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CHARACTERIZATION OF ARACHIDONIC ACID METABOLIZING ENZYMES IN THE METAZOA *SCHISTOSOMA MANSONI* AND *CAENORHABDITIS ELEGANS*

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ABSTRACT

Cyclooxygenase and lipoxygenase enzymes metabolize arachidonic acid to biologically active eicosanoids which act as potent local mediators of various physiological and pathological responses. The production of certain immunoregulatory eicosanoids may help parasites to evade host immune responses and establish chronic infections. Our analysis of arachidonic acid metabolites synthesized by the adult trematode parasite *Schistosoma mansoni* indicated the presence of a soluble, enzymatically active lipoxygenase (LOX) and the absence of any cyclooxygenase (COX) activity. Screening of several *S. mansoni* cDNA libraries using mammalian LOX sequences resulted in the cloning of the mouse (the parasite host) LOX homologue due to the high sequence homology among the different mammalian lipoxygenases. In search of a more specific probe, the phylogenetically related nematode *Caenorhabditis elegans* DNA database was searched for arachidonic acid metabolizing enzyme-like sequences. Although no COX or LOX related sequences were revealed, a small expressed sequence tag (EST) (termed cm01c7) with strong homology to the human leukotriene A_4 (LTA_4) hydrolase was found. The *C. elegans* cm01c7 clone was used to screen *S. mansoni* cDNA libraries but failed to identify any homologous sequences from *S. mansoni*. On the other hand, the utilization of cm01c7 as a probe together with conventional hybridization screening and anchored polymerase chain reaction techniques resulted in the cloning of the full-length LTA_4 hydrolase-like homologue, termed AP-1, from *C. elegans*. The AP-1 cDNA was expressed transiently in COS-7 mammalian cells and tested for LTA_4 hydrolase and aminopeptidase activities. Despite the strong homology between the human LTA_4 hydrolase and *C. elegans* AP-1 (45 % identity at the amino acid level), reverse-phase high pressure liquid chromatography and radioimmunoassay for LTB_4 production revealed the inability of *C. elegans* AP-1 to use LTA_4 as a substrate. In contrast, *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 LTA_4 hydrolase/aminopeptidase enzyme with a preference for arginyl-*p*-nitroanilide as a substrate. These results support the idea that the enzyme originally functioned as an aminopeptidase in lower metazoa and then developed LTA_4 hydrolase activity in more evolved organisms. The study of arachidonic acid oxygenases in nematodes and trematodes will extend our knowledge about the degree of evolutionary conservation of these important regulatory proteins and may also lead to novel therapeutic targets for the treatment of human parasitic diseases.

RÉSUMÉ

La cyclooxygénase (COX) et la lipoxygénase (LOX) sont des enzymes responsables de la transformation de l'acide arachidonique en composés biologiquement actifs. Ces composés, appelés eicosanoïdes, agissent comme médiateurs puissants dans diverses réponses physiologiques et pathologiques. Comme certains eicosanoïdes possèdent des propriétés immunorégulatrices, leur production par des parasites peuvent déjouer la réponse immunitaire de l'hôte et ainsi entraîner une infection chronique. L'analyse des métabolites de l'acide arachidonique produits chez le trématode adulte *Schistosoma mansoni* a révélé la présence d'une lipoxygénase soluble et active sur le plan enzymatique, de même que l'absence d'activité cyclooxygénase. Le criblage de plusieurs librairies d'ADNc de *S. mansoni* à l'aide d'amorces constituées de séquences de LOX de mammifères a donné lieu au clonage de la LOX de souris (l'hôte du parasite), cela étant dû à la forte homologie dans les séquences des LOX de mammifères. Afin d'obtenir une amorce plus spécifique, nous avons recherché des séquences possédant des similarités avec les enzymes responsables de la transformation de l'acide arachidonique dans la base de données d'ADN de *Caenorhabditis elegans,* un nématode relié sur le plan phylogénétique à *S. mansoni.* Bien qu'aucunes séquences possédant des similarités avec la COX ou la LOX n'aient été trouvées, une petite séquence exprimée avec un tag (EST) appelée cm01c7 et possédant une forte homologie avec la leucotriène A_4 (LTA_4) hydrolase humaine a été identifiée. L'utilisation du cm01c7 comme amorce n'a pas permis l'identification d'une séquence homologue à partir des librairies d'ADNc de *S. mansoni.* Toutefois, lorsque le cm01c7 a été utilisé comme amorce avec les techniques de criblage par hybridation conventionnelle et les techniques d'amplification de gènes PCR (*polymerase chain reaction*) ancrée, la séquence complète d'un homologue de la LTA_4 hydrolase appelé AP-1 a pu être clonée chez *C. elegans.* L'ADNc du clone AP-1 a ensuite été exprimé de façon transitoire dans les cellules de mammifères COS-7, et ensuite testé pour les activités de LTA_4 hydrolase et d'aminopeptidase. Malgré la forte homologie entre

la LTA_4 hydrolase humaine et le clone AP-1 de *C. elegans* (45 % des acides aminés étant identiques), l'analyse par chromatographie liquide à haute pression en phase inversée et par essais radio-immunologiques de la production de leucotriène B_4 a montré l'incapacité du clone AP-1 à utiliser la LTA_4 comme substrat. Par contre, sa capacité à hydrolyser une variété de substrats peptidiques couplés à un groupe *p*-nitroanilide a permis de demontrer que le clone AP-1 possède une activité aminopeptidase importante. Cette activité ressemble à celle de l'enzyme LTA_4 hydrolase/aminopeptidase humaine qui possède elle aussi une préférence pour le substrat arginyl-p-nitroanilide. Ces résultats appuient l'hypothèse selon laquelle l'enzyme devait à l'origine fonctionner uniquement comme aminopeptidase chez les métazoaires pour ensuite développer une activité LTA_4 hydrolase chez les organismes plus évolués. L'étude des oxygénases responsables de la transformation de l'acide arachidonique chez les nématodes et les trématodes devrait contribuer à améliorer notre compréhension du degré d'évolution de ces protéines régulatrices importantes, et également mener à la découverte de nouvelles cibles thérapeutiques pour le traitement de maladies parasitaires humaines.

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ABBREVIATIONS

AA	arachidonic acid (5,8,11,14-eicosatetraenoic acid)
AP	aminopeptidase
COX	cyclooxygenase
cDNA	complementary DNA
DMSO	dimethyl sulfoxide
ECL	enhanced chemiluminescence
EDTA	ethylenediamine-tetraacetic acid
ETYA	eicosatetraynoic acid
EETs	epoxyeicosatrienoic acids
FLAP	5-lipoxygenase activating protein
HETE	hydroxyeicosatetraenoic acid
HPETE	hydroperoxyeicosatetraenoic acid
HODE	hydroxyoctadecadienoic acid
IC_{50}	concentration of half inhibition
kb(p)	kilobase (pairs)
kDa	kilodaltons
LDL	low-density lipoproteins
LOX	lipoxygenase
LT	leukotriene
LTA_4	5,6-oxido-7,9,11,14-eicosatetraenoic acid
LTB_4	5(S),12(R)-dihydroxy-6,8,10,14-eicosatetraenoic acid
LTC_4	5(S)-hydroxy-6(R)-S-glutathionyl-7,9,trans-11,14-cis-eicosatetraenoic acid
LTD_4	5(S)-hydroxy-6(R)-S-cysteinylglycyl-7,9-trans-11,14-cis-eicosatetraenoic acid
LTE_4	5(S)-hydroxy-6(R)-S-cysteinyl-7,9-trans-11,14-cis-eicosatetraenoic acid
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	prostaglandin
PGB_2	prostaglandinB_2
PI	phosphatidylinositol
PLA_2	phospholipase A_2
PLC	phospholipase C
PMN	polymorphonuclear cells
RP-HPLC	reverse phase-high pressure liquid chromatography
RT-PCR	reverse transcriptase-polymerase chain reaction
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SRS-A	slow reacting substance of anaphylaxis
TLC	thin layer chromatography

TNF	tumor necrosis factor
TXA_2	thromboxane A_2

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1. INTRODUCTION

1.1 Eicosanoids

The term eicosanoids was coined to refer to all biologically active oxygenated metabolites of arachidonic acid and certain other C20 polyunsaturated essential fatty acids (1, 2). In mammals, eicosanoids act as potent local mediators of various physiological and pathological responses including inflammation, bronchoconstriction, pain, fever, regulation of vascular tone, and regulation of immune responses (3, 4). Three broad groups of eicosanoids are recognized, the prostanoids, all products of the cyclooxygenase pathways, the epoxyeicosatrienoic acids (EETs), products of cytochrome P450 monooxygenases, and the leukotrienes, hydroxyeicosatetraenoic acids (HETEs), and lipoxins, products of the 5, 12, and 15- lipoxygenase pathways (5). The classical substrates for the production of eicosanoids are dihomo-γ-linolenic acid (20:3w-6), arachidonic acid (20:4w-6), and eicosapentaenoic acid (20:5w-3). These precursor fatty acids contain three, four or five double sites of unsaturation and lead to the production of the 1, 2, or 3 series of prostanoids, respectively (6, 7). As the 5-6 *cis* double bond in the precursor fatty acid is required for the biosynthesis of the 3, 4, or 5 series of leukotrienes, these are therefore formed from mead acid (20:3w-9), (20:4w-6), or (20:5w-3) but not from dihomo-γ-linolenic acid (which does not carry a 5-6 *cis* double bond) (7). These essential polyunsaturated fatty acids could be obtained directly from the diet (meat, fish, or seafood) or derived from linoleic acid (18:2w-6) and γ-linolenic acid (18:3w-6) by desaturation and elongation processes which usually occur in the liver (7, 8).

Arachidonic acid is the preferred substrate for eicosanoid biosynthesis and it is the most abundant C20 polyunsaturated fatty acid found in the membranes of mammalian cells. The abundance of arachidonate lead to the predominant formation of the 4-series of leukotrienes and the 2-series of prostanoids. Cellular concentrations of free arachidonic acid are very low (6, 9) as it is extensively and preferentially incorporated into the *sn*-2 position of phospholipids in cell

membranes. Therefore, the free concentration of arachidonate available for eicosanoid biosynthesis is dependent on the release of arachidonate by phospholipases versus the rate of its re-esterification by acyl-CoA transferases (9, 10, 11).

1.2 Phospholipases and the release of arachidonic acid

Arachidonic acid is normally esterified in membrane glycerophospholipids mainly in the *sn*-2 position of phosphatidylcholine (PC), phosphatidylinositol (PI), and phosphatidylethanolamine (PE) (9). Several phospholipase pathways contribute to the release of arachidonic acid from membrane phospholipids. The direct action of phospholipase A_2 (PLA_2) mainly on PC and PE , and the activation of phospholipase C (PLC) and diacyl/monoacylglycerol lipases which release arachidonic acid from PI, are considered to be the major routes for obtaining free arachidonic acid (9, 11, 12, 13). However, phospholipase A_1 and phospholipase D (which convert PE or PC to phosphatidic acid followed by the formation of diglycerides and monoglycerides) (11, 14) as well as receptor activation of PC-specific phospholipase C and low-density lipoproteins (LDLs) can also liberate arachidonic acid. In addition, phospholipase C and D and arachidonic acid itself activate protein kinase C which can reduce the rate of reacylation of arachidonate resulting in increased free arachidonic acid levels (15, 16). Phospholipases can be activated directly by the interactions of growth factors, hormones, autocoids or other chemical mediators with cell-surface G-protein coupled receptors or through an increase in intracellular free calcium concentration in response to physical stimuli (17, 18).

In most cells, PLA_2 is the primary enzymatic activity which regulate arachidonic acid release for eicosanoid biosynthesis, accordingly, PLA_2 enzymes are considered to be the rate limiting enzymes for the formation of prostanoids and leukotrienes (9, 19). The mammalian PLA_2 enzymes include several forms: 1) the calcium-dependent secreted PLA_2 forms which are characterized by their low molecular masses of 13-15 kDa and an absolute requirement for calcium during

hydrolysis (20, 21); 2) the intracellular cytosolic PLA_2 (85 kDa enzyme) (22, 23); and 3) the calcium- independent PLA_2 enzymes which include a 40 kDa PLA_2 from myocardium (24, 25, 26) and a 80 kDa PLA_2 recently isolated from the macrophage cell line P388D1 (27, 28).

The secreted forms of ($sPLA_2$) show little if any specificity for the acyl group at the *sn*-2 position of the phospholipid (18). Proinflammatory stimuli including tumor necrosis factor (TNF), interleukin 1β and cytokines induce $sPLA_2$ (29, 30, 31) and this induction can be prevented by corticosteroids (32, 33). Secreted PLA_2 has been postulated to play a role in digestion, cell membrane homeostasis, and inflammation (18, 20).

In contrast to $sPLA_2$, the cytosolic PLA_2 ($cPLA_2$) has a preference for hydrolysis of arachidonic acid which is esterified at the *sn*-2 position of PC and PE (22). This $cPLA_2$ is coupled to receptors via G-proteins (20) and has the ability to translocate to membrane structures (upon cell activation) in a calcium-dependent manner (34, 35). This translocation is essential for the $cPLA_2$ activity and is due to a 138 amino acid terminal domain which is a calcium-dependent phospholipid binding domain. Agonist-stimulated phosphorylation further enhances $cPLA_2$ activity (36). Phosphorylation by MAP (mitogen-activated protein) kinases at serine-505 of $cPLA_2$ which can be activated by TNF has been shown (17, 37, 38). Thus, both increased intracellular calcium levels and serine phosphorylation are required for full activation of $cPLA_2$. It has also been shown that the transcription and translation of $cPLA_2$ is induced by interleukin 1β and transforming growth factor-β_2 (39).

Calcium-independent forms of PLA_2 ($iPLA_2$) are abundant in myocardium (17) and the 40 kDa myocardial $iPLA_2$ displays enzymatic selectivity for plasmalogen-containing phospholipids (the predominant phospholipid in myocardium). This enzyme may be involved in the pathogenesis of myocardial diseases (40, 41, 42).

It is not yet determined if there is one specific phospholipase which is responsible for the release of arachidonic acid utilized in the formation of prostanoids and leukotrienes (11).

1.3 Arachidonic acid metabolism

The first committed step in the metabolism of arachidonic acid involves fatty acid oxygenases (cyclooxygenases and lipoxygenases) which require hydroperoxides for initiation of oxygenation, resulting in the formation of lipid hydroperoxides. Subsequent metabolism leads to the formation of the biologically active eicosanoids (5). Upon its release from membrane phospholipids, arachidonic acid can be metabolized mainly through three distinct pathways. The cyclooxygenase pathway leads to the conversion of arachidonic acid to prostaglandins, thromboxanes, and prostacyclin (5, 43, 44). The lipoxygenase pathway results in the production of leukotrienes, HETEs, lipoxins and other related hydroxy acids (4, 5, 19, 45). The cytochrome P450 pathway produces EETs and hydroxyeicosatrienoic acid isomers through a non-regiospecific manner (5, 46).

1.3.1 The cyclooxygenase pathway

The first committed step in the metabolism of arachidonic acid involves fatty acid dioxygenases (cyclooxygenases and lipoxygenases) and results in the formation of lipid peroxides, which strongly enhance the catalytic activity of these enzymes (for review see 5, 47). Cyclooxygenase, also known as prostaglandin G/H synthase (PGHS) or prostaglandin endoperoxide synthase, is a bifunctional, membrane-bound hemeprotein that catalyzes both the bisoxygenation of arachidonic acid to form PGG_2 (cyclooxygenase activity) as well as the peroxidative reduction of PGG_2 to form the endoperoxide PGH_2 (peroxidase activity) which is the precursor to the formation of all prostanoids (48-51). Two isoforms of the cyclooxygenase (COX) enzyme with similar molecular masses (≈ 70 kDa) have been identified. The constitutively expressed COX-1 which was first characterized from sheep vesicular glands (52, 53), is involved in "housekeeping functions" including regulation of vascular homeostasis,

modulation of renal function, and protection of the gastric mucosa (54, 55). The inducible form COX-2 which is induced by various inflammatory cytokines, mitogens, and endotoxin (56-59), is involved in differentiative processes such as inflammation and ovulation (60) and is the enzyme responsible for production of prostaglandins during inflammation (61-64). Both COX isoforms are non-selectively inhibited by non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin, indomethacin and flurbiprofen (65), a property that accounts for their shared therapeutic and side effects (66). Thus, the ability of NSAIDs to inhibit COX-2 may well explain their therapeutic effect as antiinflammatory drugs, whereas inhibition of COX-1 may explain their undesirable side effects, such as gastric and renal damage.

COX-1 is a glycoprotein homodimer complex composed of two identical heme-containing subunits (70 kDa each), and is found primarily in the endoplasmic reticulum of mammalian cells (67, 68). The cDNA for COX-1 has been cloned from ram seminal vesicle, mouse, as well as different human sources (69-71) and encodes for a 600-amino acid protein with a 24-residue signal peptide and four potential N-linked glycosylation sites. COX-2 cDNA has been cloned from chicken and mouse (62, 72) and encodes a 603 (chick)/604 (mouse)-amino acid protein that is 60% identical to COX-1 at the amino-acid level. COX-2 is also a glycoprotein of 70 kDa containing five potential N-glycosylation sites (61) (N-linked glycosylation is required for maximum activity of both COX-1 and COX-2 (73). The major differences between the COX-1 and COX-2 forms are at their amino and carboxyl termini (68) , a 17-amino acid stretch within the signal peptide of COX-1 at the N-terminus is not present in COX-2, in contrast, COX-2 contains an 18-amino acid region at the carboxyl terminus not present in COX-1. All the other major features found in COX-1, like the aspirin-sensitive serine residue, epidermal growth factor-like domain, homology to peroxidases, and residues essential to catalytic activity are conserved in COX-2 (61, 70).

The human COX-1 mRNA (3 Kb) is ubiquitously expressed in most tissues (74) while very low levels of basal expression of human COX-2 mRNA

(4.5 Kb) are restricted to certain tissues (including kidney, prostate, brain, testis, and lung), with no expression in most other major organs (11, 59, 62, 72). COX-1 mRNA is subjected to low level regulation (usually 2-4 fold changes in mRNA levels) by many factors including phorbol ester, interleukin-1, progesterone, platelet-derived growth factor, EGF, cAMP, and serum (51, 56). On the other hand, COX-2 mRNA is downregulated by glucocorticoids and is highly upregulated by EGF, forskolin, phorbol ester, and serum (62, 72) (which induce a large and rapid increase in mRNA levels in mouse fibroblasts) as well as by luteinizing hormone/human chorionic gonadotropin (LH/hCG) which induces a 40-fold induction of COX-2 mRNA levels in rat granulosa cells of preovulatory follicles (75).

The human and murine COX-1 genes have been cloned (76, 77), they share the same exon/intron boundaries and are divided into 11 exons spanning about 22 Kb, while the murine COX-2 gene contains 10 exons and spans 8 Kb of genomic DNA (78). The human COX-1 gene is located on chromosome 9q32-q33.3 (70, 78) while the human COX-2 gene is located on chromosome 1q25.2-q25.3 (78).

1.3.2 Prostanoids: biological actions and receptors

Prostaglandins (PGs) are classified into 3 series based on the number of double bonds in the side chains; PGs are also classified into several types based on the substitution of the cyclopentane ring. The 2-series of PGs, which have the greatest biological importance in higher eukaryotes, are derived from arachidonic acid (the most abundant of the prostanoid precursors) and thus constitute the most abundant naturally occurring prostanoids (6). These biologically active prostanoids (which include PGs and thromboxanes (TXs)) are synthesized from the endoperoxide PGH_2 by the action of PGD_2 synthase, PGE_2 synthase, $PGF_{2\alpha}$ synthase, thromboxane synthase, and prostacyclin synthase which produce PGD_2, PGE_2, $PGF_{2\alpha}$, TXA_2, and PGI_2, respectively (11, 79). Using pharmacological and ligand binding techniques (80, 81), these prostanoids were

shown to act on specific receptors on cell membranes. Distinct receptors were identified for each of the naturally occurring prostanoids and a comprehensive classification of the receptors was developed (82). According to the relative affinities of the natural PGs for specific receptors, the receptors with highest affinities for PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and TXA_2 were designated as the DP, EP, FP, IP, and TP receptors, respectively. Several subtypes of these receptors have also been identified (83).

Once released, prostanoids have been reported to have a diverse spectrum of biological activities in a variety of cell and tissue types (for review see 47). Of the various biological activities associated with prostanoids, the most well characterized are their contractile and relaxant effects on smooth muscles. Four of the prostanoid receptor subtypes (EP_1, EP_3, TP, and FP) mediate contraction of smooth muscles via increased intracellular calcium and another four (EP_2, EP_4, DP, IP) mediate relaxation via increased intracellular cAMP (47, 83). EP_3 receptors also mediate their contractile actions indirectly via coupling to inhibition of adenylate cyclase and lowering cAMP levels (83). Depending on the type of receptor present, prostaglandin-mediated contraction or relaxation has been detected in many smooth muscle preparations including those of respiratory, gastro-intestinal, vascular, reproductive, and urinary systems as well as in the eye (83). Another important biological function which is mediated through EP_2 and EP_4 receptors is the negative regulation of the immune system by PGE_2 (83). PGE_2 efficiently induces apoptosis of thymocytes and inhibits the function and the proliferation of T cells by increasing the intracellular cAMP level (84). PGE_2 also inhibits LTB_4 synthesis and tumor necrosis factor induction in macrophage, as well as colony-stimulating factor 1-dependent growth of macrophages (85-87). PGE_2 has also been shown to induce B-cell unresponsiveness and to act as a potent feedback inhibitor of both cellular and humoral immune responses (88, 89). EP_2 and/or EP_4 receptors are also involved in the vasodilation of various blood vessels, a PGE_2 effect mediated by PGE_2-induced intracellular cAMP elevation (47, 83). PGI_2 also causes vasodilation, and through the coupling of IP receptor to stimulation of adenylate cyclase via stimulatory G-protein (G_s), PGI_2 increases

cAMP levels in human platelets leading to the inhibition of platelet aggregation (90). Recently, several prostanoid receptors and their isoforms have been cloned from mouse, bovine, and human cDNA libraries and include the EP_1 (91, 92), EP_2 (92), EP_3 (which exist as a number of alternatively spliced forms (94, 95)), EP_4 (96), FP (97), and TP (98, 99) receptors (which also exist as two alternatively spliced forms (83)). The recently isolated four isoforms of the EP_3 receptors (A-D) (95), differ only in their C-terminal tails and may exert their different functional roles either by coupling to different G-proteins to activate different second messenger systems or by showing different rates of agonist-induced desensitization (83). The identification of such isotypes for the prostaglandin receptors may help to explain the diversity of prostaglandin functions.

1.3.3 The lipoxygenase pathway

Alternative pathways of oxidation of arachidonic acid are through the lipoxygenase enzymes. There are three major lipoxygenases in mammalian cells, 5-, 12-, and 15-lipoxygenase, named for their ability to insert molecular oxygen at carbon 5, 12, and 15 of arachidonic acid, respectively (3, 19). The 5-lipoxygenase pathway leads to the formation of leukotrienes; HETES are generated by the action of the different lipoxygenases, and lipoxins are formed by the coordinate participation of 5- and 12/15-lipoxygenase via the putative intermediate 15-hydroxy-LTA_4 (19, 45). These lipid mediators have been implicated in the pathogenesis of allergy and inflammation.

1.4 Lipoxygenases

Lipoxygenases are a family of non-heme iron-containing dioxygenases which catalyze the stereospecific insertion of molecular oxygen into polyunsaturated fatty acids with 1-*cis*,4-*cis*-pentadiene structure such as arachidonic acid and linoleic acid (100). Lipoxygenases are widely distributed throughout the plant and animal kingdoms, and with the advances in molecular

biology techniques, at least 31 unique lipoxygenase sequences from various plant and animal species are currently entered into the GenBank database. The first lipoxygenase to be characterized was the soybean lipoxygenase-1 enzyme which catalyzes the synthesis of 13-hydroperoxy-9,11-octadecadienoic acid (13-HPODE) from linoleic acid (for reviews of plant lipoxygenases see 101, 102). This plant enzyme was extensively investigated and its three-dimensional X-ray crystal structure was later reported (103). In plants, the fatty acid hydroperoxides (products of the lipoxygenase pathway) are precursors of species that are involved in development, growth regulation, wound response, and pest resistance (104-106). In mammalian tissues, beside the well characterized 5-, 12-, and 15-lipoxygenases, an 8-lipoxygenase from mouse epidermis has also been described (107, 108).

1.4.1 5-lipoxygenase and leukotriene biosynthesis

The most extensively studied lipoxygenase enzyme is the 5-lipoxygenase (5-LOX) as it catalyses the first two steps in the biosynthesis of the biologically important leukotriene mediators. The first step involves the incorporation of molecular oxygen at carbon 5 of arachidonate to form 5(S)-hydroperoxy-6, 8, 11, 14-eicosatetraenoate (5-HPETE) (Figure 1.1). This intermediate is then either dehydrated to the allylic epoxide LTA_4 by the dehydrase activity of 5-LOX (the second catalytic activity of 5-LOX) (109, 110), or degraded by a peroxidase to 5-hydroxyeicosatetraenoate (5-HETE) (111). LTA_4 can then be hydrolyzed to LTB_4 by the action of LTA_4 hydrolase or conjugated with glutathione to form LTC_4 by the action of LTC_4 synthase (3). Sequential removal of glutamate and glycine by γ-glutamyltranspeptidase and cysteinylglycine dipeptidase, subsequently produces LTD_4 and LTE_4, respectively.

LTC_4, LTD_4, and LTE_4 are known as the cysteinyl leukotrienes and collectively account for the biological activity originally described as the *slow reacting substance of anaphylaxis* or *SRS-A* (112). These leukotrienes interact with high-affinity, structurally specific, G protein-coupled receptors (at least two

subtypes cys-LT_1 and cys-LT_2 are described to date) to produce contractile responses which can lead to bronchoconstriction, induction of airway secretion, and changes in microvascular permeability in the lung (19). Thus, the cysteinyl leukotrienes have been implicated as important mediators of immediate hypersensitivity reactions and allergic conditions, and leukotriene D_4 receptor antagonists have shown promise in the treatment of human bronchial asthma (113).

5-LOX was initially characterized in rabbit leukocytes (114) and its dual catalytic activity which generates both 5-HPETE and LTA_4 was subsequently demonstrated using potato tuber 5-LOX and mammalian 5-LOX (115-117). The enzyme has been purified and cloned from different mammalian sources (118-120) and was characterized as a 78 kDa soluble protein that requires calcium and ATP for enzymatic activity (116, 117, 121, 122, 123). Increases in intracellular calcium induced by agents such as A23187 result in 5-LOX activation and subsequent translocation from the cytosol or nucleus to the nuclear membrane (124-126). This translocation is associated with the activation of cellular leukotriene biosynthesis with the subsequent irreversible suicide-inactivation of 5-LOX (126).

In whole cells, 5-LOX also requires the presence of 5-lipoxygenase activating protein (FLAP) to form leukotrienes from endogenous substrate (127). FLAP is an 18 kDa membrane bound protein that resides in the nuclear envelope of PMN leukocytes and macrophages (124, 128). This small protein was originally discovered through its ability to bind with high affinity to MK-886, an indole inhibitor of leukotriene biosynthesis (129). MK-886 inhibits leukotriene biosynthesis in intact cells, but not in cell-free systems, by inhibiting the membrane association of 5-LOX (127). Thus, FLAP is thought to act as an arachidonate transfer protein that presents arachidonic acid to 5-LOX (130, 131).

cDNAs for mammalian 5-LOX have been cloned from various sources including human (119, 120), rat (118), mouse (132), and hamster(133). There is an overall 60% amino acid sequence similarity among human 5-, 12-, and 15-LOX, and each human lipoxygenase is roughly 25% identical to plant

lipoxygenases (134). A core region in the central portion of human 5-LOX molecule is highly conserved in other mammalian and plant lipoxygenases including rat 5-LOX (118), human platelet 12-LOX (135), porcine leukocyte 12-LOX (136), human 15-LOX (137), rabbit 15-LOX (138), soybean LOX isozymes 1, 2, and 3 (139-141), and pea seed LOX (142) (Figure 1.2). This core region contains the motif His-X_4-His-X_4-His-X_{17}-His-X_8-His with five conserved histidine residues (numbered with respect to 5-LOX in Figure 1.2), as well as several interspersed conserved acidic and basic residues (boxed residues in Figure 1.2). A second region of high homology with another conserved histidine and glutamine residues, closer to the C-terminal, is found in all ten LOX sequences, and a third region with a conserved histidine is found in mammalian but not in plant LOX (Figure 1.2; 143). Some of these conserved residues, as discussed below, are essential for iron-binding and/or activity of LOX enzymes.

Human 5-LOX has been expressed in various cellular systems (144-146), and the recombinant protein was used to demonstrate that the enzyme contains one mole of iron per mole of enzyme (147). Site directed mutagenesis was utilized to delineate the residues of 5-LOX which are critical for binding the iron atom. The residues of soybean 15-LOX which were shown to be involved in iron binding were four imidazole ligands and two oxygen ligands (148). Two crystal structures of the soybean 15-LOX have shown that the iron atom in the enzyme is coordinated by three histidines and the carboxylate of the C-terminal isoleucine (103, 149). The corresponding residues in human 5-LOX are His-367, His-372 (in the main core region in Figure 1.2), His-550 (in the second region in Figure 1.2), and Ile-673. Mutagenesis of His-367, His-372, His-550 and deletion of the six C-terminal residues of 5-LOX produced an inactive enzyme (150-153). The His-372 and His-550 mutants were devoid of iron while the His-367 mutant contained smaller amounts of iron than in the wild type enzyme (152, 154). The His-390 and His-399 (also found in the core region in Figure 1.2) mutants contained similar amounts of iron as the native protein but only retained a partial amount of the enzymatic activity. Additional mutational studies of other conserved residues showed that His-362, Met-435, Tyr-383, and Phe-393 are

not essential for 5-LOX activity (151, 154). Taken together, these results indicate that His-367, His-372, and His-550 are essential for 5-LOX activity (as was previously shown for the corresponding residues in the soybean enzyme; 155), and that the same residues with the possible inclusion of Ile-673 may constitute the iron binding sites for 5-LOX.

The gene encoding human 5-LOX has been cloned and determined to reside on chromosome 10 (156). The gene spans more than 82 kb and consists of 14 exons and 13 introns with similar exon/intron boundaries to the human 12-LOX and rabbit 15-LOX genes. Recently, the 5-LOX gene has been disrupted in mice, and 5-LOX 'knockout' mice have been developed and characterized. These 5-LOX$^{-/-}$ mice do not display a phenotype different from control mice and, as expected, 5-LOX was not essential for fetal development or survival (157). On the other hand, the analysis of these 5-LOX-deficient mice confirmed the role of leukotriene products as important inflammatory mediators (158). 5-LOX$^{-/-}$ mice also demonstrated a potential beneficial role of leukotrienes in the host defense reaction against parasitic and bacterial infections (159).

1.4.2 12-lipoxygenase

Arachidonate 12-LOX, found in human platelets, was the first mammalian lipoxygenase to be discovered (160). It catalyzes the stereospecific incorporation of molecular oxygen into C-12 of arachidonic acid to form 12(S)-hydroperoxy-5,8,14-*cis*-10-*trans*-eicosatetraenoic acid (12-HPETE) which is then reduced by cellular glutathione peroxidases to 12(S)-hydroxy eicosatetraenoic acid (12-HETE; Figure 1.1). There are two main classes of 12-LOX in terms of the identity of primary structure; the 'platelet-type' (the enzymes cloned from human (135, 161) and mouse platelets (162), and the 'leukocyte-type' (the enzymes cloned from porcine leukocyte (136), bovine trachea (163), rat pineal gland (164) and whole brain (165), and mouse peritoneal macrophage (166). All 12-LOX proteins consist of 662 amino acid residues and their molecular masses are about 75 kDa. The leukocyte and platelet 12-LOX isozymes share only 58% identity (at the

amino acid level) and differ in their substrate specificity, product profile, immunogenicity, sequence and gene structure (100, 167, 168). The leukocyte-type 12-LOX forms 15-HPETE in addition to 12-HPETE from arachidonic acid (169) and metabolizes both arachidonic and linoleic acids, whereas the platelet enzyme forms almost exclusively 12-HPETE and metabolizes virtually no C-18 fatty acids (for review see 170). Thus, leukocyte-type 12-LOX is more related to 15-LOX than it is to platelet 12-LOX, as both leukocyte 12-LOX and 15-LOX are dual specificity lipoxygenases and share up to 85% identity in their primary structure in higher mammals (100, 134, 136). In fact, leukocyte 12-LOX and 15-LOX cDNAs have not yet been isolated within the same species and it has been postulated that the 15-LOX found in human reticulocytes, airway epithelium, and eosinophils is the species equivalent of the leukocyte 12-LOX found in bovine tracheal epithelium, porcine leukocytes, and mouse macrophages (134,170). In view of the high amino acid sequence homology between leukocyte 12-LOX and reticulocyte 15-LOX (85%), site-directed mutagenesis was used in an attempt to convert 12-LOX to 15-LOX. A double mutation of Val-418 and -419 of porcine leukocyte 12-LOX to Ile and Met (residues found in 15-LOX) increased the ratio of 15- and 12-LOX activities from 0.1 to 5.7 (171). Similar mutations of 12-LOXs of rat brain and human platelets resulted in a partial conversion of 12-LOX to 15-LOX (172, 173).

12-LOX purified from porcine leukocyte contains about 0.7 atom of non-heme iron per mole of enzyme (174), and site-directed mutagenesis studies showed that the same histidine residues essential for iron binding and catalytic activity of 5-LOX are also essential for both leukocyte and platelet 12-LOX activities (171, 173). Thus, a loss of enzyme activity and a very low content of iron were observed with His-361, His-366, and His-541 mutants of porcine leukocyte 12-LOX (171), and the corresponding three histidine residues (His-360, His-365, and His-540) were also shown to be essential for the platelet 12-LOX activity (173).

The human platelet-type 12-LOX gene has been cloned and localized to chromosome 17p13.1. The gene spans 15 kb of DNA and consists of 14 exons

and 13 introns (175). The murine leukocyte-type 12-LOX (L-12-LOX) gene has also been cloned (162) and used to generate L-12-LOX-deficient mice (176). The L-12-LOX$^{-/-}$ mice grew normally and were fertile. Peritoneal macrophages from these L-12-LOX$^{-/-}$ mice did not produce detectable amounts of 12-HETE, but a small amount of 15-HETE production was detected and the arachidonic acid metabolism was diverted to the 5-LOX pathway (159, 176).

The biological functions of 12/15-LOXs are poorly understood, partly due to the lack of specific inhibitors for these pathways. Although the biological role of 12-LOX has not been elucidated, one report shows the induction of this enzyme in intestinal epithelial cells of patients suffering from inflammatory bowel disease (177). In addition, many reports have indicated various biological activities for 12-HPETE and 12-HETE, including the secretion of LH-RH from rat median eminence and melatonin from rat pineal gland, a possible involvement in neurotransmission, chemotactic and vasoconstrictive activities, as well as increased mucous glycoprotein release from cultured human airway preparations (for reviews see 170, 178). Leukocyte-type 12-LOX has been found in the adrenal gland, pancreas, vascular smooth muscle, tracheal epithelium, several regions of the brain, and in many other locations (100), and 12-HETE has been proposed to modulate angiotensin-induced aldosterone secretion in the adrenal gland and glucose-induced insulin secretion in the pancreas (179, 180). It is not clear how HPETEs or HETEs exert their biological actions and the question of whether they have specific receptors, either on the cell surface or in distinct intracellular locations, or they act only by incorporation into biological membranes, is yet to be answered. There is now some evidence for specific HETE binding sites (181), but no HETE receptor cDNAs have been cloned to date. Recently, a novel lipoxygenase cDNA from mouse epidermis has been cloned (182). The cDNA encodes a 662 amino acid polypeptide that is most related to human 15-LOX and displays 60% amino acid sequence identity to both murine leukocyte-type and platelet-type 12-LOXs. This epidermal-derived cDNA expressed an enzyme with 12*S*-LOX activity which produced predominantly 12*S*-HETE from arachidonic acid. Since linoleic and linolenic acids were not

substrates (182), the enzyme could be classified as a platelet-type 12-LOX. The biological rationale for the existence of three 12-LOX isozymes (platelet-type, leukocyte-type, and epidermal 12-LOXs) that synthesize the same product is not clear.

1.4.3 15-lipoxygenase

The mammalian form of 15-LOX is an iron-containing enzyme which inserts molecular oxygen primarily into the C-15 position of arachidonic acid to produce 15(S)-hydroperoxy-5,8,11-*cis*-13-*trans*-eicosatetraenoic acid (15(S)-HPETE)(183; Figure 1.3). The mechanism of this reaction is similar to that described for soybean lipoxygenase (184). The enzyme is activated by hydroperoxides which probably cause an oxidation of the active site iron from the Fe^{2+} to the Fe^{3+} (active form) state (185). A free radical mechanism then occurs in which the stereospecific abstraction of a hydrogen atom at the C-13 position of the 1,4-*cis*-pentadiene results in a radical at this position (187-189). The hydrogen is converted to a proton and an electron which then reduces Fe^{3+} to Fe^{2+}. The unpaired electron from the radical combines with one of the electrons of the neighboring double bonds and forms a 1-*cis*,3-*trans* conjugated diene. The radical then reacts with molecular oxygen to form a peroxy radical which is protonated and Fe^{2+} is re-oxidized to the active Fe^{3+} state. In addition to oxygenation at C-15, the mammalian 15-LOX can also catalyze the insertion of molecular oxygen at C-12 of arachidonic acid to produce 12(S)-HPETE (with a ratio of 15-HPETE to 12-HPETE production of 9:1) (190, 191). Studies with a variety of substrates have suggested that the alignment of the methylene groups of the substrate at the active site is a major determinant of this dual positional specificity (192). Rabbit reticulocyte 15-LOX, like the soybean 15-LOX, displays a broad substrate specificity in terms of the carbon chain length and can therefore metabolize numerous unsaturated C-18, C-20, and C-22 fatty acids (191,192). The optimal substrate for 15-LOX is linoleic acid which is converted to 13-hydroperoxy-9,11-*Z,E*-octadecadienoic acid (13-HPODE) (191), that can

subsequently be reduced to 13-hydroxyoctadecadienoic acid (13-HODE) by cellular glutathione peroxidases (Figure 1.3). 15-HPETE, the primary product of arachidonic acid metabolism by 15-LOX, can either be reduced to 15-HETE or further metabolized to a variety of epoxide, dihydroxy, and trihydroxy derivatives (193, 194). Thus, 15-LOX may undergo a second redox cycle to produce the epoxide 14,15-leukotriene A_4, which can be hydrolyzed to diastereoisomers of either 8,15-dihydroxy-5,9,11,13-eicosatetraenoic acid (8,15-diHETEs) or 14,15-diHETEs (190). In addition, dual lipoxygenase products can be formed (by the combined actions of 5-, 12- or 15-LOX) which include 8,15-diHETEs, 14,15 diHETEs, 5,15-diHETE and lipoxins (195- 197). The mammalian 15-LOX can also catalyze the oxygenation of unsaturated fatty acids attached to membrane phospholipids without their prior release by phospholipase enzymes (191, 198, 199). Based on this ability to attack biological membranes (200), a major role for 15-LOX in the maturational breakdown of mitochondria in reticulocytes has been proposed (201).

15-LOX was first purified and characterized from reticulocyte-rich blood cells obtained from anemic rabbits (202, 203). It was subsequently purified and characterized from rabbit and human leukocyte preparations, and airway epithelial cells (204-207). Like other lipoxygenases, 15-LOX is found in the cytosol and contains one iron atom per molecule of enzyme (208). Mammalian 15-LOX cDNAs have been cloned from human reticulocyte (137), airway epithelium (209), and rabbit reticulocyte (138). The human reticulocyte and tracheal epithelium cDNA sequences are identical and share 81% sequence identity with the rabbit reticulocyte homologue. The human 15-LOX cDNA (2.7 kb) encodes a protein of 661 amino acids with a molecular mass of 75 kDa. Consistent with the characteristics of the purified protein and its cytosolic localization, the predicted human polypeptide is overall hydrophilic and contains no Ca^{2+}-binding, ATP-binding, or glycosylation sites (134, 137). Human 15-LOX protein was subsequently expressed in eukaryotic and prokaryotic cells and the expressed enzyme had the characteristics of the original protein isolated from eosinophil-rich leukocyte preparations (210, 211). 15-LOX is 39% and 65%

identical in its primary structure to 5-LOX and platelet-type 12-LOX, respectively, (119, 120, 135, 161). Moreover, human 15-LOX is 86% identical to porcine leukocyte-type 12-LOX (136), and both enzymes display dual positional specificity producing both 15-HETE and 12-HETE from arachidonic acid. Site directed mutagenesis of 15-LOX has demonstrated that Met-418 in the human reticulocyte enzyme is a major determinant of the positional specificity of the enzyme (212). Conversion of this methionine residue to the corresponding valine residue of 12-LOXs results in an enzyme with equivalent 12- and 15-LOX activities which produces nearly equal amounts of 12-HETE and 15-HETE. Further mutation of the two adjacent residues Gln-416 and Ile-417 to residues found in platelet 12-LOX (Lys-416 and Ala-417) resulted in the conversion of 15-LOX into 12-LOX with a shift in the formation of 12-HETE accounting for over 90% of the products (212).

Recently, a second type of 15(S)-LOX was cloned from human skin (213). This enzyme differ from the well known reticulocyte 15(S)-LOX in that it oxygenates arachidonic acid purely at C-15 and linoleic acid is a relatively poor substrate for it. The human skin 15-LOX (which is also found in lung, prostate, and cornea) shares about 40% amino acid identity with the reticulocyte 15-LOX and other reported mammalian LOXs. The sequence of this enzyme contains the absolutely conserved iron-binding histidines (found in all other LOXs) and the C-terminal isoleucine that also functions as an iron ligand (103, 149). In contrast, the putative fifth iron ligand which is normally a histidine or an asparagine in other LOXs (His-544 in the human reticulocyte 15-LOX) is a serine (Ser-558) in the human skin 15-LOX (213). Recently, the mouse homologue of this newly identified 15-LOX has been cloned and characterized from mouse epidermis (107, 108). This mouse 8(S)-LOX (which oxygenates arachidonic acid at C-8 producing 8-HETE) is inducible by phorbol ester (108), and it remains to be established if this inducibility is also a characteristic feature of the human skin 15-LOX isozyme.

The gene encoding the rabbit reticulocyte 15-LOX has been cloned (214). The gene is 8-kb long and consists of 14 exons and 13 introns having the same

exon/intron format as the human 5- and 12-LOX genes. In addition, the human reticulocyte 15-LOX gene, like the human 12-LOX genes, has been localized to chromosome 17 (175). Since mouse leukocyte-type 12-LOX may be the species equivalent of human and rabbit reticulocyte 15-LOX, the study of the already developed leukocyte-type 12-LOX deficient mice may help to explore potential biological roles for the 12/15-LOX pathway (159).

Although a clear biological role for 15-LOX has not yet been elucidated, a possible involvement of this enzyme in the formation of atherosclerotic plaques has been suggested. The evidence supporting this theory is that 15-LOX is found in macrophages of atherosclerotic plaques (215, 216) and it can oxidize low density lipoprotein (LDL), which is proposed to be an early event in the process of atherosclerosis (217, 218). Other biological functions have also been reported for 15-LOX-catalyzed products, including an immunoregulatory role for 15-HETE (which can inhibit various lymphocyte functions and can stimulate suppressor cell activity) and lipoxins (which inhibit killer cell cytotoxicity and modulate leukocyte chemotaxis) (199, 19, 45). A definitive role for the 15-LOX enzyme awaits the development of specific 15-LOX inhibitors.

1.5 Leukotriene A_4 (LTA_4) hydrolase

1.5.1 Metabolism of LTA_4

The unstable epoxide LTA_4 (5(S)-*trans*-5,6-oxido-7,9 *trans*-11,14-*cis*-eicosatetraenoic acid), which is formed from arachidonic acid by the action of 5-LOX, is the common precursor for the synthesis of LTB_4 and LTC_4. The formation of LTB_4 is catalyzed by LTA_4 hydrolase and LTC_4 synthesis is catalyzed by LTC_4 synthase (3). As shown in Figure 1.4, LTA_4 can also be metabolized non-enzymatically to the all *trans* isomers of LTB_4 (6-*trans*-LTB_4 and 6-*trans*-12-epi-LTB_4) as well as the two isomers 5(S),6(R)-diHETE and 5(S),6(S)-diHETE (219). On the other hand, LTA_4 can be stereospecifically converted to 5(S),6(R)diHETE by cytosolic epoxide hydrolase (220, 221). A second lipoxygenation can also

occur on LTA_4 resulting in the formation of lipoxins via the intermediate formation of 15-hydroxy-LTA_4 (45, 222).

1.5.2 Leukotriene A_4 hydrolase and formation of LTB_4

LTA_4 hydrolase is the rate limiting enzyme for the formation of the pro-inflammatory LTB_4 from the unstable epoxide LTA_4 (19, 223). The enzyme was originally characterized in rabbit polymorphonuclear leukocytes and in rat basophilic cells (224, 225). This monomeric cytosolic enzyme (which is distinct from other known epoxide hydrolases) was purified from human and rat leukocytes and has a molecular mass of 69 kDa (226, 227). LTA_4 hydrolase activity has been detected in various human tissues including the liver, and in many different cells including those devoid of 5-LOX activity such as keratinocytes and erythrocytes (228-230). This widespread distribution of LTA_4 hydrolase is in contrast to the highly restricted distribution of 5-LOX (which produces the substrate LTA_4) and may account for the process of transcellular metabolism of LTA_4 to LTB_4 (231). LTA_4 hydrolase has a narrow substrate specificity for its epoxide hydrolase activity which only accepts a 5,6-*trans*-epoxide with a free carboxylic acid moiety at the C-1 position (224, 229, 232). The number of double bonds is also important since LTA_3 which lacks the C-14-C-15 double bond is a poor substrate but a good inhibitor for the enzyme (233). The enzyme is typically inactivated and covalently modified by its substrate (LTA_4), and this suicide inactivation has been clearly shown to be mechanism-based (234). LTA_5, which is obtained from eicosapentaenoic acid, is also a poor substrate and acts as an inhibitor of LTA_4 hydrolase activity (235).

LTA_4 hydrolase cDNAs have been cloned from human (236), mouse (237), rat (238), and guinea pig (239), and were expressed in both bacterial and insect cell systems. All the enzymes contain 611 amino acid residues and have a minimum sequence homology of 92%. Sequence comparison of LTA_4 hydrolase with zinc metalloenzymes, e.g. aminopeptidase M and thermolysin, led to the identification of a zinc binding motif ($HEXXHX_{18}E$) in the primary structure of the

enzyme (240-242). Further studies verified that LTA_4 hydrolase contained one catalytic zinc atom complexed to His-295, His-299, and Glu-318 (243). Subsequently, the enzyme was shown to exhibit a previously unknown zinc-dependent peptidase/amidase activity towards different synthetic amide substrates (244, 245). Unlike the epoxide hydrolase activity which does not require cofactors, the peptidase activity of LTA_4 hydrolase is specifically stimulated by monovalent anions, e.g., chloride ions (246) and also by albumin (247). Although a physiological peptide substrate for the aminopeptidase activity of the enzyme has not yet been found, LTA_4 hydrolase has been shown to efficiently hydrolyze arginyl tri- and dipeptides leading to its identification as an arginine aminopeptidase (248). The aminopeptidase active site overlapped with the epoxide hydrolase active site since the aminopeptidase inhibitor bestatin and the angiotensin converting enzyme inhibitor captopril were capable of inhibiting both activities (245, 249). The epoxide hydrolase activity was also dependent on the zinc atom. Site directed mutagenesis of the three zinc-binding ligands (His-295, His-299, and Glu-318) confirmed their importance since a change in any of these residues resulted in the loss of both aminopeptidase and epoxide hydrolase activities (243). A single mutation in Glu-296 abrogated the peptidase activity but did not affect the epoxide hydrolase activity showing that although the two activities overlap, they are distinct (250). In addition, a tyrosine residue at position 383 of LTA_4 hydrolase has been suggested to serve as a proton donor in the peptidase reaction. Site directed mutagenesis of this tyrosine demonstrated a complete loss of the peptidase activity and a 90% loss of the epoxide hydrolase activity (251).

Using the data provided by the zinc ligand dependency and the critical residues for the aminopeptidase activity, a reaction mechanism (252) for the cleavage of alanine-*p*-nitroanilide (one of the synthetic amide substrates for LTA_4 hydrolase) has been proposed (Figure 1.5). The mechanism involves a nucleophilic attack by a water molecule (which is displaced from the zinc atom by the carbonyl oxygen of the substrate) on the carbonyl carbon of the scissile peptide bond. The nucleophile is generated by Glu-296 acting as a general base

to remove a proton from the water molecule and the positively charged zinc coordinates the hydroxyl group and the carbonyl group of the peptide. Simultaneously, a proton is transferred to the nitrogen of the peptide bond from the adjacent Tyr-383 residue which acts as a proton donor. A similar reaction mechanism has been described for the peptidase activity of thermolysin (253, 254). Recently, potent inhibitors of both the peptidase and epoxide hydrolase activity of LTA_4 hydrolase have been developed and they may provide a better understanding of the physiological role of the aminopeptidase activity of LTA_4 hydrolase (255).

The mechanism of the epoxide hydrolase activity of the human LTA_4 hydrolase has been recently investigated. One report has shown that a peptide fragment (21 amino acids) of LTA_4 hydrolase contains the covalent attachment site for the suicide substrate LTA_4. The residue which may covalently bind LTA_4 was identified as Tyr-378 through sequencing of the peptide fragment by Edman degradation and the presence of a gap at the Tyr-378 residue (256). Mutation of this tyrosine residue to a phenylalanine demonstrated that suicide inactivation is almost completely abolished (257). Conversion of this tyrosine to a phenylalanine or a glutamate also resulted in mutants which could convert LTA_4 into both LTB_4 and a newly identified isomer of LTB_4, 5(S),12(R)-dihydroxy-8,14-*cis*-6,10-*trans*-eicosatetraenoic acid (257). These recent studies suggest that Tyr-378 is a critical residue in the epoxide hydrolase activity of LTA_4 hydrolase.

The genomic structure of the human LTA_4 hydrolase has recently been characterized (258). The gene consists of 19 exons, is greater than 35 kb, and is localized to chromosome 12q22. The two essential zinc-binding histidine residues (His-295, His-299) are present on exon 10 while the third zinc-binding ligand glutamate (Glu-318) is located to exon 11 (258). The fact that the zinc-binding residues that make up the zinc-binding domain are divided between two exons may provide a mechanism for the regulation of this enzyme through RNA splicing events. An aminopeptidase B (AP-B) from rat testis, which exhibits 33% amino acid sequence identity to the rat LTA_4 hydrolase, has recently been cloned (259). This AP-B contains the metallopeptidase signature ($HEXXHX_{18}E$) which is found

in LTA_4 hydrolase and can utilize LTA_4 to produce LTB_4, albeit at approximately 10% of the efficiency of LTA_4 hydrolase. Although the bifunctional LTA_4 hydrolase/aminopeptidase has only been characterized from mammalian sources, a partial sequence from the slime mold *Dictyostelium discoideum* (Jho, E., and Kopachik, W. 1995, GenBank accession number U27538) and a gene from the yeast *Saccharomyces cerevisiae* (260) have been recently identified as putative LTA_4 hydrolase homologues. Both sequences encode proteins that show 39% amino acid sequence identity to the mammalian LTA_4 hydrolase but neither of them has been expressed nor characterized. In addition, an enzyme cloned from the pathogenic yeast *Candida albicans* with 41% homology to the mammalian LTA_4 hydrolase, exhibited mainly aminopeptidase activity while its hydrolase activity converted the majority of the substrate LTA_4 to what has been putatively identified as 5,6-diHETE rather than LTB_4 (U. S. Patent 5,529,916, Cormack, B. P., and Falkow, S., June 25, 1996). The study of LTA_4 hydrolase homologues from different organisms may provide a better understanding of the dual activity of the mammalian enzyme, help in identifying a physiological substrate for the aminopeptidase activity, as well as providing new therapeutic targets for pharmaceutical intervention in the treatment of inflammatory diseases.

1.5.3 Biological actions of LTB_4

Leukotriene B_4 is the most potent chemoattractant known and it is active at nanomolar concentrations (3, 261). The process of chemotaxis refers to the recruitment of leukocytes from the circulation to the extravascular tissue. At nanomolar concentrations, LTB_4 enhances leukocyte-chemotaxis, chemokinesis, aggregation and adherence to endothelial cells, and at higher concentrations it also triggers degranulation and generation of superoxide anions (3, 261, 262). Due to these biological properties, LTB_4 is regarded as an important chemical mediator in a variety of inflammatory diseases (262, 263). In addition, LTB_4 plays a role in immunoregulation by stimulating the activation, proliferation, and differentiation of human B-lymphocytes (264, 265) and by augmenting human NK

cell cytotoxicity (266, 267). LTB_4 can also induce T suppressor cell activity and stimulate lymphocyte production of cytokines (268, 269).

The biological effects of LTB_4 in inflammation are mediated through a specific guanosine-binding-protein (G-protein) coupled receptor that has both high and low affinity states. The high-affinity receptor state mediates chemotaxis, adherence of neutrophils to endothelial wall, and transient calcium mobilization. The low-affinity receptor state mediates degranulation, lysosomal enzyme release, and long-term calcium mobilization (270, 271). The high affinity site and the low affinity site appear to correspond to G-protein coupled and uncoupled forms of the receptor (272, 273). The B-LT (LTB_4) receptor couples to a pertussis toxin-sensitive G-protein which mediates the functional responses of LTB_4 via phosphoinositol hydrolysis.

1.6 The trematode parasite *Schistosoma mansoni*

Schistosoma mansoni is a member of a group of digenetic parasites known as schistosomes which cause considerable morbidity and mortality in humans. Worms of the genus *Schistosoma* belong to the phylum Platyhelminthes (family Schistosomatidae) and comprise several blood parasites of humans and other animals. Humans can be infected by different schistosome species but the major human parasites are *Schistosoma mansoni* (*S. mansoni*), *S. haematobium*, and *S. japonicum*. These three parasites are responsible for schistosomiasis (bilharziasis), a life threatening human infection in many tropical and subtropical parts of the world (274). According to the World Health Organization estimation, over 200 million people are currently infected with these parasites and another 600 million are at risk (275). *S. mansoni* alone is estimated to cause the endemic disease schistosomiasis in at least 300 million people in many parts of the developing world. With the current rate of global warming there is even greater risk of increased prevalence and mortality due to this disease (276).

1.6.1 The life cycle of schistosome parasites

Adult schistosomes are obligatory parasites that have a complicated life cycle which include an intermediate snail host, a definitive mammalian host, and several developmental stages of the parasite (Figure 1.6). The adult worms exist as separate sexes (males and females) but are usually found paired in the vesical and portal blood vessels of the definitive host. The exact location of the final habitat of adult worms in the human definitive host is determined by the parasite species; *S. mansoni* lives in superior mesenteric veins; *S. haematobium* in vesical veins; and *S. japonicum* in inferior mesenteric veins (277). Upon sexual maturity of female schistosomes, the fertilized eggs are deposited in the small venules of the vesical or portal venous systems. When freshly deposited, schistosoma ova contain immature miracidia (one of the developmental stages of the parasite) which reach full maturity in approximately 10 days. Oviposition occurs intravascularly and eggs work their way towards the lumen of ureters, urinary bladder or intestines (*S. mansoni*) in order to be carried to the outside with the host urine or feces as they are incapable of hatching within humans or other mammalian hosts (277). The eggs have spines in various positions characteristic of the species (Figure 1.6). Following their release from the definitive host into fresh water bodies and under optimal environmental conditions, the schistosome eggs hatch leading to the liberation of actively swimming miracidia which find and penetrate different snail intermediate hosts depending on the parasite species (*S. mansoni* uses the snails of the genus *Biomphalaria*). Within the snail tissues, two generations of sporocysts develop which result in the emergence of 200 to 400 cercariae (the infective stage of the parasite) (277). Mature cercariae penetrate through the snail tissues and escape to water where they can find their human definitive host (who may be present in contaminated fresh water for a multitude of cultural, social and economic purposes). Cercariae initiate and complete their penetration of the intact host skin (the only known route of entry) within minutes

after contact (278, 279). During this process, cercariae shake off their tails and proceed to occupy tunnels in the stratum corneum parallel to the skin surface. At this stage these organisms are called schistosomulae which undergo another maturational process to become adult parasites along with their migration from the dermal sites of cercarial penetration through the lungs and finally to the liver (277).

