Analysis of the Expression, Structure and Function of Nematode Lipid-binding Proteins

Alison Elizabeth Prior

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Division of Infection and Immunity
IBLS
University of Glasgow

Unit of Mycology, Bacteriology and Nematology
Scottish Crop Research Institute

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Abstract

Several classes of lipid-binding protein (LBP) are present in nematodes. These include members of well-characterised families which are widespread in higher animals, but in nematodes have distinct features which set them apart from their counterparts in other organisms. These include an atypical, secreted protein from the FABP (fatty acid-binding) protein family which, in other organisms, are thought to be exclusively intracellular. In addition, two novel classes of LBP, the fatty acid and retinol-binding (FAR) proteins and the nematode polyprotein allergens (NPA), are present in abundance in nematodes. Database similarity searches indicate that these proteins are confined to nematodes and so are possible targets for control and diagnosis. To date, all knowledge on the FAR and NPA proteins has come from studies on animal-parasitic nematodes and nothing is known about the localisation and function of these proteins in plant-parasitic nematodes.

In-situ hybridisation studies on FAR, NPA and FABP mRNA localisation in the potato cyst nematode, *Globodera pallida*, indicated distinct spatial expression patterns for each of these proteins. Immunolocalisation studies indicated that the *G. pallida* FAR protein (Gp-FAR-1) is present on and rapidly shed from the surface of this nematode and so may interact with host tissues.

Spectrofluorimetric studies, using fluorescent probes, showed that Gp-FAR-1 binds long-chain fatty acids and retinol with similar affinities to its counterpart Ov-FAR-1 from the animal-parasitic nematode *Onchocerca volvulus* and commonly occurring LBPs from other organisms. Surprisingly, mutation of the most conserved residues in Gp-FAR-1 did not ablate ligand-binding but did alter its binding affinities.

The surface localisation and rapid shedding of Gp-FAR-1 indicated that, *in vivo*, this protein is in contact with host tissues and so may be involved in the host-parasite interaction. Lipoxygenase functions as a central enzyme in fatty acid-based defence signalling pathways in plants. Gp-FAR-1 was shown to inhibit the lipoxygenase-mediated peroxidation of fatty acids *in vitro*, indicating that Gp-FAR-1 could be involved in inhibition and/or evasion of host defence mechanisms.

Database similarity searches and structural prediction programs indicated that the FARs and NPAs have stretches of low amino acid identity with a class of vertebrate exchangeable apolipoproteins which share other features with the NPAs. Lipoprotein systems which function in the transport and storage of lipids in oocytes are present in nematodes but little is known about the mechanisms of acquisition, transport and storage of lipids destined for metabolic pathways. Since lipids are the main energy source in nematodes, these lipid transport pathways may be good targets for the development of control strategies. In the absence of recombinant *G. pallida* NPA protein for use in this work, lipoprotein-binding assays showed that ABA-1, an NPA from the animal-parasitic nematode *A. suum*, but not Gp-FAR-1, can inhibit aggregation of human low-density lipoprotein *in vitro* and therefore could function in the disruption of host lipid transport and/or play a role in a previously undescribed lipid transport pathway in nematodes. Evidence in support of the presence of such a pathway in nematodes is presented and discussed.

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Contents

List of Figures

List of Tables

Abbreviations

2XTY	2x tryptone-yeast medium
A_{234}	absorbance at 234nm
A_{600}	absorbance at 600nm
ABA-1	*Ascaris suum* npa protein monomer
ABF	*Ascaris suum* body fluid
ApoAI	apolipoprotein AI
ApoB	apolipoprotein B
ApoE	apolipoprotein E
ApoLp I	apolipophorin I
ApoLp II	apolipophorin II
ApoLpIII	apolipophorin III
Arg/R	arginine
ASM	antibiotic selection mix
As-p18	*Ascaris suum* perivitelline FABP
ATP	adenosine triphosphate
Av-FAR-1	*Acanthocheilonema viteae* FAR protein
Bm-FAR-1	*Brugia malayi* FAR protein
BSA	bovine serum albumin
CD	circular dichroism
cDNA	complementary DNA
cPnA	*cis*-parinaric acid
CRABP	cellular retinoic acid-binding protein
CRBP	cellular retinol-binding protein
cv	cultivar
DACA	dansyl-dl-α-aminocaprylic acid
DAG	diacylglycerol
dansylamide	5-(dimethylamino)naphthalene-1-sulphonamide
DAUDA	11-(((5-(dimethyl(amino)-1-naphthalenyl)sulfonyl)amino)undecanoic acid

DEPC	diethylpyrocarbonate
DIG	digoxigenin
DIG-DNA	digoxigenin-labelled DNA
DMPC	dimyristoylphosphatidylcholine
DNA	deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DSC	differential scanning calorimetry
DTT	dithiothreitol
Dva-1	NPA monomer from *Dictyocaulus viviparous*
EDTA	ethylenediaminetetraacetic acid
E/S	excretory/secretory
EST	expressed sequence tag
F67W	Gp-FAR-1 protein mutated by substitution of phenylalanine residue at position 67 with tryptophan
FA	fatty acid
FABP	fatty acid-binding protein
FAR	fatty acid- and retinol-binding protein
Fh12	*Fasciola hepatica* secreted FABP
FPLC	fast protein liquid chromatography
FRET	fluorescent resonance energy transfer
GdnHCl	guanidine hydrochloride
Gln/Q	glutamine
Glu/E	glutamic acid
Gp-FAR-1	*Globodera pallida* FAR protein
GSH	glutathione
GST	glutathione-S-transferase
GST:Gp-FAR-1	fusion protein
His/H	histidine
HR	hypersensitive response
iFABP	intracellular fatty acid-binding protein
IgG	immunoglobulin G

IgM	immunoglobulin M
IPTG	isopropyl β-D-thiogalactopyranoside
ISH	*in-situ* hybridisation
J2	free-living infective juvenile stage of PCN
JA	jasmonic acid
K78Q	Gp-FAR-1 protein mutated by substitution of lysine residue at position 78 with glutamine
K78R	Gp-FAR-1 protein mutated by substitution of lysine residue at position 78 with arginine
K80Q	Gp-FAR-1 protein mutated by substitution of lysine residue at position 80 with glutamine
K80R	Gp-FAR-1 protein mutated by substitution of lysine residue at position 80 with arginine
K_d	estimated dissociation constant
kDa	kilodalton
KOAc	potassium acetate
K_{SV}	Stern-Volmer constant
LB	Luria-Bertani medium
LBP	lipid-binding protein
LDL	low-density lipoprotein
LDS	lithium dodecyl sulphate
λ_{em}	wavelength of maximum fluorescence emission
Leu/L	leucine
λ_{ex}	wavelength of excitation
Lys/K	lysine
MES	2-(n-morpholino)ethane sulphonic acid
μM	micromolar
mM	millimolar
MOPS	3-(n-morpholino) propane sulphonic acid
mRNA	messenger RNA
MW	molecular weight

MWCO	molecular weight cutoff
NaOAc	sodium acetate
NBT	nitroblue tetrazolium salt
nM	nanomolar
nm	nanometre
NMR	nuclear magnetic resonance
NPA	nematode polyprotein allergen
ns-LTP	non-specific lipid transfer protein
OD	optical density
Ov-FAR-1	*Onchocerca volvulus* far protein
Pa1-Pa3	*Globodera pallida* pathotypes
PBS	phosphate-buffered saline
PC	phosphatidylcholine
PCN	potato cyst nematodes
PCR	polymerase chain reaction
PDB	protein data bank
PEBP	phosphatidylethanolamine-binding proteins
Phe/F	phenylalanine
PI	phosphatidylinositol
pI	isoelectric point
PLC	phospholipase C
PLTP	phospholipid transfer protein
PRD	potato root diffusate
PRP	pathogenesis-related proteins
rGp-FAR-1	recombinant Gp-FAR-1
RNA	ribonucleic acid
RNase A	ribonuclease A
Ro1-Ro5	*Globodera rostochiensis* pathotypes
rpm	revolutions per minute
RT-PCR	reverse transcription PCR
SDM	site-directed mutagenesis

SDS	sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
Sm14	*Schistosoma mansoni* secreted FABP
SDW	sterile, distilled water
TAG	triacylglycerol
TBE	tris borate EDTA buffer
TE	tris-EDTA buffer
TES-26	*Toxocara canis* PEBP
TNF-α	tumour necrosis factor- α
TRD	tomato root diffusate
Trp/W	tryptophan
Tyr/Y	tyrosine
UV	ultra-violet
Val/V	valine
Vit 1-6	vitellogenins 1-6
WT	wild-type
X-phosphate	5-bromo-4-chloro-3-indoyl phosphate toluidinium salt

Chapter 1

Introduction

1 Introduction

1.1. Plant-parasitic nematodes

Nematodes are ubiquitous on Earth, present in almost all environments from the poles to deserts and can be free-living or parasites of plants, animals or fungi. Worldwide, around 20,000 species of nematode have been described to date, although many undescribed species are thought to exist, and around 48% of these nematode genera contain parasitic species. As a result, these organisms have a huge effect on human health, mainly in developing countries, and on the world economy due to yield losses in parasitised crops and livestock (Zunke & Perry, 1997).

The most economically important nematode parasites of plants are found in the Dorylaimida, Triplonchida, Tylenchida and Aphelenchida. Nematodes from these groups cause an estimated US$77billion worth of crop damage annually (Sasser & Freckman, 1987). Some species of plant-parasitic nematode, such as the root-knot nematodes, *Meloidogyne* spp., have very broad host ranges, while others such as the potato cyst nematodes (PCN), have a much more restricted host range. Nematodes can cause direct damage to plants or may be vectors of other economically damaging pests including viruses. The process of nematode attack may also provide an entry route for secondary infection by other pathogens (Agrios, 1997).

1.2 Potato cyst nematodes

1.2.1 Host range, damage and economic importance

The potato cyst nematodes *Globodera rostochiensis* and *G. pallida,* parasitise solanaceaeous plants including the potato (*Solanum tuberosum*), tomato (*Lycopersicum esculentum*) and eggplant and cause economic losses estimated at £30 million annually

in the UK alone (M. Phillips, pers. comm.). Symptoms of infection by these parasites include growth retardation, reduction of leaf expansion, reduced ground cover, retarded flowering, abnormal branching and stunting of roots, yield reductions and in some cases premature death of the host plant. Environmental factors such as water availability can also affect the variety and degree of symptoms seen in potato plants upon PCN infection (Agrios, 1997).

Control of PCN is achieved using a combination of crop rotation, application of nematicides and use of resistant cultivars where available. Of the five *G. rostochiensis* (Ro1-Ro5) and three *G. pallida* (Pa1-Pa3) pathotypes (Kort *et al.*, 1977), potato cultivars with major gene resistance are only commercially available against *G. rostochiensis* Ro1 and Ro4 pathotypes (Phillips *et al.*, 1998). Tolerant cultivars, which withstand infection but do not suffer yield losses, are available. However, use of these cultivars has the disadvantage that they do not eradicate the nematodes but enhance the build-up of nematode populations within the soil (Phillips *et al.*, 1998). *G. pallida* presents a particular problem for control as the few resistant cultivars available are only partially resistant (Phillips *et al.*, 1998). This species also hatches over a longer period than *G. rostochiensis* making control with nematicides, which have a limited lifespan in soil and are often targeted at the hatched second stage juveniles, more difficult (Zunke & Perry, 1997).

1.2.2 Life-cycle of PCN

PCN juveniles undergo their first moult within the eggs where they remain as tightly-coiled, infective second stage juveniles (J2s) until stimulated to hatch by potato root diffusate (PRD) (Perry, 1987). PRD has been shown to induce a Ca^{2+}-mediated permeability change in the inner lipoprotein layers of the eggshell which results in the release of trehalose from the perivitelline fluid in the egg. This reduces the osmotic

pressure within the egg, causing an increase in water content and metabolic activity of the juveniles which then escape from the egg and are attracted to the roots (Perry, 1989). This strategy ensures that the J2s only hatch in the presence of host plants and provides a mechanism linking host and parasite life-cycles. During this time, the oesophageal gland cells accumulate secretions which are used in invasion and feeding (Perry 1989). The J2s penetrate the root, usually near the root tip or at the junction between the main root and a lateral root, then migrate intracellularly without feeding towards the vascular cylinder where, like other tylenchid nematodes, they pierce a single cell with their stylet (Linford, 1937). This stylet thrusting is stimulated by root diffusates (Grundler *et al.,* 1991; Clemens *et al.*, 1994) and it is widely accepted that at this stage the nematodes pump salivary secretions from their oesophageal gland cells through the stylet into the host cell (Wyss *et al.*, 1992; Wyss & Zunke, 1986). The cell is then rapidly converted into a highly metabolically-active multinucleate syncytium. This multinucleate condition is brought about by cell wall dissolution and merging of neighbouring cells. It is thought that induction and maintenance of the feeding site is brought about by changes in host gene expression induced by unidentified factors in the nematode oesophageal gland cell secretions. The survival and continued development of the nematode is dependent on maintenance of this feeding site (Burrows, 1992).

After induction of the feeding site the nematode begins to feed by extracting material from the syncytium through its stylet. The nematodes lose all body wall muscle and undergo a series of moults to the sexually mature adult at the feeding site. Under optimum conditions the molt to 3rd stage juvenile occurs approximately 7 days after invasion, with the molt to 4th stage occurring 3-4 days later. The sex of the juvenile becomes apparent immediately before the moult to the 3rd stage and is determined by environmental factors such as nutrient availability. The body of the adult

female swells and extrudes from the surface of the root as the egg-filled ovary fills the body cavity while the head remains embedded in the feeding site. Male nematodes retain their vermiform morphology and locomotory ability and emerge from the root into the soil where they are attracted by sex pheromones emitted by the females. After fertilisation, the female dies and her cuticle hardens and tans forming a highly resistant cyst containing 300-500 eggs. These cysts can remain dormant in the soil for up to 20 years until another host is detected. Under optimum environmental conditions the life-cycle is completed in 1-2 months (Zunke & Perry, 1997).

1.3 Lipids in PCN and host root tissues

Several main classes of lipids exist including neutral lipids (TAG, DAG, free sterols, free fatty acids, sterol esters of fatty acids), polar lipids (phospholipids), glycolipids sterols and waxes. These molecules are essential constituents of all living organisms and are important as structural components of cell membranes and of protective surfaces such as skin and the cuticles and surface waxes of insects, nematodes and plants. Lipids also play important metabolic roles in cells as storage and transport forms of fuel and some are highly biologically active and important as signalling molecules or their precursors (Stryer, 1988).

Lipids and their derivatives are known to play important roles in plant defences, acting as signal molecules and exogenous or endogenous elicitors of defence responses (e.g., Farmer, 1988) and nematodes, among other organisms are known to have nutritional requirements for certain classes of lipids (Chitwood & Feldlaufer, 1990).

Many typical central enzymes involved in fatty acid biosynthesis have been identified and functionally characterised in *C. elegans* (reviewed by Napier & Michaelson, 2001) and enzymes involved in catabolism of fatty acids have been demonstrated in a range of free-living and parasitic nematodes (reviewed by Barrettt &

Wright, 1998), indicating that fatty acid metabolism in nematodes is similar to that in higher metazoans. However, some evidence suggests that, unlike vertebrates, nematodes are capable of *de novo* synthesis of fatty acids (Chitwood, 1998). Also, some species of nematode have been shown to have a functional glyoxylate cycle, which provides pyruvate from β-oxidation for gluconeogenesis (Barrett & Wright, 1998). Nematodes are the only metazoans known to have this cycle, which is commonly present in plants, bacteria and fungi.

1.3.1 Lipids in PCN

Lipids are usually the predominant energy reserve in plant parasitic nematodes (Barrett, 1981) and in PCN comprise about one-third of the dry weight of J2s (Holz *et al.,* 1997, 1998a,b). Neutral lipids account for around 80% of total lipids in PCN cysts and J2s, non-acidic phospholipids around 15% and free fatty acids around 5%, the latter two values being relatively high in common with many plant-parasitic nematodes (Holz *et al.,*1998b). However, in contrast to all other plant parasitic genera studied to date which all have a lipid composition dominated by C18 fatty acids (usually C18:1 vaccenic acid), PCN are distinctive, with lipids dominated by C20 fatty acids. The most abundant fatty acids in hatched PCN J2s, accounting for over 60% of the total are C20:4 (arachidonic acid), C20:1 and C18:1 whereas in unhatched J2s the most abundant are C18:0, C18:1 and C20:1. The reasons for this large increase in polyunsaturated fatty acids after hatching are not known (Holz *et al.,* 1998a).

1.3.2 Lipids in host plant roots

The lipid composition of potato and tomato roots have been shown to be dominated by C18:2, C16:0, C18:3, C18:0 and C18:1 fatty acids, compared with C20:4, C20:1 and C18:1 in PCN. Total fatty acids in roots consist mainly of polyunsaturated and saturated fatty acids whereas PCN has mainly monounsaturated and

polyunsaturated (Holz *et al.*, 1997, 1998a,b). This difference between nematode and root lipid profiles indicates that, as roots are the only nutrient source for PCN, these nematodes are able to elongate and desaturate host-derived fatty acids or to synthesise fatty acids *de novo*. Although most animals are thought not to have this ability, it has been reported in several species of nematode (Chitwood, 1998).

1.4 Lipid/fatty acid binding proteins

1.4.1 Lipid transport

For normal cell functioning, lipids are required to travel throughout the aqueous environments of cells and tissues from their sites of synthesis to their, often distant, sites of utilization. Diffusion along the plane of membranes and vesicle transport, where newly-synthesised lipids bud off a membrane as a spherical vesicle, traverse the cell then fuse with an acceptor membrane at the site of utilisation, are two ways in which lipids can be transported within cells. Lipids can also be transported within and between cells by interaction with transfer proteins. These proteins solubilize their ligands and, often through interaction with receptors, can target these molecules to specific organelles, cells or tissues.

Many different families of specific and non-specific lipid/fatty acid-binding proteins have been characterised in animals and plants such as, the FABPs (reviewed by Van Nieuwenhoven *et al.*, 1996; Veerkamp *et al.*, 1993), the non-specific LTPs (ns-LTP) (Akeroyd *et al.*, 1981), and plant LTPs (Bouillon *et al.*, 1987). Although the exact roles of these protein families have never been conclusively demonstrated, a wide range of functions have been postulated including, intracellular transport of fatty acids and lipophilic molecules (reviewed by Londraville, 1996), transfer of lipids between organelles (reviewed by Wirtz, 1991), lipid storage in seeds (Leprince *et al.*, 1998) and adaptation of plants to environmental stress (reviewed by Kader, 1996). Several

homologues of these proteins have now been identified in a range of free-living and animal- and plant-parasitic nematodes. However, many of the homologues in parasitic nematodes are distinct in that they have signal sequences and have been shown to be secreted. Consequently, these proteins may function differently in parasitic nematodes and may, in some cases, play a role in the host-parasite interaction.

1.4.2. Lipid and fatty acid binding proteins in parasitic helminths

A variety of lipid and fatty acid-binding proteins have been described in helminths (Table1.1) with an equally diverse range of functions ascribed to them. Each class is described briefly below.

1.4.2.1. Intracellular fatty acid-binding proteins (iFABP)

Intracellular FABP's are widespread throughout the animal kingdom. Homologues from a wide range of organisms have now been extensively studied and characterised as intracellular fatty acid- or retinoid-binding proteins (reviewed by Van Nieuwenhoven *et al.*, 1996; Veerkamp *et al.*, 1997). However, a distinct group of secreted homologues of the iFABPs have recently been discovered in nematodes (Mei *et al.*, 1997; Plenefisch *et al.*, 2000) and trematodes (Rodriguez-Perez *et al.*, 1992; Moser *et al.*, 1991).

a) Genetics

In *C. elegans,* the three genes encoding secreted FABPs are clustered together and arranged in the same orientation on Chromosome X separated by approximately 1kb of intragenic sequence (Plenefisch *et al.*, 2000). In contrast, the other, non-secreted, *C. elegans* iFABPs are scattered throughout the genome. Phylogenetic analyses indicate that nematode secreted FABPs form a distinct cluster of related genes within the FABP family with homologues in the trematodes *Schistosoma mansoni* (Moser *et al.,*1991) and *Fasciola hepatica* (Rodriguez-Perez *et al.,* 1992).

Table 1.1 Lipid/fatty acid-binding proteins in parasitic helminths

Family	Protein	Size (kDa)	Organism	Localisation	Proposed functions	References
FABP	Fh12 Sm14	14	*F. hepatica* *S. mansoni*	Surface-secreted Tubercles	Acquisition of host lipids	Rodriguez-Perez *et al.*, 1992 Moser *et al.*, 1991
FABP	As-p18	14	*A. suum*	Perivitelline fluid	Sequestration of toxic metabolic by products Alteration of eggshell permeability during hatching	Mei *et al.*,1997
PEBP	TES-26	26	*T. canis* *O. volvulus*	Surface-secreted	Acqusition of host lipids Segregation of lipids on cuticle surface	Gems *et al.*, 1995
Unknown	*M.expansa* LBP	7	*M. expansa*	Cytoplasmic	Acqusition of host lipids Sequestration of anthelmintics	Janssen & Barrett.,1995, Barrett *et al.*, 1997
NPA	DvA-1 ABA-1	14	*D. viviparus* *A. suum & others*	Secreted	Internal lipid transport Evasion of host immune responses	Kennedy *et al.*, 1995
FAR-1	Ov-FAR-1	19	*O. volvulus* and other animal-parasitic nematodes. *C. elegans*	Secreted	Internal lipid transport Acquisition of host retinol &/or lipids	Kennedy *et al.*, 1997

b) Structure

All FABPs are approximately 14kDa and are composed of ten anti-parallel β-sheets and two α-helices which form a clamshell-like structure with each 'shell' composed of 5 β-strands bordered by the α-helices. These shells enclose a large, hydrophilic, water-filled cavity in which fatty acids bind. The mouth of the binding cavity is lined with hydrophobic residues which are thought to stabilise the acyl chain when bound. Binding of fatty acid has been shown to be dependent upon a direct charge interaction between the carboxylate end of the fatty acid and the residues Arg 126 and Tyr 128 which are buried deep within the binding pocket of the FABP. The hydrocarbon chain, however, protrudes from the surface of the protein through a hydrophobic patch (Xu, Bernlohr & Banaszak, 1993).

c) Expression

One secreted FABP, As-p18, is expressed exclusively by embryonic and early larval stages of *A. suum* and secreted into the perivitelline fluid (Mei *et al.*,1997). Subsequent work has shown that this protein is also expressed in the perienteric fluid of adult worms and that three *C. elegans* homologues are secreted into the perivitelline and perienteric fluids from the hypodermis and body wall muscle respectively (Plenefisch *et al.*, 2000). Nothing is known about the spatial or temporal expression patterns of the intracellular proteins of this family in nematodes.

d) Function

The iFABPs bind a wide range of saturated and unsaturated long-chain fatty acids with micromolar affinity (reviewed by van Nieuwenhoven, 1996; Londraville, 1996). Some members of this family bind retinol and retinoic acid with high affinity but do not bind fatty acids. With the exception of liver FABP (L-FABP) which binds

two ligands per molecule, all FABPs tested to date bind their ligands with a 1:1 molar stoichiometry.

Although the FABP family of proteins are among the most extensively studied lipid-binding proteins, their precise function has never been conclusively demonstrated. A range of functions have been postulated for the intracellular FABPs including, intracellular long-chain fatty acid (LCFA) transport (Stewart *et al.*, 1991; Baier *et al*, 1996), intracellular buffering and modulation of enzyme activity and modulation of mitogenesis (Sorof, 1994; Khan & Sorof, 1994).

It has been proposed that the secreted FABP, As-p18, which is present in the perivitelline fluid, and its *C. elegans* homologues may function in the sequestration of metabolic by-products (such as toxic fatty acids and lipid peroxides) which may accumulate in the egg. An alternative function for these proteins in the alteration of the impermeability of the eggshell prior to hatching has also been suggested (Mei *et al.*, 1997). Roles in transport of lipid nutrients from the intestine to the body wall muscle have been suggested for the secreted *C. elegans* FABPs present in the perienteric fluid (Plenefisch *et al.*, 2000).

1.4.2.2. Phosphatidylethanolamine-binding proteins (PEBP)

The PEBP family of proteins have been described throughout eukaryotes from yeasts to humans. These proteins are generally intracellular with the exception of murine PEBP which is abundant in epididymal fluid. However, novel secreted homologues of these proteins have been described in the nematodes *Toxocara canis*, (TcSL-2), and *O. volvulus* (Ag16) (Gems *et al.*, 1995). These proteins have several features which distinguish them from PEBPs in other organisms.

a) Genetics

Protein sequence analysis indicates that the gene encoding TcSL-2 is a hybrid gene with domains of diverse ancestry (Gems *et al.*, 1995). TcSL-2 polypeptides consist of 263 amino acid residues, the first 20 of which encode a hydrophobic signal peptide to target secretion. The presence of a signal sequence is unique to the nematode PEBPs.

b) Structure

All conserved residues and motifs present in the PEBP homologues are also present in TcSL-2 including a previously proposed PE-binding sequence around residues 245-250 (YV(W/F)LVY), which is bordered by highly charged residues (H,R and E/K/R,Q) (Schoentgen *et al.*, 1987). However, TcSL-2 also has unique features, which set the nematode PEBPs aside from homologues in other organisms. These include a paired NC6 motif, containing 6 conserved cysteine residues, which is absent in all other PEBPs but present in other unrelated *T. canis* and *C. elegans* surface-localised proteins. This motif may be involved in protein-protein interactions specific to extracellular proteins (Blaxter, 1998). Another distinct feature of TcSL-2 is a 20 residue hydrophobic domain which separates the NC6 motifs from the PE-binding motif. It has been proposed that this region may allow membrane association. In all other homologues this region contains several highly charged residues making membrane interactions highly unlikely (Gems *et al.*, 1995).

c) Expression

TcSL-2 is expressed in a stage-specific manner, being present in abundance in the developmentally-arrested infective larvae of *T. canis* but absent in adult males. Immunofluorescence studies indicate that TcSL-2 is present on the anterior surface of the worms and is found in the E/S products (Gems et al., 1995). The *O. volvulus*

homologue, Ag16, has also been reported to secreted and present on the surface of the organism (Lobos *et al.*, 1990).

d) Function

All known PEBPs bind phosphatidylethanolamine with varying degrees of specificity (e.g., Schoentgen *et al.*, 1987; Jones & Hall, 1991; Gems *et al.*, 1995).

The surface-localisation of TcSL-2 and Ag16 and the fact that parasitic helminths cannot synthesise long-chain fatty acids and cholesterol *de novo* led to the proposal that these proteins play a role in uptake and transport of host lipids. The surface of nematodes is thought to be composed of non-diffusing, discontinuous lipid domains (Kennedy *et al.*, 1987; Proudfoot *et al.*, 1993) and it has been proposed that the secreted PEBPs could function in the segregation of lipids into these biologically distinct compartments on the nematode surface (Gems *et al.*, 1995).

1.4.2.3. Nematode polyprotein allergens (NPA)

This family of nematode lipid-binding proteins were first identified as potent allergens in *Ascaris suum* (Spence *et al.*, 1993) and have since been identified in EST datasets from *C. elegans*, tylenchid nematodes and a range of strongylid and filarial nematodes (Blaxter, 1998).

a) Genetics

NPAs are translated as large polypeptides which are subsequently cleaved into 15kDa monomers. These polyproteins are encoded by long genes consisting of multiple tandem repeats forming a head to tail array of units, each approximately 400 base pairs long which encode 15kDa monomers linked by endoproteinase cleavage sites. Depending on the species, each gene encodes between ten and twenty 15kDa units. In some cases these are comparatively monomorphic (Xia *et al.*, 2000; Moore *et al.*,1999) whereas in others extensive polymorphism is observed (Britton *et al.*, 1995). Six of the

ten *A. suum* NPA (ABA-1) monomers, for example, are identical at the amino acid level, three show slight differences and one has only 49% identity (Kennedy *et al.*, 1995a). In other species, e.g, *D. viviparus* and *C. elegans*, these units are highly diverged (Britton *et al.*, 1995; Blaxter, 1998). ABA-1, the NPA from *A. suum,* was shown to be encoded by a single copy gene by Southern blotting with genomic DNA (Moore *et al.*, 1999).

b) Structure

Before proteinase cleavage, the NPA polypeptides consist of between ten and twenty units each 120-130 amino acids in length, separated by arginine tetrads. These tetrads were shown to be tetrabasic, subtilisin-like proteinase cleavage sites. Kennedy *et. al.,*(1995a), reported that this arginine tetrad is completely removed in mature, parasite-processed ABA-1 protein. All the 15kDa mature NPA proteins contain around 15% glutamic and aspartic acid and around 10% lysine and arginine, have pI's ranging from 5.0 to 6.4 and are all negatively charged at physiological pH (A. Cooper & M.W. Kennedy, unpublished work). All NPAs have the conserved residues, Gln 20, Cys 64, Cys 120 and a single tryptophan residue, Trp 15. Site-directed mutagenesis and chemical modification of these residues showed that ligand binding was not affected by substitution of Gln20 or Trp 15 but chemical disruption of the disulphide bridge between Cys64 and Cys120 did alter the proteins function (McDermott *et al.,* 2001).

Computer-based predictions for secondary structure of the NPA peptides indicate a highly helical structure. Circular dichroism (CD) gives estimates of secondary structure content of 53% α-helix and 43% β-sheet for DvA-1L, a 15kDa unit of DvA-1 the *D. viviparus* NPA (Kennedy *et al.*, 1995b). For two divergent units of ABA-1, the same technique yielded estimates of 47% and 48% α-helix and 34% and 40% β-sheet respectively (Moore *et al.*, 1999). However, CD tends to overestimate β-

sheet content in helix-rich proteins. The values obtained for helix content are therefore compatible with the helical structure predicted by computer studies.

Spectrofluorimetric studies using fluorescent ligands indicate a highly apolar binding site in these proteins and suggest that ligands are completely enclosed within this site (Kennedy *et al.*, 1995a,b; Moore *et al.*, 1999).

Some evidence exists to suggest that the NPA units are formed as the result of an ancient sequence duplication. Internal homology beween different regions of units exists and differential scanning calorimetry (DSC) analysis of DvA-1L showed evidence of two discrete domains within the structure with two distinct phases of folding and refolding observed (Kennedy et al., 1995b).

Evidence for the dimerisation of NPA units has been found using DSC and gel filtration chromatography studies on parasite-derived ABA-1 (Kennedy et al., 1995a). Further evidence for the existence of NPA dimers was obtained from sedimentation equilibration studies and isothermal titration calorimetry on the same protein (McDermott, 2001).

c) Expression

Extensive database searches indicate that the NPAs are confined to nematodes where they are widespread and probably ubiquitous, occurring in a range of free-living and animal- and plant-parasitic species. In strongylid and filarial nematodes cultured *in vitro*, NPAs are secreted into the culture medium (Kennedy *et al.*, 1995b; Xia *et al.*, 2000) and in *A. suum*, ABA-1 is one of the most abundant proteins in the pseudocoelomic fluid. ABA-1 is also highly expressed in the reproductive tissues of *A. suum* (Xia *et al.*, 2000). Timanova *et al.*, (1999), isolated an *Ascaridia galli* NPA with no predicted N-glycosylation sites which was shown to occur predominantly in the cytosol, indicating that not all NPAs are secreted.

d) Function

NPAs have been shown to bind a range of saturated and unsaturated long chain fatty acids with similar affinities to the 14kDa FABP-type proteins and to have an even greater affinity for the retinoids, retinol (vitamin A) and retinoic acid (e.g, Xia *et al.*, 2000). Despite the sequence diversity between different NPA units, all NPA monomers tested to date bind the same range of ligands with similar affinities.

The pseudocoelomic localisation of ABA-1 led to the proposal that these proteins function as extracellular transporters of hydrophobic ligands in *Ascaris suum* (Kennedy *et al.*, 1995a). It has also been proposed that the secretion of DvA-1 from the surface of the animal parasite *D. viviparus* indicates roles in host nutrient acquisition or binding and inhibition of host immune mediators (Kennedy *et al.*, 1995b).

