Improved Detection of Hydrophilic Phosphopeptides Using Graphite Powder Microcolumns and Mass Spectrometry

EVIDENCE FOR IN VIVO DOUBLY PHOSPHORYLATED DYNAMIN I AND DYNAMIN III*

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A common strategy in proteomics to improve the number and quality of peptides detected by mass spectrometry (MS) is to desalt and concentrate proteolytic digests using reversed phase (RP) chromatography prior to analysis. However, this does not allow for detection of small or hydrophilic peptides, or peptides altered in hydrophilicity such as phosphopeptides. We used microcolumns to compare the ability of RP resin or graphite powder to retain phosphopeptides. A number of standard phosphopeptides and a biologically relevant phosphoprotein, dynamin I, were analyzed. MS revealed that some phosphopeptides did not bind the RP resin but were retained efficiently on the graphite. Those that did bind the RP resin often produced much stronger signals from the graphite powder. In particular, the method revealed a doubly phosphorylated peptide in a tryptic digest of dynamin I purified from rat brain nerve terminals. The detection of this peptide was greatly enhanced by graphite micropurification. Sequencing by tandem MS confirmed the presence of phosphate at both Ser-774 and Ser-778, while a singly phosphorylated peptide was predominantly phosphorylated only on Ser-774. The method further revealed a singly and doubly phosphorylated peptide in dynamin III, analogous to the dynamin I sequence. A pair of dynamin III phosphorylation sites were found at Ser-759 and Ser-763 by tandem MS. The results directly define the in vivo phosphorylation sites in dynamins I and III for the first time. The findings indicate a large improvement in the detection of small amounts of phosphopeptides by MS and the approach has major implications for both small- and large-scale projects in phosphoproteomics. Molecular & Cellular Proteomics 3:456–465, 2004.

Proteomics studies using mass spectrometry (MS)† are focused on small- or large-scale identification of proteins and quantification of levels of protein expression. Because protein function often can be dependent on post-translational modifications of the protein, this approach may fail to reveal insights into the function of target proteins. Reversible phosphorylation of proteins is one of the most abundant post-translational modifications in nature that affects protein function. Phosphorylation is a means of regulation of protein activity, subcellular localization, or modulation of molecular interactions (1, 2).

Protein phosphorylation is not routinely surveyed in proteomic studies. Phosphopeptides produced by proteolysis are usually small and hydrophilic, due to the addition of the hydrophilic phosphate group and the preference for many protein kinases to target sequences rich in basic amino acids residues. Proteolytic digests examined by MS are often likely to fail to detect phosphopeptides because they are low in stoichiometry, are suppressed during the ionization (3), or do not bind to the media used to desalt the fraction or separate the peptides (e.g. 4). Recently, attempts have been made to identify phosphorylated proteins using proteomic methods combined with immobilized metal affinity chromatography (IMAC) to selectively bind phosphopeptides, spawning the sub-field of phosphoproteomics (5–8). After selective binding and elution of the phosphopeptides, the standard approach used in proteomics is to desalt and concentrate the eluted peptides using reversed phase (RP) chromatographic material prior to MS detection. The phosphopeptides are then eluted from the RP column with an acetonitrile gradient, either online or off-line with MS, or by batch-mode/isocratic elution from RP microcolumns (e.g. ZipTips) or spin-columns prior to MS.

An endemic problem in this approach is that phosphopeptides may not be retained by RP chromatography because they are too small and/or hydrophilic to bind to the C18 stationary phase (4, 9, 10). An alternative chromatographic material that is able to strongly bind hydrophilic molecules is graphite powder (11, 12). Porous graphitic carbon chromatography has been used recently to retain and resolve phos-

†The abbreviations used are: MS, mass spectrometry; MALDI, matrix-assisted laser desorption/ionization; RP, reversed phase; IMAC, immobilized metal affinity chromatography; GST, glutathione S-transferase; TFA, trifluoroacetic acid; 4HCCA, α-cyano-4-hydroxycinnamic acid; LC, liquid chromatography; ESI, electrospray ionization; 2DE, two-dimensional gel electrophoresis.

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phopeptides that previously eluted with the nonbinding salts and buffers from an RP column (9). This study revealed three phosphorylated peptides from mixed-lineage kinase 3 that were not retained by RP chromatography. Recently, we introduced the use of graphite powder microcolumns packed in GELoader tips as an alternative or supplement to RP material for purification of sub-picomole amounts of peptides (10). We found that graphite powder microcolumns were able to effectively retain small and hydrophilic peptides, which could be readily eluted for MS analysis. When used in combination with RP material, we gained a significant increase in sequence coverage from tiny amounts of peptides derived from proteolytic digestion of gel-separated proteins.

