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Cloning, Expression and Characterization of Plasma Platelet-Activating Factor-Acetylhydrolase from Guinea Pig[1]

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In a previous study, we purified PAF-acetylhydrolase, which converts PAF to an inactive metabolite, lysoPAF, from peritoneal fluid of guinea pigs subjected to experimental endotoxin shock and found that this purified enzyme had similar biochemical properties to the plasma enzyme [Karasawa, K., Yato, M., Setaka, M., and Nojima, S. (1994) *J. Biochem.* 116, 374-379]. In this study, we isolated a homogeneous enzyme preparation from guinea pig plasma using a similar procedure. The molecular mass of this purified enzyme, as determined by SDS-PAGE was 58-63 kDa, larger than that (43 kDa) of the human enzyme. To elucidate the molecular structure of this enzyme and clarify its relationships with PAF-acetylhydrolases of other species, we isolated and sequenced a cDNA encoding this enzyme. Its cDNA contains an open reading frame encoding 436 amino acids and its predicted molecular mass (49 kDa) is lower than that of the native enzyme, suggesting that guinea pig plasma PAF-acetylhydrolase, unlike the human enzyme, is modified post-translationally, perhaps by glycosylation.

Key words: cDNA cloning, guinea pig, PAF, PAF-acetylhydrolase, purification.

Platelet-activating factor (PAF) is a phospholipid that evokes a wide range of biological responses including hypotension, increased vascular permeability, leukocyte migration, and smooth muscle contraction (*1, 2*). PAF is degraded and converted to an inactive metabolite, lyso-PAF, by an enzyme with specific hydrolytic activity toward PAF, PAF-acetylhydrolase, which was found to be distributed widely in mammalian plasma and cytosolic fractions of various cells and tissues (*3*). Several lines of evidence suggest that PAF-acetylhydrolase can be classified into two groups, plasma and tissue cytosol types (*4, 5*). Plasma PAF-acetylhydrolase is synthesized by liver (*6*) and macrophages (*7*) and secreted with lipoprotein into blood. Not only is plasma PAF-acetylhydrolase involved in PAF degradation, it also plays a role in removing oxidized phosphatidylcholine with PAF-like biological activity (*8*). In recent years, plasma PAF-acetylhydrolase has been investigated extensively, because reduced enzyme activity in blood may lead to the development of pathological states due to accumulation of PAF and PAF-like oxidized phosphatidylcholine (*9-11*).

In a previous study, we demonstrated that PAF-acetylhydrolase activity accumulated in the peritoneal cavity of the guinea pig during endotoxin shock induced by *Escherichia coli* LPS administration (*12*). This enzyme was purified to near homogeneity and identified, using SDS-PAGE, as a monomeric polypeptide with a molecular mass of 63 kDa (*13*). The purified enzyme exhibited similar biological properties to the crude enzyme in plasma, suggesting that it migrated into the peritoneal cavity from the blood due to increased vasopermeability during the shock state. In this study, we purified this enzyme from guinea pig plasma using a procedure similar to that used to purify the enzyme from peritoneal fluid and isolated a homogeneous preparation with the same molecular mass and specific activity. The apparent molecular mass of the guinea pig enzyme differed from that of the human enzyme (43 kDa) (*14*).

To elucidate the molecular structure of the enzyme and clarify its relationships with PAF-acetylhydrolases of other species, we isolated and sequenced a cDNA encoding this enzyme. The predicted molecular mass of the enzyme was lower than that of the native enzyme, leading us to conclude that guinea pig plasma PAF-acetylhydrolase, unlike the human enzyme, is modified post-translationally, perhaps by glycosylation.

MATERIALS AND METHODS

PAF-Acetylhydrolase Activity—PAF-acetylhydrolase activity was determined by the method of Stafforini *et al.* (*14*).

Purification of Plasma PAF-Acetylhydrolase—Guinea

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Abbreviations: PAF, platelet-activating factor; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; SSC, sodium citrate-sodium chloride buffer.

pig plasma PAF-acetylhydrolase was purified using procedure similar to that described in our previous report on the purification of PAF-acetylhydrolase from the peritoneal fluid obtained after *E. coli* LPS administration to guinea pigs (*12*). Blood was collected from 50 guinea pigs into 1/10 volume of 3.8% (w/v) sodium citrate, and 275 ml of plasma was separated by centrifugation. The solution was brought to 30% saturation with solid ammonium sulfate, stirred for 30 min and centrifuged at 10,000 × *g* for 10 min. The supernatant was brought to 60% saturation with solid ammonium sulfate, stirred and centrifuged in the same manner. The precipitate was dissolved in 50 ml of Tris-HCl (pH 8.0), the resulting solution was passed through a column of Affigel-Blue (3.0 × 32 cm), which was equilibrated with the same buffer, and the Affigel-Blue-treated solution was subjected to chromatography on several different columns, including butyl-Sepharose, heparin-Sepharose, ConA-Sepharose, chelating-Sepharose, and MonoQ, as described in our previous report (*13*). The purity of the enzyme preparation was analyzed by SDS-PAGE, using a 4 to 20% gradient gel, and the proteins on the gel were visualized using a Silver Stain II kit (Wako Pure Chemical Industries).

