Phosphorylation of Dynamin I on Ser-795 by Protein Kinase C Blocks Its Association with Phospholipids

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Dynamin I is a GTPase enzyme required for the retrieval of synaptic vesicles after exocytosis. It functions in endocytosis by stimulated assembly as a helix around the neck of invaginating synaptic vesicles (1, 2). Dynamin can self-assemble as a series of rings in the absence of guanine nucleotide (3), and helices form around phospholipid vesicles in vitro (4) or around the neck of invaginating synaptic vesicles (5). The helices behave like a nanospring, with GTP hydrolysis producing an increase in the helix pitch, suggesting that endocytosis might occur by a nucleotide-dependent conformational change in dynamin that cleaves vesicles from the plasma membrane (2). Dynamin I is also a phosphoprotein found in intact nerve terminals where it is apparently phosphorylated by PKC. It is rapidly dephosphorylated by calcineurin on stimulation of endocytosis by depolarization and calcium influx (7) and blocking dephosphorylation prevents endocytosis in nerve terminals (8). It remains dephosphorylated during endocytosis of most vesicles and is rephosphorylated while endocytosis is completing (9). Therefore the phosphorylation of dynamin is not likely to play a role during endocytosis but is probably a priming step prior to endocytosis.

Many of the proteins essential for endocytosis are constitutively phosphorylated in nerve terminals at rest and are coordinately dephosphorylated by calcineurin upon a burst of exocytosis and endocytosis. These “dephosphins” include dynamin I, amphiphysins I and II, synaptojanin, epsin and Eps15 (8, 10–14). In vitro dephosphorylation of rat brain extracts promotes the assembly of dynamin I, synaptojanin, amphiphysins, clathrin, and AP-2 into complexes (15). Thus, phosphorylation appears to regulate the association and dissociation cycle of the endocytic machinery. This suggests that the role of dynamin phosphorylation may be to either prevent inappropriate protein-protein interactions (15) or to keep dynamin away from sites of exocytosis until required for endocytosis (6).

The precise role of the phosphorylation of dynamin has not been fully determined, but several biochemical correlates have been reported. In vitro phosphorylation by cPKC (the Ca2+-dependent PKC family comprising PKCa, β-, and γ-) stimulates the GTPase activity of dynamin I (10), promotes the formation of disulfide bonds and its assembly into a tetramer, and increases low affinity Ca2+ binding (16). Phosphorylation by a cyclin-dependent protein kinase, Cdc2, prevents dynamin association with microtubules in vitro (17). Phosphorylation of dynamin I by an unidentified cytosolic kinase activity inhibits its binding to amphiphysin (15), although phospho-dynamin from intact nerve terminals is capable of binding to the SH3 domains of amphiphysins I and II (8). In none of these examples was the phosphorylation site identified, and it might differ for each protein kinase.

Dynamin I is known to be phosphorylated exclusively on serine on a site within the C terminus by cPKC in vitro and by endogenous protein kinases in intact nerve terminals (10, 18, 19). Phospho-dynamin I has a subcellular localization restricted to the cytosolic fraction of intact nerve terminals, de-
spite that greater than 90% of the dynamin I is membrane-associated (19). Similarly, the larger dynamin I pool that is associated with brain membranes or cytoskeleton cannot be phosphorylated in vitro by PKC until dynamin is extracted from the membranes (19). These data suggest that phosphorylation of dynamin I may be required to generate or maintain a cytosolic pool of tetramereric phospho-dynamin which associates with proteins required to target dynamin to sites of endocytosis following a dephosphorylation stimulus. There are examples where one consequence of phosphorylation of other peripheral membrane proteins such as myristoylated alanine-rich C kinase substrate, synapsin I, and spectrin is an inhibition of their binding to phospholipid and consequent release into the cytosol (20–24). However, in the case of dynamin I this concept has not been directly tested. Therefore our aims were to examine the consequences of phosphorylation by PKC on phospholipid binding by dynamin I and to determine the phosphorylation site sequence.

