Dynamin I (dynI) is phosphorylated in synaptosomes at Ser\textsuperscript{774} and Ser\textsuperscript{778} by cyclin-dependent kinase 5 to regulate recruitment of syndapin I for synaptic vesicle endocytosis, and in PC12 cells on Ser\textsuperscript{857}. Hierarchical phosphorylation of Ser\textsuperscript{774} precedes phosphorylation of Ser\textsuperscript{778}. In contrast, Thr\textsuperscript{780} phosphorylation by cdk5 has been reported as the sole site (Tomizawa, K., Sunada, S., Lu, Y. F., Oda, Y., Kinuta, M., Ohshima, T., Saito, T., Wei, F. Y., Matsushita, M., Li, S. T., Tsutsui, K., Hisanaga, S. I., Mikoshiba, K., Takei, K., and Matsui, H. (2003) Cell Biol. 163, 813–824). To resolve the discrepancy and to better understand the biological roles of dynI phosphorylation, we undertook a systematic identification of all phosphorylation sites in rat brain nerve terminal dynI. Using phosphoamino acid analysis, exclusively phospho-serine residues were found. Thr\textsuperscript{780} phosphorylation was not detectable. Mutation of Ser\textsuperscript{774}, Ser\textsuperscript{778}, and Thr\textsuperscript{780} confirmed that Thr\textsuperscript{780} phosphorylation is restricted to \textit{in vitro} conditions. Mass spectrometry of 32P-labeled phosphopeptides separated by two-dimensional mapping revealed seven \textit{in vivo} phosphorylation sites: Ser\textsuperscript{774}, Ser\textsuperscript{778}, Ser\textsuperscript{822}, Ser\textsuperscript{851}, Ser\textsuperscript{857}, Ser\textsuperscript{852}, and Ser\textsuperscript{857}. Quantification of 32P radiation in each phosphopeptide showed that Ser\textsuperscript{774} and Ser\textsuperscript{778} were the major sites (up to 69% of the total), followed by Ser\textsuperscript{851} and Ser\textsuperscript{857} (12%), and Ser\textsuperscript{853} (2%). Phosphorylation of Ser\textsuperscript{851} and Ser\textsuperscript{857} was restricted to the long tail splice variant dynIxa and was not hierarchical. Co-purified, 32P-labeled dynIII was phosphorylated at Ser\textsuperscript{759}, Ser\textsuperscript{763}, and Ser\textsuperscript{853}. Ser\textsuperscript{853} is homologous to Ser\textsuperscript{851} in dynIxa. The results identify all major and several minor phosphorylation sites in dynI and provide the first measure of their relative abundance and relative responses to depolarization. The multiple phospho-sites suggest subtle regulation of synaptic vesicle endocytosis by new protein kinases and new protein-protein interactions. The homologous dynI and dynIII phosphorylation indicates a high mechanistic similarity. The results suggest a unique role for the long splice variants of dynI and dynIII in nerve terminals.

Synaptic vesicle endocytosis (SVE)\textsuperscript{2} is triggered by a coordinated dephosphorylation of a group of at least eight proteins called the dephosphins (1). The dephosphins are constitutively phosphorylated in nerve terminals, and their collective dephosphorylation after SVE is necessary for maintaining synaptic vesicle recycling and thus synaptic transmission. One such dephosphin is dynamin I (dynI), a large GTPase enzyme that is crucial for the fission stage of SVE (2). During SVE, dynI is dephosphorylated by the calcium-dependent phosphatase calcineurin (3) and is subsequently rephosphorylated by cyclin-dependent kinase 5 (cdk5) on Ser\textsuperscript{774} and Ser\textsuperscript{778} (4, 5). These phosphorylation sites, located in the proline-rich domain (PRD), are thought to regulate the interaction with the src-3 homology (SH3) domain-containing proteins involved in SVE. A long list of SH3 domain-containing proteins has been shown to bind the dynI PRD \textit{in vitro}. Recently, we have identified syndapin I (sdpnI) as the phosphorylation-regulated dynI partner \textit{in vivo} and that its interaction with dynI is crucial to SVE (6).

Apart from phosphorylation of Ser\textsuperscript{774} and Ser\textsuperscript{778} by cdk5, there have been a number of reports on other dynI phosphorylation sites and their potential protein kinases, both \textit{in vivo} and \textit{in vitro}. DynI is phosphorylated on Ser\textsuperscript{857} by minibrain kinase/ Dyrk1A \textit{in vitro}, and the phosphorylation regulates binding of dynI to amphiphysin I (ampl) and Grb2 (7). This phosphorylation was shown to occur in PC12 cells and was responsive to depolarizing stimuli, strongly suggesting a physiological relevance. It has also been reported that dynI is phosphorylated at Thr\textsuperscript{780} by cdk5, which apparently regulates its binding to ampl (8). This is in conflict with our previous \textit{in vivo} phosphorylation site analysis of dynI, which did not detect phosphorylation at this site (5). Other studies have suggested that there may be other kinases capable of phosphorylating dynI \textit{in vitro}, but the \textit{in vivo} relevance of these events has not been established (9–12). DynI and dynll are also substrates for the tyrosine kinase c-Src in non-neuronal cells on Tyr\textsuperscript{231} and Tyr\textsuperscript{597} (13, 14). The two sites are highly conserved between all three dynamin genes. However, the tyrosine phosphorylation of dynI has only been reported in transfected non-neuronal cells where it is not known to be normally expressed (13), whereas that of dynll occurs under endogenous conditions (13, 14). This suggests that \textit{in vivo} phosphorylation of these tyrosine phospho-sites in dynI remains an open question.

In this study, we sought to determine whether there were \textit{other in vivo} dynI phosphorylation sites, in addition to Ser\textsuperscript{774},...
Ser\textsuperscript{778}, and Ser\textsuperscript{857}, that may prove to be functionally important for SVE. We established a method that ensures maximum purification of \(^{32}\text{P}\)-labeled dynl so that none of the relevant phosphorylation sites were missed. We show that dynl was exclusively phosphorylated on serine residues by \(^{32}\text{P}\)-phosphoamino acid analysis. We identified two new dynl phosphorylation sites at Ser\textsuperscript{347} and Ser\textsuperscript{512} and two new stimulation-dependent dynl phosphorylation sites at Ser\textsuperscript{822} and Ser\textsuperscript{851}. We also showed that phosphorylation of Thr\textsuperscript{780} by cdk5 was restricted to in vitro conditions, did not occur in vivo (within detection limits), and did not regulate binding of amphl.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs and Protein Expression**—The dynl PRD (rat, amino acids 746–864) was amplified from the green fluorescent protein-tagged dynamin and subcloned into pGEX4T-1 as described previously (6). Dynl point mutants were generated using the QuikChange site-directed mutagenesis kit (Stratagene) and confirmed by DNA sequencing. GST-amphl SH3 domain was from Pietro de Camilli (Yale, New Haven, CT). GST-sdpln SH3 domain was from Markus Plomann (University of Cologne, Germany). GST-endophilin 1 (endol) SH3 domain was from Peter McPherson (McCill, Canada). GST-ckd5 and GST-p25 were from Jerry Wang (Hong Kong University, Hong Kong) and Li-Huei Tsai (Massachusetts Institute of Technology, Cambridge, MA), respectively. All GST fusion proteins were expressed in *Escherichia coli* and purified using glutathione-Sepharose beads (Amersham Biosciences) according to the manufacturer’s instructions.

