Molecular Cloning and Functional Expression of a Caenorhabditis elegans Aminopeptidase Structurally Related to Mammalian Leukotriene A₄ Hydrolases*

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In a search of the Caenorhabditis elegans DNA data base, an expressed sequence tag of 327 base pairs (termed cm01c7) with strong homology to the human leukotriene A₄ (LTA₄) hydrolase was found. The use of cm01c7 as a probe, together with conventional hybridization screening and anchored polymerase chain reaction techniques resulted in the cloning of the full-length 2.1 kilobase pair C. elegans LTA₄ hydrolase-like homologue, termed aminopeptidase-1 (AP-1). The AP-1 cDNA was expressed transiently as an epitope-tagged recombinant protein in COS-7 mammalian cells, purified using an anti-epitope antibody affinity resin, and tested for LTA₄ hydrolase and aminopeptidase activities. Despite the strong homology between the human LTA₄ hydrolase and C. elegans AP-1 (63% similarity and 45% identity at the amino acid level), reverse-phase high pressure liquid chromatography and radiolmmunoassay for LTB₄ production revealed the inability of the C. elegans AP-1 to use LTA₄ as a substrate. In contrast, the C. elegans AP-1 was an efficient aminopeptidase, as demonstrated by its ability to hydrolyze a variety of amino acid p-nitroanilide derivatives. The aminopeptidase activity of C. elegans AP-1 resembled that of the human LTA₄ hydrolase/aminopeptidase enzyme with a preference for arginyl-p-nitroanilide as a substrate. Hydrolysis of the amide bond of arginyl-p-nitroanilide was inhibited by bestatin with an IC₅₀ of 2.6 ± 1.2 μM. The bifunctionality of the mammalian LTA₄ hydrolase is still poorly understood, as the physiological substrate for its aminopeptidase activity is yet to be discovered. Our results support the idea that the enzyme originally functioned as an aminopeptidase in lower metazoa and then developed LTA₄ hydrolase activity in more evolved organisms.

Leukotriene A₄ (LTA₄)¹ hydrolase (EC 3.3.2.6) is the rate-limiting enzyme in the lipooxygenase cascade of arachidonic acid metabolism leading to the biosynthesis of the proinflammatory substance leukotriene B₄ (LTB₄) from the epoxide intermediate LTA₄ (1, 2). At nanomolar concentrations, LTB₄ elicits chemotaxis and adherence of leukocytes, and in higher doses it also triggers degranulation and generation of superoxide anions (3). Due to these biological properties, LTB₄ is regarded as an important chemical mediator in a variety of inflammatory diseases (4). Sequence comparison of LTA₄ hydrolase with other zinc metalloenzymes, e.g., aminopeptidase M and thermolysin, led to the identification of a zinc binding motif in the primary structure of the enzyme (5–7). Further studies verified that LTA₄ hydrolase contained one catalytic zinc atom coordinated by His²⁹⁵, His²⁹⁹, and Glu³¹⁵ (8). Subsequently, the enzyme was shown to exhibit a previously unknown zinc-dependent peptidase/amidase activity toward synthetic substrates (9, 10) that was specifically stimulated by monovalent anions, e.g., chloride ions (11), and also by albumin (12). Although a physiological peptide substrate for the aminopeptidase activity of the enzyme has not yet been found, LTA₄ hydrolase has been shown to efficiently hydrolyze several arginyl tri- and dipetides, leading to its identification as an arginine aminopeptidase (13). Both the aminopeptidase and the LTA₄ hydrolase activity of the enzyme are inhibited by the aminopeptidase inhibitor bestatin (10) and the angiotensin converting enzyme inhibitor captopril (14), suggesting that the active sites corresponding to the two activities are overlapping (15). Important questions regarding the dual activity of the mammalian LTA₄ hydrolase/aminopeptidase remain unanswered. For example, does the enzyme demonstrate both LTA₄ hydrolase and aminopeptidase activities in other species? Which function originated first in evolution? What is the significance of this bifunctionality? LTA₄ hydrolase/aminopeptidase is a soluble monomeric protein (M₉ ̃ 69,000) (16, 17) that has been cloned from human (18), mouse (19), rat (20), and guinea pig (21). Recently a partial sequence from the slime mold Dictyostelium discoideum and a gene from the yeast Saccharomyces cerevisiae (22) have been deposited into the GenBank™ data base as putative LTA₄ hydrolases (accession numbers U27538 and X94547, respectively). Both sequences encode proteins similar in their primary amino acid sequences to the mammalian LTA₄ hydrolase, but neither of them has been expressed or characterized. In addition, an enzyme from the pathogenic yeast Candida albicans with 41% homology to the mammalian LTA₄ hydrolase exhibited mainly aminopeptidase activity, whereas its hydrolase activity converted the ma-
minority of the substrate LTA₄ to what has been putatively identified as 5,6-diHETE (a much less potent leukotriene) rather than LTB₄ (47). We report the molecular cloning and functional expression of an aminopeptidase enzyme from Caenorhabditis elegans, named AP-1, that, despite its strong homology to the human LTA₄ hydrolase, exhibits no LTA₄ hydrolase activity and only aminopeptidase activity. The strong homology between C. elegans AP-1 and mammalian LTA₄ hydrolases (45%) suggests that these enzymes may have developed from a common ancestral precursor.

