The Use of Adenosine 5'-Triphosphate (γ-P^{18}O$_4$, 97%) for the Unambiguous Identification of Phosphopeptides

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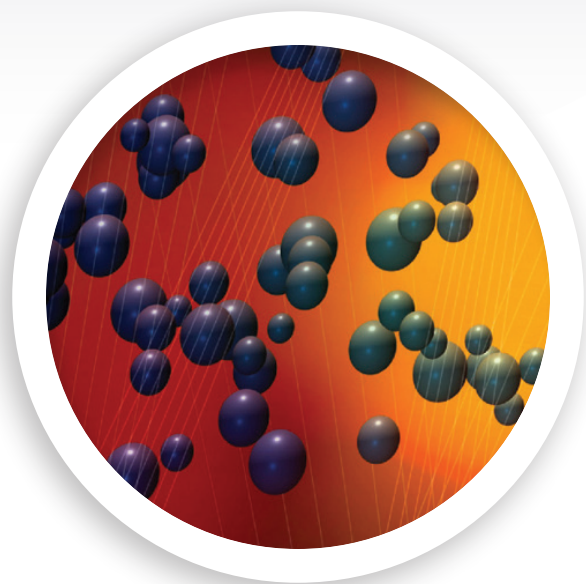
Phosphorylation is arguably the key signaling event that occurs within cells controlling processes such as metabolism, growth, proliferation, motility, differentiation and division. Current data predicts that approximately 2% of the human genome encodes for kinases represented just over 500 individual protein species.[2] The importance of kinases (and phosphorylation) is underscored by the fact that 30% of all drug-discovery efforts target this class of proteins. Determining kinase specificity has long been a key research area in molecular biology and is important to a variety of fields, including cancer research, cell and developmental biology and drug discovery.

Identifying phosphorylated residues within proteins has historically been accomplished using *in vitro* studies in which a kinase is mixed with a potential substrate in the presence of ^{32}P-labeled adenosine triphosphate ATP (γ-^{32}P). The reaction mixture is then digested into peptides that are analyzed using scintillation counting. Radioactive peptides are then sequenced to reveal the identity of the phosphopeptide. While radioactive isotopes have been used quite successfully for a number of years, they have a number of drawbacks that are not simply limited to safety and regulatory issues. To overcome the need for radioactivity, investigators have turned to mass spectrometry (MS) for the identification of phosphopeptides. While MS data is extremely useful, sequence database search engines and statistical models for data validation are not optimized for the specific MS fragmentation properties exhibited by phosphopeptides. The result is a large, indeterminable rate of false-positive and false-negative values.

In this note, we describe a stable isotope-labeling approach that provides unambiguous identification of phosphorylated peptides produced through *in vitro* kinase reactions. The method utilizes adenosine triphosphate in which four oxygen-[16] atoms of the terminal phosphate group are substituted with oxygen-[18] ATP (γ-P^{18}O$_4$). In vitro kinase reactions were conducted using a 1:1 mixture of ATP (γ-P^{18}O$_4$, 97%) and normal isotopic abundance ATP. Phosphopeptides produced during the reaction present themselves in the mass spectrum as peaks separated by 6.01 Da due to the presence of both normal and ^{18}O-labeled phosphate groups.

Results

In vitro phosphorylation using ATP (γ-P¹⁸O₄)

The utility of adenosine 5'- triphosphate (γ-P¹⁸O₄,97%) (ATP (γ-P¹⁸O₄, 97%)) (Catalog No. OLM-7858) as a novel reagent for identifying phosphopeptides was evaluated in a series of reactions using a known kinase/substrate in vitro reaction. Myelin basic protein (MBP) was phosphorylated *in vitro* using MAP kinase in the presence of a 1:1 mixture of normal isotopic abundance ATP and ATP (γ-P¹⁸O₄, 97%). The mass spectrum of the reaction products is shown in Figure 1. The MALDI-TOF/MS spectrum is dominated by singlet peaks, however, a doublet of signals at mlz ratios 1571.79 and 1577.81 (Δm/z = 6.02) was observed. The accurate mass, as well as the tandem MS data (not shown), confirmed the doublet as $^{16}O_3PO$- and $^{18}O_3PO$-labeled versions of the peptide NIVpTPRTPPPSQGK. Site-specific assignment of the phospho-threonine residue was determined by the tandem MS data.

