Communication

Synaptojanin Is the Major Constitutively Active Phosphatidylinositol-3,4,5-trisphosphate 5-Phosphatase in Rodent Brain*

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The major constitutive phosphatidylinositol-3,4,5-P$_3$ (PtdIns) 5-phosphatase activity was purified and subjected to peptide sequence analysis providing extensive amino acid sequence which was subsequently used for cloning the cDNA. Peptide and cDNA sequences revealed that the purified PtdIns(3,4,5)P$_3$ 5-phosphatase was identical to a splice variant of a recently cloned inositol polyphosphate 5-phosphatase termed synaptojanin. Since synaptojanin is not known to possess PtdIns(3,4,5)P$_3$ 5-phosphatase activity, we verified that the purified PtdIns(3,4,5)P$_3$ 5-phosphatase activity and synaptojanin are identical by Western blot using specific antibodies raised against synaptojanin sequences. Immunoprecipitation from crude lysates of rat brain tissue showed that synaptojanin accounts for the major part of the active PtdIns(3,4,5)P$_3$ 5-phosphatase activity. It is also shown that the protein is localized to the soluble fraction. Expression of a truncated recombinant protein demonstrates that the conserved 5-phosphatase region of the synaptojanin gene expresses PtdIns(3,4,5)P$_3$ 5-phosphatase activity. However, immunological analysis demonstrates that the PtdIns(3,4,5)P$_3$ 5-phosphatase activity expressed from the synaptojanin gene in brain is due to a particular splice variant which contains a 16-amino acid insert as shown by immunoprecipitation using a specific antibody raised against this particular splice variant.

The regulation of inositol phospholipid metabolism has provided a cornerstone to at least two signal transduction pathways, one involving the phospholipase C-dependent phosphodiesterase cleavage of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P$_2$) (1) and the second the phosphorylation of this lipid by PtdIns 3-kinases yielding PtdIns(3,4,5)P$_3$ (2, 3). This latter lipid is observed in many signaling pathways (PKB/akt, p70S6K, PKC, PRK) as has its metabolite PtdIns(3,4)P$_2$ (4–9). It has been suggested that the 5-phosphatase responsible for the transformation of PtdIns(3,4,5)P$_3$ to PtdIns(3,4)P$_2$ (10) is due to a recently cloned 5-phosphatase termed SIP (11) or SHIP (p145 SHIP) (12–14) which has specificity for 3-phosphorylated inositol phosphates (11–14). However, other PtdIns(3,4,5)P$_3$ 5-phosphatases have been identified (15, 16) indicating that this issue is still not resolved.

We have previously identified and purified the major constitutive PtdIns(3,4,5)P$_3$ 5-phosphatase from rodent brain tissue (16). Here we demonstrate that this protein is encoded by a particular splice variant of the recently described synaptojanin gene. It is further shown that a recombinant synaptojanin protein has intrinsic PtdIns(3,4,5)P$_3$ 5-phosphatase activity. Thus, we are able to show that synaptojanin which to date has not been associated with PtdIns 3-kinase signaling could play a major role in PtdIns 3-kinase-dependent pathways.

EXPERIMENTAL PROCEDURES

Microsequencing of Purified Rat PIP$_3$ 5-Phosphatase—The 5-phosphatase was purified (16), further fractionated by SDS-polyacrylamide gel electrophoresis, and stained with Coomassie Brilliant Blue R250. The bands were excised and digested with lysyl endopeptidase (Wako Chemicals) or with endoproteinase Asp-N (Boehringer Mannheim). Peptides were extracted for 2 h in a sonication water bath. After concentration the peptides were resolved using an Aqapore AX-300 (30 × 2.1 mm) and OD-300 (150 × 2.1 mm) columns connected in series on a Hewlett-Packard 1090/4H high performance liquid chromatography system. The columns were developed with a linear acetonitrile gradient in 0.1% trifluoroacetic acid. Peptide elution was monitored by means of diode-array detection (200–600 nm). Peptides were sequenced using an ABI 494 HT Procise sequencer employing fast cycles as described previously (17). Initial yields were in the 2–10-pmol range.

Expression of Synaptojanin Constructs in COS7 Cells—Details of the cloning of the PIP$_3$ 5-phosphatase will described elsewhere. Briefly, primers were designed based on the obtained peptide sequence and then used to amplify the corresponding cDNA sequence using the polymerase chain reaction (PCR) from a rat brain library (Stratagene). The cDNA produced was then used as a probe to search for PIP$_3$ 5-phosphatase clones.

