Identification of a new 80 k isoform of phosphatidylinositol 4-phosphate 5-kinase from bovine brain

Martina Schmidt, Magdolna Varsányi, Rolf Thieleczek and Ludwig M.G. Heilmeyer Jr.

Institut für Physiologische Chemie. Abteilung für Biochemie Supramolekularer Systeme, Ruhr-Universität Bochum, Universitätsstr. 150, 4630 Bochum 1, Germany

Phosphatidylinositol 4-phosphate 5-kinase is associated with bovine brain microsomes to an extent of approximately 65% of the total cellular enzyme activity. This membrane-associated kinase activity can be solubilized with Triton X-114. Following polyacrylamide gel electrophoresis in the presence of SDS the enzyme can be renatured from gel slices in the presence of desoxycholate and Triton X-100. Catalytic activity appears at an apparent molecular weight of 80 k. Analysis of the reaction product formed by the 80 k protein reveals the existence of a 5-phosphotransferase, identifying the 80 k polypeptide as a new phosphatidylinositol 4-phosphate 5-kinase isoform.

Phosphatidylinositol 4-phosphate 5-kinase; Bovine brain; Renaturation

1. INTRODUCTION

Phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P$_2$), a rare and uniquely polar plasma membrane lipid, acts as an extraordinarily versatile precursor in signal transduction. This phospholipid has recently been recognized as the substrate of two ubiquitous signalling enzymes, phosphoinositide-specific phospholipase C (PLC) (for review see [1]) and phosphatidylinositol (PtdIns) 3-kinase [2,3]. Agonist stimulation of several cell types leads to a rapid decrease of the cellular PtdIns(4,5)P$_2$ level within 5–30 s followed by a return to the basal level within 1 min [4]. These observations strengthen the prediction that the net synthesis of PtdIns(4,5)P$_2$ requires a strict regulation [5].

PtdIns4P 5-kinase phosphorylates PtdIns4P at the 5-position of the inositol ring. Since there is no evidence for an alternative route of PtdIns(4,5)P$_2$ synthesis, PtdIns4P 5-kinase as found both in soluble and membrane-associated form may be a key enzyme in controlling cell function [6]. A 53 k type II PtdIns4P 5-kinase has been purified to homogeneity from human erythrocyte membranes [7]. Its phosphorylation specificity as well as the identification of the catalytically active polypeptide have been described [8]. The existence of a further as yet unpurified isoform, type I, has also been demonstrated [7]. Furthermore, a 53 k protein termed PtdIns4P 5-kinase C [9] and a 110 k membrane-associated form [10] were purified to apparent homogeneity from bovine brain. The 53 k enzyme seems to be similar to type II PtdIns4P 5-kinase from human erythrocytes. A 53 k isoform from rat brain may be regulated by reversible phosphorylation of an activating protein, B50 [11]. PtdIns4P 5-kinase stimulation also seems to occur upon EGF receptor activation [12]. Furthermore, GTPγS, possibly acting through a G-protein, can also activate this enzyme [13–15]. These different regulatory mechanisms may involve different PtdIns4P 5-kinase isoforms, a conclusion which can be drawn only if all isoforms are known.

Here we describe the identification of a new PtdIns4P 5-kinase isoform from bovine brain by employing the high resolving power of polyacrylamide gel electrophoresis in the presence of SDS, combined with a new renaturation procedure.

Correspondence address. L.M.G. Heilmeyer Jr., Institut für Physiologische Chemie, Abteilung für Biochemie Supramolekularer Systeme, Ruhr-Universität Bochum, Universitätsstr. 150, 4630 Bochum 1, Germany. Fax: (49) (234) 7094 193.

Abbreviations: PtdIns, 1-(3-*sn* phosphatidyl)-D-*myo*-inositol; PtdIns4P, 1-(3-*sn* phosphatidyl)-D-*myo*-inositol 4-phosphate; PtdIns(4,5)P$_2$, 1-(3-*sn* phosphatidyl)-D-*myo*-inositol 4,5-bisphosphate; GroPIns, glycerophospho-D-*myo*-inositol; SDS-PAGE, polyacrylamide gel electrophoresis in presence of SDS; EGF, epidermal growth factor; DOC, desoxycholate; G-protein, guanine-nucleotide-dependent regulatory protein; SDS, sodium dodecyl sulfate; PMSF, phenylmethylsulfonyl fluoride; DTE, dithioerythritol.

