MOLECULAR BIOLOGY APPROACH TO INVESTIGATE ENZYMES IN TICK SALIVARY GLAND WHICH ARE IMPORTANT IN TICK FEEDING

By

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LIST OF ABBREVIATIONS

AA	Arachidonic Acid
BSA	Bovine serum albumin
cDNA	complementary DNA
d	deoxy
dd	dideoxy
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	Ethylenedinitrilo-Tetraacetic Acid
EthBr	Ethidium bromide
GAP	GTPase activating protein
Kb	Kilo base pair
KD	Kilodaltons
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGHs	Prostaglandin synthase
PGI_2	Prostacyclin
PKC	Protein Kinase C
PLA_2	Phospholipase A_2
SCD	Stearoyl-CoA Deseaturase
SDS	Sodium dodecyl sulfate
TEMED	N,N,N', N'-Tetramethylethlee Diamine
UV	Ultraviolet

CHAPTER I

INTRODUCTION

Ticks are geographically widespread; they are major pests of man and domestic animals (Sonenshine, 1991; Kaufman, 1989). Compared to many other arthropod ectoparasites, ticks feed for relatively long periods on a host and successfully evade many host defense pressures. The secretion of eicosanoids such as prostaglandin E_2 and prostacyclin in the tick's saliva are believed to assist in tick feeding by inhibiting host immune and inflammatory responses (Ribeiro et al., 1985). These two substances are known to inhibit cell-mediated processes such as mast cell degranulation (Bach et al., 1982; Davies et al., 1984), preventing release of other mediators of inflammation (Ribeiro et al., 1987).

Previous studies investigated the lipid composition in tick salivary gland and found that arachidonic acid was only in phosphatidylcholine and phosphatidylethanolamine. Additionally, approximately 75% of all arachidonic acid is in the sn-2 position of phosphatidylcholine and phosphatidylethanolamine (Shipley et al., 1993), and a potent phospholipase A_2 (PLA_2) activated at micromolar calcium is present in the salivary glands. This PLA_2 preferred phosphatidylcholine as substrate (Surdick et al., 1992).

Although prostaglandins have been detected in salivary glands of ticks, very little is known about synthesis of eicosanoids in ticks. Preliminary results suggest that prostaglandins can be synthesized in the reproductive tissues and salivary glands of unfed and partially fed *Hyalomma anatolicum* (Shamesh et al., 1979).

These findings lead to the proposed existence of prostaglandin synthase (PGHs) in tick salivary gland. PGHs can convert arachidonic acid to PGH_2, which is the immediate precursor of all series-2 prostaglandins, prostacyclins and thromboxanes.

Although phospholipase A_2 and prostaglandin synthase are two very important enzymes involved in arachidonic acid metabolism in tick salivary glands, they have not thoroughly investigated. PLA_2 activity has been detected in the tick salivary gland, and has been partially characterized (Surdicket al., 1992). No direct evidence is available of the existence of PGHs in tick salivary glands. Biochemical methods to purify and characterize these two enzymes need time and may encounter problems, for these two proteins are not naturally abundant .

The molecular biology approach was used to investigate these two enzymes in tick salivary glands. This approach should provide genetic information about these two enzymes. Further work on gene expression will make it possible to investigate catalytic action and structure of the enzyme; rescreening the genomic library with the cDNA probe will let us know how this gene is regulated. Genetic analysis of this enzyme is important for the evolutionary study since little of such work has been done on arthropods.

CHAPTER II

LITERATURE REVIEW

Ticks are major arthropod vectors of arboviruses, rickettsiae, spirochetes, and parasitic protozoa of man and domestic animals (Kaufman, 1989). They are found in almost every region of the world (Sonenshine, 1991). There are two stages of feeding in female ixodid ticks, *Amblyomma americanum* (L.). The first stage is the slow feeding phase, where the tick increases from about 4 to 100-300 mg in 8-14 days. Rapid feeding lasts 12-48 hr. during which females attain a final pre-oviposition mass of 600-900 mg (Sauer et al., 1979). Males feed little after attachment to a host (Sauer et al., 1984).

The paired salivary glands of ixodid females consist of approximately 1,400 acini (alveoli) of three types (Fawcett et al., 1986; Krolak et al., 1982). Upon initiation and progression of ixodid female tick feeding, major cytological changes are observed in cells of alveolar types II and III (Fawcett et al., 1986). During the slow feeding stage, an extensive network of rough endoplasmic reticulum forms in cells of these acini. Membranes of interstitial cells and simple granular cells begin to proliferate and interdigitate. Numerous mitochondria are observed in close association with the extensive proliferating membranous network, suggesting a transport function. No increase in cell number occurs although the size, mass and protein content of the salivary glands increase about 25-fold during tick feeding (McSwain et al., 1982; Shelby et al., 1982). Salivary secretions are essential for successful tick feeding (Fawcett et al., 1986). It is thought that pathogens develop in the salivary glands and/or are co-secreted into the host body with saliva (Stich et al., 1993). Cement is secreted to help anchor the tick to the host. Other known tick salivary secretory products are anticoagulants, antihistamine, kininase, apyrase,

and eicosanoids such as prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and prostacyclin (PGI_2) (Sonenshine et al., 1991; Higgs et al., 1976; Dickenson et al., 1976; Schemesh et al., 1979; Ribeiro et al., 1985; Ribeiro et al., 1992). Esterase and glycosidases have been identified in tick saliva but their functions are unknown (Kemp et al., 1982).

The vertebrate hosts have their system of defending themselves against tick feeding. These are normal vertebrate responses to blood vessel damage, which include vasoconstriction, platelet aggregation, blood coagulation, and immune reactions (Ribeiro et al., 1986). It has been proposed that the mechanism by which ixodid ticks overcome many of these host defenses is by secreting eicosanoids such as prostaglandin E_2 and prostacyclin in their saliva (Ribeiro et al., 1985). These two substances are known to inhibit cell-mediated processes such as mast cell degranulation (Bach et al., 1982; Davies et al., 1984), preventing release of other mediators of inflammation (Ribeiro et al., 1987). PGE_2 may increase tick feeding success by promoting hyperemia and consequently increasing the amount of blood to the tick's feeding site (Ribeiro et al., 1988). PGE_2 and PGI_2 are also good inhibitors of PAF-induced platelet aggregation (Goodwin and Cenppens, 1983). PGE_2 is known to be a vasodilator as well (Kemp et al., 1982; Ribeiro et al., 1987; Binnington et al., 1980).

Prostaglandin E_2 has been found in the saliva or salivary gland homogenates of at least four species of ixodid ticks (Higgs et al., 1976; Dickenson et al., 1976; Ribeiro et al., 1985; Ribeiro et al., 1992). Schemesh et al. noted low or undetectable levels of PGF and PGE_2 in the salivary glands of unfed ticks but concentrations increased markedly in the tick salivary glands after 6 days of feeding (Schemesh et al., 1979). Involvement of eicosanoids in tick feeding is very important. Compared to many arthropod ectoparasites, ixodid ticks feed for relatively long periods on a host and successfully evade many host defense pressures. Synthesis and secretion of eicosanoids may be key processes in this successful parasitization.

In most tissues, the synthesis of eicosanoids is limited by the availability of their common precursor, free arachidonic acid, which is liberated from membrane phospholipids (Irvine et al., 1982). The rate-limiting step in eicosanoid synthesis is the hydrolysis of arachidonic acid from the sn-2 position of cellular phospholipids by phospholipase A_2 (PLA_2) enzymes (Flower et al., 1976). Arachidonic acid also can be released by a phospholipase C-diglyceride lipase pathway, a phospholipase D-phosphatase-diglyceride lipase pathway, a phospholipase D-PLA_2 pathway, or a phospholipase A_1 lysophospholipase pathway (Holtzman et al., 1991; Dennis et al., 1991). However, the major source of free arachidonic acid for eicosanoid synthesis is believed to be phospholipase A_2 (Dennis et al., 1991). The reactions and products are shown in Figure. 1A and 1B.

Arachidonic acid, a C_{20} polyunsaturated fatty acid that has four nonconjugated double bonds, has two metabolism pathways: cyclic and linear pathways. The cyclic pathway leads to the synthesis of prostaglandins, prostacyclins and thromboxanes; the linear pathway leads to the synthesis of leukotrienes. The first step in the cyclic pathway of arachidonic acid metabolism is catalyzed by prostaglandin synthase. This enzyme contains two catalytic activities: a cyclooxygenase activity and a hydroperoxidase activity. The former catalyzes the addition of two molecules of O_2 to arachidonic acid, forming PGG_2. The latter mediates a glutathione-dependent reaction that converts the hydroperoxy function of PGG_2 to an OH group (PGH_2). PGH_2 is the immediate precursor of all series-2 prostaglandins, prostacyclins, and thromboxanes.

Phospholipase A_2 activity is ubiquitous and has been found in bacteria, amoebae, insects, plants, and many mammalian tissues (Van den Bosch, 1980). The most studied PLA_2 activities are the secreted extracellular phospholipases A_2 that are found in snake and bee venoms as well as in pancreatic secretions. Less is known about intracellular PLA_2 activities, as they are less stable and present in low quantities (Jain and Berg, 1989).

The phospholipases A_2 that have received the most attention in arthropods till now

Figure 1A. Cutting Sites of Phospholipases on Phospholipid.

A.

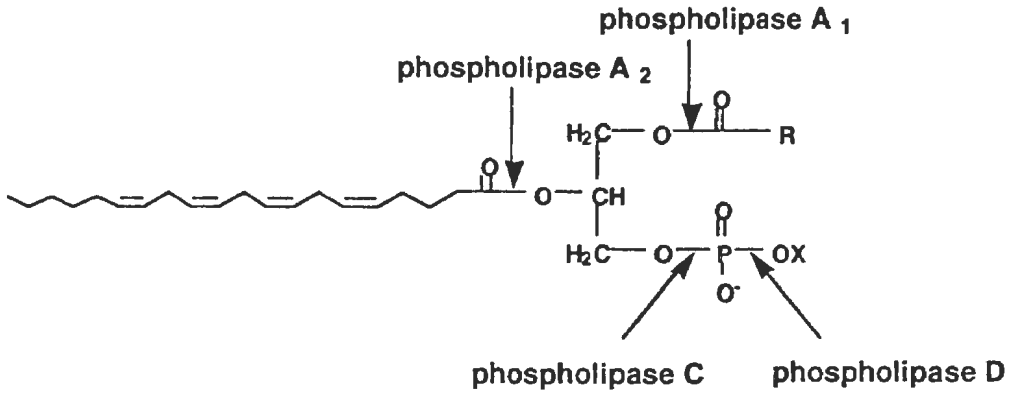

phospholipase A 1
phospholipase A 2
H2C
O
R
O
O
CH
H2C
O
P
OX
O-
phospholipase C
phospholipase D

Figure 1B. Pathways of Arachidonic Acid (AA) Libration from Phospholipids.

