A monoclonal antibody distinguishes two types of phosphatidylinositol 4-kinase

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A monoclonal antibody has been developed against the type II PtdIns 4-kinase from bovine brain. This antibody, 4C5G, causes greater than 90 % inhibition of the type II PtdIns 4-kinase from bovine brain, rat brain and human erythrocytes. However, it fails to inhibit type III PtdIns 4-kinase from bovine brain or PtdIns 3-kinase from rat liver. These results suggest that type II and type III PtdIns 4-kinases are distinct gene products, and that 4C5G will be useful in studying the function of the type II PtdIns 4-kinase.

INTRODUCTION

PtdIns 4-kinase is the first enzyme in a pathway by which a lipid component of cellular membranes is converted to PtdIns(4,5) P_2 , the immediate precursor of the second messengers Ins(1,4,5) P_3 and diacylglycerol. Although it would seem likely that regulation of this enzyme is important in determining the magnitude of the second messenger response to agonists, little is known about such regulation. PtdIns 4-kinases are membrane-associated enzymes which have been described in many tissues and species, and have been purified from A431 cells [1], bovine uterus [2], rat brain [3], pig liver [4], bovine brain myelin [5] and yeast [6].

We have previously shown that two types of PtdIns kinase can be separated from detergent extracts of bovine brain membranes by rate zonal centrifugation on sucrose gradients [7]. We found that 43 % of the total PtdIns kinase activity, termed brain type II, migrated on the gradients with an approximate size of 55 kDa, similar to the bovine uterus and A431 cell enzymes. The brain type II, bovine uterus and A431 cell activities also possess similar K_m values for ATP and PtdIns [1,2,7]. The remainder of the brain membrane activity, termed brain type III, migrates on sucrose gradients with an approximate size of 230 kDa. This activity shares certain properties with a PtdIns kinase purified from rat brain, including similar K_m values for PtdIns and poor inhibition by adenosine [3,7]. A PtdIns 4-kinase activity with similar characteristics has also been described in bovine uterus [8].

The products of the PtdIns kinase activities described above have been either demonstrated or presumed to be PtdIns4P. A PtdIns 3-kinase activity has been described which specifically associates with activated tyrosine kinases. The function of this activity, previously called type I PtdIns kinase, is unknown [9].

In order to be able to functionally distinguish these various PtdIns kinase activities *in vivo*, we have developed a monoclonal antibody which inhibits the type II, but not the type III, PtdIns 4-kinase activity from bovine brain membranes. This antibody inhibits PtdIns 4-kinase activities from other species and tissues, but does not inhibit PtdIns 3-kinase.

MATERIALS AND METHODS

Materials

Azaserine, hypoxanthine, penicillin and streptomycin were purchased from Sigma. [γ - 32 P]ATP (3000 Ci/mmol) was from Amersham, poly(ethylene glycol) from ATCC, RPMI-1640 medium and fetal bovine serum from Hyclone, and glutamine and pyruvate from Gibco. Other materials were from the sources previously described [7].

Assay of brain PtdIns kinase activities

PtdIns 4-kinase activities were assayed by measuring the incorporation of label from [γ - 32 P]ATP into PtdInsP in the presence of 0.1 % Triton X-100 exactly as described [7]. The radioactive reaction product was isolated by t.l.c. and located by autoradiography. Types II and III PtdIns kinase were fractionated from a detergent extract of bovine or rat brain membranes by centrifugation on sucrose density gradients [7]. The product of both types II and III PtdIns kinase from brain membranes has been shown to be PtdIns4P by comigration of deacylated material with glycerophosphoinositol 4-phosphate (G. Endemann, unpublished work).

Assay of rat liver cytosolic PtdIns kinase activities

Rat liver was homogenized in 2 vol. of 50 mM-Hepes (pH 7.8)/10 % (v/v) glycerol/1 mM-vanadate/0.1 mM-phenylmethanesulphonyl fluoride (PMSF)/leupeptin (1 μ g/ml)/aprotinin (1 μ g/ml). The homogenate was centrifuged at 9000 *g* for 20 min, and the resulting supernatant was centrifuged for 1 h at 120 000 *g* to obtain cytosol and microsomal fractions. PtdIns kinase activities from rat liver cytosol were assayed with sonicated PtdIns in the absence of detergent as described [10]. After isolation by t.l.c., the PtdInsP was deacylated and analysed by h.p.l.c. [9] in order to distinguish PtdIns3P and PtdIns4P.

