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Improved Enrichment Strategies for Phosphorylated Peptides on Titanium Dioxide Using Methyl Esterification and pH Gradient Elution

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Abstract

Improvements to phosphopeptide enrichment protocols employing titanium dioxide (TiO₂) are described and applied to identification of phosphorylation sites on recombinant human cyclin-dependent kinase 2 (CDK2). Titanium dioxide binds phosphopeptides under acidic conditions and they can be eluted under basic conditions. However, some nonphosphorylated peptides, particularly acidic peptides, bind and elute under these conditions as well. These nonphosphorylated peptides contribute significantly to ion suppression of phosphopeptides as well as increasing sample complexity. We show here that the conversion of peptide carboxylates to their corresponding methyl esters sharply reduces nonspecific binding, improving the selectivity for phosphopeptides, just as reported for IMAC columns. We also present evidence that mono-phosphorylated peptides can be effectively fractionated from multiply phosphorylated peptides, as well as acidic peptides, via stepwise elution from TiO₂ using pH step gradients from pH 8.5 to pH 11.5. These approaches were applied to human CDK2 phosphorylated *in vitro* by yeast CAK1p in the absence of cyclin. We confirmed phosphorylation at T160, a site previously documented and shown to be necessary for CDK2 activity. However, we also discovered several novel sites of partial phosphorylation at S46, T47, T165, and Y168 when ion suppressing nonphosphorylated peptides were eliminated using the new protocols.

Keywords

phosphorylation; phosphopeptides; enrichment; phosphoproteomics; titanium dioxide; methyl esterification; cyclin dependent kinase 2

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Introduction

Reversible phosphorylation is arguably the main mechanism that contributes to the regulation and large dynamic range of protein expression in cells. This post-translational modification influences the regulation of such fundamental cellular processes as signal transduction, modulation of kinase activity, DNA replication, transcription, translation, and protein localization [1,2,3]. It is not only ubiquitous (4,5), but dynamic by nature (6). In order to understand the role of phosphorylation on protein function, it is necessary to identify the specific sites of amino acid phosphate modifications under varying physiological and developmental conditions.

Despite the importance and widespread occurrence of phosphorylation as well as advancements in analytical instrumentation, complete identification of phosphorylation sites remains a significant challenge [7,8]. The main obstacles to be overcome in order to meet this challenge are the low stoichiometry of phosphate modifications [9] on most proteins and some shortcomings of modern mass spectrometry, the major technology for analyzing peptide and protein structures [10]. Despite the advantages (speed, sensitivity, and robustness) of mass spectrometry, this tool exhibits some fundamental challenges when analyzing phosphopeptides [11]. Phosphopeptides exhibit low relative ionization efficiency in positive mode, compared to most nonphosphorylated peptides, which suppresses their detection, particularly in complex mixtures [4,12]. Negative mode has other challenges including generally lower sensitivity [13]. In addition, when phosphopeptides are detected, identification of the peptide and determination of the site(s) of phosphorylation can be difficult due to the poor and uninformative fragmentation exhibited by phosphopeptides in tandem mass spectrometry (MSMS) [11].

These factors can be partially obviated by the increased ion intensities that can be obtained through enrichment for phosphopeptides from proteolytic digests of target proteins. Immobilized metal affinity chromatography (IMAC) has been the most widely used strategy, taking advantage of phosphate affinity for metal ions [5,14]. However, carboxylate groups on acidic amino acid residues can also exhibit significant affinities for IMAC supports and result in non-specific binding of nonphosphorylated, acidic peptides as well [5]. The collection of early eluting fractions in strong cation-exchange (SCX) chromatography at pH < 2.7 has been demonstrated to be an effective enrichment technique, although many phosphopeptides still elute in later fractions [4,15]. Anti-pY antibodies have been applied for enrichment of pY containing phosphopeptides [16,17]. This is especially beneficial since tyrosine phosphorylation is generally of much lower abundance than phosphothreonine and phosphoserine. Another strategy reported recently employs isoelectric focusing of previously methyl esterified peptide mixtures [18]. Esterification neutralizes the charge contribution of carboxylates to the isoelectric point, pI, of the peptides. This results in discrimination in pI shift between phosphopeptides and nonphosphorylated peptides allowing for the enrichment of phosphopeptides in the low pI range (pI 3–7). This approach has only been demonstrated on α - and β -casein (bovine) at this point. Recently, titanium dioxide (TiO₂) has been introduced as a useful medium for phosphopeptide enrichment [19]. In addition to its specificity for phosphopeptides, TiO₂ is relatively simple to implement. However, as in the case for IMAC, carboxylates also exhibit high affinity for TiO₂ resulting in non-specific binding of nonphosphorylated, acidic peptides [20]. It has been demonstrated using simple mixtures of α - and β -casein that samples loaded onto TiO₂ in the presence of 2,5-dihydroxybenzoic acid (DHB) resulted in reduced non-specific binding [19]. However, non-specific binding is still apparent when this approach is applied to real complex mixtures [20]. Casein phosphopeptides are phosphorylated at nearly quantitative stoichiometry and are all acidic. This gives them two modes of interaction and high affinities toward metal ions and TiO₂. Binding of acidic peptides to affinity supports is mediated predominantly via the carboxylates and efforts to reduce