A triplet of signals at m/z ratios 1651.76, 1657.77 and 1663.79 was also observed in the mass spectrum shown in Figure 1. The accurate mass and tandem MS data identified these signals as originating from the doubly phosphorylated peptide NIVpTPRpTPPPSQGK. The peak at m/z 1651.76 corresponded to the phosphopeptide containing two light-isotopic (i.e. $^{16}O_3PO$-) phosphate groups. The peak at m/z 1663.79 corresponded to the peptide containing two heavy-isotopic (i.e. $^{18}O_3PO$-) phosphate groups. The middle peak at 1657.77 was identified as the phosphopeptide containing both a light and heavy isotopic phosphate group. The phosphate modifications to Thr94 and Thr97 of MBP were readily assigned using the tandem MS data.

Evaluation of Isotope Exchange Loss

Depending on the type and structural position of the isotope, exchange loss can be a concern when utilizing stable-isotope labeling. While no obvious reason for a natural exchange loss of ^{18}O atoms of ATP (γ-P¹⁸O₄) for ^{16}O when dissolved in H_2O was clear, the effect, if any, of the kinase activity on exchange of the phosphate oxygen atoms was not known. Therefore, a second series of *in vitro* kinase reactions were performed in which a synthetic peptide (KVEKIGEGTYGVVYK), representing residues 6-20 of Cdc2, was incubated with the tyrosine kinase Src in the presence of ATP (γ-P¹⁸O₄). The reaction was performed in both native H_2O (i.e. $H_2^{16}O$) and $H_2^{18}O$. As shown in Figure 2, the MALDI-TOF/MS spectra of the phosphopeptides were identical regardless of whether the kinase reactions are run in $H_2^{16}O$ or $H_2^{18}O$ showing that no isotope-exchange loss occurs.

Discussion

Adenosine 5'-triphosphate (γ-P¹⁸O₄, 97%) (ATP (γ-P¹⁸O₄, 97%)) (Catalog No. OLM-7858) represents a new, non-radioactive proteomic reagent for identifying phosphopeptides using MS. **Other labeling methods either chemically modify the phosphorylation site, usually by removing the phosphate group first and then modifying the reactive site that is left behind with a tag. This method is the first that simply incorporates a stable-isotope label on the native phosphate.**

The data shows the first example of a stable-isotope labeling method for phosphopeptides in which the label is part of the phosphate group. Conducting *in vitro* kinase reactions using a 1:1 mixture of ATP and ATP (γ-P¹⁸O₄, 97%) produces a distinct peak signature within the mass spectrum, providing absolute confidence in the phosphorylation status of the peptide. This distinctive signature represents a tremendous benefit to peptide mapping experiments that rely solely on accurate mass measurement for phosphopeptide identification.

The use of a non-radioactive phosphate tag for the identification of phosphorylation sites eliminates a number of problems associated with ATP (γ-³²P). Besides all of the associated health issues and precautions that must be taken, experiments using ATP (γ-³²P) require extremely careful planning and timing so that the precise amount needed for a specific experiment is ordered and consumed within a relatively short period of time upon delivery. After completion of the experiment, all of the unused reagent and any consumable materials that it came in contact with must be properly disposed. In comparison to its radioactive counterpart, ATP (γ-P¹⁸O₄,97%) shelf-life is comparable to normal ATP and requires no special precautions or disposal procedures.

The identification of kinase-induced phosphorylation sites in target proteins is critical for the understanding of the biological processes mediated by these kinases. The technique described here is a novel, non-radioactive method that enables phosphorylation sites identification. Samples can be safely manipulated without the need for radioactive tags and the need for other precautions needed when working with ³²P. This reagent will have a positive impact by decreasing the false-positive rate associated with the identification of phosphopeptides using MS.

