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A novel form of glycosylphosphatidylinositol-anchor converting activity with a specificity of a phospholipase D in mammalian liver membranes

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It has been reported that rat liver membranes contain a glycosylphosphatidylinositol-specific phospholipase C (GPI-PLC) which may be involved in generation of phosphoinositol-glycan, a putative insulin second messenger (Saltiel, A.R. and Cuatrecasas, P. (1988) Am. J. Physiol. 255, C1–C11). Using GPI-anchored acetylcholinesterase (AChE) from bovine erythrocytes as substrate, we attempted to isolate GPI-PLC from bovine and rat liver membranes. A major part of the GPI-anchor converting activity present in liver could be washed away from the tissue by extraction with detergent-free buffer. Solubilisation of the washed membranes with 0.25% (v/v) Nonidet P-40 and ultracentrifugation resulted in a considerable amount of detergent soluble GPI-anchor converting activity in the supernatant. Anion-exchange chromatography on a Fractogel TSK-DEAE column of detergent-soluble GPI-anchor converting activity revealed two distinct peaks eluting at 50–80 mM and 120–170 mM NaCl, respectively. Using [^{125}I]TID-labelled mf-AChE as substrate, radiolabelled diradylglycerol was obtained with both peak activities. However, when the phosphatase inhibitors NaF and sodium orthovanadate were included in the assay systems, phosphatidic acid was detected in additon to diradylglycerol. Both GPI-anchor converting activities were Ca^{2+}-sensitive and inhibited by heavy metal chelating agents. These results suggested the presence of two isoenzymes of GPI-PLD and a phosphatase, rather than a GPI-PLC activity, in liver. Further, it could be shown that the activity in the second peak was identical to GPI-PLD, abundantly present in serum, while the activity contained in the first peak seems to be genuine for liver cells and, thus, apparently represents a novel form of a GPI-PLD which is membrane-associated and distinctly different from the serum enzyme.

Introduction

Glycosylphosphatidylinositols (GPIs) are a class of glycolipids occurring in membranes and serving as lipid anchor of an increasingly large number of membrane proteins. By now, well over 100 GPI-anchored proteins have been identified, all of them being oriented towards the extracellular space. They occur in eukaryotic cells ranging from mammals to the slime mold *Dictostelium*, the yeast *Saccharomyces cerevisiae* and protozoa like *Trypanosoma brucei*. Up to this date, there is no report on the presence of GPI-anchored proteins in prokaryotes, algae or plants (for reviews see Refs. 1–3). As known so far, the core of all GPIs and GPI-anchors has common structural features which seem to have been conserved during evolution [3,4]. In spite of the fact that this novel type of membrane anchor is widely distributed in nature, information on the possible biological significance of this anchoring principle is just starting to emerge. One possible functional consequence of it may be its hydrolysis by anchor-specific enzymes resulting in the selective release of GPI-anchored proteins from membranes. Such an enzyme was reported to exist in *Trypanosomes* and cleaved the GPI-anchor of the variant surface glycoprotein with a specificity of a phospholipase C [5–7]. Since this enzyme did not hydrolyze PI but acted only on the PI-moiety of the GPI-anchor, it was denominated as GPI-specific phospholipase C (GPI-PLC). In mammals, a similar activity with a cleavage specificity

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Abbreviations: mf-AChE, membrane form of acetylcholinesterase; sAChE, soluble AChE; DRG, diradylglycerol; GIP, glycosylinositolphosphate; GPI, glycosylphosphatidylinositol; HDL, high-density lipoproteins; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 2-morpholinoethanesulfonic acid; NP-40, Nonidet P-40; PA, phosphatidic acid; TID, 3-trifluoromethyl-3-(*m*-iodophenyl) diazirine.

Enzymes: acetylcholinesterase (EC 3.1.1.7); phosphatidylinositol-specific phospholipase C (EC 3.1.4.10); phosphatidylinositol-glycan-specific phospholipase D (EC 3.1.4.-).

of a phospholipase D (GPI-PLD) occurs in large amounts in plasma [8–11] and to a lower extent in placenta [12] and in brain [13].

It has been suggested that in mammals, a membrane bound GPI-PLC might be involved in insulin signal transduction, leading to the hydrolysis of GPI and the formation of diradylglycerol (DRG) and glycosylinositolphosphate (GIP) as products [14–20]. It was shown that isolated GIP is able to mimic some of the typical insulin effects like increased amino-acid uptake [21] and regulation of key enzymes in lipid and carbohydrate metabolism [22–24]. Despite these effects of isolated GIP, very little is known about the enzymes involved in the generation of GIP from its precursor GPI. To our knowledge, there have been three reports on the existence of a GPI-PLC in mammalian cells. In mouse brain, two forms of GPI-anchor converting activities were described [25]. Fox et al. [17] partially purified and characterized a GPI-PLC from rat liver and recently, the existence of such an activity was confirmed in isolated rat hepatocytes [26]. Since the properties of GPI-PLC described so far are ambiguous, we set out to further characterize the putative GPI-PLC in liver. In the present report, we confirm the existence of a GPI-anchor converting activity in mammalian liver. However, our results demonstrate that hydrolysis of GPI-anchored proteins occurs with the specificity of a phospholipase D rather than a phospholipase C and that there are two isoenzymes of GPI-PLD present in liver.