binding have focused on blocking the carboxylate groups or by introduction of weak competitors. Methyl esterification of peptide carboxylate groups has been shown to enhance the selectivity of IMAC for phosphopeptides by reducing non-specific binding of acidic peptides. This effect had not been demonstrated for TiO₂.

In this study, we describe two approaches for improving the selectivity for phosphopeptides on TiO₂. The first approach calls for eluting peptides from TiO₂ by a pH step gradient. Conventionally, peptides are loaded under acidic conditions (optionally with an additive like DHB) and eluted under basic conditions. It has been demonstrated that the pH of the elution buffer should be above 10.5 in order to elute multiply phosphorylated peptides [19]. This suggests that fractionation of phosphopeptides is feasible by eluting with solutions of sequentially increasing pH, providing increased enrichment. Our protocol calls for the application of 100 mM triethylammonium bicarbonate (pH 8.5) followed by subsequent elution with 3% ammonium hydroxide (NH₄OH). Our results show that the first fraction is highly enriched in mono-phosphorylated peptides while the second fraction contains predominantly multiply phosphorylated peptides, as well as an abundance of acidic, nonphosphorylated peptides. The end result is a higher level of enrichment for mono-phosphorylated peptides. The second approach applied here is enrichment of phosphopeptides using TiO₂ after peptide carboxylate groups have been converted to methyl esters. When applied in IMAC, methyl esterification results in a dramatic increase in specificity for phosphopeptides [5]. When applied to TiO₂, we observed very little non-specific binding under these conditions. This strategy resulted in the identification of a doubly phosphorylated peptide in a mapping experiment of CDK2 that was not detected without methyl esterification. Despite some drawbacks to the esterification reaction, such as incomplete reaction and side reactions, this approach shows great promise for identifying low level phosphopeptides.

We applied these strategies to characterize the phosphorylation sites of human Cyclin-dependent kinase 2 (CDK2), co-expressed with yeast CAK1p in *E. coli*, in the absence of cyclin. Like other CDKs, CDK2 plays a key role in the cell cycle and in cellular proliferation, modulating the transition from G₁ to S phase in eukaryotes [21]. CDKs are a family of enzymes whose activity is regulated by association with a cyclin subunit [22,23]. In addition, CDK2 must be phosphorylated on its activation loop segment at T160 [22,23]. In vertebrates, this phosphorylation event is carried out by CAK, a trimeric complex composed of CDK7/cyclin H/Mat1 and, currently, is the only known CAK for CDK2 [24]. It was discovered a decade ago, however, that monomeric CAK1p from yeast substitutes human CAK, activating CDK2 in vitro through phosphorylation at T160 in the presence of cyclin [25]. Recently, it was discovered that CDK2 can auto-phosphorylate (i.e., auto-activate) in the absence of CAK [26]. This suggests that multiple mechanisms of activation through phosphorylation may exist in vivo, including the possibility of other unknown CAKs. The other indispensable input for CDK2 activation in vertebrates is its association with cyclin. Surprisingly, previous experiments incubating CDK2 with CAK1p and radio-labeled ATP (in the absence of cyclin) demonstrated phosphorylation of CDK2 [25]. However, it did not result in active CDK2 and this approach did not allow for determining where phosphorylation occurs under these conditions. Hypothetically, CDK2 was phosphorylated at sites other than T160, or multiple sites in addition to T160. We present here the characterization of CDK2 phosphorylation by yeast CAK1p in the absence of cyclin using new approaches to phosphopeptide enrichment with TiO₂. The methods described here indicate that CDK2 is indeed phosphorylated at T160 under these conditions as well as at several novel sites of partial phosphorylation at S46, T47, T165, and Y168. These results demonstrate the potential of these approaches for qualitatively identifying low level phosphorylation sites in proteins.

