The in Vivo Phosphorylation Sites in Multiple Isoforms of Amphiphysin I from Rat Brain Nerve Terminals*

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Amphiphysin I (amphI) is dephosphorylated by calcineurin during nerve terminal depolarization and synaptic vesicle endocytosis (SVE). Some amphI phosphorylation sites (phosphosites) have been identified with in vitro studies or phosphoproteomics screens. We used a multifaceted strategy including 32P tracking to identify all in vivo amphI phosphosites and determine their relative abundance and potential relevance to SVE. AmphI was extracted from 32P-labeled synaptosomes, phosphopeptides were isolated from proteolytic digests using TiO2 chromatography, and mass spectrometry revealed 13 sites: serines 250, 252, 262, 268, 272, 276, 285, 293, 496, 514, 539, and 626 and Thr-310. These were distributed into two clusters around the proline-rich domain and the C-terminal Src homology 3 domain. Hierarchical phosphorylation of Ser-262 preceded phosphorylation of Ser-268, -272, -276, and -285. Off-line HPLC separation and two-dimensional trypsic mapping of 32P-labeled amphI revealed that Thr-310, Ser-293, Ser-285, Ser-272, Ser-276, and Ser-268 contained the highest 32P incorporation and were the most stimulus-sensitive. Individually Thr-310 and Ser-293 were the most abundant phosphosites, incorporating 16 and 23% of the 32P. The multiple phosphopeptides containing Ser-268, Ser-276, Ser-272, and Ser-285 had 27% of the 32P. Evidence for a role for at least one proline-directed protein kinase and one non-proline-directed kinase was obtained. Four phosphosites predicted for non-proline-directed kinases, Ser-626, -250, -252, and -539, contained low amounts of 32P and were not depolarization-responsive. At least one alternatively spliced amphI isoform was identified in synaptosomes as being constitutively phosphorylated because it did not incorporate 32P during the 1-h labeling period. Multiple phosphosites from amphI co-migrating synapitosomal proteins were also identified, including SGIP (Src homology 3 domain growth factor receptor-bound 2 (Grb2)-like (endophilin)-interacting protein 1), AAK1, eps15R, MAP6, α/β-adducin, and HCN1. The results reveal two sets of amphI phosphosites that are either dynamically turning over or constitutively phosphorylated in nerve terminals and improve understanding of the role of individual amphI sites or phosphosite clusters in synaptic SVE. Molecular & Cellular Proteomics 7: 1146–1161, 2008.

Synaptic vesicle endocytosis (SVE)1 is responsible for the retrieval of synaptic vesicles from the plasma membrane after exocytosis (1). It is activated upon nerve terminal depolarization by calcium influx that stimulates the protein phosphatase calcineurin (2). Calcineurin dephosphorylates the dephosphorylated proteins including amphI/II, dynamin I, synaptojanin, AP180, epsin, eps15, and phosphatidylinositol phosphate kinase Iγ (1). It is the dephosphorylation of these proteins that is thought to trigger SVE during synaptic transmission.

AmphI has four primary domains. The N-terminal BAR domain has the ability to sense membrane curvature and to tubulate lipid membranes in vitro and in vivo (3, 4). AmphI can bind several of the major endocytic proteins including dynamin I and synaptojanin through its C-terminal Src homology 3 (SH3) domain. It has a central proline-rich domain (PRD) that binds endophilin I and an adjoining clathrin and AP-2 binding site (CLAP) domain (5–7). AmphI migrates as a doublet in SDS-PAGE due to its phosphorylation status (8). Although both bands are phosphorylated, the upper, slower migrating band (amphIup) contains a considerably higher incorporation of radiolabeled phosphate than the lower, faster migrating band (amphIlow), suggesting a much greater phosphate stoichiometry. Calcineurin-mediated dephosphorylation collapses amphIup into amphIlow, resulting in a single band on SDS gels (8). Other splice variants of amphI are known to exist.

1 The abbreviations used are: SVE, synaptic vesicle endocytosis; amphI, amphiphysin I isoform 1; amphIiso2, amphiphysin I isoform 2; amphI putative iso3, putative amphiphysin isoform 3; amphIup, amphiphysin I upper band; amphIlow, amphiphysin I lower band; AntP, Antarctic phosphatase; CLAP, clathrin and AP-2 binding site; PRD, proline-rich domain; SGIP, Src homology 3 domain growth factor receptor-bound 2 (Grb2)-like (endophilin)-interacting protein 1; SH3, Src homology 3; TiO2, titanium dioxide; 2D, two-dimensional; BAR, Bin-amphiphysin-Rvs167; DHB, 2,5-dihydroxybenzoic acid; 1D, one-dimensional.
that produce different sized protein products, but these do not appear to account for the amphl phosphorylated doublet, nor have they yet been detected as phosphoproteins in vivo (9–11).

Protein-protein interactions also regulate SVE (1). Amphl has a number of protein partners, in particular clathrin, AP-2, dynamin I, synaptojanin I, endophilin I, and p35 (5, 6, 12–15). Some of its interactions with these proteins are regulated by phosphorylation, suggesting they may be important for stimulus-dependent SVE. Interaction with the endocytic proteins AP-2, clathrin, and endophilin I are thought to be regulated by in vitro phosphorylation of amphl (6, 16–18), but its interaction with dynamin I is not (16). Recent studies have emphasized the need to study phosphosites that occur in vivo rather than solely in vitro sites because many of the latter are artifacts that do not occur in vivo (19, 20). Thus dynam I phosphorylation at Thr-780 in vitro was reported to inhibit its interaction with amphl by preventing binding to the amphl SH3 domain (16). However, dynam I was later shown to be phosphorylated at seven sites in vivo, primarily on Ser-774 and Ser-778 but not on Thr-780, and phosphorylation inhibited dynam I interactions with syndapin I, but not amphl, in nerve terminals (20, 21). Similarly in vitro phosphorylation of dynam I by protein kinase C occurs at Ser-795 (22), yet no phosphorylation of this site occurs in respiring nerve terminals (20). Therefore, it is important to determine whether protein-protein interactions are regulated by phosphosites that occur in vivo without primary reliance on interactions with in vitro phosphosites. Protein-lipid interactions are also important for SVE, and phosphorylation of Ser-276 and Ser-285 has been suggested to regulate amphl binding to lipid membranes (23).

The protein kinase(s) that phosphorylates amphl in nerve terminals in vivo is not known, but amphl is a substrate for at least four protein kinases in vitro. Three proline-directed protein kinases phosphorylate amphl, including cdk5/p35 (Ser-262, Ser-272, Ser-276, Ser-285, and Thr-310) (15, 16, 23), mitogen-activated protein kinase (MAPK) (Ser-285 and Ser-293) (24), and Dyrk1A/minibrain kinase (Ser-293 with minor sites including Thr-310, Ser-295, and Thr-312) (17). CK2 phosphorylates amphl in vitro on Thr-350 and Thr-387 (18). The majority of the in vitro phosphosites identified to date cluster to a small region within the PRD (amphl-PRD(260–312)). The in vivo kinase for amphl was proposed to be cdk5 (16, 23), but evidence for this is contradictory, and definitive data are lacking concerning the extent of its role. In nerve terminals, amphl rephosphorylation upon recovery from a depolarization stimulus is only slightly reduced by the two cdk5 inhibitors roscovitine and Ro31-8220 despite a complete block of dynam I and synaptojanin I phosphorylation by these drugs in the same synaptosomes (sheared off nerve terminals) (19, 25). Evidence claimed to support a role for p35/cdk5 as the in vivo protein kinase came from a study using p35-deficient mice (16). However, results of that study showed that the ratio of amphl<sub>up</sub>:amphl<sub>low</sub> in synaptosomes from these mice was unaltered despite that dynam I phosphorylation was abolished. The presence of normal levels of amphl<sub>up</sub> indicates that it is still being phosphorylated to near normal levels in mice lacking cdk5 activity. This supports the hypothesis that there is a minor role for cdk5 phosphorylation of amphl in synaptosomes. Therefore, at least a second protein kinase or probably more may phosphorylate amphl in vivo.

Previous studies aimed at identifying the amphl phosphosites have used in vitro phosphorylation of predicted phosphosite mutants to show reduced in vitro <sup>32</sup>P incorporation and have used a limited amount of MSMS (15, 17). Site-directed mutagenesis approaches are also limited and can result in some sites being missed or potentially ignored. Such approaches do not provide direct evidence concerning which sites are phosphorylated in vivo or are functionally relevant to SVE. Two amphl in vivo phosphosites (Ser-496 and Ser-250) have been identified using large scale phosphoproteomics studies (26, 27), and a third in vivo phosphosite, Ser-293, was identified using a phosphospecific antibody (17). Clearly a concerted systematic approach to finding the in vivo phosphosites in amphl is lacking. To address this we have used a multifaceted strategy, largely based on <sup>32</sup>P tracking, to separate and identify phosphopeptides from synaptosomal amphl. TiO<sub>2</sub> chromatography provided a highly selective enrichment of phosphopeptides from a complex sample with minimal binding of non-phosphorylated peptides (28). Mass spectrometry was used to identify and sequence all the phosphopeptides to unambiguously assign phosphosites to a specific amino acid. Off-line HPLC fraction collection and 2D tryptic phosphopeptide mapping of <sup>32</sup>P labeling of phosphopeptides not only allowed the number of phosphosites to be determined but were used to measure the potential relative significance of each phosphorylation site to SVE. Two criteria were used to identify and assess the potential biological significance of the phosphosites we identified. The first was the relative amount of <sup>32</sup>P incorporation (“dynamic” phosphorylation), and the second was the relative sensitivity of each phosphosite to a brief depolarization stimulus. Together these provide clues as to which sites may be functionally important to SVE and which may regulate protein-protein interactions in SVE. Furthermore many phosphosites were neither <sup>32</sup>P-labeled nor stimulus-sensitive and were therefore called “constitutive” phosphosites to indicate that they were phosphorylated prior to synaptosome isolation from brain. Our approach contrasts to targeting a simple list of phosphosites, any number of which may be highly phosphorylated and yet not directly involved in rapid signaling during SVE. The results suggest a complex interplay between constitutive and rapidly turning over phosphosites in multiple amphl splice variants occurs in nerve terminals. The data implicate a role for a subset of these phosphosites only in amphl<sub>up</sub> in SVE.
**EXPERIMENTAL PROCEDURES**

