Inhibition of Phospholipase D by Clathrin Assembly Protein 3 (AP3)*

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We recently showed that rat brain cytosol contains proteins that inhibit the activity of partially purified brain membrane phospholipase D (PLD)1 stimulated by ADP-ribosylation factor (ARF) and phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) (1). Sequential chromatography of the brain cytosolic preparation yielded four inhibitory fractions, which exhibited differential sensitivity to heat. The most heat-labile inhibitor (inhibitor IA) was purified to yield a 150-kDa protein, which, in the accompanying paper (2), we showed to be synaptojanin, a nerve terminal-enriched protein with inositol polyphosphate phosphatase (IPPase) activity, and which inhibits PLD by hydrolyzing PI(4,5)P2.

We now describe the purification of PLD inhibitor IB, which has an apparent molecular mass of 165 kDa and is less susceptible to heat treatment than synaptojanin. Sequences of tryptic peptides of inhibitor IB suggest that it is identical to clathrin assembly protein 3 (AP3). We further show that AP3 binds to PLD.

EXPERIMENTAL PROCEDURES

Materials—The sources of rat brains, bovine brain phosphatidylcholine (PC), phosphatidylethanolamine (PE), GTPγS, PI(4,5)P2, choline-methyl-14C)-dipalmitoylphosphatidylcholine (ppam)PC (50 Ci/mmol), 1,2-palmitoyl-4,5,10,10-(H)pam)PC (89 Ci/mmol), and n-octyl-β-D-glucopyranoside were as described previously (2). Phytic acid (inositol hexaphosphate, or IP6) and monoclonal antibodies to AP3 (clone AP180-I) were obtained from Sigma. PLD, ARF, human PLD1 (hPLD1), phospholipase C-β1 (PLC-β1), PLC-γ1, and PLC-δ1 were purified as described (2). Purified clathrin triskelia was a gift from Drs. Evan Eisenberg and Ruo-Fan Jiang (National Institutes of Health). Rabbit antiserum specific to hPLD1 was as described (3).

Assay of PLD and PLC—Procedures for assaying the activities of PLD and PLC were as described in the accompanying paper (2).

Purification of PLD Inhibitor IB—Inhibitor IB was partially purified from the 0–35% ammonium sulfate precipitate of rat brain cytosol by sequential chromatography on DEAE-Sephal, DEAE-5PW, phenyl-Sepharose; GST, glutathione S-transferase; PBS, phosphate-buffered saline; PEtOH, phosphatidylethanol; PA, phosphatidic acid; GTPγS, guanosine 5′-O-(3′-thiotriphosphate); Mes, 4-morpholineethanesulfonic acid.

1 The abbreviations used are: PLD, phospholipase D; ARF, ADP-ribosylation factor; PI(4,5)P2, phosphatidylinositol 4,5-bisphosphate; IPPase, inositol polyphosphate phosphatase; AP3, clathrin assembly protein 3; PC, phosphatidylcholine; (ppam)PC, dipalmityloyl PC; PE, phosphatidylethanolamine; IP6, inositol hexakisphosphate; hPLD1, human PLD1; PLC, phospholipase C; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; PBS, phosphate-buffered saline; PEtOH, phosphatidylethanol; PA, phosphatidic acid; GTPγS, guanosine 5′-O-(3′-thiotriphosphate); Mes, 4-morpholineethanesulfonic acid.

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3.230 column (3.2 x 300 mm, Pharmacia Biotech Inc.) and chromatography was performed with a Pharmacia-LKB SMART System equipped with a μ separation unit and a μ precision pump. Proteins were eluted at a flow rate of 40 μl/min with a solution containing 20 mM Tris-HCl (pH 7.5) and 150 mM NaCl (Fig. 1B). Fractions of 40 μl were collected, assayed for enzymatic activity, and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE).