Materials and Methods

Materials

Triethylammonium bicarbonate (TEAB), 2,5-dihydroxybenzoic acid (DHB), iodoacetamide, dithiothreitol, 3-indoleacrylic acid, and β -casein (bovine) were all purchased from Sigma-Aldrich (St. Louis, MO). Titanium dioxide (TiO₂) particles (50 μ m) were purchased from Glygen Corp. (Columbia, MD). Sequencing grade, modified trypsin was purchased from Promega (Madison, WI). Ammonium hydroxide (NH₄OH), trifluoroacetic acid (TFA), HPLC grade water and acetonitrile (ACN) were purchased from Fisher Scientific (Fairlawn, NJ).

Cloning, transformation, and purification of CDK2

Full length human CDK2 was cloned into a pET28a vector (Novagen), modified so the Thrombin protease site is replaced with a tobacco etch virus (TEV) protease recognition site. Yeast Cak1p cloned into pGEX (GE Healthcare) was a gift of Mark Solomon [25]. The two plasmids are co-transformed into BL21(DE3) cells (Invitrogen), grown in terrific broth media (+Kanamycin/Ampicillin) supplemented with glucose and lactose to induce expression without isopropyl- β -D-thiogalactoside (IPTG). After growing the cells to OD₆₀₀ of 0.8 at 37 °C, the temperature was reduced to 16 °C for overnight expression. Cells were lysed in an Avestin cell homogenizer under ~18,000 psi and centrifuged at 30,000 rpm in an ultra-centrifuge for 30 minutes.

For CDK2 purification, supernatant was diluted into 500mM sodium chloride, 25mM sodium phosphate buffer, pH 7.4 (Buffer A) and loaded onto a 5ml HisTrap HP chelating nickel (Ni) affinity column (GE Healthcare). Protein was eluted over a 20 column volume gradient of 4–50% buffer B (Buffer A + 500mM imidazole). TEV protease was added to the pooled fractions to cleave the His affinity tag and the mixture was dialyzed overnight against 150mM sodium chloride, 25mM Tris, pH 8.5. Cleaved protein was then passed through a second Ni affinity column to remove the tagged-protease, cleaved tag, and contaminants. The protein was then diluted 1:5 in 20mM Glycine pH 10.0 and loaded onto a Source 15Q Anion exchange column (GE Healthcare) and eluted with a 3–30% gradient to 1M sodium chloride over 30 column volumes.

β -casein sample preparation

Lyophilized β -casein was weighed and dissolved in 100 mM TEAB (pH 8.5). Digestion with trypsin was carried out overnight at 37 °C at a ratio of 50:1 (w/w, protein:trypsin). The final concentration of β -casein was 1 μ g/ μ L. The stock solution was divided into 30 μ L aliquots and stored at –20 °C until needed.

CDK2 sample preparation

30 μ g of CDK2 (concentration 1.7 mg / mL) was precipitated to remove glycerol and salt by adding cold acetone (–20 °C), to the sample at a 4:1 ratio (v/v, acetone:sample) in a micro centrifuge tube. The tube was then incubated at –20 °C for 2 hours. The precipitated protein was spun down at 12,500 G for 7 minutes at 4 °C. After decanting the acetone, the protein pellet was washed twice with 100 μ L of cold acetone. The pellet was dried in a speedvap for ten minutes (no heat) to evaporate residual acetone. The pellet was solubilized in 100 mM TEAB (pH 8.5) containing 0.1% SDS. Disulfide bonds were reduced with 2 μ L of 50mM tris-(2-carboxyethyl)phosphine (incubated one hour at 60 °C) and cysteine residues were modified with 1 μ L of 200mM methylmethanethiosulfate (ten minute reaction time at room temperature). 1 μ g trypsin was added and the mixture was incubated at 37 °C overnight.

Methyl-esterification of tryptic peptides

Prior to methyl esterification, peptides were dried to completion in a Speedvac for at least six hours. Methyl esterification was performed as described previously [6]. Briefly, the peptides were dissolved in 500 μ L of 2N methanolic hydrochloride, which was prepared by slow, dropwise addition of 40 μ L of thionyl chloride to 500 μ L of anhydrous methanol (Sigma-Aldrich, St. Louis, MO). After sonication for 15 minutes, esterification was allowed to proceed for two hours at room temperature. The methyl-esterified peptides were then dried to completion in a Speedvac and reconstituted in 0.1% TFA. In some cases, the procedure was repeated after drying. Methyl and dimethyl-phosphoesters are not observed under these conditions [5].

