

# Phosphoproteomic Analysis of the Developing Mouse Brain\*<sup>§</sup>

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Proper development of the mammalian brain requires the precise integration of numerous temporally and spatially regulated stimuli. Many of these signals transduce their cues via the reversible phosphorylation of downstream effector molecules. Neuronal stimuli acting in concert have the potential of generating enormous arrays of regulatory phosphoproteins. Toward the global profiling of phosphoproteins in the developing brain, we report here the use of a mass spectrometry-based methodology permitting the first proteomic-scale phosphorylation site analysis of primary animal tissue, identifying over 500 protein phosphorylation sites in the developing mouse brain. *Molecular & Cellular Proteomics* 3:1093–1101, 2004.

Phosphorylation can dramatically change a protein's biological location or activity. The profound influence of protein phosphorylation on mammalian brain development has strong genetic support. This is exemplified by the important brain phenotypes observed in mice with loss of function mutations in genes encoding kinases such as p35/cyclin-dependent kinase 5 (Cdk5)<sup>1</sup> (1, 2), loss of tyrosine phosphorylation sites in signaling molecules such as the Reelin-stimulated adaptor protein Disabled-1 (3), or loss of genes encoding proteins that interact with phosphorylated protein motifs such as 14-3-3ε (4).

Historically, the analysis of protein phosphorylation sites has been restricted to studies at the single-protein level. Recently, larger-scale MS-based analyses have emerged. However, such studies have been challenging due to a) the immaturity of methods to enrich for low-abundance phosphoproteins or phosphopeptides and b) the reduction in quality of informative tandem mass spectra obtained from phosphopeptides subjected to CID (5). The latter challenge is due primarily to the propensity for precursor ions containing phosphoserine or phosphothreonine to undergo β-elimination of phosphoric acid with an accompanied reduction of structur-

ally informative ions from peptide backbone fragmentation. Recent advances in metal ion affinity chromatography have permitted large-scale phosphorylation analysis (200–400 sites identified) in yeast (6) and plants (7). Here, using strong cation exchange (SCX) chromatography at low pH to enrich for tryptic phosphopeptides (8), we show the first large-scale proteomic profiling of phosphorylation sites from primary animal tissue. These methods promise to greatly enrich our global view of the dynamic changes of phosphoproteins during brain development and may be applied to a variety of primary tissues or comparative states in cultured cells.

## EXPERIMENTAL PROCEDURES

**Mice and Tissue Preparation**—A timed pregnant Swiss Webster mouse was obtained from Taconic (Germantown, New York). Developing forebrains and midbrains were dissected from embryos at day 16.5 (E16.5). The tissue from four brains (10 mg) was dounce homogenized in 25 mM Tris pH 7.2, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 25 mM NaF, 10 mM Na<sub>2</sub>P<sub>2</sub>O<sub>7</sub>, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM DTT, 1 mM PMSF, 10 μg/ml leupeptin, and 1% aprotinin.

**Gel Electrophoresis and In-gel Digests**—Cleared extracts were boiled in bromophenol blue sample buffer (150 mM Tris pH 6.8, 2% SDS, 5% β-mercaptoethanol, 7.8% glycerol), and 6 mg of extract was loaded onto a hand-poured, 7.5–20% gradient SDS-polyacrylamide (37.5:1 acrylamide:bis-acrylamide) preparative gel (see Fig. 2A). The Coomassie blue-stained gel was cut into four regions and then diced into 1-mm cubes. The gel pieces were washed with water and further destained with 50% ACN, 50 mM NH<sub>4</sub>HCO<sub>3</sub> pH 8.5. Gel slices were dehydrated with ACN, dried, and subjected to in-gel digestion with sequencing-grade modified trypsin (12.5 ng/μl; Promega, Madison, WI) in 50 mM NH<sub>4</sub>HCO<sub>3</sub> overnight at 37 °C. Peptides were extracted with 50% ACN, 5% formic acid (FA) and dried.

**SCX Chromatography**—Peptides from each gel region were resuspended in 500 μl of SCX solvent A (5 mM KH<sub>2</sub>PO<sub>4</sub> pH 2.7, 33% ACN). Then 400 μl of each peptide mixture were resolved by SCX chromatography on a polysulphoethyl aspartimide (5 μm, 200 Å) column (3 × 200 mm; PolyLC Inc., Columbia, MD), against an increasing gradient of SCX solvent B (5 mM KH<sub>2</sub>PO<sub>4</sub> pH 2.7, 33% ACN, 350 mM KCl) as shown in Fig. 2, B and C. Fractions were collected every minute with a flow rate of 400 μl/min. Each fraction was lyophilized and then desalted by resuspending peptides in 0.5% TFA and loading them on a gel-loading tip column packed with 2 cm of OLIGO R3 (Applied Biosystems, Foster City, CA). After washing with 0.1% TFA, peptides were eluted from the column with 95% ACN and 0.4% FA and dried. For SCX chromatography of peptide standards shown in Fig. 1B, 200 pmol of synthetic peptides IGEGTYGVVYK with or without one phosphorylated residue at the underlined threonine or tyrosine residue (kind gifts of J. Rush, Cell Signaling Technology, Beverly MA) were analyzed using SCX chromatography. Note that “solution charge state” is used throughout when referencing peptide separation using SCX chromatography and should not be confused with the charge state of ions in the gas phase.

