cDNA Cloning and Expression of Intracellular Platelet-activating Factor (PAF) Acetylhydrolase II

ITS HOMOLOGY WITH PLASMA PAF ACETYLHYDROLASE*

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Kenshi Hattori‡, Hideki Adachi‡, Atsushi Matsuzawa‡, Kazuo Yamamoto§, Masafumi Tsujimoto§, Junken Aoki‡, Mitsuharu Hattori‡, Hiroyuki Arai‡, and Keizo Inoue‡

From the ‡Department of Health Chemistry and §Division of Cancer Biology and Molecular Immunology, Faculty of Pharmaceutical Sciences, The University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113, Japan and the ¶Laboratory of Bioorganic Chemistry, The Institute of Physical and Chemical Research (RIKEN), Wako-shi, Saitama 351-01, Japan

Platelet-activating factor (PAF) acetylhydrolase, which inactivates PAF by removing the acetyl group at the sn-2 position, is widely distributed in plasma and tissues. We previously demonstrated that tissue cytosol contains at least two types of PAF acetylhydrolase, isoforms Ib and II, and that isoform Ib is a heterotrimer comprising 45-, 30-, and 29-kDa subunits, whereas isoform II is a 40-kDa monomer.

In this study, we isolated cDNA clones of bovine and human PAF acetylhydrolase isoform II. From the longest open reading frame of the cloned cDNAs, both bovine and human PAF acetylhydrolases II are predicted to contain 392 amino acid residues and to exhibit 88% identity with each other at the amino acid level. Both enzymes contain a Gly-X-Ser-X-Gly motif that is characteristic of lipases and serine esterases. Expression of isoform II cDNA in COS7 cells resulted in a marked increase in PAF acetylhydrolase activity. An immunoblot study using an established monoclonal antibody against the bovine enzyme revealed that the recombinant protein exists in the membranous fraction as well as the soluble fraction. Isoform II is expressed most abundantly in the liver and kidney in cattle, but low levels were also observed in other tissues. The amino acid sequence deduced from the cDNA of isoform II had no homology with any subunit of isoform Ib. Interestingly, however, the amino acid sequence of isoform II showed 41% identity with that of plasma PAF acetylhydrolase. Combined with previous data demonstrating that isoform II shows similar substrate specificity to plasma PAF acetylhydrolase, these results indicate that tissue type isoform II and the plasma enzyme may share a common physiologic function.

Platelet-activating factor (PAF); 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine is a potent phospholipid mediator of inflammation (1–5) synthesized by many cell types, including macrophages, platelets, basophils, eosinophils, and endothelial cells on appropriate stimulation (6–8). It mediates a broad spectrum of biological activities, such as hypotension, smooth muscle contraction, and an increase in vascular permeability (9–12). These actions of PAF are mediated mainly through specific cell surface receptors, although accumulation of intracellular PAF may also influence cell function (13, 14).

PAF is degraded by hydrolysis of the acetyl group at the sn-2 position of the glycerol backbone to produce the biologically inactive lyso-PAF and acetate. This reaction is catalyzed by a specific enzyme, PAF acetylhydrolase (15–18). PAF acetylhydrolase is detectable in the cytosol of tissues and cells as well as plasma. The physiologic function of PAF acetylhydrolase has not yet been established, but several possible hypotheses have been proposed. Finally, PAF production may be regulated at the levels of both synthesis and degradation. This notion is based on the observation that PAF production was greatly enhanced in cells pretreated with phenylmethanesulfonyl fluoride, an inhibitor of intracellular PAF acetylhydrolase, on stimulation of platelets with thrombin (19). It was also demonstrated that plasma PAF acetylhydrolase effectively abolishes the inflammatory effects of PAF on leukocytes and the vasculature, indicating involvement of the enzyme in the maintenance of plasma PAF at certain levels (20). In addition to the degradation of PAF, PAF acetylhydrolase has the ability to hydrolyze short chain phospholipids and oxidized fragments of polyunsaturated fatty acids at the sn-2 position in a calcium-independent manner (21–23). From these biochemical properties, it was speculated that PAF acetylhydrolase may scavange oxidized phospholipids produced inside or outside of the cells during oxidative stress. It is well known that during oxidation of low-density lipoprotein in vitro, significant amounts of lyso-phosphatidylcholine are produced in the low-density lipoprotein particles (23–25). Lyso-phosphatidylcholine may be formed by sequential oxidation of phosphatidylcholine in the surface coat of the low-density lipoprotein particle and subsequent hydrolysis of the oxidized phospholipids by plasma acetylhydrolase, which is usually associated with plasma lipoproteins (26, 27).

