Isolation and Characterization of Two 3-Phosphatases That Hydrolyze Both Phosphatidylinositol 3-Phosphate and Inositol 1,3,5-Bisphosphate*

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Inositol-polyphosphate 3-phosphatase catalyzes the hydrolysis of the 3-position phosphate bond of inositol 1,3,4,5,6-pentakisphosphate (Ins(1,3,4,5,6)P5) to form inositol 1-monophosphate and inorganic phosphate (Bansal, V. S., Inhorn, R. C., and Majerus, P. W. (1987) J. Biol. Chem. 262, 9444-9447). Phosphatidylinositol 3-phosphatase catalyzes the analogous reaction utilizing phosphatidylinositol 3-phosphate (PtdIns(3)P) as substrate to form phosphatidylinositol and inorganic phosphate (Lips, D. L., and Majerus, P. W. (1989) J. Biol. Chem. 264, 19911-19915). We now demonstrate that these enzyme activities are identical. Two forms of the enzyme, designated Type I and II 3-phosphatases, were isolated from rat brain. The Type I 3-phosphatase consisted of a protein doublet that migrated at a relative Mr, 65,000 upon sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The Mr of this isoform upon size-exclusion chromatography was 110,000, suggesting that the native enzyme is a dimer. The Type II enzyme consisted of equal amounts of an Mr, 65,000 doublet and an Mr, 78,000 band upon SDS-polyacrylamide gel electrophoresis. This isoform displayed an Mr, upon size-exclusion chromatography of 147,000, indicating that it is a heterodimer. The Type II 3-phosphatase catalyzed the hydrolysis of Ins(1,3)P2 with a catalytic efficiency of one-nineteenth of that measured for the Type I enzyme, whereas PtdIns(3)P was hydrolyzed by the Type II 3-phosphatase at three times the rate measured for the Type I 3-phosphatase. The Mr = 65,000 subunits of the two forms of 3-phosphatase appear to be the same based on co-migration on SDS-polyacrylamide gels and peptide maps generated with Staphylococcus aureus protease V8 and trypsin. The peptide map of the Mr = 78,000 subunit was different from that of the Mr = 65,000 subunits. Thus, we propose that the differing relative specificities of the Type I and II 3-phosphatases for Ins(1,3)P2 and PtdIns(3)P are due to the presence of the Mr, 78,000 subunit of the Type II enzyme.

Lipid- and water-soluble molecules derived from phosphatidylinositol (PtdIns) have been shown to act as signals coupling various extracellular stimuli to intracellular responses (1-3). Five phosphorylated forms of the parent molecule, PtdIns, have been identified: phosphatidylinositol 4-phosphate (PtdIns(4)P), phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2), phosphatidylinositol 3-phosphate (PtdIns(3)P), phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P2), and phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3) (4, 5). Of these lipids, PtdIns(4)P, PtdIns(4,5)P2, and PtdIns(3,4)P2 are hydrolyzed by PtdIns-specific phospholipase C to yield inositol phosphates and 1,2-diacetylgluceral (6, 7). In contrast, the 3-phosphate-containing lipids are resistant to the action of PtdIns-specific phospholipase C (8, 9). Rather, these lipids are metabolized by a group of relatively poorly described phosphatases and kinases (4, 5). We recently identified one of these enzymes, phosphatidylinositol 3-phosphatase, in NIH 3T3 cell extracts (10). This enzyme catalyzes the hydrolysis of the phosphate bond in the 3-position of the inositol ring of PtdIns(3)P to form PtdIns and inorganic phosphate.

In addition to being derived directly from phosphatidylinositols, inositol phosphates are formed from other inositol phosphates by highly selective phosphatases and kinases (3). Over 20 inositol phosphates have been identified in various cells (3). Among these inositol phosphates is inositol 1,3-bisphosphate (Ins(1,3)P2). This molecule is formed by inositol-polyphosphate 4-phosphatase-catalyzed hydrolysis of inositol 1,3,4,5,6-pentakisphosphate (11). Ins(1,3)P2 is degraded by inositol-polyphosphate 3-phosphatase, which catalyzes the hydrolysis of the 3-position phosphate to yield inositol 1-monophosphate (Ins(1)P) and inorganic phosphate (11). We now demonstrate that inositol-polyphosphate 3-phosphatase and phosphatidylinositol 3-phosphatase copurify from rat brain. Two forms of the active enzyme, designated Type I and II 3-phosphatases, were identified.

EXPERIMENTAL PROCEDURES

Materials—Phosphocellulose (medium mesh), octyl glucoside, bovine serum albumin (fraction V, globulin-free), cytochrome c (prepared without trichloroacetic acid), protein A-Sepharose CL-4B, trypsin, and phenylmethylsulfonyl fluoride were purchased from Sigma.