EXPERIMENTAL PROCEDURES

Production of Proteins—Dynamin purification from sheep brain and cPKC was performed by Drs. S.1 and 2, respectively, from the methods of An et al. (25) and Konrad et al. (26) with some modifications. The recombinant virus was provided by Trevor Biden (Garvan Institute, Sydney, Australia). The GST fusion protein (GST-p85-SH3) was prepared from the sequence of p85

Phosphorylation of Dynamin I—Purified dynamin I (5 μg) was phosphorylated for 30 min with recombinant PKCs as described above and then precipitated using chloroform/methanol (26). The precipitated dynamin I was resuspended in 100 μl of digestion buffer (25 mM Tris buffer, pH 8.5), and trypsin (Promega, modified sequence grade) was added at a 1:50 molar ratio. The digestion was performed overnight at 4 °C. Following digestion, the sample was desalted and concentrated 10-fold in volume, and the ATP was removed by vacuum centrifugation.

Immobilized Metal Affinity Chromatography (IMAC)—An IMAC micro-column was constructed from a partially constricted GeLoader tip (Eppendorf). A small portion (7 μl) of a slurry of nickel-nitrotriacetate activated Poros Oligo R3 beads was added at a 1:50 molar ratio. The column three times, and specific antisera was eluted with 100 mM glycine, pH 2.5, into tubes containing 1 mM tris buffer, pH 7.4, to neutralize the solution. Fractions containing eluted antibody were pooled and dialyzed against six changes of 50 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl, concentrated to 5 ml (the original serum volume) using an Amicon ultra-filtration device with a PM-10 membrane, and lyophilized.

Tryptic Digestion of Dynamin I—Purified dynamin I (5 μg) was phosphorylated for 30 min with recombinant PKCs as described above and then precipitated using chloroform/methanol (26). The precipitated dynamin I was resuspended in 100 μl of digestion buffer (25 mM Tris buffer, pH 8.5), and trypsin (Promega, modified sequence grade) was added at a 1:50 molar ratio. The digestion was performed overnight at 4 °C. Following digestion, the sample was desalted by vacuum centrifugation.

Phospholipid-binding Plate Assay—For a quantifiable lipid-binding assay a plate assay was developed. The bottom of wells in plastic 96-well plates (Maxisorp, Nunc) were coated with 30 μg/ml phosphatidylserine (PS) in absolute ethanol (PS:PC ratio of 1:3), dried under vacuum, and stored under vacuum until required. Nonspecific binding sites were blocked by incubating the plates with an excess volume (200 μl/well) of assay buffer (30 mM Tris-HCl buffer, pH 7.4, plus 1 mM EGTA) containing 5% fatty acid-free bovine serum albumin (BSA, Roche Molecular Biochemicals, fraction V) for 1 h, followed by washing. Assays were performed with the plates on ice in final assay volumes of 60 μl/well. Incubations were initiated by addition of dynamin I (diluted in 5 mM Tris-HCl buffer, pH 7.4, containing 0.05% Tween 80) at 0.5–2.0 μg/well and incubated for 30 min on a shaking platform. Subsequent steps were at room temperature. Plates were rinsed twice and washed with three changes of 2 min in Tris-HCl buffer, pH 7.4, containing 1 mM CaCl₂, and 5 μg/ml phosphatidylserine hydrophobic detergent (Bio-Rad), and the absorbance was read after 1 h on microplate reader at 415 nm.

Phosphoprotein—Dynamin I rabbit antiserum was affinity purified from sheep brain and cPKC was performed by Drs. S. 1 and 2, respectively, from the methods of An et al. (25) and Konrad et al. (26) with some modifications. The recombinant virus was provided by Trevor Biden (Garvan Institute, Sydney, Australia). The GST fusion protein (GST-p85-SH3) was prepared from the sequence of p85...
isolated by IMAC and analyzed by MALDI-TOF MS were treated with alkaline phosphatase directly on the MALDI probe after the analysis (28). Alkaline phosphatase (0.17 unit in 2 μl of 0.1 M NaH₂PO₄, pH 7.8) was added directly to the matrix/sample deposit. The MALDI probe was placed for 20 min at 37 °C in a small closed plastic box containing a wet paper tissue to maintain high humidity. The buffer was allowed to evaporate for 10 min at ambient temperature before 0.6 μl of 2–5% trifluoroacetic acid and 0.3 μl of matrix (α-cyano-4-hydroxycinnamic acid in 70% acetonitrile) was added. The dry crystalline deposit was carefully washed with 0.1% trifluoroacetic acid prior to the second analysis by MALDI-TOF MS.