Materials and Methods

All reagents used were of analytical grade and either from Fluka (Buchs, Switzerland), Sigma (St. Louis, MO, USA) or Merck (Darmstadt, Germany). 3-Trifluoromethyl-3-(*m*-[^{125}I]iodophenyl)diazirine ([^{125}I]TID) was from Amersham (UK). Fractogel TSK-DEAE-650(M) and TLC silica gel 60 plates were from Merck (Darmstadt, Germany). Edrophonium (Tensilon) was a gift from Hoffmann-La Roche (Basel, Switzerland).

Biological material

Bovine liver and bovine blood were obtained from the local slaughterhouse. Rat livers and isolated rat hepatocytes which were prepared by standard procedures [27,28] with modifications as described in Ref. 29, were a kind gift of Prof. A. Jakob (Basel, Switzerland). The membrane form of AChE was purified by affinity chromatography from bovine erythrocytes as described by Brodbeck et al. [30].

To obtain bovine serum, blood was let to coagulate and then centrifuged at 4°C for 20 min at 2400 × *g* in a Cool-spin centrifuge. The resulting supernatant was dialyzed at 4°C for 3 days against 10 mM Tris-HCl (pH 8.0) containing 2 mM β-mercaptoethanol (buffer A).

Isolation and solubilization of liver membrane GPI-anchor converting activity

All the subsequent working steps were carried out over ice or at 4°C. Frozen liver tissue, either from cow, rat, or human was cut in fine pieces and thawed in a 5-fold volume (w/v) of ice-cold Tris-buffered saline (pH 8.0) containing 10 mM Tris-HCl (pH 8.0), 144 mM NaCl and 2 mM β-mercaptoethanol (buffer B). After 5 min of centrifugation at 800 × *g* in a Sorvall SS-34 rotor, the pellet was homogenized for 20 s in 5 volumes of buffer B, using a VIRTIS-type blender at low speed. The subsequent centrifugation for 30 min at 20000 × *g* gave a pellet which was rehomogenized twice for 1 min at medium speed in 5 volumes of buffer B. The homogenate was filtered through two layers of nylon gauze and centrifuged for 20 min at 130000 × *g* in a Centrikon TFT 70.38 rotor. Then, the pellet was washed twice in 5 volumes of buffer A as described above. The washed membranes were solubilized in 5 volumes of buffer A containing 0.25% (v/v) NP-40 (buffer C) with 10 strokes in a motor-driven loose-fitting teflon-glass homogenizer and centrifuged for 1 h at 100000 × *g*. In order to enhance the yield of GPI-anchor converting activity, the pellet was extracted once more with buffer C. The combined supernatants were asigned as NP-40 membrane-extract.

Anion-exchange chromatography on Fractogel TSK-DEAE 650(M)

The anion-exchange chromatography was carried out on a fast protein liquid chromatography (FPLC) system with a HR 16/10 column from Pharmacia (Uppsala, Sweden) and a Hitachi L-6200 Intelligent Pump at a constant flow rate of 1.5 ml/min in a refrigerated room. After equilibration of the column with buffer C, the NP-40 membrane-extract was percolated, and the column was washed with 200 ml buffer C. The column was developed with 300 ml of a linear 0–200 mM NaCl-gradient in buffer C, and fractions of 3 ml were collected.

Assay for GPI-anchor converting activity with mf-AChE as substrate

Unless otherwise stated, GPI-anchor converting activity was probed under standard assay conditions [26]: 4 μl of samples containing 0.25% NP-40 were diluted into 16 μl of 50 mM Mes (pH 6.1) containing 0.5 mM $CaCl_2$ and 2 mM β-mercaptoethanol and incubated at 37°C with 2 μl of a solution containing 640 fmol mf-AChE as substrate in Tris-buffered saline (pH 7.4) and 0.1% (v/v) Triton X-100. The product of the reaction (sAChE) was separated from the substrate by phase separation in Triton X-114 according to Bordier [31] as outlined in Ref. 26. Aliquots of 30 μl each of the total Triton X-114 phase (total substrate) and of the aqueous supernatant after phase separation (en-

zymatic product) were diluted 8-fold in Tris-buffered saline (pH 7.4) containing 0.1% (v/v) Triton X-100. Aliquots of 50 μl were transferred to microtitre plates and AChE activity was measured spectrophotometrically according to Ellman et al. [32]. The reaction was started by addition of 150 μl of a solution containing 1 mM acetylthiocholine, 0.25 mM 5,5'-dithiobis(2-nitrobenzoic acid) and 0.1% (v/v) Triton X-100 in 100 mM phosphate (pH 7.4). The increase in absorbance at 405 nm was measured with a Molecular Devices V_{max} Kinetic Microplate Reader interfaced to a HP-Vectra computer and rates were calculated using the SOFTMAX software (Molecular Device Corporation, Palo Alto, USA). The product (sAChE) formation was calculated as described in Ref. 13. Blanks contained about 4.5% of the total AChE activity in the aqueous phase; this value did not increase by incubation at 37°C. The GPI-anchor converting activity was expressed either as arbitrary units or as units where 1 unit equals 1 pmol sAChE produced per min (1 pmol AChE corresponds to 0.43 IU of AChE activity [26]).

