Identification of a Human cDNA Clone for Lysosomal Type Ca^{2+}-independent Phospholipase A_2 and Properties of the Expressed Protein*

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A Ca^{2+}-independent phospholipase A_2 (PLA_2) maximally active at pH 4 and specifically inhibited by the transition-state analogue 1-hexadecyl-3-trifluoroethylglycerol-sn-2-phosphomethanol (MJ33) was isolated from rat lungs. The sequence for three internal peptides (35 amino acids) was used to identify a 1653-basepair cDNA clone (HA0683) from a human myeloblast cell line. The deduced protein sequence of 224 amino acids contained a putative motif (GXSGX) for the catalytic site of a serine hydrolase, but showed no significant homology to known phospholipases. Translation of mRNA produced from this clone in both a wheat germ system and known phospholipases. Translation of mRNA produced from this clone in both a wheat germ system and

Phospholipase A_2 (PLA_2) represents a diverse family of enzymes that hydrolyze the sn-2-fatty acyl or alkyl bond of phospholipids. The best characterized member of this family is a group of low molecular mass (~15 kDa) enzymes that require Ca^{2+} for catalytic activity and show maximal activity in an alkaline (pH 8.5) medium (1). This group of enzymes, called secreted PLA_2 (sPLA_2; types 1–3), include snake and bee venoms and mammalian pancreatic and synovial PLA_2 enzymes. The more recently characterized 87-kDa cytoplasmic PLA_2 (cPLA_2) requires only μM Ca^{2+} for binding to the interface and shows maximal activity at neutral pH (2). This enzyme is widely distributed in cell types and may be specifically linked to eicosanoid metabolism. Finally, there is a group of enzymes (iPLA_2) that do not require Ca^{2+} for maximal activity. As recently reviewed (3), few of these enzymes have been purified, and in contrast to sPLA_2 and cPLA_2, little molecular information is available. Therefore, although the iPLA_2 enzymes are widely distributed, relatively little is known about their intra-group relationships and specific functions. Based on biochemical characteristics, Ackermann and Dennis (3) have divided the iPLA_2 enzymes into three subgroups: lysosomal iPLA_2, brush-border membrane iPLA_2, and intracellular (cytosolic/membrane) iPLA_2. A distinguishing characteristic of the lysosomal iPLA_2 group is maximal activity in acidic medium, and accordingly, we have designated the enzyme in this report as aIPLA_2.

iPLA_2 activity in rat lung homogenates was first described ~25 years ago (4), and activity at acid pH was subsequently demonstrated in rabbit lung (5), rat granular pneumocytes (lung alveolar type II cells) (6), and rat and human alveolar macrophages (7, 8). Activity has been localized further to the lung lamellar bodies (the surfactant secretory organelle) and the lysosomal fraction (5, 7, 9). Under our assay conditions, lung aIPLA_2 activity is inhibited by a phospholipid transition-state analogue, 1-hexadecyl-3-trifluoroethylglycerol-sn-2-phosphomethanol (MJ33), while Ca^{2+}-dependent PLA_2 activity in the lung homogenate is insensitive (7). Using MJ33 as a probe, we previously purified a protein with aIPLA_2 activity to appar-

1 The abbreviations used are: PLA_2, phospholipase A_2; sPLA_2, secreted phospholipase A_2; cPLA_2, cytoplasmic phospholipase A_2; iPLA_2, Ca^{2+}-independent phospholipase A_2; aIPLA_2, acidic Ca^{2+}-independent phospholipase A_2; PLA_2, phospholipase A_2; PC, phosphatidylcholine; AAOCOF_2, trifluoromethylarachidonoyl ketone; BEL, bromoelaidic lactone; pBPB, p-bromophenacyl bromide; DENP, diethyl p-nitrophenyl phosphate; PAGE, polyacrylamide gel electrophoresis; DPPC, 1-palmitoyl-2-palmitoyl-sn-glycero-3-phosphocholine; MES, 4-morpholineethanesulfonic acid; PAF, 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) D14662.

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ent homogeneity (10). This protein had a molecular mass of ~15 kDa and a unique N-terminal amino acid sequence. Subsequent attempts to reproduce the isolation yielded similar enzyme activity, although the molecular mass of the isolated protein was ~26 kDa. Furthermore, the amino acid sequence of the larger enzyme, as described in this report, does not contain the previously determined N-terminal sequence. Consequently, we believe that aiPLA2 described in this report is different from the previously isolated protein.