**Synaptosomal Dynl Purification**—Crude (P2) synaptosomes were prepared from rat brain and labeled with \(^{32}\text{P}\) as described previously (10). Briefly, synaptosomes were incubated with 0.75 mCi/ml \(\gamma^{32}\text{P}\)-Pi, for 1 h at 37 °C. The synaptosomes were briefly depolarized with 41 mM KCl and immediately lysed as described previously (4). Dynl was purified from synaptosome lysates using the GST-amphl SH3 domain bound to glutathione-Sepharose beads as described previously (4). GST-endol SH3 domain and GST-sdpln SH3 domain were also used as bait to pull down dynl from synaptosome lysates, as described previously (6). Beads were washed extensively, eluted in 2× SDS sample buffer, and resolved on 7.5–15% gradient SDS gels.

**Tryptic Digestion and Phosphopeptide Enrichment**—Dynl gel bands, each containing —2 \(\mu\)g of purified protein, were excised and dried from colloidal Coomassie Blue-stained SDS gels. Four untreated and four KCl-treated \(^{32}\text{P}\)-labeled dynl bands were used in each two-dimensional map. Twenty unlabeled dynl bands were TiO\textsubscript{2}-enriched (see below) and added to the untreated sample for two-dimensional mapping. Two dynl bands were used in the nano-LC-MS/MS experiments (see below). The bands were destained in three 1-ml washes of 25 mM ammonium bicarbonate in a 50% acetonitrile solution with 1 h of vortexing between washes. The gel pieces were digested in 25 mM ammonium bicarbonate aqueous solution containing 12.5 ng/\(\mu\)l trypsin at 37 °C overnight. The digest solution was made up to 50% acetonitrile, and the tryptic peptides were extracted after 15 min of vortexing. A second extraction was obtained with 50% acetonitrile solution after 15 min of vortexing. A final extraction was obtained with 80% acetonitrile, 5% formic acid solution after 15 min of vortexing. The combined extract was dried down to 2.5 \(\mu\)l.

Phosphopeptides from a tryptic digest of non-radioactive dynl were enriched using TiO\textsubscript{2} as described earlier (15). Briefly, the tryptic digest was concentrated into 5 \(\mu\)l and added to 25 \(\mu\)l of loading solution (300 mg/\(\mu\)l dihydroxybenzoic acid in 0.1% trifluoroacetic acid 80% acetonitrile or 5% trifluoroacetic acid in 80% acetonitrile in the absence of dihydroxybenzoic acid). The sample was loaded onto a GELoader tip (Eppendorf), converted to a microcolumn, packed with TiO\textsubscript{2}, and washed twice with loading solution and then twice with a wash solution of 0.1% trifluoroacetic acid/80% acetonitrile, without the presence of dihydroxybenzoic acid. The sample was eluted in 20% ammonium hydroxide/20% acetonitrile solution and immediately dried.

**Phosphoamino Acids Analysis and Two-dimensional Phosphopeptide Mapping**—The \(^{32}\text{P}\)-labeled dynl tryptic digest was analyzed by phosphoamino acid analysis as described previously (4, 10). Two-dimensional phosphopeptide mapping was done on 20 × 20 cm cellulose plates (Merck) by electrophoresis at pH 4.7 (16) followed by ascending chromatography in 35% 1-butanol, 20% pyridine, 7.5% acetic acid, 2.5% acetonitrile, and 35% water. The radioactive spots were detected by quantitative phosphorimaging (Storm 860, Amersham Biosciences), scraped from the plate, and extracted from the cellulose as described (16), except that the extraction was done in 5% formic acid solution. The phosphopeptides were concentrated using C18 or graphite microcolumns and detected by matrix-assisted laser desorption ionization-mass spectrometry (MALDI-MS, Voyager DE-PRO, Applied Biosystems) as described previously (5).

A theoretical two-dimensional phosphopeptide map was generated firstly by determining the theoretical electrophoretic mobility by predicting the charge at pH 4.7 and dividing by the mass of the phosphopeptide as described by Meisenhelder *et al.* (16). The mobility in the second dimension, which is based on relative hydrophobicity, was estimated from the reversed-phase HPLC index (17) as calculated using GPMAW version 7.01 (Lighthouse Data, Denmark). The theoretical HPLC index was decreased by two units for each additional phosphate group to account for the observed decreased mobility of multiply phosphorylated peptides (Fig. 2, B and C).

**Nano-LC-MS**—An 8-\(\mu\)l aliquot (estimated at 0.5–1.5 pmol) of TiO\textsubscript{2}-enriched dynl phosphopeptides was loaded onto the nano-LHLC system (LC Packings Ultimate HPLC system, Dionex, Netherlands) with a 75-\(\mu\)m inside diameter pre-column of C18 reversed-phase material (ReproSil-Pur 120 C18-AQ, 3-\(\mu\)m beads, Dr. Maisch, Germany) in 5 min. It was then eluted through a 50-\(\mu\)m inside diameter C18 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 increased to 10% phase B (90% acetonitrile, 0.1% formic acid, and 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-\(\mu\)m inside diameter distal coated SilicaTip (New Objective) into a QSTAR XL quadrupole-TOF (QqTOF) MS (Applied Biosystems) or a QTOF Ultima MS (Micromass/Waters) using 1900 V on the tip. For
the detection of phosphopeptides of a known molecular mass
the phosphopeptide in its most abundant charge state with a wide
$\text{m/z}$ setting (2–3 units) using consecutive 2-s scans. Information-dependent data acquisition was done by using a 1-s survey scan from which the three most abundant doubly, triply, or quadruply charged peptides were selected for product ion scans (2 s). The data for the phospho-dynI343–364 was from experiments using stable isotope labeling, the full details of which will be published elsewhere. As a consequence, the N terminus of a phospho-dynI343–364 was labeled with iTRAQ reagent (Fig. 4C). In this instance, the TiO$_2$-enriched digest was first treated with iTRAQ 116 reagent (Applied Biosystems) according to the manufacturer’s instructions before nano-LC-MS/MS analysis. All phosphopeptides reported in this study were detected in at least three independent experiments.