The abbreviations used are: PtdIns, phosphatidylinositol; PtdIns(3)P, phosphatidylinositol 3-phosphate; PtdIns(4,5)P2, phosphatidylinositol 4,5-bisphosphate; PtdIns(3,4,5)P3, phosphatidylinositol 3,4,5-trisphosphate; Ins(1)P, inositol 1-monophosphate; Ins(1,3)P2, inositol 1,3-bisphosphate; Ins(1,3,4,5)P4, inositol 1,3,4,5-tetrakisphosphate; BisTris, bis(2-hydroxyethyl)morpholinoisotris(hydroxyethyl)methane; MES, 2-(N-morpholino)ethanesulfonic acid; HEPES, N-2-hydroxyethylpiperezine-N'-2-ethanesulfonic acid; EGTA, [ethylenebis(oxyethyl)-methylammonium]tetraacetate; PMSF, phenylmethylsulfonyl fluoride; HPLC, high performance liquid chromatography.
Hydroxylapatite (Bio-Gel HTP), Dowex AG 1-X8 formate (200–400 mesh), SDS-PAGE molecular weight standards, gel filtration standards, and protein assay dye reagent were from Bio-Rad.

Aprotinin and Ins(1,3,4,5)P₄ were purchased from Boehringer Mannheim. Leupeptin and calpain inhibitors I and II were supplied by both Boehringer Mannheim and Calbiochem. Benzamidine, phenylmethylsulfonyl fluoride (PMSF), and NaClO₃ were from Sigma. The H₂[³²P]ATP (3000 Ci/mmol) was purchased from Du Pont-New England Nuclear. [³²P]Ins(1,3,4,5)P₄ was prepared by treating [³²P]Ins(1,3,4,5)P₄ with purified inositol-polyphosphate 1-phosphatase as described (12). The [³²P]Ins(1,3,4,5)P₄ and [γ-³²P]ATP (6000 Ci/mmol) were purchased from Du Pont-New England Nuclear. [³²P]Ins(3,4,5)P₃ was prepared by treating [³²P]Ins(3,4,5)P₃ with purified inositol-polyphosphate 3-phosphatase as described in Section 1.1. The protein (iodination) grade of 200 (w/v) Na₂SO₄ was purchased from Bio-Rad (Costa Mesa, CA). All other chemicals were from commercial sources.

Preparation of [³²P]PtdIns(3,4,5)P₃—NIH 3T3 fibroblasts were grown in Dulbecco's modified Eagle's medium with 10% (v/v) calf serum. The medium was removed, and the cells were stimulated for 15 min at 37 °C with human platelet-derived growth factor (PDGF) (BB homodimer form, 100 ng/ml) in Dulbecco's modified Eagle's medium containing 100 μg of bovine serum albumin/ml. The medium was removed; and the cells were washed with phosphate-buffered saline at 4 °C and then lysed with buffer that contained 50 mM NaCl, 20 mM Tris-HCl, pH 7.4, 50 mM NaF, 20 mM sodium pyrophosphate, 1 mg/ml cytochrome c, and 0.3% (w/v) octyl glucoside in 20 mM HEPES, pH 7.5, and 200 μM Na₂VO₄ and finally, 100 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 1 mM EGTA as described by Serunian et al. (9) with slight modification.

The washed immunoprecipitate was incubated for 60 min at 37 °C with 200 μM PtdIns(3,4,5)P₃, 5 μM [γ-³²P]ATP (800 Ci/mmol) in 20 mM HEPES, pH 7.5, 10 mM MgCl₂, and 0.5 mM EGTA as described by Serunian et al. (9) with slight modification. The integrated first-order rate equation [SI] = [S]₀ exp (−k t) was used to calculate a first-order rate constant (k) in units of minute⁻¹ for a given amount of enzyme. This rate constant is a measure of enzyme activity since k = V_max/K_m and V_max varies directly with enzyme concentration while K_m is constant. The fraction [SI]/[IS] was calculated from the equation [SI]/[IS] = [S]₀/V_max/K_m = [S]₀/k.

Preparation of Rat Brain Homogenate and Cytosolic Fraction—All procedures were performed at 4 °C. Rat brains (2500 brains, wet weight = 5160 g), obtained from Pel-Freeze Biologicals (Rogers, AR), were homogenized with a commercial vegetable shredder and suspended (1:3, w/v) in homogenization buffer (50 mM MES, pH 6.5, 5 mM MgCl₂, 0.5% (w/v) octyl glucoside, and 0.2 mg of cytochrome c/ml using [³²P]Ins(1,3,4,5)P₃ of a lower specific activity (170 cpm/pmol). In these assays, <20% of the substrate was consumed.

