

An integrated global strategy for cell lysis, fractionation, enrichment and mass spectrometric analysis of phosphorylated peptides†

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Recently, the field of phosphoproteomics has progressed to the point where thousands of protein phosphorylations can be analyzed simultaneously and used to address significant biological questions. However, several challenges still exist in current LC-MS/MS-based phosphoproteomics methods. Among these are the increased dynamic range of phosphoproteomics samples (due to low stoichiometry of most protein phosphorylations), insufficient inhibition of phosphatase activity, and neutral losses which occur during phosphopeptide fragmentation by MSⁿ. Here we present an improved method, free of conventional phosphatase inhibitors, for sample treatment to minimize phosphatase activity and improve the efficiency of phosphopeptide enrichment. We also present a solution-based IEF method for phosphopeptide fractionation and explore the utility of various fragmentation methods for identifying phosphopeptides and localizing phosphorylation sites.

Introduction

Reversible protein phosphorylation on serine, threonine, and tyrosine residues is believed to be among the most widespread post-translational modifications of proteins and has been shown to regulate a vast array of cellular processes.^{1,2} Recently, studying protein phosphorylation has developed from almost exclusively a reductionist approach, to global analyses capable of tracking thousands of phosphorylation events simultaneously.^{3–5} Originally, two-dimensional gel electrophoresis (2DE) utilizing ³²P-labeled proteins or colorimetric and fluorimetric phosphate specific imaging to detect and visualize phosphoproteins was the method of choice for phosphorylation analysis on a proteome scale (*i.e.*, phosphoproteomics).^{6,7} Despite the high sensitivity of these imaging techniques, relatively low throughput and sensitivity in spot identification has led to the replacement of several 2DE approaches by liquid chromatography–tandem mass spectrometry (LC-MSⁿ). However, even using these new approaches, its reversible nature, the low stoichiometry of protein phosphorylation events, and the lability of the phosphate are still considered significant challenges in phosphoproteomics.

Due to the reversible nature of protein phosphorylation, phosphatases liberated upon cell lysis can quickly and substantially reduce the signal from a given stimulus. By far the most common means of inhibiting phosphatases is through the use of commercially available inhibitors. Vanadium oxides such as

pervanadate and orthovanadate are typically used to inhibit protein tyrosine phosphatases (PTPs). Of the twenty known families of serine/threonine phosphatases, the most common are protein phosphatase 1 (PP1), PP2A and PP2B, which are targeted by common inhibitors such as calyculin A (PP1 and PP2A) and deltamethrin (PP2B). However, while treating live Hepa1–6 liver cells with pervanadate, calyculin A, and deltamethrin, Pan *et al.* compared the abundance of individual phosphorylated peptides to an untreated condition and observed that only 27% increased more than two-fold.⁸

Similarly, in an analysis of the stem cell plasma membrane phosphoproteome, Thingholm *et al.* found that pre-treating cells with calyculin A, sodium pervanadate, or two commercially available phosphatase inhibitor cocktails resulted in only a 10–40% increase in the number of phosphopeptides identified.⁹ In both cases, a majority of phosphotyrosine containing sites were preserved, while phosphoserine and phosphothreonine sites were less effectively protected by the inhibitors. Thus, while inhibitor-based methods are somewhat effective, especially when considering tyrosine phosphorylation, truly global analyses require additional techniques.

Due to the tight spatial and temporal control observed in signalling pathways, protein phosphorylation events typically occur at very low stoichiometry.^{2,10} Thus fractionation and enrichment of phosphorylated peptides and proteins prior to LC-MSⁿ analysis is indispensable for large-scale phosphoproteomics. In addition to subcellular fractionation to purify a given organelle and calcium phosphate precipitation or barium phosphate precipitation through a pH range, several chromatographic approaches have been reported to enrich phosphopeptides based on either their negative charge or polarity.^{3,11,12} These include strong-cation and strong-anion exchange chromatography (SCX and SAX), as well as hydrophilic interaction chromatography (HILIC) and electrostatic repulsion hydrophilic interaction chromatography (ERLIC).^{4,13–18}

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To a lesser extent, isoelectric focusing has also been used to enrich for phosphopeptides in the low pH range.^{19–22} SCX is by far the most commonly used technique for phosphopeptide pre-fractionation, as the strong negative charge on phosphate causes phosphopeptides to elute earlier than the majority of non-phosphorylated peptides. However, with several chromatographic matrices (*e.g.* SAX and HILIC resins where phosphopeptides bind very strongly and are difficult to elute⁷) sample is lost either in the flow through or by irreversibly binding the resin. Precipitation methods can also result in similar sample losses, thus fractionation procedures such as in-solution IEF offer the advantage of virtually complete sample recovery.

