Hepatic Ins(1,3,4,5)P$_4$ 3-Phosphatase Is Compartmentalized Inside Endoplasmic Reticulum*

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In pursuit of the physiological role of inositol 1,3,4,5-tetrakisphosphate 3-phosphatase, which also attacks inositol pentakisphosphate and inositol hexakisphosphate with much higher affinity (Nogimori, K., Hughes, P. J., Glennon, M. C., Hodgson, M. E., Putney, J. W., Jr., and Shears, S. B. (1991) J. Biol. Chem. 266, 16499–16506), we have studied the subcellular distribution of the enzyme in liver. Initially, we had to overcome the problem that potent endogenous inhibitor(s) compromise the detection of this enzyme in vitro (Hodgson, M. E., and Shears, S. B. (1990) Biochem. J. 267, 831–834). We partially purified these inhibitor(s) by anion-exchange chromatography and gel filtration; inhibitory activity co-eluted with standard inositol hexakisphosphate and was depleted by treatment with phytase. Thus, subcellular fractions were pretreated with phytase before assay of 3-phosphatase activity. Our experiments revealed that the hepatic 3-phosphatase was nearly exclusively restricted to the endoplasmic reticulum, and there was little or no activity in either the cytosol, plasma membranes, mitochondria, or nuclei. Detergent treatment of microsomes indicated that there was 93 ± 2% latency to mannose-6-phosphatase, an intraganelle enzyme activity (Vanstapel, P., Pua, K., and Blanckaert, N. (1986) Eur. J. Biochem. 156, 73–77). Similar latencies were found for the hydrolysis of inositol 1,3,4,5-tetrakisphosphate (95 ± 1%), inositol 1,3,4,5,6-pentakisphosphate (94 ± 1%), and inositol hexakisphosphate (95 ± 2%). Treatment of microsomes with either sodium carbonate or phosphatidylcholine-specific phospholipase C, to release luminal contents, led to solubilization of approximately 90% of 3-phosphatase activity. Thus, hepatic 3-phosphatase has a highly restricted access to inositol polyphosphates in vivo that needs to be accounted for in the determination of the physiological role of this enzyme.

It is now well established that agonist-stimulated phospholipase C activity results in accelerated breakdown of PtdIns(4,5)P$_2$,† releasing Ins(1,4,5)P$_3$ which mobilizes intra-

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† Inositol phosphates are abbreviated according to IUPAC nomenclature (1989) Biochem. J. 258, 1–2. The abbreviations used are: BAPTA, 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; bis-Tris, bis-(2-hydroxyethyl)iminotris(hydroxymethyl)methane; CHAPS, 3-[[(cholamidopropyl)-dimethylammonio]-1-propane sulfonate; MES, 2-(N-morpholinio)ethanesulfonic acid.

cellular Ca$^{2+}$ stores (Berridge and Irvine, 1989). The action of Ins(1,4,5)P$_3$ is terminated by its rapid metabolism, through dephosphorylation by a 5-phosphatase to yield Ins(1,3,4,5)P$_4$, and phosphorylation by a 3-kinase to produce Ins(1,3,4,5,6)P$_5$ (for review, see Shears, 1992). There are many reports that Ins(1,3,4,5)P$_4$ contributes to Ca$^{2+}$ mobilization (see Irvine, 1992), although there is still controversy concerning how widespread this phenomenon might be (Bird et al., 1991). The major route of Ins(1,3,4,5,6)P$_5$ metabolism occurs by removal of the 5-phosphate, yielding Ins(1,3,4)P$_3$. During the last few years a number of cell-free systems have been shown to actively dephosphorylate Ins(1,3,4,5)P$_4$, with a 3-phosphatase (Cunha-Melo et al., 1988; Höer et al., 1988; Cullen et al., 1989; Doughney et al., 1988; Hodgson and Shears, 1990; Hughes and Shears, 1990). Thus, this enzyme has been considered as a potentially important mechanism for regulating levels of Ins(1,3,4,5)P$_4$, and prolonging the Ins(1,4,5)P$_3$ signal (Oberdisse et al., 1990). Recently, it was shown that the 3-phosphatase attacks Ins(1,3,4,5,6)P$_5$ and InsP$_6$ with much higher affinity than Ins(1,3,4,5)P$_4$ (Nogimori et al., 1991). When this observation was considered in relation to the relatively high amounts of Ins(1,3,4,5,6)P$_5$ and InsP$_6$ in cells, it was proposed that Ins(1,3,4,5)P$_4$ was unlikely to be hydrolyzed by a 3-phosphatase to a substantial extent in vivo (Nogimori et al., 1991). Instead, we suggested that Ins(1,3,4,5,6)P$_5$ and InsP$_6$ were more likely to be the physiologically relevant substrates.