In Vitro Phosphorylation—GST-dynl PRD (1 µg) immobilized on glutathione-Sepharose beads was phosphorylated by recombinant cdk5/p25 in a total volume of 40 µl containing 30 mM Tris-HCl, pH 7.4, 1 mM EGTA, 1 mM MgCl$_2$, 0.05% Tween 20 and 40 µM [γ-32P]ATP (6.9 × 10$^{6}$ cpm/nmol) at 37 °C. Phosphorylation proceeded for 10 min, and reactions were terminated by addition of 3 × SDS sample buffer and boiling. Samples were analyzed by SDS-PAGE, and phosphoproteins were detected by autoradiography. A single band each of in vitro phosphorylated GST-dynl PRD wild-type and double mutant Ser to Ala were digested as above and enriched for phosphopeptides using Fe$^{3+}$-immobilized metal affinity chromatography as described previously (5). The phospho-box phosphopeptides were sequenced by nano-LC-MS/MS by fixing the precursor ion selection at a specific $\text{m/z}$ as described above.

Pulldown Experiments—A rat brain or synaptosomal extract was prepared as described previously (6). Various GST-dynl PRD or GST-amphl SH3 recombinant proteins were then incubated with the same amounts of tissue lysate at 4 °C for 1 h. All pulldown experiments were done in the presence of 150 mM NaCl unless stated otherwise. Beads were washed extensively, eluted in 2× SDS sample buffer and analyzed by SDS-PAGE.

Antibodies and Western Blots—The anti-amphiphysin I monoclonal antibody was from Pietro de Camilli. The anti-dynl antibodies and phosphospecific antibodies to phospho-Ser$^{774}$ and phospho-Ser$^{778}$ in dynl were reported previously (4). Immunoprecipitations from rat brain synaptosomes were performed as described previously (10). Protein samples were separated by SDS-PAGE on 10% or 12% acrylamide gels and transferred to nitrocellulose membrane. Western blots were analyzed by enhanced chemiluminescence method using the SuperSignal West Pico Chemiluminescent Substrate (Pierce).

RESULTS

Quantitative Extraction of Dynl by GST-amphl SH3 Domain—To catalogue all in vivo phosphorylation sites in dynl from rat brain synaptosomes, we used a method capable of extracting and purifying all dynl from synaptosome lysates, as judged by SDS-PAGE analysis and autoradiography. We have previously reported that the SH3 domain of amphl quantitatively extracts synaptosomal dynl independently of its phosphorylation status (6). However, another study has proposed that the interaction of GST-amphl SH3 with dynl is phospho-dependent (8). Here, we compared the efficiency of GST fusion proteins of amphl, endol, and sdpnl SH3 domains to quantitatively extract dynl by three sequential pulldown experiments. Initial pulldown experiments with GST-amphl SH3 and GST-sdpnl SH3 recovered a large amount of endogenous dynl protein (Fig. 1A) and 32P-labeled phospho-dynl (Fig. 1B), whereas GST-endol SH3 bound less dynl. A second pulldown of the same extracts with GST-amphl SH3 was performed to capture residual dynl missed in the first pulldown assay. There was <2% residual dynl protein and phospho-dynl following amphl or sdpnl in the first pulldown experiment (Fig. 1, A and B). In contrast, there was significant dynl and phospho-dynl captured following endol SH3 in the first pulldown experiment (Fig. 1, A and B, middle panels). This is in agreement with a previous report that endol SH3 is partly sensitive to dynl phosphorylation in vitro (6) and shows that amphl SH3 can indeed capture this small pool. A third sequential pulldown assay with sdpnl SH3 or endol SH3 domains recovered no residual dynl protein or phospho-dynl after two previous extractions with amphl SH3, endol SH3, or sdpnl SH3 (Fig. 1, A and B). Therefore the SH3 domain of amphl and sdpnl quantitatively extracts synaptosomal dynl from synaptosomes.

We independently performed the similar GST-amphl SH3 triple pulldown experiments using synaptosome extracts and blotted with anti-dynl antibodies. Two rounds of GST-amphl SH3 pulldown assay was enough to quantitatively extract all synaptosomal dynl, because no trace of unbound dynl was found in the second or third pulldown experiment (Fig. 1C). We thus used this system of double GST-amphl SH3 pulldown experiments in all subsequent experiments to purify all synaptosomal dynl, regardless of its in vivo phosphorylation status.

Dynl Is Phosphorylated Exclusively on Serine Residues—Dynl phosphorylation on Thr$^{780}$ has been controversial (18). It was not detected in a previous in vivo study (5) but was claimed to be found after in vitro phosphorylation with cdk5 (8). A simple way to address whether a protein is phosphorylated on serine, threonine, or tyrosine residues is by phosphoamino acid analysis after 32P labeling. There may be some phosphorylation sites that are not labeled with 32P, however, in dynl these sites are unlikely to be relevant to SVE, because they do not turnover 32P in 1 h of labeling. The first published report of phosphoamino acid analysis on synaptosomal dynl showed that only serine was phosphorylated (19). The source of dynl in that experiment was the bands cut from an SDS gel of a synaptosome lysate, which may be contaminated by another underlying protein with similar size. The same result was reported when dynl was purified by a single pulldown experiment with the GST-amphl SH3. Phosphorylation at threonine or tyrosine was absent (4). In this study, we performed phosphoamino acid analysis using dynl purified by GST-amphl-SH3 pulldown experiments from 32P-labeled synaptosomes and again found only serine was phosphorylated (Fig. 2A). We conclude that synaptosomal dynl is exclusively phosphorylated on serine residues with no detectable 32P labeling on threonine or tyrosine residues.

Separation and Detection of in Vivo 32P-Labeled Dynl Phosphopeptides—A tryptic digest of dynl purified from 32P-labeled synaptosomes was subjected to two-dimensional phos-
Similarly, a tryptic digest of dynI from synaptosomes depolarized with 41 mM KCl for 10 s was also analyzed in parallel (Fig. 2C). The \(^{32}\)P-labeled phosphopeptides were detected by autoradiography. We observed 15 clearly distinct spots on the two-dimensional map (A–O). The majority of the radiation was in spots A–D (Fig. 2B). These spots have previously been shown to contain overlapping tryptic phosphopeptides (including tryptic cleavages and missed cleavages) from the dynI phospho-box (772RRSPTSSPTPQRR784) (6).

Spots C and D were previously shown to contain almost exclusively phospho-Ser774, and spots A and B were shown to contain doubly phosphorylated peptides that are equal parts phospho-Ser774 and Ser778 (5).

**MS/MS Identification of Phosphorylation Sites in DynI Phosphopeptides**

To account for all the \(^{32}\)P that is distributed among the various dynI phosphorylation sites, the phosphopeptides contained within each spot on the two-dimen-
sional map were extracted from the cellulose and analyzed by MALDI-MS and tandem MS. Spots A–D contained the dynl phospho-box peptides (772RRSPTSSPTPQR784, data not shown) that we identified previously (6). Spots E–H had low relative radiation levels and were analyzed by MALDI-TOF MS, but no phosphopeptides were detectable (Fig. 2B). Similarly, no phosphopeptides were detected in spots J, M, and N, which had even lower levels of total radiation (~2%, 1%, and 1% respectively, Fig. 2B).