Protein Sequencing—The purified enzyme (6 μg) was blotted onto a PVDF membrane, which was blocked with PVP-40 and cut into small pieces. These were treated with 0.3 μg lysylendopeptidase in 50 μl 0.1 M Tris-HCl (pH 8.45) at 37°C overnight. The digested peptides were extracted successively with 40% CH_3CN alone and containing 0.1% TFA. The sample was applied to a reverse-phase Tosoh ODS80TS column (2.2 × 250 mm), which was equilibrated with 5% CH_3CN containing 0.1% TFA. The column was washed with the same solution for 5 min, and the peptides were eluted with a linear gradient of CH_3CN (5-38%) containing 0.1% TFA at a flow rate of 1 ml/min. Each peptide peak was monitored by measuring the absorbance of the eluate at 214 nm, the appropriate fractions were collected, and the N-terminal amino acid sequence of each peptide was analyzed with a gas phase protein sequencer, Model 473A (Perkin Elmer).

Isolation of RNA, Synthesis of cDNA, and Generation of PCR Product—RNA was isolated from the livers of male guinea pigs using guanidinium thiocyanate and CsTFA, and fractionated on oligo (dT)-cellulose to obtain the poly $(A)^+$ fraction. The first strand cDNA was produced with RNaseH-free reverse transcriptase (Gibco-BRL). Degenerated primers based on the amino acid sequences of the peptide fragments generated by lysylendopeptidase cleavage were designed. The sense primer used was 5′-ATGCA**GCGGCCGC**AA(T/C)(T/C)TIGA(A/G)ACNGA(A/G)-GA-3′ (the *Not*I restriction site is in bold type, and the targeting sequence corresponding to the amino acid sequence NLETEE is underlined), and the antisense primer was 5′-ATGCAT**GTCGAC**TT(T/C)TGIA(A/G)(A/G)AA-NGCNAA-3′ (the *Sal*I restriction site is in bold type, and the targeting sequence corresponding to the amino acid sequence LAFLQK is underlined). PCR was performed using the first strand cDNA, Taq DNA polymerase (Perkin Elmer) and these oligonucleotide primers. A 25-cycle program with each cycle comprising 94°C (1 min), 55°C (1 min), and 72°C (2 min) was carried out and the appropriate PCR product (about 600 bp) was identified by Southern blot analysis using ^{32}P-end-labeled oligonucleotides, 5′-TTIC-(T/G)(T/C)TC(T/C)TCNGT(T/C)TC-3′, corresponding to the amino acid sequence ETEERK, which is a nested amino acid sequence of NLETEE. The PCR product was purified by agarose gel electrophoresis, digested with *Not*I and *Sal*I, and then subcloned into *p*Bluescript SK(+).

Screening of cDNA Library and DNA Sequencing—The λgt 10 guinea pig liver cDNA library (Clontech) was screened with the cloned PCR product, which was labeled with ^{32}P using a random primed DNA labeling kit (Boehringer). Duplicate filters (Hybond, Amersham) were pre-hybridized at 65°C for 16 h in a solution of 5 × sodium citrate-sodium chloride buffer (SSC), 5 × Denhardt's solution [0.1% (w/v) BSA, 0.1% (w/v) Ficoll, 0.1% (w/v) polyvinylpyrrolidone], and 0.5% (w/v) SDS solution containing 100 μg/ml heat-denatured salmon sperm DNA and then hybridized under the same conditions for 16 h in the presence of the ^{32}P-labeled PCR product (1.1×10^9 cpm/μg DNA). The filters were washed twice with 0.5 × SSC containing 0.1% (w/v) SDS at 50°C for 30 min each, then with 2 × SSC containing 0.1% (w/v) SDS at room temperature for 10 min. After exposing filters to the imaging plate, the positive plaques were isolated. DNA from a positive phage was isolated by standard procedures and fragments were subcloned into *p*Bluescript II SK(−). Various deletion clones were obtained using a kilosequence deletion kit (Takara) and both strands were sequenced completely by the dideoxy chain-termination method using a DNA Sequencing System, Model 373S (Perkin Elmer).