Type I and II 3-Phosphatases—The amount of substrate converted to product was <30%. The K_m and V_max values were derived from reactions assayed (Assay 2) in the presence of 20 mM MES, pH 6.5, 2.5 mM EDTA, 3.5 mM MgCl₂, 0.3% (w/v) octyl glucoside, and 0.2 mg of cytochrome c/ml using [³²P]Ins(1,3,4,5)P₃ of a lower specific activity (170 cpm/pmol). Under these assay conditions of low (30–60 nM) substrate concentrations, enzyme activity follows first-order kinetics and is expressed as counts/minute of product formed. For comparisons of inositol-polyphosphate 3-phosphatase activity, a PtdIns(3,4,5)P₃-hydrolyzing activity, the [³²P]Ins(1,3,4,5)P₃ product formed was used to calculate a first-order rate constant (see above) for the breakdown of [³²P]Ins(1,3,4,5)P₃. The fraction [SI]/[IS] was defined as (total [³²P]Ins(1,3,4,5)P₃ in the assay − [³²P]Ins(1,3,4,5)P₃ formed)/total [³²P]Ins(1,3,4,5)P₃. The amount of substrate converted to product was <30%. The K_m and V_max values were derived from reactions assayed (Assay 2) in the presence of 20 mM MES, pH 6.5, 2.5 mM EDTA, 3.5 mM MgCl₂, 0.3% (w/v) octyl glucoside, and 0.2 mg of cytochrome c/ml using [³²P]Ins(1,3,4,5)P₃ of a lower specific activity (170 cpm/pmol).
The two peaks of enzyme activity from the DEAE column (see Fig. 1) were pooled separately and precipitated with ammonium sulfate as described above. The pellets were resuspended in 75 ml of 20 mM sodium phosphate, pH 7.0, 20 mM 2-mercaptoethanol, 4 μg each of calpain inhibitors I and II/ml, 0.5 mM benzamidine, and 1 mM PMSF. The conductivity of the sample was measured, and solid ammonium sulfate was added (as described above) to increase the conductivity to that of a 25% saturated solution of ammonium sulfate. The supernatant after centrifugation (41,400 × g for 20 min) was filtered through a 0.45-μm filter and chromatographed on a hydrophobic interaction column.

Hydrophobic Interaction Chromatography—Each sample was applied at 8 ml/min to a Bio-Rad Bio-Gel phenyl-5PW column (21.5 × 150 mm) equilibrated with 20 mM sodium phosphate, pH 7.0, 20 mM 2-mercaptoethanol, and 25% saturated ammonium sulfate. The column was washed with 165 ml of equilibration buffer and eluted with a 1565-ml gradient from 25% saturated to 0% ammonium sulfate in 20 mM sodium phosphate, pH 7.0, and 20 mM 2-mercaptoethanol at 8 ml/min. Fractions (20 ml) were collected, and EDTA was added to a 2.5 mM final concentration.

The fractions containing enzyme activity were pooled and concentrated in a Micro-ProDICon as described above and frozen at -90 °C.

Results

Mono S Chromatography—The samples from hydrophobic interaction chromatography were thawed at room temperature and then diluted 1:4 (v/v) with Mono S equilibration buffer (20 mM HEPES, pH 7.2, 0.5 mM EDTA, 20 mM 2-mercaptoethanol, 20% (v/v) glycerol, 0.5% (w/v) octyl glucoside, 1 mM PMSF, 0.5 mM benzamidine, 10 μg each of calpain inhibitors I and II/ml, and 4 μg of calpain inhibitor II/ml. Following concentration, the sample was stored at -90 °C. Both the Type I and II enzyme activities were stable for at least 6 weeks at -90 °C. The next three chromatography steps, Mono S, hydroxyapatite, and Mono Q chromatography were completed in the same buffer day.

Results

Enzyme Purification—Initial studies of phosphatidylinositol 3-phosphatase disclosed similarities to inositol-polysphosphate 3-phosphatase, including the observations that both activities: 1) displayed similar subcellular distributions, 2) were maximal in the presence of EDTA, and 3) bound to and were eluted from DEAE and Mono Q columns under similar conditions. However, preliminary experiments with crude preparations of the PtdIns(3)P-hydrolyzing activity from NIH 3T3 cells (10) indicated that there was little inositol-polysphosphate 3-phosphatase activity in fractions enriched for the PtdIns(3)P phosphatase (see “Discussion”). Thus, to determine the relationship of these enzymatic activities to each other, we performed simultaneous purification of each from rat brain. At each stage of the procedure described below, the elution profiles of the Ins(1,3)P2 and PtdIns(3)P phosphatase activities were highly similar. For the sake of clarity, the purification procedure will be detailed for Ins(1,3)P2 phosphatase until the final step of the procedure, size-exclusion chromatography.

Approximately 70% of the total Ins(1,3)P2 phosphatase activity in the rat brain homogenate was in the 20,300 × g soluble fraction. Batch elution of the cytosolic enzyme from phosphocellulose resulted in a 15-fold purification as shown in Table I. The enzyme activity was resolved into two peaks on a DEAE HPLC column (Fig. 1). We designated these activities as Type I and II 3-phosphatases based on their activities as Type I versus II 3-phosphatase. First, inclusion of a wide spectrum of protease inhibitors in all buffers did not change significantly the relative distribution of the Type I versus II 3-phosphatase. Second, fraction-
Type I and II 3-Phosphatases

Enzyme activities were expressed as first-order rate constants (k) for the total amount of 3-phosphatase activity for PtdIns(3)P or Ins(1,3)P_2. The enzyme activities were normalized for the phenyl load samples. The cumulative yields and specific activities for these samples and the final preparation are shown in parentheses.