The transformation of cercariae into schistosomulae is accompanied by a series of morphological, biochemical, antigenic, and particularly membrane changes (280). Schistosomulae continuously turnover and change their membranocalyx which may lead to loss of antigenic components (antigen shedding) that can be recognized by antibodies (277). In fact, Schistosomulae quickly lose their susceptibility to antibody-dependent killing which was originally hypothesized to be due to the acquisition of host antigens on their surface (280). The question of how schistosomulae evade the host immune and defense mechanisms has been the subject of considerable investigations and is still unanswered.

In contrast to the progress of knowledge about the morphology and other aspects of the parasite, its molecular biology is almost unknown. DNA extraction and hybridization studies demonstrated that the size of *S. mansoni* haploid genome is approximately 2.7 x 10^8 base pairs (281). *S. mansoni* is diploid, and the study of its karyotypes revealed the presence of eight chromosomes in the haploid genome which can be divided into three groups according to size (282). Furthermore, the presence of sex chromosomes was suggested by finding a heteromorphic pair in female worms (WZ) and a homomorphic pair in males (ZZ). Other than the sex chromosome, the structure and function of the schistosome genome are largely unknown and details of only approximately 100 genes had been deposited in the GenBank database.

1.6.2 Disease syndromes associated with schistosomiasis

Schistosome infection of the definitive mammalian host is initiated by cercarial penetration of the skin, and the outcome of infection varies across a wide spectrum ranging from acute dermatitis to liver fibrosis and bladder carcinoma (277). Cercarial dermatitis (swimmer's itch) is due to skin invasion and is likely a result of host sensitization as both humoral and cellular immune responses to cercarial antigens have been demonstrated in infected humans and experimental animals (283). This severe immediate hypersensitivity reaction is a constant feature of human infection by non-human schistosomes (specially avian schistosomes) which do not successfully penetrate the human skin and are entrapped in the subcutaneous tissue. A less severe form of cercarial dermatitis has been reported following skin penetration by *S. mansoni* cercariae (277).

Acute schistosomiasis or Katayama fever is a clinical syndrome that has been described a few weeks following primary infection with *S. mansoni* particularly in heavily infected individuals (284). The characteristic clinical features of this syndrome include fever, hepatosplenomegaly, lymphadenopathy, eosinophilia, and elevated immunoglobulins. Although the etiology of acute schistosomiasis is unknown, its manifestations and association with primary heavy infection may suggest a serum sickness-like syndrome (277).

The most prevalent form of schistosomiasis is the established chronic lesions which are due to the eggs being retained in different tissues and the subsequent host responses to the enzymes and antigenic materials released by the eggs during the maturation of the enclosed meracidia (277). The host granulomatous response to the retained schistosome eggs has been shown to be a form of immune reactiveness with cell-mediated hypersensitivity playing the key role in granuloma formation around *S. mansoni* eggs (285). Granuloma formation leads to a compact cellular infiltrate surrounding the eggs which is made of lymphocytes, eosinophils, macrophages, and fibroblasts. The presence of these granulomas may lead to mechanical obstruction of the urinary tract or portal circulation while their aggregation and the deposition of fibrous tissue around them lead to periportal hepatic fibrosis marked by excessive collagen

deposition along the portal tracts (286). Intestinal lesions of schistosomiasis *mansoni* include diffuse colonic polyposis and granular mucosa with hemorrhages and ulcerations (287). A major effect of liver disease in schistosomiasis *mansoni* is the block in portal blood flow with subsequent portal hypertension (leading to hepatosplenomegaly) and development of portosystemic collaterals. With the development of collateral circulation, *S. mansoni* eggs may enter the lungs causing scattered granulomas, chronic obliterative arthritis, diffuse hypertensive arteriolar changes, and a form of pulmonary hypertension known as cor pulmonale (288). Other clinical manifestations of chronic schistosomiasis include carcinoma of the bladder (*S. haematobium*), central nervous system infection characterized by convulsive attacks and psychomotor epilepsy (*S. japonicum*), and kidney diseases (*S. mansoni*) (277). Despite intensive efforts dedicated to eradicating schistosomiasis through sanitary measures, control of the intermediate snail host, and drug treatment, its prevalence remains essentially unaltered (275). There is little hope for improved control of this serious disease in the immediate future, since no vaccine is yet available (none of the well-characterized, full-length cloned antigens provide high levels of protection in experimental animals) and there is resistance to the few drugs available for treatment (277).

1.7 The production of eicosanoids by invertebrates

Although extensive research in the field of eicosanoids has focused on mammals, the production of these oxygenated compounds have been detected in many vertebrate and invertebrate species that represent all major phyla (289). Eicosanoids play an important role in the normal physiology of different species including insects, fish, and amphibians, to mention a few. They exert physiological actions in reproduction including the release of egg-laying behavior in some insects, hatching in barnacles, egg-production in snails, spawning in bivalves, oocyte maturation in sea stars, and prevention of polyspermic fertilizations in sea urchin eggs (289, 290). Eicosanoids are also involved in salt

and water transport physiology (insects and bivalves), neurophysiology (mollusks), and cellular immune defenses (insects) (289). Because of their obvious clinical implications, parasitic invertebrates such as blood flukes (nematodes, platyhelminthes), have been particularly studied for the production of eicosanoids that can mediate certain host-parasite interactions and may enable these parasites to adapt to their hostile host environment.

1.7.1 Eicosanoid production by nematode parasites

The intravascular nematodes *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori* are multicellular round worms that dwell in the human vascular system and cause human lymphatic filariasis, a chronic mosquito-borne parasitic infection that affects more than 100 million people worldwide (291). Adult filarial worms inhabits lymphatic vessels and release their larval offspring, microfilariae, into the bloodstream. Microfilariae circulate in the blood and survive for several months or more despite close encounters with host leukocyte effector cells of the immune system, platelets, and other vascular elements. Furthermore, circulating microfilariae pass through tiny capillary vessels without becoming trapped, and neither platelets nor leukocytes adhere to their surfaces (292). Most microfilaremic individuals are asymptomatic and these patients have defects in both B and T lymphocyte responses to parasitic antigens and express very low serum levels of parasite-specific antibodies (291, 293). The adaptive success of filariae, as well as other blood or tissue helminth parasites, depends upon their ability to survive recurrent encounters with surrounding host cells. Consequently, a search for parasite molecules involved in regulating host immune responses was initiated. Because of the established role of certain eicosanoids as potent modulators of immune responses in mammalian cells (88), it has been postulated that filarial parasites produce eicosanoid derivatives of arachidonic acid as a mechanism that may enable them to escape effective host immune responses and to establish chronic infections (292).

In general, helminths are not capable of *de novo* synthesis of their own long chain polyunsaturated fatty acids (PUFAs) from acetate (294). However, arachidonic acid is present at a concentration of approximately 10 μM in human plasma (predominantly bound to albumin and proteins), and other essential fatty acids are also available to parasites in human plasma. It has been demonstrated that microfilariae of *Brugia malayi* rapidly incorporate and esterify albumin-bound arachidonic acid and other exogenous fatty acids such as linoleic acid into parasite phospholipids (295, 296). Furthermore, microfilariae contain relatively abundant stores of linoleic acid and other polyunsaturated fatty acids which they can convert enzymatically to arachidonic acid (296).

Accumulating evidence now indicates that microfilariae can metabolize arachidonic acid into eicosanoids. Reverse-phase HPLC analysis demonstrated that microfilariae of *Brugia malayi* produce PGE_2 and 6-keto-$PGF_{1\alpha}$ (the stable hydrolysis product of prostacyclin) following short term incubation with exogenous radiolabelled arachidonic acid (297). Furthermore, the utilization of endogenous stores of arachidonate to produce prostanoids was evaluated by radioimmunoassay (RIA) and the major products detected were PGE_2 and 6-keto-$PGF_{1\alpha}$ with minor amounts of PGD_2. Heat-killed microfilaria did not produce these prostanoids and there was decreased production at 4°C. The production of PGE_2 and 6-keto-$PGF_{1\alpha}$ by microfilariae was also corroborated by thin-layer chromatography (TLC) of radiolabelled lipids extracted from the culture media and compared to co-chromatographed prostanoid standards (297). The formation of these prostanoids was shown to be enzymatic and was blocked by known inhibitors of mammalian COX such as indomethacin and eicosatetraynoic acid (297). Thus, it was suggested that microfilariae may possess a key eicosanoid-forming enzyme functionally similar to mammalian COX (292). As with mammalian cells, microfilarial prostaglandins were not stored within parasites, but were quickly released after their formation. By immobilizing living microfilariae of *B. malayi* and *W. bancrofti* within an albumin-agar matrix, parasite release of PGE_2 into the microenvironment around individual microfilariae has been demonstrated by immunofluorescence staining using an

anti-PGE_2 serum (298). Microfilarial PGE_2 formation was constitutive and was not dependent on serum or pharmacologic agonists (298). Preliminary data also indicate that microfilariae produce a 12-HETE-like compound when stimulated by lipid or nonlipid peroxides. Furthermore, although limited parasite numbers have prevented extensive studies of adult filariae, adult *Brugia* worms also incorporate exogenous arachidonic acid and release PGE_2 (292).

Filarial parasite production of prostacyclin, the most potent known inhibitor of platelet aggregation (90), was postulated to enable microfilariae to inhibit platelet aggregation onto their surfaces (292). In addition, circulating microfilariae may release the vasodilatory prostacyclin and PGE_2 to ease their passage through small capillary vessels, and the immunosuppressive and antiinflammatory effects of PGE_2 (84-89) may contribute to the cellular and humoral immune defects observed in infected individuals with microfilaremia (293). In addition to filarial nematodes, other nematode, cestode, and trematode parasites have also been shown to metabolically transform host fatty acids into biologically active eicosanoids (Figure 1.7) (299). Therefore, the production of eicosanoids by certain parasites may constitute a survival strategy developed by these parasites in their adaptation to parasitism.

1.7.2 Eicosanoid production by trematode parasites

It has been well established that fatty acids stimulate penetration behavior and transformation of schistosome cercariae (278, 300). Studies have shown that essential fatty acids (EFAs) which are normally found on human skin are very effective stimulants for host skin penetration by cercariae of the trematode *S. mansoni* (301, 302). This stimulation of penetration behavior by exogenous polyunsaturated fatty acids was inhibited by COX inhibitors like asprin and ibuprofen (302, 303). Furthermore, cercariae of *S. mansoni* have been shown to metabolize exogenous linoleic acid rapidly to arachidonic acid and to synthesize eicosanoids (303, 304). Based on these studies, it was hypothesized that cercarial eicosanoids (both LOX and COX products), produced as a result of

stimulation by skin surface EFAs, are important mediators for successful penetration and transformation of cercariae into schistosomulae (302-305).

Cercariae of *S. mansoni* incubated with radiolabelled linoleic acid produced metabolites that displayed the same high performance chromatographic properties as PGE_2, PGD_2, LTB_4, LTC_4, 5-HETE, and possibly PGA_2 in lipid extracts of cercarial culture media (based on co-elution with eicosanoid standards by reverse-phase HPLC) (304). In addition, RIA detected PGE_1, PGE_2, 5-HETE, and 15-HETE in cercarial extracts. RIA of lipid extracts examined at different times after the addition of 3.3 mM linoleate showed that different eicosanoids are maximized at various times following the stimulation with EFAs. Peak production of PGE_1 and 5-HETE were seen within 1 min, whereas maximum concentration of PGE_2 and 15-HETE were observed at 60 min. HPLC profiles of cercarial extracts also showed that cercariae of *S. mansoni* regulate the production of eicosanoids through time after stimulation with radiolabelled linoleic acid (304). The cercarial penetration rates of an agar matrix containing varying concentration of linoleate was correlated with increased cercarial production of leukotrienes and HETEs, while cercarial-schistosomular transformation rates were correlated with increased prostaglandin levels (305). Based on these findings, it was suggested that cercarial LTs may promote skin penetration because of their potent inflammatory properties while the vasodilatory and immunoregulatory effects of PGs may facilitate penetration into small capillary vessels and help schistosomulae to evade the initial response of the skin's immune system (305). However, until the enzymes and genes responsible for the elaboration of eicosanoids by *S. mansoni* are fully characterized, the production, regulation, and the exact role that these eicosanoids may play in the penetration response, schistosomulae transformation, and the regulation of host immune responses will remain largely speculative.

In order to explain the differences in penetration success, migration in the host and immune evasion by different species of trematode cercariae, eicosanoid secretion by *S. mansoni* was compared to those secreted by the duck

parasite *Trichobilharzia ocellata* (306). RIA performed on aliquots from culture supernatants following incubation with linoleic acid identified the presence of immunoreactive PGE_1, LTB_4, and 5-HETE in the supernatant of both cercarial species. In addition, reverse-phase HPLC analysis of culture supernatants from both species, incubated with unlabelled and labeled linoleic acid, identified PGE_1, LTB_4, LTC_4, 15-HETE, and 5-HETE by co-elution with authentic eicosanoid standards. The remarkable similarity between the eicosanoids released may indicate that the functions of cercarial eicosanoids coincide between species (306).

Although the cercarial production of eicosanoids has been the subject of several studies, there is only one report suggesting the elaboration of eicosanoids by the adult stage of *S. mansoni* parasites. Reverse-phase HPLC analysis of culture supernatants from adult *S. mansoni* worms, following their incubation with radiolabelled linoleic acid, has revealed the production of metabolites which have the chromatographic characteristics of 15-HETE, 5-HETE, and LTB_4 (307). RIA performed on aliquots from culture supernatants of adult worms following incubation with linoleic acid also identified the presence of immunoreactive LTB_4, and 5-HETE (307). With regards to arachidonic acid metabolism by cyclooxygenase pathways in adult *S. mansoni* the literature is less clear. One study has shown no formation of prostaglandins following the incubation of adult worms with radiolabelled arachidonic acid (308), whereas another study has reported the presence of immunoreactive and chromatographically characterized materials with the characteristics of prostaglandins although no definitive physicochemical or pharmacological data were obtained (307). More detailed studies are clearly needed to verify the different pathways of arachidonic acid metabolism by adult *S. mansoni*. Eicosanoids production by this medically important parasite may be required for normal parasitic physiological processes as well as helping the parasite to evade host immune responses. Thus, the characterization of the enzymes responsible for the production of these eicosanoids by adult *S. mansoni* is extremely

important and may provide potential targets for pharmacological intervention in the treatment of human schistosomiasis.

1.8 The nematode *Caenorhabditis elegans* (*C. elegans*)

Over the past two decades, the small non-parasitic soil nematode *C. elegans* has become established as a major model system for the study of a great variety of problems in biology and medicine (309). One of its most significant advantages is its simplicity, both in anatomy and in genomic organization. Unlike the complicated life cycles of parasitic nematodes and trematodes, the life cycle of *C. elegans* is simple and rapid. The worms grow from an embryo to a 1 mm long adult in three days and populations can be easily maintained on agar plates or in liquid using *E. coli* bacteria as a food source (310). These populations normally consist of only self-fertilizing hermaphrodites but cross-fertilization with the male sexual form is also possible. The option of reproduction by either selfing or crossing leads to very convenient genetics so that mutants can readily be generated, propagated, and analyzed. A simple freezing protocol permits stable storage of all strains which retain viability indefinitely in the frozen state. In addition, *C. elegans* is fully transparent at all stages of its life, allowing all cell divisions, migration, and differentiation to be seen in live animals (310). Despite the simplicity of its anatomy, the somatic cells of the adult *C. elegans* represent most major differentiated tissue types of more complicated animals, including muscles, neurons, intestine, and epidermis (310, 311).

In addition to these outstanding experimental features, the entire haploid genome of *C. elegans* which amounts to 100 million base pairs of DNA (about 1/30 the size of the human genome) has been subjected to systematic sequencing and more than 85% of the genome has been already sequenced (312). In fact, it is estimated that the *C. elegans* Genome Sequencing Project will be completed in 1998, consequently, *C. elegans* will be the first multicellular animal to have its entire genome sequenced (312). Furthermore, both forward

and reverse genetics are fast and currently there are mutants for more than 2000 genetic loci. Producing transgenic lines is also easy and the essentially complete physical map of *C. elegans* facilitates rapid gene cloning (313). These powerful genetic tools allows virtually any gene of interest to be studied at the functional level. Currently, over 50% of human gene sequences have a significant match to a *C. elegans* gene, and gene inhibition using antisense RNA or gene knockout may lead to the identification of function of these genes (314). Moreover, the use of identified sequences from *C. elegans* may facilitate the sequencing and characterization of important homologous genes from phylogenetically-related parasitic nematodes or trematodes which may lead to novel therapeutic targets for the treatment of human parasitic infections.

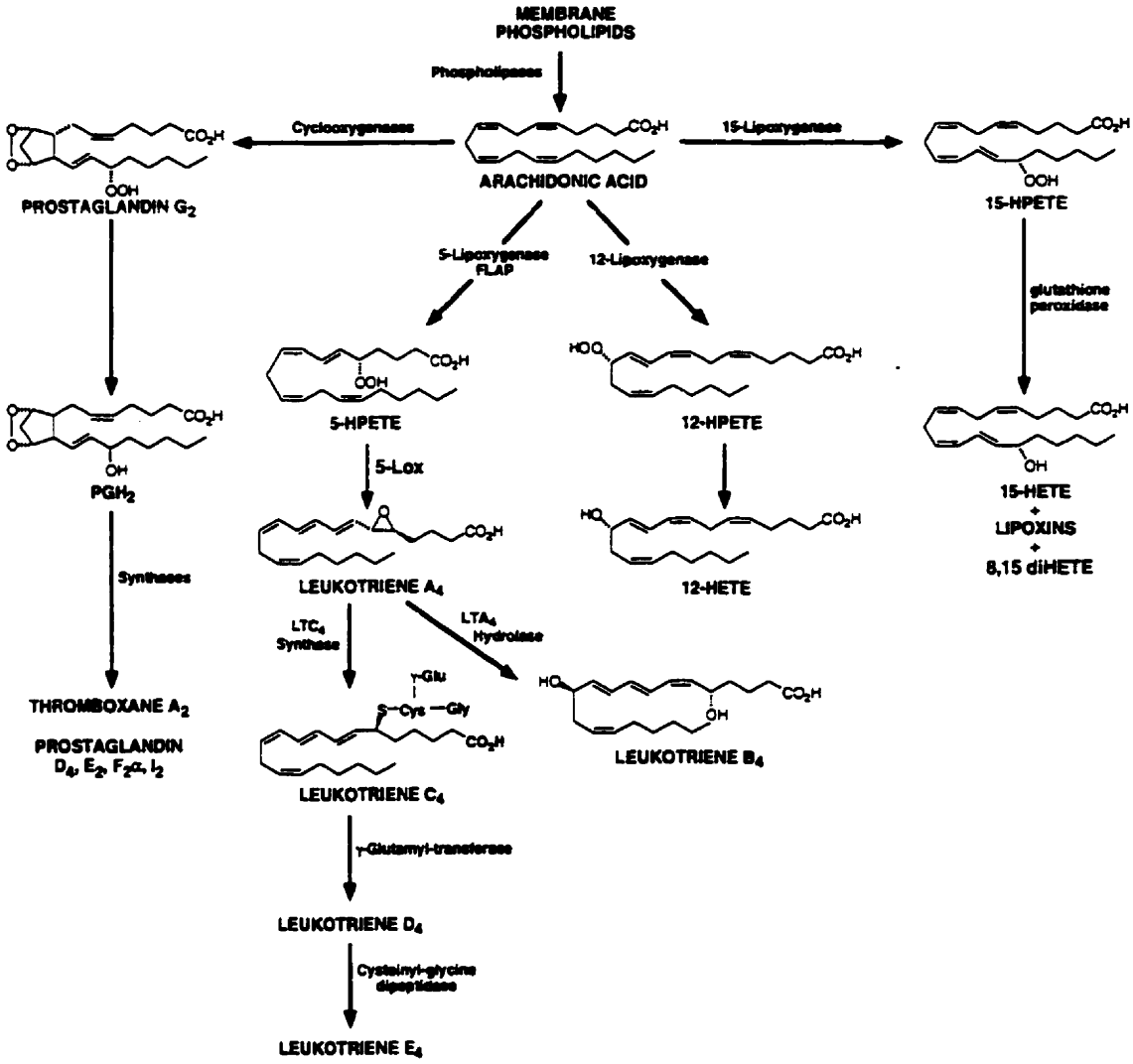
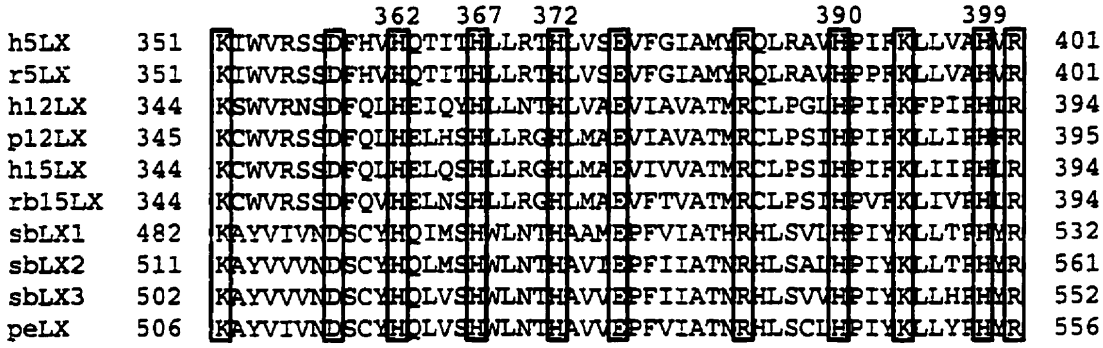
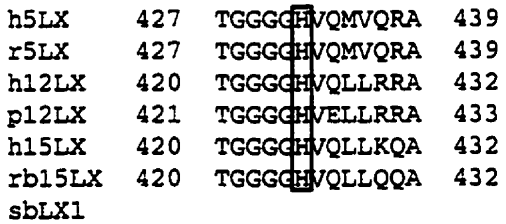


Figure 1.1. Arachidonic acid metabolism via cyclooxygenase and lipoxygenase pathways

Core Region



Third Region



Second Region; C-terminal

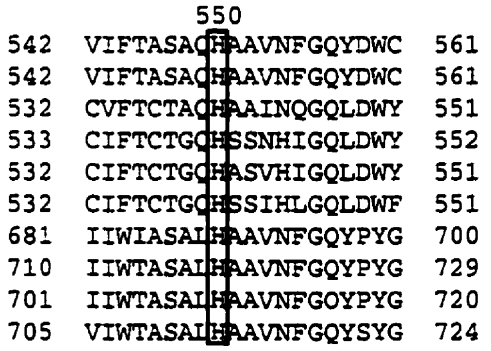


Figure 1.2. Amino acid sequence homology among different mammalian and plant lipoxygenases.

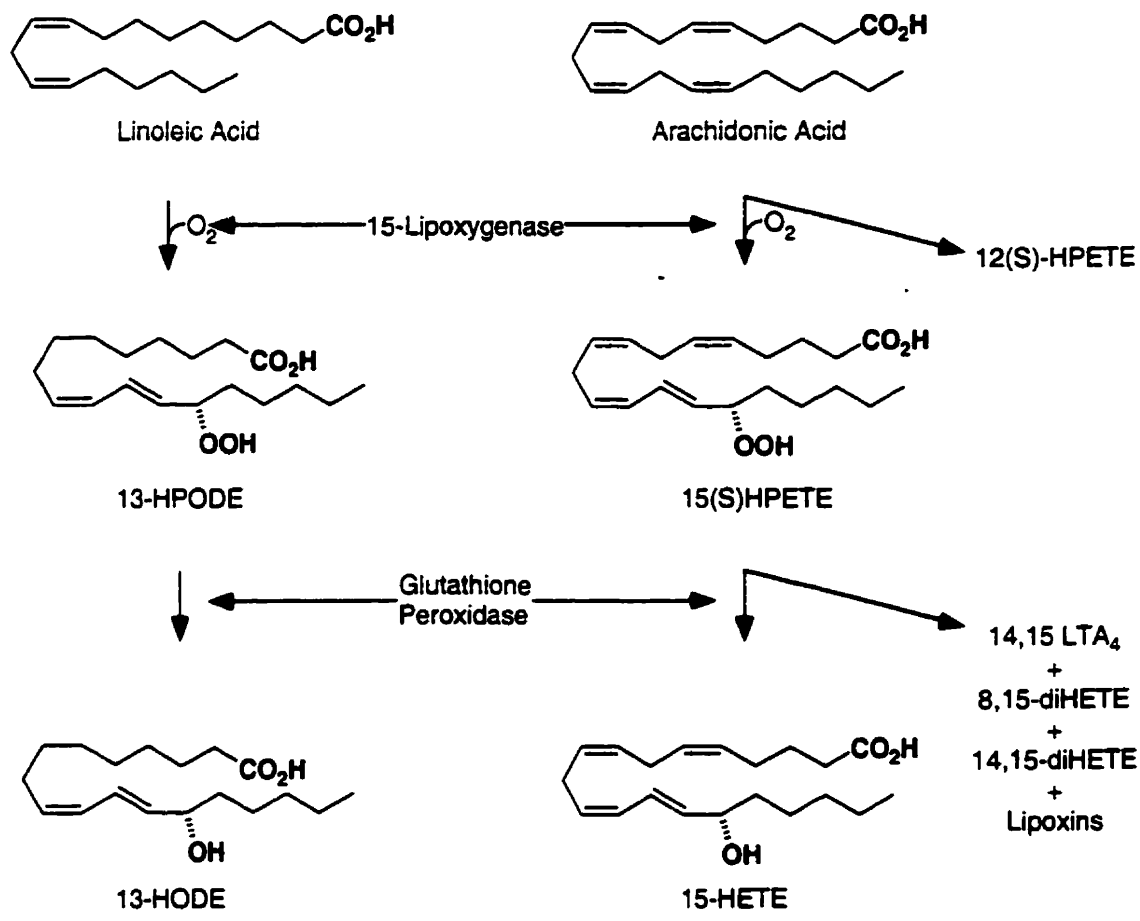


Figure 1.3. The metabolism of arachidonic acid and linoleic acid by 15-lipoxygenase

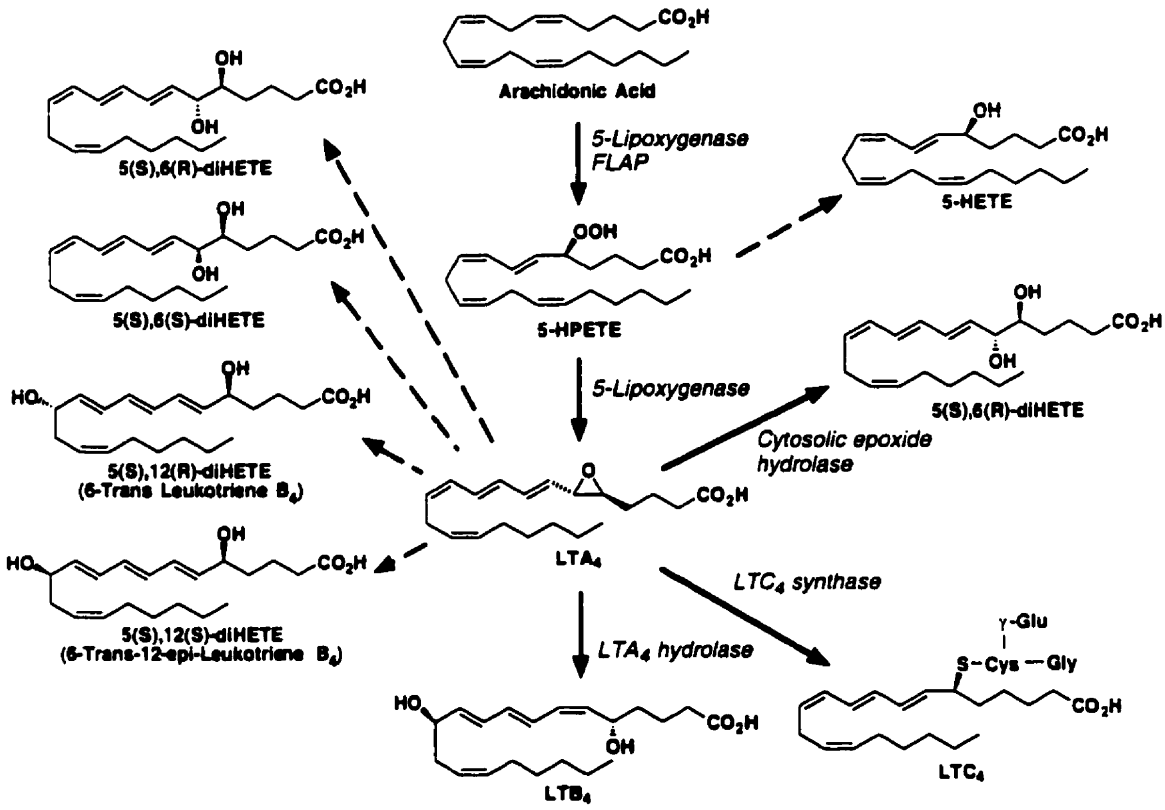


Figure 1.4. Enzymatic and non-enzymatic metabolism of LTA_4

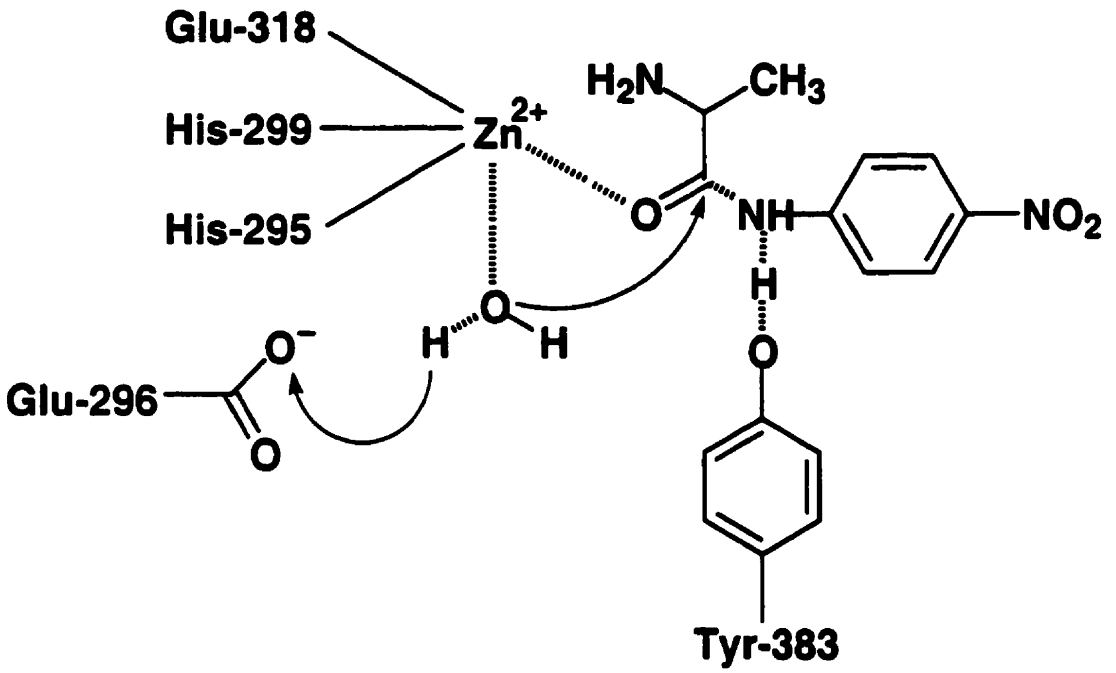


Figure 1.5. Proposed reaction mechanism for the hydrolysis of alanine-4-nitroanilide by LTA_4 hydrolase

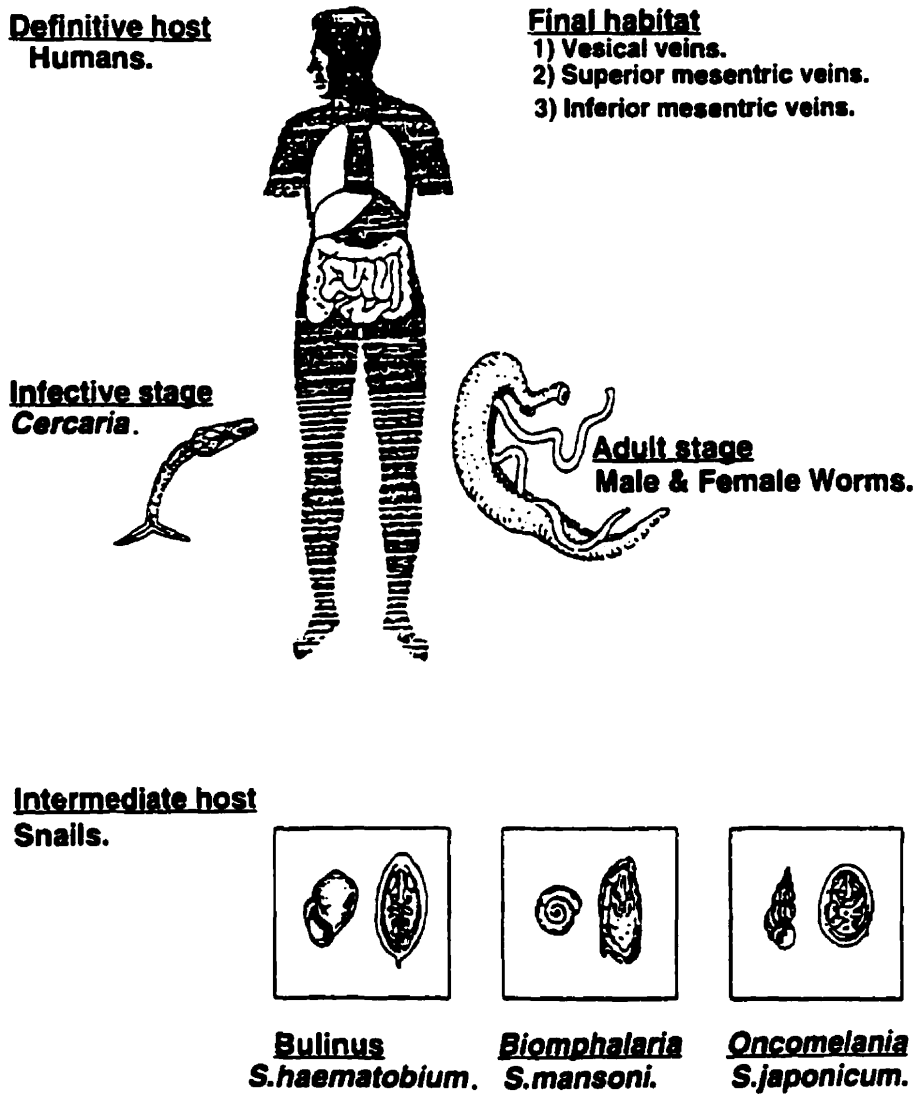


Figure 1.6. The life cycle of Schistosome parasites

PARASITE PHYLUM	EICOSANOIDS PRODUCED
TREMATODES	
Schistosoma mansoni	PGE_2, PGD_2, 5-HETE, 15-HETE, LTB_4, LTC_4
Trichobilharzia ocellata	PGE_1, 5-HETE, 15-HETE, LTB_4, LTC_4
NEMATODES	
Brugia malayi	PGE_2, PGI_2, PGD_2
Wucheraria bancrofti	PGE_2
Dirofilaria immitis	PGD_2, PGE_2, PGI_2
CESTODES	
Taenia taeniaeformis	PGE_2, PGI_2, TXA_2
Spirometra crinacei	PGE_2

Figure 1.7. Production of eicosanoids by parasites

2. RESEARCH OBJECTIVES

Cyclooxygenase and lipoxygenase enzymes metabolize arachidonic acid to a wide range of biologically active eicosanoids including prostaglandins, leukotrienes, HETEs, and lipoxins. In mammals, eicosanoids act as potent local mediators of various physiological and pathological responses including inflammation, asthma, pain, fever, regulation of vascular tone and regulation of immune responses. Recent studies have shown the production of eicosanoids by larvae of several nematode and trematode parasites including cercariae of the blood dwelling *S. mansoni* parasite which produce PGs, LTs, and HETEs upon stimulation by essential fatty acids normally present on the host skin surface leading to initiation of skin penetration. Little is known about the mechanism(s) by which adult helminth parasites overcome host immune responses to establish chronic diseases. Eicosanoids production by these parasites may be required for normal parasitic physiological processes as well as facilitating their invasion and subsequent evasion of host immune responses. Therefore, the study of the enzymes responsible for eicosanoids production in metazoa may provide potential targets for pharmacological intervention in the treatment of parasitic infections. Thus, the objectives of this study were:

1. To investigate the production of eicosanoids by the adult stage of the trematode parasite *S. mansoni.*

2. To determine which pathway(s) of arachidonic acid metabolism (cyclooxygenase and/or lipoxygenase pathways) is responsible for the formation of eicosanoids by adult *S. mansoni*

3. To identify arachidonic acid metabolizing enzyme-like sequences from *S. mansoni* or from the phylogenitically-related nematode *C. elegans* in order to study, at the molecular level, the genes involved in eicosanoids production by metazoa.

4. To clone, functionally express, and characterize an LTA_4 hydrolase-like homologue from *C. elegans*.

3. STATEMENT OF CONTRIBUTIONS

This thesis is composed of 3 manuscripts and the contributions of co-authors and myself are described below:

A. Abdel baset, H., O'Neill, G. P., and Ford-Hutchinson, A. W. (1995) Characterization of arachidonic acid metabolizing enzymes in adult *Schistosoma mansoni*. *Molecular and Biochemical Parasitology* 73, 31-41.

All of the experiments described in this paper were carefully thought about by Dr G. P. O'Neill and myself, and performed by myself. This manuscript was written by myself. The work was supervised by Drs. G. P. O'Neill and A. W. Ford-Hutchinson.

B. Abdel baset, H., O'Neill, G. P., and Ford-Hutchinson, A. W. (1996) Attempts to clone a lipoxygenase-like homologue from *Schistosoma mansoni*. (unpublished data).

All of the experiments described in this manuscript were planned and performed by myself. This manuscript was also written by myself. The work was supervised by Drs. G. P. O'Neill and A. W. Ford-Hutchinson.

C. Abdel baset, H., Ford-Hutchinson, A. W., and O'Neill, G. P. (1998) Molecular cloning and functional expression of a *Caenorhabditis elegans* aminopeptidase structurally related to mammalian leukotriene A_4 hydrolases. *Journal of Biological Chemistry* 273, 27978-27987.

All of the experiments described in this paper were planned by Dr. G. P. O'Neill and myself, and performed by myself. This manuscript was also written by myself. The work was supervised by Drs. G. P. O'Neill and A. W. Ford-Hutchinson.

4. MANUSCRIPT A

Characterization of Arachidonic Acid Metabolizing Enzymes in Adult *Schistosoma mansoni*

Molecular and Biochemical Parasitology 73, 31-41 (1995)

Eicosanoids are potent local mediators of various physiological and pathological responses. The production of eicosanoids by helminth parasites may be essential for normal physiological processes as well as helping the parasites to overcome host immune responses. This paper presents evidence for the presence of a soluble, enzymatically active LOX and the absence of any COX activity in extracts from adult *S. mansoni* parasites. The *S. mansoni* LOX activity catalyzed the formation of a 15-HETE-like species from arachidonic acid and this activity was calcium-independent and inhibitable by inhibitors of mammalian and plant LOXs. In addition, *S. mansoni* extracts efficiently metabolized linoleic acid to a 13-HODE-like product indicating that the parasite LOX-homologue is similar to mammalian 15-LOXs. Two LOX-specific immunoreactive proteins with similar molecular masses to plant and mammalian LOXs were detected in *S. mansoni* extracts. The paper also presents evidence that *S. mansoni* genomic DNA contains LOX-like sequences which hybridized to a human 15-LOX cDNA probe.

CHARACTERIZATION OF ARACHIDONIC ACID METABOLIZING ENZYMES IN ADULT *SCHISTISOMA MANSONI*

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ABBREVIATIONS

AA	Arachidonic acid
bp	base pair
IC_{50}	concentration of half inhibition
COX-2	cyclooxygenase-2
DMSO	dimethyl sulfoxide
ETYA	eicosatetraynoic acid
15-HETE	15-hydroxyeicosatetraenoic acid
13-HODE	13-hydroxyoctadecadienoic acid
LTs	leukotrienes
LOX	lipoxygenase
PCR	polymerase chain reaction
PGs	prostaglandins
RIA	radioimmunoassay
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SUMMARY

Schistosoma mansoni has previously been reported to synthesize a wide range of eicosanoids including prostaglandins, leukotrienes, and hydroxyeicosatetraenoic acids (HETEs). Our analysis of arachidonic acid metabolites synthesized by microsomal and cytosolic extracts from adult *S. mansoni* using thin layer chromatography and radioimmunoassay techniques indicate the presence of a soluble, enzymatically active lipoxygenase (LOX) and the absence of any cyclooxygenase (COX) activity. The *S. mansoni* LOX activity catalyzed the formation of a 15-hydroxyeicosatetraenoic acid (15-HETE)-like species. This activity was calcium-independent and inhibitable by inhibitors of mammalian and plant LOX. The conversion of linoleic acid to a 13-hydroxyoctadecadienoic acid (13-HODE)-like product by *S. mansoni* extracts indicates that the parasite LOX-homologue is similar to mammalian 15-lipoxygenase. Immunoblot analysis of *S. mansoni* extracts using antisera to different mammalian lipoxygenases detects two immunoreactive proteins with molecular weights similar to plant and mammalian lipoxygenases. In addition, polymerase chain reaction (PCR) amplification of LOX-like sequences from *S. mansoni* genomic DNA using degenerate primers based on conserved plant and mammalian LOX sequences, generated two PCR products which hybridized to a human 15-LOX cDNA probe. While the role of eicosanoid production in the physiology of *S. mansoni* is not known, eicosanoids may be essential for normal physiological processes as is the case in other invertebrates. Interestingly, 15-HETE has previously been shown to have immunosuppressive effects in mammals, and this may be related to the ability of the parasite to overcome host immune responses.

INTRODUCTION

The metabolism of arachidonic acid by cyclooxygenase and lipoxygenase enzymes results in the production of a wide range of biologically active oxygenated metabolites including prostaglandins, thromboxanes, leukotrienes, hydroxyeicosatetraenoic acids (HETEs), and lipoxins which are collectively referred to as eicosanoids (5, 315). In mammals, eicosanoids are potent mediators of various physiological and pathological responses including inflammation, bronchoconstriction, pain, fever, regulation of vascular tone and regulation of immune responses (3, 4).

Studies have shown the production of eicosanoids by several parasites in response to the addition of polyunsaturated fatty acids, including *Taenia taeniaeformis*, *Schistosoma mansoni*, *Wuchereria bancrofti*, and *Brugia malayi* (292, 316). In certain parasites eicosanoid production may be related to a developmental stage or process. For example, in *S. mansoni* the production of lipoxygenase products such as leukotrienes and hydroxyeicosatetraenoic acids (HETE) was suggested to correlate with ceracarial penetration, whereas prostaglandin production was associated with the transformation of cercaria into schistosomules (305). In adult *S. mansoni*, prostaglandins, leukotrienes and hydroxyeicosatetraenoic acids were all produced by both male and female worms, with 15-HETE as the major product of the lipoxygenase pathway. As certain eicosanoids, in particular PGE_2 and 15-hydroxyeicosatetraenoic acids (15-HETE) have immunosuppressant activity (5, 292, 317, 318) it has been postulated that eicosanoids synthesized by developing schistosomulae may help the parasite evade the initial response of the dermal immune system (307). In the present study we have investigated the metabolism of arachidonic acid by adult *S. mansoni* parasites, demonstrating the presence of an active lipoxygenase pathway and the absence of any cyclooxygenase-derived prostaglandins.

S. mansoni Infections and adult worm isolation.

Infected CD.01 mice were maintained in the laboratory of Dr. James M. Smith (Institute of Parasitology, McGill University, Ste.-Anne de Bellevue, Quebec, Canada) as described (319). Adult *S. mansoni* were recovered by perfusion of the hepatoportal system 7 weeks post infection using ice-cold RPMI media (GIBCO) containing 0.85% NaCl/0.75% Na_3 . citrate, washed twice in sterile saline solution, visually examined by light microscopy for host cell contamination, and immediately frozen in liquid nitrogen.

Preparation of subcellular fractions.

Adult *S. mansoni* obtained from 40 infected mice were resuspended in 10 ml homogenization buffer (100 mM Tris. HCl, pH 7.4, containing 5 mM EDTA/1 mM phenylmethylsulfonyl fluoride/10 μg ml^{-1} soybean trypsin inhibitor/1 μg ml^{-1} leupeptin/1 μg ml^{-1} pepstatin/1 mM homovanillic acid (Sigma Chemical Co., St. Louis, MO, USA). The worms were homogenized using a Dounce homogenizer followed by sonication at 4°C. The suspension was first centrifuged at 2000 x g for 10 min at 4°C to yield a large membrane fraction, followed by centrifugation of the resultant supernatant at 200,000 x g for 45 min at 4°C to prepare microsomal and cytosolic fractions. Protein concentrations were determined using a protein assay kit (Bio-Rad, Mississauga, Ontario, Canada).

Analysis of 15-HETE synthesis.

Assays were carried out in a 100 μl final volume in 0.1 M Tris, pH 8.0, containing 5 mM EDTA/1 μM hematin/1 mM homovanillic acid/15 μg total worm protein/0.2 μCi [^{14}C(U)]-arachidonic acid (866 mCi $mmol^{-1}$; 0.46 μM final concentration) or 0.4 μCi [^{14}C(U)]-linoleic acid (1045 mCi $mmol^{-1}$; 0.76 μM final

concentration) (New England Nuclear, Boston, MA, USA). Incubations were carried out for 1 h at 37°C; reactions were then quenched with 50 µl methanol and applied to silica gel thin layer chromatography plates (Whatman). The thin layer chromatography plates were developed with either ethyl acetate/acetic acid (99:1) or with ether/petroleum ether/acetic acid (50: 50:1) (320). Plates were scanned for radioactivity and the products were quantified on a Berthold LB2842 thin-layer chromatography linear analyzer and then visualized by autoradiography. Authentic prostaglandins, monohydroxy acids, monohydroperoxy acids, linoleic acid, and arachidonic acid standards (Cayman) were run in parallel. Results are expressed as percentage conversion of arachidonic acid per µg total worm protein.

Radioimmunoassay for Prostaglandin E_2 detection.

S. mansoni fractions were incubated for 1 h at 37 °C in the same buffer used for the assays of 15-HETE synthesis except for the use of nonradioactive arachidonic acid (20 µM final concentration), and 20 µg protein in an 80 µl final reaction volume. Reactions were stopped by adding 0.1 vol. of 1 M HCl followed by 0.1 vol. of 1 M NaOH. PGE_2 production was assayed using a radioimmunoassay kit (PGE_2 [^{125}I] RIA Kit, NEN, Boston, MA, USA).

Effect of cyclooxygenase / lipoxygenase inhibitors.

L-670,630 (2,3-dihydro-6-(3-phenoxypropyl)-2-(2-phenylethyl)-5-benzofuranol) (321), CPHU (*N*-(4-chlorophenyl)-*N*-hydroxy-*N'*-(3-chlorophenyl) urea (322)), and indomethacin (Merck Frosst) were dissolved in DMSO and 5,8,11,14-eicosatetraynoic acid (ETYA) (Cayman) was dissolved in 100% ethanol. Inhibitors or their respective solvents were preincubated with cytosolic fraction for 15 min and the reaction was initiated by adding an ethanol solution of the substrate (arachidonic acid or linoleic acid). 1 h after substrate addition, the reactions were quenched with methanol and spotted on thin layer chromatography plates. Human recombinant cyclooxygenase-2 enzyme (COX-2) (323) was used as a positive control for the production of prostaglandins and 15-

HETE. The IC_{50} values for the lipoxygenase inhibitors were calculated after scanning the autoradiographs using a computing densitometer (Molecular Dynamics, Sunnyvale, CA, USA).

Immunoblot analysis.

Large membrane, microsomal, and cytosolic fractions of *S. mansoni* adult worms as well as 5-, 12-, and 15-lipoxygenase enzymes standards (Oxford Biochemicals) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as previously described followed by electrophoretic transfer to nitrocellulose membranes (73, 324, 325). The nitrocellulose membranes were probed with the following antisera: 1:300 dilution of rabbit anti-human 5-lipoxygenase (324), 1:5000 dilution of rabbit anti-sheep COX-1, 1:7500 dilution of rabbit anti-sheep COX-2 (325), an 1:100 dilution of rabbit anti-human 12-lipoxygenase (Oxford Biochemicals), an 1:100 dilution of goat anti-human 15-lipoxygenase (Cayman). The secondary horse radish peroxidase-linked donkey anti-rabbit IgG antibody (Amersham Life Sciences, Oakville, Ontario, Canada) was used at a dilution of 1:3000. Immunodetection was performed using enhanced chemiluminescence according to the manufacturer's instructions (Amersham). When anti-5-lipoxygenase antiserum was used as a primary antibody, ^{125}I-protein A (NEN) was used for detection as previously described (324). Autoradiographs for chemiluminescence detection were exposed to Kodak X-OMAT X-ray films for 3 min; autoradiographs for protein A detection method were exposed for 5 days.

Polymerase chain reaction (PCR) amplification of lipoxygenase-like sequences.

For the selection of oligonucleotides for PCR, highly conserved regions of 8 lipoxygenases were identified by aligning the amino acid sequences of rabbit 15-lipoxygenase, bovine 12-lipoxygenase, pig 12-lipoxygenase human 15-lipoxygenase, human 12-lipoxygenase, and lipoxygenases from the plants soybean, pea seed and rice (137, 143, 326). Based on the alignment of the

lipoxygenases, the following degenerate oligonucleotides and their positions within the human 15-lipoxygenase cDNA (137) were chemically synthesized: lx-4, 5'-CC(A/T) (G/C)(G/T)(A/G) GAT GAG (A/C)GA TT-3', nt 481-497; lx-5, 5'-GG(T/C) (G/A)(C/T)(C/A) AAC CCC (GA)TG IT-3', nt 703-719; lx-7, 5'-AG(G/C) (C/T)(A/G)(C/T) C(T/G)(C/G) (A/T)TG GTG GC-3', nt 1135-1119. A primary PCR reaction (GeneAmp DNA PCR kit, Perkin Elmer Cetus, Norwalk,CT, USA) was done using lx-4 and lx-7 primers in a buffer containing 10 mM Tris . HCl (pH 8.3)/50 mM KCl/2.5 mM $MgCl_2$/0.2 mM deoxynucleotide triphosphates/0.5 μM primers and 40 ng of adult *S. mansoni* genomic DNA as template. The PCR cycling conditions were: 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. A secondary PCR reaction was then performed under the same conditions using 0.01 μl of the primary PCR reaction and the primers lx-5 and lx-7. PCR products were separated by electrophoresis in a 1.2% (w/v) agarose gel, visualized by ethidium bromide staining and blotted to Hybond-N^+ nylon membranes (Amersham Life Science) by overnight capillary transfer using 0.4 M NaOH. The oligonucleotides used for PCR amplification of cyclooxygenase sequences were based on conserved sequences from chicken, human, mouse and rat COX-2 (327). The COX PCR primers and their corresponding codons in human, rat, mouse and chicken COX-2 include (327): cx-1, 5'-ATG ATG TA(TC) GCI ACI ATI TGG-3', codons 285-291 ; cx-2, 5'-ATG TAC CCI CCI ACI GTI AA-3', codons 247-253; cx-3, 5'-TT(TC) AA(CT) ACI (CT)TI TA (TC) CA(TC) TGG-3', codons 367-373; cx-4, 5'-(AG)AA IA(AG) (TC)TG (TC)TC (AG)TC (AG)TC CCA-3', codons 316-309; cx-5, 5'-CCA IAT IGT IGC (AG)TA CAT CAT-3', codons 291-285; cx-6, 5'-(CT)TC (CT)TC IA(AG) (CT)TC IGC IGC CAT-3', codons 479-473.

Southern blot analysis of PCR products.

Southern blot hybridization was performed at 65°C for 16 h in a solution containing 5 x SSPE (1 x SSPE is 0.15 M NaCl/0.01 M NaH_2PO_4/0.01 M EDTA, pH 7.4), 5 x Denhardt's (1 x Denhardt's solution is 0.02% (w/v) Ficoll/0.02% (w/v) bovine serum albumin/0.02% (w/v) polyvinyl pyrrolidone)/100 μg ml^{-1} denatured

calf thymus DNA/0.5% (w/v) SDS, and randomly primed (Boehringer-Mannheim) ^{32}P-labeled human 15-lipoxygenase cDNA (137); Dr. E. Sigal, University of Califomia, San Francisco, CA, USA), or a mixture of ^{32}P-labeled human COX-1 and human COX-2 cDNAs (73). The membranes were washed twice in 2 x SSC (1 x SSC is 0.15 M NaCl/0.015 M Na_3 . citrate, pH 7)/0.1% (w/v) SDS at room temperature, then washed twice in 2 x SSC/0.1% SDS at 65°C for 30 min, and exposed to Kodak XAR-5 film for 1-5 days at -70°C.

RESULTS

Eicosanoid production by adult *S. mansoni*.

To determine the profile of arachidonic acid metabolism by adult *S. mansoni*, different fractions of homogenized worms were incubated with ^{14}C-arachidonic and the products were analyzed by thin layer chromatography. In both the cytosolic and microsomal adult *S. mansoni* fractions, the major metabolite co-chromatographed with an authentic 15-HETE standard (Fig. 1). The synthesis of the 15-HETE-like product was highest in the cytosolic fraction (68% conversion of ^{14}C-arachidonic acid per 15 µg protein) as compared to the microsomal fraction (16% conversion of ^{14}C-arachidonic acid per 48 µg microsomal protein). No ^{14}C-arachidonic acid metabolites which co-chromatographed with authentic prostaglandin standards were detected in either the microsomal or cytosolic adult *S. mansoni* fractions (Fig. 1). In contrast, using the same assay and detection methods, 15-HETE and prostaglandin products were detected in control reactions using recombinant human COX-2, as previously reported (73, 323). Since the level of prostaglandin synthesis in the *S. mansoni* fractions may be below the detection level of the thin layer chromatography method, *S. mansoni* fractions were incubated with nonradioactive arachidonic acid and analyzed using a sensitive PGE_2 radioimmunoassay (lower limit of detection is 0.5 pg PGE_2 ml^{-1}). However, no PGE_2 production was detected in any of the *S. mansoni* extracts (results not shown). These results suggest that adult *S. mansoni* express at least one lipoxygenase but no detectable cyclooxygenase activity.

The production of the 15-HETE-like metabolite is calcium-independent and heat-inactivable.

Since lipoxygenases (except for 5-lipoxygenase) are calcium independent (328), the effect of calcium addition on the production of 15-HETE in the

cytosolic fraction was determined. Addition of 2 mM calcium chloride to the extracts did not alter the production of the 15-HETE-like species (Fig. 2). Heat denaturation of the cytosolic fraction (100°C, 15 min) totally abolished the appearance of the 15-HETE-like metabolite (Fig. 2), confirming an enzymatic involvement in the production of this metabolite.

The effect of cyclooxygenase and lipoxygenase inhibitors on the production of the 15-HETE-like species.

The synthesis of 15-HETE from arachidonic acid by both 15-lipoxygenase and cyclooxygenase can be inhibited in a dose-dependent manner by specific inhibitors (73, 191, 323, 329). Indomethacin, a potent inhibitor of both 11- and 15-hydroxylation of arachidonic acid by cyclooxygenases (73, 329), did not affect the production of the 15-HETE-like metabolite in the *S. mansoni* cytosolic fraction, whereas preincubation of human recombinant COX-2 with indomethacin totally abolished the formation of both prostaglandins and 15-HETE (Fig. 3; panel A). In contrast 10 μM ETYA, a known suicide substrate inhibitor of lipoxygenases (191), completely abolished the production of the 15-HETE-like species in the *S. mansoni* cytosolic fraction (Fig. 3, panel B), with an IC_{50} of 2 ± 0.07 μM (n=5; data not shown). Since ETYA can inhibit both cyclooxygenases and lipoxygenases, two other lipoxygenase inhibitors were used which displayed dose-dependent inhibition of the *S. mansoni* cytosolic lipoxygenase-like activity. Pretreatment of the cytosolic fraction with increasing concentrations of L-670,630, a phenolic redox inhibitor of lipoxygenases (321), and CPHU, one of the N-hydroxyurea class of lipoxygenase redox inhibitors (322) resulted in dose-dependent inhibition of the production of the 15-HETE-like species with IC_{50} values of 3 ± 0.06 μM (n=5) and 0.08 ± 0.003 μM (n=5), respectively (data not shown).