Enrichment of phosphopeptides with TiO_2

Phosphopeptide enrichment was performed via phosphate affinity chromatography on TiO_2 particles (50 μ m, Glygen Corp., Columbia, MD). 1 mg of TiO_2 particles were conditioned with 100 μ L of 80% acetonitrile (ACN) and 1% trifluoroacetic acid (TFA) in a microcentrifuge tube. The suspension was vortex mixed for five minutes, spun down at 9000 G for 30 seconds, and the supernatant was discarded. All subsequent centrifugations were performed at the same speed and duration. For each experiment, an estimated 500 ng of peptides were diluted in 200 μ L DHB solution (2,5-dihydroxybenzoic acid (DHB) at a concentration of 66 mg/mL in 80% ACN/1% trifluoroacetic acid). The tube was then vortex mixed for 30 minutes, centrifuged, and the supernatant removed. The TiO_2 particles were then washed twice with 200 μ L of DHB solution followed by two washes with 80% ACN/1% TFA. Phosphopeptides were eluted from the TiO_2 particles via incubation with 20 μ L of 3% ammonium hydroxide (pH 11.5) with shaking for 15 minutes. For fractionation of monophosphorylated peptides from multiply phosphorylated peptides, elution was performed sequentially with 20 μ L of 100 mM triethylammonium bicarbonate (TEAB), (pH 8.5) followed by 3% ammonium hydroxide (pH 11.5). After centrifugation, the supernatant was immediately dried to completion. The peptides were re-dissolved in 10 μ L of 0.1% TFA.

Mass spectrometry

Peptides collected after phosphopeptide enrichment with TiO_2 were analyzed by both MALDI MSMS and nanospray-LC-MSMS. For MALDI, 0.5 μ L of sample was spotted onto a stainless steel MALDI plate followed by 0.5 μ L of indoleacrylic acid matrix solution. The matrix was prepared by mixing 66 μ L of supernatant from a saturated solution of 3-indoleacrylic acid (previously prepared in 50% ACN/0.1% acetic acid) with 125 μ L of 50% ACN/0.1% acetic acid and 8 μ L of 250 mM ammonium dihydrogenphosphate (Sigma-Aldrich). The spots were analyzed with a 4800 MALDI TOF/TOF Analyzer (Applied Biosystems / MDS SCIEX, Framingham, MA). For analysis by nanospray-LC-MSMS, peptides were separated using reversed-phase column (10cm $\times$ 50 μ m i.d.) slurry packed in a tapered tip fused silica capillary column (New Objective, Inc.) with POROS 10 R2 reversed-phase packing material (10 μ m particles; Applied Biosystems, Foster City, CA) in-line to the mass spectrometer. An ACN gradient was delivered by a binary nano-LC pump (nanoLC•2D™; Eksigent). Buffer A was composed of 0.1% formic acid and buffer B was composed of 90% ACN/0.1% formic acid. Peptides were delivered to the column at 3% buffer B and separated via a linear gradient of increasing buffer B to 60% at 45 minutes. It was held at 60% B for five minutes and then the composition was reset to 3% B for the duration (60 minute run). Eluting peptides were analyzed with an ion trap/Orbitrap hybrid mass spectrometer (LTQ Orbitrap, Thermo Fisher Scientific, San Jose, CA). Source settings included: spray voltage 1.3 kV; spray current 100 μ A; capillary voltage 35 V; ion transfer tube temperature 220 $^{\circ}$ C. The mass spectrometer was operated in the data-dependent mode to automatically switch between Orbitrap-MS and ion trap-MSMS acquisition. Survey full scan MS spectra (from m/z 300–2000) were acquired in the orbitrap at

a resolution of 30,000 (based on m/z 400). The five most intense ions were sequentially isolated for fragmentation in the linear ion trap using collisionally induced dissociation with a normalized collision energy setting of 27.0.

Data analysis and interpretation

Data acquired with the LTQ-Orbitrap was searched against the IPI-human database using MASCOT. Search parameters included: two missed cleavages from trypsin proteolysis; fixed modification of cysteine residues with MMTS; variable modifications included deamidation (N and Q), oxidation (M), and phosphorylation (S, T, and Y). No confidence thresholds were established since the bulk of the data analysis was performed via manual *de novo* interpretation of the MSMS spectra, including all MALDI MSMS spectra.

Results and discussion

pH dependent elution for fractionation of mono- from multi-phosphorylated peptides

It was emphasized by Larsen *et al.* that elution buffers of pH > 10.5 were necessary to efficiently elute multiply phosphorylated peptides from TiO₂ [19]. This suggested that fractionation, in addition to enrichment, of monophosphorylated from multiply phosphorylated peptides would be possible by eluting sequentially from low to high pH. We explored this possibility using a two-step elution strategy. Peptides were eluted from TiO₂ initially with 100 mM TEAB (pH 8.5) followed by a subsequent elution with 3% NH₄OH (pH 11.5). The sample was loaded in the presence of 2,5-dihydroxybenzoic acid (DHB) to limit nonspecific binding.

