Comparison of the activities of a multiple inositol polyphosphate phosphatase obtained from several sources: a search for heterogeneity in this enzyme

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A multiple inositol polyphosphate phosphatase (formerly known as inositol 1,3,4,5-tetrakisphosphate 3-phosphatase) was purified approx. 22000-fold from rat liver. The final preparation migrated on SDS/PAGE as a doublet with a mean apparent molecular mass of 47 kDa. Upon size-exclusion chromatography, the enzyme was eluted with an apparent molecular mass of 36 kDa. This enzyme was approximately evenly distributed between the 'rough' and 'smooth' subfractions of endoplasmic reticulum. There was a 20-fold range of specific activities of this phosphatase in CHAPS-solubilized particulate fractions prepared from the following rat tissues: liver, heart, kidney, testis and brain. However, each of these extracts contained different amounts of endogenous inhibitors of enzyme activity. After removal of these inhibitors by MonoQ anion-exchange chromatography, there

was only a 2.5-fold range of specific activities; kidney contained the most and brain contained the least. We prepared and characterized polyclonal antiserum to the hepatic phosphatase, which immunoprecipitated 85–100% of both particulate and soluble phosphatase activities. The antiserum also immunoprecipitated, with equivalent efficacy, CHAPS-solubilized phosphatase activities from heart, kidney, testis, brain and erythrocytes (all prepared from rat). Our data strengthen the case that the function of the mammalian phosphatase is unrelated to the metabolism of Ca^{2+} -mobilizing cellular signals. The CHAPS-solubilized phosphatase from turkey erythrocytes was not immunoprecipitated by the polyclonal antiserum, and is therefore an isoform that is structurally distinct, and possibly functionally unique.

INTRODUCTION

$\text{Ins}(1,3,4,5)\text{P}_4$ is generated in cells by phosphorylation of $\text{Ins}(1,4,5)\text{P}_3$, which is itself produced from the receptor-activated PLC-catalysed hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$ [1]. The function of $\text{Ins}(1,3,4,5)\text{P}_4$ is a subject of intensive analysis and debate; the contention focuses on the extent of its contribution to the regulation of Ca^{2+} entry and the sustaining of intracellular Ca^{2+} signals (see [2] and [3]). As for the metabolism of $\text{Ins}(1,3,4,5)\text{P}_4$, although it is well established that a major pathway is dephosphorylation by a 5-phosphatase, it is the unknown physiological significance of a $\text{Ins}(1,3,4,5)\text{P}_4$ 3-phosphatase that is currently a puzzling aspect of this particular field (see [1] for review). We have previously purified $\text{Ins}(1,3,4,5)\text{P}_4$ 3-phosphatase activity from rat liver, and we have demonstrated that $\text{Ins}(1,3,4,5,6)\text{P}_5$ and InsP_6 are high-affinity competing substrates [4]. For this reason, we now propose that this enzyme be termed a 'multiple inositol polyphosphate phosphatase' (MIPP).

$\text{Ins}(1,3,4,5,6)\text{P}_5$ and InsP_6 act as potent endogenous inhibitors of MIPP-catalysed $\text{Ins}(1,3,4,5)\text{P}_4$ 3-phosphatase activity, in both cell homogenates and subcellular fractions [4–6]. Only after these inhibitors are resolved from the enzyme is it possible to assay accurately its activity against $\text{Ins}(1,3,4,5)\text{P}_4$. By performing such experiments, we have shown that the hepatic phosphatase activity is compartmentalized inside endoplasmic reticulum [5]. We have not yet found a mechanism by which the phosphatase may have access to any of its known substrates *in vivo*.

In order to gain further information on the characteristics of MIPP, we have now investigated the extent to which there may be heterogeneity in this protein, either between or within a variety of cell types; information in this direction may provide clues towards identifying possible site or tissue-dependent

functions of this protein. For example, we have investigated the distribution of hepatic MIPP between the functionally different compartments of endoplasmic reticulum, by analysing enzyme activity in both 'rough' and 'smooth' subfractions.

Another approach to search for the significance of an enzyme is to compare specific activities across several tissues. This strategy has already provided useful information on the relative activities and importance of some other enzymes involved in inositol phosphate turnover. For example, $\text{Ins}(1,3,4)\text{P}_3$ 4-phosphatase activity is particularly enriched in brain, which has led to the proposal that $\text{Ins}(1,3)\text{P}_2$ might have a specific neuronal function [7]. $\text{Ins}(1,4)\text{P}_2$ 1-phosphatase and $\text{Ins}(1,4,5)\text{P}_3$ 3-kinase activities are both unusually low in heart [7,8], which has underscored the idea that inositol phosphates are not of such primary importance to Ca^{2+} signalling in this particular organ. Thus, we have now compared the specific activity of hepatic MIPP with its counterpart in several other tissues; this goal was complicated by the requirement that the enzyme from each source first had to be separated from endogenous inhibitor(s).