Following a pre-fractionation step, most studies employ one of a few approaches offering much higher selectivity for phosphopeptides. While immunoprecipitation of tyrosine phosphorylated species has proven effective, poor specificity has generally been observed for phosphoserine and phosphothreonine antibodies.^{23–26} Immobilized metal affinity (IMAC) is based on the high affinity of phosphate for metal ions such as Fe³⁺, Zn²⁺, and Ga³⁺ and has been extensively used, often incorporating methyl esterification of carboxylates which otherwise also bind strongly to the metal ions.^{27–30} Alternatively, metal oxide chromatography (MOC) offers very selective enrichment of phosphopeptides and is based on the affinity of acidified phosphoric acid for metal oxides such as TiO₂ and ZrO₂.³¹ Without requiring chemical derivatization, MOC most often utilizes dihydroxybenzoic acid (DHB) to selectively compete off carboxylate containing peptides from the TiO₂ or ZrO₂ matrix.³² However, as even small amounts of DHB can accumulate and damage electrospray systems, aliphatic hydroxyl acids such as lactic acid or β -hydroxypropanoic acid can also be used.³³

Collision induced dissociation is often considered sub-optimal for phosphoproteomics, as neutral loss of phosphoric acid (H₃PO₄ from pS and pT) can occur before backbone cleavage, resulting in insufficient backbone fragmentation for effective identification of the peptide.^{34,35} Methods such as MS³ and MultiStage Activation impose additional activation events on preselected neutral loss peaks.^{13,36} While the majority of phosphoproteomics studies use these techniques when possible, the advantages are somewhat controversial. Villen *et al.* have shown that, when compared to MS², MS³ and pseudo-MS³ schemes resulted in an overall decrease in the number of phosphopeptides identified and offered only a very minor advantage in phospho site localization.³⁷ More recently however, Ulintz *et al.* observed that MultiStage Activation did increase the number of phosphopeptides identified when compared to MS² and MS³-based methodologies.³⁸

There have been several significant technical advances in phosphoproteomics in the past few years;³⁹ nonetheless, we believe there are still significant challenges and room for improvement. To this end, we have undertaken a comprehensive re-evaluation of all the significant steps involved in a phosphoproteomics experiment. We present here a modified strategy with significant improvements at each step, beginning with improved inhibition of phosphatases during cell lysis and ending with an evaluation of different fragmentation mechanisms for the purpose of identifying phosphopeptides.

Results

Phosphatase inhibitors versus heat/chaotropic denaturation for minimizing phosphatase activity

Phosphatases are robust, highly efficient enzymes that exhibit much less specificity than kinases and will be active in most cell lysis procedures. Thus, their activity must be minimized or eliminated if one wishes to study the phosphorylated state of proteins. Biochemists have typically used broad-spectrum, competitive inhibitors of protein phosphatases to preserve phosphorylations, but recent evidence suggests that this classical approach is not as effective as one would hope.^{8,9} Besides inhibitors, another approach for eliminating phosphatase activity in a cell lysate is to quickly and completely denature all proteins in the sample. Heat denaturation alone in the absence of a chaotropic surfactant is effective for eliminating phosphatase activity but often results in precipitates that are difficult to resolubilize (results not shown). SDS is, of course, a very effective chaotrope but must be removed (*i.e.* by filtration) prior to tryptic digestion and LC-MS/MS.^{40,41} Alternatively, deoxycholate is also an effective chaotrope and, when used at 0.5–1% w/v, greatly improves protein solubility without interfering with tryptic digestion.^{42,43} After acidifying the sample to pH < 4 it can be quantitatively eliminated either by precipitation or transfer to an organic phase prior to LC-MS/MS. When we compared free phosphate levels in a post-nuclear supernatant prepared with conventional phosphatase inhibitors to a similar post-nuclear supernatant treated with 1% w/v deoxycholate and heated to 99 °C, the deoxycholate treated condition was very clearly more effective at preventing the release of free phosphate during a 1 hour incubation either on ice or at room temperature (Fig. 1A). In addition, when conventional inhibitors were used the amount of detected phosphate increased dramatically between the incubation on ice and at room temperature, whereas when deoxycholate/denaturation was used this difference was minimal. The number of phosphopeptides identified using deoxycholate alone or in combination with a cocktail of phosphatase inhibitors was also considered. However adding phosphatase inhibitors offered no improvement over the use of deoxycholate alone (Fig. 1B).

The presence of phosphatase inhibitors during phosphopeptide enrichment has one further disadvantage: since many phosphatase inhibitors work by mimicking phosphate groups, they also compete with phosphopeptides for binding to TiO₂. Indeed, when a full cocktail of phosphatase inhibitors was present during enrichment by MOC, our phosphopeptide yield was very low (Fig. 1C) and eliminating any one of β -glycerophosphate, calyculin A or pyrophosphate did not significantly improve the number of phosphopeptides identified. Only by eliminating all three phosphomimetic inhibitors were we able to improve the phosphopeptide yield to near the levels obtained by a condition where all the inhibitors were used during cell lysis and then depleted by C18 chromatography (desalting) prior to phosphopeptide enrichment.