1.4.2.4. Fatty acid and retinol-binding proteins (FAR)

The identification of OV-FAR-1, a major antigen of the nematode, *Onchocerca volvulus*, led to the discovery of the FAR family of proteins (Tree *et al.*, 1995). These proteins have since been identified in a range of rhabditid, tylenchid, strongylid and filarial nematodes (Blaxter, 1998).

a) Genetics

The six known *C. elegans* FAR proteins are encoded by genes on chromosome II. Southern blotting indicates that the *O. volvulus* FAR protein, Ov-FAR-1, is encoded by a single copy gene (Tree *et al.*, 1995). RT-PCR experiments on *G. pallida* mRNA indicate that the nematode SL1 leader sequence is *trans*-spliced to the *gp-far-1* transcript *in vivo* and that the mRNA is expressed throughout the life-cycle.

b) Structure

In all homologues the FAR proteins consist of polypeptides of around 190 amino acids with a hydrophobic signal sequence at the N-terminal. Although there is

high sequence diversity between these proteins, all homologues share a predicted consensus casein kinase II phosphorylation site at a conserved position. None of the FAR proteins identified to date contain tryptophan residues. This is unusual since most retinol/fatty acid binding proteins, including the NPAs and serum albumin, have a tryptophan residue in or near their binding sites (Kennedy *et al.*, 1997). With the exception of two diverged *C. elegans* homologues, all known FAR proteins contain predicted N-linked glycosylation sites. Ov-FAR-1 and the *Acanthocheilonema vitae* FAR protein, Av-FAR-1, were shown to be glycosylated whereas the *Brugia malayi* homologue, Bm-FAR-1, is not (Nirmalan *et al.*, 1999).

Analysis of the far-UV CD spectrum for Ov-FAR-1 indicated a secondary structure content of 60% α-helix, and 32% β-sheet. However, secondary structure computer predictions on alignments of FAR-1 homologues indicate the presence of at least four amphipathic α-helices separated by short loops with no evidence of β-structure and a high probability of coiled coil in these proteins (Kennedy *et al.*, 1997).

c) Expression

Like the NPAs, the FAR proteins are widespread throughout, but apparently confined to nematodes. Immunolocalisation and *in situ* hybridisation studies on *O. volvulus* showed that Ov-FAR-1 is present in developing embryos, L3, L4, male and microfilarial stages. Ov-FAR-1 was also found in adult females where protein was detected in the excretory/secretory (E/S) products and mRNA in the hypodermis (Tree *et al.*, 1995). To date, the expression pattern of these proteins has not been studied in free-living nematodes.

d) Function

Kennedy *et al.,* (1997), proposed sequestration of host retinol, which is thought to be a dietary requirement of this parasite, and sequestration of inflammatory mediators

such as arachidonic acid as functions of Ov-FAR-1. Bradley *et al.*, (2001) suggest that retinol-binding by Ov-FAR-1 could be responsible for the skin and eye pathology caused by *O. volvulus* infection (as retinoids play a vital role in vision, tissue differentiation and collagen synthesis) and may also be involved in the characteristic formation of subcutaneous nodules in infected hosts. No functional information on the FAR proteins in *C. elegans* is available. However, the presence of these proteins in non-parasitic nematodes suggests a role in general nematode metabolism. Recently, cDNA encoding a homologue of the Ov-FAR-1 protein was isolated from a *G. pallida* cDNA expression library and the expression and functional characterisation of the recombinant protein, rGP-FAR-1, is reported here.

Chapter 2

General Materials and Methods

2.1 Biological Material

2.1.1 Nematodes

Cysts of *Globodera pallida* were grown on potato cv Désirée in pots, extracted and stored dry at room temperature (20 °C). To obtain second stage juveniles (J2s), cysts were soaked for 5 days in sterile, distilled water (SDW) and then transferred to potato root diffusate (PRD). PRD was obtained by the method of Fenwick (1949) from 10-week-old potato plants (cv Désirée) grown in sterilised loam pot cultures in a glasshouse. Diffusate was stored at 4 °C until required, when it was diluted 1 in 4 in SDW. Juveniles which hatched in this solution were removed within 48 hours of hatching and used immediately. Fungal and algal contamination of nematode material was minimised by rinsing the cysts thoroughly each day in SDW or PRD as appropriate.

2.1.2 Bacterial cultures

Plasmids were transformed into the maintenance strain *E. coli* XL-1 Blue or the expression strain *E. coli* BL21, using standard heat shock protocols (Sambrook *et al.*, 1989) and the cells grown overnight at 37°C on LB agar containing 100 μg ml^{-1} ampicillin to select for transformants.

To amplify plasmid stocks and prepare glycerol stocks for storage of clones, single transformant colonies were inoculated into 2ml LB broth containing 100μg ml^{-1} ampicillin and incubated overnight at 37 °C with shaking at 225-250rpm. To prepare glycerol stocks, 850μl of the overnight cultures were mixed with 150 μl of sterile 30% glycerol, vortexed and stored at -80 °C.

2.2 Protein expression and purification

2.2.1 Production of recombinant proteins

The pGEX GST fusion system (Pharmacia Biotech) was used for expression of all recombinant proteins. 500ml flasks containing 100ml 2XTY with 100μg ml^{-1} ampicillin (2XTYA) were inoculated with single transformant colonies and incubated overnight at 37°C with shaking to ensure adequate aeration. The cultures were then diluted 1:50 into 500ml 2XTYA in 1 litre flasks and grown to log-phase (A600 = 0.5-2.0) at 37 °C with shaking. Protein expression was induced by addition of isopropyl β-D-thiogalactopyranoside (IPTG) (Pharmacia Biotech) to a final concentration of 100μM and the cells incubated for a further 2-6 hours at 30°C.

2.2.2 Purification of recombinant proteins

Cells were pelleted by centrifugation at 7700g, frozen at -20°C overnight then resuspended in PBS containing Complete protease inhibitor cocktail (Boehringer Mannheim). Protein was released from the cells by sonication on ice at 6-12μ amplitude in 20-60 second bursts in an MSE 150W Ultrasonic Disintegrator fitted with a titanium probe with a 9.5mm tip. Triton X-100 (BDH) was added to a final concentration of 1% to aid solubility of the fusion protein. Insoluble cell debris was removed by centrifugation at 9400g for 10 minutes at 4°C. Fusion proteins were affinity-purified from total bacterial protein by binding to glutathione-sepharose 4B (GSH) slurry (Pharmacia Biotech) then liberated from the GST moiety by overnight incubation with 50 units of thrombin per ml of resin at room temperature.

2.2.3 Protein Quantification

Samples of expressed proteins in LDS sample buffer were run alongside molecular weight markers (BioRad) on 4-12% Bis-Tris polyacrylamide gels (Novex) at 150-200V in MES or MOPS buffer in order to confirm the presence and purity of protein. Gels were immersed in Coomassie stain (0.25% Coomassie Brilliant Blue R250, 10% acetic acid, 50% methanol) for 40 minutes at room temperature then destained (10% acetic acid, 50% methanol) to remove background staining.

Protein yield was quantified by spectrophotometry using predicted extinction co-efficients at 280nm (ε_{280}), based on the amino acid composition of the recombinant proteins, generated using the ProtParam program on the ExPasy server (http://www.expasy.ch/tools/protparam.html). The absorbance of purified protein was measured at 280nm in quartz cuvettes with a 1cm path length and the concentration of protein calculated using the Beer Lambert Law:

$$A = \varepsilon cl$$

Where A = absorbance at 280nm, ε = predicted extinction co-efficient at 280nm ($M^{-1}cm^{-1}$), c = concentration of protein (M), l = path length (cm)

2.2.4 Detergent Removal Chromatography

After purification, residual detergent in the protein samples was removed using 2ml Extracti-gel D dedetergent columns (Pierce). Columns were washed and equilibrated in 2 column volumes of PBS then 200-500 µl of protein sample was applied to the column and washed through by addition of 500µl aliquots of PBS. Fractions were collected and the fractions containing protein were detected by measuring absorbance at 280nm.

2.2.5 Protein Dialysis

Proteins were dialysed using 10000MW cutoff Slide-a-Lyzer dialysis cassettes (Pierce). Dialysis cassettes were pre-wetted by immersion in the desired buffer for 30 seconds, then 0.5 – 1ml of protein sample was injected into the cassette. The cassettes were then immersed in 500-1000ml of the desired buffer and stirred slowly for 24 hours at 4°C with at least 4 buffer changes.

2.2.6 Concentration of protein samples

Protein samples were concentrated using Microcon centrifugal concentrators (Amicon). Sample was applied to the concentrator then centrifuged at 14 000g until the desired volume was reached.

2.3 Spectrofluorimetry

2.3.1 Binding of Fluorescent Ligands

All fluorescence experiments were carried out in PBS on a SPEX FluorMax spectrofluorimeter (Spex Industries, Edison, NJ) (and appropriate software) with 2ml samples in silica cuvettes. PBS blanks were used to correct for Raman and background scattering.

The ligand binding capacity of recombinant Gp-FAR-1 was investigated using the fluorescent ligands 11-((5-dimethylaminonaphthalene-1-sulfonyl)amino)-undecanoic acid (DAUDA), dansyl-DL-α-aminocaprylic acid (DACA), retinol, cis- and trans-parinaric acid and dehydroergosterol. Binding of these ligands was measured by a shift in emission wavelength and an increase in fluorescence emission intensity upon addition of protein to the ligand. To investigate retinol binding, the procedure required modification due to the inherent instability of retinol in aqueous solution, so freshly

prepared retinol dilutions were added to solutions containing protein. Binding of 5-(dimethylamino)naphthalene-1-sulphonamide (dansylamide) was also tested in order to investigate the effect of the fluorescent moiety of DAUDA and DACA on ligand binding.

The fluorescent compounds dansylamide, DAUDA, DACA, cis-parinaric acid and dehydroergosterol were prepared as 10mM stock solutions in ethanol and stored under argon at –20 °C in the dark . DAUDA, DACA, dansylamide and dehydroergosterol were diluted a thousand-fold in PBS for use in the assays, while *cis*-parinaric acid was diluted two thousand-fold in PBS. Retinol solution was freshly prepared at a concentration of 10mM in ethanol and was diluted ten-fold in PBS before use.

Excitation wavelengths and emission spectra for the fluorescent compounds tested are shown in Table 2.1.

Fluorescent compounds	**Excitation wavelength (nm)**	**Emission spectrum (nm)**
DAUDA DACA dansylamide	345	360-675
Retinol	350	360-675
cis-parinaric acid	319	370-570
Dehydroergosterol	325	350-450

Table 2.1. Excitation wavelengths and emission spectra of fluorescent compounds tested.

2.3.2 Binding of Non-fluorescent Ligands

Binding of non-fluorescent ligands was assayed by measuring the gradual reversal of the wavelength shift and decrease in fluorescence emission intensity upon successive 10µl additions of thousand-, hundred- and ten-fold dilutions of ligand to a DAUDA/protein complex.

Stock solutions of all the non-fluorescent competitors were made to a concentration of approximately 10mM in ethanol, then diluted ten-, hundred- and thousand-fold in PBS for use in the assays. Stock solutions were stored at -20 °C in the dark. All fluorescence experiments were also carried out using ribonuclease A as a control.

2.3.3 Dissociation curve titrations

rGp-FAR-1, *cis*-parinaric acid, DAUDA and retinol were quantified spectrophotometrically by measuring absorbance at 280nm, 303nm, 335nm and 325nm respectively. The concentration of each was calculated using predicted extinction co-efficients of $2560M^{-1}cm^{-1}$, $76000\ M^{-1}cm^{-1}$, $4400M^{-1}cm^{-1}$ and $52480M^{-1}cm^{-1}$ respectively.

To calculate the apparent dissociation constants (K_d) and apparent molar ratio (n) of DAUDA:rGp-FAR-1 complexes, a solution containing 111μM rGp-FAR-1 was added, in 10μl increments, to 2ml of a 9.6μM solution of DAUDA in PBS in quartz cuvettes, and the fluorescence emission at 482nm measured after each addition. The corrected data thus obtained were analysed using Microcal ORIGIN software, adapted for use in these experiments, using standard non-linear regression techniques in a simple non-competitive binding model:

$F_{max}/F = 1+K_d/(C_L-nC_pF/F_{max})$,

where F_{max}= maximal fluorescence intensity, F=observed fluorescence intensity, K_d = dissociation constant of rGp-FAR-1:DAUDA complexes, C_L = total DAUDA concentration, n = apparent molar ratio of rGp-FAR-1:DAUDA complexes at saturation (or number of DAUDA molecules bound per rGp-FAR-1 molecule) and C_p= total rGp-FAR-1 concentration. Similar experiments were also carried out to estimate the K_d for retinol and *cis*-parinaric acid and the data analysed as above.

2.4 Molecular Biology

2.4.1 Plasmid preparation

pGEX-2T plasmid, encoding Gp-FAR-1 fused to a 26kD N-terminal moiety of *Schistosoma japonicum* GST, and separated by a thrombin cleavage site, was purified from overnight cultures using a Wizard *Plus* MiniPreps DNA Purification Kit (Promega). 2ml of cells were centrifuged at 10,000g in a microcentrifuge and cell pellets were resuspended in 50mM Tris-HCl pH 7.5, 10mM EDTA containing 100μg ml^{-1} RNase A. Cells were then lysed by addition of 0.1M NaOH, 0.5% SDS to release DNA and the solution was neutralised with 0.88M KOAc (pH 4.8). Samples were centrifuged at 10,000g for 5 minutes to pellet cell debris. The supernatants were then added to 1ml of Wizard Minipreps DNA purification resin, to which plasmid DNA binds and the supernatant liquid was removed using a vacuum manifold. The resin was then washed with 2ml 80mM KOAc, 8.3mM Tris-HCl pH 7.5, 40μM EDTA in 55% ethanol. Bound plasmid DNA was eluted in 50μl of sterile, distilled water. The presence and purity of purified recombinant plasmid was confirmed by standard ethidium bromide agarose gel electrophoresis of samples (Sambrook *et al.*, 1989) alongside DNA molecular weight markers (Promega) and pGEX-2T plasmid containing no insert. Purified plasmid stocks were stored at 4°C.

2.4.2 DNA Sequencing

Purified plasmids were sequenced using an ABI PRISM Dye Primer Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems). 20ng sequencing primer and 4μl of Big Dye ReadyReaction mix were added to 200ng of purified plasmid. Samples were then run on a Perkin-Elmer 2400 PCR machine for 25 cycles under the following

conditions: 1°C/ sec ramp to 96 °C then 96 °C for 10 seconds, 1°C/ sec ramp to 50 °C then 50 °C for 5 seconds, 1°C/ sec ramp to 60 °C then 60 °C for 4 minutes. Samples were stored at 4°C until required.

1µl of 3M NaOAc (pH 4.6) and 25µl of absolute ethanol were added to the reactions then vortexed and incubated on ice for exactly 10 minutes to precipitate the extension products. Samples were spun at top speed in a microcentrifuge for 30 minutes and the supernatants aspirated. The pellets were then washed in 125µl 70% ethanol to remove residual salts and dye terminators and centrifuged as above for a further 5 minutes. After aspiration of the supernatant, the pellets were dried for 5 minutes at 65 °C. Sequencing reactions were then run on an ABI 377 Stretch Automatic Sequencer and sequences analysed using ABI Sequence Navigator software.

2.4.3 Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) was used to screen colonies to identify those containing insert of the appropriate size. PCR reactions were set up containing 1x Taq buffer, 1mM $MgCl_2$, 20µM dNTP's, 1µM forward and reverse primer, varying amounts of template and 1 unit Taq DNA polymerase (Promega). 30 cycles of synthesis were used with annealing temperature between 45 and 55°C depending on the primer combination used. 10µl of PCR reactions were run on ethidium bromide agarose gels in TBE buffer using standard protocols (Sambrook *et al.*, 1989).

2.5 General Reagents

2.5.1. Bacterial Growth Media

LB medium

10g bacto-tryptone

5g bacto-yeast extract
10g NaCl

to 900ml with distilled water

2XTY medium

16g tryptone
10g yeast extract
5g NaCl

to 900ml with distilled water.

The pH of the above media were adjusted to 7.0 by addition of 5M NaOH and the volumes made up to 1 litre with distilled water.

To prepare solid growth media, 15g agar was added to the above media prior to autoclaving.

All growth media were sterilised by autoclaving at 121°C for 15 minutes.

Where required, ampicillin was aseptically added to growth media after autoclaving to a final concentration of 100μg ml^{-1}.

2.5.2 Buffers & Stains

10X PBS

80.0g NaCl
29.0g KCl
2.0g $NaH_2PO_4.12H_2O$
2.0g KH_2PO_4 (pH 7.3)

to 1 litre with distilled water.

10X TBE

108.0gTris-HCl
55.0g Orthoboric acid
9.3g EDTA

to 1 litre with distilled water.

20X MOPS SDS running buffer (pH 7.7)

104.6g 3-(N-morpholino) propane sulphonic acid (MOPS)
60.6g Tris
10.0g sodium dodecyl sulphate (SDS)

3.0g ethylenediaminetetraacetic acid (EDTA)

to 500ml with distilled water

20X MES SDS running buffer (pH 7.3)

97.6g 2-(N-morpholino) ethane sulphonic acid
60.6g Tris
10.0g SDS
3.0g EDTA

to 500ml with distilled water

LDS sample buffer (pH 8.5)

4.00g glycerol
0.682g Tris Base
0.666g Tris HCl
0.800g lithium dodecyl sulphate (LDS)
0.006g EDTA
0.75ml of 1% Serva Blue G250 solution
0.25ml of 1% Phenol Red solution

to 10ml with distilled water

Coomassie stain

50% methanol (BDH), 10% acetic acid (BDH), 0.25% Coomassie blue R-250 (Sigma)

Destain

50% methanol, 10% acetic acid

Chapter 3

Expression Pattern of Lipid-binding Proteins in PCN

3.1 Introduction

3.1.1. Cuticle morphology

As well as functioning as an exoskeleton which maintains and defines body shape the cuticle of parasitic nematodes and its associated secretions represent the interface between the nematode and its host and, as a result, are postulated to play an important role in the host-parasite interaction.

All nematode cuticles share a common overall architecture whereby the cuticle, which is secreted by the underlying hypodermis, covers the surface of the nematode and invaginates at the mouth, amphids, secretory/excretory pore, vulva, cloaca and rectum (Bird, 1971). Electron microscopic observations show the cuticle to be composed of three distinct layers. The basal zone or endocuticle is seen as a layer of uniformly spaced lines described as "rods, canals, stripes and striations" (Bird, 1971), which are thought to be composed of closely linked collagen-like proteins which bestow resistant, protective properties on the cuticle. As mutations in collagen genes result in grossly altered body form, these proteins are also thought to be important in defining body shape (Johnstone, 1994). The median zone or mesocuticle varies between species and can be seen as a fluid-filled layer which may contain struts, rods and/or globular bodies and in other cases may be indistinguishable from the basal zone (Bird, 1971). The cortex or exocuticle is composed of several layers. The inner cortical layer, which appears to be fibrous in nature is thought to contain extensively cross-linked non-collagenous proteins. This layer is covered by a thinner layer, the epicuticle, the trilaminate appearance of which led to the proposal that it was a highly modified plasma membrane, although this is now

known not to be the case. It is now thought that the epicuticle is composed of a lipid-containing layer with surface-associated proteins and glycoproteins.

Evidence for the presence of lipids in the epicuticle came from radio-iodination studies, which indicated the presence of phospholipids and non-polar lipids in the epicuticles of the animal parasites *S. mansoni, A. viteae, Litomosoides carinii* and *A. suum* (Proudfoot *et al.,* 1991), and a glycolipid on the surface of *Dirofilaria immitis* (Scott *et al.,* 1988). Kennedy *et al.* (1987), investigated the lipidic nature of the epicuticles of *Trichinella spiralis* and *Toxocara canis* by the insertion of fluorescent lipid probes into this structure, and found insertion to be highly selective for the type of lipid tested. The lateral mobility of these probes was shown to be severely restricted, reinforcing the finding that the epicuticle does not exhibit the classical fluid properties of plasma membranes. Freeze-fracture studies of the epicuticle of *T. spiralis* showed a ribbed, particulate appearance similar to hexagonal phase lipidic particles which, it is thought, may explain the observed immobility of surface lipids and the ability of the epicuticle to exclude certain lipophilic molecules (Kennedy *et al.,* 1987). It has been postulated that these properties could serve to protect the nematode from host immune attack by excluding membrane-disrupting agents such as fatty acids and complement factors.

Nematode epicuticles, therefore, appear to be composed of a rigid, resistant lipid layer which would be expected to be relatively impermeable to hydrophilic substances. However, it is has been shown that transcuticular uptake of nutrients does occur in some nematodes and this has been taken to indicate that the otherwise impermeable epicuticle must contain factors involved in the uptake of these nutrients (Howells & Chen, 1981).

3.1.2 Cuticular secretions

It was originally thought that nematode cuticles were inert, non-immunogenic surfaces permitting immune evasion and thus aiding survival within the host. However, it has since been shown that this structure is a dynamic, highly antigenic surface which is constantly shedding and replacing material. Material shed through the cuticle forms the surface coat, a glycocalyx-like layer covering the epicuticle, seen in the juvenile stages of most parasitic and free-living nematodes. This shedding and replacement has been shown to be an extremely rapid phenomenon; Carlow *et al.* (1987) showed that an IgM monoclonal antibody against surface components of *Brugia malayi* was shed from the surface within minutes and Maizels *et al.* (1984) showed that *T. canis* 2nd stage larvae shed approximately 25% of labelled surface components within one hour *in vitro*. Endo & Wyss (1992) observed fibrillar cuticular exudates along the surface of *Heterodera schachtii* after the onset of feeding and showed that these contained saturated, even-numbered long chain fatty acids and calcium salts. These were proposed to be constituents of the sub-crystalline layer first described by Schmidt in 1871 (cited in Endo & Wyss, 1992). Similar fibrillar exudates, originating from the median zone of the cuticle, were observed around the lip region of *G. rostochiensis* in potato roots and Forrest *et al.* (1989) proposed that these may anchor the nematode at its feeding site and may be formed by an interaction of plant and nematode components. Fibrillar exudates on the cuticle surface of feeding *H. glycines* were reported to originate from the hypodermis, being secreted into a "secretion accumulation zone" at the base of the endocuticle then extruded onto the cuticle surface (Endo, 1993). These exudations were proposed to be important in feeding and in masking the nematode from host defences.

Most work on plant-parasitic nematode cuticles to date has focussed on morphological observations and, as a result, little is known about the biochemical composition of these structures. Many animal-parasitic nematodes are known to have antioxidant enzymes on their surface and these are thought to function in defence of these parasites. For example, Cookson *et al.* (1992) identified the major cuticular protein of lymphatic filarial parasites, gp29, as a secreted glutathione peroxidase. Plant-parasitic nematodes are also known to express many antioxidant enzymes (Molinari & Miacola, 1997) although it is not known whether these enzymes are secreted. However, recently, a cDNA encoding the enzyme thioredoxin peroxidase was isolated from a *G. rostochiensis* cDNA expression library and immunolocalisation studies, using antisera raised against the cloned expressed protein, indicate that this protein is surface-localised and rapidly shed from the cuticle (Robertson, *et al.*, 2000). It has been proposed that this protein plays a role in evasion of plant defences by breaking down the hydrogen peroxide produced as part of the hypersensitive reaction, an important component of plant defences. Other work (see Section 1.6) has shown that lipid-binding proteins may be present in the secretions or at the surface of parasitic nematodes (Tree *et al.*, 1995, Mei *et al.*, 1997, Kennedy *et al.*, 1995b).

In previous work, three genes encoding putative lipid-binding proteins were identified in PCN. Analysis of a *G.rostochiensis* EST dataset identified two genes encoding homologs of the nematode NPA and secreted FABP proteins, and a homolog of the nematode FAR-1 proteins was isolated by screening of a *G.pallida* cDNA library with a polyclonal antiserum raised against whole *G.pallida* J2s. This chapter describes the patterns of localisation of these proteins in PCN.

3.2. Materials and Methods

3.2.1. In-situ hybridisation of PCN with DIG-labelled antisense DNA probes

3.2.1.1. Preparation of DIG-labelled antisense DNA probes

Primers (Genosys) were designed to amplify 200 - 250bp fragments of genes encoding, PCN FAR-1, PCN NPA and PCN FABP from EST plasmid clones. PCR reactions containing, 50-100ng template DNA, 1.5mM $MgCl_2$, 0.2mM dNTPs, 1μM forward and reverse primers and 1unit Taq polymerase (Promega) in 1xTaq buffer (Promega), were run on a Perkin Elmer 9700 thermal cycler under the following conditions: 2 minutes at 94°C for 1 cycle, 30 seconds at 94°C, 30 seconds at 55°C, 30 seconds at 72°C for 30 cycles then 7 minutes at 72°C for 1 cycle. Reaction products were stored at -20°C until required. Aliquots of the PCR reactions were electrophoresed on an ethidium bromide-stained agarose gel alongside a 100bp ladder marker (Promega) to confirm specific amplification.

To synthesise DIG-labelled DNA probes, asymmetric PCR reactions containing approximately 17ng PCR product, 1.5mM $MgCl_2$, 4μM antisense (reverse) primer, 26μM digoxigenin 11 dUTP (DIG-dUTP), 49μM dTTP, 75μM dATP/dCTP/dGTP, 1unit Taq polymerase in 1xTaq buffer (Promega) were run as follows: 2 minutes at 94°C for 1 cycle, 30 seconds at 94°C, 30 seconds at 55°C, 90 seconds at 72°C for 30 cycles then 4 minutes at 72°C for 1 cycle. Reactions were stored at -20°C until required. Probe synthesis and DIG incorporation was confirmed by ethidium bromide agarose gel electrophoresis of aliquots of template synthesis and DIG-labelling reaction products alongside a 100bp ladder marker

(Promega). Confirmation of DIG-labelling was observed as a band shift, indicating an increase in the molecular weight of the probes.

3.2.1.2. In-situ labelling of nematodes

All solutions in this study were prepared with DEPC-treated, distilled water and all glassware was baked at 160°C for 6-12 hours to minimise ribonuclease contamination.

Freshly-hatched *G. rostochiensis* or *G. pallida* J2s were cleaned by flotation on 35% sucrose after centriguation at 2000 rpm for 5 minutes, then resuspended in 2% paraformaldehyde in M9 buffer and incubated at 4°C for at least 18 hours. Fixed J2s were pelleted at 8000rpm for 40s, resuspended in 0.2% paraformaldehyde in M9 buffer then pipetted onto glass slides. A razor blade attached to a vibrating aquarium pump was drawn over the nematodes until most of the worms were cut into 2-3 pieces, as determined by light microscopy. Cut nematodes were washed twice in M9 buffer to remove residual fixative then incubated in 0.5mgml^{-1} proteinase K (Roche Diagnostics) in M9 buffer for 30minutes at 22°C on a rotator. Samples were washed in M9 buffer then the nematode pellets were incubated for 15 minutes on deep frozen ice. Pellets were resuspended in -20°C methanol and incubated for 30 seconds on deep frozen ice, then spun and resuspended in -20°C acetone and incubated on deep frozen ice for 1 minute. The samples were rehydrated by the dropwise addition of an equal volume of DEPC-treated water to the acetone. Samples were pelleted and washed in hybridisation buffer to remove residual acetone, then resuspended in 150µl of fresh hybridisation buffer and prehybridised for 15 minutes at 50°C in a hybridisation oven. DIG-labelled DNA probes were heat denatured by incubating for 10 minutes at 95°C in a heating block and snap-cooled on wet ice. Up to 10µl of the probes were then added to the samples and hybridised overnight at 50°C. Hybridised samples were washed three times for 15 minutes in 4xSSC at 50°C, then three times for 20 minutes in 0.1xSSC, 0.1% SDS at 50°C to remove unbound probe. An anti-

DIG antibody was used to detect bound probe. Samples were blocked for 30 minutes at room temperature in TNB buffer (0.1M Tris pH 7.5, 0.15M NaCl, 0.5% Blocking reagent (Roche Diagnostics) then incubated in TNB buffer containing a hundred-fold dilution of anti-DIG POD conjugate (Roche Diagnostics) for 2 hours at room temperature. After three 15 minute washes in PBS, samples were incubated in DNP amplification working solution (NEN) for 15 minutes at room temperature then washed as before. The samples were incubated overnight at 4°C in TNB buffer containing a hundred-fold dilution of anti-DNP alkaline phosphatase conjugate, then washed in PBS three times for 15 minutes to remove excess antibody. The nematode pellets were then incubated in alkaline phosphate detection buffer (0.1M NaCl, 0.1M Tris, 50mM $MgCl_2.6H_2O$, (pH 9.5)) containing 496μgml^{-1} NBT and 187 μgml^{-1} X-phosphate (Roche Diagnostics) until colour development was observed. The staining reaction was stopped by washing the samples twice in 0.1% Tween-20 (Sigma). Stained samples were mounted on glass slides under coverslips sealed with nail varnish. Slides were observed under a binocular light microscope with attached camera at magnificatons of 10X, 20X and 40X.

3.2.2 Western blotting

Protein samples and nematode homogenates in lithium dodecyl sulphate (LDS) sample buffer were run alongside prestained Kaleidoscope markers (BioRad) on 4-12% Bis-Tris acrylamide gels as described in Chapter 2. Nitrocellulose membrane (Hybond) was laid over the gel and both were sandwiched between 6 pieces of 3M filter paper (Whatman). The filter paper and nitrocellulose were presoaked in transfer buffer before use. Gels were then electroblotted in a BioRad blotting apparatus at 100V for 45-60 minutes. Blots were washed three times for 5 minutes in PBS containing 0.1% Tween 20 (Sigma) (PBST) then blocked for 1 hour at room temperature in PBST containing 5%

skimmed milk powder (Marvel) (MPBST). The blots were then incubated for 1-3 hours in MPBST containing a thousand-fold dilution of a polyclonal antiserum raised against Gp-FAR-1. After a further three washes in PBST, blots were incubated for 1 hour in MPBST containing a 1/10,000 dilution of anti-rabbit IgG:alkaline phosphatase conjugate (Sigma). After washing, the blots were then soaked in 5 ml of alkaline phosphate detection buffer (0.1M NaCl, 0.1M Tris, 50mM $MgCl_2.6H_2O$, (pH 9.5)) containing 496μgml^{-1} NBT and 187 μgml^{-1} X-phosphate (Roche Diagnostics) until colour development was complete. Development reactions were terminated by washing blots in distilled water.

3.2.3 Indirect immunofluorescence

Hatched *G. pallida* J2s were collected by centrifugation at 3000rpm for 10 minutes in a benchtop microcentrifuge. Nematode pellets were then cleaned by sucrose flotation then washed three times and resuspended in 1ml PBS. Nematodes were then incubated for 1-2 hours at room temperature in a 1/50 dilution of either anti-Gp-FAR-1 polyclonal serum or, as a negative control, pre-immune rabbit serum. Samples were washed three times in PBS to remove unbound antibody then incubated for 1 hour in a 1/100 dilution of anti-rabbit IgG-Cy3 conjugate (Sigma). Nematodes were washed three times in PBS to remove unbound secondary antibody. Negative controls, where worms were incubated in secondary antibody alone, were also used. 50µl aliquots of the samples were then placed on microscope slides and observed under UV light with an Olympus fluorescence microscope at 400x magnification.

3.3 Results and Discussion

Because of the endoparasitic life-cycle of PCN and lack of *in-vitro* culture methods it is difficult to obtain sufficient quantities of life-cycle stages other than J2s for

experimental analysis. As a result, only J2s were studied in all of the following experiments.

3.3.1. NPA Expression in PCN

In-situ hybridisation (ISH) was used to determine the spatial expression pattern of the PCN NPA. As shown in Figures 3.1a & b, some hybridisation was seen in the gut of the nematodes but the strongest hybridisation was observed in the hypodermis (Figs. 3.1c & d). This agrees well with patterns of localisation of the NPAs previously identified in other parasitic nematodes. Kennedy *et al.*, (1995a) showed by Northern Blotting that NPA mRNA was present in the gut and also in the body wall of *Ascaris suum* while studies in *C. elegans* showed that the promoter controlling NPA expression was active only in the gut (Kennedy, 2000). The NPAs were originally identified in animal-parasitic nematodes as major allergens in nematode infections, indicating a secreted or surface localisation, and this finding was reinforced by the observed secretion of these proteins into culture medium *in vitro* in several animal-parasitic nematode species (Kennedy & Qureshi, 1986, McReynolds *et al.*, 1993, Kennedy *et al.*, 1995a, Kennedy *et al.*, 1995b, Kennedy *et al.*, 1995c). NPAs have been localised in the hypodermis of the animal parasites *Dirofilaria immitis* (Culpepper *et al.*,1992, Poole *et al.*,1992), *Brugia pahangi* and *B.malayi*, and in the latter two species protein was also found in the cuticle, further supporting the hypothesis of the extrusion of these proteins from the hypodermis onto the surface of the cuticle (Tweedie *et al.*, 1993).