Amino acid sequencing (35 residues) of tryptic digests of the 26-kDa protein isolated from rat lung revealed 100% identity to deduced amino acids from the nucleotide sequence (previously unpublished) of a clone isolated from a human myeloblast cDNA library (11). This report provides evidence that the protein expressed by this cDNA is aiPLA2 and provides the first molecular cloning for this group of enzymes.

**EXPERIMENTAL PROCEDURES**

**Materials**—Lipids were obtained from Avanti Polar Lipids (Birmingham, AL). All radiochemicals and x-ray film were purchased from DuPont NEN, and bisbodipy-C11-PC was from Molecular Probes, Inc. (Eugene, OR). Acrylamide, bisacrylamide, N,N’-methylenebisacrylamide (AALOCE), Ponceau S, Coomassie Blue, and then with silver staining. The protein was electrophoresing the Laemmli buffersystem (12). Bands were first visualized with SDS-PAGE (15% acrylamide) under reducing conditions (Eugene, OR). Trifluoromethylarachidonoyl ketone (AACOCF3) was a gift from Dupont NEN, and bisbodipy-C11-PC was from Molecular Probes, Inc. (Eugene, OR). Acrylamide, bisacrylamide, N,N’-methylenebisacrylamide (AALOCE) was from Calbiochem; bromoelaidic acid was from Cayman Chemical Co., Inc. (Ann Arbor, MI); and p-bromophenacyl bromide (pBPP), 2-mercaptoethanol, diethyl p-nitrophenyl phosphate (DEPNP), Naja naja PLAA, SepharacH S-100, and phenyl-Sepharose CL-4B were from Sigma. DE52 was purchased from Whatman (Maidstone, United Kingdom). Molecular mass standards for SDS-PAGE and Transblot membrane were from Bio-Rad. Nitrocellulose membrane was from Schleicher & Schuell. Sulfomass standards for SDS-PAGE and Transblot membranes were from Life Technologies, Inc. Klenow enzyme was from Boehringer Mannheim. pBluescript SK+ vector was from Stratagene (La Jolla, CA). In vitro transcription and wheat germ in vitro translation kit were from Ambion Inc. (Austin, TX). Male Sprague-Dawley rats weighing ~200 g were obtained from Charles River Breeding Laboratories (Kingston, NY).

**Isolation of Rat Lung aiPLA2**—Rats were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally). Lungs were ventilated through the trachea, perfused through the pulmonary artery in situ to clear them of blood, removed from the thorax, and stored at ~80°C. Frozen lung tissue (~80 g) was extensively homogenized with Polytron PT-10 and Potter-Elvehjem homogenizers in 200 ml of ice-cold 50 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA, 0.2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, and 10% glycerol. This buffer was used in all further steps of purification. The lung homogenate was spun at 10,000 g for 1 h. The supernatant was subjected to 55% ammonium sulfate fractionation. The precipitate was redissolved in 80 ml of buffer and dialyzed extensively against 50 mM Tris-HCl (pH 7.4) containing 1 mM EDTA and 5% glycerol. This buffer was used in all further steps of purification. The dialyzed sample was centrifuged at 1500 × g for 15 min, and the supernatant was applied to a DE52 column (38 × 2.2 cm), which was washed with 2 bed volumes of the same buffer at a flow rate of 60 ml/h and then eluted with a linear gradient of 0–50 mM NaCl. The major aiPLA2 activity was recovered after the second bed volume wash and was retarded with respect to the unbound protein peak (Fig. 1). The fractions containing aiPLA2 activity that was inhibited by MJ33 were pooled, concentrated to 7 ml on an Amicon YM-10 membrane, applied to a SepharacH S-100 HR column (120 × 2.2 cm), and eluted with buffer at a flow rate of 17 ml/h (Fig. 1). Fractions with MJ33-sensitive aiPLA2 activity (3.5 ml each) were pooled and applied to a second DE52 column (7 × 1.3 cm). The column was washed with 5 bed volumes of buffer and then eluted with a linear gradient of 10–60 mM NaCl. aiPLA2 activity eluted between 20 and 40 mM NaCl (Fig. 1). The eluted protein fraction was chromatographed on a 2-ml phenyl-Sepharose column equilibrated with buffer containing 1 mM KCl. The column was washed with a linear gradient of 1.5 to 0 KCl, followed by buffer and then water. PLAA activity was detected in the H2O wash, which was analyzed by SDS-PAGE.