Phosphopeptide pre-fractionation using solution-based isoelectric focusing

It is now widely recognized that pre-fractionating complex proteomes prior to LC-MS/MS analysis allows deeper

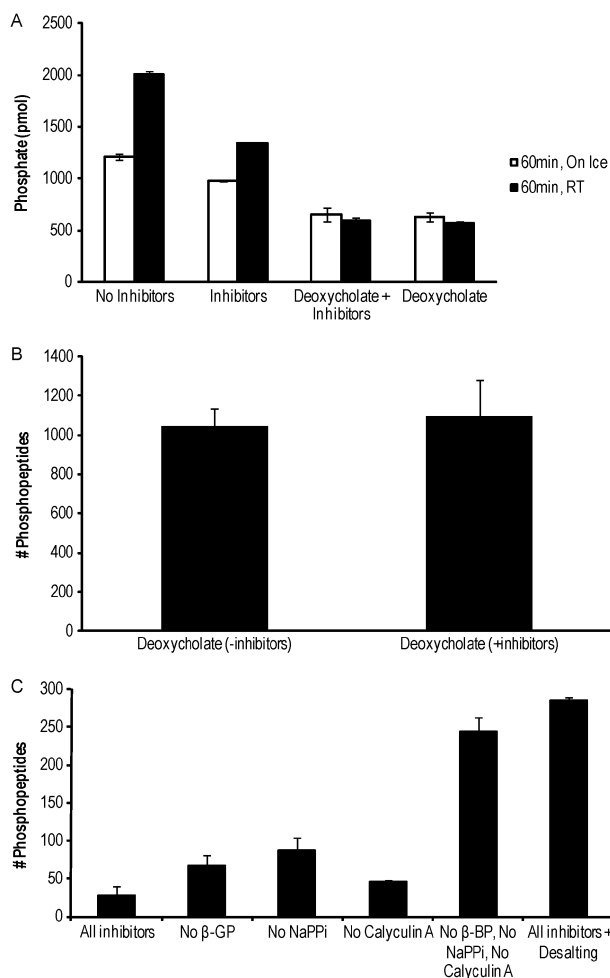


Fig. 1 Effects of phosphatase inhibitors during cell lysis and phosphopeptide enrichment. (A) Post-nuclear supernatants prepared as described in Experimental were incubated on ice or at room temperature for 60 min before phosphate levels were measured. Each bar represents the average (+ st. dev.) level of free phosphate measured for the described condition after subtraction of individual buffer controls. (B) Average (+ st. dev.) number of phosphopeptides identified from cells lysed in deoxycholate with or without inhibitors present. (C) Average (+ st. dev.) number of phosphopeptides identified when each or all phosphomimetic inhibitors are absent. β -Glycerophosphate- β -GP; Na pyrophosphate-NaPPI.

coverage of the components of that sample.^{44,45} SCX has been used for this purpose previously^{4,46} but IEF offers similar separation properties and potentially very high loading capacities. Thus, we examined the utility of IEF in this regard. Using a completely solution-based IEF instrument, we were able to load up to 30 mg of digested total protein and still obtain satisfactory resolution as, under these conditions, 60% of phosphopeptides were identified in only one of the 10 fractions collected and more than 85% were in two or fewer fractions (Fig. 2A). As expected, most phosphopeptides have a low pI so an ampholyte mix with a pH range between 3 and 10 forced most of the phosphopeptides into the first five fractions, effectively enriching them away from a significant portion of the non-phosphopeptides found at higher pIs.⁴⁷ This compression could be overcome by either a second round of

fractionation where the first five fractions are pooled and re-fractionated, or by a single fractionation run using an ampholyte mixture ranging in pH from 3 to 6 (Fig. 2B).

Binding capacity of TiO₂ for phosphopeptides

It is important to know the binding capacity of any chromatographic resin before loading a sample so that the resin is not over or underloaded, which, in the case of TiO₂, could lead to biased retention of specific phosphopeptides, needless waste of costly TiO₂ resin or undesired retention of non-phosphopeptides. By holding steady the amount of TiO₂ used and varying the total digested protein mass loaded on-column, we observed an optimal TiO₂ : protein mass ratio of approximately 6 : 1 (Fig. 3A). We also observed that when the amount of protein loaded was approximately equal to the binding capacity of TiO₂ (6 : 1), the yield of phosphopeptides was 95+ % (Fig. 3B).