**Peptide Sequence Analysis**—Purified PLD inhibitor IB (500 μg) from the Superose 12 PC 3.2/30 column was denatured and reduced by treatment with 50 mM Tris-HCl (pH 8.0), 6 M guanidine hydrochloride, and 2 mM dithiothreitol. The sulphydryl groups were labeled with 2-nitro-5-thio-chromobenzoyl by adding Ellman reagent (5′-dithiobenzoyl acid) to a final concentration of 10 mM, and the resulting conjugated protein was precipitated with 10% (w/v) trichloroacetic acid. The pellet was washed with cold acetone, suspended in 50 mM Tris-HCl (pH 8.0), and digested overnight at 37 °C with trypsin. The generated peptides were applied to a C18 column (4.6 x 250 mm, Vydac) that had been equilibrated with 0.1% trifluoroacetic acid, and were eluted at a flow rate of 1 mL/min with a 60-μl linear gradient of 0–60% (v/v) acetonitrile in 0.1% trifluoroacetic acid. Peptides were detected by measuring absorbance at 215 nm.

**Preparation of GST Fusion Proteins Containing AP3 or Various Truncated AP3 Fragments**—An expression vector (pGEX-5X-F1–20 (AS15)) that encodes a fusion protein consisting of glutathione S-transferase and the full-length rat AP3 (505 residues, described (4)). Truncated DNAs corresponding to the NH2-terminal 289 amino acid residues (AP3–1–289), NH2-terminal 320 residues (AP3–1–320), and COOH-terminal 595 residues (AP3–321–915) were prepared by polymerase chain reaction using the plasmid pGEX-5X-F1–20 (AS15) as the template. The 5′ primer (AAAAAAGCGGGCGCAAT- GTGCATTAGCCTCACGTC) was used. The polymerase chain reaction products were purified, digested with Not I and BamHI, and ligated into pGEX-4T1 (Pharmacia) to construct the respective GST fusion protein expression vector.

**Escherichia coli DH5α cells** were transfected with each of the expression vectors. The vectors obtained were retransfected into E. coli BL21 cells. The colonies containing each vector were grown at 37 °C. Expression of the fusion proteins was then induced for 6 h at 37 °C with 0.1 mM isopropyl-β-D-thiogalactopyranoside. The cells were sonicated in phosphate-buffered saline (PBS) and centrifuged at 5,000 × g for 15 min. The resulting supernatant was mixed with a 50% (w/v) slurry of glutathione-Sepharose 4B (Pharmacia) in PBS and incubated at room temperature for 30 min. After centrifugation at 5,000 × g for 30 min, the supernatant was transferred and the pellets were resuspended in 30 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5 mM MgCl2, and 1 mM CaCl2 as described (8). The assembled cages were precipitated by ultracentrifugation at 100,000 × g for 5 min at 4 °C, washed three times with 500 μl of the isolation buffer, and suspended in 150 μl of the buffer. The clathrin cage solution was centrifuged at 13,000 × g for 5 min to remove nonspecific aggregates. Complexes of AP3 with clathrin cage were formed by incubating preassembled clathrin cages at 0.45 μM (corresponding to the molarity of the triskelia) and 4.5 μM AP3 in 400 μl of isolation buffer without CaCl2 for 1 h on ice with rocking. The clathrin cage-AP3 complex was precipitated by ultracentrifugation at 100,000 × g for 4 min at 4 °C and was washed three times with 500 μl of the isolation buffer without CaCl2. The 1:1 stoichiometry of clathrin triskelia and AP3 in the complex (5) was confirmed by SDS-PAGE, followed by Coomassie Blue staining and Western blot analysis using antibodies to AP3.

**Binding of GST Fusion Proteins to PLDI—GST or GST fusion proteins containing AP3–1–289, AP3–1–320, or AP3–321–915** were incubated with hPLD1 for 30 min on ice. The final reaction mixture contained 1 μM GST or GST fusion protein and partially purified hPLD1 in 1.5 μl of total volume of 150 μl (50 mM Tris-HCl (pH 8.0), 10 mM KCl, 1 mM dithiothreitol, and 0.7% (w/v) n-octyl-β-D-glucopyranoside). Ten microliters of 50% (w/v) slurry of glutathione-Sepharose 4B was added to the mixture and incubated for 5 min on ice with rocking. After centrifugation at 12,000 × g for 2 min, the resulting supernatants were transferred and the pellets were washed three times with 1 ml each of the binding buffer. Both the pellet and supernatant fractions were subjected to electrophoresis on an 8% SDS-polyacrylamide gel, and hPLD1 was visualized by alkaline phosphatase-immunoblotting using rabbit antibodies to hPLD1 (3).