In this study, we explore the use of GELoader tip microcolumns packed with graphite powder for analysis of small amounts of phosphorylated peptides. We show that a significant number of phosphorylated peptides are not retained by conventional RP chromatographic material, or even material that is slightly stronger in hydrophobicity (Poros oligo R3, which was originally designed for purification of DNA/RNA). Standard phosphoproteins and biologically relevant phosphopeptides were both analyzed after separation by gel electrophoresis. The microcolumns packed with graphite powder efficiently retained and purified phosphorylated peptides from these samples. Application of the method to phospho-dynamin I from nerve terminals revealed the previously reported singly phosphorylated peptides, but also a doubly phosphorylated peptide not previously detected by conventional methods (13). The method also revealed singly and doubly phosphorylated dynamin III, a protein not previously characterized as an in vivo phosphoprotein. The results indicate a major improvement in detection of low-abundance phosphopeptides for sequencing by tandem MS (MS/MS).

EXPERIMENTAL PROCEDURES

Materials—Modified trypsin was obtained from Promega (Madison, WI). Endoproteinase Glu-C was obtained from Calbiochem (La Jolla, CA). ε-Glycyl-4-hydroxycinnamic acid (4HCCA), activated charcoal (C-5510), ethylenediamine tetraacetic acid (EDTA), iron (III) chloride, glutathione then affinity purified from the synaptosomes using a mixture of the phosphorylation of proteins in the nerve terminals. Dynamin I was been incubated for 1 h at 37 °C for 4–18 h. The supernatant from the digestion was used for analysis by matrix-assisted laser desorption/ionization (MALDI-MS) without peptide extraction.

In-gel Digestion—In-gel digestion was performed as described (16). Briefly, the excised gel pieces and incubated on ice for 1 h. After removing the supernatant, additional digestion buffer was added and the digestion was continued at 37 °C for 4–18 h. The supernatant from the digestion was used for analysis by matrix-assisted laser desorption/ionization (MALDI-MS) without peptide extraction.

Peptide Desalting—Custom-made chromatographic microcolumns used for desalting and concentration of the peptide mixture prior to mass spectrometric analysis were prepared using GELoader micropipette tips, as described in detail earlier (10, 17). The flow-through from the Poros R2 and/or Poros oligo R3 were desalted and concentrated on a microcolumn packed with graphite powder. The columns were washed with 20 μl of 0.1% trifluoroacetic acid (TFA). The peptides retained on the columns were eluted using 4HCCA in 70% acetonitrile/0.1% TFA (10 mg/ml).

Alkaline Phosphatase Treatment—Alkaline phosphatase treatment was performed directly on the previously analyzed samples after initial MALDI-MS, as described previously (18). The matrix (4HCCA or dihydroxybenzoic acid) was redissolved using 1.5 μl of 50 mM NH₄HCO₃, pH 7.8, containing alkaline phosphatase (0.05 unit/μl). The MALDI target was placed in a closed plastic box containing a wet tissue to prevent the samples from drying. The box was placed at 37 °C for 20 min. After incubation, the sample was acidified with 0.5 l of 5% TFA, and the matrix was allowed to recrystallize. In cases where the previously analyzed sample was desalted and concentrated on microcolumns, additional matrix solution (0.2 μl) was added before recrystallization. Prior to MALDI-MS analysis, the surface of the sample was washed gently with 10 μl of 0.1% TFA. When very low amounts of peptides were analyzed, the dephosphorylated peptide sample was redissolved on the target, transferred to a microcolumn containing Poros R2, Poros R3, or graphite powder, desalted, and
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returned to the target prior to analysis. For the dynamin 1 sample, one half of the flow-through from the R2 column was dried down and re-suspended in 2.5 μl of 50 mM ammonium bicarbonate with 1 mM magnesium sulfate and 0.25 units/μl alkaline phosphatase. The sample was incubated for 3 h before graphite microcolumn purification and MALDI-MS analysis.