Analysis of phosphopeptides by LC-ESI-MS

One property of phosphopeptides that has been assumed to limit phosphoproteomics is the relative hydrophilicity imparted by the phosphate group, which could potentially require altered chromatographic gradients or even different phases. In the process of optimizing phosphopeptide analysis, we

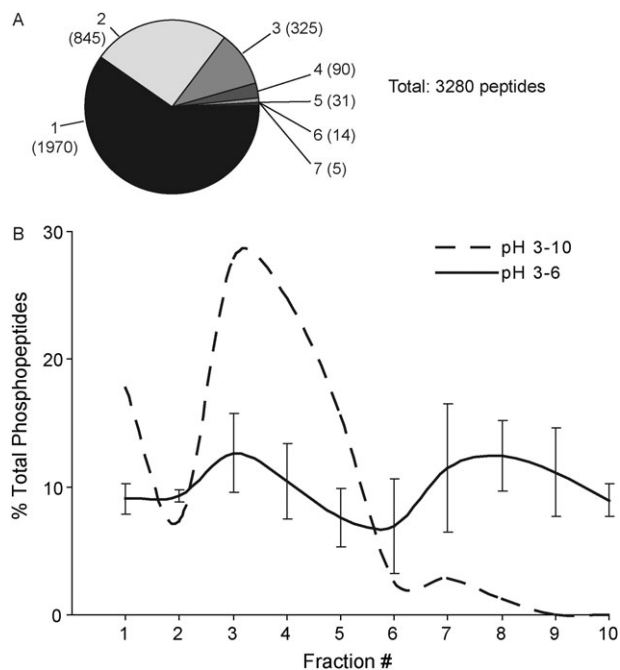


Fig. 2 Phosphopeptide fractionation by in-solution isoelectric focusing. Tryptic peptides from a HeLa cell lysate were separated by IEF. Subsequently phosphopeptides were enriched and analyzed by TiO₂ chromatography and LC-ESI-MS as described. Plotted are (A) the number of phosphopeptides identified in a single versus multiple IEF fractions, and (B) the number of peptides found in each fraction. In (A) the data represent peptide fractionation using ampholytes within a pH range of 3–6. In (B) the dashed curve represents peptide fractionation using ampholytes within a pH range from 3 to 10. The solid curve represents the average of three replicates of peptide fractionation using ampholytes within a pH range from 3 to 6. Error bars shown on the solid curve represent one standard deviation.

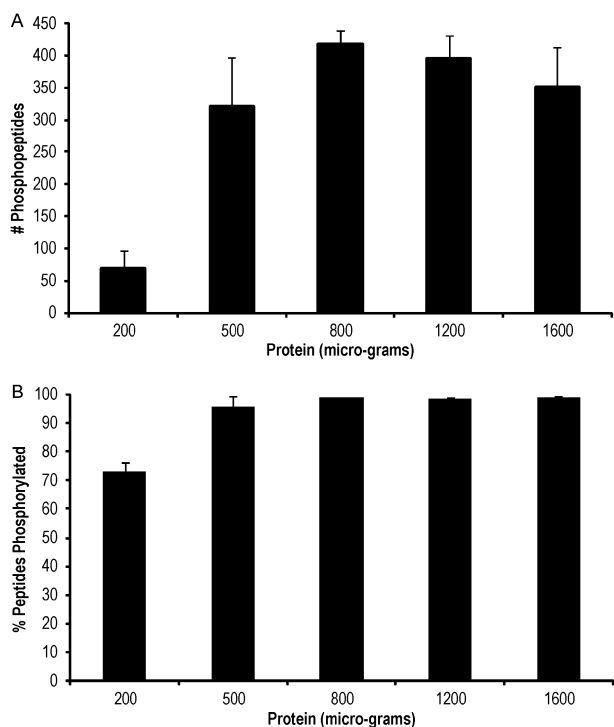


Fig. 3 Phosphopeptide enrichment by MOC. (A) Capacity of titanium dioxide matrix for phosphopeptides. Equal masses of Titansphere beads (10 μm diameter) were titrated with increasing protein amounts for phosphopeptide enrichment. Three replicates of each protein amount were averaged. Error bars represent one standard deviation. (B) The percent yield of phosphopeptides for each condition in (A) is shown.

tested several gradient profiles but found that our standard profile for conventional LC-MS/MS, which spends the majority of analysis time between 3% and 30% acetonitrile, was as good as any other, with an excellent distribution of phosphopeptides across the timescale (ESI⁺, Fig. S1).

The solvents used in most LC-MS/MS applications designed to analyze peptides have a pH of between 2.5 and 3.0, *e.g.*, pH of 0.5% acetic acid = 2.9, pH of 0.1% formic acid = 2.7. However, the $\text{p}K_{\text{a}}$ of the first proton from phosphoric acid is 2.15 and likely even lower within the physiochemical environment of a peptide, meaning that phosphates will typically carry a negative charge under LC-MS conditions. In data-dependent MS/MS mode for peptide analyses, mass spectrometers are typically directed to only select multiply charged precursor ions for fragmentation. However when we allowed singly charged ions to be sequenced as well, we observed essentially no difference in either the number of phosphopeptides identified or the numbers of phosphates per peptide (Fig. 4A and B).