The metabolism of linoleic acid by *S. mansoni* cytosol.

Certain lipoxygenases, such as erythroid 15-lipoxygenase and leukocyte 12-lipoxygenase, are distinguished by their ability to metabolize linoleic acid to

13-hydroxyoctadecadienoic acid (13-HODE; (330)). The *S. mansoni* cytosolic extract also metabolized ^{14}C-linoleic acid to a product that co-chromatographed with a 13-HODE standard (Fig. 4). The production of the 13-HODE-like product by the *S. mansoni* cytosolic extract was abolished by heat-inactivation and markedly reduced by pretreatment of the cytosol with CPHU or L-670,630 (Fig. 4).

Immunoblot analysis of *S. mansoni* fractions.

Due to the significant amino acid sequence homology among plant and mammalian lipoxygenases (143), it is reasonable to expect that antibodies to mammalian 5-, 12-, or 15-lipoxygenase might show immunological cross-reactivity to a *S. mansoni* homologue. Thus, immunoblot analyses of *S. mansoni* extracts with an anti-human 12-lipoxygenase polyclonal antiserum and an anti-human 5-lipoxygenase polyclonal antiserum were performed (Fig. 5). Both the antisera to 5- and 12-lipoxygenase detected all three purified standards, including the 78 kDa human 5-lipoxygenase, the 75 kDa porcine leukocyte 12-lipoxygenase, and the 76 kDa rabbit reticulocyte 15-lipoxygenase (143). Although both antisera to 5- and 12-lipoxygenase detected a number of immunoreactive proteins in the *S. mansoni* extracts, two proteins were detected by both antisera and corresponded to the molecular weights of mammalian and plant lipoxygenases (Fig. 5). One *S. mansoni* protein of 78 kDa co-migrated with the human 5-lipoxygenase standard and was seen in the microsomal and cytosolic fractions, and a second protein of 100 kDa was comparable in molecular weight to that of soybean-2 lipoxygenase and pea seed lipoxygenase (143). Other immunoreactive bands may represent non-specific cross-reactivity as these bands were also detected using preimmune serum (data not shown). Using an anti-ram seminal vesicle COX-1 polyclonal antiserum and an anti-sheep COX-2 polyclonal antiserum (325), no cyclooxygenase specific immunoreactive protein bands were seen in any of the different *S. mansoni* fractions (data not shown). These results suggest that adult *S. mansoni*

expresses at least one lipoxygenase that is antigenically related to mammalian and plant lipoxygenases.

Lipoxygenase-like DNA sequences in *S. mansoni*.

Southern hybridization of *S. mansoni* genomic DNA with the human 15-lipoxygenase cDNA and subsequent washing under moderately stringent conditions, revealed hybridizing bands of 8.6 kb in the *Hind*III, 6.4 kb in *Eco*RI, 6.4 kb in *Hind*III/*Eco*RI, and 7 kb in *Bam*HI digested samples (Fig. 6). Southern hybridization of *S. mansoni* genomic DNA with both full-length human cyclooxygenase-1 and -2 cDNA probes revealed two weakly hybridizing fragments of 6.5 kb and 2.3 kb in a *Bam*HI digest (data not shown).

The positive hybridization signals observed in the *S. mansoni* genomic DNA with the mammalian lipoxygenase and cyclooxygenase probes indicated that the homologous *S. mansoni* sequences might be amplifiable by the polymerase chain reaction (PCR). Oligonucleotide primers for amplification of the putative *S. mansoni* lipoxygenase and cyclooxygenase were based on highly conserved regions identified by sequence comparison of 8 different mammalian and plant lipoxygenases and six different cyclooxygenases, respectively (see Materials and Methods). In order to detect parasite lipoxygenase sequences, PCR amplification of *S. mansoni* genomic DNA was performed using the primer pair lx-4 / lx-7 in a primary PCR, followed by a second round of amplification using the nested primer pair lx-5 / lx-7 and an aliquot of the primary PCR as template, separation of the PCR products by agarose gel electrophoresis and Southern blot analysis with the human 15-lipoxygenase as a probe. As expected a 433 bp fragment was amplified using the human 15-lipoxygenase cDNA as a template. Two products were amplified from *S. mansoni* DNA, including a 433 bp fragment which co-migrated with the PCR product amplified from the human 15-lipoxygenase cDNA, and a second PCR product of 370 bp. The identity of both PCR fragments as LOX-like sequences was confirmed by their hybridization to the human 15-LOX cDNA probe. The oligonucleotides lx-4,-5, and -7 have also

been used to amplify identically-sized PCR products from an *S. mansoni* cDNA library (kindly provided by Dr. C.B. Shoemaker, Harvard School of Public Health, Boston, MA, USA) (data not shown).

DISCUSSION

Adult *S. mansoni* has previously been shown to incorporate polyunsaturated fatty acids, including arachidonic acid, and it has been suggested that they readily metabolize other polyunsaturated fatty acids, including linoleic acid, to arachidonic acid (304). Analysis of eicosanoid metabolism following addition of linoleic acid to adult worms has shown the major product to have the chromatographic characteristics of 15-HETE consistent with the present results (307). With regard to arachidonic acid metabolism by cyclooxygenase pathways in adult *S. mansoni* the literature is less clear. One publication has reported no formation of prostaglandins (308) whereas another has reported on the presence of immunoreactive and chromotographically characterized materials with the characteristics of prostaglandins although no definitive physiochemical or pharmacological data were obtained (307). Similar results were obtained with *S. mansoni* cercariae (304, 305). Our results indicate an absence of PGE_2 production by adult worm extracts as tested both by thin layer chromatography and radioimmunoassay techniques. In addition, we could find no evidence for the presence of a parasite COX homologue by PCR using degenerate primers based on conserved sequences in mammalian and avian COX-2. Furthermore, immunoblot analysis of different adult worm extracts using antisera to mammalian cyclooxygenase did not show specific COX-protein bands. Taken together, these results combined with the fact that no prostaglandin production was detected, suggest the absence of an active cyclooxygenase pathway in adult *S. mansoni* parasites. However, two weakly hybridizing bands observed by Southern blot hybridization of *S. mansoni* genomic DNA using the human cyclooxygenase-1 and -2 cDNA probes, suggests that the parasite does contain cyclooxygenase-related sequences. Our inability to detect an *S. mansoni* cyclooxygenase protein or mRNA in the adult worm may be related to a stage-specific expression of the putative *S. mansoni* cyclooxygenase gene.

As reported previously (307), our results show that a 15-HETE-like product is the major eicosanoid produced from arachidonic acid by *S. mansoni* suggesting the presence of an active lipoxygenase pathway. Calcium addition did not stimulate the activity of the parasite lipoxygenase homologue suggesting that it is regulated differently to the mammalian 5-lipoxygenase enzyme but similarly to mammalian 15-lipoxygenase. The inability of indomethacin, a potent cyclooxygenase inhibitor, to inhibit *S. mansoni* 15-HETE production and the use of different lipoxygenase inhibitors, which showed similar pharmacological profiles to that seen with different plant and mammalian lipoxygenases (191), confirmed the lipoxygenase origin of the 15-HETE-like species. Although the 15-HETE-like species migrated with the same R_f value as that of a 15-HETE standard on thin layer chromatography, its identity to the mammalian 15-HETE can only be confirmed using gas chromatography / mass spectrometry. This awaits the cloning and expression of the parasite lipoxygenase homologue in order to produce large enough quantities of eicosanoids to allow for such measurements. The efficient conversion of linoleic acid to 13-HODE by the cytosolic fraction of adult worms indicates that the parasite lipoxygenase activity is similar to the erythroid 15-lipoxygenase and the leukocyte 12-lipoxygenase. Our immunoblot analysis and PCR amplification results suggest the presence of more than one lipoxygenase protein and more than one lipoxygenase gene, respectively, in adult *S. mansoni*.

Although we cannot totally exclude the possibility that the lipoxygenase we observed in *S. mansoni* is due to contamination from murine blood cells, it is unlikely based on several lines of evidence. Since murine white blood cells contain both cyclooxygenases and lipoxygenases, if our *S. mansoni* extracts were contaminated with murine cells then the extracts and DNA preparations would be expected to contain not only lipoxygenase, but also murine cell-derived cyclooxygenase activity, cyclooxygenase immunoreactive protein, and PCR-amplifiable sequences. A second line of evidence is based on our preliminary cloning experiments, in which we have cloned and sequenced 12 unique

subclones derived from the PCR amplification of *S. mansoni* DNA with the lipoxygenase oligonucleotides. The deduced amino acid sequence of one clone displayed 52% similarity (28% identity) and 42% similarity (18% identity) with the analogous regions in human 15-lipoxygenase and soybean lipoxygenase-2 respectively (unpublished data). None of the 12 clones displayed any significant sequence homology to murine sequences in the GenBank database (release 84.0). If our PCR-generated subclones were derived from amplification of contaminating murine DNA or RNA then one might expect the putative clones to show near sequence identity to known murine lipoxygenase sequences. The origin of the lipoxygenase-like PCR product from *S. mansoni* is further strengthened by our ability to amplify by PCR the same lipoxygenase-like sequences from a characterized *S. mansoni* cDNA library obtained from an independent source.

The observation that the lipoxygenase-like PCR products generated from the *S. mansoni* genomic DNA and an *S. mansoni* cDNA library were all of the same size (unpublished observations), suggests that the relevant *S. mansoni* genomic sequences do not contain introns. This would be in contrast to known mammalian lipoxygenase genes (143), since the oligonucleotides used in the lipoxygenase PCR experiment reported here are all located in separate exons of the mammalian lipoxygenase genes (143). Primers lx-4, lx-5, and lx-7 are located in exons 4, 6, and 8, respectively, in all three genes for the human 5-, 12-, and 15-lipoxygenase genes. However, the introns sizes in these three genes are different, ranging in size from 0.2 kb to 12 kb. For example, PCR amplification of the human 5 and 12 lipoxygenases with primers lx-5 / lx-7 would generate two products of 14.4 kb and 2.2 kb, respectively.

The ability of immunoblot, Southern hybridization, and PCR methods using reagents based on mammalian lipoxygenases to detect evolutionarily distant lipoxygenases in *S. mansoni* is not surprising considering the high degree of sequence conservation in the lipoxygenase family (137, 143, 326) and in addition, the sequence conservation between human and *S. mansoni* genes

(331). The human 5-, 12- and 15-lipoxygenases exhibit an overall 60% sequence similarity, and each of the human lipoxygenases is roughly 25% identical in sequence to plant lipoxygenases (143, 328). For specific lipoxygenases even greater sequence conservation across species is observed. Thus, the human 5- and 15-lipoxygenases are 92% and 81% identical to their respective rat and rabbit homologues (143). Furthermore, the recent sequence determination of 607 *S. mansoni* cDNA sequences indicates that significant sequence identity is displayed between several human and *S. mansoni* homologues (331); for example, aldehyde dehydrogenase from human and *S. mansoni* are 59.7% identical (74% similar).

The production of 15-HETE by an *S. mansoni* lipoxygenase may be significant in both the normal physiology of *S. mansoni* and the mediation of host-parasite interactions. In other invertebrates eicosanoids have been implicated in areas such as egg production and laying, oocyte maturation, prevention of polyspermic fertilization, and salt and water transport physiology (289). Several groups have suggested that *S. mansoni* eicosanoid production is related to host-parasite interactions including cercarial penetration (305), and modulation of host immune defenses through suppression of host cell-mediated immune responses (292, 307, 316). One approach to elucidate the role of eicosanoid biosynthesis in *S. mansoni* might be to determine if developmental regulation occurs. The molecular cloning of genes involved in eicosanoid metabolism from *S. mansoni* would provide useful probes to address the expression of these genes during the parasite's life cycle.

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FIGURE LEGENDS

Figure 1. 15-HETE production by adult *S. mansoni*. Subcellular fractions of adult *S. mansoni* and recombinant human COX-2 were incubated for 1 hr in the presence of [^{14}C]-arachidonic acid under the reaction conditions described in Materials and Methods. Following incubation, the products were extracted, separated on silica gel thin-layer chromatography plates and scanned for radioactivity. Tracing 1, microsomal fraction (48 μg protein); tracing 2, cytosolic fraction (15 μg protein); tracing 3, [^{14}C]-arachidonic acid only; tracing 4, human recombinant COX-2. The migration of co-chromatographed prostaglandins and 15-HETE standards are indicated. The tracings are from one of three experiments.

Figure 2. Effect of calcium addition and denaturation on the production of the 15-HETE-like product. Adult *S. mansoni* cytosol (15 μg protein) was incubated with [^{14}C]-arachidonic acid (0.46 μM) in the absence (lane 1) or presence (lane 2) of 2 mM $CaCl_2$, or denatured by boiling for 15 min prior to incubation with the substrate (lane 3). The reaction products were analyzed using thin-layer chromatography and visualized by autoradiography. Lane 4, [^{14}C]-arachidonic only; lane 5, recombinant human COX-2 with [^{14}C]-arachidonic acid (0.46 μM). The positions of co-chromatographed standards are indicated. The autoradiogram shown is a representative of three separate experiments with similar results.

Figure 3. Effect of cyclooxygenase and lipoxygenase inhibitors on the metabolism of arachidonic acid by *S. mansoni*. **(A)** Thin layer chromatography of products formed from [^{14}C]-arachidonic acid by *S. mansoni* cytosol (15 μg; lanes 1,2, and 3) and human recombinant COX-2 (lanes 4 and 5) in the absence (lane 1) or presence (lane 2) of DMSO vehicle or 100 μM indomethacin dissolved in DMSO (lane 3 and 4). **(B)** Thin-layer chromatography

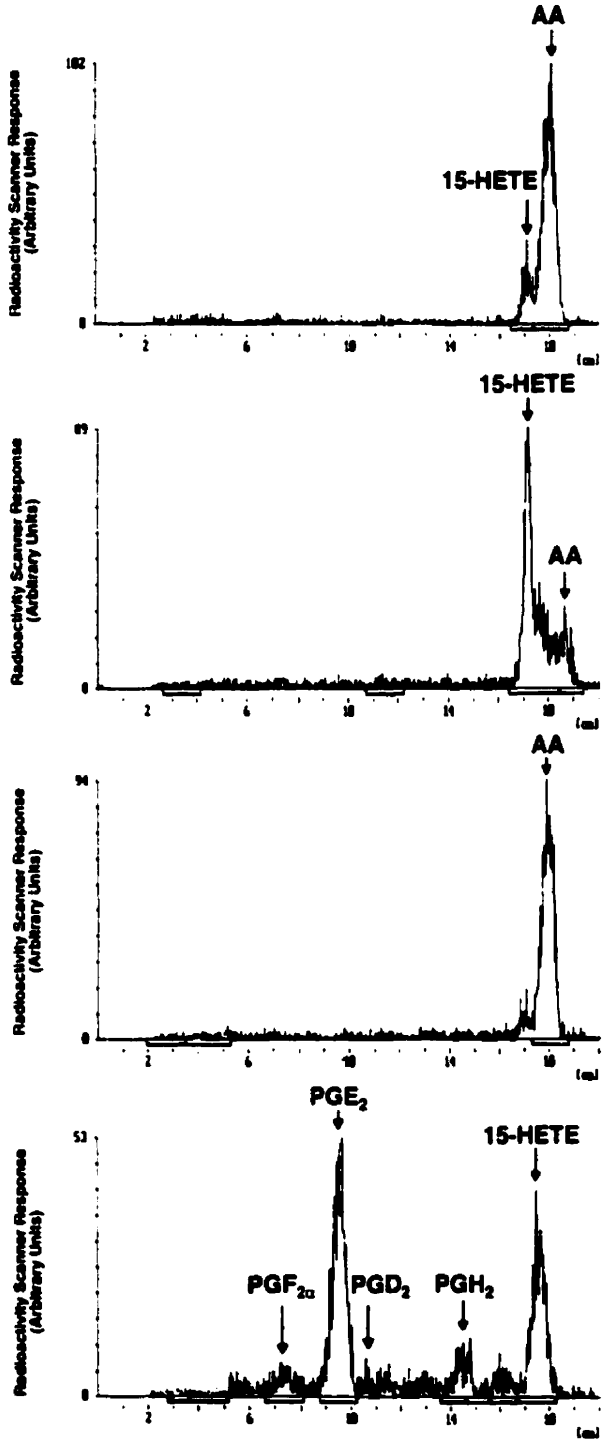
of products formed from [^{14}C]-arachidonic acid by *S. mansoni* cytosol (15 μg; lanes 1-3) in the absence (lane 1) or presence of ethanol vehicle (lane 2), in the presence of 10 μM ETYA dissolved in ethanol (lane 3). [^{14}C]-arachidonic acid in the absence of any added protein (lane 4). The reaction products were analyzed using thin-layer chromatography plates developed either with ethyl acetate/ acetic acid (panel A) or with ether/petroleum ether/acetic acid (panel B) and visualized by autoradiography. The positions of co-chromatographed standards are indicated. The autoradiograms shown in panel A and B are representative of three experiments.

Figure 4. Metabolism of linoleic acid by *S. mansoni* cytosol. Thin-layer chromatography of products formed from [^{14}C]-linoleic acid (0.76 μM) by *S. mansoni* cytosol (15 μg) in the absence of inhibitor or vehicle (lanes 1), in the presence of 1% DMSO vehicle (lane 2), treated with 5 μM CPHU (lane 3), treated with 5 μM L-670,630 (lane 4), or boiled *S. mansoni* cytosol (lane 5). [^{14}C]-linoleic acid in the absence of any added *S. mansoni* cytosol (lane 6). Thin-layer chromatography and densitometry were carried out as described in Materials and Methods. The position of co-chromatographed standards are indicated. This experiment was repeated twice with similar results.

Figure 5. Immunoblot analysis of *S. mansoni* fractions. 140 ng each of human leukocyte 5-lipoxygenase (lanes 1 and 8), porcine leukocyte 12-lipoxygenase standard (lanes 2 and 9), rabbit reticulocyte 15-lipoxygenase standard (lanes 3 and 7), 50 μg total protein of adult *S. mansoni* large membrane fraction (lanes 4 and 10), 30 μg of *S. mansoni* microsomal fraction (lanes 5 and 11), and 15 μg of *S. mansoni* cytosolic fraction (lanes 6 and 12) were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted using anti-human platelet 12-lipoxygenase antiserum (1:100 dilution) or anti-human 5-lipoxygenase antiserum (1:300 dilution) as described in Materials and Methods. Enhanced chemiluminescence (lanes 1 to 7) or ^{125}I-protein A (lanes 7 to 12)

were used for detection. The positions of molecular weight markers are indicated.

Figure 6. Detection of *S. mansoni* lipoxygenase-like DNA sequences. **(A)** Southern hybridization of a ^{32}P-labeled human 15-lipoxygenase cDNA with human (lanes 1-4) and *S. mansoni* genomic DNA (lanes 5-8). The lanes contain 10 µg human DNA or 10 µg *S. mansoni* DNA restricted with *Hind*III (lanes 1 and 5), *Eco*RI (lanes 2 and 6), *Hind*III and *Eco*RI (lanes 3 and 7), and *Bam*HI (lanes 4 and 8). Fragment sizes of *Hind*III-cleaved lambda DNA are shown in kb. **(B)** Analysis of PCR-amplified products from *S. mansoni* genomic DNA using primers based on conserved lipoxygenase sequences. *S. mansoni* genomic DNA (40 ng) and human 15-lipoxygenase cDNA (1 ng) were used as templates for PCR amplification with degenerate primers based on conserved sequences in lipoxygenases. Amplification products were electrophoresed on a 1.2% agarose gel, capillary blotted onto nylon membrane and probed with ^{32}P-random primer-labeled human 15-lipoxygenase cDNA probe. The positions of DNA size markers are indicated (in bp). Lane 1, 10 µl of PCR reaction using human 15-lipoxygenase cDNA as a template; lane 2, 10 µl of the negative control for the PCR reaction using no added template; lane 3, 10 µl of PCR reaction using *S. mansoni* genomic DNA.



① S.mansoni Microsomal Fraction

② S.mansoni Cytosolic Fraction

③ Arachidonic Acid

④ Recombinant Human COX-2

Figure 1

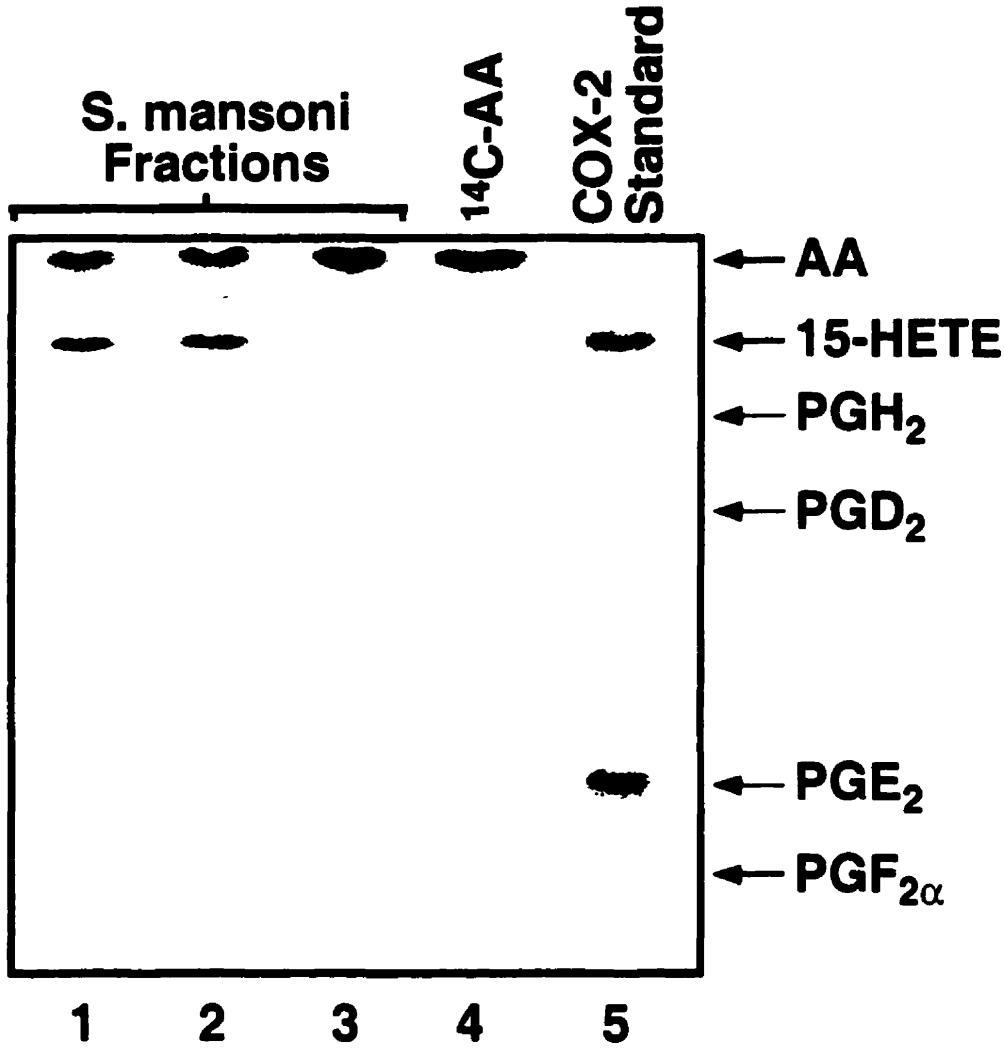

S. mansoni Fractions
14C-AA
COX-2 Standard
AA
15-HETE
PGH2
PGD2
PGE2
PGF2α
1
2
3
4
5

Figure 2

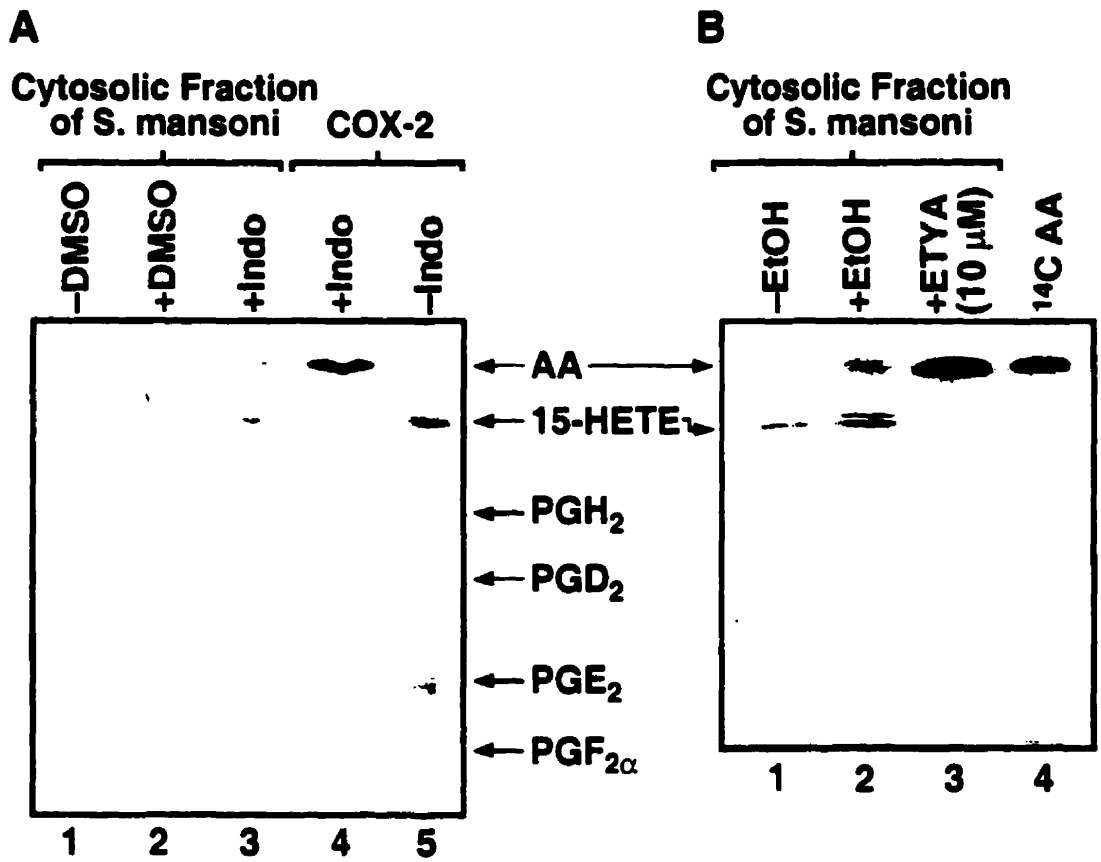

A
Cytosolic Fraction of S. mansoni
COX-2
-DMSO
+DMSO
+Indo
+Indo
-Indo
AA
15-HETE
PGH2
PGD2
PGE2
PGF2α
1
2
3
4
5
B
Cytosolic Fraction of S. mansoni
-EtOH
+EtOH
+ETYA (10 μM)
14C AA
1
2
3
4

Figure 3

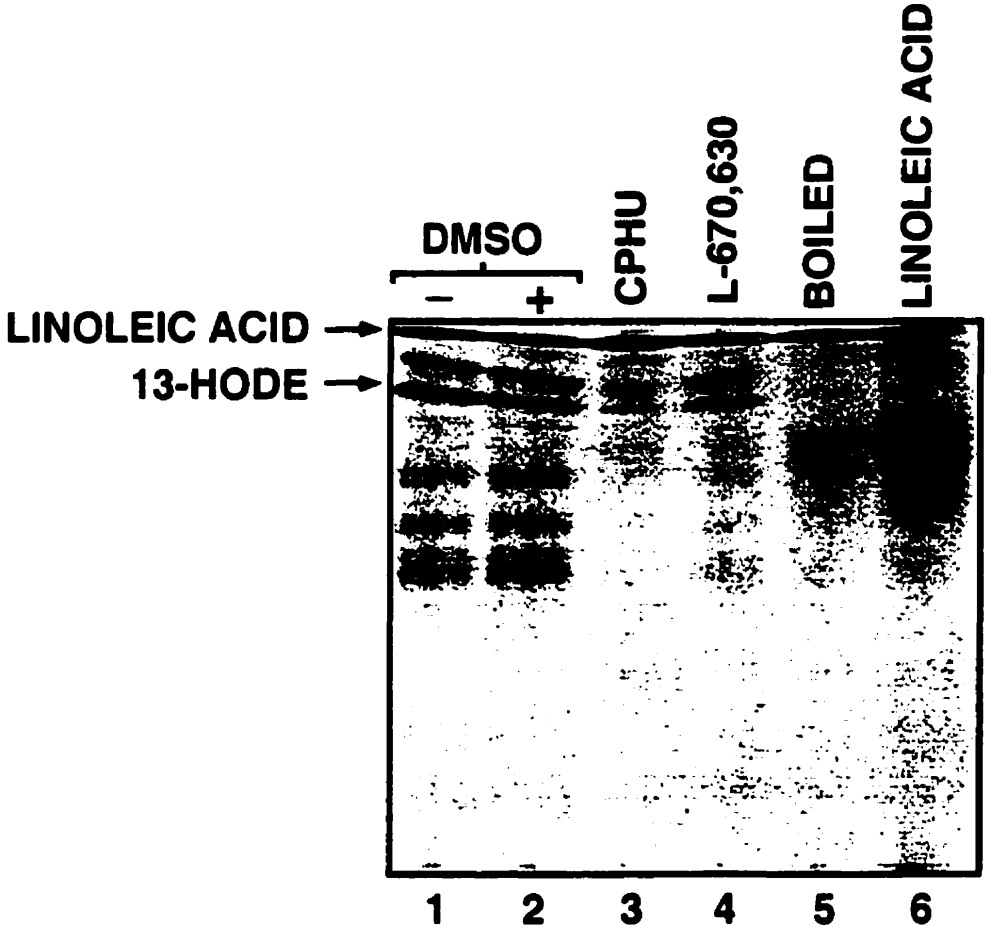

DMSO
–
+
CPHU
L-670,630
BOILED
LINOLEIC ACID
LINOLEIC ACID
13-HODE
1
2
3
4
5
6

Figure 4

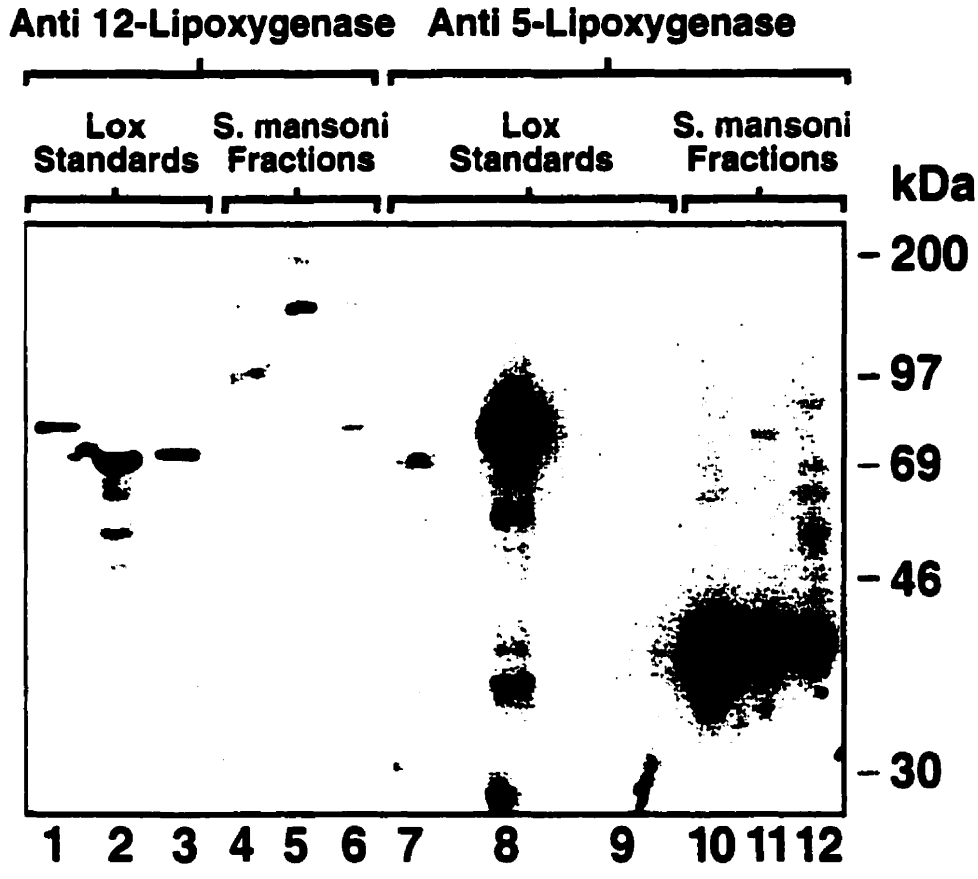

Anti 12-Lipoxygenase
Anti 5-Lipoxygenase
Lox Standards
S. mansoni Fractions
Lox Standards
S. mansoni Fractions
kDa
200
97
69
46
30
1 2 3 4 5 6 7 8 9 10 11 12

Figure 5

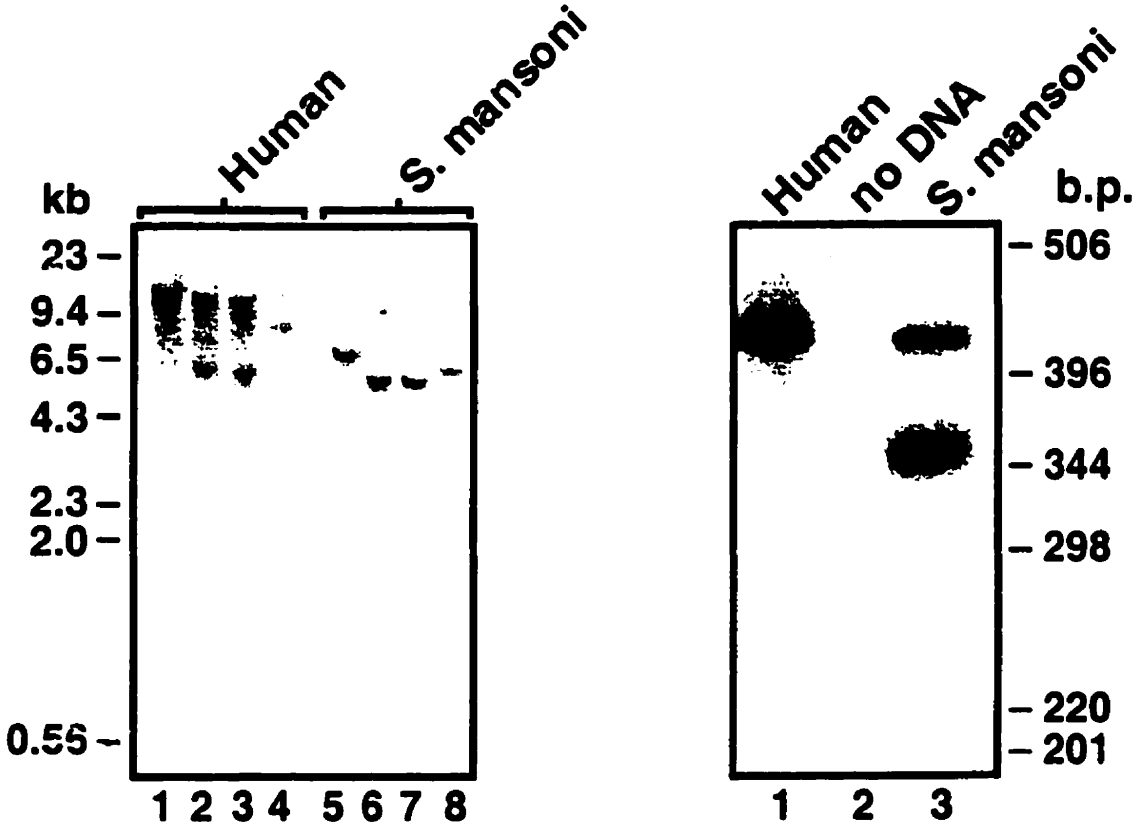

A.
B.
Human
S. mansoni
kb
23
9.4
6.5
4.3
2.3
2.0
0.56
1 2 3 4 5 6 7 8
Human
no DNA
S. mansoni
b.p.
506
396
344
298
220
201
1 2 3

Figure 6

5. MANUSCRIPT B

Attempts to Clone a Lipoxygenase-Like Homologue from *Schistosoma mansoni*

Unpublished data (1996)

In the previous manuscript we described the presence of an enzymatically active LOX pathway in extracts from adult *S. mansoni* parasites which is similar to mammalian 15-LOX. Thus, in this manuscript we attempted to clone the parasite LOX-homologue to further characterize the production of eicosanoids by *S. mansoni*. This manuscript presents evidence for the requirement of a parasite-specific probe in order to clone the LOX-homologue. The use of mammalian LOX sequences to screen several *S. mansoni* cDNA libraries resulted in the cloning of a mouse (the parasite host) LOX-cDNA due to the strong homology among different mammalian LOXs. The manuscript also describes the efforts made to try to obtain a lipoxygenase-specific probe from *S. mansoni* DNA and RNA.

ATTEMPTS TO CLONE A LIPOXYGENASE-LIKE HOMOLOGUE FROM ADULT *SCHISTOSOMA MANSONI*

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ABBREVIATIONS

BLAST	basic local alignment search tool
bp	base pair(s)
cDNA	complementary DNA
cfu	colony forming unit
EST	expressed sequence tag
15-HETE	15-hydroxyeicosatetraenoic acid
kb(p)	kilobase (pairs)
5-LOX	5-lipoxygenase
12-LOX	12-lipoxygenase
15-LOX	15-lipoxygenase
Mb	megabase (100 million bp)
mRNA	messenger RNA
PCR	polymerase chain reaction
pfu	plaque forming unit
RT-PCR	reverse transcriptase-PCR

SUMMARY

The metabolism of arachidonic acid to certain immunoregulatory eicosanoids may help parasites evade host immune responses and establish chronic diseases. Our previous analysis of eicosanoids synthesized by the adult human parasite *Schistosoma mansoni* indicated the presence of an enzymatically active lipoxygenase (LOX) pathway similar to mammalian 15-LOXs. In an attempt to clone the parasite LOX-homologue, polymerase chain reaction (PCR) amplification of LOX-like sequences from *S. mansoni* DNA, RNA, and several cDNA libraries using degenerate primers based on conserved plant and mammalian LOX sequences was performed. Several PCR products which hybridized to a human 15-LOX cDNA probe were obtained and subsequently cloned and sequenced. Ten sequenced reverse transcriptase-PCR clones had matches to different proteins in the databases but showed no sequence homology to either mammalian or plant LOXs. On the other hand, one of twenty sequenced PCR clones amplified from *S. mansoni* DNA (termed clone # 15), revealed some sequence similarity to the human 15-LOX protein. This clone was subsequently used to screen three *S. mansoni* cDNA libraries (obtained from different sources) but failed to identify any positive clones. In addition, several other probes were utilized to screen the different *S. mansoni* libraries including one of the LOX-based degenerate primers, pig 12-LOX cDNA, as well as human 5-, 12-, and 15-LOX cDNAs. These probes were also unsuccessful in identifying LOX-like positive clones following secondary screening of *S. mansoni* cDNA libraries. Moreover, an adult *S. mansoni* cDNA library in pcDNA3.1(+) plasmid was diluted into several pools and screened by PCR using LOX-degenerate primers. Several positive clones were isolated from pools by hybridization to human 15-LOX cDNA. This approach resulted in the cloning and sequencing of the mouse (the parasite host) 12-LOX due to the consistent presence of contaminating host sequences in all *S. mansoni* libraries and the high sequence homology among different mammalian LOXs. Our results reveal the difficulty of using mammalian cDNA sequences as probes to isolate metazoan genes and

indicate the requirement of a LOX-like partial sequence from *S. mansoni* or from an evolutionary-related organism in order to facilitate the cloning of the parasite LOX-homologue.

INTRODUCTION

The trematode worm *Schistosoma mansoni* (*S. mansoni*) is one of three schistosome species responsible for schistosomiasis, a debilitating human parasitic disease that is currently infecting over 300 million people worldwide (274, 275). As certain eicosanoids (oxygenated metabolites of arachidonic acid which include prostaglandins (PGs), leukotrienes (LTs), and hydroxyeicosatetraenoic acids (HETEs) (5, 315)) have immunosuppressant activity (317, 318), it has been postulated that the production of immunoregulatory eicosanoids by *S. mansoni* may help the parasites to overcome host immune responses to establish chronic diseases (301, 307). Studies have shown that cercariae (the infective stage of the parasite) produce various eicosanoids upon stimulation by essential fatty acids normally found on the host skin surface (304, 305). In addition, the cercarial production of lipoxygenase-derived eicosanoids such as LTs and HETEs was correlated with the process of penetration, whereas the production of cyclooxygenase-derived PGs was associated with the transformation of cercariae into schistosomules (305). The production of PGs by adult *S. mansoni* in response to the addition of polyunsaturated fatty acids is less clear. One study reported the presence of chromatographically characterized materials with the characteristics of PGs (307), whereas another study revealed no formation of prostaglandins by adult parasites (308).

Our previous analysis of arachidonic acid metabolites synthesized by adult *S. mansoni* extracts indicated the presence of a soluble, enzymatically active lipoxygenase (LOX) and the absence of any cyclooxygenase activity (332). The *S. mansoni* LOX activity was similar to mammalian 15-LOXs since it catalyzed the formation of a 15-HETE-like product from arachidonic acid and also metabolized linoleic acid efficiently to a 13-hydroxyoctadecadienoic acid (13-HODE)-like product (191, 332). These oxygenated metabolites migrated with the same R_f values as that of 15-HETE and 13-HODE standards on thin layer chromatography (332) but their identity to the mammalian 15-HETE and 13-

HODE can only be confirmed using gas chromatography / mass spectrometry. The cloning and expression of the parasite lipoxygenase homologue is required in order to produce large enough quantities of eicosanoids to allow for such measurements. In addition, cloning of the *S. mansoni* LOX-homologue would provide a useful probe to investigate its expression in the different developmental stages of the parasite and could also help in elucidating the exact role that eicosanoids may play in the host-parasite interactions.

During our investigation of the adult *S. mansoni* lipoxygenase pathway, we have detected LOX-specific immunoreactive proteins using antisera to different mammalian lipoxygenases (332). Furthermore, LOX-like sequences from *S. mansoni* genomic DNA were identified by hybridization to a human 15-LOX cDNA probe. These results combined with the significant sequence homology shared by plant and mammalian LOXs (143), suggested the possibility of utilizing mammalian probes to clone the parasite-LOX homologue. In the present study we have attempted to clone the putative *S. mansoni* lipoxygenase using different mammalian lipoxygenase cDNAs as probes. We also describe our efforts to obtain a *S. mansoni*-specific LOX-like partial sequence to assist in the cloning of the parasite lipoxygenase-homologue.

MATERIALS AND METHODS

Isolation of DNA and polyadenylated RNA from adult *Schistosoma mansoni* worms.

The life cycle of *S. mansoni* was maintained using CD.01 mice as the definitive host in the laboratory of Dr. James M. Smith (Institute of Parasitology, McGill University, Ste.-Anne de Bellevue, Quebec, Canada) as previously described (319). Adult worms were recovered by perfusion of the hepatoportal system 7-8 weeks post infection using ice-cold RPMI media (GIBCO) containing 0.85% NaCl and 0.75% sodium citrate, washed five times in sterile saline solution, visually examined by light microscopy for host cell contamination, and immediately frozen in liquid nitrogen. 300 mg of frozen adult worms were homogenized under liquid nitrogen (using a mortar and pestle) and genomic DNA was isolated using the Easy-DNA Isolation kit (Invitrogen, Co. San Diego, CA) according to the manufacturer's instructions. Total RNA was prepared from 500 mg of homogenized adult worms using guanidinium lysis (Total RNA Isolation kit, Invitrogen) and poly $(A)^+$ RNA was then isolated from total RNA or prepared directly from homogenized worms using oligo dT cellulose (Fast Track mRNA Isolation kit, Invitrogen) as per the manufacturer's directions.

Oligonucleotides and polymerase chain reaction (PCR).

To select oligonucleotides for PCR amplification of lipoxygenase-like sequences from adult *S. mansoni*, highly conserved regions of 8 lipoxygenases were identified by aligning the amino acid sequences of rabbit 15-lipoxygenase, bovine 12-lipoxygenase, pig 12-lipoxygenase, human 15-lipoxygenase, human 12-lipoxygenase, and lipoxygenases from the plants soybean , pea seed and rice (137, 143, 326) using the PILEUP program (Genetics Computing Group, Madison, WI) (Fig. 1). Based on these conserved regions among the different lipoxygenases, degenerate sense and antisense oligonucleotides were designed and chemically synthesized (Research Genetics, Huntsville, AL). The nucleotide

sequences of the degenerate primers, their positions within the human 15-lipoxygenase cDNA (137), and the expected sizes of PCR fragments amplified from human 15-LOX cDNA are summarized in Table 1 and Figure 2, respectively.

Reverse transcription-PCR was performed using Murine Leukemia Virus (MuLV) Reverse Transcriptase (Perkin Elmer Cetus, Norwalk, CT, USA) and 10 ng of adult *S. mansoni* poly $(A)^+$ RNA. Two rounds of PCR amplifications were used to amplify LOX-like sequences from the adult worm cDNA, or genomic DNA, while only one PCR reaction was performed when the different *S. mansoni* cDNA libraries (described below) were used as templates. The primary PCR reaction (GeneAmp DNA PCR kit, Perkin Elmer Cetus) was performed using Lox-4/Lox-7 primers (in case of amplification from genomic DNA), Lox-4/Lox-7, Lox-5/Lox-7, or Lox-5/Lox-11 primer sets (in case of amplification from RT-PCR-produced cDNA), and Lox-4/Lox-7 or Lox-5/Lox-7 primers (in case of amplification from cDNA libraries) in a buffer containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphates, 0.5 µM primers, 2.5 units/100 µl *Taq* polymerase, and 10 µl of cDNA, 40 ng of genomic DNA, or 1 µl of *S. mansoni* cDNA libraries as templates. Using a Perkin-Elmer thermal cycler, the PCR cycling conditions were: 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. The secondary PCR reaction was performed under the same conditions using 0.01 µl of the primary PCR reaction and the nested primers Lox-5 and Lox-7. Both primary and secondary PCR products were separated by electrophoresis in a 1.2% (w/v) agarose gel, visualized by ethidium bromide staining and blotted to Hybond-N^+ nylon membranes (Amersham Life Science, Oakville, Ontario) by overnight capillary transfer using 0.4 M NaOH.

Southern blot analysis of RT-PCR and PCR products.

Southern blot hybridization of *S. mansoni* RT-PCR and PCR products was performed at 65 °C for 16 h in a solution containing 5x SSPE (1x SSPE is 0.15 M

NaCl, 0.01 M NaH_2PO_4, 0.01 M EDTA, pH 7.4), 5x Denhardt's (1x Denhardt's solution is 0.02% (w/v) Ficoll, 0.02% (w/v) bovine serum albumin, 0.02% (w/v) polyvinyl pyrrolidone), 100 μg ml^{-1} denatured calf thymus DNA, 0.5% (w/v) SDS, and randomly primed (Boehringer Mannheim) ^{32}P-labeled human 15-lipoxygenase cDNA (a gift from Dr. E. Sigal, University of California, San Francisco, CA (137). The membranes were washed twice in 2x SSC (1x SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7), 0.1% (w/v) SDS at room temperature, then washed twice in 2x SSC, 0.1% SDS at 65°C for 30 min, and exposed to Kodak XAR-5 film (Eastman Kodak Co) for 1-5 days at -70°C.

Cloning and sequence analysis of *S. mansoni* RT-PCR and PCR products.

Several putative *S. mansoni* LOX-like RT-PCR and PCR fragments of ≈ 300-500 bp were identified by their hybridization to human 15-LOX cDNA, isolated on 1.9% agarose gel, and gel purified using the QIAquick Gel Extraction kit (Qiagen Inc, Santa Clarita, CA). PCR products amplified from *S. mansoni* genomic DNA were subcloned into pBlueScript II SK^+ vector (Stratagene, La Jolla, CA) and RT-PCR products were subcloned into the TA vector (Invitrogen). The ligation mixtures of PCR-generated fragments were transformed into DH5α *E.coli* (Gibco BRL) and the transformed colonies were plated, lifted, and screened (according to standard protocols (333)) using the [α-^{32}P] dCTP labeled (Boehringer-Mannheim, Germany) human 15-LOX cDNA. Plasmid DNA was then prepared from the positively-hybridized bacterial colonies using the Wizard plus kit (Promega, Madison, WI, USA) and inserts were characterized by restriction analysis and hybridization to the human 15-LOX cDNA. The PCR positive inserts were sequenced using the pBlueScript II SK^+ plasmid-based primers T3 and T7 and the Sequenase Version 2.0 sequencing kit (Amersham Corp.). RT-PCR positive inserts were sequenced using the TA vector-based M13 reverse primer and automated DNA sequencing on an Applied Biosystems model 386 utilizing T7 DNA polymerase and internal labeling with fluorescein-15-dATP (334). The BLASTN and BLASTX algorithms (National Centre for

Biotechnology Information) (335) and FASTA and TFASTA programs (the GCG program package) (336) were used for computer analysis of sequence data.

Screening of *S. mansoni* cDNA libraries.

Three cDNA libraries were obtained from different sources, an adult *S. mansoni* cDNA library in the bacteriophage λ vector gt11 (kindly provided by Dr. C.B. Shoemaker, Harvard School of Public Health, Boston, MA), a Schistosomulae cDNA library in the bacteriophage λ vector UNI-ZAP HR (a gift from Dr. James M. Smith, Institute of Parasitology, McGill University, Ste.-Anne de Bellevue, Quebec, Canada), and an adult *S. mansoni* cDNA library in the phagemid BA vector (a gift from Dr. S.D.J. Pena, Instituto de Ciências Biológicas, Belo Horizonte, Brazil (331)). Another two *S. mansoni* cDNA libraries were newly constructed from adult worm mRNA. One was bidirectional, size-selected for cDNAs of ≥ 500 bp, and custom made in the TripIEx lambdaphage vector (Clontech, Palo Alto, CA, USA), and the other was unidirectional, size-selected for cDNAs of ≥ 1000 bp and custom made in the pcDNA3.1(+) plasmid vector (Invitrogen). The probes used for screening were human 5-LOX cDNA (Merck Frosst, Canada), human 15-LOX cDNA (Oxford Biochemicals), human platelet 12-LOX and pig leukocyte 12-LOX cDNAs (Cayman Chemicals), degenerate oligonucleotide primer Lox-7 (Table 1), and *S. mansoni* clone # 15 (obtained by PCR amplification from adult worm genomic DNA). All probes were labeled by random priming using [α-^{32}P] dCTP and Klenow enzyme (labeling grade, Boehringer Mannheim) except for Lox-7 primer which was labeled with [γ-^{32}P] ATP and T4 polynucleotide kinase (Boehringer-Mannheim). Unincorporated nucleotides were removed using NucTrap probe purification columns (Stratagene).

Approximately 2 x 10^6 phage from *S. mansoni* cDNA libraries in the different bacteriophage λ vectors (λgt11, λUNI-ZAP HR, and λTripIEx) were plated, lifted using 0.45-mm nitrocellulose membranes (Schleicher & Schuell), and screened by hybridization (essentially as described in (337)) using the above

mentioned probes. The different probes used for the screening of each library and the host bacterial strains used for phage propagation and plating are summarized in Table 3. Plaque hybridization was performed in 50% deionized formamide, 0.1% SDS, 5x SSC, 5x Denhardt's solution, 100 μg ml^{-1} denatured calf thymus DNA (GIBCO), and 1-3 x 10^6 cpm/ml of [α-^{32}P]dCTP-labeled probes at 42°C. When [γ-^{32}P]ATP-labeled Lox-7 primer was used as a probe, hybridization was carried out in 6x SSC, 20 mM NaH_2PO_4, 0.4% SDS, 5x Denhardt's solution, and 500 μg ml^{-1} denatured calf thymus DNA at 42°C. After overnight hybridization, membranes were washed three times for 10 min each at room temperature in 2x SSC, 0.1% SDS, two times for 30 min each at 65°C in 1x SSC, 0.1% SDS, and exposed to X-OMAT AR film (Eastman Kodak Co) for 2-5 days at -70°C. Any putative positive plaques were rescreened twice using the same probe.

Screening of *S. mansoni* cDNA library in pcDNA3.1(+) by PCR.

A cDNA library in the pcDNA3.1(+) plasmid vector was custom made (Invitrogen) from 30 μg of adult *S. mansoni* poly $(A)^+$ RNA. First-strand cDNA synthesis was performed with a unique oligo dT (*Not*I) primer and cDNAs were cloned unidirectionally into the *BstX*I/*Eco*RI sites of the vector. The resulting cDNA library was titrated using standard protocols (333), and the titer was 9 x 10^{10} cfu/ml.

To reduce the complexity of colonies to be screened, the library was separated into 100 pools of 8000 cfu/pool and plated on 100 plates of LB agar containing 100 μg/ml ampicillin. Plates were scraped in LB and plasmid DNA was prepared from each pool using the Wizard plus kit (promega). To screen the pools for the presence of lipoxygenase-like sequences, PCR amplification using the lipoxygenase-degenerate primers Lox-5/Lox-7 and 1 μl of DNA from each pool was performed using the PCR Core kit (Boehringer-Mannheim) and a Perkin-Elmer thermal cycler. The PCR reactions were carried out using the same

buffer and cycling conditions described earlier. PCR products were separated on 1.5% (w/v) agarose gels, blotted to Hybond-N^+ nylon membranes, and hybridized with the [α-^{32}P]dCTP-labeled human 15-LOX cDNA probe. Membranes were washed twice for 30 min each under high stringency conditions in 0.5x SSC, 0.1% SDS at 65°C and exposed to films overnight at -70 °C.

Six positive pools were identified and three of them were chosen for further analysis. Each of the three positive pools was diluted into five pools of 4000 cfu/pool, plated, lifted, and screened using the human 15-LOX cDNA probe. Colony hybridization was performed in 40% deionized formamide, 0.5% SDS, 5x SSC, 5x Denhardt's solution, and 100 μg ml^{-1} denatured calf thymus DNA at 42 °C. Positive colonies were picked from each pool and diluted into 10 pools of 250 cfu/pool. The process of dilution, plating, and screening was repeated to isolate a single hybridizing colony from each pool. DNA was then prepared from the single colonies of pools # 18, 25, and 53 using Qiagen tip-500 (Qiagen) and restricted using the *Hind*III/*Xho*I sites (which flanked the *BstX*I/*Eco*RI sites of the vector in which the cDNAs were cloned) to release the inserts. The restricted DNA was electrophoresed through a 1% agarose gel, blotted, and the inserts were further characterized by their hybridization to the human 15-LOX cDNA probe. The positive inserts were then sequenced in both directions using the vector-based T7 primer as well as several other specific sense and antisense primers synthesized based on the insert sequence. Computer analysis of sequence data was performed using the BLASTN and TBLASTN algorithms (335) and FASTA and TFASTA programs (336) for nucleotide sequence searches of all available databases.

RESULTS AND DISCUSSION

PCR amplification of LOX-like sequences from adult *S. mansoni* RNA and cDNA libraries.

Our previous analysis of arachidonic acid metabolizing enzymes in adult *S. mansoni* parasites indicated that the parasites possess an active lipoxygenase pathway similar to mammalian 15-LOXs (332). Positive hybridization signals were observed following Southern hybridization of *S. mansoni* genomic DNA with the human 15-lipoxygenase cDNA which indicated that the homologous *S. mansoni* sequence could be amplified by the polymerase chain reaction (PCR) (332). To select oligonucleotide primers for amplification of the putative *S. mansoni* lipoxygenase, a multiple alignment of amino acid sequences of 8 different mammalian and plant lipoxygenases was made (Fig. 1). Degenerate sense and antisense primers were then designed and synthesized based on highly conserved regions identified from the LOXs multiple alignment (Table 1). The LOX-based degenerate primers were tested for their ability to amplify the right size PCR fragments from the human 15-LOX cDNA (Fig. 2). The primer sets chosen for both primary and secondary PCR (Fig. 2), were found to amplify fragments with the expected sizes from the human 15-LOX cDNA (results not shown).