AA: Arachidonic Acid; PLA_1: phospholipase A_1; PLA_2: phospholipase A_2; PLC: phospholipase C; PLD: phospholipase D; R: alkyl group; P: phosphate; X: head group.

B.

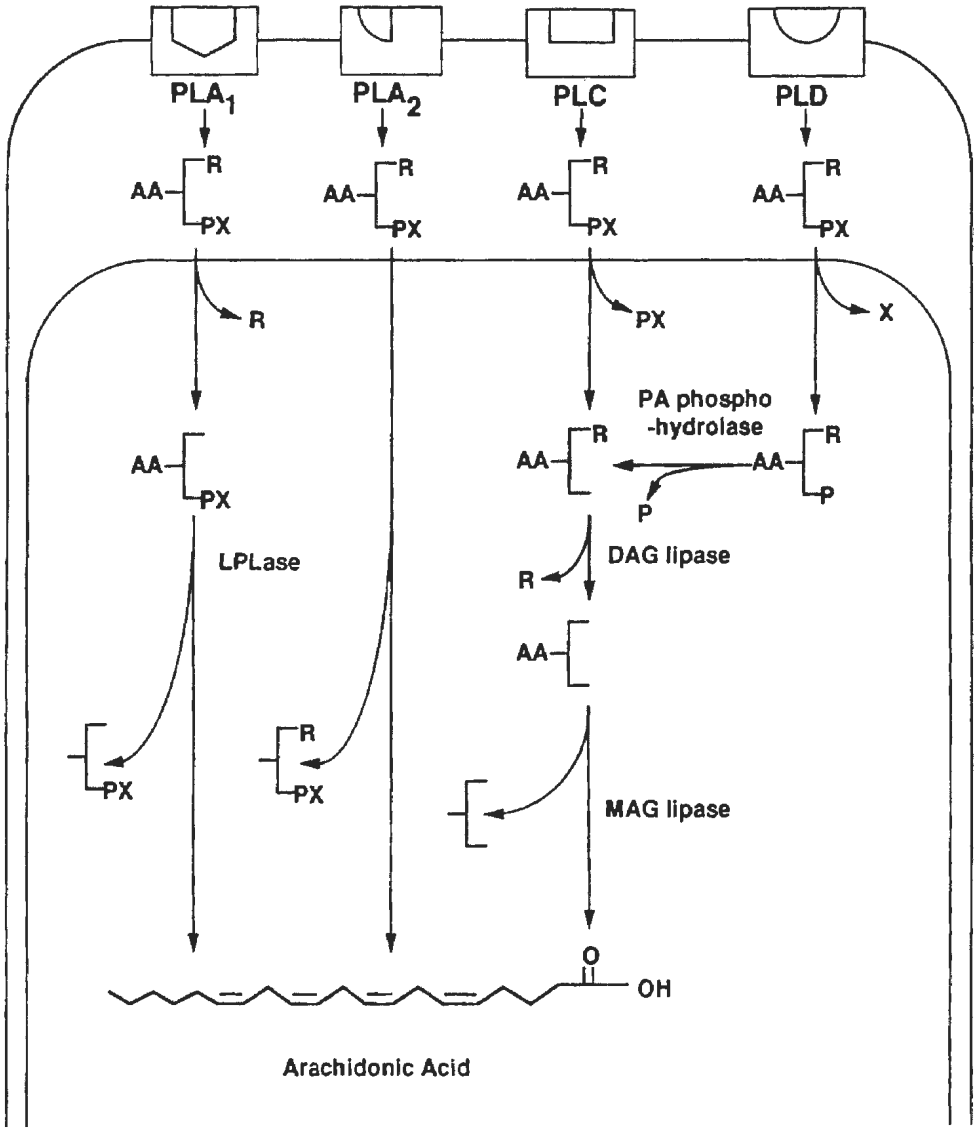
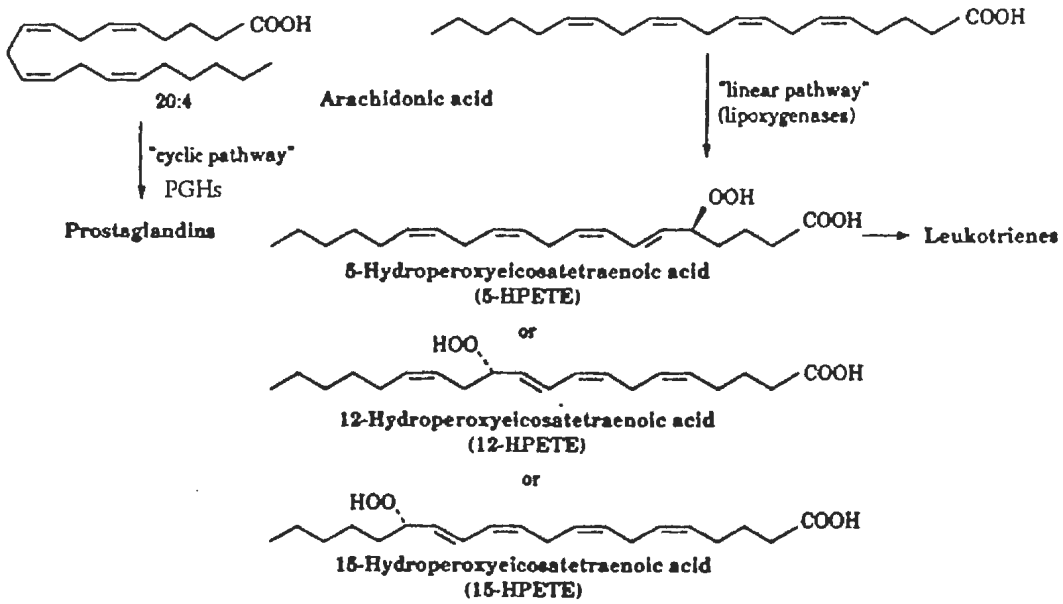

PLA1
PLA2
PLC
PLD
AA
R
PX
X
P
PA phospho
-hydrolase
LPLase
DAG lipase
MAG lipase
O
OH
Arachidonic Acid

Figure 1C. The Cyclic and Linear Pathways of Arachidonic Acid Metabolism.

PGHs: Prostaglandin Synthase.

C


COOH
20:4
Arachidonic acid
COOH
"linear pathway"
(lipoxygenases)
"cyclic pathway"
PGHs
Prostaglandins
OOH
COOH
Leukotrienes
5-Hydroperoxyeicosatetraenoic acid
(5-HPETE)
or
HOO
COOH
12-Hydroperoxyeicosatetraenoic acid
(12-HPETE)
or
HOO
COOH
15-Hydroperoxyeicosatetraenoic acid
(15-HPETE)

are the extracellular secreted PLA_2's in the venoms of stinging insects. The majority of these enzymes are very similar to each other and to mammalian digestive and reptilian venom PLA_2 as well. They have an approximate molecular weight of 14,000, a peptide chain of about 123 amino acids, high heat-stability, and dependence of calcium at millimolar levels for activation (Dijkstra et al., 1978). The amino acid sequences from two hymenopteran species have recently been determined. The structure of the PLA_2 from the honeybee, *Apis mellifera* L., shows some homology to mammalian enzymes such as bovine pancreatic PLA_2 at several residues in the active site and Ca^{2+}-binding loop (Kuchler, et al., 1989). The extremely toxic secreted PLA_2 in the venom of the hornet *Vespa orientalis* shares no homology with other known enzymes of this type (Kroneev et al., 1989).

Evidence for non-secreted PLA_2 in insects has also been found. Phospholipase A_1 and A_2 activity, determined by the release of lysophospholipid from phospholipid, has been found in such diptera species as *Musca domestic* L., *Phormia regina* (Meigen), *Glossina morsitans* Westwood, and *Culex pipiens fatigans* (L.) (Bridges, 1983; Rao and Dubrahmanyam, 1969). The PLA_2 activity in the *Culex pipiens* larva is the most extensively characterized non-secreted arthropod PLA_2 at this time. It has an optimum pH of 9 and an optimum temperature of 45°C. This PLA_2 activity is unique in that it is inhibited by calcium, which activates most other PLA_2 enzymes (Rao and Dubrahamanyam, 1969).

A new class of intracellular, cytosolic PLA_2 has been found recently. Usually, intracellular PLA_2 is difficult to obtain because of the low levels (<0.01%) of protein present in cells and the loss of activity resulting from proteolysis, aggregation, and denaturation (Kramer et al., 1991). However, there is increasing evidence to indicate that the intracellular cytosolic PLA_2 involved in signal transduction processes and receptor mediated liberation of arachidonic acid represents a different enzyme with distinct molecular and functional properties (Kramer et al., 1991). Such a cytosolic PLA_2 has been

characterized in platelets (Kramer et al., 1990; Loeb et al., 1986), renal mesangial cells (Gronich et al., 1988), macrophages (Wijkander et al., 1989), leukemia cell lines (Lesile et al., 1988; Diez et al., 1990), and human monoblast U937 cells (Kramer et al., 1991; Clark et al., 1991). It has an apparent Mr of 60,000-70,000 (by gel filtration), is unstable, requires less than millimolar concentrations of Ca^{2+} for activity, and prefers arachidonate esterified at the sn-2 position of phospholipid substrates (Kramer et al., 1991).

The kinetic mechanism of phospholipase A_2 is unique because it is a soluble enzyme acting upon an insoluble substrate. A PLA_2 enzyme does not bind to individual lipid molecules, but to the lipid-water interface (Verger and de Hass, 1976). Thus the PLA_2 activity is dependent on both the surface area of lipid exposed to aqueous media and on absolute substrate concentration.

There are two modes of action of phospholipase A_2: the hopping mode and the scooting mode. In the first one, the enzyme in the aqueous phase binds to a lipid-water interface, acts upon a substrate molecule, and then returns to the aqueous phase; in the latter one, after the enzyme has acted upon a substrate molecule in an interface, it binds to an adjacent substrate molecule in the same interface (Jain and Berg, 1989).

Recent crystal structures suggest an isostructural, desolvated and presumably isoenergetic transfer of phospholipid from the substrate aggregate to the catalytic surface through a hydrophobic channel whose opening is to the lipid-water interface (Scott et al., 1991). This access mode is required for optimal enzymatic action and can only occur at the interface. Soluble and dispersed phospholipids cannot exploit these features.

Some PLA_2 enzymes show specificity for fatty acids in the 2-position of phospholipids. Phospholipase A_2 enzymes specific for arachidonic acid in the sn-2 position include those found in human placental blood vessels (Karnachouw and Chan, 1985), human platelet cytoplasm (Kim et al., 1988), and hamster heart (Tam et al., 1984). Hamster heart phospholipase A_2 was also found to be specific not only for the fatty acid in

the sn-2 position, but also affected by that in the 1-position (Cao et al., 1987). Other PLA_2's are less specific in the 2-position.

