Efficient Identification of Phosphorylation by Mass Spectrometric Phosphopeptide Fingerprinting

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We describe a rapid and efficient method for the identification of phosphopeptides, which we term mass spectrometric (MS) phosphopeptide fingerprinting. The method involves quantitative comparison of proteolytic peptides from native versus completely dephosphorylated proteins. Dephosphorylation of serine, threonine, and tyrosine residues is achieved by in-gel treatment of the separated proteins with hydrogen fluoride (HF). This chemical dephosphorylation results in enrichment of those unmodified peptides that correspond to previously phosphorylated peptides. Quantitative comparison of the signal-to-noise ratios of peaks in the treated versus untreated samples are used to identify phosphopeptides, which can be confirmed and further studied by tandem mass spectrometry (MS/MS). We have applied this method to identify eight known phosphorylation sites of *Xenopus* Aurora A kinase, as well as several novel sites in the *Xenopus* chromosome passenger complex (CPC).

Protein phosphorylation plays a central role in the regulation of many cellular processes. In recent years, mass spectrometry (MS) has become a method of choice for the analysis of protein phosphorylation.[1−3] Although advances in instrumentation and software have made it possible to perform large-scale studies of phosphoproteomes,[4,5] the comprehensive analysis of phosphorylation in single proteins remains challenging.

Detection of phosphorylation has been difficult due to the relative suppression of phosphopeptide MS signals compared to their unphosphorylated counterparts. Thus, even when the unmodified peptide can be observed as a strong peak in the spectrum, comparable amounts of the unmodified peptide are often obscured by the background.[6,7] Because of this difficulty, several approaches to study phosphorylation involve removal of the phosphate group. For example, enzymatic treatment with phosphatase has been employed to dephosphorylate proteins, resulting in an enhanced signal,[8−14] but this method has been shown to be subject to enzyme preferences.[15,16] Alternatively, the phosphate group has been converted to moieties that enhance detection, including affinity tags[17] or lysine analogs that are recognized and cleaved by trypsin.[18,19]

Tandem MS (MS/MS) is frequently employed to confirm and map phosphorylation sites, but here too the phosphate group is often removed. Phosphate groups on amino acids are particularly unstable to collision-induced dissociation (CID) and are preferentially lost from the peptide as the elements of phosphoric acid. This loss of 98 Da provides a useful phosphopeptide signature, and many studies have taken advantage of this property to identify phosphorylation.[20] For example, a neutral loss scan can be performed to identify any species that displays this signature loss, but this approach suffers from reduction in sensitivity arising from the need to scan the entire m/z range of interest.[21] This so-called "scanning disadvantage" can be partially overcome by targeting either only discernible peaks in the spectrum[22] or by performing MS/MS on a hypothetical subset of m/z values.[23] A major drawback of the former approach is its inability to analyze species that are obscured by the background. Although the hypothesis-driven approach overcomes this difficulty, it is still relatively costly in terms of time and sample.

In an effort to overcome some of the difficulties described above, we have revisited the dephosphorylation approach, employing a chemical dephosphorylation strategy. Hydrogen fluoride has been shown to rapidly dephosphorylate phosphopeptides in solution,[24] and we have adapted this technique for use in-gel and

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(1) McLachlin, D. T.; Chait, B. T. *Curr. Opin. Chem. Biol.* **2001**, *5*, 591−602.
(2) Ptacek, J.; Snyder, M. *Trends Genet.* **2006**, *22*, 545−554.
(3) Reinders, J.; Sickmann, A. *Proteomics* **2005**, *5*, 4052−4061.
(4) Li, X.; Gerber, S. A.; Rudner, A. D.; Beausoleil, S. A.; Haas, W.; Villen, J.; Elias, J. E.; Gygi, S. P. *J. Proteome Res.* **2007**, *6*, 1190−1197.
(5) Olsen, J. V.; Blagoev, B.; Gnad, F.; Macek, B.; Kumar, C.; Mortensen, P.; Mann, M. *Cell* **2006**, *127*, 635−648.
(6) Kang, S.; Liao, P.; Gage, D. A.; Esselman, W. J. *J. Biol. Chem.* **1997**, *272*, 11588−11596.
(7) Zhou, W.; Merrick, B. A.; Khaledi, M. G.; Tomer, K. B. *J. Am. Soc. Mass Spectrom.* **2000**, *11*, 273−282.
(8) Hirschberg, D.; Jagerbrink, T.; Samskog, J.; Gustafsson, M.; Stahlberg, M.; Alvelius, G.; Husman, B.; Carlquist, M.; Jornvall, H.; Bergman, T. *Anal. Chem.* **2004**, *76*, 5864−5871.
(9) Liao, P. C.; Leykam, J.; Andrews, P. C.; Gage, D. A.; Allison, J. *Anal. Biochem.* **1994**, *219*, 9−20.
(10) Zhang, X.; Herring, C. J.; Romano, P. R.; Szczepanowska, J.; Brzeska, H.; Hinnebusch, A. G.; Qin, J. *Anal. Chem.* **1998**, *70*, 2050−2059.
(11) Larsen, M. R.; Sorensen, G. L.; Fey, S. J.; Larsen, P. M.; Roepstorff, P. *Proteomics* **2001**, *1*, 223−238.
(12) Marcantonio, M.; Trost, M.; Courcelles, M.; Desjardins, M.; Thibault, P. *Mol. Cell. Proteomics* **2007**, in press.
(13) Torres, M. P.; Thapar, R.; Marzluff, W. F.; Borchers, C. H. *J. Proteome Res.* **2005**, *4*, 1628−1635.
(14) Ishihama, Y.; Wei, F. Y.; Aoshima, K.; Sato, T.; Kuromitsu, J.; Oda, Y. *J. Proteome Res.* **2007**, *6*, 1139−1144.
(15) Hunter, A. P.; Games, D. E. *Rapid Commun. Mass Spectrom.* **1994**, *8*, 559−570.
(16) Zeller, M.; Konig, S. *Anal. Bioanal. Chem.* **2004**, *378*, 898−909.
(17) Oda, Y.; Nagasu, T.; Chait, B. T. *Nat. Biotechnol.* **2001**, *19*, 379−382.
(18) Knight, Z. A.; Schilling, B.; Row, R. H.; Kenski, D. M.; Gibson, B. W.; Shokat, K. M. *Nat. Biotechnol.* **2003**, *21*, 1047−1054.
(19) Rusnak, F.; Zhou, J.; Hathaway, G. M. *J. Biomol. Tech.* **2004**, *15*, 296−304.
(20) Carr, S. A.; Annan, R. S.; Huddleston, M. J. *Methods Enzymol.* **2005**, *405*, 82−115.
(21) Krutchinsky, A. N. *Int. J. Mass Spectrom.* **2007**, *268*, 93−105.
(22) Casado-Vela, J.; Ruiz, E. J.; Nebreda, A. R.; Casal, J. I. *Proteomics* **2007**, *7*, 2522−2529.
(23) Chang, E. J.; Archambault, V.; McLachlin, D. T.; Krutchinsky, A. N.; Chait, B. T. *Anal. Chem.* **2004**, *76*, 4472−4483.

