Hepatic Injury-Specific Conversion of Mouse Plasma Hyaluronan Binding Protein to the Active Hetero-Dimer Form

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Plasma hyaluronan binding protein (PHBP) is produced only in liver and kidney in mouse. The induction of PHBP mRNA and the conversion of pro PHBP to the active hetero-dimer form were studied after CCl4, d-galactosamine, HgCl2 or turpentine administration and after partial hepatectomy. The results indicated that the administrations of CCl4 and d-galactosamine, which caused hepatic failure, and the partial hepatectomy enhanced the conversion of pro PHBP to the active two-chain form in the plasma. On the other hand, HgCl2, which injured kidney and turpentine which led to inflammation, were not involved in the activation of PHBP. The weak induction and suppression of PHBP mRNA were observed in the liver at 3 h and 12 h, respectively, after the CCl4 administration. However, HgCl2 and turpentine did not influence the amount of PHBP mRNA. These results suggested the hepatic injury-specific activation of PHBP in plasma. PHBP may act as an early factor in the cascade for the tissue remodeling in liver following hepatic injury, i.e., PHBP activates urokinase, urokinase activates matrix metalloproteinases (MMPs) and MMPs degrade extracellular matrix for liver regeneration.

Key words PHBP; hepatic injury; tissue remodeling; serine protease; activation

MATERIALS AND METHODS

Plasma hyaluronan binding protein (PHBP) is a novel serine protease found in human plasma.1–3 It cleaved fibrinogen and fibronectin, and converted inactive single chain urokinase type plasminogen activator (scu PA) to the active two-chain form (tcu PA).3 PHBP is present in human plasma as a pro-form of 70 kDa and the purified PHBP caused auto-fragmentation to the active hetero-dimer form (50-kDa heavy chain and 27-kDa light chain, both of which are bridged by a disulfide linkage).4 This auto-activation was accelerated in the presence of dextran sulfate and phosphatidylethanolamine.4

The active hetero-dimer further advanced conversion to the inactive form, i.e., the heavy chain to two 26-kDa fragments and the light chain to the 17 and 8-kDa fragments.5 In human plasma, Cl inhibitor is a major inhibitor of PHBP.4 Other groups also reported the serine protease activity of PHBP.5–8 In particular, Romisch et al. reported that PHBP activated factor VII of the tissue factor pathway of the coagulation system independently from tissue factor.5 These results suggest that PHBP is a principal protease in retaining the homeostasis of the body. To study the biological significance of PHBP, we administered to mice CCl4, which caused hepatic failure, HgCl2, which injured kidney, and turpentine which led to inflammation, and the induction and activation of PHBP were investigated in this paper.

Treatment of Mice CCl4 (0, 10, 20 or 40% in olive oil, 5 μl/g b.w., orally), d-galactosamine (280 mg/ml in phosphate-buffered saline [PBS], 5 μl/g b.w., intraperitoneally), HgCl2 (1 mg/ml in PBS, 6 μl/g b.w., subcutaneously) or turpentine (5 μl/g b.w., subcutaneously) was administered to an ICR mouse (male, 6-weeks old, Saitama Experimental Animals). Partial hepatectomy (2/3 hepatectomy) was performed according to the methods of Sakamoto et al.9 and Higgins and Anderson.10

Preparation of Anti-recombinant-Mouse PHBP Antibody Mouse PHBP expression vector was constructed in pTrcHisC (Invitrogen) and the expression vector was transfected to Escherichia coli (DH5α, Toyobo).11 The recombinant (r-) mouse PHBP which had His-tag at its N-terminus, was purified from the transformed E. coli with Ni-NTA-agarose (QIAGEN) after the cultivation. A rabbit was immunized with 100 μg of purified r-mouse PHBP as an emulsion with Freund's complete adjuvant. Two and three weeks after the first immunization, the rabbit was boosted as above. One week after the final injection, the rabbit was bled and the anti-r-mouse PHBP antiserum was collected after the clotting.

