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Accelerated Articles

Quantitative Analysis of Protein Phosphorylation in Mouse Brain by Hypothesis-Driven Multistage Mass Spectrometry

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Determination of site-specific changes in the levels of protein phosphorylation in mammals presents a formidable analytical challenge. Here, we demonstrate a strategy for such analyses utilizing a combination of stable isotope chemical labeling and tandem mass spectrometry. Phosphoproteins of interest are isolated from two sets of animals that have undergone differential drug treatments, separated by SDS−PAGE, excised, and subjected to in-gel enzymatic digestion. Using a simple chemical labeling step, we introduce stable, isotopically distinct mass tags into each of the two sets of peptides that originate from the samples under comparison, mix the samples, and subject the resulting mixture to a procedure based on our previously reported hypothesis-driven multistage MS (HMS−MS) method (Chang, E. J.; Archambault, V.; McLachlin, D. T.; Krutchinsky, A. N.; Chait, B. T. *Anal. Chem.* 2004, *76*, 4472−4483). The method takes advantage of the dominant loss of H_3PO_4 during MS/MS from singly charged phosphopeptide ions produced by matrix-assisted laser desorption/ionization (MALDI) in the ion trap mass spectrometer. In the present work, quantitation is achieved by isolating the range of *m/z* values that include both isotopic forms of the putative phosphopeptide and measuring the relative intensities of the two resulting −98-Da fragment ion peaks. This MS/MS measurement can be repeated on the same MALDI sample for all potential phosphopeptide ion pairs that we hypothesize might be produced from the protein under study. Use of MS/MS for quantitation greatly increases the sensitivity of the method and allows us to measure relatively low levels of phosphorylation, phosphopeptides, or both that are not easily observable by single-stage MS. We apply the current method to the determination of changes in the levels of phosphorylation in DARPP-32 from the mouse striatum upon treatment of animals with psychostimulant drugs.

Quantitative measurements of changes in site-specific protein phosphorylation provide critical information for deducing the identity of signaling pathway components and for elucidating details of signaling and control networks. Traditional methods for quantification include the use of ^{32}P labeling[1] and phosphopeptide-specific antibodies.[2] While highly effective, these methods have certain practical limitations. For example, the use of antibodies presupposes knowledge of the phosphorylation site(s) of interest and the availability of the requisite site-specific antibodies. More recently, mass spectrometry (MS)-based methods have emerged as viable alternatives for quantitative phosphorylation analysis.[3−6] MS methods are attractive because they can, in principle, detect and measure any phosphorylation site within a protein. Quantification is typically achieved by MS determination of phosphopeptides that are differentially labeled with stable isotopes.[7−9] Detection of the phosphopeptides may be facilitated by selective enrichment

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(1) Boyle, W. J.; van der Geer, P.; Hunter, T. *Methods Enzymol.* **1991**, *201*, 110−149.
(2) Nishi, A.; Bibb, J. A.; Snyder, G. L.; Higashi, H.; Nairn, A. C.; Greengard, P. *Proc. Natl. Acad. Sci. (U.S.A.)* **2000**, *97*, 12840−12845.
(3) McLachlin, D. T.; Chait, B. T. *Curr. Opin. Chem. Biol.* **2001**, *5*, 591−602.
(4) Aebersold, R.; Mann, M. *Nature* **2003**, *422*, 198−207.
(5) Loyet, K. M.; Stults, J. T.; Arnott, D. *Mol. Cell. Proteomics* **2005**, *4*, 235−245.
(6) Jensen, O. N. *Curr. Opin. Chem. Biol.* **2004**, *8*, 33−41.
(7) Oda, Y.; Huang, K.; Cross, F. R.; Cowburn, D.; Chait, B. T. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 6591−6596.
(8) Gruhler, A.; Olsen, J. V.; Mohammed, S.; Mortensen, P.; Faergeman, N. J.; Mann, M.; Jensen, O. N. *Mol. Cell. proteomics* **2005**, *4*, 310−327.
(9) Julka, S.; Regnier, F. *J. Proteome Res.* **2004**, *3*, 350−363.

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with immobilized metal affinity chromatography[10] or chemically added affinity tags,[9] differential peptide mapping of samples before and after phosphatase treatment,[11] and the use of tandem MS (MS/MS).[12,13] One method combines phosphatase treatment and chemical labeling with single-stage MS to determine absolute stoichiometries of site-specific phosphorylation to within 10% accuracy.[14] Notwithstanding these substantial advances, determination of in vivo phosphorylation changes in animals remains a formidable analytical challenge because (i) it is usually not practical to metabolically label whole animals with stable isotopes, (ii) the amount of phosphoprotein is often limiting, and (iii) the stoichiometry of phosphorylation can be low.

Quantitation using MS/MS of isotopically labeled proteins has been shown to have certain advantages, as compared to quantitation using single-stage MS.[15−18] Here, we demonstrate a strategy for determination of site-specific changes in the levels of protein phosphorylation in mammals utilizing a combination of stable isotope chemical labeling and tandem mass spectrometry. We apply this method to the determination of changes in the levels of phosphorylation in DARPP-32 from the mouse striatum upon treatment of animals with psychostimulant drugs.