For expression of the PIP$_3$ 5-phosphatase in COS7 cells, a cDNA encoding residues 401–1308 (Syn N-del; including the 16-amino acid insert) was subcloned into a pCDNA3 derivative which had been modified to contain a myc epitope (MEQKLISEEDL) followed by an in-frame EcoRI site. Expression of the myc-tagged protein was driven from a cytomegalovirus promoter. The correct sequence of the myc-tagged phosphatase construct was confirmed by DNA sequence analysis. COS7 cells were transfected by electroporation.

Immunoprecipitation and Phosphatase Assay—Antibodies were raised against the C terminus of synaptojanin (C-term) or the unique splice sequence “GVGAPPSPGVTRREME” (insert) in rabbits. The corresponding peptide antigen was included in immunoprecipitation experiments or Western blots as a control. Antibodies raised against the unique insert region (insert), the C terminus (C-term), or the myc epitope were bound to protein A beads. Antbody-loaded beads were

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then incubated for 3 h with rat brain fractions at 4 °C. The supernatant was separated by centrifugation, and the beads were washed several times using phosphate-buffered saline and phosphate-buffered saline containing 0.5 M NaCl. Supernatant and washed beads were then assayed for PtdInsP$_3$-5-phosphatase activity as described (16). For Western blots, the extracts were subjected to SDS-gel electrophoresis transferred onto PVDF or nitrocellulose membranes and incubated with the indicated antibodies. Proteins were visualized by ECL (Amersham). All other methods were done as described previously (16).

RESULTS

The 145-kDa protein previously purified and identified as a PtdIns(3,4,5)P$_3$-5-phosphatase was subjected to proteolytic fragmentation and amino acid sequence analysis. A total of 23 peptide sequences covering 361 amino acids were determined (not shown). During the course of the cDNA cloning of this protein, the sequence of synaptojanin was reported (18), and it became clear that the 145-kDa protein sequenced was in fact a particular splice variant of synaptojanin containing a defined 16-amino acid insert (see Fig. 1). For simplicity the purified protein will be termed hereafter p145Isynj (145-kDa species of synaptojanin containing the 16-amino acid insert); the insert-less protein is denoted p145synj.

Synaptojanin is a component of synaptic vesicles and is thought to play a key role in vesicle traffic at the synapse (18). PtdIns 3-kinase activities are themselves implicated in vesicle traffic in non-neuronal cells. For example by analogy to the PtdIns 3-kinase activities are themselves implicated in vesicle traffic at the synapse (18). There is also circumstantial evidence for PtdIns(4,5)P$_2$ 3-kinase activities controlling endosome fusion (21–23) as well as trans-Golgi to lysosome traffic (19, 20). Synaptojanin is a key issue. However, PtdIns(3,4,5)P$_3$ 5-phosphatase activity has not been found associated with synaptojanin (18); thus it has been important to establish this identity. However, PtdIns(3,4,5)P$_3$ 5-phosphatase activity is very little p145 synj protein shows that in fact this protein represents the major constitutive activity expressed in brain tissue. The expression of recombinant proteins encompassing the catalytic domain of the protein (see Fig. 4). Transient expression in COS cells correlates with the production of a protein that was immunoreactive with p145synj antisera (panel A). Activity determination demonstrated that this protein had PtdIns(3,4,5)P$_3$ 5-phosphatase activity (panel B). Similar observations have been made for a C-terminal deletion that does not encode the splice insert (data not shown).

FIG. 1. The purified PtdInsP$_3$-5-phosphatase is identical to synaptojanin. The purified PtdInsP$_3$-5-phosphatase (16) was subjected to Edman microsequencing using Asp-N and Lys-C digests. The determined peptide sequences (light gray and black boxes) are aligned with the synaptojanin sequence (18) (shown in white). Synaptojanin consists of at least two forms due to splicing which inserts a short peptide sequence (GVGAPPSPGVTRREME) in the C-terminal part of the protein (black box). Sequences which were used as antigens for antibody production are shown as black boxes; these had also been identified by Edman microsequencing.

This was also specific as demonstrated by antigen competition (Fig. 3, panel A).

To establish that the synaptojanin gene encodes a protein with intrinsic PtdIns(3,4,5)P$_3$ 5-phosphatase activity, an N-terminally deleted expression construct was prepared that included the predicted catalytic domain of the protein (see Fig. 4). Transient expression in COS cells correlates with the production of a protein that was immunoreactive with p145synj antisera (panel A). Activity determination demonstrated that this protein had PtdIns(3,4,5)P$_3$ 5-phosphatase activity (panel B). Similar observations have been made for a C-terminal deletion that does not encode the splice insert (data not shown).