| Step               | Protein          | Total activity | Yield | Specific activity | Purification |
|--------------------|------------------|----------------|-------|------------------|--------------|
|                    |                  | mg | min^{-1} | % | min mg^{-1} | -fold |
| Homogenate         |                  | 328 | 4.9 x 10^6 | 100 | 1.5 x 10^7 | 8.0 x 10^5 |
| Phosphocellulose    |                  | 175,000 | 3.6 x 10^6 | 73 | 2.0 x 10^7 | 8.4 x 10^5 |
| DEAE load          |                  | 9855 | 2.3 x 10^6 | 47 | 2.4 x 10^7 | 5.7 x 10^5 |
| Phenyl load        |                  | 312 | 6.1 x 10^7 | 100 (12) | 2.0 x 10^7 | 2.4 x 10^5 |
| Type I             |                  | 480 | 8.4 x 10^7 | 100 (17) | 1.3 x 10^7 | 3.0 x 10^5 |
| Mono S load        |                  | 17.8 | 4.7 x 10^7 | 77 | 2.6 x 10^7 | 3.7 x 10^5 |
| Type I             |                  | 18.3 | 4.6 x 10^7 | 56 | 2.5 x 10^7 | 4.3 x 10^5 |
| HA^* load          |                  | 7.6 | 4.3 x 10^7 | 70 | 5.0 x 10^7 | 8.8 x 10^5 |
| Type II            |                  | 8.0 | 2.5 x 10^7 | 30 | 3.1 x 10^7 | 1.4 x 10^5 |
| Mono Q 1 load      |                  | 5.32 | 2.8 x 10^7 | 46 | 5.3 x 10^7 | 6.8 x 10^5 |
| Type I             |                  | 4.29 | 1.2 x 10^7 | 14 | 2.8 x 10^7 | 7.3 x 10^5 |
| Type II            |                  | 4.4 x 10^7 | 7.2 | 6.4 |
| Type I             |                  | 4.0 x 10^7 | 4.9 x 10^7 | 2.7 x 10^5 | 4.8 |
| Type II            |                  | 4.0 x 10^7 | 7.2 | 6.4 |
| Size-exclusion load|                  | 0.225 | 2.6 x 10^7 | 4.2 | 1.1 x 10^7 | 6.7 x 10^5 |
| Type I             |                  | 0.334 | 2.5 x 10^7 | 3.0 | 7.5 x 10^7 | 7.8 x 10^5 |
| Purified protein   |                  | 0.0065 | 3.2 x 10^7 | 0.52 | 5.7 x 10^7 | 2.5 x 10^6 |
| Type II            |                  | 0.0285 | 3.4 x 10^7 | 0.40 | 1.2 x 10^7 | 8.1 x 10^6 |

^a HA, hydroxylapatite.

Fig. 1. DEAE HPLC chromatography of 3-phosphatase activity. The phosphocellulose eluate was chromatographed on a DEAE HPLC column as described under "Experimental Procedures." Fractions (20 ml) containing inositol-polyphosphate 3-phosphatase activity, measured as Ins(1)P formation (○), were distributed into two pools, Type I (fractions 85–125) and Type II (fractions 126–160). Conductivities of the fractions (---) were measured at 4 °C.

Fig. 2. Mono Q anion-exchange chromatography of Type I and II 3-phosphatases. Type I (upper) and II (lower) 3-phosphatase activities were chromatographed on a Mono Q HR5/5 fast protein liquid chromatography column as described under "Experimental Procedures." The inositolpolyphosphate 3-phosphatase activity, expressed as Ins(1)P formation (○), was eluted from the column with a linear gradient of NaCl (%B (---) indicates the percentage of eluate containing 1 M NaCl that was present in the fractions (0.5 ml).

The purification of each type separately. The Type I 3-phosphatase eluted from a hydrophobic interaction chromatography column at 11% ammonium sulfate, whereas the Type II enzyme eluted at ~13% ammonium sulfate. The Type I 3-phosphatase had been purified 1900-fold, and the Type II 3-phosphatase had been purified 2200-fold at this stage when both samples were frozen.

We next carried out chromatography on Mono S, hydroxylapatite, and Mono Q columns in a single day and refreeze each preparation. Both enzyme preparations displayed cross-contamination with the other form of the enzyme. The Type I and II 3-phosphatases were best resolved on the Mono Q column (Fig. 2, upper and lower, respectively). The cross-contamination of the preparations accounts for the observed activity in fractions 21–24 in Fig. 2 (upper), which reflects contamination of calf brain homogenates by the same procedure yielded the same two peaks of activity, with the same relative distribution. In light of this, we continued the purification of each type separately.

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the Type II enzyme in the Type I preparation, and that in fractions 14-18 in Fig. 2 (lower), which reflects the Type I 3-phosphatase in the Type II preparation. SDS-PAGE of fractions from the Mono Q columns indicated that the activity of the Type I preparation correlated with a polypeptide migrating at an \( M_r \) of 65,000; however, the preparation was not homogeneous. The enzyme activity in the Type II preparation correlated with two protein bands present in approximately equal amounts, corresponding to \( M_r \) = 65,000 and 78,000. Several other proteins also were detected in these fractions. Therefore, we further subjected each preparation to repeat chromatography on Mono Q followed by size exclusion chromatography (Fig. 3, upper and center).

