Murine and Human Autotaxin α, β, and γ Isoforms

GENE ORGANIZATION, TISSUE DISTRIBUTION, AND BIOCHEMICAL CHARACTERIZATION*

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Autotaxin is a type II ectonucleotide pyrophosphatase phosphodiesterase enzyme. It has been recently discovered that it also has a lysophospholipase D activity. This enzyme probably provides most of the extracellular lysophosphatidic acid from lysophosphatidylcholine. The cloning and tissue distribution of the three isoforms (imaginatively called α, β, and γ) from human and mouse are reported in this study, as well as their tissue distribution by PCR in the human and mouse. The fate of the α isoform from human was also studied after purification and using mass spectrometry. Indeed, this particular isoform expresses the intron 12 in which a cleavage site is present, leading to a rapid catabolism of the isoform. For the human isoform γ and the total autotaxin mRNA expression, quantitative PCR is presented in 21 tissues. The isoforms were expressed in two different hosts, insect cells and Chinese hamster ovary cells, and were highly purified. The characteristics of the six purified isoforms (pH and temperature dependence, Kₘ values, and their dependence on metal ions) are presented in this study. Their sensitivity to a small molecule inhibitor, hypericin, is also shown. Finally, the specificity of the isoforms toward a large family of lysophosphatidylcholines is reported. This study is the first complete description of the reported autotaxin isoforms.

Autotaxin (ATX) is a member of the nucleotide pyrophosphatase/phosphodiesterase family of ectoenzymes (E-NPP) that hydrolyze phosphodiester bonds of various nucleotides and nucleotide derivatives (1–4). It was initially identified as a membrane-standing glycoprotein of 125 kDa and a brain-specific member of the phosphodiesterase I gene family (4). E-NPPs belong to a multigene family that currently contains five members. NPP1–3 are type II transmembrane glycoproteins characterized by a similar modular structure composed of a short N-terminal intracellular domain involved in the targeting to the plasma membrane, a single transmembrane domain, and a large extracellular domain (1) encompassing two consecutive somatomedin-B-like domains, a catalytic domain, and a poorly characterized C-terminal domain. It has recently been discovered that autotaxin also catalyzed a lysophospholipase D activity (5–7). Initially believed to be a soluble autotaxin/lysoPLD derived from a membrane-bound protein by extracellular proteolytic cleavage (8), it is now clear that autotaxin is synthesized as a prepro-enzyme and that the proteolyzed protein is secreted (9, 57). A recent report established that its secretion from adipocytes to extracellular milieu was driven by N-glycosylation and signal peptide cleavage. N-Glycosylation was a key element in the regulation of the enzyme catalytic activity, whereas the holoenzyme was mainly cytoplasmic in adipocytes (9). The two catalytic activities (phosphodiesterase and lysophospholipase D) are catalyzed by the same catalytic site (10, 11). Three isoforms of autotaxin have been described as follows: mel (from human melanoma cell line A2058), ter (from human teratocarcinoma cell line), and PD-1α (from rat brain) (12–15). Most of the biochemical data available in the literature on these autotaxin isoforms were obtained from purified, natural sources or from a single recombinant protein, probably the β isoform (5, 6, 10, 13, 16–20).

Lysophospholipid D (lyso-PLD) catalyzes the transformation of lysophosphatidylcholine (LPC) in lysophosphatic acid (LPA) (21). LPA is a bioactive phospholipid regulating a wide range of cellular responses (proliferation, survival, motility, ion flux, and secretion) through the activation of G-protein-coupled receptors as follows: LPA₁, LPA₂, and LPA₃ (22, 23) and two more distantly related receptors LPA₄ and LPA₅ (24). For instance, LPA is able to activate preadipocyte motility and proliferation, and to inhibit adipocyte differentiation by interacting preferentially with LPA₁ (25–27). Autotaxin is secreted from adipocytes (7) and its expression is strongly up-regulated during adipocyte differentiation as well as in adipocytes isolated from obese/diabetic db/db mice (28). Therefore, autotaxin may be involved in the control of adipose tissue development and/or metabolism. Furthermore, autotaxin might be the unique source of circulating LPA from LPC, at least in the early stages.
of fetal development, because mice deleted of the ATX gene are not viable (19, 29), even if autotaxin in these animals bears a single amino acid mutation in the catalytic site, rendering the enzyme inactive (58).

Concomitantly, autotaxin has been identified as a lyso-PLD from various other sources such as fetal bovine serum (5) and human plasma (6). Autotaxin catalytic activity is present at high concentrations in various biological fluids, including plasma, serum, and seminal plasma (30). Recently, a link has been established between LPA and progression of breast cancer bone metastases (31), whereas a relationship had already been shown between autotaxin and the invasiveness of breast cancer cells (32, 33). Once purified, it was shown that autotaxin was an autocrine motility factor that promotes cancer cell invasion, cell migration, and angiogenesis (2). Indeed, autotaxin was initially described from melanoma cell supernatants (1, 8).

Enhanced expression of autotaxin has been demonstrated in various malignant tumor tissues (34). It is therefore possible that in addition to its possible involvement in the physiopathology of adipose tissue, autotaxin might also play an important role in cancer.

Because LPA is a key extracellular mediator acting through its cell-surface receptors on a wide variety of cells, it seemed important to better characterize the enzyme responsible for its production. To this end, the tissue expressions of the three isoforms in mice and in human are reported, as well as a qPCR analysis of human expression of autotaxin compared with the expression of the isoform γ. Furthermore, the corresponding six isoforms were purified, and their catalytic properties and substrate specificities were described, as well as their sensitivity to various inhibitory species (metal, small molecule, and albumin). We also explain why the α isoform, from both species, was so poorly expressed. This study on autotaxin characteristics is the first completed study on these isoforms.