Historically, there has been a perception in the mass spectrometry field that phosphopeptides will fragment poorly in positive ion mode, with neutral loss of phosphoric acid (H_3PO_4 , from pS and pT) often being preferred to backbone cleavage.³⁹ We therefore tested the utility of the MultiStage activation fragmentation mechanism available on the LTQ-Orbitrap for phosphopeptide analysis. As reported by others,³⁷ the additional time required for multi-stage activation

experiments seems to have a small negative impact on the overall number of phosphopeptide identifications (Fig. 4C) and it also does not appear to improve traditional MS² with regards to the number of multiply phosphorylated peptides identified (Fig. 4D). Another potential advantage of MS³ or pseudo-MS³ methods is in providing more backbone cleavage, leading to more MSⁿ ions available for peptide identification. However, while there appeared to be a very small shift towards higher Mascot Ionscores when using MultiStage activation, the differences were well within the margin of error of the experiments, both for the initial Ionscore and the subsequent PTM Score⁴⁸ (Fig. 4E and F).

Discussion

While not using phosphatase inhibitors goes against all the training biochemists receive, two previous reports found that using a cocktail of phosphatase inhibitors does not completely abolish phosphatase activity. Thingholm *et al.* found that pre-treating cells with calyculin A, sodium pervanadate, or two phosphatase inhibitor cocktails resulted in only a 10–40% increase in the number of phosphopeptides identified by LC-MS/MS.⁹ In a similar study using pervanadate, calyculin A and deltamethrin, Pan *et al.* found only 27% of all phosphopeptides to increase more than two-fold.⁸ In both cases the authors report that inhibitors of protein tyrosine phosphatases (PTPs) were quite effective and resulted in increases in up to 88%, but tyrosine phosphorylation represents only a very small fraction of the total cellular protein phosphorylation.³ The data presented here strongly suggest that quick, harsh denaturation completely and irreversibly inactivates phosphatases, making it at least as effective as the more traditional inhibitors. Baseline levels of free phosphate still detected in deoxycholate/boiled samples (Fig. 1A) likely reflect phosphate that was not directly liberated from protein substrates (*e.g.*, from inositol phosphatases or from ATP).

A harsh denaturation method also helps to avoid downstream complications caused by inhibitors during phosphopeptide enrichment. The vast majority of phosphatase inhibitors are designed to mimic phosphate and bind the enzymes' active sites. Thus, several inhibitors contain phosphate groups and likely have a high affinity for TiO_2 . While testing a cocktail of phosphatase inhibitors present during phosphopeptide enrichment, pyrophosphate, β -glycerophosphate and calyculin A all resulted in between a three and five-fold decrease in the number of phosphopeptides identified by LC-MS/MS. Thus, for efficient phosphopeptide recovery, either an excess of costly matrix must be used, or phosphatase inhibitors should be depleted (*i.e.* using chromatographic or protein precipitation methods) prior to enrichment. However this requires additional unnecessary sample handling steps, while samples solubilized in deoxycholate can be directly used for both trypsinization and phosphopeptide enrichment. We did not test other chaotropes such as high concentrations of urea in this study, as many enzymes retain their activity at room temperature in these conditions, *e.g.*, endopeptidase LysC⁴⁹ and heating urea solutions can result in significant, undesirable carbamylation.^{50,51}