Both the Ins(1,3)P\(_2\) and PtdIns(3)P phosphatase activities in the Type I preparation eluted from the size-exclusion column with an \( M_r \) of 110,000 (Fig. 3, upper). SDS-PAGE analysis of the column fractions (data not shown) showed that the activity correlated with a protein doublet at \( M_r \) = 65,000. This gel was stained with silver to determine whether the Type I and Type I1 enzymes co-migrate, suggesting that they are the same. This hypothesis was substantiated by the results of peptide mapping (see below).

The Type I enzyme had been purified 38,000-fold, and the Type I1 3-phosphatase had been purified 8000-fold. The total yield of enzyme activity was only 0.13%. When samples were thawed for the final Mono Q and size-exclusion chromatography steps, there was a substantial loss of activity (Table I). In retrospect, a larger yield may have been obtained had the second Mono Q column been omitted and size-exclusion chromatography carried out directly after Mono Q chromatography 1. In a repeat of this procedure, we omitted the Mono Q and size-exclusion chromatography steps to achieve higher yields of protein for sequencing and only purified the Type I1 enzyme. In this preparation, we obtained ~400 \( \mu \)g of the Type II enzyme.

The copurification of the Ins(1,3)P\(_2\) and PtdIns(3)P phosphatase activities is summarized in Table I. The purification was similar as monitored by either assay with two exceptions. After separation of the Type I and II 3-phosphatases on DEAE, the -fold purification using Ins(1,3)P\(_2\) as substrate was 120-133 (Table I, phenyl load), whereas using PtdIns(3)P, the purification was only 30-38-fold. This apparent discrepancy may be explained by the fact that the PtdIns(3)P phosphatase assay is extremely sensitive to changes in octyl glucoside concentration (10). The assay conditions were optimized using crude supernatant fractions that contained undetermined amounts of lipid that may alter the optimal octyl glucoside concentration. Therefore, after DEAE chromatography, a low apparent yield of activity may be due to the use of less than optimal assay conditions compared to the crude preparation. The other discrepancy occurred in the

\[ \text{any } M_r = 78,000 \text{ polypeptide was present. None was detected in the peak fractions, which were pooled and concentrated. In these fractions, } >95\% \text{ of the protein migrated at } M_r = 65,000. \]

The Ins(1,3)P\(_2\)- and PtdIns(3)P-hydrolyzing Type II enzyme eluted from the size-exclusion column with an \( M_r \) of 147,000 (Fig. 3, center). The enzyme activity correlated with polypeptides that migrated at \( M_r \) = 65,000 and 78,000 upon SDS-PAGE (Fig. 3, lower). These two bands were present in approximately equal amounts, suggesting that the Type II enzyme is a heterodimer of these polypeptides. Samples of the pooled concentrated Type I and II enzymes were analyzed by SDS-PAGE as shown in Fig. 4. The \( M_r \) = 65,000 proteins of the Type I and II enzymes co-migrate, suggesting that they are the same.

**FIG. 3.** Size-exclusion chromatography of Type I and II 3-phosphatases. The molecular weights of the Type I (upper) and II (center) 3-phosphatases were estimated by measuring their elution from size-exclusion columns relative to the elution of protein standards: thyroglobulin (\( M_r \) = 670,000), bovine \( \gamma \)-globulin (\( M_r \) = 158,000), ovalbumin (\( M_r \) = 44,000), myoglobin (\( M_r \) = 17,000), and cyano
cobalamin (\( M_r \) = 1300). [\(^{3}H\)Ins(1,3)P\(_2\)] (●) and [\(^{32}P\)PtdIns(3)P (○) phosphatase activities were measured as described under "Experimental Procedures." Enzyme activities were expressed as a first-order rate constant (\( k \)) for the total amount of catalytic activity in the fraction. The fraction volume collected was 0.5 ml. SDS-PAGE analysis of the fractions from the Type II preparation is also shown (lower). The samples were resolved on a 7.5% polyacrylamide gel as described (22) and stained with Coomassie Blue. The relative migration of protein standards is shown in the center of the gel: myosin (\( M_r \) = 200,000), \( \beta \)-galactosidase (\( M_r \) = 116,000), phosphorylase \( b \) (\( M_r \) = 97,000), bovine serum albumin (\( M_r \) = 66,000), and ovalbumin (\( M_r \) = 45,000).

**FIG. 4.** SDS-PAGE of purified Type I and II 3-phosphatases. Purified Type I and II 3-phosphatase preparations were electrophoresed on a 7.5% polyacrylamide gel and stained with Coomassie Blue. The Type I preparation was derived from fractions 90-92, and the Type II enzyme preparation from fractions 86-88 of the respective size-exclusion column. The relative migration of protein standards (see Fig. 3 (lower) legend) is shown.
Type II preparation upon Mono Q column 1, where the yield was excellent as monitored by PtdIns(3)P phosphatase activity, whereas two-thirds of the Ins(1,3)P_2 phosphatase activity was lost. We have no explanation for this result except to speculate that some alteration in the enzyme occurred that favored the lipid substrate.