Assay for phosphatase activity

Phosphatase was measured as follows: 20 μl of sample was diluted in 80 μl of 50 mM Mes (pH 6.1) containing 0.5 mM $CaCl_2$ and 2 mM β-mercaptoethanol, incubated at 37°C for 10 min and then mixed with 10 μl of a solution of 70 mM *p*-nitrophenyl phosphate (*p*-NPP) in water. After 30 min of incubation at 37°C, the reaction was stopped by adding 0.4 ml of 0.1 M NaOH on ice. Samples containing 80 μl of the above Mes-buffer and 10 μl of *p*-NPP to which 20 μl of enzyme-solution was added after the incubation period, served as blanks. Aliquots (200 μl each) were pipetted in duplicates into microtitre plates and the absorbance at 405 nm was determined using the same equipment as described above. After subtraction of the blank, phosphatase activity was calculated from a standard curve obtained with *p*-nitrophenol.

Protein assay

Protein was determined by the method of Wang and Smith [33] adapted to the use of microtitre plates (total volume of 230 μl). Bovine serum albumin in the concentration range of 0–800 μg/ml served as standard. The samples were diluted 1:4 (v/v) with double distilled water.

Identification of products of GPI-anchor converting activity

Hydrophobic labelling of mf-AChE was carried out with [^{125}I]TID as described previously [13,34]. The resulting specific radioactivity of mf-AChE was 5580 cpm/pmol AChE. Aliquots containing GPI-anchor converting activity were incubated with [^{125}I]TID-labelled mf-AChE in a total volume of 200 μl under standard assay conditions with or without the addition of the phosphatase inhibitors NaF and sodium orthovanadate. Then, 100 μl of a suspension containing 50 nmol dipalmitoyl phosphatidic acid and 50 nmol 1-*o*-octadecyl-2-palmitoyl glycerol as carrier lipids in Tris-buffered saline (pH 7.4) containing 0.1% (w/v) sodium deoxycholate and 200 μl water were added. Lipids were extracted by vortexing with 2.15 ml chloroform/methanol/HCl (25:50:1, by vol.) at room temperature. Water and chloroform, 0.7 ml each, were added afterwards, and the turbid mixture was centrifuged for 10 min at 300 × *g*. The lower, organic phase was removed and dried under nitrogen. Lipids were redissolved in 60 μl of chloroform/methanol (2:1, by vol.) and quantitatively applied to a TLC plate. The chromatogram was developed with chloroform/methanol/conc. NH_3/water (90:54:5.5:5.5, by vol.). In order to obtain a better resolution of the lipids, two-dimensional TLC's were carried out either with TLC-system 1 (see below), which separated DRG from phospholipids, or with TLC-system 2 (see below), which separated PA from other phospholipids. Lipid spots were stained by iodine vapor. Radioactivity on the TLC plates was visualized by autoradiography using Fuji RX X-ray films, and quantified by scraping appropriate areas of the plate and counting them in a γ-counter. The [^{125}I]TID-labelled lipid products migrated in all solvent systems a little bit further than the unlabelled carrier lipids dipalmitoyl-phosphatidic acid and 1-*o*-octadecyl-2-palmitoylglycerol, which can be explained by the greater hydrophobicity of [^{125}I]TID-labelled lipids.

TLC-system 1. First dimension, benzene/diethylether/conc. NH_3 (100:80:0.1, by vol.); second dimension, chloroform/methanol/conc. NH_3/water (45:45:3.5:10, by vol.).

TLC-system 2. First dimension, chloroform/methanol/water/conc. NH_3 (100:82:10:13.3, by vol.); second dimension, chloroform/methanol/water/acetic acid/aceton (100:37.5:20:30:37.5, by vol.).

Results

To obtain further information on GPI-anchor converting activity, especially with respect to its putative role in insulin signal transduction [21–24], we aimed at characterizing this enzyme in bovine liver. Upon extraction of liver tissue as described in Materials and Methods with buffer B and buffer A, respectively, a significant amount of anchor converting activity appeared in the soluble fraction after centrifugation. In case of rat liver, successive washing decreased the extractable activity in the supernatant of the fourth extraction to less than 1% of the totally extractable GPI-anchor converting activity, whereas the supernatant of the last wash of bovine liver contained about 5% of the anchor converting activity. The remaining

pellet was subsequently extracted with buffer C (buffer B containing 0.25% NP-40 (v/v)) and after ultracentrifugation GPI-anchor converting activity was measured in the detergent soluble extract. In order to enhance the yield, two extractions were carried out. Per g of bovine liver, 48 units of GPI-anchor converting activity could be totally extracted, of which 87% were soluble in absence and 13% were soluble in the presence of detergent. Perfused rat liver contained 19 units per g of tissue of which 76% were soluble in absence and 24% in presence of NP-40.

