Phosphopeptides and their biological applications

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ABSTRACT
Protein phosphorylation/dephosphorylation plays a central role in cell signaling. In recent years, a variety of reagents have been developed for protein phosphorylation research. Among these reagents, phosphopeptides have been proven to be critical components in designing assays for protein phosphorylation. In this article, we have summarized the synthesis and purifications of phosphopeptides. The biological applications of phosphopeptides are highlighted, and the future trend in protein phosphorylation is also discussed.

INTRODUCTION

Cellular phosphorylation is a reversible, covalent modification of a protein or lipid that serves to modify the activity of the phosphorylated molecule by inducing conformational changes within the molecule. This modification occurs either through the addition of phosphate groups via the transfer of the terminal phosphate from a phosphate donor, *e.g.* ATP to an amino acid residue and/or by their removal. The function of these post-translational modifications is to alter the substrate's activity, subcellular localization, binding properties, or association with other proteins. The post-translational modification by phosphorylation is a ubiquitous regulatory mechanism in both eukaryotes and prokaryotes, and is regulated by protein kinases and phosphatases. The use of phosphate esters within the cell has a broad range of applications. These include their use as a source of energy such as in the form of adenosyl triphosphate (ATP), a means of covalently linking organic molecules synthesized in primary and secondary metabolism, or a mechanism of regulation of protein function via covalent modification of amino acid side chains. The reversible phosphorylation of proteins can result in the activation or termination of many important cellular events including cell signaling, growth, and differentiation. Intracellular phosphorylation by protein kinases, triggered in response to extracellular signals, provides a mechanism for the cell to switch on or off many diverse processes (1,2). These processes include metabolic pathways, kinase cascade activation, membrane transport, gene transcription, and motor mechanisms (3).

Kinases and phosphatases are of interest to researchers involved in drug discovery, because of their broad relevance to health and disease. Cancer and other proliferative diseases, inflammatory diseases, metabolic disorders, and neurological diseases are among those in which protein phosphorylation plays an important role. All signal transduction pathways are regulated, on some level, by phosphorylation, making phosphorylation relevant to most, if not all, areas of cell signaling and neuroscience research (4). Although the natural substrates of protein kinases and phosphatases are usually proteins (*e.g.* Myelin basic protein, GSK-3 fusion protein, and Bad fusion protein, etc.), peptides and phosphopeptides are increasingly used for analyzing protein kinases and phosphatases (instead of the natural protein substrates), particularly for high throughput screening of protein kinase and phosphatase inhibitors.

SYNTHESIS OF PHOSPHOPEPTIDES

The synthesis of high purity phosphopeptides was quite challenging. Phosphopeptides were initially synthesized using Boc chemistry or by post-phosphorylation of a regular peptide (5). These two methods require careful manipulation of reaction and purification conditions, and usually have low yields. These reactions are usually carried by experienced

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peptide chemists and difficult to be automated. In recent years, the synthesis of phosphopeptides has been significantly improved with the introduction of monobenzyl protected-phosphoamino acids, such as Fmoc-Ser[PO(OBzl)OH]-OH, Fmoc-Thr[PO(OBzl)OH]-OH, and Fmoc-Tyr[PO(OBzl)OH]-OH. Ultra pure phosphopeptides are now routinely synthesized with high yields using monobenzyl-protected phosphoamino acids and standard Fmoc chemistry (6-9). These solid-phase peptide syntheses are readily automated to generate a variety of phosphopeptides (6). The preparation of synthetic phosphopeptides by using Fmoc derivatives of phosphoamino acids has become the state-of-the-art method. The automated solid-phase synthesis has been used at AnaSpec to synthesize a number of phosphopeptides. For example, a peptide with six phosphorylation sites has been recently synthesized at AnaSpec using the monobenzyl-protected amino acids and Fmoc chemistry. Specifically, the synthesis was performed using solid phase synthesis method, employing Rink Amide resin and Fmoc chemistry. The Fmoc amino acids were activated *in situ* with HBTU, and the coupling reactions were carried out in DMF. The deprotections were performed using the TFA cocktail with thioanisole added as scavenger. The reaction was monitored by HPLC, and the crude peptides were purified using reverse phase HPLC with 0.1% TFA in water/0.1%TFA in acetonitrile as the buffer system. Figure 1 demonstrates the HPLC profiles of both the crude reaction mixture and purified desired product. The structure of the desired phosphopeptide was confirmed by MS analysis.