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on whole proteins. This chemical dephosphorylation results in enrichment of those unmodified peptides that correspond to previously phosphorylated peptides. Quantitative comparison of the signal-to-noise ratios of peaks in the treated versus untreated samples are used to identify phosphopeptides. The ability to completely dephosphorylate proteins irrespective of amino acid sequence and the improved signal-to-noise ratio (S/N) of the resulting dephosphorylated peptides permit us to capture an MS phosphorylation "fingerprint" of a given protein. We have applied this technique to characterize the phosphorylation of the Aurora A protein and the Aurora B chromosome passenger complex (i.e., Aurora B, Incenp, Dasra A, and Survivin). Aurora A is a centrosome-associated kinase whose autophosphorylation has been previously studied in detail.[25−28] Aurora B is a chromosome-associated kinase that is required for a variety of mitotic processes.[29,30]

EXPERIMENTAL SECTION

In-Gel Chemical Dephosphorylation. All peptides were produced by solid-phase peptide synthesis. Bovine casein was purchased from Sigma. Peptides or proteins were separated and immobilized in Tris−glycine gels (Invitrogen) by SDS−PAGE, followed by fixation for at least 10 min in 20% methanol/5% acetic acid solution. Fixation was sufficient to visualize precipitated peptides within the gel without the need to use stains. Protein bands were visualized by colloidal Coomassie GelCode blue stain (Pierce). Bands were excised from the gel and destained in 400 μL of 55% 100 mM ammonium bicarbonate/45% acetonitrile. Gel slices were then dehydrated in 300 μL of acetonitrile (HPLC-grade from Pierce) and dried in a Speedvac (Thermo Savant). The dry gel slices were immersed in 70 μL of 70% hydrogen fluoride (HF)−pyridine (Sigma) and incubated on ice for 1 h, then washed twice with 150 μL of acetic acid and twice with 150 μL of water. Washed gel slices were soaked in 300 μL of 0.5 M ammonium bicarbonate to raise the pH to ~8.5 before digestion with trypsin or Glu-C (Roche).

Mass Spectrometry. A comprehensive protocol for matrix-assisted laser desorption ionization (MALDI) sample preparation is available online at http://www.rockefeller.edu/labheads/chait/. Briefly, peptides were extracted from the gel slices with a slurry of 25 μg/μL POROS R2 20 reversed-phase resin (Applied Biosystems) in 5% formic acid/0.2% trifluoroacetic acid (TFA) at 4 °C for 6 h. The slurry was transferred to C18 Ziptips (Millipore) and washed with 0.1% TFA. Peptides were eluted on a MALDI compact disk sample stage[31] with one-third saturated 2,5-dihydroxybenzoic acid (Lancaster Synthesis) in 50% methanol/20% acetonitrile/0.1% TFA or on a metal plate with saturated α-cyano-4-hydroxycinnamic acid (4-HCCA) in two parts water, one part acetonitrile. MALDI-MS was performed with a modified MALDI-quadrupole−quadrupole-time-of-flight (QqTOF) mass spectrometer[32] (Centaur Technology, Sciex) or a prOTOF 2000 MALDI-time-of-flight (MALDI-TOF) mass spectrometer (Perkin-Elmer). Tandem mass spectrometry (MS/MS) was performed using a vMALDI LTQ instrument (Thermo Scientific).