This approach was evaluated using β -casein which provides two phosphopeptides upon digestion by trypsin, a monophosphorylated peptide of monoisotopic mass (MH^+) 2061.8 Da and tetra-phosphorylated peptide of mass 3122.3 Da. Figure 1a displays a MALDI MS spectrum of the digest before TiO₂ enrichment. Figure 1b displays the spectrum of the peptides eluted with 100 mM TEAB. The number of asterisks indicates the number of phosphate groups on the peptides and indicates all mono-phosphorylated peptides were eluted in the TEAB fraction. Noteworthy is that this sample was a digestion of β -casein, yet we also detected several α -casein phosphopeptides (designated appropriately as α and β in the figure). This is a known contaminant according to the distributor. The spectrum in Figure 1c represents the peptides eluted subsequently to TEAB with NH₄OH (pH 11.5). Here, the tetraphosphorylated peptide of β -casein was detected, along with some carryover of one of the α -casein monophosphorylated peptides. The peaks at m/z 3024.0 and 2925.5 represent in-source decay products of the tetra-phosphorylated peptide corresponding to neutral loss of one and two H₃PO₄ molecules respectively. These results demonstrate not only the effectiveness of TiO₂ for phosphopeptide enrichment, but the capacity to fractionate phosphopeptides according to their relative levels of phosphorylation by using pH step gradients. It is noteworthy that, although nonspecific binding was minimal, there were more peaks representing nonphosphorylated peptides in the NH₄OH fraction than the TEAB fraction. This implies that some nonphosphorylated peptides are more tightly bound to TiO₂ than monophosphorylated peptides. It also demonstrates that mono-phosphorylated peptides can be selectively enriched using lower pH elution conditions.

Enrichment/fractionation of CDK2 phosphopeptides with TiO₂

Initially, the optimized protocol by Larsen *et al.* was applied to the tryptic digestion products of CDK2 which employs sample loading in the presence of DHB and one elution step with 3% NH₄OH (pH 11.5). Before enrichment, a small portion of the CDK2 digestion products were analyzed by MALDI MS as a control (Figure 2a). Phosphopeptides are again indicated with asterisks. Figure 2b displays a MALDI MS spectrum of the peptide mixture after the one-step elution from TiO₂ with NH₄OH. Enrichment is apparent with three monophosphorylated

peptides identified, one represented by the base peak of the spectrum, and a reduction in the overall complexity of the mixture. However, there was also a greater extent of nonspecific binding in this case, indicated by the number of nonphosphorylated peptides observed.

An equivalent aliquot of the CDK2 digest was enriched using two successive pH elution steps as described above for β -casein. Figure 2c displays a MALDI MS spectrum of CDK2 peptides eluted in the first fraction with TEAB (pH 8.5). All of the monophosphorylated peptides were present in this fraction. Despite two intense peaks, m/z 1096.8 and 1489.0, representing two nonphosphorylated peptides, this spectrum exhibits a notable reduction in complexity compared to one step elution with NH_4OH (Figure 2b). Figure 2d displays a MALDI MS spectrum of the subsequent elution of peptides from TiO_2 with NH_4OH (pH 11.5). Except for some carry over of the phosphopeptide represented by m/z 1647.9, this spectrum is practically devoid of phosphopeptides. This supports the suggestion that monophosphorylated peptides can be enriched to a greater extent using lower pH elution conditions since at least some acidic peptides appear to exhibit higher affinity for TiO_2 . Although we did not detect multiply phosphorylated peptides in this particular experiment, this result is consistent with monophosphorylated peptides being selectively separated from multiply phosphorylated peptides on TiO_2 using more weakly basic pH elution.

As in the case of β -casein, there was a greater number of nonphosphorylated peptides observed in the NH_4OH fraction, suggesting that many nonphosphorylated peptides bind more strongly than mono-phosphorylated peptides. The nonphosphorylated peptides observed in both fractions were also observed in the initial enrichment of CDK2 phosphopeptides using elution with NH_4OH (Figure 2b). Binding of nonphosphorylated peptides occurs presumably through interactions between the carboxylate groups of the C-terminus as well as the aspartic acid and glutamic acid residues with TiO_2 [18]. We used DHB in the sample loading solutions which has been shown to minimize nonspecific binding when applied to β -casein [19]. However, this strategy was not as effective when applied to CDK2. This is not surprising since β -casein contains few acidic peptides beyond the phosphopeptides themselves. All of the most abundant, nonspecifically bound CDK2 peptides identified in this study contained at least one acidic residue in addition to the C-terminus.