Mono- and di-phosphopeptides matching the tail of the long splice variant dynlxa, 847SGQASPRPSPRPPFDSL864, were found in spots K and L, respectively. The mono-phosphorylated peptide in spot K was determined to be dynlxa847–864 where either Ser851 or Ser857 is phosphorylated (data not shown). Neither phosphorylation site was grossly dominant in this mixture of two phosphopeptides of equal molecular mass. The di-phosphorylated peptide in spot L was determined to be dynlxa847–864 where both Ser851 and Ser857 are phosphorylated (Fig. 3A). Phospho-Ser851 was detected between y13 and y14 and also as a dehydroalanine between b2 and b3. Phospho-Ser857 was revealed as the only possible position for the second phosphorylation site in the sequence 855PPES857 between the b8/y10 and b11/y7 ions. In contrast to the two sites in the dynl phospho-box (5, 6), there appears to be no hierarchy between phospho-Ser851 and -Ser857, suggesting independent regulation and potentially independent protein kinases.

A weak signal, that was specific to phosphatase treatment, was detected by MALDI-TOF MS in spot O. Nano-LC-MS/MS was used to sequence this peptide by selecting the quadruply charged precursor at m/z 606.3. The sequence was determined to be dynIIIxxb 849RPPPpSPRPTIIRPLESSLD869 where Ser853 is phosphorylated (Fig. 3B). DynIII was previously found to be co-purified with dyn from P2 synaposomes (5). Small y-type ions and both small and large b-type ions were detected, with little information on the middle part of the sequence. However, the position of the phosphorylation site was unequivocally determined by crucial ions such as the b2 and b3 ions, which limit the phosphorylation site to the sequence 851PS853, of which only Ser853 can be phosphorylated. In addition to the homologous phosphorylation of the dynl and dynlII phospho-box (5), we have now revealed that dynIIIxxb has a third in vivo phosphorylation site in a sequence location that is homologous to the dynlxxa sequence containing phospho-Ser851. As for dynl, this site is present only in the long-tailed splice variant of dynIII.

Spot L was analyzed by MALDI-TOF MS, and a phosphopeptide was observed, as confirmed by dephosphorylation with Antarctic phosphatase (data not shown). Nano-LC-MS/MS was required to sequence this phosphopeptide. The sequence was dynl 757GPAPGPPPAGSLGGAPPVPSPRGASDPFGPPQVPSRNR838 where Ser822 is phosphorylated (Fig. 4A). Small b-type ions and y-type ions throughout the sequence were used to determine the phosphorylation site and the identity of the phosphopeptide. Y-type ions up to y16 and b-type ions up to b16 (and then a weak signal at b25) were not phosphorylated. Because y13 and higher ions were phosphorylated, the phosphorylation site can be unequivocally localized to Ser822. This is the fifth proline-directed serine (i.e. SP sequence) in the dynlxxa PRD that was found to be phosphorylated in vivo.

Finally, we used another independent approach to maximize detection of any relatively minor phosphorylation sites. Extraction of phosphopeptides from cellulose plates after two-dimensional peptide mapping may not be completely efficient due to adsorption to surfaces. Therefore to maximize the number of phosphopeptides detected, without relying on 32P labeling, we used TiO2 to enrich for all phosphopeptides from a dynl tryptic digest. The enriched phosphopeptide was extracted from the cellulose plate and analyzed by electro-spray ionization-QqTOF MS. The triply charged precursor at m/z 695.6 was selected and produced a sequence of b- and y-type ions that describe phospho-dynlxa847–864 where Ser851 and Ser857 are phosphorylated. Ions showing neutral loss of phosphoric acid (~98 Da) are shown. B, tandem mass spectrum of the phosphopeptide detected in spot O (such as in Fig. 2B). A dynl tryptic digest enriched for phosphopeptides using TiO2 was analyzed by nano-LC-MS/MS by selecting the quadruply charged precursor at m/z 606.3. The fragment ions match the sequence of dynIIIxxb849–869 where Ser853 is phosphorylated. The m/z range from 850 to 1150 has been multiplied by a factor of 18 to improve clarity.

**FIGURE 3.** Phosphorylation sites in dynl and dynlII phosphopeptides detected by two-dimensional tryptic mapping. A, tandem mass spectrum of the phosphopeptide detected in spot I (such as in Fig. 2B). The phosphopeptide was extracted from the cellulose plate and analyzed by electro-spray ionization-QqTOF MS. The triply charged precursor at m/z 695.6 was selected and produced a sequence of b- and y-type ions that describe phospho-dynlxa847–864 where Ser851 and Ser857 are phosphorylated. B, tandem mass spectrum of the phosphopeptide detected in spot O (such as in Fig. 2B). A dynl tryptic digest enriched for phosphopeptides using TiO2 was analyzed by nano-LC-MS/MS by selecting the quadruply charged precursor at m/z 606.3. The fragment ions match the sequence of dynIIIxxb849–869 where Ser853 is phosphorylated. The m/z range from 850 to 1150 has been multiplied by a factor of 18 to improve clarity.
detected as the dehydroalanine residue between y11 and y10. In the same experiment, we also sequenced phospho-dynI510–522 and found that Ser512 was phosphorylated in this slightly larger phosphopeptide (data not shown). Automatic selection of the precursor at m/z 750.0 produced another unique fragmentation spectrum (Fig. 4C). This phosphopeptide matched dynI343RIEGSGDQIDTYELSGGARINR364 where Ser347 is phosphorylated. Non-phosphorylated y-type ions ruled out the possibility of phosphorylation near the C-terminal half of this phosphopeptide. The phosphorylation site was deduced to be at Ser347, before the residues producing phosphorylated (b5 to b11) fragments and after the residues producing non-phosphorylated fragments (b3). This is the first time that in vivo dynI sites have been detected outside of the PRD. It is possible that these sites were 32P-labeled but were not detected by MALDI-TOF MS.

It can be predicted where the peptides encompassing Ser512 and Ser347 would appear in the two-dimensional map in Fig. 2B, if they were 32P-labeled. We plotted the theoretical HPLC index versus the estimated electrophoretic mobility for each of the detected dynI and dynIII peptides (Fig. 5A). Only peptides with up to one missed trypsin cleavage are shown. The theoretical electrophoretic mobility correlated with the experimentally observed mobility of the sequenced phosphopeptides (Figs. 2B, 2C, and 5A). In the second dimension, the HPLC index was a good predictor of mobility, except for the large peptide in spot L (Figs. 2B, 2C, and 5A). This may reflect an overemphasis on peptide molecular mass when using the HPLC index. DynI511–522 has an HPLC index greater than dynI774–783 (spots A–D) and less than dynI847–864 (spots K and I, Fig. 5A). The phosphopeptides dynI511–522, dynI511–523, and dynI510–522 are predicted to migrate in the second dimension near the unidentified spots at E–H (Fig. 2B). Therefore, phosphorylated dynI510–523 theoretically accounts for at least two of the spots in the region of E–H. There are two unassigned spots (M and N, Fig. 2B) above dynI847–864 (spots K and I). Spot N (Fig. 2B) might be accounted for by dynI839–864, which would be expected to be phosphorylated at Ser851 and/or Ser857. The theoretical mobility of dynI343–361 predicts migration to a spot above and to the left of dynI847–864 (spots K and I). Therefore, dynI343–361 theoretically accounts for spot M. However, direct evidence is lacking for Ser347 and Ser512 being 32P-labeled in 1 h during synaptoosome labeling or depolarization. Moreover, the possibility of non- or semi-tryptic dynI proteolysis products and minor contaminating non-dynI phosphopeptides in the two-dimensional map cannot be ruled out.

