A monoclonal antibody to bovine brain inositol monophosphatase

Immunoaffinity purification of the brain and kidney enzymes and evidence for their structural identity

Nicolas S. GEE,* Steven HOWELL, Gordon RYAN and C. Ian RAGAN

Merck Sharp and Dohme Research Laboratories, Terlings Park, Eastwick Road, Harlow, Essex CM20 2QR, U.K.

A monoclonal IgG_{2b}(K) antibody, G-2A4, has been generated against bovine brain *myo*-inositol monophosphatase (EC 3.1.3.25). The identity of the antigen recognized by the antibody was established by using e.l.i.s.a. and Western blotting procedures, and by immunoprecipitation of enzyme activity from crude brain supernatant. In addition, the hydrolysis of Ins1P by crude brain extract was inhibited by up to 83 % by the pure antibody. Under identical conditions, the hydrolysis of Ins(1,4)P₂ was unaffected. An immunoabsorbent column containing monoclonal antibody G-2A4 covalently attached to CNBr-activated Sepharose 4B has been used for rapid purification of the brain enzyme. Elution conditions have been optimized to allow isolation of the enzyme in high yield (54 %) with full retention of column-binding capacity. The enzyme was electrophoretically homogeneous, *M*_r 30000 and of higher specific activity than that purified conventionally. Chromatography of the pure enzyme on high resolution ion-exchange columns revealed some charge heterogeneity, possibly indicative of some type of post-translational modification. The immunoabsorbent column has also been used to purify the bovine kidney cortex enzyme to homogeneity. Partial proteolytic fragmentation patterns of the brain and kidney enzymes using endoprotease glu-C were identical, suggesting that they are almost certainly products of the same gene.

INTRODUCTION

It is now well established that a variety of hormones and neurotransmitters stimulate phospholipase C-mediated breakdown of PtdIns(4,5)P₂, generating two second messengers, diacylglycerol and Ins(1,4,5)P₃ (for reviews, see [1–3]). The latter molecule mobilizes intracellular Ca²⁺ [4,5] and is metabolized to a variety of inositol phosphates including D-Ins1P, D-Ins3P [L-Ins1P], D-Ins4P and possibly *meso*-Ins5P (for review, see [6]). These metabolites are all substrates for *myo*-inositol monophosphatase (EC 3.1.3.25), first identified in mammalian tissues over 20 years ago by Eisenberg [7]. D-Ins1P may also be generated directly by phosphodiesteratic cleavage of phosphatidylinositol (see [3]), and L-Ins1P can be synthesized from glucose 6-phosphate in certain tissues, including brain [7]. Inositol monophosphatase therefore occupies a key position at the convergence of a number of metabolic pathways and is responsible for the provision of inositol required for synthesis of phosphoinositides.

In 1971, Allison & Stewart [8] showed that acute treatment with Li⁺ caused up to a 35 % reduction in rat brain inositol levels. In later work this was shown to be paralleled by a large increase in Ins1P levels [9], suggesting that Li⁺ achieved these effects through inhibition of inositol monophosphatase. This was subsequently confirmed *in vitro* by Hallcher & Sherman [10]. This work has led to speculation that inhibition of this

enzyme underlies the anti-manic and anti-depressant action of Li⁺ [11]. It is argued that a reduction in the supply of inositol may compromise inositol lipid re-synthesis and therefore attenuate phosphoinositide-mediated responses. Furthermore, the brain should be particularly sensitive since it has no access to dietary inositol, thus providing a plausible explanation of how Li⁺ produces distinctive psychological effects largely in the absence of peripheral side effects. Although the theory has yet to receive convincing experimental support, there has been considerable interest in inositol monophosphatase as a primary target for Li⁺ action.