**EXPERIMENTAL PROCEDURES**

**Compounds**—All chemicals were obtained at the highest purity grade available from Sigma. Phospholipids are as follows: LPC-hexanoyl (6:0), -octanoyl (8:0), -decanoyl (10:0), -lauroyl (12:0), -myristoyl (14:0), -palmitoyl (16:0), -stearoyl (18:0), -arachidoyl (20:0), and -lignoceryl (24:0) were purchased from Avanti Polar Lipids, Inc. Hypericin was purified from various other sources such as fetal bovine serum (5) and human plasma (6). Total RNA from human subcutaneous adipose tissue was obtained from Clontech. Total RNA from human subcutaneous adipose tissue was obtained as described previously (28). cDNA was synthesized from 1 μg of total RNA using (dT)12–18 and reverse transcriptase Superscript II (Invitrogen). A minus RT reaction was performed in parallel to ensure the absence of genomic DNA contamination. The PCR conditions were 30 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min. For the human autotaxin variants, the first set of primers, forward h1 5′-CTCACCTGCGACATCATGA-3′ and reverse h2 5′-CTCAGTTCTATCACATGTGAC-3′, was used with the oligonucleotide probe hAS, 5′-GTTGCCCTAA-GAGAGACA-3′, recognizing specifically the autotaxin α variant, or hC1 5′-GGTGTGGCTACATGTGAC-3′ based on a common region to the three human variants. The second set of primers, forward h3 5′-GGATGGACGCATCTCCTAAT-3′ and reverse h4 5′-CACTGTCAGATGTTCAGG-3′, was used with the oligonucleotide probe hGS, 5′-AGGAAAT-TCAGAGCAGCAG-3′, recognizing specifically the autotaxin γ variant, or hC2 5′-GGATGTGCTTATCCGACTAG-3′ based on a common region to the three human variants. For the murine autotaxin variants, the first set of primers, forward m1, 5′-CTCTACTATCTCAGTGCC-3′ and reverse m2 5′-CTCAGTCAGATGTGCTC-3′, was used with the oligonucleotide probes mAS, 5′-GTTGCCCTAAAGGAGGACA-3′, recognizing specifically the murine autotaxin α isoform, or mC1 5′-GACGGATGTGATTACAT-3′ based on a common region to the three murine variants. The second set of primers, forward m3 5′-GACTAAATGTCCTCCATTG-3′ and reverse m4 5′-CATCGGGCCGAACA-CATTTG-3′, was used with the oligonucleotide probes mGS, 5′-GAAAATTCAGGACAGCAGA-3′, recognizing specifically the autotaxin γ variant, or mC2 5′-GAGTGTGCTTATCCGACTAG-3′ based on a common region to the three murine variants (see complete sequences and details in supplemental Table 1). PCR products were separated by agarose (1%) gel electrophoresis and transferred onto Hybond N+ membrane (Amersham Biosciences). Hybridization was performed at 42 °C with a 32P-labeled oligonucleotide probe. The blots were washed twice at 50 °C in 2× SSC containing 0.1% (w/v) SDS for 30 min each and exposed to a x-ray film.

**Quantitative PCR for the Determination of Global Autotaxin Expression Level in Human Tissues**—Because of the structure of the mRNA corresponding to the three isoforms, no design was possible to selectively determine the amount of the three mRNAs in tissues. Therefore, we performed a global qPCR in a region common to the three isoforms. The mRNAs species of human tissues were purchased from Clontech and reverse-transcribed the SuperScript first strand synthesis system for RT-PCR (Invitrogen). The ABI 7500 PCR and detection system (Applied Biosystems) with SYBR premix Ex Taq™ kit (Takara) was used in qPCR. PCR was conducted in triplicate for each
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sample. Primers were indicated in supplemental Table 1. All primer sets satisfied the requirements for the DDCt method with the absolute value of the slope of log input amount versus Ct <0.1 and the efficiency >99%. The primer set of ATX was selected to amplify all ATX isoforms published to date. PCRs were performed according to the supplier’s instructions (Applied Biosystems) with primers at 300 nM each and 1 µl of the reverse transcription reaction in 10 µl of volume reaction. PCR protocol was as follows: 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Human GAPDH was amplified as an internal standard. Human GAPDH was amplified as an internal standard. Reported values were calculated using DDCt method normalized against endogenous GAPDH. The qPCR experiments for global ATX and ATX isoform γ were performed with the sequences reported in supplemental Table 3.

Autotaxin Production—COS-7 cells (ATCC) served as the expression system for the wild type autotaxin. Cells were maintained in culture at 37 °C under a humidified atmosphere with 5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (PPA Laboratories), penicillin, streptomycin, and glutamine 2 mM (Invitrogen). Cells were transiently transfected with control or autotaxin-expressing pMC1 plasmids using Lipofectamine (Invitrogen) and Lipofectamine TS reagent (Invitrogen), according to the manufacturer’s method. Two days after transfection, the cell layer was washed three times with red phenol-free DMEM supplemented with glutamine (2 mM) and incubated at 37 °C for 6 h in the same DMEM. After incubation, the conditioned medium was harvested, dialyzed, concentrated as described previously, and stored at −20 °C until further use.

Recombinant Autotaxin—The Bac-to-Bac baculovirus expression system (Invitrogen) was used for autotaxin production. Autotaxin cDNA fused to the FLAG-M2 sequence at the C terminus was cloned into the pFastBacI vector (Invitrogen). The resulting plasmid was then used for generating recombinant baculovirus to infect Sf9 insect cells, which were grown in SF-900 II medium (Invitrogen); the multiplicity of infection was ~2, and the time of infection was ~2, × 106 cells/ml. After 48 h of infection at 27 °C, medium containing secreted autotaxin was centrifuged (500 × g for 10 min) and frozen at −80 °C. The conditioned medium supplemented with proteases inhibitors was then centrifuged (32,000 rpm for 60 min) to remove cells debris and applied onto an anti-FLAG M2 Affinity Gel column (Sigma) equilibrated with the following buffer: 50 mM Tris, pH 7.5, 140 mM NaCl. The bound proteins were eluted with 10 µg/ml FLAG peptide. Purity of autotaxin-containing fractions was >95% as shown by SDS-PAGE and Sypro Ruby staining. All steps in enzyme purification were carried out at 4 °C. All proteins were expressed with a C-terminal FLAG-M2 tag.

SDS-PAGE—SDS-PAGE 4–20% was performed according to Laemmli (36) followed by Sypro Ruby or colloidal blue staining. After addition of the sample buffer (Novex, Invitrogen), concentrated fractions from the gel filtration step were boiled at 100 °C for 5 min. Electrophoretic separation of proteins was carried out on a 1-mm-thick 8 × 6-cm gel 10% acrylamide precasted gels (NOVEL and Invitrogen). A 40-µg portion of the total protein in sample buffer was loaded into a 4-mm well of the gel and separated at 40 mA. A total of 30 µg of standards (Mark12, Invitrogen) migrated in a neighboring lane. After coloration with colloidal Coomassie Blue (Biosafe, Bio-Rad), proteins were cut, reduced, and alkylated using dithiothreitol and iodoacetamide, respectively, and subjected to digestion with trypsin overnight. Extracted peptides were SpeedVac®-concentrated and then desalted on Poros 50 R2 chromatographic phase. Peptides were eluted by 2 µl of a 60% methanol, 40% water, 2% formic acid solution directly in nanoelectrospray needles.