Some phospholipases A_2 show specificity for the phospholipid head group. One of the two forms of human platelet PLA_2 is specific for phosphatidylcholine and the other is specific for phosphatidylethanolamine (Cheung and Ballou, 1989). A PLA_2 purified from human rheumatoid arthritic synovial fluid preferred phosphatidylethanolamine to phosphatidylcholine or phosphatidylserine (Hara et al., 1989). The PLA_2 in hamster heart was specific for phosphatidylcholine (Cao et al., 1987). There are PLA_2 activities that lack phospholipid specificity and act equally on phosphatidylcholine, phosphatidylethanolamine, or phosphatidylinositol. Horse platelet phospholipase A_2 and PLA_2 from human monocytic leukemic U937 are among this group (Van de Bosch, 1980).

Another type of substrate specificity observed in PLA_2 enzymes involves the linkage of the fatty acid in the 1-position. Most phospholipids have fatty acids esterified to the 1-position, but there are others in which the fatty acids are attached by an ether linkage. The dog myocardial cytosol PLA_2 belongs to the group that has a high specificity for ether-linked phospholipids (Hazen et al., 1990). Sheep platelet PLA_2 is extremely specific for the ether-linked substrates, showing 100x more activity for ether-linked phospholipids than for esterified phospholipids (Loeb and Gross, 1986).

The pH at which an enzyme shows greatest activity is an important parameter in its characterization. The pH optima for PLA_2 activities vary greatly between species and tissues. Most are active between pH 7-9.5 (Van den Bosch, 1980; Kramer et al., 1989; Stefanski et al., 1986). However, PLA_2 activities with acid pH optima have been observed. One of the two PLA_2 activities in rabbit polymorphonuclear leucocytes has a pH optimum of 5.5 (Franson et al., 1974). Another PLA_2 active at acid pH (6.4) is found in dog myocardial cytosol (Hazen et al., 1990).

Most PLA_2 enzymes are calcium-dependent. The majority of phospholipases A_2 are active only in the presence of calcium (Van de bosch, 1980; Jain and Berg, 1989). The concentration at which PLA_2's are active is varied. Intracellular PLA_2 enzymes are activated by submicromolar concentrations of calcium (Loeb and Gross, 1986; Diez and Mong, 1990). The secreted PLA_2's require millimolar calcium to be activated (Alonzo et al., 1986; Kramer et al., 1990; Parks et al., 1990).

Although most of the PLA_2 activities are calcium-dependent, there are also calcium-independent PLA_2 activities. The PLA_2 activity in hamster heart cytosol does not show any requirement for calcium or any other divalent metal ion (Cao et al., 1990). PLA_2 activities found in canine myocardial cytosol and secretory granular membranes in rat parotid gland are also calcium-independent (Hazen et al., 1990; Mizuno et al., 1991).

All PLA_2's that have been structurally characterized so far are extracellular forms (Davidson and Dennis, 1991). Some of the features appear to be invariant in the structure of all the extracellular and secreted PLA_2. These include a high number of disulfide bonds (up to 7), as judged from both crystallographic data (Dijkstra et al., 1981) and circular dichroism analysis (Dufton et al., 1983), a Ca^{2+} "binding loop" involving the residues at positions 28 (Tyr), 30 (Gly), 32 (Glu), and 49 (Asp), and a helical region at the $-NH_2$ terminal region (Waite, 1990).

A fundamental difference in the arrangement of the cysteines in the primary structure and therefore the cross-linking disulfide bonds formed between them has led to the creation of two classes of PLA_2's (Heinrikson et al., 1977; Renetseder et al., 1985). Group I contains mammalian pancreatic phospholipase A_2 and phospholipase A_2 from *Elapidae* and *Hydrophicdae* venoms: these enzymes possess a disulfide bridge between cysteines 11 and 77. Group II is composed of phospholipase A_2 from the venom of *viperidae* and *crotalidae* and non-pancreatic mammalian phospholipase A_2 (Hayakawa et al., 1988; Kramer et al., 1989); they have a unique disulfide bridge between cys-50 and the cys at the C-terminal end of the peptide extended by six residues (Heinrikson et al., 1977).

The amino acid sequences of all the mature phospholipase A_2 enzymes contain highly conserved amino acid residues and sequences: 1) the α helical amino-terminal segment containing the lipophilic residues Leu 2, Phe 5, and Ile 9; 2) the calcium binding loop with the typical glycine-rich sequence Tyr 25-Gly-Cys-X-Cys-Gly-X-Gly-Gly-X-X-X-Pro37 and the residue Asp 49; 3) the active site residues His 48, Asp 60, Tyr 52, and Tyr 73 (Kramer et al., 1989). Gly 32 is a key residue of the calcium binding loop (Davison and Dennis, 1990). The C-terminal half of all the PLA_2's is less well conserved (Davison and Dennis, 1990).

A greater number of residues are conserved in the more closely related subgroups of PLA_2's, especially those possessing the same overall disulfide patterns (Davidson and Dennis, 1990). This is because the particular side chains that modulate the catalytic reaction will be determined by three-dimensional arrangements that are highly influenced by disulfide positions (Brunie et al., 1985).

Most of the PLA_2 sequences contain a total of 14 Cys, but the number of Cysteine residues is also varied: the bee venom sequence has only 8 Cys, the A-chain of β–bungarotoxin has 13, the taipoxin γ chain is 16, and the PLA_2 from *Bitis nasciornis* and *Bitis garbonica* is 12 respectively (Davidson and Dennis, 1990).

Among all the PLA_2 amino acid sequences aligned by Davison and Dennis, the honeybee PLA_2 sequence is very different from the others. The bee sequence is only 22-28% identical with the other sequences. Conserved residues include a histidine-aspartic acid pair which forms part of the active site, several residues involved in the binding of calcium ions, as well as a number of disulfide bridges (Davison and Dennis, 1990; Kuchler et al., 1989). The reason that the bee sequence lacks a large and functionally important N-terminal piece relative to other PLA_2 may be due to the earlier divergence of its gene during the time of evolution (Davison and Dennis, 1990).

Most of the work concerning cloning and sequencing of PLA_2's gene has been done in mammals, especially human. The gene coding for human pancreatic PLA_2 has

been isolated, subcloned, and sequenced (Seilhamer et al., 1986). It appears to be encoded by a single 4.9 Kb gene per haploid human genome (Seilhamer et al., 1986). The 126-amino acid coding region of fully-processed PLA_2 was contained in three exons of almost equal size, and a membrane translocation peptide was partially encoded in an additional upstream exon (Johnson et al., 1990).

The gene encoding human Type II PLA_2 has recently been cloned and sequenced (Seilhamer et al., 1989; Kramer et al., 1989). The gene spans 4.5 Kb and is contained in five exons. The final two introns divide the 124 amino acid sequence encoding the fully processed PLA_2 protein at similar locations to those in Type I PLA_2. The sequence lacks the propeptide seen in the Type I PLA_2 gene, but contains a membrane translocation signal of 20 residues preceding the enzyme sequence (Johnson et al., 1990).

Several cDNAs encoding snake venom phospholipase A_2 have also been described previously (Ducancel et al., 1991; Verheiand de Hass, 1991; Pungercar et al., 1991). Recently, two cDNAs encoding a novel cytosolic PLA_2 have been cloned and sequenced (Kramer et al., 1991; Clark et al., 1991). The deduced amino acid sequences have no homology with the secreted form of the enzyme, but have homology to PKC and GAP. No cloning and sequencing work concerning tick PLA_2 has been published.

A potent PLA_2 is present in tick salivary glands (Surdick et al., 1993). This PLA_2 activity was stable during storage at low temperature (-20°C); however, it was unstable at low enzyme concentration but was stabilized by added bovine serum albumin. The temperature and pH optima of the enzyme were 37°C-47°C and 9, respectively. This PLA_2 activity was activated by micromolar calcium with half-maximal activation occurring at 0.5 μM. Enzyme activity was not changed by adding millimolar calcium to the reaction mixture. This PLA_2 which was found in *A.americanum* salivary gland appeared to be a cytosolic enzyme, for most of the activity of this PLA_2 was found in the supernatant of centrifuged homogenates. The fact that this PLA_2 activity was activated by micromolar calcium also suggests it is an intracellular enzyme. (Surdic et al., 1993). On the other

hand, there were data on the saliva of A.*americanum* suggesting the presence of a secreted PLA_2 in this substance.

CHAPTER III

MATERIALS AND METHODS

Preparation of Genomic DNA from Tick and Sheep

Tick: Tick genomic DNA was prepared from adult female ticks or eggs. For each preparation, no more than two grams of ticks or eggs was used. Ticks or eggs were kept at -70°C until use. The ticks or eggs were poured into a mortar and were ground to a fine power immediately in the presence of liquid nitrogen. After grinding, the sample was transferred to a 50 ml tube, and 40 mls of 1x NIB (10 mM Tris, 60 mM NaCl, 10 mM EDTA, 0.5% Triton-X100, 0.15 mM spermidine, 0.15 mM spermine, pH 7.4, spermine and spermidine were from Sigma, molecular biology grade) was added. The diluted sample was transferred to a 50 ml tube, and was homogenized by making several passes at moderately high speed. After homogenization, the sample was centrifuged at 3,000 rpm (table top centrifuge) for 15 sec. The pellet was discarded. This process was repeated twice to remove cuticle. The supernatant was centrifuged at 7.5 Kg for 7 minutes, then the pellet was resuspended in 50 mls 1x NIB and recentrifuged at 7.5 Kg for 7 minutes. The pellet was resuspened again in 14 mls 1xNIB, 4 mls 10% sarcosyl (Sigma) was added, and it was incubated on ice for 10 minutes. 21.26 grams of CsCl was added; the volume was brought to 26.75 mls with 1xNIB. After the CsCl was dissolved, 270 µl 10 mg/ml EthBr (Ethidium bromide) was added. The sample was centrifuged at 45Kg for 16 hours. The genomic DNA band was removed by 5ml syringe with an 18 gauge needle. The EthBr was removed by extracting several times with water-saturated butanol. CsCl was removed by dialysis against 1 liter of TE buffer (10mM Tris, 1mM EDTA pH 8.0) at 4°C overnight. DNA was precipitated overnight at -20°C after adding 1/10 volume of 3M sodium acetate

(pH4.6) and 2 volumes of absolute ethanol. DNA was centrifuged down at 15,000rpm (microcentrifuge) for 30 minutes, then the pellet was washed with 70% ethanol and air dried. The purified genomic DNA was dissolved in TE buffer and its concentration and purity were determined by measuring absorbance at 260nm and 280nm. The DNA integrity was checked by running aliquots of DNA on a 0.3% agarose gel at 4°C.

Sheep: Sheep genomic DNA was purified from sheep spleens. Fresh sheep spleens were obtained and stored at -70°C till use. The frozen spleen (about 1 gram) was blended in the presence of liquid nitrogen to fine power, then homogenized in 2mls LST buffer (20mM Tris pH7.4, 10mM NaCl, 3mM $MgCl_2$). After 1ml of TNLB buffer was added (5% sucrose, 4% Nonidet P-40 in LST), the homogenate was centrifuged at 3,000g for 10 minutes. The pellet was resuspended in 5mls of ACE (50mM NaAc, 10mM EDTA, pH5.9), and 0.45ml 10% SDS was added. The lysate was extracted twice with an equal volume of phenol (redistilled and saturated with TE pH7.6), and one time with an equal volume of chloroform. Genomic DNA was precipitated by adding 2 volumes of absolute ethanol, DNA was wound out by a clean glass rod, dried and dissolved in TE (pH8.0). The DNA integrity also was checked by the method described before.