The enzyme has been purified from rat [12] and bovine [13–15] brain by a number of groups, and there is good agreement as to its general properties. The enzyme is a dimer of *M*_r 60000, composed of two apparently identical subunits. It catalyses the Mg²⁺-dependent hydrolysis of both enantiomers of Ins1P, both enantiomers of Ins4P, Ins5P and several non-inositol-containing compounds. Hydrolysis of substrate is inhibited in an uncompetitive manner by Li⁺, which has recently been shown to bind to a phosphoryl-enzyme intermediate [16]. All of the purification schemes described to date are lengthy, involving at least five or six steps, resulting in yields of only 6–12 % [12–15]. In the present paper we report on the production and characterization of a monoclonal antibody to inositol monophosphatase, and on the development of an immunoaffinity purification procedure which allows rapid isolation of the enzyme in high yield.

Abbreviations used: PEG, poly(ethylene)glycol; ABTS, 2,2'-azino-bis(3-ethylbenzthiazolinesulphonic acid); FCS, foetal calf serum; DMSO, dimethyl sulphoxide; ECGS, endothelial cell growth factor supplement; CNBr, cyanogen bromide.

* To whom correspondence should be addressed.

MATERIALS AND METHODS

Materials

Tissue culture media and additives were obtained from Flow Laboratories, Rickmansworth, Herts., U.K. The myeloma line Sp2/O-Ag.14 [17] was a gift from Dr. G. Evan, Imperial Cancer Research Foundation, Bartholomew Close, London, U.K. Poly(ethylene)glycol (PEG 1500) and endoproteinase glu-C were obtained from Boehringer Corp. (London) Ltd. Freund's adjuvants were obtained from Sigma Chemical Company Ltd., Poole, Dorset, U.K. Peroxidase-conjugated rabbit anti-mouse IgG was obtained from Dako Ltd., High Wycombe, Bucks., U.K. Protein A-Sepharose and Q Sepharose were obtained from Pharmacia Ltd., Milton Keynes, Bucks., U.K.

Production of monoclonal antibody G-2A4

Immunization. Female Balb/c mice received subcutaneous injections of 10 µg of purified bovine brain inositol monophosphatase [13] in 0.9% saline in the following schedule (related to the day of fusion = 0 days): -122 days, emulsified with Freund's complete adjuvant; -108 and -80 days, emulsified with Freund's incomplete adjuvant. The final dose (-4 days) was given intravenously without adjuvant.

Media. The culture medium was RPMI 1640 containing 1 mM-sodium pyruvate, 4 mM-glutamine, penicillin (50 units/ml), streptomycin (50 µg/ml) and foetal calf serum (10%, v/v). HAT-medium [18] contained, in addition, 0.4 µM-aminopterin, 0.1 mM-hypoxanthine and 16 µM-thymidine. Aminopterin was omitted in HT-medium. HT-medium containing 6% (v/v) dimethyl sulphoxide (DMSO) was used for cryopreservation of cells.

Cell fusion. Sp2/0 cells were cultured in logarithmic phase and fused with splenocytes according to the protocol of Fazekas de St. Groth & Scheidegger [19]. After fusion, cells were cultivated in 20 × 24-well plates (Flow Laboratories; Cat. No. 76-033-05) containing 1 ml of HAT-medium/well and either 2 × 10⁴ rat peritoneal macrophages or 100 µg of endothelial cell growth factor supplement (ECGS)/ml.

Screening of culture supernatants. Supernatants were screened by e.l.i.s.a. using flexible polyvinyl 96-well plates (Flow Laboratories; Cat. No. 77-176-05) coated with purified bovine brain inositol monophosphatase. All operations were carried out at room temperature. Plates were prepared by incubation with 100 µl of purified enzyme (1 µg/ml) in 50 mM-Tris/150 mM-NaCl, pH 7.5, for 3 h in a humidified chamber. After blocking with 10% (v/v) foetal calf serum (FCS) in Tris buffer (blocking buffer) for 30 min, duplicate wells were incubated with 100 µl of undiluted culture supernatants for 1 h. Plates were washed five times with blocking buffer, and each well received 100 µl of peroxidase-conjugated rabbit anti-mouse IgG (Dako; code P161) diluted 1:200 in blocking buffer. After 1 h, plates were washed as before. Each well then received 100 µl of freshly prepared substrate solution [1 mg of ABTS/ml in 50 mM-sodium acetate/acetic acid, pH 5.0, containing 1 µl of 30% (v/v) H₂O₂/ml]. After 15 min, the absorbance at 405 nm was

determined using a Flow Laboratories Multiskan Plus e.l.i.s.a.-plate reader.