Mass Spectrometry—MALDI-MS was performed using a Voyager STR (PerSeptive Biosystems, Framingham, MA) equipped with delayed extraction. Spectra were obtained in positive reflector mode and positive linear mode using an accelerating voltage of 20 kV. MALDI-MS data analysis was performed using the MoverZ software (www.proteometrics.com). Electrospray ionization hybrid quadrupole time-of-flight mass spectrometry (ESI-MS/MS) was performed using a QSTAR XL (Applied Biosystems/MDS Sciex, Ontario, Canada). The dynamin I sample eluted from the graphite column was sprayed using a boro-silicate nanospray capillary (Proxeon Biosystems, Odense, Denmark) with 1 kV applied. Parent ions were selected using the unit resolution setting. The product ion spectra were acquired using the LINAC pulsar enhancement feature.

RESULTS

Analysis of Synthetic Phosphorylated Peptides Using Different Column Resins—The ability of conventional RP Poros material to retain relatively small phosphorylated peptides was tested using three different synthetic phosphorylated peptides: 1) DpSERGpSGDPGK (M+H)⁺ = 1321.45 Da, 2) VYGTKpSHLR (M+H)⁺ = 1140.56 Da, and 3) KiGEgpTYGV-VYK (M+H)⁺ = 1393.67 Da. The first two phosphopeptides (1, 2) are slightly more hydrophilic than the third one, according to Kyte-Doolittle values (19) calculated using the software GPMAW (www.welcome.to/GPMAW). The three peptides were mixed in equimolar ratio, and 500 femtomoles were applied onto a microcolumn packed with Poros R2 RP resin. The flow-through was collected and applied to a second microcolumn packed with Poros oligo R3 RP resin, which is more hydrophobic than the R2 resin. Finally, the flow-through from this column was applied to a microcolumn packed with graphite powder. Each column was washed using 20 μl of 0.1% TFA. The bound peptides were eluted from the three columns directly onto a MALDI-MS target using 0.2 μl of the matrix solution. The resulting MALDI-MS spectra obtained from the Poros R2 and R3 columns show that the least hydrophilic phosphopeptide (3) was partly retained on both microcolumns (Fig. 1, A and B). The absolute ion intensities in the two spectra indicate that this peptide is poorly retained by the R2 resin compared with the R3 resin. In fact, based on the relative intensities, 75% of the peptide molecules are not retained on the R2 column. Signals originating from the two other phosphopeptides were absent in the eluates from both RP microcolumns. In contrast, the MALDI-MS peptide mass map obtained from the graphite powder microcolumn (Fig. 1C) includes signals belonging to the two most hydrophilic phosphopeptides (1, 2) and a significant signal from the most hydrophobic one (3). The signal from the doubly phosphorylated peptide (1) is much lower compared with the monophosphorylated peptide (2), presumably because of suppression effects in the ionization process and metastable decomposi-

Fig. 1. Sequential purification of a mixture of three synthetic phosphorylated peptides (0.5 pmol each) using microcolumns packed with Poros R2, Poros R3, and graphite powder (GP), respectively. A, MALDI-MS spectrum obtained from the Poros R2 column eluted with the matrix solution (4HCCA in 70% acetonitrile/0.1% TFA). B, MALDI-MS spectrum obtained from the Poros R3 column eluted with the matrix solution. C, MALDI-MS spectrum obtained from the graphite column eluted with the matrix solution.

tion in the time-of-flight analyzer. This clearly shows that the more hydrophilic phosphopeptides are not retained by the Poros RP resin and would have been lost during normal desalting procedures employed in proteomics experiments.

Analysis of Endoproteinase Glu-C-derived Peptides from Gel-separated β-Casein—β-Casein is a phosphorylated protein containing five phosphorylation sites located on Ser-15, Ser-17, Ser-18, Ser-19, and Ser-35. It has been used extensively for developing and validating new methods for mass spectrometric characterization of phosphorylated proteins, mainly because the tryptic monophosphorylated peptide (LpFpSEEQKTEDELDQDKm, m/z 2061.82) ionizes extremely well in MS, resulting in an abundant, easily detectable signal. However, this is in strong contrast to most “real world” phosphorylated peptides, which are in general strongly suppressed in MS experiments in the presence of nonphosphorylated peptides. In addition, the tryptic phosphopeptides from β-casein are easily retained by normal Poros R2 resin. On the other hand, in-gel digestion of β-casein with endoproteinase Glu-C results in a slightly smaller monophosphorylated peptide (LpFpSEEQKTEDELDQDKm, m/z 1705.65), which is not retained by Poros R2 resin. The presence of multiple missed cleavage sites in this peptide and the peptide below is in accordance with our previous experience using endoproteinase Glu-C for in-gel digestion, which results in incomplete digestion of the protein. Fig. 2A shows a section (m/z 1500–3500) of the MALDI-MS peptide mass map of the R2 purification of one-quarter of a peptide solution derived by in-gel digestion with endoproteinase Glu-C from 2 pmol β-casein applied on the gel. The MALDI-MS peptide mass map after
on-target dephosphorylation of this sample using alkaline phosphatase is shown in Fig. 2B. The peptide containing four phosphorylation sites (amino acids 6–31, LNVPGEIVEpSPpSSEESITRINKKIE) is suppressed in MALDI-MS and consequently is only visible after dephosphorylation (Fig. 2B), whereas the monophosphorylated peptide is absent in both.