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<sup>1</sup> The abbreviations used are: Cdk5, cyclin-dependent kinase 5; SCX, strong cation exchange; SH2, Src homology 2; SH3, Src homology 3; NCBI, National Center for Biotechnology Information; FA, formic acid.

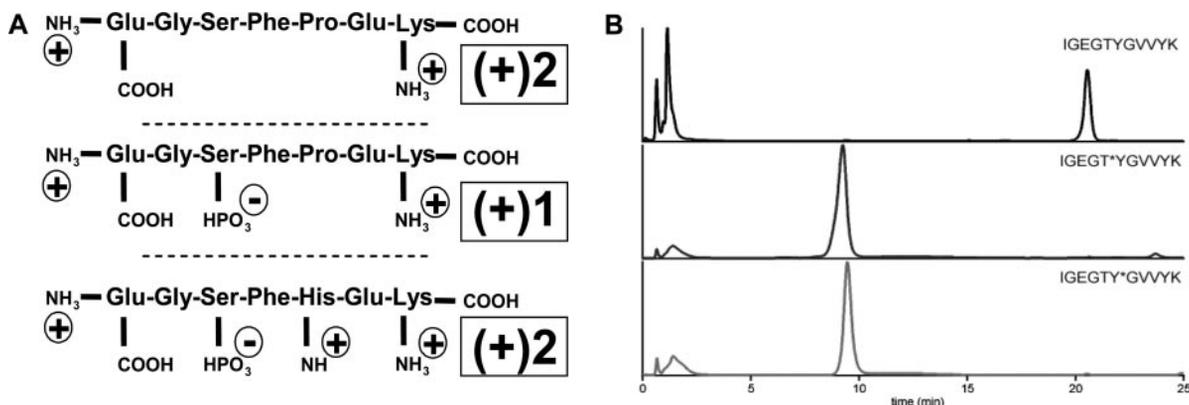


FIG. 1. Phosphorylation reduces peptide solution charge state and alters SCX elution at low pH. **A**, at pH 2.7, a theoretical tryptic peptide without histidine residues carries a net solution charge of (+)2 imparted by the amino terminus and the basic group of the carboxyl-terminal arginine or lysine. Note that the carboxyl terminus and carboxyl groups of glutamate and aspartate residues are protonated at pH 2.7. If singly phosphorylated, the solution charge state of this peptide is reduced to (+)1. Phosphorylation of a similar peptide containing a single histidine residue reduces its solution charge from (+)3 to (+)2, preventing its enrichment in early eluting SCX fractions (note depictions of protonated functional groups are simplified). **B**, SCX chromatograms of a nonphosphorylated peptide or the same sequence singly phosphorylated on either threonine or tyrosine. Phosphorylation at either residue shows a dramatic reduction in retention time, and thus the basis for phosphopeptide enrichment in early SCX fractions.

