Phosphorylation of a Synaptic Vesicle-associated Protein by an Inositol Hexakisphosphate-regulated Protein Kinase*

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Despite the fact that inositol hexakisphosphate (InsP$_6$) is the most abundant inositol metabolite in cells, its cellular function has remained an enigma. In the present study, we present the first evidence of a protein kinase identified in rat cerebral cortex/hippocampus that is activated by InsP$_6$. The substrate for the InsP$_6$-regulated protein kinase was found to be the synaptic vesicle-associated protein, pacsin/syndapin I. This brain-specific protein, which is highly enriched at nerve terminals, is proposed to act as a molecular link coupling components of the synaptic vesicle endocytic machinery to the cytoskeleton. We show here that the association between pacsin/syndapin I and dynamin I can be increased by InsP$_6$-dependent phosphorylation of pacsin/syndapin I. These data provide a model by which InsP$_6$-dependent phosphorylation regulates synaptic vesicle recycling by increasing the interaction between endocytic proteins at the synapse.

A large number of G-protein-coupled and growth factor receptors activate phospholipase C to stimulate the hydrolysis of the phospholipid, phosphatidylinositol 4,5-bisphosphate, producing inositol 1,4,5-trisphosphate (InsP$_3$) and diacylglycerol. InsP$_3$ has an established second messenger role in mobilizing intracellular calcium stores (1) and can be phosphorylated to inositol 1,3,4,5-tetrakisphosphate (InsP$_4$), which is proposed to play a role in capacitative calcium entry (2) and as an activator of ras-GAP activity (3). The sequential dephosphorylation of InsP$_3$ and InsP$_4$ gives rise to a host of inositol phosphate metabolites (5, 6), InsP$_6$ having the highest affinity to a number of proteins that are involved in exo/endoctyesis including the clathrin assembly proteins AP2 and AP3 and the synaptic vesicle calcium-sensing protein synaptotagmin (9–12).

In the present study, we have investigated the possibility of the existence of a protein kinase that is regulated by InsP$_6$. We found that the pacsin/syndapin I, a synaptic vesicle-associated protein that acts as a molecular link coupling the endocytic machinery to the cytoskeleton, is phosphorylated by a protein kinase that is regulated by InsP$_6$. In addition, InsP$_6$-regulated phosphorylation of pacsin/syndapin I increases the interaction between pacsin/syndapin I and dynamin I. These data provide a novel model by which InsP$_6$ can regulate synaptic vesicle exocytosis.

**EXPERIMENTAL PROCEDURES**

**Anion Exchange Fractionation of Soluble Rat Brain Cortex/Hippocampus Extract**—Rat brain cortex/hippocampus was homogenized in 15 ml of TE buffer (2.5 mM Tris-HCl, 2.5 mM EDTA, pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 50,000 g for 20 min. The supernatant (13 ml at ~2.5 mg of protein/ml) was applied to a 1-ml Resource Q (Amerham Pharmacia Biotech) anion-exchange column (6.4 × 30 mm).

**Fractionation of Kinase Reactions on Hydroxyapatite**—Aliquots of fractions (5 µl, ~5 µg of protein) obtained from the anion exchange column were applied to a 1 ml hydroxyapatite column (Bio-Rad) equilibrated in buffer A (10 mM MgCl$_2$, 1 mM EGTA, 0.5 mM dithiothreitol, 0.2 mM orthovanadate, 25 mM glycerol phosphate, pH 7.5) containing 10 µM [y-$^{32}$P]ATP (1–4 cpm/fmol ATP). The reactions were continued for 10 min at 37 °C and terminated by the addition of 10 µl of trichloroacetic acid (TCA) containing 2% SDS-PAGE sample buffer, and the proteins were resolved by 10% SDS-PAGE.

**Large scale reactions**—Large scale reactions that were to be fractionated on a hydroxypatite column were conducted using 100 µl of anion-exchange fraction in kinase buffer containing 10 µM [y-$^{32}$P]ATP (1–5 cpm/fmol ATP), 1% SDS-PAGE sample buffer, and the proteins were resolved by 10% SDS-PAGE.