**Amino Acid Sequencing of Rat Lung Protein**—Protein samples were analyzed by SDS-PAGE (15% acrylamide) under reducing conditions using the Laemmli buffer system (12). Bands were first visualized with Coomassie Blue and then with silver staining. The protein was electro-blotted onto polyvinylidene difluoride membrane (Transblot) in order to analyze for internal amino acid sequences. Selected protein bands were digested in situ with trypsin and then separated by high pressure liquid chromatography using a Zorbax column. Ten peaks were selected and subjected to mass spectral analysis. Based on this analysis, three peptides were selected and further purified for their amino acid sequences by Edman degradation (13). These analyses were carried out in the Protein Microchemistry Laboratory of the Wistar Institute (Philadelphia, PA). The sequences were compared with the National Center for Biotechnology Information data base for similarity to known sequences.

**Isolation and Sequencing of the cDNA Clone**—The human myeloid cell line HL-60 (1 CCL246) from the American Type Culture Collection, Rockville, MD) was used as a source of mRNA. RNA preparation, cloning, and sequencing of the HA0683 clone (referred to as the KIAA0106 gene) have been described previously (11, 14). Briefly, cytoplasmic RNA was prepared using a non-ionic detergent (15), followed by chromatography on oligo(dT)-cellulose to isolate the poly(A) RNA. First-strand synthesis was primed with a poly(dT)-Nol primer oligo-nucleotide (5’TCTCTAGGGCGCGCC(TG)3’ using Supernscript reverse transcriptase. Second-strand synthesis was performed as described (16), repaired with T4 DNA polymerase, digested with NolI, size-fractionated on a sucrose gradient, and ligated into a Nol-EcoRV-cut pBluescript SK+ vector. Automated sequencing was performed by the dideoxy method (15) using Chemical Robot DSP-240 (Seiko Instrument, Tokyo) or CATALYST800 (Applied Biosystems, Foster City, CA), and analysis was performed using an Applied Biosystems 373A sequencer and the Applied Biosystems sequence analysis system INHERIT. Large-scale preparations of plasmid were obtained by centrifugation on cesium chloride-ethidium bromide gradients (15).

**In Vitro Transcription**—The cDNA clone HA0683 was linearized by digestion with NolI, and capped mRNA was then synthesized by transcription using T7 RNA polymerase and m7GpppG. The transcripts were purified by phenol/chloroform extraction and ethanol precipitation, quantitated by comparing intensity after ethidium bromide staining with control RNA with known concentration, checked for integrity by electrophoresis on a formaldehyde-agarose (1%) gel, and subsequently used as a template for translation in wheat germ or oocytes.

**Wheat Germ in Vitro Translation**—Translation of the HA0683 mRNA transcript was performed in the wheat germ system according to the manufacturer's protocol. In brief, RNA samples of up to 2 μg were translated in a reaction mixture containing 50 mM potassium acetate, amino acid master mixture (0.16 mM creatine phosphate, 0.5 mM methionine, 0.5 mM leucine, and a 1 mM concentration of the other amino acids), and wheat germ extract. The total reaction volume was 50 μl. Each experiment included a negative control in which no RNA was added and a positive control in which XeF-1 RNA, encoding Xenopus elongation factor-1a (M, 50,300) provided by the supplier, was added. Reaction mixtures were incubated at 27 °C for 60 min. The translated products were then assayed for aiPLA2 activity.

** Autoradiography of Translated Protein with [35S]Methionine**—Labeling of the HA0683 translation product in wheat germ extract was performed with the addition of 5 μCi of translation-grade [35S]methionine (1174 Ci/mmol) and a 1 mM concentration of the other amino acids. Otherwise, the reaction mixture was as described above. Small aliquots of translated proteins were heated at 95 °C for 2 min in the sample buffer containing 5% 2-mercaptoethanol and subjected to 15% SDS-PAGE together with prestained molecular mass standards. Following electrophoresis, the gels were destained, dried, and subjected to autoradiography.

** Xenopus laevis Oocyte Injection**—Oocytes were injected with in vitro transcribed aiPLA2 mRNA (50–70 ng/oocyte) or an equal volume (50 nl) of deionized H2O (mock injection) and incubated generally for 48 h at 18°C in modified Barth’s solution (17), which contains 88 mM NaCl, 1 mM CaCl2, 2.4 mM NaHCO3, 0.82 mM MgSO4, 0.33 mM Ca(NO3)2, 0.41 mM KCl, and 10 mM NaHEPES. Oocytes were washed with saline, homogenized in pH 4 buffer, and then assayed for PLAA activity. Measurements were performed on groups containing at least five oocytes.