We (Nogimori et al., 1991) and others (Höer and Oberdisse, 1991) have pointed out that the caveat to any proposals concerning 3-phosphatase activity in vivo is the possibility of differential subcellular compartmentalization of the enzyme in relation to its potential substrates. The lack of information on this topic is impeding our understanding of the physiological significance of this enzyme. It has been reported that phosphatase activity toward both Ins(1,3,4,5,6)P$_5$ and Ins(1,3,4,5)P$_4$ may predominate on the surface of some cells (Carpenter et al., 1989), possibly inactivating putative activities of extracellular inositol phosphates (Vallejo et al., 1987; Perney and Kaczmarek 1992). However, there are no previous studies on the distribution of 3-phosphatase between the various subcellular fractions, other than a demonstration that the hepatic enzyme predominantly cosediments with a 100,000 × g particulate fraction (Nogimori et al., 1991).

Thus, it is with the aim of understanding the function of 5-phosphatase that we have investigated its subcellular distribution in liver. However, we first had to accommodate the experimental problem that cells contain potent endogenous inhibitor(s) of this particular enzyme (Hodgson and Shears, 1990; Hughes and Shears, 1990). In this study we have found that the endogenous inhibitor(s) can be digested with phytase. By assaying 3-phosphatase activity in subcellular fractions pretreated with phytase, we have discovered that the enzyme
is almost entirely restricted to the endoplasmic reticulum. Moreover, using a variety of membrane-permeabilizing techniques, we discovered that the 3-phosphatase activity resides inside this organelle. This is the first report that an enzyme involved in inositol phosphate metabolism has restricted access to its potential substrates due to subcellular compartmentalization. Our work raises important questions concerning the physiological activity of this enzyme, but additionally our data have established the direction that future studies must take in order to resolve these problems.

**EXPERIMENTAL PROCEDURES**

**Materials**—Phosphatidylcholine-specific phospholipase C, from *Bacillus cereus* (grade I), aprotinin, and In(1,3,4,5,6)P₆ were obtained from Boehringer Mannheim. Before use, the mass of In(1,3,4,5,6)P₆ was quantified by analysis of inorganic phosphate released following alkaline phosphodiesterase hydrolysis (Bartlett, 1965). [³H]Inositol (1,3,4,5,6)- and [²H]InsP₆ (12-15 Ci/mmol) were purchased from Du Pont-New England Nuclear. [³H]In(1,3,4,5,6)-P₆ was prepared from [³H]inositol-labeled AR4-25 cells (Menniti et al., 1990). AG 1-X₈ anion-exchange resin (200-400 mesh, formate form) and gel filtration standards were purchased from Bio-Rad. DEAE-Sepharose fast-flow anion-exchange resin was obtained from Pharmacia LKB Biotechnology Inc. Celluline G-CSF-90S column size exclusion resin was purchased from Amicon. Protease inhibitors were purchased from Calbiochem.

**Materials—Phosphatidylcholine-specific phospholipase C** (from *Bacillus cereus* grade I), aprotinin, and Ins(1,3,4,5,6)P₆ were obtained from Boehringer Mannheim. Before use, the mass of Ins(1,3,4,5,6)P₆ was quantified by analysis of inorganic phosphate released following alkaline phosphodiesterase hydrolysis (Bartlett, 1965). [³H]InsP₆ (21-25 Ci/mmol), and [³H]InSP₆ (12-15 Ci/mmol) were purchased from Du Pont-New England Nuclear. [³H]Ins(1,3,4,5,6)-P₆ was prepared from [³H]inositol-labeled AR4-22 cells (Menniti et al., 1990). AG 1-X₈ anion-exchange resin (200-400 mesh, formate form) and gel filtration standards were purchased from Bio-Rad. DEAE-Sepharose fast-flow anion-exchange resin was obtained from Pharmacia LKB Biotechnology Inc. Celluline G-CSF-90S column size exclusion resin was purchased from Amicon. Protease inhibitors were purchased from either Sigma or Calbiochem. InsP₆, calmodulin, and protein kinase C were purchased from Calbiochem.

**Cellulose phosphate diesterase 1** (EC 3.1.4.1) was assayed as described by Razzell (1963). Glutamate dehydrogenase (EC 1.4.1.2) was determined as described by Shephard and Hubscher (1979). Mannose-6-phosphatase was determined at 20 °C as described by Vanstapel et al. (1986).

