Characterization of membrane-associated prothrombin activator in normal and injured murine tissues

Yasuo Shikamoto, Satoshi Shibusawa, Izumi Okuyama, Takashi Morita*

Department of Biochemistry, Meiji College of Pharmacy, Yato-cho, Tanashi, Tokyo 188, Japan

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Abstract Thrombin is a multifunctional enzyme involved in coagulation, cell modulation and inflammation. We recently reported a novel membrane-associated prothrombin activator, abbreviated as MAPA, found in cultured fibroblasts and glial cell lines. In this study, we examined the physiological role of this enzyme. MAPA-like activity was detected in the liver, kidney, lung and heart but not in the spleen or brain in normal mice. To examine whether MAPA participates in biological reactions, hepatic and renal injury were induced by administration of CCl_4 and $HgCl_2$, respectively. MAPA-like activity was specifically increased in the injured tissues: the activity was elevated by about 100-fold in 48 h in the liver and increased by about 5-fold in 12 h in the kidney. Their enzymatic properties were the same as those of MAPA in 8C feline kidney fibroblast cells. Phospholipids are required for activation of prothrombin by MAPA obtained from both 8C cells and tissues. These results suggest that MAPA activates prothrombin on the cell surface in injured tissue and participates in inflammation and regeneration associated with tissue injury.

Key words: Prothrombin activator; Thrombin; Inflammation; Injury; Phospholipid

1. Introduction

Thrombin regulates a number of cellular reactions linked to inflammation, wound healing, development and differentiation [1], in addition to hemostasis and thrombosis. Thrombin affects these cells via thrombin receptors [2]. Although it was believed that its zymogen, prothrombin, is synthesized in the liver alone and circulated in the bloodstream, recent studies revealed that mRNAs encoding both prothrombin and thrombin receptor are expressed in the central nervous system [3,4]. Thrombin receptor is widely expressed in cells separated from the bloodstream such as fibroblasts, neurons, and astrocytes [4,5]. A potent thrombin inhibitor, protease nexin-1 (PN-1), is also expressed in extravascular regions [6,7]. These observations strongly suggest that thrombin regulates biological reactions not only in the bloodstream but also in other tissues. However, the mechanism of activation of prothrombin to thrombin remains unknown in these areas separated from the bloodstream. We recently reported a novel membrane-associated prothrombin activator (MAPA) that is expressed on the surface of various cell lines such as 8C (feline kidney fibroblast), MDCK (canine kidney epithelial-like) and T98G (human glioblastoma) in culture [8]. Here we show for the first time that MAPA activity is detected in the normal and injured murine tissues and the activation of prothrombin by MAPA is specifically dependent upon phosphatidylserine.

2. Materials and methods

2.1. Materials

L-α-Phosphatidylcholine (PC) from egg yolk, L-α-phosphatidylethanolamine (PE) from egg yolk, and L-α-phosphatidyl-L-serine (PS) from bovine brain, all >98% pure, and bovine serum albumin (BSA) were from Sigma. *p*-Amidinophenylmethanesulfonyl fluoride (*p*-APMSF) was obtained from Calbiochem. Di*iso*propyl fluorophosphate (DFP) was purchased from Wako (Osaka, Japan). Superdex 200 pg was from Pharmacia. Boc–Val–Pro–Arg–*p*-nitroanilide (VPR-*p*NA) was from Seikagaku Kogyo (Tokyo, Japan).

2.2. Proteins

The following proteins were prepared according to published methods: human α-thrombin [9], human prothrombin [10], bovine prothrombin fragment-1 [11], and bovine antithrombin III [12]. All of these proteins were homogeneous as judged by SDS-PAGE.

2.3. Preparation of phospholipid vesicles

Phospholipids in chloroform were mixed and evaporated under a stream of nitrogen gas. The residues were suspended in 50 mM Tris-buffered saline (TBS) and dispersed by sonication.