Data Analysis. We have devised a simple method for determining the smooth background in our mass spectra. This is accomplished by iteratively calculating the average root-mean-square deviation (rmsd) in moving windows across the mass spectrum, excluding the peaks. Initially, the rmsd is calculated across the entire spectrum in windows of 1.0 m/z units, with steps of 0.5 m/z units. The spectrum is then divided into 30 intervals and the average rmsd is calculated for each of these intervals. The average rmsd is then recalculated for each of the 30 intervals by excluding all values that deviate by more than a factor of 1.3 from the average rmsd. This recalculation is then repeated two more times to remove interference from all the peaks. The final background is the average rms in each of the 30 intervals after the fourth iteration, meaning that one value is calculated for each ~100 m/z units. Masses and S/N are then assigned to all peaks. The program for this analysis is available online at http://prowl.rockefeller.edu/software/HF.

Peaks were assigned in the above manner for spectra from samples that were untreated or treated with HF, with those with a S/N threshold above 1.1 considered for further analysis. For each peak, the ratio of S/N for treated versus untreated was computed, and the log of this ratio was scored as the "phosphopeptide index". A positive value for the phosphopeptide index indicates that the peptide is predicted to be phosphorylated, whereas a negative value indicates that the peptide is predicted to be unphosphorylated.

Aurora A Preparation and In Vitro Kinase Assay. *Xenopus* Aurora A coding sequence was amplified by PCR from a full-length cDNA clone (Open Biosystems, clone ID no. 6318106) and cloned into pET28a vector at BamHI and XhoI sites. Hexahistidine-tagged Aurora A was expressed in *E. coli* and purified as described.[33] Autophosphorylation occurred upon incubation for 30 min at 30 °C in kinase buffer (20 mM HEPES, pH 7.7, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 300 μM ATP). The reaction was stopped by boiling in SDS sample buffer, and reaction products were analyzed by SDS−PAGE.

Isolation of *Xenopus* Chromosome Passenger Complex (CPC). A volume of 150 μL of CSF-arrested *Xenopus laevis* egg extract was treated with phosphatase inhibitor (0.5 μM okadaic acid) or control (0.5% v/v DMSO) for 30 min at 20 °C, treated with nocodazole (10 μg/mL) for 10 min at 20 °C, then cooled to 4 °C for 10 min before immunoprecipitation of the CPC for 75 min on ice by incubation with affinity-purified anti-xIncenp rabbit polyclonal antibodies cross-linked to magnetic beads (Dynal). Complexes were washed five times with wash buffer (20 mM HEPES, pH 7.7, 150 mM KCl, 1 mM $MgCl_2$, 0.1% Triton-X100, 1 mM DTT, and phosphatase inhibitor cocktail), transferred to new tubes, and eluted with 30 μL of SDS sample buffer. Immunoprecipitated complexes were separated by SDS−PAGE.

Safety Considerations. Note that HF is an extremely corrosive substance and should be handled in a fume hood with

(24) Kuyama, H.; Toda, C.; Watanabe, M.; Tanaka, K.; Nishimura, O. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 1493−1496.
(25) Haydon, C. E.; Eyers, P. A.; Aveline-Wolf, L. D.; Resing, K. A.; Maller, J. L.; Ahn, N. G. *Mol. Cell. Proteomics* **2003**, *2*, 1055−1067.
(26) Littlepage, L. E.; Ruderman, J. V. *Genes Dev.* **2002**, *16*, 2274−2285.
(27) Littlepage, L. E.; Wu, H.; Andresson, T.; Deanehan, J. K.; Amundadottir, L. T.; Ruderman, J. V. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 15440−15445.
(28) Eyers, P. A.; Erikson, E.; Chen, L. G.; Maller, J. L. *Curr. Biol.* **2003**, *13*, 691−697.
(29) Ruchaud, S.; Carmena, M.; Earnshaw, W. C. *Nat. Rev. Mol. Cell Biol.* **2007**, *8*, 798−812.
(30) Sampath, S. C.; Ohi, R.; Leismann, O.; Salic, A.; Pozniakovski, A.; Funabiki, H. *Cell* **2004**, *118*, 187−202.
(31) Krutchinsky, A. N.; Kalkum, M.; Chait, B. T. *Anal. Chem.* **2001**, *73*, 5066−5077.
(32) Krutchinsky, A. N.; Zhang, W.; Chait, B. T. *J. Am. Soc. Mass Spectrom.* **2000**, *11*, 493−504.
(33) Roghi, C.; Giet, R.; Uzbekov, R.; Morin, N.; Chartrain, I.; Le Guellec, R.; Couturier, A.; Doree, M.; Philippe, M.; Prigent, C. *J. Cell Sci.* **1998**, *111* (5), 557−572.