**Phytase activity** was usually determined as described by Ullah and Gibson (1987) except that reaction mixtures were preincubated at 55 °C for 10 min prior to assay at 55 °C in a total volume of 300 μl. In addition, all enzyme incubations were terminated with 33 μl 10% (w/v) SDS, prior to the measurement of P, at 355 nm (Ullah and Gibson, 1987). One enzyme unit represents the activity that hydrolyzes 1 μmol of phytic acid in 1 min under the above experimental conditions. In some experiments, phytase activity toward [³H]InsP₆ was monitored by high-performance liquid chromatography (Nogimori et al., 1991).

**PM Ins(1,3,4,5,6)P₆ or 50 nM InsPs** was determined as described by Ullah and Gibson (1987), i.e. SP-Trisacryl M (16 × 130 mm; IBF Biotechnics (Sepracor Inc.)), followed by DEAE-Trisacryl M (10 × 140 mm; IBF Biotechnics (Sepracor Inc.).) All three steps of the purification step were run at 1 ml/min. The final purification step eluted the enzymatic activity at approximately 50 mM KCl (designated "Phytase II") by Ullah and Gibson, 1987). The final purification step used a Mono HPR 5/20 column chromatography. Phytase activity coeluted with the second major protein peak at pH 4.5-5.0. The three peak fractions of enzymatic activity were pooled and stored as 200-μ1 aliquots in the absence of glycerol at -20 °C until use.

**Removal of Endogenous Inhibitor(s)** of 3-Phosphatase in Tissue Extracts by Phytase—Phytase treatment of either DEAE-purified endogenous inhibitor(s) (prepared as described by Fig. 1), liver homogenates, or subcellular fractions (1-3 mg protein/ml) was by incubation with tissue extracts, after themselves being heat-treated, did not inhibit 3-phosphatase activity.

**Perturbation of Microsomal Integrity**—For detergent treatment, microsomes (2 mg protein/ml) were resuspended as described above for membrane sheet-like structures originating from sigmoidal (basolateral) as well as bile canalicular (apical) surfaces of the hepatocyte. For the preparation of mitochondria, livers were homogenized by 8-10 up and down strokes of a Dounce-type loose-fitting glass homogenizer (Wheaton, Melville, NJ; nominal clearance 0.00250-0.00350 mm) and the homogenate was processed according to Schneider and Hogeboom (1950). Microsomes were isolated as described by Vanstapel et al. (1986). Nuclei were isolated according to Blobel and Potter (1966). All the final preparations, with the exception of nuclei, were resuspended in 250 mM sucrose plus 10 mM Tris (pH 7.0 with HCl). Nuclei were resuspended in 50 mM Tris (pH 7.5 with HCl), 250 mM sucrose, 25 mM KCl, and 5 mM MgCl₂.

**Purification of Endogenous Inhibitor(s)—CHAPS-solubilized 100,000 × g particulate fractions** (Nogimori et al., 1991), and 100,000 × g supernatants, were prepared from homogenates of either rat liver or brain. These were chromographed on either a MonoQ HR 10/10 column (Hodgson and Shears, 1990) or a DEAE-Sepharose column (4.4 × 21 cm, Nogimori et al., 1991), using a gradient generated by mixing Buffer A (250 mM sucrose, 10 mM Tris-5 mM Na₃, 5 mM NaN₃ (pH 7.4 with HCl)) with Buffer B (Buffer A plus 500 mM NaCl). Aliquots of individual column fractions were heat-treated by incubation at 90 °C for 15 min, followed by centrifugation for 5 min at 10,000 × g. The resultant supernatant was saved; it contained heat-stable endogenous inhibitor(s) (described by Nogimori and Shears, 1990). The 3-phosphatase activity eluted from the anion-exchange column was further purified as described by Nogimori et al. (1991). For some experiments, a 4.5-ml aliquot of "heat-treated" endogenous inhibitor(s) was equilibrated with 1 × 10⁵ disintegrations/min (dpm) [³H]InsP₆ for 12 at 4 °C for 15 min). The resultant mixture was centrifuged at 10,000 × g for 30 min. The filtrate was resuspended and diluted appropriately in the buffer for use.

**Removal of Endogenous Inhibitor(s)** of 3-Phosphatase in Tissue Extracts by Phytase—Phytase treatment of either DEAE-purified endogenous inhibitor(s) (prepared as described by Fig. 1), liver homogenates, or subcellular fractions (1-3 mg protein/ml) was by incubation with tissue extracts, after themselves being heat-treated, did not inhibit 3-phosphatase activity. The trace quantities of added [³H]InsP₆ did not inhibit 3-phosphatase activity.