Western Blotting—Western blotting was performed as reported previously after protein separation with SDS-PAGE four–20% gels (Invitrogen), which were routinely run until the dye front migrated out of the gel followed either by Sypro Ruby staining or by transfer onto nitrocellulose membrane with iBlot® Gel Transfer Device (Invitrogen). The membranes were treated during 3 h at room temperature with 5% (w/v) dried skim milk in 20 mM Tris-HCl, pH 8.0, containing 0.15 mM NaCl. The membrane were washed and then incubated overnight at 4 °C with chicken polyclonal anti-autotaxin diluted at 0.1 µg/ml or with monoclonal antibody anti-FLAG-M2 diluted at 10 µg/ml, followed by extensive washing with 20 mM Tris-HCl, pH 8.0, containing 0.15 mM NaCl. Immunoreactive proteins were visualized by treatment for 1 h with anti-chicken IgG-peroxidase (1/50,000) or anti-rabbit IgG-peroxidase complex (1/30,000) (Sigma) and then visualized using a peroxidase immunostaining kit (Amersham Biosciences). Horseradish peroxidase-goat anti-rabbit or anti-chicken IgG was used as a secondary antibody for all primary antibodies. The proteins bands were run with an enhanced chemiluminescence kit (ECL, Amersham Biosciences), followed by exposure to Hyperfilm ECL.

Antibodies—Polyclonal anti-autotaxin antibody was raised against the full-length β isoform protein. Chicken antisemur was purified by affinity chromatography. The monoclonal anti-FLAG M2 was purchased from Sigma and allowed to detect the purified protein.

Phosphodiesterase Enzymatic Assay—The phosphodiesterase activity of recombinant autotaxin was measured by using pNpp substrate in test using a modification of the method of Razzell and Khorana (37). The recombinant autotaxin proteins were used as an enzyme source. Samples (10 µl) were incubated in 96-well plates in a final volume of 100 µl containing p-nitrophenyl phenylphosphonate at 5 mM final concentration in a 50 mM Tris-HCl, pH 9.0 buffer. After 30 min at 37 °C, reactions were stopped by addition of 100 µl of 0.1 M NaOH. The production of p-nitrophenol was kinetically measured by following the absorbance at 410 nm using a Pherastar plate reader (BMG, Offenburg, Germany) and after subtraction of blank.

Fluorogenic Enzymatic Assay—This assay was based on the Amplex® Red PLD assay kit (Molecular Probes, Interchim, Montluçon, France) originally designed for the measurement of the PLD activity. In this enzyme-coupled assay, PLD cleaves phosphatidylcholine (PLC) to yield choline and phosphatidic acid. The choline produced by the reaction was in turn oxidized by choline oxidase into betaine and H2O2. Finally, H2O2 reacted, in the presence of horseradish peroxidase, with Amplex Red to generate a compound, resorufin, the fluores-
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After vortexing, the mixture was allowed to stand at −20 °C and then centrifuged for 15 min at 4 °C (centrifuge Jouan MR23i). The pellet was washed once with 200 μl of cold acetone as described above. The pellet was resuspended in 50% acetonitrile, 0.1% trifluoroacetic acid. Samples were prepared by mixing 1 μl of resuspended pellet and 1 μl of sinapinic acid matrix solution (10 mg/ml acetonitrile/H2O 50/50, 0.1% trifluoroacetic acid), before loading on the target. Spectra were acquired in positive linear mode on a Voyager-DE PRO MALDI-TOF spectrometer (Applied Biosystems). The target voltage was 25 kV, first grid 92%, and the delay extraction 1300 ns. The external calibration was done with an IgG.

Biochemical Characterizations of Marine and Human Autotaxin Isoform Catalytic Activities—Purified autotaxin isoforms were incubated in the phosphodiesterase assay in the presence of increasing concentrations of substrates. Initial rates of pNP formation, measured after 30 min of incubation by light absorbance at 410 nm, are plotted against increasing substrate concentrations and fitted according to the Michaelis-Menten equation using GraphPad software. Nonlinear regression analyses of the kinetic data with calculation of Vmax and Km were performed utilizing the statistical software package Prism 4.0.

Effects of Chelating Agents in Metal Ions on PDE Activity of Autotaxin—Purified autotaxin was preincubated with EDTA or EGTA for 30 min at 37 °C in a PBC buffer (6.66 mM citric acid, 6.66 mM phosphoric acid, 11.44 mM boric acid, 68.6 mM NaOH in final concentration), and its PDE activity was assayed. Data are expressed as the mean ± S.E. of three replicates and are representative of three experiments. Similarly, purified autotaxin isoforms were incubated with increasing concentrations of metal ions. The metal solutions were prepared in the incubation buffer. Concentrations were checked from 150 mM to 100 μM, and their influence was compared with control, without added metals. All the experiments were done three times, each time in triplicate.

RESULTS

Human and Murine Autotaxin Gene Structure—The schematic organization of the murine autotaxin gene, deduced from sequence comparison between the spliced variants cDNAs and the genomic clone AC123644, is shown in Fig. 1. For the general reader, the activity catalyzed by autotaxin is schematized in the Fig. 1A. In mouse, this gene spans more than 80 kbp and contains at least 27 exons. Exon 12 encodes the 52-amino acid cence of which was measured. For the measurement of lysoplasma diacylglycerol phosphatase (lysoplasmalipase), the assay was modified by replacing phosphatidylcholine with lysopalmitoylphosphatidylcholine (Sigma). The assay was performed in 50 mM Tris-HCl, 5 mM CaCl2, pH 8.0, and purified lyso-PLD in 96-well plates. The reaction was started by the addition of 100 μl of the Amplex Red mixture (lysopalmitoylphosphatidylcholine, oleoyl 500 μM, choline oxidase, horseradish peroxidase, and Amplex Red reagent). After 30 min of incubation at 37 °C, the fluorescence was measured with a spectrofluorimeter (PolarStar Galaxy, BMG, Offenburg, Germany) using 530- and 590-nm filters for excitation and emission, respectively. Nonlinear regression analyses of the kinetic data with calculation of Vmax and Km values were performed using the statistical software package Prism 4.0 (GraphPad Software, Inc.).