**PLAA Assay Using Liposomes**—Enzyme activity was measured at pH 4 (40 mM acetate buffer with 5 mM EDTA) using either a liposome-based radiochemical assay as described previously (9) or a fluorescence assay (bisbodipy-C11-PC) for rapid screening. For the radiochemical assay, the liposomes were formulated with 1-palmitoyl-2-

\[ ^{3}H \]PPC/egg PC/egg phosphatidylglycerol/cholesterol in a molar ratio of 10:5:2:3. The specific activity of [3H]PPC was 4400 dpm/nmol. Lipids were dried under N2, resuspended in isotonic saline, repeatedly freeze/thawed by alternating liquid N2 and warm H2O, and then extruded through a 100-μm membrane to generate unilamellar liposomes. The 250-μL PLAA assay reaction
FIG. 1. Column chromatography profiles for rat lung alPLA₂. Protein in buffer (50 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 5% glycerol) was applied sequentially to DE52, Sephacryl S-100, and DE52 columns. Fractions from each column were collected and measured by the fluorescence assay for Ca²⁺-independent PLA₂ activity at pH 4 in the absence (●) and presence (△) of MJ33 and for protein content (○). A, DE52. The column was washed with 2 bed volumes of buffer (fractions 0–42) and then eluted by a linear gradient of 0–50 mM NaCl at a flow rate of 60 ml/h. B, Sephacryl S-100. MJ33-inhibitable active fractions from the DE52 columns (bracketed in A) were applied to a Sephacryl-100 column. The column was eluted with buffer at a flow rate of 17 ml/h. C, second DE52. MJ33-inhibitable active fractions from the Sephacryl-100 column (bracketed in B) were applied to a second DE52 column. The column was washed with 5 bed volumes of buffer and then eluted with a linear gradient of 10–60 mM NaCl at a flow rate of 60 ml/h (dashed line). MJ33-inhibitable fractions (bracketed) to a phenyl-Sepharose column (see text).
CHCl₃/CH₃OH (2:1, v/v); lipids were extracted by the method of Bligh and Dyer (18) for 1 h. Enzyme activity in the assay showed linear increase with incubation time between 15 and 120 min with both wheat germ- and sea urchin-expressed proteins. The reaction was stopped by the addition of 50 mM glycine (pH 3), 50 mM acetate (pH 4–5), 50 mM MES (pH 6.5), and was extracted with CHCl₃/CH₃OH/NH₄OH/H₂O (65:35:2.5:2.5 by volume) (19) to separate lyso-PC in order to assay for PLA₁ activity. In vitro, radiolabeled free fatty acids were separated by a two-step TLC procedure using hexane/ether/acetic acid as a solvent system (9). Authentic palmitic acid was co-chromatographed. The free fatty acid spots were identified using I₂ vapor, scraped from the plate, and analyzed by scintillation counting using an internal standard for quench correction. Recovered dpm was corrected for blank values obtained in the absence of enzymes. PLA₂ activity was calculated based on the specific radioactivity of DPPC. In some experiments, lipids were extracted with CHCl₃/CH₃OH (20:1) (19). Enzyme was incubated with the specific radioactivity of DPPC. In some experiments, lipids were extracted with CHCl₃/CH₃OH (20:1) (19). Enzyme was incubated with 50 mM [³H]DPPC (specific activity, 4400 dpm/nmol) and 4 mM choline-phorylcholine or its degradation products).

The 35 amino acids that are identical to those obtained by sequence analysis of peptides generated by tryptic digestion of the 26.3-kDa band shown in Fig. 2. The putative lipase motif (GXXG) (see “Discussion”) is double-underlined. i, translation initiation site.

To test substrate specificity of PLA₂, liposomes containing PC with a molar ratio of 10:3.2:4.3 (PC/PA/PS) for 1 h at 37°C. Lipid radioactivity was assayed as described above for the liposomal assay. PLA₂ Assay Using Mixed Micelles—Mixed micelles were prepared with 10 mM [³H]DPPC specific activity, 4400 dpm/nmol) and 4 mM Triton X-100 in saline by sonication using a cuphorn sonicator (Heat Systems Ultrasonics, Plainview, NY) at 70% of maximum power with 20-s bursts at 30-s intervals. For PLA₂ assay, 50 μl of enzyme, 850 μl of buffer, and 100 μl of micelles (final DPPC concentration, 1 mM) was incubated at 37°C for 60 min in the presence or absence of Ca²⁺. Lipid radioactivity was assayed as described above for the liposomal assay.