**Fractionation of Kinase Reactions on Hydroxypatite**—One ml of the above large scale kinase reaction was applied to a hydroxypatite column with a bed volume of 1 ml (Bio-Rad). The column was washed with 5 ml of buffer A and eluted with a linear gradient of 0–1 M NaCl in TE buffer over 20 ml (flow rate = 1 ml/min). One ml fractions were collected and assayed for kinase activity. The entire procedure was conducted at 4 °C.

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§ The abbreviations used are: InsP$_3$, inositol 1,4,5-trisphosphate; InsP$_4$, inositol 1,3,4,5-tetrakisphosphate; InsP$_5$, inositol 1,2,3,4,5,6-hexakisphosphate; PP-InsP$_3$, diphosphoinositol pentakisphosphate; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; MALDI-TOF, matrix-assisted laser desorption ionization-time-of-flight.
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B (5 mm Tris-HCl, 500 mm KHPO₄, pH 7.5) over 10 ml (flow rate 1 ml/min). One-ml fractions were collected. The proteins in 500 µl of each fraction were concentrated by binding to 20 µl of strataclean slurry (Stratagene). The strataclean beads were pelleted in a microcentrifuge and washed with 1 ml of TE buffer. The beads were resuspended in 2× SDS-PAGE sample buffer, and the proteins were resolved by 10% SDS-PAGE.

Gels were routinely silver stained (Bio-Rad) before autoradiography to ensure that the chromatography of each reaction was the same and that equal amounts of protein had been loaded.

**Immunoprecipitation of Pacsin/Syndapin I**—Immunoprecipitations were carried out essentially as reported previously (13). Large scale kinase reactions using fraction 9 (100 µl) from the anion-exchange fractionation of rat brain cytosolic extract were carried out as described above. The reactions were stopped by the addition of ice-cold TE buffer (500 µl). Pacsin/syndapin I specific antiserum (14) was then added (−5 µg) and incubated for 60 min. The immune complexes were isolated on protein A-Sepharose beads. The beads were washed 3× in TE buffer and resuspended in 20 µl of SDS-PAGE sample buffer. Proteins were then resolved by 10% SDS-PAGE, and an autoradiograph was obtained.

**Construction and Purification of the GST-Pacsin/Syndapin I Bacterial Fusion Protein**—Production of murine GST-pacsin/syndapin I has been previously described (14). To produce the GST-pacsin/syndapin I SH3 domain deletion mutant, site-directed mutagenesis (Transformer Site-directed Mutagenesis Kit, CLONTECH) was used to introduce two BamHI sites, one upstream of the start codon and the other at position 1153 in the murine pacsin/syndapin I coding sequence. The mutation primers used were 5′-CGCTACAGGATCCCATGTCTGGC-3′ and 5′-GATGCCAAGGGGATCCGTGTACGGGC-3′, respectively. The resulting pacsin/syndapin I was subcloned into the BamHI site of the pGEX-3X vector (Amersham Pharmacia Biotech) resulting in the production of GST-pacsin/syndapin I lacking the majority of the SH3 domain (Arg₁⁹⁰→Ile²⁴² was deleted).

**Phosphorylation of the GST-Pacsin/Syndapin I Fusion Protein**—Aliquots of each fraction from the anion exchange fractionation (5 µl containing −5 µg of protein) was added to kinase buffer containing −5 µg of GST-pacsin/syndapin I, 10 µM [γ³²P]ATP (1–4 cpm/fmol ATP), and 10% inositol polyphosphates in a final volume of 100 µl. The reactions were continued for 10 min at 37 °C and terminated by the addition of ice-cold TE buffer (100 µl). GSH-Sepharose slurry (giving 20 µl of packed beads) was added. Sepharose beads were pelleted in a microcentrifuge and washed in TE-buffer three times. SDS-PAGE sample buffer (20 µl) was added to the beads, and the proteins were resolved by 10% SDS-PAGE.

