Cloning and Expression of a cDNA Encoding the β-Subunit (30-kDa Subunit) of Bovine Brain Platelet-activating Factor Acetylhydrolase*

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Bovine brain platelet-activating factor (PAF) acetylhydrolase isoform Ib is a heterotrimeric enzyme. Its γ-subunit (which, formerly, we called the 29-kDa subunit) acts as a catalytic subunit, whereas the α-subunit (45 kDa) is the bovine homolog of the product of human LIS-1, the causative gene of Miller-Dieker lissencephaly, indicating that this intracellular PAF acetylhydrolase plays a key role in brain development.

In the current study, we cloned the cDNA for the β-subunit (30 kDa) of bovine brain PAF acetylhydrolase Ib. The predicted 229-amino acid sequence was homologous (63.2% identity) to that of the γ-subunit, especially (86% identity) in the catalytic and PAF receptor homologous domains. The recombinant β-protein produced in Escherichia coli showed significant PAF acetylhydrolase activity. A mutant protein, in which Ser48, which corresponded to the active serine residue of the γ-subunit, was replaced with cysteine showed no enzymatic activity, suggesting Ser48 is the active serine residue. Although the β- and γ-subunits form a heterocomplex in the native enzyme, both recombinant β- and γ-proteins exist as a homodimer. The purified recombinant β-protein was labeled readily with [1,3-3H]disisopropyl fluorophosphate, whereas the β-subunit in the native complex was only labeled with higher concentrations of [1,3-3H]disisopropyl fluorophosphate to a lesser extent than the γ-subunit. Combined with our previous data, the present study demonstrated that bovine brain PAF acetylhydrolase Ib is a unique enzyme possessing two catalytic subunits and another, possibly regulatory, subunit.

Moreover, PAF has been reported to serve as part of a retrograde messenger in long-term potentiation (7) and to induce differentiation of cultured neuronal cells, although high concentrations of PAF are neurotoxic (8). These data suggest that an additional role of PAF is to regulate brain development and function(s).

PAF is inactivated by a specific enzyme, PAF acetylhydrolase, which removes the acetyl moiety at the sn-2 position of the glycerol backbone (9, 10). Mammalian PAF acetylhydrolase is classified into two types (11, 12), plasma (extracellular) and tissue (intracellular). Recently, we demonstrated that there are at least three isoforms of PAF acetylhydrolase in bovine brain (13), kidney, and liver (14) and that one isoform (designated isoform Ib) consists of three different subunits, with molecular masses of 45, 30, and 29 kDa (in this paper, we call them the α-, β-, and γ-subunits, respectively) (13). We have already reported cDNA cloning of the γ- (15) and α-subunits (16). The γ-subunit was found to function as a catalytic subunit, and Ser47 of this subunit was the active serine residue, as only this residue reacted with disisopropyl fluorophosphate (DFP), a potent inhibitor of the enzyme (15). The active serine residue occurs earlier on in its sequence than it does in other serine esterases (usually 100–200 residues from the N-terminal end), and the sequence surrounding it differs from the consensus sequence (Gly-Xaa-Active Ser - Xaa-Gly) (17) of the serine esterase family (15). Even more interesting is the demonstration that the amino acid sequence of the α-subunit was almost identical (410/411) to that of the human LIS-1 gene product (16). LIS-1 was identified as the causative gene of Miller-Dieker lissencephaly (18), a brain malformation manifesting as a smooth cerebral surface and abnormal neuronal migration (19, 20). This finding indicates that the α-subunit plays an important role in brain development, probably by regulating PAF acetylhydrolase activity and thereby maintaining physiological concentrations of PAF.

In order to understand the precise role of PAF acetylhydrolase in the brain, it is very important to elucidate the particular functions of all its subunits and how they interact with each other. In this paper we report the cDNA cloning of its β-subunit and have now succeeded in revealing the primary structures of all the brain PAF acetylhydrolase Ib subunits. From the predicted amino acid sequence and transfection experiment results, we found that the β-subunit, like the γ-subunit, can function as a catalytic subunit.

EXPERIMENTAL PROCEDURES

Materials—1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine and [1,3-3H]disisopropyl fluorophosphate ([3H]DFP) were purchased from DuPont NEN. Unlabeled PAF was from Bachem Feinchemikalien AG (Bubendorf, Switzerland), and endoproteinase Lys-C (sequencing grade) was from Boehringer Mannheim. All the other materials were...
Sequences of peptides derived from the β-subunit of bovine brain PAF acetylhydrolase Ib after digestion with endoproteinase Lys-C. The sequences of HPLC-purified peptides isolated from the Lys-C-digested β-subunit of bovine brain PAF acetylhydrolase Ib were determined. Each peptide represents a pure species from a single HPLC peak. Asterisks denote ambiguous residues. The amino acid sequences of all three peptides were found in the cDNA clone (Fig. 2).