Western-Blot Analysis After each treatment of the mouse, the blood was collected from the heart and the plasma was obtained by immediate centrifugation at 1000×g for 5 min. Each sample (1 μl) was analyzed on sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE, 10% polyacrylamide gel).12 After the electrophoresis, proteins in the gel were electrically transferred to a nitrocellulose filter (Schleicher and Schuell) in 20 mM Tris–HCl, 150 mM glycine and 20% methanol. The protein-binding sites were blocked with 3% gelatin in PBS (GPBS) and the filter was incubated in 5 ml of GPBS containing 5 μl of the anti-r-mouse PHBP antiserum for 30 min at room temperature. The filter was washed with 0.05% Tween 20 in PBS (TPBS) for 5 min three times and incubated in an alkaline phosphatase-conjugated anti-rabbit IgG goat antibody (Jackson Immuno Research Laboratories, Inc.) solution (1/1000 dilution in TPBS) for 30 min at room temperature. After washings with TPBS as above, the color was developed with BCIP/NBT.13

Labeling of Mouse PHBP cDNA Probe The EcoRI fragment (2 kbp, 10 ng) of mouse PHBP cDNA was labeled with 50 μCi of [α-32P]dCTP (Muramachi Chemicals) using a cDNA labeling kit (New England Nuclear). After the labeling, the 32P-labeled cDNA probe was purified with a Nick column (Amersham Pharmacia Biotech) from free [α-32P]dCTP and used for Northern-blot hybridization. For con-
trol experiment, GAPDH cDNA was labeled as above and used for hybridization.

**Northern-Blot Analysis** After each treatment of the mouse, the liver and the kidney were removed and total RNAs were prepared using Isogen-LS (Wako Pure Chemical Ind., Ltd.) according to the manufacturer's instructions. Each RNA (25 μg) was analyzed on 1% agarose gel electrophoresis with the MOPS buffer system. After the electrophoresis, RNAs in the gel were transferred to a nylon filter (hybond-N+, Amersham) with 10X SSC. The filter was air-dried and the RNAs were cross-linked to the filter by a UV-irradiation. The filter was pre-hybridized in an H-Mix solution containing the 32P-labeled mouse PHBP cDNA probe at 42 °C overnight. The filter was washed with 2X SSC and 0.1% SDS for 15 min, followed by 0.1X SSC and 0.1% SDS for 15 min twice at 50 °C. The filter was exposed on X-ray film (RX-U, Fuji Film) at -80 °C overnight. The radioactivities of the hybridized bands were measured with a BAS-2000 (Fuji Film).

**Quantitative RT-PCR** For quantitative RT-PCR, a sense primer (#1276—#1299) and an anti-sense primer (#1355—#1376) were synthesized. RT-PCR were performed with a SYBR Green PCR core reagents kit (Perkin Elmer Applied Biosystems). Briefly, RT was done at 48 °C for 30 min and the reverse transcriptase was inactivated at 95 °C for 10 min. Then, PCR was continued for 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. The amounts of the PCR products were quantitated with a Sequence Detection System GeneAmp 5700 (Perkin Elmer Applied Biosystems).

**RESULTS**

**Conversion of Pro PHBP to the Active Hetero-Dimer Form in Response to Hepatic Injury** CCl₄, d-galactosamine, HgCl₂ or turpentine was administered to a mouse. After 24 h, the plasma PHBP was analyzed on SDS-PAGE under reducing conditions, followed by Western-blot using the anti-r-mouse PHBP antibody (Fig. 1). The plasma from the mouse treated with CCl₄, which caused hepatic injury, revealed two clear bands of 45 kDa and 30 kDa, which seemed to be the active hetero-dimer form of mouse PHBP. The plasma from the partially hepatectomized mouse also showed the two bands, but slightly weaker bands than those of the CCl₄ administered mouse. The plasma of the mice given HgCl₂ which caused renal injury and turpentine which led to inflammation contained no PHBP fragments of the active form. Therefore, we thought that pro PHBP in mouse plasma was converted to the active hetero-dimer form specifically during hepatic injury. In spite of the expectation of the presence of pro PHBP of 70 kDa in mouse plasma, no clear pro PHBP band was observed. There are two possible explanations for this result: one is that there is only a small amount of pro PHBP in mouse plasma and it.