Matrix-assisted Laser Desorption/Ionization (MALDI) Mass Spectrometry—The MALDI-TOF analysis was performed on a Voyager STR (Perkin-Elmer-Perseptive Biosystems) instrument equipped with delayed extraction. The spectra were all obtained in positive reflector mode. Two MALDI sample preparation methods were used. (i) For co-precipitation of analyte and matrix according to the dried droplet method, the analyte solution in 5% formic acid and the matrix solution (15–20 g/liter α-cyano-4-hydroxycinnamic acid in 70% acetonitrile) were mixed in equal proportions (0.4 μl) on the MALDI target and dried. The dry crystalline deposit was rinsed carefully with a small volume of 0.1% trifluoroacetic acid. (ii) Peptides were desalted by using custom micro-columns and directly deposited onto the MALDI probe by elution with matrix solution as described above.

Other Methods—Dynamin I GTPase activity was determined as described (10). Proteins were resolved by SDS-PAGE in 10% mini-gels (29) and were transferred to nitrocellulose membrane using a mini trans-blot apparatus (Bio-Rad). The transfer buffer contained 24.7 mM Tris, 191.8 mM glycine, 20% methanol, and 0.05% SDS. The membranes were blocked in 10% non-fat milk for 1 h and probed with antibodies as indicated in the legends to the figures. Immunoreactive bands were visualized by enhanced chemiluminescence according to the manufacturer’s instructions (Amersham Pharmacia Biotech).

RESULTS

To measure phospholipid-binding of dynamin I a plate-based assay was developed. The use of lipid-coated plastic or glass beads prevents the insertion of dynamin into a lipid bilayer (19, 25) and prevents dynamin from assembling as helices or vesiculating the phospholipid (4), which would otherwise confound interpretation of phospholipid association. In background experiments, the relative affinity of dynamin I for different compositions of phospholipid was assessed (Fig. 1A). Coating solutions were prepared from mixtures of two phospholipids with a fixed total concentration of phospholipid but with varying ratios. Dynamin I binding was greatest to a mixture of PS and PC and was reduced in mixtures of PI and PC or PS and PI. Dynamin I did not bind well to plates coated with any of the three individual phospholipids (data not shown). The highest binding level was achieved with a PS/PC ratio of 1:3. These conditions also best reflect the in vivo situation in regards to membrane lipid composition in cells and were used for all other experiments. In previous studies dynamin was found to dissociate from lipid-coated beads or rat brain membranes with NaCl (19). We found that NaCl also efficiently blocked dynamin I association with phospholipid in the plate assay (Fig. 1B). To demonstrate that binding was specific, we increased the detergent concentration in the assay to 1% Tween 80, which almost abolished dynamin binding (Fig. 1C). As dynamin I concentrations were increased, it bound to the phospholipid-coated plates in a linear and saturable manner (Fig. 1C). The calculated apparent affinity of dynamin for the phospholipid mixture using double-reciprocal plots was $51 \pm 51$ nM (n = 3).

To determine if the plate binding assay sensitively detects alterations in the conformational status of dynamin, we next explored the effect of adenine and guanine nucleotides on dynamin binding to phospholipids (Fig. 2). Metal ions such as Mg²⁺ (Fig. 2A) and Mn²⁺ (data not shown) had no effect on dynamin binding to phospholipid, whereas Ca²⁺ had variable effects at concentrations above 200 μM (data not shown). Mg²⁺ was included in equimolar concentrations with each guanine or adenine nucleotide. GTP potently reduced dynamin association with phospholipid (Fig. 2A). Other nucleotides also reduced binding but with greatly reduced efficacy. Concentrations pro-