Lyso phospholipase Assay—To measure lyso phospholipase activity, the [³H]lyso-PC substrate was generated by treating liposomes labeled with [choline-³H]DPPC with N. naja PLA₂ at pH 8.5 plus 10 mM Ca²⁺ to achieve complete hydrolysis. [³H]Lyso-PC was separated from the incubation mixture by TLC, and the lyso-PC spot was scraped and analyzed by scintillation counting using an internal standard for quench correction. Recovered dpm was corrected for blank values obtained in the absence of enzymes. PLA₂ activity was calculated based on the specific radioactivity of DPPC. In some experiments, lipids were extracted with CHCl₃/CH₃OH (20:1) (19). Enzyme was incubated with labeled lyso-PC as substrate at pH 4.0 or 8.5 for 1 h at 37°C. Lipids were extracted by the method of Bligh and Dyer (18), and the aqueous fraction was assayed for radioactivity (representing [³H]glycerophosphorylcholine or its degradation products).

PAP Acetylhydrolase Activity—The incubation medium contained 50 mM Tris-HCl (pH 4 or 7.4), 5 mM EDTA, and 20 nmol of lauroyl-¹⁵⁹H]PAP in a total volume of 250 μl. After 60 min of incubation at 37°C, the

| Purification step | Total protein | Total activity | Specific activity | Enzyme recovery | Purification |
|------------------|--------------|---------------|------------------|-----------------|-------------|
| 10⁵ × g supernatant | 2680 | 2230 | 0.87 | 100 | 1 |
| 55% (NH₄)₂SO₄ (precipitate) | 1820 | 2190 | 1.2 | 94 | 1.4 |
| DE52 | 19 | 198 | 10.4 | 8.5 | 12 |
| Sephacryl S-100 | 5.5 | 189 | 3.43 | 8.1 | 40 |
| DE52 | 0.17 | 24 | 141 | 1.0 | 163 |

Fig. 2. SDS-PAGE of rat lung aiPLA₂. The partially purified fractions (5 μg of protein) with aiPLA₂ activity from the second DE52 lane 1, pooled from the second DE52 column (see Fig. 1C); lane 2, pooled H₂O wash fraction from the phenyl-Sepharose column. Lane 3, pooled H₂O wash fraction from the phenyl- Sepharose column.

The incubation medium contained 50 mM sodium acetate buffer (pH 4) plus 1 mM EDTA. Incubation was at 37°C generally for 1 h. Enzyme activity in the assay showed linear increase with incubation time between 15 and 120 min with both wheat germ- and oocyte-expressed proteins. The reaction was stopped by the addition of CHCl₃/CH₃OH (2:1, v/v); lipids were extracted by the method of Bligh and Dyer (18); and radiolabeled free fatty acids were separated by a two-step TLC procedure using hexane/ether/acetic acid as a solvent system (9). Authentic palmitic acid was co-chromatographed. The free fatty acid spots were identified using I₂ vapor, scraped from the plate, and analyzed by scintillation counting using an internal standard for quench correction. Recovered dpm was corrected for blank values obtained in the absence of enzymes. PLA₂ activity was calculated based on the specific radioactivity of DPPC. In some experiments, lipids were extracted with CHCl₃/CH₃OH (20:1) (19). Enzyme was incubated with 50 mM [³H]DPPC (specific activity, 4400 dpm/nmol) and 4 mM choline-phorylcholine or its degradation products.

The 35 amino acids that are underlined are identical to those obtained by sequence analysis of peptides generated by tryptic digestion of the 26.3-kDa band shown in Fig. 2. The putative lipase motif (GXXG) (see “Discussion”) is double-underlined. i, translation initiation site.

MatTris containing 0.5 mM EDTA. The concentrations selected for testing of inhibitors have been shown previously to produce maximal effect in other systems (9, 20, 21).

TABLE I

| Purification step | Total protein | Total activity | Specific activity | Enzyme recovery | Purification |
|------------------|--------------|---------------|------------------|-----------------|-------------|
| 10⁵ × g supernatant | 2680 | 2230 | 0.87 | 100 | 1 |
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pyrocarbonate-treated H₂O, quantitated by absorbance at 260 nm, and chloroform extraction method (24). The RNA was dissolved in diethyl homogenized rat lungs using the acid guanidinium thiocyanate/phenol/chloroform extraction method. The RNA was extracted from freshly isolated granular pneumocytes or from elastase digestion of rat lungs as described previously (23). Total RNA was used to determine the amount of acetate liberated.

To determine if the human cDNA encodes a translatable protein, three internal peptides comprising 35 amino acids were sequenced (Fig. 3). Enzymatic properties of the partially purified protein from the second DE52 column. activity that eluted from the phenyl-Sepharose column showed 100% identity (Fig. 3) to a reported translated open reading frame of unknown function from a human male myeloblast cell line, KG-1 (11). Fig. 3 illustrates the nucleotide sequence of this 1653-base pair cDNA (HA0683) and its deduced amino acid sequence comprising 224 residues (calculated Mr = 25,032). In addition to the putative protein coding sequence, this clone contains 44 base pairs of upstream and 934 base pairs of downstream sequence (Fig. 3).