![Fig. 1. Hepatic Injury-Specific Conversion of Mouse Plasma PHBP to the Active Hetero-Dimer Form](image)

Mouse blood was obtained from the mouse at 24 h after the administration of turpentine (5 μl/g b.w.), CCl₄ (40% in olive oil, 5 μl/g b.w.), d-galactosamine (280 mg/ml in PBS, 5 μl/g b.w.) or HgCl₂ (1 mg/ml in PBS, 6 μl/g b.w.), or partial hepatectomy (2/3 hepatectomy). Each plasma (1 μl/lane) was analyzed on SDS-PAGE (10% polyacrylamide gel) under reducing conditions, followed by Western-blot using anti-r-mouse PHBP antibody. 1: control, 2: turpentine, 3: partial hepatectomy, 4: CCl₄, 5: d-galactosamine, 6: HgCl₂ and MWS: molecular weight standards (Gibco BRL).

![Fig. 2. Dose-Dependency of CCl₄ for the Conversion of Mouse Plasma PHBP to the Active Hetero-Dimer Form](image)

CCl₄ (0, 10, 20 or 40% in olive oil, 5 μl/g b.w.) was administered to a mouse orally and the blood was collected at 24 h after the treatment. Each plasma (1 μl/lane) was analyzed on SDS-PAGE, followed by Western-blot as described in Fig. 1. 0: olive oil only, 10: 10%, 20: 20%, 40: 40% CCl₄ in olive oil and MWS: molecular weight standards.

![Fig. 3. Time-Course Experiment on the Conversion of Mouse Plasma PHBP to the Active Hetero-Dimer Form after CCl₄ Administration](image)

CCl₄ (40% in olive oil, 5 μl/g b.w.) was administered to the mice orally and blood was collected at each time. Each plasma (1 μl/lane) was analyzed on SDS-PAGE, followed by Western-blot as described in Fig. 1. 0: 0 h, 12: 12 h, 24: 24 h, 48: 48 h, 72: 72 h after the CCl₄ administration and MWS: molecular weight standards.
is less than the detection limit of this Western-blot analysis; and the other is that there is sufficient pro PHBP in mouse plasma, but the large amount of serum albumin masked the pro PHBP making it undetectable by the analysis.

We then studied the hepatic injury-specific conversion of pro PHBP to the active form by a dose-dependency experiment (Fig. 2) and a time-course experiment (Fig. 3) using CCl₄. The amounts of the 45 and 30-kDa fragments of active PHBP increased CCl₄-dose dependently (Fig. 2). The results of the time-course experiment indicated that the plasma 12 h after CCl₂ administration contained a small amount of active PHBP (Fig. 3). At 24 h after the administration, the amount of active PHBP in the mouse plasma reached a maximum; at 48 h, the amount decreased and at 72 h, it could not be detected. These results suggested that the hepatic injury-specific activation of plasma PHBP by CCl₄ was transitory.

Quantitative Measurement of PHBP mRNA in the Liver and the Kidney of the Mouse Administered CCl₄

Fig. 4. Northern-Blot Analysis of Mouse PHBP mRNA in the Liver and Kidney after CCl₄ Administration

Total RNAs were prepared from the livers and kidneys of mice after each CCl₄ treatment. Each RNA (25 µg/lane) was analyzed on 1% agarose gel electrophoresis, followed by Northern-blot as described in Materials and Methods. The amounts of PHBP mRNA were normalized by the amounts of GAPDH mRNA, respectively. The level at 0 h was expressed as 100 and each piece of data was expressed as mean±S.D. of three independent experiments.