Relative Enzyme Activities—The absolute and relative activities of the purified Type I and II 3-phosphatases for both substrates are shown in Table II. In terms of absolute activity differences, the Type II enzyme catalyzed the hydrolysis of PtdIns(3)P three times as well as did the Type I enzyme. This is in contrast to the 5-fold greater rate of Ins(1,3)P_2 metabolism by the Type I enzyme. This difference in the rate of Ins(1,3)P_2 breakdown was even greater when more optimal assay conditions were used, in which case the Type I 3-phosphatase had a 19-fold greater catalytic efficiency than the Type II enzyme (see below). A comparison of the relative specificities of the Type I and II 3-phosphatases for the two substrates shows that the Type II enzyme is 16 times more selective for the lipid than is the Type I enzyme (ratio of relative activities = 68, cf. 4.2). These results imply that the Type II enzyme functions primarily to metabolize PtdIns(3)P and thus is most appropriately designated phosphatidylinositol 3-phosphatase. The greater catalytic efficiency of the Type I enzyme toward Ins(1,3)P_2 suggests that it regulates Ins(1,3)P_2 levels in cells; and thus, we designate it inositol-polynucleoside 3-phosphatase. These differences in specificity were documented repeatedly and are apparent from the activities in Table I and Fig. 3.

These differences in specificity may explain the apparent difference in -fold purification in Table I. Since the initial separation of Type I and II 3-phosphatases is not complete, each is initially cross-contaminated with the other. Thus, when the Type II 3-phosphatase is assayed with PtdIns(3)P as substrate, contamination with the Type I enzyme has little effect on the total activity measured. Thus, as the contaminating Type I enzyme is removed, there is little effect on the calculated yield and -fold purification for the Type II preparation. However, when Ins(1,3)P_2 phosphatase activity is measured on the same preparation, the removal of the contaminating Type I enzyme has a major effect on the total yield of activity. Thus, the apparent -fold purification and yield of Type II 3-phosphatase is less. The same argument can explain the fact that the apparent purification of the Type I enzyme assayed with Ins(1,3)P_2 as substrate is much greater than that determined using PtdIns(3)P (Table I).

Heat Inactivation Studies—As further support for the conclusion that the enzyme activities that catalyze the hydrolysis of Ins(1,3)P_2 and PtdIns(3)P were identical, we performed heat inactivation studies utilizing the purified Type I and II 3-phosphatases. Both the Type I and II 3-phosphatases were stable at 41 °C for at least 30 min (data not shown). However, the two enzyme activities displayed different sensitivities to temperatures >41 °C. Paired samples of the purified Type I and II enzymes were heated for varying times and then assayed for both Ins(1,3)P_2 and PtdIns(3)P phosphatase activities. The Type I 3-phosphatase was more heat-labile than the Type II 3-phosphatase. Type I 3-phosphatase activity (Fig. 5, upper) had a t_0.5 at 45 °C of 13 min for both Ins(1,3)P_2 and PtdIns(3)P. The t_0.5 for the Type II 3-phosphatase (Fig. 5, lower) at 49 °C was 17 and 16 min for Ins(1,3)P_2 and PtdIns(3)P, respectively. The t_0.5 for the Type I 3-phosphatase at 49 °C was 3 min (data not shown).

Peptide Mapping of Type I and II 3-Phosphatases—To assess the structural similarity of the M_r = 65,000 proteins of the Type I and II 3-phosphatases and to compare each to the M_r = 59,000 subunit of the Type II enzyme, mapping of peptides obtained from protease digests of 125I-radiolabeled proteins was undertaken (Fig. 6). Cleveland digests (19) of the M_r = 65,000 subunits of Type I and II 3-phosphatases yielded the same pattern of peptides with both S. aureus protease V8 and trypsin. Shorter exposures of the film demonstrated that the low molecular weight peptides generated from the M_r = 65,000 subunit were identical. Both the Type I and II M_r = 65,000 subunits were slightly degraded in the control (no protease) lanes to the same peptides and to approximately the same extent. These observations demonstrated that the Type I and II M_r = 65,000 subunits are the same, or very similar, and thus implicate this subunit as the catalytic subunit for Ins(1,3)P_2 and PtdIns(3)P hydrolysis. The M_r = 78,000 subunit of the Type II enzyme was less sensitive to protease V8 and trypsin digestion than the M_r = 65,000 subunit and yielded certain peptides that were not detected in the digests of the M_r = 65,000 subunit. This indicates that the M_r = 78,000 subunit of the Type II enzyme may be a regulatory subunit that inhibits the hydrolysis of Ins(1,3)P_2 and stimulates the breakdown of PtdIns(3)P.