**Chemicals and Constructs**—All chemicals were of analytical grade or higher. Milli-Q water was used in all experiments (Milli-Q UF PLUS, Millipore). All gel reagents were from Bio-Rad. EGTA, EDTA, ammonium bicarbonate, ammonium hydroxide (35% NH₄OH), TFA, formic acid, and activated charcoal were from Sigma. Antarctic phosphatase (AntP) and endoproteinase Glu-C were from New England Biosciences (Ipswich, MA). Endoproteinase Arg-C was from Roche Applied Science. ACN was from Burdick and Jackson (Muskegon, MI). POROS 10 R2 and Oligo R3 were from Applied Biosystems (Framingham, MA). Titanium dioxide (TiO₂) beads were obtained from a disassembled Titanosphere guard column (4-mm inside diameter × 100 mm, 5 μm, from Inertsil, GL Sciences). α-Cyanocinnamic acid and 2,5-dihydroxybenzoic acid (DHB) were from Fluka (St. Louis, MO). 32P[Orthophosphate] was from PerkinElmer Life Sciences. Trypsin (porcine, modified) was from Promega (Madison, WI). The plasmid depurated in three changes of 50% ACN, 50 mM ammonium bicarbonate (unstimulated synaptosomes) samples were individually excised and diced control (unstimulated synaptosomes) and four depolarization (KCl-stimulated synaptosomes) samples were prepared from adult rat brain as described previously (19) using the amphI monoclonal antibody (sc-21710, Santa Cruz Biotechnology, Inc.).

**In-vitro Phosphorylation**—All chemicals were of analytical grade or higher. Milli-Q water was used in all experiments (Milli-Q UF PLUS, Millipore). All gel reagents were from Bio-Rad. EGTA, EDTA, ammonium bicarbonate, ammonium hydroxide (35% NH₄OH), TFA, formic acid, and activated charcoal were from Sigma. Antarctic phosphatase (AntP) and endoproteinase Glu-C were from New England Biosciences (Ipswich, MA). Titanium dioxide (TiO₂) beads were obtained from a disassembled Titanosphere guard column (4-mm inside diameter × 100 mm, 5 μm, from Inertsil, GL Sciences). α-Cyanocinnamic acid and 2,5-dihydroxybenzoic acid (DHB) were from Fluka (St. Louis, MO). 32P[Orthophosphate] was from PerkinElmer Life Sciences. Trypsin (porcine, modified) was from Promega (Madison, WI). The plasmid expressing GST-endophilin I SH3 domain was from P. McPherson (McGill, Quebec, Canada), and the plasmid for GST-α-adaptin appendage domain was from R. Anderson (Dallas, TX). GST-dynamin I-PRD was the rat dynixin (long tailed splice variant) as described previously (21). Pro-Q Diamond phosphoprotein gel stain was from Invitrogen.

**32P Labeling of Synaptosomes and Pulldown Experiments**—Crude (P2) synaptosomes were prepared from adult rat brain as described previously with minor changes (19). Synaptosomes were labeled with 0.75 μCi/mg 32P-Pi for 1 h at 37 °C, washed, and depolymerized by adding 41 mM KCl for 10 s for the depolymerizing condition (reducing NaCl to maintain constant osmolarity). Synaptosomes were lysed at 4 °C in 5 mM Tris-HCl at pH 7.4 containing 1% Triton X-100, 125 mM NaCl, 1 mM EDTA, 20 μg/ml leupeptin, 1 mM PMSF, phosphatase inhibitor set II (Calbiochem), and centrifuged at 20,442 g. The supernatant was added to AntP (0.05 units/ml) and incubated for 4 h at 37 °C. The resulting doubly digested peptide solution from each sample was removed, and tryptic digestion was performed with 5% formic acid in ACN. The combined steps were concentrated to 1 μl with 0.1% (v/v) TFA aqueous solution before injection onto an HPLC SMART system (Amersham Biosciences). In some experiments a double digestion strategy was used instead of trypsin. In this case, endoproteinase Arg-C (3 μg) dissolved in 50 mM ammonium bicarbonate was used to digest the amphIlow and amphIup gel bands in a microwave for 15 min on medium-high setting (the Genius, 1,200 watts, Panasonic) (29). The total digest, including gel plugs, was dried in a rotary vacuum concentrator and then incubated with endoproteinase Glu-C (3 μg) dissolved in 50 mM ammonium bicarbonate overnight at 25 °C. The resulting doubly digested peptides were then extracted as above.

**Phosphorylation Sites**—Phosphorylated peptides from a tryptic extract from 10 unlabeled amphI bands (control) were enriched by TiO₂ as described previously (28) except that the loading solution was 80% ACN, 5% TFA and the elution buffer was 25% NH₄OH, 35% ACN (8% NH₄OH in solution). The tryptic peptide mixtures in 80% ACN, 5% TFA from amphI or amphI iso2 were loaded slowly over the column in 1 min. The column was washed two times with loading solution and eluted using four 10-μl elutions of 25% NH₄OH, 35% ACN, and the eluate was immediately dried in a rotary vacuum concentrator. Samples were resuspended in 5% formic acid and applied onto Oligo R3 and graphite microcolumns or treated with AntP and analyzed using mass spectrometry.

**Peptide Desalting**—Chromatographic GELOader microcolumns were used for desalting and concentration of the peptide mixture prior to mass spectrometry analysis were custom-made as described previously for POROS R2 (Applied Biosystems) (30), Oligo R3 (Applied Biosystems) (31), and graphite microcolumns (Sigma-Aldrich) (32). Peptides bound to each microcolumn were eluted using 20 mg/ml DHB, 1% phosphoric acid in 70% ACN, 0.1% TFA or 8 mg/ml an-cyanocinnamic acid for MALDI-TOF MS and 70% ACN, 0.1% formic acid for MSMS. A sequential desalting approach was used in which the Oligo R3 flow-through was loaded onto graphite microcolumns to detect hydrophilic phosphopeptides.

**TiO₂ Microcolumns**—Chromatographic microcolumns were packed with TiO₂ as described previously (28) except that the loading solution was 80% ACN, 5% TFA and the elution buffer was 25% NH₄OH, 35% ACN (8% NH₄OH in solution). The tryptic peptide mixtures in 80% ACN, 5% TFA from amphI or amphI iso2 were loaded slowly over the column in 1 min. The column was washed two times with loading solution and eluted using four 10-μl elutions of 25% NH₄OH, 35% ACN, and the eluate was immediately dried in a rotary vacuum concentrator. Samples were resuspended in 5% formic acid and applied onto Oligo R3 and graphite microcolumns or treated with AntP and analyzed using mass spectrometry.

**Dephosphorylation with Antarctic Phosphatase**—Samples were incubated for 4 h in AntP (0.05 units/μl) at room temperature to dephosphorylate the phosphopeptides. Samples were loaded onto Oligo R3 microcolumns, and R3 flow-through was then loaded onto graphite microcolumns. Bound peptides were eluted using 20 mg/ml DHB, 1% phosphoric acid in 70% ACN, 0.1% TFA and analyzed by MALDI-TOF MS.

**Off-line HPLC**—Phosphorylated peptides from a tryptic extract from 10 unlabeled amphI bands (control) were enriched by TiO₂ chromatography and added to the untreated 32P-labeled amphI (control) sample prior to off-line HPLC. Tryptic peptides from 32P-labeled amphIlow and amphIup bands for both control and KCl-stimulated (depolarization) samples were separated by off-line reversed phase chromatography (SMART system, Amersham Biosciences) using a 4.6 × 150-mm column (Everest C₁₈ monomeric, 5 μm, 300 Å; GraceVydac). The gradient was from 100% phase A (0.1% TFA aqueous solution) to 40% phase B (0.1% TFA in 100% ACN) in 24 min at 10 μl/min with fractions (50 μl) collected every 30 s. To quantify 32P in each fraction, 30% of each HPLC fraction was dried in a rotary vacuum concentrator and then made up to 2 μl with 10% ACN, 0.1% formic acid in water. Sample was spotted onto nitrocellulose and exposed to PhosphorImager screens for 3 weeks to detect 32P radiation. The screens were scanned using a Storm 860 PhosphorImager (GE Healthcare) and analyzed using ImageQuant 5.2 software to determine spot intensity. To identify which peptides were present in each fraction, an aliquot (10%) was subjected to MALDI-TOF MS analysis. For subsequent 2D phosphopeptide mapping (using the remaining 60% of the total sample), the HPLC fractions 9–16 were recombined while keeping the control amphIlow, depolarized amphIlow, control amphIup, and depolarized amphIup separate. HPLC fractions 17–32 and 33–46 were recombined into control amphI and depolarized amphI.
depolarized amph without keeping amphlow and amphup separate. Combined HPLC fractions were dried in a rotary vacuum concentrator.