To complement our search for possible heterogeneity of MIPP, we have characterized an immunoprecipitating antiserum which we have raised against the rat hepatic 'particulate' enzyme. This has then enabled us to undertake two important immunological comparisons. First, we have investigated the immunoreactivity of the antiserum with the small proportion of cellular MIPP that is associated with soluble fractions of the liver [4,5]. Secondly, we have investigated to what extent the hepatic antiserum will recognize 'particulate' MIPP prepared from different tissues. One cell type that received our particular attention was the erythrocyte, since, in humans at least, these cells contain $\text{Ins}(1,3,4,5)\text{P}_4$ 3-phosphatase in their plasma membranes [9]. This subcellular location is different from that of the bulk of hepatic

Abbreviations used: MIPP, multiple inositol polyphosphate phosphatase (formerly inositol 1,3,4,5-tetrakisphosphate 3-phosphatase). Inositol phosphates are abbreviated according to IUPAC nomenclature.

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MIPP (i.e. endoplasmic reticulum [5]). We have therefore investigated whether this may be a characteristic of erythrocytes from other sources, and if so, whether this may be reflected in significant structural differences that might impact on epitope recognition by our polyclonal antiserum.

MATERIALS AND METHODS

Materials

[³H]Ins(1,3,4,5)*P*₄ (21 Ci/mmol) was supplied by DuPont–New England Nuclear. Non-radioactive Ins(1,3,4,5)*P*₄ was obtained from either Boehringer Mannheim or The University of Rhode Island Foundation (Kingston, RI, U.S.A.). Killed *Staphylococcus aureus* cells (100 061) and Triton X-100 were supplied by Boehringer Mannheim. The MonoQ HR 5/5 anion-exchange column and DEAE-Sephacrose Fast-Flow were from Pharmacia LKB. CHAPS, heparin–agarose (type II), Reactive Red 120–agarose (type 3000-CL) and poly-L-lysine–agarose were purchased from Sigma Chemical Co. The poly-L-lysine column was pre-treated with 2 ml of BSA (1 mg/ml; Fraction V, Boehringer Mannheim) to maximize enzyme recovery; reversibly bound BSA was subsequently released by extensive washing with 10 mM triethanolamine/1 mM MgCl₂/0.05% (w/v) CHAPS/0.5 M KCl, pH 7.4.

Assay of MIPP

Enzyme activity towards 5 μM [³H]Ins(1,3,4,5)*P*₄ was determined either in the absence of Mg²⁺ with 1 mM EDTA at pH 6.1 (Enzyme Assay Buffer 1) during isolation of the enzyme or in the presence of 1 mM Mg²⁺ at pH 7.4 (Enzyme Assay Buffer 2) when incubations were performed with the purified enzyme (for precise assay conditions see [4]). Reactions were terminated with 1 ml of ice-cold 0.2 M ammonium formate/0.1 M formic acid containing 1 mg/ml Ins*P*₆. Samples were placed on ice for at least 5 min, and then centrifuged at 10000 *g* for 5 min. The resultant supernatants were diluted 10-fold in water and chromatographed batchwise on 0.8 ml gravity-fed anion-exchange columns (Bio-Rad AG1-X8, 200–400 mesh, formate form) to separate [³H]inositol polyphosphates [10]. Reaction rates were linear at the enzyme concentrations used, provided that less than 30% of the initial substrate was metabolized.

Purification of hepatic MIPP

The enzyme was prepared from perfused livers from ten male Sprague–Dawley rats (200–250 g) by a modification of previously described procedures [4]. Each liver was individually homogenized in 15 ml of ice-cold buffer containing 10 mM Bistris, 250 mM sucrose, 1 mM EDTA and 5 mM NaN₃, pH 7.4, and a series of protease inhibitors [4]. Homogenates from each liver were combined and centrifuged at 100000 *g* for 60 min. The resultant pellet was solubilized with CHAPS as previously described [4], except that the second pellet wash was omitted, since it did not release significant additional MIPP (results not shown). All subsequent purification procedures were performed at 0–4 °C by f.p.l.c.

The CHAPS-solubilized particulate fraction was loaded at a flow rate of 2 ml/min on to a DEAE-Sephacrose Fast-Flow column (44 mm × 210 mm) equilibrated with Buffer A (10 mM Bistris, 250 mM sucrose, 5 mM NaN₃, pH 7.4). The column was washed with 500 ml of Buffer A plus 75 mM NaCl, and bound protein was eluted in 20 ml fractions at a flow rate of 2 ml/min with a 980 ml linear gradient of 75–400 mM NaCl in Buffer A.

Peak fractions were pooled, diluted 5-fold with Buffer B (10 mM triethanolamine, pH 7.4) and applied at a flow rate of

2 ml/min to a heparin–agarose column (22 mm × 130 mm) equilibrated with Buffer B. The column was washed with 200 ml of Buffer B supplemented with 75 mM KCl. Bound protein was eluted in 20 ml fractions with a 1100 ml linear gradient of 75–400 mM KCl in Buffer B at a flow rate of 2 ml/min.