Distribution of the 32P between Phospho-sites—We next quantified the amount of phosphate in each spot (Table 1). Quantitative analysis allowed a determination of the potential in vivo significance of each site in terms of its abundance. Note that “cold phosphorylation” (i.e. that occurring prior to 32P labeling) was not quantifiable in this way. The 32P-labeled pep-
In Vivo Phosphorylation Sites in Rat Dynamin I

Table 1

| Phosphorylation Site | Context of Site | Measured % 32P Radiation | Predicted % 32P Radiation |
|----------------------|----------------|--------------------------|--------------------------|
| Ser^347              | KRIEDQGDIQD    | 1                        | 1                        |
| Ser^512              | NXRTRRGNQDE    | ≥8                       | ≥8                       |
| Ser^774              | PRGPGSPTPQR    | ≥47                      | ≥47                      |
| Ser^777              | RSPTPRTPQR     | ≥22                      | ≥22                      |
| Ser^822              | SRPGDPPDPGPSF  | 5                        | 5                        |
| Ser^851              | RSGQARGSRPE    | 12 combined              | 12 combined              |
| Ser^853              | RPPRPPRPPPPPPF | 2 combined               | 2 combined               |
| DynIII Ser^853       | RPPRPSPTRPPTF  | 2                        | 2                        |
| Unknown spots        |                |                          |                          |
| Total                | 90             | 10                       | 10                       |

Phosphorylation sites identified by MS from phosphopeptides extracted from the two-dimensional map were correlated with the 32P radiation detected in each spot to determine the percentage of total radiation. Phosphorylation sites that were not extracted from the two-dimensional map, rather than by nano-LC-MS/MS alone, were correlated with the spots in the two-dimensional map that match the theoretically predicted migration of the phosphopeptides (see Experimental Procedures and Fig. 5A). Note that it is likely that an unknown phosphopeptide contributed to part of this measured radiation predicted to be associated with Ser^347. Note that, although a small, but unknown amount of radiation is likely to be contributed by dynIII Ser^853 and Ser^857, reducing the proportion of radiation from dynIII Ser^853 and Ser^857.

Phosphorylation sites identified by MS from phosphopeptides extracted from the two-dimensional map, rather by nano-LC-MS/MS alone, were correlated with the spots in the two-dimensional map that match the theoretically predicted migration of the phosphopeptides (see Experimental Procedures and Fig. 5A). Note that it is likely that an unknown phosphopeptide contributed to part of this measured radiation predicted to be associated with Ser^347. Note that, although a small, but unknown amount of radiation is likely to be contributed by dynIII Ser^853 and Ser^857, reducing the proportion of radiation from dynIII Ser^853 and Ser^857.

**TABLE 1**

Distribution of 32P radiation on all dyn and dynIII phosphorylation sites detected from two-dimensional tryptic phosphopeptide mapping and nano-LC-MS/MS

Phosphorylation sites identified by MS from phosphopeptides extracted from the two-dimensional map were correlated with the 32P radiation detected in each spot to determine the percentage of total radiation. Phosphorylation sites that were not extracted from the two-dimensional map, rather than by nano-LC-MS/MS alone, were correlated with the spots in the two-dimensional map that match the theoretically predicted migration of the phosphopeptides (see Experimental Procedures and Fig. 5A). Note that it is likely that an unknown phosphopeptide contributed to part of this measured radiation predicted to be associated with Ser^347. Note that, although a small, but unknown amount of radiation is likely to be contributed by dynIII Ser^853 and Ser^857, reducing the proportion of radiation from dynIII Ser^853 and Ser^857.

To predict the potential biological significance of each phosphorylation site we next determined which sites were responsive to depolarization (Fig. 5C). This provides a crude measure...
of the relevance of each site to depolarization-induced SVE. The amount by which each phosphopeptide in spots A–D was decreased upon depolarization was quantified (Fig. 5B) and correlated to the phosphorylation site identified (found here and previously). The amount of $^{32}$P-labeled phospho-Ser$^{774}$ was decreased by 30% ($\pm 2\%$ S.E.), and phospho-Ser$^{778}$ was decreased by 47% ($\pm 3\%$ S.E.) following depolarization (Fig. 5C). Again, the small amount of radiation from co-purified dynIxin was ignored. Although the phosphopeptide containing the sites Ser$^{851}$ and Ser$^{857}$ were not highly $^{32}$P-labeled, the phosphorylation was reduced by 34% (range of $\pm 1\%$) following depolarization (Fig. 5C). Therefore, the two C-terminal phosphorylation sites in dynIxia are dephosphorylated upon depolarization. The phosphopeptide containing Ser$^{822}$ was significantly dephosphorylated by 56% ($\pm 0.5\%$ S.E.) following depolarization (Fig. 5, B and C). No depolarization-dependent change in the phosphorylation of Ser$^{521}$ could be measured, because there was no $^{32}$P label directly associated with this site. However, if it was part of spots E–H as we surmise, then it was depolarization-sensitive (Fig. 5B). There was no depolarization-dependent change in the phosphopeptide surmised to contain Ser$^{347}$.