Amino Acid Sequence Analysis and Mass Spectrometry Analysis—These analyses were custom performed at Proteodynamics (Clermont-Ferrand, France). Purified human autotaxin β was separated by 8% SDS-PAGE, transferred to polyvinylidene difluoride membrane, and stained by Coomasie Blue before N-terminal sequencing. The 105- and 66-kDa bands were cut out separately and analyzed. Amino acid sequence analysis was performed by the Edman degradation procedure using an Applied Biosystems Procise 492A protein sequencer. After affinity purification, Tris buffer was removed on 4-ml aliquot of purified human autotaxin by acetone precipitation (3 ml of cold acetone (−20 °C) were added per ml of protein). The N-terminal amidation procedure was then performed using an Applied Biosystems Procise 492A protein sequencer. After N-terminal sequencing, the activity catalyzed by autotaxin is schematized in the Fig. 1B. The activity catalyzed by autotaxin is schematized in the Fig. 1C. The activity catalyzed by autotaxin is schematized in the Fig. 1D.
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<table>
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<tr>
<th>Current name</th>
<th>Source of historical cloning (Ref.)</th>
<th>No. of amino acids</th>
<th>Exon characteristics</th>
<th>Historical name</th>
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</thead>
<tbody>
<tr>
<td>ATX α, human</td>
<td>From A2058 cells (12)</td>
<td>915</td>
<td>Lacks exon 21</td>
<td>ATX NPP2α, ATX mel, PDN2α, E-NPP2β</td>
</tr>
<tr>
<td>ATX β, human</td>
<td>From Ntera2D1 cells (13)</td>
<td>863</td>
<td>Lacks exons 12 and 21</td>
<td>ATX NPP2β</td>
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<tr>
<td>ATX γ rat</td>
<td>From human retina cDNA library (38)</td>
<td>885</td>
<td>Lacks exon 12</td>
<td>PD-1 α, NPP2γ</td>
</tr>
<tr>
<td></td>
<td>From rat brain (15)</td>
<td>889</td>
<td></td>
<td>E-NPP2γ</td>
</tr>
</tbody>
</table>

Insertion of the murine autotaxin α isoform (amino acids 324–375), whereas exon 21 encodes the additional 25 amino acids of the murine autotaxin γ isoform (amino acids 593–617). Surprisingly, this complex gene organization was maintained throughout evolution. Indeed, the human gene (as seen from the genomic clone 107960) is identical to that of the mouse with their common 27 exon and 26 intron organization.

Cloning of the Human and Murine Autotaxin Isoforms—Autotaxin (also known as NPP2α) was originally cloned from the human melanoma cell line A2058 (12). A splice variant, now termed NPP2β, was reported in human teratocarcinoma (13) and human retina cDNA libraries (38). The major difference between these two isoforms NPP2α and NPP2β is a 156-bp insert coding for 52 amino acids in the central domain of the melanoma autotaxin. A third variant, PD-1α or NPP2γ, isolated from rat brain, lacks the same 52-amino acid insertion found in melanoma autotaxin but contains an additional 25 residues in the C-terminal third of the protein (15) (see Table 1 for nomenclature and details). To see whether this third variant was also present in human tissues, RT-PCR was conducted using human brain cDNA as template. The resulting PCR products were cloned, and PCR screening was performed using primers spanning the region encoding for the putative 25-amino acid insertion. Sequence analysis revealed that all the isolated clones were identical and displayed an open reading frame of 2667 bp throughout evolution. Indeed, the human gene (as seen from the genomic clone 107960) is identical to that of the mouse with their common 27 exon and 26 intron organization.

Table 1.

Summary of autotaxin isoforms nomenclature

Accession numbers for human isoforms are as follows: hATX α (NM_006209; L35594), hATX β (BC034961; L46720), hATX γ (DD192842; DD192839); for murine isoforms are as follows: mATX α (AK088491; EU131009), mATX β (NM_015744; AF123542, AK161144, BC030264, AK163881, EU131010); and for mATX γ (EU131011).

Tissue Distribution of Alternatively Spliced Autotaxin Transcripts—We next analyzed the expression patterns of α, β, and γ isoforms of autotaxin in various murine and human tissues (see supplemental Table 2 for primer sequences). RT-PCR was carried out using two sets of primers as depicted in Fig. 2, A and B. The PCR products were then characterized by Southern

![Image](http://www.jbc.org/)

**FIGURE 2.** Tissue distribution of alternatively spliced autotaxin mRNA transcripts in human and murine tissues. Reverse transcription was performed on total RNA of murine (A) and human (B) tissues. PCR products from the cDNA are shown and were obtained in RT experiments. The corresponding controls (without RT) were run in parallel and are presented. They all correspond to empty lanes on the figure. The primer sets m1 and m2 and m3 and m4 were designed to distinguish the murine autotaxin variants: α and γ, respectively; whereas the primer sets h1 and h2 and h3 and h4 were designed to distinguish the human autotaxin variants α and γ, respectively. PCR products were characterized with the following oligonucleotide probes: for murine tissues, mc1 and mc2 based on a common region to the three autotaxin transcripts, mg5 based on the 52-amino acid insertion of the autotaxin α sequence, and mgS based on the 25-amino acid insertion of the autotaxin γ variant. For human tissues, the PCR products were characterized with the following oligonucleotide probes: hc1 and hc2 based on common regions to the three autotaxin transcripts, hs5 based on the 52-amino acid insertion of the autotaxin α sequence, and hgS based on the 25-amino acid insertion of the autotaxin γ variant.
blotting with different oligonucleotide probes; C1 and C2 were based on common regions to the three autotaxin transcripts, and AS was based on the 52-amino acid insertion of the autotaxin α sequence, and GS was based on the 25-amino acid insertion of the autotaxin γ variant. In mouse (Fig. 2A), autotaxin β is widely expressed in both brain and peripheral tissues, whereas the autotaxin γ variant showed little variation in its distribution, the various levels of expression were apparently lower. Despite a lower expression, murine autotaxin α was still detected in brain and adipose tissue. In human (Fig. 2B), autotaxin β and γ isoforms displayed different expression patterns. High levels of autotaxin β mRNA expression were detected in peripheral tissues, whereas lower expression levels were observed in the central nervous system. In contrast, the highest levels of mRNA expression for the autotaxin γ variant were detected in total brain, whereas significantly lower expression levels were observed in peripheral tissues. In the central nervous system, this isoform was expressed at similar levels across all the brain regions studied (results not shown). The autotaxin isoform α exhibited the lowest expression levels both in the central nervous system and peripheral tissues among the three isoforms. All these data were confirmed by qPCR experiments.