**RESULTS**

We previously showed that chromatography of rat brain cytosol on a DEAE-Sepahcel column yielded three partially resolved peaks when fractions were assayed for the ability to inhibit PLD activation by ARF and PI(4,5)P2 (1). Fractions corresponding to the first peak were further purified by high performance liquid chromatography on DEAE-5PW and phenyl-5PW columns, the latter of which yielded two well separated peaks, termed IA and IB. Chromatography of peak fractions containing inhibitor IB on a heparin-5PW column yielded one major and two minor inhibitor peaks (1). We have now subjected fractions corresponding to the major peak to sequential chromatography on a Mono-S column (Fig. 1A) and a Superose 12 gel filtration column (Fig. 1B). These procedures yielded single peak of PLD-inhibitory activity corresponding to an estimated molecular size of 370 kDa. Analysis of the peak fractions by SDS-PAGE revealed three broad bands with apparent molecular masses of 165, 155, and 135 kDa, as well as several minor bands (Fig. 1C). Repeating the purification procedure revealed that the relative abundance of the three proteins varied (as the relative intensity of the 165-kDa band decreased, those of the 155- and 135-kDa bands increased) and that the inhibitory activity correlated with none of the three bands alone but rather with the sum of all three. These results suggest that the three proteins are related and components of the PLD inhibitor IB.

The inhibitor IB preparation from the gel filtration column was digested with trypsin, and the resulting peptides were isolated on a C18 column (Fig. 2). Four of the purified peptides were identified and all corresponded to deduced N-terminal sequences of rat AP3 (9, 10). Peptides 1–4 corresponded to AP3 residues 150–157, 789–795, 113–128, and 872–886, respectively. Immunoblot analysis of inhibitor IB from the Mono-S column with a monoclonal antibody to AP3 detected not only the protein with 165-kDa protein but also the 155- and 135-kDa proteins (see Fig. 4), suggesting that the three proteins correspond to AP3 and its proteolytic fragments. The PLD inhibitor IB is

**Measurement of Proteins**—The amounts of AP3, GST-AP3, GST-AP3–1–289, GST-AP3–1–320, GST-AP3–321–915, and GST were determined spectrophotometrically with extinction coefficients (εmax) of 39,970, 80,650, 60,100, 60,100, 65,590, and 40,680, respectively (7).

**Complex of Clathrin Cage and AP3—Clathrin triskelia were assembled into cages by dialyzing overnight against isolation buffer (0.1 M NaCl, 10 mM MgCl2, and 1 mM CaCl2) as described (8). The assembled cages were precipitated by ultracentrifugation at 100,000 × g for 20 min at 4 °C, washed three times with 500 μl of the isolation buffer, and suspended in 150 μl of the buffer. The clathrin cage solution was centrifuged at 13,000 × g for 5 min to remove nonspecific aggregates. Complexes of AP3 with clathrin cage were formed by incubating preassembled clathrin cages at 0.45 μM (corresponding to the molarity of the triskelia) and 4.5 μM AP3 in 400 μl of isolation buffer without CaCl2 for 1 h on ice with rocking. The clathrin cage-AP3 complex was precipitated by ultracentrifugation at 100,000 × g for 4 min at 4 °C and was washed three times with 500 μl of the isolation buffer without CaCl2. The 1:1 stoichiometry of clathrin triskelia and AP3 in the complex (5) was confirmed by SDS-PAGE, followed by Coomassie Blue staining and Western blot analysis using antibodies to AP3.

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therefore subsequently referred to as AP3. It was previously shown that AP3 migrates anomalously on SDS-PAGE because the high density of acidic residues in the central domain interferes with SDS binding. Its apparent molecular mass ranges from 155 to 185 kDa depending on the conditions, whereas its molecular mass predicted from the corresponding cDNA is 91.4 kDa (9, 10). Purified AP3 appears as multiple bands on SDS-PAGE because it is highly susceptible to proteolytic attack (5, 6). In addition, AP3 is phosphorylated (9, 11) and glycosylated (11), both of which may cause broadening of AP3 bands on SDS-PAGE.