PAF acetylhydrolases from several sources have recently been purified and their cDNAs have been cloned. Plasma PAF acetylhydrolase is a 45-kDa monomeric enzyme (20) that is usually associated with plasma lipoproteins, such as low- and high-density lipoproteins (20, 26, 27). The predicted amino acid sequence deduced from its isolated cDNA is unique and unrelated to that of any known lipase or phospholipase (20, 28). We previously demonstrated that the PAF acetylhydrolase present in the soluble fraction of bovine brain cortex can be separated into three isoforms, designated isoforms Ia, Ib, and II (29). In contrast to plasma PAF acetylhydrolase, isoform Ib, which is the most abundant form in bovine brain, is a heterotrimeric
enzyme composed of 29- (γ), 30- (β) and 45-kDa (α) subunits (29). A heterodimer of the β and γ subunits forms a catalytic unit in the native complex. These two catalytic subunits are homologous with each other (63% identity), but share no homology with any other protein, including plasma PAF acetylhydrolase, except that a sequence of about 30 amino acids located 6 residues downstream from the active serine exhibits significant homology to the first transmembrane region of the PAF receptor (30–32). The α subunit has a γ tandem WD-40 repeat (33, 34), which is often found in proteins that function through interaction with other protein components and is identical to the product of the causative gene for Miller-Diecker lissencephaly, a malformation of the brain cortex (35). Thus, this enzyme appears to play an important role in the signal transduction system for development of the central nervous system. Very recently, we also succeeded in purifying isoform II and revealed that it differs from isoform Ib with respect to its polypeptide composition, substrate specificity, and tissue distribution, suggesting that it serves a different physiologic function from isoform Ib in tissues (36).

In this paper, we report the cloning of isoform II of tissue type PAF acetylhydrolase and demonstrate that it is distinct from isoform Ib but shows significant homology to the plasma PAF acetylhydrolase at the amino acid sequence level.

EXPERIMENTAL PROCEDURES

Materials—1-O-Hexadecyl-2-[1H-acetyl-sn-glycero-3-phosphocholine was purchased from DuPont NEN (Boston, MA) and unlabeled PAF was purchased from Bachem Feinchemikalien AG (Bubendorf, Switzerland). Horseradish peroxidase-conjugated goat anti-mouse Ig polyclonal antibody and goat anti-rabbit Ig polyclonal antibody were obtained from DuPont NEN (Boston, MA) and unlabeled PAF acetylhydrolase-7F7 antibody was purchased from DuPont NEN (Boston, MA) and unlabeled PAF acetylhydrolase-containing monoclonal antibodies was named 7F7.

bent assay. Positive cells were cloned by limiting dilution. One of the established monoclonal antibodies was named 7F7.

The cells were grown in 96-well culture plates. Ten days after fusion, the supernatants of the hybridomas were tested for production of anti-PAF acetylhydrolase II antibodies using an enzyme-linked immunosorbent assay. Positive cells were cloned by limiting dilution. One of the established monoclonal antibodies was named 7F7.