Isolation of the 5′-End Fragment of Guinea Pig cDNA—To obtain a full-length cDNA of guinea pig PAF-acetylhydrolase, the rapid amplification of cDNA ends (RACE) method was performed as follows. Poly $(A)^+$ RNAs from guinea pig liver were reverse-transcribed using the primer, 5′-CTGTCTTCTACAGCAGCAAC-3′ (corresponding to nucleotides 554-573), then a poly (C)-tail was added to the 3′ end using terminal deoxynucleotidyl transferase. The 5′-end of the cDNA encoding guinea pig PAF-acetylhydrolase was amplified by PCR using a poly (G) tailed sense primer, and an antisense primer, 5′-CAUCAUCAUCAUGAATGC-TCCAAGACCATGAG-3′ (sequence corresponding to nucleotides 625-644 is underlined). The 5′-RACE amplification product was treated with uracil DNA glycosidase and subcloned into *p*AMP1 (Gibco BRL), and both strands were sequenced completely by the dideoxy-termination method, as described above. The full-length cDNA was constructed by joining the 5′-portion of the 5′-RACE product and the 3′-portion of clone λ10 at the internal Cfr10-I (see "RESULTS") site.

Expression of Protein—To preserve the reading frame of the protein in the 6 × His tag reading frame in the *E. coli* cloning vector, *p*ProEX-1 (Gibco-BRL), the gene coding the peptide region was obtained by PCR using a sense primer, 5′-ATGCA**GCGGCCGC**GATGGCCCCACCAAAACTGC-3′ (the *Not*I restriction site is in bold type, and the sequence corresponding to nucleotides 105-124 is underlined) and an antisense primer, 5′-ATGCAT**CTCGAG**CTGCCTTTAAT-CTGGATTCC-3′ (the *Xho*I restriction site is in bold type, and the sequence corresponding nucleotides 1403-1422 is underlined). The PCR product was digested with *Not*I and *Xho*I, the resulting fragment was ligated into *p*ProEX-1, and *E. coli* strain, DH5α was transfected with the resulting plasmid, designated *p*PAFAH216. Transformants containing plasmids were grown in LB containing 50 μg/ml am-

picillin until absorbance at 590 nm reached 0.5. Then, protein expression was induced with 0.6 mM IPTG, the cells were cultured at 37°C for 3 h, collected by centrifugation and resuspended in lysis buffer [50 mM Tris-HCl (pH 8.5) containing 10 mM 2-mercaptoethanol]. The cell suspension was disrupted by sonication. The supernatant was obtained by centrifugation at 10,000 × *g* for 10 min, and the recombinant protein was purified using a column containing Ni-NTA resin (Gibco-BRL).

RESULTS

Enzyme Purification—In a previous study, we purified PAF-acetylhydrolase from guinea pig peritoneal fluid (*13*). In the present study, we purified this enzyme from guinea pig serum by a similar method, obtaining approximately 40 μg of homogeneous protein from 275 ml of serum obtained from 50 guinea pigs. The chromatographic behavior of this serum enzyme on each type of column was similar to that of the previously purified peritoneal fluid enzyme (data not shown), SDS-PAGE revealed that both preparations had the same molecular mass (Fig. 1) and their specific activities were of the same order of magnitude. Therefore, we concluded that these enzymes were identical and the enzyme activity found in the peritoneal fluid was derived from the plasma. The molecular mass of the guinea pig plasma PAF-acetylhydrolase was estimated to be 58-63 kDa, whereas that of human plasma PAF-acetylhydrolase was reported by Stafforini *et al.* to be 43 kDa (*14*). Guinea pig plasma PAF-acetylhydrolase was strongly bound to a column of Con A-Sepharose, and methyl-α-D-mannopyranoside was required for eluting the enzyme activity, suggesting that the enzyme contains carbohydrates. On the contrary, human plasma PAF-acetylhydrolase was not adsorbed to this column under the same conditions (data not shown).

In spite of this difference in apparent molecular masses, human and guinea pig plasma PAF-acetylhydrolases showed similar biochemical properties. The serine protease inhibitors DFP and PMSF inhibited both enzymes (data not shown), and oxidized phosphatidylcholines with PAF-like biological activity, such as succinoyl and glutaroyl PAF, were degraded by the purified enzyme, as well as human plasma PAF-acetylhydrolase (data not shown).

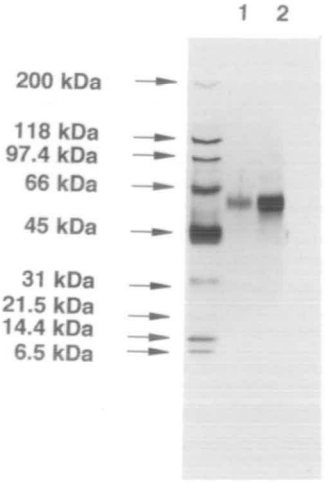
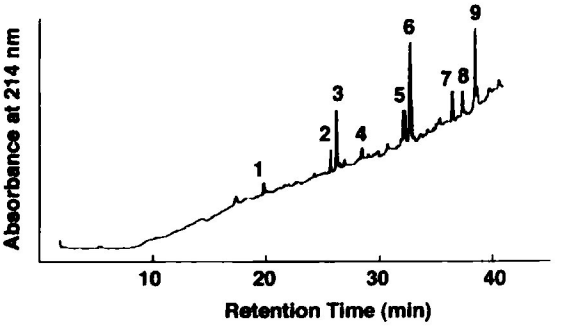


Fig. 1. **SDS-PAGE.** The purified enzymes from the peritoneal fluid (lane 1) and plasma (lane 2) of guinea pigs were analyzed by SDS-PAGE, as described in "MATERIALS AND METHODS." Molecular markers: myosin (200 kDa), β-galactosidase (118 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), and aprotinin (6.5 kDa).