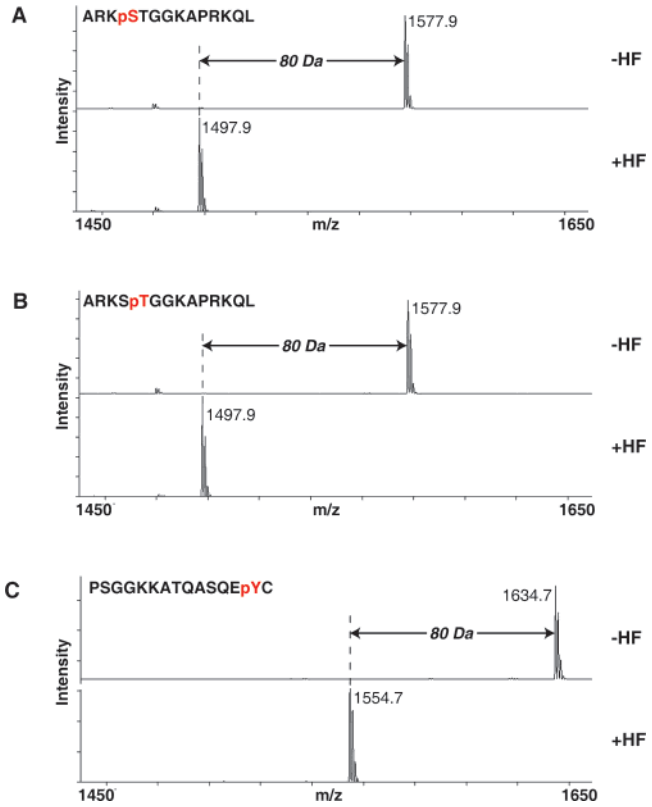

Figure 1. In-gel dephosphorylation of peptides. Polyacrylamide gel slices containing peptides were dehydrated and incubated with 70% HF−pyridine for 1 h on ice: (A) ARKpSTGGKAPRKQL, (B) ARKSpTGGKAPRKQL, (C) PSGGKATQASQEpYC. Each spectrum shows before treatment (−HF) and after (+HF).

appropriate care, including use of a lab coat, nitrile gloves, and safety goggles. After use, the HF solution and pipet tips were neutralized in calcium chloride solution prior to disposal.

RESULTS AND DISCUSSION

As a test of our method for in-gel chemical dephosphorylation, 1 μg aliquots of three synthetic histone tail phosphopeptides (phosphorylated, respectively, on serine, threonine, or tyrosine) were immobilized in a polyacrylamide gel by electrophoresis and fixed with methanol−acetic acid. The precipitated peptides were visualized directly in the gel without staining and were cut out as 2 mm slices. The gel slices were dehydrated and treated with 70% HF−pyridine for 1 h on ice, resulting in complete dephosphorylation of the phosphoserine-, phosphothreonine-, and phosphotyrosine-containing peptides, respectively (Figure 1). MS analysis of the reaction products revealed that the phosphopeptides were quantitatively converted to the unmodified backbone peptides with no detectable side products.

Previously, this chemistry had been applied to peptides.[24] Here, we tested its ability to dephosphorylate whole proteins using the model phosphoprotein ovalbumin. The protein was denatured by boiling in SDS sample buffer, purified by SDS−PAGE, and visualized by Coomassie staining. Gel slices were excised and destained, then dehydrated and treated with HF as before. The gel slices were then washed in 0.5 M ammonium bicarbonate to bring the pH to ~8 prior to trypsin digestion. Tryptic peptides were collected and analyzed by MALDI-MS, which demonstrated that the HF-treated protein had been efficiently dephosphorylated

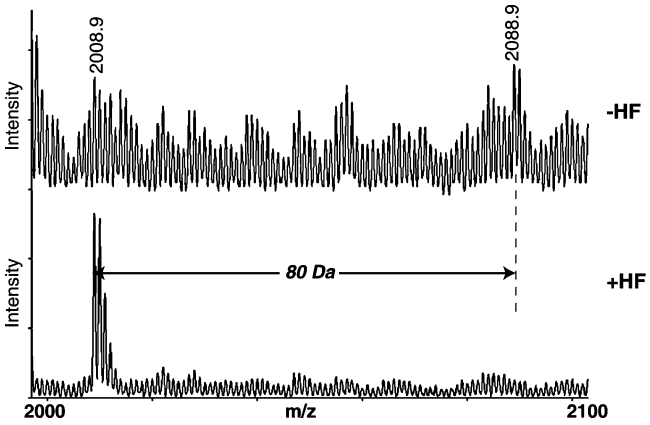

Figure 2. In-gel dephosphorylation of protein. An amount of 5 μg of ovalbumin was purified by SDS−PAGE, and gel slices containing the whole protein were either not treated (−HF) or treated (+HF) with 70% HF−pyridine for 1 h on ice. The phosphopeptide EVVGpSAEAGVDAASVSEEFR (m/z = 2088.9) was dephosphorylated to yield the unmodified peptide (m/z = 2008.9).