A polyclonal antibody against the γ subunit of bovine isoform Ib was prepared as follows. The cDNA for the γ subunit of isoform Ib was inserted into pET21a (Invitrogen) and transfected into BL21-competent cells. The recombinant γ subunit was expressed in histidine-tagged fusion protein form. The fusion protein was then purified by manganese chelate affinity chromatography and hydroxylapatite column chromatography. One hundred micrograms of the purified recombinant protein was homogenized with an equal volume of Freund’s adjuvant solution and injected intradermally into the back of an adult female New Zealand white rabbit. Two weeks after the first injection, a second injection was carried out using Freund’s incomplete adjuvant. The third and fourth immunizations were carried out at 2-week intervals. One week after the final immunization, all blood was collected from the rabbit and the serum was used for polyclonal antibody analysis. The polyclonal antibody thus obtained was named 453.

cDNA Cloning—A bovine kidney cDNA library was synthesized from poly(A) RNA using a cDNA synthesis kit from Life Technologies, Inc. The cDNA was ligated into pSPORT1 and then transfected into Electro Max DH 10B-competent cells (Life Technologies, Inc.). The human brain library was obtained from Life Technologies, Inc. Isolation and DNA Sequencing of PAF Acetylhydrolase II—The reverse transcription polymerase chain reaction (PCR) was performed to clone the cDNA. Two degenerate oligonucleotides, CRRT-GNGGDATCCAYTC and CARGGCGNCGARAGC, were synthesized based on peptide sequences EWIPHR and EEAEARET, respectively. These oligodeoxynucleotides were used as PCR primers. Initial screening of total bovine kidney cDNA revealed the presence of a 380-base pair amplified product. This PCR product was sequenced and used as a basis for the synthesis of two oligodeoxynucleotides, GAAGTGATCCCCACCG and CAAGGAGCAGAGGAC. These specific primers were used for further screening, using PCR as described previously (39, 40) with a slight modification. Briefly, the cDNA plasmid library was distributed into 96-well plates (2,000 clones/well), and the supernatants were pooled in every column and row. PCR was carried out with the specific primers to identify the wells containing cDNA for isoform II. The positive pools were then plated and screened by colony hybridization method (41). The colonies were transferred to a Hybond N+ nylon membrane (Amersham Life Science) and then treated as described previously (41). The PCR product was used as a probe. A fragment of the PCR product was subcloned into pUC18 (Pharmacia Biotech Inc.) and labeled with ECL™ probe-amp reagents (Amersham Life Science). The hybridized probe was detected using the Fluorescin Gene Images™ labeling and detection system (Amersham Life Science).

The DNA sequence was determined by the method of Sanger et al. (42) using a Taq Dyeoxygen Terminal Cycle Sequence kit and an Applied Biosystems model 373A DNA sequencer.

Human Isoform II cDNA Cloning—The coding region of bovine isoform II cDNA clone was isolated by screening a human isoform II cDNA clones by colony hybridization. After recloning into M13 mp19 vector, the cDNAs of positive clones were deleted using a double-stranded nested deletion kit (Pharmacia). DNA sequencing was carried out using a Taq Dyeoxygen Terminal Cycle Sequence kit and an Applied Biosystems model 373A fluorescence DNA sequencer.

Expression of PAF Acetylhydrolase II—Full-length isoform II cDNA was subcloned into pcDNA3 (Invitrogen) using a restriction enzyme (EcoRI/VNotI). COS7 cells (7.5 × 10⁶ cells), grown in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum, were collected in K-PBS (30.8 mM NaCl, 120.8 mM KCl, 8.1 mM NaH₂PO₄, 1.46 mM KH₂PO₄, and 5 mM MgCl₂) and then transfected with 30 μg of pcDNA3 containing the cDNA for isoform II by electroporation using a Gene Pulser system (Bio-Rad). After 48 h of culture in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, the cells were collected in phosphate buffered saline. The cells were washed twice with buffer A (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 250 mM sucrose), suspended in 500 μl of buffer A, and disrupted by sonication in a vessel surrounded by ice for five periods of 5 s at 15-s intervals using a Branson sonifier. The same procedure was carried out for control cells transfected only with pcDNA3. The cell lysates were assayed for PAF acetylhydrolase activity as described previously (36). The cell lysate was then centrifugated at 100,000 × g for 1 h to separate the soluble and pellet fractions. The pellet fraction was suspended in buffer A using sonication to disrupt the cells. Western blot analysis was performed on the soluble (16 μg of protein) described above and the protein fractions of the transfected cells using the monoclonal antibody 7F7.