The amplified product and TTAGCAGCGGATCTCAGTAGGTTG were then used as PCR primers, and the resulting amplified product was ligated into the pET-21a(+) vector using the (EcoRI/SalI) restriction enzymes. The nucleotide sequences of this mutant cDNA were confirmed by sequencing. The protein expressed by this system had a 16-amino acid leader peptide (T7-Tag)\(^\text{14}\) on its N-terminal. Vectors containing cDNA for the wild type β-subunit (T7-β-wt) or cDNA for the mutant (T7-β-S48C) were introduced into Escherichia coli BL21 and cultured in LB medium containing 50 μg/ml ampicillin. For expression, isopropyl-1-thio-β-D-galactoside at a final concentration of 2 mM was added, the cells were cultured for 4 h at 37 °C, and the cytosol fraction was prepared as described above.

Antibodies (13) and internal antibodies against the γ-subunit were prepared as follows. The partially purified recombinant β-protein was used to immunize BALB/c mice once every 2 weeks for a total of 5 times, after which the spleenocytes obtained from the mice were fused with mouse myeloma cells (PA1) (25). An enzyme-linked immunosorbent assay and immunoblotting were utilized for screening, and two monoclonal antibodies (1F4 and 6D8) against the γ-subunit were established. Polyclonal antiserum against the α- and β-subunits were obtained by immunizing New Zealand White rabbits with the purified α-subunit from bovine brain (16) and partially purified recombinant β-protein, respectively.

Immunoblotting—After SDS-PAGE was carried out, the proteins were transferred onto a nitrocellulose membrane, which was blocked with Tris-buffered saline containing 5% skim milk powder for 2 h. It was incubated at room temperature with the indicated antibodies for 2 h and then washed with Tris-buffered saline containing 0.05% Tween 20. It was then incubated with horseradish peroxidase-conjugated secondary antibodies in Tris-buffered saline for 2 h and washed with Tris-buffered saline containing 0.05% Tween 20, and the color was developed using a ECL coloring kit (Amersham Corp.).

Cross-linking of the Native HeterotrimERIC Complex and the Recombinant Proteins—Purified PAF acetylhydrolase Ib, purified recombinant β-protein, and purified recombinant γ-protein were dialyzed against 100 mM sodium phosphate buffer (pH 8.0) and 10% (v/v) glycerol. The samples (100 μg/ml) were incubated at 37 °C for 30 min at room temperature. To the reaction mixtures were added half of the volume of 3 × Laemmli sample buffer, and SDS-PAGE was carried out, as described previously (13).

RESULTS
cDNA Cloning of the β-Subunit and Its Homology to the γ-Subunit—The α-subunit of bovine brain PAF acetylhydrolase Ib was separated by heparin-Sepharose CL-4B column chromatography as described previously (data not shown) (13, 16). The complex of β- and γ-subunits thus obtained was reduced, S-alkylated, and separated by reverse-phase HPLC (Fig. 1). J udging from the absorbance at 214 nm, the amounts of β- and γ-subunits were almost equal, supporting the idea that the β- and γ-subunits exist in a 1:1 ratio in the complex. The β-subunit was digested with endoproteinase Lys-C, and the peptide fragments were purified by HPLC (data not shown). The N-terminal amino acid sequence could not be obtained, possibly because of some modification. Table I shows the sequences of the three peptides obtained from the HPLC. On the basis of
these sequences, degenerate oligonucleotides were synthesized and used in a series of PCR using bovine brain cDNA as a template. One set of primers yielded an amplified product containing sequences corresponding to the peptides. Therefore, we concluded that this amplified product was generated from the β-subunit cDNA and utilized it to screen a bovine brain cDNA library. The nucleotide sequence of the β-subunit cDNA is shown in Fig. 2. The amino acid sequence deduced from the nucleotide sequence contained all the sequences of the three peptides (see Table I). Interestingly, and unexpectedly, the deduced amino acid sequence of the β-subunit exhibited a high degree of similarity to that of the γ-subunit (63.2% identity and 82.9% similarity), which previously we had concluded to be the catalytic subunit of this enzyme (15) (Fig. 3). Conservativeresidues were spread over almost all the sequence. The most conserved lay between the active serine residue (circled in Fig. 3) and the PAF-receptor-like domain (double underlined in Fig. 3), in which 31 of 36 residues (86%) are identical. The total amino acid sequence of the β-subunit is homologous to no other proteins reported so far, including human plasma PAF acetylhydrolase (26).