**Quantitative PCR for Autotaxin Expression in Human Tissue**—This work was custom-made by GenomeXpress (Grenoble, France). As pointed out earlier, because of the organization of the autotaxin gene, and of the sequences of the three isoforms, it is not possible to design primers that would specifically recognize the β isoform. The α isoform, however, was represented at a low level, whereas the γ isoform is more expressed. Therefore, we decided to design a set of primers common to all the reported human isoforms (α, β and γ) and a set specific for the human γ isoform. The results are presented in Fig. 3. They are clearly similar to those obtained by regular PCR (Fig. 2). In brief, whereas the γ isoform expression is limited to all the regions of the brain, the autotaxin isoforms are overall particularly expressed in adipocytes and in retina and, to a certain extent, in lung. In the last organ, autotaxin is expressed comparably with the expression in the regions of the brain. When compared with the autotaxin γ expression, it becomes clear, as stated above, that autotaxin in the brain is mainly because of the γ isoform. Furthermore, we demonstrated, by cloning, that adipocytes express only the β isoform. It is worth pointing out that autotaxin is not expressed at a detectable level in human leukocytes. These data are overall comparable with what was reported in the literature, for probably the β isoform (13).

**Expression and Catalytic Activity of the Human and Murine Autotaxin Isoforms**—The six isoforms (three human and three murine) were transiently expressed in COS cells under identical experimental conditions. As a control, we performed Western blot and activity measurements on the unpurified conditioned media from empty vector-transfected cells (supplemental Fig. 1, B and C). In these conditioned media, the lyso-PLD and PDE activities were measured. As shown on supplemental Fig. 1A, the isoforms β and γ were expressed and fully active, whereas the isoform α was barely detectable by Western blot. Lyso-PLD (supplemental Fig. 1C) and PDE (supplemental Fig. 1B) activities of the isoforms β and γ were easily measurable with no significant difference between the two isoforms. On the contrary, the catalytic activity of the α isoform was also low, although this may be due to expression levels. Therefore, the three isoforms, from both human and murine origins, were expressed in S9 insect cells. The conditioned media from these cell cultures were individually purified by affinity chromatography. The final purifications were analyzed by SDS-polyacrylamide gels (Fig. 4A). The β and γ isoforms were nicely expressed and were the major proteins purified from these media. For the α isoform, a 55–66-kDa double band was visible and was the major protein with a small quantity of the expected full-length protein at the right molecular weight (~100 kDa). We performed two Western blots using either chicken anti-autotaxin antibodies (Fig. 4B) or anti-M2 FLAG antibodies (Fig. 4C). The two antibodies detected the full-length β and γ isoforms, with some contaminations of shorter versions of the β isoform (from both species) at ~90 kDa. For the α isoform, a major band was detected at 66 kDa, using both antibodies. Because the FLAG-M2 was positioned at the autotaxin isoform C termini of the protein, these observations demonstrated that a cleavage occurred in this region of the α isoform. The mass spectrom-
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A, mass spectrometry analysis of purified human autotaxin α isoform revealed a large peak around 105,300 Da (Fig. 5A). This could be due to the presence of glycan structures on the protein. The presumed cleavage site is detected only by SDS-PAGE under reducing conditions. The 66-kDa band disappeared under nonreduced conditions and was not detected by mass spectrometry analysis of the native molecule. The N-terminal sequencing analysis of the 105-kDa band gave two sequences as follows: a primary sequence AEGWEEG and a secondary sequence DSPWTKN showing heterogeneity of the purified human autotaxin. The latter sequence corresponds to the published N-terminal sequence of the secreted autotaxin (12). The N-terminal sequencing analysis of the second band (66 kDa) gave the sequence KVAPKR corresponding to position 341 of the protein (see Fig. 5B). This cleavage site is only detected by SDS-PAGE after reducing disulfide bridges. It then became obvious that the α isoform was indeed cleaved between Arg340 and Lys341. This amino acid sequence corresponds to the exon 12, the exon only expressed in the α isoform. The catalytic activities of the three isoforms in both species were measured (Fig. 6). Furthermore, the catalytic activity of both α isoforms (human and mouse) was poor but significantly different from the base line, showing that, although the α isoforms were cleaved off, their activities remained measurable.

Biochemical Characterization of Autotaxin Isoforms from Human and Mouse—The autotaxin proteins, whatever the isoforms, were unstable in standard conditions, with a peculiar affinity for plastic. This feature translated in a systematic disappearance of the protein after a single freezing/thawing cycle. We had to run a series of experiments using freshly purified pro-
in comparison with a protein preparation supplemented when a series of additives meant to help the solubilization and stability of the protein were compared. The following additives routinely used for crystallography (see for example Ref. 40) were tested alone or in various combinations as follows: hexadecyltrimethylammonium bromide, MgCl₂, NaCl, ethanol, ammonium sulfate, MES, polyethylene glycol, Hepes, and MPD. Fig. 7 shows some of the results obtained. With 30%MPD, a solubilized, stable protein could be handled without major loss of activity. Furthermore, this adjuvant was activating the catalytic activity. These enzyme preparations were then ready for standard biochemical characterization. We started by