In order to detect expressed parasite lipoxygenase-like sequences, primary PCR amplification of *S. mansoni* cDNA (obtained by reverse-transcription of the worm poly $(A)^+$ RNA, see Materials and Methods) was performed using the indicated primer pairs (Fig. 3) followed by a second round of amplification using the nested primer pair Lox-5/Lox-7. For PCR amplification from *S. mansoni* cDNA libraries, one round of amplification was performed using the indicated primer pairs (Fig. 3). After separation of the PCR products by agarose gel electrophoresis and Southern blot analysis with the human 15-LOX cDNA as a probe, several LOX-hybridizing PCR products were identified (Fig. 4). Two fragments of ≈ 320 and 344 bp(s) were amplified from *S. mansoni* mRNA

when the primer pair Lox-5/Lox-7 was used for primary amplification (Fig. 4, Panel A, lane 3) while only a 320-bp fragment was amplified when either Lox-4/Lox-7 or Lox-5/Lox-11 primers were used (Fig. 4, Panel A, lanes 2 and 4). These fragments were smaller in size than the 432-bp fragment expected to be amplified from the human 15-LOX cDNA using the same primer pair Lox-5/Lox-7 (Fig. 2). This may reflect a difference in the primary structure between the human 15-LOX (137) and the putative *S. mansoni* LOX-homologue. The degenerate oligonucleotides Lox-4/Lox-7 and Lox-5/Lox-7 also amplified a 344-bp fragment from three different *S. mansoni* cDNA libraries (Fig. 4, Panel B, lanes 2, 4, 6, and 7). The size of this 344-bp fragment was again smaller than the 654-bp or the 432-bp fragments expected to be amplified from human 15-LOX cDNA using the primer pairs Lox-4/Lox-7 and Lox-5/Lox-7, respectively (Fig. 2). The amplification of other PCR products smaller than 220 bp or larger than 500 bp (Fig. 4, Panel B, lanes 4, and 6, 7, respectively) is likely to be due to the degenerate nature of the primers which would allow them to anneal to non-lipoxygenase-like sequences resulting in amplification of PCR products that may hybridize non-specifically to the human 15-LOX cDNA probe.

Cloning and sequence analysis of *S. mansoni* RT-PCR and PCR products.

To obtain a *S. mansoni*-specific partial LOX-like sequence in order to facilitate the cloning of the putative parasite lipoxygenase homologue, RT-PCR products amplified from *S. mansoni* mRNA (Fig. 4, Panel A) were subcloned and analyzed by their hybridization to the human 15-LOX cDNA probe (see Materials and Methods for details). Ten RT-PCR clones with positive hybridization signals were subsequently sequenced, translated using the BLASTX algorithm (335), and their peptide sequences were compared to other proteins in the SwissProt database. All 10 clones had matches with certain protein entries from different organisms in the databases (Table 2) but none of them displayed any significant amino acid sequence identity to either mammalian or plant lipoxygenases. Clearly, the use of degenerate oligonucleotide primers led to the amplification of

non-lipoxygenase-specific sequences from *S. mansoni* mRNA which may have hybridized non-specifically to the human 15-LOX cDNA probe under the moderate stringency conditions used for both hybridization and washing (as described in Materials and Methods). On the other hand, analysis of the secondary *S. mansoni* RT-PCR by agarose gel electrophoresis revealed a smear of PCR products which indicated that a mixture of a large number of fragments was amplified. Thus, it is possible that lipoxygenase-specific fragments were also amplified from the parasite mRNA and hybridized specifically to the human 15-LOX cDNA but were not efficiently subcloned due to their small quantities in the PCR mixture compared to the non-LOX-specific fragments which might have been produced in larger quantities and were therefore preferentially subcloned.

Two PCR fragments amplified from *S. mansoni* genomic DNA with the LOX-based degenerate primers were previously identified as putative LOX-like sequences by their hybridization to the human 15-LOX cDNA (Chapter 4, ref. 332). Following the cloning and sequencing of 20 subclones derived from the PCR amplification of *S. mansoni* DNA (see Materials and Methods for details), the sequences were compared to other DNA sequences in the different databases by sequence homology comparisons using the BLASTN and TBLASTN algorithms (335) and FASTA and TFASTA programs (336). Several *S. mansoni* clones displayed significant DNA sequence homology to different cosmid clones derived from the nematode worm *Caenorhabditis elegans* (*C. elegans*) which may indicate a phylogenetic relatedness between the two worms (data not shown). Five clones had no significant database matches and may represent unidentified *S. mansoni* specific genes. Although none of the 20 *S. mansoni* DNA clones showed any significant nucleotide sequence homology to lipoxygenases, one clone (termed clone # 15), when translated, revealed a low degree of homology to the human 15-LOX protein. The deduced amino-acid sequence of *S. mansoni* clone # 15 displayed only 28% identity over a limited region of 55 amino acids in the human 15-LOX protein (data not shown).

The potential of *S. mansoni* PCR-amplified subclone # 15 as a probe.

Despite the low amino acid sequence homology between *S. mansoni* clone # 15 and human 15-LOX protein, the potential use of this clone as a useful probe to screen *S. mansoni* cDNA libraries for LOX-like sequences was examined. Southern hybridization of both human and *S. mansoni* genomic DNA with *S. mansoni* clone # 15 revealed strongly hybridizing bands of 8 and 4.3 kb in *Hind*III, 6.5, 4.3, and 2.3 kb in *Eco*RI, 6.5, 4.3, 3.3, and 2.3 kb in *Hind*III+*Eco*RI and 7, 4.3, and 2.7 kb in *Bam*HI-digested *S. mansoni* DNA samples (Fig. 5, Panel A, lanes 5-8). No hybridization to human DNA samples digested with the same restriction enzymes was seen (Fig. 5, Panel A, lanes 1-4). Lipoxygenase-like DNA hybridizing bands of 8.6, 6.4, and 7 kb were previously identified from *S. mansoni* DNA (restricted with the same enzymes) using the human 15-LOX cDNA as a probe (Chapter 4, Fig. 6). The inability of clone # 15 to hybridize to human genomic DNA indicated that the sequence of this clone is an authentic *S. mansoni* sequence with no apparent sequence similarity to any human gene.

Southern blot analysis of secondary *S. mansoni* RT-PCR products with clone # 15 as a probe revealed two hybridizing fragments of ≈ 400 and 432 bp amplified from *S. mansoni* mRNA using the primer sets Lox-5/Lox-11, Lox-5/Lox-7, and Lox-4/Lox-7 for primary amplification and the primer pair Lox-5/Lox-7 for secondary PCR (Fig. 5, Panel B, lane 2-4). These hybridizing fragments were slightly larger than the two fragments of ≈ 320 and 344 bp that were previously identified from secondary *S. mansoni* RT-PCR amplification with the same primer pairs when the human 15-LOX cDNA was used as a probe (Fig. 4, Panel A). Clone # 15 also hybridized to a 432 bp fragment amplified from a *S. mansoni* cDNA library in the λUNI-ZAP vector using the degenerate primer pair Lox-5/Lox-7 (Fig. 5, Panel C, lanes 2 and 3). The 432-bp fragment identified from both *S. mansoni* secondary RT-PCR and cDNA library amplifications using clone # 15 as a probe (Fig. 5, Panel B and C, respectively) had an identical size to the PCR fragment expected to be amplified from the human 15-LOX cDNA (Fig. 2). Taken together, these results indicated that clone # 15 was capable of detecting *S.*

mansoni sequences amplified using LOX-based primers and therefore suggested that this clone could be utilized as a probe to identify putative lipoxygenase-like sequences from *S. mansoni* cDNA libraries.

Screening of *S. mansoni* cDNA libraries.

Several lines of evidence suggested that mammalian lipoxygenase sequences might detect an evolutionary distant lipoxygenase homologue from *S. mansoni.* There is a high degree of sequence conservation among members of the lipoxygenase family (137, 143, 326); for example, human 5-, 12-, and 15-lipoxygenases share an overall 60% sequence similarity, and each of the human lipoxygenases is roughly 25% identical in sequence to plant lipoxygenases (143, 328). There is an even greater sequence conservation across species for specific LOXs; for example, human 5- and 15-LOXs are 92 and 81% identical to their respective rat and rabbit homologues (143). In addition, some recently-identified *S. mansoni* cDNA sequences displayed significant sequence homology to their mammalian counterparts (331). Furthermore, our immunoblot, Southern hybridization, and PCR analyses using reagents based on mammalian lipoxygenases, detected LOX-like sequences in *S. mansoni* (Chapter 4, ref. 332). Based on these lines of evidence, we speculated that mammalian lipoxygenase sequences could be used as useful probes to clone the putative *S. mansoni* LOX-homologue.

As summarized in Table 3, human 5-, 12- and 15-LOX cDNAs, pig 12-LOX cDNA, *S. mansoni* clone # 15, as well as the degenerate primer Lox-7 were all used as probes to screen several *S. mansoni* cDNA libraries by conventional hybridization screening (see Materials and Methods for details). Following secondary screening, no positive hybridization clones were identified from *S. mansoni* cDNA libraries in either λgt11, λUNI-ZAP or λTriplEx vectors using any of the indicated probes (Table 3). Both the λgt11 and the λUNI-ZAP libraries were old (synthesized in the early 1980s), poorly stored (as indicated by their very low titer of 5 X 10^4 pfu/ml), and amplified several times before we received

them. As a result, certain transcripts (especially the low-abundant, non-redundant ones) might have been lost. However, the absence of identifiable positive clones from the newly constructed, high titer (2 X 10^9 pfu/ml) λTriplEx cDNA library, could also be due to a relative low abundance of the putative *S. mansoni* lipoxygenase transcript in the library. The inability of the different probes to detect positive clones from the libraries might also be due to a lack of sufficient nucleotide sequence identity between these probes and the putative *S. mansoni* LOX-homologue.

PCR-screening of an adult *S. mansoni* cDNA library in pcDNA3.1(+).

Since the transcript for the putative *S. mansoni* LOX-homologue might have a low abundance in the different libraries and could therefore escape detection by conventional hybridization screening, we used PCR amplification to improve the representation of LOX-like sequences in one *S. mansoni* library. The newly constructed *S. mansoni* library in pcDNA3.1(+) vector (Table 3) was diluted into 100 pools to reduce its complexity, and PCR amplification using the LOX-degenerate primer pair Lox-5/Lox-7 was performed on DNA prepared from each pool (as described in Materials and Methods). Following the separation of PCR products by agarose gel electrophoresis and Southern blot analysis with the human 15-LOX cDNA, a 432 bp LOX-hybridizing fragment was detected in six pools (Fig. 6, lanes 2-7). Three of the hybridizing pools (pools # 18, 25, and 53) were further diluted and DNA was prepared from a single hybridizing colony from each pool followed by the characterization of the positive inserts using restriction analysis, hybridization to the human 15-LOX cDNA, and sequencing (for details see Materials and Methods).

The nucleotide sequence of a 2.5 kb positive insert isolated from pool # 53 was compared to other sequences in the databases using the BLASTN and TBLASTN algorithms (335) and FASTA and TFASTA programs (336). The insert displayed 100% nucleotide sequence identity to the mouse leukocyte-type 12-lipoxygenase mRNA (Fig. 7). The same result was seen with the positive inserts

isolated from pools # 18, and 25, respectively. This result indicated that we have cloned and sequenced the previously characterized mouse peritoneal macrophage 12-LOX (166).

The mouse 12-LOX transcript could have originated from mouse blood cells contaminating the adult worm mRNA preparation (from which the library was constructed), since the parasites were grown in mice as their definitive host (see Materials and Methods). Although the parasites were extensively washed and visually examined for host cell contamination by light microscopy, they can still harbor some mouse contaminating cells, since adult parasites living in the host blood as their final habitat are known to ingest some host blood cells during their life span (277). As a result, any genetic material prepared from adult parasites may contain some host contaminating sequences. In fact, the presence of minute amounts of contaminating murine sequences (≈ 0.2% of all transcripts) is a constant feature in all *S. mansoni* libraries prepared from adult worms grown in mice (331).

The cloning of the mouse 12-LOX using the human 15-LOX cDNA is not surprising since leukocyte-type 12-LOXs and 15-LOXs share up to 85% identity in their primary structure in higher mammals (134, 143). In fact, leukocyte 12-LOX and 15-LOX cDNAs have not yet been isolated within the same species and it is postulated that the leukocyte-type 12-LOX found in bovine tracheal epithelium, porcine leukocytes, and mouse macrophages is the species equivalent of the 15-LOX found in human reticulocytes, airway epithelium, and eosinophils (134, 170).

There were very few contaminating LOX-murine sequences in the libraries used in our study as judged by the failure of the different mammalian lipoxygenase probes to detect positive clones using conventional hybridization screening (Table 3). In addition, only 6 out of 100 pools from one library contained the contaminating mouse LOX-homologue which was only detected after extensive dilution of the library and the use of the powerful amplification of PCR techniques. Moreover, none of the 20 clones amplified from *S. mansoni* DNA displayed any significant sequence homology to murine sequences and

only 1 out of 10 translated RT-PCR clones showed some homology to a mouse peptide (Table 2). Taken together, these results suggest that the contaminating murine sequences should not present a problem in identifying *S. mansoni*-specific genes from these libraries if a specific probe that can differentiate between parasite and mammalian sequences is to be used.

Despite a small genome size of 270 Mb (comparable to the 100 Mb genome size of the genetically well-characterized nematode worm *C. elegans*), the structure and function of the schistosome genome are still largely unknown (277). Based on its genome size and evolutionary position (281, 338), *S. mansoni* is expected to contain roughly 20 000 expressed genes but little more than 100 cDNA sequences have been deposited in the databases and only a few dozen genes were characterized in details (GenBank, release 84.0) (331). Due to this obvious lack of genetic information, no partial *S. mansoni* lipoxygenase-like sequence (that could have facilitated the cloning of the parasite LOX-homologue) was identified from the databases. In contrast, more than 85% of the genome of the phylogenetically-related worm *C. elegans* has been sequenced as a part of the *C. elegans* Genome Sequencing Project (313). Based on its accessible genetic information (both *C. elegans* expressed sequence tags (ESTs) and DNA databases are available), we speculated that a lipoxygenase-like sequence from *C. elegans* could be identified and consequently used as a more specific probe to isolate the putative *S. mansoni* LOX-homologue.

The *C. elegans* DNA database was searched for arachidonic acid metabolizing enzymes-like sequences but no cyclooxygenase nor lipoxygenase-related sequences were found. On the other hand, an EST of 327 bp (termed cm01c7) with 51% sequence homology to the human leukotriene A_4 (LTA_4) hydrolase was identified (GenBank accession number M88793). In a previous study, adult *S. mansoni* parasites have been shown to produce a metabolite displaying the same high performance chromatographic properties as leukotriene B_4 (LTB_4) following incubation with radiolabeled linoleic acid (307). In addition, an immunoreactive protein with the same size as 5-LOX (the enzyme that produces LTA_4, the substrate for LTA_4 hydrolase) was identified from adult *S. mansoni*

extracts using antisera to mammalian LOXs (Chapter 4, ref. 332). Thus, we postulated that adult *S. mansoni* may possess an LTA_4 hydrolase activity leading to the production of the proinflammatory substance LTB_4 (3). Consequently, the complete sequence (0.95 kb) of the *C. elegans* LTA_4 hydrolase-like EST was obtained (for details refer to Chapter 6), radiolabeled, and used as a probe to screen *S. mansoni* cDNA libraries in λUNI-ZAP and λTriplEx vectors. No positive plaques were identified from the *S. mansoni* libraries by conventional hybridization screening with the *C. elegans* LTA_4 hydrolase-like sequence. Furthermore, oligonucleotide primers synthesized based on conserved sequences between mammalian LTA_4 hydrolases and the *C. elegans* LTA_4 hydrolase-like homologue (see Chapter 6, Fig. 2), did not amplify any PCR fragments from either *S. mansoni* mRNA or cDNA libraries (data not shown). These results could either suggest that the *C. elegans* sequence may not share a high degree of homology to a putative *S. mansoni* LTA_4 hydrolase-like homologue, or such a homologue does not exist in adult *S. mansoni* parasites.

The recent adaptation of the approaches being used in the Human Genome Project for the study of the schistosome genome (331, 339) will lead to rapid identification of new *S. mansoni* genes in the near future. The identification of a partial *S. mansoni* sequence homologous to any of the arachidonic acid-metabolizing enzymes would provide an ideal probe to clone such enzymes from adult parasites. Until these enzymes are cloned and characterized, their existence in *S. mansoni*, the role they might play in the host parasite relationship, as well as their possible use as future targets for chemotherapy and immunoprophylaxis will remain highly speculative.

In conclusion, our use of mammalian lipoxygenase sequences as probes to clone the putative *S. mansoni* LOX-homologue resulted in the cloning of the mouse (the parasite host) 12-LOX homologue due to the presence of some host contaminating sequences in all *S. mansoni* libraries and the significant sequence identity shared by mammalian LOXs. Our results indicate the problems associated with using mammalian cDNA sequences to isolate genes from mammalian parasites and strongly suggest that the cloning of the putative *S.*

mansoni LOX-homologue will have to await the future identification of a LOX-like partial sequence from *S. mansoni*.

FIGURE LEGENDS

Figure 1. Amino acid sequence alignment of selected regions of mammalian and plant lipoxygenases. A multiple alignment of amino acid sequences of rabbit reticulocyte 15-lipoxygenase (Rab15-LOX), bovine tracheal epithelium 12-lipoxygenase (Bov12-LOX), porcine leukocyte 12-lipoxygenase (Pig12-LOX), human reticulocyte 15-lipoxygenase (Hum15-LOX), human platelet 12-lipoxygenase (Hum12-LOX), soybean lipoxygenase-2 (Soy-LOX), pea seed lipoxygenase (Pea-LOX), and rice lipoxygenase (Rice-LOX) was made using the PILEUP function of the GCG program (336). Amino acid sequences are shown in the one-letter code. Regions containing identical amino acid (boxed residues on a gray background) were selected for the synthesis of LOX-based degenerate oligonucleotide primers. The polarity of the different primers and their amino acid positions in the human 15-LOX protein are indicated above the aligned amino acid sequences. The sense and antisense directions of the primers are represented by forward and backward arrows, respectively.

Figure 2. Map of the human 15-Lipoxygenase protein and the different PCR fragments. (A) A map of the human 15-LOX protein indicating both the length of the protein and the amino acid positions of the LOX-based degenerate primers. Sense and antisense oligonucleotides are indicated by open and dashed boxes, respectively. The numbers below and beside the boxes represent amino acid numbers in the human 15-LOX protein. **(B)** A map representing the different PCR products expected to be amplified when the indicated sets of degenerate oligonucleotide pairs are used. The expected sizes of primary and secondary PCR products when human 15-LOX is used as a template are indicated in base pairs (bp).

Figure 3. Summary of PCR amplification from *S. mansoni* RNA and cDNA libraries. cDNA was either prepared from *S. mansoni* RNA by reverse-transcription or obtained from the different *S. mansoni* cDNA libraries (in λ UNI-

ZAP, λ gt11, and BA phagemid) as described in Materials and Methods. The primer pairs Lox-4/Lox-7, Lox-5/Lox-7, or Lox-5/Lox-11 were used for a primary PCR reaction utilizing 10 µl of the worm cDNA as a template, followed by a second round of PCR amplification using Lox-5/Lox-7 primers and 0.01 µl of the primary PCR reaction. For amplification from cDNA libraries, only one round of PCR amplification was performed using 1 µl of the different *S. mansoni* cDNA libraries and the primer pairs Lox-4/Lox-7 or Lox-5/Lox-7. Both primary and secondary PCR amplification products were Southern-blotted and probed with ^{32}P-labeled human 15-lipoxygenase cDNA.

Figure 4. Detection of 15-LOX hybridizing PCR fragments amplified from adult *S. mansoni* RNA. (A) Analysis of RT-PCR-amplified products from *S. mansoni* mRNA using primers based on conserved lipoxygenase sequences. *S. mansoni* primary RT-PCR reaction (0.01 µl) was used as a template for a second round of PCR amplification with the degenerate primers Lox-5/Lox-7. Secondary PCR-amplification products were electrophoresed on a 1.2% agarose gel, capillary blotted onto nylon membrane and probed with ^{32}P-random primer-labeled human 15-lipoxygenase cDNA probe. The positions of DNA size markers are indicated (in bp). Lane 1, 10 µl of the negative control for the secondary PCR reaction using no added template; lane 2, 10 µl of secondary PCR reaction using primary RT-PCR reaction amplified by Lox-4/Lox-7 primers as a template; lane 3, 10 µl of secondary PCR reaction using primary RT-PCR reaction amplified by Lox-5/Lox-7 primers as a template; lane 4, secondary PCR reaction using primary RT-PCR reaction amplified by Lox-5/Lox-11 primers as a template. **(B)** Analysis of PCR-amplified products from *S. mansoni* cDNA libraries. *S. mansoni* cDNA libraries (1µl each) were used as templates for PCR amplification using the degenerate primers Lox-5/Lox-7 or Lox-4/Lox-7. PCR amplification products were analyzed as described earlier using human 15-LOX cDNA as a probe. The positions of DNA size markers are indicated (in bp). Lane 1, 3, and 5, 10 µl of the negative control for the PCR reaction using no added template; lane 2, 10 µl of

PCR reaction using λ UNI-ZAP cDNA library as a template and primers Lox-5/Lox-7; lane 4, 10 µl of PCR reaction using λ gt11 cDNA library as a template and primers Lox-4/Lox-7; 10 µl of PCR reaction using BA phagemid cDNA library as a template and primers Lox-4/Lox-7 (lane 6); or primers Lox-5/Lox-7 (lane 7).

Figure 5. The potential of *S. mansoni* PCR-amplified subclone 15 as a probe. *S. mansoni* clone # 15 was PCR-amplified from *S. mansoni* genomic DNA (40 ng) using degenerate primers based on conserved lipoxygenase sequences as described in Material and Methods. **(A)** Southern hybridization of ^{32}P-labeled *S. mansoni* clone 15 with human (lanes 1-4) and *S. mansoni* genomic DNA (lanes 5-8). The lanes contain 10 µg human DNA or 10 µg *S. mansoni* DNA restricted with *Hind*III (lanes 1 and 5), *Eco*RI (lanes 2 and 6), *Hind*III and *Eco*RI (lane 3 and 7), and *Bam*HI (lanes 4 and 8). Fragment sizes of *Hind*III-cleaved lambda DNA are shown in kilobases (kb). **(B)** ^{32}P-labeled *S. mansoni* clone # 15 was tested as a potential probe to detect LOX-like sequence by hybridization to Southern-blotted secondary *S. mansoni* RT-PCR products amplified using degenerate primers Lox-5/Lox-7. The positions of DNA size markers are indicated (in bp). Lane 1, 10 µl of the negative control for the secondary PCR reaction using no added template; lane 2, 10 µl of secondary PCR reaction using primary RT-PCR reaction amplified by Lox-5/Lox-11 primers as a template; lane 3, 10 µl of secondary PCR reaction using primary RT-PCR reaction amplified by Lox-5/Lox-7 primers as a template; lane 4, secondary PCR reaction using primary RT-PCR reaction amplified by Lox-4/Lox-7 primers as a template. **(C)** Hybridization of ^{32}P-labeled *S. mansoni* clone # 15 to Southern-blotted PCR product(s) amplified from *S. mansoni* λ UNI-ZAP cDNA library. The positions of DNA size markers are indicated (in bp). Lane 1, 10 µl of the negative control for the PCR reaction using no added template; lanes 2 and 3, 10 µl of PCR reaction using λ UNI-ZAP cDNA library as a template and primers Lox-5/Lox-7.

Figure 6. Detection of 15-LOX-hybridizing PCR fragments amplified from separate pools of an adult *S. mansoni* cDNA library in pcDNA3.1(+). The *S. mansoni* cDNA library in the plasmid vector pcDNA3.1(+) was separated into 100 pools of 8000 cfu/pool and plasmid DNA was prepared from each pool as described in Materials and Methods. 1 µl of DNA from each pool was used as a template for PCR amplification with degenerate primers Lox-5/Lox-7. Amplification products were electrophoresed on a 1.5% agarose gel, capillary blotted onto nylon membrane and probed with ^{32}P-random primer-labeled human 15-lipoxygenase cDNA probe. The positions of DNA size markers are indicated (in bp). Lane 1, 10 µl of the negative control for the PCR reaction using no added template; lanes 2-7, 10 µl of the PCR reaction using DNA prepared from pools # 10, 12, 18, 20, 25, and 54, respectively, as a template.

Figure 7. Sequence analysis of *S. mansoni* 15-lipoxygenase-hybridizing pools. DNAs from *S. mansoni* 15-LOX-hybridizing pools # 18, 25, and 53 were prepared and the positive inserts were sequenced in both directions as described in Materials and Methods. Computer analysis of sequence data was performed using the BLASTN and TBLASTN algorithms (335) and the FASTA and TFASTA programs (336) for nucleotide sequence searches of all available databases. The top sequence represents the sequence of *S. mansoni* pool # 53 and the bottom sequence represents the sequence of the mouse leukocyte-type 12-lipoxygenase mRNA.

Table 1. Nucleotide sequences of synthetic oligonucleotide primers used in RT-PCR and PCR amplification of lipoxygenase-like sequences from adult *S. mansoni*. Degenerate sense and antisense primers were designed and chemically synthesized based on conserved sequences in mammalian and plant lipoxygenases as described in Materials and Methods. Letters enclosed in parentheses indicate degeneracy at that position. I indicates inosine residues. The length of the primers in nucleotides and their corresponding amino acid positions in the human 15-lipoxygenase protein are also indicated.

Table 2. Amino acid sequence identity between *S. mansoni* RT-PCR clones and different protein entries in the SwissProt database. BLASTX algorithm (335) was used to translate both strands of 10 *S. mansoni* RT-PCR clones in all 6 reading frames and the translated peptides were compared to other protein entries in the SwissProt database (release 96). Database protein matches with best amino acid sequence alignment to each *S. mansoni* clone are listed. Percent identity represents the percentage of identical amino acids over the length of each alignment.

[a] The number of identical amino acid residues in each *S. mansoni* clone over the total number of compared amino acids in each of the database proteins.

Table 3. *S. mansoni* cDNA libraries and the probes used for their screening. The different origins, parasite sources, and bacterial host strains for four *S. mansoni* cDNA libraries (three in the bacteriophage λ vectors gt11, UNI-ZAP HR, and TripIEx , and one in the plasmid vector pcDNA3.1(+)) are summarized. A list of the different probes used to screen each library for LOX-like sequences (as described in Materials and Methods) is also indicated.

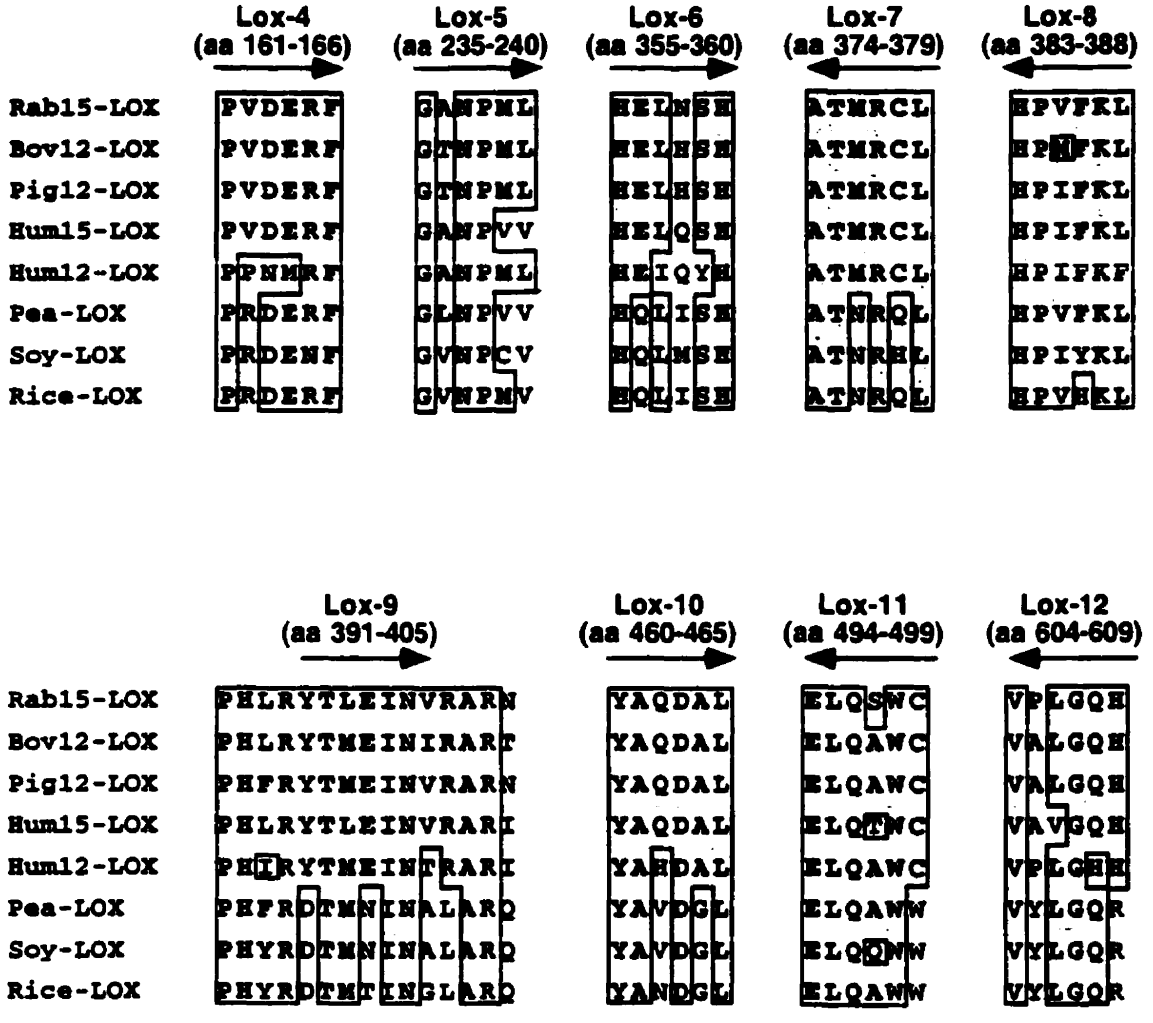
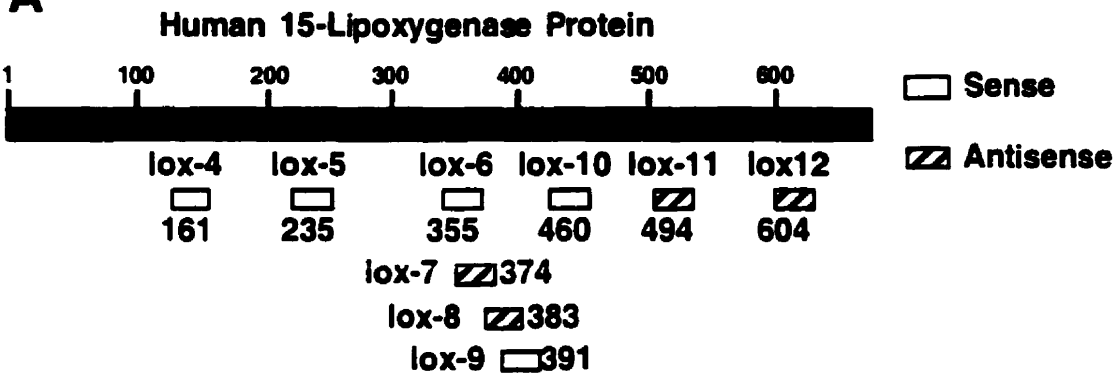
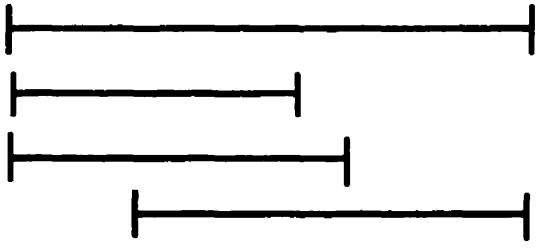

Lox-4
(aa 161-166)
Lox-5
(aa 235-240)
Lox-6
(aa 355-360)
Lox-7
(aa 374-379)
Lox-8
(aa 383-388)
Rab15-LOX
Bov12-LOX
Pig12-LOX
Hum15-LOX
Hum12-LOX
Pea-LOX
Soy-LOX
Rice-LOX
Lox-9
(aa 391-405)
Lox-10
(aa 460-465)
Lox-11
(aa 494-499)
Lox-12
(aa 604-609)

Figure 1

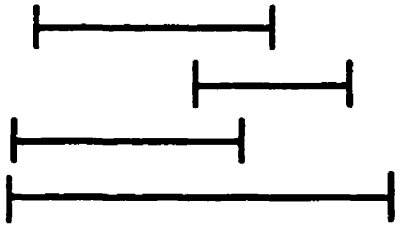
A



B



Primary PCR products	
Primers	size
lox-4, lox-11	1014 b.p
lox-4, lox-7	654 b.p
lox-4, lox8	681 b.p
lox-5, lox-11	792 b.p



Secondary PCR products	
Primers	size
lox-5, lox-7	432 b.p
lox-9, lox-11	324 b.p
lox-5, lox-8	459 b.p
lox-5, lox-11	792 b.p

Figure 2

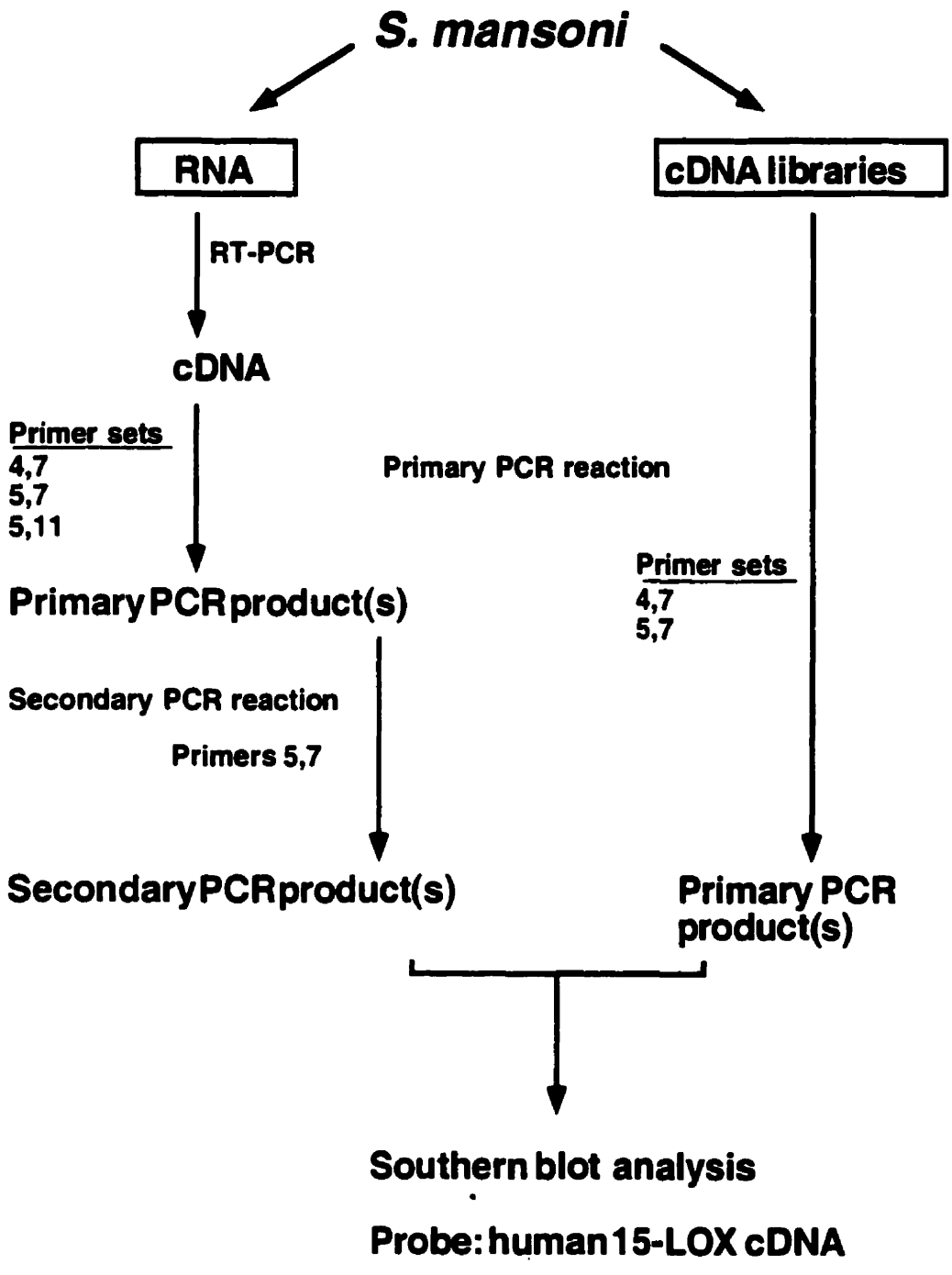
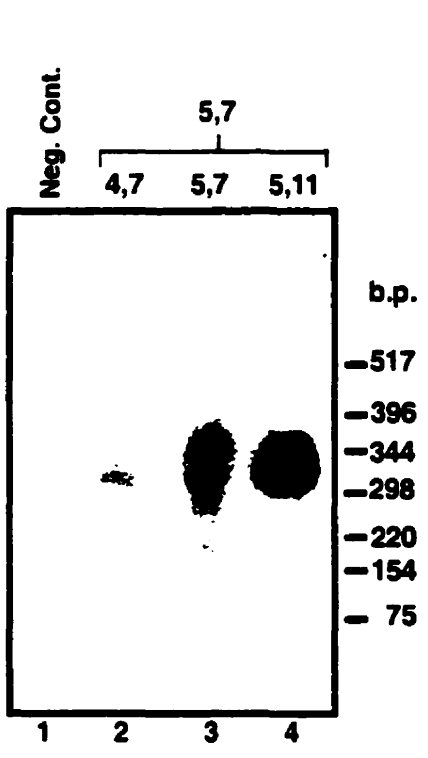

S. mansoni
RNA
cDNA libraries
RT-PCR
cDNA
Primer sets
4,7
5,7
5,11
Primary PCR reaction
Primer sets
4,7
5,7
Primary PCR product(s)
Secondary PCR reaction
Primers 5,7
Secondary PCR product(s)
Primary PCR product(s)
Southern blot analysis
Probe: human 15-LOX cDNA

Figure 3

A

mRNA

Secondary PCR amplification of primary S. mansoni RT-PCR products

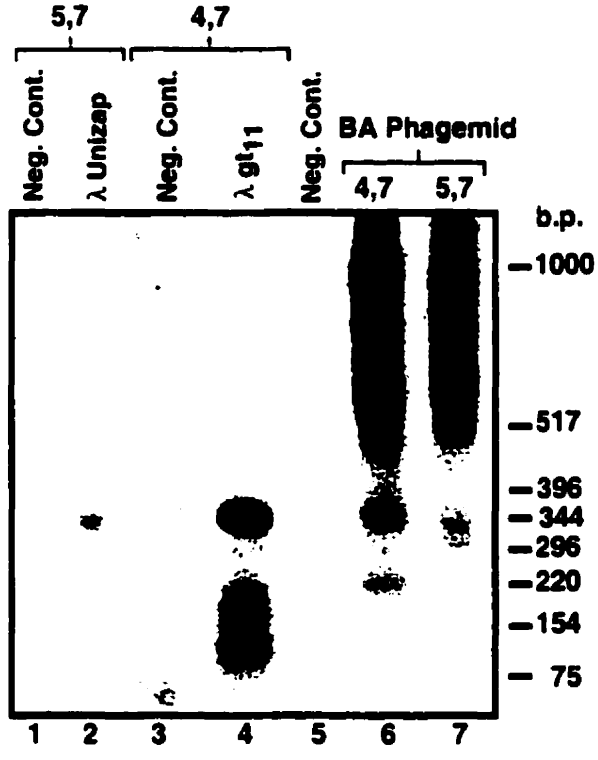


Exposure: 48 hrs at -70°C

B

Libraries

Primary PCR amplification of S. mansoni cDNA libraries



Exposure: 48 hrs at -70°C

Figure 4

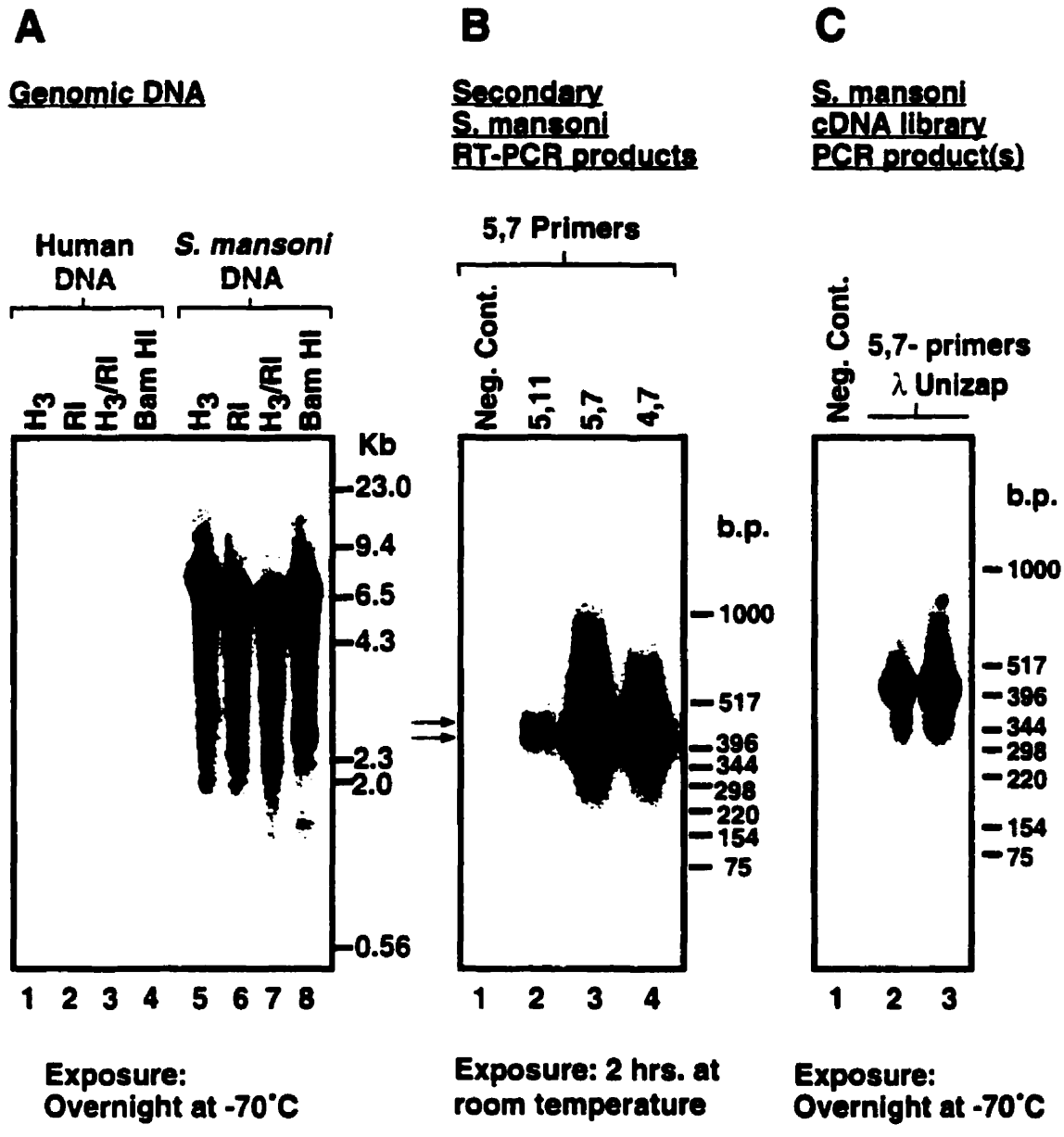

A
Genomic DNA
Human DNA
S. mansoni DNA
H3
RI
H3/RI
Bam HI
Kb
23.0
9.4
6.5
4.3
2.3
2.0
0.56
1 2 3 4 5 6 7 8
Exposure: Overnight at -70°C
B
Secondary S. mansoni RT-PCR products
5,7 Primers
Neg. Cont.
5,11
5,7
4,7
b.p.
1000
517
396
344
298
220
154
75
1 2 3 4
Exposure: 2 hrs. at room temperature
C
S. mansoni cDNA library PCR product(s)
Neg. Cont.
5,7- primers
λ Unizap
b.p.
1000
517
396
344
298
220
154
75
1 2 3
Exposure: Overnight at -70°C

Figure 5

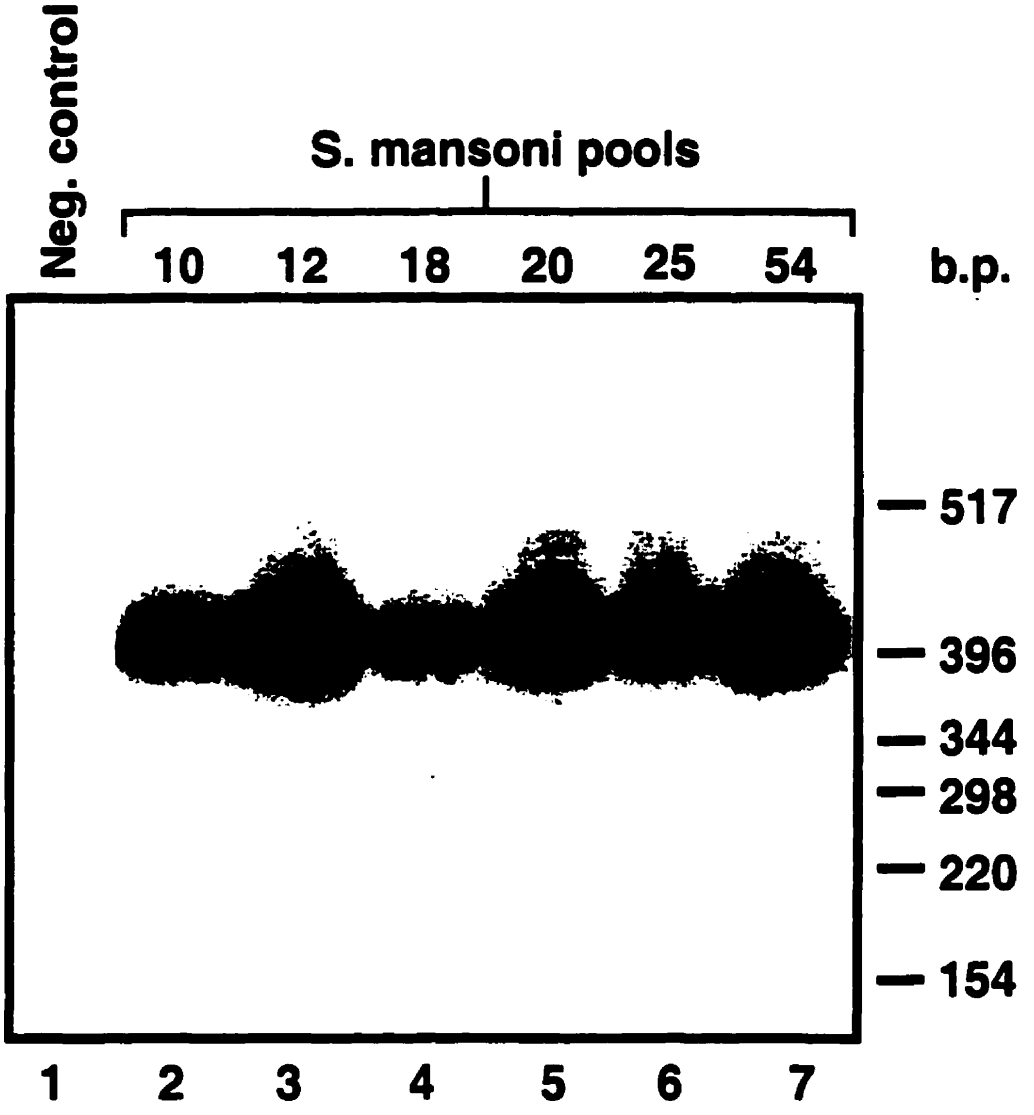

Neg. control
S. mansoni pools
10
12
18
20
25
54
b.p.
517
396
344
298
220
154
1
2
3
4
5
6
7

Figure 6

```
gb|U04331|MMU04331 Mus musculus C57BL/6 and ICR leukocyte-type
          12-lipoxygenase mRNA, complete cds.
          Length = 1992

  Plus Strand HSPs:

 Score = 1855 (512.6 bits), Expect = 1.7e-254, Sum P(3) = 1.7e-254
 Identities = 371/371 (100%), Positives = 371/371 (100%), Strand = Plus / Plus

Query:    52 GGGCCCCGGAGACCAGGGATCGGAGTACACGTTCCCCTGTTACCGATGGGTTCAGGGCAC 111
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   252 GGGCCCCGGAGACCAGGGATCGGAGTACACGTTCCCCTGTTACCGATGGGTTCAGGGCAC 311

Query:   112 CAGCATCCTGAACCTCCCTGAGGGCACTGGCTGCACCGTGGTTGAAGACTCTCAAGGCCT 171
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   312 CAGCATCCTGAACCTCCCTGAGGGCACTGGCTGCACCGTGGTTGAAGACTCTCAAGGCCT 371

Query:   172 GTTCAGGAACCACAGGGAGGAGGAACTGGAAGAAAGGAGGAGTCTGTACAGGTGGGGCAA 231
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   372 GTTCAGGAACCACAGGGAGGAGGAACTGGAAGAAAGGAGGAGTCTGTACAGGTGGGGCAA 431

Query:   232 CTGGAAGGATGGCACAATCCTGAACGTGGCGGCGACCAGTATCTCTGACCTCCCTGTAGA 291
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   432 CTGGAAGGATGGCACAATCCTGAACGTGGCGGCGACCAGTATCTCTGACCTCCCTGTAGA 491

Query:   292 CCAGCGATTTCGAGAGGACAAAAGACTTGAATTTGAAGCTTCACAGGTTCTGGGGACAAT 351
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   492 CCAGCGATTTCGAGAGGACAAAAGACTTGAATTTGAAGCTTCACAGGTTCTGGGGACAAT 551

Query:   352 GGACACCGTTATTAACTTCCCTAAAAACACTGTGACCTGCTGGAAAAGCCTAGATGACTT 411
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   552 GGACACCGTTATTAACTTCCCTAAAAACACTGTGACCTGCTGGAAAAGCCTAGATGACTT 611

Query:   412 CAACTATGTTT 422
             ||||||||||| 
Sbjct:   612 CAACTATGTTT 622

 Score = 791 (218.6 bits), Expect = 1.7e-254, Sum P(3) = 1.7e-254
 Identities = 159/160 (99%), Positives = 159/160 (99%), Strand = Plus / Plus

Query:   420 TTTCAAGAGTGGCCACACCAAGATGGCTGAGCGGGTTCGAAACTCCTGGAAAGAGGATGC 479
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   621 TTTCAAGAGTGGCCACACCAAGATGGCTGAGCGGGTTCGAAACTCCTGGAAAGAGGATGC 680

Query:   480 TTTCTTTGGGTACCAGTTTCTCAATGGTGCTAACCCCATGGTGCTGAAGCGGTCTACTTG 539
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   681 TTTCTTTGGGTACCAGTTTCTCAATGGTGCTAACCCCATGGTGCTGAAGCGGTCTACTTG 740

Query:   540 TCTCCCTGCCCGCCTGGTATTCCCTCCGGGGATGGAGGAG 579
             ||||||||||||||||||||||||||||||||||||| ||
Sbjct:   741 TCTCCCTGCCCGCCTGGTATTCCCTCCGGGGATGGAGAAG 780
```

Figure 7

Primer name	Length (nt)	Nucleotide Sequence	Amino acid position in human 15-LOX protein
LOX-4	17	5' CC[AT]-[gC][gT][Ag]-gAT-gAg-[AC]gA-TT 3'	161
LOX-5	17	5' gg[TC]-[gA][CT][CA]-AAC-CCC-[gA]Tg-IT 3'	235
LOX-7	17	5' Ag[gC]-[CT][Ag][CT]-C[Tg][Cg]-[AT]Tg-gTg-gC 3'	374
LOX-8	17	5' AgC-TT[gA]-[AT]A[gC]-A[TC]I-gg[gA]-Tg 3'	383
LOX-9	21	5' CCI-CAC-[TC][TA][Cg]-CgI-[Tg]AC-ACC-ATg 3'	391
LOX-11	17	5' CAC-CA[gC]-g[CT][TC]-Tg[gC]-AgC-TC 3'	494

Table 1

Clone Number	Database proteins with best sequence alignment	% identity	Number of identical amino acids
Clone 1	protein tyrosine phosphatase epsilon cytoplasmic	100%	17/17
Clone 2	extensin-Volvox carteri (Fragment)	40%	15/37
Clone 3	proline-rich protein-Mouse (Fragment)	53%	7/13
Clone 4	eye development protein canoe-Drosophila melanogaster	75%	6/8
Clone 5	Xylose isomerase precursor-Streptomyces rochei	56%	9/16
Clone 6	Leukocyte antigen-related protein precursor-Human	43%	10/23
Clone 7	Twitchin-Caenorhabditis elegans	33%	6/18
Clone 8	DR pLA protein-Human	29%	10/34
Clone 9	Human T-cell leukemia virus type 1	43%	7/16
Clone 10	FdoG protein-Escherichia coli	53%	8/15

Table 2

Library	Parasite Source	Origin	E. coli Host Strain	Probes Used for Screening	Clones Identified
λgt11cDNA Library	Adult parasites	Dr. J. Smith	Y1090	Human 15-LOX cDNA-primer Lox-7-Human 5- and 12-LOX cDNAs-pig 12-LOX cDNA	None
λUNI-ZAP HR cDNA Library	Schistosomulae	Dr. C.B. Shoemaker	XL-1 blue	S. mansoni clone #15-primer Lox-7-Human 15-LOX cDNA	None
λTriplEx cDNA Library	Adult parasites	Custom made (Clontech)	XL-1 blue	S. mansoni clone #15-Human 15-LOX cDNA-5- and 12-LOX cDNAs-primer Lox-7-pig 12-LOX cDNA	None
Phagemid cDNA Library in pcDNA 3.1 (+)	Adult parasites	Custom made (Invitrogen)	Top 10	Human 15-LOX cDNA	6

Table 3

6. MANUSCRIPT C

Molecular Cloning and Functional Expression of a *Caenorhabditis elegans* Aminopeptidase Structurally Related to Mammalian Leukotriene A_4 Hydrolases

Journal of Biological Chemistry 273, 27978-27987 (1998)

In the previous manuscript we described the identification of a small expressed sequence tag (EST) from *C. elegans* with strong homology to the human LTA_4 hydrolase which failed to recognize a homologous sequence from *S. mansoni* libraries. In this manuscript, we used this EST as a probe to clone the full length LTA_4 hydrolase-like homologue from *C. elegans*. Since mammalian LTA_4 hydrolases are bifunctional enzymes exhibiting both LTA_4 hydrolase and aminopeptidase activities, the *C. elegans* LTA_4 hydrolase-like cDNA was expressed in mammalian cells and tested for both activities. This paper presents evidence that the *C. elegans* enzyme functions as an aminopeptidase with broad substrate specificity but lacks any LTA_4 hydrolase activity despite the strong homology it shares with other mammalian LTA_4 hydrolases.

MOLECULAR CLONING AND FUNCTIONAL EXPRESSION OF A *CAENORHABDITIS ELEGANS* AMINOPEPTIDASE STRUCTURALLY RELATED TO MAMMALIAN LEUKOTRIENE A_4 HYDROLASES

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ABBREVIATIONS

LTA_4	leukotrieneA_4;(5*S*)-*trans*-5,6-*oxido*-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid
LTA_4 hydrolase	leukotriene A_4 hydrolase
LTB_4	leukotriene B_4; ((5*S*,12*R*)-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid
AP	aminopeptidase
5,6-diHETE	5,6-dihydroxyeicosatetraenoic acid
HPLC	high pressure liquid chromatography
EST	expressed sequence tag
PCR	polymerase chain reaction
kb	kilobase(s)
bp	base pair(s)

Summary

In a search of the *Caenorhabditis elegans* DNA database, an expressed sequence tag of 327 base pairs (termed cm01c7) with strong homology to the human leukotriene A_4 (LTA_4) 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 *C. elegans* LTA_4 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 LTA_4 hydrolase and aminopeptidase activities. Despite the strong homology between the human LTA_4 hydrolase and *C. elegans* AP-1(63 % similarity and 45 % identity at the amino acid level), reverse-phase high pressure liquid chromatography and radioimmunoassay for LTB_4 production revealed the inability of the *C. elegans* AP-1 to use LTA_4 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 LTA_4 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 IC_{50} of 2.6 ± 1.2 μM. The bifunctionality of the mammalian LTA_4 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 LTA_4 hydrolase activity in more evolved organisms.