Taken together, these results could indicate that in non-parasitic species such as *C. elegans* the NPAs are expressed only in the gut whereas the NPAs in parasitic species have evolved a secondary function on the surface or exterior of the nematode.

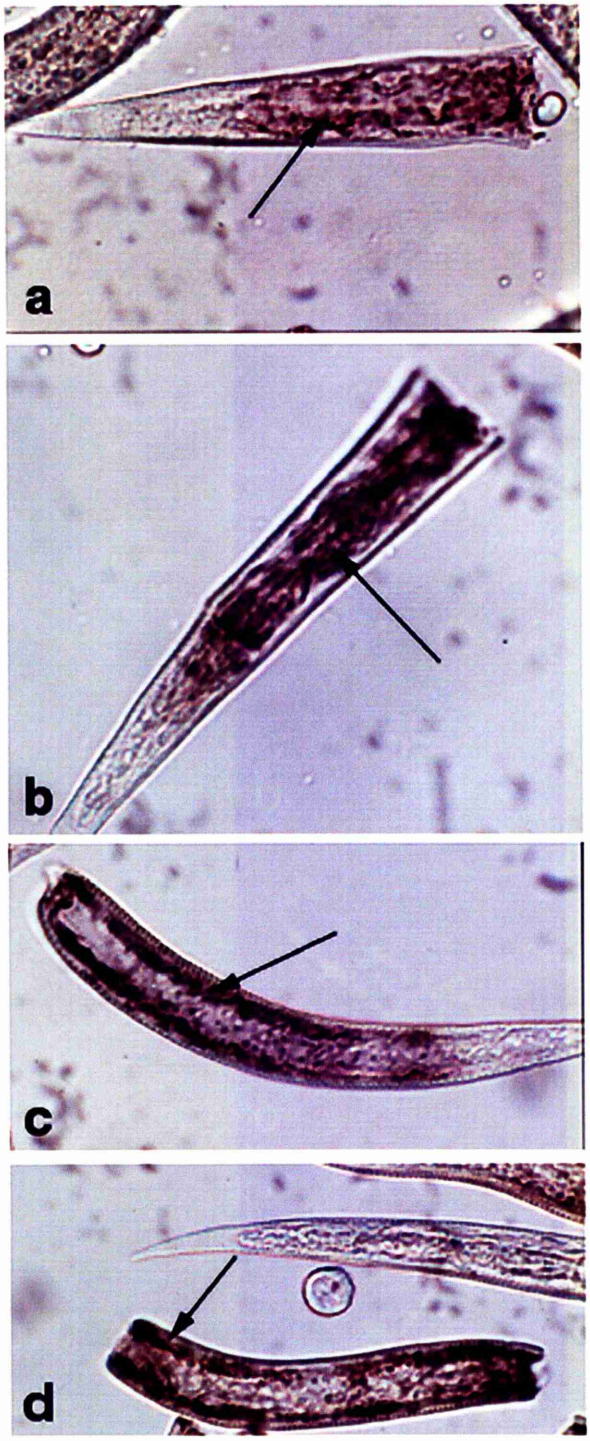


Figure 3.1. *In-situ* hybridisation of an antisense DIG-labelled NPA probe to fragments of PCN J2s. A positive reaction is indicated by a purple-brown deposit (arrows). Staining was observed in the gut (a & b) and the hypodermis (c & d). A complementary sense probe showed no staining (not shown).

Expression of NPAs in the gut may indicate a primary role for these proteins in endogenous nutrient (lipid) transport from the gut to sites of storage or utilisation (e.g., Kennedy *et al.*, 1995a). It has also been hypothesised that the secreted cuticular NPAs in animal-parasitic species may function in transcuticular nutrient acquisition from host tissues (Tweedie *et al.*, 1993, Poole *et al.*, 1992). It is perhaps unlikely that this function applies in PCN because, unlike most animal-parasitic nematodes which are bathed in nutrients in host tissues, the stylet is the only part of the nematode in contact with the feeding site, the nutrient source in this host-parasite interaction. Cuticular NPAs have also been postulated to play a role in protection of nematodes from lipid-based host immune reactions (Kennedy *et al.*, 1995b). This could also apply to endoparasitic plant-parasitic nematodes such as PCN because, as described in more detail in Chapter 4, plant and animal immune reactions, particularly lipid-based pathways, are very similar and lipid-based defence reactions are amongst the first defences plant-parasitic nematodes encounter upon invasion of a host (Farmer, 1988; Baker & Orlandi, 1995; Creelman & Mullet, 1997). However, in the absence of detailed information on NPA protein localisation in *C. elegans* or other non-parasitic nematodes makes it difficult to reach firm conclusions on the relevance of these observations in parasitic forms.

Interestingly, an NPA recently identified in the chicken parasite *Ascaridia galli* was reported to be exclusively cytosolic (Timanova *et al.*, 1999) and an *A. suum* NPA protein was also found to be abundantly expressed in reproductive tissue (Timanova *et al.*, 1999). If correct, these results indicate that different expression patterns may exist within parasitic nematode species and, could therefore indicate the evolutionary adaptation of these proteins to the different habitats (or hosts) and life-cycles of the individual parasitic species.

Other work in this thesis has revealed that these nematode-specific NPAs share striking structural similarities to insect and vertebrate exchangeable apolipoproteins which function in extracellular mass lipid transport. The results of work undertaken to investigate whether the NPAs have similar functional properties to these proteins are presented and discussed in Chapter 6.

3.3.2. FABP Expression in PCN

The spatial expression pattern of the PCN FABP was also examined. Like other nematode FABPs, this protein is predicted to have an N-terminal signal sequence implying that this protein is secreted. When ISH was performed with a probe from the PCN FABP, staining was observed in the genital primordia (Fig 3.2a). Some FABPs have previously been shown to be present in reproductive tissues in other nematodes. Asp-18 mRNA (derived from the *A.suum* FABP homolog) was found to be present in the ovaries of adult females of *Ascaris suum*, although the presence of the protein could not be demonstrated in these tissues by immunoblotting (Mei *et al.,* 1997).

As shown in Figure 3.2b, the PCN FABP probe also hybridised to mRNA in the hypodermis. Expression of *C. elegans* FABP *lbp*-1 mRNA has been shown to occur in the hypodermal precursor cells in developing embryos with the protein being apically secreted into the perivitelline fluid before the cuticle is deposited. However, this expression ceased after hatching and was not observed in later stages (Plenefisch *et al.*, 2000). Surface-secreted FABPs, albeit without leader sequences, are also known to be present in the trematodes *S. mansoni* (Moser *et al.*, 1991) and *F. hepatica* (Rodriguez-Perez *et al.*, 1992) although no information is available on the route of secretion of these proteins. The hypodermal localisation of the PCN *fabp* mRNA, therefore, could indicate that the protein is secreted onto the nematode surface. This has not been previously reported for the

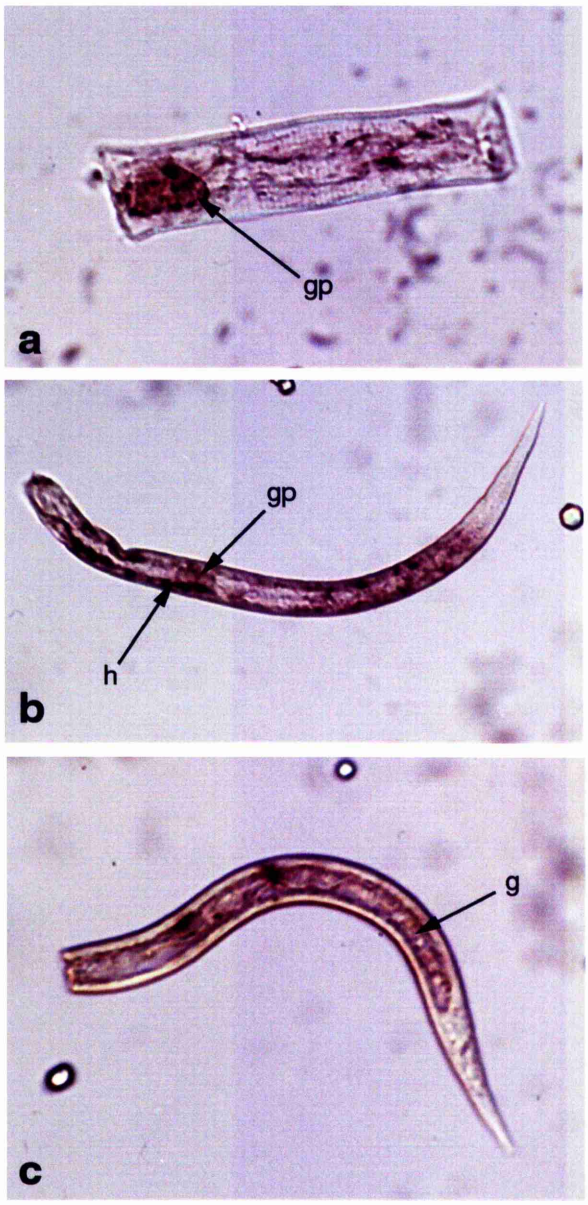


Figure 3.2. *In-situ* hybridisation of an antisense DIG-labelled FABP probe to fragments of PCN J2s. A positive reaction is indicated by a purple-brown deposit (arrows). Staining was observed in; a) genital primordia (gp); b) hypodermis (h); and c) gut (g). Sense probes showed no observable staining (not shown).

secreted FABPs in hatched nematodes and so may indicate a novel expression pattern in PCN.

In *C.elegans*, expression of two secreted FABP genes, *lbp-2 and lbp-3,* occurred in posterior body wall muscle cells abutting the pseudocoelom and it was suggested that these proteins are secreted into the perienteric fluid. In *A. suum*, Asp-18, or a closely related protein, was also found in the perienteric fluid (Plenefisch *et al.*, 2000). Although no expression in muscle tissue was observed in PCN, staining was observed in the gut (Figure 3.2c). The absence of staining of muscle cells may be attributable to the fact that a single gene was used in these experiments. PCN may have other genes encoding FABPs, as is known to be the case in *C. elegans* and other nematodes, and these genes may be expressed in the muscle tissue. Alternatively, PCN FABP may not be expressed in muscles but in the hypodermis implying a different functional role. This is the case for another group of LBPs, the FAR proteins (see below). A further interpretation of our results also needs to be considered. As most organisms contain several distinct FABP genes, it is feasible that the probe used for our ISH reactions does not distinguish between different PCN FABP mRNA. The expression patterns observed may therefore represent a hybrid of more than one PCN FABP gene.

The classical intracellular FABPs are thought to function in the cytosolic transport of fatty acids and also in the protection of these labile molecules from oxidation. Less is known about the function of the nematode secreted FABPs. Perivitelline fluid-localised Asp-18 has been proposed to function in maintenance of the impermeability of the lipid layer of the eggshell or in sequestration of toxic lipid by-products which accumulate in the PF during larval development (Mei *et al.*, 1997). It is likely that this important, if not essential, function is conserved in PF-localised FABPs in other nematodes. In our studies, the presence of PCN FABP mRNA in the gut of the nematode may indicate a role in the

sequestration and transport of dietary fatty acids to their sites of storage or utilisation and this could also be the function of the *C. elegans* and *A. suum* FABPs found in perienteric fluid. The observed hypodermal localisation of PCN FABP mRNA could indicate a role in epicuticle maintenance or in acquisition of fatty acid nutrients from the external environment. It is also possible that PCN FABP is not secreted to the cuticle but remains in the hypodermis, perhaps functioning like the classic iFABPs in intracellular fatty acid transport and protection.

3.3.3. FAR-1 Expression in PCN

3.3.3.1 In-situ Hybridisation

The ISH studies undertaken in this work showed that the Gp-FAR-1 gene was strongly expressed exclusively in the hypodermis of the J2s of *G. pallida* (Figure 3.3). As a full-length clone of the Gp-FAR-1 gene was available, this protein was expressed in *E.coli* and used to raise an antiserum for immunlocalisation studies.

3.3.3.2. Recombinant GP-FAR-1 Expression

Protein was expressed with (L) and without the hydrophobic leader peptide (NL) and as shown in Figure 3.4a, the purity of the recombinant protein was confirmed by SDS-PAGE analysis. This showed the presence of single bands migrating at around 21kDa in the sample expressed with leader sequence and 19kDa in the sample without the leader peptide. The size of these bands corresponded well with the size of the proteins predicted from their amino acid sequences (20922kDa with and 18,845kDa without the leader peptide). In all subsequent experiments, rGp-FAR-1 without the leader peptide was used.

3.3.3.3. Immunolocalisation

Western blot analysis of proteins extracted from G. pallida J2s probed with a polyclonal antiserum raised against rGp-FAR-1 confirmed the presence of Gp-FAR-1 in

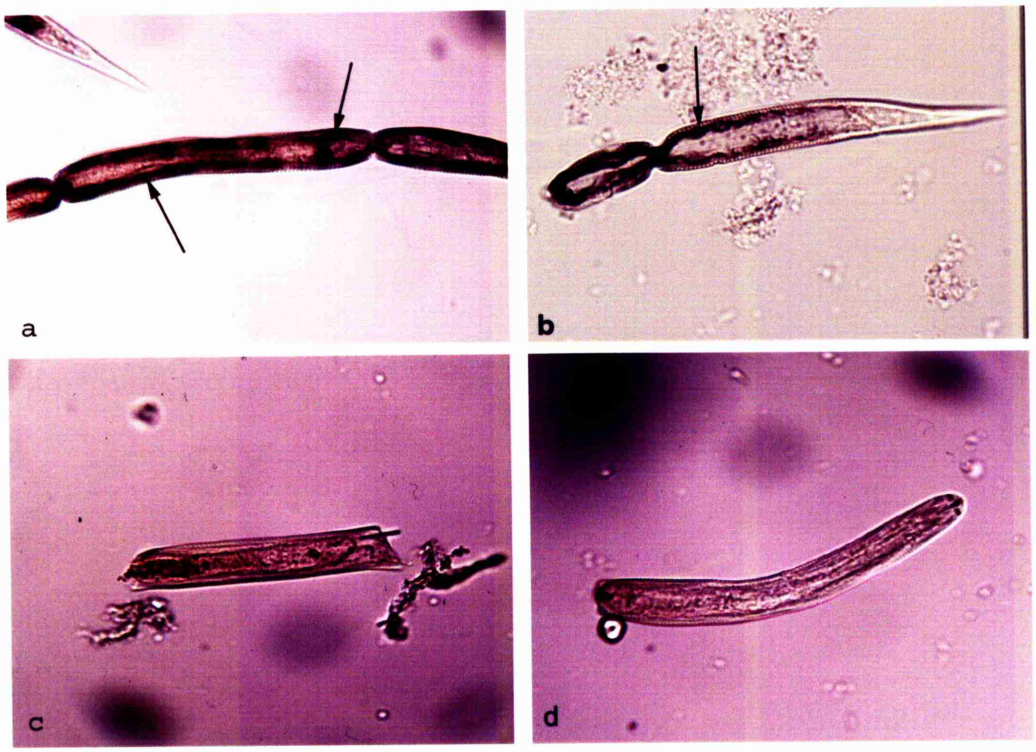
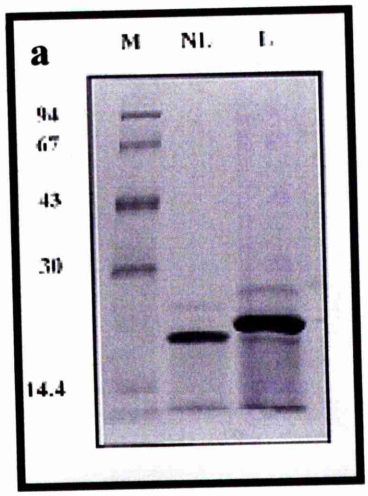


Figure 3.3. *In-situ* hybridisation of an antisense DIG-labelled Gp-FAR-1 probe to fragments of PCN J2s. A positive reaction is indicated by a purple-brown deposit (arrows). Staining was observed exclusively in the hypodermis (a & b). No staining was observed in samples probed with a Gp-FAR-1 sense probe (c & d).



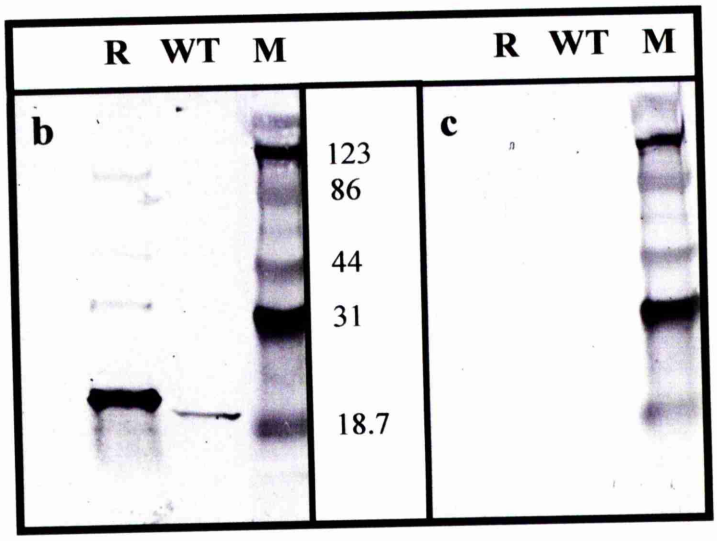


Figure 3.4. (a) SDS-PAGE analysis of recombinant Gp-FAR-1 with (L) and without (NL) leader peptide. Molecular masses of standard proteins (M) in kilodaltons are indicated at the left. (b) Western blot analysis of *G. pallida* J2 total protein extracts (WT) and recombinant Gp-FAR-1 (R) probed with an antiserum raised against rGp-FAR-1. (c) Western blot analysis of *G. pallida* J2 total protein extracts (WT) and recombinant Gp-FAR-1 (R) probed with pre-immune antiserum. Molecular masses of standard proteins (M) in kilodaltons are indicated at the centre.

these nematodes (Figure 3.4 b&c) and also confirmed the specificity of the antiserum for Gp-FAR-1. In the total protein lane a single band of approximately 20kDa was observed indicating that native Gp-FAR-1 undergoes only minor, if any post-translational modification as this size is in broad agreement with the calculated molecular mass of the predicted mature protein.

The location of the Gp-FAR-1 protein was studied by indirect immunofluorescence, using the polyclonal anti-serum raised against Gp-FAR-1. The antibody reacted with the surface of the nematodes as shown in Figure 3.5 with the fluorescence evenly distributed across the entire surface of the worms. Labelling was particularly intense in the annulations of the parasite surface. After 15-20 minutes, material shed from the nematode, stained with the serum, was deposited onto the slides. Imprints, presumably made by cuticular annulations, were seen in the shed material (Fig 3.5e). These observations suggest that Gp-FAR-1 is secreted from the parasite surface. Worms which appeared to be dead retained the surface fluorescence although no further studies were made of this phenomenon. Controls, treated with pre-immune sera, showed only very faint autofluorescence of internal regions of the worms and controls incubated in secondary antibody alone showed no observable fluorescence.

The FAR-1s have been identified in a wide range of free-living and parasitic nematodes. Parasitic species appear to contain only one FAR-1 gene whilst *C.elegans* has at least six genes encoding FAR-1s (Blaxter, 1998). Studies in parasitic nematodes show similar expression patterns to those observed here for PCN. In *O. volvulus*, FAR-1 mRNA was shown to be present in the hypodermis and biosynthetic labelling experiments showed that Ov-FAR-1 is secreted into the surrounding medium (Bradley *et al.*, 2001). This protein was also shown to be present as a circulating antigen in serum of infected individuals, consistent with it being secreted (Tree *et al.*, 1995). Similar observations have

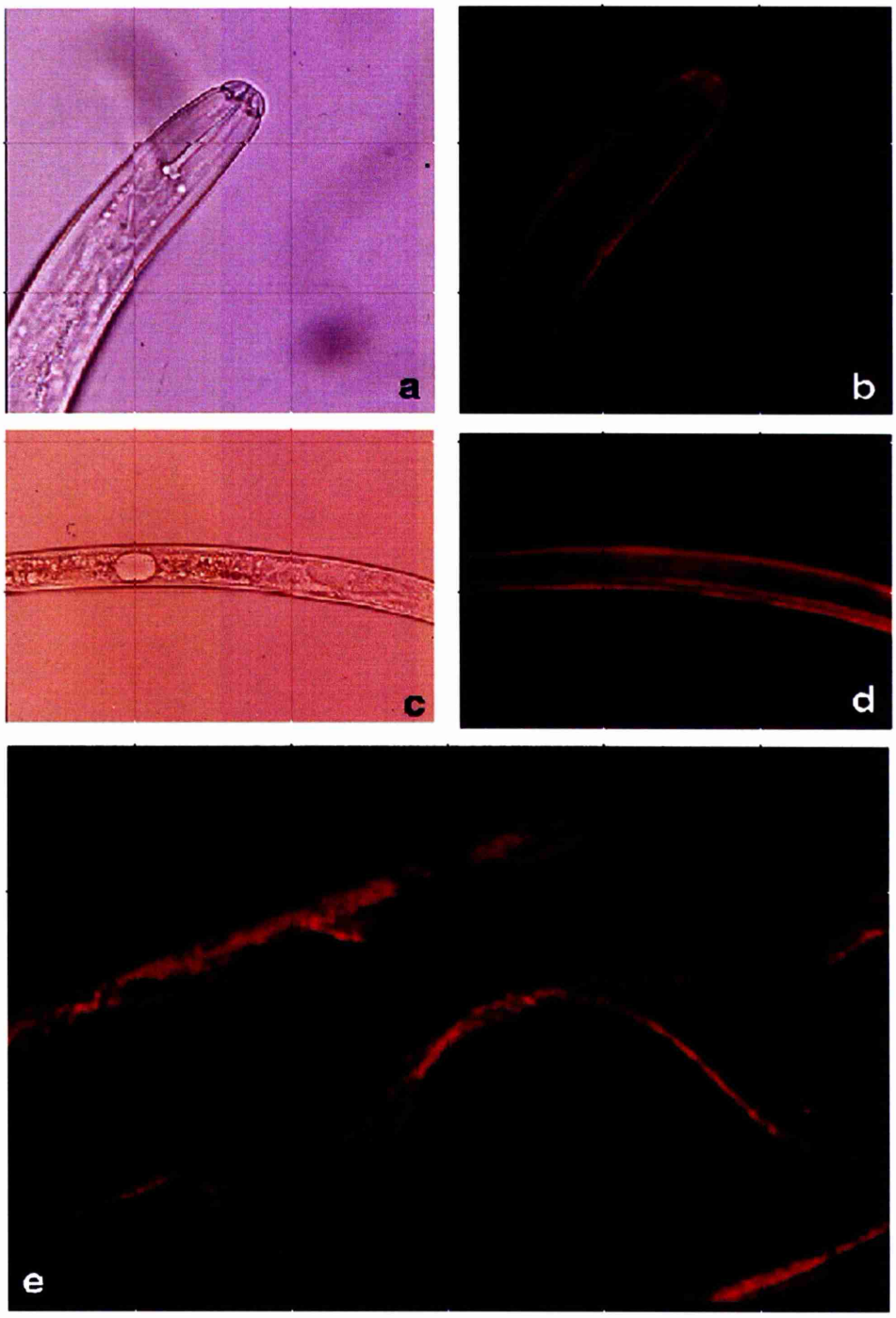


Figure 3.5. Immunolocalisation of Gp-FAR-1 on PCN J2s . Bright field images (a & c) of nematodes shown in (b & d) respectively. Staining is seen at the surface of the nematode (b & d) and on material shed from the surface (e). Nematodes treated with pre-immune antisera or secondary antibody alone showed no staining (not shown).

been made with other animal-parasitic nematodes (Bradley *et al.*, 1995). Kennedy *et al.*, (1997), speculated that Ov-FAR-1 may be involved in sequestration of retinol from host tissues or that it may modulate host defence responses by binding lipid-based immune mediators.

Surface-secreted nematode proteins are often implicated in the host-parasite interaction and several have been identified and implicated in a range of survival strategies such as evasion of host defences. For example, a surface-localised thioredoxin peroxidase from *G. rostochiensis* has been postulated to play a role in evasion of plant defences by breaking down the hydrogen peroxide produced as part of the hypersensitive reaction, an important component of plant defences (Robertson *et al.*, 2000) and Cookson *et al.* (1992) identified gp29, the major cuticular protein of lymphatic filarial parasites as a secreted glutathione peroxidase. This has also been proposed to be involved in antioxidant activity against host defence responses. Forms of both of these proteins are also expressed inside these nematodes. Therefore, proteins that have ancestral forms within nematodes may also evolve different roles outwith the nematode body.

In order to investigate the possibility that Gp-FAR-1 has been similarly adapted from a general internal lipid transport protein to a surface protein involved in pathogenesis we undertook a detailed examination of the biochemical properties of the rGp-FAR-1. These data are presented in subsequent chapters.

Chapter 4

Ligand-binding by rGp-FAR-1

4.1 Introduction

4.1.1 Plant defences

Many molecules present on the surfaces of parasitic nematodes are thought to mask the parasite from its host or to modify host defence responses (e.g, Robertson *et al.*, 2000, Kennedy *et al.*, 1997, Cookson *et al.*, 1992). Plants have evolved a wide range of defence mechanisms to protect themselves from wounding, pathogen attack and environmental stresses (Agrios, 1997). The outcome of any plant-pathogen interaction, therefore, depends on the pathogen's ability to overcome the host plant defences and surface and secreted molecules may play a vital role in these interactions.

4.1.1.1 Susceptible & resistant responses in plant-pathogen interactions

Successful infection of a plant by a pathogen is known as a susceptible response (compatible interaction). In some cases the susceptible plant does not mount a strong defence response against the pathogen. In other susceptible interactions the plant does mount a strong response but the pathogen is able to overcome them (Greenberg, 1997). Where a plant is tolerant to a pathogen, little or no symptoms appear after pathogen attack and the pathogen is free to feed and develop (e.g., Phillips, 1998).

Plant resistance to pathogens can be monogenic (gene-for-gene resistance) or polygenic in origin. In the classic gene-for-gene model of resistance proposed by Florr (cited in Hammond-Cosack & Jones, 1997), resistance results from an interaction between the products of a complementary pair of dominant pathogen avirulence (Avr) and host plant resistance (R) genes. Such a gene-for-gene relationship has been shown to be responsible for *H1* gene-mediated resistance of *S. tuberosum* ssp *andigena* to *G. rostochiensis* pathotype Ro1 (Janssen *et al.*, 1991). Four classes of disease resistance genes have been characterised to date including the NBS/CheY-LRR superfamily which

includes several nematode resistance genes e.g., *Gpa2*, *Cre3* and *Mi* (reviewed by Davis *et al.*, 2000) and these are thought to function in signal transduction pathways involved in specific pathogen recognition and subsequent induction of defence responses (Gebhardt & Valkonen, 2001). As yet, no avirulent gene products have been identified from parasitic nematodes (Davis *et al.*, 2000).

Polygenic resistance is seen as a quantitative variation in resistance levels within a plant population i.e., partial resistance. The molecular basis of polygenic resistance is not well understood but is thought to involve multiple genes (Gebhardt & Valkonen, 2001).

4.1.1.2. Hypersensitive response

Upon pathogen attack in a resistant (incompatible) interaction the host defence response is mounted in response to the detection of pathogen-derived factors or products generated as a result of breakdown of host tissues. This can lead to the hypersensitive response (HR) which has been described as a co-ordinated form of programmed cell death and is observed as necrosis and browning of cells around the site of infection which prevents further penetration of the pathogen (Collinge & Slusarenko, 1987).

4.1.1.3. Oxidative burst

One of the earliest effects detected after pathogen attack is an oxidative burst caused by activation (by phosphorylation) of membrane-bound NADPH oxidase which causes accumulation of reactive oxygen species (ROS) such as, superoxide and hydrogen peroxide (and others). As well as direct roles in protection (by damaging the pathogen), these ROS also function in cross-linking of the cell wall (to encapsulate the pathogen or prevent its further penetration), as triggers for cell death and as diffusible signals for induction of cellular protection genes in other cells (reviewed by Lamb &

Dixon, 1997). The oxidative burst occurs in two phases. Phase I results in a weak transient burst and can occur in both susceptible and resistant interactions whereas Phase II is a more sustained burst which results in accumulation of AOS and occurs in resistant reactions (Lamb & Dixon, 1997).

Other early events after pathogen attack include the generation of signalling molecules such as salicylic acid, ethylene and jasmonic acid. These signalling pathways are complex and can result in an HR or may lead to induction or repression of target genes through a membrane-bound receptor kinase and phosphorylation cascade (Reymond & Farmer, 1998). Transcription factors, which control the expression of other response genes, including those encoding pathogenesis-related proteins and those involved in wound responses are among the targets of these pathways (Delagi *et al.*, 2000).

4.1.1.4. Defence signalling in plants

Plants, therefore, have a range of inter-related defence reactions and complex signalling mechanisms induce and co-ordinate these anti-pathogen responses. Several signalling molecules involved in defence have been identified including, hydrogen peroxide, salicylic acid (SA), ethylene and jasmonic acid (Reymond & Farmer, 1998; O'Donnell *et al.,* 1996). Salicylic acid has been shown to be required for systemic acquired resistance (SAR) (Gaffney *et al.*, 1993). SAR occurs when a resistance response at one part of a plant causes distant sites of plants to become more resistant to subsequent attack by pathogens which normally cause a susceptible reaction.

Fatty-acid signalling has long been known to play a crucial role in many plant processes (Farmer, 1988) and in recent years much information has been gathered on the roles of jasmonic acid in developmental and defence responses of plants. Jasmonic acid (JA) was originally identified as a signalling molecule involved in growth

regulation and development but is now also known to function in defence signalling reactions (reviewed by Creelman & Mullet, 1997). Application of jasmonate to plants causes changes in the levels of transcription and translation of many defence genes including, protease inhibitors, phytoalexin biosynthesis genes, pathogenesis-related proteins and antifungal genes such as osmotin and thionin. However, the JA signal transduction pathway remains largely unknown (Creelman & Mulllet, 1997). It is thought that as yet unknown signals interact with a membrane receptor which causes lipase- or phospholipase-mediated release of linolenic acid (LA) from the membrane which is then converted by lipoxygenase to 13-hydroperoxylinolenic acid. Wounding is also thought to results in LA release from membranes. 13-hydroperoxylinolenic acid can then be catabolised by hydroperoxy lyase or peroxygenase which leads to the synthesis of the wound hormone traumatin or cutin monomers respectively. Alternatively, JA can be synthesised from this intermediate via an allene oxidase- and allene oxidase cyclase-dependent pathway (reviewed by Creelman & Mullet, 1997). JA also accumulates systemically in plants upon wounding. Wounding is thought to trigger the release of a polypeptide wound signal, systemin, which is transported, via the phloem throughout the plant where it activates an intracellular lipase which releases linolenic acid from membranes. This is subsequently converted, via the octadecanoid pathway, to phytodienoic acid and jasmonic acid (Pearce *et al.*, 1991).

4.1.1.5. Plant defences in response to nematode infection

Most knowledge of plant defence responses to pathogen attack comes from studies on bacterial or fungal pathogens. In contrast, little is known about the specific details of plant responses to nematode attack. Plant defence genes known to be produced locally as a result of root knot nematode infection include, lipoxygenase, chitinase, proteinase inhibitors, catalase, phenylalanine lyase and anionic peroxidases

(Niebel *et al.*, 1995; Williamson & Hussey, 1996) and hydrogen peroxide is produced by *A. thaliana* as a result of cyst nematode infection (Waetzig *et al.*, 1999). Systemic defence responses also occur upon nematode infection. Infection of potato roots by *G. rostochiensis* and *G. pallida* have been shown to result in changes in gene expression and induction of PRPs in potato leaves (Hammond-Kosack *et al.*, 1989; Rahimi *et al.*, 1993).