2D Tryptic Phosphopeptide Mapping by Thin-layer Chromatography—2D tryptic phosphopeptide mapping on thin-layer cellulose plates (Merck) was carried out as described previously (33) using amph tryptic peptides in HPLC fractions 9–16, 17–32, and 33–46. The first dimension electrophoresis was carried out at pH 1.9 in 88% formic acid, glacial acetic acid, water (50:156:1,794). Samples were run in the first dimension at constant voltage (1,000 V) for 32 min at 10 °C. The second dimension chromatography was carried out either in the phosphopeptide chromatography buffer containing n-butyl alcohol/pyridine/glacial acetic acid/water (75:50:15:70) to separate the combined HPLC fractions 17–32 and 33–46 or in a second chromatography buffer consisting of n-butyl alcohol/pyridine/glacial acetic acid/ACN/water (55:35:10:15:85) that was used to separate small hydrophobic phosphopeptides in HPLC fractions 9–16. Chromatography was run for 14 h, and then the plate was air-dried. Autoradiography on PhosphorImager screens was used to detect 32P after exposure of the thin-layer chromatography plates for 3 weeks. The cellulose containing the 32P-phosphopeptide spots of interest was scraped from the thin-layer chromatography plates, and the phosphopeptides were recovered using a custom-cut microspin column attached to a vacuum pump as described previously (33). Peptides were recovered using three 10-min extractions of the cellulose with 5% formic acid. The peptide mixture was dried, then further purified using Oligo R3 and graphite microcolumns, and analyzed using mass spectrometry. The quantitative 32P radiation in each 2D spot was calculated from the original 32P radiation in the HPLC fraction.

Mass Spectrometry—MALDI-TOF MS analysis was carried out using a Voyager-DE PRO mass spectrometer (Applied Biosystems). Spectra were obtained in positive reflector mode and positive linear mode using an accelerating voltage of 20 kV. Static electrospray ionization hybrid quadrupole time-of-flight mass spectrometry was carried out using a QSTAR XL mass spectrometer (Applied Biosystems). Samples were loaded into borosilicate nanospray capillaries (Proxeon Biosciences, Odense, Denmark), and 1,100 V was applied. Phosphopeptides of known molecular mass were selected for fragmentation. Nano-LC-MSMS was carried out using the QSTAR XL or a Q-TOF Ultima mass spectrometer (Waters/Micromass, Manchester, UK) with automated data-dependent acquisition. A nano-HPLC system (LC Packings Ultimate HPLC system, Dionex, Amsterdam, The Netherlands) was used for chromatographic separation of the peptide mixture prior to MS detection. The peptides were concentrated and desalted on a precolumn (75-μm inner diameter, 2-cm length, Reprosil-Pur 120 C18-AQ, 3-μm beads; Dr. Masch) in 5 min. They were then eluted through a 50-μm-inner diameter C18 analytical column of the same material at 100 nL/min. The gradient was from 100% phase A (0.1% formic acid in water) during loading, then to 10% phase B (90% ACN, 0.1% formic acid, 9.9% water) in 3 min, then to 50% phase B in 28 min, then to 60% phase B in 3 min, and finally to 100% phase B in 1 min. The eluate was sprayed through a 10-μm-inner diameter distal coated SilicaTip (New Objective) into the mass spectrometer. Data-dependent acquisition was done using a 1-s survey scan from which the three most abundant doubly, triply, and quadruply charged peptides were selected for product ion scans (2 s). For the detection of specific/known amph phosphopeptides, the precursor ion was set to select and fragment the highest abundance charge state for the entire chromatographic run. All experiments were done using a relatively low resolution (2–3 unit) m/z range for precursor selection.

Database Searching—Raw data files from the QSTAR XL Q-TOF mass spectrometer were processed into peak lists in Mascot format using the Analyst QS program version 1.1 (Applied Biosystems/MDS Sciex) and the mascot.dll script version 1.6b13 (Applied Biosystems/MSD Sciex and Matrix Science, London, UK). The parameters/settings for creating peak lists in the mascot.dll script were: precursor mass tolerance for grouping, 1; maximum number of cycles between groups, 1; minimum number of cycles per group, 1; centroid all MSMS data; deisotope MSMS data; report peak area; remove peaks if less than 0.1% of maximum; reject spectra if less than 10 peaks; try to determine charge state from survey scan; and default precursor charge states, 2+, 3+, and 4+. Raw data files from the Q-TOF Ultima mass spectrometer were processed into .pkf files using the Protein Lynx program. On each spectrum the background was subtracted (40%), and smoothing was performed (Savitzky-Golay; iteration, 2; window, three channels). In addition deisotoping was performed using the following parameters: minimum peak width, four channels; centroid top, 80%; TOF resolution, 10,000; Np (number of pushes correction factor) multiplier, 0.7. All MSMS peak lists produced are provided as supplemental data.

Database searching was performed using a local copy of Mascot version 2.1 (Matrix Science). The searched databases were National Center for Biotechnology Information (NCBI) (NCBIinr.20060727 (4,900,652 sequences; 1,692,193,060 residues)) with the taxonomy limited to rodents (164,870 sequences) or NCBI (NCBIinr.20060727 (3,813,612 sequences; 1,314,502,086 residues)) with the taxonomy limited to mammals (485,335 sequences). The database searches were performed with the following variable modifications: deamidation (NQ, oxidation (Met) and phosphorylation (STY) with 250-ppm precursor ion mass tolerance and 0.1-Da mass tolerance for fragment ions. Enzyme specificity for tryptic digests was selected to semitryptic with one missed cleavage. Enzyme specificity for the double Arg-C/Glu-C digest was searched in Mascot such that the peptides could have resulted from cleavage at either end by either enzyme and could have up to three missed cleavages.

We were able to remove the reporting of redundant proteins in our results. Often the proteins analyzed were brain-specific. Peptides were sequenced from the total trypic digests, rather than only the phosphopeptide-enriched fractions, to ensure confident protein identification. This allowed specific alternatively spliced isoforms to be distinguished and eliminated redundancy in protein names reported.

Amph phosphopeptides and some of the phosphopeptides from co-migrating phosphoproteins were first confirmed using Antarctic phosphatase treatment before sequencing by MSMS. No further attempt was made to differentiate between phosphorylation and sulfation. After sequencing, each identification was aided by Mascot searching. However, each spectrum was ultimately validated manually, and as such no threshold was placed on the Mascot score for any of the phosphopeptides. The Mascot scores were particularly low when the spectrum was from a long phosphopeptide >/=3.5 kDa with multiple phosphorylation sites or from a non-tryptic phosphopeptide that yielded more random fragmentation patterns. For the long phosphopeptides, the noise in the low m/z region of the spectrum was typically greater in intensity than the legitimate peptide fragment ions in the high m/z region of the spectrum. The high m/z fragment ions were typically excluded in the creation of peak lists. Therefore, manual validation of these peak lists was appropriate. The spectrum for each phosphopeptide is shown and annotated (see “Results” and supplemental data). For non-phosphopeptides, the threshold for accepting individual MSMS spectra was set at a Mascot score of 25. Those spectra with a score less than 35 (four spectra) were manually validated. All the manually validate spectra were inspected to ensure that they had sufficient y and b ions to for identification and for unambiguous assignment of the phosphorylation site. The Mascot search engine uses both the Mascot search engine uses both the +80-DA mass shift and the 98-DA neutral loss for the assignment of a phosphorylation site. We searched for both the +80-DA mass shift and the 98-DA neutral loss in our manual validation of phosphorylation sites using the Biolynx version 1.1.
Amphiphysin I in Vivo Phosphorylation Sites

Fig. 1. Extraction of phosphorylated amph from 32P-labeled synaptosomes. A triple pulldown using GST-α-adaptin appendage domain, GST-endothelin I SH3 domain, and GST-dynamin I-PRD were used to isolate amph from synaptosome lysates, and the bound proteins were analyzed using SDS-PAGE. Proteins were visualized using colloidal Coomassie G-250 (lanes 1 and 2). Peptides identified using MALDI-TOF MS and MSMS are shown on the left. Amph isolated from 32P-labeled intact synaptosomes is constitutively phosphorylated in resting (unstimulated) rat brain nerve terminals (CON) and is dephosphorylated after stimulation (depolarization) with 41 mM KCl (DEP) for 10 s. Amph is labeled as a doublet in which amphiso2 is shifted up due to phosphorylation causing its slower migration in SDS-PAGE. Western blotting (WB) using an amph antibody confirmed that the upper and lower bands are amph and reveals a second isoform, amphiso2 (lanes 3 and 4). Autoradiography (Auto- rad) detected 32P that was rapidly incorporated into the dephosphins and their stimulus-dependent response to KCl depolarization (lanes 5 and 6). Phosphoproteins were also visualized using the Pro-Q Diamond fluorescent stain that detects total phosphate (both dynamically labeled and constitutive; lanes 7 and 8). Note that all lanes (except lanes 3 and 4) were derived from the same gel. The position of molecular mass standards is shown on the right. Similar results were obtained from at least three experiments.