**Table II**

| Activity Remaining (%) | Ins(1,3)P_2 | PtdIns(3)P | PtdIns(3)P/Ins(1,3)P_2 |
|------------------------|-------------|------------|------------------------|
| Time (min)             | 5           | 10         | 15                     | 20         | 25         | 30         |
| Type I                 | 100         | 70         | 50                     | 30         | 20         | 10         |
| 100                     | 80         | 60         | 40                     | 30         | 20         | 10         |
| Type II                | 100         | 70         | 50                     | 30         | 20         | 10         |

**Fig. 5.** Thermal inactivation of [32P]PtdIns(3)P- and [3H]Ins(1,3)P_2-hydrolyzing activities of purified Type I (upper) and II (lower) 3-phosphatases. Paired samples of each preparation were heated for varying lengths of time at either 45 °C (Type I) or 49 °C (Type II). [32P]PtdIns(3)P phosphatase was measured using 59 and 19 pg of Types I and II, respectively, as described under “Experimental Procedures.” [3H]Ins(1,3)P_2-hydrolyzing activity was measured (140 and 1000 pg of Types I and II, respectively) in the presence of 100 mM KCl and 20 μM PtdIns. Linear regression analysis of the data yielded correlation coefficients of 0.95 and 0.94 for Type I [32P]PtdIns- and [3H]Ins(1,3)P_2-hydrolyzing activities, respectively, and 0.96 and 0.98, respectively, for the Type II 3-phosphatase. The calculated t_0.5 values for both substrates were 13 min at 45 °C for Type I 3-phosphatase and 16–17 min at 49 °C for Type II 3-phosphatase.
**Kinetics of Ins(1,3)P₂ Hydrolysis**—The Michaelis constant ($K_m$) and maximum velocity ($V_{max}$) for the hydrolysis of Ins(1,3)P₂ by the purified Type I and II 3-phosphatases were derived from the measurement of reaction velocities at varying concentrations of Ins(1,3)P₂. These assays were performed in the presence of 1 mM unbound Mg²⁺ and 0.3% (w/v) octyl glucoside to optimize enzyme activity. In the experiment shown (Fig. 7, upper and lower), the $K_m$ for the Type I enzyme was 0.80 μM, and the $V_{max}$ was 64 μmol of Ins(1)P formed per min/mg of protein. The $K_m$ for the Type II 3-phosphatase was 3.7 μM, and the $V_{max}$ was 16 μmol of Ins(1)P formed per min/mg of protein. The catalytic efficiency of the Type I enzyme is 19-fold higher than that of the Type II enzyme ($V_{max} / K_m = 80$ versus 4.3). This result indicates that the $M_r = 78,000$ subunit of the Type II enzyme exerts an inhibitory action on the putative catalytic ($M_r = 65,000$) subunit.

**Specificity of Type I and II 3-Phosphatases**—Several watersoluble inositol phosphates containing a phosphate in the 3-position of the inositol ring have been identified (3). Hallcher and Sherman (21) and our laboratory (22) have demonstrated that Ins(3)P is metabolized solely by inositol monophosphatase. Of the inositol polyphosphates, Ins(1,3)P₂ (11, 23), inositol 1,3,4,5-tetraakisphosphate (24–28), and inositol 1,3,4,5,6-pentakisphosphate (29, 30) have been reported to be dephosphorylated by a 3-phosphatase activity.

As detailed above, both Type I and II 3-phosphatases catalyze the hydrolysis of Ins(1,3)P₂. However, we found that neither Type I nor II 3-phosphatase catalyzed the hydrolysis of [3H]inositol 1,3,4,5-tetraakisphosphate (data not shown). In addition, [3H]inositol 1,3,4-trisphosphate and [3H]inositol 3,4-bisphosphate were not substrates for either form of the enzyme (data not shown). In these experiments, we used 1 μM substrate and added 10 times the amount of enzyme that would be needed to degrade 100% of [3H]Ins(1,3)P₂. These results indicate that any activity of Type I or II 3-phosphatase with these inositol polyphosphates is <1% of that with Ins(1,3)P₂.

**DISCUSSION**

The ratio of the activity of the Type I 3-phosphatase to that of the Type II 3-phosphatase for PtdIns(3)P hydrolysis was markedly different than that for Ins(1,3)P₂ hydrolysis (Table II). Whereas the rate of PtdIns(3)P hydrolysis by the Type II enzyme was ~5-fold greater than that by the Type I enzyme, the Type I enzyme catalyzed the hydrolysis of Ins(1,3)P₂ approximately five times better than did the Type II enzyme. The latter value underestimates the differences between the two enzymes since the kinetic data in Fig. 7 indicate that the Type I enzyme hydrolyzes Ins(1,3)P₂ 19 times better than does the Type II enzyme (catalytic efficiency = $V_{max} / K_m$). The demonstration that peptide maps of protease digests of the putative catalytic ($M_r = 65,000$) subunits for the two enzymes were the same (Fig. 6) suggests that the substrate specificity differences between the Type I and II 3-phosphatases are due to the presence of the $M_r = 78,000$ subunit in the Type II isomorph.