Peak fractions were pooled, diluted 4-fold with Buffer C (10 mM triethanolamine, 1 mM MgCl₂, 0.02% CHAPS, pH 7.4) and loaded on to a column of Reactive Red 120–agarose (10 mm × 90 mm) at a flow rate of 2 ml/min. The column was washed at a flow rate of 1 ml/min with 60 ml of Buffer C, and bound protein was released with a 210 min linear 0–500 mM KCl/0.02–0.05% CHAPS gradient at 1 ml/min, generated by the mixing of Buffer C and Buffer D (10 mM triethanolamine, 1 mM MgCl₂, 0.05% CHAPS, 0.5 M KCl, pH 7.4). Fractions were collected at intervals of either 10 min (Load and Wash protocols) or 6 min (Gradient protocol). The conditions used for the Reactive Red–agarose column were different from those previously employed [4], in that CHAPS was present (to improve enzyme recovery) and MgCl₂ was added (which augments polypeptide binding to dye–ligand matrices [11]). In ten preparations, all of the enzyme bound to the column, and this was always eluted as a single peak of activity by 300 mM KCl (Figure 1a). When CHAPS and MgCl₂ were not present, the latter peak was still observed ([4]; results not shown), but in those conditions a variable proportion of enzyme activity then failed to bind to the Reactive Red–agarose column.

The enzyme was substantially concentrated by chromatography on a BSA-conditioned poly-L-lysine column (Figure 1b). Pooled peak fractions were diluted 2.5-fold with Buffer E (10 mM triethanolamine, 1 mM MgCl₂, 0.05% CHAPS, pH 7.4) and applied to a poly-L-lysine–agarose affinity column (5 mm × 60 mm). This column was then washed at a flow rate of 0.2 ml/min with 5 ml of Buffer E supplemented with 50 mM KCl, and bound protein was eluted in 1 ml fractions with 10 ml of Buffer E plus 500 mM KCl at a flow rate of 0.2 ml/min. Fractions containing MIPP were stored for 1–2 days at 4 °C before analysis by SDS/PAGE. Longer-term storage of pooled fractions (for up to 12 months with no loss of activity) was at –20 °C after the addition of 20% (v/v) glycerol and 1 mg/ml BSA.

Solubilization of MIPP from various tissues and separation of the enzyme from endogenous inhibitor(s)

CHAPS-solubilized 100000 *g* particulate fractions were prepared from several rat tissues essentially as described above for liver. A sample of each detergent-solubilized membrane fraction (approx. 25 mg of protein) was applied at a flow rate of 1 ml/min to a MonoQ HR5/5 column equilibrated with Buffer F (Buffer A supplemented with 0.5% CHAPS). The column was washed with 5 ml of Buffer F, and bound protein was eluted at the same flow rate with a gradient generated by the mixing of Buffer F with Buffer G (Buffer F plus 500 mM NaCl) as follows: 0–40 min, 0–60% Buffer G; 40 min, 60–100% Buffer G; 40–45 min, 100% Buffer G. The three fractions containing the peak of enzyme activity were pooled and desalted.

Turkey and rat erythrocyte membranes were prepared from fresh blood essentially as described previously [12], except that the membranes were finally resuspended in 50 mM Bistris, pH 6.1. A CHAPS-solubilized membrane fraction was prepared by mixing 1 vol. of packed membranes with 1 vol. of 50 mM Bistris, pH 6.1, containing 2% CHAPS. After incubation on ice for 60 min, the membrane lysate was centrifuged at 100000 *g* for 60 min to precipitate the insoluble pellet.

Preparation of Ins(1,4,5) P_3 /Ins(1,3,4,5) P_4 5-phosphatase activities

Partially purified Ins(1,4,5) P_3 /Ins(1,3,4,5) P_4 5-phosphatase activities were prepared by MonoQ anion-exchange chromatography (see above) of either 100 000 *g* soluble (Type 1 or 2 enzyme activities) or CHAPS-solubilized particulate fractions (membrane-associated enzyme activity). Enzyme assays were performed with 25 μ M [3 H]Ins(1,4,5) P_3 as a substrate.

Preparation of polyclonal antiserum to MIPP and its immunoprecipitation of the enzyme

A 20 μ g portion of the purified native enzyme (47 kDa doublet) was emulsified with an equal volume of complete Freund's adjuvant and injected into a single male New Zealand White rabbit (2–3 kg) at both multiple intradermal and subcutaneous sites. The rabbit was injected again after 3 weeks with a fresh preparation of isolated enzyme (45 μ g) mixed with an equal volume of incomplete Freund's adjuvant. Blood was withdrawn 10 days later, from which serum was prepared [13]. Similar amounts of MIPP prepared from various sources (see above) were mixed with 0–5 μ l of polyclonal antiserum (10-fold dilution in Enzyme Assay Buffer 1) and the total amount of serum per tube was made constant with an appropriate quantity of pre-immune serum. After incubation for 2 h at 4 °C on a nutator, 25 μ l of a 10% (v/v) killed *Staph. aureus* cell suspension was added, which had previously been pre-equilibrated with Enzyme Assay Buffer 1. After brief vortex-mixing, the mixture was incubated on a nutator at 4 °C for a further 2 h. The immune complexes were pelleted by centrifugation at 10 000 *g* for 1 min and the supernatants removed for assay of residual enzyme activity.