Peak 1	GSLDRSK
Peak 2	VGNLETEERK
Peak 3	WNSPLK
Peak 4	MIAVK
Peak 5	LLYGSVK
Peak 6	FYQPQK
Peak 7	ASLAFLQK
Peak 8	IDSEVAMDLINK
Peak 9	GSVHHNFVDFTFATGK

Fig. 2. **Separation of peptides produced by lysylendopeptidase digestion of guinea pig plasma PAF-acetylhydrolase.** The purified enzyme was blotted onto a PVDF membrane, which was treated with lysylendopeptidase. The resulting peptides were extracted and separated on a Tosoh ODS80TS column with a linear gradient system as described in "MATERIALS AND METHODS."

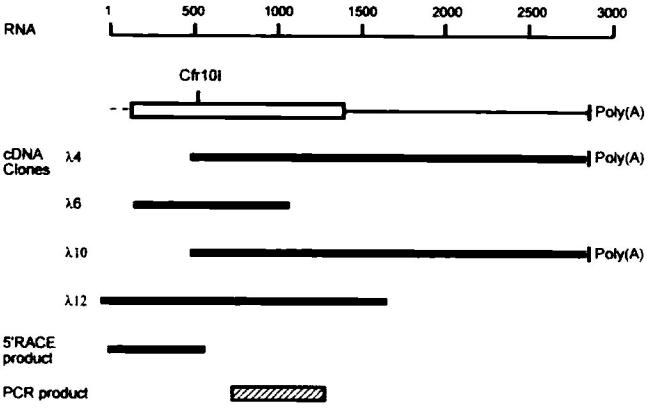


Fig. 3. **Guinea pig plasma PAF-acetylhydrolase mRNA.** The structure of guinea pig plasma PAF-acetylhydrolase mRNA, deduced from cDNA clones, is depicted below a size scale in nucleotides. The transcription direction is from left to right. The features of the message are a 5′-untranslated region (dotted line), a peptide-coding region (open box), and a 3′-untranslated region ending in a poly (A) site (line with bar). The site for the restriction enzymes, Cfr10I, is shown. The horizontal bars below this line indicate the extents of the characterized cDNA clones (λ4, λ6, λ10, λ12, and 5′RACE product). The hatched bars on the bottom line depict the regions of the PCR product used for screening the cDNA library.