Over 25 major R genes against nematodes have now been mapped in plant genomes and most of these appear to be typical NBS/LRR-type genes that function in classic gene-for-gene interactions such as the potato cyst nematode resistance gene, *Gpa2* (reviewed by Davis *et al.*, 2000). However, no avirulent gene products have yet been identified from parasitic nematodes (Davis *et al.*, 2000).

4.1.2. FAR proteins

Over 40 genes encoding FAR-like proteins have been identified in a wide range of free-living, animal- and plant-parasitic nematodes (Blaxter, 1998 and http://nema.cap.ed.ac.uk/genes-of-interest/LBP-20.html) and database similarity searches indicate that these proteins are confined to nematodes. The first FAR protein to be described, Ov-FAR-1, was identified as a vaccine candidate due to its secretion by the river blindness parasite, *O. volvulus*, and the immune response to it mounted by infected individuals (Tree *et al.*, 1995; Bradley *et al.*, 1995). Kennedy *et al.*, (1997) showed that recombinant Ov-FAR-1 bound retinol and fatty acids with high affinities.

The work described in the first section of this chapter was undertaken to investigate the ligand-binding characteristics of Gp-FAR-1 in order to allow a comparison with that found for Ov-FAR-1 and other unusual lipid-binding proteins from nematodes. The purpose was to investigate whether the different lifestyles and host environments of these nematodes were reflected in the ligand-binding

characteristics of the FAR proteins and whether Gp-FAR-1 was in any way unusual. It has also been postulated (Kennedy *et al.*, 1997) that, as the protein is secreted by *O. volvulus* into host tissues (Tree *et al.*, 1995), it may be involved in modulation of host defences and the pathogenesis of *Onchocerca* infection in its human host (Kennedy *et al.*, 1997. Gp-FAR-1 is present on, and shed from, the surface of *G. pallida* (Chapter 3), and therefore in contact with host tissues. Experiments were therefore undertaken to obtain experimental evidence that this protein could interfere with defence responses in a plant host and data suggesting a role for Gp-FAR-1 in manipulating plant defence signalling pathways is presented.

4.2 Materials and Methods

4.2.1 Spectrofluorimetry

4.2.1.1 Binding of fluorescent ligands

Binding of fluorescent ligands was measured as described in Section 2.3.1.

4.2.1.2 Binding of non-fluorescent ligands

Binding of non-fluorescent ligands was measured as described in Section 2.3.2.

4.2.1.3 Dissociation curve titrations

Dissociation curve titrations were carried out as described in Section 2.3.3 to estimate dissociation constants.

4.2.1.4 Chain length study

Experiments were carried out to determine whether binding of fatty acids by rGp-FAR-1 was affected by fatty acid chain length. This was done by measuring the percentage decrease in fluorescence at 488nm of a DAUDA:rGp-FAR-1 complex upon addition of saturated fatty acids, with chain lengths ranging from C6 to C22. Dilution factor corrections were made by measuring the percentage decrease in fluorescence

upon addition of PBS alone, then subtracting this from the data gathered. A similar experiment was carried out using ethanol, rather than PBS, as the solvent to ensure that results for the larger fatty acids were not affected by their insolubility in PBS. All fatty acids used in this experiment were dissolved in ethanol as 10mM stock solutions then diluted hundred-fold in PBS for use in the assay.

4.2.2 Lipoxygenase inhibition assay

Soybean lipoxygenase (Sigma) was diluted in ice-cold 0.2M borate buffer (pH 9.0) and used at a concentration of 100 units per reaction. rGp-FAR-1 and the control proteins, BSA (Northumbria Biologicals Ltd.) and ribonuclease A (Boehringer Mannheim) stock solutions were made to a concentration of 100μM in PBS for use in the assays. Linoleic acid (Sigma) was emulsified in 95% ethanol and dissolved in sterile, distilled water then diluted in filter-sterilised 0.2M borate buffer (pH 9.0) to a final concentration of 100-150μM for use in these assays. This experiment was carried out at a pH of 9.0 as at this pH, linoleic acid remains in a monomeric form up to a concentration of ~200μM and soybean lipoxygenase I is known to have a preference for monomeric linoleic acid. 600μl of the above linoleic acid solution was added to the cuvette and the absorbance at 234nm measured every 5 seconds for 5 minutes in an Ultrospec 2000 UV/visible spectrophotometer (Pharmacia) and used as a blank. Then, 100 units of lipoxygenase were quickly added and the absorbance measured as above and the blank subtracted. To test for inhibition of the lipoxygenase reaction, rGp-FAR-1 or the control proteins were added to the linoleic acid solution and used as a blank. Lipoxygenase was then added quickly and the absorbance measured as above. To quantify any dilution effect, another reaction was set up where PBS was added in place of protein and the absorbance measured as before and subtracted from the samples.

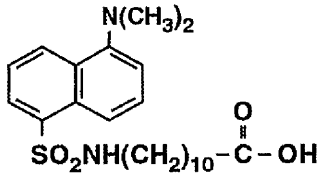
4.3 Results

4.3.1 Binding of Ligands by rGp-FAR-1

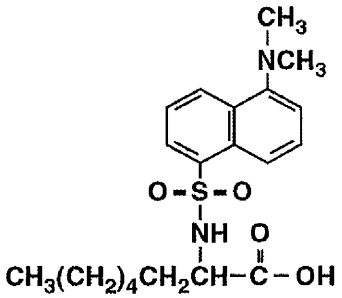
4.3.1.1. Fluorescent Ligands

Ligand-binding by rGp-FAR-1 was investigated using spectrofluorimetry, by comparing the emission spectra of fluorescent hydrophobic ligands in aqueous solvent in the presence and absence of protein, an alteration in which indicates interaction between ligand and protein. The fluorescence emission of DAUDA, a hydrophobic molecule consisting of undecanoic acid conjugated to the dansyl fluorophore at the methyl terminus of the fatty acid, (Figure 4.1a), is polarity-sensitive and shifts to a shorter emission wavelength, along with an increase in emission intensity, when moved from a polar environment such as an aqueous solution, to an apolar environment such as a hydrophobic binding site (MacGregor & Weber, 1986). In addition to indicating the capacity of a protein to bind hydrophobic ligands, this property can be used to give a measure of the apolarity of the protein's binding site (e.g. Kennedy *et al.*, 1997).

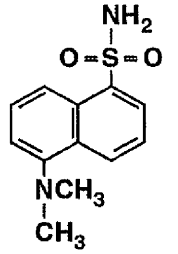
The peak wavelength of fluorescence emission of DAUDA in buffer occurs at 543nm but upon addition of rGp-FAR-1 a 62nm blue-shift to 482nm was observed (Figure 4.2a). To put this in context, the peak fluorescence emissions of DAUDA in the organic solvents ethanol, dimethylformamide and cyclohexane were previously reported to be 506, 505 and 475nm respectively (Kennedy *et al.*, 1995b), so the shift in the presence of rGp-FAR-1 is indicative of a highly apolar environment in the binding site of rGp-FAR-1. The blue shift observed for rGp-FAR-1 was almost identical to that reported for the *O. volvulus* homologue, rOv-FAR-1, at 483nm (Kennedy *et al.*, 1997), but was greater than the 498nm reported for *Ascaris suum* perivitelline FABP, As-p18 (Mei *et al.*, 1997). The *A. lumbricoides* polyprotein allergen, ABA-1, however, has a particularly apolar binding site with peak emission of DAUDA occurring at 475nm



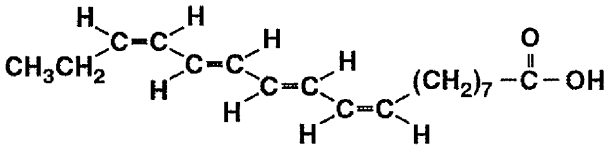
a. 11-(((5-(dimethyl(amino)-1-naphthalenyl)sulfonyl)amino)-undecanoic acid (DAUDA)



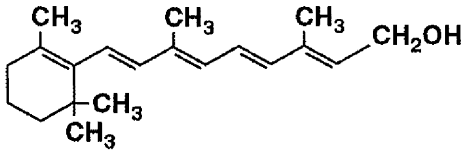
b. dansyl-DL-α-aminocaprylic acid (DACA)



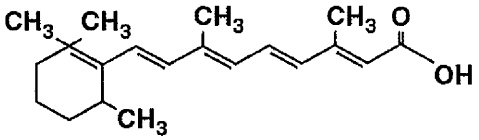
c. 5-(dimethylamino)naphthalene-1-sulphonamide (Dansylamide)



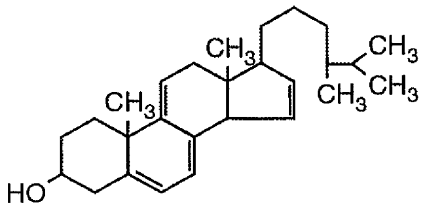
d. *cis*-parinaric acid



e. all *trans*-retinol



f. retinoic acid



g. Dehydroergosterol

Figure 4.1. Chemical structures of compounds tested for binding to Gp-FAR-1

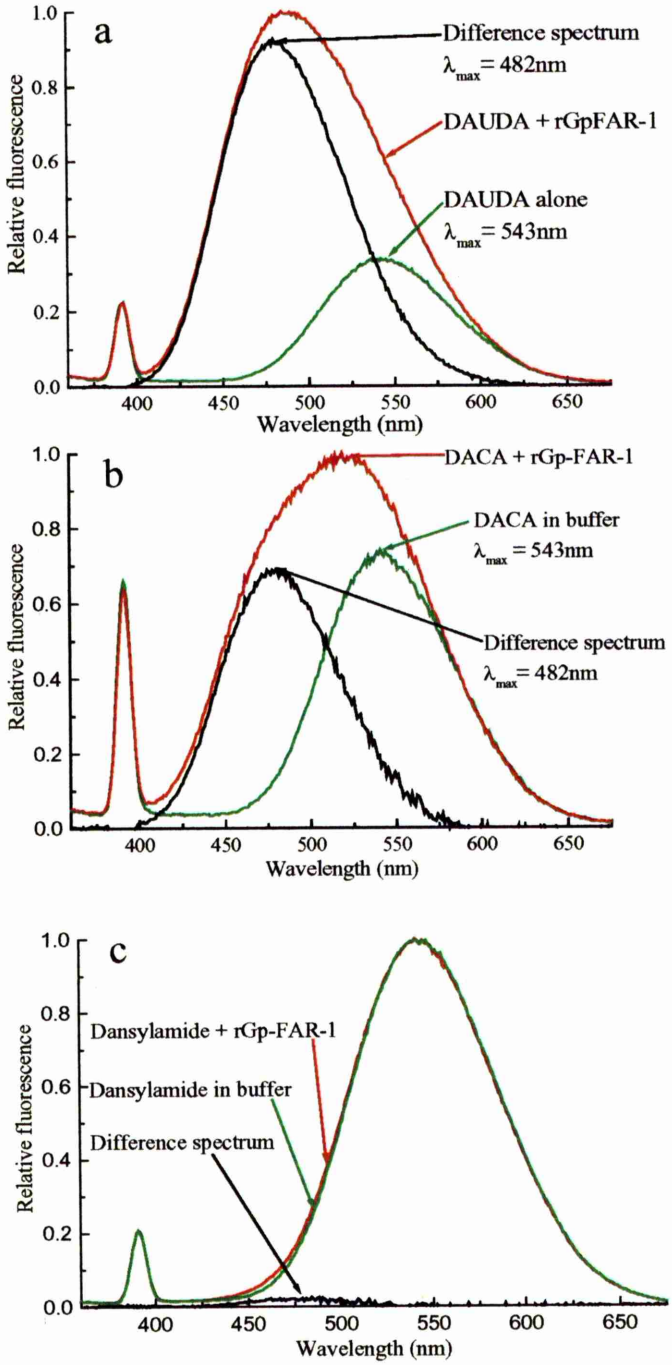


Figure 4.2. (a) Fluorescence emission spectra of 0.1μM DAUDA in buffer (green) showing characteristic maximum fluorescence emission (λ_{max}) at 543nm in aqueous solution, in the presence of approximately 1.4μM rGP-FAR-1 (red) and difference spectrum (black) showing emission of rGP-FAR-1-bound DAUDA with λ_{max} at 482nm. (b) Fluorescence emission spectra of 0.1μM DACA in buffer (green) showing characteristic λ_{max} at 543nm in aqueous solution, in the presence of approximately 1.4μM rGP-FAR-1 (red) and difference spectrum (black) showing emission of rGP-FAR-1-bound DACA with λ_{max} at 482nm. (c) Fluorescence emission spectra of 0.1μM dansylamide in buffer (green) showing characteristic λ_{max} at 543nm in aqueous solution, in the presence of approximately 1.4μM rGP-FAR-1 (red) and difference spectrum (black) indicating that rGp-FAR-1 does not bind dansylamide.

(Moore *et al*, 1999) under the same conditions. By comparison, the peak emission of DAUDA with human serum albumin and liver FABP occurs at 495nm (Wilton, 1990) and 500nm respectively (Wilkinson & Wilton, 1986).

rGp-FAR-1 was also shown to bind DACA with a wavelength shift of 62nm, identical to that seen for DAUDA (Figure 4.2b). The dansyl fluorophore of DACA is attached to the α-carbon of an 8-carbon fatty acid chain whereas that of DAUDA is located at the aliphatic end (Figure 4.1a). Binding of both DAUDA and DACA with the same blue shift in emission, therefore, implies that either the protein can bind fatty acids in either orientation, or that the whole fatty acid chain is enclosed within the rGp-FAR-1 binding site. However, the increase in emission intensity upon addition of DACA to rGp-FAR-1 was significantly lower than that of the same concentration of DAUDA indicating differences in the binding of these two molecules. This is probably seen because DACA has an 8-carbon chain and, as shown later in this chapter (Section 4.3.4), this is below the optimum chain length preference of rGp-FAR-1. Control experiments were carried out using dansylamide, which consists of the fluorescent head group without a fatty acid chain (Figure 4.1c) which showed no binding of this molecule to rGp-FAR-1 (Figure 4.2c), indicating that the fluorescent head group does not contribute significantly to the binding of DAUDA and DACA.

Retinol (vitamin A) and the naturally-occurring fatty acids, *cis*- and *trans*-parinaric acid (Figure 4.1d) are intrisically fluorescent and so can be used in binding experiments in a manner similar to DAUDA and DACA. As retinol (Figure 4.1e) is poorly soluble and prone to oxidation in aqueous solution, binding of this molecule to rGp-FAR-1 was tested by addition of aliquots of retinol in ethanol to cuvettes containing rGp-FAR-1 in PBS. The addition of successive aliquots of retinol led to incremental increases in emission intensity, indicating that retinol does bind to rGp-

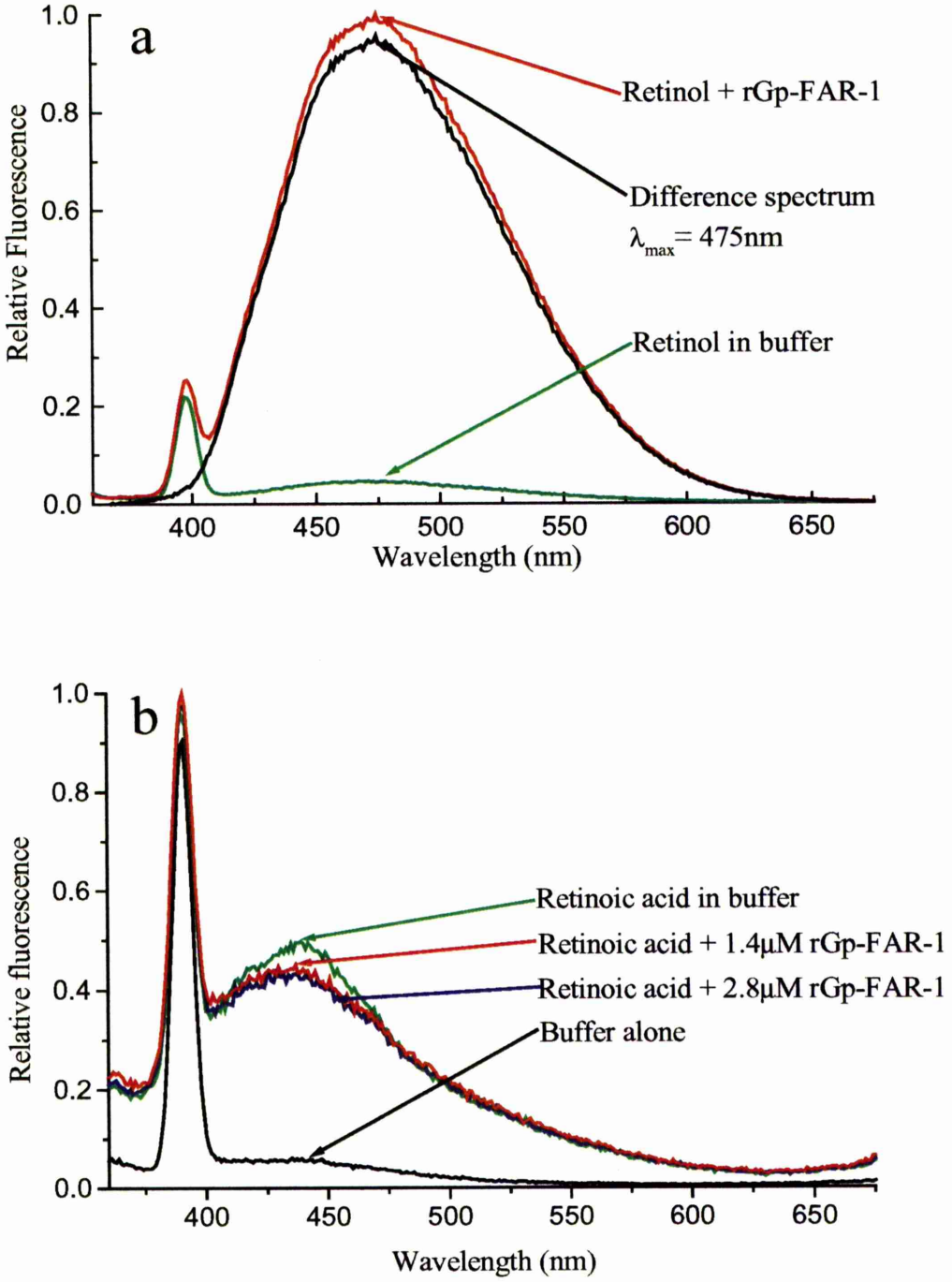


Figure 4.3. (a) Fluorescence emission spectrum of 2μM retinol in PBS (green), in the presence of approximately 1.4μM rGp-FAR-1 (red), and difference spectrum (black) showing emission of rGp-FAR-1-bound retinol with λ_{max} at 475nm. (b) Fluorescence emission spectrum of 2μM retinoic acid in buffer (green) and in the presence of approximately 1.4μM (red) and 2.8μM (blue) rGp-FAR-1 indicating that rGp-FAR-1 does not bind retinoic acid.

FAR-1 (Figure 4.3a). A related molecule, retinoic acid showed no evidence of binding to rGp-FAR-1 in similar experiments (Figure 4.3b). Considering the structure of these molecules it is perhaps surprising that rGp-FAR-1 did not bind retinoic acid given the presence of a carboxylate group on this molecule (Figure 4.1f). In contrast, the NPAs bind both compounds (Kennedy *et al.*, 1995a,b).

Cis-parinaric acid showed an increase in fluorescence emission intensity upon addition of rGp-FAR-1 with maximum fluorescence emission at 414nm indicating that this molecule binds to rGp-FAR-1 (Figure 4.4a). rGp-FAR-1 was found not to bind to the naturally fluorescent sterol, dihydroergosterol (Figure 4.1g, Figure 4.4b) in common with rOv-FAR-1, which did not bind cholesterol (Kennedy *et al.*, 1997). No binding was observed with the fluorescent amino acid, tryptophan. The control protein ribonuclease A showed no signs of binding to any of the above ligands.

4.3.1.2. Non-fluorescent Ligands

The binding of non-fluorescent fatty acids and other hydrophobic ligands was investigated by their effect on the fluorescence emission spectra of preformed complexes of rGp-FAR-1 with DAUDA, retinol or *cis*-parinaric acid. Binding was observed as a reversal of the fluorescence effect due to the competitive displacement of fluorescent ligand from the binding site. A range of fatty acids of different chain lengths (C6-C22) and different degrees of unsaturation were tested to investigate whether rGp-FAR-1 had any preference for the type of fatty acid bound.

Binding observed (λ_{max})	***No binding observed***
DAUDA (482nm)	Dansylamide
DACA (482nm)	Dehydroergosterol
cis-parinaric acid (414nm)	Retinoic acid
Retinol (475nm)	Tryptophan

Table 4.1 Binding of fluorescent ligands by rGp-FAR-1

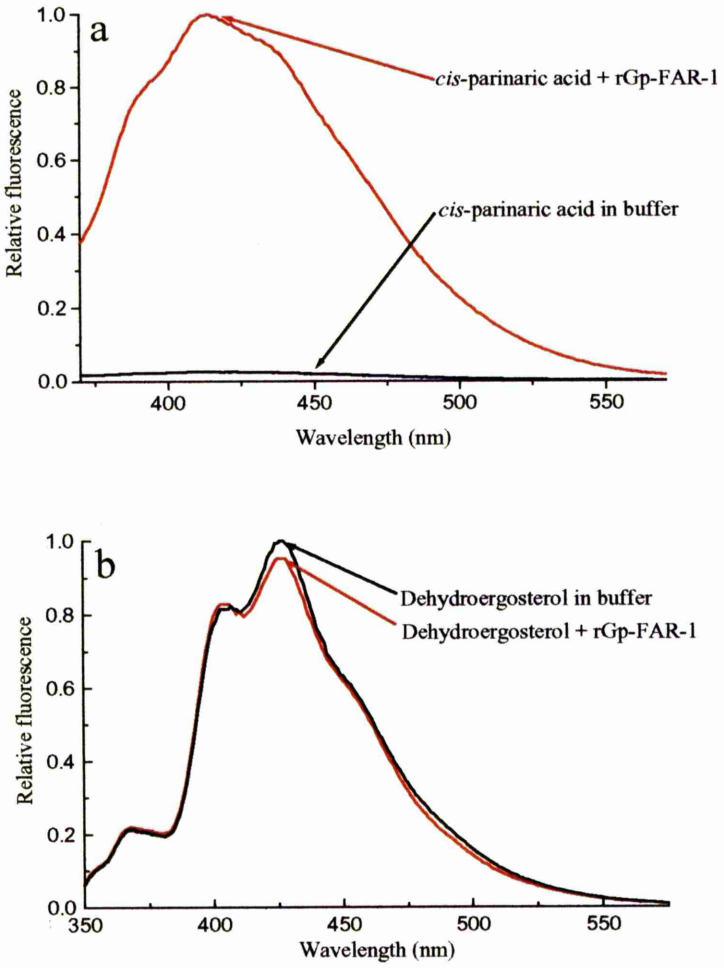


Figure 4.4. (a) Fluorescence emission spectra of 0.05μM *cis*-parinaric acid in buffer (black) and in the presence of approximately 1.2μM rGp-FAR-1 (red) indicating that rGp-FAR-1 binds *cis*-parinaric acid. (b) Fluorescence emission spectra of 0.1μM dehydroergosterol in buffer (black) and in the presence of approximately 1.2μM rGp-FAR-1 (red) indicating that rGp-FAR-1 does not bind this compound.

As shown in Figure 4.5a, upon addition of aliquots of thousand- and hundred-fold dilutions of an arachidonic acid solution to a cuvette containing pre-formed DAUDA:Gp-FAR-1 complexes, the fluorescence emission intensity was seen to decrease as the emission spectrum shifted back towards that of free DAUDA in water. This indicates that arachidonic acid competitively displaces DAUDA from the binding site. Using this method, a range of saturated and unsaturated fatty acids from undecanoic acid (C11:0) to docosahexaenoic acid (C22:6) were shown to bind to rGp-FAR-1 (Table 4.2) including all the major fatty acids found in PCN J2s (C18:1, C20:1, C20:4) and potato roots (C16:0, C18:2 and C18:3) (Holz *et al.,* 1997, 1998a,b).

It has been proposed that one function of fatty acid/lipid binding proteins is the transport of fatty acids and/or other hydrophobic ligands through aqueous environments in which they are otherwise insoluble (e.g., Stewart *et al.*,1991; Veerkamp *et al.*, 1997). The observed lack of binding of caproic (C6:0) and caprylic acids (C8:0) fits this hypothesis as both are soluble in aqueous solvents and do not, therefore, require this form of transport. It was, however, somewhat surprising that no binding was seen with the C8:0 fatty acid as DACA (which has an 8 carbon chain) was earlier shown to bind to rGp-FAR-1, so it is possible that the hydrophobic dansyl group on DACA contributed to the binding of this molecule despite the fact that dansylamide did not bind.

Addition of fatty acids to complexes of both retinol (Figure 4.5b) or *cis*-parinaric acid (Figure 4.5c) with Gp-FAR-1 gave similar results. Displacement of these probes occurred with the same range of fatty acids as that shown for DAUDA. The competitive displacement of retinol by these fatty acids indicates that retinol and fatty acids share the same binding site on rGp-FAR-1. Alternatively, it is feasible that fatty

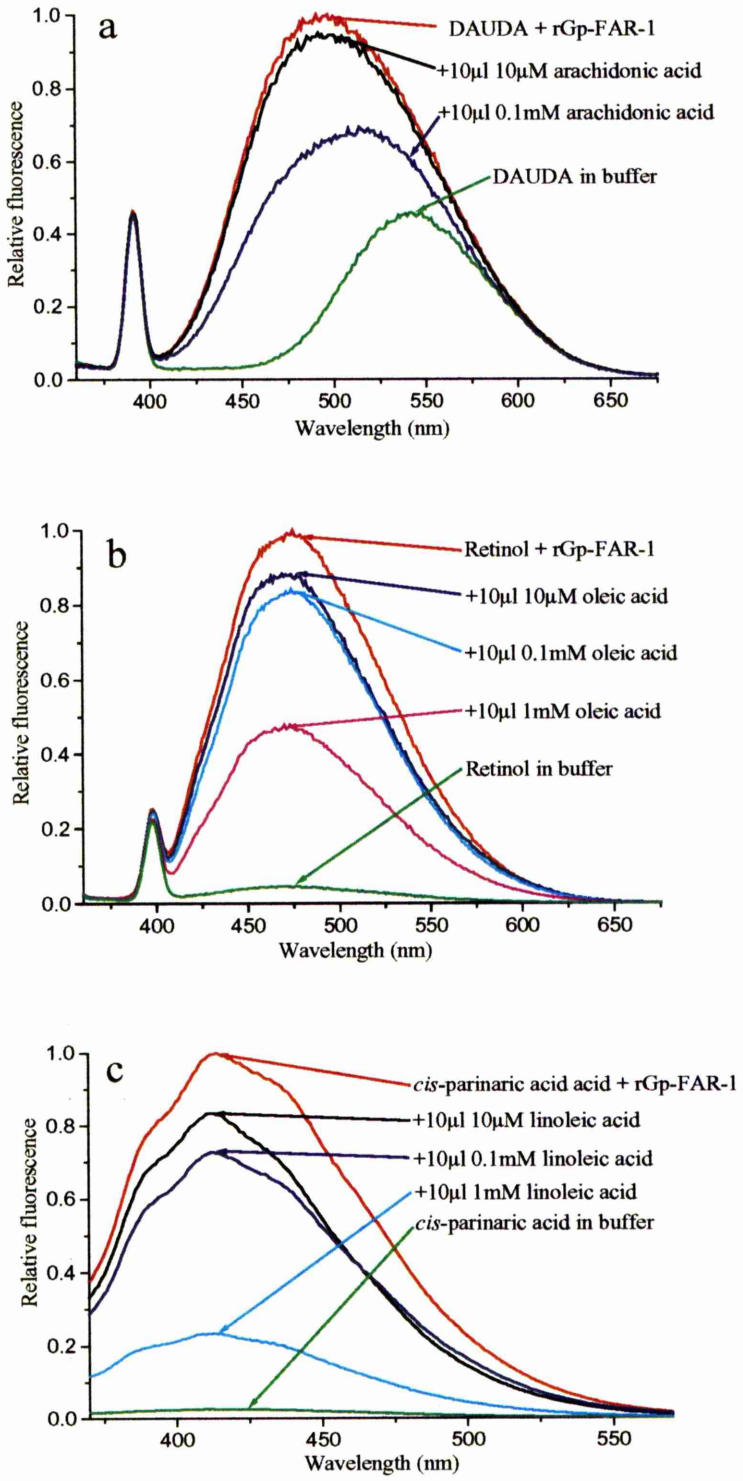


Figure 4.5. (a) Fluorescence emission spectra of DAUDA in buffer (green), in the presence of approximately 1.2µM rGp-FAR-1 (red) and upon addition of 10µl of, 10µM (black) and 0.1mM (blue) of arachidonic acid indicating that rGp-FAR-1 binds arachidonic acid. (b) Fluorescence emission spectra of 2.5µM retinol in buffer (green), in the presence of approximately 1.4µM rGp-FAR-1 (red) and upon addition of 10µl of 10µM (blue), 0.1mM (cyan) and 1mM (pink) oleic acid indicating that rGp-FAR-1 binds oleic acid. (c) Fluorescence emission spectra of *cis*-parinaric acid in buffer (green), in the presence of approximately 1.2µM rGp-FAR-1 (red) and upon addition of 10µl of, 10µM (black), 0.1mM (blue) and 1mM (cyan) of linoleic acid indicating that rGp-FAR-1 binds linoleic acid.

Ligand Type	*Binding observed*	*No binding observed*
Fatty acids	C11:0 Undecanoic acid	C6:0 Caprylic acid
	C12:0 Lauric acid	C8:0 Caproic acid
	C14:0 Myristic acid	
	C15:0 Pentadecanoic	
	C16:0 Palmitic acid	
	C16:1 Palmitoleic acid	
	C17:0 Heptadecanoic acid	
	C18:0 Stearic acid	
	C18:1 Oleic acid	
	C18:1 Elaidic acid	
	C18:2 Linoleic acid	
	C18:3 Linolenic acid	
	C19:0 Nonadecanoic acid	
	C20:0 Arachidic acid	
	C20:1Eicosanoic acid	
	C20:4 Arachidonic acid	
	C21:0 Heneicosanoic acid	
	C22:0 Behenic acid	
	C22:1 Erucic acid	
	C22:6 Docosahexaenoic acid	
		Jasmonic acid
Phenylpropanoids		Caffeic acid
		Chlorogenic acid
		Ferulic acid
Glycoalkaloids		α-chaconine
		α-solanine

Table 4.2. Binding of non-fluorescent ligands to rGp-FAR-1

acids and retinol have different binding sites in rGp-FAR-1 and that binding of a ligand in one binding site prevents or inhibits binding to the other site.

4.3.2 Dissociation curve titrations

4.3.2.1 Fluorescent Ligands

Fluorimetric titrations were carried out to determine dissociation constants (K_d) of DAUDA, retinol and *cis*-parinaric acid for rGp-FAR-1 (Table 4.3.). The K_d is defined as the concentration at which a protein reaches its half saturation point, saturation being the point where addition of more protein does not increase the number of protein:ligand complexes, seen in this study as an increase in fluorescence emission. This constant is routinely used as a measure of how tightly proteins bind their ligands. Data were analysed with Microcal Origin software using a simple, non-competitive binding model specifically adapted for use in these experiments as described in Chapter 2.