To exclude the possibility that a contaminating protein accounted for the inhibition of PLD activity, we compared the effects of GST-AP3 and purified rat brain AP3 (from the final gel filtration column) on hPLD1 activity. Bacterially expressed GST-AP3 was purified with the use of glutathione-Sepharose beads and gel filtration chromatography. Both AP3 (from rat brain) and GST-AP3 inhibited hPLD1 activity stimulated by ARF and PI(4,5)P2 in a concentration-dependent manner and exhibited similar potencies. GST alone had no effect on hPLD1 activity (Fig. 3).

AP3 binds inositol polyphosphates with high affinity (7, 12). Because many inositol polyphosphate-binding proteins also bind PI(4,5)P2, it was possible that AP3 inhibited PLD activity by masking PI(4,5)P2. We therefore evaluated the ability of AP3 to bind PI(4,5)P2 with the use of lipid vesicles of identical composition to those used for the PLD assay. The amount of AP3 that associated with the PI(4,5)P2-containing vesicles was markedly greater than that associated with lipid vesicles containing only PE and PC (Fig. 4). When the amount of AP3 added to lipid vesicles was reduced by one-half, the ratio of the amount of AP3 associated with the pellet to that present in the supernatant was not affected (data not shown), suggesting that the number of binding sites was not a limiting factor. When the pellets were washed more than once, the amount of AP3 associated with the vesicles devoid of PI(4,5)P2 decreased to virtually undetectable levels, and the amount of AP3 associated with the PI(4,5)P2-containing vesicles was also reduced proportionally (data not shown). These results suggest that AP3 interacts with PI(4,5)P2 with low affinity.

The AP3-dependent inhibition of hPLD1, which is stimulated by ARF and PI(4,5)P2, was measured with substrate vesicles containing PE and [choline-methyl-3H]pam, PC and either containing or not containing PI(4,5)P2. The ARF-dependent activity of hPLD1 in the absence of PI(4,5)P2 is ~5% of that

![Fig. 1. Purification of PLD inhibitor IB.](http://www.jbc.org/)

![Fig. 2. Isolation and sequences of tryptic peptides of PLD inhibitor IB.](http://www.jbc.org/)
in its presence, so the amount of hPLD1 used to measure activity in the absence of PI(4,5)P₂ was 20 times that used in presence. AP3 inhibited the generation of [3H]choline with similar efficacies and potencies in the presence and absence of PI(4,5)P₂ (Fig. 5A). To confirm this result, we measured the production of phosphatidylethanol (PEtOH), as well as that of phosphatidic acid (PA), from [2-palmitoyl-9,10-3H](pam)₂PC in the presence of ethanol. The production of both PEtOH and PA was inhibited by 43 nM AP3 to similar extents in the absence and presence of PI(4,5)P₂ (Fig. 5B). These results suggest that the inhibition of hPLD1 by AP3 is not attributable to the weak interaction of AP3 with PI(4,5)P₂.

We next investigated the effects of AP3 on the activities of PLC-β₁, PLC-γ₁, and PLC-δ₁ with substrate vesicles identical to those used for the PLD assay, with the exception that the