![FIGURE 7. Selection of additives for the conservation of autotaxin. The recombinant autotaxin isoform \( \beta \) was tested before (column 1) or after (column 2) freezing/thawing cycle. Several additives were tested for their capacity to maintain the protein in solution after freezing (A, control, no adjuvant; B, 0.25 M NaCl, 5 mM MgCl₂, 5 mM hexadecyltrimethylammonium bromide (CTAB); C, 0.75 M NaCl, 5% ethanol; D, 0.1 M (NH₄)₂SO₄, 50 mM MES (pH 6.5), 15% polyethylene glycol MME 5,000; E, 0.166 M (NH₄)₂SO₄, 33 mM Hepes Na-salt (pH 7.5), 10% MPD; F, 50 mM Hepes Na-salt (pH 7.5), 35% MPD). An aliquot of autotaxin was diluted at the half with the adjuvant. Equal amounts of recombinant human \( \beta \) autotaxin were separated as described under "Experimental Procedures." The gel was stained in Sypro Ruby. The data are representative for at least three independent experiments.](image)

![FIGURE 8. Effect of pH and temperature on enzyme activity of the purified recombinant autotaxin in standard assay conditions. Ten \( \mu \)l of recombinant autotaxin were incubated in a 96-well plate containing 5 mM of pNppp in a 50 mM PBC buffer at different pH values (A and B). The pH of reaction was controlled at the beginning and the end of the reaction for each point. The reaction was stopped by boiling at 95 °C for 10 min. Ten \( \mu \)l of recombinant autotaxin were incubated in 96-well plates containing 5 mM of pNppp in a 50 mM Tris, pH 9.4 buffer, in a Thermocycler to control temperature (C and D). A and C, human isoforms; B and D, murine isoforms (black box, autotaxin \( \alpha \); black triangle, autotaxin \( \beta \); open circle, autotaxin \( \gamma \)). Activities for the pH dependence were conducted at 37 °C, and experiments for the temperature were conducted at pH 9.0.](image)
Characteristics of Autotaxin Isoforms measuring the influence of pH (Fig. 8, A and B) on the catalytic activity. If the murine isoforms all have a similar peak of activity at pH 8, the human isoforms have a different behavior as follows: the α isoform was almost insensitive to pH, whereas the γ and β isoforms presented a maximal activity at pH 8 and 9, respectively. Of note was their capacity to still be active at pH 9.5, a remarkable feature for many mammalian enzymes. In the same line, the observations were done on their temperature sensitivity (Fig. 8, same line, the observations were done on their temperature sensitive (Fig. 8, C and D). Again, the murine isoforms β and γ were maximally active at 45–55 °C, whereas the α isoform was not, with no obvious maximal temperature for activity. For the human isoforms, however, the γ isoform was still active at high temperature (60 °C), and the β isoform plateaued from 45 to 60 °C, whereas the α isoform remained poorly sensitive to temperature and poorly active overall.

The standard characterization of enzyme involves the measure of $K_m$ and $V_{max}$ values. Table 2 summarized the data obtained with pNppp as a substrate. All the isoforms showed an allosteric behavior, with an $n_H > 1$, suggesting positive cooperation. Apart from that, the α isoforms were poorly active, and the β and γ isoforms have similar $V_{max}$ and $K_m$ values under those experimental conditions.

Finally, the specificity of the various isoforms toward their substrates was documented. Using a series of lysophosphatidylcholine, bearing fatty acids of different lengths and saturation degrees, we took advantage of the indirect, multienzymatic method linking the appearance of free choline in the incubation medium with a fluorescent output through the catalytic activity of added choline oxidase (10) to check the various specific activities using those substrates. Conversely to what has been reported in the literature so far, the main substrate seems to be of C12 chain length (Fig. 9). There were no major differences in the respective specificities of the various isoforms, and from both species, the main substrate remaining was lauroyl lysophosphatidylcholine.

Autotaxin Isoform Sensitivities to Chelating Agents and Metals—We wanted to check the sensitivity of the isoforms to EDTA and EGTA (Fig. 10). All six isoforms behaved similarly by being strongly inhibited by increasing concentrations of both EGTA and EDTA; 100% inhibition was reached for 100 mM of both chelating agents. This observation led us to check the sensitivity to metals for the human and murine isoforms (Fig. 11) as such sensitivity were already reported in the literature (14, 21, 41, 42). Nickel, cobalt, calcium, zinc, copper, manganese, and magnesium were studied at concentrations ranging from 0.1 to 1000 mM as it has been reported from the hen egg white isolated autotaxin (17). If cobalt and nickel activated the catalytic activities, zinc and manganese seemed to inhibit it. There are minute variations in the sensitivities to metal for all isoforms.

Autotaxin Inhibitions—We then checked the inhibition of the isoforms by several albumin preparations (Table 3). Initially, a common belief of our laboratory was that albumin could be a regulator of the circulating autotaxin (see also Ref. 17). By incubating human isoforms with human serum albumin, we observed IC$_{50}$ in 100 μM range, but when the albumin was a fatty acid-free preparation, this slight inhibition disappeared. For the murine isoform, incubations with murine serum albumin led to the same observation with inhibitions in the 200 μM range that were not observed anymore with fatty acid-free preparations. Because LPA has been reported as an inhibitor of its own production, we tested its capacity to inhibit the various isoforms (Table 3). Interestingly, all the isoforms were inhibited with IC$_{50}$ in the 20 nM range, suggesting a fine-tuning of LPA production by the product of the reaction. Hypericin, a new, albeit modest, inhibitor of autotaxin β (43) was tested on all the isoforms and showed a good potency under these conditions of their catalytic activities in the 2 μM range, a feature that might permit the use of this compound in physiopathological conditions.

**DISCUSSION**

Autotaxin plays a significant role in initiating and sustaining tumor metastasis (44). LPA stimulates cell proliferation, migration, and survival by acting on its cognate G-protein-coupled receptors. Aberrant LPA production, receptor expression, and signaling probably contribute to cancer initiation, pro-

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**TABLE 2**

Kinetic constants of murine and human autotaxin isoforms

<table>
<thead>
<tr>
<th>Specific activity</th>
<th>$V_{max}$ (nmol pNp/min/μg protein)</th>
<th>$K_m$ (mM)</th>
<th>$n_H$</th>
<th>0.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human autotaxin α</td>
<td>8.47 ± 0.81</td>
<td>0.67 ± 0.06</td>
<td>5.7 ± 0.6</td>
<td>1.2 ± 0.09</td>
</tr>
<tr>
<td>Human autotaxin β</td>
<td>135 ± 7</td>
<td>1.9 ± 0.1</td>
<td>11.3 ± 0.2</td>
<td>1.8 ± 0.36</td>
</tr>
<tr>
<td>Murine autotaxin α</td>
<td>16.3 ± 0.1</td>
<td>3.5 ± 0.01</td>
<td>4.4 ± 0.9</td>
<td>1.5 ± 0.09</td>
</tr>
<tr>
<td>Murine autotaxin β</td>
<td>91.9 ± 5.7</td>
<td>1.9 ± 0.1</td>
<td>12.9 ± 0.8</td>
<td>1.5 ± 0.1</td>
</tr>
</tbody>
</table>