EXPERIMENTAL SECTION

Striatal Slice Preparation/DARPP-32 Protein Isolation. Six 6−8-week-old male C57/Bl6 mice were used to prepare striatal brain slices as described.[19] Half of the slices were treated with 5 μM forskolin (Tocris) for 5 min, and half were untreated. Control and forskolin treated slices were pooled into separate tubes and sonicated in sodium citrate buffer (10 mM Na_2HPO_4, 10 mM citric acid, pH 2.8) with phosphatase inhibitors added at 1:100 dilution (Phosphatase Inhibitor Cocktail Sets I and II, Calbiochem). Samples were centrifuged at 15 000 rpm for 15 min to remove insoluble material. The supernatant was adjusted to pH 6.0 using 0.5 M Na_2HPO_4, and 20 μg of total DARPP-32 monoclonal antibody was added. Samples were rotated at 4 °C for 1 h, and 200 μL of protein G magnetic beads (Dynal Biotech) was added for an additional hour. Bound protein was isolated using a magnetic particle concentrator (Dynal Biotech), washed three times in PBS, and eluted by boiling in 2× NuPAGE sample buffer. Ten percent of the final immunoprecipitated sample was run on a gel and analyzed by immunoblotting using polyclonal phospho-Thr34 and monoclonal total DARPP-32 antibodies. The remaining sample was used for mass spectrometric analysis.

(10) Ficarro, S. B.; McCleland, M. L.; Stukenberg, P. T.; Burke, D. J.; Ross, M. M.; Shabanowitz, J.; Hunt, D. F.; White, F. M. *Nat. Biotechnol.* **2002**, *20*, 301−305.
(11) Larsen, M. R.; Sorensen, G. L.; Fey, S. J.; Larsen, P. M.; Roepstorff, P. *Proteomics* **2001**, *1*, 223−238.
(12) Qin, J.; Chait, B. T. *Anal. Chem.* **1997**, *69*, 4002−4009.
(13) Garcia, B. A.; Shabanowitz, J.; Hunt, D. F. *Methods* **2005**, *35*, 256−264.
(14) Zhang, X.; Jin, Q. K.; Carr, S. A.; Annan, R. S. *Rapid Commun. Mass Spectrom.* **2002**, *16*, 2325−2332.
(15) Arnott, D.; Kishiyama, A.; Luis, E. A.; Ludlum, S. G.; Marsters, J. C., Jr.; Stults, J. T. *Mol. Cell. Proteomics* **2002**, *1*, 148−156.
(16) Berger, S. J.; Lee, S. W.; Anderson, G. A.; Pasa-Tolic, L.; Tolic, N.; Shen, Y.; Zhao, R.; Smith, R. D. *Anal. Chem.* **2002**, *74*, 4994−5000.
(17) Ong, S. E.; Blagoev, B.; Kratchmarova, I.; Kristensen, D. B.; Steen, H.; Pandey, A.; Mann, M. *Mol. Cell. Proteomics* **2002**, *1*, 376−386.
(18) Venable, J. D.; Dong, M.-Q.; Wohlschlegel, J.; Dillin, A.; Yates, J. R., III. *Nat. Methods* **2004**, *1*, 39−45.
(19) Nishi, A.; Snyder, G. L.; Nairn, A. C.; Greengard, P. *J. Neurochem.* **1999**, *72*, 2015−2021.

In Vivo Drug Treatments. Eight to twelve 6−8-week-old male C57/Bl6 mice were used for each in vivo experiment, in which the effect of the drug was compared with that of a control (saline). Mice were injected intraperitoneally with saline, amphetamine (7.5 mg/kg, Sigma), cocaine (30 mg/kg, Sigma), or SKF 81297 (5 mg/kg, Tocris) and killed 15-min postinjection by focused microwave irradiation (4.5 kW for 1.4 s) using a small animal microwave (Muromachi Kikai, Tokyo, Japan). Brains were rapidly removed, bilateral striata were dissected on a cold plate, and samples were placed in microcentrifuge tubes and kept frozen in liquid nitrogen until processed. All saline- and drug-treated striata were pooled separately, and DARPP-32 protein was isolated for each treatment using the acid extraction/immunoprecipitation protocol described above.