Enrichment of phosphopeptides after conversion of carboxylate groups to methyl esters

As described in the study by Larsen et al., phosphate and carboxylate groups have similar binding constants to TiO_2 [19]. However, they are known to bond with TiO_2 in non-competing modes [19]. This served as the basis for using DHB as an inhibitor to carboxylate interactions with TiO_2 since it binds in the same mode as carboxylate groups. These two binding modes are proposed to result from heterogeneity on the surfaces of the TiO_2 particles yielding two different types of adsorption sites. Although DHB competes with carboxylate groups for binding sites on TiO_2 , it does not appear to be as effective at inhibiting nonspecific binding when applied to samples other than casein. We explored the possibility of minimizing nonspecific binding by blocking carboxylate interactions through methyl esterification.

After converting carboxylate groups to methyl esters and passing the peptides over TiO_2 , peptides were eluted in one step with 3% NH_4OH (pH 11.5). Figure 3a displays the MALDI MS spectrum of the methyl esterified peptides eluted from TiO_2 . As exhibited in the figure, this procedure resulted in a sharp increase in specificity for phosphopeptides (marked by asterisks). Very little nonspecific binding was observed. This is consistent with nonspecific binding arising primarily through carboxylate interactions and demonstrates the potential for maximizing selectivity for phosphopeptides when carboxylates are modified by methyl esterification.

The spectrum shown in Figure 3a is diagnostic for partial methyl esterification and was selected to illustrate that even when all peptides do not undergo complete reaction, the procedure is effective at minimizing non-specificity. A case where complete methyl esterification was achieved is shown in Figure 3b for comparison, where methyl esterification was performed twice in succession. Partial esterification is often observed [27, 28] and results in a single peptide appearing as an array of ions separated by 14 Da (for +1 ions), which is the mass difference of the peptide each time a carboxylate group is modified. One example in Figure 3A is the pair of peaks at m/z 1596.4 and 1610.4, which represent the same peptide. A second source of peptide heterogeneity, exhibited in both Figures 3a and 3b, includes potential side reactions of the methyl esterification chemistry [26, 28]. Notable in this case, are the single and double oxidations of tryptophan (W) side chains, represented by nominal masses 1691 and 1707 in the spectra. The doubly oxidized W residue (on mass 1707) was confirmed by MSMS (data not shown). Evidence to support the singly oxidized W on mass 1691 was lacking, but it's assumed to be an oxidized W based on the presence of the doubly oxidized analog. An additional methyl group was observed on doubly oxidized W, represented by nominal mass 1721 in Figure 3 and confirmed by MSMS (data not shown), although we could not elucidate the resulting structure or the chemistry by which it occurred. As with incomplete esterification, these side reactions could potentially reduce the signal to noise ratios for phosphorylated peptides containing W by spreading the signals across multiple ions. Despite side reactions and incomplete esterification of carboxylate groups, a remarkable increase in phosphopeptide selectivity is achieved. Although the incompleteness of the esterification reaction, as well as side reactions, might reduce sensitivity, this is countered by the reduction in nonspecific binding of more abundant nonphosphorylated peptides which greatly lowers the ion suppression effect in MALDI. It is worth pointing out that we identified a doubly phosphorylated peptide, represented by the peak at m/z 1755.4 in Figure 3a, which was not detected without methyl esterification.

Figure 3b shows that it is possible to drive methyl esterification further towards completion simply by performing sequential methyl esterifications. The emergence of m/z 1624.9 in Figure 3b, and disappearance of the incompletely methylated analogs represented by m/z 1596.4 and 1610.4 in Figure 3a demonstrate the effect of two tandem esterifications. However, the additional handling of the sample can result in some yield loss indicated by the overall reduction in ion intensity observed in Figure 3b and the absence of the peak representing the doubly phosphorylated peptide at m/z 1755.4.

Phosphorylation site identification using tandem mass spectrometry

The approaches described in this study identified a total of five phosphorylation sites on CDK2, four of which are novel. These are summarized in Table 1. Peptide 1 represents a previously identified phosphopeptide on CDK2 with phosphorylation at T160 [22,23] (data not shown). T160 is located on the activation segment, or T loop, of CDK2 and phosphorylation at this site is one input necessary for activation [22,23]. This finding verifies that CAK1p phosphorylates CDK2 at the activation site (T160) in the absence of cyclin.