Fig. 2, C and D show the same region of the MALDI-MS peptide mass map of the purification of one-quarter of the peptide mixture using a graphite microcolumn before and after on-target alkaline phosphatase treatment, respectively.

To determine if the monophosphorylated peptide is suppressed in the ionization process or lost upon purification using R2 resin, another one-quarter aliquot of the digest was purified on an R2 microcolumn, and the flow-through was collected and purified on a graphite microcolumn. The MALDI-MS peptide mass map from this preparation is shown in B. The insets show the region where the signal for the monophosphorylated peptide should be observed.

In vivo Phosphorylation Site in Dynamin I—Dynamin I is a phosphoprotein that is rapidly dephosphorylated upon initiation of synaptic vesicle endocytosis. Recently, cdk5 was shown to be the kinase responsible for its phosphorylation in vivo (13). Two phosphorylation sites (Ser-774 and Ser-778) were identified by a combination of MALDI-MS and phospho-site-specific antibodies. These sites are situated in a proline-rich domain, which binds to a number of other proteins involved in endocytosis, including amphiphysin. Phosphorylation of dynamin I by cdk5 did not prevent the SH3 domain of amphiphysin binding to dynamin I; however, phosphorylation was blocked when amphiphysin is bound to dynamin I. Dephosphorylation of Ser-774 and Ser-778 on dynamin I is essential for endocytosis to occur (13). Both
Phosphorylation sites are present on the same tryptic peptide. However, the doubly phosphorylated tryptic phosphopeptide was not observed in the previous analysis, where peptides were observed from direct MALDI-MS of IMAC beads. This raises the question of whether dynamin I is doubly phosphorylated, or whether Ser-774 and Ser-778 phosphorylation might represent mutually exclusive events, as proposed previously (13).

We have taken a sample of in vivo phosphorylated dynamin I from nerve terminals, subjected it to in-gel tryptic digestion, and applied it to IMAC, followed by Poros R2 and graphite microcolumn separation. Fig. 4A shows a region of the MALDI-MS spectrum of the peptides eluted from the IMAC column. A monoisotopic peak matching the singly phosphorylated peptide dynamin I 774–783 (SPTSSPTPQR +80 Da, theoretical monoisotopic m/z 1137.49) is present at m/z 1137.52. There are also peaks at m/z 1293.64 and 1373.54 matching singly and doubly phosphorylated dynamin I 773–783 or 774–784, respectively (RSPTSSPTPQR or SPTSSPTPQR +80 and +160 Da, should appear at 1293.60 and 1373.56, respectively), overlapping the sequence of 774–783 with a missed trypsin cleavage site. This situation arises because the peptide is flanked on both sides by two Arg residues, producing alternative tryptic cleavage sites. For simplicity, dynamin I 774–784 is not referred to again, although the peaks in the spectra could be either peptide. The doubly phosphorylated peptide was not observed in a previous study when a similar sample was analyzed by direct MALDI-MS of IMAC beads (13). However, the singly phosphorylated peptides were easily detected in that study. In the present study, similar samples were used, but eluted from the IMAC beads. We found all of the phosphopeptides were extremely low in abundance, having signal-to-noise ratios ranging from six for the peak at m/z 1137.52 to three for the peak at m/z 1373.54. The ionization of these phosphopeptides was probably suppressed by neighboring peptides, most of which can be matched to nonphosphorylated tryptic peptides of dynamin I.