**Removal of Phytase Inhibitors**—*Aegyptia fuscata* phytase was purified from 100 mg of crude culture filtrate (Sigma catalog no. P9792; 41 Sigma units/ml). All procedures were conducted at 0-4 °C. The filtrate was resuspended in 100 ml of buffer containing 10 mM sodium acetate, 2 mM 2-mercaptopoethanol, 5% (v/v) glycerol, pH 4.3 (with acetic acid), and incubated on ice for 30 min. The filtrate was centrifuged at 10,000 × g for 45 min, and the enzyme in the supernatant was purified using fast protein liquid chromatography and the procedures described by Ullah and Gibson (1987), i.e. SP-Trisacryl M (16 × 130 mm; IBF Biotechnics (Sepracor Inc.).) followed by DEAE-Trisacryl M (10 × 140 mm; IBF Biotechnics (Sepracor Inc.).) All three steps of the purification step were run at 1 ml/min. The final purification step eluted the enzymatic activity at approximately 50 mM KCl (designated "Phytase II") by Ullah and Gibson, 1987). The final purification step used a Mono HPR 5/20 column chromatography. Phytase activity coeluted with the second major protein peak at pH 4.5-5.0. The three peak fractions of enzymatic activity were pooled and stored as 200-μ1 aliquots in the absence of glycerol at -20 °C until use.
and incubated on ice with the addition of a range of concentrations of either CHAPS (0–4 mM for 60 min) or Triton X-100 (0–2 mM for 30 min).

Na₂CO₃ treatment was performed by incubation of microsomes (0.5–1 mg protein/ml) in 1 ml of 100 mM Na₂CO₃ (pH 11.4) on ice for 30 min (Fujiki et al., 1982). The pH was then adjusted to 7.0 with an appropriate volume of 400 mM MES. Phospholipase C treatment of 0.5–1 mg microsomal protein/ml was performed as described by Urade et al. (1992) except that their buffer was replaced with 1 ml of 250 mM sucrose, 10 mM Tris (pH 7.0 with HCl). Following these two treatments, samples were centrifuged (100,000 g, 20 min, 4 °C). Both the supernatants and the pellets were saved and then assayed for mannose-6-phosphatase and Ins(1,3,4,5)P₄ 3-phosphatase as described above.

**RESULTS**

Separation of Ins(1,3,4,5)P₄ 3-Phosphatase from Endogenous Inhibitor(s)—During the anion-exchange chromatography of Ins(1,3,4,5)P₄ 3-phosphatase, its apparent activity increased severalfold (see legend to Fig. 1A, and Nogimori et al., 1991). We previously proposed that this activation was due to separation of the enzyme from endogenous inhibitory activity. Thus, a CHAPS-solubilized particulate fraction was chromatographed on either a MonoQ column (Fig. 1A) or a DEAE-Sepharose column (data not shown). The resultant fractions were heat-treated (see "Experimental Procedures") and added to incubations containing purified 3-phosphatase. A broad peak of inhibitory activity toward this enzyme was clearly demonstrated and, moreover, was separated from the peak of 3-phosphatase itself (Fig. 1A). From the enzyme activities that were expressed in the soluble and particulate fractions after anion-exchange chromatography, we determined that 94 ± 1% (n = 2) of total hepatic Ins(1,3,4,5)P₄ 3-phosphatase activity was originally associated with the particulate fraction (data not shown). In contrast, it was the soluble fraction that contained the bulk (approximately 90%) of total heat-stable inhibitory activity toward 3-phosphatase. This was established by incubating aliquots of heat-treated soluble and particulate fractions with purified 3-phosphatase (data not shown). Nevertheless, the minor proportion of inhibitory activity that associates with hepatic membranes is still sufficient to substantially inhibit the 3-phosphatase activity that is also present in this fraction of the cell (see above). Therefore, in order to study the distribution of 3-
phosphatase activity associated with these membranes was endogenous inhibitor(s) that are released into the soluble fraction of liver homogenates (see above, and Hodgson and et al., 1991). We considered that if InsP₆ and/or a closely related polyphosphate, was responsible for endogenous inhibition of 3-phosphatase, then it should be possible to eliminate such inhibition by treatment with phytase. To test this possibility, inhibitor(s) purified from the CHAPS-solubilized particulate fraction (see "Experimental Procedures") were incubated with sufficient internal standard of [³H]InsP₆ to completely hydrolyze [³H]InsP₆. Phytase was then itself inactivated by heat treatment (Table I). Heat treatment alone did not affect the activity of the inhibitor (Table I). However, sequential treatment of the inhibitor, first with phytase and then with heat, completely eliminated its ability to inhibit Ins(1,3,4,5,6)P₆ 3-phosphatase (Table I). Thus, we next investigated the intracellular distribution of 3-phosphatase in subcellular fractions treated with phytase (see below).