Titration of DAUDA with rGp-FAR-1 gave an apparent K_d value of 1.16μM (Table 4.3.) which is of a similar order of magnitude to the 0.97μM reported for rOv-FAR-1 (Kennedy *et al.*, 1997) and the 1.8μM for ABA-1 (Moore *et al.*, 1999). However, to obtain this result the apparent molar ratio (n) had to be fixed at 1 in the K_d calculation and it was also noted that DAUDA:rGp-FAR-1 titrations yielded non-saturable curves (Figure 4.6a). This could indicate that DAUDA is not an ideal ligand for these studies, perhaps due to interference by the bulky fluorophore. This also occurs upon titration of the *A. suum* NPA, ABA-1 with DAUDA and was suggested to be due to the presence of *E.coli*-derived non-fluorescent fatty acids, bound during purification of the recombinant proteins, which would competitively displace DAUDA from the binding site (McDermott, 1999). However, in the present study both *cis*-parinaric acid

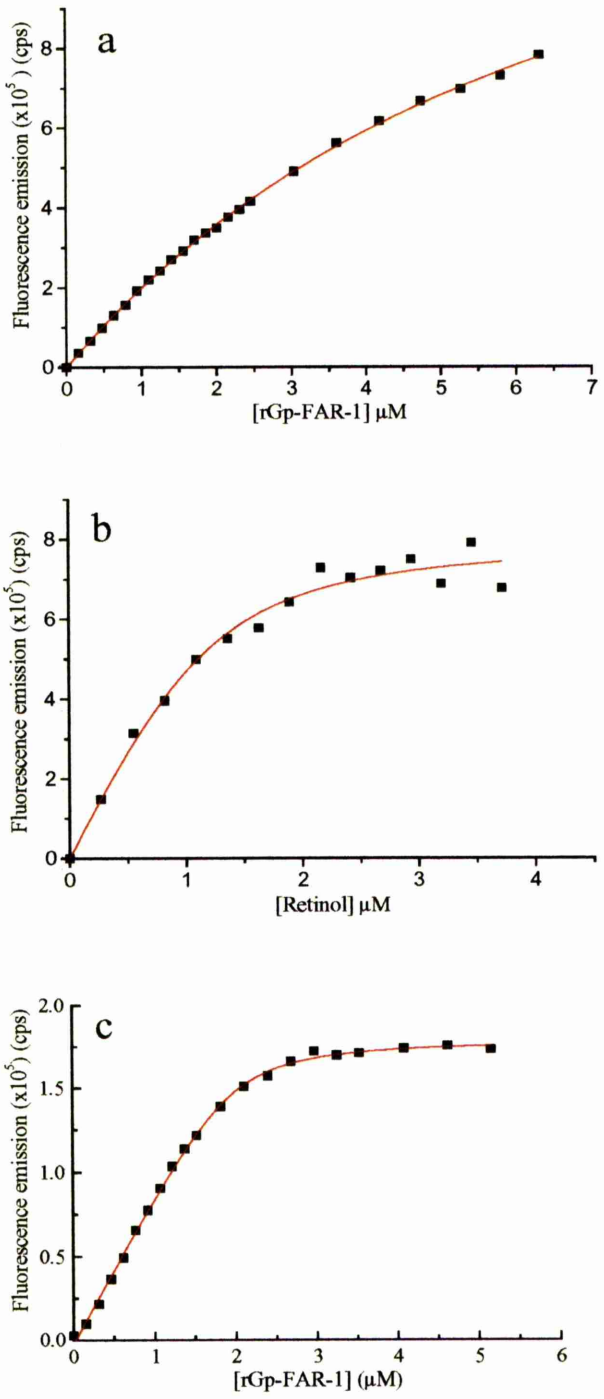


Figure 4.6. (a) Fluorescence titration of 9.6µM DAUDA with rGp-FAR-1 yielding a dissociation constant (K_d) of 1.16µM. (b) Fluorescence titration of 3.22µM rGp-FAR-1 with retinol (after subtraction of free retinol) yielding a K_d of 0.14µM. (c) Fluorescence titration of 1µM cis-parinaric acid with rGp-FAR-1 yielding a K_d of 0.08µM.

Ligand tested	Dissociation constant (K_d) (μM)	Apparent molar ratio (n)
cis-parinaric acid	0.08	1.97
Retinol	0.14	0.66
DAUDA	1.16	1*
Linolenic: *cis*-parinaric acid	2.9	1*
Linolenic: DAUDA	0.99	0.33
Linoleic: *cis*-parinaric acid	5.2	1*
Linoleic: DAUDA	0.62	0.33

* Kds obtained when n fixed at 1

Table 4.3. Dissociation constants (K_d) and apparent molar ratios (n) of rGp-FAR-1:ligand complexes.

and retinol saturated upon titration with rGp-FAR-1 which, if the above hypothesis is correct, would indicate that the pre-bound fatty acids are unable to compete out these ligands. This is highly unlikely because in the competitive displacement studies above (Section 4.3.1.2) a wide range of fatty acids were found to displace all the fluorescent ligands tested, including DAUDA. Also, when rGp-FAR-1 was subjected to detergent removal chromatography before titration, which could be expected to remove such contaminants, the reaction remained unsaturable. Other sources of error could also have affected the above result, such as, inaccuracies in the estimation of rGp-FAR-1 concentration, because to obtain reliable dissociation constants accurate concentrations of protein and ligand must be known. Another possibility is that some of the protein may be inactive, perhaps due to the rigours of the purification process (e.g., sonication or cleavage of the fusion protein), or due to the expression of a eukaryotic protein in a heterologous, prokaryotic system which could affect protein folding. rGp-FAR-1 has a predicted glycosylation site but prokaryotes lack the post-translational machinery required for glycosylation so it is possible that unglycosylated Gp-FAR-1 is less active than the native protein. Freeze-thawing of the recombinant protein could also result in loss of activity.

Titration of rGp-FAR-1 with retinol, which proceeded to saturation (Figure 4.6b), yielded a K_d value of 0.14μM with an apparent molar ratio of 0.66 indicating a 3:2 rGp-FAR-1:retinol binding stoichiometry. Alternatively, a binding stoichiometry of 1:1 may be the true value but one-third of the protein present was inactive or the protein concentration was overestimated. This K_d value was of the same order as those reported for the NPA monomer, ABA-1 at 0.1μM (Moore *et al.*, 1999) and the *A. galli* NPA at 0.56μM (Timanova *et al.*, 1999). This titration was also open to other sources of error. First, the titration had to be adapted to take account of the inherent instability

of retinol, which is prone to oxidation, and the resultant fluorescence emission interference in aqueous solution. To minimise this effect, the retinol was titrated against rGp-FAR-1. With the other fluorescent ligands, where protein was added to a constant amount of ligand, corrections to account for the fluorescence emission of unbound ligand were made by subtracting the fluorescence emission of the ligand before addition of protein from subsequent data. However, as retinol was incrementally added to protein in these titrations, it was necessary to run a control reaction where retinol was incrementally added to a cuvette containing no protein to obtain data for unbound ligand thus introducing another potential source of error.

Upon titration of *cis*-parinaric acid with rGp-FAR-1 (Figure 4.6c), which also proceeded to saturation, a K_d value of 0.08μM with an apparent molar ratio of 1.97 was obtained. This indicates either a 2:1 rGp-FAR-1: *cis*-parinaric acid binding stochiometry, or that the protein concentration was overestimated by a factor of two or, more likely, that half of the protein was inactive. The K_d value reported for rOv-FAR-1 with *cis*-parinaric acid was a factor of ten higher than that reported here at 0.86μM (Kennedy *et al.*, 1997) while that reported for ABA-1 was even higher at 1.9μM (Moore *et al.*, 1999) indicating that rGp-FAR-1 binds this molecule very tightly.

It is difficult to conclude with any certainty the number of fatty acid-binding sites per rGp-FAR-1 monomer unit from the above results. However, like other nematode lipid-binding proteins (NPAs and FABPs) (e.g, Moore *et al.*, 1999, Mei *et al.*, 1997) several studies have shown that rOv-FAR-1 binds one fatty acid per monomer unit (Kennedy *et al.*, 1997, Mpagi *et al.*, 2000) and, given the similarities in the binding site environments of, and range of ligands bound by, rGp-FAR-1 and rOv-FAR-1 it is likely that this is also the case for rGp-FAR-1.

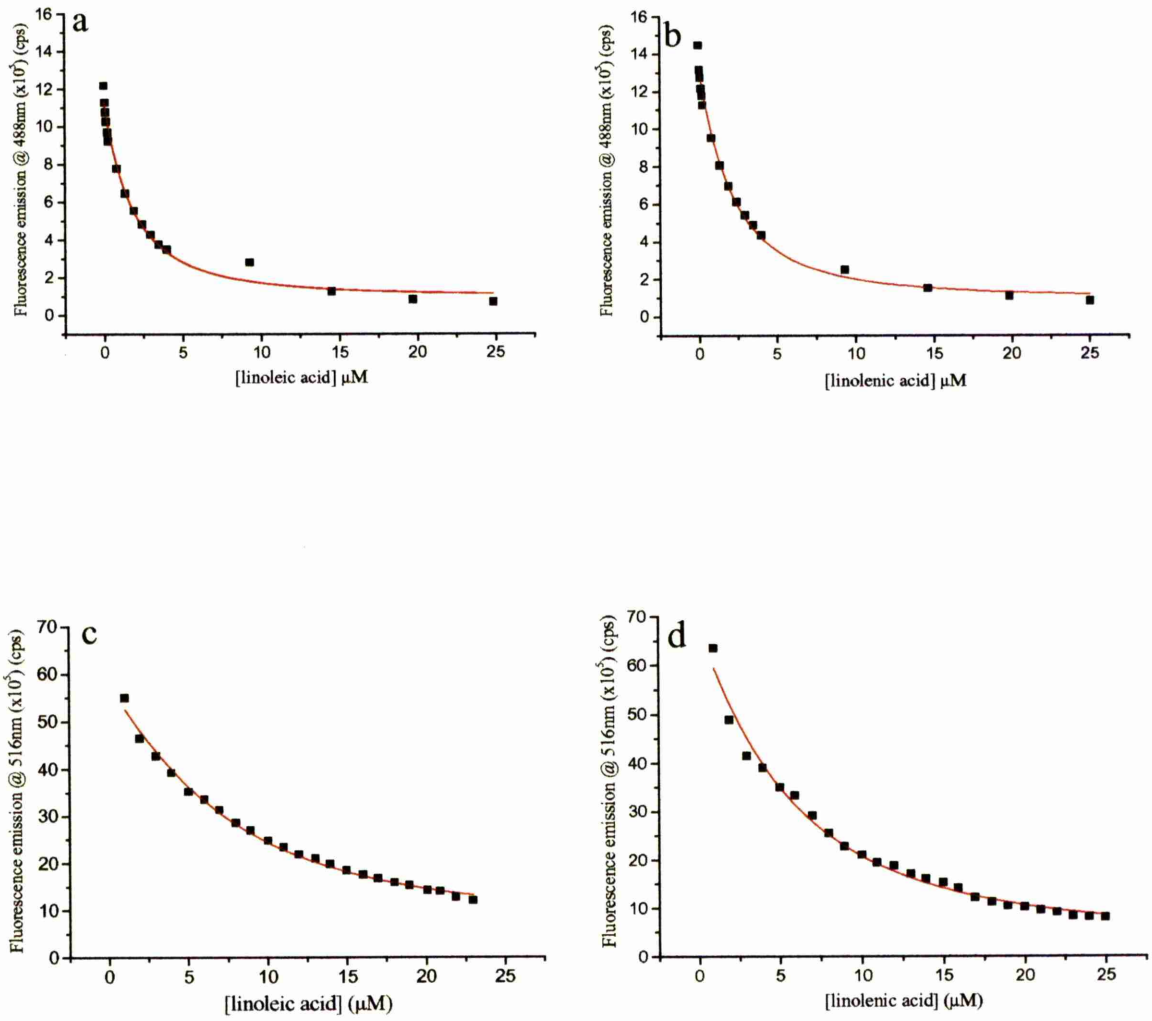


Figure 4.7 a) Back titration of 24µM rGp-FAR-1 and 1.7µM DAUDA with linoleic acid yielding an apparent K_i of 0.62µM. b) Back titration of 24µM rGp-FAR-1 and 1.7µM DAUDA with linolenic acid yielding an apparent K_i of 0.99µM. c) Back titration of 6µM rGp-FAR-1 and 11µM *cis*-parinaric acid with linoleic acid yielding an apparent K_i of 5.2µM. d) Back titration of 6µM rGp-FAR-1 and 11µM *cis*-parinaric acid with linolenic acid yielding an apparent K_i of 2.9µM.

All values obtained above fall within the range of dissociation constants of approximately 0.1-10μM reported for mammalian FABPs (Van Nieuwenhoven *et al.*, 1996).

4.3.2.2 Non-fluorescent Ligands

The dissociation constants for complexes of rGp-FAR-1 and the non-fluorescent fatty acids, linoleic and linolenic acids were determined in competitive displacement titrations by measuring the decrease in fluorescence of a DAUDA or cPnA:rGp-FAR-1 solution upon addition of competing non-fluorescent ligand. These titrations gave K_d values of 0.62μM and 0.99μM respectively for linoleic and linolenic acids in competition with DAUDA with apparent molar ratios of 0.33, and in competition with *cis*-parinaric acid K_d values of 5.2μM and 2.9μM were respectively obtained when the apparent molar ratio was fixed at 1 (Figures 4.7c & d). The Kd of linoleic acid for rOv-FAR-1:DAUDA was reported to be 0.18μM (Mpagi *et al.*, 2000) while for the *Ascaridia* NPA the Kd was 0.2μM (Timanova *et al.*, 1999) and these are of the same order of that found for rGp-FAR-1.

4.3.3 Chain length study

In order to determine whether rGp-FAR-1 binding was affected by fatty acid chain length the percentage decrease in fluorescence of a DAUDA:rGp-FAR-1 mixture in PBS upon addition of a range of saturated fatty acids was measured (Figure 4.9). To ensure that results obtained for the larger fatty acids had not been affected by their lack of solubility the experiment was repeated with ethanol as the solvent. In PBS, rGp-FAR-1 showed maximal binding to saturated fatty acids in the range C14:0 to C16:0, with DAUDA displacement apparently greatest with C15:0. When ethanol was used as the solvent maximal binding occurred in the range C14:0 to C19:0 but the greatest

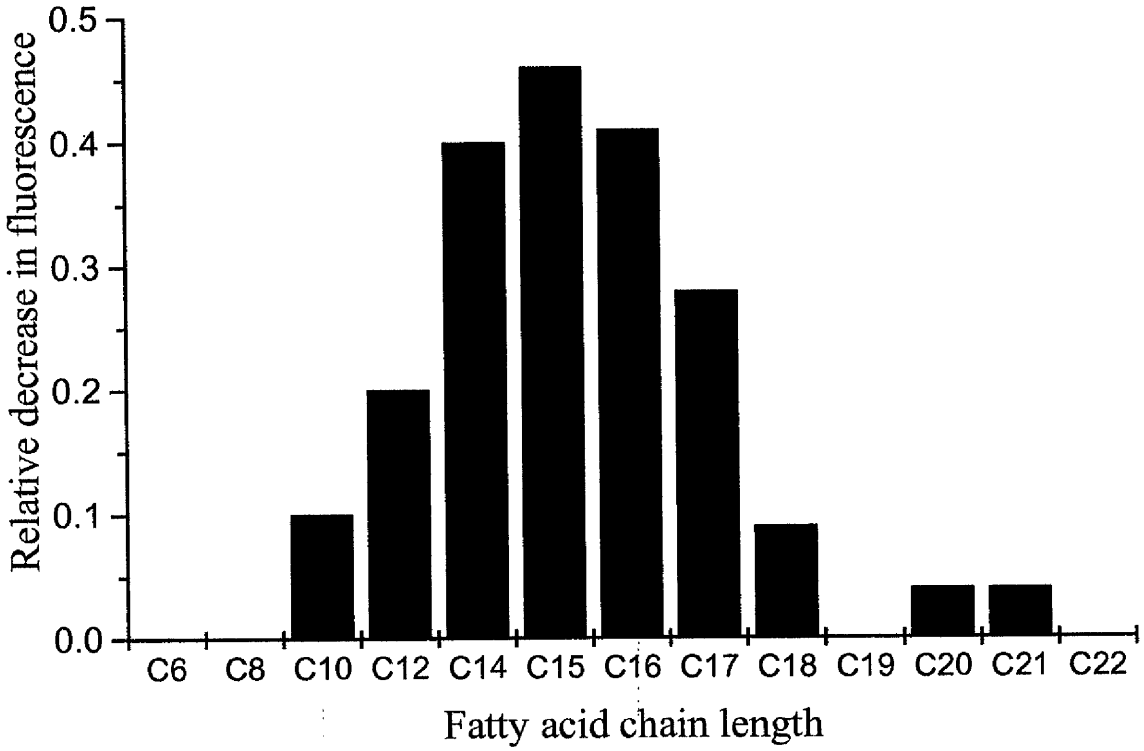


Figure 4.8. Relative decrease in fluorescence at 488nm upon addition of 1μM of saturated fatty acids to a solution containing 0.1μM DAUDA and approximately 1.2μM rGp-FAR-1. Results shown represent the mean of two replicates.

DAUDA displacement still occurred with C15:0. Recent work by Holz *et al.* (1998a), studying the fatty acid and lipid content of PCN, found C15:0 was the only odd-numbered saturated fatty acid identified. These results suggest that rGp-FAR-1 has a low affinity for saturated fatty acids above C19 yet, in contrast, appeared to bind the highly unsaturated fatty acids arachidonic (C20:4) and docosahexaenoic (C22:6) acids well. This may indicate that rGp-FAR-1 has a preference for unsaturated fatty acids. However, these results may have been affected by the lack of solubility of the larger saturated fatty acids. In experiments on the *A. galli* NPA with C11-20 fatty acids, Timanova *et al.*, 1999, obtained similar results which showed maximal binding with chain lengths of C16 –18 and no binding of chain lengths of C10 or less.

In this experiment only saturated fatty acids were tested, but it is possible that the chain length preference of Gp-FAR-1 is affected by the degree of unsaturation of the fatty acid. Future work should therefore include comparative chain length studies with polyunsaturated fatty acids.

Overall, it would appear that rGp-FAR-1 binds a similar range of fatty acids and retinol as its homologue rOv-FAR-1, the NPAs and nematode FABPs and with similar affinities.

4.3.4. Binding of plant defence compounds

The surface localisation of rGp-FAR-1 means that it is in close contact with the host environment after the nematode enters the plant and so is likely to interact with host-derived molecules. A range of plant defence compounds were therefore tested for binding to rGp-FAR-1 to investigate the possibility that rGp-FAR-1, like surface-secreted molecules from animal-parasitic nematodes, functions in evasion of host defences (Table 4.4.). No binding was observed with the plant phenylpropanoids caffeic, ferulic and chlorogenic acids (Figure 4.9a,b & c), the glycoalkaloids α-

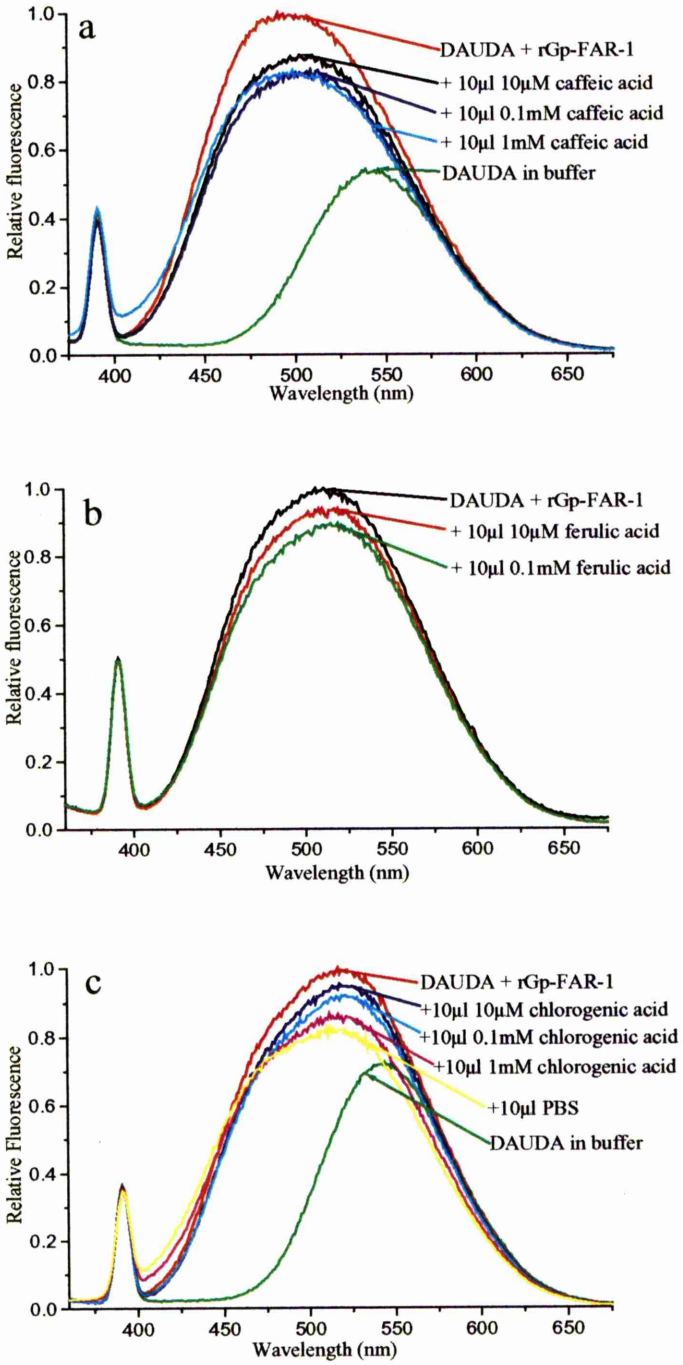


Figure 4.9. (a) Fluorescence emission spectrum of, 0.1µM DAUDA in buffer (green), in the presence of approximately 1.4µM rGp-FAR-1 (red) and upon addition of 10µl of 10µM (black), 0.1mM (blue) and 1mM (cyan) caffeic acid indicating that rGp-FAR-1 does not bind caffeic acid. (b) Fluorescence emission spectrum of, 0.1µM DAUDA in buffer (green), in the presence of approximately 1.4µM rGp-FAR-1 (red) and upon addition of 10µl of 10µM (black), 0.1mM (blue) and 1mM (cyan) ferulic acid. Addition of 10µl buffer (pink) showing that the decrease in fluorescence is due to dilution and that rGp-FAR-1 does not bind ferulic acid. (c) Fluorescence emission spectrum of, 0.1µM DAUDA in buffer (green), in the presence of approximately 1.4µM rGp-FAR-1 (red) and upon addition of 10µl of 10µM (blue), 0.1mM (cyan) and 1mM (pink) chlorogenic acid. Addition of 10µl buffer (yellow) showing that the decrease in fluorescence is due to dilution and that rGp-FAR-1 does not bind chlorogenic acid.

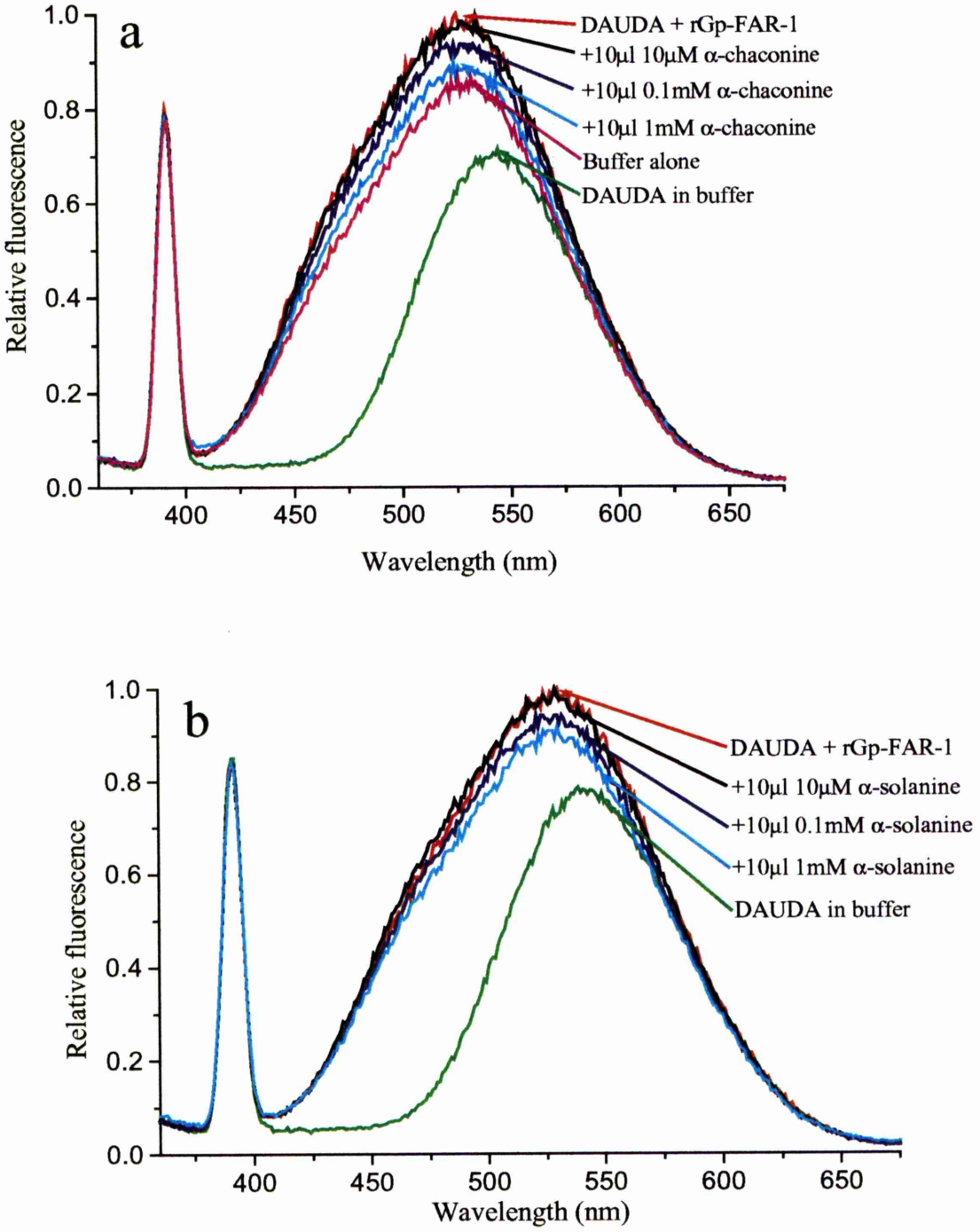


Figure 4.10. (a) Fluorescence emission spectrum of 0.1µM DAUDA in buffer (green), in the presence of approximately 1.4µM rGp-FAR-1 (red) and upon addition of 10µl of 10µM (black), 0.1mM (blue) and 1mM (cyan) α-chaconine. Addition of 10µl buffer (pink) showing that the decrease in fluorescence is due to dilution and that rGp-FAR-1 does not bind α-chaconine. (b) Fluorescence emission spectrum of 0.1µM DAUDA in buffer (green), in the presence of approximately 1.4µM rGp-FAR-1 (red) and upon addition of 10µl of 10µM (black), 0.1mM (blue) and 1mM (cyan) α-solanine indicating that rGp-FAR-1 does not bind α-solanine.

chaconine and α-solanine (Figure 4.10a & b), or the plant defence signalling compound jasmonic acid. Therefore, if Gp-FAR-1 plays a role in defence it probably does so in an indirect manner rather than by direct sequestration of these defence compounds (or compounds exclusively used in plant defences).

4.3.5 Lipoxygenase inhibition assay

Two of the fatty acids shown to bind to rGp-FAR-1, linoleic and linolenic acids, are important precursors in plant lipid peroxidation and defence signalling pathways and are the main substrates for lipoxygenase in plants. Experiments were conducted to investigate whether rGp-FAR-1 could bind these fatty acids and inhibit their breakdown by lipoxygenase. Inhibition of lipoxygenase has previously been shown to occur with human liver-FABP (Ek *et al.*, 1997). Lipoxygenase-mediated peroxidation of linolenic acid is an early step in the octadecanoid signalling pathway which leads to the synthesis of the systemic plant defence signal transducer jasmonic acid. Peroxidation of fatty acids containing 1,4 *cis, cis*-pentadienyl moieties by lipoxygenase results in the formation of toxic, membranolytic lipid hydroperoxides and free radicals which are among the first line of plant defence that nematodes will encounter upon invasion of the plant (Baker & Orlandi, 1995).

Lipoxygenase-mediated breakdown of fatty acids to their hydroperoxides can be quantified spectrophotometrically by measuring absorbance at 234nm (A_{234}), an increase in which indicates formation of double bonds. Under the conditions used, this indicates formation of hydroperoxides. To test for inhibition of lipoxygenase-mediated hydroperoxide formation by rGp-FAR-1, the A_{234} of a lipoxygenase/linoleic acid mixture was measured in the presence and absence of rGp-FAR-1.

A pronounced inhibition of lipoxygenase-mediated breakdown of linoleic acid was observed in the presence of rGp-FAR-1 (Figure 4.11). BSA showed an even

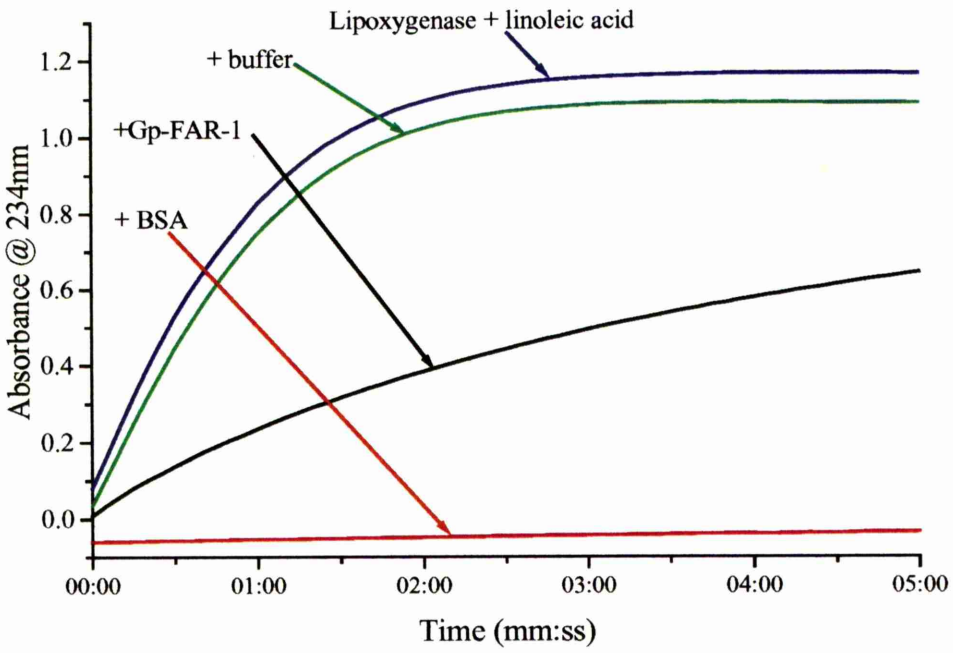


Figure 4.11. Lipoxygenase-mediated peroxidation of linoleic acid (blue) and upon addition of 100µl of PBS (green), 100µM rGp-FAR-1 (black) and 100µM BSA (red) indicating that rGp-FAR-1 inhibits the lipoxygenase-mediated peroxidation of linoleic acid *in vitro*. The results shown represent the mean of three experiments.

greater ability to inhibit breakdown of linoleic acid which could be expected as this protein is known to have several fatty-acid binding sites per BSA molecule (Curry *et al.*, 1998). Another control protein, ribonuclease A, had no observable effect on hydroperoxide formation.

4.4. Discussion

The results presented in this work show that Gp-FAR-1 binds retinol and long-chain fatty acids with affinities similar to those found for other classes of fatty acid and retinol-binding proteins from nematodes, such as NPAs (Kennedy, 1995a,b,c), and from higher organims such as FABPs (van Nieuwenhoven, 1996) and serum albumin (Curry *et al.*, 1998). However, a major difference between these nematode and mammalian proteins are apparent since individual FABP proteins either bind retinol (CRBP, CRtBP) or fatty acids (FABP) but not both (Bass, 1993) and this is also the case with the extracellular mammalian proteins serum albumin and retinol-binding protein. The relevance of this is not clear but it may merely represent evolutionary adaptation of these proteins in higher animals. However, the possibility that this may also indicate functional differences cannot be ruled out.

Since only a limited range of ligands were tested for binding in this study, it is possible that Gp-FAR-1 binds other ligands, such as branched chain fatty acids, fatty acid hydroperoxides, phospholipids, carotenoids, di- and tri-glycerides. Future work should, therefore, include such studies.

The presence of FAR genes in *C. elegans* as well as parasitic species indicates a general role for these proteins in nematodes. However, the observed surface localisation of Gp-FAR-1 and its rapid shedding indicate that when the nematode enters root tissues Gp-FAR-1 will be in contact with the host environment, and thus may be involved in the host-parasite interaction. This, coupled with the fact that fatty acids are

known to play important roles in plant defences as exogenous inducers/elicitors of defence responses (eg Altamura *et al.*, 1994) and also as precursors of systemic defence signalling molecules such as jasmonic acid (e.g., Creelman & Mullet, 1997), suggest that Gp-FAR-1 could be involved in disruption or evasion of these host plant defences.