DARPP-32 In-Gel Digestion, Propionylation, and Extraction. Immunoprecipitated DARPP-32 protein from mouse striatum was dissolved in NuPAGE sample buffer, reduced with 10 mM DTT at 70 °C for 10 min, and then alkylated with 50 mM iodoacetamide at room temperature in the dark for 30 min. The sample was separated on SDS−PAGE (NuPAGE Bis-Tris gel, Invitrogen, Carlsbad, CA), stained with GelCode blue staining reagent (Pierce, Rockford, IL) and excised from the gel. The protein in the gel band was oxidized with 0.01% performic acid reagent in acetonitrile/water (1:1) for 15 min at room temperature, destained in acetonitrile/50 mM NH_4HCO_3 (1:1), and dried. (Performic acid reagent was generated by reacting 100 μL of 88% formic acid and 900 μL of 30% hydrogen peroxide at room temperature for 1 h.) Trypsin (250 ng) in 50 mM NH_4HCO_3 was then added, and the digestion was allowed to proceed for 6 h at 37 °C. Propionylation and peptide extraction was achieved by sequentially treating the gel with the following solutions: (1) 50 μL of 1% d_0- or d_{10}-propionic anhydride (Sigma) in acetonitrile/50 mM NH_4HCO_3 (1:1); (2) 30 μL of 1% d_0- or d_{10}-propionic anhydride in acetonitrile; (3) twice with 50 μL of 0.1% TFA in acetonitrile/water (1:1); (4) 30 μL of acetonitrile. The sample was sonicated for 15 min in each solution, and the supernatant from each extraction step was combined and dried in a Speedvac.

In Vitro phosphorylation of rrDARPP-32. For our studies of the linearity of the present method, recombinant rat DARPP-32 (rrDARPP-32) was phosphorylated to completion on Thr34 using purified PKA. For a 50-μL reaction, we used 5 μL of 10× PKA reaction buffer (500 mM HEPES, 100 mM $Mg(OAc)_2$, 10 mM EGTA final concentration), 34 μL of dd-H_2O, 5 μL of ATP/$MgCl_2$ cocktail (Upstate Catalogue No. 20-113), 5 μg of rrDARPP-32 protein, and ~1 μg of PKA catalytic subunit.[20] The reaction was carried out at 37 °C for 1 h. Incorporation of phosphate was verified with a radioactive side reaction containing the same reaction mixture plus 0.5 mCi of hot (^{32}P) ATP.

Mass Spectrometry. Single-stage and MS/MS mass spectra were collected on a MALDI-QqTOF mass spectrometer and MALDI-ion trap mass spectrometer, respectively, both fitted with in-house-constructed ion sources.[21,22] The MALDI matrix used was 2,5-dihydroxybenzoic acid (DHB) (Sigma). MS/MS spectra were

(20) Kaczmarek, L. K.; Jennings, K. R.; Strumwasser, F.; Nairn A. C.; Walter, U.; Wilson, F. D.; Greengard, P. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 7487−7491.
(21) Krutchinsky, A. N.; Zhang, W.; Chait, B. T. *J. Am. Soc. Mass Spectrom.* **2000**, *11*, 493−504.
(22) Krutchinsky, A. N.; Kalkum, M.; Chait, B. T. *Anal. Chem.* **2001**, *73*, 5066−5077.