Partial purification of type II PtdIns kinase

Bovine brain membranes were prepared as described [7], and a soluble extract was made with 0.5 % Triton X-100 in buffer A [50 mM-Tris, pH 8, 5 % (w/v) sucrose, 10 mM-MgCl $_2$,

Abbreviations used: PMSF, phenylmethanesulphonyl fluoride.

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1 mM-dithiothreitol, 0.5 mM-ATP and proteinase/phosphatase inhibitors (1 mM-EDTA, 0.2 mM-ammonium vanadate, 0.2 mM-PMSF, 1 μ g of soybean trypsin inhibitor/ml and 1 μ g of aprotinin/ml). A 50 ml portion of the extract was run over a 500 ml column of DEAE-Sephacel in buffer A containing 1% Triton X-100, and the flow-through fractions containing type II PtdIns kinase [7] were pooled and dialysed against buffer B (25 mM-Mes, pH 6.0, 20% (w/v) glycerol, 1% Triton X-100, 5 mM-MgCl₂, 0.1 mM-ATP, 1 mM-dithiothreitol and proteinase/phosphatase inhibitors). The preparation was loaded on to a 50 ml column of SP-Sephadex C-50 (Pharmacia), and proteins were eluted with a 0–0.3 M-NaCl gradient in buffer B. Fractions containing PtdIns kinase activity were dialysed against buffer B and loaded on to a 5 ml column of SP-Sephadex. The column was eluted with 0.5 M-NaCl in buffer B containing 0.1% Triton X-100. The eluate was diluted to < 0.01% Triton X-100 in buffer B containing 0.5 M-NaCl and mixed with 35 ml of phenyl-Sepharose (Pharmacia) equilibrated with the same buffer. After a 30 min incubation, the gel was washed with buffer B containing 0.5 M-NaCl and 0.01% Triton X-100, and eluted with 6 $\times$ 60 ml portions of buffer B containing 0.5% Triton X-100. The PtdIns kinase activity was concentrated on a 1 ml column of SP-Sephadex and eluted with 0.5 M-NaCl/0.1% Triton X-100.

Monoclonal antibody production and characterization

The SP-Sephadex eluate was concentrated and washed in a Centricon 30 (Amicon) to a final composition of 625 μ g of protein/ml in 0.25% Triton X-100/0.15 M-NaCl/10 mM-Hepes (pH 7.1)/20% glycerol/1 mM-dithiothreitol/1 mM-EDTA. An equal volume of Ribi adjuvant (Ribi Immunochemicals) was vortex-mixed with the PtdIns kinase for 1 min. A 25 μ l portion of this mixture was injected (using a Hamilton syringe with a 25G, pt 4 needle) into the rear footpads of 4-week-old Balb/C female mice (Charles River), once a week for 7 weeks.

Popliteal lymph nodes were the source of cells used for the production of antibody-producing hybridoma lines. Lymph node cells from one mouse were mixed with SP2/0-Ag14 myeloma cells [11] at a ratio of 2:1, and suspended in 50 μ l of phosphate-buffered saline (8.2 mM-Na₂HPO₄/1.5 mM-KH₂PO₄/138 mM-NaCl). Fusion was carried out according to Galfre *et al.* [12] with the following modifications. A 1 ml portion of RPMI containing 40% poly(ethylene glycol) and 10% (v/v) dimethyl sulphoxide at 37 °C was added with shaking over 30 s. After 2 min, 2 ml of 10% (v/v) dimethyl sulphoxide in RPMI was added dropwise with shaking over the next 2 min, followed by 7 ml over the next 3.5 min. Then 15 ml of RPMI was slowly added, and the cells were gently pelleted and resuspended in 1 ml of RPMI. Fusion medium [RPMI 1640/20% (v/v) fetal bovine serum/penicillin/streptomycin/2 mM-L-glutamine/1 mM-pyruvate/0.1 mM-hypoxanthine/azaserine (2 μ g/ml)] and the cells from one mouse thymus (dissociated by rubbing gently between two ground glass slides) were added, and the suspension was plated in a 96-well plate.