Cloning of hybridomas. Hybridomas were cloned by limiting dilution in 96-well plates (Flow Laboratories; Cat. No. 76-008-04) in HT-medium containing 20% FCS.

Ascites tumours. These were induced by injecting 5 × 10⁶ twice-cloned cells into the peritoneal cavity of Balb/c mice, primed 1–2 weeks earlier with 0.5 ml of pristane. After 6–10 days, mice were killed and the fluid was removed by using a syringe and 18-gauge needle. Cells were pelleted by centrifugation and the supernatant was decanted and stored at -20 °C.

Purification of monoclonal antibody

Ascites fluid was dialysed against 50 mM-Tris/HCl, pH 8.0, at 4 °C and subjected to ion-exchange chromatography on Q Sepharose (Pharmacia). Bound proteins were eluted with a linear gradient of 0–300 mM-NaCl in the above buffer. Fractions containing monoclonal antibody were identified by the e.l.i.s.a. procedure, and purity was assessed by SDS/polyacrylamide-gel electrophoresis.

Western blotting

All operations were carried out at room temperature. Samples were run on 12% polyacrylamide gels with a 3% stacking gel. Polypeptides were transferred to nitrocellulose as described by Towbin *et al.* [20] using the Tris/glycine/methanol buffer system at 60 V for 18 h. Transfer was checked by staining with 0.1% Ponceau S in 50 mM-sodium acetate/acetic acid, pH 5.0. Nitrocellulose strips were blocked by incubation with 50 mM-Tris/150 mM-NaCl, pH 7.5, containing 10% FCS and 0.2% Tween 20 for 30 min, and were then incubated with undiluted spent culture supernatant for 1 h. Strips were washed five times with the above buffer and incubated with 1:200 diluted peroxidase-conjugated rabbit anti-mouse IgG for 1 h. Strips were washed five times before addition of substrate solution [0.2 mg of 3-amino-9-ethylcarbazole/ml in 50 mM-sodium acetate/acetic acid, pH 5.0, containing 1 µl of 30% (v/v) H₂O₂/ml].

Immunoabsorption of enzyme activity using Sepharose-Protein A

Protein A-Sepharose 4B (200 µl of 50% suspension) was incubated with 200 µl of rabbit anti-mouse IgG (Deiko, code Z259) for 15 min at room temperature. The gel was washed five times with 1.4 ml of 50 mM-Tris/150 mM-NaCl, pH 8.0, and resuspended in the same buffer (final vol. 300 µl). Aliquots of 100 µl were incubated for 1 h at 4 °C with spent culture medium (9 ml) from G-2A4 hybridomas or the myeloma parent Sp2/0. After washing three times as above, the gel was mixed with 50 µl of a 40–60%-satd. ammonium sulphate fraction (500 munits/ml) prepared from bovine brain supernatant ('crude brain extract') [13]. After 30 min at 4 °C, the gel was washed five times and assayed for inositol monophosphatase activity.

Enzyme assays

Inositol monophosphatase and Ins(1,4)P₂/Ins(1,3,4)P₃ 1-phosphatase activities were measured by radiochemical

assays as described previously [21]; 1 unit hydrolyses 1 μ mol of substrate/min at 37 °C.

Limited proteolysis

Samples of purified inositol monophosphatase (0.1 mg/ml in 50 mM-Tris/150 mM-NaCl, pH 8.0) were subjected to limited proteolysis with 0.2 μ g of endoproteinase glu-C (Boehringer; code 791156) in a final volume of 25 μ l for 1 h at 37 °C. Digestion was terminated by heating in boiling water for 5 min and products were analysed by SDS/polyacrylamide-gel electrophoresis.

