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A general strategy for studying multisite protein phosphorylation using label-free selected reaction monitoring mass spectrometry

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ABSTRACT

The majority of eukaryotic proteins are phosphorylated in vivo, and phosphorylation may be the most common regulatory posttranslational modification. Many proteins are phosphorylated at numerous sites, often by multiple kinases, which may have different functional consequences. Understanding biological functions of phosphorylation events requires methods to detect and quantify individual sites within a substrate. Here we outline a general strategy that addresses this need and relies on the high sensitivity and specificity of selected reaction monitoring (SRM) mass spectrometry, making it potentially useful for studying in vivo phosphorylation without the need to isolate target proteins. Our approach uses label-free quantification for simplicity and general applicability, although it is equally compatible with stable isotope quantification methods. We demonstrate that label-free SRM-based quantification is comparable to conventional assays for measuring the kinetics of phosphatase and kinase reactions in vitro. We also demonstrate the capability of this method to simultaneously measure relative rates of phosphorylation and dephosphorylation of substrate mixtures, including individual sites on intact protein substrates in the context of a whole cell extract. This strategy should be particularly useful for characterizing the physiological substrate specificity of kinases and phosphatases and can be applied to studies of other protein modifications as well.

The function of most proteins is regulated in some way by enzymatic posttranslational modifications (PTMs)[1]. A large number of covalent PTMs exist in nature and can affect many aspects of protein function, including enzymatic activity, binding partners, stability, structure, and cellular localization. Most PTMs are reversible and, therefore, allow dynamic control of protein function. Reversible phosphorylation of serine, threonine, and tyrosine, controlled by the opposing activities of kinases and phosphatases, is the most abundant and heavily studied regulatory PTM in eukaryotic cells. At least 75% of human proteins are phosphorylated in vivo [1], and many proteins are phosphorylated on multiple sites that can be subject to differential regulation [2], often by more than one kinase and/or phosphatase. As an example, more than 100 in vivo phosphorylation sites have been mapped on the human anaphase-promoting complex (APC), a large E3 ubiquitin ligase complex that regulates progression through mitosis [1,3,4]. Evidence suggests that the APC is regulated, both positively and negatively, by at least four different kinases [5–12]. This complexity makes functional studies of protein phosphorylation challenging. There is a need for improved methods for detection and quantification of individual PTM sites on multiply modified proteins.

Very few analytical methods employed to study phosphorylation, or PTMs in general, are effective at quantitatively detecting multiple sites independently on the same protein. Mass spectrometry (MS) is one exception. Reduction of proteins to peptides is an effective way to separate the PTMs on a single protein into distinct molecules for mass spectral analysis. Tandem MS can provide the sequence of individual peptides and the exact location of any PTMs. Although the complexity of biological systems poses a constant challenge to the detection and quantification of target molecules, the development of affinity enrichment strategies, multidimensional chromatographic separations, improved instrument designs, and various other methodological advances are steadily allowing

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[1] *Abbreviations used:* PTM, posttranslational modification; APC, anaphase-promoting complex; MS, mass spectrometry; SRM, selected reaction monitoring; HPLC, high-performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethlysulfonyl fluoride; DTT, dithiothreitol; Clb2-PrA, Clb2-protein A; IgG, immunoglobulin G; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CE, collision energy; EMV, electron multiplier voltage.

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more comprehensive study of the proteome. Selected reaction monitoring (SRM) MS, which relies on the filtering capabilities of a triple quadrupole mass spectrometer, has become a popular method for direct detection of specific target proteins within complex biological samples. Because of its sensitivity and specificity, SRM is also a useful approach for the quantitative study of PTM regulation in living systems.

To fully understand the regulation and function of PTMs, it is necessary to measure their dynamic changes under different conditions and in response to physiological and environmental signals. A variety of methods exist for the absolute and relative quantification of proteins and PTMs from peptide-based mass spectral data [13,14]. Most quantitative MS approaches employ some form of stable isotope dilution, which has the advantage of allowing simultaneous analysis and comparison of multiple samples or a single sample with an internal standard [15–17]. However, the use of stable isotopes is not essential for quantification, and a number of "label-free" approaches have also been developed [14]. Label-free quantification requires comparison of separate MS experiments, and this necessitates exquisite care in sample preparation and the appropriate controls or standards to account for run-to-run signal variations. Although stable isotope-based methods are generally thought to be more accurate than label-free approaches [13], there are important advantages to label-free methods such as simplicity and general applicability. With label-free quantification, there are no specialized reagents required and no extra chemical reaction and sample processing steps, and the methods are suitable for any experimental system. For these reasons, label-free quantitative MS methods have important advantages for the study of PTMs and are becoming more common.