Detergent extracts of bovine, rat and human liver containing anchor-converting activity were chromatographed on a Fractogel TSK-DEAE column and the eluted fractions were assayed for GPI-anchor converting activity, phosphatase activity and protein. As can be seen from Fig. 1, this procedure resolved the anchor-converting activity of bovine liver-membrane extracts into two distinct peaks (P1 and P2), eluting in the range of 50–80 mM NaCl and 120–170 mM NaCl, respectively. The purification factor in both peaks was 3–4-fold. When the two peaks were pooled individually and rechromatographed under identical conditions in separate runs, they eluted as single peaks at their respective NaCl concentrations, indicating that the two peaks represent distinct forms of GPI-anchor-converting activities (results not shown). Comparable elution profiles were obtained with detergent extracts from rat and human liver (results not shown). The distribution of anchor-converting activity between the two peaks was strongly influenced by the preceeding washing procedure of the membranes. If the pellets were washed three times with buffer A containing 1 M NaCl and twice more with buffer A before NP-40 solubilization, the relative amount of anchor-converting activity in peak 2 decreased considerably as compared to that in peak 1, indicating that peak 2 represents an anchor-converting activity only loosely associated with the membranes while the activity in peak 1 is membrane-bound.

It is well established that bovine serum contains large amounts of GPI-PLD [9]. Since liver extracts are usually contaminated with blood, we investigated the possibility whether one of the two resolved peaks of anchor-converting activity could be due to contamination by serum. This was done by adding increasing amounts of serum to detergent extracts of washed liver membranes before anion-exchange chromatography. As shown in Fig. 2, panels A–C, addition of serum did not significantly effect the amount of anchor-converting activity present under peak 1, while the activity under peak 2 increased with increasing amounts of added serum. When serum alone was subjected to anion-exchange chromatography, serum GPI-PLD eluted at the same ionic strength as peak 2 of the anchor-converting activity from liver and no activity was detected in a position corresponding to peak 1 (Fig. 2, panel D). Thus, according to the elution behaviour of serum GPI-PLD, the GPI-anchor-converting activity under peak 2 of liver detergent extracts can be attributed to serum contamination. Since in the preparation of hepatocytes livers are extensively perfused and, thus, practically freed from contaminating blood, we determined the distribution of anchor-converting activity of detergent extracts from rat hepatocytes after anion-exchange chromatography. As with liver extracts, two peaks of activity were detected in hepatocytes (Fig. 3). The distribution of anchor converting activity between the two peaks was about 3:2. With bovine liver, a similar distribution could only be obtained when liver membranes were extensively washed with 1 M NaCl salt solution prior to solubilisation.

Our routine assay for GPI-anchor-converting activity relies on the conversion of amphiphilic substrate

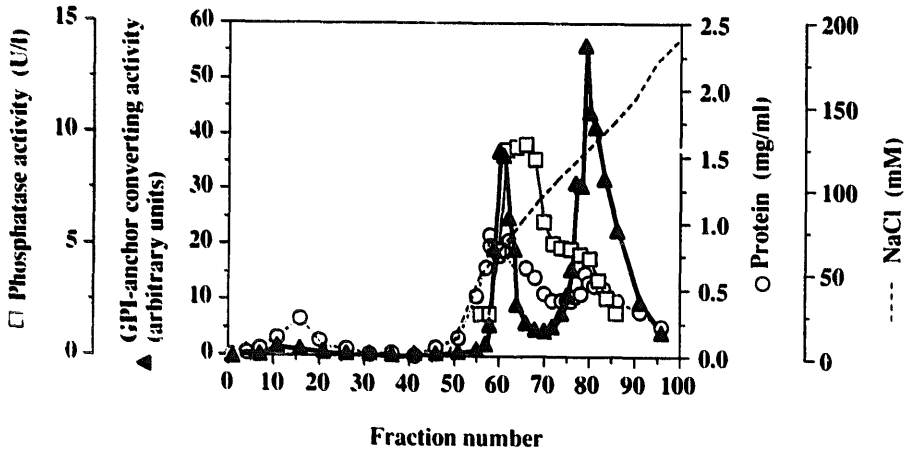

Fig. 1. Separation of GPI-anchor converting activity from bovine liver into 2 different forms by anion-exchange chromatography. The combined detergent soluble supernatants (152 ml containing 240 mg protein and 90 units GPI-anchor-converting activity) obtained from 15 g bovine liver were chromatographed on a 30-ml column of Fractogel TSK-DEAE at a constant flow rate of 1.25 ml/min. The column was washed with 300 ml of buffer C and developed with 400 ml of a linear 0–200 mM NaCl-gradient in buffer C. Fractions of 8 ml were collected.