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1     CAAACAGACCATCACTGGGTCAGTAACACCTAAGTGTCTCTTGGCAGCTTGTTTAAATTACGGATCTGT  69
70    CACCTTGAATATCCAGACGGCTCAACTGAACCCAGGATGGCCCCACCAAAACTGCACACGCTCTTCTGC  138
1                                    M  A  P  P  K  L  H  T  L  F  C          11
139   CTCTCTGGATTCCTGGCACTGGTCCACCCTTTTGACTGGCGAGACCTAGATCCTGTCACTTATATCCAA  207
12    L  S  G  F  L  A  L  V  H  P  F  D  W  R  D  L  D  P  V  T  Y  I  Q    34
208   TCATCAGTATGGATCCAAAGAATACAATCGGAGCTGCTCATTACCAGCTTTGGACACACAACAATCCCC  276
35    S  S  V  W  I  Q  R  I  Q  S  E  L  L  I  T  S  F  G  H  T  T  I  P    57
277   AAGGGAAATGGACCTTACTCCGTGGGTTGTACAGACCTGATGTCTGGTTACACTAACCAGAGCAGCTTT  345
58    K  G  N  G  P  Y  S  V  G  C  T  D  L  M  S  G  Y  T  N  Q  S  S  F    80
346   TTGCGCTTGTATTATCCATCGCAAGATAATGATTTCCCTGATGCCCTGTGGATCCCAAATGAAGAGTAT  414
81    L  R  L  Y  Y  P  S  Q  D  N  D  F  P  D  A  L  W  I  P  N  E  E  Y   103
415   TTCCAGGGTCTTACTGAAACCCTTGGAGCATCTAGTTTCCTGGGCAAACTTTTGAAGCTGTTATACGGC  483
104   F  Q  G  L  T  E  T  L  G  A  S  S  F  L  G  K  L  L  K  L  L  Y  G   126
484   TCAGTGAAGGTTCCTGCAAAGTGGAATTCTCCTCTGAAGACCGGTGAAAAATACCCACTAATTATCTTT  552
127   S  V  K  V  P  A  K  W  N  S  P  L  K  T  G  E  K  Y  P  L  I  I  F   149
553   TCTCATGGTCTTGGAGCATTCAGGTCAATTTATTCTGCTATAGGCATTGAGCTGGCGTCTCATGGGTTC  621
150   S  H  G  L  G  A  F  R  S  I  Y  S  A  I  G  I  E  L  A  S  H  G  F   172
622   ATAGTTGCTGCTGTAGAACACAGAGATGAATCTGCCGCTGCTACTTACTACTTCCAGGACGCTCCTGCA  690
173   I  V  A  A  V  E  H  R  D  E  S  A  A  A  T  Y  Y  F  Q  D  A  P  A   195
691   GCTGAATCAGGCAACAGGTCTTGGATTTACTACAAAGTTGGAAACCTAGAGACAGAAGAGCGAAAAAGG  759
196   A  E  S  G  N  R  S  W  I  Y  Y  K  V  G  N  L  E  T  E  E  R  K  R   218
760   CAGTTACGGCAGCGAGGGGAGGAGTGTTCCCAGGCTCTCAGTTGGCTTCTCAGCATTGACGAAGGAGAG  828
219   Q  L  R  Q  R  G  E  E  C  S  Q  A  L  S  W  L  L  S  I  D  E  G  E   241
829   CCAGTGAAGAACGTGTTGGATTTGAACTTTGACATTCAGCAACTGAAGGGCTCTCTTGATAGGAGTAAA  897
242   P  V  K  N  V  L  D  L  N  F  D  I  Q  Q  L  K  G  S  L  D  R  S  K   264
898   GTAGCGATCATTGGACATTCATTTGGTGGAGCAACAGTTATCCAGACCCTCAGTGAAGACCAGAGATTC  966
265   V  A  I  I  G  H  S  F  G  G  A  T  V  I  Q  T  L  S  E  D  Q  R  F   287
967   AGGTGTGGCATTGCCCTGGATCCATGGATGTTTCCGGTGGGGGAGGACGTCCATTCCAAAATCCCACAA  1035
288   R  C  G  I  A  L  D  P  W  M  F  P  V  G  E  D  V  H  S  K  I  P  Q   310
1036  CCCCTCTTTTTTATCAACTCTGAATACTTCCAAAGTGCTAATGATACCAAAAAAATAGAAAAGTTCTAC  1104
311   P  L  F  F  I  N  S  E  Y  F  Q  S  A  N  D  T  K  K  I  E  K  F  Y   333
1105  CAACCTCAAAAAGAGAGAAAAATGATTGCAGTCAAGGGTTCAGTCCATCACAATTTTGTTGACTTCACT  1173
334   Q  P  Q  K  E  R  K  M  I  A  V  K  G  S  V  H  H  N  F  V  D  F  T   356
1174  TTTGCCACTGGGAAAATTATTGGACAGATGTTATCATTGAAAGGAAAGATAGACTCAGAGGTAGCTATG  1242
357   F  A  T  G  K  I  I  G  Q  M  L  S  L  K  G  K  I  D  S  E  V  A  M   379
1243  GACCTCATCAACAAAGCTTCGTTAGCATTCTTACAGAAATACTTAGGACTTGATAAAAATTTTGACCAG  1311
380   D  L  I  N  K  A  S  L  A  F  L  Q  K  Y  L  G  L  D  K  N  F  D  Q   402
1312  TGGAACTCTCTGATGGAAGGAGATGATGAGAATCTTATTCCAGAGTTCACAATACCCACCACTATGCAG  1380
403   W  N  S  L  M  E  G  D  D  E  N  L  I  P  E  F  T  I  P  T  T  M  Q   425
1381  AGCTCTACAGGAACAGAACAAAGGAATCCAGATTAAAGGCAGTTTTTTTTTTTTAAAGAAAAGTTCATT  1449
425   S  S  T  G  T  E  Q  R  N  P  D  *
1450  GAAAAACTATCTAAAACAGAGCGAGTGCATGTGTGAAGAGACTTCAATGTATTTTCTGAGGTGACTCAT  1518
1519  ATTTTAAAATGTGGGCTATTCAGTAACACTGGCAAAGGCTTGAGTTAAGAAAATTTTCCCTTACGAGTA  1587
1588  TGTAGTGACCATCTTTGGAGAGTCAGCAGGAGGTGAATGTGTCTCTGTTCTTGTGCTATCTCCCCTGGC  1656
1657  CAAGAACCTCTTCCACAGGGCCATTTCAGAGAGTGGTGTGGTCTTCACTCCTAGTCTGTTAAGCAAGGA  1725
1726  TGTCAAGCCTCTGGCTAAGAAAATTGCAGGTGCTGCTGGGTGTAAAACTACTTCCTCAGCTGTCATGGT  1794
1795  TCACTGCCTGCGCCAGAAGACAGAGGATGAACTCTTGGAGGTCACACAGAACTTGAAATTTGCTTCTGT  1863
1864  GGATATTTTTGAAGACCCCAGAAAGAAGTATATGTTTGTGCCCACTGTTGTTGACGGTGTGCTGCTGCC  1932
1933  CAAAGCACCTGAAGAGATGATGGCTGAAAAGAATTTCAACCTCGTTCCCTATATGGTAGGATTCAACAA  2001
2002  GCAAGAGTTTGGCTGGGTCATTCCCATGATGCTTGGAGGTGTAATCCCTGAAGACAAACTGGATCAGAA  2070
2071  GATGGCCGCATCACTCTTGTGGACGTTGTATCCCTTTGCTAATGTTCCTGAGGAACTGACAGCAGTGGT  2139
2140  CACTGAGATGTATTTAGGAGGGACAGATGACCTCGTCAAAAAGAGAGAACTGTTCCTGGACTTAATGGG  2208
2209  AGATTTGATGTTTGGTATCCCATCTGTGATTGCAGCTCGTGGCCACAGAGATGCTGGAGCCCCCACCTA  2277
2278  CATGTATGAGTTCCAGTACTGTCCAACTTTCATATCAGACATGAGACCCAAGACAGTGAAAGGAGACCA  2346
2347  CGGGGATGAGATTTTCTCAGTGTTTGGGAGTCCACTTGTGAAAGAGGGTGCTTCCGAGGAGGAGATCAA  2415
2416  CCTCAGCAAGATGGTGATGAAATTCTGGGCCAACTTTGCTCGGACTGGGAACCCCAATGGGGAAGGTCT  2484
2485  GCCTCACTGGCCAGAGTATGACCAAAAGGAAGGATACCTGCAGATTGGTGTCACCACCCAGGCTGCCCA  2553
2554  GAAACTGAAGGGCAAAGAAATGGCTTTCTATACTGAGGTTCTAGCCAAGAAGACTGTGGGGAATCCACA  2622
2623  CCAGCAAGAACACATAGAGCTCTGAAACGACTATGCAGCCAGTTTCAGGGTCCTGAGACAGTTAACATG  2691
2692  GGTGTATCTATAGGAGCGTTTGTAAGCTAAGCTTGCATAAGTTTGTAGTGGCTGAAGCATGGTTTGTTT  2760
2761  TATGATGGGAGGAGTCCGGGAATGGGGAATGTTTGTGCAATGACTTTAATTTGAAGATAAGTGTTAATT  2829
2830  TTGACAATAAAATCAATGTCTGTGTTGAAATGTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA       2894
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Fig. 4. **DNA sequence analysis.** The nucleotides are numbered from the 5′-terminus of cDNA and the complete predicted amino acid sequence of guinea pig PAF-acetylhydrolase is shown under the DNA sequence. Solid underlines indicate the amino acid sequences of the nine purified peptides that were determined directly (Fig. 2). Possible *N*-linked glycosylation sites are marked by closed circles above the peptide sequence, the polyadenylation signal is double underlined and the active site sequences for serine esterases and lipases are boxed.