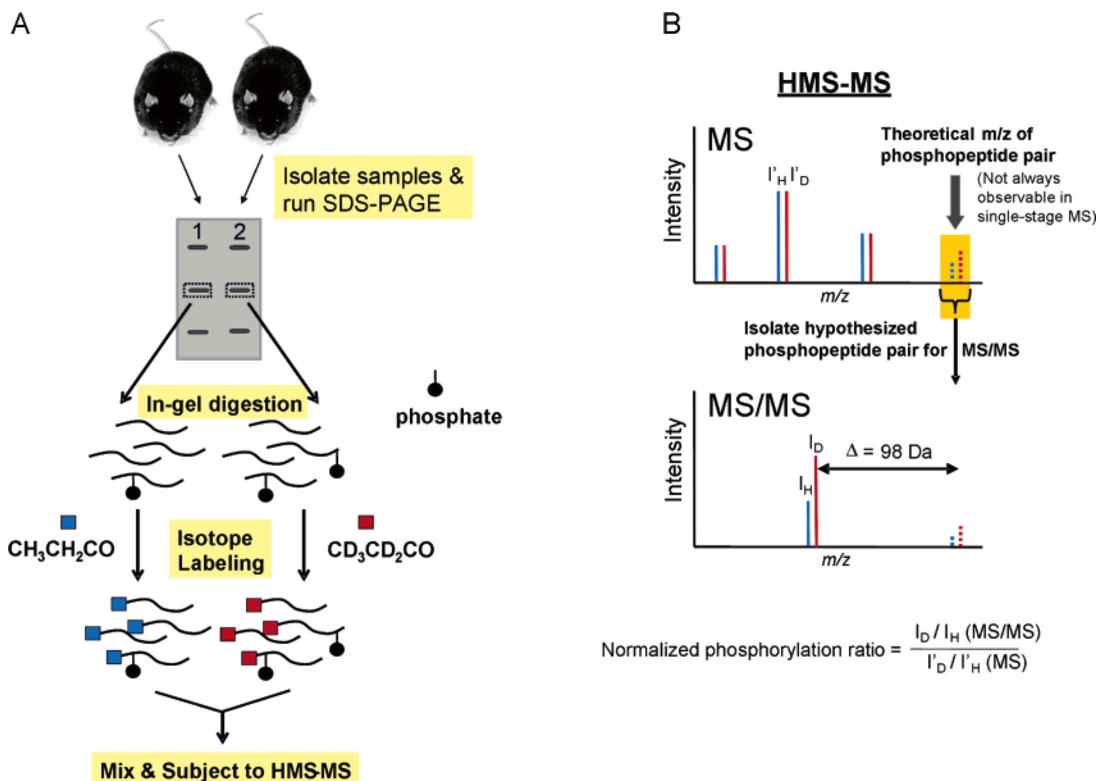

Figure 1. Strategy for measuring site-specific changes in the levels of protein phosphorylation. (A) Proteins from differentially treated mice are isolated, chemically labeled with stable, isotopically distinct mass tags, mixed, and subjected to the HMS−MS shown in (B). I_H and I_D designate the intensities of the 98-Da loss fragment ions from the hydrogen- and deuterium-labeled phosphorylated peptide. I'_H and I'_D designate the intensities of the hydrogen- and deuterium-labeled unphosphorylated peptide ion peaks that are used to normalize the phosphorylation ratio.

collected for 0.5−2 min, using injection times of 100−1000 ms, activation time of 300 ms, and relative collision energy of 30−40%. The isolation width was 15−20 *m/z* units, where the range was set to include all isotope peaks of the hypothesized phosphopeptide pair of interest.

Linearity Measurements. In the large range study, samples of dephospho-rrDARPP-32 and rrDARPP-32 with Thr34 fully phosphorylated in vitro were reduced, alkylated, and mixed to obtain samples with the following phosphorylation stoichiometries: 1.25, 2.5, 5, 10, and 20%. Total protein (10 pmol) from each sample was separated on SDS−PAGE in duplicate. In-gel digestion, propionic anhydride labeling, and peptide extraction were performed as described above, except for each duplicate, the sample was labeled with d_0- or d_{10}-propionic anhydride. The extracted tryptic peptides from each sample were dried and reconstituted in 20 μL of 1/6 saturated DHB solution in methanol/acetonitrile/water at 5:2:3 (v/v/v) containing 0.1% TFA final concentration. MALDI samples containing the various ratios of heavy- to light-labeled phosphopeptide were obtained by mixing 2-μL samples from each of the two respective reconstituted solutions on the MALDI sample plate. The phosphorylation ratios were determined as $[I_H/I_D \text{ (MS/MS)}]/[I'_H/I'_D \text{ (MS)}]$. In the small range study, samples were mixed as above to give phosphorylation stoichiometries of 1.0, 1.2, 1.4, 1.6, 1.8, and 2.0%. The 1.2, 1.4, 1.6, 1.8, and 2.0% stoichiometry samples were labeled with d_{10}-propionic anhydride and individually compared with 1.0% stoichiometry samples labeled with d_0-propionic anhydride. The resulting phosphorylation ratios were determined as $[I_D/I_H \text{ (MS/MS)}]/[I'_D/I'_H \text{ (MS)}]$. The labeling was then reversed, and the ratios were determined as $[I_H/I_D \text{ (MS/MS)}]/[I'_H/I'_D \text{ (MS)}]$. Averaged ratios were calculated as the mean of these two results for each data point.

RESULTS AND DISCUSSION

To address the challenges outlined above for determining in vivo phosphorylation changes in animals, we developed the strategy summarized in Figure 1. Here, the proteins of interest are isolated from two sets of animals that have undergone differential drug treatments, separated by SDS−PAGE, excised, and subjected to in-gel enzymatic digestion. Using a simple chemical labeling step, we introduce stable, isotopically distinct mass tags into each of the two sets of peptides that originate from the samples under comparison, mix the samples, and subject the resulting mixture to a procedure based on our previously reported hypothesis-driven multistage MS (HMS−MS) method.[23] With this procedure, it is not necessary to observe the phosphopeptides directly by single-stage MS. We need only hypothesize that a given phosphopeptide may be present and test this hypothesis by looking for the neutral loss of H_3PO_4 (i.e., −98 Da) in an MS/MS experiment. The method takes advantage of this dominant loss of H_3PO_4 during MS/MS from singly charged phosphopeptide ions produced by matrix-assisted laser desorption/ionization

(23) Chang, E. J.; Archambault, V.; McLachlin, D. T.; Krutchinsky, A. N.; Chait, B. T. *Anal. Chem.* **2004**, *76*, 4472−4483.