Genomic DNA Southern Blot

Enzyme digestion and electrophoresis

There were three different genomic blots. One of them was probed by a plasmid probe containing part of the sheep prostaglandin synthase (PGHs) cDNA. The second one was probed by a snake full length phospholipase A_2 cDNA encoding a secreted PLA_2 enzyme. The third one was probed by a full length cDNA from human monoblast cells which encodes a novel intracellular phospholipase A_2 (Clark et al., 1991). On the first blot, sheep genomic DNA was used as positive control. On the second one, no positive control was used. Sheep genomic DNA was also used on the third blot.

Tick genomic DNA was digested by EcoRI, BamHI, and HindIII (all from BRL) separately, as was sheep genomic DNA. In each digestion, 10μg of genomic DNA was used. In each tube, 10μg of DNA, 1/10 of total volume of enzyme buffer (supplied by BRL with its enzyme) and 2.5 μl of one of the enzymes (25units) was mixed. The digestions were carried on at 37°C for 3-4 hours. After digestion, an appropriate amount of dye was added. The samples were loaded on a 0.8% agarose gel and run at 1.4V/cm overnight at room temperature with the λ DNA cut by BstEI (from BRL) as MW standard.

Capillary Transferring of DNA to Nylon Membrane

After electrophoresis, the DNA was depurinized, denatured and neutralized, following the standard protocol (Maniatis et al., 1987). The DNA was then transferred to the nylon membrane (Sartolon nylon 66) by capillary transfer overnight, using 20x SSPE (2.8M NaCl, 0.2M NaH_2PO_4, 0.02M EDTA, pH 7.4) as transfer buffer. DNA was fixed on the membrane by UV-crosslinking (UV Stratalinker 1800).

Preparation of the Radioisotope (^{32}P) Labeled Probe

PGHs probe: the PGHs probe was a pure insert of 1.6 Kb cDNA . It was a part of the full length sheep PGHs cDNA, and was purchased from Oxford Biomedical Research Inc (Oxford Biomedical Research Inc).

The ^{32}P labelled probe was made by random priming (Manaiatis et al., 1987). The DNA (50ng in a total volume of 7.1μl) was boiled for 5 minutes and then immediately cooled on ice for 5 minutes. Then the following was mixed with the denatured DNA: 11.4μl LS (HEPES/DTM/OL in a ratio of 25/25/7, HEPES: 1 M, pH6.6; DTM: 100μM dATP, 100μM dGTP, 100μM dTTP in 250mM Tris pH8.0, 25mM $MgCl_2$, 50mM β-mercaptoethanol; OL: 1mM Tris pH7.5, 1mM EDTA pH8.0, 90 OD units/ml of oligonucleotide hexamers), 1μl 10mg/ml BSA (bovine serum albumin), 5μl ^{32}P-dCTP (3,000Ci/mmol, NEN), 0.5μl Klenow (2.5 units, BRL). The reaction was incubated at

room temperature for at least 3 hours. The unincorporated nucleotides were separated by a Sephadex G-50 spin column (Maniatis et al., 1987).

Prehybridization and Hybridization

PGHs as probe: The prehybridization and hybridization buffer was: 50% formamide, 5x SSPE (diluted from 20x SSPE stock), 5x Denhardt's (diluted from 50x stock, 50x Denhardt's contains 1% Ficol, Type 400 (Pharmacia), 1% polyvinylpyrolidine, 1% bovine serum albumin, Fraction V, (Sigma)), 0.5% SDS, and 100μg/ml denatured salmon sperm DNA. Prehybridization was carried out at 37°C for 2 hours.

The hybridization solution was the same as the prehybridization except it contained radioactive probe. The column purified probe was denatured by boiling for 10 minutes, then immediately cooled on ice. Hybridization was carried out at 37°C overnight.

PLA_2 as probe: The prehybridization and hybridization conditions for both PLA_2 membranes were the same as described previously except that the formamide concentration was 35% instead of 50%.

Washing

After hybridization, the membranes were washed in buffer at increasing stringency to decrease the background. The PGHs probed membrane was washed at 55°C in 2x SSPE, 1% SDS twice, 15 minutes per time. For the PLA_2 probed membrane, washing was at 52°C in 5x SSPE, 0.1% SDS twice, 30 minutes per time, then the membranes were washed at 62°C in 5x SSPE, 0.1% SDS for 15 minutes.

Screening the Uni-ZAP™ XR cDNA Library

One million clones from the Uni-ZAP™ XR cDNA library were screened. XL1-Blue bacteria were grown in L-broth (1% NaCl, 1% Bacto-Tryptone, 0.5% Yeast Extract)

containing 4% maltose at 37°C overnight. The cells were centrifuged at 5 Kg for 5 minutes, and resuspended in sterile 10mM $MgSO_4$. 0.3ml of cells was mixed with an appropriate amount of phage solution to give 10,000-50,000 plaques/150mm plate. The mixture was incubated at 37°C for 15 minutes with constant shaking, then plated in 7ml top agar (0.7% agarose in L-broth). Plates were incubated at 37°C for 11 hours to let phages grow, then cooled at 4°C for 3 hours, then plaques were lifted onto nitrocellulose filters (MagnaGraph, Westboro, MA). Two duplicate membranes were used for each plate. Three pieces of Whatman 3MM paper were soaked with denaturation buffer (0.5N NaOH, 1.5 M NaCl), neutralization buffer (0.5 Tris pH 8.0, 1.5 M NaCl), and 2x SSC (diluted from 20x stock, 2.8M NaCl, 0.3M sodium citrate, pH 7.0), respectively. Membranes were laid on the denaturation paper for 5 minutes, the neutralization paper for 5 minutes, then the 2x SSC paper for 2 minutes with the DNA side up. Membranes were allowed to dry 10 minutes, then DNA was fixed on the membrane by UV-crosslinking (UV Stratalinker 1800).

The processed membranes were prehybridized for 2 hours in 35% formamide, 5x SSPE, 5x Denhardt's, 0.5% SDS, 100μg/ml salmon sperm DNA at the amount of 4ml/150mm filter. The prehybridization solution was replaced by fresh hybridization buffer and the radioisotope labeled PLA_2 and PGHs probes (made by random priming as above) were added to make the concentration of each of them to $0.5\text{-}0.75 \times 10^6$cpm/ml. Hybridization was carried out at 37°C overnight.

The membranes were washed first at 42°C then at 52°C, and finally at 62°C in 5x SSPE 0.1% SDS, 30 minutes per time. Membranes were autoradiographed for two days.

The clones which showed positive spots on both of the duplicate filters were removed from the plates, put in 1ml SM buffer (0.1M NaCl, 0.017M $MgSO_4$, 0.05M Tris-Cl, 0.01% gelatin, pH 7.5) with 20μl chloroform, and incubated 2 hours at room temperature to release the phage. The phages from positive clones were replated and

screened a second and third time to purify. The final positive clones were removed from the plates and resuspended as before to use for subcloning

In vivo excision of pBluescript from Uni-ZAP™ XR (Subcloning)

This subclone step followed the protocol supplied by Stratagene. After the phagemid pBluescript was rescued, 200 µl and 20 µl of phagemid were mixed with 200µl XL1-Blue at $O.D._{600}$ =1 respectively, and 100µl of each mixture was plated on plate containing 100 µg/ml ampicilline separately. The colonies grown on the plate after incubating at 37°C overnight contained pBluescript double stranded phagemid with the cloned DNA insert, and could be used for plasmid DNA preparation.

Clone Blot

Plasmid Midi Preparation

Single clonies were inoculated in 150ml L-broth/Ampicillin culture, grown overnight at 37°C with shaking. The cells were harvested by centrifugation at 4,000 g for 30 minutes, and plasmid DNA was purified by QIAGEN kit (QIAGEN Inc., Studio City, CA). The protocol followed is supplied with the kit. After the DNA was redissolved in TE buffer, its concentration was determined with a DNA fluorometer (Model TKO100, San Francisco, CA).

Clone blot

About 1 µg of plasmid DNA from the Midi preparation was used for the clone blot. The DNA was mixed with 1µl of EcoRI (10 unit), 1µl of XhoI (10 unit) and 2µls of universal restriction buffer (330mM Tris acetate, pH7.9, 600mM K^+ acetate, 100mM Mg^{2+} acetate, 5mM DTT, 5µg/ml BSA, 40mM spermidine) in a final volume of 20µl. Digestion was carried out at 37°C for 2 hours. After adding the dye, the DNA was run on

a 0.8% agarose gel with λ DNA cut by BstEI as the standard. After electrophoresis, the gel was prepared for capillary transfer, and the DNA was transferred to nitrocellulose membrane as above. Prehybridization and hybridization were carried out under the same conditions as stated in screening the library, and the membrane was then exposed to film (Kodak film, X-OMAT™ AR).

Sequencing

Sequencing reaction

Sequencing reactions were done according to the protocol supplied with the Sequenase Version 2.0 kit (from USB). All the reagents for the sequencing are from the kit. For each reaction, 6μg of purified supercoiled DNA was used. It was denatured in 0.2M NaOH, 0.2mM EDTA for 5 minutes at room temperature, then neutralized with 0.3M sodium acetate (pH4.6) and precipitated with ethanol at -70°C for 15 minutes. The sequencing reaction followed the protocol except that: the annealing reaction was incubated at 37°C for 30 minutes; 1 μl of S-α^{35} dATP (1000Ci/mmol, NEN) was added to the labelling reaction; after termination, the reaction was incubated at 37°C for 8 minutes, 1 μl of dNTP/TdT cocktail (all four dNTP, each at a concentration of 1 mM in 1x sequenase buffer. Terminal deoxynucleotide transferase, TdT, was diluted at 1:8 with the dNTP mixture before use, TdT was 1.6 u/μl after dilution) was added to the termination mixture, and the reaction was incubated at 37°C for another 30 minutes. The reaction was stopped by adding 4 μl stop solution. Completed reactions were stored at -20°C till use.

Polyacrymide gel electrophoresis

SEQUAGEL (ultra pure, from National Diagnostics, Atlanta, Georgia) was used when doing DNA polyacrymide gel electrophoresis. An 8% gel was used throughout all electrophoreses. The gel was polymerized for 1 hour, then it was prerun at 50W for 30

minutes. Samples were heated at 75°C for 3 minutes to denature before loading. The temperature was kept at 50-55°C during electrophoresis. The DNA samples were run for 2, 5, and 8 hours to read about 250-300 base pairs.