Molecular Cloning of Guinea Pig PAF-Acetylhydrolase—Sequencing attempts revealed that the protein was blocked at the N-terminus, so the protein was cleaved by lysylendopeptidase and the amino acid sequences of the nine peptides produced were determined for generating PCR primers (Fig. 2). The PCR was performed using reverse-transcribed cDNA from the guinea pig liver poly $(A)^+$ RNA as a template and degenerated primers designed from the amino acid sequences of the peptide fragments. When the oligonucleotides from the amino acid sequences NLETEE and LAFLQK were used as sense and antisense primers, respectively, a fragment of approximately 600 bp was amplified. Southern blot analysis using an internal sequence as a probe suggested that this PCR product was a cDNA fragment of the protein. We used this 600-bp cDNA fragment to screen a guinea pig liver λgt10 cDNA library and identified four clones. The extents of these sequenced clones and the deduced structure of the mRNA are shown in Fig. 3. Clones λ4 and λ10 contained poly $(A)^+$ tails, whereas clones λ6 and λ12 did not extend back to the poly $(A)^+$ tail. As these four clones had identical and overlapping nucleotide sequences, they were derived from the same gene. The longest clones, λ4 and λ10, lacked nucleotides encoding the N-terminus of the protein, so the 5′-end of the cDNA was isolated by the 5′-RACE method and a full-length cDNA was constructed by ligating the two fragments using Cfr10-I restriction sites located in both DNA fragments.