2. MATERIALS AND METHODS

2.1. Preparation of PtdIns4P 5-kinase-containing fraction

Membranes carrying PtdIns4P 5-kinase activity were prepared from fresh bovine brain as previously described in detail [16]. Briefly, ca. 100 g of brain was homogenized to 10% (w/v) in a buffer containing 10 mM Tris-HCl, 1 mM DTE, 0.1 mM EDTA, 0.32 M saccharose, 2 mM benzamidine, 2 mM aminocaproic acid, 0.1 mM EGTA, 0.1 mM PMSF, 2 μg/ml aprotinin, 2 μM leupeptin, pH 7.5 and centrifuged at 1,000 × g for 10 min. The resulting crude extract was centrifuged at 100,000 × g for 70 min. The sediment was resuspended in 10 mM Tris-HCl, 1 mM DTE, 0.1 mM EDTA, pH 7.5 (buffer A) contain-

ing 2 μM leupeptin, 0.9% NaCl, and homogenized again. The membranes were then collected by centrifugation at 100,000 × g for 60 min and resuspended in 300 ml buffer A containing 1 mM benzamidine, 1 mM aminocaproic acid, 0.05 mM EGTA, 0.05 mM PMSF, 1 μg/ml aprotinin and 1 μM leupeptin PtdIns4P 5-kinase was solubilized from these membranes at a protein concentration of 2 mg/ml with 0 1% Triton X-114 by analogy to the solubilization of the PtdIns 4-kinase described in [16]. The solubilized PtdIns4P 5-kinase was concentrated by adding solid ammonium sulfate up to 30% saturation and dialysed against 5 mM Tris-HCl, pH 7.5, 0.5 mM DTE, 0 05 mM EDTA and 0.1% (w/v) Triton X-100 containing 1 μg/ml aprotinin, 0.05 mM PMSF and 1 μM leupeptin. The enzyme was stored at −20°C for several months without any significant loss of activity.

2 2 Assay for PtdIns4P 5-kinase

The assay was performed at 25°C in a volume of 150 μl containing 100 mM KCl, 100 mM HEPES-KOH, pH 7.5, 1 mM DTE, 1 mM EDTA, 1 mM EGTA, 1 mM [γ-^{32}P]ATP-Tris (2 μCi/nmol), pH 7.5, 5 mM MgCl$_2$, 840 μM PtdIns4P (using an average molecular weight of 926) and 10–40 μg protein of PtdIns4P 5-kinase-containing fractions. To prevent decomposition the PtdIns4P was stored desiccated at −70°C and was dissolved just before the assay in buffer B composed of 10 mM HEPES-KOH (pH 7.5), 100 mM KCl and 2.4% Triton X-100 Lipid incorporated radioactivity was determined according to [17] with the following modifications. Prior to starting the assay by addition of PtdIns4P 5-kinase containing fractions an aliquot of 25 μl was transferred into 1 ml of chloroform/methanol (3.1, v/v) To this mixture 150 μl of 2.4 N HCl was added and the radioactivity extracted into the organic phase was determined. After starting the phosphorylation reaction, further aliquots of 25 μl were quenched with chloroform, methanol and HCl as described above Following phase separation 400 μl of the chloroform phase was added to 1 ml of methanol/1 N HCl (1:1, v/v) for further separation of hydrophilic components like inorganic phosphate, ADP and ATP from the lipophilic acidic phospholipids. Lipid-incorporated radioactivity was determined by scintillation counting of 100 μl of the chloroform phase.

2.3. Identification of the phosphorylation position on the inositol ring of PtdIns(4,5)P$_2$

An aliquot of the PtdIns4P 5-kinase assay was transferred to 1.15 ml of chloroform/2 4 N HCl (6 7:1; v/v). After phase separation the volume of the chloroform phase (ca. 900 μl) was reduced to approximately 100 μl in vacuo. This sample was subjected to deacylation by monomethylamine according to the modified method of [18]. After mixing the chloroform phase with 300 μl 25% monomethylamine reagent the sample was incubated in a sealed glass tube at 53°C for 40 min. Thereafter, 150 μl n-propanol was added and the sample was dried in vacuo. The resulting pellet was suspended in a mixture of 1 ml water and 1.2 ml n-butanol/petrolether/ethyl formate (20:4:1, v/v). The aqueous phase (950 μl) containing the GroPIns derivatives was collected and dried in vacuo. If necessary, the sample was stored at −70°C. The formed GroPIns polyphosphates were dissolved in sample buffer, separated by high performance anion exchange chromatography and detected by an on-line metal-dye detection system as described by [19] with the modification that potassium chloride instead of tetramethylammonium chloride was used in the chromatography Alternatively, the [^{32}P]phosphate radioactivity of the fractions following HPLC was counted to detect the [^{32}P]GroPIns phosphate distribution.

2.4. Preparation of phosphoinositide standards for calibrating the HPLC system

0.5 mg [^{3}H]PtdIns4P or 0.5 mg [^{3}H]PtdIns(4,5)P$_2$ (0 5 nCi/nmol each) was dissolved in 100 μl buffer B and transferred into 400 μl of double distilled water Extraction of phospholipid by chloroform and deacylation were carried out as described above. Radioactivity was determined from an aliquot of 0.75 ml dissolved in 2 ml Hydroluma cocktail.