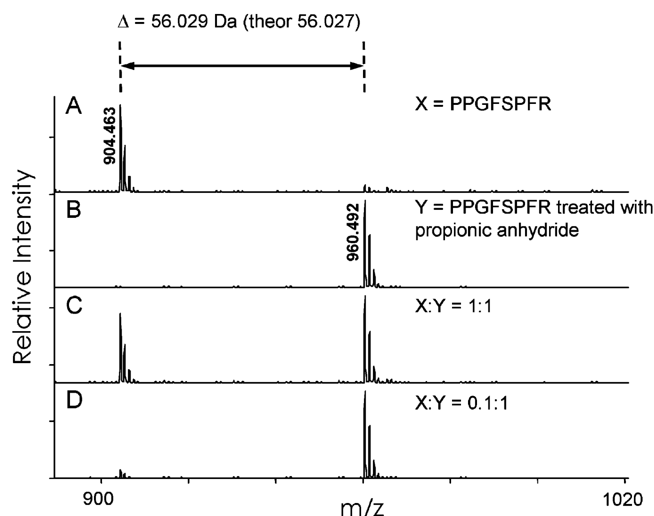

Figure 2. Fifteen-minute treatment of a peptide with propionic anhydride yielding quantitative propionylation of the free amine with no significant side reactions. MALDI-TOF spectra of (A) control peptide sample treated with a 1:1 (v/v) mixture of acetonitrile and 50 mM NH_4HCO_3 for 15 min; (B) peptide treated with 1% propionic anhydride in a 1:1 (v/v) mixture of acetonitrile and 50 mM NH_4HCO_3 for 15 min; (C) dried and resuspended samples of (A) and (B) mixed at 1:1; (D) dried and resuspended samples of (A) and (B) mixed at 1:10 (v/v).

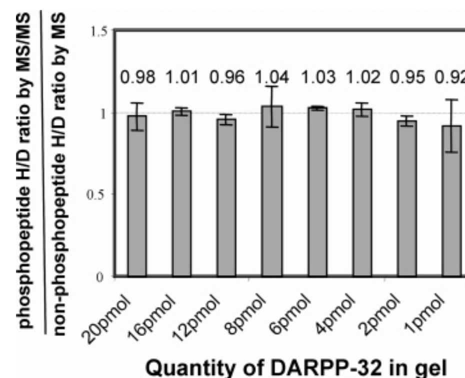

Figure 3. H-labeled to D-labeled phosphopeptide ratio for phosphorylated Thr34 measured by MS/MS accurately reflects the ratio measured using the unphosphorylated peptide, [9−23] IQFSVPAPPSQLDPR at m/z 1707.9 and 1712.9 (MH^+), observed in the single-stage mass spectrum of rrDARPP-32 tryptic peptides. The same peptide was used for normalization in the mice experiments. The error bars represent the standard deviations of three independent measurements of different H/D mixing ratios.

(MALDI) in the ion trap mass spectrometer.[12] Quantitation is achieved by isolating the range of m/z values that include both isotopic forms of the putative phosphopeptide and measuring the relative intensities of the two resulting −98-Da fragment ion peaks. This MS/MS measurement can be repeated on the same MALDI sample for all potential phosphopeptide ion pairs that we hypothesize might be produced from the protein under study. Use of MS/MS for quantitation greatly increases the sensitivity of the method and allows us to measure relatively low levels of phosphorylation, phosphopeptides, or both that are not easily observable by single-stage MS. Here, we apply this method to determine site-specific changes in the levels of phosphorylation in DARPP-32 (an integrator of signaling in the mammalian brain[24] from mouse striatum) upon treatment of live animals with cocaine, amphetamine, or a D1 dopamine receptor agonist.

For stable isotope labeling, we use propionic anhydride to introduce propionyl groups (containing either 5 hydrogens or 5 deuteriums) to the peptide amino termini and the free amines on lysine side chains (Experimental Section). This conversion was observed to be quantitative after a simple 15-min treatment with no significant side reactions (Figure 2). The reaction was carried out subsequently to in-gel trypsin digestion of the protein of interest and was combined with the normal peptide extraction steps. The mass differences between light and heavy isotopically labeled tryptic peptides were generally either 5 or 10 Da (corresponding to the presence of either 0 or 1 lysine residue). The present quantitation consists of three steps. First, assuming that all potential sites can be phosphorylated in a given protein, we calculate the m/z values of all possible singly charged phosphopeptide ions that could theoretically be generated from the particular digestion and isotope labeling procedures used. Second, employing a MALDI ion trap mass spectrometer, we set an isolation window that encompasses the calculated m/z for both the heavy- and light-isotope labeled forms of a given phosphopeptide. Typically, an isolation window between 15 and 20 m/z units is sufficient to include all isotope peaks of the peptide pairs. Third, we subject peptide ions in this mass range to MS/MS, where the fragment ion peak (pair) generated from the neutral loss of H_3PO_4 indicates the presence of a phosphopeptide and also serves as the basis for quantitation (Figure 1).

To validate our method, we tested whether the phosphopeptide ratio measured using MS/MS accurately reflects the ratio measured using single-stage MS. Equal aliquots of rrDARPP-32 with 10% phosphorylation on Thr34 (a site previously demonstrated, when phosphorylated, to convert DARPP-32 into a potent inhibitor of protein phosphatase-1[24]) were subjected to SDS−PAGE, in-gel digestion, and labeling with heavy or light propionic anhydride, respectively. The extracted peptides from the two samples were mixed in various ratios and subjected to both single-stage MS and MS/MS analysis. The ratio of the heavy- versus light-labeled Thr34-containing phosphopeptide measured in the MS/MS experiment consistently reflected the ratio of heavy- versus light-labeled nonphosphorylated peptides from rrDARPP-32 measured in the single-stage MS for all amounts tested (Figure 3). This result validates the current approach and demonstrates that the ratio of non-phosphopeptide peaks measured in single-stage MS can be used to normalize the total sample loading. The linearity of the method was tested by mixing rrDARPP-32 that was fully phosphorylated on Thr34 with unphosphorylated rrDARPP-32 at different ratios to simulate different phosphorylation stoichiometries. We found that HMS−MS quantitated relatively large changes in phosphorylation level with as little as 12.5 fmol of phosphopeptide present (Figure 4A), as well as relatively small changes in phosphorylation level (in the range of 1−2-fold) when as little as 10 fmol of phosphopeptide was present (Figure 4B).