Other methods

Protein was determined with either a Bio-Rad protein microassay kit [14], or an ISS Protein Gold assay kit (Enprotech Corp., Hyde Park, MA, U.S.A.). BSA was the protein standard. SDS/PAGE was performed with a Mighty Small II mini-gel system (Hoefer Scientific Instruments, San Francisco, CA, U.S.A.) with a 10% resolving gel, according to Laemmli [15]. Polypeptide bands were revealed with a silver diamine stain. Size-exclusion chromatography was performed with a Superose 6 FPLC column (10 mm $\times$ 300 mm; Pharmacia LKB Biotechnology Inc.) equilibrated at a flow rate of 0.2 ml/min with 10 mM triethanolamine/200 mM KCl, pH 7.4, in either the presence or the absence of 10 mM 2-mercaptoethanol/0.5% CHAPS. The gel-filtration column was calibrated under each specific elution protocol with Blue Dextran 2000 (Pharmacia LKB Biotechnology) and the following standards (Bio-Rad, Richmond, CA, U.S.A.): thyroglobulin (670 kDa), γ -globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa) and vitamin B₁₂ (1.35 kDa). 'Rough' and 'smooth' subfractions of endoplasmic reticulum were prepared as described in [16]. NADPH-cytochrome *c* reductase was assayed as described in [17]; RNA was measured as described in [18].

RESULTS

Purification of hepatic MIPP

Our procedures for purifying hepatic MIPP [previously called Ins(1,3,4,5) P_4 3-phosphatase] vary only slightly from those described previously [4]. One difference in our current work was the inclusion of CHAPS and MgCl₂ during the chromatography on Red-agarose, which facilitated total binding of enzyme

Figure 1 Purification of MIPP

(a) Pooled peak fractions (fractions 65–70) from the heparin-agarose column were diluted and applied to a Reactive Red-agarose column, and then eluted as described in the Materials and methods section. Bound protein was eluted with a gradient (broken line) of 0–0.5 M KCl/0.02–0.05% CHAPS. Fractions were collected and assayed for MIPP-dependent Ins(1,3,4,5) P_4 3-phosphatase activity (■) or protein (□) by using a Protein Gold assay kit. Similar chromatographic profiles were observed with nine additional preparations of MIPP. (b) Pooled peak fractions (fractions 52–56) from the Reactive Red 120 dye-ligand affinity column were diluted and loaded on to a poly-L-lysine-agarose column as described in the Materials and methods section. Bound material was eluted with a 0–0.5 M KCl step gradient (broken line). Fractions were collected and assayed for either MIPP-dependent Ins(1,3,4,5) P_4 3-phosphatase activity (■) or protein (□). Similar elution patterns have been observed with seven different enzyme preparations. Inset to (b): 15 μ l portions of fractions 16–23 from the poly-L-lysine-agarose column were incubated for 5 min with 15 μ l of SDS sample buffer [final concn. 0.7 M 2-mercaptoethanol plus 2% (w/v) SDS] at 95–100 °C, and 25 μ l samples were applied to a 10% resolving minigel. Polypeptides were separated under constant-current conditions (15 mA/gel). The gel was developed with a silver-diamine stain. Individual lanes are denoted as their column fraction numbers from the poly-L-lysine-agarose column. The position of the molecular-mass standard is indicated. Similar gel profiles were observed in six independent enzyme preparations.

activity to the column (compare Figure 1a with [4]). One further difference was that the final chromatographic step used a poly-L-lysine column (Figure 1b) instead of a hydroxyapatite column [4]. The final preparations of MIPP were enriched approx. 22 000-fold relative to the CHAPS-solubilized particulate fraction with an overall recovery of 12% (Table 1). These purification parameters are similar to those reported in our previous study [4], although such data are approximate because of the presence

Table 1 Purification of MIPP

A 100 000 g CHAPS-solubilized particulate fraction was prepared from 10 perfused rat livers as described in the Materials and methods section. The apparent enzyme activity against $\text{Ins}(1,3,4,5)\text{P}_4$ in the CHAPS-solubilized particulate fraction was 172 pmol/min, but this was constrained by endogenous inhibitor(s) [5]. Thus initial total enzyme activity was estimated from the sum of the activity in individual fractions, after DEAE-Sepharose anion-exchange chromatography. A 10–20-fold increase in apparent activity was typically observed after separation from its endogenous inhibitor(s). Protein was quantified by using either a Bio-Rad protein microassay (lines 1–3) or an ISS Protein Gold assay kit (lines 4 and 5). The data are representative of eight different enzyme preparations.

