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Kinase-Catalyzed Biotinylation for Phosphoprotein Detection

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Protein phosphorylation is a ubiquitous post-translational modification involved in cell signaling.[1] Because of its fundamental role in cellular functions, monitoring phosphoproteins and phosphopeptides is a fruitful area of proteomics studies. For example, phosphotyrosine specific antibodies are used routinely to detect and enrich phosphoproteins;[2] however, antibodies recognizing phosphoserine and phosphothreonine residues have been less successful.[3] To overcome the limitations of immunoreactive reagents, several strategies for phosphopeptide labeling and enrichment have been developed, including immobilized metal affinity chromatography,[4] covalent phosphate modification,[5] and bio-orthogonal affinity purification.[6] In addition, phosphate stains have been commercialized for general phosphoprotein detection, including the Pro-Q diamond stain.[7] In spite of these chemical tools, the labeling and detection of phosphoproteins in cell lysates is challenging.[5]

We describe here a novel enzymatic approach for phosphoprotein labeling and detection in cellular lysates. After studying the structures of various kinase enzymes,[8] we realized that the adenosine 5′-triphosphate (ATP, **1a**) required for phosphate transfer is partially solvent-exposed in the catalytic active site, particularly near the γ-phosphate (Figure 1A). On the basis of this structural analysis, we hypothesized that γ-phosphate modified ATP analogues (Figure 1B) will bind to kinases and act as cosubstrates, allowing transfer of the γ-modified phosphate group (Figure 1C). Consistent with this analysis, Wang et al. recently developed a kinase inhibitor screen using protein kinase A (PKA) and an ATP-biotin conjugate (**1b**) to produce biotinylated peptides in vitro.[9,10] Therefore, we envisioned use of ATP-biotin (**1b**) to selectively biotinylate full-length phosphoproteins from cellular lysates to aid in their detection.

As a first step, we explored the scope and limitations of kinase-mediated biotinylation using three synthetic peptides containing serine (**3**), threonine (**4**), or tyrosine (**5**), and three kinases, PKA, CK2, and Abl. Each kinase was incubated with the peptide containing its corresponding consensus sequence and ATP-biotin (**1b**) (Table 1). In all cases, ATP-biotin (**1b**) served as a cosubstrate, converting each peptide to its corresponding biotinylated phosphopeptide (Figure 1C), as assessed by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) (Table 1). The results indicate that the kinase-catalyzed biotinylation reaction is generally compatible with the three natural hydroxyl-containing residues and three kinase enzymes.

To determine the efficiency of the enzymatic biotinylation reactions, quantitative MS analysis was employed. Briefly, each peptide substrate was incubated with its corresponding kinase and either ATP (**1a**) or ATP-biotin (**1b**) as the cosubstrate. The resulting phosphorylated or biotinylated peptides were isotopically labeled through carboxylic acid esterification with either d_0-methanol or d_4-methanol, as previously described (see Supporting Information).[11] Subsequently, the biotinylated peptides were converted to phosphopeptide products under acidic conditions (Figure 1C). The two isotopically differentiated phosphopeptide samples were quantitatively compared using MALDI-TOF MS to calculate a relative

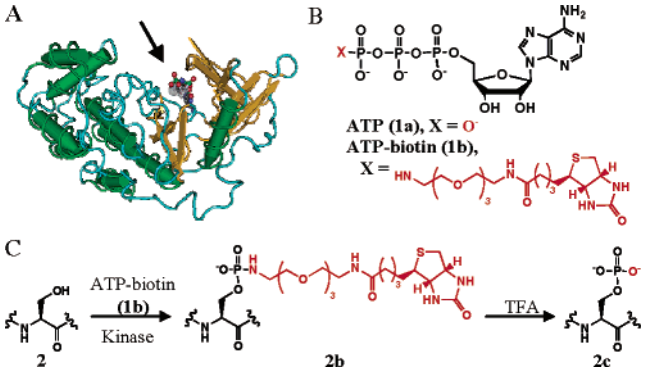

Figure 1. (A) CK2 in complex with the ATP analogue, AMPPnP (Pubmed pdb: 1DAW). The arrow points to the solvent-exposed γ-phosphate of AMPPnP. (B) General structure of γ-phosphate modified ATP analogues. (C) ATP-biotin (**1b**) acts as a kinase cosubstrate to promote phosphorylation-dependent biotinylation of peptides and proteins. Incubation with 50% TFA cleaves the biotin label.