INTRODUCTION

Leukotriene A_4 (LTA_4) hydrolase (EC 3.3.2.6) is the rate-limiting enzyme in the lipoxygenase cascade of arachidonic acid metabolism leading to the biosynthesis of the proinflammatory substance leukotriene B_4 (LTB_4) from the epoxide intermediate LTA_4 (19, 261). At nanomolar concentrations, LTB_4 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, LTB_4 is regarded as an important chemical mediator in a variety of inflammatory diseases (263). Sequence comparison of LTA_4 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 (240-242). Further studies verified that LTA_4 hydrolase contained one catalytic zinc atom coordinated by His^{295}, His^{299}, and Glu^{318} (243). Subsequently, the enzyme was shown to exhibit a previously unknown zinc-dependant peptidase/amidase activity toward synthetic substrates (244, 245) that was specifically stimulated by monovalent anions, *e.g.*, chloride ions (246), and also by albumin (247). Although a physiological peptide substrate for the aminopeptidase activity of the enzyme has not yet been found, LTA_4 hydrolase has been shown to efficiently hydrolyze several arginyl tri- and dipeptides, leading to its identification as an arginine aminopeptidase (248). Both the aminopeptidase and the LTA_4 hydrolase activity of the enzyme are inhibited by the aminopeptidase inhibitor bestatin (245) and the angiotensin converting enzyme inhibitor captopril (249), suggesting that the active sites corresponding to the two activities are overlapping (340). Important questions regarding the dual activity of the mammalian LTA_4 hydrolase/aminopeptidase remain unanswered. For example, does the enzyme demonstrate both LTA_4 hydrolase and aminopeptidase activities in other species? Which function originated first in evolution? What is the significance of this bifunctionality? LTA_4 hydrolase/aminopeptidase is a soluble monomeric protein ($M_r \approx 69{,}000$) (341, 342) that has been cloned from human (236), mouse (237), rat (238), and guinea pig (239). Recently a partial sequence from the slime

mold *Dictyostelium discoideum* and a gene from the yeast *Saccharomyces cerevisiae* (260) have been deposited into the GenBank™ data base as putative LTA_4 hydrolases (accession numbers U27538 and X94547, respectively). Both sequences encode proteins similar in their primary amino acid sequences to the mammalian LTA_4 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 LTA_4 hydrolase exhibited mainly aminopeptidase activity, whereas its hydrolase activity converted the majority of the substrate LTA_4 to what has been putatively identified as 5,6-diHETE (a much less potent leukotriene) rather than LTB_4 (355). 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 LTA_4 hydrolase, exhibits no LTA_4 hydrolase activity and only aminopeptidase activity. The strong homology between *C. elegans* AP-1 and mammalian LTA_4 hydrolases (45%) suggests that these enzymes may have developed from a common ancestral precursor.

MATERIALS AND METHODS

Cloning of a LTA_4 Hydrolase-like cDNA Homologue from *C. elegans*.

The cm01c7 phage clone from a *C. elegans* mixed stage hermaphrodite cDNA library (made by Chris Martin) containing the LTA_4 hydrolase-like EST in SHLX2 λ phage vector (343) was obtained from Dr. R. H. Waterston (344). MC1061 $recA^-$ tet^R (used for plating λ SHLX2) and the pop-out *Escherichia coli* cam^R Kan^R 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 (333). Five colonies were picked and plasmid DNA was prepared using either the Wizard plus kit (Promega, Madison, WI) or Qiagen tip-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 plasmid was then sequenced using T7 and SP6 primers and automated DNA sequencing on an Applied Biosystems model 386 DNA sequencer utilizing T7 DNA polymerase and internal labeling with fluorescein-15-dATP (334).

Approximately 2×10^6 phage from a mixed stage *C. elegans* cDNA library in bacteriophage λ vector UNI-ZAP XR (Stratagene) were plated and screened by hybridization as previously described (337) using the [α-^{32}P]dCTP-labeled (Boehringer-Mannheim) 0.95-kb *ApaI/ScaI C. elegans* fragment obtained from the cm01c7 clone as a probe. Hybridization was performed in 50% deionized formamide, 0.1% SDS, 5x SSC, 5x 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 2x SSC, 0.1% SDS, two times for 30 min each at 65 °C in 1x 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 pBluescript SK phagemid-based primers T3 and T7. Five positive plaques were isolated and confirmed to be related to the cm01c7 EST by PCR using *C. elegans* cm01c7 EST-based primers. Following *in vivo* excision of the pBluescript phagemid from the UNI-ZAP vector, plasmid DNA was prepared, and the positive inserts were sequenced.

The longest LTA_4 hydrolase-like clone obtained, termed C5 (≈ 1.4 kb), lacked the 5'-end of the coding region (as predicted from the size of cDNA of all previously cloned LTA_4 hydrolases). To isolate the 5'-end of the *C. elegans* cDNA, several anchored PCR amplifications using the phagemid-based primers T3, SK, and pSK (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 $MgCl_2$, 0.2 mM deoxynucleotide triphosphates, 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 M NaOH. The PCR-amplified fragments were screened by overnight hybridization using the nested [γ-^{32}P]ATP-labeled primer C5T3 (5'-CCA GAC GGC GCA TCT TTC GCT-3') (based on the most 5' sequence in clone C5) and T4 polynucleotide kinase (Boehringer-Mannheim) in 6x SSC, 20 mM NaH_2PO_4, 0.4% SDS, 5x Denhardt's solution, 500 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 Qiaquick 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 inserts 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 *Eco*RI restriction site at the 5'-end of clone C5 and the 3'-end of the PCR-amplified NH_2-terminal sequence.

Expression of the Recombinant *C. elegans* AP-1 Protein.

A 1.8-kb *NotI/XbaI* fragment representing the entire AP-1 coding sequence was amplified by PCR using Expand high fidelity *Taq* Polymerase (Boehringer-Mannheim) and the primers flag-1 (5'-CAT GCA TGC ATG GCG GCC GCG GCA CCT CCA CAT CCG AGA GAT CCC-3') and flag-2 (5'-CAT GCA TGC ATG TCT AGA TTA TTT GAG AAG ACT TTG GAT TGC-3'). The flag-1 primer introduces an NH_2-terminal *NotI* site (the *C. elegans* translation initiation codon was abolished to force translation to start from the ATG supplied by the pFLAG CMV2 expression vector), and the flag-2 primer introduces a COOH-terminal *XbaI* site immediately after the stop codon (thus eliminating the 3'-untranslated region). The AP-1 *NotI/XbaI* fragment was then subcloned into the *NotI/XbaI* 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 cells), 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 an atmosphere of 6% CO_2. 10 x 10^6 cells per 600-cm^2 culture dishes were seeded in 100 ml of media and transiently transfected at 80% confluence with 94 µg of pFLAG.celAP-1 or pFLAG control plasmid and 283 µl of LipofectAMINE reagent (Life Technologies, Inc.), following the recommendations of the manufacturer. Two days after transfection, cells were harvested in phosphate-

buffered saline, centrifuged at 1100 x g, resuspended in TBS (50 mM Tris/HCl, pH 7.4, 150 mM NaCl), and recentrifuged at 10,000 x g for 10 min. Both the 10,000 x 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. McGhee (University of Calgary, Alberta, Canada). 5 ml of wet worms (resuspended in phosphate-buffered saline) were homogenized under liquid nitrogen (using a mortar and pestle) and resuspended in 5 ml of 0.1 M Tris, pH 7.0, and then in 10 ml of TBS (50 mM Tris pH 7.4, 150 mM NaCl) to a total volume of 20 ml. The homogenate was then sonicated at 4 °C (three times, 20 s each). The suspension was first centrifuged at 2000 x g for 10 min at 4 °C to yield a large membrane fraction, followed by centrifugation of the resultant supernatant at 200,000 x g for 60 min at 4 °C to prepare microsomal and cytosolic fractions. Protein concentrations were determined using a protein assay kit (Bio-Rad).

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 glycine/HCl at pH 3.5, followed by washing three times with 3 ml of TBS. A total of 30 mg (10 ml of 3 mg/ml) of the 10,000 x g supernatants of COS-7 cells transfected with either pFLAG vector or pFLAG.celAP-1 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 columns, 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; NH_2-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 columns 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 x g COS-7 cell supernatants (lysates of COS-7 cells transfected with either pFLAG vector or pFLAG.cel AP-1 construct), the anti-FLAG M2 affinity columns flow-through, combined washes, and the different FLAG peptide eluted fractions, as well as the NH_2-terminal FLAG fusion protein of *E.coli* bacterial alkaline phosphatase control (Kodak) were separated electrophoretically on 10 % polyacrylamide gels according to the method of Laemmli (345). 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 antibody (Amersham Pharmacia Biotech) was used at a dilution of 1:1000. Immunodetection was performed using enhanced chemiluminescence according to the manufacturer's instructions (Amersham Pharmacia Biotech). Autoradiographs for chemiluminescence detection were exposed to Kodak X-OMAT X-ray films for 3 min and then developed. A polyclonal anti-rabbit human LTA_4 hydrolase antiserum (raised against the entire protein) (346) was also used for immunoblot analysis (Merck Frosst, Pointe Claire-Dorval, Quebec).

LTB_4 Assays.

LTA_4 ethyl ester was synthesized at the Merck Frosst Centre for Therapeutic Research (Pointe Claire-Dorval, Quebec). LTB_4, prostaglandin B_2, 6-*trans*-LTB_4, 6-*trans*-12-*epi*-LTB_4, and (5*S*,6*S*)-diHETE, (5*S*,6*R*)-diHETE standards were purchased from Cayman Chemical Co., Inc. (Ann Arbor, MI). The soluble supernatant fraction following a 100,000 x g centrifugation (100S fraction) of Sf9 cells infected with a recombinant human LTA_4 hydrolase construct (346) was used as a positive control (Merck Frosst Centre for Therapeutic Research). Alkaline hydrolysis of LTA_4 ethyl ester was carried out as described (347). LTA_4 hydrolase assays on *C. elegans* cytosolic fraction (125 µg) , anti-FLAG M2 purified COS-7 lysates (200 µl of 10,000 x g supernatant of COS-7 cells transfected with pFLAG vector and 0.8 µg of FLAG-tagged *C. elegans* AP-1 protein), or recombinant human LTA_4 hydrolase (25 µg) were performed as described previously (233). Samples in 250-µl reactions were incubated with 25 µM LTA_4 in 0.1 M Tris/HCl, pH 8.0, 1 mg/ml BSA for 10 min at room temperature. Reactions were terminated with the addition of an equal volume of methanol containing 1 nmol/ml prostaglandin B_2 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 x 150 mm NovaPak C18 column (Waters). The MeOH/H_2O/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 eicosanoid standards.

Radioimmunoassay detection of LTB_4 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 25 µM LTA_4 in 0.1 M Tris/HCl, pH 8.0, 1 mg/ml BSA for 10 min at room temperature. Reactions were stopped by the addition of 20 % methanol followed by a brief centrifugation at 10,000 x g for 20 s. LTB_4 production was assayed using a

radioimmunoassay kit ([^{3}H]leukotriene B_4 assay system) (Amersham Pharmacia Biotech). The human recombinant LTA_4 hydrolase was used as a positive control as described above.

Aminopeptidase Assays.

Amino acid *p*-nitroanilides (Sigma or Bachem Bioscience Inc.) were incubated (final concentration, 0.05-5 mM) at room temperature with 0.17 μg of purified *C. elegans* AP-1 FLAG-fusion protein, anti-FLAG M2 affinity gel-purified fractions of lysates of COS-7 cells transfected with pFLAG vector, or 0.17 μg of purified human recombinant LTA_4 hydrolase/aminopeptidase in 250 μl of buffer containing 0.1 M Tris, pH 8, 200 mM NaCl, 1 mg/ml BSA. The assays were performed in 96-well microtiter plates (path length, 0.7 cm), and the formation of the product (*p*-nitroaniline, ϵ = 10,800 M^{-1} cm^{-1}) was monitored for 60 min at 405 nm using a kinetic microplate reader spectrophotometer (Molecular Devices). Spontaneous hydrolysis of the substrate (≈ 0.03 milli-absorbance units/min) was corrected for by subtracting the absorbance of control incubations without enzyme. Kinetic constants (K_m and k_{cat}) were determined by nonlinear regression (Kaleidagraph software) of the data to the Michaelis-Menten equation.

Inhibition of Aminopeptidase Activity.

Bestatin, an inhibitor of aminopeptidases (Sigma), was evaluated as an inhibitor of the *C. elegans* aminopeptidase activity. Bestatin (final concentration, 0-50 mM) in 250 μl of buffer containing 0.1 M Tris, pH 8, 200 mM NaCl, 1 mg/ml BSA was incubated at room temperature with 0.17 μg of purified *C. elegans* AP-1 (FLAG) enzyme for 10 min. L-Arginine-*p*-nitroanilide (1 mM) was then added, and substrate hydrolysis was monitored as described above. IC_{50} values were estimated from a log dose-response curve of initial velocity *versus* inhibitor concentration.

RESULTS AND DISCUSSION

Molecular Cloning of a LTA_4 Hydrolase-like cDNA Homologue from *C. elegans*.

A search of the annotated files in GenBank using the key words "leukotriene hydrolase" yielded an EST (GenBank accession number M88793) clone termed cm01c7 (327bp) from a *C. elegans* mixed stage hermaphrodite cDNA library. When translated, the EST revealed strong homology (51%) to the human LTA_4 hydrolase. The complete sequence of a 0.95-kb *ApaI/ScaI* restriction fragment containing the *C. elegans* LTA_4 hydrolase-like EST was obtained from the phage clone cm01c7 (see under "Material and Methods" for details) and was confirmed as a mammalian LTA_4 hydrolase-like sequence by sequence homology comparisons using the BLASTN and TBLASTN algorithms (National Centre for Biotechnology Information) (335) and FASTA and TFASTA programs (336). The 0.95-kb fragment was radiolabeled with ^{32}P and used as a probe to screen a *C. elegans* λ phage cDNA library, resulting in the isolation of clone C5 (≈ 1.4 kb), which showed a strong sequence homology (≈ 45%) to the human LTA_4 hydrolase but was missing the 5' part of the gene (as predicted from the sizes of cDNAs encoding previously cloned LTA_4 hydrolases). A series of anchored PCRs with the *C. elegans* cDNA library as template and using pBluescript SK phagemid-based primers, as well as several antisense primers based on the most 5' sequence of clone C5, was carried out to isolate the missing 5'-end of clone C5. The full-length *C. elegans* cDNA sequence and the deduced amino acid sequence, named AP-1, are shown in Fig. 1. The *C. elegans* AP-1 cDNA sequence is 2152 bp long, consisting of a short 15-bp 5'-untranslated region, an open reading frame encoding a 609-amino acid protein, a 282-bp-long 3'-UTR, and a 28-bp-long poly(A^+) tail. No consensus N-glycosylation sites, targeting signals, or putative phosphorylation sites were found in the sequence. The first ATG triplet (starting at nucleotide 1 in Fig. 1) in the sequence was considered to be the initiation codon of protein translation

because 1) it matches the location of the translation initiation codon from both the human and the mouse LTA_4 hydrolase cDNAs (236, 237) with only one extra codon in the *C. elegans* sequence (Fig. 2), 2) the nucleotide sequence flanking it (AATATGG) is in agreement with Kozak's rule for translational initiation consensus sequence (348), and 3) the open reading frame starting from this methionine and ending at the TAA terminator codon (nucleotide 1828) encodes a 68,248-kDa protein, corresponding to the molecular mass of the recombinant *C. elegans* AP-1 protein estimated by SDS-polyacrylamide gel electrophoresis (see below). The length of the AP-1 open reading frame (1827 bp), the number of the deduced amino acids (609 residues), and the molecular mass of the encoded protein (68,248 kDa) are comparable to the human (1830 bp, 610 residues, 69,140 kDa) and the mouse (1830 bp, 610 residues, 68,917 kDa) LTA_4 hydrolases (236, 237).

Amino Acid Sequence Comparison of the Human and the Mouse LTA_4 Hydrolases to Their *C. elegans* Homologue.

Data base searches identified the human LTA_4 hydrolase as the most closely related protein to the *C. elegans* AP-1 translation product (63% similarity and 45% identity) (results not shown). His^{297}, His^{301}, and Glu^{320} in the *C. elegans* amino acid sequence (the underlined residues in Fig. 1) conform to a catalytic zinc site of mammalian LTA_4 hydrolases and zinc metallopeptidases (240, 349) and match His^{295}, His^{299}, and Glu^{318} in both the human and the mouse LTA_4 hydrolase sequences. These three residues are likely to be involved in the coordination of the zinc atom as described previously for the mouse LTA_4 hydrolase (243) and for certain peptidases and neutral proteases (240). A multiple alignment of the amino acid sequences of the human LTA_4 hydrolase, the mouse LTA_4 hydrolase, and their putative *C. elegans* homologue and the conserved residues in the three sequences are shown in a consensus (Fig. 2). A high degree of homology is seen in the region between amino acid residues 246 and 320 in the *C. elegans* sequence, the homology then decreases towards

either the amino or the carboxyl terminus of the protein. A typical consensus zinc binding motif $HEXXHX_{18}E$ is indicated starting from amino acid 298 to 321 in the *C. elegans* 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 (350). Members of this family also include aminopeptidase A, aminopeptidase N, cysteine aminopeptidase, and LTA_4 hydrolase. The tyrosine residue Tyr^{383} in both the human and the mouse sequences, which is essential for the peptidase activity of the mammalian LTA_4 hydrolase/aminopeptidase enzyme and may act as a proton donor in a general base mechanism (251), is conserved in the *C. elegans* AP-1 (Fig. 2, Tyr^{387} in the *C. elegans* sequence). Glu^{296} in both human and mouse sequences, the mutation of which to Gln^{296} abolishes the aminopeptidase activity of the mammalian enzyme (250), is also conserved in the *C. elegans* sequence (Fig. 2, Glu^{298} in the *C. elegans* sequence). On the other hand, Tyr^{378} in both the human and the mouse LTA_4 hydrolase sequences, which is known to be involved in the covalent binding of LTA_4 (257), is replaced by a phenylalanine (Phe^{382}) in the *C. elegans* sequence.

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 Centre *C. elegans* data base, and two cosmid clones were identified (cosmid C42C1and 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. 3*A*). 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 (351). The sequences of exon-intron boundaries were determined by comparing

the cDNA sequence and the genomic sequence (Fig. 3*B*). The exon-intron junction in intron 2 follows the GT/AG rule and agrees with consensus sequences for the donor and acceptor sites (352). 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 (353). 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 (351). The proposed zinc-binding histidine residues (His^{297} and His^{301}) and glutamate residue (Glu^{320}) which constitute the zinc-binding domain ($HEXXHX_{18}E$), are located on one exon (Fig. 3, *exon 3*), unlike the structure of the human LTA_4 hydrolase/aminopeptidase gene (258), in which the two essential zinc-binding histidine residues (His^{295} and His^{299}) are present on exon 10, whereas the third zinc-binding ligand glutamate (Glu^{318}) 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 ATG, followed by an amino-terminal FLAG epitope (NH_2-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, and harvested, followed by preparation of membrane and soluble protein fractions and analysis by SDS-polyacrylamide gel electrophoresis and Western blotting using anti-FLAG M2 antibody. As expected from its calculated molecular mass, a 69-kDa immunoreactive protein was detected using the anti-FLAG antibody in the supernatant of COS-7 cells transfected with pFLAG.cel AP-1 cDNA (Fig. 4, *lane 2*) but not in the supernatant of mock-transfected cells (results not shown). As described previously for the mammalian LTA_4 hydrolase/aminopeptidase (341), 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 x

g and 2000 x 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 10% SDS-polyacrylamide gel electrophoresis, and detected by the monoclonal antibody anti-FLAG M2. All of the expressed *C. elegans* AP-1 protein was bound to the anti-FLAG M2 affinity resin. No protein was detected in either the flow-through from the column or the column washes with the *C. elegans* FLAG-tagged AP-1 eluting mainly in fractions 4-7 (Fig. 4, *lanes 8-11*). Despite the significant amino-acid sequence homology between the *C. elegans* AP-1 and the human LTA_4 hydrolase/aminopeptidase (45% identity), a rabbit anti-human LTA_4 hydrolase polyclonal antiserum (346) failed to detect any LTA_4 hydrolase 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 $HEXXHX_{18}E$ 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 NH_2-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 K_m of 0.43 ± 0.01 mM and a V_{max} of 0.18 ± 0.01 µmol/min/mg enzyme. These values can be compared to a K_m of 0.09 ± 0.01 mM and a V_{max} of 0.47 ± 0.01 µmol/min/mg obtained for

the human LTA_4 hydrolase/aminopeptidase (Fig. 5). The K_m and k_{cat} 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 k_{cat}/K_m 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 NH_2-terminal substitutions, and amino acids with *D*-stereochemistry were poor substrates. The human recombinant LTA_4 hydrolase/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 tri-peptides with L-arginine at the NH_2-terminal position (248). In the absence of a physiological substrate for the aminopeptidase activity of the human enzyme and its high catalytic efficiency for several synthetic tri-peptides (exceeding the k_{cat}/K_m for LTA_4 by 10-fold), the enzyme was suggested to be involved in the metabolism of dietary peptides and neuropeptides (248). This role can also be proposed for the *C. elegans* AP-1 enzyme. Bestatin, a potent inhibitor of human LTA_4 hydrolase/aminopeptidase (245), as well as other aminopeptidases, inhibited the hydrolysis of L-arginine *p*-nitroanilide by AP-1. The concentration for half-maximal inhibition (IC_{50}) 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 Ala^{117} to Val^{117} 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 Tyr^{383} and Glu^{296} may be important for the aminopeptidase activity of the mammalian LTA_4 hydrolase/aminopeptidase enzyme. It is also interesting to note that this alanine residue (Ala^{114} in the human sequence) is conserved evolutionary as it is found in the *C. albicans* LTA_4 hydrolase (which mainly exhibits aminopeptidase activity), the *S. cerevisiae* proposed LTA_4 hydrolase (which is yet to be characterized), all

cloned mammalian LTA_4 hydrolases, including human, mouse, rat, and guinea pig, and the *C. elegans* AP-1 (data not shown).

Measurement of LTA_4 Hydrolase activity of *C. elegans* AP-1 Enzyme.

The *C. elegans* AP-1 protein was analyzed for epoxide hydrolase activity using LTA_4 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 LTA_4 revealed no production of LTB_4 (Fig. 6, peak *4, tracing 6 and 2*, respectively). In contrast, the human LTA_4 hydrolase (used as a positive control) produced mainly peak 4 (Fig. 6, *tracing 1*), which eluted with the retention time of the expected enzymatic product LTB_4. Small amounts of the other peaks (peaks 2, 3, 5, and 6) represent the nonenzymatic hydrolysis of LTA_4. In the absence of LTA_4 hydrolase activity (Fig. 6, all tracings except *tracing 1*), LTA_4 was mostly converted to the nonenzymatic hydrolysis products, the all-*trans*-LTB_4 epimers at C12 (6-*trans*-LTB_4, and 12-*epi*-6-*trans*-LTB_4, peaks 2 and 3 respectively), as well as (5*S*,6*R*)-diHETE (peak 5), and (5*S*,6*S*)-diHETE (peak 6). These species are normally formed in small and equal amounts in aqueous solutions by spontaneous hydrolysis (354), and their peaks eluted with the retention times and spectra of known eicosanoid standards. Peaks 2, 3, 5, and 6 were also produced by boiled *C. elegans* cytosol (Fig. 6, *tracing 4*), as well as boiled FLAG-tagged AP-1 (data not shown), confirming their nonenzymatic origin. *C. elegans* cytosol pretreated with 100 μM bestatin (an inhibitor of both LTA_4 hydrolase and aminopeptidase activities of the human enzyme (245)), showed the same chromatographic profile (Fig. 6, *tracing 3*) as the untreated or the boiled *C. elegans* cytosol. The inability of the *C. elegans* AP-1 enzyme to synthesize LTB_4 from LTA_4 was also confirmed by the absence of any LTB_4 production (data not shown) using a LTB_4 radioimmunoassay (lower limit of detection is 16 pg of LTB_4 ml^{-1}).

Although *C. elegans* AP-1 does not appear to hydrolyze LTA_4, the high degree of identity between the active sites of AP-1 and mammalian LTA_4

hydrolases (Fig. 2) suggests that LTA_4 may bind in the active site of AP-1 without being a substrate for catalysis. To test this possibility, we examined the effect of LTA_4-ethyl ester on the aminopeptidase activity of AP-1 using L-arginine-*p*-nitroanilide as a substrate. We chose the LTA_4-ethyl ester as it is a suicide inactivator of the mammalian LTA_4 hydrolases and is much more resistant than LTA_4 to nonenzymatic hydration. The LTA_4-ethyl ester had no effect on the aminopeptidase activity of AP-1 at concentrations up to 100 μM, 10 times the K_m of mammalian LTA_4 hydrolase for LTA_4-ethyl ester (results not shown).

The bifunctional human LTA_4 hydrolase/aminopeptidase enzyme is suicide-inactivated during catalysis via an apparently mechanism-based irreversible binding of LTA_4 to the protein (234), with tyrosine at position 378 identified as the site for covalent binding of LTA_4. Interestingly, the mutation of Tyr^{378} to Phe^{378} in the human LTA_4 hydrolase yielded an enzyme with increased turnover and resistance to mechanism-based inactivation (257), thus dissociating catalysis and covalent modification/inactivation events. This tyrosine residue is a phenylalanine in the *C. elegans* AP-1 enzyme sequence (Phe^{382}), but the *C. elegans* enzyme does not hydrolyze LTA_4 indicating that other residues (lacking in the *C. elegans* AP-1 sequence) must also be important for LTA_4 binding and catalysis.

That the cloned *C. elegans* AP-1 enzyme functions as an aminopeptidase with no LTA_4 hydrolase activity is interesting, as its primary structure resembles LTA_4 hydrolases more than it does aminopeptidases (Table II). Comparison of the *C. elegans* AP-1 enzyme and other proteins in the SwissProt data base revealed 45% identity to the human, mouse, rat, and guinea pig LTA_4 hydrolases at the amino acid level, with lower identity to the human, rat, and pig aminopeptidase-N (28-30%). Moreover, the identity between *C. elegans* AP-1 and mammalian LTA_4 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 $HEXXHX_{18}E$). It is interesting to note that the same identity (an average of 30%)

that is shown between *C. elegans* AP-1 and other aminopeptidases is also seen between mammalian LTA_4 hydrolases and any given aminopeptidase enzyme (Table II). The structural similarity between *C. elegans* AP-1 and mammalian LTA_4 hydrolases suggests an evolutionary relationship. Recently, three other proteins that are structurally related to mammalian LTA_4 hydrolases have been identified in lower invertebrates. These proteins include a *C. albicans* LTA_4 hydrolase-related enzyme (41% identity to human LTA_4 hydrolase) that functions mainly as an aminopeptidase but fails to hydrolyze LTA_4 to LTB_4 (355), a gene from the yeast *S. cerevisiae* (39% identity to human LTA_4 hydrolase) (260), and a partial amino acid sequence (316 residues) of a *D. discoideum* cDNA (GenBank accession number U27538, Jho, E., and Kopachik, W. 1995, unpublished data) with 38% identity to human LTA_4 hydrolase. Biochemical studies to clarify the enzymatic activity (or activities) of both the yeast and the *Dictyostelium* LTA_4 hydrolase-like proteins await their expression and characterization. LTB_4 production has not been reported in either *C. albicans* or *D. discoideum*, and our analysis of mixed stage *C. elegans* worms failed to detect any LTA_4 hydrolase activity. The high primary sequence identity of the *C. elegans* AP-1 to mammalian LTA_4 hydrolases suggests that AP-1 may represent an evolutionary precursor of the mammalian LTA_4 hydrolases. Thus, mammalian LTA_4 hydrolases may have originated from aminopeptidases like AP-1, retaining their aminopeptidase activity and developing a LTA_4 hydrolase function in higher eukaryotes. In support of this hypothesis, an aminopeptidase B has recently been cloned from rat testes (259) that shows highest homology to mammalian leukotriene A_4 hydrolases (44%), intermediate homology to *C. elegans* AP-1 (38%), and lowest homology to mammalian N-type aminopeptidases (21-24%) Table II) and can catalyze the conversion of LTA_4 to LTB_4 (259).

In conclusion, we have cloned and functionally expressed a 69-kDa protein from *C. elegans*, the primary structure of which is more homologous to mammalian LTA_4 hydrolases than other zinc aminopeptidases. This protein functions as an aminopeptidase with broad substrate specificity but lacks any LTA_4 hydrolase activity. The primary sequence identity of *C. elegans* AP-1

enzyme to mammalian LTA_4 hydrolases and rat aminopeptidase B suggests that these enzymes are evolutionarily related.

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FIGURE LEGENDS

Figure 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 NH_2-terminal methionine residue. Amino acids involved in the putative zinc binding site are *underlined*. GenBank accession number AF068201.

Figure 2. A multiple alignment of amino acid sequences of the human leukotriene A_4, the mouse leukotriene A_4 hydrolase, and the *C. elegans* AP-1. The alignment was made using the Pretty Plot function of the GCG program (336). 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 ($HEXXHX_{18}E$), 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 LTA_4 hydrolase/aminopeptidase, are indicated on a *black background*. The conserved glutamate residue necessary for peptidolysis of mammalian LTA_4 hydrolases (Glu^{296} in both human and mouse sequences) is *underlined*. The tyrosine residue Tyr^{378} (in both the human and the mouse LTA_4 hydrolase sequences) involved in the covalent binding of LTA_4 to the human LTA_4 hydrolase is indicated by an *asterisk* and is replaced by a phenylalanine (Phe^{382} in the *C. elegans* sequence).

Figure 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 *lowercase letters*

representing nucleotides present in introns. Amino acids (in one-letter code) are indicated by the single *uppercase letters* present above the first nucleotide of each codon.

Figure 4. Immunoblot analysis of the *C. elegans* FLAG-tagged AP-1 protein purified using anti-FLAG M2 affinity gel. Shown are 50 ng of FLAG-tagged bacterial alkaline phosphatase (*BAP*) standard (*lane 1*), 50 μg of total protein of the 10,000 × g supernatant of COS-7 cells transfected with pFLAG.celAP-1 (*lane 2*), 30 μl each of anti-FLAG M2 affinity column flow-through (*lane 3*), anti-FLAG M2 affinity column washes (*lane 4*), and FLAG octapeptide eluted fractions 1-11 (*lanes 5 to 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.

Figure 5. Comparison of aminopeptidase activity of the cloned *C. elegans* AP-1 (*A*) with that of the recombinant human LTA_4hydrolase/aminopeptidase (*B*). Purified FLAG-tagged *C. elegans* AP-1 (0.17 μg) (*A*) and human LTA_4 hydrolase/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 *K*m values were determined by nonlinear fit of the experimental values to the Michaelis-Menten equation.

Figure 6. Reverse-phase HPLC analysis of products formed by *C. elegans* cytosolic extracts and COS-7 cells transfected with the pFLAG.cel AP-1 construct. Reverse-phase HPLC chromatograms of products formed following the incubation of 25 μM LTA_4 with human recombinant LTA_4 hydrolase standard (25 μg of the S100 fraction of Sf9 cells, see under "Materials and Methods" and Ref. 346) (*tracing 1*) , 125 μg of *C. elegans* cytosol in the absence of bestatin (*tracing 2*) or pre-treated with 100 μM bestatin (*tracing 3*), boiled *C. elegans* cytosol (*tracing 4*), 200 μl of anti-FLAG M2 purified 10,000 x 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*). LTA_4 hydrolase assay and analysis

of eicosanoid products were carried out as described previously (346). Peaks were identified by elution with co-chromatographed standards and their characteristic absorbance spectrum. *Peak 1*, PGB_2 (internal standard); *peak 2*, 6-*trans*-LTB_4; *peak 3*, 6-*trans*-12-*epi*-LTB_4; *peak 4*, LTB_4; *peak 5*, (5*S*,6*R*)-diHETE; *peak 6*, (5*S*,6*S*)-diHETE. The chromatograms are representative of three experiments with identical results.

Table I. Kinetic constants for hydrolysis of amino acid *p*-nitroanilides. The different amino acid *p*-nitroanilides (0.05-5 mM) were incubated at room temperature with 0.17 μg of either purified *C. elegans* AP-1 (FLAG) enzyme or human LTA_4 hydrolase/aminopeptidase enzyme in 250 μl of 0.1 M Tris, pH 8.0, 200 mM sodium chloride containing BSA (1 mg/ml). *p*-nitroaniline formation was monitored spectrophotometrically for 60 min at 405 nm. Kinetic constants (K_m and k_{cat}) were determined by nonlinear regression analysis. Activities of purified vector construct with each substrate were less than 10% of values obtained with purified *C. elegans* AP-1 enzyme and were comparable to the observed nonenzymatic hydrolysis rates.

Table II. Amino acid sequence identity (%) between *C. elegans* AP-1, LTA_4 hydrolases and aminopeptidases. A BLASTP search of SwissProt data base (release 96) identified LTA_4 hydrolases followed by aminopeptidases as the most closely related proteins to the *C. elegans* AP-1. The Bestfit algorithm in the GCG sequence analysis software (gap weight of 12, length weight of 4, gap creation penalty of 12, and gap extension penalty of 4) was used to determine percentage sequence identity. $hLTA_4$, human LTA_4 hydrolase; $mLTA_4$, mouse LTA_4 hydrolase; $rLTA_4$, rat LTA_4 hydrolase; $gpLTA_4$, guinea pig LTA_4 hydrolase; rAP-B, rat aminopeptidase B; hAP-N, human microsomal aminopeptidase N; rAP-N, rat microsomal aminopeptidase N; pgAP-N, pig microsomal aminopeptidase N.

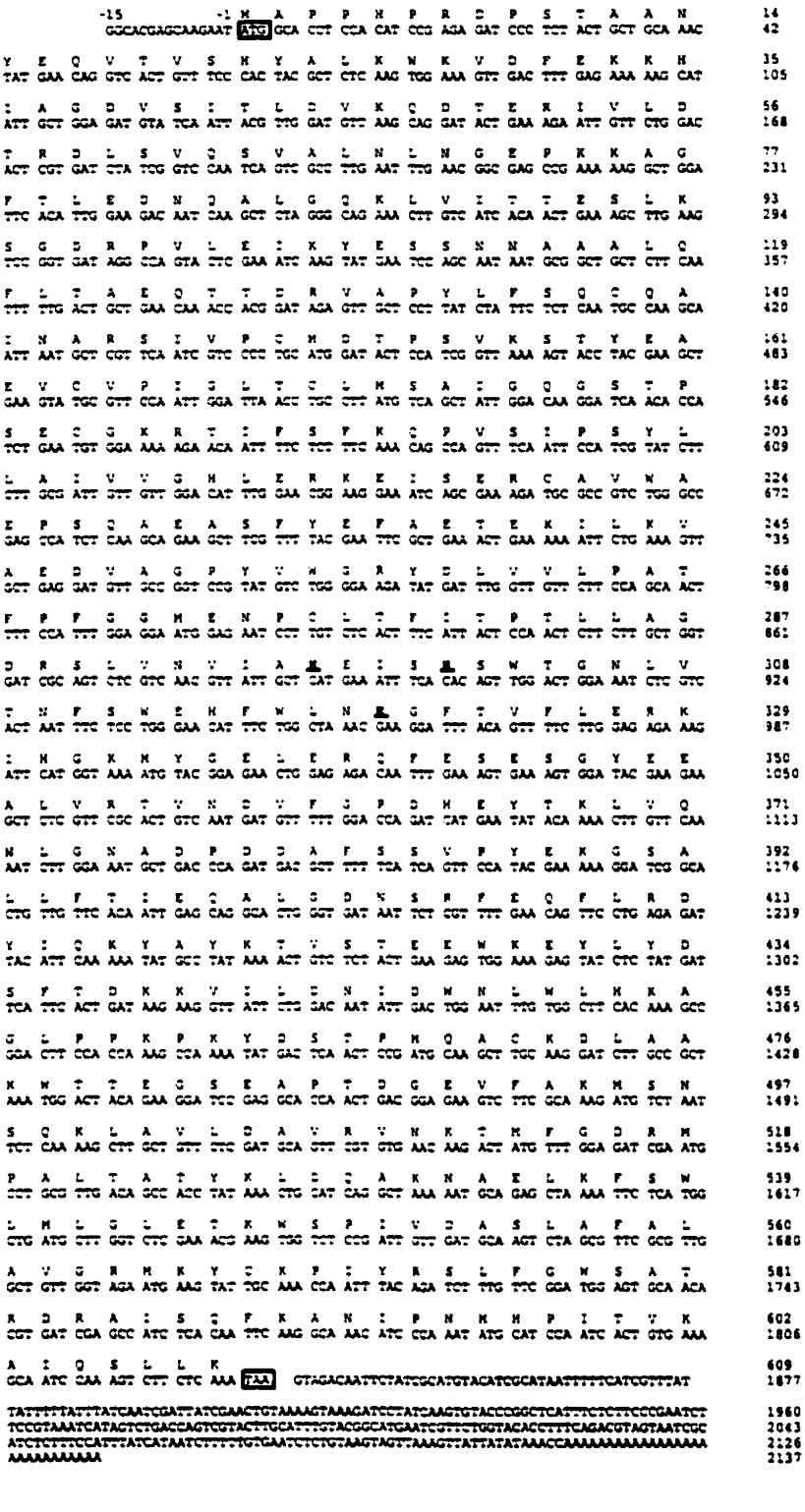


Figure 1

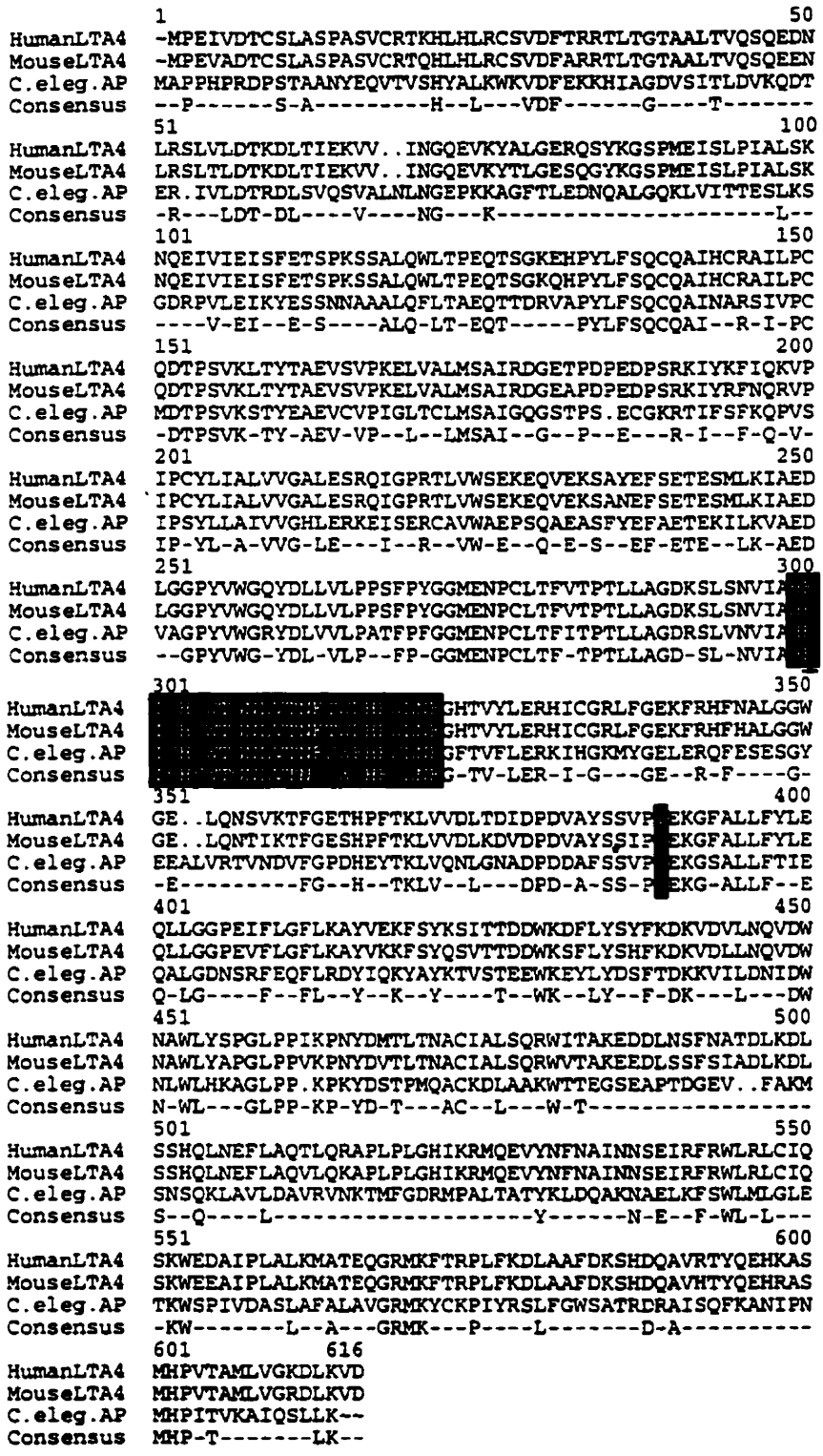
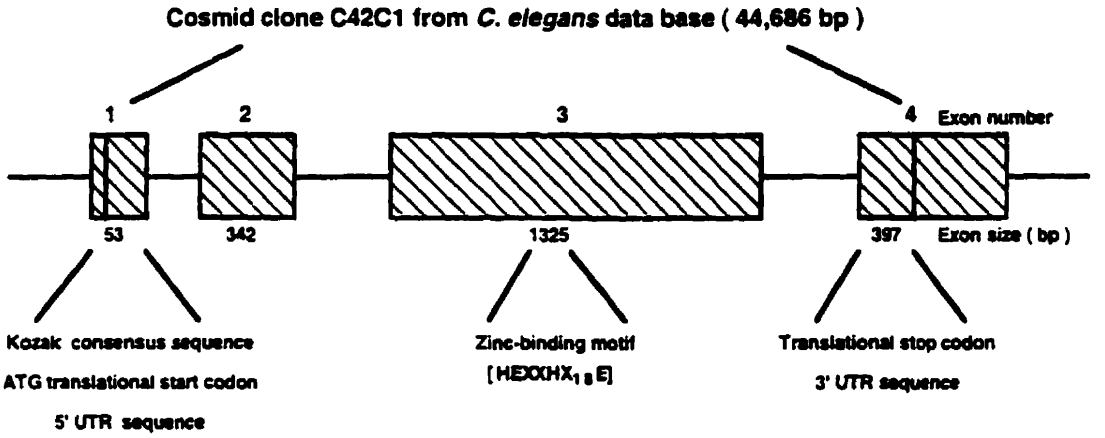

1 50
HumanLTA4 -MPEIVDTCSLASPASVCRTKHLHLRCSVDFTRRTLTGTAALTVQSQEDN
MouseLTA4 -MPEVADTCSLASPASVCRTQHLHLRCSVDFARRTLTGTAALTVQSQEEN
C.eleg.AP MAPPHPRDPSTAANYEQVTVSHYALKWKVDFEKKHIAGDVSITLDVKQDT
Consensus --P------S-A---------H--L---VDF------G----T-------
51 100
HumanLTA4 LRSLVLDTKDLTIEKVV..INGQEVKYALGERQSYKGSPMEISLPIALSK
MouseLTA4 LRSLTLDTKDLTIEKVV..INGQEVKYTLGESQGYKGSPMEISLPIALSK
C.eleg.AP ER.IVLDTRDLSVQSVALNLNGEPKKAGFTLEDNQALGQKLVITTESLKS
Consensus -R---LDT-DL----V----NG---K----------L--
101 150
HumanLTA4 NQEIVIEISFETSPKSSALQWLTPEQTSGKEHPYLFSQCQAIHCRAILPC
MouseLTA4 NQEIVIEISFETSPKSSALQWLTPEQTSGKQHPYLFSQCQAIHCRAILPC
C.eleg.AP GDRPVLEIKYESSNNAAALQFLTAEQTTDRVAPYLFSQCQAINARSIVPC
Consensus ----V-EI--E-S----ALQ-LT-EQT-----PYLFSQCQAI--R-I-PC
151 200
HumanLTA4 QDTPSVKLTYTAEVSVPKELVALMSAIRDGETPDPEDPSRKIYKFIQKVP
MouseLTA4 QDTPSVKLTYTAEVSVPKELVALMSAIRDGEAPDPEDPSRKIYRFNQRVP
C.eleg.AP MDTPSVKSTYEAEVCVPIGLTCLMSAIGQGSTPS.ECGKRTIFSFKQPVS
Consensus -DTPSVK-TY-AEV-VP--L--LMSAI--G--P--E---R-I--F-Q-V-
201 250
HumanLTA4 IPCYLIALVVGALESRQIGPRTLVWSEKEQVEKSAYEFSETESMLKIAED
MouseLTA4 IPCYLIALVVGALESRQIGPRTLVWSEKEQVEKSANEFSETESMLKIAED
C.eleg.AP IPSYLLAIVVGHLERKEISERCAVWAEPSQAEASFYEFAETEKILKVAED
Consensus IP-YL-A-VVG-LE---I--R--VW-E--Q-E-S--EF-ETE--LK-AED
251 300
HumanLTA4 LGGPYVWGQYDLLVLPPSFPYGGMENPCLTFVTPTLLAGDKSLSNVIA[redacted]
MouseLTA4 LGGPYVWGQYDLLVLPPSFPYGGMENPCLTFVTPTLLAGDKSLSNVIA[redacted]
C.eleg.AP VAGPYVWGRYDLVVLPATFPFGGMENPCLTFITPTLLAGDRSLVNVIA[redacted]
Consensus --GPYVWG-YDL-VLP--FP-GGMENPCLTF-TPTLLAGD-SL-NVIA[redacted]
301 350
HumanLTA4 [redacted]GHTVYLERHICGRLFGEKFRHFNALGGW
MouseLTA4 [redacted]GHTVYLERHICGRLFGEKFRHFHALGGW
C.eleg.AP [redacted]GFTVFLERKIHGKMYGELERQFESESGY
Consensus [redacted]G-TV-LER-I-G---GE--R-F----G-
351 400
HumanLTA4 GE..LQNSVKTFGETHPFTKLVVDLTDIDPDVAYSSVP[redacted]EKGFALLFYLE
MouseLTA4 GE..LQNTIKTFGESHPFTKLVVDLKDVDPDVAYSSIP[redacted]EKGFALLFYLE
C.eleg.AP EEALVRTVNDVFGPDHEYTKLVQNLGNADPDDAFSSVP[redacted]EKGSALLFTIE
Consensus -E---------FG--H--TKLV--L---DPD-A-SS-P[redacted]EKG-ALLF--E
401 450
HumanLTA4 QLLGGPEIFLGFLKAYVEKFSYKSITTDDWKDFLYSYFKDKVDVLNQVDW
MouseLTA4 QLLGGPEVFLGFLKAYVKKFSYQSVTTDDWKSFLYSHFKDKVDLLNQVDW
C.eleg.AP QALGDNSRFEQFLRDYIQKYAYKTVSTEEWKEYLYDSFTDKKVILDNIDW
Consensus Q-LG----F--FL--Y--K--Y----T--WK--LY--F-DK---L---DW
451 500
HumanLTA4 NAWLYSPGLPPIKPNYDMTLTNACIALSQRWITAKEDDLNSFNATDLKDL
MouseLTA4 NAWLYAPGLPPVKPNYDVTLTNACIALSQRWVTAKEEDLSSFSIADLKDL
C.eleg.AP NLWLHKAGLPP.KPKYDSTPMQACKDLAAKWTTEGSEAPTDGEV..FAKM
Consensus N-WL---GLPP-KP-YD-T---AC--L---W-T----------------
501 550
HumanLTA4 SSHQLNEFLAQTLQRAPLPLGHIKRMQEVYNFNAINNSEIRFRWLRLCIQ
MouseLTA4 SSHQLNEFLAQVLQKAPLPLGHIKRMQEVYNFNAINNSEIRFRWLRLCIQ
C.eleg.AP SNSQKLAVLDAVRVNKTMFGDRMPALTATYKLDQAKNAELKFSWLMLGLE
Consensus S--Q----L-------------------Y------N-E--F-WL-L---
551 600
HumanLTA4 SKWEDAIPLALKMATEQGRMKFTRPLFKDLAAFDKSHDQAVRTYQEHKAS
MouseLTA4 SKWEEAIPLALKMATEQGRMKFTRPLFKDLAAFDKSHDQAVHTYQEHRAS
C.eleg.AP TKWSPIVDASLAFALAVGRMKYCKPIYRSLFGWSATRDRAISQFKANIPN
Consensus -KW-------L--A---GRMK---P----L-------D-A----------
601 616
HumanLTA4 MHPVTAMLVGKDLKVD
MouseLTA4 MHPVTAMLVGRDLKVD
C.eleg.AP MHPITVKAIQSLLK--
Consensus MHP-T-------LK--

Figure 2

A



B

Intron number	Intron size (bp)	Splice position	Exon	Intron donor	Intron acceptor	Exon
1	44	48/49	Y E TAT GAA	caggtataaa.....aaattaactc		Q V CAG GTC
2	49	390/391	R V AGA GTT	gtgattttta.....gataattcag		A P GCT CCT
3	49	1715/1716	P I Y CCA ATT TA	caggtgaaaa.....atttataatt		R S C AGA TCT

Figure 3

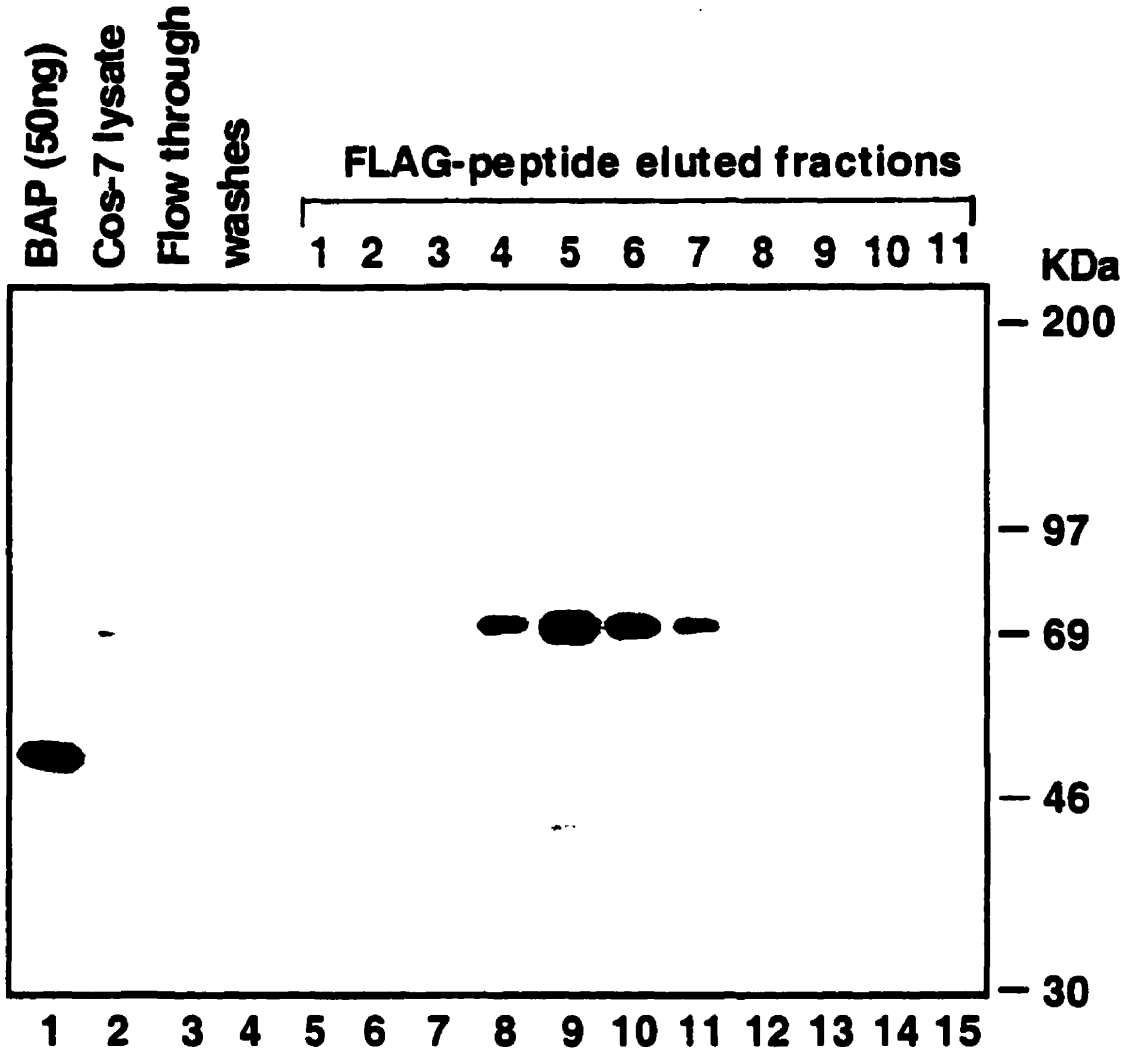
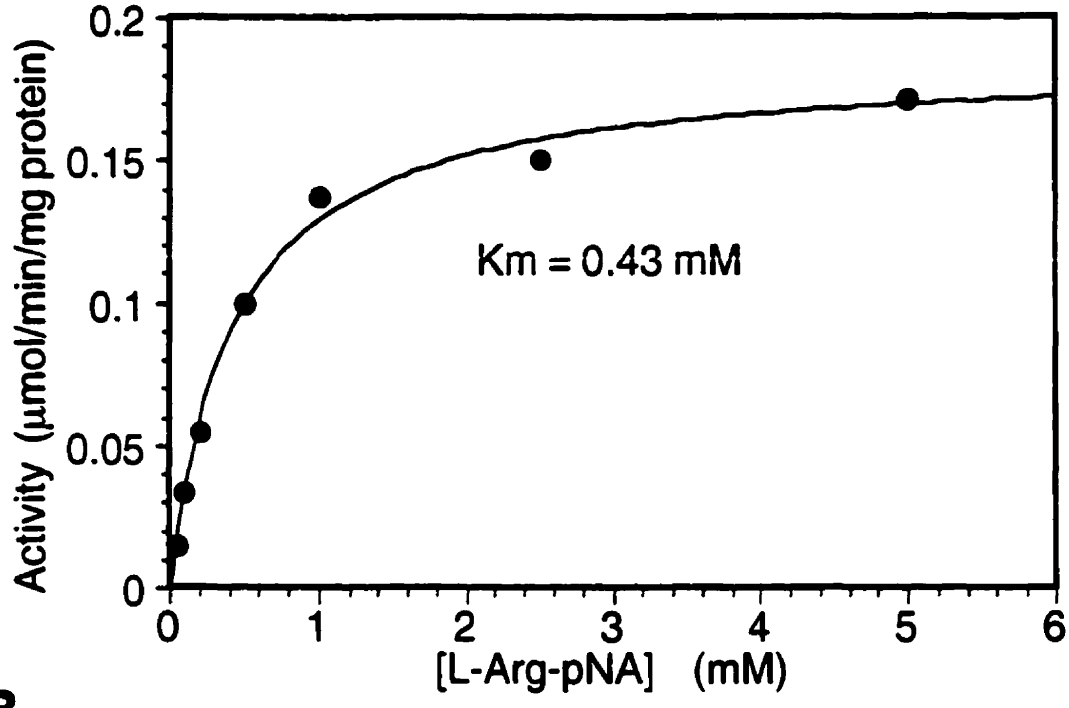

BAP (50ng)
Cos-7 lysate
Flow through
washes
FLAG-peptide eluted fractions
1 2 3 4 5 6 7 8 9 10 11
KDa
200
97
69
46
30
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure 4

A


0.2
0.15
0.1
0.05
0
Activity (µmol/min/mg protein)
Km = 0.43 mM
0
1
2
3
4
5
6
[L-Arg-pNA] (mM)

B

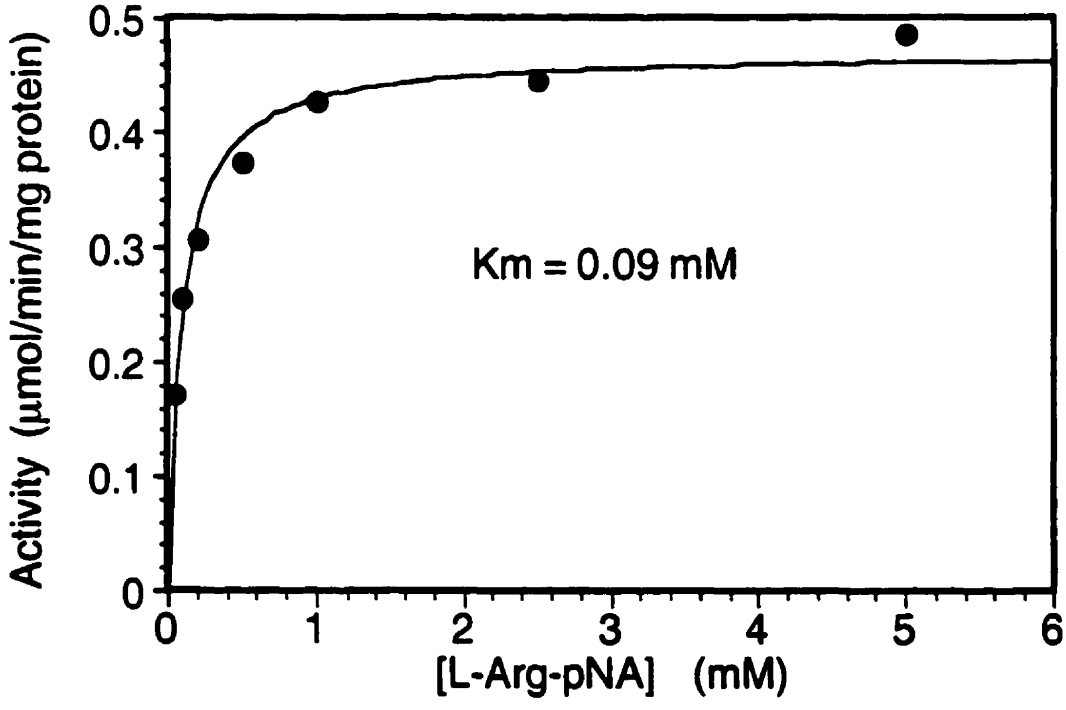

0.5
0.4
0.3
0.2
0.1
0
Activity (µmol/min/mg protein)
Km = 0.09 mM
0
1
2
3
4
5
6
[L-Arg-pNA] (mM)

Figure 5

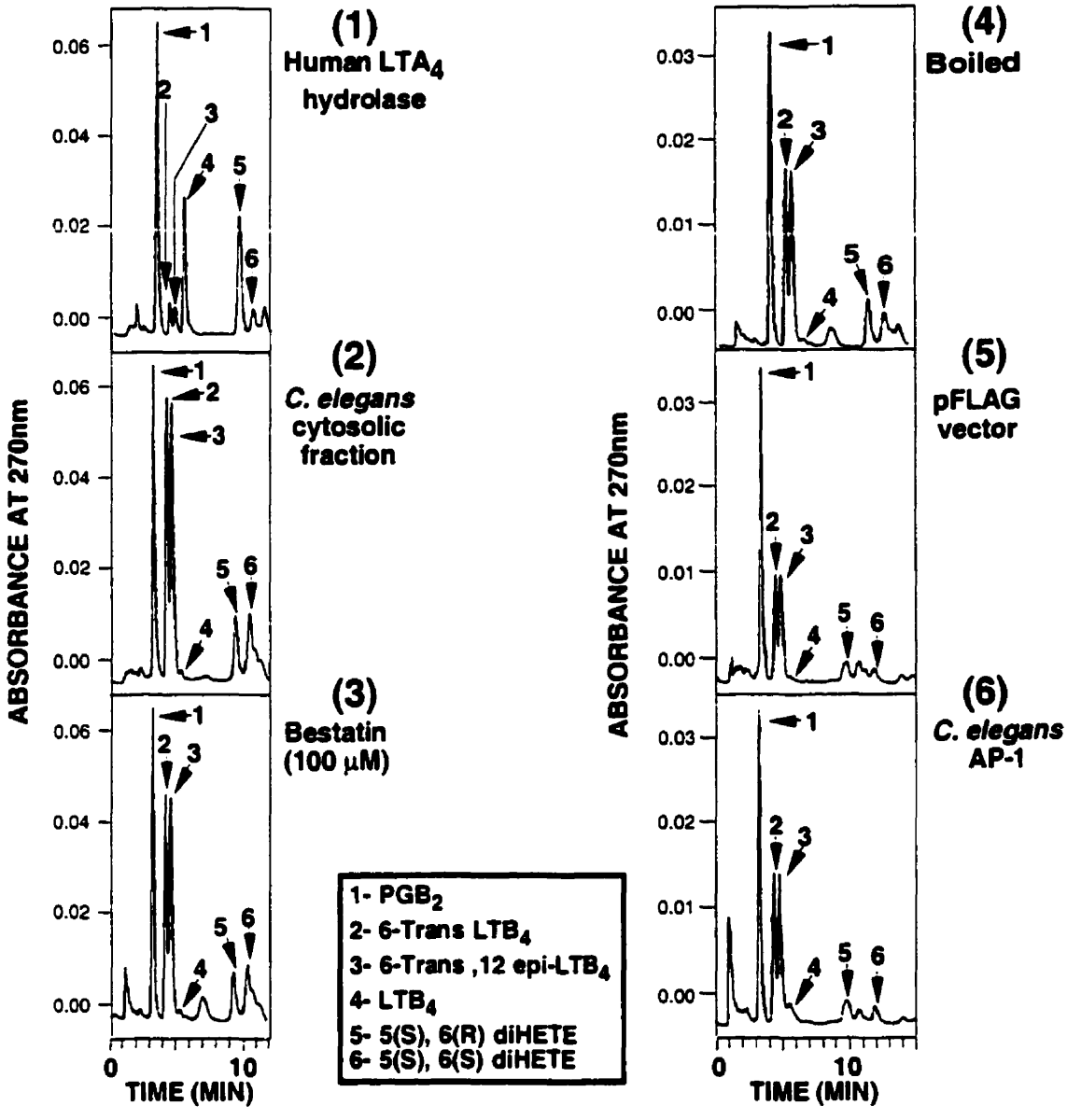

(1)
Human LTA4 hydrolase
(2)
C. elegans cytosolic fraction
(3)
Bestatin (100 μM)
(4)
Boiled
(5)
pFLAG vector
(6)
C. elegans AP-1
ABSORBANCE AT 270nm
TIME (MIN)
1- PGB2
2- 6-Trans LTB4
3- 6-Trans ,12 epi-LTB4
4- LTB4
5- 5(S), 6(R) diHETE
6- 5(S), 6(S) diHETE

Figure 6

Kinetic constants for hydrolysis of amino acid *p*-nitroanilides

	Human LTA_4 hydrolase			*C. elegans* AP-1		
	K_m	k_{cat}	k_{cat}/K_m	K_m	k_{cat}	k_{cat}/K_m
	mM	s^{-1}	$s^{-1}\ M^{-1}$	*mM*	s^{-1}	$s^{-1}\ M^{-1}$
L-Arg-pNA	0.09	0.55	6.10×10^3	0.43	0.21	0.48×10^3
L-Ala-pNA	1.44	3.11	2.20×10^3	5.53	0.78	0.14×10^3
L-Leu-pNA	0.25	0.52	2.08×10^3	2.00	0.25	0.13×10^3
L-Lys-pNA	0.05	0.09	1.80×10^3	0.39	0.05	0.13×10^3
L-Pro-pNA	0.24	0.45	1.87×10^3	1.90	0.23	0.12×10^3
L-Met-pNA	0.40	0.24	0.60×10^3	0.46	0.03	0.06×10^3
L-Val-pNA	0.80	0.08	0.10×10^3	0.90	0.01	0.01×10^3
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		

Table I

Amino acid sequence identity (%) between *C. elegans* AP-1, LTA_4 hydrolases, and aminopeptidases.