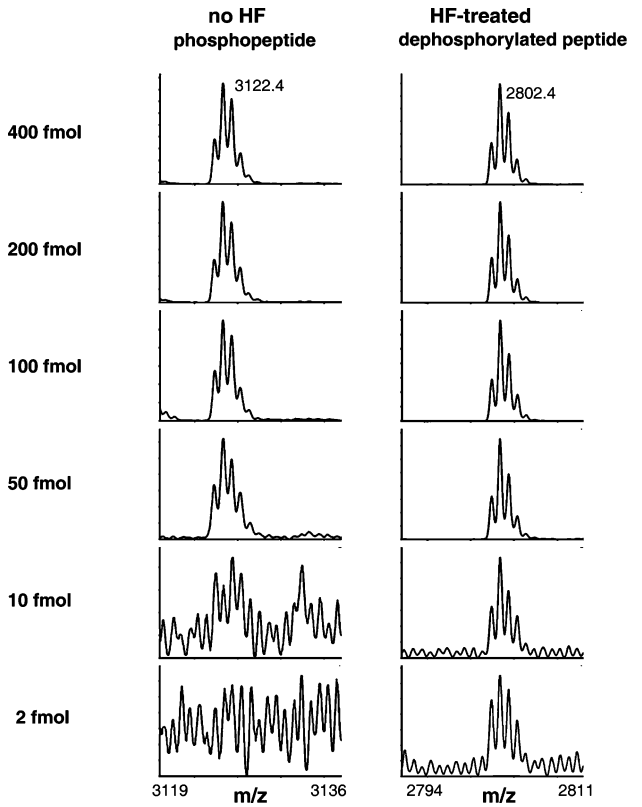

Figure 3. Sensitivity of HF dephosphorylation for detecting phosphorylation. Varying amounts of β-casein were either untreated or treated with HF. The peak corresponding to the singly protonated phosphopeptide, RELEELNVPGEIVEpSLpSpSpSEESITR (m/z = 3122.4), is shown for the untreated samples, and the unmodified peak (m/z = 2802.4) is shown for the treated samples.

(Figure 2). From these experiments, we conclude that HF−pyridine can efficiently dephosphorylate peptides, as well as whole proteins, in gel. We observed that the S/N for the dephosphorylated peak was significantly greater than the sum of the S/Ns of the unmodified and phosphorylated peaks prior to HF treatment (Figure 2). Dephosphorylated peptides should therefore be detectable down to lower levels than the corresponding phosphorylated peptides.

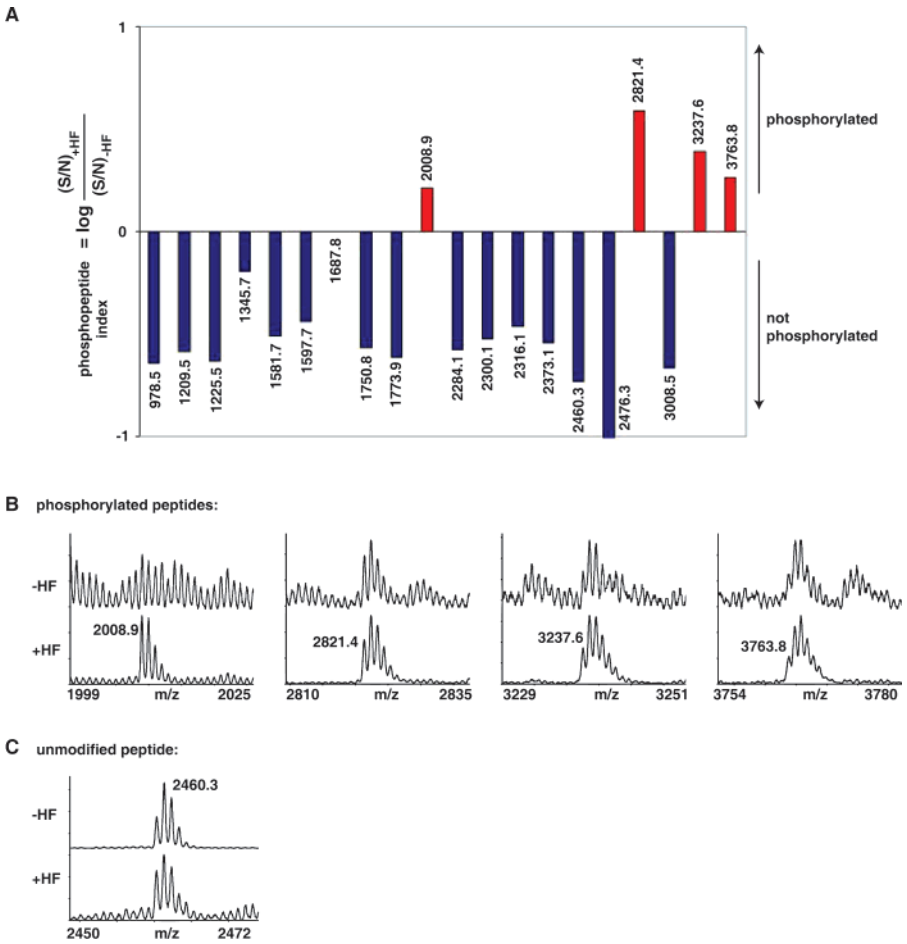

Figure 4. Observed increase in S/N of unmodified peptides upon HF treatment provides an "MS phosphopeptide fingerprint", indicating the likelihood that a given peptide is phosphorylated. (A) The S/N was calculated for peaks corresponding to unmodified peptides from ovalbumin in HF-treated and untreated samples. The log of the ratio of S/Ns is plotted for each peptide to yield the "phosphopeptide index". Positive values were found to represent previously phosphorylated peptides, whereas negative values represented unphosphorylated peptides. (B) Peaks corresponding to dephosphorylated versions of four phosphopeptides increased in S/N upon HF treatment. The four phosphopeptides are EVVGpSAEAGVDAASVSEEFR (singly protonated unmodified $m/z = 2008.9$), DKLPGFGDpSIEAQCGTSVNVHSSLR (unmodified $m/z = 2821.4$), EVVGpSAEAGVDAASVSEEFRADHPFLFCIK (unmodified $m/z = 3237.6$), and ISQAVHAAHAEINEAGREVVGpSAEAGVDAASVSEEF (unmodified $m/z = 3763.8$). (C) Unmodified peptide peaks showed a decrease in S/N upon HF treatment. The example shown here is NVLQPSSVDSQTAMVLVNAIVFK ($m/z = 2460.3$).