MATERIALS AND METHODS

Cloning of a LTA₄ Hydrolase-like cDNA Homologue from C. elegans—The cm01c7 phage clone from a C. elegans mixed stage hermaphroditic cDNA library (made by Chris Martin) containing the LTA₄ hydrolase-like EST in SHLX2 λ phage vector (25) was obtained from Dr. R. H. Waterston (24). MC1061 recA⁻ tetR⁺ (used for plating λ SHLX2) and the pop-out Escherichia coli camR² KanR² strain (used to convert λ SHLX2 clones to plasmid clones) were also generously provided by Dr. R. H. Waterston. The pop-out strain was infected with the λ SHLX2 phage containing cm01c7 EST using standard protocols (25). Five colonies were picked, and plasmid DNA was prepared using either the Wizard PCR Prep Kit (Promega, Madison, WI) or Qiagen tips-500 (Qiagen Inc., Santa Clarita, CA). DNA was then used to transform XL-1 blue E. coli strain (Stratagene, La Jolla, CA) followed by DNA preparation and verification by restriction analysis. The resulting 0.95-kb C. elegans fragment in the pRAT II phasmid was then sequenced using T7 and SP6 primers and automated DNA sequencing on an Applied Biosystems model 386 Data sequencer utilizing T7 DNA polymerase and internal labeling with fluorescent-15-dATP (26).

Approximately 2 × 10⁹ phage from a mixed stage C. elegans cDNA library in bacteriophage λ vector UNI-ZAP XR (Stratagene) were plated and screened by hybridization as described previously (27) using the [α-³²P]CTP-labeled (Boehringer Mannheim) 0.95-kb Apal/Sca I C. elegans fragment obtained from the cm01c7 clone as a probe. Hybridization was performed in 50% deionized formamide, 0.1% SDS, 5 × SSC, 5 × Denhardt’s solution, and 100 μg ml⁻¹ denatured calf thymus DNA at 42 °C. After overnight hybridization, filters were washed three times for 10 min each at room temperature in 2 × SSC, 0.1% SDS, two times for 30 min each at 65 °C in 1 × SSC, 0.1% SDS, and exposed to X-OMAT AR film (Eastman Kodak Co.). Positive plaques were rescreened twice with the same probe, and the size of positive inserts was determined by PCR amplification using the pfXBlueScript phagemid as template and cycling conditions of 35 cycles of 1 min at 94 °C, 1 min at 55–62 °C, and 1 min at 72 °C. PCR products were separated by electrophoresis in 1% agarose gels, visualized by ethidium bromide staining, and Southern blotted to Hybond-N (+) film (Amersham Pharmacia Biotech) by overnight capillary transfer using 0.4 × sodium citrate. The positive inserts were sequenced. The longest LTA₄ hydrolase-like clone obtained, termed C5 (>1.4 kb in size), started from the 5' end of the coding region (as predicted from the size of cDNA of all previously cloned LTA₄ hydrolases). To isolate the 5'-end of the C. elegans cDNA, several anchored PCR amplifications using the phagemid-based primers T3, SK, and pSR (Stratagene) and a series of antisense primers based on the most 5' sequences in clone C5 were performed using the PCR Core kit (Boehringer Mannheim) and a Perkin-Elmer thermal cycler. PCRs were carried out in a buffer containing 10 mM Tris/HCl, pH 8.3, 50 mM KCl, 2.5 mM MgCl₂, 0.2 mM deoxynucleotidetriphosphates, 0.5 μM primers and 1 μl of the C. elegans cDNA library or 1 μl of the primary or secondary PCR amplification products as templates and cycling conditions of 35 cycles of 1 min at 94 °C, 1 min at 55–62 °C, and 1 min at 72 °C. PCR products were separated by electrophoresis in 1% agarose gels, visualized by ethidium bromide staining, and Southern blotted to Hybond-N⁺ nylon membranes (Amersham Pharmacia Biotech) by overnight capillary transfer using 0.4 × sodium hydroxide. The PCR-amplified fragments were sequenced by overnight hybridization using the nested [γ-³²P]ATP-labeled primer C5T3 (5'-GCC GGC CCA TCT TCT GCT-3') (based on the most 5' sequence in clone C5) and T4 polynucleotide kinase (Boehringer Mannheim) in 6 × SSC, 20 μg/ml Na₃PO₄, 0.4% SDS, 5 × Denhardt’s solution, 50 μg ml⁻¹ denatured calf thymus DNA at 42 °C. PCR products of ~ 600–800 bp were identified by their hybridization, isolated on 1.9% agarose gel, purified using Qiagen spin columns (Qiagen), and subcloned into the TA vector (Invitrogen, Co. San Diego, CA). The ligation mixtures of PCR-generated fragments were transformed into DH5α E. coli, and insertions were characterized by restriction analysis, PCR, and DNA sequencing. From this cloning approach, several clones encoding the missing 5' coding region of clone C5 were identified. The full-length C. elegans cDNA clone (2.1 kb) was then reconstructed using a common EcoRI restriction site at the 5'-end of clone C5 and the 3'-end of the PCR-amplified NH₂-terminal sequence.

Expression of the Recombinant C. elegans AP-1 Protein—A 1.8-kb NotI/BalI fragment representing the entire AP-1 coding sequence was amplified by PCR using Expand high fidelity Taq Polymerase (Boehringer Mannheim) and the primers 5'-CAT GCA TGC AGC GGC GCC GGC GCT CCT CCA CAT CGG AGA GAT CCC-3' and 5'-CAT GCA TGC ATG TCT ATG AAT TTA GAG ACT TTG GTC GTC-3'. The 5'-end primer introduces an NH₂-terminal NotI site (the C. elegans translation initiation codon was abolished by a synthetic oligonucleotide to start from the ATG, supplied by the pFLAG CMV2 expression vector), and the 3'-end primer introduces a COOH-terminal BalI site immediately after the stop codon (thus eliminating the 3'-untranslated region). The AP-1 NotI/BalI fragment was then subcloned into the NotI/BalI restricted mammalian expression vector pFLAG CMV2 (Kodak), and the resulting clone, pFLAG celAP-1, was verified by sequencing.