Table 1. MALDI-TOF MS Data of Peptides **3**−**5** after Incubation with PKA, CK2, or Abl Kinase and the ATP-Biotin (**1b**)

		biotinylated peptide		
peptide substrate	kinase	calcd	obsd[d]	conversion[e]
(**3**) LRRA**S**LG	PKA	1308.27[a]	1308.48	79%
(**4**) RRREEE**T**EEE	CK2	1885.76[b]	1886.19	56%
(**5**) EAI**Y**AAPFAKKK	Abl	1816.94[c]	1816.39	80%

[a] Calculated mass $[M + Fe]^+$. [b] Calculated mass $[M + 2Na]^+$. [c] Calculated mass $[M + H]^+$. [d] Observed mass based on MALDI-TOF MS analysis. [e] Percentage conversion was determined using quantitative MS by comparing to ATP phosphorylation (100%).

conversion percentage. Quantitative MS analysis revealed that each peptide was efficiently phosphorylated using ATP-biotin (**1b**) with 79%, 56%, or 80% conversion relative to ATP (Table 1 and Figures S1, S2, and S3). The data indicate that kinase-catalyzed biotinylation proceeds with conversion percentages comparable to ATP phosphate transfer reactions.

As the next step, the kinase-catalyzed biotinylation reaction was validated for labeling of proteins. Full-length β-casein was incubated with CK2 and ATP-biotin (**1b**) and the reaction product was separated by SDS-PAGE (Figure 2A). The presence of a biotin label on β-casein was assessed by blotting with a streptavidin−horse radish peroxidase conjugate (SA−HRP). Full-length β-casein was visualized by the SA−HRP conjugate (Figure 2A, lane 4), indicating that β-casein is labeled with biotin. The efficiency of β-casein biotinylation was determined using quantitative MALDI-TOF MS after trypsin digestion, as described for the peptide reactions. The conversion efficiency of the β-casein-derived phosphopeptide (FQpSEEQQQ**pT**EDELQDK) was 73% relative to the corresponding ATP reactions (Figure S4). The data indicate that a full-length protein is efficiently biotinylated in the presence of ATP-biotin (**1b**).

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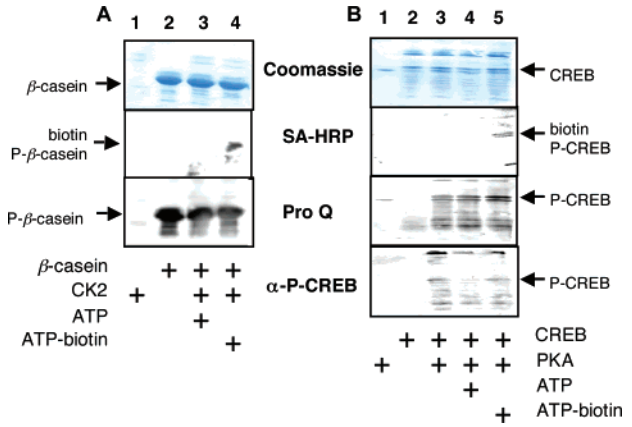

Figure 2. Kinase-catalyzed biotinylation of purified β-casein (A) and recombinant CREB in bacterial lysates (B). The proteins were visualized with Coomassie, SA−HRP, Pro-Q diamond phosphate stain (Pro-Q), and α-P-CREB (B only) as indicated. The proteins are indicated with arrows. The contents of each reaction are indicated below each lane. The gels are representative of at least three independent experiments.