To test this hypothesis, we analyzed varying amounts (400−2 fmol) of the model phosphoprotein casein, comparing HF-treated and untreated samples. Casein is quadruply phosphorylated on its N-terminal tryptic peptide, and we observed, as before, that HF treatment efficiently dephosphorylated the protein to yield the unmodified tryptic peptide (Figure 3). The dephosphorylated peptide peak was readily discernible at 2 fmol, whereas the phosphopeptide peak did not rise significantly above the background at 10 fmol. Thus, dephosphorylated peptide peaks are detected with considerably enhanced S/N compared with their phosphorylated counterparts.

We sought to take advantage of this enhancement of dephosphorylated peptide signals upon HF treatment in order to efficiently identify phosphopeptides. As a test case, we examined tryptic peptides of ovalbumin, including four phosphopeptides corresponding to two known phosphorylation sites. We calculated the S/Ns for all ovalbumin peaks before and after HF treatment and found a striking division of the peaks into two populations (Figure 4A). The formerly phosphorylated peptides showed an increase in S/N upon HF treatment, whereas unphosphorylated peptides exhibited reduced S/N after HF treatment. This reduction appears to reflect a consistent loss of protein during exposure to HF and pyridine solvent. The detailed mechanism of this loss remains to be explored. Based on this observation, we calculated a "phosphopeptide index" for each tryptic peptide corresponding to the log of the ratio of S/N with and without HF treatment. Positive values for the phosphopeptide index were found to correspond to phosphorylated peptides, and negative values corresponded to unphosphorylated peptides. Visual examination of the spectra confirmed that peaks corresponding to dephosphorylated phosphopeptides showed an increase in S/N upon HF treatment (Figure 4B), whereas unmodified peaks showed a decrease in S/N (see, for example, Figure 4C).

Next, we tested our approach on the recombinant *X. laevis* phosphoprotein, Aurora A kinase. Subsequent to in vitro autophosphorylation in the presence of ATP, SDS−PAGE analysis showed an additional mobility shift for Aurora A (Figure 5A). The shifted band was excised from the gel and split into four samples, which were digested with trypsin or Glu-C and either treated or not treated with HF. These four samples were then analyzed by

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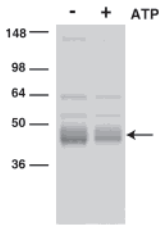

B

residues	peptide sequence	phosphopeptide index	S/N -H_3PO_4	phosphorylation site
[49-58]	ILGPSNVPQR	0.47	149	Ser-53
[344-352]	TYRRISKVE	0.09	104	Ser-349
[293-311]	RTTLCGTLDYLPPEMIEGR[a]	0.07	37	Thr-295
[113-129]	KKGSTDQGKTSAVPKEE	0.11	34	S116, T122
[348-364]	ISKVEFQYPPYVSEEAR	0.13	23	Ser-349
[149-158]	GKFGNVYLAR	0.22	5.3	Tyr-155
[99-113]	NPQQTSHSSTPNVEK[a]	0.12	4.3	Thr-103
[7-25]	ENHKPSNVKIFHPMTEGAK	0.08	3.1	Thr-21
[16-25]	IFHPMTEGAK	0.43	2.1	Thr-21
[246-257]	QLAEALLYCHSK[b]	0.21	<0.5	-
[228-234]	ELQKCTR	0.00	1.3	-
[134-148]	QWCLEDFEIGRPLGK	-0.31	1.1	-
[234-245]	FDDQRSAMYIK	-0.10	1	-
[263-278]	DIKPENLLLGSNGELK	-0.01	1	-
[170-178]	VLFKSQLEK	-0.03	0.9	-
[279-292]	IADFGWSVHAPSSR	-0.47	0.8	-
[279-293]	IADFGWSVHAPSSRR	-0.17	0.5	-
[197-202]	HPNILR	0.00	<0.5	-
[383-407]	GVLEHPWIIKNSQLKKKDEPLPGAQ	0.00	<0.5	-
[213-227]	VYLILDYAPGGELFR	-0.06	<0.5	-
[179-186]	AGVEHQLR	-0.09	<0.5	-
[351-364]	VEFQYPPYVSEEAR	-0.27	<0.5	-
[161-169]	ESKFILALK	-0.36	<0.5	-
[203-212]	LYGYFHDASR	-0.46	<0.5	-
[351-369]	VEFQYPPYVSEEARDLVSK	-0.61	<0.5	-
[27-48]	IPVNQPQSTQFRPPGTAVSAQR	-0.02	<0.5	-
[59-98]	VLAQAQKPILSSQKPTTQIPLRPATQGHQSSKPQGPNENR	-	-	-
[312-346]	MHDETVDLWSLGVLCYEFLVGKPPFETDTHQETYR	-	-	-