FIG. 1. A phospholipid-binding plate assay detects dynamin I (Dyn I) binding to phospholipids. A, the wells of 96-well plates were coated with varying mixtures of PS and PC, PS and PI, and PI and PS. In this figure, ratios are expressed as the amount of the first named phospholipid in each pair (e.g. the PS/PC value plotted at 25 = 25% PS, 75% PC; 0 = no PS or 100% PC). Plates were coated with phospholipid and blocked with 5% fatty acid-free BSA. Dynamin I (0.8 μg per well) was incubated in the wells for 30 min on ice. B, at a fixed lipid coating of PS:PC of 1:3 the effect of increasing the concentration of NaCl is shown. C, at PS:PC of 1:4, increasing amounts of dynamin were bound to the plate in the presence of 0.05% Tween 80 (control) or 1% Tween 80. In all panels bound dynamin I was detected with affinity-purified anti-dynamin I antibody followed by a horseradish peroxidase-conjugated second antibody and visualized with 2,2'-azino-di[3-ethylbenz-thiazoline-6-sulfonic acid] substrate. Values plotted have had the blank reading (no dynamin I) subtracted and are the means of three experiments, and error bars indicate S.E.
ducing 50% inhibition of binding (IC₅₀) were 0.30 mM for GTP, and 2.5, 4.3, and 14 mM for GDP, GMP, and cGMP respectively. This suggests specificity in the action of GTP, as 0.3 mM is close to the physiological concentration of GTP in cells (30). To determine whether GTP binding or GTP hydrolysis was responsible for the reduced binding, two non-hydrolyzable GTP analogues were used, and both potently reduced the ability of dynamin I to bind to phospholipid (Fig. 2B). GTP₃S had a very similar response to GTP, and GMP-PNP was less potent. IC₅₀ values for the analogues were 0.35 mM (Mg/GTP₃S and 0.9 mM (Mg/GMP-PNP). This demonstrates that the effect of nucleotides on dynamin I binding to phospholipid is a consequence of GTP binding, rather than GTP hydrolysis. Qualitatively similar results were obtained with ATP and its analogues, except that the potency of all adenine nucleotides was much less than guanine nucleotide analogues (Fig. 2C and D). The IC₅₀ values were (in mM) ATP 2.0, ADP 5.1, AMP 9.5, ATP₃S 2.9, and AMP-PNP 4.8.

The effect of phosphorylation by cPKC on the ability of dynamin I to bind to phospholipid was then determined in the phospholipid-binding plate assay. Binding of non-phosphorylated dynamin I to phospholipid-coated plates increased in a linear manner, whereas phosphorylated dynamin I essentially failed to bind (Fig. 3). Some minor binding was achieved with the phosphorylated preparation as the concentration of dynamin was increased, the amount of which varied between different preparations of purified dynamin I. This variability might result from different amounts of phospho-dynamin contaminating each preparation of the protein. Phosphorylation of dynamin I was confirmed by either inclusion of [γ-³²P]ATP in some experiments and autoradiography or by the ability of phosphorylated dynamin I to form tetramers in non-reducing conditions.
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**Fig. 4.** Binding of dynamin I to phospholipid-coated controlled-pore glass beads is reduced by phosphorylation. Purified dynamin I (1.0 μg) was phosphorylated with cPKC (lanes 3 and 4) or CK2 (lanes 5 and 6) and repurified. Non-phosphorylated (lanes 1 and 2) and phosphorylated dynamin I were mixed with phospholipid-coated CPG beads for 30 min. Samples were separated into supernatant (S) or pellet (P) fractions by brief centrifugation and applied to SDS-PAGE (7.5–15% gradient acrylamide gels), and the protein was stained with Coomassie Blue. The same results were obtained in a second, independent experiment. Ctrl, control.