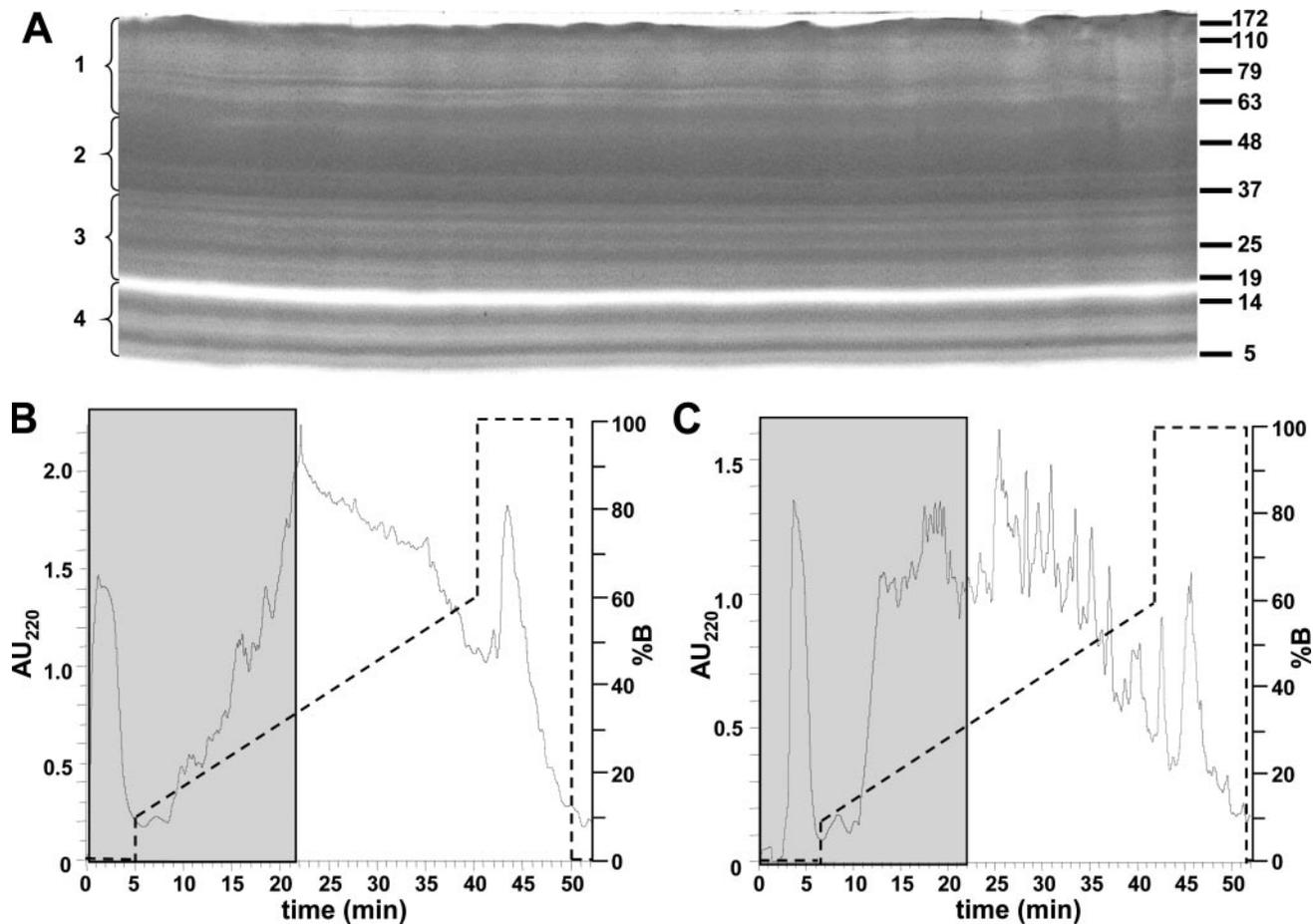


FIG. 2. Separation of embryonic brain proteins and peptides by sequential preparative SDS-PAGE and SCX chromatography. **A**, 6 mg of embryonic day 16.5 murine brain extract were separated by SDS-PAGE. The gel was cut into four regions, minced, and digested with trypsin. **B** and **C**, tryptic peptides were subjected to SCX chromatography and chromatograms for peptides from regions 1 and 4, respectively, are shown depicting UV absorbance at 220 nm. The gradient of SCX solvent B is indicated with a dashed line. The shaded regions indicate the analyzed early fractions with solution charge states of up to (+)2. Alternative phosphopeptide enrichment strategies may prove useful on later fractions.