[a] Phosphorylation sites were not localized by ms-ms, but have been previously described

[b] False positive

Figure 5. Determination of Aurora A autophosphorylation sites. (A) Recombinant *Xenopus* Aurora A displays reduced mobility in SDS−PAGE upon incubation with ATP. (B) Autophosphorylation sites of Aurora A were found by MS phosphopeptide fingerprinting and confirmed by MS/MS and MS/MS/MS. The S/Ns for all unmodified Aurora A peptides are shown in the table, along with the S/Ns for the peaks corresponding to the loss of phosphoric acid in the MS/MS of the corresponding phosphopeptides. The residues that were phosphorylated are indicated in red.

single-stage MS, and a phosphopeptide index was calculated for each unmodified Aurora A peak (Figure 5B). The phosphopeptide indexes generally correlated well with phosphorylation, as gauged by the S/N of the peak for neutral loss of phosphoric acid (98 Da) in MS/MS. Thus, of the 10 peptides with a positive phosphopeptide index, 9 were verified as phosphorylated by clear detection of the 98 Da signature loss (i.e., with a S/N > 2). The phosphorylation modifications on these nine phosphopeptides were mapped by tandem MS, yielding eight phosphorylation sites that all agreed with previous characterizations of Aurora A phosphorylation.[25−28] None of the peptides with a negative phosphopeptide index was found to be phosphorylated.

We compared our MS phosphopeptide fingerprinting approach to two other established methods using phosphorylated Aurora A as a test case and found that it was the most efficient for finding phosphopeptides (Figure 6). Our method found all 9 phosphopeptides in the first 10 peptides tested. By comparison, analysis of all hypothesized Aurora A phosphopeptides in mass order (i.e., the hypothesis-driven multiple-stage MS approach[23]) yielded all 9 phosphopeptides in the sample, but only after examining 84 peptides. A neutral loss scan approach that analyzed the top 300 most intense peaks in the spectrum found only 4 of the 9 phosphopeptides, indicating that many phosphopeptides are not well-detected directly by MS.

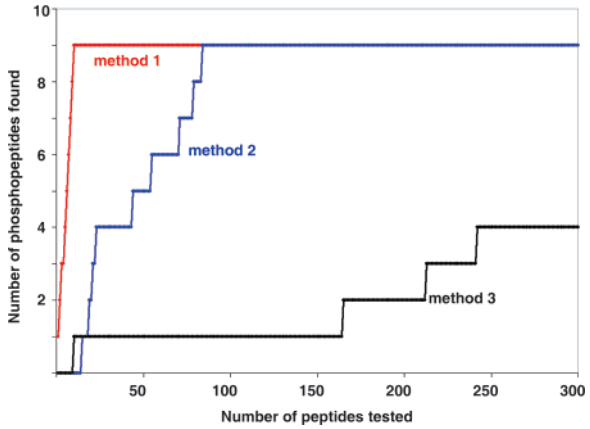

Figure 6. MS phosphopeptide fingerprinting (method 1, red) identifies phosphopeptides more efficiently than hypothesis-driven multistage MS (method 2, blue) and fragmentation of the 300 most intense peaks in the spectrum (method 3, black).

Finally, we applied the MS phosphopeptide fingerprinting approach to an in vivo sample, the *X. laevis* CPC. This complex consists of the Aurora B kinase as well as three other members, Incenp, Dasra A, and Survivin.[29,30] Aurora B has been previously shown to phosphorylate itself as well as the other CPC complex members by incorporation of ^{32}P, but not all of the in vivo

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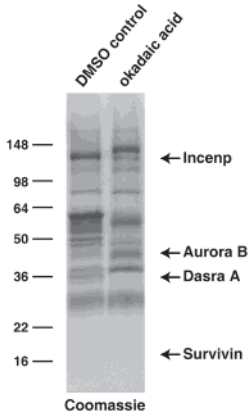

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residues	peptide sequence	phosphopeptide index	s/n -H_3PO_4	phosphorylation site
Incenp				
[847-862]	RTSSAVWHSPPLSSNR	0.45	79	T848, S849, S850
[275-287]	RASLASQFSLASK	0.18	20	Ser-277
Aurora B				
[2-15]	EYKENLNPSSYTSK	0.62	22	Y3, S10, S11, Y12, T13, or S14
[55-66]	ITPSASSSVPGR	0.17	15	Thr-56
[248-264]	TMCGTLDYLPPEMIEGK	0.20	5.7	Thr-248
Dasra A				
[60-70]	DLESLSTTADK	0.36	5.5	S63, S65, T66, T67
[194-206]	LLVTNSSSKPAQR	0.50	4.3	T197, S199, S200, or S201

Figure 7. Chromosome passenger complex (CPC) phosphorylation. (A) CPC was immunoprecipitated from *Xenopus* egg extracts treated with DMSO control or okadaic acid (phosphatase inhibitor) and analyzed by SDS−PAGE. (B) Phosphopeptides were identified for Incenp, Aurora B, and Dasra A. Phosphorylation sites confirmed by tandem MS are indicated in red.

phosphorylation sites have been mapped.[34−38] Phosphorylation serves to activate the CPC, which is normally suppressed in the cytoplasm but active on chromatin. This phosphorylation and activation can be induced either by the addition of phosphatase inhibitors or by clustering of the complex on chromatin.[39] We therefore examined the phosphorylation of CPC members in *Xenopus* egg extracts that had been treated with a phosphatase inhibitor (okadaic acid) or a control (DMSO).