RESULTS

Multisite Phosphorylation of Amph in Synaptosomes—Amph was affinity-purified from 32P-labeled rat brain synaptosomes using a combination of three amph-binding GST fusion proteins: α-adaptin appendage domain, endothelin I SH3 domain, and dynamin I-PRD. We term this approach a “triple pulldown” because the recombinant proteins attached to GSH-Sepharose were mixed and used together. Six of the dephosphins were simultaneously isolated using this pulldown: amph, amphiphysin II, dynamin I, synaptojanin I, AP180, and epsin (19) (Fig. 1 and data not shown). Western blotting using an amph monoclonal antibody (sc-21710) detected three bands: amphiso2, amphiso2, and the alternatively spliced amphiso2 (Fig. 1, lanes 3 and 4). In the case of the amphiso2, it was barely detectable with Coomassie staining (Fig. 1, lanes 1 and 2). Depolarization of synaptosomes using 41 mM KCl for 10 s decreased the 32P in at least four of the dephosphins (amph, amphiphysin I, synaptojanin I, and AP180), but the total protein level remained unchanged (Fig. 1, lanes 5 and 6). The phosphorylation of synaptojanin I increased (data not shown, but see also Tan et al. (19)). This demonstrated that the synaptotagmin I were not stimulated for too long, which can result in a decrease in synaptotagmin I phosphorylation (34). Note that amphiso2 was barely labeled with 32P. Phosphoproteins were additionally detected using Pro-Q Diamond phosphoprotein stain (Fig. 1, lanes 7 and 8). Several proteins including amphiso2 did not show significant 32P incorporation but were shown to be phosphorylated using the Pro-Q Diamond stain. This suggests that these proteins are primarily constitutively phosphorylated in nerve terminals rather than dynamically turning over their phosphate and that they are not responding to depolarization. Phosphorylation that occurs in vivo and is detected with Pro-Q Diamond, mass spectrometry, or phosphosite-specific antibodies yet is not well labeled with 32P after 1 h is termed constitutive and is likely to be subject to long term rather than acute regulation. The sequential staining method allowed total protein, total phosphoprotein, and the 32P turnover to be analyzed from one gel (Fig. 1, lanes 1, 2, and 5–8). It is the depolarization-induced change in amph phosphorylation that is most likely to influence protein-protein interactions involved in SVE because SVE is activated by depolarization. As reported previously, amphiso2 was phospho-phosphorylated to a much higher stoichiometry than amphiso2, and amphiso2 was dephosphorylated to a much greater extent during depolarization.

Isolation of Amph Phosphopeptides Using TiO2 Chromatography—To identify all the in vivo phosphosites we initially performed a tryptic digest on amph isolated from control (unstimulated) synaptosomes after 60-min incubation in a Krebs-like solution. The eluted peptides from TiO2 microcolumns were analyzed by MALDI-TOF MS. The mass spectrum for the analysis of amphiso2 is shown in Fig. 2A. Several mass peaks were candidate phosphopeptides because their detected mass was characteristically mass-shifted by 80 Da or multiples of 80 Da; this was confirmed by AntP treatment (Fig. 2B). All the masses marked by asterisks were later demonstrated to be phosphopeptides and were sequenced.

Six of the detected phosphopeptides could be assigned as tryptic peptides corresponding to amph. These included signals for the phosphopeptides at m/z: 1,463.6, 2,866.3, 3,128.4, 3,406.7, 3,486.7, and 3,566.7 (Fig. 2A and Table I). It was previously shown that cdk5 can phosphorylate the latter tryptic peptide amphiso2 (260–292) on three or four sites in vitro. We now show that it is phosphorylated on at least four sites in vivo as evident from Fig. 2C. Of note was the much lower abundance of the triply and quadruply phosphorylated peptides of amphiso2 (260–292) in the amphiso2 analysis (data not shown). It is important to note that the signal intensity in mass spectrometry is not an accurate indication of the relative phosphorylation levels.
Small phosphopeptides may not bind the C18 chromatography material that is used in desalting samples prior to mass spectrometry. However, such peptides can be captured on graphite microcolumns (32). Therefore the flow-through from our C18 microcolumns was captured on graphite. Two short tryptic phosphopeptides were found at m/z 625.3 (m/z 545.3 amphI-(309–313)) and at m/z 755.3 (675.3 amphI-(293–298)) after sequential use of TiO2, C18, and graphite microcolumns (Fig. 2D). The results clearly indicate that amphI is phosphorylated on multiple sites within nine phosphopeptides. Although some of the actual phosphorylation site sequences can be deduced when these phosphopeptides contained only a single Ser or Thr residue, most of them contained multiple Ser or Thr residues in their sequence. Therefore, in the following experiments MSMS was used to directly identify all the phosphosites in all peptides.

**Identification of 13 Phosphorylation Sites Using MSMS**—To unequivocally assign the phosphosites for all amphI phosphopeptides, the eluates from TiO2 microcolumns were subjected to LC-MSMS. The MSMS spectra of each phosphopeptide from Fig. 2 are shown in Fig. 3 and supplemental Figs. S1–S5. All 13 phosphopeptide sequences had the appropriate b and y ions to unambiguously determine the site(s) of phosphorylation. A summary of all the phosphopeptides and their phosphorylation sites is shown in Table I.

The monophosphorylated peptide amphI-(260–292) encompassing most of the amphI-PRD sequence was phosphorylated on Ser-262 (Fig. 3A). The doubly phosphorylated peptide, 3,486.7 Da (supplemental Figs. S1–S3), was phosphorylated on Ser-262 accompanied by one of the following: Ser-268, Ser-276, Ser-272, or Ser-285. This demonstrates that phosphorylation is hierarchical to the extent that Ser-262 phosphorylation preferentially precedes phosphorylation at Ser-276, Ser-268, Ser-272, and/or Ser-285. The triply phosphorylated peptide, 3,566.7 Da, revealed a mixture of at least five phosphosites but contained two dominant species: Ser-262/Ser-268/Ser-276 and Ser-262/Ser-268/Ser-272 (supplemental Figs. S1B and S4). There was also evidence for phosphorylation at Ser-285. The quadruply phosphorylated peptide, 3,646.7 Da, was a mixture of at least two phosphopeptides. It was phosphorylated on Ser-262/Ser-268/Ser-272/Ser-276 (supplemental Fig. S5). There was also evidence for phosphorylation at Ser-285 (therefore one of the following sites must have been absent in this minor species: Ser-268/
**Table I**

**Summary of amphI phosphorylation sites identified in three splice variants from adult rat brain nerve terminals**

Thirteen phosphosites were identified in amphI by TiO₂ enrichment and MSMS. Seven sites were found in amphI iso2, and four sites were found in amphI putative iso3. The phosphosite in each peptide is underlined. **Mods**, modifications; **phos**, phosphorylation.

| Amino acid residues | Peptide sequence | Phosphorylation site(s) | Mods (phos) | Precursor mass/charge m/z | Peptide mass [M + H]^+ | MASCOT score | Expect |
|---------------------|------------------|-------------------------|-------------|---------------------------|------------------------|--------------|--------|
| **AmphI**           |                  |                         |             |                           |                        |              |        |
| 242–256             | AFSIQGAPSGGGLR   | Major Ser-250, minor Ser-252 | 1           | 791.9, 2+                 | 1,582.8                | 82           | 0.0000056 |
| 260–292             | TPSPPEASPLPSPTAQPNTLAPA_SAPAPVRPR | Ser-262 | 1           | 852.4, 4+                 | 3,406.7                | 11           | 790    |
| 260–292             | TPSPPEASPLPSPTAQPNTLAPA_SAPAPVRPR | Major Ser-262 + Ser-276; also Ser-268, Ser-272, and Ser-285 | 2           | 872.4, 4+                 | 3,486.7                | 49           | 0.12   |
| 260–292             | TPSPPEASPLPSPTAQPNTLAPA_SAPAPVRPR | Major Ser-262 + Ser-268 + Ser-276; also Ser-272 and Ser-285 | 3           | 892.4, 4+                 | 3,566.7                | 20           | 92     |
| 260–292             | TPSPPEASPLPSPTAQPNTLAPA_SAPAPVRPR | Major Ser-262 + Ser-268 + Ser-276 + Ser-272; and Ser-285 | 4           | 912.4, 4+                 | 3,646.7                | 7            | 1500   |
| 293–298             | SPSQTR           | Ser-293                 | 1           | 378.1, 2+                 | 755.3                  | 23           | 1.2    |
| 309–313             | VPTK             | Ser-310                 | 1           | -                         | 625.3                  | N/A          | N/A    |
| 299–314             | KGPPVPPLKPVTK**  | Ser-310                 | 1           | 588.9, 3+                 | 1,765.0                | 1            | 850    |
| 487–501             | AALPAGEGESPEGAK  | Ser-496                 | 1           | 732.3, 2+                 | 1,463.6                | 40           | 0.52   |
| 502–528             | IDVESTELASSGEQPAAELEGAPQEK | Ser-514 | 1           | 956.1, 3+                 | 2,866.3                | 65           | 0.0021 |
| 529–556             | VIPSVVIEPAGNEGEHEQETTTGTETR | Ser-539 | 1           | 782.6, 4+                 | 3,128.4                | 54           | 0.84   |
| 615–633             | VETLHDEAANGDELTLQR | Ser-626 | 1           | 756.7, 3+                 | 2,288.0                | 56           | 1.6    |
| 257–266             | IAARTPPPEEP**    | Ser-262                 | 1           | 574.8, 2+                 | 1,148.5                | 52           | 0.43   |
| 260–278             | TPSPPEASPLPSPTAQSPN** | Ser-262 | 1           | 652.7, 3+                 | 1,954.9                | 23           | 11     |
| 262–292             | SPPPEASPLPSPATASPN** | Ser-262 | 1           | 802.9, 4+                 | 3,208.6                | 58           | 0.3    |
| **AmphI iso2**      |                  |                         |             |                           |                        |              |        |
| 260–292             | TPSPPEASPLPSPTAQPNTLAPA_SAPAPVRPR | Ser-262 | 1           | 852.4, 4+                 | 3,406.7                | 26           | 19     |
| 260–292             | TPSPPEASPLPSPTAQPNTLAPA_SAPAPVRPR | Ser-262 + Ser-276; also Ser-268, Ser-272; or Ser-272 | 2           | 872.4, 4+                 | 3,486.7                | 13           | 410    |
| 446–460             | AALPAGEGESPEGAK  | Ser-455                 | 1           | 732.3, 2+                 | 1,463.6                | 42           | 0.3    |
| 461–487             | IDVESTELASSGEQPAAELEGAPQEK | Ser-473 | 1           | 956.1, 3+                 | 2,866.3                | 74           | 0.00026 |
| 488–515             | VIPSVVIEPAGNEGEHEQETTTGTETR | Ser-498 | 1           | 782.6, 4+                 | 3,128.4                | 42           | 2500   |
| **AmphI putative iso3** |                  |                         |             |                           |                        |              |        |
| 930–944             | AALPAGEGESPEGAK  | Ser-939                 | 1           | 732.3, 2+                 | 1,463.6                | 49           | 0.018  |
| 945–971             | IDVESTELASSGEQPAAELEGAPQEK | Ser-957 | 1           | 956.1, 3+                 | 2,866.3                | 29           | 5.3    |
| 972–999             | VIPSVVIEPAGNEGEHEQETTTGTETR | Ser-982 | 1           | 782.6, 4+                 | 3,128.4                | 55           | 0.017  |
| 1051–1068           | ETIHDEAANGDELTLQR | Ser-1061 | 1           | 543.3, 4+                 | 2,170.3                | 26           | 0.0032 |

*a* This phosphopeptide results from a double Arg-C/Glu-C digestion.