Expression of the β-Subunit cDNA in E. coli—Two vectors, pUC-P-β and pET-21a(+) were used for expression of the β-protein in E. coli. Cultured at 42°C, E. coli transfected with the β-subunit cDNA in the former vector generated a protein that migrated to the same position as the β-subunit of the native enzyme when subjected to SDS-PAGE (Fig. 4A, lanes 1 and 3). This recombinant β-protein showed significant PAF acetylhydrolase activity (Fig. 4B, column 3). E. coli transfected

FIG. 1. Separation of the β-subunit from the γ-subunit by reverse-phase HPLC. About 200 μg of the α-subunit-depleted bovine brain PAF acetylhydrolase Ib, purified as described previously (16), was reduced and S-alkylated as described previously (15). The reaction mixture was then applied to a reverse-phase HPLC system with a 4.6 × 250-mm Yvidac 304-1251 C4 column previously equilibrated with 5% acetonitrile containing 0.1% trifluoroacetic acid. Proteins were eluted with a linear gradient of acetonitrile (5–50%) containing 0.1% trifluoroacetic acid. The effluent was monitored by absorbance at 214 nm. The peak fractions were collected manually and analyzed by SDS-PAGE. Inset, the peak 1 and 2 fractions were applied to SDS-PAGE using 12% acrylamide slab gel, and the gel was stained with silver.

FIG. 2. Nucleotide and predicted amino acid sequences of the cDNA encoding the β-subunit of brain PAF acetylhydrolase Ib. Nucleotide residues are numbered on the right; amino acid residues are numbered on the left. Residue 1 is the putative initiator methionine. Underlined sequences represent the sequences of peptides obtained from the purified β-subunit digested by endoproteinase Lys-C. The putative active serine residue is circled.
with the \( \beta \)-subunit cDNA in the latter vector generated a \( \beta \)-subunit with 16 extra amino acids on its N-terminal end (T7-\( \beta \)-wt), which also possessed PAF acetylhydrolase activity (Fig. 4, A and B, lane and column 6). We utilized the pET-21a(+) vector for site-directed mutagenesis.

Replacement of the Putative Active Serine Residue with Cys-

We inferred that Ser48 was the active serine residue of the \( \beta \)-subunit of bovine brain PAF acetylhydrolase Ib. To confirm this, we replaced Ser48 of the \( \beta \)-subunit protein with cysteine by site-directed mutagenesis. This mutation resulted in complete loss of enzyme activity, whereas expression levels in that column were not affected significantly (Fig. 4, A and B, lanes and columns 7 and 8). We confirmed that the expression level was indeed unaffected by immunoblotting with an anti-T7-Tag monoclonal antibody (data not shown).

Immunoblotting and DFP Labeling Analysis—We purified the recombinant \( \beta \)-protein (Fig. 4, A, lane 4) and raised an antisera against it in rabbits. This antisera recognized the \( \beta \)-, but not the \( \gamma \)-subunit (Fig. 5B, left). We also established two monodonal antibodies against the \( \gamma \)-subunit, neither of which recognized the \( \beta \)-subunit (Fig. 5B, right), using monoclonal antibody 1F4. Data using monoclonal antibody 6D8 not shown.

The PAF acetylhydrolase activity of the recombinant \( \beta \)-protein was abolished by 0.1 mM DFP (data not shown). When incubated with \(^{3} \text{H} \)DFP at a concentration of 17 \( \mu \)M, the purified recombinant \( \beta \)-protein was covalently labeled by this reagent (Fig. 5C, lane 2). The purified recombinant \( \gamma \)-protein was also labeled under the same conditions (Fig. 5C, lane 3).

However, as shown in our previous study (13), the \( \beta \)-subunit of the native heterotrimeric enzyme was hardly labeled at all under these conditions (Fig. 5C, lane 1).