Immunoblotting—The test samples were resolved by SDS-PAGE on a 12% acrylamide gel by the method of Laemmli (43) and blotted onto a nitrocellulose membrane. The membrane was blocked with PBS containing 5% skim milk for 2 h at room temperature and then incubated in the supernatant of the 7F7 hybridoma or rabbit anti-γ subunit serum...
diluted to 1/1,000 in PBS containing 5% skim milk for 12 h at 4°C. The filter was washed four times with PBS containing 0.05% Tween 20 (T-PBS) and incubated for 1 h with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit Ig polyclonal antibodies, diluted to 1/2,000 in PBS containing 5% skim milk. After washing the filter six times with T-PBS, the blots were detected by an enhanced chemiluminescence method using an ECL Western blotting detection set from Amersham Life Science. The results were visualized by fluorography using Hyperfilm ECL (Amersham Life Science).

Preparation of Tissue Homogenate of Isoform II—Fresh bovine tissues were obtained from a local slaughterhouse and were processed within 3 h of slaughter. All procedures were carried out at 0–4°C. The tissues were homogenized with a Waring blender in 2 volumes of buffer A, and then the homogenates were centrifuged at 10,000 \( \times g \) for 30 min to remove the bulk of the solid material. The protein concentrations of the homogenates were determined using BCA protein assay reagent with BSA as a standard. Western blot analysis was performed on the tissue homogenate (100 \( \mu \)g of protein) with the monoclonal antibody 7F7 or the polyclonal antibody 453.

Analysis of DNA and Protein Sequences—Nucleotide and predicted amino acid sequences were analyzed using GENETYX programs (Software Development, Tokyo, Japan). Computer searches for protein sequences were performed using the GenomeNet World-Wide Web server (address, http://www.genome.ad.jp/).

RESULTS

Sequence of the Peptide Fragments of PAF Acetylhydrolase II—As previously reported, the N-terminal amino acid sequence of the purified 40-kDa polypeptide could not be determined. One fragment obtained by digestion of the polypeptide with lysyl endopeptidase showed no perfect match with any sequence ever reported, indicating that this is a new enzyme (36). In this study, purified PAF acetylhydrolase II was again digested with lysyl endopeptidase and cyanogen bromide, and the peptide fragments were separated by reverse-phase high-performance liquid chromatography. Five new peptide sequences were determined.

Cloning of the PAF Acetylhydrolase II Gene—Using the pep-
Comparison of the amino acid sequences of bovine and human PAF acetylhydrolase II. The upper and lower sequences represent PAF acetylhydrolase II from cattle and humans, respectively. Amino acid residues are numbered from the left. The asterisks and dots denote identical and similar residues, respectively. The large dot indicates the active serine residue of bovine isoform II.

FIG. 2. Comparison of the amino acid sequences of bovine and human PAF acetylhydrolase II. The upper and lower sequences represent PAF acetylhydrolase II from cattle and humans, respectively. Amino acid residues are numbered from the left. The asterisks and dots denote identical and similar residues, respectively. The large dot indicates the active serine residue of bovine isoform II.
and the vector containing isoform II cDNA, respectively.

The pyruvate dehydrogenase complex. The pyruvate dehydro-

cytosolic and membrane fractions (16 and 10 μg of protein, respectively) were subjected to SDS-PAGE and blotted onto nitrocellulose membrane. Immunoblotting was performed with the monoclonal antibody 7F7. Lanes 1 and 3, cytosolic fraction transfected with control vector and the vector containing isoform II cDNA, respectively, lanes 2 and 4, membrane fraction transfected with vector control with the vector containing isoform II cDNA, respectively.

A, and ultimately the reduction of NAD⁺. Dihydrolipoamide

catalyzes the second reaction. This enzyme also has some homology with isoform II around the catalytic serine residue. NK-4 protein was identified as the product of a gene the expression of which is increased after activation of T cells, although its precise role in the cell remains unknown. The N-terminal region of this protein exhibits less, but significant, homology with the N-terminal region of isoform II.