	C. elegans AP-1	$hLTA_4$	$mLTA_4$	$rLTA_4$	$gpLTA_4$	rAP-B	hAP-N	rAP-N	pgAP-N
C. elegans AP-1	100	45	45	45	45	38	29	28	30
$hLTA_4$	45	100	92	92	92	44	30	29	29
$mLTA_4$	45	92	100	97	90	44	30	32	29
$rLTA_4$	45	92	97	100	90	44	29	31	29
$gpLTA_4$	45	92	90	90	100	42	33	32	33
rAP-B	38	44	44	44	42	100	24	21	23
hAP-N	29	30	30	29	33	24	100	77	79
rAP-N	28	29	32	31	32	21	77	100	77
pgAP-N	30	29	29	29	33	23	77	77	100

Table II

7. DISCUSSION AND CONCLUSION

Arachidonic acid is metabolized via cyclooxygenase and lipoxygenase enzymes to various eicosanoids including prostaglandins (PGs), leukotrienes (LTs), hydroxyeicosatetraenoic acids (HETEs) and lipoxins. Eicosanoids are important local mediators of numerous physiological and pathological responses including inflammation, bronchoconstriction, pain, fever, regulation of vascular tone, and regulation of immune responses. While eicosanoids are very well studied in mammalian systems, mostly due to their pharmaceutical interest, there is increasing recognition of the significance of these oxygenated compounds in invertebrates. Recent studies have established the ability of various insects and parasites to produce eicosanoids in response to the addition of arachidonic acid. Certain nematode and trematode helminth parasites cause considerable mortality in humans in many tropical and subtropical parts of the world. An estimated 200 to 300 million people worldwide are currently infected with the trematode parasite *Schistosoma mansoni* (*S. mansoni*) and another 600 million are at risk. Little is known about the mechanism(s) by which helminth parasites overcome host immune responses to survive and establish chronic diseases. Eicosanoids production by these parasites is believed to facilitate their invasion of the host and their subsequent evasion of the host cellular immune responses. In addition, eicosanoids may be essential for normal parasitic physiological processes (e.g oocyte maturation, egg production, salt and water transport) as is the case in other invertebrates. To further our understanding of the roles eicosanoids may play in lower organisms, we investigated arachidonic acid metabolizing enzymes responsible for the production of these important mediators in the trematode parasite *S. mansoni* and the nematode worm *Caenorhabditis elegans* (*C. elegans*).

7.1 Characterization of arachidonic acid metabolizing enzymes in adult *S. mansoni* parasites

Several recent studies have aimed at elucidating the production of eicosanoids by nematode and trematode parasites. Microfilariae of the intravascular nematode parasites *Wuchereria bancrofti* and *Brugia malayi* have been shown to produce vasodilatory, anti-aggregatory, and immunomodulatory PGE_2 and prostacyclin following incubation with exogenous arachidonic acid (297, 298). Thus, it was postulated that filarial parasites may exploit the effects of these eicosanoids to inhibit platelet aggregation onto their surfaces, to facilitate their passage through small capillary vessels, and to modulate and escape host defenses. Similarly, cercariae of the blood dwelling trematode *S. mansoni* have been demonstrated to synthesize a wide variety of eicosanoids including PGs, LTs, and HETEs upon stimulation by essential fatty acids (EFAs) normally found on the host skin surface (304). In addition, these EFAs stimulated host skin penetration by cercariae, an effect which was inhibited by cyclooxygenase inhibitors, suggesting an involvement of prostanoids in cercarial penetration (301-303). Furthermore, successful cercarial penetration and transformation into schistosomulae, *in vitro*, was correlated with eicosanoid biosynthesis (305). Although limited parasite numbers have prevented extensive examination of adult *S. mansoni*, one study has suggested the elaboration of certain eicosanoids by developing schistosomulae and adult male and female parasites (307). To further elucidate the production of eicosanoids by adult *S. mansoni*, we investigated the metabolism of arachidonic acid in these medically important parasites.

Following incubation with radiolabeled arachidonic acid, cytosolic and microsomal extracts of adult *S. mansoni* worms synthesized mainly a major metabolite which co-chromatographed with an authentic 15-HETE standard on thin-layer chromatography (TLC) (chapter 4, ref. 332). The synthesis of the 15-HETE-like product was confirmed to be enzymatic as it was totally abolished by heat denaturation of the *S. mansoni* extracts. This result is in agreement with a

previous study which demonstrated that a metabolite with the chromatographic characteristics of 15-HETE was the major eicosanoid produced by adult parasites in response to the addition of linoleic acid (307). On the other hand, in the literature, the formation of prostaglandins by adult *S. mansoni* via cyclooxygenase (COX) pathways is subject to controversy. One study, which established the ability of adult parasites to incorporate arachidonic acid into their various membrane structures, detected no prostaglandin biosynthesis using TLC (308). Another study reported the production of a metabolite which displayed the same high performance chromatographic properties as PGE_2, but no definitive pharmacological or physiochemical data were obtained (307). Consistent with the first study, our analysis of arachidonic acid metabolism indicated the absence of PGE_2 production by adult worm extracts using both TLC and radioimmunoassay techniques. In addition, immunoblot analysis of the different *S. mansoni* extracts using antisera to mammalian cyclooxygenases revealed no specific COX-protein bands. Moreover, no evidence for the presence of a parasite COX homologue was demonstrated by polymerase chain reaction (PCR) analysis using degenerate primers based on conserved sequences in mammalian and avian cyclooxygenases (chapter 4, ref. 332). These results, combined with our inability to detect any prostaglandin formation, suggested the absence of an active cyclooxygenase pathway in adult *S. mansoni* parasites. Nonetheless, the weakly hybridizing bands revealed by Southern blot hybridization of *S. mansoni* genomic DNA using human COX-1 and COX-2 probes, implied that the parasite may contain cyclooxygenase-related sequences. Since cercarial production of COX-derived prostaglandins has been well documented (301-305), our incapability to detect an *S. mansoni* cyclooxygenase protein or mRNA in the adult worm could therefore be related to a stage-specific expression of the putative *S. mansoni* cyclooxygenase gene.

Since cyclooxygenase-2 as well as 12/15-lipoxygenases (LOXs) are capable of metabolizing arachidonic acid to 15-HETE (356, 170, 199), we consequently used both COX and LOX inhibitors to delineate the pathway responsible for the enzymatic activity detected in adult *S. mansoni* extracts. The

inability of indomethacin, a potent COX inhibitor, to inhibit *S. mansoni* 15-HETE production and the observed inhibitory effects of different LOX inhibitors, which displayed similar pharmacological profiles to those previously seen with different plant and mammalian LOXs (191), confirmed the lipoxygenase origin of the 15-HETE-like metabolite. Resembling the cytosolic localization of other previously characterized mammalian lipoxygenases, the *S. mansoni* lipoxygenase activity was mainly detected in the cytosolic fraction of adult worms. This activity was not stimulated by calcium addition suggesting that the parasite lipoxygenase homologue is regulated differently to the mammalian 5-LOX enzyme but similarly to other mammalian LOXs (123). The efficient metabolism of linoleic acid to 13-HODE displayed by the cytosolic fraction of adult worms indicated that the parasite lipoxygenase activity is similar to that of the erythroid 15-LOX and the leukocyte 12-LOX (170, 191). Collectively, these results indicated the presence of a soluble, enzymatically active lipoxygenase pathway in adult *S. mansoni* parasites which demonstrated a similar activity to mammalian 15-lipoxygenases. Both of the *S. mansoni*-produced 15-HETE and 13-HODE-like species migrated with the same R_f values as those of 15-HETE and 13-HODE standards on TLC, however, the identity of these metabolites to their mammalian counterparts can only be confirmed using gas chromatography/mass spectrometry. Presently, the small amounts of eicosanoids produced by adult *S. mansoni* worms preclude such measurements, and hence, the full characterization of these metabolites would have to await the cloning and expression of the parasite lipoxygenase homologue.

In view of the significant amino acid sequence homology shared among plant and mammalian lipoxygenases (143), it was reasonable to anticipate that antibodies to mammalian lipoxygenases might show immunological cross-reactivity to a *S. mansoni* LOX-homologue. Our immunoblot analyses of the different *S. mansoni* extracts using antisera to mammalian lipoxygenases unveiled two LOX-specific protein bands with molecular masses comparable to those of mammalian and plant lipoxygenases (chapter 4, ref. 332). In addition, positive hybridization signals were detected by Southern hybridization of *S.*

mansoni genomic DNA using a human 15-lipoxygenase cDNA probe. Moreover, two lipoxygenase-like PCR fragments (which hybridized to the human 15-LOX probe) were amplified from the worm DNA using degenerate primers based on conserved sequences in mammalian and plant lipoxygenases. These results suggested the presence of more than one lipoxygenase protein and more than one lipoxygenase gene in adult *S. mansoni* parasites. On the other hand, our immunoblot, Southern hybridization, and PCR results suggested the potential of mammalian lipoxygenase cDNAs as useful probes to clone the *putative S. mansoni* lipoxygenase homologue.

As previously noted in other invertebrates, eicosanoids produced by *S. mansoni* parasites may have some important physiological actions in areas such as reproduction, neurophysiology, and salt and water transport physiology (289, 290). In particular, the production of 15-HETE by an *S. mansoni* lipoxygenase may be significant in the mediation of host-parasite interactions. 15-HETE has been reported to have immunosuppressant effects in mammals including the inhibition of mitogen-induced lymphocyte proliferation, the inhibition of neutrophil migration across cytokine-activated endothelium (a pivotal event in their recruitment to sites of inflammation), as well as the inhibition of various lymphocyte functions and the stimulation of suppressor cell activity (199, 318). In addition, 15-HETE has also been shown to decrease the formation of the proinflammatory LTB_4 by leukocytes, to antagonize neutrophil chemotaxis by LTB_4, and to suppress leukocyte activation (199, 357, 358). Thus, it could be postulated that adult *S. mansoni* parasites may use these immunoregulatory and anti-inflammatory effects of 15-HETE to suppress host cell-mediated immune responses. Since eicosanoids synthesized by cercariae of *S. mansoni* have been demonstrated to facilitate cercarial skin penetration and transformation, it is reasonable to suggest that host immunomodulation via eicosanoid production may also be utilized by adult parasites to help them establish chronic infections. Therefore, the full characterization of the enzymes and genes involved in eicosanoid metabolism in *S. mansoni* is imperative to our understanding of the exact role that these metabolites may play in the host-parasite relationship.

7.2 Attempts to clone a lipoxygenase-like homologue from *Schistosoma mansoni*

In the previous investigation, a lipoxygenase pathway with similar activity to mammalian 15-lipoxygenases was identified from adult *S. mansoni* extracts but no cyclooxygenase activity was detected (chapter 4, ref. 332). Developmental regulation of eicosanoid biosynthesis may occur in *S. mansoni* since the larval stage of the parasite has been shown to produce both cyclooxygenase and lipoxygenase-derived eicosanoids (304, 305). Thus, the molecular cloning of genes involved in arachidonic acid metabolism from *S. mansoni* is required to address the expression of these genes during the different stages of the parasite life cycle. In addition, following the cloning and expression of these genes, sufficient quantities of eicosanoids could be produced permitting an accurate determination of the chemical structure of the parasite-generated metabolites. Furthermore, the identification of *S. mansoni* genes implicated in eicosanoid production may also provide potential therapeutic targets for the treatment of schistosomiasis.

PCR amplification using degenerate primers based on conserved sequences in mammalian and plant lipoxygenases was utilized in order to obtain a partial LOX-like sequence from *S. mansoni* to assist in the cloning of the putative parasite lipoxygenase homologue. Several lipoxygenase-like PCR fragments which hybridized to a human 15-LOX cDNA probe were amplified from both *S. mansoni* mRNA and cDNA libraries (chapter 5). Subsequent to the cloning and sequence analysis of ten reverse transcription-PCR subclones, their deduced amino acid sequences revealed no significant homology to either mammalian or plant lipoxygenases. Similarly, none of the twenty subclones derived from the PCR amplification of *S. mansoni* DNA displayed any significant amino acid sequence identity to lipoxygenases. Evidently, the degenerate nature of the LOX-based primers allowed for the amplification of other non-lipoxygenase-specific sequences from *S. mansoni* mRNA and DNA. However, the specific hybridization of certain PCR fragments to the human 15-LOX probe strongly implied that some lipoxygenase-specific sequences had also been

amplified. In fact, a mixture of many PCR products was obtained and the difficulty in capturing LOX-specific sequences may have been due to the predominant presence of non-LOX-like sequences in this mixture leading to their preferential subcloning.

Taking into account the homology shared by plant and mammalian LOXs in their primary structures (143), and based on our previous identification of LOX-like proteins and sequences from *S. mansoni* using reagents based on mammalian lipoxygenases (chapter 4, ref. 332), we speculated that mammalian lipoxygenase sequences might detect an evolutionarily distant LOX-homologue from *S. mansoni*. However, conventional hybridization screening of three different *S. mansoni* cDNA libraries using several mammalian lipoxygenase cDNA probes failed to recognize any positive hybridization clones (chapter 5). Similarly, no LOX-like positive clones were detected from *S. mansoni* libraries when other probes including one of the LOX-based degenerate primers as well as a PCR clone amplified from *S. mansoni* DNA (which showed some sequence similarity to the human 15-LOX protein) were used for screening. The absence of identifiable LOX-like positive clones may have been due to a relative low abundance of the putative *S. mansoni* lipoxygenase transcript in the different *S. mansoni* libraries. On the other hand, some recently-cloned *S. mansoni* proteins such as glucose transporter proteins displayed only 30-35% amino acid sequence identity to their mammalian counterparts (359). Therefore, the inability of mammalian lipoxygenase probes to detect positive clones from *S. mansoni* libraries could have also been a result of insufficient sequence identity between mammalian lipoxygenases and the putative *S. mansoni* LOX-homologue.

Since low abundant transcripts could escape detection by conventional hybridization screening, PCR amplification using the lipoxygenase-based degenerate primers was utilized in order to ameliorate the representation of LOX-like sequences in *S. mansoni* libraries (chapter 5). The screening of one *S. mansoni* cDNA library by pool separation, PCR amplification, and hybridization to the human 15-LOX probe resulted in the cloning and subsequent sequencing of the mouse (the parasite host) leukocyte-type 12-lipoxygenase (166). Mice are

generally used as the definitive host to propagate *S. mansoni* parasites in experimental animals (304, 305, 307). It is well established that adult *S. mansoni* parasites ingest some host blood cells during their life span in the host blood as their final habitat (277). Consequently, any genetic material prepared from adult parasites could potentially carry some host contaminating sequences. In fact, minuscule amounts of contaminating murine sequences (representing ≈ 0.2% of all transcripts) are constantly present in all *S. mansoni* libraries prepared from adult parasites grown in mice (331). Since the parasites used in our study were also grown in mice, it is conceivable that the mouse 12-LOX transcript originated from mouse blood cells contaminating the adult *S. mansoni* mRNA preparation used to construct the cDNA library.

That the human 15-lipoxygenase cDNA probe was able to clone the mouse leukocyte-type 12-lipoxygenase is not unexpected considering the 85% sequence identity that they share in their primary structures (134, 143). On the other hand, the human 15-LOX probe as well as the other mammalian lipoxygenase probes were unsuccessful in detecting any contaminating LOX-murine sequences from *S. mansoni* libraries by conventional hybridization screening (chapter 5). This result indicated that the libraries used in our study contained only minute quantities of contaminating LOX-murine sequences. In fact, the contaminating mouse LOX-homologue was only detected in 6 out of 100 pools from one *S. mansoni* library only after extensive dilution of the library and the utilization of the powerful amplification of PCR techniques. In addition, no significant sequence homology to murine sequences was displayed by any of the 20 clones amplified from *S. mansoni* DNA. Furthermore, only 1 out of 10 translated *S. mansoni* RT-PCR clones revealed low amino acid sequence homology to a known murine peptide present in the SwissProt database (chapter 5). In all, these results strongly suggested the presence of only negligible amounts of contaminating murine sequences in either *S. mansoni* DNA, mRNA, or cDNA libraries. Hence, this minor contamination with murine sequences should not hinder the isolation of specific *S. mansoni* genes providing the use of

a specific probe that can differentiate between parasite and mammalian sequences.

In an attempt to obtain a specific *S. mansoni* probe in order to facilitate the cloning of the putative *S. mansoni* LOX-homologue, the different databases were searched for a partial *S. mansoni* lipoxygenase-like sequence. However, the small schistosome genome (270 Mb) is still poorly characterized with only 1% of its total potential genetic information being described (331). Therefore, the inability to identify a partial *S. mansoni* LOX-like sequence was not surprising considering the limited number of *S. mansoni* cDNA sequences currently deposited in the databases. In contrast, the phylogenetically-related nematode worm *C. elegans* is one of the best genetically-characterized organisms with more than 85% of its 100 Mb genome already sequenced as a result of the *C. elegans* Genome Sequencing Project (313). Consequently, both *C. elegans* DNA and expressed sequence tags (ESTs) databases are now available for accessible genetic information. Since several *S. mansoni* PCR clones displayed significant DNA sequence homology to different *C. elegans* cosmid clones (chapter 5), we speculated that if a LOX-like sequence could be identified from *C. elegans*, it could then be utilized as a useful probe to clone the putative *S. mansoni* LOX-homologue.

No sequences related to mammalian lipoxygenases or cyclooxygenases were found following the search of the *C. elegans* databases for arachidonic acid metabolizing enzyme-like sequences. On the other hand, a small (327 bp) EST with 51% amino acid-sequence homology to the human leukotriene A_4 (LTA_4) hydrolase was identified. We have previously described an immunoreactive protein with the same size as 5-LOX (the enzyme that synthesizes LTA_4, the substrate for LTA_4 hydrolase) from adult *S. mansoni* extracts using antisera to mammalian LOXs (chapter 4, ref. 332). In addition, adult *S. mansoni* parasites have also been shown to produce a metabolite displaying the same chromatographic characteristics as leukotriene B_4 (307). Thus, it was reasonable to postulate that adult *S. mansoni* may contain an LTA_4 hydrolase activity resulting in the production of the proinflammatory substance LTB_4 (3).

Subsequent to obtaining the complete sequence (0.95 kb) of the *C. elegans* LTA_4 hydrolase-like EST (chapter 6, ref. 363) and its use as a probe to screen two *S. mansoni* libraries, no positive plaques were identified by conventional hybridization screening (chapter 5). Furthermore, no PCR fragments were amplified from either *S. mansoni* mRNA or cDNA libraries following the use of oligonucleotide primers generated based on conserved sequences between mammalian LTA_4 hydrolases and the *C. elegans* LTA_4 hydrolase-like homologue (chapter 6, ref. 363). These results implied that either the *C. elegans* sequence did not share significant sequence homology to a putative *S. mansoni* LTA_4 hydrolase-like homologue, or that such a homologue was not present in adult *S. mansoni* parasites.

Although it could be argued that the lipoxygenase activity we observed in adult *S. mansoni* extracts was a result of a possible contamination from murine blood cells, it is unlikely based on several lines of evidence. Our analysis of arachidonic acid metabolites synthesized by *S. mansoni* extracts indicated the presence of an active LOX and the absence of any COX activity despite the fact that murine blood cells contain both cyclooxygenases and lipoxygenases. Thus, if our *S. mansoni* extracts were contaminated with murine blood cells, then the extracts, DNA, and mRNA preparations would be expected to contain not only lipoxygenase, but also murine cell-derived cyclooxygenase activity, cyclooxygenase immunoreactive protein, and PCR-amplifiable sequences. However, our results showed no production of prostaglandins and no specific COX-protein bands or sequences were detected using immunoblot and PCR analysis (chapter 4, ref. 332). In addition, none of our PCR clones displayed any significant sequence homology to murine sequences and mammalian lipoxygenase probes were unable to detect any contaminating LOX-murine sequences from *S. mansoni* libraries by conventional hybridization screening. Our results further indicated that only negligible amounts of contaminating LOX-murine sequences were found in one *S. mansoni* library. Furthermore, lipoxygenase-derived eicosanoids were shown to be produced by cercariae of *S. mansoni* which had no previous contact with any mammalian host suggesting

that *S. mansoni* parasites may in fact possess a genuine lipoxygenase activity (304, 305).

Since arachidonic acid metabolites produced by *S. mansoni* cercariae have been shown to participate in cercarial skin penetration and transformation (302, 304, 305), a recent study explored the effects of two COX inhibitors (ibuprofen and diclofenac sodium) on the pathogenesis of experimental murine schistosomiasis. Treatment of *S. mansoni*-infected mice with either of these two non-steroidal anti-inflammatory drugs (NSAIDs) was effective in reducing the severity of infection (as indicated by a significant decrease in both liver weights and worm loads) and in attenuating hepatic fibrosis (360). The importance of such beneficial effects on the course of schistosomiasis in humans is unclear, however, their demonstration in mice given drug doses close to those used in human therapy seems to hold considerable promise. Nonetheless, during the early stages of schistosomiasis, the granulomatous response to viable ova trapped in the host tissues involves an immunological cellular reaction of the delayed-hypersensitivity type (277). NSAIDs are known to inhibit monocyte chemotaxis and leukocyte migration to inflammatory sites (361), and some NSAIDs have been shown to suppress the inflammatory delayed-hypersensitivity reaction in schistosome-infected mice leading to a reduction in the size of schistosomal pulmonary granulomas (362). Therefore, the reduction in the severity of hepatic fibrosis in infected mice treated with ibuprofen or diclofenac sodium (360) could have been the result of the NSAIDs' modulation of the host immune responses to schistosome eggs trapped in the liver. Hence, any significant effect that *S. mansoni*-derived eicosanoids might have on the pathogenesis of schistosomiasis will remain questionable till these parasitic metabolites as well as the enzymes involved in their biosynthesis are completely described.

Altogether, our results revealed the difficulty of using mammalian cDNA sequences to isolate genes from mammalian parasites and strongly emphasized the requirement of a *S. mansoni* LOX-like partial sequence in order to clone the putative *S. mansoni* lipoxygenase-homologue. In 1992, the *Schistosoma*

mansoni Genome Project started in Brazil with the systematic sequencing of cDNA clones selected at random from *S. mansoni* libraries. In the first two years, the initiative yielded more than 600 useful ESTs (331). These results encouraged researchers from other countries to join the project with the consequence that more than 2000 ESTs have been sequenced and deposited in the databases. With the adaptation of the different strategies being used in the Human Genome Project for the study of the schistosome genome, a speedy identification of new *S. mansoni* genes is anticipated in the near future. Therefore, the potential future identification of partial *S. mansoni* sequences homologous to any of the arachidonic acid metabolizing-enzymes would unquestionably provide ideal probes for the cloning of such enzymes from adult parasites. Until these enzymes are cloned and thoroughly characterized, their presence in *S. mansoni*, the part they might play in the mediation of host-parasite interactions, along with their prospect as future targets for immunoprophylaxis and chemotherapy will continue to be uncertain.

7.3 Cloning and functional expression of a *C. elegans* aminopeptidase structurally related to mammalian leukotriene A_4 hydrolases

During our search of the *C. elegans* databases for arachidonic acid metabolizing enzyme-like sequences, an EST of 327 bp (termed cm01c7) with strong homology to the human LTA_4 hydrolase was identified, but failed to recognize any homologous sequences from *S. mansoni* libraries. In contrast, the use of the complete sequence of cm01c7 (0.95 kb) as a probe, together with conventional hybridization screening, resulted in the isolation of a 1.4 kb clone from a *C. elegans* mixed stage cDNA library that revealed a strong sequence homology (≈ 45%) to the human LTA_4 hydrolase (chapter 6, ref. 363). Sequence homology comparisons with previously cloned LTA_4 hydrolases (236-239) indicated that the *C. elegans* clone was missing the 5' part of the cDNA. The subsequent use of a series of anchored PCR reactions led to the isolation of the missing 5'-end of the *C. elegans* clone. The full-length 2.1-kb *C. elegans* LTA_4

hydrolase-like homologue (termed AP-1) consisted of a short 15-bp 5'-untranslated region, an open reading frame encoding a 609-amino acid protein, a 282-bp-long 3'-untranslated region, and a 28-bp-long poly(A^+) tail (chapter 6, ref. 363). In agreement with the sequences of the previously identified mammalian LTA_4 hydrolases, no consensus N-glycosylation sites, targeting signals or putative phosphorylation sites were detected in the *C. elegans* AP-1 sequence. In addition, the length of the AP-1 open reading frame (1827 bp), the number of the deduced amino acids (609 residues), and the calculated molecular mass of the encoded protein (68,248 kDa) were comparable to the human (1830 bp, 610 residues, 69,140 kDa) and the mouse (1830 bp, 610 residues, 68,917 kDa) LTA_4 hydrolases (236, 237).

Data base searches demonstrated that the human LTA_4 hydrolase was the most closely related protein to the *C. elegans* AP-1 translation product with 63% similarity and 45% identity at the amino acid level. In addition, a multiple alignment of the amino acid sequences of the human LTA_4 hydrolase, the mouse LTA_4 hydrolase, and the putative *C. elegans* homologue revealed the conservation of the zinc-binding motif ($HEXXHX_{18}E$) in the primary structure of the *C. elegans* AP-1 protein. The presence of this motif, which is also located in several metallopeptidases, allowed for the classification of the *C. elegans* enzyme under the M1 family of metalloexopeptidases (350). Members of this family also include LTA_4 hydrolase, aminopeptidase A, aminopeptidase N, and cysteine aminopeptidase. The three zinc-binding residues His^{295}, His^{299}, and Glu^{318} (in both the human and the mouse LTA_4 hydrolase sequences), shown previously to be essential for both the epoxide hydrolase and the aminopeptidase activity of the mammalian LTA_4 hydrolase (243), were conserved in the *C. elegans* sequence. Accordingly, His^{297}, His^{301}, and Glu^{320} (in the *C. elegans* amino acid sequence) corresponded to the previously-identified catalytic zinc site of mammalian LTA_4 hydrolases and zinc metallopeptidases (240). Hence, these three residues were postulated to be involved in the coordination of the zinc atom as described earlier for the mouse LTA_4 hydrolase (243) and for certain peptidases and neutral proteases (240). Furthermore, Tyr^{383}

(conserved in both human and mouse LTA_4 hydrolases), which is essential for the peptidase activity of the mammalian LTA_4 hydrolase/aminopeptidase and is proposed to act as a proton donor in a general base mechanism (251), was conserved in the *C. elegans* AP-1 sequence. Similarly, Glu^{296} in both human and mouse sequences, the mutation of which to Gln^{296} has been shown to abolish the aminopeptidase activity of the mammalian enzyme (250), was also conserved in the *C. elegans* sequence. Conversely, Tyr^{378} in both human and mouse LTA_4 hydrolases, which is known to be involved in the covalent binding of LTA_4 (257), was replaced by a phenylalanine (Phe^{382}) in the *C. elegans* sequence.

Following the identification of two cosmid clones (from the *C. elegans* genomic data base) that showed a 100% match to the cloned *C. elegans* AP-1 cDNA, a map of the structure of the *C. elegans* aminopeptidase gene was constructed (chapter 6, ref. 363). The gene was localized to *C. elegans* chromosome 4 with the entire open reading frame of the *C. elegans* AP-1 cDNA contained within four exons ranging in size from 53 to 1325 bp. The small size of the introns, the determined sequences of the exon-intron boundaries, and the elevated A-U content of all three introns, were characteristic of previously described *C. elegans* introns (351). The proposed zinc-binding histidine residues (His^{297} and His^{301}) and glutamate residue (Glu^{320}), which constituted the zinc-binding domain ($HEXXHX_{18}E$), were located on exon 3 in the *C. elegans* gene. This segregation of the zinc-binding motif to one exon is not seen in the structure of the human LTA_4 hydrolase/aminopeptidase gene (258), in which the two essential zinc-binding histidine residues (His^{295} and His^{299}) are present on exon 10, whereas the third zinc-binding ligand glutamate (Glu^{318}) is located on another exon (exon 11).

Subsequent to the transient expression of the *C. elegans* AP-1 cDNA as an epitope-tagged (FLAG) recombinant protein in COS-7 cells, and immunoblot analysis using an anti-FLAG antibody, the 69-kDa-*C. elegans* AP-1 was mainly detected as a soluble protein in the cell cytosol with minimal presence in either the microsomal or the membrane fractions. This was in agreement with the

cytosolic localization of other previously described mammalian LTA_4 hydrolase/aminopeptidase enzymes (226, 227). On the other hand, despite the significant amino-acid sequence homology shared by the *C. elegans* AP-1 and the human LTA_4 hydrolase/aminopeptidase, the use of an antiserum to the human LTA_4 hydrolase, failed to detect any LTA_4 hydrolase specific immunoreactive proteins in either the supernatant of COS-7 cells transfected with the FLAG-tagged *C. elegans* AP-1 cDNA or the cytosolic extract from *C. elegans* worms. Following the purification of the expressed FLAG-tagged *C. elegans* AP-1 protein using an anti-FLAG antibody affinity resin, the fractions containing the purified protein (as detected by an anti-FLAG antibody on Western blots) were tested for aminopeptidase and LTA_4 hydrolase activities.

The purified *C. elegans* AP-1 FLAG fusion protein possessed an intrinsic aminopeptidase activity as manifested by its ability to efficiently hydrolyze a variety of amino acid *p*-nitroanilide derivatives. In addition, the recombinant *C. elegans* AP-1 had a similar substrate specificity to the human LTA_4 hydrolase/aminopeptidase with a preference for the arginyl-*p*-nitroanilide derivative as a substrate, whereas acidic amino acids, amino acids with NH_2-terminal substitutions, or with *D*-stereochemistry were poor substrates. Moreover, the hydrolysis of the amide bond of L-arginine-*p*-nitroanilide by *C. elegans* AP-1 was inhibited by bestatin, a potent inhibitor of human LTA_4 hydrolase/aminopeptidase (245), as well as other aminopeptidases. In contrast, the *C. elegans* AP-1 had no LTA_4 hydrolase activity as detected by both reverse-phase HPLC and radioimmunoassay for LTB_4 production which revealed the inability of AP-1 to use LTA_4 as a substrate (chapter 6, ref. 363). Furthermore, the cytosolic extract prepared from *C. elegans* worms was also devoid of any LTA_4 hydrolase activity. Although the high degree of identity shared between the active sites of AP-1 and mammalian LTA_4 hydrolases implied that LTA_4 may bind in the active site of AP-1 without being a substrate for catalysis, the inability of LTA_4-ethyl ester to inhibit the aminopeptidase activity of AP-1 strongly suggested that LTA_4 did not bind to the active site of AP-1.

During catalysis, the bifunctional human LTA_4 hydrolase/aminopeptidase enzyme is suicide inactivated by a mechanism-based irreversible binding of the substrate LTA_4 to the protein (234), and Tyr^{378} has been identified as the site for the covalent binding of LTA_4 (256). The mutation of this Tyr^{378} to Phe^{378} in the human LTA_4 hydrolase produced an enzyme that had an increased turnover but was resistant to mechanism-based inactivation, therefore dissociating catalysis and covalent modification/inactivation events (257). Interestingly, although this tyrosine residue is also a phenylalanine in the *C. elegans* AP-1 (Phe^{382}), the *C. elegans* enzyme failed to hydrolyze LTA_4. This result clearly indicated that residues, other than the previously identified Tyr^{378}, which are present in the human LTA_4 hydrolase but are lacking in the *C. elegans* AP-1 sequence, must also be essential for LTA_4 binding and catalysis. On the other hand, the PCR-introduced mutation of Ala^{117} to Val^{117} in the *C. elegans* AP-1 abrogated the aminopeptidase activity of the *C. elegans* enzyme (chapter 6, ref. 363). This alanine residue (Ala^{114} in the human LTA_4 hydrolase) is evolutionary conserved since it is found in the *Candida albicans* LTA_4 hydrolase (which mainly exhibits aminopeptidase activity), the *Saccharomyces cerevisiae* proposed LTA_4 hydrolase (which is not yet characterized), all cloned mammalian LTA_4 hydrolases including human, mouse, rat, and guinea pig (236-239), as well as in the *C. elegans* AP-1. Thus, it is conceivable that certain conserved residues, other than the previously reported Tyr^{383} and Glu^{296}, may also be important for the aminopeptidase activity of the mammalian LTA_4 hydrolase/aminopeptidase.

It was interesting to observe that the cloned *C. elegans* AP-1 enzyme only functioned as an aminopeptidase with no LTA_4 hydrolase activity since its primary structure is more homologous to LTA_4 hydrolases than it is to other zinc aminopeptidases. In fact, the *C. elegans* AP-1 enzyme is 45% identical (at the amino acid level) to other mammalian LTA_4 hydrolases and this identity extends over their entire primary structures. Conversely, the *C. elegans* AP-1 shares lower identity (28-30%) with other aminopeptidases and this identity is limited to a small region which contains the canonical zinc-binding motif $HEXXHX_{18}E$ (chapter 6, ref. 363). Moreover, the same identity that was shown between *C.*

elegans AP-1 and other aminopeptidases was also seen between LTA_4 hydrolases and any given aminopeptidase enzyme. This structural similarity between *C. elegans* AP-1 and mammalian LTA_4 hydrolases suggested an evolutionary relationship. Furthermore, three other proteins that are structurally related to mammalian LTA_4 hydrolases have recently been identified in lower invertebrates. These proteins include an enzyme from the pathogenic yeast *Candida albicans* (with 41% identity to human LTA_4 hydrolase) that displayed mainly aminopeptidase activity but failed to hydrolyze LTA_4 to LTB_4 (355), a gene from the yeast *Saccharomyces cerevisiae* (with 39% identity to human LTA_4 hydrolase) (260), and a partial amino acid sequence (316 residues) from the slime mold *Dictyostelium discoideum* (with 38% identity to human LTA_4 hydrolase) (GenBank accession number U27538). However, the enzymatic activity(s) of both the *S. cerevisiae* (260) and the *D. discoideum* LTA_4 hydrolase-like proteins is still unknown since they have not yet been expressed or characterized. On the other hand, neither *C. albicans* nor *D. discoideum* has been reported to produce LTB_4, and our analysis of mixed stage *C. elegans* worms revealed no LTA_4 hydrolase activity. Therefore, the LTA_4 hydrolase-like enzymes that have been cloned and characterized to date from lower invertebrates appear to only display aminopeptidase activity. Based on the high primary sequence identity between the *C. elegans* AP-1 and mammalian LTA_4 hydrolases, it could be postulated that AP-1 may represent an evolutionary predecessor of the mammalian LTA_4 hydrolases. Consequently, mammalian LTA_4 hydrolases may have originated from aminopeptidases like AP-1, maintaining their aminopeptidase function and developing a LTA_4 hydrolase activity in higher organisms. This hypothesis is further strengthened by the recent cloning of an aminopeptidase B from rat testes which shows highest homology to mammalian LTA_4 hydrolases (44%), intermediate homology to *C. elegans* AP-1 (38%), and lowest homology to mammalian N-type aminopeptidases (21-24%) (chapter 6, ref. 363), and can utilize LTA_4 to produce LTB_4, albeit at ≈ 10% of the efficiency of LTA_4 hydrolase (259).

The dual activity of the mammalian LTA_4 hydrolase/aminopeptidase remains poorly understood since no physiological substrate for its aminopeptidase activity has been discovered. However, based on the ability of the human LTA_4 hydrolase/aminopeptidase enzyme to efficiently hydrolyze several synthetic tri-peptides, the enzyme was suggested to be involved in the metabolism of dietary peptides and neuropeptides (248), a role that could also be proposed for the *C. elegans* AP-1 enzyme. *C. elegans* provides an attractive model to study any gene at the functional level since it is one of the best genetically characterized organisms with a complete physical map and mutants for more than 2000 genetic loci (313, 314). Thus, as previously shown for other *C. elegans* genes, the use of genetic techniques such as gene knockout and gene inhibition using antisense RNA can easily generate loss of function mutants for the *C. elegans* AP-1 gene (314). The subsequent study of the phenotype of AP-1 null mutants could lead to the discovery of the peptide substrate for the *C. elegans* enzyme which may also help in identifying the physiological peptide substrate for the aminopeptidase activity of the mammalian LTA_4 hydrolase/aminopeptidase enzyme. In view of the potent proinflammatory effects of LTB_4 (3, 261, 262), LTA_4 hydrolase is regarded as a potential pharmacological target for the treatment of a variety of inflammatory disorders. Therefore, the study of LTA_4 hydrolase homologues from different organisms may also lead to a better understanding of the structural and functional properties of the mammalian enzyme which can ultimately result in the development of novel anti-inflammatory drugs.

Overall, our analysis of arachidonic acid metabolism by the adult trematode parasite *S. mansoni* led to the identification of an enzymatically active lipoxygenase pathway similar in its activity to mammalian 15-lipoxygenases. On the other hand, no cyclooxygenase activity was detected in *S. mansoni* extracts and there was no evidence to suggest the presence of a cyclooxygenase homologue in adult parasites. Our results further revealed the difficulty of using mammalian cDNA sequences as probes to isolate metazoan genes and indicated the requirement of a lipoxygenase-like partial sequence from *S. mansoni* in order

to clone the parasite lipoxygenase-homologue. We have also cloned and functionally expressed a 69-kDa protein from the free-living nematode worm *C. elegans*, the primary structure of which is more homologous to mammalian LTA_4 hydrolases than other zinc aminopeptidases. This protein displayed an efficient aminopeptidase activity with broad substrate specificity but lacked any LTA_4 hydrolase activity. Hence, the study of arachidonic acid metabolizing enzymes in nematodes and trematodes can expand our understanding of the evolutionary conservation of the structure and function of these important regulatory proteins in lower organisms. Finally, the characterization of arachidonic acid oxygenases from parasitic nematodes and trematodes may also lead to novel therapeutic targets for the treatment of human parasitic diseases.

8. ORIGINAL CONTRIBUTION TO THE LITERATURE

This thesis demonstrates for the first time that:

1. Adult *S. mansoni* parasites possess a soluble lipoxygenase activity similar to mammalian 15-lipoxygenases which can catalyze the formation of a 15-hydroxyeicosatetraenoic acid-like-species from arachidonic acid and a 13-hydroxyoctadecadienoic acid-like product from linoleic acid. This *S. mansoni* lipoxygenase activity was calcium-independent and was inhibited by inhibitors of mammalian and plant lipoxygenases.

2. Two immunoreactive proteins with molecular masses similar to plant and mammalian lipoxygenases were detected from adult *S. mansoni* extracts using antisera to different mammalian lipoxygenases. Several lipoxygenase-like PCR fragments were amplified from adult *S. mansoni* DNA, mRNA, and cDNA libraries using degenerate primers based on conserved plant and mammalian lipoxygenase sequences.

3. There was no cyclooxygenase activity in *S. mansoni* extracts and no evidence for the presence of a cyclooxygenase homologue in adult parasites.

4. A 69-kDa protein with 45% identity to the human LTA_4 hydrolase (at the amino acid level) was cloned from *C. elegans* and functionally expressed in mammalian cells.

5. The primary structure of the cloned *C. elegans* AP-1 protein is more homologous to mammalian LTA_4 hydrolases than it is to other zinc aminopeptidases.

6. The *C. elegans* AP-1 enzyme functioned as an aminopeptidase with broad substrate specificity but lacked any LTA_4 hydrolase activity.

7. The aminopeptidase activity of the *C. elegans* AP-1 was inhibited by bestatin and was similar to that of the human LTA_4 hydrolase/aminopeptidase enzyme with a preference for arginyl-*p*-nitroanilide as a substrate.

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APPENDIX A-Supplemented data to chapter 4

This appendix describes data that was previously mentioned in the original manuscript (chapter 4, ref. 332) as data not shown.

The inhibitory effect of three different classes of lipoxygenase inhibitors on the production of the 15-HETE-like species by adult *S. mansoni* extracts

The incubation of adult *S. mansoni* cytosolic extracts with radiolabeled arachidonic acid followed by the analysis of the synthesized products using thin-layer chromatography indicated the formation of a major metabolite which co-chromatographed with an authentic 15-HETE standard. Since 15-HETE could be produced from arachidonic acid by the action of both cyclooxygenase-2 and 12/15-lipoxygenases, both cyclooxygenase and lipoxygenase inhibitors were used to delineate the pathway responsible for the enzymatic activity detected in adult *S. mansoni* extracts. Unlike the inability of indomethacin (a potent COX inhibitor) to inhibit *S. mansoni* 15-HETE production, the use of three different LOX inhibitors resulted in a dose-dependent inhibition of the formation of the 15-HETE-like species by *S. mansoni* extracts which confirmed the lipoxygenase origin of this metabolite. These three inhibitors included ETYA (5,8,11,14-eicosatetraynoic acid), a known suicide substrate inhibitor of lipoxygenases which can also inhibit cyclooxygenases, L-670,630 (2,3-dihydro-6-(3-phenoxypropyl)-2-(2-phenylethyl)-5-benzofuranol), a phenolic redox inhibitor of lipoxygenases, and CPHU (*N*-(4-chlorophenyl)-*N*-hydroxy-*N'*-(3-chlorophenyl) urea), one of the N-hydroxyurea class of lipoxygenase redox inhibitors (Table 1). The three LOX inhibitors are non specific and can inhibit 5-, 12-, and 15-lipoxygenases. The three inhibitors showed similar pharmacological profiles to those previously seen with different plant and mammalian lipoxygenases as indicated by their calculated IC_{50} values (Table 1). Figure 1 represents the dose-response study of the inhibitory effect of L-670,630 which was repeated for the other two LOX inhibitors ETYA and CPHU with similar results.

L-670,630 (Merck Frosst) was dissolved in DMSO. Adult *S. mansoni* cytosolic fractions (15 µg total protein/fraction) were preincubated with either DMSO or different concentrations of L-670,630 (0.05 to 10 µM) for 15 min and the reaction was initiated by adding an ethanol solution of 0.2 µCi [^{14}C(U)]-arachidonic acid (866 mCi/mmol; 0.46 µM final concentration) (New England Nuclear, Boston, MA). Assays were carried out in a 100 µl final volume in 0.1 M Tris, pH 8.0, containing 5 mM EDTA, 1 µM hematin, and 1 mM homovanillic acid. Following 1 hour incubations at 37°C, the reactions were then quenched with 50 µl methanol and applied to silica gel thin-layer chromatography plates (Whatman). Authentic 15-HETE and arachidonic acid standards (Cayman) were run in parallel and the thin layer chromatography plates were developed with ether/petroleum ether/acetic acid (50:50:1) and visualized by autoradiography. The IC_{50} was calculated after scanning the autoradiographs using a computing densitometer (Molecular Dynamics, Sunnyvale, CA).

Figure 1. Dose-response study of the inhibitory effect of the lipoxygenase inhibitor L-670,630. Thin layer chromatography of products formed from [^{14}C]-arachidonic acid (0.46 µM) by *S. mansoni* cytosol (15 µg) in the absence (lane 1) or presence (lane 2) of DMSO vehicle or 0.5-10 µM L-670,630 dissolved in DMSO (lanes 3-7). The reaction products were extracted, separated on silica gel thin-layer chromatography plates, developed with ether/petroleum ether/acetic acid and visualized by autoradiography. The positions of co-chromatographed standards are indicated. The autoradiogram shown is a representative of five separate experiments with similar results.

Table 1. Inhibitory potencies of three different classes of lipoxygenase inhibitors. Different concentrations (ranging from 0.05 to 10 µM) of ETYA, L-670,630, and CPHU were used to estimate their concentration of half inhibition (IC_{50}) of the production of 15-HETE. Adult *S. mansoni* cytosolic fractions (15 µg total protein/fraction) were treated with the different concentrations of the inhibitors for 15 minutes followed by incubation with [^{14}C]-arachidonic acid under

the reaction conditions described earlier. The reaction products were spotted on thin-layer chromatography plates and the plates were subjected to autoradiography. Autoradiographs were scanned for 15-HETE production and the IC_{50} for each inhibitor was calculated following the construction of dose-response curves. The chemical structure of the inhibitors and the number of experiments performed (N) are indicated.

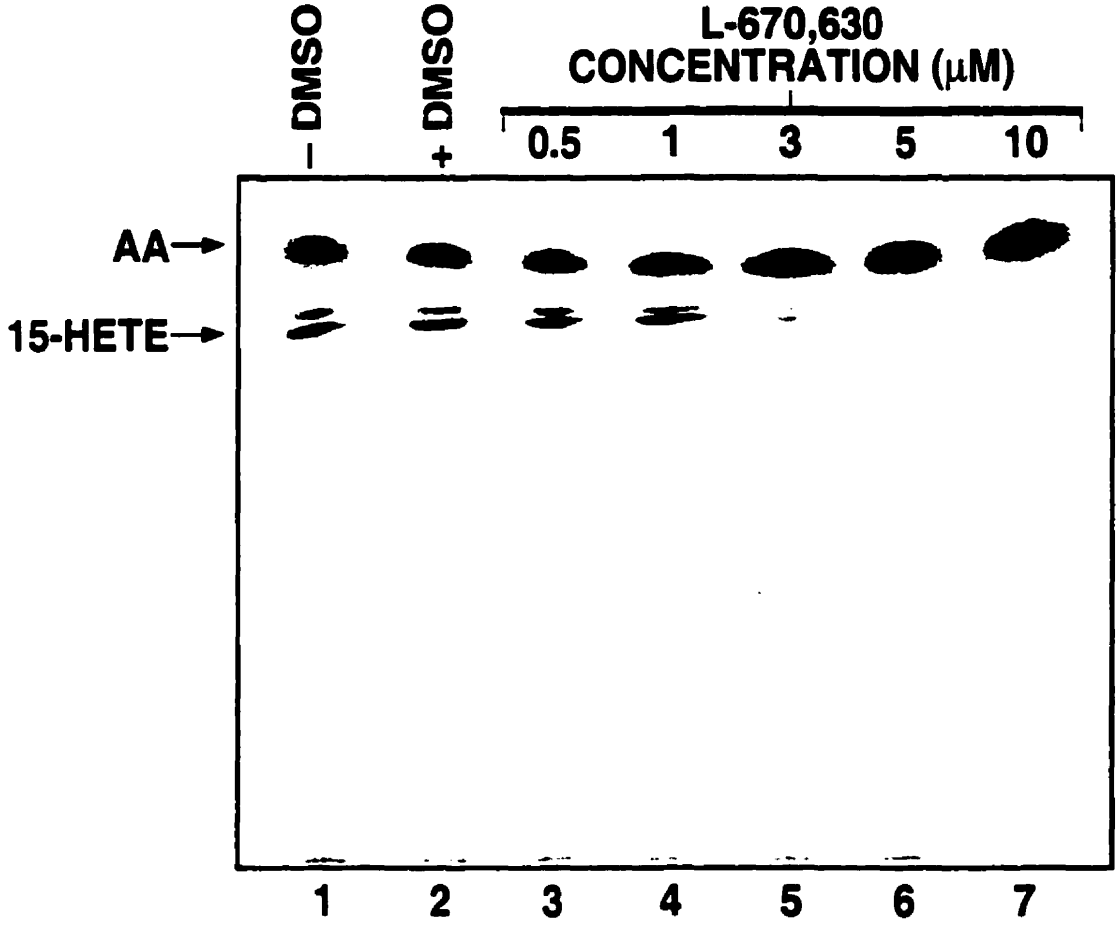

- DMSO
+ DMSO
L-670,630
CONCENTRATION (μM)
0.5
1
3
5
10
AA
15-HETE
1
2
3
4
5
6
7

Figure 1

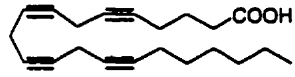
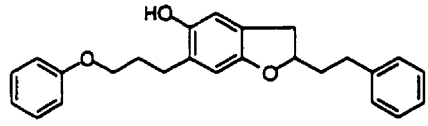
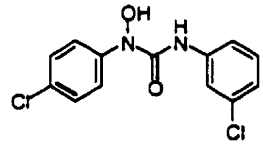
Inhibitor	Chemical structure	IC_{50} (μM)	N
ETYA		2 ± 0.07	5
L-670,630-00E		3 ± 0.06	5
CPHU L-685,015-000S		0.08 ± 0.003	5

Table I

This appendix describes data that was not mentioned in the original manuscript (chapter 6, ref. 363) or was mentioned as data not shown.

Identification of a *C. elegans* expressed sequence tag (EST) as a putative leukotriene A_4 hydrolase-like sequence

In a search of the *C. elegans* DNA data base for arachidonic acid metabolizing-enzymes, a small EST (GenBank accession number M88793) clone of 327 base pairs (termed cm01c7) was identified from a *C. elegans* mixed stage hermaphrodite cDNA library (made by Chris Martin in the lambdaphage vector SHLX2). The nucleotide sequence of cm01c7 was translated using the GCG sequence analysis software (Genetic Computing Group, Madison, WI) and the resulting peptide was compared to protein sequences in the SwissProt data base (release 96) using the BLASTP algorithm. The BLASTP search (National Centre for Biotechnology Information) identified the human LTA_4 hydrolase as the most closely related protein to the *C. elegans* cm01c7 translated product (with 51% identity) followed by LTA_4 hydrolases from mouse, rat, and guinea pig (Figure 1). The homology between the *C. elegans* EST and the human LTA_4 hydrolase was seen in a region that contained the tyrosine residues Tyr^{383} (in the human sequence) which is essential for the peptidase activity of the human LTA_4 hydrolase/aminopeptidase and Tyr^{378} which is involved in the covalent binding of LTA_4 to the human LTA_4 hydrolase.