**Pacsin/Syndapin I Association with Dynamin I**—Kinase reactions were performed as described above except with 2 mM ATP. The GST-pacsin/syndapin (~5 µg) bound to glutathione-Sepharose beads was then washed in ice-cold TE buffer and incubated with rat cytosol (1 mg protein) in a final volume of 1 ml for 60 min at 4 °C. The Sepharose beads were then washed three times in ice-cold TE buffer, and the associated proteins were resolved by 10% SDS-PAGE. The gels were transferred to nitrocellulose, and the membranes were probed with anti-dynamin I polyclonal antibody (Santa Cruz Biotechnology). To determine whether there was equal transfer and loading of proteins, membranes were stained with Ponceau before being probed with dynamin I antibodies. Quantification of protein bands in the immunoblots was determined using NIH Image.

**Mass Spectrometry**—Samples were separated on 10–20% tricine SDS-polyacrylamide gel prior to staining with GELCODE colloidal Coomassie stain (Pierce) or a mass spectrometry-compatible silver stain. Excised bands were destained and carbamidomethylated and digested with trypsin prior to analysis of a portion of the digested supernatant by MALDI-TOF mass spectrometry on a TOFSPEC-S.E. instrument (Micromass). Nanoelectrospray mass spectrometry was performed on an API-III triple quadrupole instrument (PE-Sciex).

**RESULTS**

**Identification of the Synaptic Vesicle-associated Protein, Pacsin/Syndapin I, as a Substrate for an InsP₆-regulated Protein Kinase**—The strategy we adopted to identify the presence of an InsP₆-regulated protein kinase was based on the fact that many signaling molecules, including protein kinases and their substrates, form complexes with each other that are robust enough to be purified using techniques such as co-immunoprecipitation (15, 16). It was, therefore, considered possible that the protein substrates for a putative inositol polyphosphate-
regulated protein kinase may form a complex with the kinase that will allow them to co-purify over at least one chromatographic step.

A low speed supernatant extract from rat brain cortex/hippocampus was fractionated over an anion-exchange column. The presence of an InsP₆-regulated protein kinase was assessed by incubating an aliquot of each fraction with [P³²]ATP, in the absence or presence of InsP₆ (50 μM) and resolving the phosphoproteins by SDS-PAGE. The level of phosphorylation of the majority of phosphoproteins contained in these kinase reactions were not affected by the presence of InsP₆ (Fig. 1). However, in fractions 9 and 10, the phosphorylation state of three proteins (Fig. 1, labeled 1–3) appeared to be altered by InsP₆. Interestingly, band 3 appears to have decreased in its level of phosphorylation, whereas the other two protein bands have increased levels of phosphorylation in the presence of InsP₆. We decided to center our attention on the identity of the 52-kDa protein band labeled 1 in Fig. 1.

To determine the identity of the 52-kDa phosphoprotein, large scale kinase reactions containing vehicle, InsP₃, or InsP₆, were performed. The kinase reactions were then fractionated on a hydroxyapatite column, and the phosphoproteins were resolved by 10% SDS-PAGE. Before the gels were exposed to film, they were silver stained to establish the fidelity of the chromatography. The silver stain demonstrates that the chromatography of each of the three kinase reactions on the hydroxyapatite matrix and the amount of protein loaded on each lane were identical (Fig. 2). The autoradiograph of the silver-stained gels revealed that the phosphorylation state of a 52-kDa protein band eluting in fractions 14 and 15 was increased in the presence of InsP₆ but not InsP₃ (Fig. 2). By aligning the silver-stained gel with the autoradiograph, it was possible to determine that the 52-kDa phosphoprotein in fraction 14 lined up exactly with a 52-kDa protein on the silver-stained gel (Fig. 2).

MALDI-TOF analysis of tryptic peptide fragments of the 52-kDa protein band excised from the SDS-PAGE gel showed 28 peptides (marked with a diamond) matched exactly with that of pacsin/syndapin I, covering 63% of the amino acid sequence. B, rat pacsin/syndapin I showing the sequence of five tryptic peptides (in bold) as determined by MS/MS.