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\text{FIG. 3. Effects of various concentrations of rat brain AP3 (○), GST-AP3 (●), and GST (■) on PLD activity stimulated by ARF and PI(4,5)P₂. Data are expressed as a percentage of the PLD activity (75 nmol/mg/min) apparent in the absence of test-protein and are mean (± S.E.) values of three independent experiments.}
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\text{FIG. 4. Binding of AP3 to PI(4,5)P₂. Rat brain AP3 (from Mono-S column) was incubated at 37 °C for 30 min and on ice for 5 min with lipid vesicles containing PE and PC or with lipid vesicles containing PE, PI(4,5)P₂, and PC, as indicated. The final concentration of AP3 was 104 nM. After centrifugation of the incubation mixtures, pellets (P) and supernatants (S) were subjected to SDS-PAGE on 8% gels and subsequent immunoblot analysis with the monoclonal antibody to AP3. The 165-, 155-, and 135-kDa forms of AP3 are indicated by arrows on the right.}
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\text{FIG. 5. Effects of AP3 on hPLD1 activity in the absence or presence of PI(4,5)P₂. A, hPLD1 (216 ng (●) or 11 ng (○)) was incubated in the presence of the indicated amounts of purified rat-brain AP3 and phospholipid vesicles containing either PE and PC or [choline-methyl-3H](pam)₂PC in a molar ratio of 16:1 (●) or PE, PI(4,5)P₂, and [choline-methyl-3H](pam)₂PC in a molar ratio of 16:1.4:1 (○). The final concentration of PC was 3.4 μM. After incubation for 30 min at 37 °C, the reaction was stopped and the released [3H]choline was quantitated as a measure of PLD activity as described (2). Data are expressed as a percentage of the respective PLD activity observed in the absence of AP3 and the means (± S.E.) of three independent experiments. The activity corresponding to 100% was 1.2 nmol/mg/min in the absence of PI(4,5)P₂ and 80 nmol/mg/min in the presence of PI(4,5)P₂. B, hPLD1 (11 ng for lanes 2 and 3, and 216 ng for lanes 5 and 6) was incubated with phospholipid vesicles in a reaction mixture containing 0.67% (v/v) ethanol in the absence (lanes 2 and 5) or presence (lanes 3 and 6) of 86 nM AP3. The substrate vesicles comprised either PI(4,5)P₂ and [2-palmitoyl-9,10-3H](pam)₂PC in a molar ratio of 16:1 (lanes 1–3) or PE and [2-palmitoyl-9,10-3H](pam)₂PC in a molar ratio of 16:1 (lanes 4–6). The final concentration (3.4 μM) and specific radioactivity of PC were identical in the two substrate systems. After incubation for 1 h at 37 °C, the lipid products were analyzed by thin layer chromatography and visualized by autoradiography as described (2). The positions of PC, PA, and PEtOH are indicated. Lanes 1 and 4 represent analysis of the reaction mixture before the addition of hPLD1. Data are representative of two similar experiments.}
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Fig. 6. Effects of various concentrations of AP3, preassembled clathrin cages, or AP3 bound to preassembled cages on hPLD1 activity stimulated by ARF and PI(4,5)P2. The assembly of clathrin cage and the preparation of complexes containing AP3 and clathrin cages were described under “Experimental Procedures.” hPLD1 was incubated in the presence of the indicated concentration of AP3 (○), clathrin cages (□), or AP3 bound to clathrin cages (●). The concentrations of clathrin cages and AP3 bound to clathrin cages were expressed as nanomolar amounts of clathrin triskelia or bound AP3, respectively. Assay conditions were the same as those for Fig. 3 except that 50 mM Mes-NaOH (pH 6.5) was used instead of 50 mM Hepes-NaOH (pH 7.0). Data are expressed as a percentage of the PLD activity (60 nmol/mg/min) apparent in the absence of test protein and are mean (± S.E.) values of three independent experiments, each performed in duplicate.

was measured at three different concentrations of ARF and PC. The inhibition profiles were virtually identical when the concentrations of ARF and PC were reduced by up to 95 and 75%, respectively, suggesting that inhibition does not result from competition of AP3 with either ARF or PC for binding to PLD (data not shown).

AP3 binds IP₆ with a dissociation constant of 0.24–1.2 μM, and IP₆ inhibits the interaction of AP3 with clathrin (7, 12). At a concentration of 15 μM, however, IP₆ had no effect on the inhibition of PLD by AP3 measured in the absence or presence of PI(4,5)P₂ (data not shown). AP3 also binds clathrin cages with a high affinity (8, 13). However, the capacity of the cage-bound AP3 to inhibit PLD was similar to that of free AP3 (Fig. 6). Clathrin cages alone did not affect PLD significantly. These results suggest that the region of the AP3 that interacts with clathrin cages differs from that responsible for interaction with PLD and that the inhibitory function of AP3 is not affected by its cage binding.

Previous studies demonstrated that AP3 is readily cleaved by trypsin into a 33-kDa (often a mixture of 30- and 33-kDa) NH₂-terminal fragment and a 58-kDa COOH-terminal fragment (6, 8). The clathrin assembly activity is associated with the 58-kDa fragment, whereas the 33-kDa fragment contains a high affinity binding site for IP₆ (8). To localize the region responsible for interaction with hPLD1, we prepared GST fusion proteins containing the NH₂-terminal sequence of AP3. These results suggest that the region of the AP3 that interacts with clathrin cages differs from that responsible for interaction with PLD and that the inhibitory function of AP3 is not affected by its cage binding.