(mf-AChE) to soluble product (s-AChE). It does not discriminate between the action of a phospholipase C or a phospholipase D, i.e., it is not able to determine which phosphodiester bond of PI in the GPI-anchor is hydrolyzed. Furthermore, the routine assay is unable to distinguish between hydrolysis of the substrate by a phospholipase or conversion of mf-AChE to sAChE by a proteinase. In order to verify that the two resolved GPI-anchor converting activities were due to the action of phospholipases and to assess their cleavage specificities, we analyzed the hydrophobic products, i.e., diradylglycerol or phosphatidic acid, of the reactions by using [^{125}I]TID-labelled mf-AChE. [^{125}I]TID is a hydrophobic photolabel which can be covalently bound to the diradylglycerol moiety of GPI-anchored AChE [35,36]. Labelled substrate was incubated with aliquots from both peaks of anchor converting activities and the [^{125}I]TID-labelled cleavage products were extracted and analyzed by TLC. The radiolabelled, hydrophobic products were identified in parallel experiments in

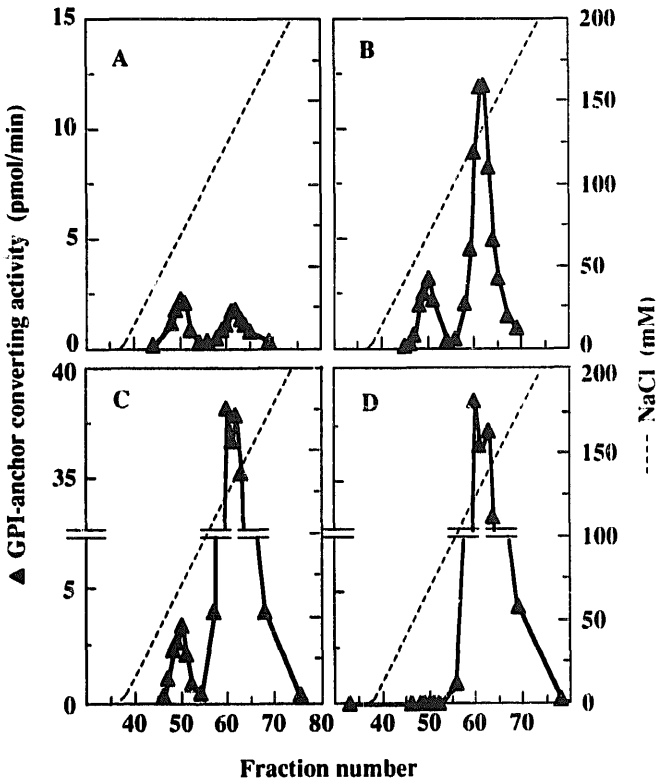

Fig. 2. Distribution of GPI-anchor-converting activities of combined NP-40 extracts from bovine liver membrane containing increasing amounts of serum. NP-40 extracts from 30 g of bovine liver were divided in several parts of 60 ml each. To the first portion of 60 ml NP-40 extract, 1.2 ml buffer C was added and 50 ml of the resulting mixture was subjected to anion-exchange chromatography as detailed in Materials and Methods (panel A). To the second portion, 0.24 ml of previously dialysed bovine serum in 0.96 ml buffer C was added and chromatographed identically (panel B). To the third portion, 1.2 ml dialysed bovine serum was added and treated as above (panel C). Panel D shows the elution profile obtained when 1.0 ml of bovine serum alone was subjected to anion-exchange chromatography.

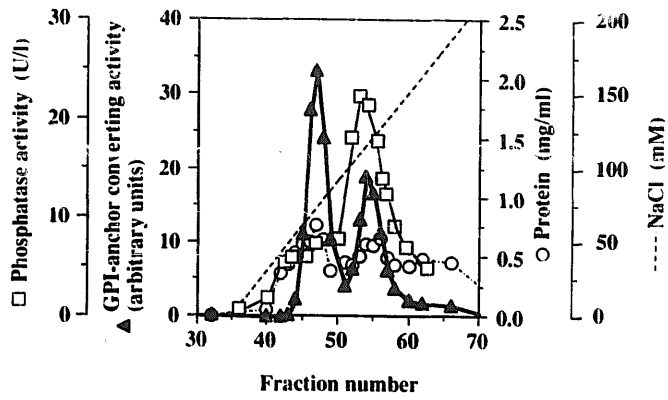

Fig. 3. Distribution of GPI-anchor-converting activities of combined NP-40 extracts of isolated rat hepatocytes. Isolated rat hepatocytes (5 ml of a packed suspension in 20 mM Hepes-buffered (pH 7.4) salt medium) were washed twice with 25 ml buffer B and centrifuged at 4°C for 10 min at 800× *g*. The washed cells were homogenized with a loose fitting teflon-glass homogenizer in 25 ml buffer A and centrifuged at 4°C for 20 min at 130000× *g*. The membrane pellet was subsequentely solubilized twice in 25 ml of buffer C and centrifuged at 4°C for 50 min at 130000× *g*. The combined supernatants were subjected to anion-exchange chromatography using a 7.8 ml Fractogel TSK-DEAE column at a constant flow rate of 1.0 ml/min. The column was washed with 150 ml of buffer C and developed with 100 ml of a linear 0–200 mM NaCl-gradient in buffer C. Fractions of 3 ml were collected.