2 5 Renaturation of PtdIns4P 5-kinase activity following SDS-PAGE

Preparation of a PtdIns4P 5-kinase containing sample and electrophoresis in 7.5% polyacrylamide gel at 4°C for 5 h were carried out as described by [20]. The gel lanes were cut in 2 mm segments, after disruption, they were incubated overnight at 4°C by gently shaking in 100 μl of renaturation buffer (10 mM Tris-HCl, 1 mM DTE, 0 1 mM EDTA, 0.1% DOC, 0.2% Triton X-100, 0.03% sodiumazide, pH 7 5). Lipid kinase activity was assayed in supernatants of 113 μl as described above The recovery of enzyme activity was nearly 50% Protein in an unprocessed parallel gel lane was silver stained according to [21]

2.6 Other methods

Protein was determined by the method of [22] employing bovine serum albumin as standard.

2.7. Materials

Analytical grade HCl and Hydroluma cocktail were from Baker Chemicals (Deventer, The Netherlands) Triethanolamine, monomethylamine (33% in ethanol) and 4-(2-pyridylazo)resorcinol (PAR)-dye (analytical grade) and Triton X-114 were from Fluka (FRG). Potassium chloride was from Merck (FRG). The MonoQ anion exchange column and the Act 100 autosampler were from Pharmacia (Uppsala, Sweden). An inert HPLC system consisting of pumps type 2150, LC-controller type 2152 and monitor type 2151 was from LKB (FRG). Yttrium trichloride hexahydrate (99 9%) was from Janssen (Beerse, Belgium). PtdIns4P, PtdIns(4,5)P$_2$, ATP, Triton X-100, DOC, the molecular weight standards for SDS-PAGE as well as the protease inhibitors were from Sigma (FRG). [^{3}H]PtdIns4P and [^{3}H]PtdIns(4,5)P$_2$ were from BioTrend (St Louis, USA) [γ-^{32}P]ATP was from NEN DuPont de Nemours (FRG) and ICN Biomedicals (FRG).

3. RESULTS

Under the employed experimental conditions PtdIns4P 5-kinase activity has been found in soluble and in microsomal fractions (up to 65% of the total
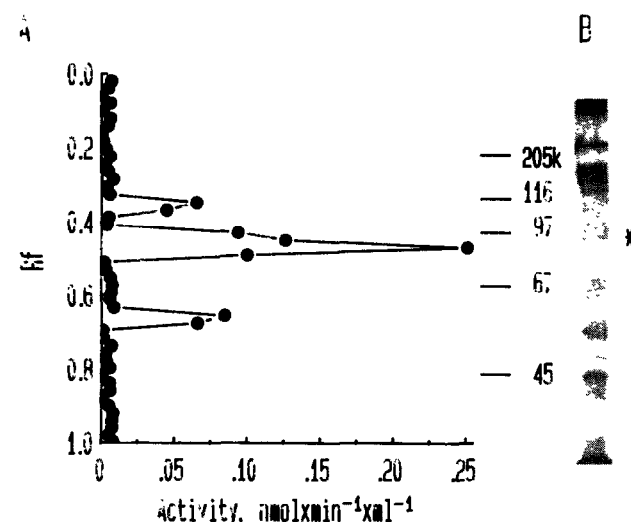

Fig. 1 Localization of PtdIns4P 5-kinase activity after SDS-PAGE. PtdIns4P 5-kinase-containing fraction (152 μg protein; 0.78 nmol · min^{-1}) was separated electrophoretically in the presence of SDS. (A) Following renaturation PtdIns4P 5-kinase activity was tested as described in Section 2 (B) In a parallel slot 19 μg protein was separated and silver stained. The 80 k protein is marked. Molecular weight standards were run in parallel
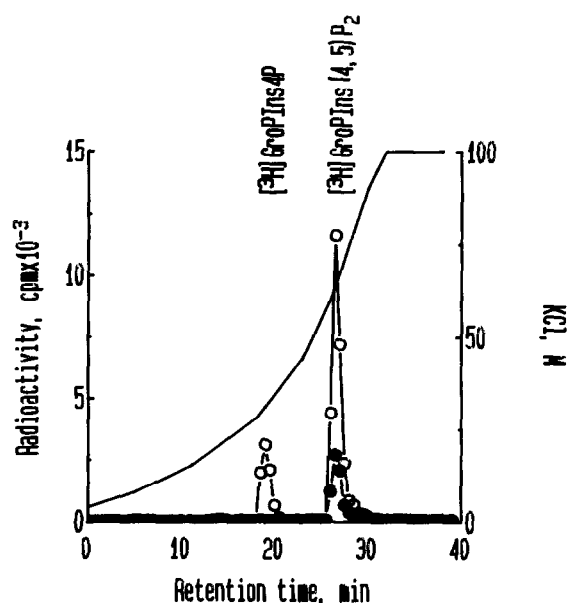
Fig. 2. Identification of the phosphorylation position on the inositol ring. PtdIns4P was phosphorylated in presence of $[^{32}P]ATP-Mg^{2+}$ with the electrophoretically separated and renaturated kinase. ^{32}P-labeled phosphoinositides (●) were analysed as described in section 2. Calibration of the HPLC system was carried out with $[^3H]$phosphoinositides (○) as described in Section 2.