*b* Semitryptic or non-tryptic phosphopeptides from the tryptic digest.
MALDI-TOF MS spectrum (Fig. 2) that Thr-260 was phosphorylated in vivo potential sites ((S/T)P) in this PRD peptide are phosphorylated at Thr-260. The MSMS data provided proof that five of the six Ser-272/Ser-276. There was no evidence for phosphorylation of Ser-262 (shown as pS in the sequence above the panel) as the major phosphorylation site. B, phosphopeptide 2,268.1 Da (amphI-(615–633)) was selected for LC-MSMS analysis as the parent ion 756.7 [M + 3H]+, and the phosphorylation site identified as Ser-626. Fragment ions showing neutral loss of phosphoric acid (−98 Da) are shown.

Ser-272/Ser-276. There was no evidence for phosphorylation at Thr-260. The MSMS data provided proof that five of the six potential sites (S/T)P in this PRD peptide are phosphorylated in vivo but to very different extents. We did not find evidence that Thr-260 was phosphorylated in vivo.

Three of the most prominent phosphopeptides from the MALDI-TOF MS spectrum (Fig. 2A) were all located near the amphI SH3 domain. Tandem MS identified the phosphopeptide in the peptide at m/z 1,463.6 (amphI-(487–501)) as Ser-496 (supplemental Fig. S6A). The phosphosite of the peptide at m/z 2,866.2 (amphI-(502–528)) was identified as Ser-514 (supplemental Fig. S6B), and the peptide at m/z 3,128.4 (amphI-(529–556)) was identified as Ser-539 (supplemental Fig. S7A). Ser-539 was the first phosphosite to be identified that did not contain a proline residue directly after the phosphosite, providing evidence that amphI is phosphorylated by a non-proline-directed protein kinase in vivo. Four low abundance phosphopeptides that were not observed in the MALDI-TOF MS spectrum (Fig. 2A) were sequenced by MSMS. The phosphopeptide at m/z 1,582.8 (amphI-(242–256)) was found to be phosphorylated at Ser-250 with a minor level of phosphorylation at Ser-252 (supplemental Fig. S7B). The phosphosite of the peptide at m/z 2,268.0 (amphI-(615–633)) was found to be Ser-626 (Fig. 3B), which is also a non-proline-directed protein kinase site (Ser-Asp). The phosphosite of the peptide at m/z 755.3 (amphI-(293–298)) from Fig. 2D was identified as Ser-293 (supplemental Fig. S8A). Tandem MS sequencing of the TiO2 sample also revealed a non-tryptic 1,954.9-Da phosphopeptide (amphI-(260–278)) and a 3,208.6-Da phosphopeptide (amphI-(262–292)) as semitryptic peptides both containing Ser-262 as the major phosphosite (supplemental Fig. S9, A and B).

The tryptic digest resulted in a phosphopeptide at m/z 625.3 (amphI-(309–313)) containing the suspected Thr-310 phosphorylation site that was not easily detected using MALDI-TOF MS (see Fig. 2D) or LC-MSMS (data not shown). We utilized endoproteinases Arg-C and Glu-C sequentially to provide a set of amphI peptides different from those of a tryptic digest. This resulted in a larger phosphopeptide encompassing Thr-310, a 1,765.0-Da phosphopeptide (amphI-(299–314)). This non-tryptic phosphopeptide was sequenced by nanospray ESI-MSMS and revealed the in vivo phosphorylation at Thr-310 (supplemental Fig. S8B). A phosphopeptide resulting from the same sequential Arg-C and Glu-C digestion resulted in a peptide that contained both Thr-260 and Ser-262 but not any other site in the PRD. The predominance of ions assigning phosphorylation to Ser-262 confirmed that Ser-262 is the major phosphorylation site and that Thr-260 is not phosphorylated in vivo (supplemental Fig. S9C and Table I).

Overall amphI was found to be phosphorylated on 13 phosphorylation sites in nerve terminals (Table I), and the complete list of all phosphosites are highlighted on the amphI schematic (see Fig. 3A). The phosphorylation sites clustered around the amphI-PRD and the SH3 domains. These results provide a list of all major and minor phosphosites in the amphI tryptic digest. Through the use of various endoproteinases including trypsin, the double Arg-C/Glu-C digest, and a slightly less specific Asp-N digestion, no further phosphorylation sites in amphI were identified. Using the different combination of endoproteinases, we are confident we did not miss any phosphorylation sites in amphI that are above the detection limit of the mass spectrometer.

Co-migrating Synaptosomal Phosphoproteins—It is highly likely that there are several co-migrating phosphoproteins in the amphI band from the 1D SDS-PAGE gel. Several proteins that bind GST-α-adaptin appendage domain have been reported to have a migration in 1D SDS gels similar to that of amphI (35). Several low abundance phosphopeptides in the MALDI-TOF MS spectra (Fig. 2A) (2,030.0, 2,157.0, 2,397.3, 3,066.4, and 3,082.4) did not match any theoretical tryptic peptides from amphI. We sought to identify all the phosphopeptides from the gel bands because they needed to be.

**Fig. 3.** Phosphorylation site assignment of two representative amphI phosphopeptides by LC-MSMS. AmphI tryptic peptides were purified using TiO2 microcolumns (Fig. 2A) and subjected to direct sequencing of the phosphorylation sites using MSMS. A, the monophosphorylated peptide at m/z 3,406.7 (amphI-(260–292)) was selected for LC-MSMS sequencing as the precursor ion 852.4 [M + 4H]+. Analysis of the y and b product ions allowed the unambiguous assignment of Ser-262 (shown as pS in the sequence above the panel) as the major phosphorylation site. B, phosphopeptide 2,268.1 Da (amphI-(615–633)) was selected for LC-MSMS analysis as the parent ion 756.7 [M + 3H]+, and the phosphorylation site identified as Ser-626. Fragment ions showing neutral loss of phosphoric acid (−98 Da) are shown.
accounted for in the subsequent $^{32}$P label quantification of amph phosphosites (see below), and they may also be of importance to SVE. LC-MSMS sequencing of the phosphorylated peptides from the TiO$_2$ eluate from an amph tryptic digest identified phosphorylation sites from AAK1 (Thr-607, Thr-621, and Thr-625), auxilin (Ser-66, Ser-625, Ser-713, and Ser-764), $\alpha$-adducin (Ser-12, Thr-358, and Thr-610), $\beta$-adducin (Ser-594 and Ser-602), eps15R (Ser-255, Ser-329, and Ser-355), MAP6 (Ser-681), and SGIP (Src homology 3 domain growth factor receptor-bound 2 (Grb2)-like (endophilin)-interacting protein 1) (Ser-149, Ser-169, Thr-259, Ser-265, Ser-319, Thr-409, and Thr-492) (supplemental Figs. S10–S13 and supplemental Tables S1, S4, and S5). Several of these phosphosites have not been reported previously in nerve terminals, and auxilin, eps15R, and SGIP are proposed to be involved in SVE.

**Ranking Amph Phosphosites for Abundance and Stimulus Response**—The phosphosites in amph are proposed to mediate protein-protein interactions required for SVE (6). However, previous studies focused predominately on in vitro sites that were not known to occur in vivo. Now that we have established through an exhaustive analysis what is likely to be a complete list of all in vivo phosphosites, our next aim was to determine which sites are the most abundant and which are stimulus-responsive. This may provide insights as to the relative physiological significance of each site. We used $^{32}$P labeling to provide a measure of the turnover of incorporated phosphate and to measure the response of the synaptosomes to depolarization (41 mM KCl for 10 s). This is a preferred method of quantification because it disregards constitutive protein phosphorylation (at sites that are not turning over during the 1-h labeling period). Also other methods that measure the absolute or relative amount of phosphopeptides may not be sensitive enough to measure changes in the potentially small pool of dynamic protein phosphorylation involved in turnover in a background of high endogenous level of protein phosphorylation. We undertook $^{32}$P tracking by measuring the $^{32}$P label throughout different stages of purification (1D SDS-PAGE, off-line HPLC, and 2D phosphopeptide mapping) to account for it all. Two types of phosphosites were identified in this study: dynamic phosphosites that were labeled with $^{32}$P during the 1-h incubation and constitutive phosphosites (with no/low level of $^{32}$P labeling) that were phosphorylated prior to $^{32}$P labeling of the synaptosomes.

Amph migrates on SDS-PAGE as a doublet, indicative of a phosphorylation-induced mobility shift (Fig. 1, lanes 5 and 6). Amph$_{up}$ contained 55 ± 2% (S.E., $n = 5$) of the $^{32}$P incorporated in synaptosomes at rest, whereas amph$_{low}$ contained 45 ± 2% (S.E.). Stimulation of the synaptosomes with 41 mM KCl for 10 s resulted in a 41 ± 3% (S.E.) decrease in amph$_{up}$ and a 19 ± 2% (S.E.) decrease in the amph$_{low}$. Because the Coomassie Blue protein staining of amph$_{up}$ versus amph$_{low}$ was at least 1:50, we conclude that the specific activity of amph$_{up}$ is far greater than that of amph$_{low}$, suggesting that phosphosites in the former are more likely to be relevant to the triggering of SVE by depolarization.