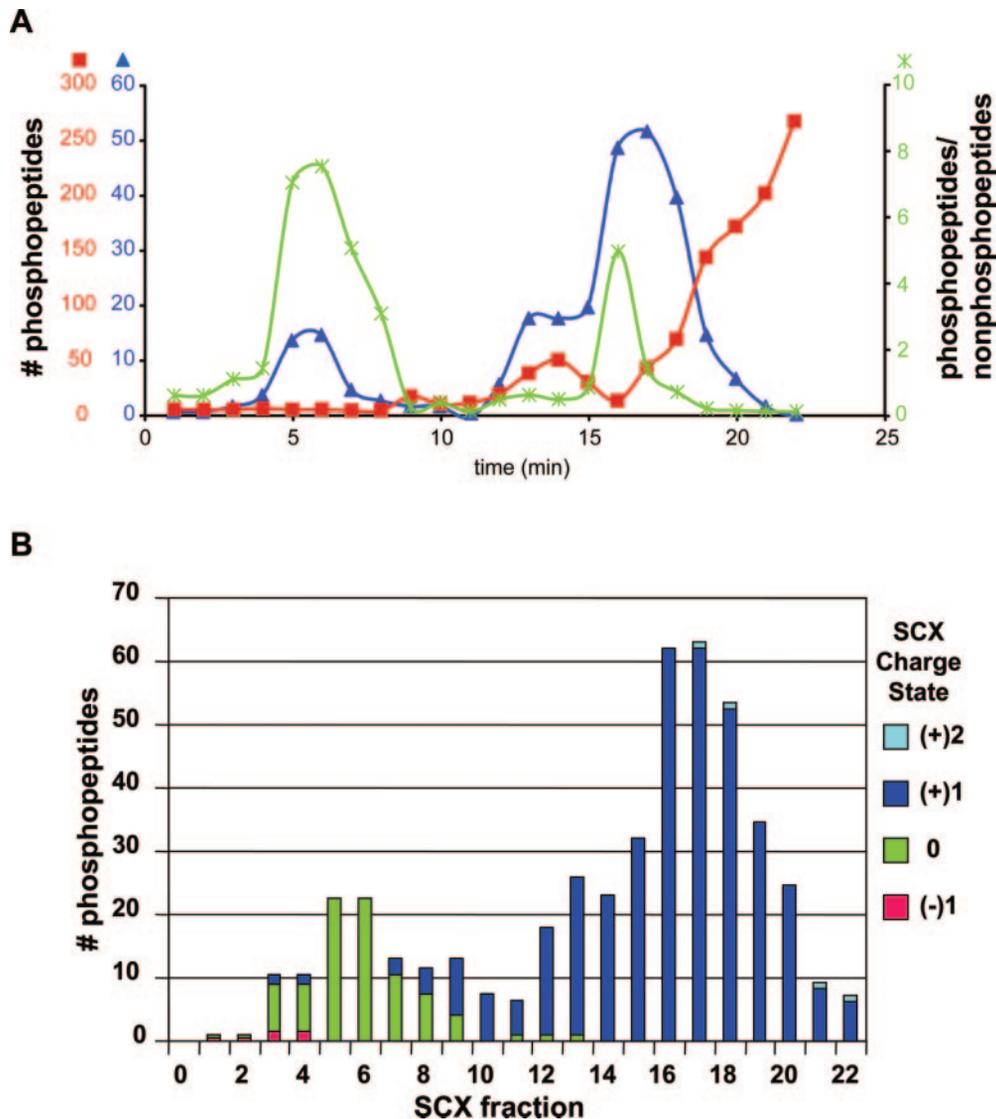


FIG. 3. **SCX chromatography at low pH permits strong enrichment of phosphopeptides of distinct solution charge states.** *A*, the number of phosphopeptides (*blue triangles*) and nonphosphorylated peptides (*red squares*) identified from gel region 1 (see Fig. 2, *A* and *B*) were plotted against their SCX elution time, and their ratio to each other is overlaid (*green stars*). Note distinct scales for each plot. *B*, phosphopeptides from the entire dataset show distinct solution charge state distributions in early SCX eluates.

**MS and Manual Validation of Phosphorylation Sites**—Following desalting, individual SCX fractions from each gel region were resuspended in 0.5% FA and subjected to LC-MS/MS runs. Samples were loaded using an autosampler onto a microcapillary column (100  $\mu\text{m} \times 12$  cm) packed with reverse phase MagicC18 material (5  $\mu\text{m}$ , 200  $\text{\AA}$ ; Michrom Bioresources, Inc., Auburn, CA). Elution was achieved with a 5–35% ACN (0.1% FA) gradient over 100 min, after a 20-min isocratic loading at 0% ACN, 0.5% FA. Mass spectra were acquired on a LCQ-Deca XP (Thermo Electron, Woburn, MA) over the entire run using eight MS/MS scans following each survey scan. Approximately 3,000 sequencing events were performed for each run. Raw data were searched for fully tryptic peptides against the NCBI nonredundant mouse database using Sequest software, permitting a dynamic modification of 80 Da on serine, threonine, and tyrosine residues and a mass allowance of 2 Da. Database matches were filtered for XCorr values higher than 2.5 and 3.3 for doubly and triply charged ions, respectively. Resulting sequences were inspected manually and val-

idated with the assistance of in-house software to assign prominent peaks unmatched by Sequest such as doubly charged ions, neutral losses, and the same with accompanying water losses (see Supplemental Fig. 1). As the vast majority of phosphopeptides identified showed significant loss of phosphoric acid, they can be distinguished from sulfonation, which also imparts a mass addition of 80 Da to serine, threonine, and tyrosine residues (9). When the exact site of phosphorylation could not be assigned for a given phosphopeptide, it was tabulated as ambiguous. Motif analysis of identified phosphorylation sites was performed using “phosphomotif” software ([deer.med.harvard.edu/dan/phosomotif.html](http://deer.med.harvard.edu/dan/phosomotif.html)).