Expression in Wheat Germ In Vitro Translation System—To determine if the human cDNA encodes a translatable protein with a molecular mass similar to that of rat lung protein, HA0683 cDNA was transcribed in vitro with T7 polymerase and translated in vitro using a wheat germ system. SDS-PAGE reaction was stopped by the addition of 2.5 ml of CHCl₃/CH₃OH (4:1) and 250 µl of H₂O (22). The aqueous phase was assayed for radioactivity to determine the amount of acetate liberated.

RESULTS

Isolation of Rat Lung PLA₂—By the assay used to identify fractions during the isolation procedure, the isolated rat lung protein was active at acid pH in the absence of Ca²⁺ and was inhibited by MJ33. The sequential use of the DE52, Sephacryl S-100, and repeat DE52 columns resulted in 163-fold increase in PLA₂ specific activity, although 99% of the starting activity was lost (Table I). The subsequent phenyl-Sepharose column resulted in further purification as determined by SDS-PAGE (Fig. 2), although insufficient protein was recovered for enzymatic characterization. Enzymatic properties of the preparation were analyzed with the partially purified protein from the second DE52 column.

Identification of the Human cDNA Clone Corresponding to Rat Lung aiPLA₂—SDS-PAGE of the fraction with aiPLA₂ activity that eluted from the phenyl-Sepharose column showed two proteins bands with apparent molecular masses of 26.3 and 25.0 kDa (Fig. 2), and these were used for internal amino acid sequencing. Fragments produced by tryptic digests of these two proteins were subjected to amino acid sequence analysis. For the 25.0-kDa protein, two fragments (25 amino acids total) were sequenced and found to have significant homology to rat brain thiol-specific antioxidant (25). Therefore, this protein was not further evaluated. For the 26.3-kDa protein, three internal peptides comprising 35 amino acids were sequenced and showed 100% identity (Fig. 3) to a reported translated open reading frame of unknown function from a human male myeloblast cell line, KG-1 (11). Fig. 3 illustrates the nucleotide sequence of this 1653-base pair cDNA (HA0683) and its deduced amino acid sequence comprising 224 residues (calculated Mr = 25,032). In addition to the putative protein coding sequence, this clone contains 44 base pairs of upstream and 934 base pairs of downstream sequence (Fig. 3).

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with autoradiography of the translated [35S]methionine-labeled protein (Fig. 4) showed an apparent molecular mass of 25.8 kDa, similar to the mass of the predicted protein and of the PLA2 enzyme isolated from rat lung (Fig. 2). [35S]Methionine-labeled protein expressed by the wheat germ system increased as a function of RNA concentration, with saturation at 1.0 mg in a 50-μl reaction volume (Fig. 4). Saturation of the wheat germ system at higher concentrations of RNA has been shown previously (26).

Ten separate in vitro translations were carried out with the wheat germ expression system; although these used varying concentrations of cRNA, in each we found the expression of an enzyme activity with the characteristics of aPLA2 (assay at pH 4 in Ca2+-free buffer). PLA2 activity of the expressed protein measured by the radiochemical assay increased linearly as a function of the cRNA concentration used for expression up to 1 μg (Fig. 5). For translation with 1 μg of cRNA, expressed PLA2 activity was 1556 ± 20 pmol/h (mean ± S.E.; n = 7). Control incubation with the wheat germ system in the absence of cRNA showed zero PLA2 activity. To confirm PLA2 activity, generation of free fatty acid from [3H]palmitoyl-labeled DPPC and of [3H]lyso-PC from [3H]choline-labeled DPPC was measured in the same assay using protein expressed in the wheat germ system. Liberation of the free fatty acid was 1288 ± 32 pmol/h, and generation of lyso-PC was 1273 ± 27 pmol/h (mean ± range; n = 2). Blank values for PLA2 assays were <250 dpm, and the usual value for expressed enzyme was ~6000 dpm, giving a satisfactory signal-to-noise ratio of ~24.

