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Proteolytic activation of a bovine brain protein with phosphatidylinositol transfer activity

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We have purified a 38 kDa protein from bovine brain which is cross-reactive with an affinity purified antibody against the 35 kDa phosphatidylinositol transfer protein from the same source. Controlled trypsinization of the 38 kDa protein yielded an immunoreactive protein of 35 kDa which displayed a 6-fold increase in phosphatidylinositol transfer activity and a 10-fold higher affinity for this phospholipid. The possibility that the 38 kDa protein is a precursor of the phosphatidylinositol transfer protein is discussed.

Phosphatidylinositol transfer protein; Protein purification; Trypsinization; Phospholipid transfer; Bovine brain

1. INTRODUCTION

Phosphatidylinositol transfer proteins (PI-TP) facilitate the transfer of phosphatidylinositol (PI) and, to a lesser extent, phosphatidylcholine (PC) between membranes in vitro [1-3]. The proteins are present in cytosolic fractions from mammalian, avian, reptile, amphibian and insect tissues, and have an apparent molecular mass ranging from 33 kDa to 35 kDa depending on the species [4]. Cross-reactivity of antibodies raised against bovine brain PI-TP, with PI-TP from other sources indicates its conserved nature. However, it is noteworthy that PI-TP purified from yeast is not cross-reactive with this antibody [5]. Recently, it was demonstrated that deletion of the PI-TP gene is lethal for the yeast cell [6]. cDNA analysis has indicated that yeast is identical to the SEC14 protein which plays an essential role in vesicle-mediated protein secretion [7]. Binding of either PI or PC to PI-TP results in two forms of the protein (PI-TP I and II) with different isoelectric points (5.5 and 5.7, respectively). Moreover, the affinity of PI-TP for PI is 16 times higher than for PC [8]. Using pyrenylacyl-labeled PI and PC analogs with acyl chains of different length, it was shown that PI-TP has a preference for pyrenyl-octanoyl and -decanoyl chains [9]. Furthermore, it was shown that PI-TP has different binding sites for the 1- and 2-acyl chains.

Recently, it was reported that, in addition to the 35 kDa PI-TP, mature mammalian testis contain a PI-TP with a molecular mass of 41 kDa [10]. This PI-TP is able to transfer PI and PC but its structural and catalytic relationship with the 35 kDa PI-TP is unclear. The physiological role of mammalian PI-TP still remains to be determined. It has been suggested that PI-TP is involved in the control of PI levels in the membranes of cellular organelles and in the plasma membrane [11].

In the present paper we report the purification of a 38 kDa protein from bovine brain which is cross-reactive with an affinity purified polyclonal antiserum against bovine brain PI-TP (35 kDa). Compared to PI-TP this cross-reactive protein has a very low ability to bind and transfer PI. However, upon mild trypsinization of the 38 kDa protein a 35 kDa protein is produced, which shows a distinct increase in PI binding and PI transfer activity. These findings suggest that the 38 kDa protein may be a precursor protein of PI-TP. Analysis of the 38 kDa protein under various physiological conditions may provide information about a possible precursor/PI-TP relationship and, thus, on the physiological role of PI-TP.

2. MATERIALS AND METHODS

2.1. *Purification of PI-TP and the 38 kDa protein from bovine brain*

Purification of PI-TP and the related 38 kDa protein has been carried out following a procedure for the isolation of PI-TP from bovine brain, as described by van Paridon et al. [8]. The difference in molecular mass made it possible to discriminate between the cross-reactive 38 kDa protein and the 35 kDa PI-TP by SDS-PAGE immunoblotting. During the purification the fractions were routinely tested for PI-transfer activity and immunoreactivity (ELISA and immunoblotting, see below).

Briefly, a 25% homogenate (w/v) of the cerebral cortex of 15 fresh bovine brains was made in a buffer containing 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM PMSF and 0.9% NaCl. Following cen-

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trifugation at 14000 × *g* for 1 h, ammonium sulphate precipitation (70% saturation) of the supernatant was carried out overnight. After centrifugation the pellet was resuspended in a sodium phosphate buffer (5 mM Na-phosphate pH 7.2, 10 mM 2-mercaptoethanol) and extensively dialysed against this phosphate buffer. The dialysate was applied to a DEAE-cellulose Whatman (DE52) column (6 × 80 cm) and eluted with a linear gradient of 0–300 mM NaCl in the sodium-phosphate buffer. The fractions which showed transfer activity and immunoreactivity were identical. These fractions were combined and applied to a Sephadex G-75 column (Pharmacia, 4 × 160 cm). The active fractions were combined, glycerol was added to a final concentration of 10% (v/v) and the sample was applied to a hydroxyapatite column (Biogel, HTP, Biorad; 2.2 × 31 cm) and eluted with a 5–200 mM potassium-phosphate gradient, pH 6.8, in 10% glycerol. The active fractions identified by the transfer assay and by immunoreactivity assays were purified further on a chromatofocusing column (PBE94, Polybuffer 74-HCl, Pharmacia; 30 ml). Ampholines present in the positive fractions were removed on a Sephadex G-75 column. Proteins were stored in 50% glycerol (v/v) at −20°C.