activity) of bovine brain. The membrane-associated activity can be solubilized with Triton X-114; after phase separation approximately 80% of the enzyme activity appears in the aqueous phase. Ammonium sulfate precipitation of the aqueous phase results in a ca. 2-fold enhancement of the specific activity to ca. 5 $nmol \cdot min^{-1} \cdot mg^{-1}$ (data not shown). In order to identify the polypeptide carrying PtdIns4P 5-kinase activity we have developed a renaturation procedure which allows the determination of the enzyme activity after gel-electrophoretic separation of the proteins in presence of SDS. Renaturation occurs in a buffer containing 0.1% DOC and 0.2% Triton X-100 which has been added to gel slices (Fig. 1). One main PtdIns4P 5-kinase activity peak appears exhibiting an apparent molecular weight of 80 k. A protein band with identical apparent molecular weight of 80 k is also detectable following silver staining of a slot running in parallel. Ca. 50% of the applied activity can be recovered. Minor activity peaks exhibit apparent molecular weights of 110 k and 53 k. Employing the 80 k polypeptide after SDS-PAGE and renaturation as PtdIns4P 5-kinase the lipids present in the assay mixture have been extracted. They have been deacylated and subjected to analysis as described in Section 2. Fig. 2 shows that the ^{32}P-labeled reaction product of this lipid kinase comigrates with a $[^3H]GroPIns(4,5)P_2$ standard showing that the analysed PtdIns4P kinase is a 5-phosphotransferase.

4. DISCUSSION

Proteins solubilized with Triton X-114 from NaCl washed membranes were separated by high resolving polyacrylamide gel electrophoresis in presence of SDS. Renaturation of PtdIns4P 5-kinase is possible in DOC/Triton X-100 containing buffer which allows to identify a 80 k protein carrying the catalytic activity. Employing this procedure the 80 k protein represents the major PtdIns4P 5-kinase activity in brain microsomes, the other isoforms type I, 110 k, and type II, 53 k, seem to be minor components. However, the renaturation conditions might influence this activity distribution. Renaturation of membrane-associated PtdIns4P 5-kinase from human erythrocytes has been attempted by blotting SDS-PAGE-separated proteins to nitrocellulose and analysing the blotted proteins for activity [8]. Employing this approach only ca. 0.01% of the applied activity was recovered. Additionally, the enzyme activity was detected over a broad range from ca. 40 k to 70 k. The combination of DOC with Triton X-100 in the renaturation assay described here improves the efficiency of this process ca. 5,000-fold (yielding a recovery of ca. 50%) probably due to an enhanced replacement of SDS from the protein surface by these activating detergents. The method of renaturation of lipid metabolizing enzymes, as demonstrated here for the PtdIns4P 5-kinase, seems to be a general method which has been applied successfully for renaturation of type 2 and type 3 PtdIns 4-kinases from bovine brain [16] and PLC from rabbit fast skeletal muscle [23].

In principle, an 80 k isoform could be generated by partial proteolysis from a higher molecular weight form such as the 110 k enzyme. However, all preparation steps were carried out in the presence of a variety of protease inhibitors. No variations were observed in the relation of activities determined by renaturation following SDS gel-electrophoresis which makes formation of the 80 k form from the 110 k protein unlikely. Sequence determination, which is in progress, coupled with the new renaturation assay described here, will allow a final answer to this question.

Several natural and synthetic lipids, such as PtdIns, $PtdIns(4,5)P_2$, PtdIns- and PtdIns4P-phosphatases, have been tested as potential substrates of this newly detected 80 k kinase. This enzyme accepts only PtdIns4P as substrate (data not shown). The phosphorylated product runs following deacylation like a $GroPIns(4,5)P_2$ standard in the HPCL system (Fig. 2). Moreover, it is well established that PtdIns 3-kinase could also accept $PtdIns(4,5)P_2$ as substrate [24]. However, a trisphosphorylated glycerophosphoinositol derivative has not been detected with the chromatographic system. Furthermore, the activity assays were carried out in the presence of high concentrations of Triton X-100, above the critical micellar concentration, which strongly inhibits the PtdIns 3-kinase activity [24]. Con-

sequently, the assumption that PtdIns 3-kinase might be responsible for this lipid phosphorylation can be excluded.

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