AP3 (data not shown), suggesting that under non-denaturing conditions parts of the 30- and 33-kDa fragments remain associated with the acidic 58-kDa fragment. The proteins in both the first and second peaks inhibited hPLD1, and the inhibitory activity of the second peak correlated better with the elution profile of the 33-kDa protein than with that of the 30-kDa protein. The 30- and 33-kDa proteins were eluted from excised gel bands, and the sequences of their NH₂-terminal 12 residues were determined; both sequences were the same as the NH₂-terminal sequence of AP3. These results suggest that the NH₂-terminal 33-kDa contains the region required for PLD inhibition and its COOH-terminal 3-kDa region might be critical for interaction with hPLD1.

To further delineate the AP3 sequence involved in hPLD1 inhibition, we prepared GST fusion proteins containing the NH₂-terminal 289 amino acid residues (AP3-(1–289)), NH₂-terminal 320 residues (AP3-(1–320)), or COOH-terminal 595 residues (AP3-(321–915)) and tested their abilities to inhibit hPLD1. GST-AP3-(1–320) was nearly as potent as AP3, whereas GST-AP3-(321–915) and GST-AP3-(1–289) had almost...
no effect (Fig. 8). It appears, therefore, that NH₂-terminal 320 residues but not the COOH-terminal 576 residues of AP3 are important for interaction with hPLD1 and that the sequence extending from residues Pro-290 to Lys-320 of AP3 is critical for the interaction.

The GST fusion proteins were also tested for the ability to bind hPLD1 by incubation with hPLD1, followed by precipitation with glutathione-Sepharose 4B beads and immunoblot analysis of hPLD1 in precipitate and supernatant fractions (Fig. 9). The ability of GST-AP3 and GST-AP3-(1–320) coupled with the inability of GST-AP3-(321–915), GST-AP3-(1–289), and GST to retain hPLD1 in the precipitate support the notion that the sequence extending from residues Pro-290 to Lys-320 of AP3 is involved in interaction with hPLD1. GST-AP3 partially retained hPLD1 and the ratio of hPLD1 in precipitate and supernatant varied (data not shown). This was probably because the GST-AP3 preparations used in the experiment were proteolyzed, to different extents, to AP3 fragments that were detached from the GST moiety but able to bind hPLD1.

DISCUSSION

AP3 was independently discovered in a variety of contexts and has been termed AP 180 (14), AP3 (15), NP185 (16), and F1–20 (9). It binds to clathrin triskelion and promotes their assembly into a homogeneous population of 60–70-nm coats (5, 14). Clathrin-coated vesicles are dynamic organelles that participate in intracellular membrane trafficking, including the biogenesis and recycling of synaptic vesicles. In addition to AP3, clathrin-coated vesicles contain one or more other assembly proteins, including AP1, AP2, and auxilin (17, 18). AP1 and AP2 are tetramers and widely distributed, whereas AP3 and auxilin are monomers and present only in neuronal cells and tissues. In bovine brain, AP3 is localized predominantly in cytosol, with only 30% associated with membranes (19).

AP3 binds IP₆ with high affinity (7, 12). Like many other inositol polyphosphate-binding proteins, AP3 also binds PI(4,5)P₂ as judged from the observation that AP3 associated with lipid vesicles containing PI(4,5)P₂ but not with those vesicles lacking PI(4,5)P₂. However, the interaction of AP3 with PI (4,5)P₂ is of low affinity and was not responsible for inhibition of PLD. Furthermore, at a concentration that maximally inhibits PLD, AP3 had no effect on the PI(4,5)P₂-hydrolyzing activities of three PLC isozymes assayed with substrate vesicles of lipid composition identical to that of those used for the PLD assay. This result suggests that the amount of PI(4,5)P₂ that is bound to AP3 on the surface of substrate vesicles is negligible and that the inhibition of PLD by AP3 is not due to nonspecific interaction of AP3 with lipid vesicles.