Analysis of the DNA sequences

After the complete sequences of the two clones were obtained by primer walking, they were analyzed with the MacVector 3.5 computer program. The complete sequences were put into the computer and open reading frames (ORF) were found within them. After the open reading frames were found, the 5' and 3' flanking were removed from clone 1 and clone 2, and the ORFs were aligned with the known phospholipase A_2 nucleotide sequences encoding secreted PLA_2s. Also, the ORF of clone one was aligned to ORF of clone two to analyze their relationship.

The ORFs from clone one and clone two were translated into amino acid sequences, and basic properties such as molecular weight, pI and amino acid composition were analyzed by the computer. The highly conserved sequences and residues such as calcium binding loop and active site were searched for in the two translated ORFs by the protein subsequence program. There is another group of calcium-binding proteins like calmodulin, which contain another type of highly conserved calcium-binding region called the EF hand. The amino acid sequences of this region were searched for in the translated ORFs of these two clones by using the protein subsequence program. The nucleotide sequences of these two clones were aligned to the snake DNA probe using a Pustell DNA Matrix.

Finally, the translated open reading frames of clone one and two were compared to the protein data base.

CHAPTER IV

RESULTS AND DISCUSSION

Prostaglandin synthase

Prostaglandin synthase in tick salivary glands has not been characterized. The presence of prostaglandin and its precursor arachidonic acid in tick saliva led people to propose the existence of this enzyme. So, it was interesting to investigate this problem using a molecular biology approach.

One indirect way to investigate if the tick has prostaglandin synthase is to check if tick has the PGHs gene in its genome. Genomic southern blotting is a powerful method to investigate if a particular gene is present in a genome. Analysis is based on the fact that DNAs which are homologous to each other can hybridize under certain condition.

Tick genomic DNA was digested with EcoRI, BamHI, and HindIII separately. The resulting fragments were separated according to size by electrophoresis through an agarose gel. The DNA was then denatured in situ and transferred from the gel to a solid support (nylon membrane). The DNA attached to the filter was hybridized to a radiolabeled fragment of sheep prostaglandin synthase cDNA. Bands will show up on the autoradiograph if the probe hybridizes to the DNA on the filter. This gives evidence of the presence of the PGHs gene in tick genome.

Hybridization specificity depends on the stringency of the conditions used during incubation and subsequent washes. Temperature, the ionic strength of hybridization solutions and the presence of detergents or blocking agents affect the degree of specific annealing of the probe to the immobilized molecule. DNAs with low homology can only hybridize at lower stringency (i.e. less formamide, lower temperature, higher ionic

strength), while those with high homology can hybridize at higher stringency. On the genomic blot, several bands were seen on sheep genomic DNA, but no band was found in tick genomic DNA (Fig. 2), suggesting no hybridization between the sheep PGHs probe and tick DNA. The reason for this result could be: the tick does not have PGHs gene in its genome; or there is not enough homology between the sheep PGHs gene and the tick PGHs gene because these two species are too far apart in evolution; or the hybridization condition was too stringent for these two genes to hybridize each other. A lower hybridization stringency was used, which contained 35% formamide instead of 50% formamide. This time, again, only sheep genomic DNA had bands (Fig. 3). The hybridization stringency could not be made lower than 35% formamide, because the PGHs gene is a single copy gene in sheep genome, and a lower stringency could not give good bands on the control sheep DNA (on the 35% formamide blot, backgrounds are already high, making it difficult to see bands on it).

These results suggested: the ticks do not have the prostaglandin synthase, or the prostaglandin synthase is very different from that in the sheep.

Phospholipase A_2

A phospholipase A_2 activity has been found in tick salivary gland (Surdick et al., 1992). It hydrolyzes arachidonic acid from the membrane phospholipid. This free arachidonic acid could be used for prostaglandin synthesis. The PLA_2 activity found in tick salivary gland is likely to be an intracellular one, because it can be activated by micromolar calcium and most of the observed activity occurred in the supernatant of centrifuged salivary gland homogenates. On the other hand, there was also evidence suggesting the existence of secreted PLA_2 in tick saliva (Gengla et al., unpublished)

Unfortunately, this PLA_2 enzyme has not been purified from the salivary glands of *A americanum*. Our knowledge about the properties, regulation and structure of this enzyme is very limited. It would be valuable to investigate this enzyme by a molecular

Figure 2. Genomic Southern Blot with Sheep PGHs cDNA Probe (50% formamide).
S: molecular weight marker; 1, 2 and 3 were sheep genomic DNA, 4, 5 and 6 were tick genomic DNA. DNA was digested by BamHI (lanes 1&4), EcoRI (lanes 2&5), and HindIII (lanes 3&6), electrophoresised on 0.8% agarose gel, blotted to nylon filters and hybridized with ^{32}P-labeled DNA. Hybridization was at 50% formamide, 5xSSPE 37°C, washing was at 55°C, 2xSSPE 1% SDS.

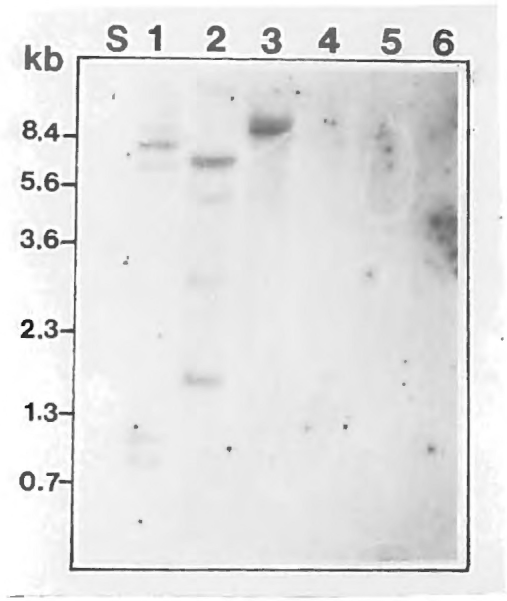
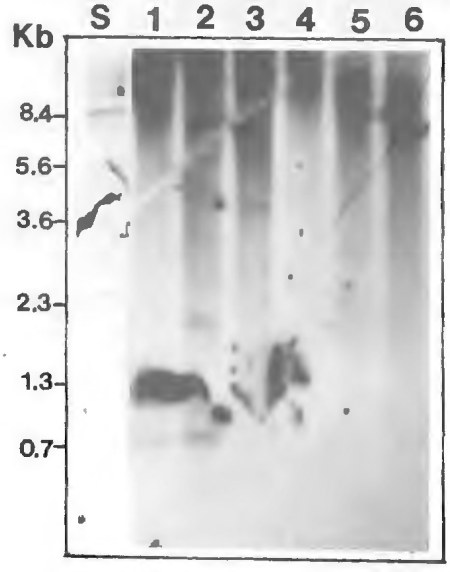

S 1 2 3 4 5 6
kb
8.4
5.6
3.6
2.3
1.3
0.7

Figure 3. Genomic Southern Blot with Sheep PGHs cDNA (35% formamide).

S: molecular weight marker; lanes 1, 2 and 3 were sheep genomic DNA, lanes 4, 5 and 6 were tick genomic DNA. DNA was digested by BamHI (lanes 1&4), EcoRI (lanes 2&5), and HindIII (lanes 3&6), electrophoresised on 0.8% agarose gel, blotted to nylon filters and hybridized with ^{32}P-labelled DNA. Hybridization was at 35% formamide,5xSSPE, 37°C, washing was at 5xSSPE, 0.1% SDS, 52°C.


S 1 2 3 4 5 6
Kb
8.4
5.6
3.6
2.3
1.3
0.7

biology approach, as this could give us information about the structure of this PLA_2 gene. Also, the deduced amino acid sequence can be obtained, which will give us some structural and catalytic information. Further work on gene regulation and expression will allow the extensive study of this enzyme and provide some clues for controlling this effective disease vector.

A full length cDNA encoding the basic subunit of Mojave toxin was used as the probe to identify tick PLA_2 gene. This cDNA probe consisted of 675 base pairs and was cloned in the EcoRI site of the Bluescript vector. The PLA_2 protein this cDNA encoded belongs to a secreted type. Also there is a PLA_2 probe from human monoblast U937 cells. This cDNA encodes a novel type of intracellular cytosolic PLA_2 enzyme. It has a size of 2.5Kb and the protein it encodes is about 85.2 KDa (Clark et al., 1991).

A genomic southern blot was carried out before screening the tick cDNA library to test if these two probes could hybridize with the tick gene and to determine the conditions under which they hybridize. On the two blots probed by the snake and human probe, bands only showed up on the one probed by the snake cDNA (Fig. 4A). This demonstrates the snake cDNA probe is homologous to a tick gene and can be used to screen the tick cDNA library. The hybridization condition (for both Southern blot and screening library) was 35% formamide and 37°C, with washing up to 62°C at 5x SSPE, 0.1% SDS. Although this is not very high stringency screening, it is reasonable because the snake probe may not have much homology with the tick gene

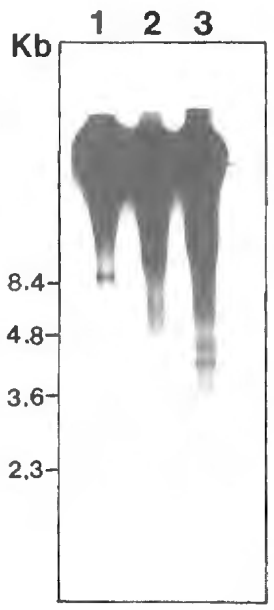
A tick Uni-ZAP™ cDNA library was screened, using the snake cDNA probe with the hybridization conditions used on genomic blot. After screening one million plaques, 2 positive clones were obtained. Usually, after screening $1x10^6$ plagues, it is possible to get one to twenty positive clones (for a gene of low or moderate expression).

This Uni-ZAP™ cDNA library was designed to allow in vivo excision of pBluescript containing the insert from the phage clones. The phagemid containing the insert was digested by EcoRI and XhoI to release the insert. Agarose gel electrophoresis

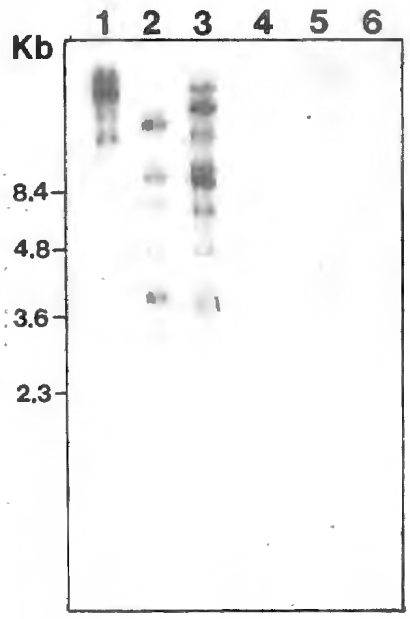
Figure 4. Genomic Southern Blot with PLA_2 probe.