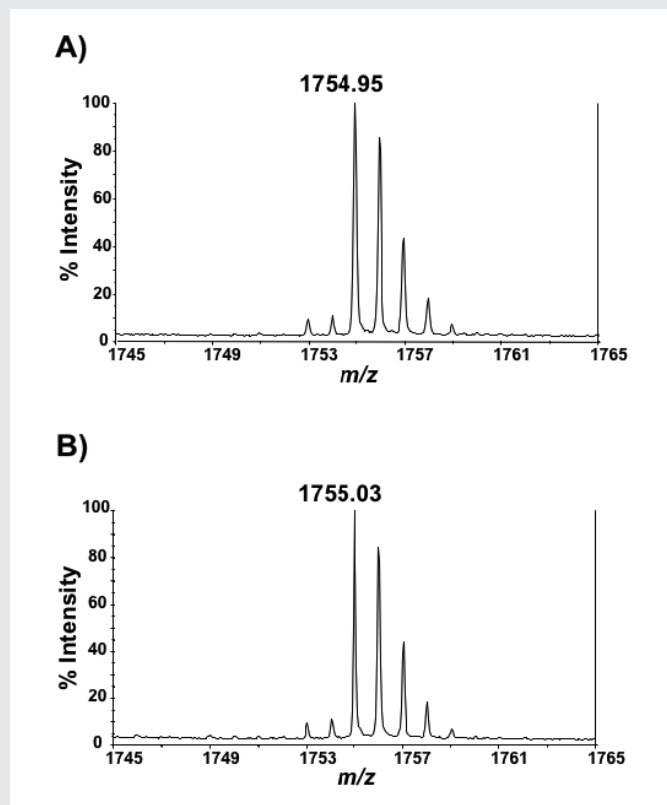## Acknowledgements

Competing Interests Statement

Figure 1. Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry spectra of *in vitro* kinase reaction products of MAP kinase and myelin basic protein in the presence of a 1:1 mixture of ATP (γ-P^{18}O$_4$). A) Mono- and di-phosphorylated versions of the tryptic peptide NIVTPRTPPPSQGK were observed in the mass spectrum (insets).
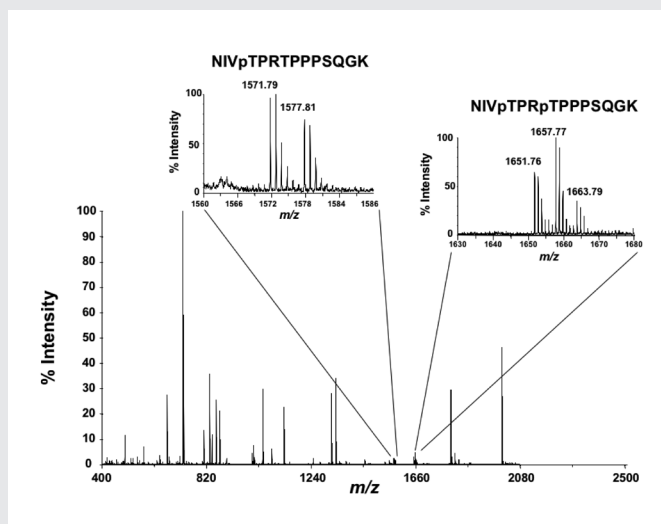

Figure 2. Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry spectra of the synthetic peptide (KVEKIGEGTYGVVYK) phosphorylated by Src protein tyrosine kinase using ATP (γ-P^{18}O$_4$) in (A) H$_2$O and (B) H$_2$^{18}O.

Additional products of interest

CLM-2265	L-Arginine•HCL (U-^{13}C$_6$, 98%)
CNLM-539	L-Arginine•HCL (U-^{13}C$_6$, 99%; U-^{15}N$_4$, 98%)
CDNLM-6801	L-Arginine•HCL (U -^{13}C$_6$, 98%; U-D$_7$, 98%; U-^{15}N$_4$, 98%)
DNLM-7543	L-Arginine•HCL (D$_7$, 98%; ^{15}N$_4$, 98%)
CLM-2247	L-Lysine•2HCL (U-^{13}C$_6$, 98%)
CNLM-291	L-Lysine•2HCL (U-^{13}C$_6$, 98%; ^{15}N$_2$, 98%)
CDNLM-6810	L-Lysine•2HCL (U-^{13}C$_6$, 98%; U-D$_9$, 98%; U-^{15}N$_2$, 98%)
DNLM-7545	L-Lysine•2HCL (D$_9$, 98%; ^{15}N$_2$, 98%)
CDLM-760	L-Methionine (1-^{13}C, 99%; methyl-D$_3$, 98%)

For additional technical information please visit:

http://stke.sciencemag.org/cgi/content/abstract/sigtrans;2005/267/pl2

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