Figure 1. Identification of the *C. elegans* cm01c7 EST as a putative leukotriene A_4 hydrolase-like sequence. A search of the annotated files in GenBank using the key words "leukotriene hydrolase" yielded an EST clone termed cm01c7 from a *C. elegans* cDNA library. The nucleotide sequence (327 bp) of the identified *C. elegans* cm01c7 EST is indicated on top. A description of the *C. elegans* cDNA library from which the EST was obtained is also shown. An

alignment of the translated cm01c7 peptide with the human LTA_4 hydrolase is shown at the bottom. Amino acid sequences are shown in one-letter code. The top sequence represents the sequence of the translated cm01c7 *C. elegans* EST and the bottom sequence represents the human LTA_4 hydrolase protein.

***In vitro* transcription/translation of the full-length *C. elegans* LTA_4 hydrolase-like homologue and purification using nickel affinity chromatography**

Following the cloning of the full-length (2.1 kilobase) *C. elegans* LTA_4 hydrolase-like cDNA clone (termed clone 11), different recombinant expression systems were used to express the full-length clone as a wild-type and an epitope-tagged protein before it was successfully expressed as a FLAG-tagged recombinant protein in COS-7 mammalian cells. *C. elegans* clone 11 was originally subcloned in three prokaryotic plasmid expression vectors including the pKK388-1 vector (Clontech) (which contains no epitope tags), and the 6 histidine-tag-containing vectors pPRoEX HTa (Life Technologies Inc.) and PTrcHis C (Invitrogen). Expression of the recombinant proteins was analyzed in two *E.coli* strains including DH5α (Gibco BRL) and XL-1 blue (Stratagene) cells (according to the manufacturer's instructions). No protein expression was seen following SDS-polyacrylamide gel electrophoresis and Coomassie staining. In the case of the histidine-tagged proteins, no expression was detected by immunoblot analysis using an anti-hisitdine tag antibody (Invitrogen) before or after affinity chromatography purification of bacterial cell lysates using the metal-chelating ProBond resin (Invitrogen).

In another attempt to express the *C. elegans* protein, *C. elegans* clone 11 was subcloned into the mammalian expression vector pcDNA 3.1/His A (Invitrogen) (which contains the T7 promoter to allow for *in vitro* transcription, an Anti-Xpress epitope tag for protein detection, and a polyhistidine metal-binding tag for purification of the recombinant histidine-tagged protein by nickel-affinity chromatography), and expressed transiently in different mammalian cells

including COS-7, CHO-K1, and HEK-293 cells (American Type Culture Collection). Cells transfected with the control plasmid pcDNA3.1/His/*lacZ* expressed the histidine-tagged β-galactosidase gene whereas the pcDNA 3.1/His/clone 11-transfected cells did not express the expected histidine-tagged *C. elegans*-fusion protein as verified by immunoblot analysis using an Anti-Xpress antibody (invitrogen) before or after the purification of cell lysates using nickel Ni-NTA affinity columns (Qiagen).

In order to confirm that the sequence of the cloned *C. elegans* clone 11 was complete and could be transcribed and translated, *in vitro* transcription/translation was performed using the TNT®T7 Coupled Reticulocyte Lysate System (Promega). Assays were carried out for 2 h at 30°C using 1 µg of pcDNA 3.1/His/clone 11, 1 µg of pcDNA3.1/His/*lacZ*, or 1 µg of the luciferase-encoding control plasmid, 25 µl rabbit reticulocyte lysate, 2 µl reaction buffer, 1 µl T7 RNA polymerase, 20 µM amino acid mixture minus methionine, 40 units of Rnasin® ribonuclease inhibitor, and 40 µCi ^{35}S-methionine (1000Ci/mmol) (Amersham) in a 50 µl final volume of nuclease-free water. Small aliquots of each reaction were then separated on 10% polyacrylamide gels (Novex) and analyzed by SDS-PAGE. The gels were fixed in 10% methanol/7% acetic acid, dried for 4 h at 60°C, exposed to Kodak X-OMAT X-ray films for 3-5 h and then developed. As expected from the calculated molecular mass for the translation product of *C. elegans* clone 11, a 69-kDa protein was transcribed and translated *in vitro* (Figure 2A, lanes 4 and 8), the 121-kDa β-galactosidase (Lac Z, lanes 3 and 7) and the 61-kDa luciferase (lanes 6 and 10) control proteins were also expressed. To verify the presence of the histidine tag in the *in vitro*-expressed *C. elegans* protein, the *in vitro* transcription/translation reactions were purified on nickel Ni-NTA spin columns (Qiagen) under non-denaturing conditions and eluted using 250-500 mM imidazole (as described by the manufacturer). The columns eluates were counted for radioactivity and also analyzed by SDS-PAGE. As shown in Figure 2B, almost all of the control histidine-tagged Lac Z fusion protein was bound to the nickel affinity resin column since only a small amount

was detected in the flow-through from the column (lane 3) and most of the protein was detected in the eluate (lane 4). On the other hand, almost all of the *C. elegans in vitro*-translated protein was detected in the flow-through from the nickel column (lane 6) with minimal detection in the column eluate (lane 7). This result indicated that the expressed *C. elegans* protein did not contain a histidine tag to allow for its efficient binding to the nickel column. Since the cloning strategy of *C. elegans* clone 11 in either the prokaryotic vectors or in the mammalian vector pcDNA 3.1/His A was such that the translation initiation codon from the *C. elegans* clone 11 sequence was not removed, it is likely that protein translation was preferentially initiated from the *C. elegans* ATG rather than the ATG supplied by the different expression vectors which precede the histidine tag. This could explain the absence of the histidine tag from the *in vitro*-translated *C. elegans* clone 11 protein and possibly from the *C. elegans* protein expressed in bacterial cells. The *C. elegans* translation initiation codon was later abolished to force translation to start from the ATG supplied by the pFLAG CMV2 expression vector leading to the successful expression of the *C. elegans* FLAG-fusion protein.

Figure 2. *In vitro* transcription/translation of the full-length *C. elegans* cDNA clone 11 and the use of nickel affinity resin for purification. (A) Autoradiogram of the *in vitro* transcription/translation products of 1 µg pcDNA 3.1/His/clone 11, 1 µg pcDNA3.1/His/*lacZ*, and 1 µg luciferase-encoding control plasmid. 2.5 µl (lanes 3-6) or 7.5 µl (lanes 7-10) aliquots of the *in vitro* transcription/translation reactions were separated on a 10% polyacrylamide gel, analyzed by SDS-PAGE, and visualized by autoradiography. The translated 121-kDa Lac Z (lanes 3 and 7), the 69-kDa *C. elegans* (lanes 4 and 8), and the 61-kDa luciferase (lanes 6 and 10) proteins are indicated. The negative control for the *in vitro* transcription/translation assay with no added DNA (lanes 5 and 9) and the positions of the radioactive ^{14}C-molecular mass markers (lane 1) are also indicated. **(B)** Autoradiogram of Lac Z and *C. elegans* proteins purified using nickel Ni-NTA affinity resin. The *in vitro* transcription/translation reactions (50 µl)

of Lac Z and *C. elegans* clone 11 were loaded on two separate nickel Ni-NTA spin columns and the columns were eluted using 200 µl of 500 mM imidazole. 10 µl aliquots of the loaded *in vitro* transcription/translation reactions (lanes 2 and 5), the flow-through from the columns (lanes 3 and 6), and the column eluates (lanes 4 and 7) were separated on a 10% polyacrylamide gel, analyzed by SDS-PAGE, and visualized by autoradiography. The positions of the ^{14}C-molecular mass markers (lane 1) are indicated. Both the autoradiograms shown in (A) and (B) are representative of three separate experiments with similar results.

Aminopeptidase activity of *C. elegans* cytosolic extracts

In order to confirm that *C. elegans* worms did indeed contain aminopeptidase activity, we tested the *C. elegans* extracts for aminopeptidase activity. 5 ml of mixed stage hermaphrodite *C. elegans* worms (resuspended in phosphate-buffered saline) were homogenized under liquid nitrogen (using a mortar and pestle) and resuspended in 5 ml of 0.1 M Tris, pH 7.0, and then in 10 ml of TBS (50 mM Tris pH 7.4, 150 mM NaCl) to a total volume of 20 ml. The homogenate was then sonicated at 4 °C (three times, 20 s each). The suspension was first centrifuged at 2000 x g for 10 min at 4 °C to yield a large membrane fraction, followed by centrifugation of the resultant supernatant at 200,000 x g for 60 min at 4 °C to prepare microsomal and cytosolic fractions. Protein concentrations were determined using a protein assay kit (Bio-Rad). Aminopeptidase assays were carried out using the chromogenic amide substrate L-alanine-*p*-nitroanilide (Sigma) and the microsomal porcine kidney L-leucine aminopeptidase as a positive control (Sigma). 1 mM L-alanine-*p*-nitroanilide was incubated at room temperature with *C. elegans* cytosolic fractions (115 µg total protein/fraction), heat-denatured (by boiling for 15 min) cytosolic fractions, cytosolic fractions pretreated for 10 min with 100 µM bestatin, or with 0.01 units of porcine kidney L-leucine aminopeptidase in 250 µl of buffer containing 0.1 M Tris, pH 8, 200 mM NaCl, and 1 mg/ml BSA. The assays (three reactions for each sample) were performed in 96-well microtiter plates (path length, 0.7 cm), and the formation of the product (*p*-nitroaniline, ϵ = 10,800 M^{-1} cm^{-1}) was

monitored at different time points ranging from 10 min to 2 h at 405 nm using a kinetic microplate reader spectrophotometer (Molecular Devices). Spontaneous hydrolysis of the substrate (≈0.03 milli-absorbance units/min) was corrected for by subtracting the absorbance of control incubations containing no *C. elegans* cytosolic extracts. The concentrations of the hydrolyzed L-alanine-*p*-nitroanilide were calculated (in nmoles) and plotted against the different time points (Figure 3). *C. elegans* cytosolic extracts possessed an intrinsic aminopeptidase activity that was increased with proportion to the incubation time with the L-alanine-*p*-nitroanilide substrate. This aminopeptidase activity was totally abolished by both heat denaturation and incubation of the extracts with the aminopeptidase inhibitor bestatin.

Figure 3. Aminopeptidase activity of *C. elegans* cytosolic extracts. *C. elegans* cytosolic extracts, boiled extracts, or extracts pretreated with 100 μM bestatin (115 μg total protein/extract) were assayed for aminopeptidase activity using 1 mM L-alanine-*p*-nitroanilide. The rate of amide bond hydrolysis was monitored by spectrophotometry of *p*-nitroaniline at 405 nm. The absorbance (in OD units) was used to calculate the nmoles of L-alanine-*p*-nitroanilide hydrolyzed at 10, 20, 30, 60, 90, and 120 min.

SEQUENCE

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ATAAAACTGTCTCTACTGAAGAGTGGAAAGAGTATCTCTATGATTCATTCACTGATAAGA
AGGTTATTCTGGACAATATTGACTGGG

Entry Created: Sep 16 1992
Last Updated: Dec 2 1992

COMMENTS

single read

PUTATIVE ID Assigned by submitter
leukotriene-A4 hydrolase homologous peptide

LIBRARY

Lib Name: Chris Martin sorted cDNA library
Organism: Caenorhabditis elegans

Strain: Bristol N2
Lab host: MC1061
Vector: lambdaphage SHLX2
Description: Mixed stage hermaphrodite cDNA library. Partially normalized by successively picking groups of clones that didn't hybridize to previously picked clones. Vector: lambdaphage SHLX2 (Lipshitz, D.H. et al., Gene 88:25-36 (1990)) Host: MC1061

NCB **BLAST Search Results** Entrez ?

Sequences producing High-scoring Segment Pairs:		Reading Frame	High Score	Smallest Sum Probability P(N)	N
sp|P09960|LKHA_HUMAN	LEUKOTRIENE A-4 HYDROLASE (LTA-4 ...	+3	260	6.7e-29	1
sp|P24527|LKHA_MOUSE	LEUKOTRIENE A-4 HYDROLASE (LTA-4 ...	+3	255	3.2e-28	1
sp|P30349|LKHA_RAT	LEUKOTRIENE A-4 HYDROLASE (LTA-4 ...	+3	254	4.4e-28	1
sp|P19602|LKHA_CAVPO	LEUKOTRIENE A-4 HYDROLASE (LTA-4 ...	+3	241	1.4e-26	1
sp|O10740|LKHA_YEAST	PROBABLE LEUKOTRIENE A-4 HYDROLAS...	+3	211	3.7e-22	1
sp|P52922|LKHA_DICDI	LEUKOTRIENE A-4 HYDROLASE (LTA-4 ...	+3	110	1.4e-16	2
sp|Q00721|VN34_ROTS4	NONSTRUCTURAL RNA-BINDING PROTEIN...	+3	54	0.49	2
sp|P03536|VN34_ROTS1	NONSTRUCTURAL RNA-BINDING PROTEIN...	+3	54	0.49	2
sp|P37593|NARU_SALTY	NITRITE EXTRUSION PROTEIN 2 (NITR...	-2	57	0.55	2

sp|P09960|LKHA_HUMAN LEUKOTRIENE A-4 HYDROLASE (LTA-4 HYDROLASE) (LEUKOTRIENE A(4) HYDROLASE)
Length = 611

Plus Strand HSPs:

Score = 260 (117.9 bits), Expect = 6.7e-29, P = 6.7e-29
Identities = 46/89 (51%), Positives = 66/89 (74%), Frame = +3

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Query:    60 FGPDHEYTKLVQNLGNADPDDAFSSVPYEKGSALLFTIEQALGDNSRFEQFLRDYIQKYA 239
             FG  H +TKLV +L + DPD A+SSVPYEKG ALLF +EQ LG    F  FL+ Y++K++
Sbjct:   357 FGETHPFTKLVVDLTDIDPDVAYSSVPYEKGFALLFYLEQLLGGPEIFLGFLKAYVEKFS 416

Query:   240 YKTVSTEEWKEYLYDSFTDKKVILDNIDW 326
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Sbjct:   417 YKSITTDDWKDFLYSYFKDKVDVLNQVDW 445
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Figure 1

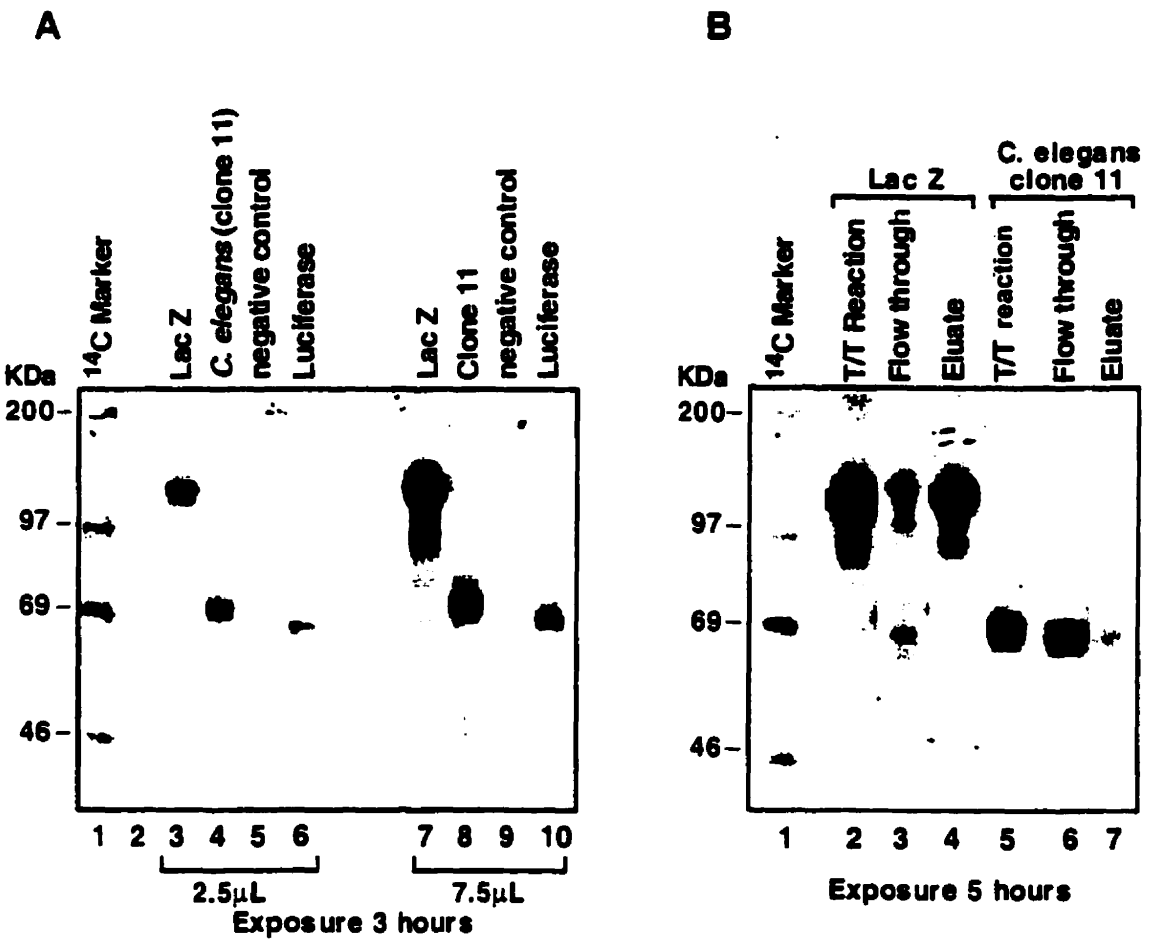
A
B
KDa
200
97
69
46
^{14}C Marker
Lac Z
C. elegans (clone 11)
negative control
Luciferase
Lac Z
Clone 11
negative control
Luciferase
1 2 3 4 5 6 7 8 9 10
2.5µL
7.5µL
Exposure 3 hours
Lac Z
C. elegans clone 11
T/T Reaction
Flow through
Eluate
T/T reaction
Flow through
Eluate
1 2 3 4 5 6 7
Exposure 5 hours

Figure 2

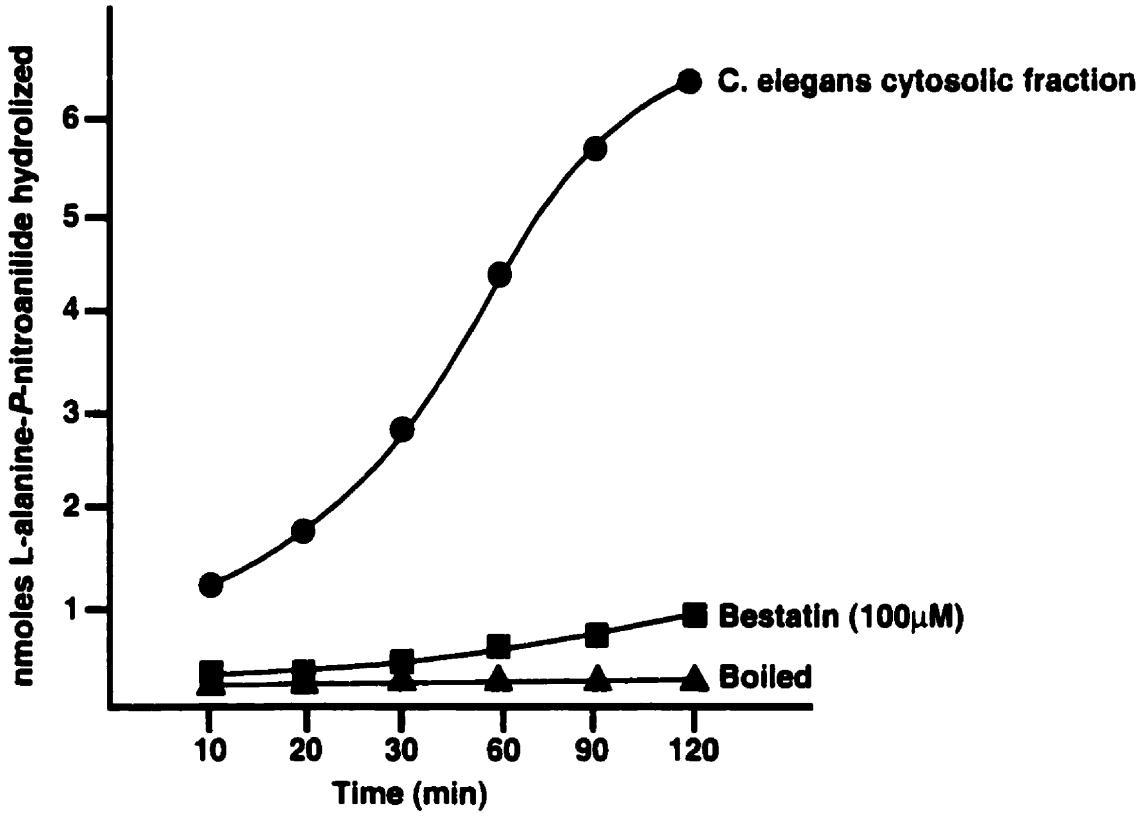
nmoles L-alanine-P-nitroanilide hydrolized
1
2
3
4
5
6
10
20
30
60
90
120
Time (min)
C. elegans cytosolic fraction
Bestatin (100μM)
Boiled

Figure 3

APPENDIX C-Official copyright waivers

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MOLECULAR AND BIOCHEMICAL PARASITOLOGY

Molecular and Biochemical Parasitology 73 (1995) 31–41

Characterization of arachidonic-acid-metabolizing enzymes in adult *Schistisoma mansoni*

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ELSEVIER

MOLECULAR AND BIOCHEMICAL PARASITOLOGY

Aims and Scope
The journal provides a medium for the rapid publication of investigations of the molecular biology, molecular immunology and biochemistry of parasitic protozoa and helminths and their interactions with both the definitive and intermediate host. The main subject areas covered are: chemical structure, biosynthesis, degradation, properties and function of small molecular weight substances, DNA, RNA, proteins, lipids and carbohydrates – intermediary metabolism and bioenergetics – molecular and biochemical studies on the mode of action of antiparasitic drugs – molecular and biochemical aspects of membrane structure and function – molecular and biochemical aspects of host–parasite relationships including analysis of parasitic escape mechanisms – characterisation of parasite antigen and parasite and host cell surface receptors – characterisation of genes by biophysical and biochemical methods, including recombinant DNA technology – analysis of gene structure, function and expression – mechanisms of genetic recombination.
Papers will only be accepted for publication if they fall within these areas, if they contain original work of high scientific quality, and if they are well presented.

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Received 27 February 1995; accepted 8 May 1995

Abstract

Schistosoma mansoni has previously been reported to synthesize a wide range of eicosanoids including prostaglandins, leukotrienes and hydroxyeicosatetraenoic acids (HETEs). Our analysis of arachidonic acid metabolites synthesized by microsomal and cytosolic extracts from adult *S. mansoni* using thin-layer chromatography and radioimmunoassay techniques indicate the presence of a soluble, enzymatically active lipoxygenase (Lox) and the absence of any cyclooxygenase (Cox) activity. The *S. mansoni* Lox activity catalyzed the formation of a 15-hydroxyeicosatetraenoic acid (15-HETE)-like species. This activity was calcium-independent and inhibitable by inhibitors of mammalian and plant Lox. The conversion of linoleic acid to a 13-hydroxyoctadecadienoic acid (13-HODE)-like product by *S. mansoni* extracts indicates that the parasite Lox-homologue is similar to mammalian 15-Lox. Immunoblot analysis of *S. mansoni* extracts using antisera to different mammalian lipoxygenases detects two immunoreactive proteins with molecular weights similar to plant and mammalian lipoxygenases. In addition, polymerase chain reaction (PCR) amplification of Lox-like sequences from *S. mansoni* genomic DNA using degenerate primers based on conserved plant and mammalian Lox sequences, generated two PCR products which hybridized to a human 15-Lox cDNA probe. While the role of eicosanoid production in the physiology of *S. mansoni* is not known, eicosanoids may be essential for normal physiological processes as is the case in other invertebrates. Interestingly, 15-HETE has previously been shown to have immunosuppressive effects in mammals, and this may be related to the ability of the parasite to overcome host immune responses.

Keywords: *Schistosoma mansoni*; Arachidonic acid; Cyclooxygenase; Eicosanoid; 15-HETE; Lipoxygenase; Prostaglandin

1. Introduction

The metabolism of arachidonic acid by cyclooxygenase and lipoxygenase enzymes results in the production of a wide range of biologically active oxygenated metabolites including prostaglandins, thromboxanes, leukotrienes, hydroxyeicosatetraenoic acids

Abbreviations: AA, arachidonic acid; Cox-2, cyclooxygenase-2; DMSO, dimethylsulfoxide; ETYA, eicosatetraynoic acid; 15-HETE, 15-hydroxyeicosatetraenoic acid; 13-HODE, 13-hydroxyoctadecadienoic acid; IC_{50}, concentration of half inhibition; LT, leukotriene; Lox, lipoxygenase; PG, prostaglandin.

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0166-6851/95/$09.50
SSDI 0166-6851(95)00085-2

(HETEs) and lipoxins which are collectively referred to as eicosanoids [1,2]. In mammals, eicosanoids are potent mediators of various physiological and pathological responses including inflammation, bronchoconstriction, pain, fever, regulation of vascular tone and regulation of immune responses [3,4].

Studies have shown the production of eicosanoids by several parasites in response to the addition of polyunsaturated fatty acids, including *Taenia taeniaeformis*, *Schistosoma mansoni*, *Wuchereria bancrofti* and *Brugia malayi* [5,6]. In certain parasites eicosanoid production may be related to a developmental stage or process. For example, in *S. mansoni* the production of lipoxygenase products such as leukotrienes and hydroxyeicosatetraenoic acids (HETE) was suggested to correlate with cercarial penetration, whereas prostaglandin production was associated with the transformation of cercaria into schistosomules [7]. In adult *S. mansoni*, prostaglandins, leukotrienes and hydroxyeicosatetraenoic acids were all produced by both male and female worms, with 15-HETE as the major product of the lipoxygenase pathway. As certain eicosanoids, in particular PGE_2 and 15-hydroxyeicosatetraenoic acids (15-HETE) have immunosuppressant activity [1,6,8,9], it has been postulated that eicosanoids synthesized by developing schistosomulae may help the parasite evade the initial response of the dermal immune system [10]. In the present study we have investigated the metabolism of arachidonic acid by adult *S. mansoni* parasites, demonstrating the presence of an active lipoxygenase pathway and the absence of any cyclooxygenase-derived prostaglandins.

2. Materials and methods

2.1. S. mansoni infections and adult worm isolation

Infected CD.01 mice were maintained in the laboratory of Dr. James M. Smith (Institute of Parasitology, McGill University, Ste.-Anne de Bellevue, Quebec, Canada) as described [11]. Adult *S. mansoni* were recovered by perfusion of the hepatoportal system 7 weeks post infection using ice-cold RPMI media (GIBCO) containing 0.85% NaCl/0.75% Na_3 · citrate, washed twice in sterile saline solution, visually examined by light microscopy for host cell contamination and immediately frozen in liquid nitrogen.

2.2. Preparation of subcellular fractions

Adult *S. mansoni* obtained from 40 infected mice were resuspended in 10 ml homogenization buffer (100 mM Tris · HCl, pH 7.4, containing 5 mM EDTA/1 mM phenylmethylsulfonyl fluoride/10 μg ml^{-1} soybean trypsin inhibitor/1 μg ml^{-1} leupeptin/1 μg ml^{-1} pepstatin/1 mM homovanillic acid (Sigma, St. Louis, MO, USA). The worms were homogenized using a Dounce homogenizer followed by sonication at 4°C. The suspension was first centrifuged at $2000 \times g$ for 10 min at 4°C to yield a large membrane fraction, followed by centrifugation of the resultant supernatant at $200\,000 \times g$ for 45 min at 4°C to prepare microsomal and cytosolic fractions. Protein concentrations were determined using a protein assay kit (Bio-Rad, Mississauga, Ontario, Canada).

2.3. Analysis of 15-HETE synthesis

Assays were carried out in a 100 μl final volume in 0.1 M Tris, pH 8.0, containing 5 mM EDTA/1 μM hematin/1 mM homovanillic acid/15 μg total worm protein/0.2 μCi [^{14}C(U)]arachidonic acid (866 mCi $mmol^{-1}$; 0.46 μM final concentration) or 0.4 μCi [^{14}C(U)]linoleic acid (1045 mCi $mmol^{-1}$; 0.76 μM final concentration) (New England Nuclear, Boston, MA, USA). Incubations were carried out for 1 h at 37°C; reactions were then quenched with 50 μl methanol and applied to silica gel thin-layer chromatography plates (Whatman). The thin-layer chromatography plates were developed with either ethylacetate/acetic acid (99:1) or with ether/petroleum ether/acetic acid (50:50:1) [12]. Plates were scanned for radioactivity and the products were quantified on a Berthold LB2842 thin-layer chromatography linear analyzer and then visualized by autoradiography. Authentic prostaglandins, monohydroxy acids, monohydroperoxy acids, linoleic acid and arachidonic acid standards (Cayman) were run in parallel. Results are expressed as percentage conversion of arachidonic acid per μg total worm protein.

2.4. Radioimmunoassay for prostaglandin E_2 detection

S. mansoni fractions were incubated for 1 h at 37°C in the same buffer used for the assays of 15-HETE synthesis except for the use of nonradioactive arachidonic acid (20 μM final concentration), and 20 μg protein in an 80 μl final reaction volume. Reactions were stopped by adding 0.1 vol. of 1 M HCl followed by 0.1 vol. of 1 M NaOH. PGE_2 production was assayed using a radioimmunoassay kit (PGE_2 [^{125}I] RIA Kit, NEN, Boston, MA, USA).

2.5. Effect of cyclooxygenase / lipoxygenase inhibitors

L-670,630 (2,3-dihydro-6-(3-phenoxypropyl)-2-(2-phenylethyl)-5-benzofuranol) [13], CPHU (*N*-(4-chlorophenyl)-*N*-hydroxy-*N'*-(3-chlorophenyl) urea [14] and indomethacin (Merck Frosst) were dissolved in DMSO and 5,8,11,14-eicosatetraynoic acid (ETYA) (Cayman) was dissolved in 100% ethanol. Inhibitors or their respective solvents were preincubated with cytosolic fraction for 15 min and the reaction was initiated by adding an ethanol solution of the substrate (arachidonic acid or linoleic acid). 1 h after substrate addition, the reactions were quenched with methanol and spotted on thin-layer chromatography plates. Human recombinant cyclooxygenase-2 enzyme (Cox-2) [15] was used as a positive control for the production of prostaglandins and 15-HETE. The IC_{50} values for the lipoxygenase inhibitors were calculated after scanning the autoradiographs using a computing densitometer (Molecular Dynamics, Sunnyvale, CA, USA).

2.6. Immunoblot analysis

Large membrane, microsomal and cytosolic fractions of *S. mansoni* adult worms as well as 5-, 12-, and 15-lipoxygenase enzymes standards (Oxford Biochemicals) were analyzed by SDS-PAGE as previously described followed by electrophoretic transfer to nitrocellulose membranes [16,17,18]. The nitrocellulose membranes were probed with the following antisera: 1:300 dilution of rabbit anti-human 5-lipoxygenase [16], 1:5000 dilution of rabbit anti-sheep Cox-1, 1:7500 dilution of rabbit anti-sheep Cox-2 [17], an 1:100 dilution of rabbit anti-human 12-lipoxygenase (Oxford Biochemicals), an 1:100 dilution of goat anti-rabbit 15-lipoxygenase (Cayman). The secondary horse radish peroxidase-linked donkey anti-rabbit IgG antibody (Amersham Life Sciences, Oakville, Ontario, Canada) was used at a dilution of 1:3000. Immunodetection was performed using enhanced chemiluminescence according to the manufacturer's instructions (Amersham). When anti-5 lipoxygenase antiserum was used as a primary antibody, ^{125}I-protein A (NEN) was used for detection as previously described [16]. Autoradiographs for chemiluminescence detection were exposed to Kodak X-OMAT X-ray films for 3 min; autoradiographs for protein A detection method were exposed for 5 days.

2.7. Polymerase chain reaction (PCR) amplification of lipoxygenase-like sequences

For the selection of oligonucleotides for PCR, highly conserved regions of 8 lipoxygenases were identified by aligning the amino acid sequences of rabbit 15-lipoxygenase, bovine 12-lipoxygenase, pig 12-lipoxygenase human 15-lipoxygenase, human 12-lipoxygenase, and lipoxygenases from the plants soybean, pea seed and rice [19–21]. Based on the alignment of the lipoxygenases, the following degenerate oligonucleotides and their positions within the human 15-lipoxygenase cDNA [21] were chemically synthesized: lx-4, 5'-CC(A/T)(G/C)(G/T)(A/G)-GATGAG(A/C)GATT-3', nt 481–497; lx-5, 5'GG-(T/C)(G/A)(C/T)(C/A)AACCCC(GA)TGIT-3', nt 703–719; lx-7, 5'-AG(G/C)(C/T)(A/G)(C/T)-C(T/G)(C/G)(A/T)TGGTGGC-3', nt 1135–1119. A primary PCR reaction (GeneAmp DNA PCR kit, Perkin-Elmer Cetus, Norwalk, CT, USA) was done using lx-4 and lx-7 primers in a buffer containing 10 mM Tris · HCl (pH 8.3)/50 mM KCl/2.5 mM $MgCl_2$/0.2 mM deoxynucleotide triphosphates/0.5 μM primers and 40 ng of adult *S. mansoni* genomic DNA as template. The PCR cycling conditions were: 35 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C. A secondary PCR reaction was then performed under the same conditions using 0.01 μl of the primary PCR reaction and the primers lx-5 and lx-7. PCR products were separated by electrophoresis in a 1.2% (w/v) agarose gel, visualized by ethidium bromide staining and blotted to Hybond-N$^+$ nylon membranes (Amersham Life Science) by overnight capillary transfer using 0.4 M NaOH. The

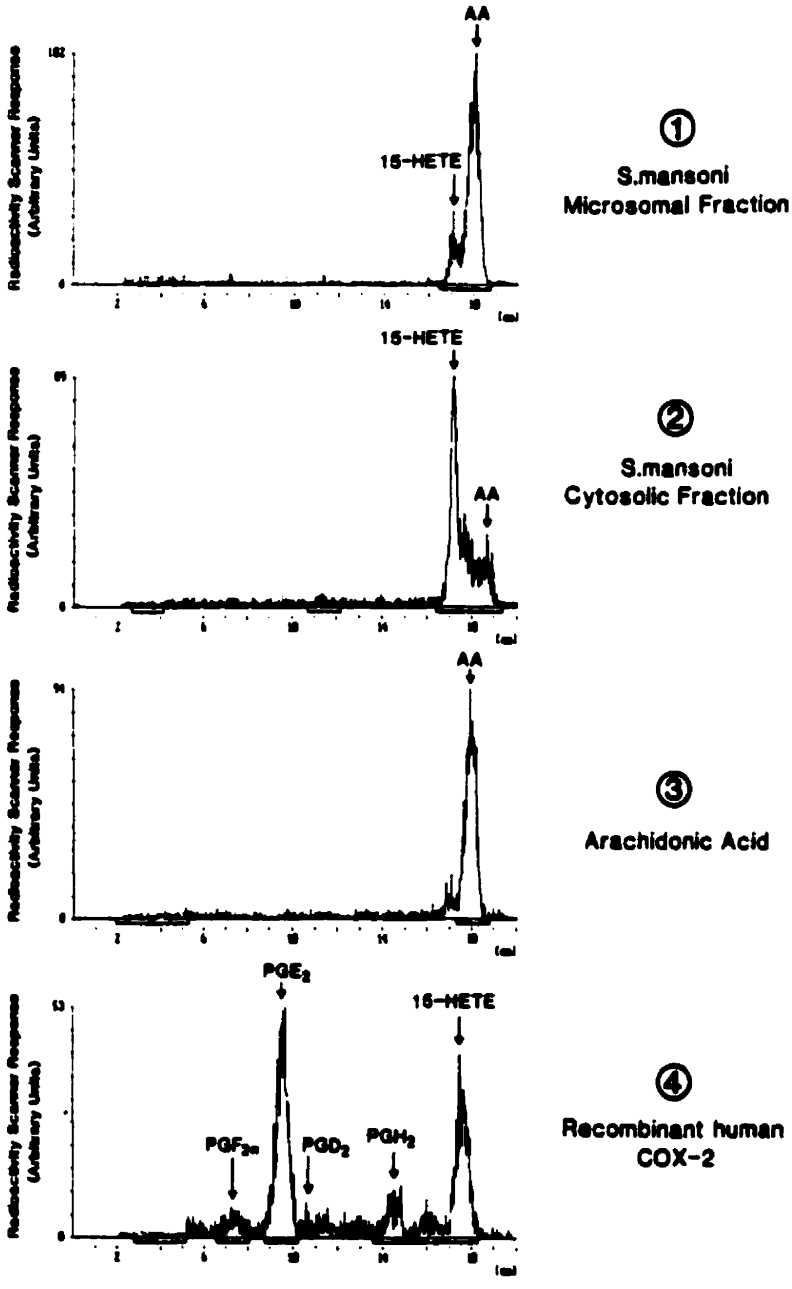

Fig. 1. 15-HETE production by adult *S. mansoni*. Subcellular fractions of adult *S. mansoni* and recombinant human Cox-2 were incubated for 1 h in the presence of [^{14}C]arachidonic acid under the reaction conditions described in Materials and methods. Following incubation, the products were extracted, separated on silica gel thin-layer chromatography plates and scanned for radioactivity. Tracing 1, microsomal fraction (48 μg protein); tracing 2, cytosolic fraction (15 μg protein); tracing 3, [^{14}C]arachidonic acid only; tracing 4, human recombinant Cox-2. The migration of co-chromatographed prostaglandins and 15-HETE standards are indicated. The tracings are from one of three experiments.

oligonucleotides used for PCR amplification of cyclooxygenase sequences were based on conserved sequences from chicken, human, mouse and rat COX-2 [22]. The COX PCR primers and their corresponding codons in human, rat, mouse and chicken COX-2 include [22]: cx-1, 5'-ATGATGTA(TC) GCIACIATITGG-3', codons 285–291; cx-2, 5'-ATGTACCCICCIACIGTIAA-3', codons 247–253; cx3, 5'-TT(TC)AA(CT)ACI(CT)TITA(TC)CA(TC)-TGG-3', codons 367–373; cx4, 5'-(AG)AAIA(AG)-(TC)TG(TC)TC(AG)TC(AG)TCCCA-3', codons 316–309; cx5, 5'-CCAIATIGTIGC(AG)-TACATCAT-3', codons 291–285; cx6, 5'-(CT)TC-(CT)TCIA(AG)(CT)TCIGCIGCCAT-3', codons 479–473.

2.8. Southern blot analysis of PCR products

Southern blot hybridization was performed at 65°C for 16 h in a solution containing 5 × SSPE (1 × SSPE is 0.15 M NaCl/0.01 M NaH_2PO_4/0.01 M EDTA, pH 7.4), 5 × Denhardts (1 × Denhardts solution is 0.02% (w/v) Ficoll/0.02% (w/v) bovine serum albumin/0.02% (w/v) polyvinyl pyrrolidone)/100 μg ml^{-1} denatured calf thymus DNA/0.5% (w/v) SDS, and randomly primed (Boehringer-Mannheim) ^{32}P-labeled human 15-lipoxygenase cDNA ([21]; Dr. E. Sigal, University of California, San Francisco, CA, USA), or a mixture of ^{32}P-labeled human Cox-1 and human Cox-2 cDNAs [18]. The membranes were washed twice in 2 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M Na_3 · citrate, pH 7)/0.1% (w/v) SDS at room temperature, then washed twice in 2 × SSC/0.1% SDS at 65°C for 30 min, and exposed to Kodak XAR-5 film for 1–5 days at −70°C.

3. Results

3.1. Eicosanoid production by adult S. mansoni

To determine the profile of arachidonic acid metabolism by adult *S. mansoni*, different fractions of homogenized worms were incubated with [^{14}C]arachidonic and the products were analyzed by thin-layer chromatography. In both the cytosolic and microsomal adult *S. mansoni* fractions, the major metabolite co-chromatographed with an authentic 15-HETE standard (Fig. 1). The synthesis of the 15-HETE-like product was highest in the cytosolic fraction (68% conversion of [^{14}C]arachidonic acid per 15 μg protein) as compared to the microsomal fraction (16% conversion of [^{14}C]arachidonic acid per 48 μg microsomal protein). No [^{14}C]arachidonic acid metabolites which co-chromatographed with authentic prostaglandin standards were detected in either the microsomal or cytosolic adult *S. mansoni* fractions (Fig. 1). In contrast, using the same assay and detection methods, 15-HETE and prostaglandin products were detected in control reactions using recombinant human COX-2, as previously reported [15,18]. Since the level of prostaglandin synthesis in the *S. mansoni* fractions may be below the detection level of the thin-layer chromatography method, *S. mansoni* fractions were incubated with nonradioactive arachidonic acid and analyzed using a sensitive PGE_2 radioimmunoassay (lower limit of detection is 0.5 pg PGE_2 ml^{-1}). However, no PGE_2 production was detected in any of the *S. mansoni* extracts (results not shown). These results suggest that adult

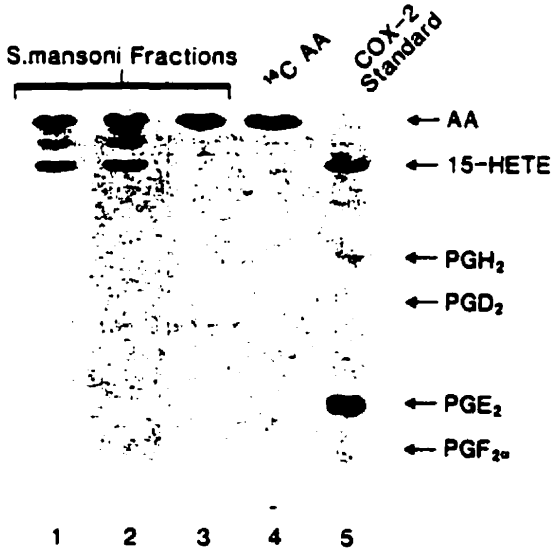

Fig. 2. Effect of calcium addition and denaturation on the production of the 15-HETE-like product. Adult *S. mansoni* cytosol (15 μg protein) was incubated with [^{14}C]arachidonic acid (0.46 μM) in the absence (lane 1) or presence (lane 2) of 2 mM $CaCl_2$, or denatured by boiling for 15 min prior to incubation with the substrate (lane 3). The reaction products were analyzed using thin-layer chromatography and visualized by autoradiography. Lane 4, [^{14}C]arachidonic only; lane 5, recombinant human Cox-2 with [^{14}C]arachidonic acid (0.46 μM). The positions of co-chromatographed standards are indicated. The autoradiogram shown is a representative of three separate experiments with similar results.

S. mansoni express at least one lipoxygenase but no detectable cyclooxygenase activity.

3.2. The production of the 15-HETE-like metabolite is calcium-independent and heat-inactivable

Since lipoxygenases (except for 5-lipoxygenase) are calcium independent [23], the effect of calcium addition on the production of 15-HETE in the cytosolic fraction was determined. Addition of 2 mM $CaCl_2$ to the extracts did not alter the production of the 15-HETE-like species (Fig. 2). Heat denaturation of the cytosolic fraction (100°C, 15 min) totally abolished the appearance of the 15-HETE-like metabolite (Fig. 2), confirming an enzymatic involvement in the production of this metabolite.

3.3. The effect of cyclooxygenase and lipoxygenase inhibitors on the production of the 15-HETE-like species

The synthesis of 15-HETE from arachidonic acid by both 15-lipoxygenase and cyclooxygenases can be inhibited in a dose-dependent manner by specific inhibitors [15,18,24,25]. Indomethacin, a potent inhibitor of both 11- and 15-hydroxylation of arachidonic acid by cyclooxygenases [18,24], did not affect the production of the 15-HETE-like metabolite in the *S. mansoni* cytosolic fraction, whereas preincubation of human recombinant Cox-2 with indomethacin totally abolished the formation of both prostaglandins and 15-HETE (Fig. 3A). In contrast 10 μM ETYA, a known suicide substrate inhibitor of lipoxygenases

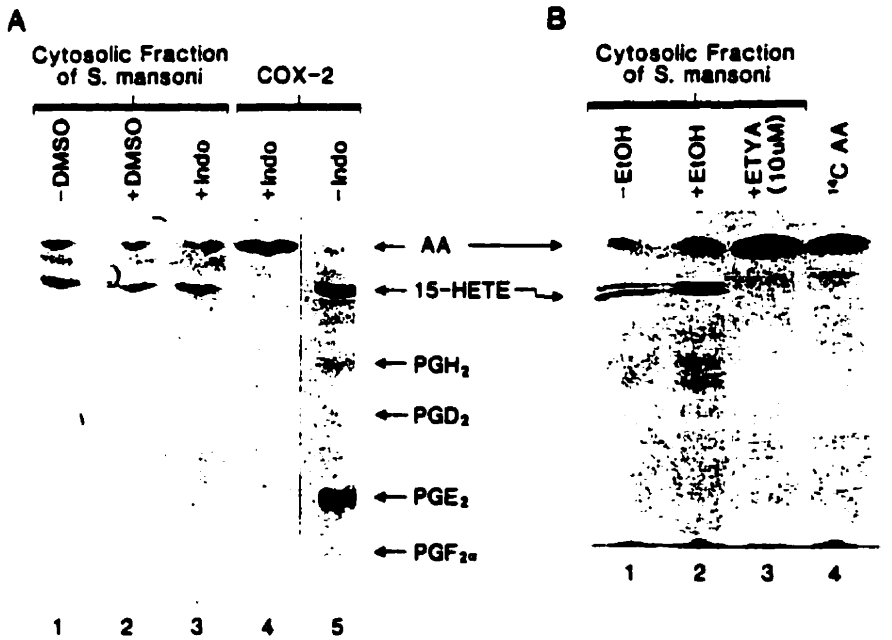

Fig. 3. Effect of cyclooxygenase and lipoxygenase inhibitors on the metabolism of arachidonic acid by *S. mansoni*. (A) Thin-layer chromatography of products formed from [^{14}C]arachidonic acid by *S. mansoni* cytosol (15 μg; lanes 1, 2 and 3) and human recombinant COX-2 (lanes 4 and 5) in the absence (lane 1) or presence (lane 2) of DMSO vehicle or 100 μM indomethacin dissolved in DMSO (lane 3 and 4). (B) Thin-layer chromatography of products formed from [^{14}C]arachidonic acid by *S. mansoni* cytosol (15 μg; lanes 1–3) in the absence (lane 1) or presence of ethanol vehicle (lane 2), in the presence of 10 μM ETYA dissolved in ethanol (lane 3). [^{14}C]arachidonic acid in the absence of any added protein (lane 4). The reaction products were analyzed using thin-layer chromatography plates developed either with ethyl acetate/acetic acid (panel A) or with ether: petroleum ether/acetic acid (panel B) and visualized by autoradiography. The positions of co-chromatographed standards are indicated. The autoradiograms shown in panel A and B are representative of three experiments.

[25], completely abolished the production of the 15-HETE-like species in the *S. mansoni* cytosolic fraction (Fig. 3B), with an IC_{50} of 2 ± 0.07 μM ($n = 5$; data not shown). Since ETYA can inhibit both cyclooxygenases and lipoxygenases, two other lipoxygenase inhibitors were used which displayed dose-dependent inhibition of the *S. mansoni* cytosolic lipoxygenase-like activity. Pretreatment of the cytosolic fraction with increasing concentrations of L-670,630, a phenolic redox inhibitor of lipoxygenases [13], and CPHU, one of the N-hydroxyurea class of lipoxygenase redox inhibitors [14] resulted in dose-dependent inhibition of the production of the 15-HETE-like species with IC_{50} values of 3 ± 0.06 μM ($n = 5$) and 0.08 ± 0.003 μM ($n = 5$), respectively (data not shown).

3.4. The metabolism of linoleic acid by S. mansoni cytosol

Certain lipoxygenases, such as erythroid 15-lipoxygenase and leukocyte 12-lipoxygenase, are distinguished by their ability to metabolize linoleic acid to 13-hydroxyoctadecadienoic acid (13-HODE; [26]).

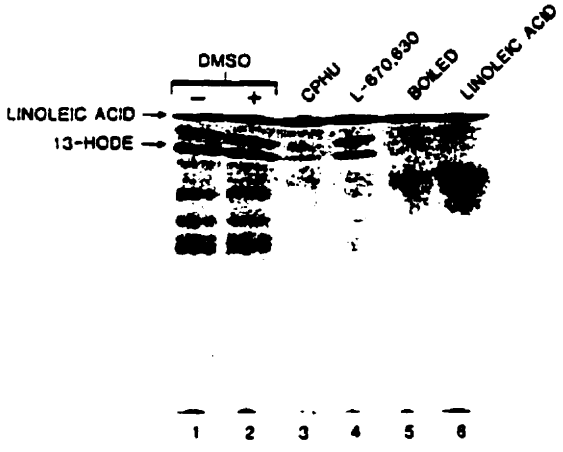

Fig. 4. Metabolism of linoleic acid by S. *mansoni* cytosol. Thin-layer chromatography of products formed from [^{14}C]linoleic acid (0.76 μM) by *S. mansoni* cytosol (15 μg) in the absence of inhibitor or vehicle (lanes 1), in the presence of 1% DMSO vehicle (lane 2), treated with 5 μM CPHU (lane 3), treated with 5 μM L-670,630 (lane 4), or boiled *S. mansoni* cytosol (lane 5). [^{14}C]Linoleic acid in the absence of any added *S. mansoni* cytosol (lane 6). Thin-layer chromatography and densitometry were carried out as described in Materials and methods. The position of co-chromatographed standards are indicated. This experiment was repeated twice with similar results.

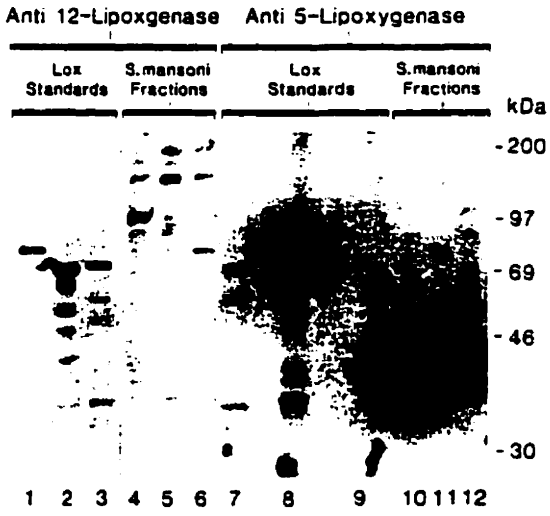

Fig. 5. Immunoblot analysis of *S. mansoni* fractions. 140 ng each of human leukocyte 5-lipoxygenase (lanes 1 and 8), porcine leukocyte 12-lipoxygenase standard (lanes 2 and 9), rabbit reticulocyte 15-lipoxygenase standard (lanes 3 and 7), 50 μg total protein of adult *S. mansoni* large membrane fraction (lanes 4 and 10), 30 μg of *S. mansoni* microsomal fraction (lanes 5 and 11), and 15 μg of *S. mansoni* cytosolic fraction (lanes 6 and 12) were separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted using anti-human platelet 12-lipoxygenase antiserum (1:100 dilution) or anti-human 5-lipoxygenase antiserum (1:300 dilution) as described in Materials and methods. Enhanced chemiluminescence (lanes 1 to 7) or ^{125}I-labeled protein A (lanes 7 to 12) were used for detection. The positions of molecular mass markers are indicated.

The *S. mansoni* cytosolic extract also metabolized [^{14}C]linoleic acid to a product that co-chromatographed with a 13-HODE standard (Fig. 4). The production of the 13-HODE-like product by the *S. mansoni* cytosolic extract was abolished by heat-inactivation and markedly reduced by pretreatment of the cytosol with CPHU or L-670,630 (Fig. 4).

3.5. Immunoblot analysis of S. mansoni fractions

Due to the significant amino-acid sequence homology among plant and mammalian lipoxygenases [19], it is reasonable to expect that antibodies to mammalian 5-, 12-, or 15-lipoxygenase might show immunological cross-reactivity to a *S. mansoni* homologue. Thus, immunoblot analyses of *S. mansoni* extracts with an anti-human 12-lipoxygenase polyclonal antiserum and an anti-human 5-lipoxygenase polyclonal antiserum were performed (Fig. 5). Both

the antisera to 5- and 12-lipoxygenase detected all three purified standards, including the 78-kDa human 5-lipoxygenase, the 75-kDa porcine leukocyte 12-lipoxygenase and the 76-kDa rabbit reticulocyte 15-lipoxygenase [19]. Although both antisera to 5- and 12-lipoxygenase detected a number of immunoreactive proteins in the *S. mansoni* extracts, two proteins were detected by both antisera and corresponded to the molecular masses of mammalian and plant lipoxygenases (Fig. 5). One *S. mansoni* protein of 78 kDa co-migrated with the human 5-lipoxygenase standard and was seen in the microsomal and cytosolic fractions, and a second protein of 100 kDa was comparable in molecular weight to that of soybean-2 lipoxygenase and pea seed lipoxygenase [19]. Other immunoreactive bands may represent non-specific cross-reactivity as these bands were also detected using preimmune serum (data not shown). Using an anti-ram seminal vesicle Cox-1 polyclonal antiserum and an anti-sheep Cox-2 polyclonal antiserum [17], no cyclooxygenase specific immunoreactive protein bands were seen in any of the different *S. mansoni* fractions (data not shown). These results suggest that adult *S. mansoni* expresses at least one lipoxygenase that is antigenically related to mammalian lipoxygenases.

3.6. Lipoxygenase-like DNA sequences in S. mansoni

Southern hybridization of *S. mansoni* genomic DNA with the human 15-lipoxygenase cDNA and subsequent washing under moderately stringent conditions, revealed hybridizing bands of 8.6 kb in the *Hin*dIII, 6.4 kb in *Eco*RI, 6.4 kb in *Hin*dIII + *Eco*RI, and 7 kb in *Bam*HI-digested samples (Fig. 6). Southern hybridization of *S. mansoni* genomic DNA with both full-length human cyclooxygenase-1 and -2 cDNA probes revealed two weakly hybridizing fragments of 6.5 and 2.3 kb in a *Bam*HI digest (data not shown).