which [^{125}I]TID-labelled mf-AChE was hydrolyzed either by PI-PLC from *Bacillus cereus* producing [^{125}I]TID-labelled diradylglycerol or purified GPI-PLD from bovine serum producing [^{125}I]TID-labelled phosphatidic acid. As shown in Fig. 4, lane 1, the product of GPI-anchor-converting activity in peak 1 was almost exclusively diradylglycerol. The faint trace of radioactivity seen below the diradylglycerol spot was also seen in untreated [^{125}I]TID-labelled mf-AChE and represents an impurity. Generation of [^{125}I]TID-labelled diradylglycerol from mf-AChE can result either from the action of a GPI-PLC or from the combined action of a GPI-PLD and a phosphatase. Since we noted phosphatase activity in both peaks eluting from the anion-exchange column (Fig. 1), we analyzed the products of [^{125}I]TID-labelled mf-AChE hydrolysis by GPI-anchor-converting activity in presence of the phosphatase inhibitor NaF. As seen in Fig. 4, hydrolysis of [^{125}I]TID-labelled mf-AChE by anchor-converting activity from both peaks of bovine liver extracts in presence of increasing amounts of NaF in the incubation mixture, yielded decreasing relative amounts of DRG with concomitant increases in PA. Since peak 2 contained considerably lower amounts of phosphatase activity than peak 1 (cf., Fig. 1), small amounts of PA were detected with GPI-anchor converting activity from peak 2 even in the absence of NaF (Fig. 4, lane 4 and Table I). Similar results were obtained with GPI-anchor-converting activity extracted from isolated hepatocytes. In absence of NaF, 93% of the label was

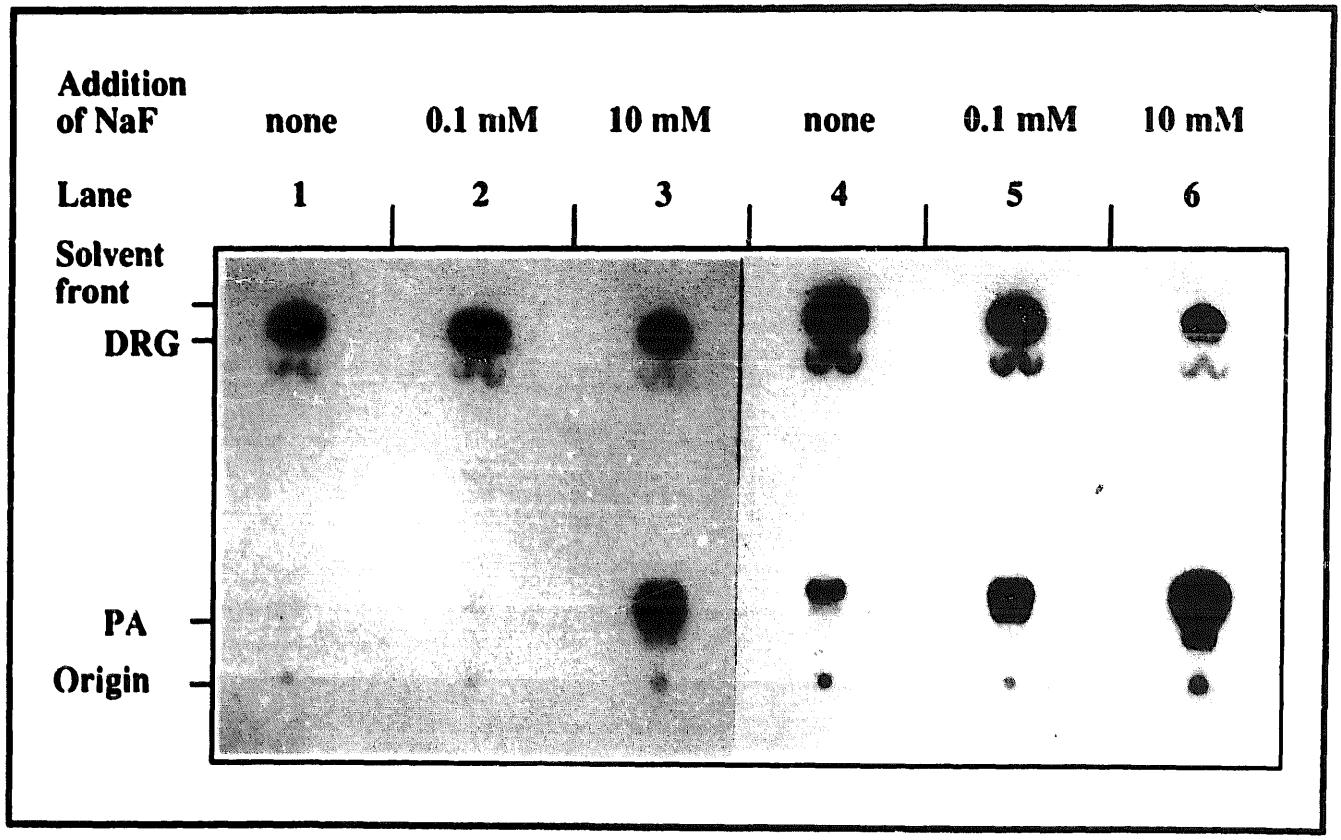

Fig. 4. Identification of hydrophobic products after treatment of [^{125}I]TID-labelled mf-AChE with GPI-anchor-converting activity. [^{125}I]TID-labelled mf-AChE was incubated at 37°C for 3 h with GPI-anchor-converting activity from peak 1 (lanes 1–3) and peak 2 (lanes 4–6) obtained after anion-exchange chromatography of a bovine liver NP-40 extract (cf., Fig. 1). Under these conditions, the amount of conversion of [^{125}I]TID-labelled mf-AChE from membrane form to soluble form was at least 50%. To inhibit phosphatase activity, NaF in increasing concentrations was added to the incubation media. After addition of carrier lipids, the hydrophobic products were extracted and analyzed by one-dimensional TLC in chloroform/methanol/conc. NH_3/water (90:54:5.5:5.5, by vol.) as described in Materials and Methods.