The probes used were full length cDNA from snake PLA_2 (A), and full length cDNA from human monoblast U937 cells(B). lanes 1, 2 and 3 on A were tick genomic DNA; lanes 1, 2, 3 on B were sheep genomic DNA, lanes 4, 5, 6 on B were tick genomic DNA. DNA was digested by BamHI (1a,1b and 3), EcoRI (2a,2b and 4), and HindIII (3a,3b and 6), electrophoresised on 0.8% agarose gel, blotted to nylon filters and hybridized with ^{32}P-labeled probe. Hybridization was at 35% formamide, 5xSSPE, 37°C, washing was at 5xSSPE 0.1% SDS, 62°C.

A



B



showed the insert size of these two clones to be about 1Kb for clone 1 and 1.5Kb for clone 2 (Fig. 5).

In order to check for homology between these two clones and to test for homology of these two clones to the snake cDNA probe, a clone blot was performed before sequencing. The plasmid DNA was digested by EcoRI and XhoI which separates the insert and the vector and the DNA was separated by agarose gel electrophoresis. The DNA was then transferred to a nitrocellulose membrane and hybridized to the snake cDNA probe and to clone 1. The membrane was washed first at 52°C, then at 62°C in 5x SSPE 0.1% SDS, 30 minutes per time. Clone 1 gave a darker band than clone 2 did (Fig. 6). When clone 1 was used as the probe, hybridization was under 50% formamide at 37°C, washing was at 52°C 5x SSPE, 0.1% SDS, then at 62°C 2x SSPE 0.1% SDS, finally at 62°C 0.1x SSPE 0.1% SDS, 30 minutes per time. A faint band showed up on clone 2 (Fig.7). This may suggest those two clones have some homology to each other.

For clone 1, sequencing was difficult from the T7 primer direction because of a long poly A tail. This clone was sequenced from the T3 primer direction first, then the T3 sequence was used to design primers to get around the poly A tail, and sequenced from the T7 direction. For clone 2, sequencing could be carried on from both direction by primer walking.

Figure.8 gives the complete sequence of clone one. The insert of this clone is 1084 base pairs, and has a polyadenylation signal (AATAAA) 22 base pairs upstream of the poly (A^+) tract.

The largest open reading frame of clone one (ORF-1) is 827 base pairs. The start codon (ATG) is 33 base pairs downstream of the EcoRI site, the stop codon is 222 base pairs upstream of the XhoI site.

The translated sequence of ORF-1 is 275 amino acids, with a calculated molecular weight of 31.7 KDa, and a pI of 9.19. There is one Cys in this sequence.

Figure 5. Insert Size of the Two Positive Clones.

Lane1: clone 1; lane 2: clone 2. Plasmid DNA was purified by small scale preparation, cut by EcoRI and XhoI to release insert, and run on 0.8% agarose gel.

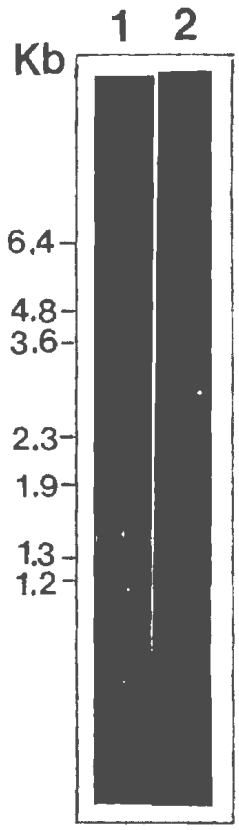

1
2
Kb
6.4
4.8
3.6
2.3
1.9
1.3
1.2

Figure 6. Clone Blot.

1μg of plasmid DNA from clone 1 (lane 1) and clone 2 (lane 2) were digested by EcoRI and XhoI to release insert, run through a 0.8% agarose gel and blotted to a nitrocellulose membrane, hybridized with the snake PLA_2 probe, washing was at 62°C, 2xSSPE, 0.1% SDS.

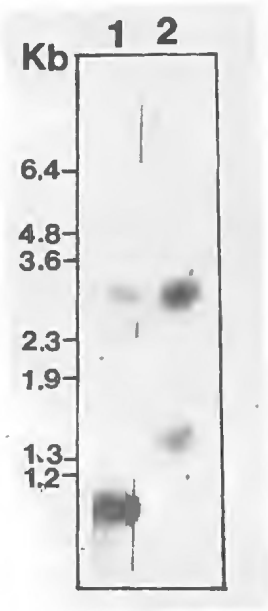

1 2
Kb
6.4
4.8
3.6
2.3
1.9
1.3
1.2

Figure 7. Cross-hybridization of the Two Positive Clones.

Lane 1: clone 1, lane 2: clone 2. 1μg of plasmid DNA was digested by EcoRI and XhoI, run through a 0.8% agarose gel, blotted to a nitrocellulose membrane, hybridized with clone 1 at 50% formamide, 5x SSPE, 37°C, and washed at 0.1x SSPE 0.1% SDS, 62°C.

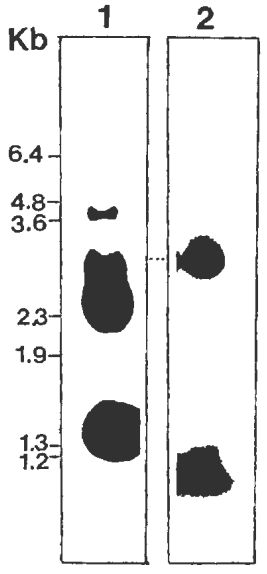

1
2
Kb
6.4
4.8
3.6
2.3
1.9
1.3
1.2

Figure 8. Nucleotide and Translated Amino Acid Sequences of Clone 1.

The sequence shown is the nucleotide sequence of clone 1 and the corresponding amino acid sequence specified by a single open reading frame between positions 35 and 859. The underlined sequence is the consensus polyadenylation sequence.

```
          GAATTCGGCACGAGCTGACGCGCTTCAGTGGGAC ATG GCC AAG TAC CCC ATC AAG CAG TCA
                                              M   A   K   Y   P   I   K   Q   S

CTG AAG AAC ATC ACT GAC ATC ATC AGC AAG CAA GTG GGC CAG ATT GAC GCA GAT CTC AAG
 L   K   N   I   T   D   I   I   S   K   Q   V   G   Q   I   D   A   D   L   K

AGC AAG TCG GCC GCG TAC AAC AAC CTG AAG ACG CAG CTG CAG TCC ATC GAG CGC AAG TCC
 S   K   S   A   A   Y   N   N   L   K   T   Q   L   Q   S   I   E   R   K   S

ACG GGC AGC TTG CTA GTG CGC AGC CTT GCA GAC CTG GTG CGG AAG GAG CAC TTT GTG CTT
 T   G   S   L   L   V   R   S   L   A   D   L   V   R   K   E   H   F   V   L

GGC TCC GAG TAC CTC ATC ACC CTG CTG GTC GTG GTG CCC AAG ATG ATG TAC AAG GAC TGG
 G   S   E   Y   L   I   T   L   L   V   V   V   P   K   M   M   Y   K   D   W

TAC TCC AAG TAT GAG AAG CTC TCG GAC ATG GTG GTG CCG TCA TCT TCC CAG CTT GTC TAT
 Y   S   K   Y   E   K   L   S   D   M   V   V   P   S   S   S   Q   L   V   Y

GAG GAC AAC GAC CAC GGC CTG TTC ACG GTC ACC CTC TTC CAG AAG GTG GTG GAC ACG TTC
 E   D   N   D   H   G   L   F   T   V   T   L   F   Q   K   V   V   D   T   F

AAG CAT CAT TGC CGA GAG AAC AAG TTT GTG GTG CGG GAT TTC GTG TAT GAC GAG AGT GCG
 K   H   H   C   R   E   N   K   F   V   V   R   D   F   V   Y   D   E   S   A

CTG CTG GCC GGC AAG AAC GAG ATT GCC AAG CTG GAG AGC GAC AAG AAG AAG CAA TAT GGG
 L   L   A   G   K   N   E   I   A   K   L   E   S   D   K   K   K   Q   Y   G

CTG TTG GTA CGC TGG CTG AAA GTG AAT TTC AGT GAA GCC TTC ACG GCC TGG GTA CAC GTC
 L   L   V   R   W   L   K   V   N   F   S   E   A   F   T   A   W   V   H   V

AAG GCC CTG AGG CTT TTT GTT GAG TCC GTG CTG AGG TAC GGC CTG CCG GTG AAT TTC CAG
 K   A   L   R   L   F   V   E   S   V   L   R   Y   G   L   P   V   N   F   Q

GGC ATG CTG CTG CAG CCG CAG AAG AAG ACT GCC AAG CGG CTG CGT GAA GTG TTG AAC CAG
 G   M   L   L   Q   P   Q   K   K   T   A   K   R   L   R   E   V   L   N   Q

CTG TAC AGC CAT CTG GAC ACA AGC ATT GCC CAG GGT CCC GTT GAC GAC ATT CCT GGC CTG
 L   Y   S   H   L   D   T   S   I   A   Q   G   P   V   D   D   I   P   G   L

AAC CTC GGT CAG CAG GAG TAC TAC CCA TAC GTC TAC TTC AAG ATC AGC ATC GAC ATG GCT
 N   L   G   Q   Q   E   Y   Y   P   Y   V   Y   F   K   I   S   I   D   M   A

GAC ACA CAC GTG CGG ATC TGA GGGGGGCCACACCTGCTGCTTCTGTGCATGCCTCCTGTGGHGGCCGTACAT
 D   T   H   V   R   I

TCCAGATGTTTTATTGTCTAGCAGTGTACAGATTAGCGTTGTCGTCGTCACTTTGGTGTCACGCAATAACCAAAAAAAT

ATATAAATGTATAAACAATAAACGAGGGCCACGCCCTCCGCAAGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

AAAAAAAACTCGAG
```

The nucleotide sequence of ORF-1 was aligned to the known PLA_2 sequences encoding secreted PLA_2s from other species. The first 150 base pairs of ORF-1 have homology with *Laticauda laticaudata* PLA_2 in region from 410 to 540 base pairs. This alignment got a score of 142. The homology between these two sequences in this region is about 56%.

When ORF-1 was aligned to the snake PLA_2 probe using the Pustell DNA Matrix, setting window size at 40, min % score at 55, and hash value at 4, six short pieces of DNA sequences, each with a size of 30-40 base pairs, were found to have homology with the probe sequence.

Among all the secreted PLA_2 proteins sequenced to date, the Ca^{2+}-binding loop and active sequences are highly conserved, so these two were searched in the translated ORF-1 using subsequence analysis in MacVector 3.5. It seems there are no such domains in ORF-1. Because the Ca^{2+}-binding structure in calmodulin is another important type of conserved calcium binding structure, this type of structure was also searched for in ORF-1. The calmodulin Ca^{2+}-binding loop consists of an α-helix, a loop about the calcium ion, and another α helix, the so-called EF hand (Tufty and Kretsiger, 1975). The primary structure of EF hand sequences has a conserved pattern of

X Y Z -Y-X -Z
L*xxL*L*xxL*DxD*xD*GxID*xxEL*xxL*L*xxL*, L*is a hydrophobic residue, D* is an amino acid which has an oxygen atom as a calcium ligand, and x can be any of other amino acid. X, Y, Z, -X, -Y, -Z refer to verticals of calcium coordination octahedron. There is one region found in ORF-1 which is likely to form an EF hand structure (Fig.9) (Tufty and Kretsiger, 1975).