## RESULTS

SCX chromatography separates peptide ions based on solution charge state (resulting from protonation/deprotonation of basic and acidic groups). As much as 68% of an *in silico*

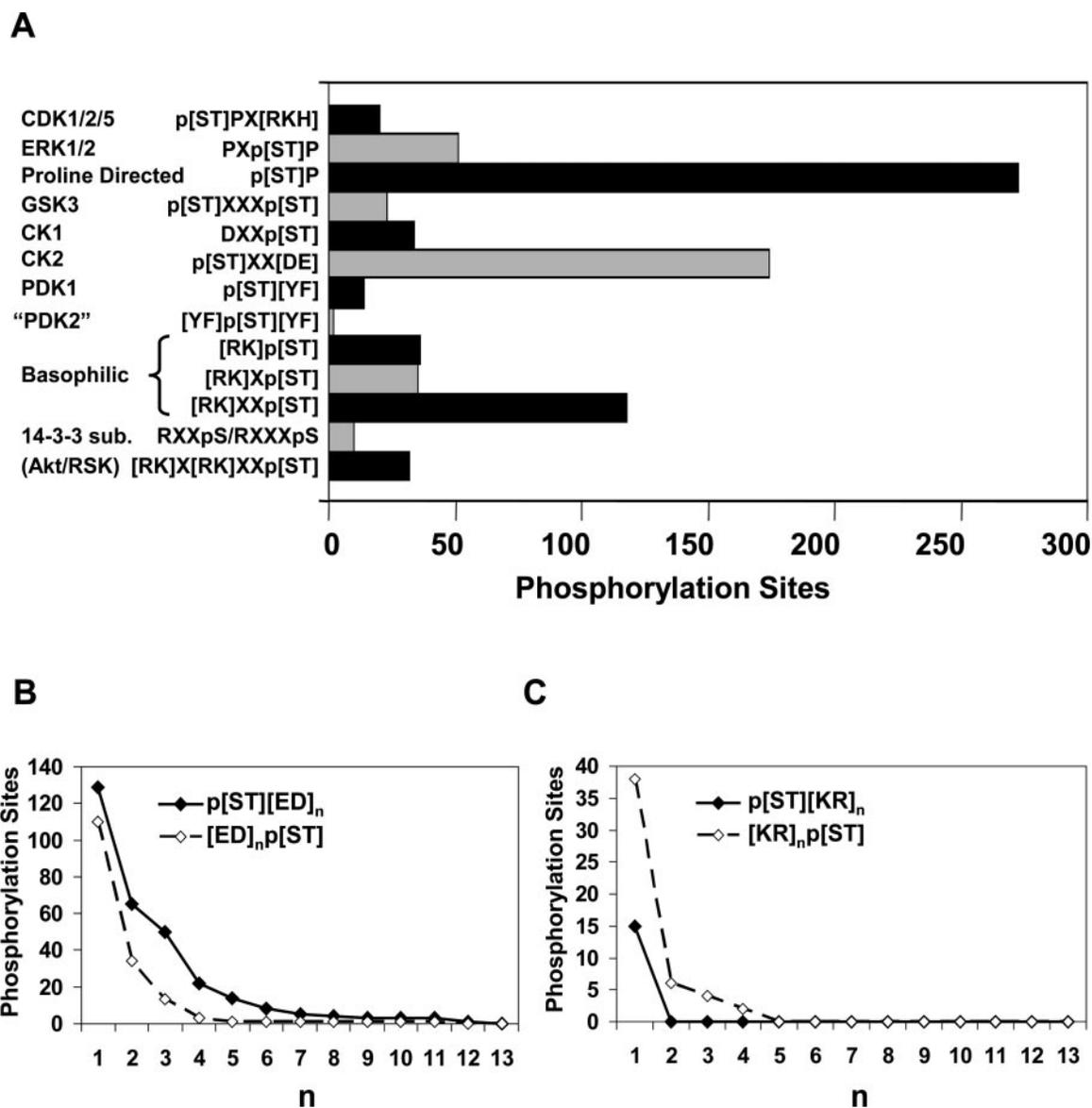


FIG. 4. **Survey of phosphorylation motifs.** A, a query of the primary sequences surrounding the identified phosphorylation sites reveals potential links to distinct kinase activities. In addition to proline-directed phosphorylation events, strings of acidic residues (primarily downstream) of the phosphorylation site (B) or basic residues (primarily upstream) of the phosphorylation site (C) were prominent.

tryptic digest of the human NCBI protein database generates peptides with a predicted solution charge state of (+)2 at pH 2.7, and fewer than 3% with a solution charge state of less than (+)2 (8). Phosphorylation at serine, threonine, or tyrosine reduces the solution charge state of peptides at pH 2.7, allowing (+)2 phosphopeptides to be enriched into a far less complex fraction (Fig. 1). This greatly increases the probability of their favorable analysis by MS.