The positive hybridization signals observed in the *S. mansoni* genomic DNA with the mammalian lipoxygenase and cyclooxygenase probes indicated that the homologous *S. mansoni* sequences might be amplifiable by the polymerase chain reaction (PCR). Oligonucleotide primers for amplification of the putative *S. mansoni* lipoxygenase and cyclooxygenase were based on highly conserved regions identified by sequence comparison of 8 different mammalian and

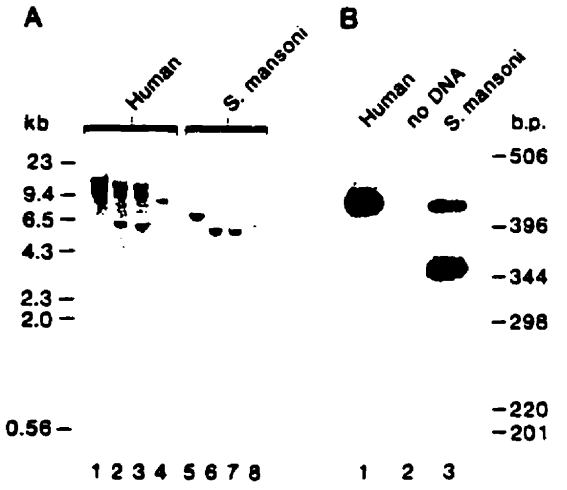

Fig. 6. Detection of *S. mansoni* lipoxygenase-like DNA sequences. (A) Southern hybridization of a ^{32}P-labeled human 15-lipoxygenase cDNA with human (lanes 1–4) and *S. mansoni* genomic DNA (lanes 5–8). The lanes contain 10 μg human DNA or 10 μg *S. mansoni* DNA restricted with *Hin*dIII (lanes 1 and 5), *Eco*RI (lanes 2 and 6), *Hin*dIII + *Eco*RI (lanes 3 and 7) and *Bam*HI (lanes 4 and 8). Fragment sizes of *Hin*dIII-cleaved lambda DNA are shown in kb. (B) Analysis of PCR-amplified products from *S. mansoni* genomic DNA using primers based on conserved lipoxygenase sequences. *S. mansoni* genomic DNA (40 ng) and human 15-lipoxygenase cDNA (1 ng) were used as templates for PCR amplification with degenerate primers based on conserved sequences in lipoxygenases. Amplification products were electrophoresed on a 1.2% agarose gel, capillary blotted onto nylon membrane and probed with ^{32}P-random primer-labeled human 15-lipoxygenase cDNA probe. The positions of DNA size markers are indicated (in bp). Lane 1, 10 μl of PCR reaction using human 15-lipoxygenase cDNA as a template; lane 2, 10 μl of the negative control for the PCR reaction using no added template; lane 3, 10 μl of PCR reaction using *S. mansoni* genomic DNA.

plant lipoxygenases and six different cyclooxygenases, respectively (see Materials and methods). In order to detect parasite lipoxygenase sequences, PCR amplification of *S. mansoni* genomic DNA was performed using the primer pair lx-4/lx-7 in a primary PCR, followed by a second round of amplification using the nested primer pair lx-5/lx-7 and an aliquot of the primary PCR as template, separation of the PCR products by agarose gel electrophoresis and Southern blot analysis with the human 15-lipoxygenase as a probe. As expected a 433-bp fragment was amplified using the human 15-lipoxygenase cDNA as a template. Two products were amplified from *S. mansoni* DNA, including a 433-bp fragment which co-migrated with the PCR product amplified

from the human 15-lipoxygenase cDNA, and a second PCR product of 370 bp. The identity of both PCR fragments as Lox-like sequences was confirmed by their hybridization to the human 15-Lox cDNA probe. The oligonucleotides lx-4, lx-5 and lx-7 have also been used to amplify identically-sized PCR products from an *S. mansoni* cDNA library (kindly provided by Dr. C.B. Shoemaker, Harvard School of Public Health, Boston, MA, USA) (data not shown).

4. Discussion

Adult *S. mansoni* has previously been shown to incorporate polyunsaturated fatty acids, including arachidonic acid, and it has been suggested that they readily metabolize other polyunsaturated fatty acids, including linoleic acid, to arachidonic acid [27]. Analysis of eicosanoid metabolism following addition of linoleic acid to adult worms has shown the major product to have the chromatographic characteristics of 15-HETE consistent with the present results [10]. With regard to arachidonic acid metabolism by cyclooxygenase pathways in adult *S. mansoni* the literature is less clear. One publication has reported no formation of prostaglandins [28], whereas another has reported on the presence of immunoreactive and chromatographically characterized materials with the characteristics of prostaglandins although no definitive physiochemical or pharmacological data were obtained [10]. Similar results were obtained with *S. mansoni* cercariae [7,27]. Our results indicate an absence of PGE_2 production by adult worm extracts as tested both by thin-layer chromatography and radioimmunoassay techniques. In addition, we could find no evidence for the presence of a parasite Cox homologue by PCR using degenerate primers based on conserved sequences in mammalian and avian COX-2. Furthermore, immunoblot analysis of different adult worm extracts using antisera to mammalian cyxlooxygenase did not show specific Cox-protein bands. Taken together, these results combined with the fact that no prostaglandin production was detected, suggest the absence of an active cyclooxygenase pathway in adult *S. mansoni* parasites. However, two weakly hybridizing bands observed by Southern blot hybridization of *S. mansoni* genomic DNA using the human cyclooxygenase-1 and -2 cDNA probes, suggests that the parasite does contain cyclooxygenase-related sequences. Our inability to detect an *S. mansoni* cyclooxygenase protein or mRNA in the adult worm may be related to a stage-specific expression of the putative *S. mansoni* cyclooxygenase gene.

As reported previously [10], our results show that a 15-HETE-like product is the major eicosanoid produced from arachidonic acid by *S. mansoni*, suggesting the presence of an active lipoxygenase pathway. Calcium addition did not stimulate the activity of the parasite lipoxygenase homologue suggesting that it is regulated differently to the mammalian 5-lipoxygenase enzyme but similarly to mammalian 15-lipoxygenase. The inability of indomethacin, a potent cyclooxygenase inhibitor, to inhibit *S. mansoni* 15-HETE production and the use of different lipoxygenase inhibitors, which showed similar pharmacological profiles to that seen with different plant and mammalian lipoxygenases [25], confirmed the lipoxygenase origin of the 15-HETE-like species. Although the 15-HETE-like species migrated with the same R_f value as that of a 15-HETE standard on thin-layer chromatography, its identity to the mammalian 15-HETE can only be confirmed using gas chromatography/mass spectrometry. This awaits the cloning and expression of the parasite lipoxygenase homologue in order to produce large enough quantities of eicosanoids to allow for such measurements. The efficient conversion of linoleic acid to 13-HODE by the cytosolic fraction of adult worms indicates that the parasite lipoxygenase activity is similar to the erythroid 15-lipoxygenase and the leukocyte 12-lipoxygenase. Our immunoblot analysis and PCR amplification results suggest the presence of more than one lipoxygenase protein and more than one lipoxygenase gene, respectively, in adult *S. mansoni*.

Although we cannot totally exclude the possibility that the lipoxygenase we observed in *S. mansoni* is due to contamination from murine blood cells, it is unlikely based on several lines of evidence. Since murine white blood cells contain both cyclooxygenases and lipoxygenases, if our *S. mansoni* extracts were contaminated with murine cells then the extracts and DNA preparations would be expected to contain not only lipoxygenase, but also murine cell-derived cyclooxygenase activity, cyclooxygenase immunoreactive protein, and PCR-amplifiable sequences. A second line of evidence is based on our

preliminary cloning experiments, in which we have cloned and sequenced 12 unique subclones derived from the PCR amplification of *S. mansoni* DNA with the lipoxygenase oligonucleotides. The deduced amino-acid sequence of one clone displayed 52% similarity (28% identity) and 42% similarity (18% identity) with the analogous regions in human 15-lipoxygenase and soybean lipoxygenase-2, respectively (unpublished data). None of the 12 clones displayed any significant sequence homology to murine sequences in the GenBank database (release 84.0). If our PCR-generated subclones were derived from amplification of contaminating murine DNA or RNA then one might expect the putative clones to show near sequence identity to known murine lipoxygenase sequences. The origin of the lipoxygenase-like PCR product from *S. mansoni* is further strengthened by our ability to amplify by PCR the same lipoxygenase-like sequences from a characterized *S. mansoni* cDNA library obtained from an independent source.

The observation that the lipoxygenase-like PCR products generated from the *S. mansoni* genomic DNA and an *S. mansoni* cDNA library were all of the same size (unpublished observations), suggests that the relevant *S. mansoni* genomic sequences do not contain introns. This would be in contrast to known mammalian lipoxygenase genes [19], since the oligonucleotides used in the lipoxygenase PCR experiment reported here are all located in separate exons of the mammalian lipoxygenase genes [19]. Primers lx-4, lx-5 and lx-7 are located in exons 4, 6 and 8, respectively, in all three genes for the human 5-, 12- and 15-lipoxygenase genes. However, the introns sizes in these three genes are different, ranging in size from 0.2 to 12 kb. For example, PCR amplification of the human 5 and 12 lipoxygenases with primers lx-5/lx-7 would generate two products of 14.4 and 2.2 kb, respectively.

The ability of immunoblot, Southern hybridization and PCR methods using reagents based on mammalian lipoxygenases to detect evolutionarily distant lipoxygenases in *S. mansoni* is not surprising considering the high degree of sequence conservation in the lipoxygenase family [19–21] and in addition, the sequence conservation between human and *S. mansoni* genes [29]. The human 5-, 12- and 15-lipoxygenases exhibit an overall 60% sequence similarity, and each of the human lipoxygenases is roughly 25% identical in sequence to plant lipoxygenases [19,23]. For specific lipoxygenases even greater sequence conservation across species is observed. Thus, the human 5- and 15-lipoxygenases are 92 and 81% identical to their respective rat and rabbit homologues [19]. Furthermore, the recent sequence determination of 607 *S. mansoni* cDNA sequences indicates that significant sequence identity is displayed between several human and *S. mansoni* homologues [29]; for example, aldehyde dehydrogenase from human and *S. mansoni* are 59.7% identical (74% similar).

The production of 15-HETE by an *S. mansoni* lipoxygenase may be significant in both the normal physiology of *S. mansoni* and the mediation of host-parasite interactions. In other invertebrates eicosanoids have been implicated in areas such as egg production and laying, oocyte maturation, prevention of polyspermic fertilization, and salt and water transport physiology [30]. Several groups have suggested that *S. mansoni* eicosanoid production is related to host-parasite interactions including cercarial penetration [7], and modulation of host immune defenses through suppression of host-cell-mediated immune responses [5,6,10]. One approach to elucidate the role of eicosanoid biosynthesis in *S. mansoni* might be to determine if developmental regulation occurs. The molecular cloning of genes involved in eicosanoid metabolism from *S. mansoni* would provide useful probes to address the expression of these genes during the parasites' life cycle.

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Molecular Cloning and Functional Expression of a *Caenorhabditis elegans* Aminopeptidase Structurally Related to Mammalian Leukotriene A_4 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 A_4 (LTA_4) 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* LTA_4 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 LTA_4 hydrolase and aminopeptidase activities. Despite the strong homology between the human LTA_4 hydrolase and *C. elegans* AP-1(63% similarity and 45% identity at the amino acid level), reverse-phase high pressure liquid chromatography and radioimmunoassay for LTB_4 production revealed the inability of the *C. elegans* AP-1 to use LTA_4 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 LTA_4 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 IC_{50} of 2.6 ± 1.2 μM. The bifunctionality of the mammalian LTA_4 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 LTA_4 hydrolase activity in more evolved organisms.

Leukotriene A_4 (LTA_4)[1] hydrolase (EC 3.3.2.6) is the rate-limiting enzyme in the lipoxygenase cascade of arachidonic acid metabolism leading to the biosynthesis of the proinflammatory substance leukotriene B_4 (LTB_4) from the epoxide intermediate LTA_4 (1, 2). At nanomolar concentrations, LTB_4 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, LTB_4 is regarded as an important chemical mediator in a variety of inflammatory diseases (4). Sequence comparison of LTA_4 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 LTA_4 hydrolase contained one catalytic zinc atom coordinated by His^{295}, His^{299}, and Glu^{318} (8). Subsequently, the enzyme was shown to exhibit a previously unknown zinc-dependant 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, LTA_4 hydrolase has been shown to efficiently hydrolyze several arginyl tri- and dipeptides, leading to its identification as an arginine aminopeptidase (13). Both the aminopeptidase and the LTA_4 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 LTA_4 hydrolase/aminopeptidase remain unanswered. For example, does the enzyme demonstrate both LTA_4 hydrolase and aminopeptidase activities in other species? Which function originated first in evolution? What is the significance of this bifunctionality? LTA_4 hydrolase/aminopeptidase is a soluble monomeric protein (M_r ~ 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 LTA_4 hydrolases (accession numbers U27538 and X94547, respectively). Both sequences encode proteins similar in their primary amino acid sequences to the mammalian LTA_4 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 LTA_4 hydrolase exhibited mainly aminopeptidase activity, whereas its hydrolase activity converted the ma-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF068200 and AF068201.

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[1] The abbreviations used are: LTA_4, leukotriene A_4 (5*S*)-*trans*-5,6-*oxido*-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid); LTA_4 hydrolase, leukotriene A_4 hydrolase; LTB_4, leukotriene B_4 ((5*S*,12*R*)-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid); AP, aminopeptidase; 5,6-diHETE, 5,6-dihydroxyeicosatetraenoic acid; HPLC, high pressure liquid chromatography; EST, expressed sequence tag; PCR, polymerase chain reaction; kb, kilobase pair(s); bp, base pair(s).

jority of the substrate LTA_4 to what has been putatively identified as 5,6-diHETE (a much less potent leukotriene) rather than LTB_4 (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 LTA_4 hydrolase, exhibits no LTA_4 hydrolase activity and only aminopeptidase activity. The strong homology between *C. elegans* AP-1 and mammalian LTA_4 hydrolases (45%) suggests that these enzymes may have developed from a common ancestral precursor.

MATERIALS AND METHODS

Cloning of a LTA_4 Hydrolase-like cDNA Homologue from C. elegans—The cm01c7 phage clone from a *C. elegans* mixed stage hermaphrodite cDNA library (made by Chris Martin) containing the LTA_4 hydrolase-like EST in SHLX2 λ phage vector (23) was obtained from Dr. R. H. Waterston (24). MC1061 $recA^-$ tet^R (used for plating λ SHLX2) and the pop-out *Escherichia coli* cam^R Kan^R 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 plus kit (Promega, Madison, WI) or Qiagen tip-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 plasmid was then sequenced using T7 and SP6 primers and automated DNA sequencing on an Applied Biosystems model 386 DNA sequencer utilizing T7 DNA polymerase and internal labeling with fluorescein-15-dATP (26).

Approximately 2×10^6 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-^{32}P]dCTP-labeled (Boehringer Mannheim) 0.95-kb *ApaI/ScaI 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 $\mu g\ ml^{-1}$ 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 pBluescript SK phagemid-based primers T3 and T7. Five positive plaques were isolated and confirmed to be related to the cm01c7 EST by PCR using *C. elegans* cm01c7 EST-based primers. Following *in vivo* excision of the pBluescript phagemid from the UNI-ZAP vector, plasmid DNA was prepared, and the positive inserts were sequenced.

The longest LTA_4 hydrolase-like clone obtained, termed C5 (~1.4 kb), lacked the 5'-end of the coding region (as predicted from the size of cDNA of all previously cloned LTA_4 hydrolases). To isolate the 5'-end of the *C. elegans* cDNA, several anchored PCR amplifications using the phagemid-based primers T3, SK, and pSK (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 $MgCl_2$, 0.2 mM deoxynucleotide triphosphates, 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 M NaOH. The PCR-amplified fragments were screened by overnight hybridization using the nested [γ-^{32}P]ATP-labeled primer C5T3 (5'-CCA GAC GGC GCA TCT TTC GCT-3') (based on the most 5' sequence in clone C5) and T4 polynucleotide kinase (Boehringer Mannheim) in 6× SSC, 20 mM NaH_2PO_4, 0.4% SDS, 5× Denhardt's solution, 500 $\mu g\ ml^{-1}$ 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 Qiaquick 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 inserts 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 NH_2-terminal sequence.

Expression of the Recombinant C. elegans AP-1 Protein—A 1.8-kb *NotI/XbaI* fragment representing the entire AP-1 coding sequence was amplified by PCR using Expand high fidelity *Taq* Polymerase (Boehringer Mannheim) and the primers flag-1 (5'-CAT GCA TGC ATG GCG GCC GCG GCA CCT CCA CAT CCG AGA GAT CCC-3') and flag-2 (5'-CAT GCA TGC ATG TCT AGA TTA TTT GAG AAG ACT TTG GAT TGC-3'). The flag-1 primer introduces an NH_2-terminal *NotI* site (the *C. elegans* translation initiation codon was abolished to force translation to start from the ATG, supplied by the pFLAG CMV2 expression vector), and the flag-2 primer introduces a COOH-terminal *XbaI* site immediately after the stop codon (thus eliminating the 3'-untranslated region). The AP-1 *NotI/XbaI* fragment was then subcloned into the *NotI/XbaI* 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 cells), 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 an atmosphere of 6% CO_2. 10×10^6 cells per 600-cm^2 culture dish were seeded in 100 ml of media and transiently transfected at 80% confluence with 94 μg of pFLAG.celAP-1 or pFLAG control plasmid and 283 μl of LipofectAMINE reagent (Life Technologies, Inc.), following the recommendations of the manufacturer. Two days after transfection, cells were harvested 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.

Preparation of C. elegans Extracts—Frozen mixed stage hermaphrodite *C. elegans* worms were a generous gift from J. McGhee (University of Calgary, Alberta, Canada). 5 ml of wet worms (resuspended in phosphate-buffered saline) were homogenized under liquid nitrogen (using a mortar and pestle) and resuspended in 5 ml of 0.1 M Tris, pH 7.0, and then in 10 ml of TBS (50 mM Tris pH 7.4, 150 mM NaCl) to a total volume of 20 ml. The homogenate was then sonicated at 4 °C (three times, 20 s each). The suspension was first centrifuged at 2000 × *g* for 10 min at 4 °C to yield a large membrane fraction, followed by centrifugation of the resultant supernatant at 200,000 × *g* for 60 min at 4 °C to prepare microsomal and cytosolic fractions. Protein concentrations were determined using a protein assay kit (Bio-Rad).

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 glycine/HCl at pH 3.5, followed by washing three times with 3 ml of TBS. A total of 30 mg (10 ml of 3 mg/ml) of the 10,000 × *g* supernatants of COS-7 cells transfected with either pFLAG vector or pFLAG.celAP-1 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 columns, 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; NH_2-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 columns 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 (lysates of COS-7 cells transfected with either pFLAG vector or pFLAG.cel AP-1 construct), the anti-FLAG M2 affinity columns flow-through, combined washes, and the different FLAG peptide eluted fractions, as well as the NH_2-terminal FLAG fusion protein of *E. coli* bacterial alkaline phosphatase control (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

antibody (Amersham Pharmacia Biotech) was used at a dilution of 1:1000. Immunodetection was performed using enhanced chemiluminescence according to the manufacturer's instructions (Amersham Pharmacia Biotech). Autoradiographs for chemiluminescence detection were exposed to Kodak X-OMAT x-ray films for 3 min and then developed. A polyclonal anti-rabbit human LTA_4 hydrolase antiserum (raised against the entire protein) (29) was also used for immunoblot analysis (Merck Frosst, Pointe Claire-Dorval, Quebec, Canada).

LTB_4 Assays—LTA_4 ethyl ester was synthesized at the Merck Frosst Center for Therapeutic Research (Montreal, Quebec, Canada). LTB_4, prostaglandin B_2, 6-*trans*-LTB_4, 6-*trans*-12-epi-LTB_4, and (5*S*,6*S*)-diHETE, (5*S*,6*R*)-diHETE standards were purchased from Cayman Chemical Co., Inc. (Ann Arbor, MI). The soluble supernatant fraction following a 100,000 × *g* centrifugation (100S fraction) of Sf9 cells infected with a recombinant human LTA_4 hydrolase construct (29) was used as a positive control (Merck Frosst Center for Therapeutic Research). Alkaline hydrolysis of LTA_4 ethyl ester was carried out as described (30). LTA_4 hydrolase assays on *C. elegans* cytosolic fraction (125 μg), anti-FLAG M2 purified COS-7 lysates (200 μl of 10,000 × *g* supernatant of COS-7 cells transfected with pFLAG vector and 0.8 μg of FLAG-tagged *C. elegans* AP-1 protein), or recombinant human LTA_4 hydrolase (25 μg) were performed as described previously (31). Samples in 250-μl reactions were incubated with 25 μM LTA_4 in 0.1 M Tris/HCl, pH 8.0, 1 mg/ml BSA for 10 min at room temperature. Reactions were terminated with the addition of an equal volume of methanol containing 1 nmol/ml prostaglandin B_2 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/H_2O/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 eicosanoid standards.

Radioimmunoassay detection of LTB_4 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 25 μM LTA_4 in 0.1 M Tris/HCl, pH 8.0, 1 mg/ml BSA for 10 min at room temperature. Reactions were stopped by the addition of 20% methanol followed by a brief centrifugation at 10,000 × *g* for 20 s. LTB_4 production was assayed using a radioimmunoassay kit ([^{3}H]leukotriene B_4 assay system) (Amersham Pharmacia Biotech). The human recombinant LTA_4 hydrolase was used as a positive control as described above.

Aminopeptidase Assays—Amino acid *p*-nitroanilides (Sigma or Bachem Bioscience Inc.) were incubated (final concentration, 0.05–5 mM) at room temperature with 0.17 μg of purified *C. elegans* AP-1 FLAG-fusion protein, anti-FLAG M2 affinity gel-purified fractions of lysates of COS-7 cells transfected with pFLAG vector, or 0.17 μg of purified human recombinant LTA_4 hydrolase/aminopeptidase in 250 μl of buffer containing 0.1 M Tris, pH 8, 200 mM NaCl, 1 mg/ml BSA. The assays were performed in 96-well microtiter plates (path length, 0.7 cm), and the formation of the product (*p*-nitroaniline, ϵ = 10, 800 M^{-1} cm^{-1}) was monitored for 60 min at 405 nm using a kinetic microplate reader spectrophotometer (Molecular Devices). Spontaneous hydrolysis of the substrate (~ 0.03 milli-absorbance units/min) was corrected for by subtracting the absorbance of control incubations without enzyme. Kinetic constants (K_m and k_{cat}) were determined by nonlinear regression (Kaleidagraph software) of the data to the Michaelis-Menten equation.

Inhibition of Aminopeptidase Activity—Bestatin, an inhibitor of aminopeptidases (Sigma), was evaluated as an inhibitor of the *C. elegans* aminopeptidase activity. Bestatin (final concentration, 0–50 μM) in 250 μl of buffer containing 0.1 M Tris, pH 8, 200 mM NaCl, 1 mg/ml BSA was incubated at room temperature with 0.17 μg of purified *C. elegans* AP-1(FLAG) enzyme for 10 min. L-Arginine-*p*-nitroanilide (1 mM) was then added, and substrate hydrolysis was monitored as described above. IC_{50} values were estimated from a log dose-response curve of initial velocity *versus* inhibitor concentration.

RESULTS AND DISCUSSION

Molecular Cloning of a LTA_4 Hydrolase-like cDNA Homologue from C. elegans—A search of the annotated files in GenBank using the key words "leukotriene hydrolase" yielded an EST (GenBank accession number M88793) clone termed cm01c7 (327 bp) from a *C. elegans* mixed stage hermaphrodite cDNA library. When translated, the EST revealed strong homology (51%) to the human LTA_4 hydrolase. The complete sequence of a 0.95-kb *ApaI*/Sca I restriction fragment containing the *C. elegans* LTA_4 hydrolase-like EST was obtained from the phage clone cm01c7 (see under "Materials and Methods" for details) and was confirmed as a mammalian LTA_4 hydrolase-like sequence by sequence homology comparisons using the BLASTN and TBLASTN algorithms (National Center for Biotechnology Information) (32) and FASTA and TFASTA programs (33). The 0.95-kb fragment was radiolabeled with ^{32}P and used as a probe to screen a *C. elegans* λ phage cDNA library, resulting in the isolation of clone C5 (~ 1.4 kb), which showed a strong sequence homology (~ 45%) to the human LTA_4 hydrolase but was missing the 5′ part of the gene (as predicted from the sizes of cDNAs encoding previously cloned LTA_4 hydrolases). A series of anchored PCRs with the *C. elegans* cDNA library as template and using pBluescript SK phagemid-based primers, as well as several antisense primers based on the most 5′ sequence of clone C5, was carried out to isolate the missing 5′-end of clone C5. The full-length *C. elegans* cDNA sequence and the deduced amino acid sequence, named AP-1, are shown in Fig. 1. The *C. elegans* AP-1 cDNA sequence is 2152 bp long, consisting of a short 15-base 5′-untranslated region, an open reading frame encoding a 609-amino acid protein, a 282-bp-long 3′-untranslated region, and a 28-bp-long poly(A$^+$) tail. No consensus *N*-glycosylation sites, targeting signals, or putative phosphorylation sites were found in the sequence. The first ATG triplet (starting at nucleotide 1 in Fig. 1) in the sequence was considered to be the initiation codon of protein translation because 1) it matches the location of the translation initiation codon from both the human and the mouse LTA_4 hydrolase cDNAs (18, 19) with only one extra codon in the *C. elegans sequence* (Fig. 2), 2) the nucleotide sequence flanking it (AATATGG) is in agreement with Kozak's rule for translational initiation consensus sequence (34), and 3) the open reading frame starting from this methionine and ending at the TAA terminator codon (nucleotide 1828) encodes a 68,248-kDa protein, corresponding to the molecular mass of the recombinant *C. elegans* AP-1 protein estimated by SDS-polyacrylamide gel electrophoresis (see below). The length of the AP-1 open reading frame (1827 bp), the number of the deduced amino acids (609 residues), and the molecular mass of the encoded protein (68,248 kDa) are comparable to the human (1830 bp, 610 residues, 69,140 kDa) and the mouse (1830 bp, 610 residues, 68,917 kDa) LTA_4 hydrolases (18, 19).

Amino Acid Sequence Comparison of the Human and the Mouse LTA_4 Hydrolases to Their C. elegans Homologue—Data base searches identified the human LTA_4 hydrolase as the most closely related protein to the *C. elegans* AP-1 translation product (63% similarity and 45% identity) (results not shown). His^{297}, His^{301}, and Glu^{320} in the *C. elegans* amino acid sequence (the underlined residues in Fig. 1) conform to a catalytic zinc site of mammalian LTA_4 hydrolases and zinc metallopeptidases (5, 35) and match His^{295}, His^{299}, and Glu^{318} in both the human and the mouse LTA_4 hydrolase sequences. These three residues are likely to be involved in the coordination of the zinc atom as described previously for the mouse LTA_4 hydrolase (8) and for certain peptidases and neutral proteases (5). A multiple alignment of the amino acid sequences of the human LTA_4 hydrolase, the mouse LTA_4 hydrolase, and their putative *C. elegans* homologue and the conserved residues in the three sequences are shown in a consensus (Fig. 2). A high degree of homology is seen in the region between amino acid residues 246 and 320 in the *C. elegans* sequence, the homology then decreases toward either the amino or the carboxyl terminus of the protein. A typical consensus zinc binding motif $HEXXHX_{18}E$ is indicated starting from amino acid 298 to 321 in the *C. elegans*

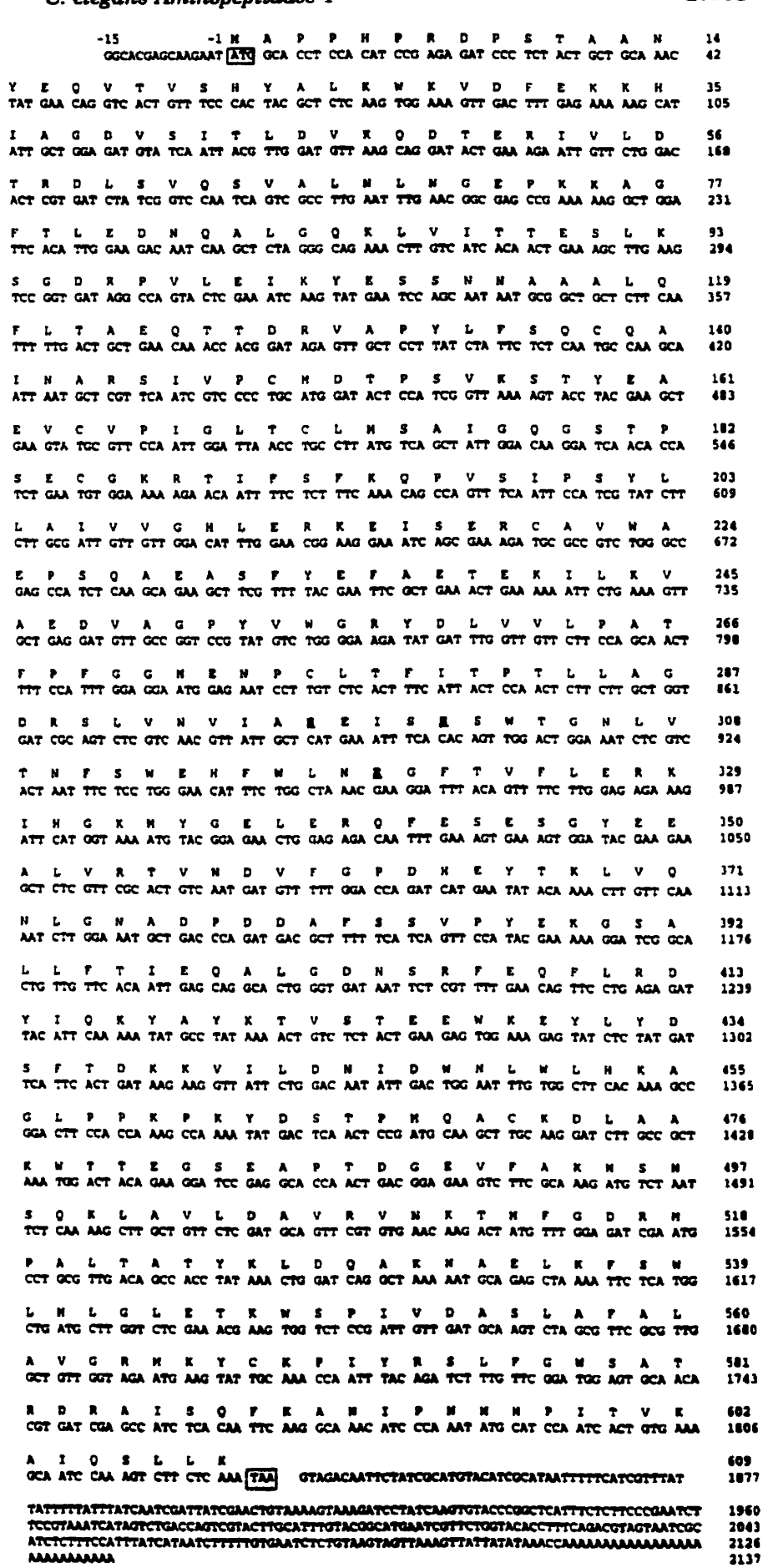


FIG. 1. **Nucleotide sequence of the *C. elegans* AP-1 cDNA and deduced amino acid sequence.** Nucleotides are numbered beginning with the first residue of the ATG translation initiation codon. Nucleotides 5′ of the ATG are designated by negative numbers. The deduced amino acid sequence is shown in the one-letter code above the nucleotide sequence. Amino acids are numbered from the NH_2-terminal methionine residue. Amino acids involved in the putative zinc binding site are *underlined*.

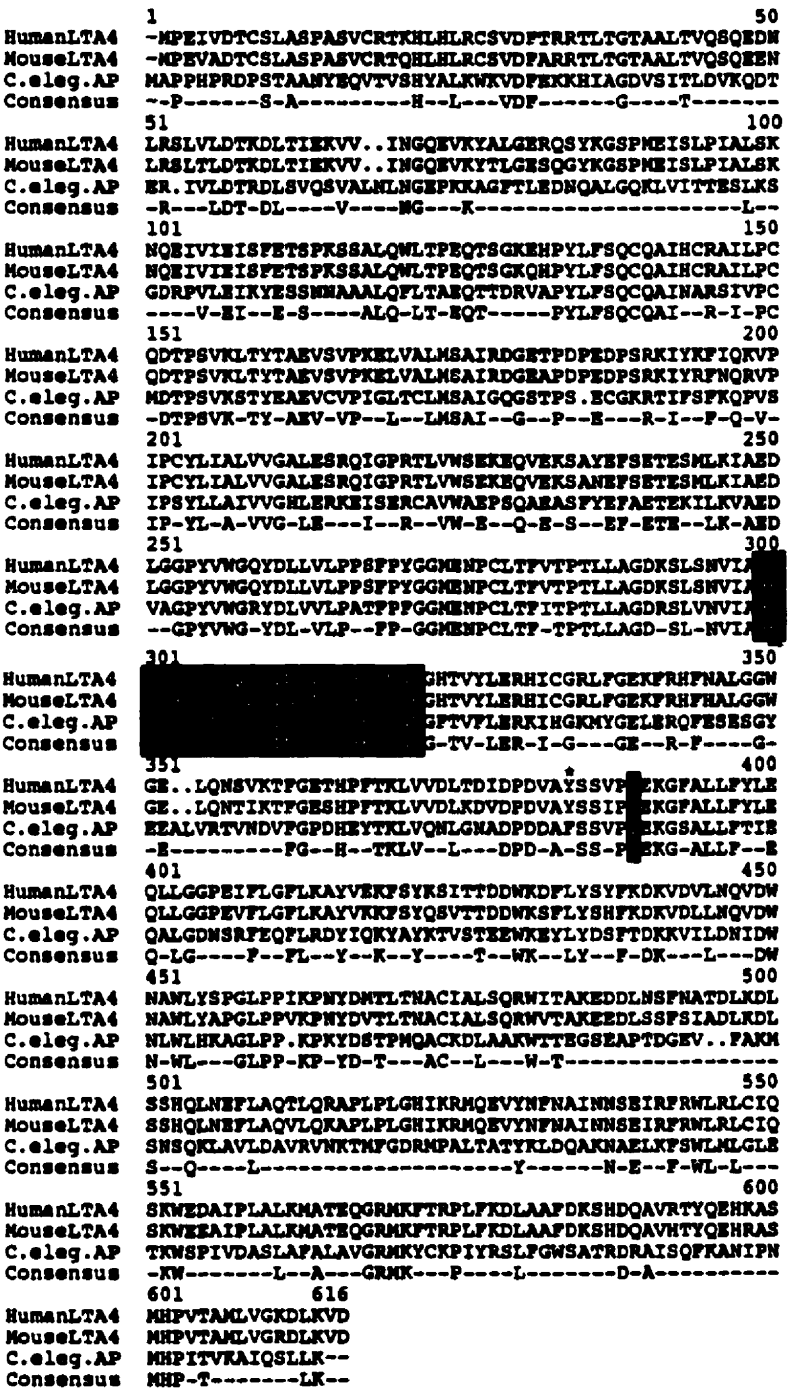
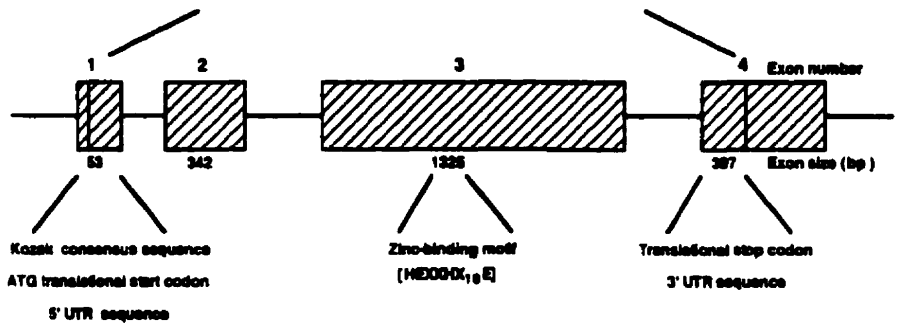


FIG. 2. **A multiple alignment of amino acid sequences of the human leukotriene A_4, the mouse leukotriene A_4 hydrolase, and the *C. elegans* AP-1.** The alignment was made using the Pretty Plot function of the GCG program (33). Amino acids are numbered beginning with the first methionine residue in 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 ($HEXXHX_{18}E$), 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 LTA_4 hydrolase/aminopeptidase, are indicated on a *black background*. The conserved glutamate residue necessary for peptidolysis of mammalian LTA_4 hydrolases (Glu^{296} in both human and mouse sequences) is *underlined*. The tyrosine residue Tyr^{378} (in both the human and the mouse LTA_4 hydrolase sequences) involved in the covalent binding of LTA_4 to the human LTA_4 hydrolase is indicated by an *asterisk* and is replaced by a phenylalanine (Phe^{382} in the *C. elegans* sequence).

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 LTA_4 hydrolase. The tyrosine residue Tyr^{383} in both the human and the mouse sequences, which is essential for the peptidase activity of the mammalian LTA_4 hydrolase/aminopeptidase enzyme and may act as a proton donor in a general base mechanism (37), is conserved in the *C. elegans* AP-1 (Fig. 2, Tyr^{387} in the *C. elegans* sequence). Glu^{296} in both human and mouse sequences, the mutation of which to Gln^{296} abolishes the aminopeptidase activity of the mammalian enzyme (38), is also conserved in the *C. elegans* sequence (Fig. 2, Glu^{298} in the *C. elegans* sequence). On the other hand, Tyr^{378} in both the human and the mouse LTA_4 hydrolase sequences, which is known to be involved in the covalent binding of LTA_4 (39), is replaced by a phenylalanine (Phe^{382}) in the *C. elegans* sequence.

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 C42C1and 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. 3*A*). 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

A



B

Intron number	Intron size (bp)	Splice position	Exon	Intron donor	Intron acceptor	Exon
1	44	48/49	Y TAT E GAA	cagg/atcaa.....aaattaactc		Q CAG V GTC
2	49	390/391	R AGA V GTT	gtgat/tta.....gatcatt/cag		A GCT P CCT
3	49	1715/1716	P CCA I ATT Y TA	cagg/gaaaa.....attta/tatt		C AGA S TCT

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 indicated by the single *uppercase letters* present above the first nucleotide of each codon.

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. 3*B*). 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^{297} and His^{301}) and glutamate residue (Glu^{320}), which constitute the zinc-binding domain ($HEXXHX_{18}E$), are located on one exon (Fig. 3, *exon 3*), unlike the structure of the human LTA_4 hydrolase/aminopeptidase gene (43), in which the two essential zinc-binding histidine residues (His^{295} and His^{299}) are present on exon 10, whereas the third zinc-binding ligand glutamate (Glu^{318}) 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 ATG, followed by an amino-terminal FLAG epitope (NH_2-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 and harvested, followed by preparation of membrane and soluble protein fractions and analysis by SDS-polyacrylamide gel electrophoresis and Western blotting using anti-FLAG M2 antibody. As expected from its calculated molecular mass, a 69-kDa immunoreactive protein was detected using the anti-FLAG antibody in the supernatant of COS-7 cells transfected with pFLAG.cel AP-1 cDNA (Fig. 4, *lane 2*) but not in the supernatant of mock-transfected cells (results not shown). As described previously for the mammalian LTA_4 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 10% SDS-polyacrylamide gel electrophoresis, and detected by the monoclonal antibody anti-FLAG M2. All of the expressed *C. elegans* AP-1 protein was bound to the anti-FLAG M2 affinity resin. No protein was detected in either the flow-through from the column or the column washes with the *C. elegans* FLAG-tagged AP-1 eluting mainly in fractions 4–7 (Fig. 4, *lanes 8–11*). Despite the significant amino acid sequence homology

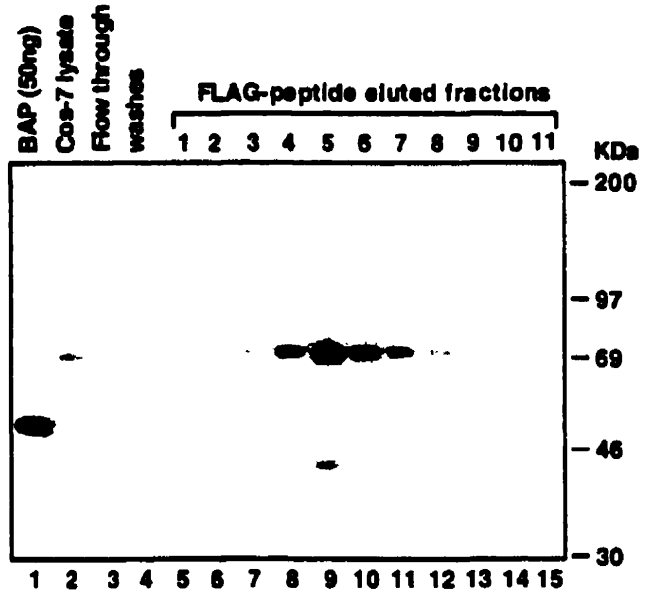


FIG. 4. **Immunoblot analysis of the *C. elegans* FLAG-tagged AP-1 protein purified using anti-FLAG M2 affinity gel.** Shown are 50 ng of FLAG-tagged bacterial alkaline phosphatase (*BAP*) standard (*lane 1*), 50 μg of total protein of the 10,000 × g supernatant of COS-7 cells transfected with pFLAG.celAP-1 (*lane 2*), 30 μl each of anti-FLAG M2 affinity column flow-through (*lane 3*), anti-FLAG M2 affinity column washes (*lane 4*), and 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.

TABLE I
Kinetic constants for hydrolysis of amino acid p-nitroanilides

The different amino acid p-nitroanilides (0.05–5 mM) were incubated at room temperature with 0.17 μg of either purified *C. elegans* AP-1 (FLAG) enzyme or human LTA_4 hydrolase/aminopeptidase enzyme in 250 μl of 0.1 M Tris, pH 8.0, 200 mM sodium chloride containing BSA (1 mg/ml). *p*-Nitroaniline formation was monitored spectrophotometrically for 60 min at 405 nm. Kinetic constants (K_m and k_{cat}) were determined by nonlinear regression analysis. Activities of purified vector construct with each substrate were less than 10% of values obtained with purified *C. elegans* AP-1 enzyme and were comparable to the observed nonenzymatic hydrolysis rates.

	Human LTA_4 hydrolase			*C. elegans* AP-1		
	K_m	k_{cat}	k_{cat}/K_m	K_m	k_{cat}	k_{cat}/K_m
	mM	s^{-1}	$s^{-1}M^{-1}$	mM	s^{-1}	$s^{-1}M^{-1}$
L-Arg-pNA	0.09	0.55	6.10×10^3	0.43	0.21	0.48×10^3
L-Ala-pNA	1.44	3.11	2.20×10^3	5.53	0.78	0.14×10^3
L-Leu-pNA	0.25	0.52	2.08×10^3	2.00	0.25	0.13×10^3
L-Lys-pNA	0.05	0.09	1.80×10^3	0.39	0.05	0.13×10^3
L-Pro-pNA	0.24	0.45	1.87×10^3	1.90	0.23	0.12×10^3
L-Met-pNA	0.40	0.24	0.60×10^3	0.46	0.03	0.06×10^3
L-Val-pNA	0.80	0.08	0.10×10^3	0.90	0.01	0.01×10^3
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	

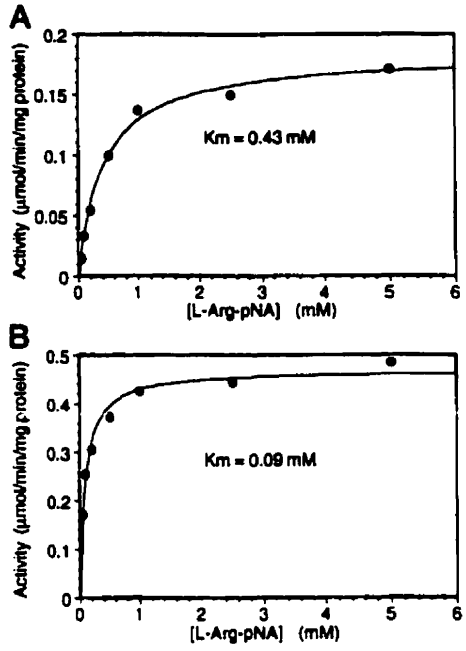


FIG. 5. **Comparison of aminopeptidase activity of the cloned *C. elegans* AP-1 (*A*) with that of the recombinant human LTA_4 hydrolase/aminopeptidase (*B*).** Purified FLAG-tagged *C. elegans* AP-1 (0.17 μg) (*A*) and human LTA_4 hydrolase/aminopeptidase (0.17 μg) (*B*) in 250 μl of 0.1 M Tris, pH 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 K_m values were determined by nonlinear fit of the experimental values to the Michaelis-Menten equation.

between the *C. elegans* AP-1 and the human LTA_4 hydrolase/aminopeptidase (45% identity), a rabbit anti-human LTA_4 hydrolase polyclonal antiserum (29) failed to detect any LTA_4 hydrolase 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 $HEXXHX_{18}E$ 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*-nitroanalide derivatives as chromogenic amide substrates. These compounds represent acidic, basic, and neutral amino acids, as well as amino acids with NH_2-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 K_m of 0.43 ± 0.01 mM and a V_{max} of 0.18 ± 0.01 μmol/min/mg enzyme. These values can be compared with a K_m of 0.09 ± 0.01 mM and a V_{max} of 0.47 ± 0.01 μmol/min/mg obtained for the human LTA_4 hydrolase/aminopeptidase (Fig. 5). The K_m and k_{cat} 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 k_{cat}/K_m 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 NH_2-terminal substitutions, and amino acids with *D*-stereochemistry were poor substrates. The human recombinant LTA_4 hydrolase/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 NH_2-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 k_{cat}/K_m for LTA_4 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 LTA_4 hydrolase/aminopeptidase (10), as well as other aminopeptidases, inhibited the hydrolysis of L-arginine *p*-nitroanilide by AP-1. The concentration for half-maximal inhibition (IC_{50}) 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 Ala^{117} to Val^{117} 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 Tyr^{383} and Glu^{296} may be important for the aminopeptidase activity of the mammalian LTA_4 hydrolase/aminopeptidase enzyme. It is also interesting to note that this alanine residue (Ala^{114} in the human sequence) is conserved evolutionary as it is found in the *C. albicans* LTA_4 hydrolase (which mainly exhibits aminopeptidase activity), the *S. cerevisiae* proposed LTA_4 hydrolase (which is yet to be characterized), all cloned mammalian LTA_4 hydrolases, including human, mouse, rat, and guinea pig, and the *C. elegans* AP-1 (data not shown).

Measurement of LTA_4 Hydrolase Activity of C. elegans AP-1 Enzyme—The *C. elegans* AP-1 protein was analyzed for epoxide hydrolase activity using LTA_4 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 LTA_4 revealed no production of LTB_4 (Fig. 6, *peak 4, tracings 6* and *2*, respectively). In contrast, the human LTA_4 hydrolase (used as a positive con-

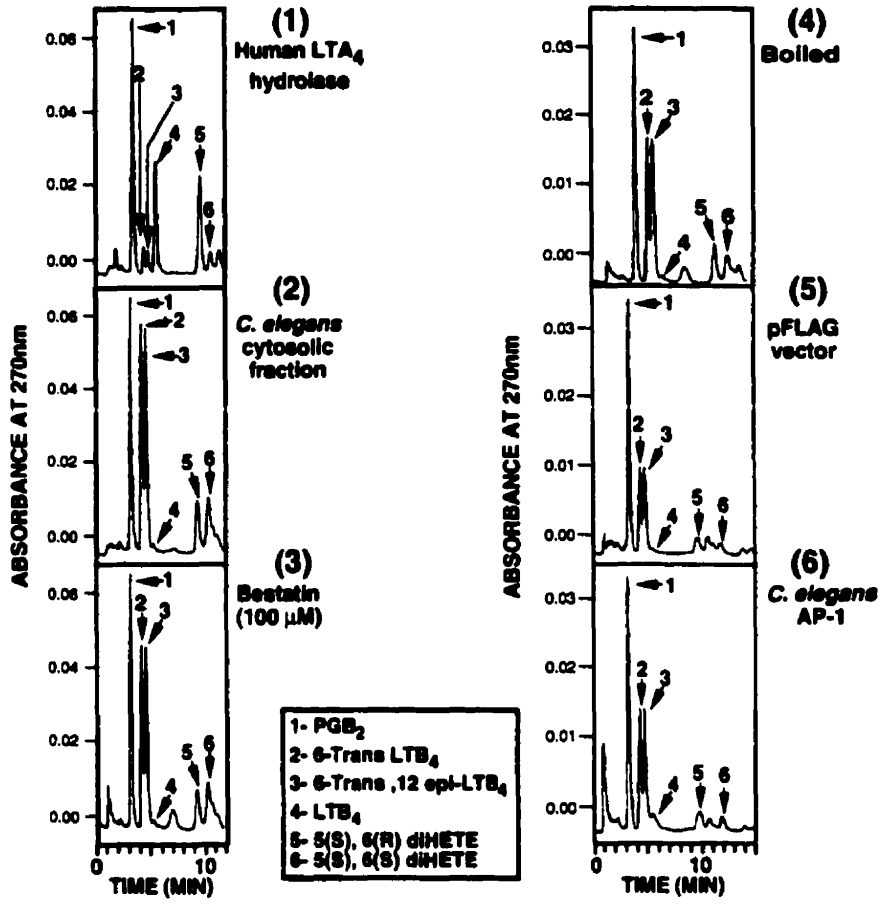


FIG. 6. **Reverse-phase HPLC analysis of products formed by *C. elegans* cytosolic extracts and COS-7 cells transfected with the pFLAG.cel AP-1 construct.** Shown are reverse-phase HPLC chromatograms of products formed following the incubation of 25 μM LTA_4 with human recombinant LTA_4 hydrolase standard (25 μg of the S100 fraction of Sf9 cells; see under "Materials and Methods" and Ref. 29) (*tracing 1*), 125 μg of *C. elegans* cytosol in the absence of bestatin (*tracing 2*) or pretreated with 100 μM bestatin (*tracing 3*), boiled *C. elegans* cytosol (*tracing 4*), 200 μl 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*). LTA_4 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 B_2 (internal standard); *peak 2*, 6-*trans*-LTB_4; *peak 3*, 6-*trans*-12-epi-LTB_4; *peak 4*, LTB_4; *peak 5*, (5*S*,6*R*)-diHETE; *peak 6*, (5*S*,6*S*)-diHETE. The chromatograms are representative of three experiments with identical results.

trol) produced mainly peak 4 (Fig. 6, *tracing 1*), which eluted with the retention time of the expected enzymatic product LTB_4. Small amounts of the other peaks (peaks 2, 3, 5, and 6) represent the nonenzymatic hydrolysis of LTA_4. In the absence of LTA_4 hydrolase activity (Fig. 6, all tracings except *tracing 1*), LTA_4 was mostly converted to the nonenzymatic hydrolysis products, the all-*trans*-LTB_4 epimers at C12 (6-*trans*- LTB_4 and 12-*epi*-6-*trans*- LTB_4, peaks 2 and 3, respectively), as well as (5*S*,6*R*)-diHETE (peak 5), and (5*S*,6*S*)-diHETE (peak 6). These species are normally formed in small and equal amounts in aqueous solutions by spontaneous hydrolysis (44), and their peaks eluted with the retention times and spectra of known eicosanoid standards. Peaks 2, 3, 5, and 6 were also produced by boiled *C. elegans* cytosol (Fig. 6, *tracing 4*), as well as boiled FLAG-tagged AP-1 (data not shown), confirming their nonenzymatic origin. *C. elegans* cytosol pretreated with 100 μM bestatin (an inhibitor of both LTA_4 hydrolase and aminopeptidase activities of the human enzyme (10)), showed the same chromatographic profile (Fig. 6, *tracing 3*) as the untreated or the boiled *C. elegans* cytosol. The inability of the *C. elegans* AP-1 enzyme to synthesize LTB_4 from LTA_4 was also confirmed by the absence of any LTB_4 production (data not shown) using a LTB_4 radioimmunoassay (lower limit of detection is 16 pg of LTB_4 ml^{-1}).

Although *C. elegans* AP-1 does not appear to hydrolyze LTA_4, the high degree of identity between the active sites of AP-1 and mammalian LTA_4 hydrolases (Fig. 2) suggests that LTA_4 may bind in the active site of AP-1 without being a substrate for catalysis. To test this possibility, we examined the effect of LTA_4-ethyl ester on the aminopeptidase activity of AP-1 using L-arginine-*p*-nitroanilide as a substrate. We chose the LTA_4-ethyl ester as it is a suicide inactivator of the mammalian LTA_4 hydrolases and is much more resistant than LTA_4 to nonenzymatic hydration. The LTA_4-ethyl ester had no effect on the aminopeptidase activity of AP-1 at concentrations up to 100 μM, 10 times the K_m of mammalian LTA_4 hydrolase for LTA_4-ethyl ester (results not shown).

The bifunctional human LTA_4 hydrolase/aminopeptidase enzyme is suicide-inactivated during catalysis via an apparently mechanism-based irreversible binding of LTA_4 to the protein (45), with tyrosine at position 378 identified as the site for covalent binding of LTA_4. Interestingly, the mutation of Tyr^{378} to Phe^{378} in the human LTA_4 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 (Phe^{382}), but the *C. elegans* enzyme does not hydrolyze LTA_4, indicating that other residues (lacking in the *C. elegans* AP-1 sequence) must also be important for LTA_4 binding and catalysis.

That the cloned *C. elegans* AP-1 enzyme functions as an aminopeptidase with no LTA_4 hydrolase activity is interesting, as its primary structure resembles LTA_4 hydrolases more than it does aminopeptidases (Table II). Comparison of the *C. elegans* AP-1 enzyme and other proteins in the SwissProt data base revealed 45% identity to the human, mouse, rat, and guinea pig LTA_4 hydrolases at the amino acid level, with lower identity to the human, rat, and pig aminopeptidase-N (28–30%). Moreover, the identity between *C. elegans* AP-1 and mammalian LTA_4 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 $HEXXHX_{18}E$). It is interesting to note that the same identity (an average of 30%) that is shown between *C. elegans* AP-1 and

TABLE II

Amino acid sequence identifity (%) between C. elegans AP-1, LTA_4 hydrolases, and aminopeptidases

A BLASTP search of SwissProt data base (release 96) identified LTA_4 hydrolases followed by aminopeptidases as the most closely related proteins to the *C. elegans* AP-1. The Bestfit algorithm in the GCG sequence analysis software (gap weight of 12, length weight of 4, gap creation penalty of 12, and gap extension penalty of 4) was used to determine percentage sequence identity. $hLTA_4$, human LTA_4 hydrolase; $mLTA_4$, mouse LTA_4 hydrolase; $rLTA_4$, rat LTA_4 hydrolase; $gpLTA_4$, guinea pig LTA_4 hydrolase; rAP-B, rat aminopeptidase B; hAP-N, human microsomal aminopeptidase N; rAP-N, rat microsomal aminopeptidase N; pgAP-N, pig microsomal aminopeptidase N.

	C. elegans AP-1	$hLTA_4$	$mLTA_4$	$rLTA_4$	$gpLTA_4$	rAP-B	hAP-N	rAP-N	pgAP-N
C. elegans AP-1	100	45	45	45	45	38	29	28	30
$hLTA_4$	45	100	92	92	92	44	30	29	29
$mLTA_4$	45	92	100	97	90	44	30	32	29
$rLTA_4$	45	92	97	100	90	44	29	31	29
$gpLTA_4$	45	92	90	90	100	42	33	32	33
rAP-B	38	44	44	44	42	100	24	21	23
hAP-N	29	30	30	29	33	24	100	77	79
rAP-N	28	29	32	31	32	21	77	100	77
pgAP-N	30	29	29	29	33	23	77	77	100

other aminopeptidases is also seen between mammalian LTA_4 hydrolases and any given aminopeptidase enzyme (Table II). The structural similarity between *C. elegans* AP-1 and mammalian LTA_4 hydrolases suggests an evolutionary relationship. Recently, three other proteins that are structurally related to mammalian LTA_4 hydrolases have been identified in lower invertebrates. These proteins include a *C. albicans* LTA_4 hydrolase-related enzyme (41% identity to human LTA_4 hydrolase) that functions mainly as an aminopeptidase but fails to hydrolyze LTA_4 to LTB_4 (47), a gene from the yeast *S. cerevisiae* (39% identity to human LTA_4 hydrolase) (22), and a partial amino acid sequence (316 residues) of a *D. discoideum* cDNA (GenBank accession number U27538)[2] with 38% identity to human LTA_4 hydrolase. Biochemical studies to clarify the enzymatic activity (or activities) of both the yeast and the *Dictyostelium* LTA_4 hydrolase-like proteins await their expression and characterization. LTB_4 production has not been reported in either *C. albicans* or *D. discoideum*, and our analysis of mixed stage *C. elegans* worms failed to detect any LTA_4 hydrolase activity. The high primary sequence identity of the *C. elegans* AP-1 to mammalian LTA_4 hydrolases suggests that AP-1 may represent an evolutionary precursor of the mammalian LTA_4 hydrolases. Thus, mammalian LTA_4 hydrolases may have originated from aminopeptidases like AP-1, retaining their aminopeptidase activity and developing a LTA_4 hydrolase function in higher eukaryotes. In support of this hypothesis, an aminopeptidase B has recently been cloned from rat testes (46) that shows highest homology to mammalian leukotriene A_4 hydrolases (44%), intermediate homology to *C. elegans* AP-1 (38%), and lowest homology to mammalian N-type aminopeptidases (21–24%) (Table II) and can catalyze the conversion of LTA_4 to LTB_4 (46).

In conclusion, we have cloned and functionally expressed a 69-kDa protein from *C. elegans*, the primary structure of which is more homologous to mammalian LTA_4 hydrolases than other zinc aminopeptidases. This protein functions as an aminopeptidase with broad substrate specificity but lacks any LTA_4 hydrolase activity. The primary sequence identity of *C. elegans* AP-1 enzyme to mammalian LTA_4 hydrolases and rat aminopeptidase B suggests that these enzymes are evolutionarily related.

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