Distribution of PAF Acetylhydrolase II in Bovine Tissues—Next, we examined the tissue distribution of isoform II by Western blot analysis using an established monoclonal antibody against purified bovine isoform II. As shown in Fig. 6, among the bovine tissues tested, isoform II was most abundant in the liver and kidney, although other tissues expressed detectable levels of the protein. We also performed Western blot analysis with a polyclonal antibody against the γ subunit of isoform Ib. Significant levels of the γ subunit were expressed in the brain, kidney, adrenal, ovary, and intestine. Interestingly, the catalytic subunit of isoform Ib was almost undetectable in bovine liver, in which isoform II is most abundant. These data thus indicate that these two intracellular PAF acetylhydrolases show a marked contrast in tissue distribution.

DISCUSSION

We previously demonstrated that intracellular PAF acetylhydrolase isoforms Ib and II are different from each other with respect to substrate specificity, tissue distribution, and polypeptide composition (36). In the current study, it was shown by cDNA cloning that these two intracellular PAF acetylhydrolases are also distinct from each other at the amino acid sequence level, indicating a different origin for the two enzymes. Interestingly, the amino acid sequence of isoform II exhibits 43% identity to that of plasma PAF acetylhydrolase. The plasma form of acetylhydrolase has been cloned from humans (20) and several other species (28). The human enzyme encodes a 441-amino acid protein that includes a predicted signal peptide for secretion. The deduced amino acid sequence is unique except for the Gly-X-Ser-X-Gly motif found around the active serine residue of most serine esterases and lipases. Ser273 of the human plasma PAF acetylhydrolase was identified as an active site. The active serine residue of intracellular isoform II was inferred to be Ser236, as this apparently corresponds to Ser273 of the plasma enzyme and the Gly-X-Ser-X-Gly motif occurs around this amino acid (Fig. 4). Complete loss of enzyme activity after mutation of this Ser residue to Cys also supports this hypothesis. The region surrounding the active serine residue (231–243 of isoform II) exhibited an almost complete match with plasma acetylhydrolase (Fig. 4). The active site of plasma PAF acetylhydrolase, as well as those of other lipases, forms a catalytic triad comprising the nucleophilic residues Ser, Asp, and His. Using site-directed mutagenesis, it was proposed that Ser273, Asp296, and His351 form a catalytic triad in human plasma PAF acetylhydrolase. Although we have not yet confirmed this, Asp296 and His351 of isoform II correspond to the amino acids comprising the catalytic triad of plasma PAF acetylhydrolase. Interestingly, the sequences surrounding these Asp and His residues exhibit high homology between the plasma and intracellular enzymes, suggesting that the structures of the catalytic domains of these two enzymes are highly homologous. Crystallization and x-ray structural analysis of both enzymes should provide a definite conclusion.

The other region showing extensive homology between plasma and intracellular isoform II was observed in the N-terminal half (101–142, Fig. 4). A computer search showed that this region also exhibited some homology with lipases of bacterial origin (Fig. 5). Although the function of this region is unknown at present, it may be assumed to be involved in substrate binding or recognition of lipid-water interfaces, since it is relatively hydrophobic on hydrophathy plot analysis (data not shown).

As described above, isoform II and plasma PAF acetylhydrolase appear to be typical members of the serine esterase family and are possibly derived from a common ancestral gene. In contrast, several features of isoform Ib, the other form of intra-
cellular PAF acetylhydrolase, suggest that it does not belong to this family. First, it showed no significant homology with other lipases and esterases on computer search analysis. Second, it does not contain the typical Gly-X-Ser-Gly pentapeptide motif around its catalytic serine residue. We have recently succeeded in crystallizing the $\gamma$ subunit, one of the catalytic subunits of isoform Ib, and have revealed its structure on x-ray.2 According to this analysis, Ser47, Asp192, and His195 form a catalytic triad. In most serine esterases, the Asp and His are separated by more than 20 amino acid residues (28, 42), whereas the distance in the $\gamma$ subunit is unusually short (only 4 residues). Finally, the tertiary folding of the catalytic subunit is strikingly similar to that of p21ras and the GTP-binding domain of the $\alpha$ subunit of trimeric G proteins. All of these experimental data support the notion that isoform Ib is a novel serine esterase.