Characterization of aPLA2 Activity—The standard PLA2 assay was carried out in the absence of Ca2+. The activities of both the expressed PLA2 at pH 3–8.5 as well as the partially purified rat lung enzyme at pH 4 were not affected by the addition of Ca2+ (Fig. 6). The pH versus activity profile for the isolated lung enzyme showed maximal activity at pH 4 and essentially no activity at pH 6 and above (Fig. 6). The pH profile for the expressed protein was similar (Fig. 6). The pH profile and Ca2+-independence for the expressed protein were similar using either the liposomal or micellar assay system (Fig. 6).

aiPLA2 expressed in vitro showed similar activity using dipalmitoyl-PC or palmitoylarachidonoyl-PC as substrate (Fig. 7 and Table II). The apparent K_m for the substrates was ~250 μM, PLA2 activity using the alkyl ether phospholipid, O-hexadecylarachidonoyl-PC, as substrate at 1 μM was lower by ~50% compared with DPPC (Table II), although the apparent K_m was similar (Fig. 7). In vitro translated aiPLA2 did not show any PLA1 or lyso phospholipase activity, and PAF acetylhydrolase activity was negligible (Table II).

Potential inhibitors (AACOCF3 (100 μM), BEL (100 μM), pBPB (20 μM), and 2-mercaptoethanol (5 mM)) were tested for their effect on the activity of the isolated rat lung enzyme and in vitro translated aiPLA2 (Table III). ATP (10 mM), an activator of myocardial iPLA2 (27), was also evaluated. None of these agents had a significant effect on the activity of the rat lung or expressed enzyme. MJ33 significantly inhibited the activity of
aiPLA₂ (Table III), with a maximal effect at 1 mol % (Fig. 8). Because of the “lipase” motif (Fig. 3), the effect of the serine protease inhibitor DENP was evaluated and was found to significantly inhibit aiPLA₂ activity, with 80% inhibition at 0.5 mM (Fig. 8 and Table III).

**Expression of Human aiPLA₂ in Xenopus Oocytes**—To further evaluate the protein product of the HA0683 clone, cRNA generated from the clone was expressed in Xenopus oocytes and assayed for aiPLA₂ activity. Expressed aiPLA₂ activity increased as a function of time after oocyte injection, reaching a maximum at 30 h, and maintained this level at 48 h post-injection (Fig. 5). Further studies were carried out using oocytes at 48 h after injection. In each of seven separate injection experiments, we observed an increase in aiPLA₂ activity compared with oocytes injected with deionized H₂O, with a mean increase of ~40% (Table IV). Similar results were obtained using both the fluorescence and radiochemical assays. For the radiochemical assay, the blank value (~250 dpm representing ≤50 dpm/oocyte) was subtracted from the measured PLA₂ activity; this gave a signal-to-noise ratio of ~10 for the expressed PLA₂ activity or ~4 for the difference between cRNA- and deionized H₂O-injected oocytes. To confirm PLA₂ activity, liberation of free fatty acid and generation of lyso-PC were compared in the same assay; liberation of free fatty acid was 580 ± 2 dpm/oocyte, and generation of lyso-PC was 580 ± 6 dpm/oocyte (mean ± range; n = 2). Activity was not changed in the presence of 10 mM Ca²⁺, but was decreased to the basal value in the presence of 3 mol % MJ33 (Table V). When assayed at pH 8.5 in the presence of Ca²⁺, activity decreased by 90% compared with pH 4. These results parallel those obtained with the wheat germ expression system and the isolated lung enzyme.

**Northern Analysis**—Since the cDNA clone was isolated from a human myeloblast cell line, it was of interest to test whether this mRNA is present in rat lung, the source of the original protein isolation. Northern analysis of aiPLA₂ RNA demonstrated high levels of expression of a hybridizing mRNA, ~1.7 kilobase pairs in size, in both rat lung and granular pneumocytes isolated from rat lung (Fig. 9).

**DISCUSSION**

iPLA₂ enzymes have been shown to be widely distributed and ubiquitously expressed in most mammalian tissue, underlining their potential importance in cellular functions (3). Although mammalian iPLA₂ enzymes have been isolated and characterized from various sources, there is scant molecular information due to difficulties associated with purification and insufficient yield. To date, the only iPLA₂-type enzyme that has been sequenced is a PAF hydrolase (28), which also has properties of a low density lipoprotein-associated PLA₂ (29). Therefore, little is known about the structure or mechanisms of iPLA₂ enzymes or their relationship to other PLA₂ enzymes. Here, we describe the cloning, sequencing, and characterization of a human iPLA₂ (aiPLA₂) that shows maximal activity in an acidic medium.