Cell Culture and Transfection—The African green monkey SV40 transformed kidney cell line (COS-7), obtained from the American Type Culture Collection, was grown in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum (Sigma), 50 units/ml penicillin, 50 μg/ml streptomycin (Flow Laboratories, McLean, VA), and 2 mM glutamine (Flow Laboratories) at 37 °C under 5% CO₂. Cells were plated at 10 000 × g for 10 min. Both the 10 000 × g pellet and supernatant were assayed for recombinant expression of AP-1 protein by immunoblot analysis.

Preparation of C. elegans Extracts—Frozen mixed stage hermaphrodite C. elegans worms were a generous gift from J. McGehee (University of Calgary, Alberta, Canada). 5 ml of wet worms (resuspended in phosphate-buffered saline, centrifuged at 1100 × g, resuspended in TBS (50 mM Tris/HCl, pH 7.4, 150 mM NaCl), and recentrifuged at 10 000 × g for 10 min. Both the 10 000 × g pellet and supernatant were assayed for recombinant expression of AP-1 protein by immunoblot analysis.

Affinity Chromatography Purification of the Recombinant C. elegans AP-1 Protein—Chromatography columns were packed with 3 ml each of anti-FLAG M2 affinity resin (Kodak), equilibrated three times with 3 ml of TBS, and activated by washing three times with 3 ml of glycerine/ HCl at pH 3.5, followed by washing three times with 3 ml of TBS. A 10 μl aliquot of the 10 μl aliquots of COS-7 cells transfected with either pFLAG vector or pFLAG cel-AP1 construct were incubated with 3 ml of the activated anti-FLAG M2 affinity gel in 15-ml polypropylene tubes and left to rotate at 4 °C overnight. Each slurry was transferred back to chromatography column, and the flow-through samples from the columns were drained the next day, followed by washing three times with 3 ml of TBS. Columns were then eluted using 11 ml (0.5 ml/fraction) of the FLAG octapeptide (0.5 mg/ml in TBS; NH₂-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-COOH) (Kodak). Fractions containing the C. elegans FLAG fusion protein (as assessed by immunoblot analysis using the anti-FLAG M2 monoclonal antibody) were pooled. Aliquots of the flow-through, combined washes, and the different eluted fractions were kept for immunoblot analysis, and the rest of the samples were frozen at 80 °C when not used immediately for functional assays.

Immunoblot Analysis—The 10 000 × g COS-7 cell supernatants (lyses of COS-7 cells transfected with either pFLAG vector or pFLAG cel-AP1 construct), the anti-FLAG M2 affinity flow-through, combined washes, and the different FLAG peptide eluted fractions, as well as the NH₂-terminal FLAG fusion protein of E. coli bacterial alkaline phosphatase (Kodak) were separated electrophoretically on 10% polyacrylamide gels according to the method of Laemmli (28). This was followed by electrophoretic transfer to nitrocellulose membranes using a Novex immunoblot system according to the manufacturer’s instructions (Novex). The nitrocellulose membranes were developed using a 1:300 dilution of mouse anti-FLAG M2 monoclonal antibody (Kodak). The secondary horse radish peroxidase-linked donkey anti-mouse IgG
H2O/acetic acid solvent was pumped isocratically at a flow rate of 1 ml/min and then adjusted to a pH of 7.4. The isocratic elution was continued from affinity columns loaded with mock-transfected cell extracts using a Beckman lysate reader spectrophotometer (Molecular Devices). Spontaneous hydrolysis was determined by incubating 100,000 × g centrifugation fraction (100S fraction) of SF9 cells infected with a recombinant human LT A4 hydrolase construct (29) was used as a positive control (Merck Frpest Center for Therapeutic Research). Alkaline hydrolysis of LT A4 ethyl ester was carried out as described (30). LT A4 hydrolase assays on C. elegans cytosolic fraction (125 μg) extracted from an equal volume of methanol containing 1 nmol/ml prostaglandin B2 standard. Eicosanoid products were extracted with an equal volume of chloroform, evaporated under nitrogen and resuspended in 100 μl of the HPLC solvent methanol/water/acetic acid (75:25:0.1) (32). Eicosanoid products were analyzed by reverse-phase HPLC on a 3.9 × 150 mm NovaPak C18 column (Waters). The MeOH/ H2O-acetic acid solvent was pumped isocratically at a flow rate of 1 ml/min. The effluent was monitored at 270 nm by a photodiode array detector. Products were compared with the retention times and spectra of known eicosanoids standards.

Radioimmunooassay detection of LT B4 production was performed by incubating 100 ng of either purified C. elegans AP-1 (FLAG) or protein eluted from affinity columns loaded with mock-transfected cell extracts with 100 ng of either purified human LT A4 hydrolase or Tris/HCl, pH 8.0, 1 mg/ml BSA for 10 min at room temperature. Reactions were terminated by addition of an equal volume of methanol containing 1 nmol/ml prostaglandin B2 standard. Eicosanoid products were extracted using an equal volume of chloroform, evaporated under nitrogen and resuspended in 100 μl of the HPLC solvent methanol/water/acetic acid (75:25:0.01). Eicosanoid products were analyzed by reverse-phase HPLC on a 3.9 × 150 mm NovaPak C18 column (Waters). The MeOH/ H2O-acetic acid solvent was pumped isocratically at a flow rate of 1 ml/min. The effluent was monitored at 270 nm by a photodiode array detector. Products were compared with the retention times and spectra of known eicosanoids standards.