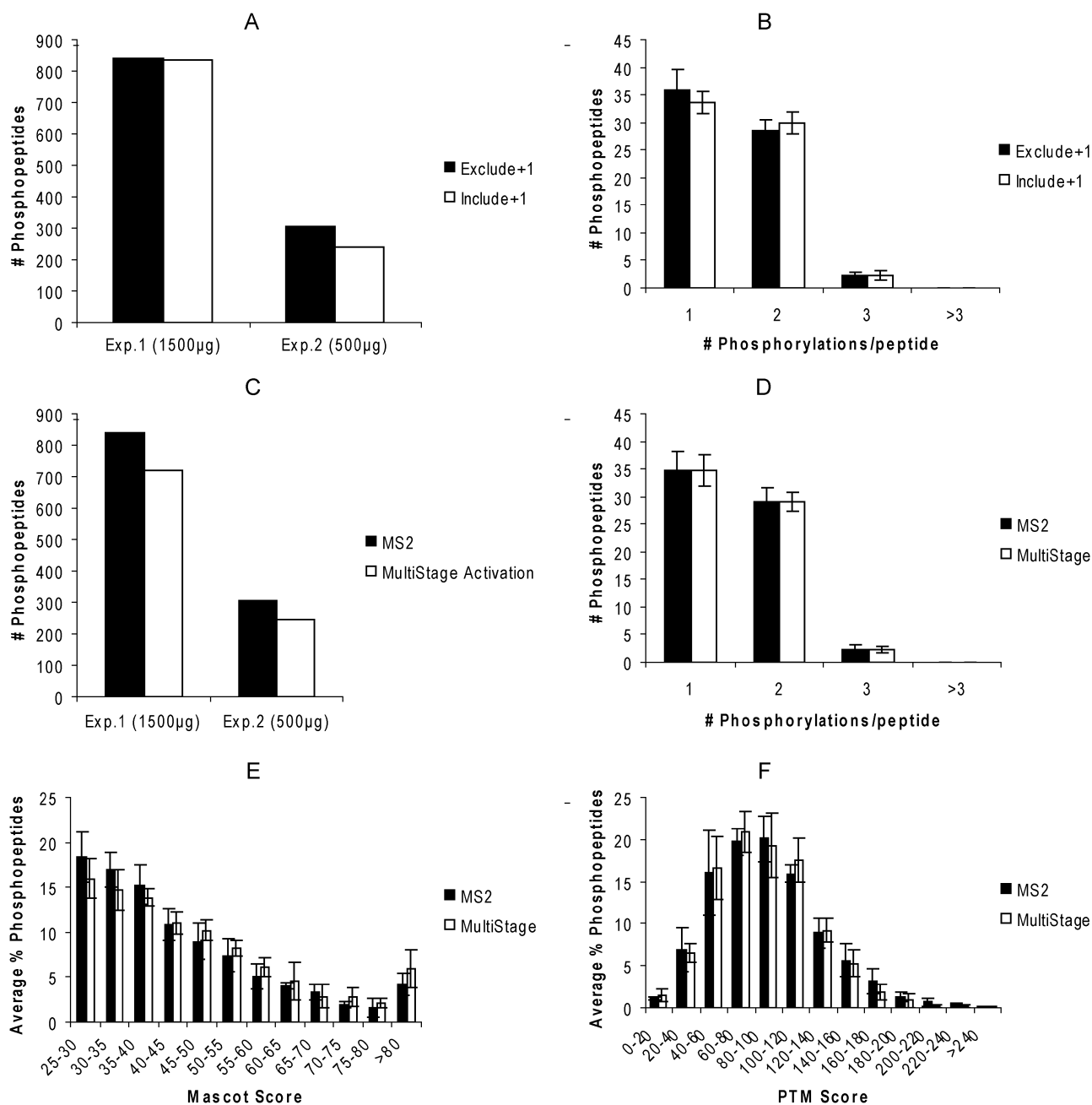


Fig. 4 Analyzing phosphopeptides by LC-ESI-MS. (A) Number of phosphopeptides identified by including *versus* excluding singly charged peptides for MSⁿ. (B) Distribution of multiply phosphorylated peptides identified by including *versus* excluding singly charged peptides for MSⁿ. (C) Number of phosphopeptides identified using MS² *versus* MultiStage Activation. (D) Distribution of multiply phosphorylated peptides identified using MS² *versus* MultiStage Activation. (E) Distribution of Mascot scores using MS² *versus* MultiStage Activation. (F) Distribution of PTM scores from MSQuant using MS² *versus* MultiStage Activation. Data in (A) and (C) represent results from two independent experiments that started with 1500 or 500 µg of protein. Data in (B), (D), (E) and (F) represent the average value from two independent replicates. Error bars represent one standard deviation.

The dynamic range of a mammalian cell's proteome can span over ten orders of magnitude, while current LC-MS/MS systems can handle only four at best.^{52,53} Thus, the need for sample pre-fractionation is widely acknowledged by researchers in proteomics. Here we use in-solution IEF with a loading capacity of 30 mg to fractionate phosphopeptides effectively and uniformly across a pH range of 3 to 6 within a few hours. An additional advantage of solution-based IEF is the virtually

complete sample recovery, in contrast to IPG-based IEF and several chromatographic systems where peptides with extreme pIs, charge states,⁵⁴ *etc.* migrate off the strip, are not retained on-column or bind irreversibly to the resin. Solution-based IEF is also considerably faster to perform than IPG-based IEF, which can require run times of 20 to 24 h.⁴⁷

Two previous reports evaluating MS³ and pseudo-MS³ for phosphopeptide analysis both agree that MS³ methods

perform poorly compared to the others. However, Villen *et al.* found that MultiStage Activation outperforms MS² while Ulintz *et al.* found exactly the opposite. As our data clearly favour MS² over MultiStage Activation, we feel that the balance of evidence now suggests that conventional MS² of precursors with two or more charges outperforms MS³ and pseudo-MS³ approaches.^{37,38} This is likely due to the fact that higher-order scans are slower and generate ion fragments with lower intensities than those generated by an MS² scan.³⁷ The previous concerns that the negative charges on phosphates would cancel out the positive charges on the rest of a peptide molecule are unfounded as charge state in the gas phase is clearly not equivalent to solution charge state calculated from the pK_a's of all ionizable groups. Undoubtedly, for complete analysis of a phosphoproteome where every single phosphopeptide is fragmented and quantified, a combination of methods will be required, including electron transfer dissociation,^{55,56} MS², MS³ and even some of the methods that are not as compatible with LC timescales, *e.g.*, electron capture dissociation and infrared multiphoton dissociation. In addition, the potential problems of phosphosite scrambling in ion trap fragmentation requires significant work in order to allow accurate assignment of the specific residues that are modified.⁵⁷ Several factors still limit our ability for a truly complete phosphoproteomic study,³⁹ but while those are still being addressed the methods described here can be implemented immediately and applied to answer an array of biological questions.