2.4. Assays for MAPA activity

In a typical assay, 90 μl aliquots of 1 μM human prothrombin in TBS containing 5 mM $CaCl_2$ and 1 mg/ml BSA were incubated with samples at 37°C for 20 min in 96-well microtiter plates. Then, the samples were mixed with 10 μl of 2.5 mM VPR-*p*NA. The initial rate of *p*-nitroaniline liberation was monitored at 405 nm with a kinetic plate reader (Well reader SK-601, Seikagaku Kogyo). The activity of the enzyme giving an absorbance of 0.5 at 5 min under these conditions was defined simply as 1 unit.

2.5. Preparation of plasma membranes from normal and injured tissues

Hepatic and renal injury were induced according to the published methods [13,14]. Young adult female ddY mice weighing 18–24 g were used in all experiments. Three mice were treated for each period. Hepatic injury was induced by intraperitoneal injection of 1 ml of 20% CCl_4 in olive oil/100 g mouse. Renal injury was induced by subcutaneous injection of 0.6 mg $HgCl_2$/100 g mouse. After appropriate periods, tissues were removed and minced. Control mice were administrated only olive oil or saline. The livers and kidneys were removed and minced after 0 and 48 h. Then, the tissues were homogenized in Tris-buffered saline (TBS; 50 mM Tris-HCl, 100 mM NaCl, pH 8.0) with a motor-driven Teflon homogenizer. The homogenates were treated with 1 mM DFP and 0.1 mM *p*-APMSF for 1 h, then centrifuged at 100000×*g* for 60 min to remove excess inhibitors and soluble proteins. The pellets were suspended in TBS and assayed for MAPA activity.

2.6. Cell culture

8C feline kidney fibroblast cells were maintained in plastic culture dishes (Iwaki, Tokyo, Japan) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 70 μg/ml kanamycin in a humidified atmosphere of 95% air and 5% CO_2 at 37°C, and cells were transferred after trypsinization every 5–6 days.

*Corresponding author. Tel: (81) 424-21-0429; Fax: (81) 424-21-1489.

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2.7. Solubilization and gel filtration

8C cells were rinsed twice with TBS and cells were scraped off the dishes. Harvested cells were suspended in TBS and the plasma membrane fractions were prepared as described [8]. MAPA was extracted and fractionated as follows. The plasma membrane fractions containing 100 μg protein were homogenated in the presence of 1% CHAPS, incubated for 1 h on ice, and then centrifuged at 100000×*g* for 60 min. Then, the supernatant was applied to a Superdex 200 pg column (2.6×60 cm) equipped with a fast protein liquid chromatography system (Pharmacia) that had been pre-equilibrated with TBS containing 0.3% CHAPS and eluted with the same buffer at a flow rate of 3 ml/min. Fractions (3 ml) were collected and subjected to the assay for MAPA activity. These procedures were all performed at 4°C.

3. Results

3.1. Detection of MAPA activity in murine liver and kidney

Since the membrane-associated prothrombin activator (MAPA) activity has been detected in several cell lines derived from kidney fibroblasts and glioma cells, we examined whether similar MAPA-like activity is present in the plasma membrane fractions of the kidney and other organs. Murine tissues of the liver, kidney, lung and heart had MAPA-like activities, whereas no activity of the enzyme was detected in other tissues such as the spleen and brain (Fig. 1). The activity detected in the liver was about 1/10 of that of 8C based on the amount of protein in the lysates. The properties of the enzyme detected in the kidney were characterized in detail. The activity detected in murine tissues was recovered mainly in the plasma membrane fraction. The activation derivatives and fragments generated by incubation of prothrombin with plasma membranes of the kidney were indistinguishable from those generated by incubation with plasma membrane fractions of 8C on SDS-PAGE. Activation of prothrombin was dependent on Ca^{2+} ions. The enzyme had a similar K_m value for prothrombin (0.17 μM), compared with that of the 8C-enzyme (0.20 μM). The inhibitors had essentially identical effects to the enzyme of the kidney. For example, di*iso*propyl fluorophosphate (10 mM), *p*-amidinophenylmethane-sulfonyl fluoride (1 mM), dansyl–Glu–Gly–Arg–chloromethyl ketone (1 μM), benzamidine (10 mM), pepstatin (1 mM), and iodoacetamide (10 mM) could not abolish the activity. On the other hand, exposure of the plasma membrane fraction to EDTA (1 mM) resulted in irreversible loss of the activity. Thus, the MAPA-like activity found in the murine kidney was essentially the same as that in the cultured cell lines.