Step	Volume (ml)	Total activity (nmol/min)	Protein (mg)	Specific activity (nmol/min per mg)	Purification (fold)	Yield (%)
CHAPS solubilization	312	1588	7181	0.22	1	100
DEAE-Sepharose	120	1588	489	3.2	15	100
Heparin-agarose	80	677	8	84	380	43
Reactive Red-agarose	30	400	0.075	5298	24100	25
Polylysine-agarose	2	187	0.038	4291	22400	12

Figure 2 SDS/PAGE of purified MIPP

A preparation of purified enzyme (2 μg) was separated on a 10% resolving gel under constant-current conditions (30 mA/gel), and the gel was developed with Coomassie Brilliant Blue R-250. The positions of the molecular-mass standards (kDa) are indicated as shown.

of endogenous inhibitor(s) of phosphatase activity in the CHAPS-solubilized particulate fraction [4–6]. The final preparations of enzyme were also similar to those of Nogimori et al. [4] in terms of specific activity, substrate specificity and kinetic parameters (results not shown).

Molecular mass of MIPP

The molecular mass of the enzyme was determined by SDS/PAGE. The purified preparation of enzyme was resolved as a doublet, the two bands of which migrated approx. 1 kDa from each other; their average apparent molecular mass was 47 kDa (Figures 1b and 2). The relative intensities of both bands of the

Figure 3 Size-exclusion chromatography of MIPP

Purified enzyme (200 μl) was applied to a Superose 6 FPLC column (10 mm $\times$ 300 mm) equilibrated with 10 mM triethanolamine/200 mM KCl/10 mM 2-mercaptoethanol/0.5% CHAPS, pH 7.4. The column was eluted at a flow rate of 0.2 ml/min, and fractions were collected at intervals of either 5 min (fractions 1–10) or 1 min (fractions 11–90) and assayed for enzyme activity ($\blacktriangle$). The Superose 6 FPLC column was calibrated ($\bullet$) with thyroglobulin (670 kDa), γ -globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa) and vitamin B₁₂ (1.35 kDa). The recovery of applied activity was 83%. Similar values for the molecular mass of this protein were determined with two independent enzyme preparations in either the absence or presence of a reducing agent (results not shown). No significant difference in the molecular mass of the enzyme was observed in the absence of CHAPS, in either the absence or presence of 2-mercaptoethanol (results not shown), although enzyme recoveries were then typically less than 20%.

doublet correlated with the distribution of enzyme activity in the peak fractions eluted from the poly-L-lysine column (Figure 1b); the lower-molecular-mass band in the doublet was consistently the more intense of the two (Figures 1b and 2). Neither the poly-L-lysine column (Figure 1b) nor a hydroxyapatite column (results not shown) were able to separate the two bands of the doublet from each other. We investigated whether these two bands were differentially phosphorylated forms of the same enzyme, by incubating 100 ng of a preparation of the doublet with 0.1 Sigma Unit of potato acid phosphatase (Type VII) for up to 30 min at 37 °C. This treatment did not shift either the R_f values or relative intensities of the two bands on SDS/PAGE (results not shown). It is likely that the doublet is composed of two slightly different forms of MIPP, but we cannot exclude the possibility that an unrelated protein is contaminating our final preparations.

Partially purified preparations of 'Ins(1,3,4,5) P_4 3-phosphatase' activity isolated from pig brain were eluted with an apparent molecular mass of 36 kDa on size-exclusion chromatography

Table 2 Comparison of MIPP specific activity in rough and smooth subfractions of endoplasmic reticulum

The various assays were performed as described in the Materials and methods section, except that the MIPP activities were assayed against $\text{Ins}(1,3,4,5)\text{P}_4$ after phytase digestion of endogenous inhibitors [5].

	Ratio (rough/smooth)
MIPP	1.5 ± 0.2 (4)
NADPH-cytochrome <i>c</i> reductase	0.56 ± 0.04 (5)
RNA	5.1 ± 0.5 (5)

[19]. This was also the apparent molecular mass that we obtained on gel filtration of our purified MIPP, in both the presence and absence of mercaptoethanol (Figure 3). In the current studies we never observed the 66 kDa species of MIPP that we described in our previous experiments [4]; we are at present unable to explain this difference.

Distribution of hepatic MIPP between rough and smooth subfractions of endoplasmic reticulum

Almost all of the MIPP in a liver homogenate co-sediments with a 100 000 *g* particulate fraction [4,5]. Subsequent studies demonstrated that the enzyme is inside the endoplasmic reticulum [5]. We have now investigated the distribution of MIPP between the functionally different smooth and rough subfractions of this heterogeneous organelle. RNA was about 5 times more enriched in our rough subfractions, as compared with the smooth subfraction (Table 2). NADPH-cytochrome *c* reductase was preferentially concentrated by a factor of approx. 2 in the smooth subfraction (Table 2). The distribution of these markers in rat liver subfractions compares favourably with those seen by other workers [16,20]. MIPP-dependent $\text{Ins}(1,3,4,5)\text{P}_4$ 3-phosphatase activity in each subfraction was measured after digestion of endogenous inhibitors by phytase (see [5]). The distribution of MIPP did not parallel either of our specific markers, and instead it was approximately uniformly distributed between both subfractions of endoplasmic reticulum.