These properties were applied toward the identification of phosphorylation events occurring during mouse brain development. As the relative abundance of rare mRNA species in mammalian cells represents as much as 95% of unique message expressed per cell (10), we reasoned that milligram amounts of protein were required to successfully identify

phosphorylation events achieving as high as 10% stoichiometry (Supplemental Table I). Six milligrams of embryonic brain extract were separated on a preparative polyacrylamide gel. The gel was cut into four large regions, and each region was subjected to in-gel digestion with trypsin (Fig. 2A). Extracted peptides from each region were separated by SCX chromatography at pH 2.7 (Fig. 2, B and C), desalted, and analyzed by reverse-phase LC-MS/MS.

More than 250,000 MS/MS spectra were acquired while analyzing the first 40% of SCX fractions from all four gel regions. Following interrogation of the nonredundant NCBI murine database using the Sequest algorithm, results were conservatively filtered by requiring top-hit phosphopeptides to have XCorr values of more than 2.5 and 3.3 for doubly and

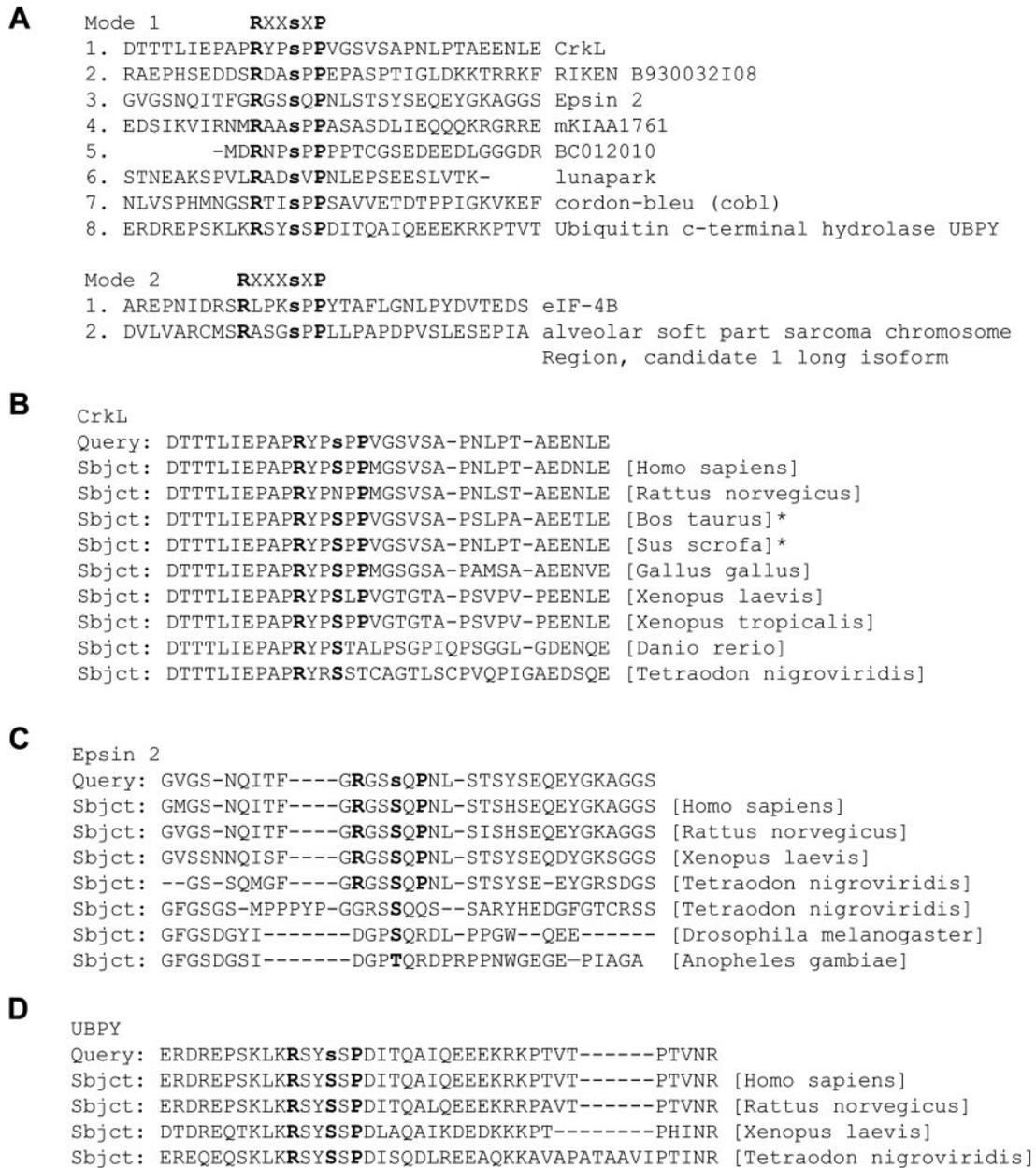


FIG. 5. **Phosphopeptides identified harboring minimal 14-3-3 binding motifs.** 14-3-3 proteins employ two modes of binding to phosphoproteins based on primary amino acid sequence surrounding a phosphoserine residue. *A*, phosphopeptides identified containing minimal binding motifs for 14-3-3 family members. *B* and *D*, potential 14-3-3 binding sites found in CrkL, Epsin 2 and UBPY show different degrees of conservation relative to surrounding amino acids found in orthologous proteins from other members of the animal kingdom. Similar alignments for all other potential 14-3-3 binding sites identified are presented in Supplemental Fig. 2. Motif residues are in *bold* with the identified phosphorylated serine residue in lowercase. *Asterisks* indicate the sequence was identified from the translated EST database.

triply charged ions, respectively. All spectra passing these criteria were manually examined to explain intense (>15% of the most intense peak) ions left unexplained by conventional b- and y-type ions. We found manual evaluation an essential step for proper identification of phosphorylation sites due to the frequent unassigned loss of phosphoric acid and ambiguity found between top-hit peptides containing multiple serine and threonine residues, represented by low  $\Delta$ Corr values

(see Supplemental Fig. 1). After validation we identified 460 unique phosphorylation sites and 86 more for which the precise site of phosphorylation was ambiguous. This entire dataset is provided as Supplemental Table II and as well on our laboratory website with interactive Sequest links at [gygi.med.harvard.edu/pubs/brain/PhosphoBrain.xls](http://gygi.med.harvard.edu/pubs/brain/PhosphoBrain.xls).

To evaluate the relative enrichment of phosphopeptides afforded by SCX chromatography, we plotted the number of