3.2. Increase of MAPA activity in hepatic or renal injured murine tissues

Hepatic or renal injury was induced in mice by intraperitoneal injection of CCl_4 or subcutaneous injection of $HgCl_2$. Livers and kidneys were excised at various times after administration of CCl_4 to induce hepatic injury and MAPA activities of plasma membrane fractions were analyzed. MAPA activity of control murine tissues did not change following administration of olive oil (data not shown). MAPA-like activity of the liver rapidly increased to about 100-fold in 48 h compared with controls (Fig. 2A). However, MAPA-like activity of the kidney did not change after hepatic injury (Fig. 2B). Next, mice were treated with $HgCl_2$, and MAPA activity was analyzed in the liver and kidney after renal injury. MAPA activity of control murine tissues also did not change following administration of saline (data not shown). MAPA-like activity of the kidney was increased by about 5-fold in 12 h compared with controls, while the enzymatic activity in the liver did not change after renal injury (Fig. 2).

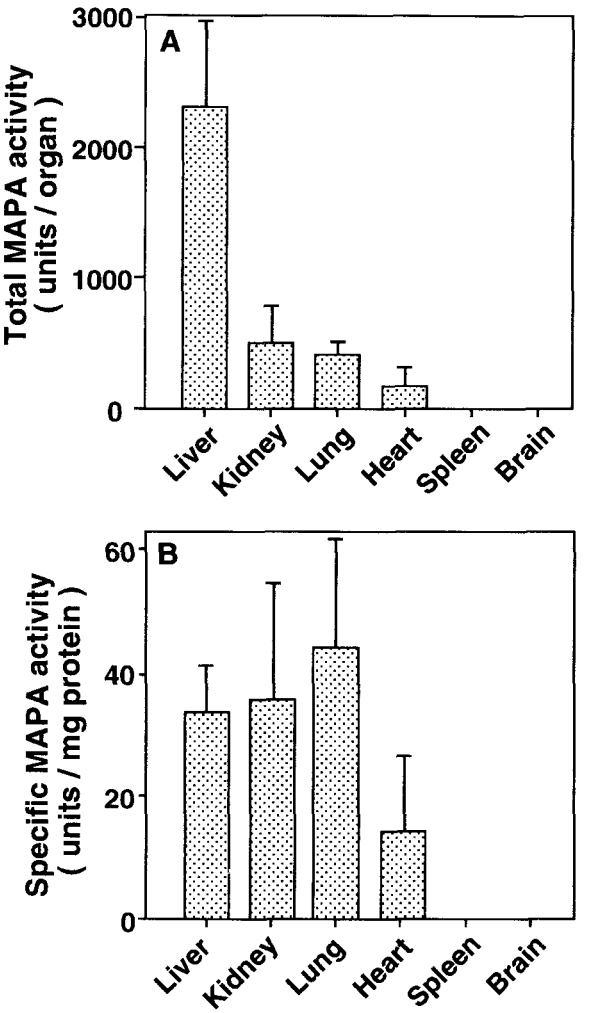

Fig. 1. Distribution of MAPA activity in normal murine tissues. Murine tissues were removed, minced, washed in TBS, and lysed by freezing/thawing. The lysates were homogenized with a Teflon homogenizer. The homogenates were assayed for MAPA activity ($n=3$) after treatment with *p*-APMSF and DFP. A: Total MAPA activity (units/organ). B: Specific MAPA activity (U/mg protein).

Enzymatic properties of the MAPA-like activities in both injured tissues were compared with those of 8C cells. These enzymatic properties were indistinguishable from those in the tissues and cell lines, indicating that an enzyme similar to MAPA detected in 8C is induced in both liver and kidney by tissue injury.