Comparison of the specific activities of MIPP isolated from several rat tissues

We have compared MIPP in the CHAPS-solubilized particulate fractions of five different organs: kidney, heart, testis, liver and

brain (Table 3). Across this range of tissues, there was a 20-fold difference in apparent specific enzyme activities towards $\text{Ins}(1,3,4,5)\text{P}_4$. However, we anticipated that the interpretation of these data would be compromised by the CHAPS-solubilized extracts containing various amounts of $\text{Ins}(1,3,4,5,6)\text{P}_5$, InsP_6 and possibly other endogenous inhibitors of MIPP-dependent $\text{Ins}(1,3,4,5)\text{P}_4$ dephosphorylation. Indeed, in extracts prepared from brain, liver and testis, the specific activities of MIPP increased up to 6-fold when the protein concentration in our assays (see Table 3) was decreased by up to 16-fold (results not shown). Interestingly, the specific activities of MIPP in extracts prepared from kidney and heart were independent of the same range of protein concentrations, indicating the absence of significant endogenous inhibition (results not shown). Thus, in order accurately to compare MIPP specific activities across various tissues, we routinely separated the enzyme from any inhibitors by anion-exchange chromatography [4–6]. The enzyme activity obtained from each tissue was eluted from the MonoQ column as a single peak by approx. 200 mM NaCl (see [5]).

Some loss of enzyme activity during chromatography is inevitable. The extent of this loss can be ascertained by comparing the apparent MIPP specific activities, pre- and post-chromatography, in extracts prepared from kidney and heart, since in these cases there was no significant endogenous inhibition (see above). These procedures resulted in a 10–20% decrease in specific enzyme activity (Table 3). We have made the reasonable assumption that there was not a significant tissue-dependent variation in this apparent loss of enzyme activity. Therefore, it is likely that the varying degree to which apparent enzyme activity was changed after anion-exchange chromatography (Table 3) predominantly reflects differences in the levels of endogenous inhibitor(s) of the enzyme that were originally present in the CHAPS-solubilized fractions.

Once endogenous inhibitors were resolved from MIPP, there was only a 2.5-fold range in specific activities across the range of tissues that we have analysed (Table 3). The highest specific activity was observed in kidney, and the lowest was expressed in brain (Table 3).

Characterization and application of polyclonal immunoprecipitating antiserum against MIPP

The polyclonal antiserum that we have prepared against hepatic particulate MIPP (see the Materials and methods section) was able to immunodeplete 85–100% of the enzyme activity that was present in CHAPS-solubilized particulate fractions of rat liver (Figure 4; Table 4). About 70% of the immunoprecipitated activity was recovered from the immune pellet (results not shown); the incomplete recovery presumably reflects partial

Table 3 Comparison of MIPP activities towards $\text{Ins}(1,3,4,5)\text{P}_4$ in CHAPS-solubilized particulate fractions prepared from various tissues, before and after anion-exchange chromatography

MIPP-dependent $\text{Ins}(1,3,4,5)\text{P}_4$ 3-phosphatase activities in CHAPS-solubilized particulate fractions from various tissues were compared before and after chromatography on a MonoQ HR 5/5 anion-exchange column. The specific activities in both cases relate to the amount of protein in the assays before chromatography, i.e. 0.2–0.5 mg of protein/ml (also see the text).

Tissue	Specific activity before chromatography (pmol/min per mg of protein)	Specific activity after chromatography (pmol/min per mg of protein)	Activation (fold)
Kidney	1370	1072	0.8
Heart	893	811	0.9
Testis	170	635	3.7
Liver	130	577	4.4
Brain	66	424	6.4

Figure 4 Extent of immunoprecipitation of cytosolic and particulate species of MIPP and Ins(1,4,5) P_3 5-phosphatase by polyclonal antiserum

5-Phosphatase activities towards 25 μ M Ins(1,4,5) P_3 were determined at pH 7.4 with 1 mM $MgCl_2$; MIPP-dependent 3-phosphatase activity towards 5 μ M Ins(1,3,4,5) P_4 was measured in 1 mM EDTA with no added Mg^{2+} (see the Materials and methods section). The extent of immunoprecipitation of MIPP or 5-phosphatase activity was determined as described in the Materials and methods section. Original 5-phosphatase activities were 12.2 ± 0.5 pmol/min (soluble type 1; ■), 7.9 ± 0.5 pmol/min (soluble type 2; □), 16.1 pmol/min (detergent-solubilized particulate fractions; ●). Original MIPP-dependent 3-phosphatase activities were 0.8 ± 0.03 pmol/min (soluble; ▲) or 0.6 ± 0.01 pmol/min (CHAPS-solubilized particulate; △).

inhibition by the immune complexes. The antiserum did not immunoprecipitate either soluble (types 1 or 2) or CHAPS-solubilized particulate Ins(1,4,5) P_3 5-phosphatase activities (Figure 4). Pre-immune serum (obtained from the same rabbit before immunization) did not immunoprecipitate MIPP (results not shown).