The amino acid sequence of ORF-1 was aligned to the protein data base. but no sequences with significently high score were found after the search.

The fact that the nucleotide sequence of ORF-1 has short homology regions with the snake PLA_2 probe could explain why this clone was identified from the cDNA library under low stringency hybridization and washing.

Figure 9. Comparison of EF Hand Sequence with One Region in ORF-1 Amino Acid Sequence.

The amino acid sequence between 226 and 256 of ORF-1 is compared to the conserved EF hand sequence. L* is a hydrophobic residue, D* has an oxygenation as a calcium ligand, X, Y, Z, -X, -Y, -Z refer to vertices of the calcium coordination octahedron.

```
                    X   Y    Z  -Y  -X   -Z
E L*- - L*L*- - L*D - D* - D*G - I D*- -E L*- - L*L*- - L*
    226V L N Q L Y S H  L D T S I^Q G PV D D I P G L N L G Q Q E256
                                 a
```

Previous data shows that tick PLA_2 is activated by micromolar calcium while the secreted PLA_2 is activated by millimolar calcium. This difference may suggest that the tick PLA_2 has a very different Ca^{2+}-binding structure from that existing in all secreted PLA_2's. Thus the conserved PLA_2 Ca^{2+}-binding loop was not found in ORF-1. On the other hand, the lack of Cys and consequently disulfide bonds in ORF-1 may suggest it is not a secreted type of PLA_2. This is also possible because previous data also indicated there is an intracellular PLA_2 existing in tick saliva. Comparison of ORF-1 with other known intracellular PLA_2 is not feasible because the sequence and structural information about these proteins is very little.

ORF-1 could be the phospholipase A_2 we are looking for. This PLA_2 in tick may be so different that regular sequence analysis to compare ORF-1 with other known PLA_2 sequences could not tell anything.

Information from cloning, sequencing and sequence analysis only is not enough to draw a conclusion that ORF-1 is or is not PLA_2. Efforts could be made to get active over-expressed ORF-1 protein and to check PLA_2 activity; or to get the pure form of tick PLA_2 protein, and to sequence it, then compare the deduced amino acid sequence with the protein sequence.

Figure.10 shows the complete sequence of clone two. The insert of this clone is 1488 base pairs. The 3'-untranslated regions of the cDNA contain a consensus polyadenylation signal (AATAAA) 24 base pairs upstream from the poly (A+) tract. The largest open reading frame (ORF-2) in clone two is 954 base pairs. The start codon (ATG) is 113 base pairs downstream of the EcoRI site, the stop codon (TAG) is 369 base pairs upstream of the XhoI site.

This ORF-2 was translated into an amino acid sequence. ORF-2 has 317 amino acids, the molecular weight is 37 KDa, the calculated pI is 9.76. It has four cys.

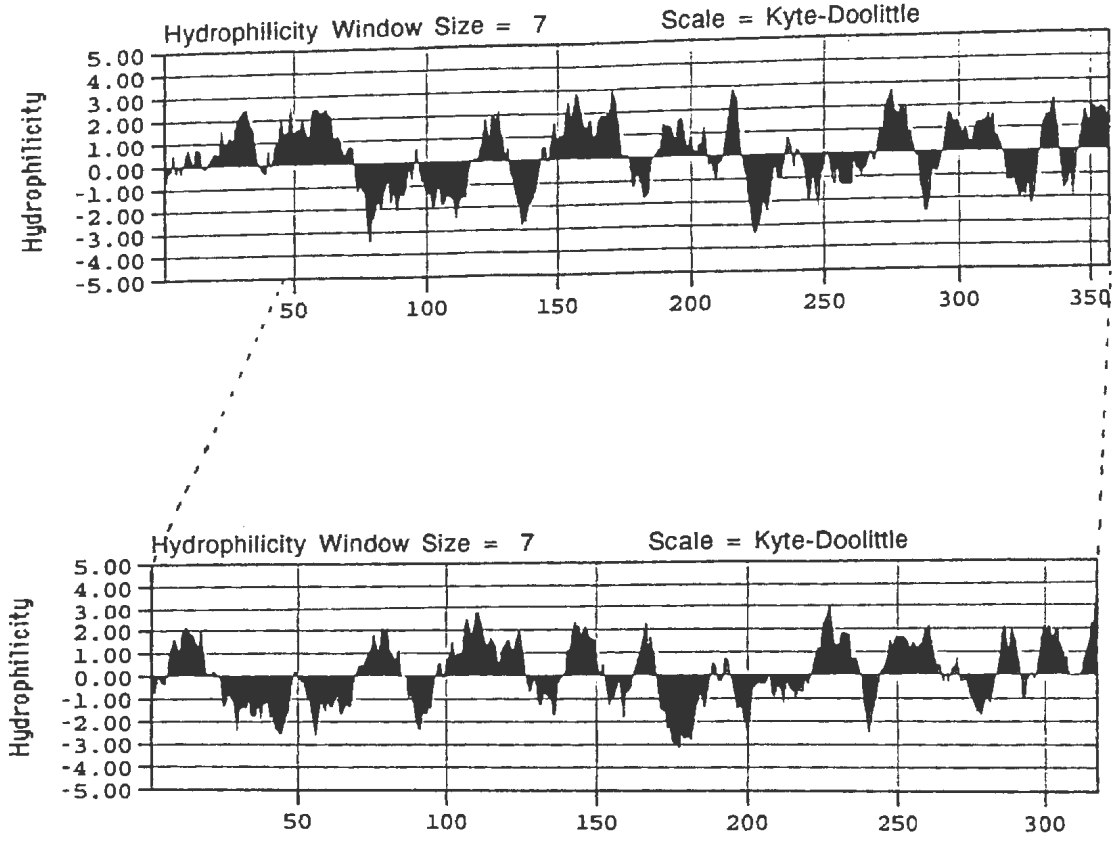

Hydrophilicity Window Size = 7
Scale = Kyte-Doolittle
Hydrophilicity
5.00
4.00
3.00
2.00
1.00
0.00
-1.00
-2.00
-3.00
-4.00
-5.00
50
100
150
200
250
300
350
Hydrophilicity Window Size = 7
Scale = Kyte-Doolittle
Hydrophilicity
5.00
4.00
3.00
2.00
1.00
0.00
-1.00
-2.00
-3.00
-4.00
-5.00
50
100
150
200
250
300

Figure 12. Comparison of the Hydrophilicity of Stearoyl-CoA Desaturase with that of ORF-2.

The upper figure is the hydrophilicity of Stearoyl-CoA desaturase, the lower figure is the hydrophilicity of ORF-2. The pattern of these two are very similar begin at amino acid residue 50 in the sequence of SCD, this similarity suggests a structural similarity between these two proteins.

hydrophilicity, this similarity suggests a similar structrue between these two proteins (Fig. 12).

The function of stearoyl-CoA desaturase is the synthesis of unsaturated fatty acids as well as the regulation of this process. This enzyme catalyzes the Δ^9-cis desaturation of fatty acyl-CoA, the major products being palmitoleoyl- and oleoyl-CoA. Shipley et al. noticed that in the salivary glands of partially fed *A. americanum* female ticks, oleoylic acid is the most abundant (51.7% of all the fatty acids), palmitoleoylic acid is about 3.4% of all the fatty acids. The existence of stearoyl-CoA desaturase in the salivary glands may be the reason of the high content of oleoylic acid.

The fact that ORF-2 is highly homologous with stearoyl-CoA desaturase, and the highest homology lies in the C-terminal of the protein, which is possibly the active site of this enzyme, may suggest that this ORF-2 is a stearoyl-CoA desaturase. Similar structure between ORF-2 and SCD based on hydrophilicity may also suggest ORF-2 is a stearoyl-CoA desaturase in tick.

The reason that this clone was identified by a snake PLA_2 probe is that the nucleotide sequence of ORF-2 does have short pieces of sequence which are homologous with the probe, and under low stringency hybridization and washing, these short pieces may hybridize to the corresponding sequences in the probe, and thus give positive spots when the library is screened.

The overall results of ORF-2 analysis suggest that ORF-2 is likely a stearoyl-CoA desaturase, which is an important enzyme in unsaturated fatty acid synthesis and regulation. Again, information from gene expression can provide direct evidence.

```
ORF-2     1 MSTVTETVTE ERPASKPHKM EIVWRNVILM GSLHLISIYG FYLIFFAAQW   50
 1       49 MREDIHDPSY QDEEGPPPKL EYVWRNIILM ALLHVGALYG ITLIPSSKVY   98
 2       49 MREDIHDPSY QDEEGPPPKL EYVWRNIILM ALLHVGALYG ITLIPSSKVY   98
 3       49 MKEDIHDPTY QDEEGPPPKL EYVWRNIILM VLLHLGGLYG IILVPSCKLY   98
 4       65                   PPKL EYVWRNIILM ALLHLGALYG ITLV-PSCKL   98

ORF-2       KTVLAAYIFY TISGIGVTAG SHRLWSHRSY KAKLPYRIML MIFQTMAFQN  100
 1           TLLWGIFYY LISALGITAG AHRLWSHRTY KARLPLRIFL IIANTMAFQN  148
 2           TLLWGIFYY LISALGITAG AHRLWSHRTY KARLPLRIFL IIANTMAFQN  148
 3          TALFGIF-YY MTSALGITAG AHRLWSHRTY KARLPLRIFL IIANTMAFQN  148
 4          YTCLFAYLYY VISALGITAG AHRLWSHRTY KARLPLRLFL IIANTMAFQN  148

ORF-2       DIYDWARDHR MHHKFSETTA DPHDATRGFF FSHVGWLLVR KHPDVRNKGK  150
 1          DVYEWARDHR AHHKFSETHA DPHNSRRGFF FSHVGWLLVR KHPAVKEKGG  198
 2          DVYEWARDHR AHHKFSETHA DPHNSRRGFF FSHVGWLLVR KHPAVKEKGG  198
 3          DVYEWARDHR AHHKFSETHA DPHNSRRGFF FSHVGWLLVR KHPAVKEKGG  198
 4          DVYEWARDHR AHHKFSETHA DPHNSRRGFF FSHVGWLLVR KHPAVKEKGG  198

ORF-2       SIDLSDVLAD PVVRFQRRYY LPLMVTICFI VPALLPWWLW GETLWNSFVV  200
 1          KLDMSDLKAE KLVMFQRRYY KPGLLLMCFI LPTLVPWYCW GETFLHSLFV  248
 2          KLDMSDLKAE KLVMFQRRYY KPGLLLMCFI LPTLVPWYCW GETFLHSLFV  248
 3          KLDMSDLKAE KLVMFQRRYY KPGLLLMCFI LPTLVPWYCW GETFVNSLFV  248
 4          KLDMSDLKAE KLVMFQRRYY KPDLLLMCFV LPTLVPWYCW GETFVNSLCV  248

ORF-2       CSLTRYCFTL NMTWLVNSAA HIWGNRPYDR HISPRQNLVT IVGAHGEGFH  250
 1          STFLRYTLVL NATWLVNSAA HLYGYRPYDK NIQSRENILV SLGAVGEGFH  298
 2          STFLRYTLVL NATWLVNSAA HLYGYRPYDK NIQSRENILV SLGSVGEGFH  298
 3          STFLRYTLVL NATWLVNSAA HLYGYRPYDK NIQSRENILV SLGAVGEGFH  298
 4          STFLRYAVVL NATWLVNSAA HLYGYRPYDK NISSRENILV SMGAVGERFH  298

ORF-2       NYHHTFPYDY RTSELGCRIN TTTWFIDFFA WLGQVYDRKE VPTSVVEGRM  300
 1          NYHHAFPYDY SASEYRWHIN FTTFFIDCMA ALGLAYDRKK VSKAAVLARI  348
 2          NYHHAFPYDY SASEYRWHIN FTTFFIDCMA ALGLAYDRKK VSKAAVLARI  348
 3          NYHHTFPFDY SASEYRWHIN FTTFFIDCMA ALGLAYDRKK VSKATVLARI  348
 4          NYHHAFPYDY SASEYRWHIN FTTFFIDCMA LLGLAYDRKR VSRAAVLARI  348

ORF-2       KRTGDGSRGL TAGTRSW  317
 1          KRTGDGSH            357
 2          KRTGDGSH            357
 3          KRTGDGSH            357
 4          KRTGDGS             357
```