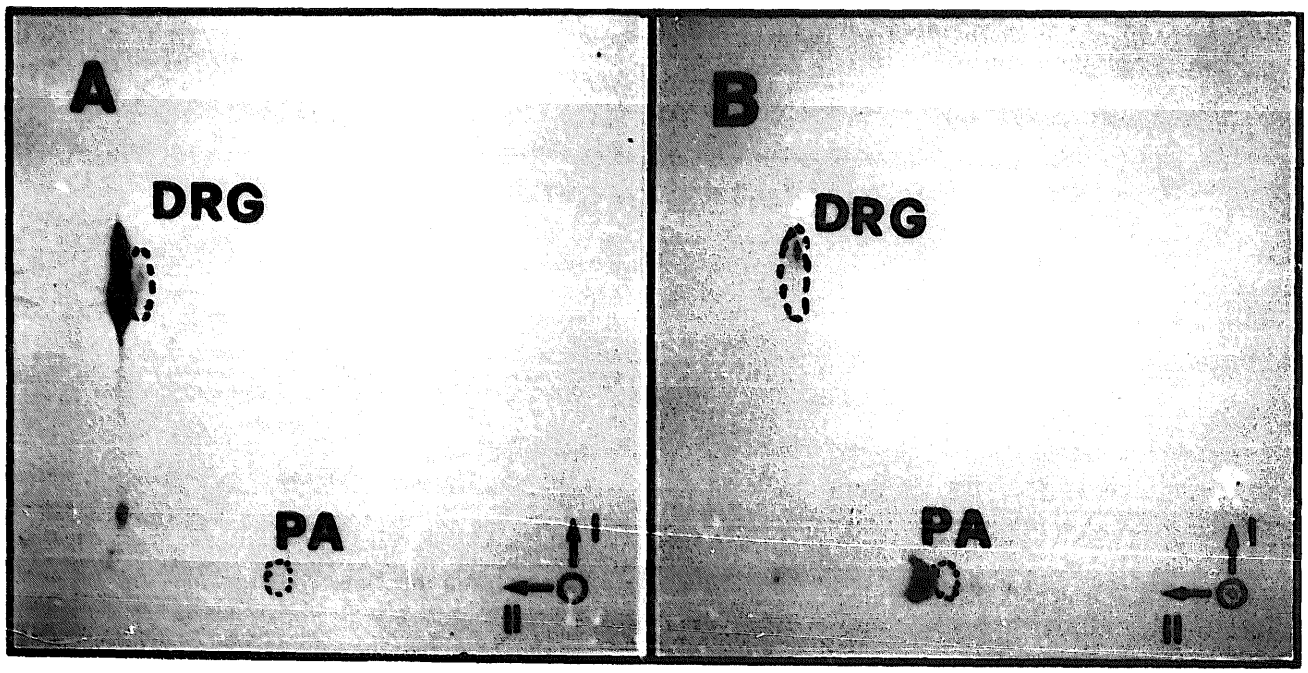

Fig. 5. Product identification of GPI-anchor-converting activity of peak 1 by two-dimensional TLC in presence of 50 mM NaF and 2.6 mM sodium orthovanadate. [^{125}I]TID-labelled mf-AChE was treated with GPI-anchor-converting activity of peak 1 obtained after anion-exchange chromatography of a bovine liver NP-40 extract (cf., Fig. 1) without any phosphatase inhibitors (A), and in presence of 50 mM NaF and 2.6 mM sodium orthovanadate (B). The hydrophobic products were extracted and analysed by two-dimensional TLC in system 1 as described in Materials and Methods.

TABLE I

Effect of NaF and sodium orthovanadate on product formation by GPI-anchor-converting enzymes

Product identification was carried out as described in Materials and Methods in the absence or presence of the phosphatase inhibitors NaF and sodium orthovanadate. Radioactive spots on the TLC plates were scraped and quantified by counting on a γ-counter.

Enzyme source	Phosphatase inhibitors		Products	
	NaF (mM)	vanadate (mM)	PA (%)	DRG (%)
Bovine liver, peak 1	–	–	0.3	98.5
	10	–	33.0	67.0
	50	2.6	96.0	4.0
Bovine liver, peak 2	–	–	5.8	92.0
	10	–	85.0	14.0
	50	2.6	97.0	3.0
Rat liver, peak 1	–	–	0.0	100.0
	50	2.6	93.9	5.5
Rat liver, peak 2	–	–	0.6	99.4
	50	2.6	100.0	0.0
B. cereus PI-PLC	–	–	0.3	99.6
	50	–	0.2	99.6
Serum GPI-PLD	–	–	99.5	0.4
	50	–	98.6	0.6