2.2. *Phospholipid transfer and binding assays*

Phosphatidylinositol transfer activity was measured by the fluorescent lipid transfer assay as described by Somerharju et al. [12]. Donor vesicles consisted of 2-pyrenyl-decanoyl-PI/egg PC/ trinitrophenol-phosphatidylethanolamine (30:60:10 mol%). Acceptor vesicles consisted of PC and phosphatidic acid (95:5 mol%).

Binding studies between protein and pyrene-labeled phospholipids were carried out as described [9]. Donor vesicles consisted of 2-pyrenyl-decanoyl-PI/egg PC/trinitrophenolphosphatidyl-ethanolamine (45:45:10 mol%).

2.3. *Antisera and enzyme-linked immunosorbent assay*

A polyclonal antiserum directed against purified bovine PI-TP was produced in rabbits according to standard procedures. IgG specific antibodies were isolated by DEAE-cellulose chromatography followed by affinity chromatography with pure bovine brain 35 kDa PI-TP coupled to a carrier. An enzyme-linked immunosorbent assay (ELISA) was used to determine the presence of immunoreactive proteins.

2.4. *Electrophoresis and immunoblotting*

Proteins were separated by SDS-polyacrylamide gel electrophoresis, using a 11% acrylamide running gel. Proteins were visualized by Coomassie blue staining or silver staining. For immunological detection the proteins were transferred electrophoretically to nitrocellulose membranes (Schleicher and Schuell) and developed immunochemically.

2.5. *Trypsinization*

Tryptic digestion (EC 3.4.21.4, Boehringer) was carried out in a sucrose buffer (0.25 M sucrose, 1 mM EDTA and 5 mM Tris-HCl, pH 7.4) for 90 min at 37°C. The ratio protein/trypsin was 2.5 (w/w). Digestion was terminated by chilling on ice.

3. RESULTS

Fractionation of cytosolic proteins from bovine brain on DEAE-cellulose and Sephadex G-75 yielded one immunoreactive peak which coincided with PI-transfer activity. Subsequent fractionation on a hydroxyapatite column gave two immunoreactive peaks of which one eluted at 60 mM sodium phosphate (fractions 36–55, peak I) and another at 100 mM sodium phosphate (fractions 56–80, peak II, see Fig. 1). Determination of the PI transfer activity revealed that peak II contained two active peaks whereas peak I was virtually inactive. Chromatofocusing of peak II again yielded two fractions with transfer activity. This separation can be ascribed to the phospholipid (PI or PC) bound to PI-TP (see introduction). Chromatofusing showed that peak I also consisted of two immunoreactive fractions. Their isoelectric points differed 0.2 units. This is the same as observed for PI-TP I and II. Moreover, the inactive proteins had an isoelectric point of 0.2–0.4 pH units lower than observed for PI-TP. Immunoblot analysis of the 4 fractions obtained from chromatofocusing revealed that peak II consisted mainly of PI-TP (M_w 35 kDa), whereas peak I consisted of a cross-reactive protein with a molecular mass of 38 kDa.

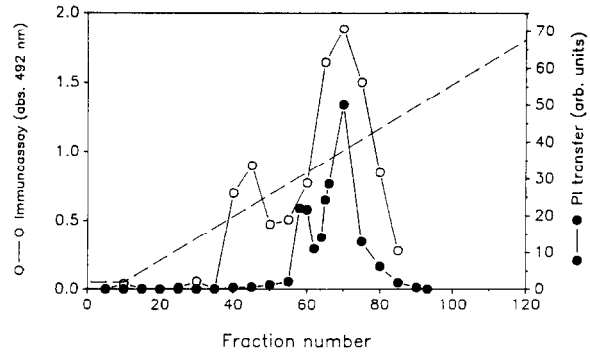

Fig. 1. Separation of PI-TP and the 38 kDa protein by chromatography on a hydroxyapatite column. For experimental details see section 2.1. (– – –) 5-200 mM potassium-phosphate gradient.