**Fig. 5.** Dephosphorylation of dynamin I (*Dyn I*) restores phospholipid binding. Dynamin I was phosphorylated in vitro with cPKC and [γ-32P]ATP and was repurified. Dephosphorylation with calcineurin plus calmodulin (CaM), Ca²⁺, and Mn²⁺ was performed at 30 °C for 30 min, and samples were applied to SDS-PAGE for Coomassie staining (B) autoradiography (A) or were used in the phospholipid-binding plate assay (C). A, autoradiograph and B, Coomassie blue-stained gel demonstrating the dephosphorylation of dynamin. Dynamin I without phosphorylation (lane 1 and 6) or after cPKC phosphorylation (lanes 2–5). Non-phosphorylated dynamin I prior to phosphorylation (lane 1), phosphorylated dynamin I (lane 2), dephosphorylation of phosphorylated dynamin I with calcineurin and calmodulin (CaM, lane 3), phosphorylated dynamin I with calcineurin but no calmodulin (lane 4), phosphorylated dynamin I with calmodulin, Ca²⁺, and Mn²⁺, but no calcineurin (lane 5), and non-phosphorylated dynamin I with calcineurin and calmodulin (lane 6). C, dynamin or PKC-phosphorylated dynamin I (2.0 μg per well) were added to phospholipid-coated wells and incubated with the phosphatase calcineurin (0.15 or 0.75 μg) plus its activators (calmodulin, calcium, and manganese) for 30 min at 30 °C. Results have had the blank reading (no dynamin I) subtracted and are the means of three experiments ± S.E.

Identification of the phosphopeptides by MALDI MS of the radioactive fraction after high pressure liquid chromatography separation. The tryptic peptides of dynamin I and phosphorylated dynamin I were analyzed by MALDI-TOF MS, and the observed peptides were identified based on their predicted masses determined from the known sequence. Comparison of the peptide mass map of phosphorylated dynamin I and dynamin I revealed a single peptide of mass-over-charge ratio (m/z) of 1175.74 Da that was unique to dynamin I and was not found in phosphorylated dynamin I (data not shown). However, the corresponding phosphorylated peptide predicted at an m/z 1255.75 Da was not observed in the spectrum of the tryptic digest of the phosphorylated sample, probably due to suppression effects frequently observed in...
MALDI-TOF MS. Another peptide signal at m/z 1256.69 Da could be correlated to a peptide derived from trypsin (amino acids 4–14) (Fig. 6A). Treatment of the mixture with alkaline phosphatase resulted in a very weak peak at m/z 1175.74, indicating that the phosphorylated peptide might have been present (data not shown). To demonstrate this, the phosphopeptides in the tryptic digest of phosphorylated dynamin I were selectively purified by immobilized metal affinity chromatography (IMAC) using Fe$^{3+}$-charged nitrilotriacetic acid resin and were desalted prior to MALDI-TOF MS analysis. A major mass peak at m/z 1255.74 Da was found, accompanied by a characteristic metastable fragment ion, diagnostic of the loss of H$_2$PO$_4^-$ by post-source decay in the first field free region in the MALDI (Fig. 6B). A minor peak at m/z 1411.86 Da was also observed, with its accompanying fragment ion, corresponding to alternative tryptic cleavage after Arg-694 instead of Arg-695. To confirm further that these phosphopeptides were indeed phosphorylated, they were dephosphorylated with alkaline phosphatase directly on the MALDI target, resulting in a mass shift of −80 Da to m/z 1175.75 Da concomitant with the disappearance of the metastable species (Fig. 6C). This mass corresponds to the dynamin 785–796 fragment APAPVPPARPGSR, which contains a single serine at Ser-795. In the experiment shown in Fig. 6B, the m/z 1411.86-Da peptide (B) did not appear at m/z 1331.86 Da (C) probably due to its low abundance. However, a weak signal corresponding to this peptide was detected in two additional experiments (data not shown). Furthermore, a radioactively labeled peak detected by high pressure liquid chromatography was found by MALDI-TOF MS to represent the m/z 1255.74-Da peptide, accompanied by the metastable fragment ion signal. This peak shifted to m/z 1175.74 Da after alkaline phosphatase treatment. Since we previously demonstrated that dynamin I is exclusively phosphorylated on serine residues by PKC (10, 18, 19), this result confirms Ser-795 as the PKC phosphorylation site in dynamin I.

A synthetic peptide encompassing the Ser-795 PKC phosphorylation site (Dyn$_{785-803}$ VPPARPGSRGAPAGPP) was not appreciably phosphorylated by PKC in an in vitro kinase assay (data not shown), nor was another peptide encompassing the adjacent serines in dynamin$_{770-784}$ (AGRRSPTSSPTPQQR) phosphorylated by PKC in vitro. This result contrasts with the high affinity phosphorylation of dynamin previously reported for the intact protein (19) and indicates a role for dynamin tertiary structure in defining its interaction with PKC.