phosphopeptides and nonphosphorylated peptides (many of which were carboxyl termini) identified from gel region 1. Intriguingly, we noticed two distinct peaks of phosphopeptides eluting in the early SCX fractions. These peaks represented phosphopeptides to nonphosphorylated peptides ratios of 8:1 and 5:1, respectively, and this ratio tapered quickly to 0:260 in the last SCX fractions analyzed by MS (Fig. 3A). To determine if the two peaks of phosphopeptides were the result of a distinct separation of solution charge states by SCX, we plotted the expected solution charge states for the phosphopeptides from the entire dataset as a function of the SCX fraction from which they were derived. In excellent agreement with theoretical elution patterns, we determined that the two peaks represent phosphopeptides with a predicted net solution charge of 0 and (+)1, respectively. Additionally, a minor peak with a net solution charge of (-)1 was observed in the earliest fractions. All phosphopeptides with net solution charges of less than (+)1 were the result of multiple phosphorylation events and/or phosphorylation of carboxyl termini. These peptides had little to no retention on the SCX column, owing to disproportionate charge distribution along the lengths of the peptides.

Protein kinases target substrates based on primary sequence proximal to the serine, threonine, or tyrosine residue(s) they phosphorylate (11). There are over 500 protein kinases (12), many of which share overlapping sequence specificities. To provide a preliminary affiliation between kinases with known target sequences and the phosphorylation sites in our database, we queried our dataset with 11 different phosphorylation motifs and tallied the results (Fig. 4A). In addition to proline-directed (p(S/T)P) phosphorylation sites, both phosphorylation sites upstream of acidic or downstream of basic residues constituted a large fraction of the dataset (Fig. 4, A–C).

Basophilic kinases capable of phosphorylating a serine residue lying three or four residues downstream of arginine and two residues upstream of proline (RXXpSXP or RXXXpSXP) create a minimal potential binding site for the 14-3-3 family of phosphoprotein regulators (13). This is of particular interest as loss of a single member of this family (14-3-3e) leads to abnormalities in neuronal migration during brain development in mice (4) and is tightly linked to the severity of lissencephalies resulting from genetic loss of *Pafah1b1* (the gene encoding Lis1) in humans (4). A query of our dataset for phosphorylation sites creating these two modes of 14-3-3 binding identified 10 phosphopeptides (Fig. 5A). Only one of these was contained in a protein (CrkL) already reported to interact with 14-3-3, although the respective sites of recognition were not reported (14). Interestingly, CrkL was recently implicated

in signaling pathways downstream of Reelin (15), a ligand essential for proper brain development (16).