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FIG. 1. Nucleotide sequence of the C. elegans AP-1 cDNA and deduced amino acid sequence. Nucleotides are numbered beginning with the first residue of the ATG translation initiation codon. Nucleotides 5’ of the ATG are designated by negative numbers. The deduced amino acid sequence is shown in the one-letter code above the nucleotide sequence. Amino acids are numbered from the NH2-terminal methionine residue. Amino acids involved in the putative zinc binding site are underlined.
AP-1 sequence. This motif is found in several reported metallopeptidases and allows the classification of the *C. elegans* enzyme under the M1 family of metalloexopeptidases (36). Members of this family also include aminopeptidase A, aminopeptidase N, cysteine aminopeptidase, and LTA4 hydrolase. The tyrosine residue Tyr383 in both the human and the mouse sequences, which is essential for the peptidase activity of the human and mouse LTA4 hydrolase/aminopeptidase, are indicated on a black background. The conserved glutamate residue necessary for peptidolysis of mammalian LTA4 hydrolases (Glu296 in both human and mouse sequences) is underlined. The tyrosine residue Tyr293 (in both the human and the mouse LTA4 hydrolase sequences) involved in the covalent binding of LTA4 to the human LTA4 hydrolase is indicated by an asterisk and is replaced by a phenylalanine (Phe382) in the *C. elegans* sequence.

**Fig. 2.** A multiple alignment of amino acid sequences of the human leukotriene A4, the mouse leukotriene A4 hydrolase, and the *C. elegans* AP-1. The alignment was made using the Pretty Plot function of the GCG program (33). Amino acids are numbered beginning with the first methionine residue in the *C. elegans* sequence. Conserved residues in all three sequences are shown in the consensus. Both the zinc-binding motif, common among members of the M1 family of metallopeptidases (HEXXH_E), and the tyrosine residue (number 383 in both the human and the mouse sequences), which is essential for the peptidase activity of the human and mouse LTA4 hydrolase/aminopeptidase, are indicated on a black background. The conserved glutamate residue necessary for peptidolysis of mammalian LTA4 hydrolases (Glu296 in both human and mouse sequences) is underlined. The tyrosine residue Tyr293 (in both the human and the mouse LTA4 hydrolase sequences) involved in the covalent binding of LTA4 to the human LTA4 hydrolase is indicated by an asterisk and is replaced by a phenylalanine (Phe382) in the *C. elegans* sequence.

**C. elegans Aminopeptidase-1**

| Human | Mouse | C. elegans | Consensus |
|-------|-------|------------|-----------|
|      |       |            |           |
| AP-1 | AP-1  | AP-1       |           |
| 1     | 1     | 1          |           |
| -MPEIVDCSTLASAPVSCTKHLRLSVCVD=TPLTRLTLGAATLVQGQEDN | -MPEIVDCSTLASAPVSCTKHLRLSVCVD=TPLTRLTLGAATLVQGQEDN | -MPEIVDCSTLASAPVSCTKHLRLSVCVD=TPLTRLTLGAATLVQGQEDN | -MPEIVDCSTLASAPVSCTKHLRLSVCVD=TPLTRLTLGAATLVQGQEDN |
| 50    | 50    | 50         |           |
| LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. |
| 100   | 100   | 100        |           |
| LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. |
| 150   | 150   | 150        |           |
| LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. |
| 200   | 200   | 200        |           |
| LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. |
| 250   | 250   | 250        |           |
| LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. |
| 300   | 300   | 300        |           |
| LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. |
| 350   | 350   | 350        |           |
| LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. |
| 400   | 400   | 400        |           |
| LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. |
| 450   | 450   | 450        |           |
| LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. |
| 500   | 500   | 500        |           |
| LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. |
| 550   | 550   | 550        |           |
| LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. |
| 600   | 600   | 600        |           |
| LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. |
| 650   | 650   | 650        |           |
| LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. |
| 700   | 700   | 700        |           |
| LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. |
| 750   | 750   | 750        |           |
| LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. |
| 800   | 800   | 800        |           |
| LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. |
| 850   | 850   | 850        |           |
| LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. |
| 900   | 900   | 900        |           |
| LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. |
| 950   | 950   | 950        |           |
| LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. |
| 1000  | 1000  | 1000       |           |
| LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. | LLSIVDLDTGKLIEKVR. |