As summarized in Table 1, CAK1p also phosphorylates CDK2 at other sites under these conditions. We also identified sites of partial phosphorylation at Y168 and T165 as indicated by peptides 2 and 3 respectively. These are residues near the activating site of phosphorylation at T160. Figure 4 displays a MSMS spectrum of the doubly charged precursor ion, m/z 878.38 (MH^+ 1755.76) for this doubly phosphorylated peptide obtained from an Orbitrap mass spectrometer. This peptide was only detected after methyl esterification the sample prior to phosphopeptide enrichment. The observed mass matches the theoretical mass of this peptide (assuming two phosphorylation sites and complete esterification) accurately to two decimal points. The base peak of the spectrum indicates a loss of 80 Da from the precursor mass,

indicating loss of HPO_3 from the peptide. This is a common neutral loss product of peptides phosphorylated at Tyr residues and indicates the presence of at least one phosphorylated Tyr residue. The inset of the figure summarizes the b and y fragment ions observed in the spectrum. The asterisks refer to ions with one or two phosphate moieties attached. The fragment ion b_2 indicates no sites of phosphorylation on the first two residues on the N-terminus. Taking the observed b_3^* ion into consideration, this places a phosphorylation site at T160. At the b_{10} fragmentation site, we observed both the b_{10}^* and b_{10}^{**} fragment ions. This implies that some copies of this peptide are phosphorylated at T165, since this is the only possible site of phosphorylation between the b_3 and b_{10} fragmentation sites, while other copies are not. This also means that the other site of partial phosphorylation must be at Y168. This is verified by the presence of the b_{10}^* and b_{11}^{**} ions. The y fragment ions provide evidence for phosphorylation at T160 and Y168. The interpretation of this is that two isobaric phosphopeptides of the same amino acid sequence and with two sites of phosphorylation (albeit phosphorylation at different sites) co-eluted and were isolated and fragmented together in the mass spectrometer. The evidence suggests that both phosphopeptides were phosphorylated at T160, but some copies were phosphorylated at T165 and others at Y168.

Monophosphorylated peptides 4 through 7 in Table 1 provide evidence for two phosphorylation sites at S46 and T47. Figure 5 displays a MSMS spectrum of a doubly charged precursor ion of m/z 919.46 representing a monophosphorylated peptide of sequence IRLDTETEGVPSTAIR. The observed mass matches the theoretical mass of this peptide with one phosphate moiety. The base peak of the spectrum represents neutral loss of H_3PO_4 , common for peptides with phosphorylated serine or threonine residues. As indicated by the sequence in the inset of Figure 5, two distinct b-ion series emerge on the C-terminal side of the b_{11} fragment ion. The b_{13}^* , b_{14}^* , and b_{15}^* ions indicate phosphorylation at T47, while the presence of ($b_{12}^* - 98$) through ($b_{15}^* - 98$) fragment ions indicates phosphorylation at S46. This interpretation suggests the co-elution, isolation, and co-fragmentation of two isobaric, monophosphorylated peptides in the ion trap with the same amino acid sequence but with phosphorylation on different residues. This indicates the protein is partially phosphorylated at S46 and T47 by CAK1p. We have not detected this peptide with both sites phosphorylated and this could be either because the two sites are mutually exclusive or that the doubly phosphorylated peptide is present below our level of detection. The same conclusion was made for peptides 4 and 5 in Table 1 (data not shown).

Discussion

Greater selectivity for enrichment of phosphopeptides on titanium dioxide supports can be attained by moderating buffer pH (for monophosphorylated peptides) and methyl esterification. These methods were employed in the characterization of CDK2 phosphorylation by yeast CAK1p in the absence of cyclin. We initially evaluated the protocol developed by Larsen et al. [19] which utilized DHB, as an additive to deter nonspecific binding to TiO_2 , and elution with NH_4OH ($\text{pH} > 10.5$). We found that nonspecific binding was still prevalent when applied to CDK2, although phosphopeptide enrichment was also evident. We demonstrated the ability to fractionate mono-phosphorylated peptides from multiply phosphorylated peptides by applying differential pH elutions: a TEAB solution buffered at pH 8.5 followed by a typical NH_4OH solution at pH 11.5. This approach was first applied to a tryptic digest of β -casein. The first fraction was composed almost exclusively of mono-phosphorylated peptides, including several from the contaminant protein α -casein. The tetra-phosphorylated peptide of β -casein was detected exclusively in the second fraction. This demonstrates the feasibility of fractionating peptides with TiO_2 which provides the potential for detecting and identifying more phosphopeptides compared to elution at a single, high pH. We also noted that more nonspecifically bound peptides elute in the high pH fraction than in the first, indicating that many nonphosphorylated peptides possess higher affinities for TiO_2 than mono-

phosphorylated peptides. Elution at lower pH values (~ 8.5) results in improved selectivity (thus, enrichment) for mono-phosphorylated peptides. This was confirmed in application of this approach to CDK2. The TEAB fraction contained all of the monophosphorylated peptides with few nonphosphorylated peptides. The remainder of the bound nonphosphorylated peptides eluted in the second fraction with the multiply phosphorylated peptides. The mono-phosphorylated peptides were therefore, more efficiently enriched by elution at pH 8.5 than a single elution at pH 11.5.