The *in vitro* ability of Gp-FAR-1 to inhibit or prevent the lipoxygenase-mediated peroxidation of these fatty acids observed in this work indicate that Gp-FAR-1 could disrupt the plant defence response in at least two ways. Inhibition of the formation of fatty acid hydroperoxides could firstly, lessen the overall toxic, membranolytic effects of these molecules, and secondly inhibit the formation of jasmonic acid by limiting the availability of its precursors.

Another mechanism by which Gp-FAR-1 may inhibit host defences is also possible. Fatty acids are known to be present on the epicuticle and/or the surface coat of nematodes, which are subject to rapid shedding. Holz *et al.*, (1998a), found that the most abundant fatty acid in PCN is arachidonic acid, the levels of which increased 33-fold upon hatching of the J2s. This fatty acid has been shown to induce resistance-related responses when applied exogenously to potato tubers (Altamura *et al.*, 1994), so it is possible that Gp-FAR-1, which was observed in this study to bind arachidonic acid, and indeed all of the major nematode and root fatty acids, could sequester fatty acids on the nematode surface, thus preventing or reducing elicitation of the plant defence responses.

The soybean lipoxygenase used in this study is known to have a preference for monomeric linoleic acid but it has been reported that a potato tuber lipoxygenase is activated by fatty acid aggregates and this has been shown to occur with other water-soluble enzymes with hydrophobic or membrane bound substrates (Bru & Garcia-Carmona, 1997). It is possible that ligand binding by Gp-FAR-1 prevents or inhibits the

formation of fatty acid aggregates, thereby preventing the activation of potato lipoxygenase.

Plant and animal defence responses share striking similarities, especially in fatty acid-based signalling defence systems (Bergey *et al.*, 1996). Interactions between plants and pathogens trigger the release of a polypeptide wound signal, systemin, which is transported, via the phloem throughout the plant where it activates an intracellular lipase which releases linolenic acid from membranes. This is subsequently converted, via the octadecanoid pathway, to phytodienoic acid and jasmonic acid, which activate a range of defence-related genes. In higher animals, macrophage-pathogen interactions result in the release of a polypeptide cytokine TNF-α which travels throughout the animal via the bloodstream in a manner analogous to that of systemin in phloem. TNF-α then triggers the release of an intracellular phospholipase A_2 which releases arachidonic acid from membranes, and this fatty acid is then converted to prostaglandins, which trigger fever to fight the infection. These prostaglandins are structurally very similar to, jasmonic acid and phytodienoic acid of plants (Bergey *et al.*, 1996). It is therefore possible that the functions proposed for Gp-FAR-1 in evasion or inhibition of host defences could also apply for the animal-parasitic nematode counterpart, Ov-FAR-1, and the fact that this protein is secreted into host tissues by *O. volvulus* (Tree *et al.*, 1995; Bradley *et al.*, 2001) lends support to this suggestion.

Overall, the binding characteristics of Gp-FAR-1 appear to be very similar to those of the FABP and NPA proteins, both in the ligands bound and in their affinities for these ligands. However, these proteins have no sequence similarity and, assuming the secondary structure predictions for Gp-FAR-1 and its homologues are accurate, are structurally dissimilar to the FABPs. The FABPs have a well-characterised clam-shell structure composed mainly of β-sheet (Xu *et al.*, 1993) whilst Gp-FAR-1 and its

homologues are predicted to be composed mainly of amphipathic α-helix. The structure of NPAs are also predicted to be composed of at least six amphipathic α-helices (McDermott, 2001). These structural predictions are supported by circular dichroism experiments on Ov-FAR-1 and ABA-1 which indicated a highly helical structure in both of these proteins with very little β-structure (Kennedy *et al.*,1997, Kennedy *et al.*,1995a). The structural properties of Gp-FAR-1 were investigated by site-directed mutagenesis and these studies are reported in Chapter 5.

Chapter 5

Site-directed mutagenesis of rGp-FAR-1

5.1 Introduction

Site-directed mutagenesis (SDM) is a technique widely used for the investigation of the role of single amino acids in the function or structure of a protein. For example, this technique was recently used to engineer disulfide bridges between the helices of the insect exchangeable apoliprotein, ApoLpIII, imposing a structural constraint that prevented ApoLpIII from forming discs with dimyristoylphosphatidylcholine (DMPC), a property characteristic of ApoLpIIIs (Narayanaswami *et al.*, 1996). This showed that the helices in these proteins require a certain degree of motional freedom for the normal functioning of the protein.

The value of this technique in studying the structure and function of proteins has also been shown with the *Ascaris suum* nematode polyprotein allergen monomer, rABA-1A (McDermott, 2001). Site-directed mutagenesis was used to substitute a conserved leucine residue with an arginine residue. This substitution was shown to alter the structural integrity and ligand-binding function of the mutant protein, indicating that this residue may be involved in ligand binding (McDermott *et al.*, 2001).

No empirical structural information yet exists for the FAR proteins, apart from circular dichroism analysis which showed that at least one member of the class is rich in α-helix with little evidence of beta structures (Kennedy *et al.*, 1997). Secondary structure prediction algorithms for Gp-FAR-1 also predict extensive helicity, and helical wheel projections indicate the presence of amphipathic alpha-helices. The hydrophobic faces of these helical stretches probably fold towards the interior of the protein and may also comprise the sides of a binding pocket or groove for hydrophobic ligands, a common feature of α-helical lipid-binding proteins such as plant non-specific LTPs (Tassin *et al.*, 1998).

Three highly conserved and thus potentially functionally or structurally important residues predicted to lie within the hydrophobic faces of GP-FAR-1 were subjected to SDM in order to attempt to identify and characterise side chains that are crucial for ligand binding or the structural integrity of the binding site of Gp-FAR-1.

5.2 Materials and Methods

5.2.1 Site-directed mutagenesis

5.2.1.1 Alkaline denaturation of template DNA

pGEX-2T plasmid containing a GST:*Gp-FAR-1* gene fusion was transformed into *E. coli* JM109 cells, then plasmid was harvested from overnight cultures as described in Chapter 2. The purity of the plasmid was verified by standard ethidium bromide agarose gel electrophoresis and the DNA quantified by comparison with known DNA standards.

The template DNA was denatured by incubation in 0.2M NaOH, 0.2mM EDTA for 5 minutes at room temperature. Denatured plasmid was then precipitated by addition of 0.1vol 2M ammonium acetate (pH 4.6) and 3.75vols 100% ethanol followed by incubation at -70°C for 30 minutes. Samples were then precipitated by centrifugation at 14000g for 15 minutes at 4°C. After washing in 250µl 70% ethanol and spinning as above, pellets were resuspended in 100 µl TE buffer (pH 8.0) and an aliquot analysed by agarose gel electrophoresis to verify recovery of denatured template

5.2.1.2 5'-Phosphorylation of mutagenic oligonucleotides

The mutagenic primers used in this study were:

K78Q: - atggaagcgctg**c**aggcaaagagcgag K80R: - gcgctgaaggcaa**g**gagcgagaagctg

K80Q: - gcgctgaaggca**c**agagcgagaagctg F67W: - cgccatgaagaat**gg**cagactgaagaa

K78R: - atggaagcgctga**g**ggcaaagagcgag

The above primers were obtained from Genosys and were 5'- phosphorylated before use as follows: 100pmol of oligonucleotide was incubated for 30 minutes at 37 °C with 5 units T4 polynucleotide kinase (Promega) and 1mM ATP in kinase buffer (70mM Tris-HCl (pH 7.6), 10mM $MgCl_2$, 5mM dithiothreitol) (Promega). Reactions were then incubated at 70 °C for 10 minutes to inactivate the enzyme. Samples were stored at -20 °C until required.

5.2.1.3 Oligonucleotide hybridisation reactions

Oligonucleotides were annealed to template DNA by addition of 0.25pmol of Selection Oligonucleotide and 1.25pmol of mutagenic oligonucleotide to 0.05pmol of denatured template DNA in annealing buffer (20mM Tris-HCl (pH 7.5), 10mM $MgCl_2$, 50mM NaCl) (Promega). Reactions were incubated for 5 minutes at 75 °C then cooled to 37 °C at a rate of 1.5 °C per minute on a Hybaid Omnigene thermal cycler.

5.2.1.4 Mutant strand synthesis and ligation

To drive mutant strand synthesis and ligation, the annealing reactions were incubated overnight at 4°C with 5-10 units of T4 DNA polymerase (Promega) and 1-3 units of T4 DNA ligase (Promega) in Synthesis buffer (10mM Tris-HCl (pH 7.5), 0.5mM dNTPs, 1mM ATP, 2mM DTT) (Promega).

5.2.1.5 Transformation of mutant clones

To select for cells containing mutated plasmid, BMH 71–18*mut*S cells transformed with the mutagenesis reactions using standard heat shock protocols

(Sambrook *et al.*, 1989) were grown overnight in LB broth containing ampicillin and Antibiotic Selection Mix (ASM) (Promega) at 37°C with vigorous shaking to ensure adequate aeration. Plasmid was then purified from these cultures using standard protocols and transformed into *E. coli* JM109 cells, a stable host suitable for long term maintenance of the mutated plasmids, then plated on LB with 100μgml^{-1} ampicillin and 50μl ASM and incubated overnight at 37 °C to select for transformants.

5.2.2 Sequencing of mutant clones

Mutated plasmids were sequenced (see 2.4.2) to confirm the presence of mutations.

5.2.3 Expression of mutant forms of Gp-FAR-1

Mutated plasmids were transformed into *E. coli* BL21 cells and the mutant proteins expressed and purified as described in 2.2. The presence and purity of mutant proteins was confirmed by SDS-PAGE analysis (see 2.2.3).

5.2.4 Ligand binding by mutant proteins

5.2.4.1 Binding of fluorescent and non-fluorescent ligands

To assess whether any of the mutations affected the function of Gp-FAR-1, fluorescent ligand binding studies were carried out on the mutant forms of Gp-FAR-1 with fluorescent and non-fluorescent ligands, as described in 2.3.1 and 2.3.2 respectively.

Fluorimetric dissociation curve titrations were carried out on the mutants to assess the effect of the mutations on the binding affinity of Gp-FAR-1 for ligand. Successive 5μl additions of ~50μM wild-type and Gp-FAR-1 mutants were made to 35μM solutions of *cis*-parinaric acid in PBS and fluorescence emission intensity at 413nm (the wavelength of maximum fluorescence emission) was recorded. To obtain

dissociation constants for retinol with these proteins, 5µl additions of a 115µM solution of all *trans*-retinol were made to cuvettes containing 3µM solutions of wild-type and mutant Gp-FAR-1 and the fluorescence emission intensity at 472nm was recorded. After dilution factor corrections, the above data were analysed to estimate dissociation constants as described in 2.3.3.

5.2.4.2 Gp-FAR-1 F67W mutant intrinsic tryptophan fluorescence

5.2.4.2.1 Intrinsic tryptophan fluorescence and ligand-binding

The intrinsic fluorescence of tryptophan was utilised to investigate the proximity of the tryptophan residue to the binding site of Gp-FAR-1 F67W mutant . Solutions of ~10µM F67W in PBS were excited at 290nm and maximum fluorescence emission values were noted. 10µl additions of thousand-, hundred- and ten-fold dilutions of solutions of 12µM linolenic and 2.5µM oleic acids were made and fluorescence emission spectra recorded.

5.2.4.2.2 Succinimide quenching of intrinsic tryptophan fluorescence

The solvent accessibility of the tryptophan residue in F67W was analysed by succinimide quenching using the Stern-Volmer equation:

$$F_0/F = 1 + K_{sv}[Q],$$

Where F_0 = fluorescence intensity in the absence of quencher, F = fluorescence intensity in the presence of quencher, K_{sv} = Stern-Volmer constant and [Q] = concentration of quencher.

To obtain values for F, 7µM solutions of Gp-FAR-1 F67W or rABA-1 in PBS were excited at 290nm and the fluorescence emission intensity at 335nm and 306nm (the wavelengths of maximum fluorescence emission) respectively were measured upon

successive 20µl additions of a 2.5M solution of recrystallised succinimide (Sigma). The above experiment was repeated with 7µM solutions of F67W and ABA-1 which had been denatured by pre-incubation in 6M guanidine hydrochloride to allow comparison of tryptophan accessibility in the denatured and non-denatured proteins. Maximal fluorescence emission occurred at 351nm and 356nm, respectively, in the denatured proteins. Succinimide quenching was also measured as above in a 2.5µM solution of L-tryptophan (L-Trp) (Sigma) representing free, fully accessible tryptophan, the wavelength of maximum fluorescence emission of which occurred at 356nm.

To obtain values for F_0 , 20µl aliquots of PBS were substituted for succinimide in all the above samples and fluorescence emission measured at the relevant wavelengths. Values obtained for F_0/F were plotted against [Q] and the K_{sv} values obtained by measuring the slopes produced.

5.2.4.2.3 Fluorescence resonance energy transfer

Fluorescence resonance energy transfer (FRET) was used to investigate the proximity between the tryptophan residue and bound ligand in rGp-FAR-1 F67W. A 50µM solution of rGp-FAR-1 F67W was excited at 290nm and the wavelength spectra and emission recorded at 333nm. Successive 10µl additions of 65µM *cis*-parinaric acid were made and the emission measured as before. This experiment was repeated using wild-type rGp-FAR-1 and the data subtracted from the above sample to deduct any inherent background emission present in the tryptophan-less wild-type protein. Dilution factor corrections were made as described in 2.3.3.

5.3 Results

5.3.1 Generation and Expression of Mutant Forms of GP-FAR-1

After discussion with crystallographers (J. Beauchamp, pers. comm.), several residues in Gp-FAR-1 were selected for mutagenesis. Residues within a highly conserved region between residues 65 and 95 (Figure 5.1) were selected as a target for mutation and substitutions were recommended which would be unlikely to disrupt the overall structure of the protein. Since Gp-FAR-1 has no tryptophan residues, a tryptophan was substituted for the phenylalanine residue at position 67 (Phe67) which lies within the most conserved region of this protein. Fluorescence quenching experiments, should, therefore, give information on the environment surrounding the Trp residue. Such experiments could help to locate the protein's binding site, test the accuracy of the structural predictions and provide information on the tertiary structure of Gp-FAR-1.

Two amino acids close to Phe-67 which are either absolutely conserved (Lys-78) or conserved in all but one sequence (Lys-80, conservatively substituted by an Arg in the *A. viteae* FAR protein (Blaxter, 1998), were also targeted for further site directed mutagenesis. It was postulated that the observed strong conservation across species may indicate importance for maintaining the structural integrity and/or function of Gp-FAR-1. These residues were substituted by either arginine (which, like the naturally occurring residue lysine, is basic and positively charged at neutral pH, but has a bulkier side chain), or glutamine (which is of a similar size to lysine but charge neutral). It was hoped that by selecting these relatively conservative substitutions the stability of the protein structure would not be jeopardised. Selection of residues also took into account the likelihood that certain residues would be crucial to the overall stability of the protein structure and so mutation of these would be likely to disrupt the protein structure.

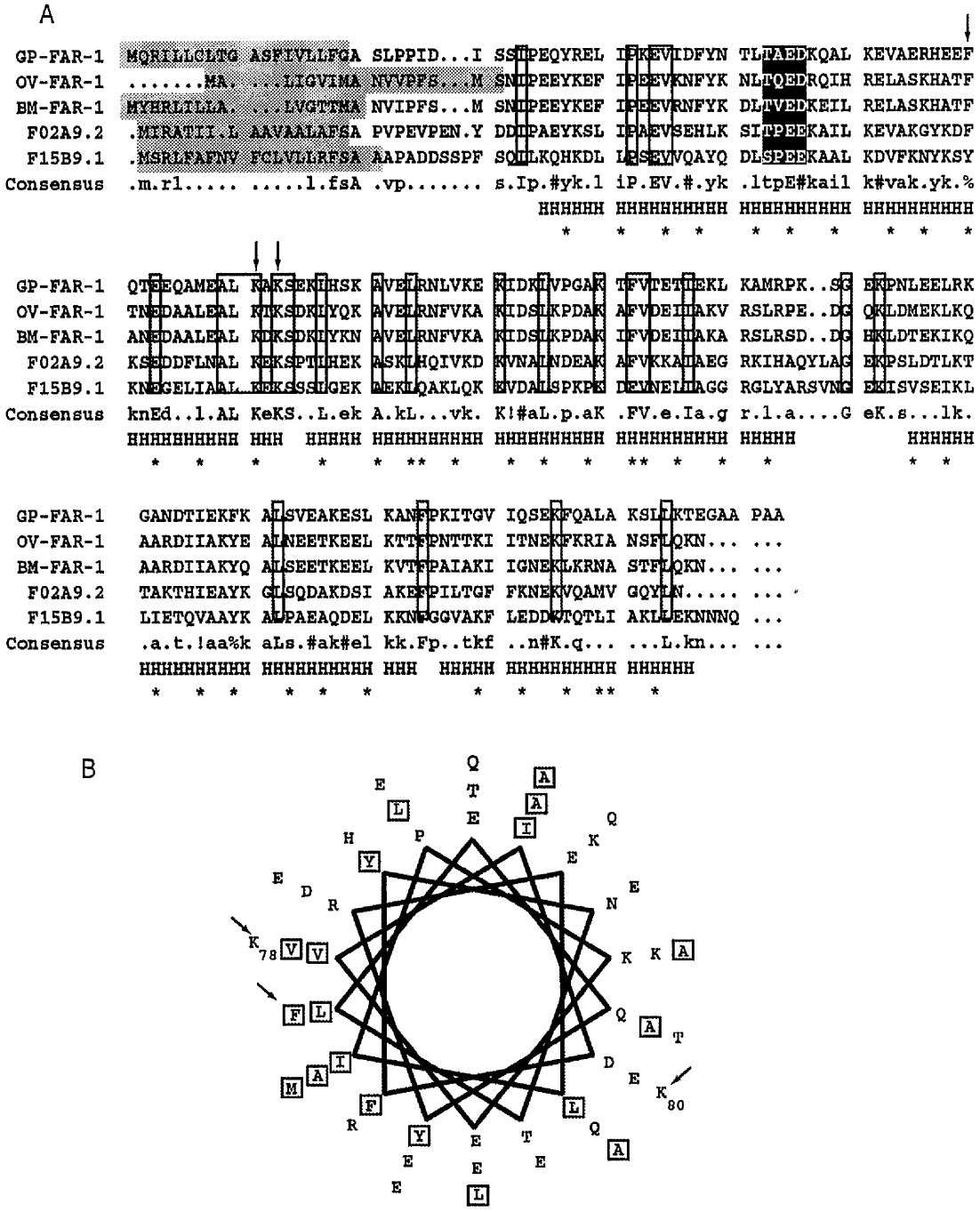


Figure 5.1. Sequence analysis of the FAR family of proteins. **A.** Multiple alignment with selected FAR sequences to illustrate the diversity of the sequences from different species of nematode and the regions and sites of greatest conservation. Identical amino acids are indicated by clear boxes, and conserved positions predicted to lie at the hydrophobic face of amphipathic helices are indicated by asterisks below the alignment. The grey boxes indicate potential signal peptides as predicted by SignalP (Nielsen *et al.*, 1997), and the black boxes indicate a potential casein kinase II site, the position of which is conserved in all counterparts studied so far (Kennedy *et al.*., 1997). The consensus line indicates absolutely conserved amino acid positions as well as amino acids considered to be functionally conserved with the Dayhoff substitiution matrix. Absolutely conserved residues are given as capitals, lower case represents positions conserved in more than half of the sequences, ! represents I or V (single-letter amino acid codes), % represents F or Y and # represents any one of N, D, Q or E. The secondary structure line is derived from an analysis of the sequences by the PHD program (http://cubic.bioc.columbia.edu./predictprotein/), in which H represents a predicted a-helix and gaps indicate regions for which no structural prediction emerged. Amino acids selected for site-directed mutagenesis are indicated by arrows. Ov-FAR-1, *Onchocerca volvulus* homolog (Tree *et al.*., 1995); Bm-FAR-1, *Brugia malayi* homolog; F02A9.2 and F15B9.1, two of six potential *C. elegans* counterparts predicted from the genome sequence of this species. **B.** Helical wheel projection of the region between Glu-31 and Lys-80 to show the strong amphipathicity of the predicted helix. Conserved hydrophobic positions are boxed and the positions selected for site-directed mutagenesis are indicated by arrowsindicated by arrows.

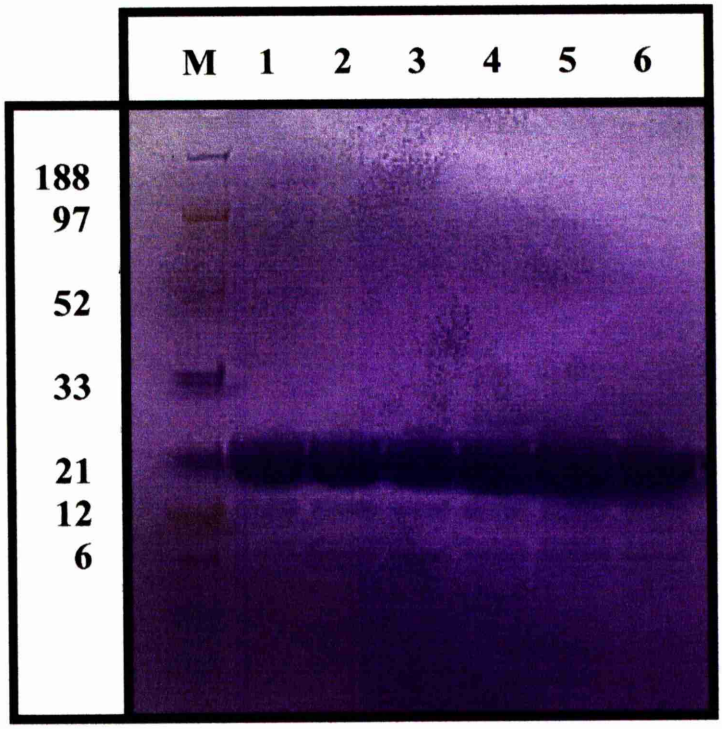


Figure 5.2. SDS-PAGE analysis of recombinant Gp-FAR-1 and its mutant forms. M - Molecular weight markers (kDa); 1 - wild-type Gp-FAR-1; 2 - K78Q; 3 - K80Q; 4 - K78R; 5 - K80R; 6 - F67W.

Sequence analysis confirmed that the desired mutations had been introduced and all mutant proteins were successfully expressed, as described previously for Gp-FAR-1, and purified to homogeneity as assessed by the presence of single bands upon SDS-PAGE analysis (Figure 5.2).

5.3.2 Functional analysis of Gp-FAR-1 mutants

5.3.2.1 Ligand-binding of rGp-FAR-1 mutants

All fluorescent probes and fatty acids which bound to rGp-FAR-1 (C11-C22) (see Chapter 4) were tested for binding to the mutant forms of Gp-FAR-1 using the spectrofluorimetry techniques described in Chapter 4. All the mutant proteins bound the same range of ligands, and where relevant with the same emission maxima, as wild-type Gp-FAR-1, although no quantitative measurements of K_d were made for the ligands other than for *cis*-parinaric acid. Typical fluorescence emission spectra obtained for Gp-FAR-1 and its mutant forms are given in Figures 5.3 & 5.4.

5.3.2.2 Dissociation constant estimation of rGp-FAR-1 mutants

When Lys-78 was substituted with the similarly sized but charge neutral amino acid Gln, there was a slight increase in binding strength in titration experiments with *cis*-parinaric acid, changing the K_d from 77 nM for the wild-type protein to 47nM indicating that the ligand is bound more tightly in this mutant (Table 5.1 & Figure 5.5). This may indicate that a positive charge is required in this position for efficient dissociation of ligand from the binding site of the protein. Substitution of Lys-78 instead with the larger amino acid Arg resulted in a higher mean K_d value of 200nM, indicating that ligand binding is weakened in this mutant (Table 5.1 & Figure 5.6). One interpretation of this data is that the bulkier arginine side-chain prevents efficient entry

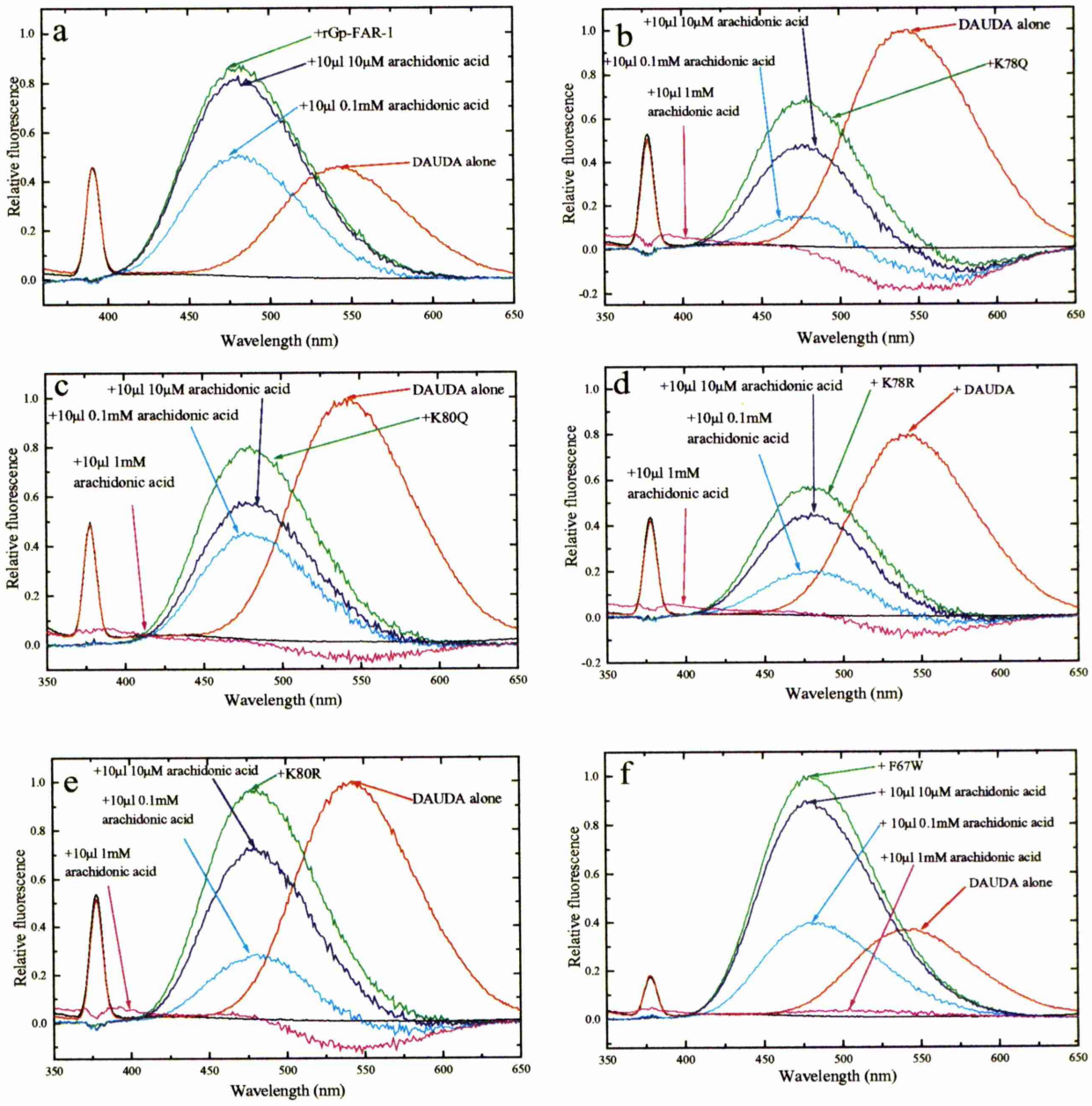


Figure 5.3. Binding of DAUDA to rGp-FAR-1 and its mutant forms and competitive displacement of DAUDA by arachidonic acid. Fluorescence emission spectra of DAUDA in buffer (red), in the presence of 1.2µM protein (green), upon addition of 10µl of 10µM (blue), 0.1mM (cyan) and 1mM (pink) arachidonic acid. a) rGP-FAR-1, b) K78Q, c) K80Q, d) K78R, e)K80R and f) F67W.

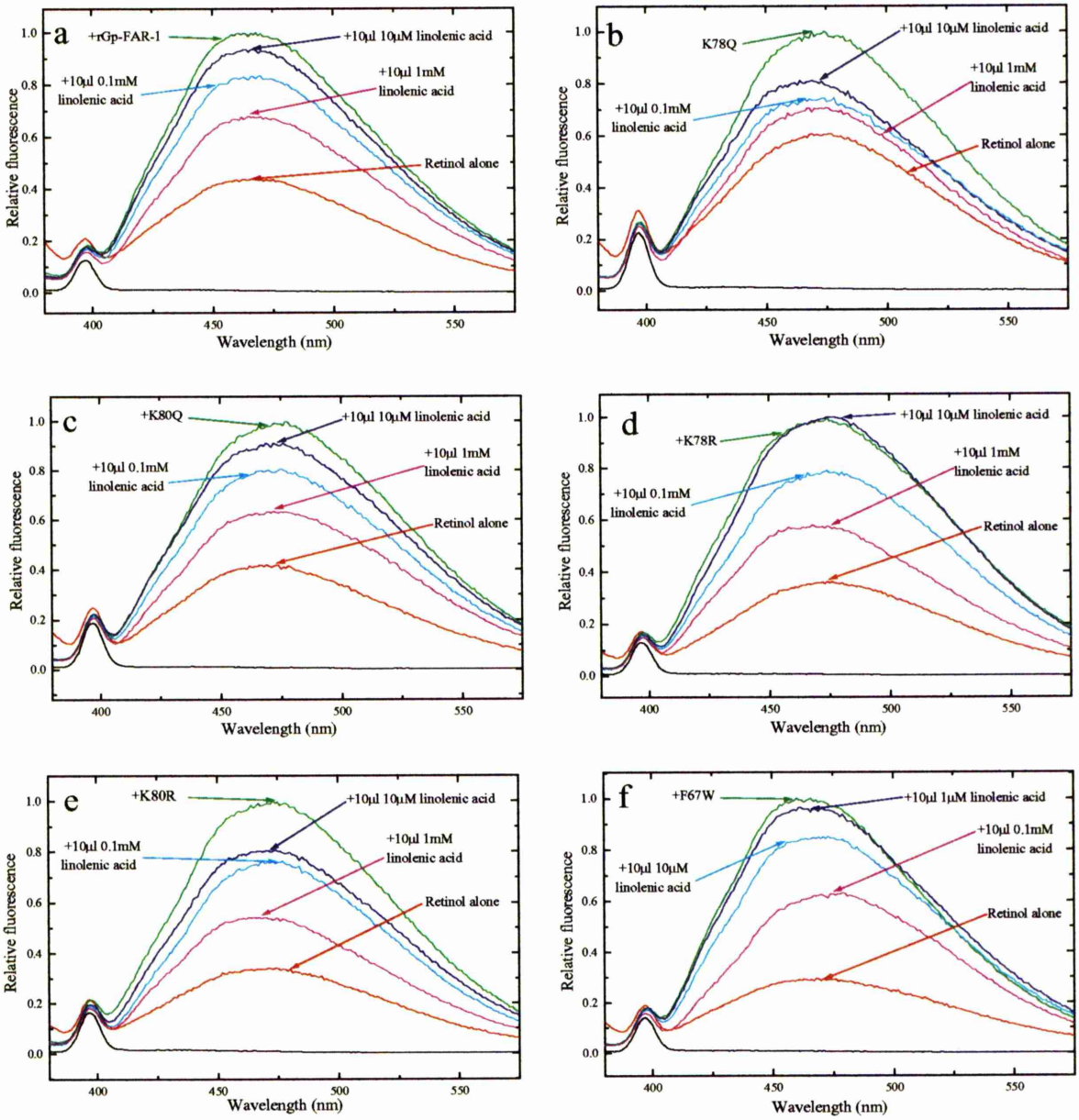


Figure 5.4. Binding of retinol to rGp-FAR-1 and its mutant forms and competitive displacement of retinol by linolenic acid. Fluorescence emission spectra of retinol in buffer (red), in the presence of 1.2µM protein (green) and upon addition of 10µl of 10µM (blue), 0.1mM (cyan) and 1mM (pink) linolenic acid. a) rGP-FAR-1, b) K78Q, c) K80Q, d) K78R, e)K80R and f) F67W.

of ligand into the binding site or in someway disrupts the binding of ligand within the binding site.