3.3. Requirement of phospholipids for the MAPA activity of 8C cells

To isolate and characterize MAPA of the 8C plasma membrane fraction, the solubilized plasma membrane fraction of 8C was applied to a column of Superdex 200 pg. The results are shown in Fig. 3. Little MAPA activity was detected in any fraction over a 20 min incubation period. Low activity of MAPA was detected in the fraction C even after 60 min pre-incubation with prothrombin. Total MAPA activity recovered from the column was less than 1% of that of the initial solubilized plasma membrane used. This low recovery suggested that an unknown factor(s) required for the activa-

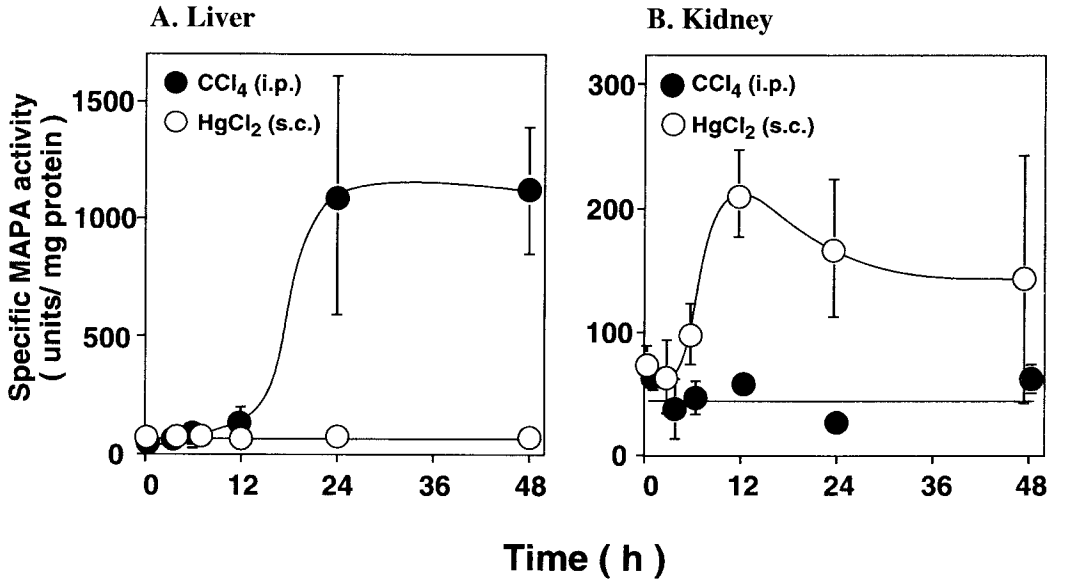

Fig. 2. Induction of MAPA activity by tissue injury. MAPA activity in the liver (A) and kidney (B) after hepatic or renal injury. Hepatic injury was induced by intraperitoneal injection of 1 ml of 20% CCl_4 in olive oil/100 g mouse (●). Renal injury was induced by subcutaneous injection of 0.6 mg $HgCl_2$/100 g mouse (○). Tissues were removed at each period and homogenized, then centrifuged at 100 000×*g* for 60 min. MAPA activities of homogenates were assayed after treatment with *p*-APMSF and DFP.