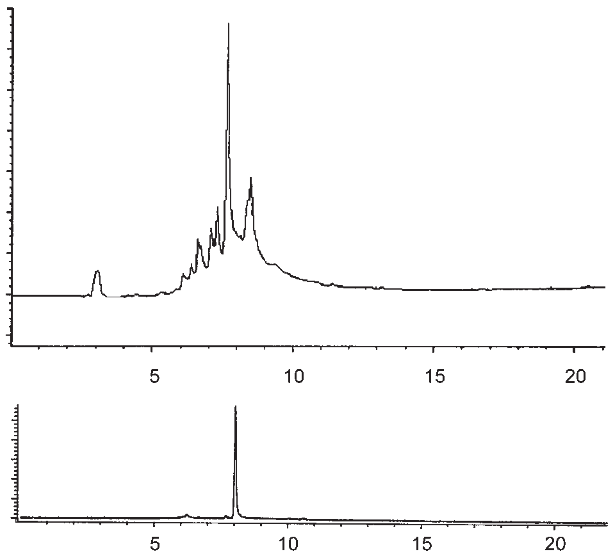

Figure 1 – HPLC analysis of the crude reaction mixture (top) and purified desired phosphopeptide (bottom) of Biotin-Gly-pSer-Ala-Pro-pSer-Ser-Gly-pSer-Ala-Pro-Ser-Pro-pSer-Gly-Pro-pSer-Ala-pSer-Gly-Pro-Ser-Gly-NH$_2$.

APPLICATIONS OF FLUORESCENT LABELED AND BIOTINYLATED PHOSPHOPEPTIDES

The reversible phosphorylation of proteins is a key mechanism whereby signaling cascades involved in response to extracellular stimuli bring about changes in cellular function. These proteins include the kinases/phosphatases that form such signaling pathways as well as the transcription factors involved in inducible changes in gene expression. Phosphorylation induces changes in the function of these proteins either by induction of allosteric conformational changes in the protein itself or in the regulation of its interaction with other cellular factors (1,2,4). The study of phosphorylation in cells can involve a range of methods, but the primary technique for determining the extent of phosphate incorporation into specific sites *in vivo* involves labeling cells with [^{32}P] phosphate followed by phospho amino acid analysis or peptide sequencing of the protein of interest. Apart from the practical problems associated with this technique, the incubation of cells with radioactive precursors (^{32}P and ^{3}H) can in itself activate growth arrest and stress-responsive signaling pathways, which obviously perturb protein phosphorylation. Thus, unless phosphorylation at specific sites can be studied indirectly for a protein, *e.g.* a mobility shift on an sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, there is a clear need for non-invasive methodologies that can be used to complement the well-established radioactive labeling techniques (10,11). Safety concerns and economic considerations have fueled the growing trend of avoiding radioactivity. Fluorescent labeled and biotinylated peptides and corresponding phosphopeptides are increasingly used for analyzing protein kinases and phosphatases (to replace the radioactive phosphopeptides and phosphoproteins). In particular, fluorescent labeled peptides (substrates for protein kinases) and phosphopeptides (substrates for protein phosphatases and reference standards/competing tracers for the kinase reactions) are critical components of many commercial assay kits for protein kinases and phosphatases. A few examples are summarized in Table I.

ELISA-Based Analysis of Protein Kinase Activities (12,13)

So far, ELISA might still be the most sensitive method for analyzing protein kinases. A number of companies offer ELISA-based kinase assays. A versatile kit from Calbiochem of San Diego can be used to selectively measure the activity of either PKC or PKA. The kit contains micro strips coated with an immobilized peptide based on a pseudo substrate sequence along with the reagents needed for both types of kinase reactions and subsequent ELISA. PKC or PKA reactions are performed in the micro strip wells and kinase activity is detected via subsequent incubations with a biotinylated antibody specific for the phosphorylated pseudo substrate and streptavidin-horseradish peroxidase (HRP). Oxidation of the chromogenic HRP substrate *o*-phenylenediamine (OPD) is monitored using an ELISA plate reader. Pierce's ELISA-based PTK assay kits utilize a similar strategy. These kits use a different biotinylated peptide substrate that is derived from the phosphorylation sites of cdc2, gastrin, or c-Src. The peptide substrate is immobilized to NeutrAvidin-coated microplate wells. The PTK reaction gives the phosphorylated substrate that is detected by using an HRP-labeled anti-phosphotyrosine antibody.

Oncogene Research Products of Boston offers an ELISA-based PTK assay that employs a random polymer substrate. This polymer contains multiple Tyr residues but is devoid of Ser and Thr residues, and can be phosphorylated by a wide range of PTKs. Reactions are performed in microplate wells coated with the polymer substrate. A HRP-conjugated anti-phosphotyrosine antibody is used to detect Tyr-phosphorylated substrate. 3,3',5,5'-tetramethylbenzidine (TMB) is used to monitor the HRP conjugate. Sigma of St. Louis also offers similar PTK assay kit, but Sigma's kits use a different anti-phosphotyrosine mAb and a different HRP substrate (OPD instead of TMB). The ELISA-based PTK assay from Roche Molecular Biochemicals of Indianapolis employs a biotinylated peptide substrate that is captured in streptavidin-coated wells. Two biotinylated peptide substrates are supplied with the kit. One is based on the amino-terminal region of cdc2, and the other on a portion of gastrin. After the PTK reaction, the resultant phosphorylated substrate is immunodetected via binding to a HRP-conjugated anti-phosphotyrosine.