AP3 purified from brain and bacterially expressed GST-AP3 were equally potent inhibitors of PLD. In an inhibition assay utilizing a partial trypsin digestion of AP3, the NH₂-terminal 33-kDa fragment appeared to inhibit hPLD1 while the NH₂-terminal 30-kDa fragment did not. Furthermore, the GST fusion protein comprising the NH₂-terminal 320 residues of AP3 was able to inhibit hPLD1, whereas the GST fusion protein comprising the COOH-terminal 595 residues was ineffective. We were able to further localize the inhibitory site to 31 amino acid residues extending from the Pro-290 to Lys-320 of AP3 on the basis of the ability of GST-AP3-(1–320) and the inability of GST-AP3-(1–289) to inhibit hPLD1. Direct binding of AP3 to hPLD1 and the essential role of the 31 residues in the binding were also demonstrated by the fact that GST-AP3 and GST-AP3-(1–320) retain hPLD1 during sedimentation while GST, GST-AP3-(1–289), and GST-AP3-(321–915) do not. However, the AP3 binding does not appear to interfere with the binding of ARF or PC to hPLD1, as indicated by the observation that the inhibition by AP3 was not affected by changes in the concentration of ARF or PC.

The predicted primary structure and characterization of the products of limited proteolysis suggest a three-domain organization for AP3: a central 42-kDa domain flanked by 33-kDa NH₂-terminal and 16-kDa COOH-terminal domains (6, 8, 10). The 33-kDa NH₂-terminal domain of AP3 binds to clathrin triskelion but neither promotes the assembly of nor binds to preassembled clathrin cages, whereas the 58-kDa COOH-terminal region (the 42-kDa central domain plus the 16-kDa COOH-terminal domain) binds clathrin, induces assembly of a homogeneous population of clathrin cages in a manner similar to that of the full-length protein, and binds to preassembled clathrin cages (8). AP3 also binds IP₆ with high affinity through the 33-kDa NH₂-terminal domain, and IP₆ binding inhibits clathrin assembly (7, 12). Our data show that IP₆ binding does not affect inhibition of PLD by AP3. Our data also show that AP3 molecules that are associated with clathrin cages through their 58-kDa COOH-terminal region can inhibit PLD via their 33-kDa NH₂-terminal region.
AP3 shares no significant sequence similarities with other known clathrin-binding proteins, such as the subunits of AP1 and AP2, or auxilin (9, 10). Murphy et al. (11) therefore suggested that AP3 performs an additional cellular function that distinguishes it from other clathrin assembly proteins. Although AP3 is a high affinity receptor for inositol polyphosphates, interaction of the latter with AP2 also modulates its clathrin assembly activity (20–22). Inhibition of PLD might therefore be the additional function that is specific to AP3. Immunoblot analysis with antibodies to AP1 or AP2 suggests that none of the PLD-inhibitory fractions from column chromatography of rat brain proteins contained AP1 or AP2 (data not shown).

Although the presence of PLD in synaptic vesicles has not been established, ARF-activated PLD is highly enriched in Golgi membranes (23) and the production of PA by PLD is a key event in the formation of coatomer-coated vesicles (24). Under certain conditions, increased concentrations of PA and PI(4,5)P₂ are sufficient to promote the binding of coatomer to membranes (24). The generation of PA and that of PI(4,5)P₂ are related events; PI(4,5)P₂ stimulates PA production by activating PI(4)P₅-kinase, constituting a positive feedback loop for the generation of these two lipid messenger molecules (25, 26).

In the accompanying paper (2), we show that synaptojanin inhibits PLD by hydrolyzing PI(4,5)P₂. Synaptojanin is a nerve terminal-specific IPPase and has been implicated in the trafficking of synaptic vesicles (27). Golgi membranes also contain an IPPase known as OCRL (28), which we showed is capable of inhibiting PLD. We therefore proposed that the hydrolysis of PI(4,5)P₂, and consequent inhibition of PLD, by IPPases such as synaptojanin and OCRL might constitute a common mechanism for interrupting the positive feedback loop during membrane invagination and act as a signal for uncoating (2). The present study suggests that direct inhibition of PLD by AP3 without the involvement of PI(4,5)P₂ might constitute an additional mechanism to halt the positive feedback loop and to signal uncoating. Because AP3 is a synapse-specific protein (29, 30), such a mechanism would be specific to synaptic vesicles.

The synaptic vesicle cycle at the nerve terminal shares many features with membrane trafficking in nonneuronal cells (31). However, synaptic transmission requires rapid transitions through each step of the cycle (32). For example, the membranes of empty synaptic vesicles are taken up within 1–5 s after exocytosis (33), which is much faster than most endocytotic events. This special requirement for speed might necessitate the additional, AP3-dependent mechanism for inhibition of PLD.

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REFERENCES