Lys80 is conserved in all but one of the the FAR family of proteins (but is not in the hydrophobic face of the helix if the helical wheel prediction is accurate), and a Lys-80 to Gln substitution resulted in an increased K_d of 226 nM, suggesting that a positive charge may be required for efficient ligand binding in this position or to maintain the structure of the protein (Table 5.1 & Figure 5.5). Indeed, the K80R substitution had no significant effect on ligand-binding (Table 5.1 & Figure 5.6), yielding a K_d of 73nM, indicating that the positive charge is more important at this position in the protein than the size of the residue, whether involved in structure maintenance or direct interaction with ligand. This is supported by the presence of a natural Lys to Arg substitution at this position in the FAR-1 homolog from *A. viteae*. Given the binding inhibition seen on ablation of the positive charge in this position coupled with the lack of effect of the larger positively-charged residue arginine at this position, it is tempting to speculate that the positive charge at this position may be involved in the binding of ligand, perhaps by interaction with the negatively charged carboxyl group of the fatty acid. However, further work would be required to conclusively demonstrate this. It is possible then that the K80 residue is in the binding site in an area where a where a positive charge is required but a larger side-chain does not interfere with ligand-binding. Alternatively, it is possible that the K80 is not in the binding site, but its positive charge is involved in maintaining the stability of the binding site structure.

Attempts to estimate dissociation constants for retinol binding to rGp-FAR-1 mutants proved unsuccessful so it is possible that binding of retinol was more affected by these mutations. However, this is unlikely as all mutants did bind retinol in the ligand-binding assays performed and competitive displacement of retinol by non-

Mutant	Effect of substitution	Dissociation constant (K_d) (nM)	Apparent molar ratio (n)
rGp-FAR-1 (wild-type)	n.a.	74 80 (mean 77)	1.59 1.97
K78Q	Abolition of positive charge	58 45 38 (mean 47)	1.03 1.06 2.78
K80Q	Abolition of positive charge	226	2.14
K78R	Increase in size of residue	199 207 (mean 203)	1.47 2.14
K80R	Increase in size of residue	62 84 (mean 73)	2.28 2.10
F67W	Introduction of intrinsically fluorescent residue	90 96 (mean 93)	1.77 0.76

Table 5.1 Dissociation constants (Kd) of wild-type rGp-FAR-1 and its mutant forms with *cis*-parinaric acid.

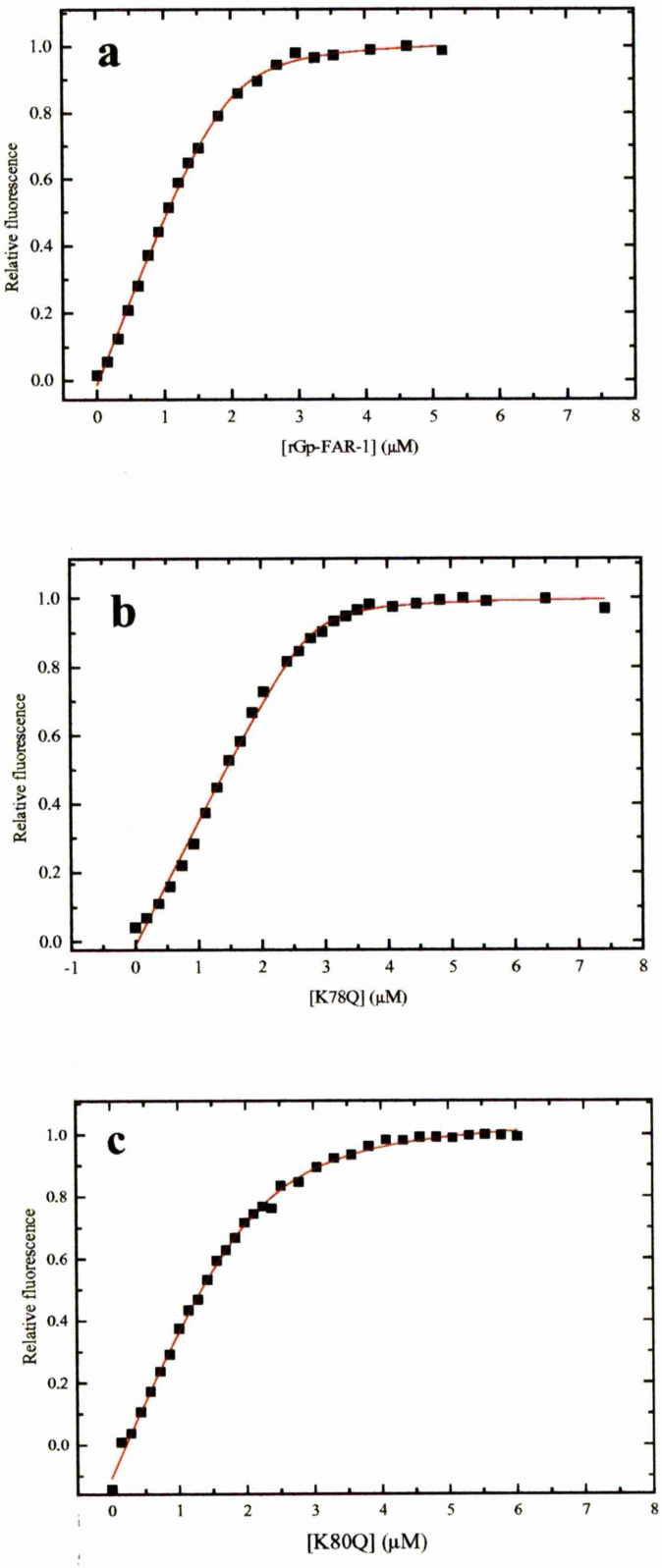


Figure 5.5. (a) Fluorescence titration of 1μM *cis*-parinaric acid with rGp-FAR-1 yielding a mean dissociation constant (K_d) of 77nM. (b) Fluorescence titration of 1μM *cis*-parinaric acid with K78Q yielding a mean K_d of 38nM. (c) Fluorescence titration of 1μM *cis*-parinaric acid with K80Q yielding a mean K_d of 226nM. K_d values reported represent the means of two independent titrations

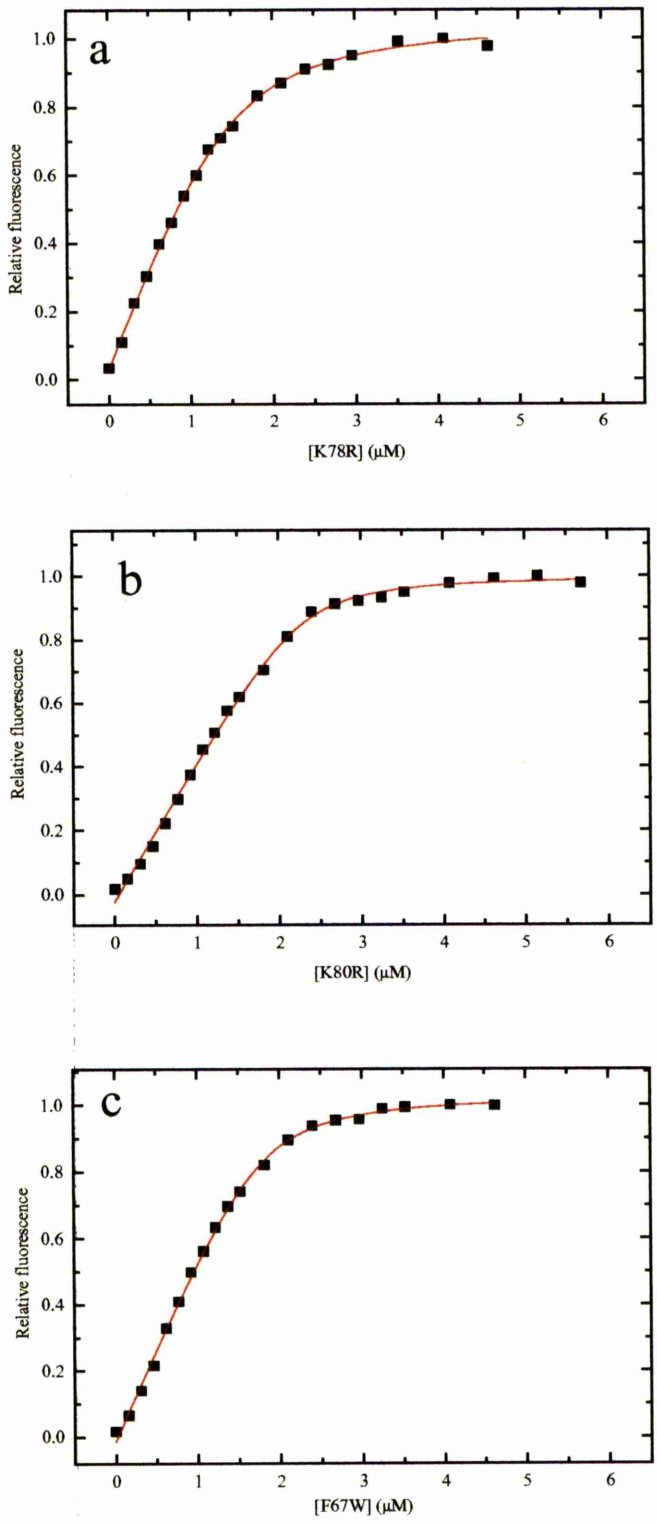


Figure 5.6. (a) Fluorescence titration of 1μM *cis*-parinaric acid with K78R yielding a mean dissociation constant (K_d) of 199nM. (b) Fluorescence titration of 1μM *cis*-parinaric acid with K80R yielding a mean K_d of 62nM. (c) Fluorescence titration of 1μM *cis*-parinaric acid with F67W yielding a mean K_d of 90nM. K_d values reported represent the means of two independent titrations.

fluorescent fatty acids was observed in all mutants. Since dissociation constants were only obtained for *cis*-parinaric acid, it is possible that the mutant proteins have altered affinities for other ligands. However, any such effects would likely be small as no significant differences were observed in the emission spectra of the ligands tested.

None of the substitutions produced a radical change in the ligand binding i.e., complete abolition of binding. Given the absolute or strong conservation of the positions in an otherwise sequence diverse set of proteins, it therefore seems likely that these residues are essential for some purpose other than direct and essential involvement in ligand binding.

It is possible that the amino acid substitutions used were too conservative and if the residues had been subjected to more radical mutations such as changing to negatively charged or hydrophobic amino acids a larger effect would have been observed. Such radical substitutions, however, would be more likely to destabilise the protein structure rather than ablating function in protein with native structure and would require parallel structural studies, such as CD or NMR to be performed. Since recombinant protein was used it was not post-translationally modified and this may have affected the protein's function. For example, Soulages *et al*, (1998) found differences in the activity of glycosylated and unglycosylated forms of ApoLpIII. It is thus possible that, due to a lack of post-translational modification, the function of recombinant Gp-FAR-1 is inhibited. Unfortunately, because of the small size and endoparasitic life-cycle of these nematodes it was not possible to obtain native protein for use in these studies. Another possibility is that the conservation of the mutated residues has arisen by chance and that a hydrophobic environment is all that is required for ligand binding. However, this is perhaps unlikely because although ligand-binding was not ablated, the dissociation constants were altered in some cases.

5.3.3 Insertion of a tryptophan reporter probe

To confirm that, as predicted by helical wheel projections, the predicted hydrophobic faces of the helices are internally orientated i.e., away from polar solvent, a tryptophan residue was substituted for Phe 67, a residue located in the most highly conserved region of the protein with strong amino acid conservation amongst all species and for which amphipathicity was strongly predicted (Figure 5.1). This conservative substitution should not disrupt the structure and function of the protein, but provides a reporter probe to study the predicted interior of the protein by using the fluorescence emission characteristics of the introduced (and solitary) tryptophan. Like wild-type Gp-FAR-1, the F67W mutant protein bound the fluorescent ligands, DAUDA, retinol and *cis*-parinaric acid with the same emission maxima as wild-type protein. F67W also bound the same range of non-fluorescent ligands as the wild-type. Typical emission spectra obtained are shown in Figures 5.3 & 5.4 and upon titration with *cis*-parinaric acid yielded an apparent K_d value of 90nM which is of the same order of magnitude as that obtained for wild-type rGp-FAR-1 (77nM) (Figure 5.6). These results indicate that the overall structure and function of the protein was essentially unchanged in F67W and thus could be used in further experiments to represent wild-type rGp-FAR-1.

5.3.3.1 Intrinsic Tryptophan Fluorescence

As wild-type Gp-FAR-1 contains no tryptophan (Trp) residues, it was possible to study the fluorescence emission properties of the inserted Trp in isolation. The maximum fluorescence emission When excited at 290 nm, the maximum wavelength emission of the first batch of F67W protein produced was 338 nm (Fig 5.7) indicating that the introduced Trp is not exposed to solvent water but is buried to a significant extent within the protein (Etfink & Ghiron, 1976) as predicted. A blue shift of 6nm to 332nm was observed upon addition of linoleic acid to F67W indicating that upon ligand

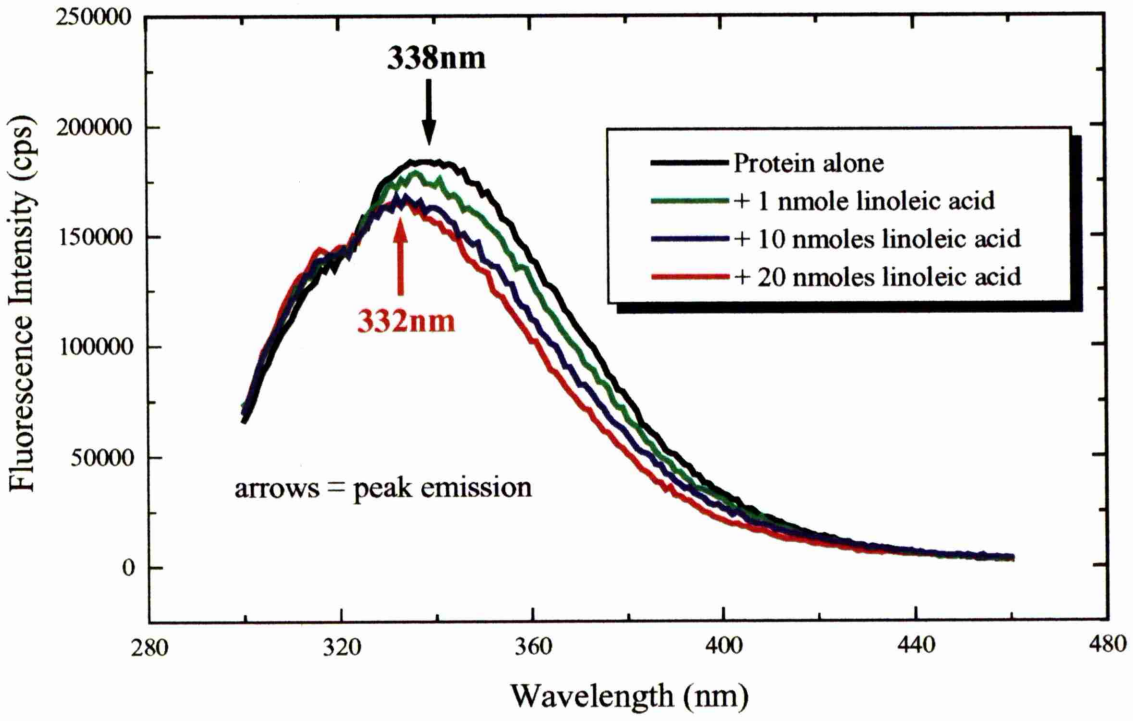


Figure 5.7. Fluorescence emission of the F67W reporter Trp residue in the absence and presence of ligand showing a clear 6nm blue shift upon ligand binding (λ_{ex}.290nm)

binding, the environment surrounding the Trp residue becomes more apolar. This indicates that the Trp residue lies within or in close proximity to the ligand binding site. However, this result could not be reproduced with fresh batches of F67W which had emission maxima at 335nm that did not shift upon addition of oleic or linoleic acids. It is possible that the observed blue shift upon addition of ligand was an erroneous result caused by contamination of the F67W during expression and purification, perhaps with Trp-containing *E.coli* proteins or the GST fusion moiety. Alternatively, it is possible that this was a true result but that subsequent batches of the F67W protein contained incorrectly folded or inactive recombinant protein. Indeed, this was thought to be the source of inaccuracies in the apparent molar ratio estimations for the FAR-1 titrations reported in Chapter 4.

These results show that the Trp residue is buried within the protein interior away from solvent water given the emission maxima between 335 and 338nm (compared to 356nm for free tryptophan in solution). However, it is not possible to conclude with any certainty whether the Trp is indeed in the binding site or involved in interaction with ligand.

To attempt to answer this question, fluorescent resonance energy transfer (FRET) was carried out which could give a measure of the proximity of the Trp residue to bound ligand (in this case *cis*-parinaric acid). Unfortunately, results from these experiments were not conclusive, presumably due to fluorescence interference from the two tyrosine residues present in this protein, even after subtraction of the fluorescence emission of wild-type rGp-FAR-1 (i.e., with no tryptophan residue) under the same conditions.

5.3.3.2 Succinimide Quenching

To further study the environment surrounding the introduced Trp, succinimide quenching was carried out on F67W and denatured F67W, which had been incubated in 6M guanidinium chloride to unfold the protein. For comparison this technique was also carried out on the *A. suum* NPA monomer, rABA-1, and L-Trp to represent free tryptophan in polar solvent (Figure 5.8). The Stern-Volmer constant (K_{sv}) obtained for L-Trp was $14.4M^{-1}$ showing a high degree of quenching by succinimide and indicating that, as expected, the tryptophan was freely accessible to the succinimide molecules. The Stern-Volmer constant obtained for F67W was $0.74M^{-1}$ indicating that the Trp probe is indeed internally located, where the large succinimide molecules have difficulty in gaining access. Unfolding of the F67W protein in 6M GdmCl led to a red shift in the emission peak from 335nm to 351nm and resulted in a K_{sv} for succinimide quenching of 2.64 M^{-1} (Figure 5.8) indicating the increased accessibility of the Trp residue to succinimide. This is of a similar order to that observed for other single Trp-containing proteins when unfolded (e.g., Idziak *et al.*, 1997, Boam *et al.*, 1997 & Kennedy *et al.*, 1995).

The Stern-Volmer constants of $0.34M^{-1}$ and $4.4M^{-1}$ obtained for the positive control protein rABA-1 and denatured ABA-1 respectively, indicate that the tryptophan is more deeply buried in this protein than the Trp residue in F67W and upon denaturation the tryptophan is more accessible to the succinimide than that of denatured F67W. To place these results in context, the K_{sv} (for succinimide quenching) of various proteins containing a single Trp range from 0.26 M^{-1} for a highly buried Trp in Type I dehydroquinase from *Salmonella typhi* (Moore *et al.*, 1993), to 4.8 M^{-1} and 3.2 M^{-1} for shikimate kinase of *Erwinia chrystanthemi* and Type II dehydroquinase from *Streptomyces coelicolor*, respectively (Idziak *et al.*, 1997, Boam *et al.*, 1997). These

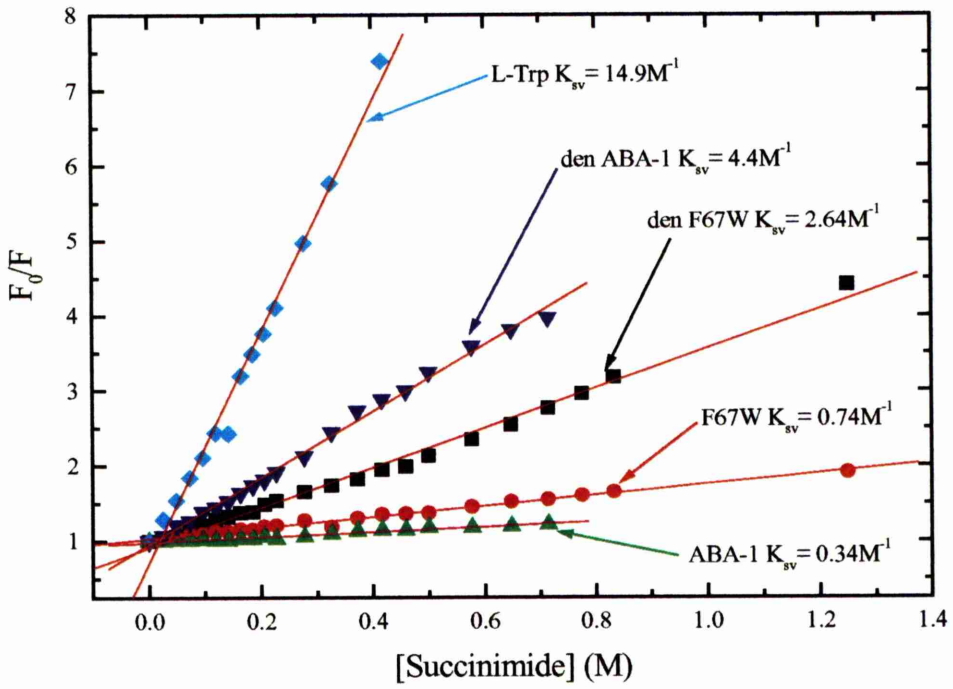


Figure 5.8. Exposure of tryptophan side chains to solvent in F67W and ABA-1. Stern-Volmer plot of native and denatured F67W where F_0 is the fluorescence emission of protein alone in solvent and F is the fluorescence emission of protein in the presence of succinimide yielding K_{sv} values of 0.74M^{-1} and 2.64M^{-1} respectively. Stern-Volmer plots of native and denatured rABA-1 yielding K_{sv} values of 0.34M^{-1} and 4.4M^{-1} respectively are shown for comparison and that of L-Trp, yielding a K_{sv} of 14.9M^{-1} is shown to represent free tryptophan in solution.

results indicate that the Phe-67 position, which lies in the region of strongest amino acid conservation amongst this highly sequence-diverse family of proteins (Figure 5.1), is as predicted, interior to the protein. This supports the secondary structure predictions obtained for Gp-FAR-1. However, in the absence of empirical structural information about the FAR-1 proteins, it is difficult to conclude with any certainty that the conserved residues studied do lie within, or are otherwise involved in, the fatty acid and retinol binding site. Crystals of Gp-FAR-1 have now been produced and X-ray crystallographic studies are underway (M. Kennedy, pers. comm.) and data obtained from this work should give a much clearer picture of the binding site and the overall function of this protein. It would therefore be useful to revisit this data when the crystal structure is elucidated.

5.3 Discussion

The presence of so many different proteins which bind fatty acids and retinol in nematodes and the fact that mutation of the most conserved residues in the FAR (this work) and NPA proteins (McDermott, 2001) did not ablate ligand-binding may indicate that at least some of these proteins have other functions and that the observed binding of fatty acids and retinol is related to that. So, it is possible, although perhaps unlikely, that fatty acid and retinol binding is not the true function of Gp-FAR-1, or that the ligands used are not the true ligands, and that the true function is ablated but is not seen in the assays used. It is also possible that fatty acids and retinol are the true ligands but that the protein has other active domains involved in other functions such as receptor binding or signal transduction and that these functions were ablated but not tested in this assay. For example, fatty acids could play a regulatory role by activating or inhibiting the proteins function upon binding.

Several precedents exist to support this hypothesis. For example, a protein from *D. melanogaster* was originally identified as a retinol and fatty acid-binding protein and was shown to bind fatty acids and retinol with similar affinities to those reported here for Gp-FAR-1 (Kutty *et al.*, 1996). However, similiarity searches showed that this protein was in fact a proapolipophorin, a protein involved in mass transport of lipids and the retinol and fatty acid binding may have a regulatory role. Also, the 21-23kDa lipid-binding proteins (PLTPs) in higher eukaryotes were known to bind phospholipids with high affinity and this was widely held to be the function of these proteins. However, Bernhard & Sommerville (1989) reported amino acid co-identity of these proteins with amylase inhibitors and Bruun *et al.*, (1998) showed that a yeast PLTP, I_c , was a high affinity inhibitor of the protease, carboxypeptidase Y. Also, plant non-specific lipid transfer proteins (nsLTPs) bind lipids with high affinity and this was widely held to be responsible for the reported anti-microbial activity of these proteins. However, Tassin *et al*, (1998) isolated an nsLTP from onion seed, Ace-AMP-1, which did not bind lipids but had much higher anti-microbial activity indicating that lipid-binding was not responsible for this activity. Tassin concluded that considering the various proteins from micro-organisms, animals and plants with different sequences, molecular weights, ligand specificities and structures which can bind lipids this property seems to be general and not clearly related to function. Tassin also concluded that given the various expression and localisation patterns of the nsLTPs these appear to be multi-functional proteins with several different substrates and mechanisms of binding. Thus it is possible that this also applies to Gp-FAR-1.

Similarity searches carried out with the sequences of Gp-FAR-1 and its homologues show that these proteins share low levels of amino acid similarity with the vertebrate exchangeable apolipoprotein apo-AI and to a lesser extent the insect

exchangeable apolipophorin ApoLpIII. These proteins are involved in mass lipid transport and are thought to function in stabilising the surface of lipid-loaded lipoprotein particles. The next chapter in this thesis describes work carried out to investigate the possibility that the FAR-1 proteins have an analogous function in nematodes.

Chapter 6

Potential exchangeable apolipoprotein activity in nematode LBPs

6.1 Introduction

For normal cell functioning, lipids are required to traverse the aqueous environments of cells and tissues from their sites of synthesis to their, often distant, sites of utilisation. Diffusion along the plane of membranes and vesicle transport, where newly-synthesised lipids bud off a membrane as a spherical vesicle, traverse the cell then fuse with an acceptor membrane at the site of utilisation, are two ways in which lipids can be transported within cells (Stryer, 1988). In vertebrates and insects extracellular mass lipid transport is achieved by interaction with lipoproteins. These proteins solubilize their ligands and, often through interaction with receptors, may target these molecules to specific organelles, cells or tissues. The vertebrate lipoprotein system is more complex than that of insects and, as a result, the exact roles of the different exchangeable apolipoprotein classes in vertebrates are not well understood. However, since insects appear to contain only one exchangeable apolipoprotein, ApoLpIII, it has been easier to elucidate the function of these proteins in the insect system. Although functionally indistinguishable, vertebrate and insect lipoproteins exhibit no amino acid identity, but share striking similarities in their secondary and tertiary structure (Borhani *et al.*, 1997, Breiter *et al.,* 1991).

6.1.1 Exchangeable Apolipoprotein Structure

Mammalian plasma lipoproteins are stable complexes of lipids and apolipoproteins, held together by non-covalent forces. Two classes of apolipoprotein are involved in formation of lipoproteins, the exchangeable and non-exchangeable apolipoproteins. The non-exchangeable apolipoproteins and lipids form a spherical assembly with a hydrophobic core of cholesterol esters and triglycerides surrounded by a monolayer of phospholipids, free cholesterol and protein (Shen *et al.,* 1977).

Exchangeable apolipoproteins are defined as those capable of moving from one lipoprotein particle to another and these exist in two forms, lipid-free and lipid-bound. These proteins are members of a class of amphipathic α-helical proteins, which bind reversibly to hydrophobic regions or defects on the surface of lipoprotein particles and regulate their metabolism. The non-exchangeable apolipoproteins are so called because they form the core of these particles and cannot therefore move between lipoprotein particles.

Insects have a single, high density haemolymph lipoprotein, lipophorin, which is analogous to the plasma lipoproteins in mammals. This is composed of a hydrophobic core consisting of diacylglycerols, sterols, carotenoids and hydrocarbons, covered with a surface monolayer of phospholipids and the non-exchangeable lipoproteins, lipophorin I & II, which are synthesised in, and secreted from the fat body into the haemolymph in response to adipokinetic hormone, the lipid mobilizing hormone of insects, which is the functional equivalent of human hormone-sensitive lipase (reviewed by Ryan & Van der Horst, 2000). This insect hormone activates a lipase which converts triacylglycerol into diacylglycerols, which leave the fat body and associate with high density lipophorin (HDLp) in the haemolymph. As the diacylglycerol content increases, hydrophobic lipid patches form on the surface of the lipoprotein, reducing its solubility in the haemolymph. Apolipophorin III (ApoLpIII) molecules, insect analogs of the apoE, apoA-I, apoA-IVand apoC family of mammalian exchangeable apolipoproteins, which, in the absence of hormone, exist in a soluble, monomeric, lipid-free state in the haemolymph, attach to these patches, forming low density lipophorin (LDLp). It is thought that the stabilising effect of ApoLpIII's retains the solubility of the lipophorin particles and permits further diacylglycerol loading of lipophorin for delivery to sites of utilisation (reviewed by Ryan & Van der Horst, 2000).

ApoLpIII's have now been identified in several insect species representing five orders and twelve families e.g. *Manduca sexta*, *Locusta migratoria*, *Acheta domesticus*, *Galleria melonella* and *Derobrachus germinatus,* and all have been shown to share common characteristics.

ApoLpIII's are small 17-22kDa proteins composed of 160-170 amino acids with putative cleavable signal peptides 18-23 amino acids long. In some species, e.g., *Locusta migratoria* and *D. germinatus*, ApoLpIII is glycosylated, but in others, e.g., *A. domesticus* and *Manduca sexta*, it is not (Soulages *et al.*, 1998, Weers *et al.*, 2000). The 3-D structure of *L. migratoria* apoLpIII has been determined by X-ray crystallography (Breiter *et al.*, 1991) and is composed of five amphipathic α-helices connected by short loops and arranged as simple up and down helical bundles which are oriented with their hydrophobic residues facing inwards to the centre of the protein and the hydrophilic residues facing outward to the aqueous environment. Although the insect apoLpIII's have low amino acid sequence similarity with one another, the distribution of hydrophobic and hydrophilic residues are similar, and these proteins are functionally indistinguishable. The tertiary structure of the 22kDa N-terminal lipid-binding fragment of human apolipoprotein E (apoE), which consists of an amphipathic four-helix bundle, is also similar to apoLpIII. Indeed, Singh *et al.* (1992), showed that *M.sexta* apoLpIII can associate with human LDL, and Lui *et al.* (1993), reported that human apoA-I can replace apoLpIII on insect LDLp. This was an important finding which, given the lack of amino acid similarity between the mammalian and insect apolipoproteins, showed that secondary and tertiary structure is, in this case at least, more important than primary structure (ie amino acid identity) in defining the functionality of these proteins (Kiss *et al.*, 2001).

Thus, although insect and vertebrate exchangeable apolipoproteins share a common function in assisting lipid transport, precise amino acid identity is less important than the common structural feature of multiple amphipathic helices.

The model for binding of ApoLpIII to lipophorin as proposed by Breiter *et al.*, (1991) consists of binding of apoLpIII to an exposed lipid patch on the lipoprotein surface via two leucines which project into the haemolymph between helices 1 and 2, and 3 and 4, and these leucines are conserved around residues 32 and 93 in the apoLpIII's characterised to date. Upon binding to the lipid surface, the apoLpIII molecules undergo a conformational alteration and it is postulated that the helices separate and spread over the lipophorin surface, with the hydrophobic residues in contact with the lipid surface and the polar residues facing out to the haemolymph. Indeed, it has been shown that by engineering disulfide bridges between the helices, the ability of apoLpIII to bind to lipophorin is lost (Narayanaswami *et al.*, 1996). The mammalian lipoprotein system is more complex than that of insects, with several different types of exchangeable apolipoproteins. The function of several of these is not well understood. However, X-ray crystallographic data indicates that the apoAI and ApoE apolipoproteins function in a similar manner to ApoLpIII.

6.1.2 Proposed functions of apolipoproteins

Originally, the main function of apoLpIII was thought to be in the transfer of diacylglycerols from the fat body to muscles to fuel prolonged flight (Chino, 1997). However, Smith *et al.* (1994), contend that, as ancestral apoLpIII arose before the development of insect flight, and as several non-flying insects have apoLpIII, these proteins probably have a more general function in delivery of lipids to various tissues for a range of physiological functions. An example of this is apoLpIII in larval *M.sexta*,

which is not bound to lipophorin in normal conditions, but does bind if the larva is subject to starvation (Wiesner *et al.*, 1997).