**Evidence for aiPLA₂ Activity of Expressed Protein**—Expression of the HA0683 cDNA using both the wheat germ and Xenopus oocyte systems demonstrated PLA₂ activity. In vitro translated aiPLA₂ did not show any PLA₁ or lysophospholipase activity, excluding the possibility that the combined activities of those two enzymes could have accounted for the measured PLA₂ activity. Furthermore, assays of activity with both wheat germ- and oocyte-expressed enzyme based on recoveries of labeled free fatty acid from sn-2-fatty acyl-labeled DPPC and labeled lyso-PC from choline-labeled DPPC were nearly identical, indicating PLA₂ for the lipolytic activity. The pH 4 optimum for activity and the Ca²⁺ independence indicated that the
protein encoded by the HA0683 cDNA clone has enzymatic properties that correspond to the activity profile for the partially purified rat lung enzyme as well as the activity previously demonstrated in homogenates of rat lungs and granular pneumocytes (7, 30). Northern analysis demonstrated that this gene is highly expressed in rat lung and granular pneumocytes (Fig. 9). aiPLA₂ activity was significantly inhibited by MJ33, a competitive inhibitor of acidic Ca²⁺-independent PLA₂ activity in lung homogenates and subcellular organelles (lysosomes and lamellar bodies) (7, 30), and by a serine protease inhibitor, DENP. Unlike other iPLA₂ enzyme activities that have been characterized, the activity of the expressed protein was not affected by the presence of detergent (Triton X-100), and neither it nor the isolated rat lung enzyme was affected by the inhibitor BEL or the activator ATP (27, 31). Thus, it appears that aiPLA₂ presented in this report is distinct from the iPLA₂ enzymes previously purified from the P388D₁ macrophage cell line (20) and from cardiac muscle (27). The relative activity of PAF acetylhydrolase, a Ca²⁺-independent enzyme that can cleave short-chain acyl groups at the sn-2-position (22), was negligible. The isolated rat enzyme and the expressed protein are differentiated from sPLA₂ and cPLA₂ by their Ca²⁺-independence and pH profile as well as by insensitivity to inhibitors (pBPB, 2-mercaptoethanol, and AACOCF₃).

Properties of the Predicted Protein—The HA0683 cDNA encodes a mature protein of 224 amino acids with a calculated molecular mass of 25.0 kDa. We have shown in a wheat germ in vitro translation system that mRNA transcribed from this clone results in the expression of a protein of 25.8 kDa in size, in reasonable agreement with the predicted mass of the deduced amino acid sequence and with the estimated mass of aiPLA₂ isolated from rat lung. The predicted protein has 32 (14.3%) negatively charged and 30 (13.4%) positively charged residues, with no predicted charge clusters and a predicted pI of 6.0 (32). Nonpolar residues account for 107 (48%) of the 224 amino acids in the predicted sequence. A hydrophobicity plot of the predicted protein did not indicate any extended regions of
high hydrophobicity, consistent with the fact that aiPLA2 was isolated as a soluble protein.

**Sequence Homology to Other Proteins**—Searches for similarity to the HA0683 cDNA sequence at the protein (SWISSPROT protein data base) and DNA (GenBankTM data base) levels did not identify any phospholipases. Therefore, aiPLA2 is apparently not related to any of the phospholipases previously sequenced. The predicted aiPLA2 enzyme contains a 5-amino acid sequence, GXSGX (double underline in Fig. 3), that has been described as a lipase motif and may represent the active serine of the catalytic triad SDH (33). Inhibition by DENP supports a serine-based mechanism for aiPLA2 activity. The motif is present in both iPLA2 enzymes (aiPLA2 and PAF acetylhydrolase) that have been cloned, although it is not observed in sPLA2 or cPLA2.

From the protein data base, the amino acid sequence with the most similarity to an HA0683-encoded protein was a hypothetical 29.5-kDa protein from yeast (SWISSPROT ID YBG4) (34), which matched at 99 out of 261 amino acid positions. A search for similarity to the deduced N-terminal sequence of aiPLA2 revealed homology to two human unknown proteins isolated by two-dimensional gel electrophoresis from liver (SWISSPROT ID P30041) (35) and red blood cells (SWISSPROT ID P32077) (36). These sequences, consisting of 14 and 12 amino acids, respectively, showed 100% homology to the predicted aiPLA2 sequence following its deduced start site. Electrophoresis showed molecular masses of 23.5 kDa for the liver protein (35) and 26 kDa for the red blood cell protein (36), with an identical pI of 6.2. The similarities in N-terminal sequence, size, and pI to the parameters predicted for the aiPLA2 RNA using a 5’ rapid amplification of cDNA ends (Proehm, M. A. (1995) Methods Enzymol. 218, 340–358) and found an additional 24 nucleotides but no upstream ATG translational start codon.

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**Note Added in Proof**—We have now cloned and sequenced the actual 5’ end of the aiPLA2 RNA using a 5’ rapid amplification of cDNA ends (Proehm, M. A. (1995) Methods Enzymol. 218, 340–358) and found an additional 24 nucleotides but no upstream ATG translational start codon.

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