The relative $^{32}$P distribution of amph$_{up}$ and amph$_{low}$ phosphopeptides from control synaptosomes was determined using off-line reversed phase HPLC in combination with $^{32}$P detection (Fig. 4, A and B, black and gray bars). Similarly amph$_{up}$ and amph$_{low}$ phosphopeptides from depolarized synaptosomes were analyzed in parallel to identify stimulus-sensitive phosphosites (Fig. 4, A and B, open and cross-hatched bars). Phosphopeptides in each fraction were iden-
tified by MALDI-TOF MS analysis or MSMS and correlated with the data in Table I. AmphI phosphopeptides were separated into four groups according to their relative HPLC elution and 32P distribution (Fig. 4C). Pooled HPLC fractions 9–16 contained the highest percentage of 32P in control synaptosomes (39% of amphIup and 37% of amphIlow) and were dephosphorylated by 51% in amphIup and 16% in amphIlow upon depolarization. The phosphopeptides containing Ser-293 and Thr-310 were identified in fractions 9–16. The second major group, HPLC fractions 32–38, contained 27% of amphIup and 31% of amphIlow control 32P and were dephosphorylated by 40% in amphIup and 16% in amphIlow upon depolarization. The relative amount of 32P could not be assigned to each individual phosphopeptide as most co-eluted over several HPLC fractions, including the phosphorylation sites Ser-250, Ser-252, Ser-262, Ser-268, Ser-276, Ser-272, Ser-285, Ser-514, and Ser-539. The third group in fractions 24–25 contained Ser-496 (3% amphIup and 4% amphIlow) with little change after depolarization. Fraction 31 contained Ser-626 (2% amphIup and 1% amphIlow) with only minor changes after depolarization. Overall these experiments did not fully resolve the 13 phosphosites; however, the data suggest a major abundance of Ser-293 and Thr-310.

Because the separation of phosphopeptides by HPLC and fraction collection did not provide a complete understanding, we used 2D phosphopeptide mapping to improve the separation and quantification. Two-dimensional mapping and MS were used to determine which phosphosite, Ser-293 or Thr-310, accounted for the majority of 32P in HPLC fractions 9–16 from Fig. 4. AmphIup and amphIlow, HPLC fractions 9–16 were analyzed separately using samples from both control and depolarized synaptosomes (Fig. 5A). MALDI-TOF MS of peptides extracted from the 2D spots revealed the upper migrating spot (labeled spot 01) contained only Thr-310 (625.3-Da phosphopeptide), whereas the lower spot 02 contained only Ser-293 (755.3-Da phosphopeptide) (supplemental Fig. S14, A and B). Quantitative analysis indicated that these two phosphosites contained equivalent levels of total 32P incorporation (20% of total control 32P, n = 3) in amphIup and were both dephosphorylated by 50% after depolarization (Fig. 5B). In the amphIlow band, however, Ser-293 contained 30% of the total 32P incorporation and was dephosphorylated by 16%, whereas Thr-310 contained only 8% of control 32P and was dephosphorylated by 24%. Although Ser-293 has a greater overall amount of 32P (23% of total 32P; Table II), Thr-310 may be the most depolarization-responsive phosphosite because it is mainly present in hyperphosphorylated amphIup, whereas Ser-293 is still abundant in amphIlow. This raises the possibility that Thr-310 contributes to the mobility shift observed in amphIup in SDS-PAGE. We conclude that Thr-310 and Ser-293 are the major stimulus-responsive sites in amphI.

We next used 2D tryptic phosphopeptide mapping to separate the remaining peptides in the HPLC fractions 17–32 and 33–46 that contained 23 and 36% of the total control 32P. Because the separation of phosphopeptides by HPLC and fraction collection did not provide a complete understanding, we used 2D phosphopeptide mapping to improve the separation and quantification. Two-dimensional mapping and MS were used to determine which phosphosite, Ser-293 or Thr-310, accounted for the majority of 32P in HPLC fractions 9–16 from Fig. 4. AmphIup and amphIlow, HPLC fractions 9–16 were analyzed separately using samples from both control and depolarized synaptosomes (Fig. 5A). MALDI-TOF MS of peptides extracted from the 2D spots revealed the upper migrating spot (labeled spot 01) contained only Thr-310 (625.3-Da phosphopeptide), whereas the lower spot 02 contained only Ser-293 (755.3-Da phosphopeptide) (supplemental Fig. S14, A and B). Quantitative analysis indicated that these two phosphosites contained equivalent levels of total 32P incorporation (20% of total control 32P, n = 3) in amphIup and were both dephosphorylated by 50% after depolarization (Fig. 5B). In the amphIlow band, however, Ser-293 contained 30% of the total 32P incorporation and was dephosphorylated by 16%, whereas Thr-310 contained only 8% of control 32P and was dephosphorylated by 24%. Although Ser-293 has a greater overall amount of 32P (23% of total 32P; Table II), Thr-310 may be the most depolarization-responsive phosphosite because it is mainly present in hyperphosphorylated amphIup, whereas Ser-293 is still abundant in amphIlow. This raises the possibility that Thr-310 contributes to the mobility shift observed in amphIup in SDS-PAGE. We conclude that Thr-310 and Ser-293 are the major stimulus-responsive sites in amphI.

We next used 2D tryptic phosphopeptide mapping to separate the remaining peptides in the HPLC fractions 17–32 and 33–46 that contained 23 and 36% of the total control 32P.
synaptosomes were analyzed in parallel (Fig. 5, D and F). MALDI-TOF MS analysis and AntP treatment identified the majority of phosphopeptide(s) corresponding to each \(^{32}\)P radioactive spot (supplemental Figs. S14–S16) with the identified phosphosite(s) shown in Table I. The majority of \(^{32}\)P radiation was in 2D spots 07, 08, and 09 (Fig. 5, E and F, and Table II). Spot 07 was a mixture of Ser-262 and Ser-539 and had 5% of the total \(^{32}\)P but was not responsive to depolarization. 2D spot 08 was the doubly phosphorylated amphI-(260–292) peptide and contained 3% of the total \(^{32}\)P and was dephosphorylated by 33%. 2D spot 09 contained the doubly, triple, and quadruply phosphorylated amphI-(260–292) (Ser-262, Ser-268, Ser-272, Ser-276, and Ser-285) peptides and had 19% of the total \(^{32}\)P incorporation and was dephosphorylated by 53% after depolarization. This indicates that the cluster of four phosphosites, Ser-262, Ser-268, Ser-272, Ser-276, and Ser-285 (Fig. 7A), in the amphI-PRD peptide are collectively the second-major depolarization-sensitive sites. The total \(^{32}\)P labeling accounted for in the amphI-PRD was 66%, and these phosphosites were dephosphorylated by 26–55% after depolarization (Table II).

Phosphosites that were not in the PRD had low amounts of \(^{32}\)P labeling and did not respond to depolarization (Table II). Spot 03 contained Ser-496 and had 2% of the total \(^{32}\)P and was not dephosphorylated after depolarization; this was the same result as the HPLC data (Fig. 4C). Spot 06 contained Ser-514 and had 1% of the total \(^{32}\)P with no change after depolarization. Spot 04 contained Ser-539 and had 1% of the total \(^{32}\)P. Spot 05 was identified as Ser-250 and Ser-252 and had 1% of the total \(^{32}\)P with no depolarization change. The total \(^{32}\)P that could be attributed to amphI was 72% of the total with an overall 35% decrease after depolarization. This leaves a significant amount of \(^{32}\)P unaccounted for. However, 2D spots (10–19) were identified as low abundance phosphoproteins that co-migrated with amphI in the 1D SDS-PAGE. These phosphoproteins included SGIP, AAK1, and HCN1 (supplemental Table S1). Quantitative analysis of 2D spots from phosphoproteins other than amphI accounted for another 22% of all the \(^{32}\)P radiation in amphI (supplemental Table S2). SGIP contained seven phosphopeptides with a total of 13% of the total \(^{32}\)P incorporation, and each either was not changed or was dephosphorylated by up to 50% after depolarization. This is the first report that SGIP is dynamically phosphorylated (\(^{32}\)P-labeled) in nerve terminals and that it responds to depolarization. It is therefore a new dephosphin candidate.

Using the 2D phosphopeptide mapping in combination with the HPLC data we accounted for 94% of the total \(^{32}\)P incorporation. AmphI phosphopeptides represented 72% of the total \(^{32}\)P incorporation with the remaining 22% \(^{32}\)P radiation attributed to a low level of co-migrating phosphoproteins. We note that the total decrease in dephosphorylation after depolarization was 34% once all the \(^{32}\)P was accounted for in the 2D phosphopeptide maps (Table II). A similar decrease in dephosphorylation was obtained from quantitative measure-
Amphiphysin I in Vivo Phosphorylation Sites

Fig. 6. Alternatively spliced amphI iso2 is phosphorylated in nerve terminals. GST-α-adaptin appendage domain and GST-endophilin I SH3 domain were used in a double pulldown experiment to isolate 32P-labeled amph. Western blotting (WB) using an anti-phospho-Ser-262 antibody detected two major bands corresponding to amphI, and a lower band at 110 kDa (lanes 5 and 6). Coomassie Blue protein stain (lanes 1 and 2) and autoradiography (autorad; lanes 3 and 4) indicated a low level of protein staining and 32P incorporation in the amph iso2. CON, control; DEP, depolarization.