Western blot analysis of recombinant bovine isoform II expressed in COS7 cells revealed that the enzyme was distributed in both cytosolic and membranous fractions in transfected cells. Although PAF acetylhydrolase activity in rat liver is highest in the cytosolic fraction, a significant level of activity was also present in the membranous fraction.

FIG. 4. Comparison of amino acid sequences between human PAF acetylhydrolase plasma form and isoform II. The upper and lower sequences represent human isoform II and human plasma form, respectively. Amino acid residues are numbered from the left. The asterisks and dots denote identical and similar residues, respectively. The dashes indicate gaps inserted to optimize alignment. The signal sequence is underlined. The arrows denote the N terminus of the purified PAF acetylhydrolase from human plasma. The large dots under the serine, aspartic acid, and histidine residues indicate the amino acid residues that form a putative catalytic triad.

FIG. 5. Alignment of the amino acid sequences of bovine isoform II and homologous proteins. The sequences of homologous proteins were derived from the GenBank and SwissProt protein sequence databases. One-letter amino acid notation is used. Amino acid residue numbers are shown at both sides. Residues identical with those of isoform II are shaded. A, bovine PAF acetylhydrolase II; B, 28k lipase from Streptomyces sp.; C, lipase from Streptomyces albus; D, dihydrolipoamide acetyltransferase of Pseudomonas putida; E, lipase from Rhigomucor miehei; F, natural killer cell protein 4.

FIG. 6. Western blot analysis of isoform II in bovine tissues. Homogenates (100 $\mu$g of protein) from bovine brain (lane 1), heart (lane 2), lung (lane 3), spleen (lane 4), liver (lane 5), kidney (lane 6), adrenal gland (lane 7), ovary (lane 8), stomach (lane 9), intestine (lane 10), and skeletal muscle (lane 11) were subjected to SDS-PAGE and blotted onto a nitrocellulose membrane. Immunoblotting was performed using the monoclonal antibody 7F7 against isoform II (A) or polyclonal antibody 453 against the $\gamma$ subunit of isoform Ib (B) as described under “Experimental Procedures”.

2 Ho, Y.S., Swenson, L., Derewenda, U., Serre, L., Wei, Y., Dauter, Z., Hattori, M., Adachi, T., Aoki, J., Avaii, H., Inoue, K., and Derewenda, Z. S. Nature, in press.
detected in the light and heavy membrane fractions (16). Although the PAF acetylhydrolase activity present in membrane fraction has not yet been characterized, isoform II associated with the membrane fraction may at least in part contribute this activity. In contrast, the catalytic subunits of isoform I, which is expressed in MDBK cells, are exclusively located in the cytosolic fraction (data not shown). It is notable that the majority of PAF acetylhydrolase in human plasma is associated with plasma lipoproteins such as low- and high-density lipoproteins. Thus, both intracellular isoform II and plasma PAF acetylhydrolase appear to be relatively hydrophobic in nature and to have the ability to associate with membranous components inside or outside cells.

It has been proposed that one of the physiologic functions of plasma PAF acetylhydrolase is to degrade oxidized phospholipid produced in circulating lipoproteins (21). The substrate specificity of isoform II resembles that of plasma PAF acetylhydrolase, which can hydrolyze oxidatively fragmented fatty acyl chains attached to phospholipid molecules as well as to PAF. The liver, in which isoform II is most abundant, is known to lack the ability to produce PAF (4, 5), indicating that isoform II in liver cells is not involved in the regulation of PAF production. Several investigators have reported that oxidized phospholipids are preferentially hydrolyzed by cellular phospholipase A2 (47–51). Housekeeping protective enzymes are thought to be necessary both inside and outside cells, since reactive oxygen species can be formed both intracellularly and extracellularly. The molecular nature of the enzyme responsible for this activity, however, remains to be determined. Intra- cellular PAF acetylhydrolase II and plasma PAF acetylhydrolase may be possible candidates for this reaction.

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