Certain of the immunoprecipitated CPC components from extracts that were treated with okadaic acid showed shifts in SDS−PAGE, compared to DMSO control, consistent with phosphorylation (Figure 7A). The regions containing the phosphorylated CPC proteins were excised from the gel, and the phosphorylated peptides were identified using our MS phosphopeptide fingerprinting method (Figure 7B). CPC peptides that yielded a positive phosphopeptide index were subjected to MS/MS and MS/MS/MS analysis, confirming that these peptides were phosphorylated. In several cases, we were also able to identify the precise sites of phosphorylation. Aurora B is known to bind and phosphorylate the C-terminal region of Incenp, resulting in a positive feedback loop that further stimulates Aurora B activity via autophosphorylation of its activation loop.[36,37] Our analysis indicates that these phosphorylation events occur in *Xenopus* egg extracts, after treatment with okadaic acid, but not DMSO. Incenp was found to be triply phosphorylated on a peptide containing Thr-848, Ser-849, and Ser-850, and Aurora B was phosphorylated at Thr-248.

In addition to these known sites, we found phosphorylation on Incenp at Ser-277 and on Aurora B at Thr-56. Interestingly, Thr-56 is a cyclin-dependent kinase (Cdk) consensus site, which may indicate Cdk regulation of Aurora B, as has been previously suggested for Incenp.[40] We also found a peptide in the N-terminal region of Dasra A that was quadruply phosphorylated on Ser-63, Ser-65, Thr-66, and Thr-67, though the functional significance of this nonconserved region has yet to be determined. In addition, two other phosphopeptides (one each for Aurora B and Dasra A) were found and confirmed by MS/MS/MS, but the precise phosphorylation sites within these peptides were not localized. From our analysis of the CPC, we conclude that MS phosphopeptide fingerprinting can successfully identify phosphopeptides isolated in vivo.

CONCLUSIONS

In summary, we show that chemical dephosphorylation is an effective method for generating an "MS phosphopeptide fingerprint" that permits rapid identification of phosphopeptides by MS.

(34) Honda, R.; Korner, R.; Nigg, E. A. *Mol. Biol. Cell* **2003**, *14*, 3325−3341.
(35) Sessa, F.; Mapelli, M.; Ciferri, C.; Tarricone, C.; Areces, L. B.; Schneider, T. R.; Stukenberg, P. T.; Musacchio, A. *Mol. Cell* **2005**, *18*, 379−391.
(36) Yasui, Y.; Urano, T.; Kawajiri, A.; Nagata, K.; Tatsuka, M.; Saya, H.; Furukawa, K.; Takahashi, T.; Izawa, I.; Inagaki, M. *J. Biol. Chem.* **2004**, *279*, 12997−13003.
(37) Bishop, J. D.; Schumacher, J. M. *J. Biol. Chem.* **2002**, *277*, 27577−27580.
(38) Wheatley, S. P.; Henzing, A. J.; Dodson, H.; Khaled, W.; Earnshaw, W. C. *J. Biol. Chem.* **2004**, *279*, 5655−5660.
(39) Kelly, A. E.; Sampath, S. C.; Maniar, T. A.; Woo, E. M.; Chait, B. T.; Funabiki, H. *Dev. Cell* **2007**, *12*, 31−43.
(40) Goto, H.; Kiyono, T.; Tomono, Y.; Kawajiri, A.; Urano, T.; Furukawa, K.; Nigg, E. A.; Inagaki, M. *Nat. Cell Biol.* **2006**, *8*, 180−187.

This approach uses MS data to determine candidate phosphopeptides that can be confirmed with efficient consumption of sample by tandem MS analysis. It takes advantage of the S/N enhancement that is generally observed upon dephosphorylation of phosphopeptides; smaller phosphopeptide signals are converted to larger unphosphorylated peptide signals. We have demonstrated this technique using MALDI, but we believe it should be an effective method for electrospray ionization as well. We have applied our technique to the analysis of model phosphoproteins, as well as in vitro and in vivo phosphorylated proteins.

A phosphopeptide index can be calculated for any peptide without prior knowledge of the sample. Thus, even when the protein sample is unknown, MS phosphopeptide fingerprinting should be an effective method for the identification of phosphopeptides. The HF-treated samples can also provide additional peptides for protein identification, especially in cases where the identification of a phosphoprotein might be complicated by heavy modification.

Although our method is highly effective for finding phosphopeptides, the confirmation and unambiguous localization of the precise phosphorylation sites can still be very difficult. Although the neutral loss of phosphoric acid in CID provides useful confirmation of a phosphopeptide, the loss of the phosphate group often makes it difficult to pinpoint its location. In the future, we plan to combine the current approach with fragmentation techniques such as electron-transfer dissociation (ETD) or electron capture dissociation (ECD), which promote peptide bond breakage without loss of phosphate groups.

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