Ins(1,3,4,5,6)P₆ 3-Phosphatase Activity in Homogenates and Subcellular Fractions Which Were Depleted of Endogenous Inhibitor(s)—In incubations containing liver homogenates (2.6-4.6 mg protein/ml) the apparent Ins(1,3,4,5,6)P₆ 3-phosphatase activity was 48 ± 6 pmol/min/mg protein (mean ± S.E., n = 10). After separation of the particulate fraction from the homogenates by centrifugation at 100,000 × g, the apparent 3-phosphatase activity increased 12-fold (to 57 ± 8 pmol/min/mg protein, n = 10). This reflects the removal of those endogenous inhibitor(s) that are released into the soluble fraction of liver homogenates (see above, and Hodgson and Shears, 1990). Nevertheless, substantial inhibitory activity persists in the 100,000 × g particulate fraction (see above). When homogenates were pretreated with phytase to digest endogenous inhibitor(s) (see "Experimental Procedures") and then the particulate fraction was isolated, the apparent 3-phosphatase activity associated with these membranes was 31-fold greater (148 ± 21 pmol/min/mg protein, n = 10) than that originally expressed in homogenates. The latter was therefore the appropriate value to refer to when estimating the enrichment of 3-phosphatase in subcellular fractions.

Table II describes the fold enrichment of selected markers in our preparations of subcellular fractions. Markers for plasma membranes (alkaline phosphodiesterase), mitochondria (glutamate dehydrogenase), and nuclear fractions (DNA) were enriched 25-, 5-, 3-, and 16-fold, respectively, which are all typical of subcellular preparations from liver (Ali et al., 1990; Schneider and Hogeboom, 1950; Joseph and Williams, 1985; Widnell and Tata, 1964). Each of these fractions were treated with phytase, which was then itself removed by two cycles of washing and centrifugation (see "Experimental Procedures"). Importantly, aliquots of all the phytase-treated subcellular fractions were, after heat treatment, found not to inhibit purified 3-phosphatase in incubations containing CHAPS (data not shown). This result indicates the absence of any residual endogenous inhibitory activity, including any that might have been intravesicular and escaped attack by phytase which out of necessity was added to subcellular preparations in the absence of detergent. Among the various subcellular fractions, the microsomes were the only organelles to contain an enrichment of 3-phosphatase (approximately 4.5-fold) that quantitatively matched the enrichment of the appropriate specific marker (Table II). Furthermore, the percentage yield in the microsomal preparations of total cellular 3-phosphatase and arylesterase were very similar: 15 ± 2% and 17 ± 2%, respectively. Finally, the relatively small amounts of 3-phosphatase activities that were found in other subcellular fractions were closely matched by contamination of the marker for endoplasmic reticulum, with the possible exception of a small amount of 3-phosphatase activity in plasma membranes. Note also that the contaminating 3-phosphatase activities, as well as that in microsomes, were all increased by between 1.5- and 3-fold by phytase treatment (Legend to Table I), suggesting that endogenous inhibitors were somewhat uniformly distributed throughout the different particulate fractions of the cell.

**Topography of Ins(1,3,4,5,6)P₆ 3-Phosphatase in Microsomes**—We compared the apparent microsomal 3-phosphatase activity in intact vesicles with the activity upon permeabilization by CHAPS. Mannose-6-phosphatase activity was the control marker for the latency of our microsomal preparations (Vanstapel et al., 1986), i.e. 93% (Table III); presumably about 7% of our microsomal vesicles were inherently leaky. Unexpectedly, Ins(1,3,4,5,6)P₆ 3-phosphatase was also latent to the same extent (Table III). We considered the possibility that the apparent latency of 3-phosphatase might be alternately explained by a nonspecific detergent-induced activation of the enzyme. However, this is extremely unlikely, since over a range of concentrations of two different types of detergent (CHAPS, which is zwitterionic, and Triton X-100, which is non-ionic), the curves that describe the progressive increases in activities of 3-phosphatase and mannose-6-phosphatase (Fig. 2) are virtually superimposable. The remote possibility that 3-phosphatase and mannose-6-phosphatase were the same enzyme was excluded since purified 3-phosphatase did not hydrolyze mannose-6-phosphate (data not shown). Thus, Ins(1,3,4,5,6)P₆ 3-phosphatase is apparently compartmentalized inside endoplasmic reticulum. This enzyme also attacks Ins(1,3,4,5,6,8)P₆ and InsP₆ (Nogimori et al., 1991). We found that these alternative substrates were also not significantly hydrolyzed by microsomal vesicles until these membranes were disrupted with detergent (Table III).