**Phosphorylation of Thr$^{780}$ on DynI Is Restricted to in Vitro Conditions**—It was previously reported that dynI is phosphorylated by cdk5 only at Thr$^{780}$ in vitro and that all phosphorylation is blocked by mutation of Thr$^{780}$ to Ala (8). These data strongly suggest Thr$^{780}$ is the sole (cdk5-mediated) phosphorylation site in the dynI PRD and conflicts with our main findings. We previously found no evidence for Thr$^{780}$ phosphorylation in vivo (5). Mass spectrometry is not a good tool for resolving this discrepancy because it cannot be used to definitively prove the absence of a phosphorylation site. Therefore we used a site-directed mutagenesis strategy to determine how a single mutation on Thr$^{780}$ might abolish $[\gamma-\text{32P}]$ATP phosphorylation of dynI on Ser$^{773}$ or Ser$^{778}$ by cdk5 in vitro. The results contradict those of Tomizawa et al. (8). We generated a series of single, double, or triple point mutations of Ser$^{774}$, Ser$^{778}$, and/or Thr$^{780}$ to Ala in GST-dynI PRD for the purpose of testing the circumstances under which phosphorylation of the phospho-box by recombinant cdk5/p25 could be prevented (Fig. 6A). Single or double mutation of Ser$^{773}$ and Ser$^{778}$ had little effect on the extent of phosphorylation by cdk5, as determined by autoradiography (Fig. 6B) and by quantitative phosphorimaging analysis (Fig. 6C). A similar result was obtained with a single mutation on Thr$^{780}$ (Fig. 6, B and C). However, when all three sites in the phospho-box were mutated to Ala, phosphorylation was greatly reduced (32 $\pm 3\%$ of dynI WT, $p < 0.001$, Fig. 6, B and C). It appears that mutation of any one of these three sites results in a compensatory increase in phosphorylation at one or more of the remaining sites. So it is only when all three sites are mutated that a significant drop in phosphorylation was detected in the PRD. However, note that 30% of the total phosphorylation remained in the triple mutant, suggesting a major contribution from other sites in the PRD. The dynI PRD construct used in these studies was based on the long splice variant dynIxa, therefore there are at least three additional sites available for phosphorylation by cdk5; Ser$^{822}$, Ser$^{851}$, and Ser$^{857}$.

![FIGURE 6. In vitro phosphorylation of dynI PRD by cdk5.](image)

A. dynI consists of four distinct domains: the GTP hydrolysis domain (GTPase), a pleckstrin homology (PH) domain, an assembly domain (AD), and a proline-rich domain (PRD). Point mutations were made in phospho-box residues at the indicated positions (arrows). B. GST-dynI PRD either WT or Ala mutants were phosphorylated by recombinant cdk5/p25 in the presence of $[\gamma-\text{32P}]$ATP in vitro. An autoradiograph is shown (top panel). The protein load was visualized by Coomassie Blue staining (bottom panel). C. The amount of incorporated $[\gamma-\text{32P}]$ATP in experiments such as that in panel B was quantified by using a Storm PhosphorImager ($n = 3$). Data were expressed as a percentage of dynI WT $\pm$ S.E. One-way analysis of variance was applied ($**$, $p < 0.001$ against dynI-WT). D. GST-dynI PRD double mutant Ala was phosphorylated in vitro with cdk5/p25, as in lane 5 of B, was digested with trypsin, enriched for phosphopeptides using Fe$^{3+}$-immobilized metal affinity chromatography, and the mutated phospho-box phosphopeptide was sequenced by tandem mass spectrometry. Fragmentation of the triply charged precursor at m/z 421.2 produced a spectrum that matched the sequence of dynI$^{773-783}$, where Ser$^{774}$ and Ser$^{778}$ are each substituted for Ala and Thr$^{780}$ is phosphorylated. The diagnostic $y_5^{2+}$ ion was the most intense at a height of 6.4 counts.
Some or all of these may account for the remaining phosphorylation in Fig. 6B.

Using tandem mass spectrometry, we confirmed that the phospho-box of the in vitro phosphorylated wild-type GST-dynI PRD was indeed phosphorylated at Ser774, Ser778, and Thr780 (supplemental Fig. S1). As expected, in vitro phosphorylation at Thr780 was most easily observed by analyzing the double mutant dynI PRD (Fig. 6D). The phosphorylation at Thr780 was determined by the y3 and y4-98 ions. The relatively strong signal for the phosphorylated y5 fragment ion, owing to the favorable cleavage of an X-P bond (20), was a clear signal that the phosphorylation site on this peptide was C-terminal to Pro779, i.e. it must only be Thr780, because no other part of 779PTPQR783 can be phosphorylated. This y5 ion was also a strong signal in the spectra of the wild-type sequences (supplemental Fig. S1), alternately conferring and denying phosphorylation to Thr780. Curiously, the phosphorylated y6 ion was absent from the spectrum of a similar in vitro analysis by Tomizawa et al. (8).

The results demonstrate that a single mutation in a protein that is multiply phosphorylated is insufficient to detect reduced overall in vitro phosphorylation. While Thr780 is easily detected as an in vitro phosphorylation site, it does not necessarily follow that Thr780 is phosphorylated in vivo. Our systematic approach contradicts the overall results and conclusions of Tomizawa et al. (8).

**AmphI Binding Is Independent of DynI Phosphorylation**— There are several reports that amphI binding to dynI is regulated by the in vitro phosphorylation status of dynI (8, 21). To extend these in vitro observations, we also generated a series of Glu mutations at Ser774, Ser778, and Thr780 in GST-dynI PRD (Fig. 6A). Pulldown experiments with GST-dynI PRD wild-type and GST-dynI Ala mutants (non-phosphorylatable) or Glu mutants (pseudo-phosphorylation) did not significantly alter the binding of native full-length amphI as shown by Western blot (Fig. 7A). The results were confirmed by quantitative densitometry analysis of multiple experiments (Fig. 7B).

We then performed a reverse pulldown experiment, using recombinant GST-amphI SH3 to capture native dynI in the presence of different sodium chloride concentrations. As the salt concentration increased from 0 to 1 M, the amount of total dynI protein bound to GST-amphI SH3 decreased (Fig. 7C). However, the amount of phosphorylated dynI bound was unchanged, even at high salt concentration (Fig. 7C). This suggests that amphI SH3 binding to dynI is not regulated by dynI phosphorylation (see Fig. 1). The observation that GST-amphI SH3 has an apparently higher binding affinity toward phosphodynI than non-phospho-dynI is interesting but not likely to have any physiological relevance, because it only occurred at 0.5–1 M NaCl.

We have shown how the dynI PRD interacts with full-length amphI and how amphI SH3 interacts with full-length dynI. Next, we used an immunoprecipitation experiment to ask whether the association of native full-length dynI with native full-length amphI might be phosphorylation-regulated. Pretreatment of synaptosomes with Ba2+ for 1 h was previously shown to produce massive dynI dephosphorylation (22) by chronic depolarization (23). We prepared Ba2+-treated synap-