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**FIGURE 9. Substrate specificity of autotaxin isoforms.** The substrate specificity of autotaxin with regard to the acyl group of LPC was determined using LPC with various acyl groups. Each substrate was subjected to the autotaxin reaction, and the activities were evaluated by choline release. The substrate used were LPC (6:0, 8:0, 10:0, 12:0, 14:0, 16:0, 18:0, 18:1, 20:0, 22:0). The results are the mean ± S.E. from three separate experiments.
gression, and metastasis. LPA production could prove to be an attractive target for therapy either directly (production of LPA) or indirectly (binding of LPA to its receptor(s)) (45). Furthermore, as shown previously (7, 21, 25, 26, 28) adipocytes secrete large amounts of autotaxin, at least in culture. Therefore, the role of autotaxin might also be linked to the development of adipose tissue, through an as yet unknown mechanism.

The isoforms of autotaxin have been described previously for human or murine tissues (12, 13, 38). Nevertheless, this study is the first to report all these isoforms from both human and murine origins, their cloning and expression patterns, as well as the organization of the autotaxin gene. This gene is located on chromosome 8 at position 8q24.1 in human and on chromosome 15 in the mouse and is of high complexity. Indeed, the presence of >20 introns could lead to many alternative splicings. Interestingly, despite this complexity, no more than three isoforms have been reported so far as follows: a form deleted of two exons (exon 12 and 21, H1 isoform), a form with only one of these deletions (exon 12, form α), and another with the second deletion only (exon 21, form γ). The isoforms of autotaxin are expressed differentially. In mouse, the β form seems to be the major one, although its expression is rather limited to peripheral tissues. On the contrary, the γ isoform seems to be mainly expressed in the brain, a situation somewhat similar in human.

It is worth pointing out that qPCR cannot be used to compare the respective levels of expression of the isoforms in different tissues. Indeed, the use of a single set of identical primers to perform these quantitative experiments is not possible, because common primers would recognize the three isoforms in every tissue. Primers designed to be specific for the corresponding region of amino acids 323–375 would recognize the α isoform only, whereas those designed for the corresponding region of amino acids 592–617 would recognize the γ isoform only, but none could be designed to recognize the β isoform without recognizing the others. Furthermore, the choice of different primers would not allow the comparison between quantitative data, because of the intrinsic properties of each primer, which would be, by definition, different in sequence from each other. The results presented for qPCR are similar to those in tissue distribution, and somehow validated them. Nevertheless, one particular point merits a further comment; the level of autotaxin expression in leukocytes seems to be low, if any. Indeed, this observation was already reported by Lee et al. (13) using another approach. This seems to be also in contradiction with the role of autotaxin in metastasis process, as suggested by Boucharaba et al. (31), although the expression of the protein in the other circulating blood cells can be hypothesized. This expression will be addressed using other approaches, particularly Western blot, if one has access to potent (and specific?) antibodies, particularly if autotaxin expression is induced in platelets during metastasis processes.

Interestingly, we found that if the β and γ isoforms were easily expressed in at least three transgenic systems (bacteria, Chinese hamster ovary cells, COS, and Sf9), the α isoform seemed to be poorly expressed in any of those systems under identical conditions. We showed that the sequence corre-
sponding to exon 12 bears a site (340R↓KVAPK) that was cleaved by endogenous protease(s), leading to a shortened peptide, starting at amino acid 341. Note that the cleavage appears in all the host systems we tested (mammalian, bacteria, and insect) suggesting that the cleavage is because of a nonspecific protease. The shortened protein should be devoid of catalytic
activity, because the core of this site (Thr210) is in the remaining cleaved fragment. Therefore, any activity measured for the α isoform preparations is likely because of the full α isoform prior cleavage. It is also interesting to note that under nonreducing conditions, the apparent molecular mass of the α isoform remains in the 100-kDa range, whereas under reducing conditions it dropped to 66 kDa, in line with the predicted cleavage of the enzyme. In this study, we describe the characterization of the three recombinant isoforms from human and mouse origins.

Recombinant human and murine autotaxin isoforms were incubated with various concentrations of inhibitor substrates (diluted in 1.9% Me2SO) during 15 min before adding the substrate for phosphodiesterase activity, as described under “Experimental Procedures.” The experiments were run independently at least three times. ND means not determined.

**Characteristics of Autotaxin Isoforms**

<table>
<thead>
<tr>
<th>Human or murine serum albumin</th>
<th>Human or murine serum albumin, fatty acid-free</th>
<th>Hypericin</th>
<th>LPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human autotaxin α</td>
<td>None</td>
<td>ND</td>
<td>38 ± 17</td>
</tr>
<tr>
<td>Human autotaxin β</td>
<td>187,000</td>
<td>1.6 ± 0.9</td>
<td>38 ± 12</td>
</tr>
<tr>
<td>Human autotaxin γ</td>
<td>317,000</td>
<td>2.8 ± 0.3</td>
<td>18 ± 9</td>
</tr>
<tr>
<td>Human autotaxin γ</td>
<td>251,000</td>
<td>2.6 ± 0.2</td>
<td>20 ± 9</td>
</tr>
</tbody>
</table>

ND means not determined.

The biochemical characteristics of these α, β, and γ autotaxin isoforms were not known, because most of the data available in the literature were issued from either a form purified from human plasma (5, 6), most probably the β isoforms, an undetermined isoform cloned from rat (13, 16), from mouse (10), or from human (47), as well as from hen egg white (17) or from homogenates of various origins as follows: rat liver (20) and rat brain (18), to cite the main ones. It should be kept in mind that before 2002, autotaxin was not known as a lyso-PLD enzyme. In this study, we describe the characterization of the three recombinant isoforms from human and mouse origins. Both the β and γ isoforms are well expressed and catalytically active, whereas the α isoform, which lacks exon 21, is expressed in this system but rapidly degraded in smaller, inactive forms, suggesting a new level of regulation of this particular isoform. Nevertheless, the remaining α isoform (see above) was active enough to permit its biochemical characterization.