In our microsomal vesicles, the latency of 3-phosphatase was

**Table I**

**Addition** | **3-Phosphatase activity** |
--- | --- |
None | 15.9 ± 0.4 pmol/min/mg protein |
Inhibitor | 0.9 ± 0.4 pmol/min/mg protein |
Inhibitor, after heat treatment | 1.7 ± 0.3 pmol/min/mg protein |
Inhibitor, after treatment with phytase, then with heat | 16.2 ± 0.5 pmol/min/mg protein |
Compartmentalization of Ins(1,3,4,5)P_4 3-Phosphatase

Details of the preparation of subcellular fractions, and the enzyme assays, are given under "Experimental Procedures"; 4 mM CHAPS was included in all assay media. Data are means ± standard errors, followed in parentheses by the number of separate preparations of subcellular fractions. The fold enrichment of subcellular markers relate to assays performed on homogenates, except for the 3-phosphatase, where enrichment in phytase-treated subcellular fractions was compared with activity in a phytase-treated 100,000 × g particulate fraction (see under "Results" and "Experimental Procedures"). The apparent 3-phosphatase activities in subcellular fractions increased following phytase treatment by the following fold values: 3.2 ± 0.3 (plasma membranes), 3.4 ± 0.8 (mitochondria), 3.2 ± 1.7 (nuclei), or 1.4 ± 0.1 (microsomes).

Activity toward either Ins(1,3,4,5)P_4, Ins(1,3,4,5,6)P_5, or InsP_6 were all unaffected (data not shown) if our routine 3-phosphatase assay medium (no ATP or Mg^{2+}, see "Experimental Procedures") was replaced by a medium closer to the physiological milieu (i.e., 100 mM KCl, 10 mM HEPES (pH 7.0 with KOH), 1 mM dithiothreitol, 3 mM MgCl_2, 2.5 mM ATP, 10 mM phosphocreatine, 5 μM GTP, 5 mM K^+ succinate, 10 μg/ml creatine kinase). Enzyme latency was also unaffected (data not shown) by the further addition to this medium of Ca^{2+}/BAPTA buffers to set the free [Ca^{2+}] to either 0.1 μM, 0.5 μM or 1 μM in the presence of 10 μM calmodulin, or by 30 min pretreatment of microsomes by 2.5 units/ml protein kinase C, or by 200 μM AlCl_3, which in some circumstances may activate 3-phosphatase (Loomis-Husselbee et al., 1991).

We further investigated the location of 3-phosphatase in microsomes by two additional methods that do not use detergents, i.e. treatment with either Na_2CO_3 (pH 11.4; Fujiki et al., 1982) or phosphatidylycholine-specific phospholipase C (Urade et al., 1992). These methods have the further advantage that they have been used to specifically release proteins that are either free in the microsomal lumen, and possibly those that are loosely associated with the luminal face of the membrane. Following such procedures (see "Experimental Procedures" for details) the disrupted microsomes were centrifuged at 100,000 × g, and both the resultant soluble and particulate fractions were separately assayed for 3-phosphatase. In both cases over 90% of total 3-phosphatase was recovered in the soluble fraction (Table IV). As a control, we also investigated the distribution of membrane-bound mannose-6-phosphatase in the disrupted microsomes. Unfortunately, phospholipase C treatment totally inactivated mannose-6-phosphatase activity (Table IV). While the Na_2CO_3 treatment reduced mannose-6-phosphatase activity by 45%, the activity that remained was all associated with the particulate fraction that has been used to specifically release proteins that are either free in the microsomal lumen, and possibly those that are loosely associated with the luminal face of the membrane.