We also investigated the efficacy of methyl esterification to minimize interactions of nonphosphorylated, acidic peptides with TiO₂ via their carboxylate groups. Methyl esterification resulted in almost complete elimination of binding by nonphosphorylated peptides and provided enhanced enrichment even in cases when the methyl esterification was incomplete. The gain in selectivity and overall sensitivity provide the potential for detecting lower level or ion suppressed phosphopeptides that may not be detected with conventional TiO₂ protocols. This was demonstrated with identification of a doubly phosphorylated peptide only when enrichment was performed after esterification.

Collectively, the improvements in the use of TiO₂ to enrich phosphorylated peptides allowed identification of four novel sites of phosphorylation on CDK2: S46, T47, T165, and Y168. Previous studies of cyclin independent phosphorylation of CDK2 by yeast CAK1p confirmed phosphorylation using radio labeled ATP, although phosphorylation under these conditions did not result in activated CDK2 [25]. This suggests that perhaps other residues were phosphorylated as well, resulting in inhibition of CDK2 activity. However, specific sites of phosphorylation were not determined in that study. Future work includes determining the stoichiometry of the phosphorylation events identified in this study.

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Figure 1. MALDI MS spectra of β -casein tryptic digestion products before enrichment with TiO_2 (A), after elution from TiO_2 with TEAB (pH 8.5) (B), and after subsequent elution with NH_4OH (pH 11.5) (C). Peaks corresponding to phosphopeptides are marked with asterisks, the number of which represents the number of phosphates on the peptide. Contaminating α -casein phosphopeptides (marked with α) were detected with β -casein peptides (marked with β).

Figure 2. MALDI MS spectra of human CDK2 tryptic digestion products before enrichment with TiO_2 (A), after one-step elution with NH_4OH (pH 11.5) (B), after elution from TiO_2 with TEAB (pH 8.5) (C), and after subsequent elution with NH_4OH (pH 11.5) (D). Peaks corresponding to phosphopeptides are marked with asterisks.

Figure 3. MALDI MS spectra of CDK2 peptides eluted from TiO_2 after conversion of carboxylate groups to methyl esters. Methyl esterification was performed once (A) and twice (B). Asterisks indicate the number of phosphorylation sites on the peptide.

Figure 4.

Nanospray-LC-MS/MS spectrum of precursor ion m/z 878.38 (charge state +2) representing the doubly phosphorylated peptide of sequence 158-TYTTHEVVTLLWYR-169. The sequence ion coverage is summarized in the inset. The spectrum exhibits evidence for phosphorylation at T160 and partial phosphorylation at T165 and Y168. Asterisks indicate the number of phosphorylation sites on the peptide.

Figure 5.

Nanospray-LC-MSMS spectrum of precursor ion m/z 919.46 (charge state +2) representing the phosphopeptide 35-IRLDTETEGVPSTAIR-50. The inset summarizes the b-ion types observed between and including the b_{11} and b_{15} fragmentation sites. A $b_n + 80$ series indicates phosphorylation at T47. However, the presence of a $b_n - 98$ series identifies phosphorylation at S46.

Table 1
Summary of phosphopeptides and phosphorylation sites identified

	Sequence	Phos. Site(s)	MH ⁺ ^{a,d}	MH ⁺ (m.e.) ^b	MH ⁺ (m.e.) obs.
1	TYpTHEVVTLWYR	T160	1647.8	1691.8 ^c 1707.8 ^d	1691.8 ^c 1707.8 ^d
2	TYpTHEVVTLWpYR	T160, Y168	1727.7	1755.8	1721.8 ^e 1755.4
3	TYpTHEVVpTLWYR	T160, T165	1727.7	1755.8	1755.4
4	LDTEGVPpSTAIR	S46	1568.7	1624.8	1624.9
5	LDTEGVPSpTAIR	T47	1568.7	1624.8	1624.9
6	IRLDTEGVPpSTAIR	S46	1837.9	1894.0	1894.0
7	IRLDTEGVPSpTAIR	T47	1837.9	1894.0	1894.0

^aTheoretical monoisotopic mass.
^bTheoretical monoisotopic mass after complete methyl esterification.
^cMass includes a single oxidation of tryptophan.
^dMass includes a double oxidation of tryptophan.
^eMass includes a double oxidation plus methylation of tryptophan.