Ser-795 resides within a region of dynamin I previously shown to be required for association with the SH3 domains of the p85 subunit of PI 3-kinase and phospholipase C$_\gamma$ (31, 32) and which is distant from the binding site for amphiphysin and Grb2 SH3 domains (32–34). However, PKC phosphorylation of dynamin I did not alter the ability of dynamin to associate with the SH3 domain of p85. By using “pull-down assays” with GST-SH3-p85 attached to glutathione-agarose beads, dynamin was found to bind to the SH3 domain of p85 but not to GST alone (Fig. 7A). When dynamin was phosphorylated with PKC and incubated with the p85-SH3 domain, it also bound strongly. An autoradiograph of the Coomassie-stained gel in A confirmed the appropriate phosphorylation status of dynamin (Fig. 7B). Thus phospho-dynamin binds the SH3 domain of p85 as well as native dynamin.

DISCUSSION

Non-phosphorylated dynamin I binds phospholipid, but the functional pool of dynamin in nerve terminals is a relatively small pool of phospho-dynamin I localized to the cytosol (19). The mechanism of this differential subcellular distribution is defined here by revealing that phosphorylation on Ser-795 by PKC abolishes dynamin I binding to phospholipids. Phosphorylation may be a mechanism for keeping dynamin away from the plasma membrane until required for endocytosis. The cytosolic pool may specifically allow more rapid recruitment of free dynamin for endocytosis or may allow dynamin to form specific associations with other proteins required for endocytosis.

The demonstration that phosphorylation blocks phospholipid binding by dynamin I was determined using two different assay systems. In both, a mixture of acidic and neutral phospholipids was coated onto a solid support. This prevented the potential insertion of dynamin I into the phospholipid mem-

**Fig. 6.** Dynamin I (dyn I) phosphorylation site by PKCα determined by MALDI-MS. A, dynamin I was phosphorylated with recombinant PKCα, digested with trypsin, and the digest subjected to MALDI-TOF MS analysis. The mass of each peak is indicated in Da. B, the same digest was subjected to micro-purification on IMAC followed by R3 to purify the phosphopeptides. Only two peaks (1255.79 and 1411.86 Da) and peaks corresponding to their metastable loss of H$_2$PO$_4^-$ were detected. Both masses represented two cleavage variants derived from the same sequence stretch (the amino acid sequence is shown), separated only by the mass of a single arginine residue. C, the samples from B were incubated on the sample plate (on-target) with alkaline phosphatase and subjected to MALDI-TOF MS again. A single mass was detected, corresponding to the non-phosphorylated peptide shown. Results are from 1 of 3 experiments with qualitatively the same results.
mitogen-activated protein kinase phosphorylate dynamin I kinases, CK2, the cell cycle protein kinase (Cdc2 kinase), and previously shown that the cytosolic pool of dynamin I in the nerve terminal was phosphorylated, whereas the majority of nerve terminal dynamin I (90%) was not phosphorylated and was associated with the particulate fraction (19). Thus a major biological consequence of the phosphorylation is to cause it to dissociate from the plasma membrane, which would remove it from sites of endocytosis. This might serve as a termination signal for recycling of synaptic vesicles or as a priming signal for dynamin tetramer formation prior to depolarization-dependent endocytosis. Other nerve terminal proteins can cycle between membrane-bound and cytosolic states in a phosphorylation-dependent manner, such as myristoylated alanine-rich C kinase substrate (22, 44) or synapsin I (45–47).