An equal amount of IMAC eluate was applied to a Poros R2 microcolumn. The flow-through from the R2 column was applied to a graphite column. The peptides eluting from the Poros R2 column are shown in Fig. 4B. A low-abundance peak matching the singly phosphorylated peptide dynamin I 774–783 is present at m/z 1137.62. Occasionally, more significant peaks matching both singly phosphorylated peptides dynamin I 774–783 and 773–783, but not the doubly phosphorylated peptides, were observed in the Poros R2 eluate (data not shown). It may be possible to encourage the retention of these singly phosphorylated peptides by altering the mobile/stationary phase. The MALDI-MS spectrum of the graphite microcolumn eluate, Fig. 4C, shows four peaks matching dynamin I phosphopeptides. These peaks are at m/z 1137.50, 1217.48, 1293.64, and 1373.52, matching both the singly and doubly phosphorylated dynamin I 774–783 and 773–783, respectively. The doubly phosphorylated dynamin I

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**Fig. 4.** MALDI-MS analysis of in vivo phosphorylated dynamin I from rat brain synaptosomes. A, the tryptic digest was analyzed after purification by IMAC, and B, an equal amount of IMAC-purified sample was further purified using a Poros R2 microcolumn. C, one-half of the flow-through from the Poros R2 was applied to a graphite microcolumn. D, the other half was treated with alkaline phosphatase (AP) before purification with a graphite microcolumn. The overlapping dynamin I phosphopeptides 774–783 SPTSSPTPQR +80 and +160 Da (theoretical m/z 1137.49 and 1217.46, respectively) and 773–783 RSPTSSPTPQR +80 and +160 Da (theoretical m/z 1293.60 and 1373.56, respectively) are enhanced after graphite microcolumn purification. The four phosphopeptides were dephosphorylated to two peptides (theoretical m/z 1057.53 and 1213.63, respectively). Insets show the zoomed m/z range from 1370 to 1380 in each spectrum drawing attention to the amount of doubly phosphorylated dynamin I 773–783 detected after purification. Peaks with asterisks are singly and doubly phosphorylated peptides, also differing by the mass of an arginine, that were coincidentally copurified with dynamin I. They are further analyzed in Fig. 5 and discussed in the text.
774–783 was not observed in any of the previous spectra (theoretical m/z 1217.46). Upon treatment with alkaline phosphatase, these four phosphopeptides could be dephosphorylated to the two peptides at m/z 1057.54 and 1213.56, matching dynamin I 774–783 (SPTSSPTPQR) and 773–783 (RSPTSSPTPQR), respectively (Fig. 4D). Note that the peak at m/z 1137.50 (Fig. 4C) is part of an unusual isotopic distribution for a peptide of this molecular mass. Dephosphorylation of this sample revealed a peptide with a monoisotopic peak at m/z 1138.49 (Fig. 4D) believed to be a GST peptide (28–35, theoretical m/z 1138.52), accounting for the unusual isotopic distribution. The GST originated from the GST fusion proteins used to isolate dynamin (see “Experimental Procedures”).

The dephosphorylation of this sample confirms that the two peptides not reported previously (13) are doubly phosphorylated dynamin I 773–783 peptides.

The lack of retention of the doubly phosphorylated peptides and poor retention of the singly phosphorylated peptides demonstrates that these phosphopeptides are too hydrophilic to bind to the Poros R2. Of course, it is difficult to rule out that their ionization was suppressed by the other peptides that were retained. Ion suppression is the most likely reason for the low-abundance detection of phosphopeptides in the IMAC eluate (Fig. 4A). The peak at m/z 1373.52 in the graphite eluate (Fig. 4D) has a 10 times higher signal-to-noise ratio than the corresponding peak in the IMAC eluate. This improved signal could be due to the separation of this phosphopeptide from the nonphosphopeptides that bound to the IMAC column (e.g. the peptide at m/z 1375.70 matching dynamin I 584–594, see insets in Fig. 4, A and C) or due to the removal of low molecular mass contaminants from the IMAC eluate (e.g. salt). Nevertheless, the improved signal, demonstrates that a second major benefit of using graphite microcolumns is improved sensitivity.