Fig. 7. Summary of all in vivo phosphorylation sites in amphl and amphI iso2. A, domain structure of amphl (amphIup) and amphIiso2 showing the location of all 13 in vivo phosphorylation sites. In amphl, the endophilin binding site resides within residues 298–307, the AP-2 binding site resides within residues 322–340, the clathrin binding site resides within residues 347–386, and the dynamin/synaptojanin binding site resides in the SH3 domain. Phosphorylation sites are color-coded to indicate the highest 32P turnover sites in green with highest stimulus sensitivity in bold green (Thr-310 and Ser-293) and medium stimulus sensitivity in non-bold green (Ser-285, Ser-276, Ser-272, and Ser-268). Red indicates low 32P turnover constitutive phosphorylation sites (Ser-262, Ser-496, Ser-514, and Ser-539) with bold red indicating highest abundance. Remaining sites are in black text and indicate low 32P incorporation as well as low abundance (Ser-250, Ser-252, and Ser-626). B, domain structure of amphI iso2 splice variant that has a 41-amino acid deletion in the C terminus (residues 421–461). The location of the seven in vivo phosphorylation sites identified in this isoform included Ser-262, Ser-269, Ser-272, and Ser-268. Red indicates low 32P turnover constitutive phosphorylation sites (Ser-262, Ser-496, Ser-514, and Ser-539) with bold red indicating highest abundance. Remaining sites are in black text and indicate low 32P incorporation as well as low abundance (Ser-250, Ser-252, and Ser-626).

Other Isoforms of Amphl in Nerve Terminals—We produced a phosphospecific antibody to the phosphosite Ser-262. This phosphosite had low amounts of 32P incorporation but was highly abundant using enrichment methods and responded poorly to depolarization. This suggests that it is a constitutive phosphosite, turning over slowly with the 32P labeling conditions, but is highly phosphorylated. We used this phosphoantibody to examine amphl isolated from nerve terminals using GST-α-adaptin and GST-endophilin I SH3 domain in a double pulldown. The phospho-Ser-262-phosphospecific antibody detected a strong band in amphIlow (Fig. 6, lanes 5 and 6) with a minor signal in the amphIup at longer exposures. This is consistent with the relative amount of protein in the upper band compared with the amount of 32P incorporation (Fig. 6, lanes 1 and 2 and lanes 3 and 4). There was no difference between the control and depolarized sample, suggesting that Ser-262 is constitutively phosphorylated and not responsive to depolarization.

The Ser-262-phosphospecific antibody also detected a strong signal under amphIlow that corresponded to amphIiso2 that had a low level of Coomassie Blue staining (Fig. 6A, lanes 1 and 2) and 32P incorporation (Fig. 6A, lanes 3 and 4). Likewise the Pro-Q Diamond phosphoprotein stain detected a corresponding protein band in the triple pulldown with low 32P incorporation (Fig. 1, lane 5 and 6 and lanes 7 and 8). The amphIiso2 tryptic digest did not contain the tryptic peptide at m/z 944.76 [M + 3H]3+ corresponding to a region only present in amphl as a result of alternative splicing, confirming the identity of isoform 2 (supplemental Fig. S17A). Next we analyzed the amphIiso2 tryptic digest using TiO2 chromatography, MALDI-TOF MS, and LC-MSMS to determine the phosphorylation sites in this isoform (supplemental Fig. S18). AmphI iso2 was found to be phosphorylated on sites equivalent to those of amphI (Table I and summarized in Fig. 7B). We also observed signals in the MALDI-TOF MS spectrum (supplemental Fig. S17B) that were identified as five phosphopeptides from auxilin (at m/z 2,157.0, 1,242.6, 1,398.7, 1,932.9, and 1,919.0) and one phosphopeptide from eps15R at m/z 2,030.0 Da (supplemental Fig. S10–S12 and supplemental Table S1). No evidence for Ser-293 and Thr-310 was observed in the MS spectra, although we cannot rule out that this may have resulted from the difficulty with the ionization of these phosphopeptides as noted for amphI. These results reveal seven phosphosites in amphI iso2, none of which were dynamically labeled with 32P and all of which were constitutively phosphorylated. Four phosphosites in a putative amphI isoform 3 with the same migration as synaptojanin 1 in the SDS gel were also identified (supplemental information, supplemental Figs. S20–S23, and Table I). However, we were...
unable to determine whether there was any $^{32}\text{P}$ associated with this isoform due to the abundance of co-migrating phosphorylated synaptojanin; therefore we assume that it is constitutively rather than dynamically phosphorylated. In ongoing work in parallel with the present report we have found that the majority of the $^{32}\text{P}$ associated with this region of the gel is related to synaptojanin I and have found none associated with amphI putative iso3 (data not shown). Therefore, all three isoforms of amphI are phosphorylated in nerve terminals, but only amphI dynamically incorporates $^{32}\text{P}$ radiation or exhibits depolarization-induced dephosphorylation (summarized in supplemental Fig. S24), indicating that among the three isoforms it is the major variant most likely to be involved in SVE.

**DISCUSSION**

A multifaceted strategy was undertaken to gain a complete analysis of the phosphorylation sites in amphI from adult rat brain synaptosomes. (a) The pulldown approach was used to isolate amphI based on the individual properties of each fusion protein to bind different regions of amphI and to negate any potential effect of phosphodependent interactions. (b) The sequential staining using Pro-Q Diamond and total Coomassie stain was influential in identifying constitutively phosphorylated proteins when compared with the autoradiograph that represented $^{32}\text{P}$-labeled proteins. (c) The use of TiO$_2$ chromatography provided a relatively specific enrichment of amphI phosphopeptides and improved the sensitivity to such an extent that extremely low abundance sites were detected and sequenced by mass spectrometry. (d) The combined approaches of off-line HPLC separation followed by 2D phosphopeptide mapping allowed the quantification of amphI phosphorylation sites based on the amount of in vivo $^{32}\text{P}$ labeling. Careful $^{32}\text{P}$ tracking proved pivotal to the success of this project because the two most predominant phosphopeptides were unable to bind reversed phase material and were difficult to detect by mass spectrometry due to the chemical composition of the short hydrophilic peptides. Hence they were only captured on graphite microcolumns. (e) We then determined which specific phosphosites are physiologically responsive to depolarization in nerve terminals. All phosphosites in amphI were found, and for the first time the phosphosites could be categorized as dynamic versus constitutive. Among the dynamic sites we revealed the subset that were stimulus-responsive. The overall comprehensive strategy has produced major new insights to understanding the functional relevance of complex specific multisite phosphorylation in a key endocytic protein.

We have identified 13 unique phosphorylation sites in amphI and have also mapped several of these phosphosites to two alternatively spliced isoforms. Previous studies relied on mutagenesis and in vitro phosphorylation and found a total of eight sites that were phosphorylated by four protein kinases. We confirmed that six of these in vitro sites were also phosphorylated in vivo, two were not observed, and seven new phosphorylation sites were identified. The lack of ability to detect two of the known in vitro sites suggests that their biological significance to the nerve terminal from adult rats is unlikely. This study has highlighted the importance of systematically identifying phosphosites from individual proteins in nerve terminals to obtain a complete list of in vivo phosphorylation sites. Prior large scale phosphoproteomics studies have identified only two of the sites reported here (Ser-496 and Ser-250) (27), but such shotgun approaches provide no relevant data as to their potential significance, relative abundance, or correlation to function and should be seen primarily as a partial catalogue.

Previous quantitative analysis showed that amphI phosphorylation was reduced by 45% after depolarization (8). Similarly this study found a decrease in $^{32}\text{P}$ levels by 30–50% after depolarization, but individual phosphorylation sites can now be assigned to account for the majority of the decreased phosphorylation. By individual analysis of amphI$_{up}$ and amphI$_{low}$, we revealed that the phosphosites Thr-310, Ser-293, Ser-272, Ser-276, Ser-285, and Ser-268 in the PRD (Fig. 7) were more prevalent in the upper band. These six sites were the most physiologically sensitive to depolarization and most likely are responsible for this reduced mobility in amphI$_{up}$. These six sites contained 66% of the control $^{32}\text{P}$ and were dephosphorylated by 26–55% after depolarization, whereas the remaining phosphorylation sites had little or no change after depolarization. We were able to further classify these amphI phosphosites based on their relative $^{32}\text{P}$ incorporation and their relative abundance and found an inverse relationship. Qualitatively we observed that the five phosphosites with high $^{32}\text{P}$ levels had a low relative abundance (MS signal intensity) after TiO$_2$ enrichment but were highly depolarization-sensitive (supplemental Table S3). This ratio corresponded to the hyperphosphorylated upper band containing low protein levels but high $^{32}\text{P}$ incorporation. This suggests that these depolarization-sensitive phosphosites may regulate transient interactions during the initial steps of Ca$^{2+}$-dependent SVE in which only a small pool of amphI is involved. We propose that amphI$_{up}$ is the major dephosphin involved in stimulated SVE, whereas amphI$_{low}$ is minor. The role of amphI$_{low}$ in SVE may better relate to signaling events other than calcineurin-mediated dephosphorylation. The seven remaining sites with low $^{32}\text{P}$ labeling are constitutive phosphosites that may have functions other than in rapid stimulus-dependent SVE.