Fig. 5. Northern-Blot Analysis of Mouse PHBP mRNA in the Liver and Kidney after HgCl₂ Administration

Total RNAs were prepared from the livers and kidneys of mice after each HgCl₂ treatment. Each RNA (25 µg/lane) was analyzed on agarose gel electrophoresis, followed by Northern-blot, and the data was expressed as described in Fig. 4.
To study whether the increase of the fragments of the active form of PHBP in the mouse plasma after the CCl₄ treatment was due to an induction of the PHBP gene transcription, we measured PHBP mRNA in the liver and the kidney of the mouse by Northern-blot analysis (Fig. 4); the reason was that only liver and kidney were known as the tissues in which PHBP was produced. The results indicated 50% induction of PHBP mRNA 3 h after the CCl₄ administration in the liver and 50% suppression 12 h after the treatment. In the kidney, only suppression 12 h after the CCl₄ administration was observed. These results did not interpret the increased amount of the fragments of the active form of PHBP in the mouse plasma after the CCl₄ treatment, because the increase was significant. Therefore, the augmentation of the fragments of the active form of PHBP in the mouse plasma after the CCl₄ administration can be explained by increase of the conversion of pro PHBP to the active hetero-dimer in the plasma rather than by induction of the PHBP gene transcription. On the other hand, HgCl₂ (Fig. 5) and turpentine (data not shown) did not influence the amount of PHBP mRNA in either tissue. To confirm these results, PHBP mRNAs in the two tissues were measured by quantitative RT-PCR (Fig. 6). The results revealed similar results with those of Fig. 4.

DISCUSSION

The amino acid sequence of human PHBP showed that it has a significant homology to that of hepatocyte growth factor activator (HGFA), and it has three EGF domains, a kringle domain and a serine protease domain from its N-terminus. HGFA is known to be a serine protease which cleaves and activates hepatocyte growth factor (HGF). HGF binds to its receptor (c-Met) and expresses its function. Macrophage stimulating protein (MSP) is the other functional protein which has a sequence similarity to HGF. MSP is also converted to the active form by a proteolytic cleavage and it functions via binding to its receptor (STK/Ron/c-Sea) whose sequence is also homologous to that of c-Met. On the basis of these sequence similarities, we assumed that PHBP might be a serine protease responsible for the activation of MSP, however, the results were negative (unpublished observations). Then, we searched the natural substrates in human plasma and found that fibrinogen and fibronectin, known to be major plasma proteins and components of extracellular matrix (ECM), were cleaved by PHBP, and that PHBP converted inactive scu-PA to active tcu-PA. Tcu-PA converts plasminogen to plasmin and plasmin acts not only as a fibrinolytic factor but also as an activator for pro matrix metalloproteinases (pro MMPs). MMPs digest collagens in ECM and facilitate the tissue remodeling. Therefore, PHBP may act as an early factor in this cascade (Fig. 7).

In this paper, we described the hepatic injury-specific conversion of mouse PHBP to the active hetero-dimer form. Although the initial activator for pro PHBP has not been identified, one possibility is the negatively-charged substances released from the damaged liver cells, because we elucidated that dextran sulfate and phosphatidylethanolamine accelerated the auto-activation of the purified human PHBP.
The active form of human PHBP showed the 50-kDa heavy chain and the 27-kDa light chain on SDS-PAGE under reducing conditions, while the hetero-dimer form of mouse PHBP revealed the 45 and 30-kDa fragments. These results may originate in the different post-translational modification, e.g., there are two N-glycosylation sites on the human heavy chain but there is no N-glycosylation site on mouse PHBP.\(^{1(1)}\) The other remarkable finding is that there is the RGD sequence, which is known as the binding site for the integrin family, in the first EGF domain of mouse PHBP.\(^{1(1)}\) At the present time, it is not clear whether this sequence functions in mouse.

Recently, we identified C1 inhibitor as a major inhibitor of PHBP in human plasma.\(^{4(5)}\) C1 inhibitor complexed with active PHBP and inhibited its protease activity effectively. The active PHBP in plasma may be inactivated immediately by C1 inhibitor. The results of Western-blot analysis of this paper revealed the existence of only a small amount of the active PHBP and inhibited its protease activity effectively. The active PHBP in plasma may be inactivated immediately by C1 inhibitor. This small amount of active PHBP may be due to the disposition by C1 inhibitor.

Acknowledgement We are grateful to Ms. Izumi Ito and Ms. Maiko Yamamoto for their excellent technical assistance. This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science, Sports, Culture and Technology of Japan.

REFERENCES