Fluorescence Polarization (FP)-Based Analysis of Protein Kinases (14)

Fluorescence polarization is one of the best methods suitable for high throughput analysis of protein kinases and phosphatases. For a competition assay mode, a fluorescent phosphopeptide tracer competes with non-fluorescent phosphopeptides generated during a kinase assay for binding to an anti-phos antibody. Fluorescent probes (that are free in solution) rotate and tumble, causing the emitted light to become depolarized. When bound to a larger molecule, such as anti-phos antibody, the tumbling rates of the fluorescent molecules are greatly decreased, and the emitted light remains highly polarized. If there are no kinase reaction products present, most of the phosphopeptide tracer binds to anti-phos antibody and the polarization value is high (15). PanVera Corp. of Madison offers a variety of homogeneous competition FP-based assays for the detection of PKA, PKC, PTK, and other protein kinase activities. In Panvera's PTK kit, as a PTK phosphorylates a selected peptide substrate, the resultant unlabeled phosphopeptide competes with the fluorescent tracer for anti-(P)Tyr binding. As the reaction progresses, increasing amount of the fluorescent tracer is displaced, resulting in a proportional decrease in polarization that is easily quantified. PanVera's kinase assay kits are also used in kinetic studies to monitor phosphorylation events in real time. As the reaction proceeds and phosphotyrosines are generated, the polarization of the sample decreases. Recently Molecular Devices of Sunnyvale has launched a series of protein kinase assay kits called IMAP (16). Molecular Devices' IMAP kits are based on the direct FP measurement of kinase-mediated phosphopeptide formation. In the IMAP kinase assay kits, a fluorescent labeled peptide is used as a tracer. The fluorescent tracer is converted to phosphopeptide that is captured by chemically modified microspheres called IMAP nanoparticles. The kinase reaction causes a significant increase in FP since the nanoparticle-binded phosphopeptide is much larger than the free fluorescent tracer. IMAP kits are more generic than Panvera's kits since the kits used chemically modified microspheres to specifically bind phosphopeptides (instead of anti-phospho antibodies). In theory, the IMAP kits are more readily adapted to analyzing different kinases if a proper fluorescence peptide substrate is identified.

Table I

Company	Product Name	Assay Type	Peptide Used	Labeling Type
Calbiochem-Novabiochem Corp. www.calbiochem.com	Protein kinase assay kit, non-radioactive	ELISA	RFARKGSLRQKNV	Immobilized to microplate wells
Molecular Devices Corp. www.moleculardevices.com	IMAP kinase assay kits	Fluorescence polarization	Fluorescent crosstide, kemptide, and other fluorescent peptides and phosphopeptides	Fluorescence
Packard Instrument Company/Perkin Elmer www.packardinst.com	Alpha screen c-Src kinase assay kit	FRET/BRET	KVEKIGEGTYGVVYK	Biotinylated
PanVera Corp. www.panvera.com	Protein tyrosine kinase assay kit	Fluorescence polarization	Various peptides are included in the kit	Fluorescence
Pierce Biotechnology, Inc. www.piercenet.com	Colorimetric PKA assay kit	Affinity-based separation	LRRASLG RFARKGSLRQKNV PLSRTLSVAAKK EKRPSQRSKYL AAKIQASFRGHMARKK	Fluorescence
	Tyrosine assay kit	ELISA	KVEKIGEGTYGVVYK EGPWLEEEEEAYGWMDF RRLIEDAEYAARG	Biotinylated
Promega Corp. www.promega.com	Pep Tag non-radioactive PKA assay kit	Electrophoresis separation	LRRASLG	Fluorescence
	Pep Tag non-radioactive PKA assay kit	Electrophoresis separation	PLSRTLSVAAK	Fluorescence
Roche Molecular Biochemicals Biochem.roche.com	Tyrosine kinase assay kit, non-radioactive	ELISA	KVEKIGEGTYGVVYK EGPWLEEEEEAYGWMDF	Biotinylated
Sigma-Aldrich Fine Chemicals www.sigma-aldrich.com	Protein tyrosine kinase assay kit	ELISA	Random polymer (poly-E;Y)	Immobilized to microplate wells

FRET-Based Analysis of Protein Kinase (17)