Experiments were next performed to determine the phosphorylation sites within the singly and doubly phosphorylated dynamin I peptides. Previous studies reported phosphorylation of this singly phosphorylated peptide by cdk5 on either Ser-774 or Ser-778 (13), but THR-780 resides in an excellent context to be predicted as a cdk5 substrate. In a somewhat conflicting report, Tomizawa et al. (20) suggested that the recombinant proline-rich domain of dynamin I is not phosphorylated by recombinant cdk5 in vitro when THR-780 is mutated to Ala, suggesting Ser-774 or Ser-778 are not in vitro substrates. However, re-evaluation of their published MS/MS data (in Fig S5B of that article) suggests the presence of the phosphate group on Ser-778 rather than Thr-780. A dynamin I sample from rat brain nerve terminals was purified using graphite, following IMAC and Poros R2, as above, then analyzed by ESI-MS/MS. A peptide at m/z 569.28, corresponding to doubly charged, singly phosphorylated dynamin I 774–783 was fragmented to produce the spectrum shown in Fig. 5A. Analysis of the spectrum revealed two possible interpretations. A highly abundant series of ions described dynamin I 774–783, where only Ser-774 was phosphorylated. The b4 ion is helpful in placing the phosphorylation site at the N terminus, because this ion corresponds to the mass of a pSer-Pro N-terminal fragment. The y4 ion also places the pSer at the N-terminal position. A second interpretation of the spectrum places the phosphate group at Ser-778 for the same dynamin I peptide. Some of the ions overlap, i.e. they could have originated from either sequence. All ions marked with a ‘ (except y4, which coincides with y3-NH3) are exclusive to this second series. The transition from y5 to y6, or b5 to b6, confirms the presence of a pSer at position 778. Many of the ions of this series are very low in abundance, when compared with the pSer-774 series, but have significant signal-to-noise ratios (e.g. S/N for b5 is 20 in Fig. 5A). There was no evidence that Thr-780 was phosphorylated. The simplest interpretation is the presence of two peptides: a highly abundant peptide phosphorylated on Ser-774 and a very-low-abundance peptide phosphorylated on Ser-778.

Next, the peak at m/z 609.26, corresponding to doubly charged, doubly phosphorylated dynamin I 774–783, was fragmented to produce the spectrum shown in Fig. 5B. The spectrum shows a near complete y ion series and a number of contributing b ions that describe the sequence pSPTSpSPTPQR. Similar to the singly phosphorylated peptide, the transition from y6 to y5, or b6 to b5, confirms the presence of a pSer at position 778 and the b5 and y5 ions place the second phosphorylation site at the N-terminal Ser-774. This confirms that dynamin I is phosphorylated in vivo on Ser-774 and Ser-778. There was no evidence to support the presence of pThr-780 or phosphorylation of any other site within this peptide.

As well as dynamin I singly and doubly phosphorylated peaks, four additional phosphopeptide peaks were detected in the dynamin I sample at m/z 1147.58, 1227.62, 1303.56, and 1383.57 (Fig. 4C, marked with asterisks). They also appear to be phosphopeptides because they can be dephosphorylated to peaks at m/z 1067.51 and 1223.61 (Fig. 4D, asterisks). As found for dynamin I, the dephosphorylated peaks appear to be related, because they differ only by the mass of an Arg residue. The identity of the peaks at 1147.58 and 1303.56 were previously proposed to represent a polymorphism in the sequence of dynamin I, because a Ser-Pro mutation could hypothetically account for such a series of peaks 10 units higher in m/z (13). However, this was not experimentally tested. To resolve this question, the doubly charged, singly and doubly phosphorylated peptides at m/z 574.29 and 614.26, corresponding to the peaks at m/z 1147.58 and 1227.62, respectively (Fig. 4C), were sequenced by ESI-MS/MS (Fig. 5, C and D, respectively). The sequence did not match dynamin I, but clearly matched a dynamin III tryptic peptide, which is similar in size and sequence to dynamin I. Dynamin III derives from a different gene and is known to be expressed at lower levels in the brain than dynamin I (21). The spectrum of the singly phosphorylated
Dynamin III peptide provided a near complete y ion series and some contributing b ions that unambiguously describe the sequence pSPPPSPTTQR of dynamin III 759–768, where Ser-759 is phosphorylated. However, the ion designated b$_2$ suggests that b$_2$ does not belong to this sequence and suggests an alternative sequence, where an amino acid other than Ser-759 is phosphorylated. There was insufficient information to describe this alternative sequence. The spectrum of the doubly phosphorylated peptide in Fig. 5D was found to describe the sequence pSPPPpSPTTQR. A complete y ion series enabled the sites of phosphorylation to be assigned to Ser-759 and Ser-763. There was no evidence that the two remaining potential sites, Thr-765 or Thr-766, were phosphorylated.