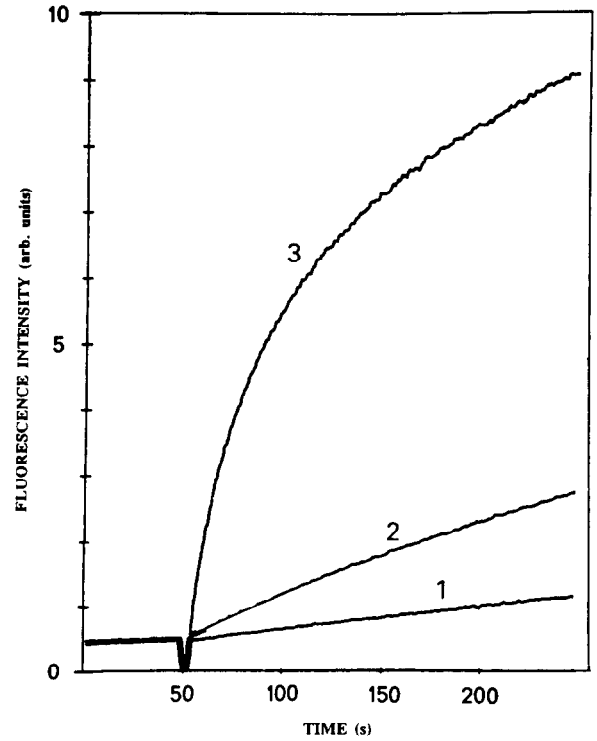

Fig. 2. Proteolytic activation of the 38 kDa protein. PI-transfer activity before and after controlled trypsinization (90 min, 37°C) of the 38 kDa protein, curve 1 and curve 2, respectively. Transfer activity of a comparable amount of PI-TP is shown in curve 3.

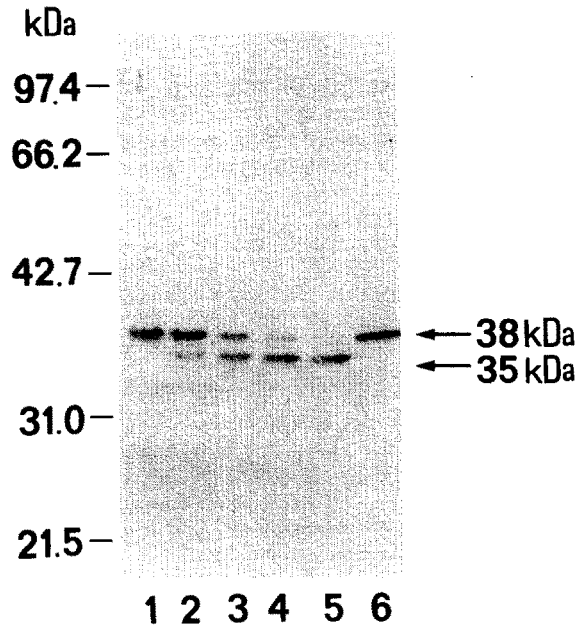

Fig. 3. Time-dependent proteolysis of the 38 kDa protein as determined by immunoblot analysis. Lane 1: 0.15 mg 38 kDa protein. Lane 2–5: 0.15 mg 38 kDa protein after 10, 30, 60, and 120 min trypsin treatment, respectively. Lane 6: 0.15 mg 38 kDa protein incubated under identical conditions for 120 min without trypsin.

As shown in Fig. 2, PI transfer activity of the 38 kDa protein (curve 1) is about 1–2% of the activity of PI-TP (curve 3). However, a 6-fold increase of PI-transfer activity was observed when the 38 kDa protein was submitted to controlled trypsin degradation (curve 2). It is shown in Fig. 3 that due to trypsin degradation the 38 kDa protein is converted into a 35 kDa protein which is cross-reactive with the antibody against PI-TP.
The affinity of PI-TP, of the 38 kDa protein and its 35 kDa degradation product for PI was compared in binding experiments. As can be seen from Fig. 4 (panel A and B) the affinity of the 38 kDa protein for PI is about 2% of that observed for PI-TP. After controlled trypsinization of the 38 kDa protein the affinity for PI was 10-fold increased (panel C).