We first applied the current method to the quantitation of cAMP-induced phosphorylation changes of DARPP-32 using an in vitro model system—i.e., slices from mouse striatum treated with forskolin. We observed a 6-fold increase in phosphorylation at Thr34, consistent with results obtained from a portion of the

(24) Svenningsson, P.; Nishi, A.; Fisone, G.; Girault, J. A.; Nairn, A. C.; Greengard, P. *Annu. Rev. Pharmacol. Toxicol.* **2004**, *44*, 269−296.

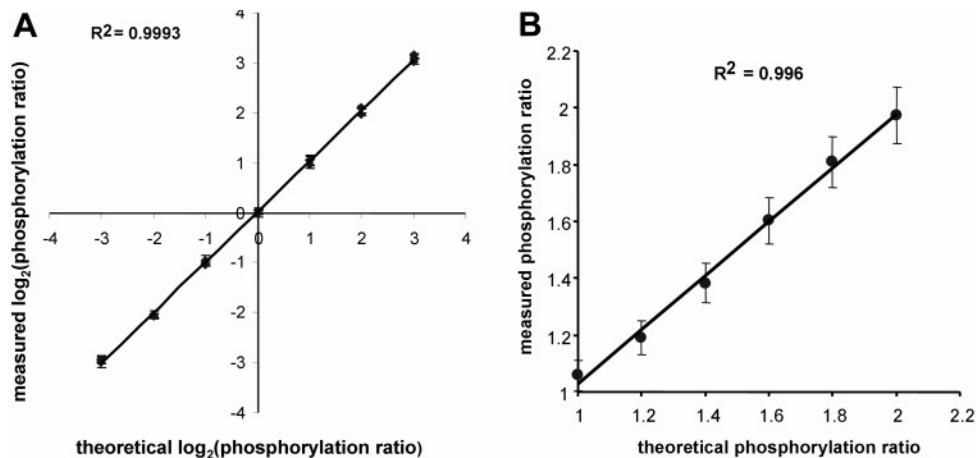

Figure 4. Accurate measure of site-specific changes in phosphorylation provided by HMS–MS. Measured versus theoretical ratios of phosphorylated Thr34 from rrDARPP-32 over a (A) large range and (B) small range. The lowest amount of phosphoprotein tested in (A) was 12.5 fmol and in (B) was 10 fmol (the latter present in 1% abundance compared to unphosphorylated rrDARPP-32). The error bars in (A) reflect the standard deviations of three independent experiments and in (B) reflect the average errors of measurements done in two independent experiments.

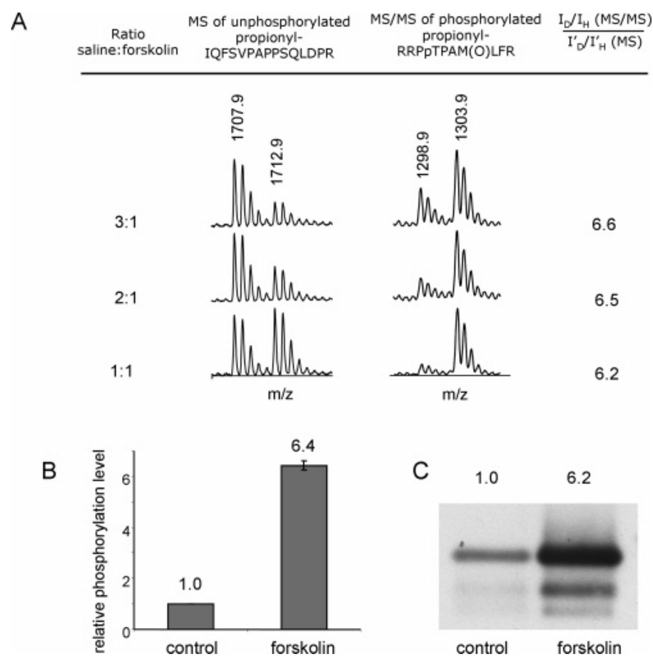

Figure 5. Quantitation of forskolin-induced phosphorylation changes of DARPP-32 using an in vitro model system—i.e., slices from mouse striatum. (A) Determination of the phosphorylation ratio (see Figure 1) for three different ratios of the saline to forskolin-treated samples. (B) Summary of the results from the measurements shown in (A). (C) Traditional immunoblot analysis with phospho-Thr34 antibody of the same samples. The numbers in (B) and (C) provide the measured fold induction of Thr34 phosphorylation.