Alternatively spliced amphI isoforms were shown to be phosphorylated in this study. Neither were $^{32}\text{P}$-labeled, indicating they are not involved in rapid stimulus-dependent SVE but may have some other role in the nerve terminal or in endocytosis poststimulus. AmphI iso2 was shown to be phosphorylated in nerve terminals using the Pro-Q phosphoprotein stain and the phosphospecific antibody to Ser-262. AmphI iso2 was shown to contain all the low turnover phosphosites as observed in amphI including Ser-262, Ser-455, Ser-473,
and Ser-498. These were the equivalent sites in amphl that had a low level of $^{32}$P labeling and were not dephosphorylated after depolarization. The identification of phosphorylation sites in the amphl putative iso3 in rat brain nerve terminals introduces more complexity for amphl. Previously, the amphl larger isoform was thought to be restricted to the retina and to consist of three large insertions after residue 394 (11). We have shown that four phosphosites are present in the C-terminal region of amphl putative iso3, and the site equivalent to Ser-626 (Ser-1061) is present at a much higher intensity than in the smaller isoforms (amphl and amphl iso2). Interestingly, the phosphopeptides from the PRD of amphl putative iso3, including that containing Ser-262, were below the level of MS detection. Because the phosphopeptides were easily detected in the other isoforms this indicates a general absence of PRD phosphorylation in amphl putative iso3.

The overall data suggest that synaptosomal amphl phosphosites can be grouped into two sets. The first are dynamic: those that are highly labeled with $^{32}$P during the 1-h labeling period and include the subset that respond to depolarization by a large dephosphorylation. The second are the constitutive sites. The comparative study of amphl iso2 and putative iso3 provides a basis for better understanding which phosphosites may be most related to SVE. Both these isoforms were essentially not dynamically phosphorylated, yet multisite phosphorylation was detected. These sites were also labeled in amphl at very low levels that might otherwise have been considered "noise." However, the conservation of phosphorylation of these phosphosites among all three isoforms and the markedly different ratio of phosphorylation within them strongly suggests that these sites are specific and not "background noise." Their constitutive phosphorylation would have occurred in the rat brain prior to synaptosomal isolation, and their phosphorylation must be quite stable for long periods of time. Hence constitutive phosphosites such as these may be particularly interesting targets for exploration of other possible roles of amphl in long term neuronal signaling, such as synaptic plasticity.

Amphl was reported previously to be phosphorylated using four protein kinases in vitro. In this study, we identified nine phosphosites that are candidates for phosphorylation by a proline-directed protein kinase and four that are candidates for a non-proline-directed protein kinase. Analysis of the amino acids surrounding each proline-directed phosphosite indicates that at least one or more proline-directed kinases would be involved. All the stimulus-sensitive phosphosites were located in the PRD region and are predicted substrates for proline-directed kinases (supplemental Table S3).

Hierarchical phosphorylation of the amphl-PRD appears to depend on Ser-262 as it was the most abundant phosphorylation site identified and was present in all the phosphopeptides in this region. The second major site is either Ser-268 or Ser-276; after these two sites it is Ser-272 and Ser-285. Phosphosite Ser-262 may direct phosphorylation onto the stimulus-sensitive sites to rephosphorylate amphl after SVE. Ser-262 was constitutively phosphorylated in vivo with only minor changes in $^{32}$P labeling. The sequence surrounding Ser-262, Ser-496, and Ser-514 contained several acidic amino acids, whereas cdk5 usually phosphorylates (S/T)P motifs in a basophilic context. Other proline-directed kinases do not require basic amino acids, suggesting that a second distinct protein kinase controls the phosphorylation of these sites. The sequence around the four phosphorylation sites Ser-250, Ser-252, Ser-539, and Ser-626 suggests a role for a non-proline-directed protein kinase in amphl phosphorylation in nerve terminals (supplemental Table S3). The four phosphosites have several acidic amino acids in the surrounding sequence. None of the phosphosites detected in this study that exhibited strong stimulus-dependent dephosphorylation were located in a local acidic context.

A number of phosphosites were identified in other proteins that bound to the GST fusion proteins in the triple pulldown experiment and co-migrated with amphl or amphl iso2 in SDS-PAGE. Three of these proteins (eps15R, SGIP, and AAK1) have functional links to endocytosis, and two have links to the cytoskeleton. Their ability to incorporate $^{32}$P in 1 h requires the protein to be located within the intact nerve terminal. Therefore, it was not only necessary to establish the identity of co-migrating phosphoproteins in the amphl bands to properly assign mass peaks and $^{32}$P incorporation, but it was also an opportunity to expand the phosphoproteome of proteins potentially involved in SVE. The clathrin coat-associated protein kinase AAK1 was found to be phosphorylated on three sites (Thr-608, Thr-621, and Thr-625) with the two latter sites containing a low level of $^{32}$P incorporation. Auxilin is involved in the uncoating of clathrin-coated vesicles and was found to contain five in vivo phosphorylation sites in the nerve terminal. SGIP is a protein containing an extensive PRD region and an adaptor complex medium subunit domain, which interacts with endophilin I, eps15, AP-2, and phospholipids, suggesting an essential role in vesicle formation during endocytosis (36, 37). The large scale phosphoproteomics studies have recently identified SGIP phosphosites (Ser-265 and Thr-409), AAK1 phosphosites (Thr-607, Thr-621, and Ser-625), an HCN1 phosphosite (Thr-39), and eps15R phosphosites (Ser-229 and Ser-355) (27). For eps15R we confirm these two sites and add a third at Ser-255. Although eps15 is a known dephosphin, it is not known whether the related gene, eps15R, is also a dephosphin. We identified seven in vivo phosphorylation sites for SGIP that contained a total of 13% of the control $^{32}$P labeling. At least two SGIP phosphosites, Ser-149 and Thr-409, were dephosphorylated by 50%, and Ser-169 was dephosphorylated by 77% after nerve terminal depolarization. This is the first report for SGIP phosphorylation in presynaptic nerve terminals, and the $^{32}$P labeling and stimulus-sensitive response reveals a potential new dephosphin and suggests a potential functional link to SVE. The $\alpha/\beta$-adducins are 120-kDa proteins with a myristoylated
alanine-rich protein kinase C substrate (MARCKS)-related domain and that recruit spectrin to actin filaments (38). α-Adducin is highly enriched in brain regions with a high density of synapses (39), and β-adducin knock-out mice have defective synaptic plasticity (40). Increased phosphoadducin has been reported in a mouse model of amyotrophic lateral sclerosis (41). We identified three α-adducin phosphorylation sites (Ser-12, Thr-358, and Thr-610) and two β-adducin phosphosites (Ser-594 and Ser-602). Overall the additional 24 in vivo synapticosomal phosphosites identified in this study provide in vivo confirmation that they are phosphorylated in rat brain nerve terminals.

The majority of the amphI phosphosites clustered around the main protein-protein-interacting domains. Seven of the 13 sites containing all the major stimulus-sensitive sites were identified within the PRD (amphI-(260–311)), and four sites were located near the SH3 domain (Fig. 7A). Notably there were no phosphorylation sites detected in the N-terminal BAR domain that is responsible for lipid tubulation, and previous in vitro evidence suggests that this heterodimerization is indeed not dependent on phosphorylation (6). This suggests that the stimulus-sensitive sites Thr-310, Ser-293, Ser-285 Ser-272, Ser-268, and Ser-276 may be regulating protein-protein interactions within the PRD region and/or the CLAP domain. Interestingly Ser-276 and Ser-285 were recently shown to inhibit lipid binding in vitro (23). However, the results of our study suggest that a re-evaluation of these findings is required to include more detailed analysis of the other adjacent phosphosites. The two phosphosites that contained the highest ³²P incorporation and were the most stimulus-sensitive were Thr-310 and Ser-293 that flank the endophilin I binding site. We propose that Ser-293 and Thr-310 may coordinately regulate endophilin I binding to amphI in SVE. In vitro phosphorylation of amphI-PRD by Dryk1A was recently shown to reduce endophilin binding (17). The other phosphosites in this region, including Ser-285, Ser-276, and Ser-272, were also stimulus-sensitive and may contribute to regulating amphI protein interactions or other functions of amphI. Species conservation of specific phosphosites is often thought to strengthen the potential physiological significance of that site. The amino acid sequence surrounding several of the amphI phosphorylation sites identified in this study provide in vivo confirmation that they are phosphorylated in rat brain nerve terminals.

The sequences of 11 of the phosphosites were conserved in mice, rats, and not in humans or monkeys (supplemental Fig. S25). The sequences of 11 of the phosphosites were conserved in all mammals, but two of the constitutive low abundance phosphosites, Ser-496 and Ser-514, were only present in rodents and not in humans or monkeys (supplemental Fig. S26). The central regions of Drosophila and Caenorhabditis elegans had little amino acid conservation with mammals compared with homology in their BAR and SH3 domains. These results show the potential for each of the phosphorylation site in these species, but their actual phosphorylation status cannot yet be confirmed.

The identification of four phosphorylation sites surrounding the SH3 domain may have implications for dynamin and synaptotagmin binding. The GST-amphI SH3 domain binding to dynamin I has been shown to be insensitive to dynamin I phosphorylation in nerve terminals (21). This suggests that amphI phosphorylation may regulate this interaction. Interestingly Ser-626 is located directly in the RT loop of the amphI SH3 domain that is responsible for dynamin I binding (42). Mutating acidic residues in this region abolished dynamin I binding to the corresponding sequence in amphiphysin II SH3 domain (42) indicating that this region may indeed regulate dynamin I binding. Ser-626 was a low abundance site in vivo in amphI, but the relative abundance of the phosphopeptide containing Ser-626 was much higher in amphI putative iso3 compared with the other isoforms, suggesting that it is only found in a small pool of amphI in vivo and is not a rapidly turning over site.

The identification of the major stimulus-sensitive and constitutive phosphosites of amphI can now be used to better understand its function in nerve terminals. AmphI phosphorylation is concentrated around its two protein-protein-interacting domains. The stimulus-sensitive phosphosites cluster primarily within the PRD. The focus of future work should be to determine how these phosphosites individually and collectively regulate amphI interaction with its binding partners during SVE.
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