![FIGURE 7. DynI phosphorylation has no effect on amphI interaction. A, interaction of pseudo-phosphorylated dynI PRD with amphI. GST-dynI PRD WT or Glu mutants were bound to GSH-Sepharose and used in pulldown experiments from rat brain lysates. The samples were blotted with anti-amphI antibodies (top panel). The amount of GST-dynI PRDs used in the pulldown experiments are shown (bottom panel). B, the amount of amphI bound to GST-dynI PRD mutants was quantified by densitometric analysis of Western blots such as in panel A (n = 4). Data were expressed as a percentage of dynI PRD WT ± S.E. One-way analysis of variance was applied, but no statistically significant differences were found. C, GST-amphI SH3 binds phospho-dynI with high affinity. GST-amphI SH3 was used in pulldown experiments from P2 synaptosomes lysed in Triton X-100 in the absence or presence of 0.15, 0.5, or 1 M NaCl. DynI was detected by Coomassie Blue staining of the gel (top panel). Lysates from the same experiment were probed with antibodies to phospho-Ser774 and phospho-Ser780. Immunoblots are displayed (bottom two panels). Data are representative of two independent experiments. D, interaction of phospho-dynI with amphI. Synaptosomes were incubated for 60 min in Krebs-like buffer containing 0.1 mM Ca2+ or 2.5 mM Ba2+, lysed, and immunoprecipitated with anti-dynI antibodies. The complexes were subjected to Western blotting analysis with antibodies to amphI and phospho-Ser774 (top two panels). The amount of dynI immunoprecipitated by the dynI antibodies was visualized by Peroxidase staining of nitrocellulose membrane. Blots are representative of three independent experiments. E, quantiative analysis from Western blots of the amount of amphI co-immunoprecipitated with dynI (D). Results are from three independent experiments.**
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**DISCUSSION**

**Dynl Has Seven Phosphorylation Sites**—An exhaustive strategy was used to identify and rank all the phosphorylation sites in dynl. Mass spectrometry of synaptosomal $^{32}$P-labeled dynl phosphopeptides separated by two-dimensional tryptic mapping revealed seven in vivo phosphorylation sites at Ser$^{274}$, Ser$^{778}$, Ser$^{822}$, Ser$^{851}$, Ser$^{857}$, and Ser$^{347}$ (Fig. 8A). Thr$^{780}$ phosphorylation was not detected. It was shown previously that a peptide containing Ser$^{857}$ was phosphorylated in vitro by Mnb/Dyrk1A (7), whereas a peptide containing Ser$^{851}$ was not. In this study, we confirmed the existence of Ser$^{857}$ as an in vivo phosphorylation site (7) and identified Ser$^{851}$ as a new in vivo phosphorylation site within the same tryptic peptide. These sites are restricted to the tail of the longer splice variant, dynlx (Fig. 8B). We found that Ser$^{851}$ and Ser$^{857}$ were phosphorylated to a similar extent, and there was no hierarchy to their phosphorylation. It has been shown that Ser$^{857}$ phosphorylation reduced dynl binding to amphI SH3 domain (7). However, our data suggested that dynl phosphorylation does not affect binding of amphI. It may be difficult to discern the true effect of phosphorylation at Ser$^{857}$ on amphI binding from our experiments, because it is $<12\%$ of the total radiation and we focused mainly on cdk5 substrates within the phospho-box.

The phosphorylation site found at Ser$^{512}$ is near the start of the pleckstrin homology domain (Fig. 8A). It is not known how much the phosphate on this site is turning over, because we were unable to correlate a radioactive spot with the sequenced phosphopeptides. Nonetheless, some of the unidentified spots were good candidates for containing phospho-Ser$^{512}$, based on peptide mobility prediction. If Ser$^{512}$ is involved in SVE, then it is most likely involved in regulating dynl interaction with phospholipids. The phosphorylation at Ser$^{347}$ is flanked on either side by PXAP motifs, which bind SH3 domain of dynl. This suggests that dynl$^{347}$ may be phosphorylated by one of the same protein kinases that phosphorylate dynl$^{347}$, or dynl$^{347}$ may not be phosphorylated by the same protein kinase. However, both Ser$^{347}$ and Ser$^{512}$ share some common amino acid sequences (SGXQ) followed closely by an acidic residue on the C-terminal side. Hence, we conclude that there is at least one other protein kinase, apart from cdk5 (and potentially minibrain kinase/Dyrk1A (7)), that phosphorylates dynl in vivo.

Ser$^{822}$ (RPGASPDPDF) is located in front of a proline and might be phosphorylated by one of the same protein kinases that phosphorylate Ser$^{774}$, Ser$^{778}$, Ser$^{851}$, or Ser$^{857}$. Ser$^{822}$ is flanked on either side by PXAP motifs, which bind SH3 domain of synaptosomes and compared them to resting (0.1 mM Ca$^{2+}$) synaptosomes. Dynl was immunoprecipitated from each of these preparations. Dynl was massively dephosphorylated upon Ba$^{2+}$ treatment, as shown by Western blot analysis with the antiphospho-Ser$^{774}$ antibody (Fig. 7D). However, the interaction between amphl and dynl was not affected (Fig. 7, D and E). From these interactions studies we conclude that amphl is not a phosphorylation-dependent partner for dynl, in vivo or in vitro.
domain-containing proteins. The motifs immediately C-terminal to Ser^{822} are involved in binding grb2 and amphl (these are called Site 8 and Site 9, respectively, Fig. 8B) (25). Therefore, it is possible that this binding may be phospho-regulated by Ser^{822}.

New information about the hierarchical nature of Ser^{774} and Ser^{778} was revealed when the extent of dephosphorylation of each site was quantified. These data showed that following depolarization, phospho-774 and phospho-778 are dephosphorylated to a similar extent, perhaps phospho-778 slightly more. More importantly, the pool of doubly phosphorylated dynl (with both Ser^{774} and Ser^{778} phosphorylated) was dephosphorylated, in preference to the singly phosphorylated pool (with only Ser^{774}). This implies a greater relative importance on the removal of phospho-Ser^{778}. We propose that dynl phosphorylation at Ser^{774} has a different regulatory role to phosphorylation at Ser^{778} in SVE.

**Phosphorylation at Thr^{780} Is an in Vitro Artifact—**Controversially (18), it was reported that cdk5 phosphorylates dynl at Ser^{774} and Ser^{778} in two studies (4, 5) and solely at Thr^{780} in another study (8). However, we have now demonstrated that, although Thr^{780} is phosphorylated *in vitro* by cdk5 and phosphorylation mutants may be able to produce a functional effect *in vivo* (8), it is unlikely that dynl is phosphorylated at Thr^{780} *in vivo* in synaptosomes within the ~100-fold detection limits of our methods. The most likely explanation is that phosphorylation at Thr^{780} is restricted to *in vitro* conditions; i.e. it is simply an artifact of cdk5 phosphorylation *in vitro*. Three experiments supported our conclusion that Thr^{780} is not phosphorylated by cdk5 in synaptosomes. Firstly, phosphoamino acid analysis of purified dynl from ^32P-labeled synaptosomes revealed exclusive phosphorylation of dynl on serine residues. This experiment alone, within the limits of detection, restricts Thr^{780} or any tyrosine phosphorylation to being a static phosphorylation site (*i.e.* not labeled with ^32P after 1 h) in synaptosomes or to not being present in synaptosomes at all. Secondly, we were unable to detect any Thr^{780} phosphorylation by exhaustive two-dimensional phosphopeptide mapping of ^32P-labeled dynl and MS analysis. This experiment, within the limits of detection, rules out the presence of a major static pool of dynl phosphorylated on Thr^{780}. Any such pool must contain <1% of the phosphorylation in dynl. Thirdly, a single mutation of Thr^{780} to Ala did not abolish cdk5 phosphorylation in the dynl-PRD, in contrast to observations reported by Tomizawa et al. (8). Like Thr^{780}, a single Ala mutation on Ser^{774} and Ser^{778}, or in combination, had no effect on dynl phosphorylation by cdk5 *in vitro*. It was only when all three sites at Ser^{774}, Ser^{778}, and Thr^{780} were mutated to Ala that phosphorylation by cdk5 was significantly reduced. This suggests that there is a compensatory phosphorylation of alternative sites within the PRD when any single site is mutated. In fact, we have previously shown in phosphoamino acid analysis that phospho-threonine only appeared when dynl was phosphorylated by cdk5 *in vitro* but was absent when dynl was phosphorylated from ^32P-labeled synaptosomes (4). Potential differences between the two PRD splice variants are possible. The long form was used in our study (containing six SP or TP motifs, while there are only four in the short form), but the form used by Tomizawa et al. (8) was not reported (see Fig. 8). Placing further focus on *in vitro* phospho-