Table II

| Fraction          | Marker enrichment relative to the bulk particulate fraction (fold) |
|-------------------|---------------------------------------------------------------|
|                   | 3-Phosphatase | Arylesterase | Alkaline phosphodiesterase | Glutamate dehydrogenase | DNA |
| plasma membrane   | 1.8 ± 0.3 (4) | 0.9 ± 0.1 (3) | 24.5 ± 2.0 (5) | 0.2 ± 0.02 (3) | 0.43 ± 0.04 (3) |
| mitochondria      | 0.9 ± 0.1 (3) | 0.6 ± 0.1 (3) | 0.3 ± 0.04 (6) | 2.5 ± 0.23 (3) | 0.29 ± 0.05 (3) |
| nuclei            | 0.3 ± 0.1 (2) | 0.3 ± 0.04 (3) | 0.3 ± 0.06 (4) | 0.05 ± 0.003 (3) | 16.3 ± 1.7 (3) |
| microsomes        | 4.4 ± 0.7 (4) | 4.9 ± 0.7 (4) | 1.2 ± 0.2 (4) | 0.02 ± 0.001 (3) | 0.67 ± 0.14 (3) |

Table III

Latency of inositol phosphatase and mannose-6-phosphatase in microsomes

Microsomes were prepared as described under "Experimental Procedures." Enzyme latency in these preparations was determined by comparing, in the presence and absence of 4 mM CHAPS, the rate of hydrolysis of either 2 mM mannose-6-phosphate, 5 μM Ins(1,3,4,5)P_4, 1 μM Ins(1,3,4,5,6)P_5, or 50 mM InsP_6 as described under "Experimental Procedures." Data are means ± standard errors with the number of preparations in parentheses.

| Enzyme activity | Latency (%) |
|-----------------|-------------|
| Mannose-6-phosphatase | 93 ± 2 (7) |
| Ins(1,3,4,5)P_4-phosphatase | 96 ± 1 (6) |
| Ins(1,3,4,5,6)P_5-phosphatase | 94 ± 1 (3) |
| InsP_6-phosphatase | 93 ± 2 (3) |

FIG. 2. Exposure of Ins(1,3,4,5)P_4 3-phosphatase and mannose-6-phosphatase in microsomes permeabilized with either CHAPS or Triton X-100. Freshly prepared microsomes (2 mg/ml) were treated on ice with the indicated concentrations of either CHAPS for 60 min (panel A) or Triton X-100 for 30 min (panel B). Both mannose-6-phosphatase (A) and 3-phosphatase (B) activities were assayed (see "Experimental Procedures") at 20 °C in 50 mM Tris, 250 mM sucrose, 2 mM EDTA (pH 6.8 with HCl). Maximum activities obtained in the presence of detergents were taken as 100%. Data shown from a single experiment are typical of three experiments performed on separate preparations.
TABLE IV

|                                  | Pellet | Supernatant | Pellet | Supernatant |
|----------------------------------|--------|-------------|--------|-------------|
| **3-Phosphatase**                | 5 ± 2 (4) | 88 ± 4 (4) | 2 ± 0.3 (3) | 94 ± 4 (3) |
| **Mannose-6-phosphatase**       | 54 ± 4 (4) | 1 ± 0.6 (4) | ND     | ND          |


dicate fraction of the microsomes, in contrast to the soluble nature of Ins(1,3,4,5,P) 3-phosphatase (Table IV).

DISCUSSION

Some workers have suggested that Ins(1,3,4,5)P, 3-phosphatase activity represents a physiological means of sustaining Ins(1,4,5)P, levels during cell stimulation (Oberdisse et al., 1990). More recently, we have shown that Ins(1,3,4,5,6)P, and particularly InsP, are both higher affinity substrates of this enzyme (Nogimori et al., 1991). These observations led us to propose that the higher polyphosphates were more likely to be the physiologically relevant substrates. However, Oberdisse and co-workers (Hoer et al., 1991), and our laboratory (Nogimori et al., 1991), have emphasized that we are unlikely to understand the physiological activity of this enzyme in vivo until we determine where in the cell the enzyme and its substrates are located.

Current evidence is consistent with Ins(1,3,4,5)P, Ins(1,3,4,5,6)P, and InsP, all being concentrated in the cytosol. For example, the enzymes that synthesize these compounds are all soluble: the Ins(1,4,5)P, 3-kinase that forms to be the physiologically relevant substrates. However, Oberdisse and co-workers (Hoer et al., 1991), and our laboratory (Nogimori et al., 1991), have emphasized that we are unlikely to understand the physiological activity of this enzyme in vivo until we determine where in the cell the enzyme and its substrates are located.

While phytase undoubtedly hydrolyzed membrane-associated InsP, this enzyme will also have removed any other inositol polyphosphates (and conceivably other unsuspected polyphosphates) that might contribute to endogenous inhibition of 3-phosphatase.