After homogenization of rat liver, about 5% of total cellular MIPP activity is recovered from the 100000 g soluble fraction of the homogenate [4,5]. We found that the cytosolic MIPP activity was immunoprecipitated by polyclonal antiserum raised against the particulate enzyme (Figure 4). Moreover, there was no

Figure 5 Comparison of the reactivity of polyclonal antiserum against hepatic MIPP with the Ins(1,3,4,5) P_4 3-phosphatase activity from turkey erythrocytes

Similar quantities of MIPP-dependent Ins(1,3,4,5) P_4 3-phosphatase activity purified from CHAPS-solubilized rat liver membranes (2.7 ± 0.08 pmol/min; ■) or Ins(1,3,4,5) P_4 3-phosphatase prepared from turkey erythrocyte membranes (2.7 ± 0.03 pmol/min; □) were immunotitrated with polyclonal antiserum (see the Materials and methods section). After centrifugation at 10000 g to precipitate the immune complexes, residual phosphatase activity in the supernatant was assayed. Data are the means of triplicate incubations. Similar results were obtained in one additional experiment.

significant difference in the relationship between the amount of antiserum added and the proportion of either soluble or particulate enzyme activity that was immunodepleted (Figure 4). The CHAPS-solubilized particulate MIPP activity in brain, heart, kidney and testis was immunoprecipitated with antiserum prepared against the hepatic enzyme (Table 4). Furthermore, the relationship between the amount of antiserum added and the quantity of enzyme activity immunodepleted was not significantly different in each of the tissues examined (Table 4).

In human erythrocytes, an Ins(1,3,4,5) P_4 3-phosphatase activity has been localized to the inner face of the plasma

Table 4 Immunotitration of MIPP-dependent Ins(1,3,4,5) P_4 3-phosphatase activities prepared from particulate fractions of various tissues, by using polyclonal antiserum raised against the hepatic enzyme

Expt. 1: equivalent amounts of partially purified MIPP-dependent Ins(1,3,4,5) P_4 3-phosphatase activities prepared by MonoQ anion-exchange chromatography of CHAPS-solubilized particulate fractions from different rat tissues were immunotitrated with polyclonal antiserum as described in the Materials and methods section, and the supernatant fractions were assayed for residual activity. The data are derived from immunotitration curves in which triplicate determinations were made with 0, 0.025, 0.05, 0.075, 0.1, 0.15, 0.2 or 0.5 μ l of antiserum. One additional experiment gave similar results. Expt. 2: this was performed as for Expt. 1, except that the erythrocyte Ins(1,3,4,5) P_4 3-phosphatase was prepared from CHAPS-solubilized plasma membranes. One additional experiment gave similar results.

	Enzyme activity (pmol/min)	Quantity of antiserum required to immunoprecipitate 50% of enzyme activity (μ l)	Immunoprecipitation by 0.5 μ l of antiserum (%)
Expt. 1			
Liver	3.8 ± 0.26	0.09	100
Testis	3.4 ± 0.03	0.09	100
Kidney	4.0 ± 0.14	0.10	100
Brain	3.7 ± 0.05	0.11	99
Heart	4.6 ± 0.08	0.10	99
Expt. 2			
Liver	2.1 ± 0.10	0.08	85
Erythrocyte	1.8 ± 0.12	0.10	95

membrane [9]. We have now found that there is also a plasma-membrane $\text{Ins}(1,3,4,5)P_4$ 3-phosphatase activity in erythrocytes obtained from both rat (Table 4) and turkey (Figure 5), which in each case were solubilized with CHAPS. Although the rat erythrocyte 3-phosphatase is in a different subcellular location from the hepatic enzyme, it was immunodepleted by antiserum raised against hepatic MIPP (Table 4). Thus these two enzymes probably share many common epitopes. In contrast, the enzyme from CHAPS-solubilized turkey erythrocytes was not precipitated by the antiserum, reflecting significant differences in antigenic determinants and presumably primary sequence (Figure 5).

DISCUSSION

The main focus of this study has been to examine whether there is heterogeneity of MIPP activity within or between tissues that may provide information leading to the identification of the function(s) of this enigmatic enzyme. One aspect of this study has been to consider the possibility that liver itself might contain different isoforms of this enzyme. It may be relevant to this particular issue that our final preparations of purified MIPP migrated as a doublet on SDS/PAGE (Figure 2). However, further work is required in order to distinguish whether or not one of these bands was an unrelated contaminant, as opposed to the doublet being composed of two different isoenzymes. We have extended our earlier observation [5] that hepatic MIPP was inside endoplasmic reticulum, by investigating the distribution of the enzyme between the 'rough' and 'smooth' subfractions of this organelle. We demonstrated that MIPP activity was evenly distributed between each subfraction (Table 2). This is a notable finding in relation to our previous suggestion that this enzyme may be a luminal enzyme [5], since many resident luminal proteins are also uniformly distributed throughout this organelle [21,22]. Important functions of members of this class of proteins include: (i) the regulation of post-translational modification, folding and assembly of secreted proteins; (ii) retention and commitment of certain proteins to degradative processes; (iii) the detoxification of harmful substances (for reviews see [21,22]). Perhaps MIPP contributes to one or more of these vital physiological processes.