**The Structure of the C. elegans Aminopeptidase Gene**—The cloned *C. elegans* AP-1 cDNA was compared with sequences in the genomic section of the Sanger Center *C. elegans* data base, and two cosmid clones were identified (cosmids C42C1 and Y39C12) that showed a 100% match to the cDNA sequence using a BLASTN search. A map of the structure of the *C. elegans* aminopeptidase gene was then constructed (Fig. 3A). Cosmid clone Y39C12 (GenBank accession number AL009026) is localized to *C. elegans* chromosome 4, and the entire open reading frame of the *C. elegans* AP-1 cDNA is contained within four exons ranging in size from 53 to 1325 bp. The exons are separated by three small introns of 44, 49, and 49 bp. The small
size of the introns is expected as most introns in the nematode *C. elegans* are very short (40). The sequences of exon-intron boundaries were determined by comparing the cDNA sequence and the genomic sequence (Fig. 3B). The exon-intron junction in intron 2 follows the GT/AG rule and agrees with consensus sequences for the donor and acceptor sites (41). On the other hand, introns 1 and 3 lack an AG at the 3' splice acceptor site, which agrees with the finding that splicing in *C. elegans* does not require this AG (42). As shown for over 98% of *C. elegans* introns, all three introns have an elevated A-U content just upstream of the 3' splice site with a U present at position -5 relative to the cleavage site (40). The proposed zinc-binding histidine residues (His<sup>297</sup> and His<sup>319</sup>) and glutamate residue (Glu<sup>320</sup>), which constitute the zinc-binding domain (HEXXHX<sub>3</sub>E), are located on one exon (Fig. 3, exon 3), unlike the structure of the human LTA<sub>4</sub> hydrolase/aminopeptidase gene (43), in which the two essential zinc-binding histidine residues (His<sup>295</sup> and His<sup>299</sup>) are present on exon 10, whereas the third zinc-binding ligand glutamate (Glu<sup>319</sup>) is located on another exon (exon 11).

**Expression of Recombinant C. elegans AP-1 and Immunoblot Analysis**—The *C. elegans* AP-1 open reading frame (1.8 kb) was subcloned into the mammalian expression vector pFLAG CMV2, which provides a translation initiation codon and a FLAG epitope (NH<sub>2</sub>-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-COOH). COS-7 cells were transiently transfected with the pFLAG.cel AP-1 or the vector control DNA (Fig. 4, lane 2) but not in the supernatant of mock-transfected cells (results not shown). As described previously for the mammalian LTA<sub>4</sub> hydrolase/aminopeptidase (16), the *C. elegans* AP-1 is a soluble protein expressed in the cell cytosol with minimal detection in either the microsomal or the membrane fractions (100,000 × g and 2000 × g pellets, respectively) (results not shown). The expressed FLAG-tagged *C. elegans* AP-1 protein was partially purified (∼ 30% purity) using anti-FLAG M2 affinity chromatography, eluted with the FLAG octapeptide, resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunoblotted using anti-FLAG M2 antibody. All of the expressed *C. elegans* AP-1 protein was bound to the anti-FLAG M2 affinity column or the column washes with the FLAG octapeptide eluted fractions 1–11 (lanes 5–15). The samples were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunoblotted using anti-FLAG M2 antiserum (1:300 dilution). Enhanced chemiluminescence was used for detection. The positions of molecular mass markers are indicated.

**Fig. 3.** Map of the structure of the *C. elegans* AP-1 gene with a description of the exon/intron junctions. The cosmid clone representing the sequence of the *C. elegans* AP-1 gene (GenBank accession number AF068200) was retrieved from the Sanger *C. elegans* data base as cosmid numbers C42C1 and Y39C12 (GenBank accession number AL009026) and compared with the cloned cDNA sequence. A, exons are indicated by rectangles separated by a single line, representing introns. The numbers below the boxes indicate the number of nucleotides in each exon. B, size and position of introns in the AP-1 gene, with the uppercase letters in the DNA sequences representing nucleotides present in exons and the lowercase letters representing nucleotides present in introns. Amino acids (in one-letter code) are indi-
C. elegans Aminopeptidase-1

between the C. elegans AP-1 and the human LTA₄ hydrolyase/aminopeptidase (45% identity), a rabbit anti-human LTA₄ hydrolyase polyclonal antiserum (29) failed to detect any LTA₄ hydrolyase specific immunoreactive proteins in either the supernatant of COS-7 cells transfected with pFLAG.cel AP-1 or the cytosolic extract from C. elegans worms (results not shown).

Aminopeptidase Activity of the C. elegans AP-1 Enzyme and Its Inhibition by Bestatin—Based on the conservation of the catalytic zinc binding motif HEXXHX₃E in the primary structure of C. elegans AP-1 protein and several other zinc proteases and peptidases, the C. elegans AP-1 FLAG fusion protein was assayed for aminopeptidase activity using 11 different amino acid p-nitroanilide derivatives as chromogenic amide substrates. These compounds represent acidic, basic, and neutral amino acids, as well as amino acids with NH₂-terminal substitutions and D-stereochemistry. The purified C. elegans AP-1 FLAG fusion protein contained an intrinsic aminopeptidase activity that was absent in the anti-FLAG M2 affinity gel purified fractions of supernatant from mock-transfected COS-7 cells (Table I). The rate of hydrolysis of L-arginine p-nitroanilide was dependent on protein and substrate concentrations with a Kₘ of 0.43 ± 0.01 mM and a Vₘₐₓ of 0.18 ± 0.01 μmol/min/mg enzyme. These values can be compared with a Kₘ of 0.09 ± 0.01 mM and a Vₘₐₓ of 0.47 ± 0.01 μmol/min/mg obtained for the human LTA₄ hydrolyase/aminopeptidase (Fig. 5). The Kₘ and kₐₙ values for the hydrolysis of the amino acid p-nitroanilides by C. elegans AP-1 enzyme were dependent on the amino acid substituent (Table I). Comparison of the specificity constant kₐₙ/Kₘ for all 11 compounds tested reveals that the recombinant C. elegans AP-1 preferentially hydrolyzed the L-arginine derivative. Acidic amino acids, amino acids with NH₂-terminal substitutions, and amino acids with D-stereochemistry were poor substrates. The human recombinant LTA₄ hydrolyase/aminopeptidase enzyme had a similar substrate specificity for the selected p-nitroanilides. The human enzyme is considered an arginine aminopeptidase, despite its wide cleavage specificity, because it preferentially hydrolyzes tripeptides with l-arginine at the NH₂-terminal position (13). In the absence of a physiological substrate for the aminopeptidase activity of the human enzyme and its high catalytic efficiency for several synthetic tripeptides (exceeding the kₐₙ/kₘ for LTA₄ by 10-fold), the enzyme was suggested to be involved in the metabolism of dietary peptides and neuropeptides (13). This role can also be proposed for the C. elegans AP-1 enzyme. Bestatin, a potent inhibitor of human LTA₄ hydrolyase/aminopeptidase (10), as well as other aminopeptidases, inhibited the hydrolysis of L-arginine p-nitroanilide by AP-1. The concentration for half-maximal inhibition (IC₅₀) of p-nitroaniline formation was 2.6 ± 1.2 μM (results not shown).