Fig. 3. Amino acid sequence analysis of the 52-kDa protein identified to be phosphorylated in an IP₆-dependent manner. A, mass spectra analysis (MALDI-TOF) of tryptic peptide fragments of the 52-kDa protein shown to be phosphorylated in an InsP₆-dependent manner. 28 peptides (marked with a diamond) matched exactly with that of pacsin/syndapin I, covering 63% of the amino acid sequence. B, rat pacsin/syndapin I showing the sequence of five tryptic peptides (in bold) as determined by MS/MS.
PP-InsP$_5$, also known as InsP$_7$, and bis(diphospho)inositol pyrophosphates termed diphosphoinositol pentakisphosphate (bis-PP-InsP$_5$) to determine the ability of inositol ring (18). We used this isomer and an isomer that does not where the pyrophosphate is in the 5-position on the inositol ring (18). The possibility that InsP$_6$ may be exerting its activity via nonspecific effects such as low affinity charge interactions or chelation of divalent or trivalent cations. The polyphosphoinositides, phosphatidylinositol (4,5)-bisphosphate and phosphatidylinositol (3,4,5)-trisphosphate, were found to be unable to stimulate the phosphorylation of GST-pacsin/syndapin I (Fig. 7B). Concentration-response analysis demonstrated that InsP$_6$ was able to stimulate protein kinase activity in a dose-dependent manner with maximal effects at 25–50 $\mu$M (Fig. 7C).

Cellular InsP$_6$ can be phosphorylated to produce the inositol pyrophosphates termed diphosphoinositol tetrakisphosphate (PP-InsP$_4$, also known as InsP$_5$$_2$) and bis(diphospho)inositol tetrakisphosphate (bis-PP-InsP$_4$ or InsP$_8$) (7, 8). The predominant PP-InsP$_4$ isomer found in mammalian cells is 5-PP-InsP$_4$, where the pyrophosphate is in the 5-position on the inositol ring (18). We used this isomer and an isomer that does not naturally occur (2-PP-InsP$_4$) to determine the ability of inositol pyrophosphates to stimulate phosphorylation of pacsin/syndapin I. We found that both isomers of PP-InsP$_4$ stimulated phosphorylation of GST-pacsin/syndapin I (Fig. 8A). Analysis of the concentration-response curve demonstrated that the potency of 5-PP-InsP$_4$ was similar to that of InsP$_6$ (Fig. 8B).

**Phosphorylation of Pacsin/Syndapin I Regulates Its Interaction with Dynamin I**—Pacsin/syndapin I is proposed to regulate synaptic vesicle recycling by acting as a molecular link between the cytoskeleton and the endocytosis machinery (14, 17). In vitro binding studies have demonstrated that one of the binding partners for pacsin/syndapin I is dynamin I (17, 19). Consistent with these previous studies, we show here that GST-pacsin/syndapin I interacts with dynamin I present in a cytosolic rat brain preparation, whereas GST alone was unable to interact (Fig. 9A). Also consistent with earlier studies we found that this interaction appears to be via the SH3 domain because the SH3 domain deletion mutant of pacsin/syndapin I was unable to associate with dynamin I (data not shown).

The possibility that phosphorylation of pacsin/syndapin I contributes to its ability to interact with proteins involved in...
synaptic vesicle endocytosis was tested by analysis of the association of the phosphorylated form of pacsin/syndapin I and dynamin I. InsP$_6$-dependent phosphorylation of GST-pacsin/syndapin I increased the association between pacsin/syndapin I and dynamin I by 3.28 ± 0.99-fold ($n$ = 3 ± S.E.; Fig. 9B).

**DISCUSSION**

In the present study, we have shown that the synaptic vesicle-associated protein, pacsin/syndapin I, is phosphorylated by a novel protein kinase that is regulated by InsP$_6$. Furthermore, InsP$_6$-dependent phosphorylation of pacsin/syndapin I increases the ability of pacsin/syndapin I to interact with dynamin I.