Homodimer Formation of the Recombinant \( \beta \)- and \( \gamma \)-Proteins—We estimated the apparent molecular masses of the recombinant \( \beta \)- and \( \gamma \)-proteins by gel filtration column chromatography. The apparent molecular masses of the native isofrom Ib and the complex from which the \( \alpha \)-subunit was dissociated (i.e. heterodimer of the \( \beta \)- and \( \gamma \)-subunits) were about 100 and 60 kDa, respectively (Fig. 6, a and b). On the other hand, those of both recombinant \( \beta \)- and \( \gamma \)-proteins were about 60 kDa, respectively (Fig. 6, c and d). These data suggest that both recombinant \( \beta \)- and \( \gamma \)-proteins form a homodimer. In order to confirm dimer formation of the recombinant proteins, a cross-linking experiment was also performed using the cross-linking reagent BS3. By mixing each recombinant protein with 2 mM BS3, two major cross-linked products were seen on SDS-PAGE; a broad band between about 80 and 100 kDa and a faint 60-kDa band (Fig. 7A, lane 1). The cross-linked product of the 100-kDa band reacted with all the antibodies against the \( \alpha \)-, \( \beta \)-, and \( \gamma \)-subunits, suggesting that this band is a product after cross-linking of these subunits (Fig. 7B, lanes 1, 4, and 7). The cross-linked product of ~80 kDa reacted with the antibodies against \( \alpha \)- and \( \gamma \)-subunits. The cross-linked product of the 60-kDa band reacted with the an-
The recombinant molar ratio of \([3H]DFP\) to the protein was changed from 1.7 to with \([3H]DFP\) (Fig. 8, protein and DFP concentration of 1 mg/ml (about 10 labeled by DFP under the conditions we used previously, pro-

[55x148]portion, bovine brain PAF acetylhydrolase Ib is the only one under these conditions. antibodies against \(\beta\)- and \(\gamma\)-subunits. These results suggest that the 80- and 60-kDa bands are composed of the \(\alpha\)- and \(\gamma\)-subunits and the \(\beta\)- and \(\gamma\)-subunits, respectively. These results may indicate that the \(\alpha\)-subunit interacts only with the \(\gamma\)-subunit but not with the \(\beta\)-subunit in the heterotrimeric complex. When the native PAF acetylhydrolase Ib was cross-linked with BS\(^3\), all the \(\alpha\)-subunits migrated to a larger form on SDS-PAGE (Fig. 7B, lane 1), while some \(\beta\)- and \(\gamma\)-subunits still remained at their original positions (Fig. 7B, lanes 4 and 7). This results may indicate that some \(\alpha\)-subunit got dissociated from the complex and was cross-linked with each other during cross-linking reaction.

[55x370]Immunoblotting and DFP labeling analysis. Partially purified bovine brain PAF acetylhydrolase Ib (lane 1), the recombinant \(\beta\)-protein (lane 2), and the recombinant \(\gamma\)-protein (lane 3) were loaded onto 12% SDS-PAGE (40). A, the gel was stained with silver. B, immu-

DISCUSSION

Of the many types of phospholipases that have been re-

that consists of heterogeneous subunits. It consists of three subunits with estimated molecular masses of 45 (\(\alpha\)), 30 (\(\beta\)), and 29 (\(\gamma\)) kDa, which, presumably, are present in a relative sto-

Fig. 5. Immunoblotting and DFP labeling analysis. Partially purified bovine brain PAF acetylhydrolase Ib (lane 1), the recombinant \(\beta\)-protein (lane 2), and the recombinant \(\gamma\)-protein (lane 3) were loaded onto 12% SDS-PAGE (40). A, the gel was stained with silver. B, immu-

[55x337]bodies against \(\beta\)- and \(\gamma\)-subunits. These results suggest that

2 M. Hattori, H. Adachi, J. Aoki, M. Tsujimoto, H. Arai, and K. Inoue, unpublished observation.
whereas the recombinant γ-protein eluted with about 30 mM. The concentrations of NaCl and KH$_2$PO$_4$ required for elution of the α-subunit-depleted PAF acetylhydrolase Ib (i.e. the heterodimer of β- and γ-subunits) on DEAE-Sepharose and hydroxyapatite column chromatographies were about 100 and 50 mM, respectively. These concentrations were just in between those of the recombinant proteins. These results suggest that PAF acetylhydrolase Ib is not a mixture of two co-purified enzymes (i.e. α-, β-, β-subunits and α-, γ-, γ-subunits) but a heterotrimeric enzyme.