The phosphorylation site for PKC was Ser-795. This is consistent with previous reports that dynamin is phosphorylated exclusively within the proline-rich domain on serine in *vitro* by PKC and in intact nerve terminals by endogenous protein kinases (10, 18, 19). Although the *in vivo* protein kinase for dynamin is reported to be PKC (6), it is unclear how PKC might perform this role under cellular conditions where there is no rise in intracellular Ca^{2+} (9). A synthetic peptide encompassing Ser-795 was not appreciably phosphorylated by PKC *in vitro*, indicating that factors other than the linear sequence around the phosphorylation site determine the substrate specificity in this case. This result was surprising, given that PKC has an unusually high affinity for dynamin as a substrate *in vitro* (19). This suggests that the interaction between dynamin and PKC might involve protein-protein interactions at sites additional to the phosphorylation site, such as the PH domain. Indeed, the PH domain of some other proteins can interact with specific forms of PKC (48–50), and we have also found that dynamin binds cPKC isoforms via its PH domain.

Another potential role for phosphorylation of dynamin I could be to alter its protein-protein interactions. Since Ser-795 is adjacent to a defined binding site for the SH3 domain of p85, we explored this interaction. Dynamin and phospho-dynamin both bound the SH3 domain of p85. Phospho-dynamin I from intact nerve terminals also associates with the SH3 domains of amphiphysin I (8), Grb2, and the p85 subunit of PI 3-kinase when prepared as GST fusion proteins coupled to GSH-agarose (data not shown). In contrast to this, a recent study suggested that dynamin phosphorylated by “cytosolic extracts” lost its ability to associate with the SH3 domain of amphiphysin (15). However, it is not clear whether this effect related to dynamin phosphorylation or to phosphorylation of another component in rat brain cytosol, and it is not known what protein kinase phosphorylated dynamin in that study. Dynamin also associates with other proteins, and a potential role for phosphorylation in these interactions remains to be investigated.

The phospholipid binding study revealed that unassembled dynamin I interacts with a variety of nucleotides, but the highest affinity interaction was with GTP. GTP binding induced conformational changes in dynamin that mimicked phosphorylation, in that phospholipid binding was abolished. This effect that was four times more potent than ATP, GDP, or GMP, and the order of potency was similar to dynamin binding to microtubules (51). The results are distinct from those reported by others (36, 52, 53), since we used dynamin that was not assembled as ring or helix structures. Addition of dynamin I to phospholipid potently stimulates its GTPase activity, particularly in the presence of the SH3 domains of a variety of proteins. However, these events are a result of stimulated dynamin ring assembly and disassembly (4), which is not regulated by guanine nucleotides in the absence of salt (3) but is

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**Fig. 7.** Phosphorylation of dynamin I (Phos-Dyn I) does not alter its association with the SH3 domain of p85. Dynamin I was phosphorylated with cPKC in the presence of [γ-32P]ATP and was repurified. Dynamin and phospho-dynamin (1.0 μg each) were incubated with p85-SH3-GST beads or with GST beads alone and then were separated into supernatant and pellet fractions by brief centrifugation. Samples were applied to an SDS gel and stained with Coomassie Blue (CBB) (A) followed by autoradiography (Autorad.) (B). Phospho-dynamin bound poorly to GST beads (lanes 3 and 7) but strongly to p85-SH3-GST beads (lanes 1 and 5).

| Supernatant | Pellet |
|-------------|--------|
| p85-SH3     | GST    | p85-SH3 | GST |
| +           | -      | +        | -   |

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regulated by guanine nucleotide binding in the presence of salt (54). This potentially relates to the mechanism whereby dynamin I assembles into rings around the necks of recycling synaptic vesicles, which is promoted by GTP binding and not hydrolysis. In contrast, hydrolysis was proposed to shear off the free synaptic vesicle (5). In another study, a guanine nucleotide-dependent conformational change in dynamin I was detected by electron microscopy, where the inter-helix spacing of dynamin spirals was 11 nm in the absence of nucleotide or in the presence of non-hydrolyzable GTP analogues but increased to 20 nm with GDP (2). This suggested that GTP hydrolysis to GDP may be likened to releasing a nanospring, to pop vesicles off the membrane. However, that study and the work of others (3, 54) on dynamin rings may not be related to the present study, where GTP binding induces the changes in dynamin that prevent its association with phospholipids. Therefore PKC phosphorylation and guanine nucleotide binding may coordinately maintain a pool of dynamin in a cytosolic location, possibly to prevent premature dynamin helix assembly.

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