same sample using traditional immunoblot analysis (Figure 5) as well as previously reported results.[2] We then applied HMS–MS quantitation to the study of the acute effect of three different drugs (cocaine, amphetamine, and the D1 agonist SKF81297) on DARPP-32 phosphorylation in the brains of living mice (Figure 6). Hypothesizing the presence of phosphorylation changes at all possible Ser and Thr residues in DARPP-32, we used HMS–MS to determine whether these were indeed phosphorylated and the levels of change in phosphorylation upon drug treatment (87 hypothetical phosphopeptides were assayed in this manner). In addition to the known phosphorylation sites at Thr34, Ser97, and Ser130, we detected two additional phosphopeptides (Table 1); one contains the phosphorylation site Ser192 and the other contains a phosphorylation site either on Ser45 or Ser46 (in this case, the site was not unambiguously identified). We observed a statistically significant increase in phosphorylation levels at Thr34 (50–100%) for all three drug treatments, consistent with prior studies.[2,25] By contrast, the other four phosphorylation sites remained unchanged within the error of the measurement (±10%).

Quantitation by HMS–MS is achieved at the MS/MS level, substantially increasing the sensitivity and accuracy compared with quantitation using single-stage MS. This improvement was clearly apparent for quantitation of Thr34 phosphorylation on DARPP-32 from mouse brain, where the basal level of phosphorylation was determined to be 0.5% (i.e., 0.005 mol/mol) (data not shown). Thus, although the phosphopeptide containing phosphorylated Thr34 is not readily discernible over background in the single-stage mass spectrum, a signal-to-noise ratio of >10 is achieved using MS/MS (Figure 7). Reasons for this marked improvement in signal-to-noise ratio include the following: (i) our ability to trap more precursor ions in the MS/MS experiment compared to the single-stage MS experiment because we are able to overfill the trap without adversely affecting the MS/MS spectrum; (ii) the 98-Da neutral loss dominates the fragmentation of singly charged phosphopeptides, effectively channeling the quantitative information from the parent ion pair into the corresponding fragment ion pair; (iii) the chemical noise does not yield much fragmentation in the −98-Da region of the MS/MS spectrum; and (iv) the neutral loss provides an additional constraint on the data.

The present method differs from LC–MS/MS strategies that employ electrospray ionization mass spectrometry in that all theoretically possible phosphopeptides can be explored using a

(25) Svenningsson, P.; Tzavara, E. T.; Carruthers, R.; Rachleff, I.; Wattler, S.; Nehls, M.; McKinzie, D. L.; Fienberg, A. A.; Nomikos, G. G.; Greengard, P. *Science* **2003**, *302*, 1412–1415.

