotinylation with peptides and subsequent MS analysis can be completed within one day (approximately 8 hours). The kinase-catalyzed biotinylation of full-length protein and cell lysates protocols require roughly two days to complete; day one will require roughly 6 hours for kinase reaction, gel separation, electrotransfer, and blocking overnight, while day two will require roughly 5 hours to complete the western blotting.

Acknowledgments

We thank the National Institutes of Health (GM079529) and Wayne State University for funding.

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Figure 2. Quantitative Analysis of Kinase-Catalyzed Biotinylation using peptide substrates

The kinase peptide substrate (4) is split into two equal samples (path A and B). After reaction of one sample with ATP-biotin (path A), unreacted (5) and biotinylated phosphopeptide product (6) are labeled by incubating with anhydrous hydrochloric acid in methanol (produced by mixing methanol (CH₃OH) and acyl chloride). The acidic conditions of the labeling reaction result in the in situ cleavage of the biotin group, which generates a methylated phosphopeptide product (8). To compare the efficiency of the ATP-biotin reaction to the natural ATP reaction, the second peptide sample (path B) is incubated with kinase and ATP to create a phosphopeptide product (9), followed by isotopic labeling with anhydrous hydrochloric acid in deuterated methanol (produced by mixing deuterated methanol (CD₃OD) and acyl chloride), which generates a methylated phosphopeptide product (10). The net result of the kinase and labeling reactions is generation of two chemically identical phosphopeptide products (8 and 10) that differ only by 3 mass units (per methylation event). After combining the two reactions and obtaining a MALDI-TOF spectrum, the percent conversion is calculated by comparing the peak area of the two

chemically identical, but isotopically differentiated peptides. Examples of the MS spectra generated are displayed in Figure 3.

Figure 3. Quantitative MALDI Analysis of Kinase-Catalyzed Biotinylation

(A) PKA model reaction: The peak at 866.588 m/z corresponding to mono-methylated phosphopeptide due to biotinylation by ATP-biotin and PKA enzyme, while the peak at 869.601 m/z corresponding to deuterated mono-methylated phosphopeptide due to phosphorylation of ATP with PKA peptide and PKA enzyme. The areas under the peaks (shaded regions) were calculated to be 1957 and 2378, respectively. The percentage conversion for this reaction is 82%. (B) CK2 model reaction: The peaks at 1541.661 m/z corresponding to the hepta-methylated phosphopeptide due to biotinylation of ATP-biotin and CK2 enzyme, while the peak at 1562.806 m/z corresponding to deuterated, hepta-methylated phosphopeptide due to phosphorylation of ATP with CK2 peptide and CK2 enzyme. For CK2, the difference of 21 mass units is observed because the peptide substrate contains 6 glutamic acids, in addition to the C-terminus. The areas under the peaks (shaded regions) were calculated to be 418 and 792, respectively. The percentage conversion for this reaction is 53%.

Figure 4. Analysis of kinase-catalyzed biotinylation using protein substrates

Recombinant CK2 enzyme (lanes 1) was incubated with full-length β -casein (lanes 2) and either ATP (lanes 3) or ATP-biotin (lanes 4). The crude reactions were separated by 12% SDS-PAGE and visualized using either Coomassie Blue stain to detect all proteins (top) or streptavidin-horseradish peroxidase (SA-HRP) to detect biotinylated proteins (bottom). (A) The reactions were run under standard conditions using 0.2 mM β -casein. (B) To show the detection sensitivity of biotinylation, a dilution experiment was performed using a final concentration of 17 μ M (lane 4), 1.7 μ M (lane 5), 0.17 μ M (lane 6), and 0.017 μ M (lane 7) β -casein. Biotinylated β -casein was observed at concentrations in the sub- μ M range.

Figure 5. Analysis of kinase-catalyzed biotinylation using cell lysates

Hela cell lysates (lane 2) were incubated with ATP (lane 4 and 6) or ATP-biotin (lane 5 and 7) in the presence (lane 4 and 5) or absence (lane 6 and 7) of PKA (lane 1). The proteins in the reactions were separated by 12% SDS-PAGE. The protein bands were visualized using either Coomassie Blue stain (top) to visualize all proteins or streptavidin-horseradish peroxidase (SA-HRP, bottom) to detect biotinylated proteins. A molecular weight marker is included in the Coomassie stained top gel (170 kDa, 100 kDa, 72 kDa, 40 kDa, and 17 kDa).

Table 1

Troubleshooting table

Protocol	Problem	Possible Cause	Solution
1	High background noise in MALDI spectrum	Residual acetyl chloride or methanol present in the sample	Make sure that the acidic methanol solution is completely removed by evaporation prior to MALDI-TOF analysis
1	No peptide peaks observed in the MALDI spectrum,	Re-suspension of the peptides in water after evaporation of acidic methanol was not complete. Dried peptide material remained in the eppendorf tube.	During re-suspension, flick the reaction tube such that water covers all walls of the tube. To collect the sample, pulse-spin the tube in a micro centrifuge.
1	No peptide peaks observed in the MALDI spectrum,	Heating in the speedvac during methanol evaporation cleaved the biotin group off of the peptide	Evaporate the acidic methanol without heating
2 or 3	High background signal on membrane after western blotting for biotin	The low-fat dry milk that is typically used as a block agent in western blotting contains biotin, which can interfere with the interaction between streptavidin and the biotinylated protein	Use bovine serum albumin (BSA) instead of milk as a blocking reagent
2 or 3	No signal on membrane after western blotting for biotin	The ATP-biotin has been used for 3–6 months and has degraded over that time.	Use a new stock of ATP-biotin
2 or 3	No signal on membrane after western blotting for biotin	Extended heating of the reaction sample in SDS gel loading buffer resulted in cleavage of the biotin group from the protein.	Heat the sample after addition of the SDS loading buffer for only 1 minute at 95°C.
2 or 3	Bands present on membrane in negative control lanes after western blotting for biotin	Streptavidin-based western blotting reagents have high background binding if incubated too long or at too high a concentration.	Reduce the length of incubation or increase the dilution of the streptavidin detection reagent. Be sure to monitor the presence of the non-specific signal in a lane where only substrate (no kinase or no ATP-biotin) was added.
3	Bands present on membrane in negative control lanes after western blotting for biotin	Biotin is a naturally occurring molecule and some proteins are naturally biotinylated in the cell. These naturally biotinylated proteins would be visible in all lanes.	Control experiments where kinase-catalyzed biotinylation should not occur (for example, reactions without ATP-biotin or with inactivated kinases) should be included to identify naturally biotinylated proteins in the lysates.
3	Bands present on membrane in negative control lanes after western blotting for biotin	Degradation of the ATP-biotin liberated free biotin-amine, which resulted in non-specific labeling in lysates. Degradation can occur if the ATP-biotin stock is 3–6 months old.	Use a new stock of ATP-biotin