We previously demonstrated that only the γ-subunit in the native complex was labeled by [3H]DFP (13). According to the present results, however, the recombinant β-protein was labeled well by this reagent. There are two possible explanations for these results. One is that the catalytic center of the β-subunit may be modified so that DFP cannot react with the active serine residue. The other is that although the β-subunit is catalytically active, the active serine residue of it is not accessible to DFP. The observation that increasing the ratio of [3H]DFP to the enzyme resulted in the labeling of not only the γ-subunit but also the β-subunit may support the latter hypothesis. Generally speaking, incorporation of DFP does not necessarily correlate with the extent of catalytic activity. For example, although the $K_m$ value for the plasma PAF acetylhydrolase (13.7 μM) (28) and brain PAF acetylhydrolase Ib (12.5 μM) are almost equal, their sensitivities to DFP are very different (plasma PAF acetylhydrolase is not completely inhibited by 1 mM DFP (31) whereas brain PAF acetylhydrolase Ib is...
completely inhibited by less than 0.1 mM DFP (13).

It should be noted that the number of deduced amino acid residues and calculated molecular mass of the β-subunit (229 and 25,569 Da, respectively) are smaller than those of the γ-subunit (232 and 25,865 Da, respectively), whereas the apparent molecular mass determined by SDS-PAGE of the former (about 30 kDa) is larger than that of the latter (about 29 kDa). This discrepancy does not result from errors during peptide sequencing, since the β-subunit was separated completely from the γ-subunit (Fig. 1) and the amino acid sequence predicted from the cDNA contained the peptide sequences obtained from the purified β-subunit (Table I and Fig. 2). Furthermore, the recombinant β- and γ-proteins migrated to exactly the same positions as the native subunits when subjected to SDS-PAGE (Fig. 5A), and a specific antibody against the γ-subunit did not recognize the β-subunit and vice versa (Fig. 5B). These results also suggest that the discrepancy does not result from post-transcriptional modification. According to our unpublished observations,² the native β-subunits and the recombinant β-protein were eluted by almost the same concentrations of acetonitrile on reverse-phase HPLC. Therefore, it appears that the discrepancy is due to the proteinous natures of the β- and γ-subunits. It is noteworthy that, despite intensive and repeated trials, we have not yet succeeded in raising an antibody or antiserum that recognizes both β- and γ-subunits. Immunogenicity may be poor, probably because the conserved region may be buried deep under a folded structure. An alternative possibility is that the region may also be conserved in immunizing animals, such as the rabbit and mouse. In fact, all the subunits of brain PAF acetylhydrolase are highly conserved among mammalian species (Ref. 32).²

Cross-linking of the native PAF acetylhydrolase Ib resulted in generation of high molecular bands on SDS-PAGE. The ~100-kDa band resulted from cross-linking of the α-, β-, and γ-subunits, supporting the idea that this enzyme is a heterotrimer of these three subunits. The ~80-kDa band resulted from cross-linking of the α- and γ-subunits, and the 60-kDa band resulted from cross-linking of the β- and γ-subunits. The simplest explanation for these results is that the β-subunit may not be directly attached to the α-subunit. At present, however, we cannot neglect the possibility that there are few residues available for the cross-linking reagent in the β-subunit of the native complex.

There is ample evidence showing that PAF and intracellular PAF acetylhydrolase play important role(s) in brain development and function. PAF receptors exist in both pre- and postsynaptic cells (6), and PAF has been suggested to be a potential retrograde messenger in CA1 hippocampal long term potentiation (7). Exposure of cultured neuronal cells to low concentrations of PAF has been reported to induce neuronal differentiation, whereas at high concentrations, PAF was toxic to the cells (8). Furthermore, we found that LIS-1, a causative gene of Miller-Dieker lissencephaly (18), is a human homolog of the bovine brain PAF acetylhydrolase α-subunit (16). Miller-Dieker lissencephaly manifests itself as a smooth cerebral surface and abnormal neuronal migration (19, 20). Putting the data together, we conclude that it is important for neuronal cells to maintain the PAF concentration within a certain range, according to their status, and degradation by PAF acetylhydrolase is essential to this. Interestingly, the α-subunit has a 7-tandem WD-40 repeat (33, 34), which is often found in proteins that function through interaction with other protein components. One candidate for the counterpart of this repeat is the PH-domain (35–37). Recently, we found that the α-subunit interacts strongly with β-spectrin (38), a cytoskeletal protein with a PH domain (39). In this context, the present finding that PAF acetylhydrolase Ib has two different catalytic units indicates that its activity is modulated in a delicate and complicated manner. Further investigation into the function of its subunits and their interactions with other cellular components is needed.
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