Effect of antibody binding on inositol monophosphatase activity

Various amounts of monoclonal antibody (in 50 μ l of monophosphatase assay buffer [21] containing 0.1 mg/ml of bovine serum albumin) were preincubated for 1 h at 4 °C with 100 μ l of diluted crude brain extract containing 0.6 munits of enzyme activity. Residual activity was then determined as described [21].

Other methods

Inositol monophosphatase used for immunizations was prepared as described previously [13]. SDS/polyacrylamide-gel electrophoresis was performed in 12% polyacrylamide gels (0.75 mm $\times$ 8 cm $\times$ 7 cm) using the Laemmli buffer system [22]. Fluorographs were prepared as described by Bonner & Lasky [23]. Protein concentration was measured by the method of Bradford [24].

RESULTS AND DISCUSSION

Production of monoclonal antibody G-2A4

After cell fusion, hybridoma growth was noted in 87 out of 288 wells. Of these, 77 developed in plates containing rat peritoneal macrophages. Omission of macrophages and inclusion of ECGS resulted in only a low yield of hybridomas (12 out of 144 wells). Initially, three of the 87 hybridoma supernatants were scored positive by the e.l.i.s.a. procedure. Two of these hybridomas proved to be unstable and only one, G-2A4, was successfully cloned twice.

Characterization of monoclonal antibody G-2A4

Analysis of radiolabelled secreted products from the G-2A4 hybridoma by SDS/polyacrylamide gel electrophoresis followed by fluorography revealed a single sharp light chain, consistent with the presumed monoclonal nature of the twice-cloned cell line (Fig. 1). The heavy chain appeared to run as a doublet and this was confirmed for antibody purified from ascites fluid (results not shown). Using an antibody isotyping kit (Amersham International) the antibody was found to be an IgG_{2b}(K).

Western blotting

In order to check that antibody G-2A4 bound to inositol monophosphatase, rather than to a trace contaminant in the e.l.i.s.a. procedure, Western blots of apparently homogeneous enzyme were probed with G-2A4 cultured supernatant. Specific binding to an M_r -30000 polypeptide was observed (Fig. 2).

Effect of antibody binding on enzyme activity

Titration of the monoclonal antibody against enzyme present in a crude brain extract gave the inhibition curve shown in Fig. 3. Binding of the antibody resulted in up to

Fig. 1. Metabolic labelling of G-2A4 by [¹⁴C]leucine

Hybridoma cells (5×10^6) were washed once with leucine-free Eagle's minimum essential medium containing 5% (v/v) dialysed FCS. Cells were resuspended in 0.5 ml of the same medium containing 1 μ Ci of L-[U-¹⁴C]leucine and incubated for 16 h at 37 °C in an atmosphere of 5% CO₂ in air. Radiolabelled secreted products were subjected to SDS/polyacrylamide-gel electrophoresis and a fluorograph of the gel was prepared.

83% inhibition of enzyme activity, whereas under identical conditions no loss of activity was observed for Ins(1,4)*P*₂/Ins(1,3,4)*P*₃ 1-phosphatase. Identical results were obtained with immunoaffinity-purified inositol monophosphatase, indicating that the crude brain extract did not contain an enzyme fraction which was unable to bind antibody. It is therefore unclear at present why full inhibition cannot be achieved with the monoclonal antibody. However, the residual enzyme activity of the immunocomplex was fully inhibited by 100 mM-Li⁺ with an IC₅₀ of 0.9 mM, similar to that reported for free enzyme [21].

Immunoabsorption of enzyme activity

Further evidence that inositol monophosphatase was indeed the antigen recognized by the antibody was obtained from immunoabsorption experiments. Inositol monophosphatase was adsorbed from crude brain extract using Protein A-Sepharose which had been coated successively with rabbit anti-(mouse IgG) and then with G-2A4 monoclonal antibody. The control with no monoclonal antibody showed weak activity (< 0.1 munits), whereas the experimental sample contained 2.6 munits. Formation of an immunocomplex under these conditions indicated that the antibody may be useful for large-scale immunoaffinity purification of the enzyme.