Packard Instrument Co. of Meriden offers homogenous time-resolved fluorescence (HTRF) PTK assays. HTRF assays exploit fluorescence resonance energy transfer (FRET) between europium cryptate and XL665, a modified allophycocyanin. Europium cryptate becomes excited at 337 nm, and normally fluoresces at 620 nm. However, if this fluorophore is proximal to XL665, FRET occurs, and the output signal is shifted to 665 nm. Critical components in Packard's kits are biotin-labeled Tyr-containing polypeptide substrates. After the kinase reaction, a europium cryptate-labeled anti-phosphotyrosine antibody is added along with a streptavidin-XL665 conjugate. The europium-antibody conjugate binds the fraction of biotinylated substrate that has become Tyr-phosphorylated and the biotinylated peptide substrate binds streptavidin-XL665. This brings the europium cryptate-labeled antibody into close proximity with streptavidin-XL665, which results in a long-lived signal at 665 nm via FRET. Packard Instrument has also developed AlphaScreen (Amplified Luminescent Proximity Homogeneous Assay Screen) assays. AlphaScreen assays utilize "donor" and "acceptor" beads. Upon irradiation with laser light (680 nm), a photosensitizer in the donor bead converts ambient oxygen to singlet-state oxygen. The excited singlet-state oxygen molecules normally diffuse approximately 250 nm (one bead diameter) and decay. However, if the donor bead is in close proximity to an acceptor bead, the singlet-state oxygen molecules react with chemiluminescent groups in the acceptor beads, transferring energy to fluorescent acceptors in the same bead, and shifting the emission wavelength to 520-620 nm. Beads are brought into proximity by biological interactions between proteins bound to the surfaces of the beads. For example, the AlphaScreen c-Src tyrosine kinase assay kit employs a biotinylated peptide substrate based on a region of cdc2. After the kinase reaction, streptavidin-labeled donor beads are added along with an anti-phosphotyrosine conjugated to acceptor beads. The streptavidin-labeled donor beads bind the biotinylated substrate. Anti-phosphotyrosine/acceptor bead conjugates bind the phosphorylated substrate fraction, thus bringing acceptor beads into close contact with donor beads.

Electrophoresis Analysis of Protein Kinases

Change in net charge (caused by the phophorylation of peptide substrates) provides an excellent property for separating phosphopeptides (the products of kinase reactions) from peptide substrates by electrophoresis. This principle is demonstrated by Promega's PepTag assay kits. In the Pep Tag kits, fluorescent labeled Kemptide peptide, a cationic fluorescent peptide substrate, is used for protein kinase A (PKA), whereas its PKC assay uses an analog of the amino-terminal portion of glycogen synthase (18,19).
Monophosphorylation of either peptide substrate alters its net charge from +1 to -1 allowing the phosphorylated and non-phosphorylated substrate fractions to be separated by gel electrophoresis.
The PepTag assays are claimed to detect sub nanomolar levels of PKC or PKA.

Affinity Separation of Phosphopeptides for Analysis of Protein Kinase and Phosphatase Activities

Most of affinity separation methods are based on the metal ion-mediated phosphate binding. For example, the SpinZyme colorimetric assay kits from Pierce of Rockford employ fluorescent labeled peptide substrates for the assay of PKA or PKC. Pierce's kits utilize affinity membrane to separate phosphorylated and non-phosphorylated substrate fractions. After the PKA or PKC reaction has been performed, an aliquot is applied to the metal chelate affinity membrane of a SpinZyme separation unit. The membrane is washed to remove non-phosphorylated substrate. The phosphorylated product is then eluted and quantified via absorption or fluorescence measurements.

One-Stop Shop of Kinase/Phosphatase Assay Components

Although there are many commercial kits developed for analyzing specific protein kinases and phosphatases, there are still strong demands for researchers to develop their own assay methods to meet their specific needs since protein kinases and phosphatases consist of numerous enzymes and isomers. Collaborating with HiLyte Biosciences of San Jose, AnaSpec (www.anaspec.com; San Jose, CA) has developed a series of peptides and related products for protein phosphorylation research so that biologists can readily develop their own kits by purchasing these critical components. For example, AnaSpec has a large collection of native peptides that are proven to be excellent substrates for protein kinases and phosphatases. AnaSpec also provide fluorescent labeled and biotinylated peptides and phosphopeptides that are used to develop various kits for assaying protein kinases and phosphatases. HiLyte Biosciences have demonstrated that the traditional ELISA assay sensitivity can be significantly improved by using 10-acetyl-3,7-dihydroxyphenoxazine (for HRP detection) and fluorescein diphosphate (for alkaline phosphatase detection) instead of the traditional OPD or TMB detections (for HRP) and BCIP or MUP (for alkaline phosphatase).

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