counted in the spot corresponding to DRG, while in presence of NaF 40% PA and 60% DRG were formed (results not shown). NaF had no effect on hydrolysis of [^{125}I]TID-labelled mf-AChE by PI-PLC from *B. cereus* or by GPI-PLD from bovine serum (Table I). We noted that the combination of NaF and sodium orthovanadate is more effective in inhibiting phosphatase activity than NaF alone. The phosphatase activity present in peaks 1 and 2 was inhibited to 97% by including 50 mM NaF and 2.6 mM sodium orthovanadate in the assay system. In this condition, GPI-anchor-converting activity in both peaks was also inhibited to 85%. However, product identification was still possible, although the amount of [^{125}I]TID-labelled mf-AChE which was converted to product, was much lower than in absence of sodium orthovanadate. As shown in Fig. 5A, DRG was again the only product obtained with GPI-anchor-converting activity in absence of inhibitors. In presence of 50 mM NaF and 2.6 mM sodium orthovanadate however, PA was the only product (Fig. 5B). The trace of radioactivity seen in position of DRG was also present in untreated [^{125}I]TID-labelled mf-AChE (result not shown) With GPI-anchor-converting activity contained in peak 2 as well as with the activities of peaks 1 and 2 of rat liver NP-40 extracts, similar results were obtained (Table I).

Discussion

In comparison to most tissues, GPI-PLD in serum exists in extraordinary high amounts [9]. This raises the question on how much of GPI-anchor converting activity assayed in a given tissue extract arises from serum contamination, and how much of it is due to tissue specific activity. In case of liver, an organ rich in blood vessels, this is of main concern as we could demonstrate with the results presented in this paper. NP-40 extracts of washed liver membranes contain two forms of anchor-converting activity which can be separated by anion-exchange chromatography. While the activity under peak 2 is likely due to contamination by serum GPI-PLD, that under peak 1 represents a novel form of anchor converting activity distinctly different from the one present in serum.

Fox et al. reported the presence of a membrane associated GPI-anchor converting activity in rat liver with a cleavage specificity of a phospholipase C [17]. In agreement with the above report, Stieger et al. demonstrated the presence of a GPI-anchor-converting activity in isolated rat hepatocytes and plasma membranes derived therefrom [26]. Based on their findings that DRG was the only hydrophobic product detected after cleavage of GPI-anchored AChE, they concluded that the anchor-converting activity was that of phospholipase C type. Our experiments, carried out in the absence of phosphatase inhibitors, confirmed the above mentioned reports in as much as hydrolysis of [^{125}I] TID-labelled mf-AChE mainly resulted in the generation of radiolabelled DRG. However, DRG can not only be produced by the action of a GPI-PLC but also by the combined action of a GPI-PLD and a phosphatase. Since phosphatase activity was present in all fractions showing GPI-anchor converting activity we performed product identification in presence of the known phosphatase inhibitors NaF and sodium orthovanadate. Addition of NaF alone resulted in decreased generation of DRG with a concomitant increase in PA, whereas addition of both inhibitors resulted in the exclusive generation of PA (Fig. 5). From these results, we concluded that the anchor-converting activities under peaks 1 and 2 in mammalian livers were of a GPI-PLD type. The elution patterns after anion-exchange chromatography indicated that the two anchor-converting activities differed in net negative charge, and, since the two activities catalyze the same reaction, the two forms may be regarded as isoenzymes of GPI-PLD.

GPI-PLD from serum [9,10,26] and brain [13] was shown to be Ca^{2+}-dependent and required heavy metals for activity. We noted similar properties for the two forms of liver GPI-PLD (results not shown) sustaining the notion that the two forms of GPI-PLD may be isoenzymes. The calcium sensitivity of liver GPI-PLD reported here differs from the results on liver GPI-PLC reported by Fox et al. [17]. Based on this observation, we can not completely exclude that in liver the GPI-anchor-converting activities reported by Fox et al. [17]

and that described presently, are due to different enzymes. Up to now however, we found no evidence of a GPI-PLC in liver membranes.

The finding that isolated hepatocytes also contained the two isoenzymes is of special interest. Hepatocytes were prepared from intact liver by extensive perfusion with buffer containing collagenase and hyaluronidase [29] which should remove blood contamination from vessels and capillaries. According to our present results, the GPI-anchor-converting activity in peak 2 likely resulted from contamination of the preparation by GPI-PLD from serum and consequently, this activity was expected to be missing in isolated hepatocytes.

As shown in Fig. 3, this was, however, not the case, as again both peaks were seen, although the activity under peak 1 relative to peak 2 was clearly increased in hepatocytes as compared to liver extracts. This finding raises the question on the origin of the two forms of GPI-PLD. A GPI-PLD from bovine liver has been cloned and sequenced recently [37]. Therefore, it might be that one or even both forms are derived from the mRNA of GPI-PLD present in liver. Recently, GPI-PLD from serum was shown to be an amphiphilic protein which in serum occurs in association with the high density lipoprotein fraction [38]. Furthermore, it is well established that HDL are targeted towards and taken up by liver cells. Thus, it is tempting to assume that the GPI-PLD under peak 2 was endocytosed by hepatocytes together with HDL. This would direct GPI-PLD to a secondary lysosome where the degradation of GPI-anchors may take place.

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