Fig. 2. Immunoblotting of inositol monophosphatase

See the Materials and methods section for details. Purified inositol monophosphatase was subjected to SDS/polyacrylamide-gel electrophoresis and transferred on to nitrocellulose membrane. Track 1, probed with 1:1000 diluted ascites fluid and stained by peroxidase reaction after addition of peroxidase-conjugated rabbit anti-(mouse IgG). Track 2, control for track 1 with omission of monoclonal antibody.

Preparation of G-2A4-Sepharose 4B immunoabsorbent

Monoclonal antibody was partially purified from 12 ml of ascites fluid by ion-exchange chromatography on Q Sepharose. This yielded a fraction containing 40 mg of protein. Analysis by SDS/polyacrylamide-gel electrophoresis revealed that the antibody was approx. 60% pure (i.e. approx. 25 mg) with one major contaminant (M_r 67000), presumed to be serum albumin (results not shown). The immunoaffinity column was prepared by coupling this fraction to CNBr-activated Sepharose 4B. Coupling efficiency was 90%, giving 38 mg of protein/ml of gel (approx. 10 mg of antibody/ml of gel).

Immunoaffinity purification of inositol monophosphatase

The choice of possible desorbing agents was necessarily limited since preservation of the biological activity of both enzyme and monoclonal antibody was desirable. Elution with low pH buffers was precluded since the enzyme is irreversibly denatured between pH 4 and pH 5 [10]. Exposure of the free antibody to chaotropic salts (4 M-MgCl₂ or 3 M-NaSCN) or urea resulted in a total loss of activity in the e.l.i.s.a. procedure (results not shown). Initial attempts to dissociate the antibody-

Fig. 3. Effect of antibody binding on enzyme activity

See the Materials and methods section for details. Various amounts of monoclonal antibody were preincubated for 1 h at 4 °C with crude brain extract. Residual enzyme activity was determined as described [21]. Activities are expressed relative to that for crude brain extract in the absence of antibody.

Table 1. Recovery of inositol monophosphatase from the immunoaffinity column with various elution buffers

Small portions of the immunoabsorbent column were saturated with enzyme by incubating with excess crude brain extract. After washing, aliquots were eluted with one of the buffers indicated below. Enzyme activity was determined as described [21] and yields are expressed relative to that obtained with pure water.

Eluent	Relative yield
Pure water	100
1 mM-Tris/2 M-urea	62
1 mM-Tris/50% ethylene glycol	16
50 mM-NaHCO ₃ /NaOH, pH 11.0	83
50 mM-NaHCO ₃ /NaOH/50% ethylene glycol, pH 11.0	216
1 mM-NaHCO ₃ /NaOH/50% ethylene glycol, pH 11.0	224

antigen complex using the exceptionally mild hypo-osmotic elution technique [25] with pure water resulted in a 17% yield in activity from the fully saturated immunoabsorbent. Other possible eluents were compared with pure water in pilot scale experiments (Table 1). Low-ionic-strength buffers containing denaturing agents (urea) or polarity-reducing agents (ethylene glycol), or high pH buffer (50 mM-NaHCO₃/NaOH, pH 11) were less effective. However, the elution efficiency of the latter buffer was markedly improved by inclusion of 50% ethylene glycol, and further improved by reducing the buffer concentration to 1 mM. In subsequent experiments we found that the concentration of ethylene glycol could be reduced to 10% without adversely affecting elution efficiency. In addition, β -alanine was used instead of bicarbonate buffer in order to facilitate concentration of the eluted enzyme by anion-exchange chromatography (see below).