Colonies from lymph node hybridomas were screened for production of anti-(PtdIns kinase) antibodies in two ways. First, hybridoma supernatants were incubated for 4–15 h at 4 °C with partially purified type II PtdIns kinase before assay of PtdIns kinase activity, in order to detect inhibitory antibodies. Secondly, antibodies which could immunoprecipitate PtdIns kinase activity were detected by the subsequent addition of goat anti-(mouse Ig)-Agarose (Hyclone) in immunoprecipitation buffer [50 mM-Tris (pH 8)/20% glycerol/1 mM-EDTA/1% Triton X-100/1 mM-dithiothreitol, 50 μ M-ATP/0.2 mM-PMSF/soybean trypsin inhibitor (1 μ g/ml)/aprotinin (1 μ g/ml)] to the hybridoma supernatant/PtdIns kinase mixture. After 2 h at 4 °C with mixing, the pellets were washed five times with buffer (the

Table 1. Partial purification of type II PtdIns 4-kinase

Fraction	Recovery (nmol/min)	Specific activity (nmol/min per mg of protein)
Brain membranes	45.7*	0.035*
0.5% Triton X-100 extract	50.4*	0.840*
DEAE-Sephacel flow-through	85.5	2.18
SP-Sephadex eluate	51.0	6.5
Second SP-Sephadex eluate	37.6	11.4
Phenyl-Sepharose	–	–†
Third SP-Sephadex eluate	10.1	24.0

* Calculated fraction of total PtdIns kinase activity.

† Activity not measured.

second and fourth washes contained 0.5 M-NaCl), and assayed for PtdIns kinase activity.

Colonies which were positive in either assay were cloned by limiting dilution. The subisotype of 4C5G was determined to be IgG1 using a kit from Hyclone. The 4C5G hybridoma line was used to obtain ascitic fluid from mice primed with Freund's incomplete adjuvant. IgG1 was purified from ascites fluid by binding to Protein A-Sepharose (Sigma) at pH 8.0, and eluting at pH 6.0 [13]. 4C5G was stored in 0.01% NaN₃ or at –80 °C.

RESULTS

Production of a monoclonal antibody, 4C5G, which recognizes type II PtdIns 4-kinase

Type II PtdIns 4-kinase was purified 686-fold from bovine brain membranes to a specific activity of 24 nmol/min per mg (Table 1). This material was used to immunize mice, and B cells producing antibody in response to this antigen were obtained from the popliteal lymph nodes. Hybridoma cell lines made from these B cells were screened for the production of antibodies which could either inhibit type II PtdIns kinase activity or immunoprecipitate type II PtdIns kinase activity. Hybridomas which were positive in either assay were cloned by limiting dilution, and the same clone, 4C5G, was strongly positive in both assays. This apparent contradiction was explained on quantification of PtdIns kinase activity in both the supernatant and the pellet following immunoprecipitation with 4C5G. Only a small fraction of the activity which is cleared from the supernatant is recovered in the pellet. PtdIns kinase appears to operate at 1–2% of its maximal rate after binding of 4C5G. Attempts to immunoblot a peptide with this antibody were not successful. Of the 40 hybridoma colonies obtained from the lymph nodes of one mouse, 16 others also tested positive initially as well as 4C5G; however, the cells ceased producing detectable antibody following expansion and cloning.

4C5G inhibits type II, but not type III, PtdIns 4-kinase from bovine brain

4C5G was purified from ascites in order to characterize the inhibition of PtdIns kinases by this antibody. Types II and III PtdIns kinase from a detergent extract of bovine brain membranes were separated by density-gradient centrifugation as described [7]. As shown in Fig. 1, purified 4C5G inhibited type II PtdIns kinase from bovine brain; inhibition was half-maximal at approx. 0.6 μ g of 4C5G/ml. In contrast, type III PtdIns kinase was not inhibited by 4C5G, except at very high antibody concentrations.

4C5G was tested to determine whether the antibody could inhibit PtdIns kinase in intact native membranes as well as in

Fig. 1. Inhibition of type II, but not type III, PtdIns4-kinase from brain by the antibody 4C5G

Types II (●) and III (○) PtdIns 4-kinase were separated from a detergent extract of bovine brain membranes by sucrose-density-gradient centrifugation and incubated with purified 4C5G before assay of PtdIns kinase activity.

Fig. 2. Inhibition of PtdIns kinase in intact native membranes by 4C5G

○, Intact native membranes from bovine brain were incubated with the indicated levels of purified 4C5G, and PtdIns kinase activity was then measured in the absence of detergent. ●, Detergent-solubilized type II PtdIns kinase from bovine brain was incubated with purified 4C5G, and PtdIns kinase activity was then measured in the standard assay with 0.1 % Triton X-100.

detergent micelles (Fig. 2). 4C5G inhibited approx. 50 % of the PtdIns kinase activity in bovine brain membranes, the expected proportion of type II PtdIns kinase [7].