An important problem in cell biology is to understand the mechanisms by which resident luminal proteins of the endoplasmic reticulum avoid being exported in the bulk flow through this organelle. In general, it appears that luminal proteins possess a characteristic C-terminal retention sequence that permits them to be salvaged from an intermediate endoplasmic-reticulum/Golgi subcompartment, by means of a vesicular recycling process [21]. We should consider that MIPP might be retrieved in a similar manner.

We have prepared immunoprecipitating polyclonal antiserum which was raised against the hepatic particulate MIPP. We found that this antiserum immunoprecipitated with identical efficacy the small proportion (approx. 5%) of total cellular MIPP activity that is present in the 100000 *g* soluble fraction (Figure 4). Thus, the two species of enzyme share common epitopes. It is probable that the two enzyme activities are identical, since tissue homogenization inevitably results in an unavoidable loss of a small proportion of luminal contents as the endoplasmic-reticulum fragments into microsomes [23]. The data obtained with our polyclonal antiserum are consistent with the latter idea.

We have also shown that polyclonal antiserum raised against the hepatic enzyme recognizes MIPP activity in other rat tissues, namely brain, heart, kidney, testis and erythrocytes. In each case,

the amount of enzyme immunodepleted per unit of antiserum was identical (Table 4). Clearly, these species of MIPP share common epitopes. This finding is of particular significance to the rat erythrocyte enzyme; its alternative subcellular location was not reflected by any substantial epitope differences that impact on its recognition by our polyclonal antiserum. Although there are probably not any inositol phosphates for this phosphatase to metabolize in mature erythrocytes, the situation is probably different during erythropoiesis [24]. Indeed, under such circumstances the erythrocyte MIPP activity may not be restricted by subcellular compartmentalization of enzyme and substrates.

The $\text{Ins}(1,3,4,5)P_4$ 3-phosphatase activity in turkey erythrocyte membranes was not recognized by antiserum against hepatic MIPP (Figure 5), and it would not be surprising if the epitope differences between these two proteins were simply a consequence of inherent phylogenetic variation. However, mature turkey erythrocytes are unusual in that, even after they have developed, they retain an active receptor-regulated phospholipase C and an array of inositol phosphate-metabolizing enzymes [25]. Another aspect of inositol phosphate physiology that is unique to avian erythrocytes is utilization of its substantial $\text{Ins}(1,3,4,5,6)P_5$ pool for the regulation of the affinity of haemoglobin for oxygen [26]. $\text{Ins}(1,3,4,5,6)P_5$ is also a substrate of the mammalian MIPP [4]. It would be interesting to study to what extent $\text{Ins}(1,3,4,5,6)P_5$ levels were regulated by the phosphatase in intact avian erythrocytes, since this could impact on haemoglobin function. Perhaps some of the structural differences between the enzymes of turkey erythrocytes and rat liver reflect specialization of physiological activity.

The specific activities of particulate MIPP in brain, heart, kidney, liver and testis varied over a fairly narrow 2.5-fold range (Table 3); brain contained the least activity, whereas heart and kidney possessed the highest levels. This is a strikingly different pattern of distribution from that of other enzymes with well-defined roles in the metabolism of $\text{Ins}(1,4,5)P_3$ and $\text{Ins}(1,3,4,5)P_4$. For example, there are relatively low levels of $\text{Ins}(1,4,5)P_3$ 3-kinase in heart, an organ in which inositol phosphate-mediated Ca^{2+} signalling is not of such primary importance [8]. Moreover, the specific activity of $\text{Ins}(1,4,5)P_3$ 5-phosphatase is 3-fold higher in brain than in liver [27], largely due to a specific enrichment in cerebellum of this enzyme and other elements of the $\text{Ins}(1,4,5)P_3/\text{Ca}^{2+}$ signalling system [28]. The implication that follows from this comparison of enzyme distribution is that the role of the particulate MIPP is not directly related to the turnover of $\text{Ins}(1,3,4,5)P_4$ and $\text{Ins}(1,4,5)P_3$. This conclusion should be considered in conjunction with our previous kinetic studies, which led to the proposal that the function of this phosphatase is more likely related to its separate ability to convert $\text{Ins}(1,3,4,5,6)P_5$ and $\text{Ins}P_6$ to $\text{Ins}(1,4,5,6)P_4$ and $\text{Ins}P_5$ respectively [4,5]. The latter metabolic reactions have, for some time, been believed to be largely independent of short-term alterations to levels of $\text{Ins}(1,4,5)P_3$ and $\text{Ins}(1,3,4,5)P_4$ [29]. However, we are unlikely to understand fully the contribution that MIPP makes to cellular biology until we unravel a mechanism by which at least one of its potential substrates can cross the membrane of the endoplasmic reticulum and gain access to the enzyme.

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