Ins(1,3,4,5)P, to Ins(1,4,5)P, does not contribute to the regulation of Ca*+ mobilization in intact liver cells. An important practical application of this discovery relates to the potential for using hepatocytes, and other intact cells in which 3-phosphatase might be similarly compartmentalized, for studies into the effects upon Ca*+ signaling of microinjection of Ins(1,3,4,5)P. Irvine (1992) has emphasized that a major problem with such an approach lies in the interpretation of positive effects of Ins(1,3,4,5)P, upon Ca*+ mobilization, in view of the possibility that such phenomena arise from generation of Ins(1,4,5)P, by the 3-phosphatase. Our results make this a much less likely scenario.

Our data also indicate that the alternative substrates of the 3-phosphatase, Ins(1,3,4,5,6)P, and InsP, also have a very restricted access to this enzyme in vivo. These results provide an explanation for the otherwise paradoxical observation that intact mammalian cells usually contain only very low steady-state levels of both Ins(1,4,5,6)P, (Stephens et al., 1988a; Balla et al., 1988; McConnell et al., 1990; Wong et al., 1992) and Ins(1,2,4,5,6)P, (Menniti et al., 1990; Mattingly et al., 1991; Nogimori et al., 1991; Stephens et al., 1991; Wong et al., 1992), despite their immediate metabolic precursors being high affinity substrates of the 3-phosphatase in vivo (Nogimori et al., 1991). In liver, only 5-7% of total cellular activity was associated with 100,000 x g soluble fractions (see "Results").  Yet even this small value probably overestimates the proportion that might be present in liver cytosol in vivo. We have obtained evidence that the enzyme is either soluble inside the lumen of the endoplasmic reticulum or at least loosely bound to the inner membrane face (Table IV). Upon cell homogenization, which fragments the endoplasmic reticulum into microsomes, there is an inevitable loss to the soluble fraction of a proportion of loosely associated luminal contents (Beckers et al., 1987). Thus, none of the hepatic 3-phosphatase may be genuinely cytosolic. In this event, perhaps when small amounts of Ins(1,4,5,6)P and Ins(1,2,4,5,6)P are observed in cell preparations, they are actually only derived from a minor proportion of cells that have begun to lose some structural integrity. It will therefore be of physiological interest to understand why levels of either Ins(1,4,5,6)P, or Ins(1,2,4,5,6)P, are atypically high in certain transformed cells, such as the AR4-2J pancreatic tumor (Menniti et al., 1990), src-transformed rat fibroblasts (Mattingly et al., 1991), Epstein-Barr virus-transformed human B-lymphocytes (McConnell et al., 1991), and Jurkat T-lymphocytes (Guse and Emmrich, 1991). Perhaps transformation has perturbed the barrier that normally separates the 3-phosphatase from its substrates. Alterately, the subcellular location of 3-phosphatase, and hence its function, may be different in some non-hepatic tissues. In this respect, the Ins(1,3,4,5)P, 3-phosphatase in human erythrocytes is an integral protein on the inner face of the plasma membrane (Estrada-Garcia et al., 1991). Our marker enzyme data do not entirely exclude the possibility that a very minor proportion of 3-phosphatase might be associated with hepatic plasma membranes (Table II). Nevertheless, the erythrocyte, being an atypical, highly differentiated cell type with a dearth of intracellular membranes, is an inappropriate model for predicting the intracellular distri-

While phytase undoubtedly hydrolyzed membrane-associated InsP, this enzyme will also have removed any other inositol polyphosphates (and conceivably other unsuspected polyphosphates) that might contribute to endogenous inhibition of 3-phosphatase.
bution of 3-phosphatase in most other cell types. The bulk of Ins(1,3,4,5)P4/Ins(1,3,4,5,6)P5 phosphatase activity has also been reported to reside on the surface of NIH-3T3 fibroblasts (Carpenter et al., 1989) where it has been suggested to inactive putative extracellular signaling activities of inositol phosphates (Perney and Kaczmarek, 1992). However, the specificity of this putative extracellular enzyme has not been determined.

The conclusion that hepatic 3-phosphatase does not make a large contribution to inositol phosphate turnover in vivo should lead us to consider that this enzyme may perform other functions. Although our studies do not directly address this issue, our identification of the intracellular location of this protein does represent important groundwork that should form the basis of further investigations into this topic. For example, luminal enzymes of the endoplasmic reticulum are frequently either exported from the cell in a secretory pathway or they participate in the processing of such secreted proteins (for review, see Pelham, 1989). Future research into the function of the 3-phosphatase should be directed at possible contributions to this, and probably other, physiological processes that may not even be directly related to the turnover of inositol polyphosphates.

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