Conclusions

We have demonstrated several significant improvements in the methods used for isolating and analyzing phosphopeptides on a large-scale. Of paramount importance, based on the data presented here, we advise that researchers move away from the use of conventional phosphatase inhibitors in phosphoproteomic experiments and instead use quick denaturing conditions to eliminate the enzyme activity more completely and effectively.

Experimental

Cell culture and lysis

HeLa cells were maintained in Dulbecco's modified Eagle serum (DMEM) containing 4500 mg L⁻¹ glucose and 4 mM l-glutamine (Thermo Fisher Scientific), supplemented with 10% v/v qualified fetal bovine serum (FBS) (Invitrogen), an additional 2 mM l-glutamine (Thermo Fisher Scientific) and 0.1 units L⁻¹ penicillin and streptomycin (Thermo Fisher Scientific). For harvesting and lysis, cells were placed on ice, washed 3× with cold phosphate buffered saline (PBS) and harvested with a scraper. Cells were pelleted at 600 relative centrifugal force (rcf) for 5 min at 4 °C. For all experiments described, excluding the phosphatase activity assay and experiments analyzing the inhibition of phosphopeptide enrichment by phosphatase inhibitors, the cell pellet was resuspended in 200 μL 1% w/v Na deoxycholate, 50 mM NH₄HCO₃ per 10 cm plate. The lysate was then immediately placed in a heating block at 99 °C for 5 min. Samples were

removed and cooled to room temperature, MgCl₂ was added to a final concentration of 1.5 mM and 2.5 × 10⁻³ units μL⁻¹ benzonase (Novagen) were added to cleave DNA and decrease viscosity.

Phosphate assay

For each condition, one 10 cm plate of HeLa cells was washed 3× with cold 20 mM tris, 150 mM NaCl, pH 7.5, harvested with a scraper, and pelleted at 600 rcf for 5 min at 4 °C. Equally sized cell pellets were subjected to each of the following lysis conditions. Cell pellets subjected to the 'No Inhibitors' conditions were resuspended and lysed in 1% v/v Nonidet P40, 20 mM tris, 150 mM NaCl, and protease inhibitor cocktail, pH 7.5. Cell pellets subjected to the 'Inhibitors' conditions were resuspended and lysed in 1% Nonidet P40, 20 mM tris, 150 mM NaCl, and protease inhibitor cocktail, pH 7.5, with the addition of 1 mM Na₃VO₄, 10 mM NaF, 0.5 mM pervanadate, 10 μM deltamethrin, and 100 nM calyculin A. Cell pellets subjected to 'Deoxycholate + Inhibitors' conditions were resuspended in 20 mM tris, 150 mM NaCl, protease inhibitor cocktail, pH 7.5, and identical phosphatase inhibitors and concentrations listed above. Cell pellets subjected to the 'Deoxycholate' conditions were resuspended in 20 mM tris, 150 mM NaCl, and protease inhibitor cocktail, pH 7.5. Cell pellets subjected to the 'Deoxycholate + Inhibitors' and 'Deoxycholate' conditions were mechanically lysed by 5–6 passages through a 22G needle. Nuclei were pelleted at 600 rcf for 5 min at 4 °C and supernatants were retained. Nonidet P40 post-nuclear supernatants were immediately placed on ice, while Na deoxycholate and NH₄HCO₃ were added to mechanically lysed post-nuclear supernatants to a final concentration of 1% w/v and 50 mM, respectively. Na deoxycholate samples were immediately heated at 99 °C for 5 min. All samples were then placed on ice or at room temperature as indicated for 60 min. Sample volumes corresponding to 100 μg protein (as measured by Bradford assay) were all adjusted to 25 μL. Levels of free phosphate in all samples and buffers were assayed exactly as described in the manufacturer's instructions (Ser/Thr Phosphatase Assay Kit 1 (K-R-pT-I-R-R), Millipore). Absorbance readings at 620 nm were determined using a microtiter plate reader (Spectra Fluor Plus, TECAN).

Tryptic digest and desalting

Protein samples solubilized in 1% w/v Na deoxycholate, 50 mM NH₄HCO₃ and heated at 99 °C for 5 min were reduced, alkylated and digested as described.⁵⁸ For desalting of tryptic peptide samples, a procedure similar to STAGE tips was utilized, excepting that C₁₈ packing material was used to increase binding capacity (WP C₁₈ Prep HPLC Packing, Baker Analyzed).⁵⁹ Briefly, trypsinized samples were diluted 3× with 1% v/v trifluoroacetic acid (TFA), 5% v/v CH₃CN and clarified at 16000 rcf for 10 min at room temperature. 100 μL of a 1 : 1 v/v slurry of C₁₈ beads in methanol were added to a STAGE tip. Each tip was first conditioned with 100 μL methanol and washed with 300 μL 1% v/v TFA, 5% v/v acetonitrile before the sample was passed through. The binding capacity of each tip for peptides was estimated at 500 μg. Tips were washed 3× with 300 μL 1% v/v TFA,

5% v/v CH₃CN and eluted with 300 µL 0.5% v/v CH₃COOH, 80% v/v CH₃CN.