**DynIII Has Three Phosphorylation Sites—**We previously found that a small amount of dynIII co-purifies during the dynamin purification from P2 synaptosomes and were able to identify the homologous phospho-box phosphorylation sites to Ser^{774} and Ser^{778} for dynIII (5). The amino acid sequences around these sites are highly conserved between dynl and dynIII. In this study we found a new dynIII phosphorylation site at Ser^{853} that was ^32P-labeled. DynIII has previously been reported to be mainly expressed in the post-synapse (26). It is unlikely that the P2 synaptosome preparation contained postsynaptic dynIII that could acquire ^32P label. Therefore, we conclude that the ^32P-labeled dynIII was presynaptic. Ser^{853} is homologous to Ser^{851} of dynl. Like dynl, this site is only present in the long splice form. We note that Ser^{857} from dynl is not present in dynIII (Fig. 8B). This third homologous phosphorylation site, Ser^{853}, suggests that dynIII may perform an analogous function to dynl in the presynaptic nerve terminal. Its phosphorylation on the same sites in synaptosomes suggests that the same protein kinases may be involved and that there is a physiologically significant role for Ser^{853} (dynIII) and Ser^{851} (dynl). The amino acid sequence of the tail of the long splice variants of dynl and dynIII are not similar except for five amino acids, SPXR, that encompass the phosphorylation site motif (Fig. 8B).

It remains to be determined whether all the phosphorylation sites in dynIII have now been identified, although the results suggest that the main dynIII sites have been found. Among the seven dynl sites, only serines 774, 778, 851, and 347 are conserved in the sequence of dynIII. Three of them have now been identified, and although phosphorylation at Ser^{347} in dynIII is possible, it would be well below detection limits in our current approaches. No sites were detected that were not homologous to dynl. However, the amino acid sequences of dynIII are sufficiently different as to indicate that different protein partners are phospho-regulated by these sites. The results suggest dynIII has a role in nerve terminals; however, it must be a highly specialized role.
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DynII Phosphorylation?—Perhaps surprisingly, we found no evidence for phosphorylation of dynII in this study. DynII was present in the synaptosomes, as judged by Western blot and mass spectrometry data (data not shown). The GST-amphiphilic SH3 domain pulldown experiment also extracts dynII and III (data not shown). Although we have not directly determined that the extraction of dynII or III is quantitative, it is likely to be so. In fact, our laboratory purifies dynII by pulldown experiment on GST-amphiphilic SH3 beads. The absence of any dynII phosphorylation sites in synaptosomes is not conclusive evidence that none are present but is compelling because sites in dynIII were detectable at low levels. Only two of the seven dynI phospho-sites are conserved in the sequence of dynII. Ser774 is missing in dynII, although Ser778 and Ser347 are conserved. Ser822 and Ser512 are missing in dynII, although the surrounding amino acid sequences are highly conserved. The tail of dynII is not subjected to the same alternative splicing as dynI and III, however it is highly related in sequence to the long tail of dynIII, both of which are missing Ser857 (dynI). The main difference is that dynII specifically lacks Ser851 (dynI, or Ser853 in dynIII), hence it has none of the sites present in either of the other dynamins and would not be expected to be phosphorylated by a proline-directed protein kinase in this region. The absence of detection of phosphorylation of dynII at Tyr231 and Tyr597 (13, 14) is not surprising, because the synaptosomes were not stimulated with any growth factor receptors. However, these sites may be phosphorylated in other cellular compartments or after the appropriate stimulation.

A Complete Description of dynI in Vivo Phosphorylation?—We have attempted to map all the in vivo phosphorylation sites in dynI. However, no technology is yet sufficiently sensitive to allow unequivocal conclusions that all sites in any protein have been identified. Other previously reported sites were not found: Thr280 (8), Tyr231 and Tyr597 in dynI (13, 27) and dynII (14), or Ser795 (28). We conclude that phosphorylation of Thr280 and Ser795 is restricted to in vitro conditions only. We found no evidence for phosphorylation on tyrosine but cannot rule out several possibilities. Phosphorylation of Tyr231 and Tyr597 was first observed in dynI transfected into non-neuronal cells, thus phosphorylation occurred in an inappropriate in vivo context. Although we found no evidence for phosphorylation of dynI on Tyr, it, and other sites, cannot be ruled out completely for a variety of reasons. Firstly, other sites in dynI may be phosphorylated in different subcellular compartments or at different stages of development. Secondly, it is possible (although unlikely) that some phosphopeptides do not bind TiO2 or Fe3+-immobilized metal affinity chromatography and therefore would have been missed. A number of phosphopeptides are not mass spectrometry “friendly” (i.e. do not produce sufficient signal), although the specific properties of such phosphopeptides have yet to be described. Within such limitations we conclude that any new sites discovered in the future must represent <2% of the total phosphorylation, because the majority of the 32P label on dynI has been accounted for. The caveats on this conclusion are the possibility that a co-migrating, poorly detected phosphopeptide could have been missed in one of the spots or the prior existence of high stoichiometry phosphorylation sites that may not be labeled with 32P after 1 h. The potential physiological significance of the latter hypothetical sites would be questionable in the context of SVE. A thorough and deliberate strategy of maximized phosphoprotein capture, 32P labeling, and highly sensitive mass spectrometry has been utilized to avoid missing significant in vivo phosphorylation sites.

The discovery of four new in vivo dynI phosphorylation sites at Ser512, Ser822, Ser851, and Ser347 in addition to the three already identified sites (Ser774, Ser778, and Ser857) provides a basis for further study of the phosphorylation of dynI and SVE. It remains to be shown whether these new phosphorylation sites in the PRD regulate binding of other SH3 domain-containing proteins, besides syndapin I (6), or whether they have a supplementary role in the same process of endocytosis or perhaps a major part in a mechanistically distinct mode of endocytosis.

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