For large-scale purification, the column was loaded with a crude ammonium sulphate fraction from bovine

Fig. 4. Chromatography of immunoaffinity-purified enzyme on Mono Q

The column was loaded with neutralized material from the immunoaffinity column and eluted with a linear salt gradient (0–200 mM-NaCl) in 50 mM-Tris/HCl, pH 8.0 (total gradient volume, 30 ml). (a) Absorbance profile at 280 nm. (b) Analysis of peak fractions by SDS/polyacrylamide-gel electrophoresis (5 μ l/track). Proteins were stained with Coomassie Blue. M, marker proteins.

brain supernatant containing a large excess of inositol monophosphatase (66400 munits). The non-bound and wash fractions contained a combined total of 29000 munits, indicating a column-binding capacity of 37400 munits. Elution with 1 mM- β -alanine/10% ethylene glycol, pH 10.8, yielded 20200 munits, 54% of the bound activity. The enzyme was essentially homogeneous, although in some operations of the column a faint band (M_r 60000) was observed in the peak fraction. The eluted and neutralized material was applied directly on to a HR5/5 Mono Q column (Pharmacia) which was then eluted with a linear salt gradient [Fig. 4]. This step allowed (i) concentration of the enzyme, (ii) removal of ethylene glycol, and (iii) removal of any trace contaminants. The yield was 90%, giving an overall yield of 47%, considerably better than the 8% yield reported by our laboratory [13] and others [12,14,15] for the conventionally purified enzyme.

The specific activity of the immunoaffinity-purified enzyme (8–16 units/mg; three preparations) was higher than that for the conventionally purified material (6 units/mg, [13]). However, K_m values for D(+)-Ins1P and (+)-Ins4P were essentially identical to those reported previously [13] (results not shown). The absorbance profile from the Mono Q column showed a major peak and several minor peaks (Fig. 4a), although analysis of

Fig. 5. Partial proteolytic maps of brain and kidney inositol monophosphatase using endoproteinase glu-C

See the Materials and methods section for details. Proteins were analysed by SDS/polyacrylamide-gel electrophoresis and stained with Coomassie Blue. Track 1, marker proteins (Pharmacia); track 2, 2 μ g of undigested brain enzyme; track 3, 2 μ g of undigested kidney enzyme; track 4, digested brain enzyme; track 5, digested kidney enzyme. 'E' indicates band corresponding to the protease.

these fractions by SDS/polyacrylamide-gel electrophoresis [Fig. 4b] failed to reveal any contaminants or different- M_r forms of the enzyme. A similar profile was observed with conventionally purified enzyme (results not shown). Although little is known about the regulation of inositol monophosphatase, this heterogeneity may be indicative of some sort of post-translational modification, possibly phosphorylation. We have not investigated this possibility further, although it is interesting to note that human platelet Ins(1,4,5) P_3 -5-phosphatase is phosphorylated by protein kinase C, leading to an alteration in its kinetic parameters [26].

Purification of the bovine kidney enzyme

Evidence for at least a limited immunological similarity of the brain and kidney enzymes was provided by the successful purification of the bovine kidney cortex enzyme using the same procedure. In addition, the two enzymes had identical electrophoretic mobilities on SDS/polyacrylamide gels and showed identical partial proteolytic fragmentation patterns with endoproteinase glu-C in the presence of SDS (Fig. 5). Thus the two enzymes are almost certainly products of the same gene.

Conclusions

In order to extend our studies on inositol monophosphatase, the development of a simple and rapid purification procedure allowing isolation of the enzyme in high yield was a matter of high priority. The development of a monoclonal-antibody-based immunoaffinity purification procedure represents a major improvement over existing conventional procedures and should be amenable to further scale-up if required. The availability of monoclonal antibody G-2A4 also opens up other areas of investigation. For example, we have

identified Li⁺-sensitive inositol monophosphatase activity in the bovine kidney cell line NBL-1 (N. S. Gee, S. Howell, G. Ryan & C. I. Ragan, unpublished work). The use of this cell line for metabolic labelling studies should allow us to investigate possible regulatory mechanisms (e.g. phosphorylation). Finally, one particularly exciting prospect would be to introduce the purified antibody into appropriate cell lines. Such a specific inhibitor of the enzyme may allow us to delineate those Li⁺-induced phosphoinositide cycle changes (direct and indirect) for which the key initial event is blockade of inositol monophosphatase, and those which require the interaction of Li⁺ with other targets.

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