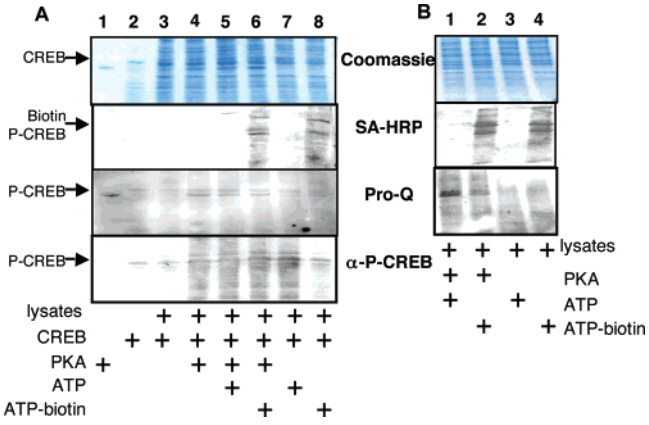

Figure 3. Kinase-catalyzed biotinylation with HeLa cell lysates containing recombinant CREB in bacterial lysates (A) or alone (B). Proteins were visualized as indicated (see Figure 2 legend). The CREB protein is indicated with arrows. The contents of each reaction are indicated below each lane. The gels are representative of at least three independent experiments. See Figure S5 in Supporting Information for the full gels of (B).

The kinase-catalyzed biotinylation reaction was also tested with a second full-length protein, CREB, which contains a single PKA consensus sequence.[12] In this case, bacterial lysates containing overexpressed CREB were used to simultaneous test compatibility with cellular lysates. The CREB protein in bacterial lysates was incubated with PKA and ATP-biotin (**1b**), and the proteins were separated by SDS-PAGE (Figure 2B). While many protein bands were observed by coomassie staining, a protein of the same molecular weight as CREB was visualized by using SA−HRP (Figure 2B, lane 5). Use of an antibody recognizing CREB phosphorylated at S133 (α-P-CREB) confirmed the identity of the biotinylated phosphoprotein. The data establish that the biotinylation reaction is compatible with full-length proteins and will tolerate the presence of endogenous ATP and nonspecific proteins from bacterial lysates. Importantly, the strategy is sensitive enough to visualize proteins with only a single phosphorylation site.

To further test kinase-catalyzed biotinylation, recombinant CREB was biotinylated in the presence of mammalian HeLa cell lysates (Figure 3A). Staining with SA−HRP revealed that CREB was biotinylated in the presence or absence of recombinant PKA (Figure 3A, lanes 6 and 8), indicating that endogenous kinase proteins present in the lysates efficiently label CREB. In addition, the data further establish that ATP-biotin (**1b**) competes with the endogenous ATP in lysates as a kinase cosubstrate.

As a final step in validating the phosphoprotein biotinylation reaction for proteomics applications, ATP-biotin (**1b**) was incubated with HeLa cell lysates with or without recombinant PKA and biotinylated proteins were visualized using SA−HRP. As seen in Figure 3B, phosphoproteins were strongly detected in the lysates after biotinylation. In fact, the visualization of phosphoproteins via the biotin label is more robust with cell lysates than the commercially available Pro-Q diamond phosphoprotein stain (Figure 3A, lanes 7 and 8 and Figure 3B, lanes 3 and 4). The reactions with HeLa cell lysates demonstrate the utility of the kinase-catalyzed biotinylation reaction to monitor phosphoproteins in cellular lysates. Because ^{32}P labeling with ATP analogues in cell lysates has been used to identify novel phosphoproteins,[13] the kinase-catalyzed biotinylation reaction provides an alternative to radioactive methods for phosphoprotein detection and identification.

In summary, kinase-catalyzed biotinylation is a general method for monitoring phosphopeptides and phosphoproteins in biological samples. Given that the biotin tag is suitable for protein purification, the kinase-catalyzed biotinylation reaction provides a flexible handle for multiple phosphoproteomics applications. Significantly, by revealing the natural cosubstrate promiscuity of kinase enzymes, these studies pioneer the use of γ-phosphate modified ATP analogues to link phosphoproteins to multiple functional tags, such as fluorophores or cleavage reagents, and promote development of unprecedented phosphoproteomics tools. Therefore, establishing the kinase-catalyzed biotinylation reaction with cellular proteins represents a first step toward the creation of new chemical tools targeting the phosphoproteome.

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Supporting Information Available: Detailed reaction procedures and MALDI-TOF MS data. This material is available free of charge via the Internet at http://pubs.acs.org.

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