During the cloning of the 5′-end of the C. elegans AP-1 cDNA, a PCR error introduced a point mutation at amino acid position 117, changing an alanine residue to a valine. When clones containing the Ala¹¹⁷ to Val¹¹⁷ PCR mutation were analyzed for aminopeptidase activity, they failed to hydrolyze the amide bond of any amino acid p-nitroanilide tested. This raises the possibility that certain conserved residues other than the previously documented Tyr³⁵⁵ and Glu³⁶⁶ may be important for the aminopeptidase activity of the mammalian LTA₄ hydrolyase/aminopeptidase enzyme. It is also interesting to note that this alanine residue (Ala¹¹⁴ in the human sequence) is conserved evolutionarily as it is found in the C. albicans LTA₄ hydrolase (which mainly exhibits aminopeptidase activity), the S. cerevisiae proposed LTA₄ hydrolase (which is yet to be characterized), all cloned mammalian LTA₄ hydrolases, including human, mouse, rat, and guinea pig, and the C. elegans AP-1 (data not shown).

Measurement of LTA₄ Hydrolase Activity of C. elegans AP-1 Enzyme—The C. elegans AP-1 protein was analyzed for epoxide hydrolase activity using LTA₄ as a substrate. Reverse-phase HPLC analysis of products formed when the purified FLAG-tagged C. elegans AP-1 enzyme or the cytosolic extract of C. elegans worms was incubated with LTA₄ revealed no production of LTB₄ (Fig. 6, peak 4, tracings 6 and 2, respectively). In contrast, the human LTA₄ hydrolase (used as a positive con-

### TABLE I

| Human LTA₄ hydrolyase | C. elegans AP-1 |
|-----------------------|-----------------|
| Kₘ (μM)      | kₐₙ (s⁻¹) | kₐₙ/Kₘ |
| L-Arg-pNA   | 0.09  | 0.55  | 6.10 × 10⁻³ |
| L-Ala-pNA   | 1.44  | 3.11  | 2.20 × 10⁻³ |
| L-Leu-pNA   | 0.25  | 0.52  | 2.08 × 10⁻³ |
| L-Lys-pNA   | 0.05  | 0.09  | 1.80 × 10⁻³ |
| L-Pro-pNA   | 0.24  | 0.45  | 1.87 × 10⁻³ |
| L-Met-pNA   | 0.40  | 0.24  | 6.00 × 10⁻³ |
| L-Val-pNA   | 0.90  | 0.08  | 10.0 × 10⁻³ |
| L-Asp-pNA   | No activity | No activity |
| L-Glu-pNA   | No activity | No activity |
| N-Acetyl-Ala-pNA | No activity | No activity |
| d-Leu-pNA   | No activity | No activity |

FIG. 5. Comparison of aminopeptidase activity of the cloned C. elegans AP-1 (A) with that of the recombinant human LTA₄ hydrolase/aminopeptidase (B). Purified FLAG-tagged C. elegans AP-1 (0.17 μg) (A) and human LTA₄ hydrolyase/aminopeptidase (0.17 μg) (B) in 250 μl of 0.1 M Tris, pH 8.0, 200 mM sodium chloride containing BSA (1 mg/ml) were incubated with 0.05-5 mM L-arginine p-nitroanilide. The rate of amide bond hydrolysis was monitored by measuring the absorbance of p-nitroaniline at 405 nm. The indicated Kₘ values were determined by nonlinear fit of the experimental values to the Michaelis-Menten equation.
LTB4; LTA4 was mostly converted to the nonenzymatic hydration by C. elegans cytosol in the absence of bestatin cytosol extracts and COS-7 cells transfected with pFLAG vector (tracing 4, 200 μM of anti-FLAG M2 purified 10,000 g supernatant of COS-7 cells transfected with pFLAG vector (tracing 5), and 0.8 μg of FLAG-tagged C. elegans AP-1 protein purified using anti-FLAG M2 affinity gel (tracing 6). LTA4 hydrolase assay and analysis of eicosanoid products were carried out as described previously (29). Peaks were identified by elution with co-chromatographed standards and their characteristic absorbance spectrum. Peak 1, prostaglandin B2 (internal standard); peak 2, 6-trans-LTB4; peak 3, 6-trans-12-epi-LTB4; peak 4, LTB4; peak 5, (5S,6R)-diHETE; peak 6, (5S,6S)-diHETE. The chromatograms are representative of three experiments with identical results.