Rat liver PtdIns 4-kinase, but not PtdIns 3-kinase, is inhibited by 4C5G

Rat liver PtdIns kinase activity consists of approx. 97 % PtdIns 4-kinase, which is primarily membrane-associated, and 3 % PtdIns 3-kinase, which is primarily cytosolic (C. Carpenter & L. C. Cantley, unpublished work). In order to directly compare the effects of 4C5G on PtdIns 3-kinase and PtdIns 4-kinase, we measured both activities in rat liver cytosol. When cytosol was incubated with sonicated PtdIns and [γ - 32 P]ATP in the absence of detergent, approx. 60 % of the labelled PtdIns P formed is PtdIns4 P , and 40 % is PtdIns3 P . 4C5G is able to block with high affinity about 75 % of the cytosolic PtdIns 4-kinase activity, but

Fig. 3. 4C5G inhibits PtdIns 4-kinase, but not PtdIns 3-kinase, from rat liver cytosol

Rat liver cytosol was incubated overnight at 4 °C with the indicated levels of 4C5G. PtdIns kinase activity was then assayed in the absence of detergent. The formation of PtdIns3 P (▲) and PtdIns4 P (●) was measured by h.p.l.c. fractionation of the two isomers as described in the Materials and methods section. Data are the averages of the results of two separate experiments.

it is unable to inhibit the cytosolic PtdIns 3-kinase activity, even at high concentrations (Fig. 3). A rat liver microsomal PtdIns 4-kinase activity, assayed in 0.1 % Triton X-100, is also inhibited by 4C5G (results not shown).

4C5G recognizes a domain common to PtdIns 4-kinases from several species

When a detergent extract of rat brain membranes is fractionated by sucrose-density-gradient centrifugation, types II and III PtdIns 4-kinases are resolved, with mobilities exactly the same as those of the bovine enzymes. These activities are also distinguished by 4C5G as for bovine activities (Table 2). Both the human erythrocyte [7] and *Xenopus laevis* oocyte (results not shown) PtdIns 4-kinases migrate on sucrose gradients as a single peak with the apparent size of the brain type II activity. These activities are inhibited to various degrees by 4C5G (Table 2).

Table 2. Inhibition of PtdIns 4-kinases from various species by 4C5G

Values are the maximal inhibitions observed at 100–200 μ g of 4C5G/ml under standard assay conditions in the presence of 0.1 % Triton X-100, and are the averages of two separate experiments which differed by no more than 10 %. Types II and III PtdIns kinase were separated by sucrose-density-gradient centrifugation of detergent extracts of brain membranes. Human erythrocyte PtdIns kinase was partially purified from a 0.1 % Triton X-100 extract of erythrocyte ghosts on DEAE-Sephacel as described in the Materials and methods section. *Xenopus laevis* oocyte PtdIns kinase was extracted with 0.1 % Triton X-100 and partially purified by centrifugation on sucrose density gradients.

PtdIns 4-kinase	Inhibition by 4C5G (%)
Bovine brain type II	98
Bovine brain type III	14
Human erythrocyte	94
Rat brain type II	94
Rat brain type III	0
<i>Xenopus laevis</i> oocyte	46

DISCUSSION

The monoclonal antibody we describe in this study, 4C5G, distinguishes two PtdIns 4-kinase activities in brain membranes. These activities have previously been separated and biochemically characterized, and on the basis of this characterization were thought to represent unique enzymes. The two activities are most easily distinguished by their inhibition by adenosine; the types II and III activities possess K_i values for adenosine of 18 and 1520 μM respectively [7]. The presence of an epitope recognized by 4C5G in the brain type II PtdIns 4-kinase, which is not accessible in the brain type III activity, lends further support to the existence of two distinct gene products. The conservation of the domain recognized by 4C5G across species, including human, cow, rat and *Xenopus laevis*, argues for the functional significance of this domain, and suggests that the absence of this domain in the type III enzyme reflects a significant difference in function or regulation.

Type II PtdIns 4-kinase in intact native membranes is 90% inhibited by 20 μg of 4C5G/ml; thus it will be possible to use 4C5G to study the involvement of this enzyme in the elevated metabolism of membrane polyphosphoinositides which occurs in response to various agonists. The presence of more than one type of PtdIns 4-kinase in membranes raises several possibilities as to their functions. First, the two enzymes may be activated in response to different ligands, either through regulation by second messengers or by co-localization in membrane domains. Secondly, one of the PtdIns 4-kinases may participate in ligand-stimulated polyphosphoinositide turnover, while the other is a housekeeping enzyme, necessary to maintain membrane PtdIns4P levels. Elucidation of the roles of the PtdIns kinases should facilitate the study of polyphosphoinositide metabolism in signal transduction pathways.

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