In-solution isoelectric focusing

Desalted tryptic peptide samples were dried completely in a vacuum centrifuge (Vacufuge Plus, Eppendorf) and resuspended in a 5% v/v glycerol in distilled water containing 2% w/v ampholytes of pH range 3–10 (Bio-Lyte Ampholyte 3/10 (BIO-RAD) or 3–6 (Fluka Analytical)). Peptide solutions were separated by in-solution isoelectric focusing according to the manufacturer's instructions (MicroRotor Cell, BIO-RAD, referred to as solution-based IEF throughout the text) using a power of 1.0 W until the voltage stabilized (~3 h). 10 fractions were harvested from each IEF run exactly as described in the manufacturer's instructions.

Phosphopeptide enrichment

Trypsinized samples or fractions harvested from solution-based IEF were diluted 3× with 1% v/v TFA acid, 5% v/v CH₃CN, the pH was adjusted to ~1, and lactic acid was added to a final concentration of 300 mg mL⁻¹.³³ In parallel, 10 mg of titanium dioxide (TiO₂) beads (Titansphere, GL Sciences) for every milligram of starting protein material were washed with 1 mL solution A (70% v/v CH₃CN, 0.1% v/v TFA, 300 mg mL⁻¹ lactic acid). The TiO₂ beads were incubated with the acidified peptide sample for at least 1 h at room temperature shaking at 1400 rpm. A STAGE tip was constructed as originally described, except that C₈ material was used as a frit to retain the TiO₂ beads (C₈ Empore Disks, 3 M).⁵⁹ The C₈ STAGE tip was washed with solution A and the sample was passed through by centrifugation at 1000 rcf at room temperature. Similarly the tip was washed at least 5× with 300 µL solution A and subsequently 5× with 300 µL solution B (80% v/v CH₃CN, 0.1% v/v TFA). Peptides were eluted stepwise with 50 µL 0.5% v/v NH₄OH, followed by 50 µL 0.5% v/v NH₄OH, 30% v/v CH₃CN and 50 µL 0.5% v/v NH₄OH, 50% v/v CH₃CN. CH₃CN was diluted from the eluate to <5% with 1% v/v TFA acid, 5% v/v CH₃CN and the sample was concentrated on a STAGE tip as previously described.⁶⁰

Testing interference of phosphopeptide enrichment by phosphatase inhibitors

Confluent, 10 cm plates of HeLa cells were cultured as described above. Cells were washed 3× with PBS, harvested with a scraper, and pelleted at 600 rcf for 5 min at 4 °C. Cell pellets corresponding to one 10 cm plates were resuspended in 200 µL of the following lysis buffers and used for each of the 6 conditions described. For the 'All inhibitors' and 'Desalting' conditions, cells were resuspended in 1× PBS, 1 mM Na₃VO₄, 5 mM sodium pyrophosphate, 10 mM NaF, 20 mM β-glycerophosphate, 0.5 mM pervanadate, 10 µM deltamethrin, 100 nM calyculin A, and protease inhibitor cocktail (Complete, Roche Diagnostics). Each of the other conditions were identical except for the exclusion of the indicated phosphatase inhibitor(s). Cells were lysed mechanically by 5–6 passages through a 22G needle, and nuclei were pelleted at 600 rcf for 5 min at 4 °C. Post-nuclear supernatants were retained and Na deoxycholate and

NH₄HCO₃ were immediately added to final concentrations of 1% w/v and 50 mM, respectively. Samples were immediately placed on a heating block at 99 °C for 5 min, cooled to room temperature. All samples were then subjected to an in-solution digest as described above. For the 'Desalting' condition, tryptic peptide samples were desalted as described above and phosphopeptide enrichment was performed exactly as described above.

Mass spectrometry

For LC-ESI-MS, samples were analyzed on a LTQ-OrbitrapXL (Thermo Fisher Scientific) coupled on-line to an Agilent 1100 Series nanoflow HPLC instrument using a nanospray ionization source (Proxeon Biosystems) as previously described.⁵⁸ For conditions utilizing MultiStage Activation, neutral loss masses of 33 Da, 49 Da, and 98 Da were selected when within the top $n = 3$. For conditions including singly charged peptides, charge state rejection was only enabled for cases of unassigned charge states. Monoisotopic peaks and charge states in the data were extracted and corrected using DTA Supercharge (<http://msquant.sourceforge.net>), and peak lists were searched against a database containing all human protein sequences in the International Proteome Index using Mascot (v2.2, Matrix Science). Search parameters included one missed cleavage, cysteine carbamidomethyl fixed modification, and variable modifications methionine oxidation and phosphorylation on serine, threonine, and tyrosine residues. The peptide mass tolerance was 10 parts-per-million and the MS/MS tolerance was 0.6 Da. MSQuant v1.5b7⁶¹ was used for parsing Mascot result files, iterative mass recalibration, and assigning PTM scores.

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