tion of prothrombin by MAPA may have been separated by this column. To test this hypothesis, reconstitution experiments with fraction C and other fractions were performed under the conditions described in the Methods section. Strong MAPA activity appeared only in the combined mixture of fraction C and D, indicating that both fractions are essential for development of MAPA activity. Activity in fraction D was not abolished by heat treatment (80°C for 30 min) and was extracted with 1:1 methanol/chloroform solution. These results clearly indicated that fraction D contained phospholipid vesicles from the plasma membrane. Thus, we examined the effects of phospholipids on the activation of prothrombin by MAPA. Prothrombin was activated by MAPA in fraction C in the presence of various concentrations of phospholipid vesicles (Fig. 4A). The presence of phospholipid vesicles was indispensable for the activation of prothrombin by MAPA. The activation rate reached a maximum at about 100 μM phospholipid vesicles. We next investigated the effects of composition of the phospholipid vesicles. Prothrombin activation was measured in the presence of phospholipid vesicles of various compositions (Fig. 4B), and phosphatidylserine was found to be essential for activation of prothrombin by MAPA. The increase in the activation rate was dependent on the proportion of phosphatidylserine in the vesicles. No activation of prothrombin was observed in the absence of phosphatidylserine in the vesicles even in the presence of phosphatidylcholine or phosphatidylcholine/phosphatidylethanolamine, indicating that MAPA-mediated prothrombin activation is specifically dependent upon phosphatidylserine. When the proportion of phosphatidylserine was varied in the vesicles, a pronounced leftward shift of the required phosphatidylserine proportion was observed in the presence of phosphatidylethanolamine.

4. Discussion

We previously reported that some culture cell lines have membrane-associated prothrombin activator on their surface [8]. We showed here the existence of MAPA activity in normal tissues in addition to culture cell lines. The enzymatic properties in tissue(s) are essentially identical to those of culture cell lines, indicating that MAPA activity is not unique to culture cell lines but also exists in vivo. This can be explanation by the necessity of supply of thrombin to cells separated from the bloodstream such as fibroblasts. Recently, it was clearly shown that prothrombin and protease nexin-1 (PN-1) were synthesized locally and thrombin activity was increased with sciatic nerve injury [15]. The present study showed that MAPA induced in hepatic or renal injury could activate prothrombin to thrombin both in hepatic and renal injuries.

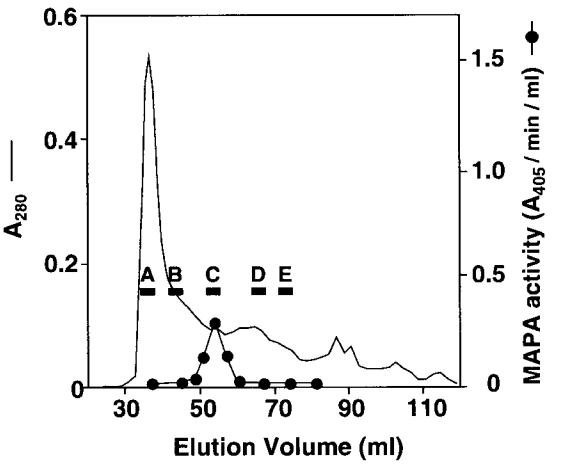

Fig. 3. Elution profile of solubilized 8C plasma membranes with gel filtration. 8C plasma membrane fraction was solubilized with 1% CHAPS for 1 h and centrifuged. The supernatants were applied to a Superdex 200 pg column. Fractions (10 μl) were incubated with prothrombin in the presence of 5 mM Ca^{2+} and 2.5 mM VPR-*p*NA and 1 mg/ml BSA in 90 μl TBS for 1 h at 37°C. Then, the absorbance was measured at 405 nm (●).