The positions of the two identified phosphoserines from dynamin I (Fig. 5A) are retained in analogous sequence contexts in dynamin III as Ser-759 and Ser-763. This sequence is also flanked by pairs of Arg residues, accounting for the appearance of two peptides in the MALDI spectra. Dynamin III was recently reported to be present at low abundance in presynaptic nerve terminals, but is greatly enriched in postsynaptic spines (21). The ESI-MS/MS analysis has unambiguously identified both Ser-759 and Ser-763 as in vivo phosphorylation sites, and this result is the first evidence that dynamin III is an in vivo phosphoprotein.

DISCUSSION

Characterization of phosphorylated proteins is now predominantly performed using mass spectrometric methods, due to the high sensitivity of these instruments, and their ability to deal with complex protein or peptide mixtures. Despite the increasing number of strategies developed for characterization of phosphoproteins by MS, the biggest remaining problem is one of low sensitivity, due to the nature of the phosphopeptides. Low sensitivity is a consequence of ion suppression effects during the initial ionization process in MS, low phosphorylation stoichiometry, and, more noteworthy, loss of the phosphopeptides during different purification techniques. Presently, no single strategy has proven adequate for routine characterization of phosphoproteins.

The results presented in this study clearly show that a large proportion of phosphopeptides derived from phosphorylated proteins by proteolytic digestion can be lost during sample purification employing RP chromatography. We have tested...
the ability of Poros RP resins to retain phosphopeptides, because we have previously found that this resin has a high binding capacity and consequently a high sensitivity when used in GELoader tip microcolumns. We have shown that some synthetic phosphopeptides and phosphopeptides derived by proteolytic digestion of gel-separated phosphoproteins were not retained by Poros R2 RP chromatography. However, they were efficiently retained on microcolumns packed with graphite powder. Preliminary results have shown that other C_{18} RP chromatographic material used for capillary high-pressure liquid chromatography on-line to MS/MS (Zorbax, SB-C_{18}, 3.5 µm; Agilent Technologies, Wilmington, DE) also lack the ability to retain some phosphorylated peptides (data not shown). It is, however, likely that other types of RP material will have a different retention for these types of peptide, and that this may also be dependent on the mobile-phase buffer used for binding the peptides to the column.

Recently we proposed the use of sequential application of chromatographic material with increasing hydrophobic material for obtaining significantly higher sequence coverage from gel-separated proteins (10). We are currently applying peptide digests sequentially to microcolumns packed with Poros R1, R2, R3, and graphite powder, respectively, which are increasingly hydrophobic stationary phases. In addition to yielding simpler peptide mixtures to analyze by MS, the separation achieved by these stationary phases increases the probability for detection of phosphorylated peptides, due to reduced suppression effects and minimal losses. However, this procedure is an off-line method more suitable to analysis of a single protein and is therefore not easily applicable to high-throughput proteomics.

Traditional proteomic strategies employing two-dimensional gel electrophoresis (2DE) are slowly being overtaken by SDS-PAGE followed by liquid chromatography tandem mass spectrometry (LC-MS/MS) of in-gel-derived peptides or entirely gel-free systems (LC/LC-MS/MS). The latter strategies are currently not matching the resolution obtained by 2DE for large-scale analysis of modified proteins, as the sequence coverage obtained from the LC-MS/MS analysis is relatively low, decreasing the possibility to detect protein isoforms and post-translational modifications. However, several groups have attempted to perform such analysis. The results presented in this study raise a major concern for large-scale phosphoproteomics studies that rely heavily on sample preparation by RP chromatography (e.g. 7, 22). These studies typically use a RP cleanup after elution from IMAC. In on-line experiments, they allow the salts used in IMAC elution to go to waste as they load the RP column, to prevent the salts from entering the mass spectrometer, as in LC/LC experiments using ion exchange media (23). Typically, the solvent flow is reconnected to perform LC-MS/MS of the bound material. This would result in some phosphopeptides going to waste, reducing the extent of the phosphoproteome.

Graphite powder is recommended as an aid in achieving a significant improvement in sequence coverage of proteins, thereby increasing the probability to detect phosphopeptides derived from phosphorylated proteins. We suggest that the waste from RP cleanup should be applied to a graphite column, and that the eluate from the RP and graphite columns should be analyzed in turn. Graphite powder (capillary) columns are commercially available or can be prepared from graphite material (e.g. Hypercarb; Thermo Hypersil-Keystone, Bellefonte, PA). Preliminary results using such capillary graphite columns in our laboratory have shown recovery of hydrophilic peptides in LC-MS/MS experiments using normal gradient runs. However, these experiments show lower sensitivity than if the elution was performed using MALDI matrix solution, as previously demonstrated for the GELoader tip graphite microcolumns (10). Applied as a second column after the C_{18} capillary column, the sequence coverage could be increased in LC-MS/MS experiments and allow detection of more peptides. In addition, the use of a C_{18} column prior to the graphite capillary column could extend the lifetime of such columns.