The strategy we adopted for the discovery of the InsP$_6$-regulated protein kinase and its protein substrate was based on the hypothesis that many signaling proteins form complexes with each other that are robust enough to allow them to be co-purified. This phenomenon has been described for the platelet-derived growth factor receptor, which forms a so-called "signal transduction particle" with PLC$_{y}$, phosphatidylinositol 3-kinase, GAP, and raf (15). Other signal transduction protein kinases, such as those of the MAP kinase pathway, form signaling complexes that require the presence of scaffolding proteins (16). In the present study we analyzed chromatographic fractions from rat brain for the presence of co-purifying InsP$_6$-regulated protein kinase and its substrate proteins. We found that a 52-kDa protein eluting from an anion exchange column was phosphorylated in response to InsP$_6$. This protein was determined to be the synaptic vesicle protein, pacsin/syndapin I, on the basis of MALDI-TOF and amino acid sequence analysis and immunoprecipitation experiments. The identification of the phosphorylation of pacsin/syndapin I is the first demonstration of a protein phosphorylation that is regulated by InsP$_6$. We are currently using the InsP$_6$-dependent phosphorylation of the GST-pacsin/syndapin I fusion protein in an assay to purify the InsP$_6$-regulated protein kinase.

Pacsin/syndapin I is a 52-kDa protein that is highly enriched in brain and associated with presynaptic terminals where it co-localizes with dynamin I (14, 17). *In vitro* binding studies have demonstrated that the SH3 domain of pacsin/syndapin I is able to bind to the proline-rich C terminus of dynamin I, as
increase the ability of pacsin/syndapin I to interact with dynamin I. This process may either contribute to the assembly of the endocytic machinery or tether the endocytic multiprotein complex to the cytoskeleton.

Our study, therefore, adds to a growing body of evidence to suggest that InsP₆ is involved in synaptic vesicle trafficking and membrane trafficking. The clathrin assembly proteins AP2 and AP3, together with the visual and non-visual arrestins and synaptotagmin, have been shown to bind InsP₆ with high affinity (9–12, 23). The binding of InsP₆ to these proteins regulates their interaction with the endo/exocytotic pathways. The present study extends a role for InsP₆ in endocytosis by demonstrating that InsP₆-dependent phosphorylation is a mechanism that can increase the interaction between specific endocytic proteins.

The discovery of an InsP₆-regulated protein kinase supports a signaling role for this inositol polyphosphate. Importantly, none of the lower inositol phosphates stimulated kinase activity. Furthermore, kinase activity was not stimulated by the polyphosphoinositides. This is of significance because a number of high affinity InsP₆-binding proteins have also been shown to bind the polyphosphoinositides, and it is in fact the binding of polyphosphoinositides that is thought in some instances to be the physiological regulators of these proteins (11, 24). We also tested the ability of the inositol pyrophosphates to stimulate kinase activity. Both isomers of PP-InsP₆ stimulated kinase activity with a concentration-response curve that was very similar to InsP₆. Thus, the InsP₆-regulated protein kinase is activated by inositol that contains a phosphate group on all 6 positions of the inositol ring and that addition of a further phosphate to make a pyrophosphate at positions 5 or 2 does not change the activation profile. It is, therefore, possible that both InsP₆ and/or 5-PP-InsP₆ could be the physiological activators of the protein kinase identified here. However, it is interesting to note that the concentration-response curve for InsP₆ is consistent with a protein kinase that would be expected to respond to cellular concentrations of InsP₆, which are thought to be in the μM range (5, 6).

Early work on InsP₆ demonstrated that it is ubiquitously present at high cellular levels, and it was thought to be involved in metal ion chelation or act as a phosphate store (25, 26). However, studies demonstrating that InsP₆ levels can change in response to receptor stimulation suggested that InsP₆ may have a signaling role (27, 28). Recently, InsP₆ has been shown to be involved in the regulation of mRNA export from the nucleus (29), the control of phosphatase activity in pancreatic β cells (28), and to act as a key factor in DNA repair (30). Our studies demonstrating the presence of a protein kinase that is specifically activated by InsP₆ adds a novel signaling pathway for this inositol polyphosphate.

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