Sequence Analysis of PAF-Acetylhydrolase cDNA—Figure 4 shows the DNA sequence and translated sequence of guinea pig plasma PAF-acetylhydrolase cDNA. The nine peptides generated by lysylendopeptidase digestion were found to be in complete accord with the deduced protein sequence. Guinea pig PAF-acetylhydrolase cDNA comprises 2,894 nucleotides, and the cDNA sequence contained an open reading frame of 1,308 nucleotides encoding a protein of 436 residues with a molecular mass of 49,062 Da and a pI of 5.47 (Fig. 4). The guinea pig plasma PAF-acetylhydrolase cDNA sequence contains 105 nucleotides of the 5′-untranslated region and 1,484 nucleotides of the 3′-untranslated region with a polyadenylation signal, AATAAA, 22 nucleotides upstream from the beginning of the poly (A) tail. The first ATG of the cDNA would appear to be a favorable initiation codon, because its sequence context, AGGAUGG, is commonly found in vertebrates as functional initiation codons (*15*). The predicted guinea pig PAF-acetylhydrolase possesses three potential *N*-glycosylation consensus sites (Asn-Xaa-Ser or Asn-Xaa-Thr) at residues 76, 200, and 324, and the deduced amino acid sequence includes the Gly-Xaa-Ser-Xaa-Gly motif found in most serine esterases and lipases. Hydropathy analysis of the predicted amino acid sequence indicated that hydrophobic segments were present in the first 20 residues. Northern blot analysis showed that the mRNA for this enzyme was not detectable in the liver, but the expected 2.9-kb mRNA was present in the lung (data not shown). The identities of the predicted amino acid sequence of guinea pig plasma PAF-acetylhydrolase with those of other previously reported mammalian and avian enzymes (*16, 17*) are 67.7% (human), 65.7% (bovine), 58.9% (mouse), 64.3% (dog), and 52.1% (chicken).

Expression of the PAF-Acetylhydrolase Gene in E. coli—To confirm that the cDNA encodes the PAF-acetylhydrolase, the open reading frame was cloned into bacterial vectors for gene expression in *E. coli*. As shown in Fig. 5A, PAF-acetylhydrolase activity in cell extracts of cells transfected with the expression vector containing the open reading frame of the cDNA was increased remarkably by inducing the expression with IPTG, whereas PAF-acetylhydrolase activity in the control cells, transfected with the vector alone, was unaffected by IPTG. Furthermore, extracts from bacteria that had been transformed with an expression vector designed to produce a fusion protein with His-tag fused to the N-terminus were purified on a Ni^+ column. The fractions eluted from the Ni^+ column had PAF-acetylhydrolase activity and each yielded one major band with an electrophoretic mobility identical to the molecular mass (56 kDa) of the deduced amino acid sequence (49 kDa) plus 40 amino acid residues from the vector DNA (4.5 kDa), as shown in Fig. 5B. Furthermore, the purified recombinant protein also exhibited hydrolytic activity toward oxidized phosphatidylcholines, as well as