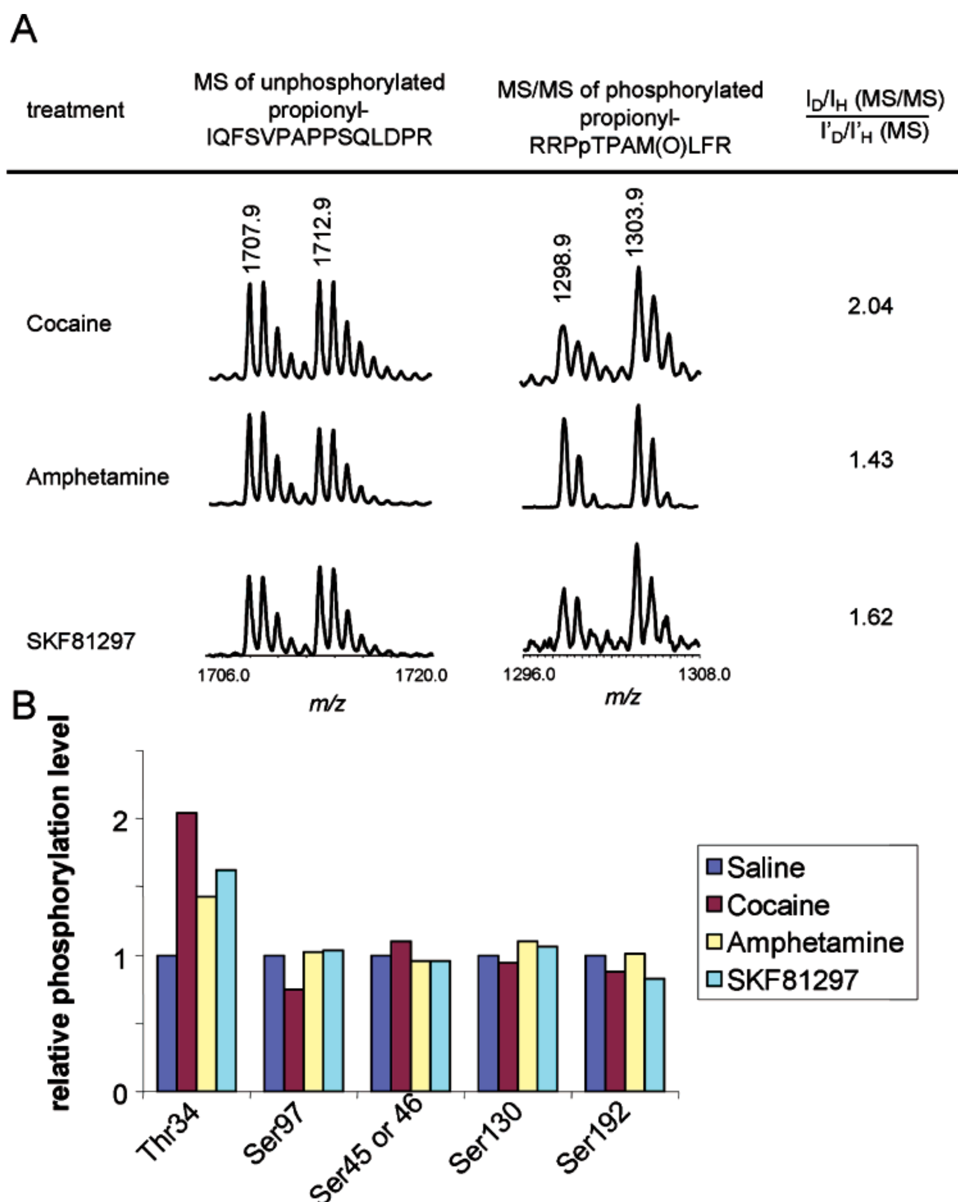

Figure 6. (A) HMS–MS data used to determine the effect of three different drugs on the phosphorylation of Thr34 in DARPP-32 in the brains of living mice, (B) Effect of the drugs on the phosphorylation of five specific sites of DARPP-32 in the brains of living mice.

Table 1. Amino Acid Sequences of the DARPP-32 Phosphopeptides Characterized in the Present Work

amino acid sequence	residue number from	residue number to	phosphorylation site
RRPTPAM(O)LFR	31	40	Thr34
AVQHLQTISNLSENQASEEEDELGELR	81	107	Ser97
ELGYPQEDDEEDEDEEEDEEEDSQAEVLK	108	136	Ser130
VSEHSSPEEEASPHQR	41	56	Ser45 or Ser46
ATLSEPGEEPQHPSPP	179	194	Ser192

single MALDI sample. While traditional immunoblot methods for quantitation of site-specific phosphorylation on proteins are typically more sensitive than the present MS-based method, the former require the availability of appropriate antibodies and can only measure one phosphorylation site at a time. By contrast, HMS–MS can provide highly accurate quantitative information on multiple phosphorylation sites in a single experiment without the need for phosphospecific antibodies. Potential limitations of the method include (1) possible interferences in the 10–20-Da wide MS/MS window and (2) low-mass spectrometric response for a given phosphopeptide. Although no instances of interference were observed in the present work, the tryptic phosphopeptide resulting from the known phosphorylation at T75 in DARPP-32 had a sufficiently low response to preclude its measurement by

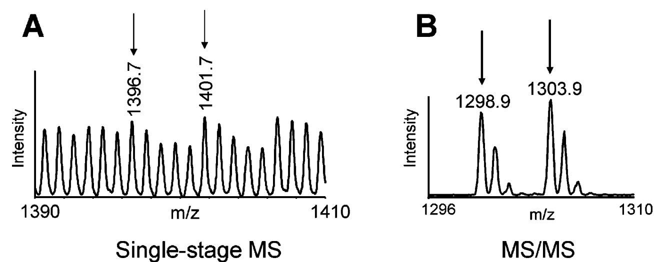

Figure 7. HMS−MS quantitation at the MS/MS level (see B) substantially increases the signal-to-noise ratio, sensitivity, and accuracy compared with quantitation using single-stage MS (see A). Arrows indicate (A) the predicted positions of the heavy and light propionylated singly protonated ions from the Thr34-containing phosphopeptide [RRPpTPAM(O)LFR], where M(O) designates methionine sulfoxide and (B) the positions of the fragment ions from this peptide pair resulting from the loss of H_3PO_4 (i.e., 98 Da). The sample was obtained from amphetamine-treated mice and contained ~70 fmol of phosphoprotein.

the present method. Indeed, propionylation of the lysine residues may, in certain cases, be expected to reduce the MS response, especially for peptides that do not contain other basic residues. In such cases, it may be advantageous to guanidylate the lysines residues, as previously described.[14,26]

(26) Brancia, F. L.; Oliver, S. G.; Gaskell, S. J. *Rapid Commun. Mass Spectrom.* **2000**, *14*, 2070−2073

CONCLUSIONS

In summary, we find HMS−MS to be a viable method for quantitating site-specific phosphorylation changes in mammalian brain proteins. It is a general method in that it can be applied to the measurement of any phosphorylation site provided that we have available a sufficient quantity of phosphoproteins (more than a few tens of femtomoles) and that the phosphopeptides yield an adequate MS response. The method should be particularly useful for following changes in phosphorylation for sites against which antibodies have not yet been raised.

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