These results contradict our previous conclusion (10) that the PtdIns(3)P⁻ and Ins(1,3)P₂-hydrolyzing activities are distinct. Under the assay conditions used previously to measure PtdIns(3)P⁻-hydrolyzing activity (100 mM KC, 20 μM PtdIns), the breakdown of Ins(1,3)P₂ by the Type I and II enzymes is inhibited by 95 and 80%, respectively (data not shown). Thus, little Ins(1,3)P₂ phosphatase activity would have been detected. Indeed, we did note that Ins(1,3)P₂ was broken down at ~6% of the rate measured for the hydrolysis of PtdIns(3)P⁻ (10).
The large purification that was achieved indicates that Type I and II 3-phosphatases are trace proteins in rat brain. Furthermore, both Type I and II 3-phosphatases are highly specific for Ins(1,3)P_2 and PtdIns(3)P since neither hydrolyzed other 3-phosphate-containing inositol phosphates including Ins(3,4,5)P_3, Ins(1,3,4,5)P_4, and Ins(1,3,4,5,6)P_5. Gee and co-workers (23) have identified a Mg^2+-independent form of Ins(1,3)P_2 phosphatase in bovine brain cytosolic preparations. We have identified two forms of 3-phosphatase in bovine brain that resemble the rat brain enzymes based on chromatographic properties and substrate specificity.

The regulation of the substrate specificity of the catalytic subunit of the 3-phosphatase by the M_= 78,000 subunit is similar to two previously described activities: the regulation of galactosyltransferase activity by α-lactalbumin and the regulation of thrombin activity by thrombomodulin. α-Lactalbumin decreases the apparent Km for galactosyltransferase for both glucose and N-acetylgalcosamine while increasing the V_max for glucose and decreasing the V_max for N-acetylgalcosamine (31). Binding of thrombomodulin to thrombin produces a complex that activates protein C rather than factors V and VIII (32, 33).

PtdIns(3)P has been implicated as a mediator of cell growth and transformation based on the association of phosphatidylinositol 3-kinase with growth factor receptors (13, 39) and oncogenes (40). However, cellular levels of PtdIns(3)P inferred from incorporation of ^32P into PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 increases markedly (41). The flux through PtdIns(3)P as well as the actual mass remains to be determined, although they are presumably controlled by the relative activities of three or more enzymes: PtdIns 3-kinase (42, 43), PtdIns(3)P phosphatase, and kinases that further phosphorylate the molecule (5, 16). PtdIns(3)P is a constituent of nonproliferating and nontransformed cells such as cultured fibroblasts (34), human vascular smooth muscle cells (35), blood platelets (16, 36), and Saccharomyces cerevisiae (38). Thus, it likely plays a role in normal as well as transformed cellular function.

Interestingly, PtdIns 3-kinase and PtdIns(3)P phosphatase share several features. Both may exist either in a form containing only the catalytic subunit (Type I) or as a heterodimer (Type II) that contains a regulatory subunit (Ref. 42 and this study). In the case of PtdIns 3-kinase, the heterodimeric Type II enzyme is five times less active than the monomeric catalytic subunit (42). The regulatory subunit is M_= 85,000 and is phosphorylated on tyrosine in response to cellular activation by PDGF (43) or insulin (44). In stimulated cells, formation of 3-phosphate-containing phosphatidylinositols is markedly enhanced transiently (41), implying increased PtdIns 3-kinase activity or reduced PtdIns(3)P phosphatase activity. PtdIns(3)P phosphatase also exists in two forms; and in this case, the heterodimeric form is more active than the homodimeric catalytic subunit. It is therefore possible that cellular activation by a growth factor such as PDGF stimulates modification of the regulatory subunits of both enzymes, which promotes dissociation into forms containing the catalytic subunit only, with active kinase and inactive phosphatase. Later, upon dephosphorylation, the heterodimeric enzymes could re-form, yielding a state where phosphatase activity predominates. Further characterization of PtdIns(3)P phosphatase and PtdIns 3-kinase will answer this and other questions regarding their role in cellular signaling.

The physiological actions of Ins(1,3)P_2 are unknown. It is formed by inositol-polyphosphate 4-phosphatase-catalyzed hydrolysis of inositol 1,3,4-triphosphate (11, 12). Nahorski and co-workers (37, 44) have demonstrated that Ins(1,3)P_2 increases several-fold in rat cerebral cortex slices following carbamyl treatment of K^-dependent depolarization. The molecular basis and physiological consequences of these increases are unknown. However, the mechanism of this effect could be analogous to the dissociation mechanism postulated for the degradation of PtdIns(3)P, with the dissociated catalytic (M_= 65,000) subunit more efficiently degrading Ins(1,3)P_2 (since the Type I enzyme is more active toward Ins(1,3)P_2). By such an interactive process, the signal(s) elicited by altered Ins(1,3)P_2 and PtdIns(3)P metabolism may be integrated. Further insight into these mechanisms will be gained by the application of molecular cloning and immunological techniques to characterize the isoforms of 3-phosphatase.

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