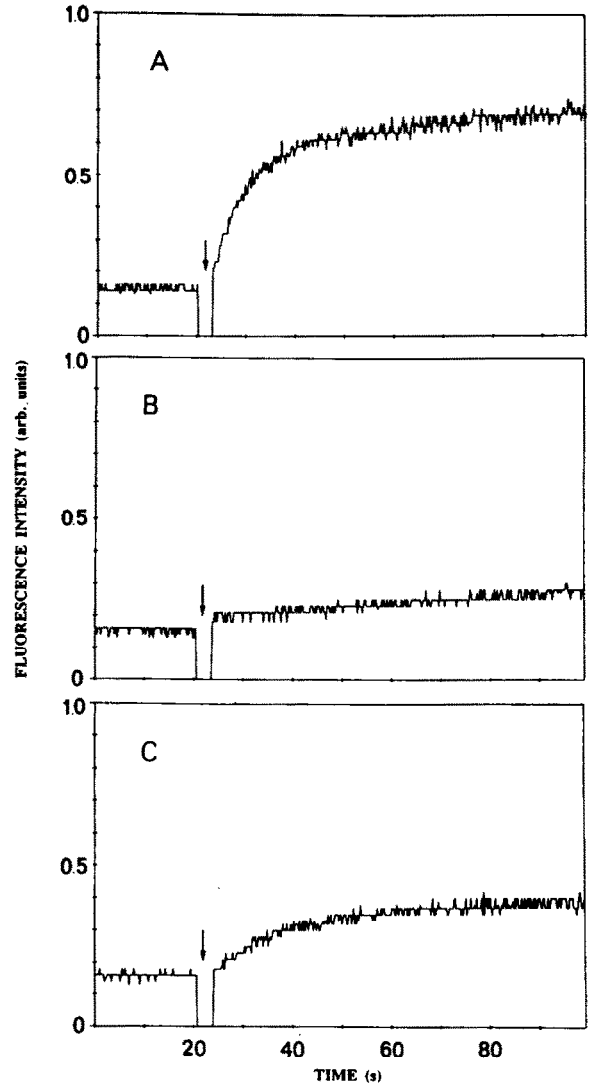

Fig. 4. Binding of pyrene-labeled PI to PI-TP (A), the 38 kDa protein (B) and its 35 kDa proteolytic product (C). Comparible amounts of protein were added to quenched donor vesicles containing pyrene-labeled PI (see arrow) and the fluorescence intensity was recorded as a function of time (emission at 377 nm.).

4. DISCUSSION

In the present study we show that bovine brain cytosol contains a 38 kDa protein which is related to PI-TP. This 38 kDa protein is cross-reactive with the affinity-purified polyclonal antibodies against bovine brain PI-TP. Its close relationship with PI-TP was further demonstrated by its cross-reactivity with a polyclonal antiserum against synthetic peptides designed on the basis of predicted B and T cell epitopes of rat brain PI-TP (I.S.C. de Wit, personal communication). Both the 38 kDa protein and PI-TP occur in two forms which can be distinquished on the basis of their different isoelectric points. The two forms of PI-TP are due to the fact that binding of their PI or PC causes a difference of one negative charge [8]. At this point we have no explanation for the occurrence of two forms of the 38 kDa protein.

Evidence that the 38 kDa protein is an inactive precursor of PI-TP, followed from trypsin degradation experiments. Controlled trypsinization of the 38 kDa protein results in an immunoreactive 35 kDa protein which has a 6-fold increased PI-transfer activity. It follows from Fig. 2 that the newly formed 35 kDa protein is not as active in PI transfer as PI-TP. This could be due to the fact that the cleavage site for trypsin is different from that for the putative endogenous cellular protease. Moreover, incubation of PI-TP with trypsin under comparable conditions leads to a 2-fold decrease in PI-transfer activity (data not shown, [1]).

Recently, it was reported that the cytosolic fraction of mature testis contains an additional PI-TP species of

41 kDa [10]. This immuno cross-reactive PI-TP is exclusive for testis and has both PI and PC transfer activity comparible to that of the 35 kDa PI-TP. It remains to be established in what way the 41 kDa PI-TP, the 38 kDa protein and PI-TP are related.

A cDNA encoding for rat brain PI-TP has been sequenced and the deduced amino acid sequence defines a protein with a molecular mass of 35 kDa [4]. However, DNA and mRNA hybridization experiments indicate that related genes and/or introns are present. It is therefore expected that one of these related genes encodes for the 38 kDa protein.

It is generally accepted that the ability of PI-TP to bind and transfer PI/PC is directly connected with its physiological role [13]. The conversion of an inactive precursor PI-TP into an active form is an efficient way to increase the pool of active PI-TP on short notice.If the 38 kDa protein described here is indeed a precursor of the 35 kDa PI-TP, information about the cellular conditions affecting the relative presence of the precursor protein and the active PI-TP, may increase our knowledge on the physiological role of PI-TP.

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