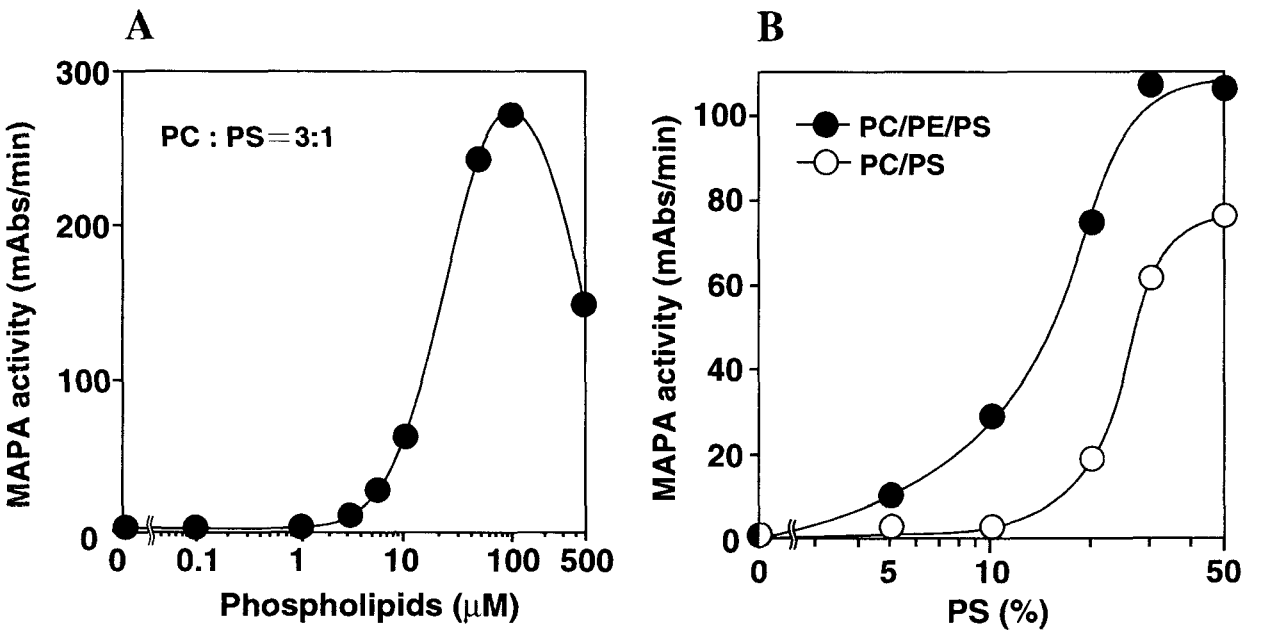

Fig. 4. Requirement of phospholipids for MAPA activity. A: MAPA activity was analyzed in the presence of the indicated concentrations of phospholipid vesicles. Aliquots of 10 μl of gel-filtrated fraction C were incubated in the presence of 5 mM Ca^{2+}, 1 μM prothrombin and the indicated concentrations of phospholipids vesicles. Phospholipids vesicles contained 25% PS and the remainder was PC. B: Effects of phospholipids on MAPA activity. Aliquots of 10 μl of gel-filtrated fraction C were assayed in the presence of 100 μM phospholipids vesicles. Phospholipids vesicles contained PS at the indicated percentages and contained (●) or lacked (○) 50% PE. The remainder was PC.

HGF/scatter factor is well known to play a critical role in regeneration of the liver and kidneys [16]. HGF/scatter factor is activated by HGF activator (HGFA) when the tissues are injured. HGFA is also produced from an inactive precursor, and the precursor of HGFA is activated by thrombin [17]. Since MAPA activity is specifically increased in injured tissues, the level of thrombin would be increased in injured tissues. Thus, MAPA is most likely to participate in the generation of thrombin in the injured tissues. Although we have no data to explain whether thrombin generated by MAPA activates the HGFA and/or thrombin receptor, the results of the present study suggest that MAPA may participate in regeneration of injured tissues in consideration of the diverse biological reactions of thrombin.

The results of recombination experiments show that the activation of prothrombin by MAPA requires phospholipid vesicles. Phosphatidylserine is a crucial component of the phospholipid vesicles. Some coagulation factors activate their substrates bound on phospholipids, especially phosphathidylserine [18]. Recently, it was found that phosphatidylethanolamine also enhances the reaction rates of coagulation/anticoagulation factors [19,20]. However, the activation of prothrombin by prothrombinase complex is not affected by phosphatidylethanolamine [19]. These observations support the hypothesis that MAPA is not the same as the prothrombinase complex. Phosphatidylserine is exposed on the outer leaflet at inflammation, while it is present in the inner leaflet [21,22]. The exposure of phosphatidylserine in the outer leaflet is responsible for the regulation of coagulant activity on the surface of platelets and endothelial cells. A similar mechanism could occur in other cells isolated from the bloodstream, and phosphatidylserine could regulate the thrombin generation by MAPA associated with injury.

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