If these phosphoproteins were to interact with 14-3-3 in a biologically meaningful way, the binding motif would be expected to show high evolutionary conservation in the class of organisms benefiting from the interaction. As an indication of the degree of conservation, the full-length protein sequences containing these phosphopeptides were analyzed by BLAST searches against both the nonredundant protein and translated nucleotide NCBI databases. Alignments were made where sequence homology to other members of the animal kingdom could be unambiguously determined. Alignments of the 14-3-3 binding motifs are shown for CrkL, Epsin 2, and UBPY (Fig. 5, B–D). Similar alignments for all other potential 14-3-3-interacting proteins are provided in Supplemental Fig. 2. Low-energy CID spectra are shown for the more conserved phosphorylation sites identified in Epsin 2 and UBPY (Fig. 6).

#### DISCUSSION

Large-scale phosphoproteomic studies have been hindered by the need to develop reliable methods to selectively enrich for low-abundance phosphoproteins and phosphopeptides. Recent advances in phosphopeptide enrichment strategies are making possible large-scale phosphorylation site analyses. Here, using SCX chromatography to enrich for tryptic phosphopeptides, we describe the identification of over 500 phosphorylation sites from the developing mouse brain.

SCX-based phosphopeptide enrichment strategies hold great promise toward contributing to an eventual global profiling of phosphorylation sites in cells and tissues. Notwithstanding their success, inherent caveats with phosphopeptide enrichment by SCX will make requisite complementary approaches to achieve a truly global profile. Histidine-containing phosphopeptides carry an additional positive solution charge at low pH (Fig. 1A), hampering their enrichment by SCX chromatography. Additionally, SCX-based enrichment is only effective when using tryptic peptides, and a number of tryptic peptides are too short or long for standard MS/MS analysis. SCX enrichment of phosphopeptides may also be confounded by post-translational modifications that impart a charge at low pH or alter trypsin hydrolysis due to the relative position of basic residues to proline or modified residues. Independent of the enrichment strategy, the significant range in phosphoprotein abundance generates additional challenges. Despite the known importance of tyrosine phosphorylation in numerous cellular processes regulating brain development, only one tyrosine phosphorylation site was identified in this study (see Accession SW:MK08 entry 1 in Supplemental Table II). As phosphotyrosyl-peptides are also

FIG. 6. Low-energy CID spectra of phosphopeptides identified from Epsin 2 and UBPY. Phosphopeptides participating in the generation of potential 14-3-3 binding sites were identified in Epsin 2 (A) and UBPY (B) by subjecting SCX eluates to LC-MS/MS analysis and database interrogation.

enriched in early SCX fractions (see Fig. 1A), this suggests that phosphotyrosine-containing phosphopeptides are falling below the limit of detection even when using 6–8 mg of starting material. These results are consistent with the rarity of phosphotyrosine (17) or its potentially low phosphorylation stoichiometry (18). Future studies may thus require higher amounts of starting material and/or enrichments with anti-phosphotyrosine antibodies.

Given the important role of 14-3-3 $\epsilon$  in the developing brain (4), we searched our dataset for phosphorylation events creating potential 14-3-3-binding motifs. We identified eight mode 1 (RXXpSXP) and two mode 2 (RXXXpSXP) 14-3-3-binding motifs (13) (Fig. 5A). An alignment of these motifs and their surrounding amino acids across animal kingdom orthologues (Fig 5, B and C, Supplemental Fig. 2) showed variable conservation (indicative to some degree of potential biological relevance). For the motif identified in CrkL, located carboxyl-terminal to the phosphotyrosine-binding SH2 domain (19), strong conservation is seen for all residues aligned from humans to *Xenopus* species, with the notable divergence of the phosphorylated serine residue mutated to asparagine in rat. The motif identified in Epsin 2, located in the carboxyl-region of the ENTH domain involved in vesicle trafficking (20), was conserved through pufferfish and showed much higher conservation than surrounding residues. The motif found in the ubiquitin isopeptidase UBPY is located near coiled-coiled domains and is amino-terminal to a proline-rich SH3 domain-binding region (21, 22). Like the motif in Epsin 2, it is conserved through pufferfish with surrounding amino acids showing much less conservation. Such analyses (here performed on previously unidentified phosphorylation sites) will be important evolutionary indices when considering phosphorylation site relevance in specific classes of organisms.

The rich and complex regulatory nature of protein phosphorylation offers an exciting and challenging opportunity for future proteomic studies. Here, using emerging technologies for the enrichment of phosphopeptides, we present the first large-scale phosphoproteomic analysis of primary animal tissue. The further refinement of these and complementary technologies, including the use of high mass-accuracy instrumentation and quantification strategies, will provide an increasingly global profiling of the unique sets of phosphorylation sites occurring across mammalian brain development.

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□ The on-line version of this manuscript (available at <http://www.mcponline.org>) contains supplemental material.

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