Although C. elegans AP-1 does not appear to hydrolyze LTA4-ethyl ester as it is a suicide inactivator of the mammalian LTA4 hydrolases and is much more resistant than LTA4 to nonenzymatic hydration. The LTA4-ethyl ester had no effect on the aminopeptidase activity of AP-1 at concentrations up to 100 μM, 10 times the Km of mammalian LTA4 hydrolase for LTA4-ethyl ester (results not shown).

The bifunctional human LTA4 hydrolase/aminopeptidase enzyme is suicide-inactivated during catalysis via an apparently mechanism-based irreversible binding of LTA4 to the protein (45), with tyrosine at position 378 identified as the site for covalent binding of LTA4. Interestingly, the mutation of Tyr378 to Phe in the human LTA4 hydrolase yielded an enzyme with increased turnover and resistance to mechanism-based inactivation (39), thus dissociating catalysis and covalent modification/inactivation events. This tyrosine residue is a phenylalanine in the C. elegans AP-1 enzyme sequence (Phe382), but the C. elegans enzyme does not hydrolyze LTA4, indicating that other residues (lacking in the C. elegans AP-1 sequence) must also be important for LTA4 binding and catalysis.

That the cloned C. elegans AP-1 enzyme functions as an aminopeptidase with no LTA4 hydrolase activity is interesting, as its primary structure resembles LTA4 hydrolases more than it does aminopeptidases (Table II). Comparison of the C. elegans AP-1 enzyme and other proteins in the SwissProt database revealed 45% identity to the human, mouse, rat, and pig aminopeptidase-N (28–30%). Moreover, the identity between C. elegans AP-1 and mammalian LTA4 hydrolases extends over their entire primary structures, with some divergence in the N and C termini. In contrast, C. elegans AP-1 only overlaps a limited region of about 300 amino acids with other aminopeptidase enzymes (a region that contains the canonical zinc-binding motif HEXXXH15X6). It is interesting to note that the same identity (an average of 30%) is that shown between C. elegans AP-1 and human LTA4 hydrolase. The C. elegans enzyme retains 30% of the aminopeptidase activity of human LTA4 hydrolase (Table II).
Amino acid sequence identity (%) between C. elegans AP-1, LTA₄ hydrolases, and aminopeptidases

| C. elegans AP-1 | hLTA₄ | mLTA₄ | rLTA₄ | gpLTA₄ | rAP-B | hAP-N | rAP-N | pgAP-N |
|----------------|-------|-------|-------|--------|-------|-------|-------|--------|
| C. elegans AP-1 | 100   | 45    | 45    | 45     | 45    | 38    | 29    | 28     |
| hLTA₄          | 45    | 100   | 92    | 97     | 90    | 44    | 30    | 29     |
| mLTA₄          | 45    | 92    | 100   | 97     | 90    | 44    | 30    | 29     |
| rLTA₄          | 45    | 92    | 97    | 100    | 90    | 44    | 30    | 29     |
| gpLTA₄         | 45    | 92    | 97    | 100    | 90    | 44    | 30    | 29     |
| rAP-B          | 38    | 44    | 44    | 44     | 42    | 100   | 24    | 21     |
| hAP-N          | 29    | 30    | 30    | 29     | 31    | 21    | 77    | 79     |
| rAP-N          | 28    | 32    | 32    | 31     | 32    | 21    | 77    | 77     |
| pgAP-N         | 30    | 29    | 29    | 29     | 33    | 23    | 77    | 100    |

In conclusion, we have cloned and functionally expressed a 69-kDa protein from C. elegans Aminopeptidase-1 (GenBank accession number U27538) with 38% identity to mammalian LTA₄ hydrolase activity. The primary sequence identity of C. elegans AP-1 to mammalian LTA₄ hydrolases suggests that AP-1 may represent an evolutionary precursor of the mammalian LTA₄ hydrolases. Thus, mammalian LTA₄ hydrolases may have originated from aminopeptidases like AP-1, retaining their aminopeptidase B activity. The primary sequence identity of C. elegans AP-1 to mammalian LTA₄ hydrolases and rat aminopeptidase B suggests that these enzymes are evolutionarily related.