A variety of strategies have been developed for quantitative MS-based analysis of phosphorylation and other PTMs (see, e.g., Refs. [18–24]). The majority of these methods rely on stable isotope labeling or the addition of specific standards for quantification. Some are limited to studies of synthetic peptide substrates or highly purified protein preparations or require phosphopeptide enrichment steps. We set out to establish a strategy that will be generally useful for the quantitative measurement of changes in phosphorylation on intact proteins. Our approach uses label-free quantification of phosphorylation stoichiometry [21,25,26] for simplicity, compatibility with all experimental systems, and practicality for studies of large numbers of phosphorylation sites. We used SRM MS of peptides generated by enzymatic digest because the sensitivity, specificity, and dynamic range of SRM [27] make it suitable for analysis of highly complex biological samples, and it is also capable of simultaneous monitoring of up to hundreds of target molecules in a single sample run. Related SRM MS methods have been developed and used recently. In one case, changes in phosphorylation of the Pho4 transcription factor in budding yeast were measured [28], but without a rigorous quantification strategy. In another case, autophosphorylation sites on the Lyn tyrosine kinase in mouse xenograft tumors were monitored [26]. Our strategy is equally useful for studying phosphatases and kinases, and it can be applied in vitro as an enzymatic assay or in vivo to quantify changes in many phosphorylation sites in response to experimental variables. Among other potential applications, we predict that it will be very useful for the exploration of kinase and phosphatase specificity. The general method is also suitable for studying other PTMs.

In this article, we describe proof-of-principle experiments that validate this strategy. We demonstrate that SRM MS combined with label-free quantification is comparable to conventional assays for measuring reaction rates and steady-state kinetic parameters of phosphatases and kinases. We also demonstrate the utility of the method for simultaneously monitoring multiple phosphorylation sites on intact protein substrates, including in a whole cell extract, and the potential to reveal different responses of individual sites to experimental variables. Possible experimental designs and practical aspects of applying this method to large-scale studies in complex biological mixtures and in vivo are discussed.

Materials and methods

Peptide synthesis and purification

Peptides were synthesized using CLEAR-Amide resin (Peptides International) at a 50-μmol scale by solid-phase Fmoc chemistry on a Prelude peptide synthesizer (Protein Technologies) essentially as described previously [29]. The only modification was that coupling times for Fmoc-phosphoserine and Fmoc-phosphothreonine (Anaspec) were increased to 3 h. Unphosphorylated Fmoc-protected amino acids were obtained from Peptides International. Synthesis products were resuspended in 5% acetonitrile/0.1% trifluoroacetic acid (TFA), fractionated by high-performance liquid chromatography (HPLC) using a preparative C18 column (Agilent Technologies), and evaluated by matrix-assisted laser desorption/ionization time-of-flight (MALDI–TOF) MS (Voyager 4800, Applied Biosystems). Fractions exhibiting the highest product purity were pooled, lyophilized, and resuspended in 50 mM Tris–HCl (pH 8.0) at approximately 5 mM based on weight. Precise concentrations of phosphopeptides were determined by measuring the inorganic phosphate released after ashing in a magnesium nitrate solution and reaction with an ammonium molybdate/malachite green mixture as described previously [30]. Unmodified peptide concentrations were determined by amino acid analysis at the Purdue Proteomics Facility.

Protein purification

The *Saccharomyces cerevisiae FIN1* gene was cloned into pGEX-6P-1 (GE Healthcare) to create an in-frame fusion with the vector GST sequence. The N-terminal fusion protein GST-Fin1 was overexpressed in 3 L of *Escherichia coli* culture by 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) induction at 37° C for roughly 4 h. Unless otherwise stated, all purification steps were performed at 4° C. Washed cells were suspended in 5 pellet volumes of 50 mM Hepes (pH 8.0), 1% Triton X-100, 250 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 10% glycerol, 1 mM phenylmethlysulfonyl fluoride (PMSF), 100 μM leupeptin, and 1 μM pepstatin, treated with 1 mg/ml lysozyme on ice for 30 min, and lysed by sonication on ice. The extract was clarified by centrifugation at 35,000*g* for 30 min and then incubated with 500 μl of GST-Bind resin (EMD Biosciences) for 30 min. Unbound proteins were then removed by extensive washing with 50 mM Hepes (pH 8.0), 0.1% Triton X-100, 250 mM NaCl, 1 mM EDTA, and 10% glycerol. GST-Fin1 was then eluted with the same buffer supplemented with 10 mM reduced glutathione and dialyzed against 10 mM Hepes (pH 7.5), 10 mM $MgCl_2$, 50 mM NaCl, 0.5 mM dithiothreitol (DTT), and 10% glycerol overnight. His6-Cdc14 was purified as described previously [31]. Following purification, pooled Cdc14 fractions were exchanged into 50 mM Tris–HCl (pH 7.5), 300 mM NaCl, 2 mM EDTA, and 0.1% β-mercaptoethanol using a G-25 desalting column.

Saccharomyces cerevisiae Cdk1 (Clb2-Cdc28) was purified from yeast strain BY4741 *sic1Δ* overexpressing a Clb2-protein A (Clb2-PrA) fusion protein from BG1805-CLB2 (Open Biosystems). Clb2-PrA expression was induced for 4 h at 30° C by the addition of 2% galactose to the YP-raffinose medium when cells had reached mid-log phase. Washed cells were lysed in 5 pellet volumes of 50 mM Tris–HCl (pH 8.0), 250 mM NaCl, 10% glycerol, 0.1% Triton X-100, 20 mM NaF, 1 mM PMSF, 100 μM leupeptin, and 1 μM pepstatin