The improved detection of hydrophilic phosphopeptides using graphite has resulted in significant advances in understanding the regulation of dynamin and synaptic vesicle endocytosis. In a previous study, the phosphorylation sites in dynamin I both in vitro and in vivo were found to be Ser-774 and Ser-778. However, because only a singly phosphorylated peptide was observed with IMAC alone in that study, it was not possible to determine which amino acid was the specific phospho-acceptor. Ser-774 and Ser-778 were revealed by production of specific phosphorylation-state antibodies, while Thr-780 was not specifically investigated because phospho-amino acid analysis revealed low abundance of the pThr in vivo and in vitro. It was proposed that dynamin I might be phosphorylated on either site in a mutually exclusive fashion (13). Using the graphite column here, we have now revealed the presence of doubly phosphorylated dynamin peptides and directly confirmed phosphorylation of the two serines in dynamin I in vivo. Because blocking cdk5 activity in neurons also inhibits endocytosis after repetitive cycles, it can now be proposed that both phosphorylation sites might play a central role in synaptic vesicle endocytosis. These sites are adjacent to a number of short proline-rich motifs that may regulate protein-protein or protein-lipid interactions with dynamin. It is possible that dual phosphorylation of dynamin I regulates its interactions with specific proteins involved in endocytosis.

In addition to revealing doubly phosphorylated dynamin I, the new techniques revealed phosphorylation sites within the homologous sequence of the C-terminal tail of dynamin III. This form of dynamin is known to be expressed at low levels in nerve terminals, but is enriched in postsynaptic spines in hippocampal neurons (21). The discovery of the presence of dynamin III in pull-downs from nerve terminals is not unexpected and is consistent with the likelihood that dynamin III interacts with similar endocytic proteins as dynamin I or that dynamin III forms heterocomplexes with dynamin I. Little is
known about the function or regulation of dynamin III. Its enrichment in postsynaptic spines is strongly indicative of an endocytic function in mediating postsynaptic receptor signaling. Our discovery of singly and doubly phosphorylated dynamin III provides direct evidence that it is an in vivo phosphoprotein. This raises the possibility that it may be regulated in a similar manner to dynamin I. However, it remains to directly demonstrate whether dynamin III might be a substrate for cdk5. Additionally, the phosphorylation sites at Ser-759 and Ser-763 are equally spaced and appear in a very similar context with respect to the dynamin I sites. It is interesting that these two serines, as in dynamin I, immediately precede a proline residue, suggesting they would be targeted by proline-directed protein kinases such as cdk5. We found no evidence for phosphorylation of Thr-780 in dynamin I despite that it also precedes a proline, although this cannot be definitively ruled out. In contrast, dynamin III diverges from dynamin I in this sequence and the proline is absent. This reduces the probability that it represents a phosphorylation site and raises the interesting possibility that there may be no major differences in its phosphorylation from dynamin I.

A further observation is that for both the dynamin I and dynamin III singly phosphorylated peptides, the sequence with the phosphorylation site at the N-terminal Ser produced much more abundant ions (Ser-774 and Ser-759, respectively). Care must be taken when using MS data for quantitative purposes because small changes to a sequence can affect the efficiency of ionization (3). However, the much larger relative abundance, particularly for singly phosphorylated dynamin III, which is almost exclusively Ser-759 phosphorylated, suggests that there may be a preference for phosphorylation of the N-terminal Ser to precede phosphorylation of the second site. This raises the possibility of hierarchical phosphorylation of dynamin I and III (24), which will require further investigation.

In conclusion, the use of graphite microcolumns clearly provides a significantly more complete coverage of phosphoproteomes. The method is simple, inexpensive, and readily amenable to automation. The method also greatly improves the signal-to-noise ratio for many peptides, thus providing a significantly more complete coverage of phosphoprotein. This raises the possibility of hierarchical phosphorylation of the N-terminal Ser to precede phosphorylation of the second site. This raises the interesting possibility that there may be no major differences in its phosphorylation from dynamin I.

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