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REFERENCES

1. Samuelsson, B., Dahlén, S. E., Lindgren, J. A., Rouzer, C. A., and Serhan, C. N. (1987) Science 237, 1171–1176
2. Ford-Hutchinson, A. W., Bray, M. A., Doiy, M. V., Shipley, M. E., and Smith, M. J. H. (1980) Nature 286, 264–265
3. Samuelsson, B. (1983) Science 220, 568–575
4. Ford-Hutchinson, A. W., and Evans, J. F. (1986) The Leukotrienes: Their Biological Significance (Piper, P. J., ed) pp. 141–150, Raven Press, New York
5. Valle, B. L., and Auld, D. S. (1989) Biochemistry 28, 5647–5659
6. Haeggstrom, J. Z., Wetterholm, A., Shapiro, R., Valle, B. L., and Samuelsson, B. (1990) Biochem. Biophys. Res. Commun. 172, 965–970
7. Toh, H., Minami, M., and Shimizu, T. (1990) Biochem. Biophys. Res. Commun. 171, 216–221
8. Medina, J. F., Wetterholm, A., Radmark, O., Shapiro, R., Haeggstrom, J. Z., Valle, B. L., and Samuelsson, B. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 7620–7624
9. Haeggstrom, J. Z., Wetterholm, A., Valle, B. L., and Samuelsson, B. (1990) Biochem. Biophys. Res. Commun. 173, 411–417
10. Orning, L., Krivi, G., and Fitzpatrick, F. A. (1991) J. Biol. Chem. 266, 1375–1378
11. Wetterholm, A., and Haeggstrom, J. Z. (1992) Biochin. Biochem. Acta 1123, 275–281
12. Orning, L., and Fitzpatrick, F. A. (1992) Biochemistry 31, 4218–4223
13. Orning, L., Giese, J. K., and Fitzpatrick, F. A. (1994) J. Biol. Chem. 269, 11209–11217
14. Orning, L., Krivi, G., Bild, G., Giese, J., Aykent, S., and Fitzpatrick, F. A. (1991) J. Biol. Chem. 266, 16507–16511
15. Wetterholm, A., Blomster, M., and Haeggstrom, J. Z. (1996) Ecosinoids: From Biotechnology to Therapeutic Applications (Folco, G. C., Samuelsson, B., Maclouf, J., and Velo, G. P., eds) pp. 1–12, Plenum Press, New York
16. Radmark, O., and Haeggstrom, J. Z. (1990) Adv. Prostaglandin Thromboxane Leukotriene Res. 20, 35–45
17. Minami, M., Minami, Y., Ohno, S., Suzuki, K., Ohishi, N., Shimizu, T., and Seyama, Y. (1989) Adv. Prostaglandin Thromboxane Leukotriene Res. 19, 478–482
18. Funk, C. D., Radmark, O., Fu, J. Y., Matsumoto, T., Jornvall, H., Shimizu, T., and Samuelsson, B. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 6677–6681
19. Medina, J. F., Radmark, O., Funk, C. D., and Haeggstrom, J. Z. (1991) Biochem. Biophys. Res. Commun. 176, 1516–1524
20. Makita, N., Funk, C. D., Imai, E., Hoover, R. I., and Badr, K. F. (1992) FEBS Lett. 299, 273–277
21. Minami, M., Mutoh, H., Ohishi, N., Honda, Z., Bito, H., and Shimizu, T. (1995) Gene 161, 249–251
22. Naar, F., Becam, A. M., and Herbert, C. J. (1996) Yeast 12, 483–499
23. Palazzolo, M. J., Hamilton, B. A., Ding, D., Martin, C. H., Mead, D. A., Mierendorf, R. C., Raghavan, K. V., Meyerowitz, E. M., and Lipshitz, H. D. (1990) Genes 8, 25–36
24. Waterston, R., Martin, C., Craze, M., Huyah, C., Coulson, A., Hillier, L., Durbin, R., Green, P., Showenken, R., Halloran, N., Metzstein, M., Hawkins, T., Wilson, R., Berks, M., Du, Z., Thomas, K., Thierry-Mieg, J., and Sulston, J. (1992) Nat. Genet. 1, 114–123
25. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
26. Wiemann, S., Bopp, T., Zimmermann, J., Voss, H., Schwager, C., and Ansorge, W. (1995) BioTechniques 18, 688–697
27. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. M., and Struhl, K. (1994) Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc, New York
28. Laemmli, U. K. (1970) Nature 227, 680–685
29. Mancini, J. A., and Evans, J. F. (1993) Eur. J. Biochem. 218, 477–484
30. Carrier, D. J., Bogi, T., Coe, D. J., Guse, L., Rakbit, S., and Singh, K. (1988) Prostaglandins Leukotrienes Essent. Fatty Acids 34, 27–30
31. Evans, J. F., Nathaniel, D. J., Zamboni, R. J., and Ford-Hutchinson, A. W. (1985) J. Biol. Chem. 260, 10966–10970
32. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) J. Mol. Biol. 215, 403–410
33. Pearson, W. R., and Lipman, D. J. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 2444–2448
34. Kozak, M. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 2662–2666
35. Valle, B. L., and Auld, D. S. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 220–224
36. Rawlings, N. D., and Barrett, A. J. (1990) Biochem. J. 290, 205–218
37. Blomster, M., Wetterholm, A., Mueller, M. J., and Haeggstrom, J. Z. (1995) *Eur. J. Biochem.* 231, 528–534
38. Wetterholm, A., Medina, J. F., Radmark, O., Shapiro, R., Haeggstrom, J. Z., Vallee, B. L., and Samuelsson, B. (1992) *Proc. Natl. Acad. Sci. U. S. A.* 89, 9141–9145
39. Mueller, M. J., Blomster, M., Oppermann, U. C. T., Jornvall, H., Samuelsson, B., and Haeggstrom, J. Z. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 5931–5935
40. Fields, C. (1990) *Nucleic Acids Res.* 18, 1509–1512
41. Paggett, R. A., Grabewski, P. J., Konarska, M. M., Seller, S., and Sharp, P. A. (1986) *Annu. Rev. Biochem.* 55, 1119–1150
42. Aroian, R. V., Levy, A. D., Koga, M., Ohshima, Y., Kramer, J. M., and Sternberg, P. W. (1993) *Mol. Cell. Biol.* 13, 626–637
43. Mancini, J. A., and Evans, J. F. (1995) *Eur. J. Biochem.* 231, 65–71
44. Borget, P., and Samuelsson, B. (1979) *J. Biol. Chem.* 254, 7865–7869
45. Orning, L., Gierse, J., Duffin, K., Bild, G., Krivi, G., and Fitzpatrick, F. A. (1992) *J. Biol. Chem.* 267, 22733–22739
46. Cadel, S., Foulon, T., Viron, A., Balogh, A., and Midol-Monnet, S. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 2963–2968
47. Cormack, B. P., and Falkow, S. (June 25, 1998) U. S. Patent 5,529,916