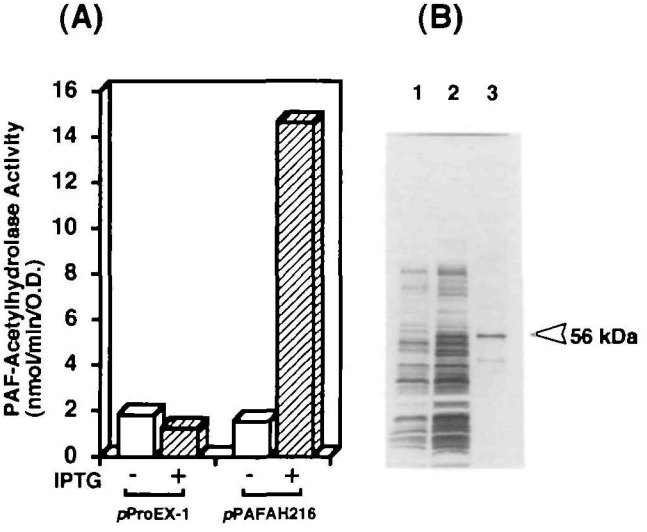

Fig. 5. **Expression of the PAF-acetylhydrolase gene in *E. coli*.** (A) *E. coli* strain DH5α was transfected with *p*PAFAH216, and grown in LB medium containing 50 μg/ml ampicillin until the A_{590} reached 0.5. Three hours after adding IPTG (0.6 mM), an aliquot was removed and the absorbance of each cell suspension was adjusted to 0.2 with the same medium. The cells were collected by centrifugation, resuspended in lysis buffer consisting of 50 mM Tris-HCl (pH 8.0), 5 mM EDTA, and 1 mg/ml lysozyme and the enzyme activities of the resulting lysates were measured, as described in "MATERIALS AND METHODS." (B) Recombinant protein expression was induced by culturing *E. coli* DH5α/*p*PAFAH216 in LB-ampicillin medium in the presence of 0.6 mM IPTG for 3 h, after which the cells were collected by centrifugation and resuspended with lysis buffer consisting of 50 mM Tris-HCl (pH 8.5) and 10 mM 2-mercaptoethanol. The cell suspensions were sonicated and the resulting lysates were centrifuged at 10,000×*g* for 10 min. The resulting supernatant was loaded onto a column containing Ni-NTA resin, which was washed with 20 mM Tris-HCl (pH 8.5), 100 mM KCl, 20 mM imidazole, 10 mM 2-mercaptoethanol, and 10% (v/v) glycerol, and the adsorbed protein was eluted with 20 mM Tris-HCl (pH 8.5), 100 mM KCl, 100 mM imidazole, 10 mM 2-mercaptoethanol, and 10% (v/v) glycerol. SDS-PAGE was performed using 10% acrylamide gel and the protein bands were visualized by silver staining. Lane 1, *E. coli* lysate before induction; lane 2, *E. coli* lysate after induction; lane 3, purified recombinant protein.

PAF, as does the native enzyme (data not shown). These results indicate that the cDNA sequence does indeed code PAF-acetylhydrolase.

DISCUSSION

In the present study, we cloned a guinea pig plasma PAF-acetylhydrolase cDNA by screening a liver cDNA library using a probe obtained from the partial amino acid sequences of the purified enzyme. Guinea pig plasma PAF-acetylhydrolase cDNA contained an open reading frame of 1,308 base pairs and coded a protein of 436 amino acids, and the deduced protein contained all of the sequences of the lysylendopeptidase-digested peptide fragments. The predicted molecular mass was lower than that of the native enzyme, suggesting that guinea pig plasma PAF-acetylhydrolase was subjected to post-translational modification.

We were unable to identify the N-terminal amino acid of the guinea pig purified plasma enzyme. Ile^{42} was identified as the NH_2-terminus of the human enzyme and the preceding peptide was thought to be cleaved as a signal sequence or fragmented during purification (*16*). Guinea pig plasma PAF-acetylhydrolase may be also produced as a propeptide, like the human enzyme, because a core of hydrophobic amino acids is contained within its first 20 residues, and Ala^{17}-Leu^{18} is a sequence which fits the "(-3, -1) rule" for the signal cleavage site of eukaryocytes (*18*).

SDS-PAGE revealed the apparent molecular mass of the purified PAF-acetylhydrolase was 58-63 kDa, about 10 kDa greater than the molecular mass of the putative propeptide predicted from the cDNA sequence (about 49 kDa), whereas the native molecular size of the human enzyme (about 43 kDa) agreed well with the predicted size of the processed protein (about 45 kDa) (*16*). A possible explanation for this anomalous electrophoretic behavior of the guinea pig enzyme is that this protein may be glycosylated post-translationally. Indeed, there is some evidence that the guinea pig enzyme contains carbohydrate. First, this enzyme showed high affinity for a column of Con A-Sepharose, which is known to bind to proteins with carbohydrates such as α-D-mannopyranosyl and α-D-glucopyranosyl residues. Second, three predicted glycosylation sites (Asn-Xaa-Thr or Asn-Xaa-Ser) (*19*) were observed in the amino acid sequences. In particular, Asn^{76} lies in a β-turn region, in which glycosylated Asn residues are frequently found (*20, 21*). In contrast to the guinea pig enzyme, the human enzyme neither bound to Con A resin under the same conditions (our unpublished result) nor possesses glycosylation sites located in a β-turn. Thus, the guinea pig and human enzymes may be generated by different processing mechanisms. However, further studies are needed to elucidate the details of the processing mechanism and its significance in enzyme function.

PAF-acetylhydrolases are classified into two, plasma and intracellular, groups according to a variety of criteria (*4, 5*). The overall homology at the amino acid level of guinea pig plasma and bovine brain intracellular PAF-acetylhydrolases is very low (*22*), but it is noteworthy that the homologous sequence domains of the PAF receptor (*23*) and bovine brain intracellular PAF acetylhydrolase (*22*) are also present in guinea pig plasma PAF-acetylhydrolase with 25.0% identity and 80.0% similarity among 20 amino acids (F^{156}-V^{174}), supporting the proposal that this sequence domain is a structure indispensable for PAF recognition (*22*).

The activity of PAF-acetylhydrolase in plasma has been shown to be altered in various diseases, such as bronchial asthma (*9*), systemic lupus erythematosus (*10*), and septic shock (*11*). A reduction in PAF-acetylhydrolase activity in blood may lead to the development of some pathological states due to accumulation of PAF and PAF-like oxidized phosphatidylcholine. We are now examining whether overexpression of the enzyme by gene transfer into the blood vessel walls can prevent these phospholipid mediators from accumulating in the blood.

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