DISCUSSION

The results presented here demonstrate that p145synj has intrinsic PtdIns(3,4,5)P$_3$ 5-phosphatase activity. The immunodepletion of PtdIns(3,4,5)P$_3$ 5-phosphatase activity, along side of p145synj protein shows that in fact this protein represents the major constitutive activity expressed in brain tissue. The expression of recombinant proteins encompassing the catalytic domain (with or without the splice insert) indicates that all the known splice variants from this gene would retain PtdIns(3,4,5)P$_3$ 5-phosphatase activity. However, the p145synj variant itself appears to be the major form present in brain based upon the immunodepletion data. This conclusion is entirely consistent with the purification of this activity from rat brain, since only a single peak of constitutive activity was observed (16), and as shown here this activity purified to near homogeneity after SDS-polyacrylamide gel electrophoresis yielding p145synj.

The properties of the native purified 5-phosphatase protein have been characterized and provide evidence of a much higher affinity for PtdIns(3,4,5)P$_3$ over PtdIns(4,5)P$_2$. Indeed the pro-
tein was purified on the basis of activity determined against 32P-labeled PtdIns(3,4,5)P3 in the presence of a 10-fold excess of unlabeled PtdIns(4,5)P2 (16). This relative preference for PtdIns(3,4,5)P3 contrasts with the related p145SHIP (12–14) which has been reported to be devoid of 5-phosphatase activity toward PtdIns(4,5)P2 and the 43-kDa inositol polyphosphate 5-phosphatase which does not hydrolyze inositol lipids (26, 27). Interestingly p145SHIP itself has poor activity toward PtdIns(3,4,5)P3 compared with the 75-kDa type II phosphatase (11, 15). The molecular basis for these distinctions must await structural analysis.

The PtdIns(3,4,5)P3 5-phosphatase activity of synaptojanin implicates the complex regulation of PtdIns(4,5)P2-PtdIns(3,4,5)P3-PtdIns(3,4)P2 inositol lipid metabolism in the directional cycle of events required for neurotransmitter granule recycling in the synapse. However it is clear that both p145synj and p145synj are largely cytosolic in brain extracts although there is a higher proportion of synaptojanin in the particulate fraction of synaptosomes (data not shown) which is

FIG. 3. Antibodies against synaptojanin immunoprecipitate PtdInsP3 5-phosphatase activity from rat brain cytosol. Rat brain cytosol and membrane fractions were prepared as described earlier (16). A, antibodies raised against the unique insert region (insert) and the C terminus (C-term.) were bound to protein A beads. For comparison, protein A beads were loaded with a bovine IgG fraction (control). Antibody-loaded beads were then incubated for 3 h with rat brain cytosol at 4 °C in the presence (+) or absence (−) of competing peptide antigen. The supernatants were separated by centrifugation, and the beads were washed and then assayed for PtdInsP3 5-phosphatase activity as described (16). The autoradiograph of a thin layer chromatography separation of the lipid products of the phosphatase assay is shown; the position of the phosphoinositides are indicated by arrows. B, aliquots of the supernatants from the immunoprecipitation experiment (see panel A) were separated by SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membranes (Western blot). The membranes were incubated with insert or C terminus antibodies. The position of the 145-kDa band is indicated. C, aliquots of the supernatants from the immunoprecipitation experiment (see panel A) were separated by SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membranes (Western blot). The membranes were incubated with insert or C terminus antibodies. The position of the 145-kDa band is indicated. C, aliquots of the supernatants from the immunoprecipitation experiment (see panel A) were separated by SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membranes (Western blot). The membranes were incubated with insert or C terminus antibodies. The position of the 145-kDa band is indicated.
in agreement with recent studies (28). Hence, a dynamic interaction of synaptojanin with the membrane has to be proposed. Such behavior would be compatible with many other "membrane-interacting" proteins (e.g. protein kinase C (29)) and "membrane-metabolizing" proteins (e.g. PtdIns-phospholipase C (30)) involved in regulatory processes. The ability of amphiphasin to interact with synaptojanin and so tether it to neurotransmitter granules may be a crucial element in the protein's role (18).

The involvement of 3-phosphorylated polyphosphoinositides in vesicle traffic processes in mammalian cells has been implied through the use of the fungal metabolite wortmannin (5, 22, 31) which is a potent inhibitor of a number of the phosphoinositide 3-kinases (32, 33). However, the specific role of PtdIns(3,4,5)P₃ is unknown in this context. Indeed it is not clear whether PtdIns(3,4,5)P₃ or actually PtdIns(3,4)P₂ is the key element. The identification of p145tyr as a PtdIns(3,4,5)P₃ 5-phosphatase provides evidence that in neuronal cells the metabolism of PtdIns(3,4,5)P₃ in granules or following fusion at synaptic termini is a critical component in their cycling. Whether this is a part of the cycling itself, for example by marking the vesicle at one stage in its cycle, or whether this is simply a negative regulatory device to effectively remove PtdIns(3,4,5)P₃ from the vesicle remains to be determined.

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