It is important to point out another fact. The manipulation of peptides or nearly pure proteins is often a source of complications. Indeed, pure proteins might have the peculiarity to bind to plastic or to glass, to aggregate, or to precipitate. The case of autotaxin is almost a textbook case. This protein does not stay very long in solution, once pure. A close and complete study was necessary, and time-consuming, to approach and solve this problem. By adding various partners in the autotaxin solution, conditions were found under which the protein stability was enhanced. Nevertheless, it came rapidly obvious that these additives had a significant effect on the kinetic and catalytic constants of the enzyme. Therefore, taking advantage of the many available additives usable in crystallography, we tested a mixture that would help the solubility and the stability of the autotaxin isoforms, without changing their affinity for their substrate.

The assays available to measure autotaxin activity are limited. They can either measure the phosphodiesterase activity of autotaxin using pNppp, the cleavage of [14C]lysophosphatidylcholine, or the amount of choline released during the autotaxin activity (through choline oxidase (10, 48)). More recent work provided a fluorescent assay using an LPC-derived compound bearing a fluophore at the end of the lipid chain of LPC (49). Although these assays correlate well with one another, none of them are easy to use or of adequate sensitivity. We chose to use the pNppp approach, because the literature on autotaxin clearly states that all the observations obtained on the lysophospholipase D activity could be confirmed with the phosphodiesterase activity, particularly those studying the catalytic site of autotaxin (10, 11).

To describe the enzymatic characteristics, we evaluated the pH and temperature dependences of autotaxin. We obtained similar results, with the peak activity at pH 8, and an optimum temperature activity of ~40 °C. These data were similar for the γ isoform (not shown). It might also be important to notice that at rather high temperatures such as 60 °C, autotaxin β and γ are still able to catalyze a significant portion of their maximal activities. Because enzymes are proteins, they are very sensitive to changes in pH. Each enzyme has its own optimum range for pH where it will be most active, resulting from the effect of pH on a combination of the following factors: 1) the binding of the enzyme to substrate; 2) the catalytic activity of the enzyme; 3) the ionization of the substrate; and 4) the variation in protein structure. These structural features will be clearer when the crystallography of autotaxin becomes available.

The substrate specificity of autotaxin was described in the recent paper of Tokumura et al. (6) using a purified isoform from human plasma. In brief, the substrates bearing fatty acid of 6–18 carbons are substrates of autotaxin, with a particular preference for C12 and C14. Interestingly, these activities are enhanced in the presence of 30 μM of cobalt.

It is known for a long time that cations can stimulate enzyme activities through various mechanisms. Early publications indicated that lyso-PLD from rat liver requires Mg2+ and is inhibited by Zn2+, Mn2+, and high concentrations of Ca2+ (25 mM) (41). In contrast, Mg2+ (5 mM) has little stimulatory effect in rabbit kidney (14). Instead, the rabbit kidney lyso-PLD requires Ca2+ (5 mM) for normal function. Based on these results, the microsomal lyso-PLDs are divided into Mg2+-dependent and Ca2+-dependent enzymes. The lyso-PLD in rat plasma requires a metal ion for optimal activity (42). Co2+ is most effective, followed in decreasing order by Zn2+, Mn2+, and Ni2+. The lyso-PLDs from human plasma and from adipocytes were likewise acti-
vated by Co²⁺ (21). Although the mechanism for the cation effects on lyso-PLD-PLD are not well understood, this cation sensitivity is shared by autotaxin. Co²⁺ enhances the phosphodiesterase activity of recombinant human and rat autotaxin as well as purified plasma lyso-PLD (6). Furthermore, the PDE activity of autotaxin is enhanced by Ca²⁺ and Mg²⁺ in a concentration-dependent manner. For autotaxin, cations may act either to stabilize structure or as part of the catalytic center (50). In addition, cations protect autotaxin from thermal denaturation and proteolysis (51). Cations may exert their stimulatory effect by interacting with a region other than the EF-hand loop region in the autotaxin structure. Note that although Co²⁺ stimulates autotaxin activity, it is not absolutely required for this activity.

As recently discussed (43), the molecular pharmacology of autotaxin is rather scarce. Indeed, apart from compounds issued from lipid chemistry, such as LPA phosphonate analogs (52, 53), fatty alcohol phosphates (54), cyclic LPA (55), or Darmstoff analogs of LPA (56), there are no small molecules described as the potent inhibitor of this enzyme. During our screening on autotaxin β partially purified from COS-transfected cells, we identified several compounds that in our hands were good candidates for such a purpose. Among them, was the inositol 1,4,5-trisphosphate kinase inhibitor, hypericin, a feature rendering this molecule an attractive candidate for validation in more complex models. We also tested an hypothesis according to which serum albumin was a possible regulator of autotaxin (see also Ref. 17). Not surprisingly, the fatty acid-free albumin was devoid of any capacity to inhibit significantly the autotaxin activities, whereas the “regular,” probably fatty acid-loaded albumin, presents IC₅₀ in the 100 μM range. In line with this observation, we tested free fatty acids, either in mixture or free ones, for their capacity to inhibit autotaxin. None shown any significant capacity to inhibit the enzyme (not shown).

In conclusion, this study establishes the structure of the autotaxin gene human. If the same three isoforms are expressed in this organ. Furthermore, we report on hypericin, a polycyclic polyether and a possible regulator of autotaxin (see also Ref. 17). Not surprisingly, the fatty acid-free albumin was devoid of any capacity to inhibit significantly the autotaxin activities, whereas the “regular,” probably fatty acid-loaded albumin, presents IC₅₀ in the 100 μM range. In line with this observation, we tested free fatty acids, either in mixture or free ones, for their capacity to inhibit autotaxin. None shown any significant capacity to inhibit the enzyme (not shown).

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Characteristics of Autotaxin Isoforms

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Murine and Human Autotaxin α, β, and γ Isoforms: GENE ORGANIZATION, TISSUE DISTRIBUTION, AND BIOCHEMICAL CHARACTERIZATION
Adeline Giganti, Marianne Rodriguez, Benjamin Fould, Natacha Moulharat, Francis Cogé, Pascale Chomarat, Jean-Pierre Galizzi, Philippe Valet, Jean-Sébastien Saulnier-Blache, Jean A. Boutin and Gilles Ferry

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