Figure 11. Comparison of the Amino Acid Sequence of Clone 2 with the Amino Acid Sequences of Stearoyl-CoA Desaturase from Different Tissues of Rat and Mouse.

The amino acid sequence of clone 2 is aligned with those of rat stearoyl-CoA desaturases (SCD) (1&2) and mouse SCDs (3&4). The overall homology is about 70%, while in some regions is higher (80%).

The nucleotide sequence of ORF-2 was aligned to the nucleotide sequences of secreted PLA_2 from other species. The first 630 base pairs of ORF-2 has homology with the corresponding region of *durissus* crotoxin A. It has a homology of about 50%.

When ORF-2 was aligned to the snake probe using the Pustell DNA Matrix with the window size, min % score and hash value set at 40, 55, 4 respectively, there were 9 short pieces which had homology with snake probe, each piece having a size of 30-50 base paris.

The well known Ca^{2+}-binding loop and active site was also searched for in ORF-2 using subsequence analysis in MacVactor 3.5, but no such region was found. Also there is no EF-hand found in ORF-2.

Interestingly, when the translated amino acid sequence of ORF-2 was aligned to sequences in the protein data base, the first four highest scores came from stearoyl-CoA desaturase (SCD) from different tissues of rat and mouse. Two of the SCDs are from rat (Mihara et al., 1990; Thiede et al., 1986), the other two are from mouse (Ntambiet et al., 1988; Kaestner et al., 1989). The two SCDs in the same species are closely related, highly homologous (87% identical). They are different in tissue distribution. Also, their mRNA expression induced by dietary alteration are different (Kaestner et al., 1989). Similarities between ORF-2 and all four stearoyl-CoA desaturases lie in: 1) the molecular weights are close, the four mature stearoyl-CoA desaturases having amino acid sequences of either 358 or 355, and ORF-2 having 317 amino acids; 2) the overall homology of the aligned sequences is about 70%, while in some regions, the homology is higher (80%) (Fig. 11). The region which has higher homology between ORF-2 and stearoyl-CoA desaturase lies in the carboxyl-terminal half of the protein sequence, which agrees with the C-terminal half of the enzyme being highly conserved, and possibly containing the catalytic active site (Kaestner et al., 1989). The nucleotide sequence of ORF-2 and stearoyl-CoA desaturase share about 50-60% homology. 3) Stearoyl-CoA desaturase and ORF-2 have similar

GAATTCGGCACGAGGCCGGCCTTCGCACGTCTCTCGAATACTGCTA

CGGCAGCGCTTTGGAAACTGTCACATTGGATACTGCCCGCCGCCTAAAATAACCTCAGAGGCGAGCC ATG TCG ACC
M S T

GTG ACG GAG ACT GTG ACG GAG GAG CGG CCG GCC TCC AAG CCG CAC AAG ATG GAG ATA GTG
V T E T V T E E R P A S K P H K M E I V

TGG CGC AAT GTG ATT CTC ATG GGA AGC CTG CAC CTT ATC TCC ATC TAC GGC TTC TAC CTG
W R N V I L M G S L H L I S I Y G F Y L

ATA TTT TTC GCT GCG CAG TGG AAG ACC GTG CTT GCC GCA TAC ATT TTC TAC ACA ATT TCC
I F F A A Q W K T V L A A Y I F Y T I S

GGC ATT GGA GTG ACA GCT GGC TCT CAC CGC CTG TGG TCT CAC CGG TCG TAC AAG GCT AAA
G I G V T A G S H R L W S H R S Y K A K

TTG CCT TAT CGT ATC ATG CTG ATG ATT TTC CAG ACC ATG GCA TTC CAA AAT GAC ATC TAT
L P Y R I M L M I F Q T M A F Q N D I Y

GAC TGG GCA AGA GAT CAT AGG ATG CAC CAC AAG TTC TCG GAG ACA ACT GCA GAT CCA CAT
D W A R D H R M H H K F S E T T A D P H

GAC GCG ACA CGG GGC TTT TTC TTC TCG CAC GTG GGC TGG CTT CTC GTG CGC AAG CAC CCG
D A T R G F F F S H V G W L L V R K H P

GAT GTG CGT AAC AAG GGC AAA TCG ATC GAC CTG AGT GAT GTT CTT GCT GAC CCA GTT GTC
D V R N K G K S I D L S D V L A D P V V

CGC TTC CAG AGA AGG TAT TAC CTG CCT CTG ATG GTG ACC ATT TGC TTC ATT GTT CCT GCA
R F Q R R Y Y L P L M V T I C F I V P A

CTG CTG CCC TGG TGG CTG TGG GGG GAG ACA CTG TGG AAC TCA TTT GTG GTC TGC TCC CTG
L L P W W L W G E T L W N S F V V C S L

ACA CGC TAC TGC TTT ACA CTC AAC ATG ACC TGG CTG GTG AAC AGC GCG GCA CAC ATC TGG
T R Y C F T L N M T W L V N S A A H I W

GGC AAC CGA CCC TAT GAT CGG CAC ATT AGC CCG CGG CAA AAC CTG GTG ACG ATT GTT GGG
G N R P Y D R H I S P R Q N L V T I V G

GCA CAC GGC GAA GGT TTT CAC AAC TAC CAC CAC ACG TTT CCC TAC GAC TAC CGC ACC AGC
A H G E G F H N Y H H T F P Y D Y R T S

GAG CTG GGC TGC CGC ATC AAC ACG ACC ACA TGG TTC ATC GAC TTC TTT GCA TGG CTT GGC
E L G C R I N T T T W F I D F F A W L G

CAG GTG TAC GAC CGC AAG GAG GTG CCT ACG AGT GTG GTG GAA GGG CGC ATG AAG CGC ACT
Q V Y D R K E V P T S V V E G R M K R T

GGC GAC GGC TCC CGG GGT CTG ACG GCT GGC ACG CGC AGC TGG TGA TGGCATACAGCCTGTCATG
G D G S R G L T A G T R S W

GCCACCTGTCTCTTTTTCCTTTTCTTTTCCTCTTCTGTTCCTCTTCTAGGTGGGCGTGATCTGACAAGACAACCACCTC

CACCCTTTTCTGTCTTGTGGGACATGTTATTTATAGTTGTCACTGTGTGAAGTAATGTCAGTTGGCTGATGCAGGCTTG

ATAATAATCAGAAAGTGTGTGAACTCTTGCACTGTTATGATGCCAAGAAGGGCAGCCTGGCTGTCAACCATGTTGCGTG

GACTGTTCAGTATCTGCAGCTGGACGCCACCTTGTTCAGACCCGGTGAGAGTGTCATGGCCAACGTTGTACCCCTCCAT

GTCAAAGCACTTCTGAAATAGTCAGTGAAATAAAGAGACCCTGTTAGAGTCCACTGGAAAAAAAAAAAAAAAAAAAAA

ACTCGAG

Figure 10. Nucleotide and Amino Acid Sequences of Clone 2 .

The sequence shown is the nucleotide sequence of clone 2 and the corresponding amino acid sequence specified by a single open reading frame between positions 114 and 1064. The underlined sequence is the consensus polyadenylation sequence.

CHAPTER V

SUMMARY AND CONCLUSION

Ticks are effective disease vectors of man and domestic animals. Compared to many arthropod ectoparasites, ticks feed for longer periods of time and successfully evade many host defense pressures. It is proposed that the synthesis and secretion of eicosanoids may be key processes in this successful parasitization.

The immediate precursor of eicosanoids is free arachidonic acid, which is released from membrane phospholipid by phospholipase A_2. Arachidonic acid can be converted to eicosanoids by prostaglandin synthase in a cyclic pathway. We therefore used available cDNA to try to isolate and sequence these two enzymes in prostaglandin synthesis.

Genomic Southern blotting had been carried out to check if PGHs and PLA_2 genes exist in the tick genome. With the cDNA probes available now, genomic southern blots probed by PGHs cDNA probe gave a negative result. Genomic southern blots probed by a snake PLA_2 probe gave a positive result, the one probed by human intracellular probe yielded a negative result. The snake PLA_2 probe was used to screen a tick Uni-ZAP^{TM} XR cDNA library and two positive clones were obtained.

The complete sequences of these two clones are 1084 and 1488 base pairs respectively. Both of them have a consensus polyadenylation signal in the 3'-untranslated region. The two largest open reading frames of these two clones are 828 and 954 base pairs respectively. The translated amino acid sequences of these two open reading frames contain 275 and 317 amino acids respectively.

ORF-1 could be the phospholipase A_2 in tick. It could form an EF-hand structure for its Ca^{2+}-binding region, instead of the usual Ca^{2+}-binding loop existing in all secreted

PLA_2. The fact that tick PLA_2 is activated by micromolar concentrations of calcium may suggest this structural difference. This ORF-1 could be the intracellular type of PLA_2 in tick salivary gland, which has a very different structure and property from the secreted type. Also, evolutionary distance may be one of the reasons why ORF-1 is so different from all known PLA_2 sequences from mammals and reptiles.

ORF-2 was found to have high homology with stearoyl-CoA desaturase from different tissues of rat and mouse. The highly conserved sequence between ORF-2 and stearoyl-CoA desaturase lies in the C-terminal part of the protein sequence, which is in agreement with the C-terminal part of the enzyme being likely to have the active site. Results from analysis of ORF-2 suggest it is likely to be the stearoyl-CoA desaturase in tick, but more direct evidence from gene expression is needed to draw a conclusion.

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