Weisner *et al.*, (1997) showed that following intrahaemocoelic injection of apoLpIII into *G.mellonella* larvae, a dose-dependent increase in antibacterial activity in cell-free haemolymph was observed and, *in vitro*, enhanced phagocytic activity of isolated haemocytes was shown in the presence of pure apoLpIII. These results led to the proposal of a role for apoLpIII in induction of insect immune responses. Another role for lipophorin in transport of lipids &/or hydrocarbons to sites of cuticle synthesis has also been proposed (Pho *et al.*, 1996).

Nothing is known about mass lipid transport in nematodes. Insects and higher animals each have a distinct system which are relatively well understood. This begs the question as to how mass lipid transport is achieved in nematodes. Given the nutritional requirement of nematodes for sterols, unlike vertebrates and insects, it is possible that they require some specialised form of mass lipid transport.

The apparently promiscuous retinol/ fatty acid binding characteristics of Gp-FAR-1 reported in Chapter 4 of this work, and the fact that mutation of the most highly conserved residues in Gp-FAR-1 did not ablate ligand-binding may indicate that Gp-FAR-1 can bind much larger hydrophobic ligands such as phospholipids. As discussed in Chapter 5, similarity searches of FAR-1 and NPA proteins indicate that both families contain stretches of amino acid sequence with low similarity to the lipid-binding domain of vertebrate exchangeable apolipoproteins. Also, structural prediction programs indicate that both families have structural similarities to both vertebrate and insect exchangeable apolipoproteins and similarity searches indicate that no obvious homologues of vertebrate or insect exchangeable or non-exchangeable apolipoproteins are present in nematodes.

The work in this chapter was therefore carried out to determine whether the nematode FAR and/or NPA proteins function as exchangeable apolipoproteins or are otherwise involved in lipoprotein-mediated lipid transport in nematodes.

6.2 Materials and methods

6.2.1 Bioinformatics

Similarity searches and sequence comparisons were made against a range of databases using BLAST searches through the European Bioinformatics Institute (EBI) Internet pages (http://www.ebi.ac.uk/ebi_home.html). Structural predictions and sequence alignments were carried out using programs available through the ExPASy molecular biology server (http://expasy.hcuge.ch/www/tools.html) including PHD, Jpred, 3D-PSSM, Propsearch, Multalin.

6.2.2 Biological material

6.2.2.1 Nematode homogenates

Nematode homogenates were freshly prepared immediately before each experiment in order to preserve the activity of potential LDL-like molecules. Freshly-hatched G. pallida J2s were washed three times in PBS then resuspended in a minimal quantity of assay buffer in 1.5ml microcentrifuge tubes in the presence and absence of Complete protease inhibitor cocktail (Pharmacia). The nematodes were then ground with a small pestle until microscopic inspection indicated sufficient homogenisation. Homogenates were then centrifuged at 13000rpm for 10 minutes in a microcentrifuge to remove insoluble debris.

6.2.2.2 Fractionation of A. suum body fluid

To isolate native ABA-1, fractionation of *Ascaris* body fluid (ABF) proteins was carried out by gel filtration chromatography on a BioRad Biologic FPLC system. A Superdex 75 (Pharmacia) size exclusion column was prewashed by isocratic flow of 50ml of deionised water, then the column was equilibrated by isocratic flow of 50ml of degassed 0.05M phosphate buffer (pH 7.0), 0.15M NaCl (FPLC buffer) at a flow rate of 0.5ml min^{-1}. A vial of gel filtration standards (Pharmacia) containing bovine thyroglobulin (670kDa), bovine gammaglobulin (158kDa), chicken ovalbumin (44kDa), horse myoglobin (17kDa) and vitamin B12 (1.35kDa) was resuspended in 500µl deionised water then passed down the column at a flow rate of 0.5ml min^{-1} and the UV trace at 280nm and elution volumes (V_e) recorded.

ABF samples were spun at 13000rpm in a microcentrifuge for 10 minutes to remove insoluble particles prior to chromatography to prevent blockage of the column. After isocratic flow of 2ml FPLC buffer ABF was applied to the column in 200µl aliquots followed by isocratic flow for 50ml FPLC buffer at a flow rate of 0.5ml min^{-1}. 0.5ml fractions were collected and aliquots run on 4-12% Bis-Tris polyacrylamide gels (Novex) then Coomassie-stained to confirm the presence of protein. Protein-containing fractions were then tested for fatty acid binding using spectrofluorimetry as described in Chapter 2. Recombinant ABA-1 used in these experiments was produced from an *E. coli* clone expressing a GST:ABA-1A fusion protein and was obtained from Dr. Joyce Moore, University of Glasgow (Moore *et al.*, 1999).

6.2.3 Lipoprotein substitution assay

This assay was carried out to determine whether the FAR-1 and/or the NPA proteins have exchangeable apolipoprotein activity. The following protocol was

adapted from the method of Lui *et al.*, (1993). Control reactions were set up containing 50μg human low-density lipoprotein (LDL) (Sigma-Aldrich) in 600μl assay buffer (50mM Tris (pH 7.5), 150mM NaCl, 2mM $CaCl_2$) in the presence and absence of 50μg human apolipoprotein-AI in quartz cuvettes. The reaction was initiated by the addition of 160 milliunits of *Bacillus cereus* Type IV phosphatidylcholine-specific phospholipase C (Sigma-Aldrich), then the absorbance at 600nm (A_{600}) was measured for 2 hours in an Ultrospec spectrophotometer (Pharmacia). It should be noted that this experiment quantified the degree of light-scattering as opposed to absorbance. The absorbance of negative controls containing LDL with no phospholipase C was measured and subtracted from all samples before analysis.

All test (rGp-FAR-1, rABA-1) and control proteins (BSA, ribonuclease A) were dialysed into assay buffer, as described in Chapter 2 before use in these experiments. Test reactions were set up as follows: 20-150μM of rGp-FAR-1, rABA-1 or the control proteins apolipoprotein AI, BSA and ribonuclease A were added to 50μg LDL then the reaction was initiated by the addition of 160milliunits of phospholipase C. The A600 of the samples were then measured for 2 hours at 25°C in an Pharmacia Ultrospec spectrophotometer. Data were corrected by subtraction of the absorbance of the reactions before addition of phospholipase C.

To test for the presence of exchangeable apolipoprotein activity in ABF or PCN homogenates 200μl of nematode extracts were added to the reactions and the absorbance monitored as above. ABF HPLC fractions, alone or in combination with other fractions were also tested in this way to investigate whether more than one protein was required for exchangeable apolipoprotein activity in nematodes.

6.2.4 SDS-PAGE of Lp:protein complex

Reactions which had proceeded to completion in the above assays were then incubated overnight at 25°C to allow LDL aggregation to go to completion. Samples were then centrifuged at 14000rpm in a microcentrifuge for 2 hours then the supernatants removed and saved for later use. Pellets were washed three times in 1ml of lipoprotein assay buffer then resuspended in 600µl of assay buffer. Aliquots of the supernatant and pellet samples were then run on a 4-12% Bis-Tris polyacrylamide gel alongside Kaleidoscope pre-stained molecular weight markers (BioRad) then visualised by Coomassie staining.

6.3 Results

6.3.1 Binding of phospholipids

As Gp-FAR-1 appears to be localised on the cuticle surface and has been shown to be capable of disrupting host defence responses in vitro, as has been reported with insect lipophorin, experiments were carried out to investigate whether Gp-FAR-1 could also bind larger hydrophobic ligands (phospholipids), and function in a similar manner to these insect proteins. However, in preliminary experiments the poor solubility of the fluorescent phospholipids used caused the aggregation of these molecules in solution and this led to undesirable interference in the fluorescence emission, namely self-quenching and the inner filter effect. As a result, it was not possible, using this assay, to conclude whether these molecules bound to rGp-FAR-1 and/or rABA-1 and thus it was necessary to devise an alternative procedure to test these proteins for binding to larger lipids.

6.3.2 Bioinformatics

Similarity searches (Blast) showed that stretches of the FAR proteins and NPA sequences have low (18-21%) amino acid sequence similarity with apolipoprotein-AI and apolipoprotein-E3 respectively ($p<0.01$) (Figure 6.1). Structural predictions for NPAs using the 3D PSSM fold library database also indicated secondary structure similar to insect apolipophorin III. This, coupled with the known lipid-binding activity of these proteins, led to the hypothesis that the FAR-1 and/or NPAs may function as exchangeable apolipoproteins in nematodes.

Searches of the *C.elegans* genome database (Wormbase) and parasitic nematode databases indicated that no homologs of vertebrate or insect exchangeable or non-exchangeable apolipoproteins are present in nematodes. However, nematodes are known to encode several proteins which are involved in mass lipid transport in vertebrates including, low density lipoprotein receptor (LDLR) (Bennet & Caulfield, 1991), lipoprotein receptor-related protein (LRP) (Yochem & Greenwald, 1993), lecithin cholesterol acyltransferase (LCAT) and vitellogenins (Kimble & Sharrock, 1983), which are involved in the storage of lipids in egg yolks and are evolutionarily related to the vertebrate non-exchangeable apolipoprotein, apoB. The presence of LDL superfamily sequences in *C. elegans* may indicate that similar lipid transport systems are or have been present in nematodes but may have diverged for functional reasons.

The main function of the lipoprotein systems in mammals and insects is sterol and hydrocarbon transport. As nematodes have a sterol auxotrophy (Chitwood & Feldlaufer, 1990), unlike higher animals, it is likely that they require some specialised form of mass lipid transport since all forward sterol transport will occur

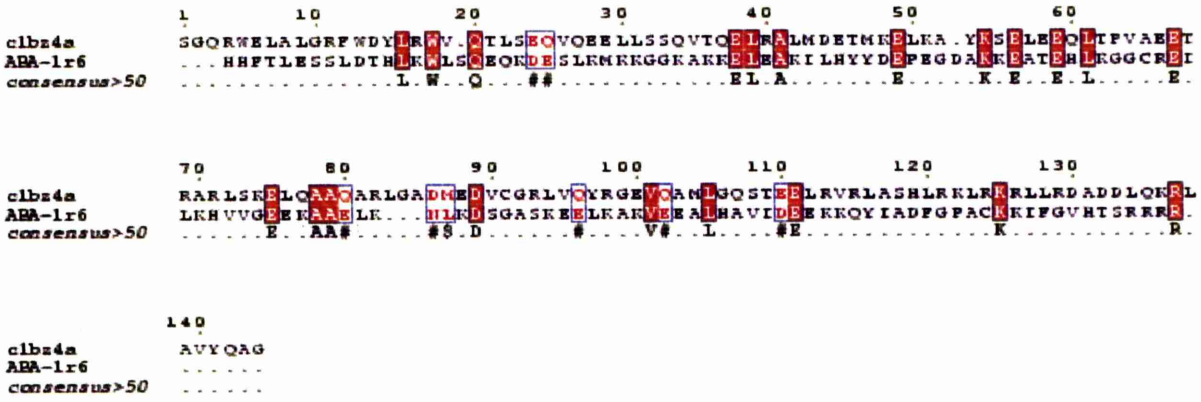


Figure 6.1. Amino acid sequence alignment of NPA (rABA-1r6) with human apolipoprotein E3 (clbz4a) showing low similarity between these proteins. Alignments were carried out using the Multalin program available through the ExPasy molecular biology server (http://www.expasy.hcuge.ch/www.tools.html).

from the gut, because all sterols in nematodes have a dietary origin. Other similarities between the NPAs and apolipoproteins include the fact that, as shown in the present study (Table 6.1), the NPAs are synthesised in the gut (See Chapter 3) as are some vertebrate apolipoproteins and nematode vitellogenins (Sharrock *et al.*, 1990). Also, the apoAI/apoAIV/apoE family of exchangeable apolipoproteins contain multiple tandem repeats within their sequence, forming repeated amphipathic α-helices, 11 or 2x11 amino acids long, which make up the lipid-binding moieties of these proteins (Segrest *et al.*, 1992). A similar repetitive structure is also seen in the sequences of all NPAs studied to date although this has not been extensively studied. Also, an N-terminal fragment (residues 1-91 representing the first three α-helices) of ApoLpIII was still able to bind lipids and to associate with LDL (Dettloff *et al.*, 2001). Similarly, a C-terminal fragment of ABA-1 did not lose the ability or affinity to bind fatty acids and retinol (McDermott, 2001). Finally, insect apolipophorin was shown to be present in the pigmented glial cells of the lamina underlying the locust retina and this was taken to suggest that ApoLps may be involved in transport of retinoids and or fatty acids to the retina (Bogerd et al., 2000). The NPAs are known to bind retinol and fatty acids with high affinity (McDermott, 2000) again indicating possible similarities in the functions of these proteins.

Nematode polyprotein allergens	Insect exchangeable apolipophorins	Vertebrate exchangeable apolipoproteins
Synthesised in gut (site of acquisition)	Synthesised in fat body (site of lipid storage)	Synthesised in gut, liver and other sites of utilisation (sites of sterol acquisition and synthesis)
Monomers composed of repeat units		Genes composed of 2x11 tandem repeats
Predicted highly amphipathic α-helical structure	Highly α-helical structure	Highly α-helical structure
	Prevents PL-C-mediated aggregation of LDL	Prevents PL-C-mediated aggregation of LDL
C-terminal fragment retains function	N-terminal fragment retains function	N-terminal fragment retains function

Table 6.1 Similarities between the NPAs and insect and vertebrate exchangeable apolipoproteins

We therefore tested the capacity of two nematode-specific lipid-binding proteins to function as exchangeable apolipoproteins using a lipoprotein substitution assay adapted from Liu *et al.,* (1993).

6.3.3 Lipoprotein substitution assay

As shown in Figure 6.2, upon addition of phospholipase C (PL-C) to a reaction mixture containing LDL, a gradual increase in turbidity was observed which plateaued after approximately 1 hour indicating the aggregation of the lipoprotein particles upon removal of the polar head groups of the surface-associated phosphatidylcholine (PC). In the presence of the positive control protein apolipoprotein-A1 (apo-A1), the absorbance remained constant throughout the assay indicating that the apo-A1 was binding to the lipoprotein surface, protecting the polar head groups and thus preventing the PL-C-induced aggregation of the particles. Upon addition of rGp-FAR-1 to the reaction mixture the absorbance increased in line with that seen for LDL and PL-C alone indicating that rGp-FAR-1 does not exhibit exchangeable apolipoprotein activity under the conditions tested. As expected, the negative control proteins BSA and RNAse H gave similar results. Addition of rABA-1 to the reaction mixture resulted in partial inhibition of LDL aggregation but addition of increasing quantities of rABA-1 failed to increase the level of inhibition to that of ApoA1. This could indicate that ABA-1 does have exchangeable apolipoprotein activity but that in the heterogeneous (mammalian) system used it was not possible to obtain complete inhibition of aggregation. It is also possible that the recombinant ABA-1 used in this study was not fully active due to incorrect folding of the recombinant protein or lack of eukaryotic post-translational modifications required for activity (as it was expressed in a prokaryotic system).

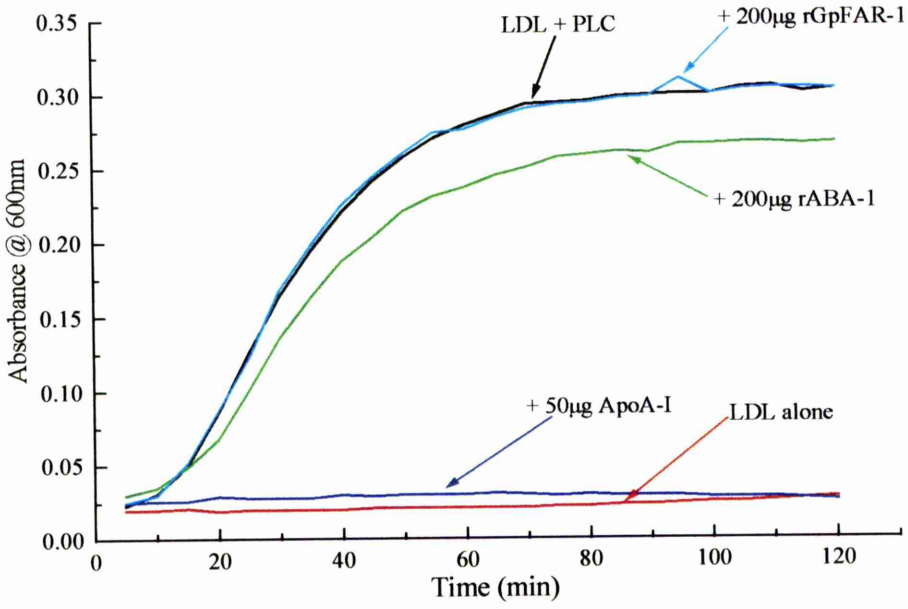


Figure 6.2. Effect of nematode LBPs on PLC-mediated aggregation of human LDL. Absorbance of 50µg human LDL in assay buffer (red) and increase in turbidity in the presence of 160 milliunits of *B. cereus* PC-specific phospholipase C (black). Inhibition of aggregation in the presence of 50µg human apolipoprotein AI (blue) or 200µg rABA-1 (green). Lack of inhibition of aggregation in the presence of 200µg rGpFAR-1 (cyan).

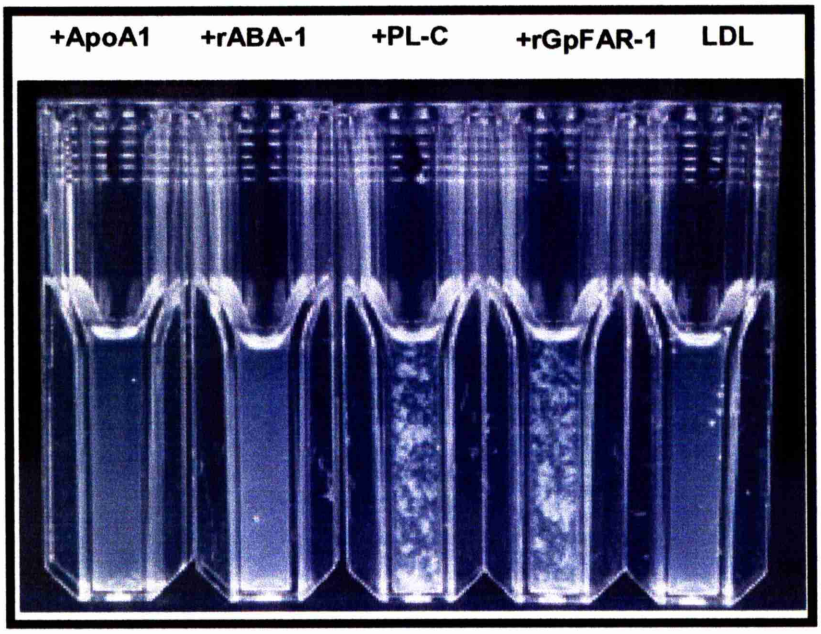


Figure 6.3. Effect of nematode LBPs on PLC-mediated aggregation of human LDL after overnight incubation. LDL particle aggregation upon incubation of 50µg human LDL in the presence of 160milliunits of PC-specific phospholipase C (+PLC). In the presence of 200µg rGpFAR-1 aggregation occurs in line with the PLC sample (+GpFAR-1). Inhibition of aggregation in the presence of, 50µg apolipoprotein AI (+ApoAI), 200µg rABA-1 (+ABA-1) where the turbidity remains similar to that of 50µg LDL alone (LDL).

Alternatively, it is possible that some other nematode component is required in addition to rABA-1 for apolipoprotein activity (such as different NPA monomers). The fatty acid binding protein, BSA, was used as a negative control, showing that the observed interaction of ABA-1 and LDL was not due to non-specific hydrophobic interactions.

Increasing concentrations of rABA-1 did not enhance the inhibition of LDL aggregation. However, overnight incubation of the above samples led to continued aggregation in the negative control samples which proceeded to a point where the LDL aggregate sank to the bottom of the cuvette leaving a clear supernatant. However, in the ApoA1 sample this degree of aggregation did not occur and the solution remained turbid and this effect was also observed in the cuvette containing ABA-1, further confirming that aggregation was indeed inhibited in this sample (Figure 6.3).

To study this effect further, samples were removed from the cuvettes and centrifuged at 14,000 rpm in a benchtop microcentrifuge for 1 hour to separate the aggregated LDL from the solution. After washing in assay buffer, the pellets and supernatant were subjected to SDS-PAGE analysis and Coomassie-stained. As shown in Figure 6.4., ApoA1 could be seen in the pellet indicating that, as expected, it was associated with the LDL molecules. This was also seen with the ABA-1 sample, further confirming that the protein interacts with LDL. However, the negative control protein BSA was also seen to be associated with the LDL pellet even though aggregation occurred as normal in the cuvettes. This may be due to non-specific hydrophobic interactions which occurred between the BSA and LDL but which were not able to prevent aggregation. More stringent washing of the pellets may have removed such non-specifically bound material. Interestingly, a band was of approximately 44kD was observed in the ABA-1 samples which was not present in any of the other samples. As

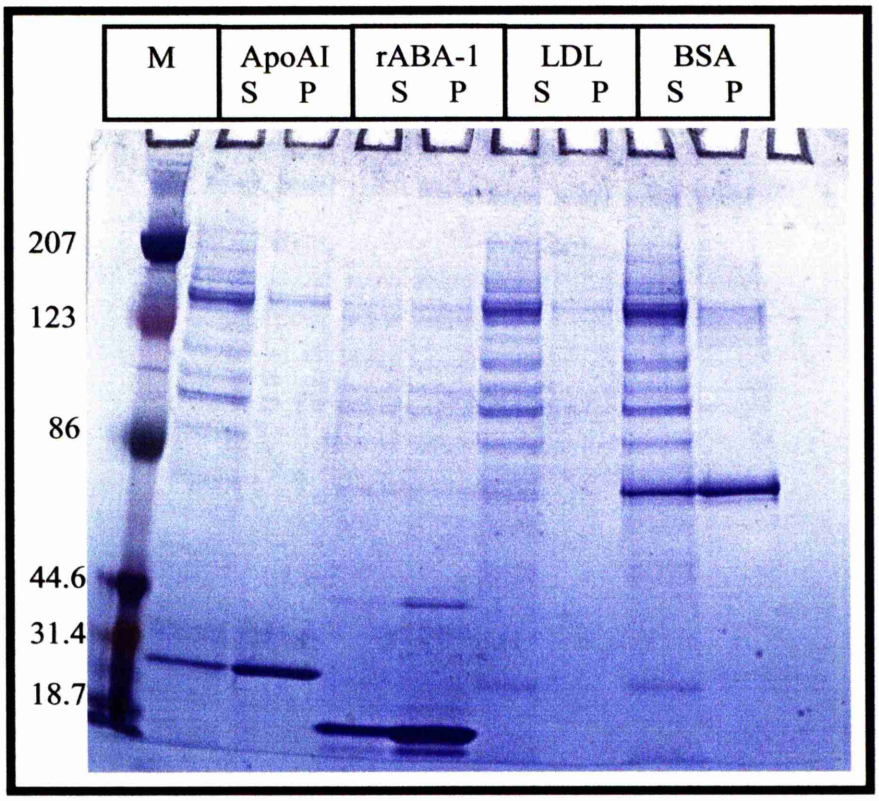


Figure 6.5. SDS-PAGE of pellets and supernatants of overnight incubated lipoprotein substitution assay samples. Molecular weight markers (M), apolipoprotein-AI supernatant (ApoAI S) and pellet (ApoAI P), rABA-1 supernatant (ABA-1 S) and pellet (ABA1 P), LDL + phospholipase C supernatant (LDL S) and pellet (LDL P) and BSA supernatant (S) and pellet (P).

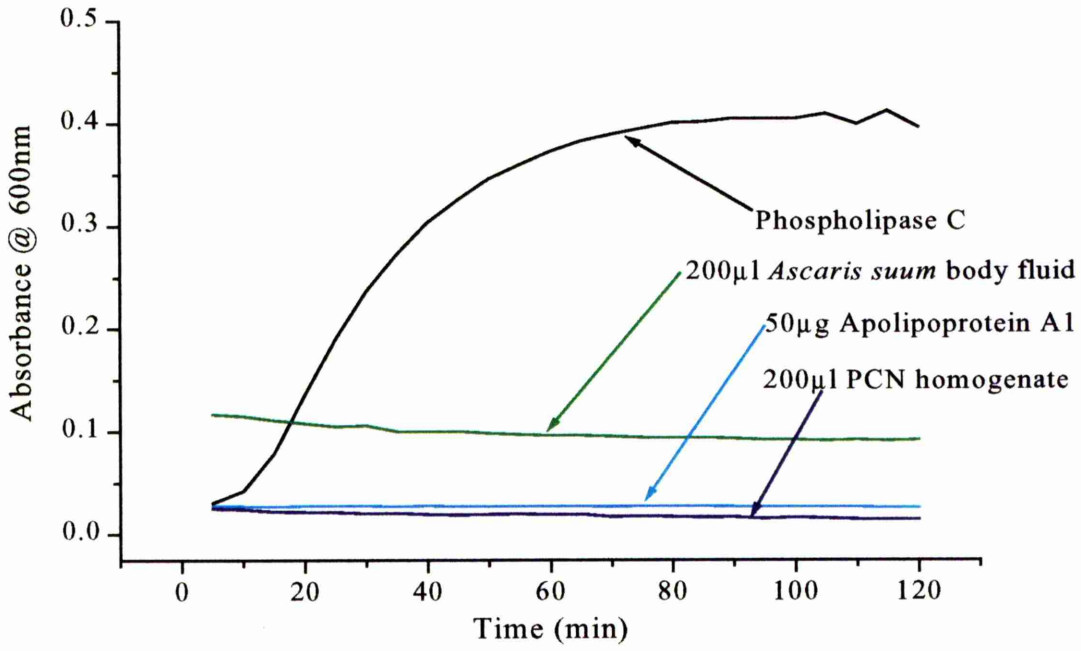


Figure 6.5. Effect of *A.suum* body fluid and *G.pallida* whole nematode homogenates on PLC-mediated aggregation of human LDL. Absorbance of 50µg human LDL in assay buffer and increase in turbidity in the presence of 160milliunits of *B. cereus* PC-specific phospholipase C (black). Inhibition of aggregation in the presence of 50µg human apolipoprotein AI (cyan), 200µl *A.suum* body fluid (green) or 200µl *G.pallida* whole nematode homogenates (blue). All values represent the mean of three separate experiments +/- SEM.

ABA-1 has previously been shown to self-associate, (McDermott, 2000), it is possible that this band represents an oligomer of ABA-1 and the size could indicate a trimer or tetramer. It is therefore possible that for maximum activity ABA-1 is required to oligomerise but being recombinant it is unable to do so efficiently. Interestingly, lipid-free mammalian apoAI has been shown to oligomerise in solution in a reversible, concentration-dependent manner (Barbeau *et al.*, 1979). Alternatively, as some NPA monomers have diverged sequences, although all studied to date bind fatty acids with similar affinities (eg., Kennedy *et al.*, 1995a, b, c; Moore *et al.*, 1999), it is possible that a combination of different polyprotein monomers are required for efficient oligomerisation and activity. It is also possible that monomers are not cleaved *in vivo* resulting in expression of single polypeptides encoding several monomers which are cleaved at different stages in the life-cycle or at different sites within the nematode body. In this scenario the entire polyprotein may become loaded with lipid in the gut and then cleaved at various sites in the nematode body with specific peptides removed at various nematode tissues.

In order to determine whether another nematode component was required to obtain maximum inhibition of aggregation, *Ascaris* body fluid or PCN homogenate were added to the reaction mixture. As shown in Figure 6.5, in both cases aggregation was completely inhibited indicating either that other nematode components are required, or that the recombinant protein is indeed partially inactive and native protein in the nematode extracts is responsible for the aggregation inhibition. However, as these were very crude extracts it is also possible that they contained components which interfered with the assay, such as PL-C inhibitors. To further refine this experiment, *Ascaris suum* body fluid was size-fractionated using FPLC to obtain purer native ABA-1 (Figures 6.6 & 6.7). The fractions were analysed by SDS-PAGE to confirm the presence of protein.

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Method Name: rif1.6 **Run Name:** Alison/Standard **Run Date:** 02:44:16 PM 09-02-99

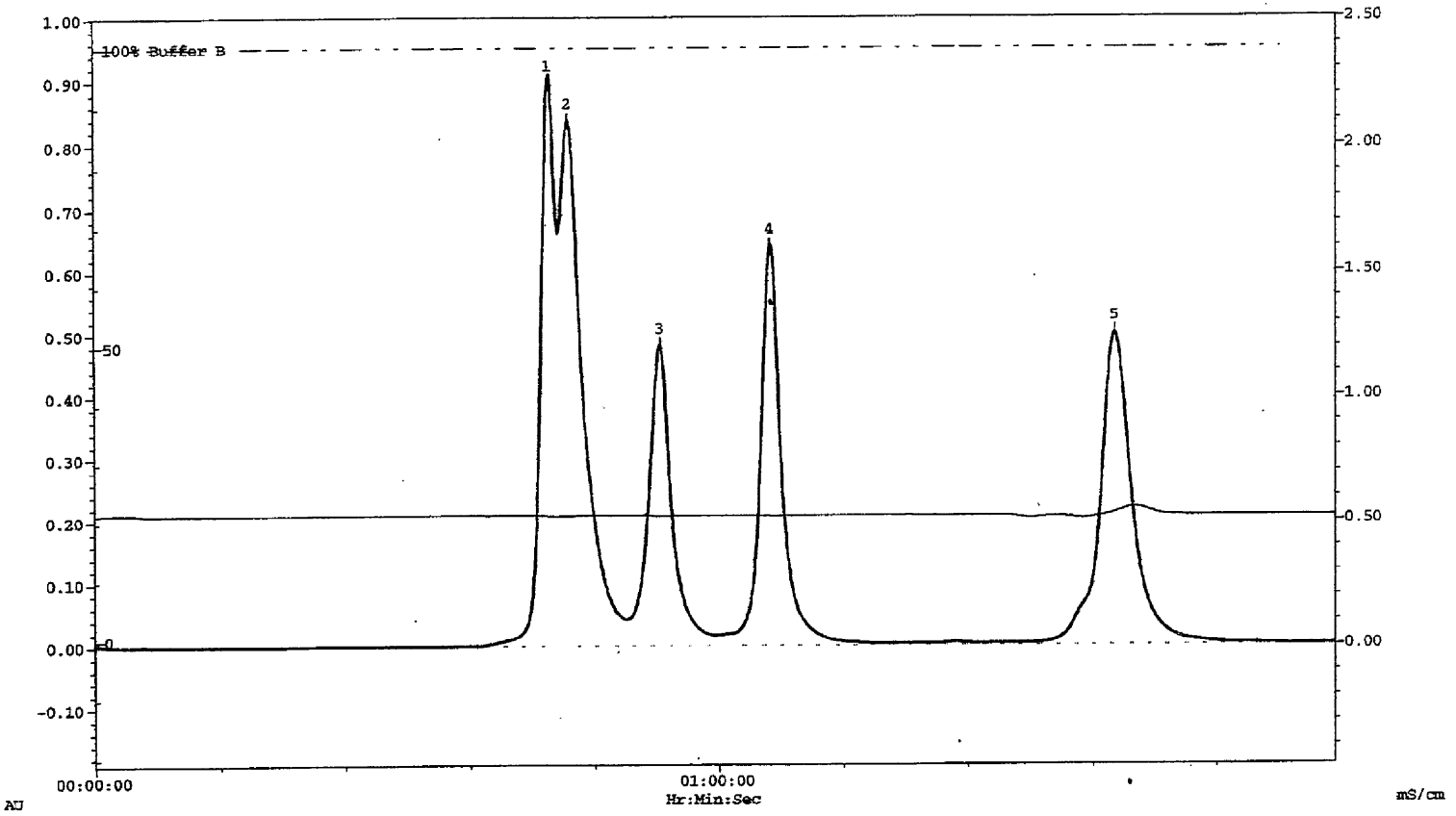

Figure 6.6. FPLC trace of of protein molecular weight standards. Peak 1. Vitamin B12 - 1.35kDa: Peak 2. Horse myoglobin - 17kDa: Peak 3. Chicken ovalbumin - 44kDa: Peak 4. Bovine gamma--globulin - 158kDa: Peak 5. Bovine thyroglobulin - 670kDa.

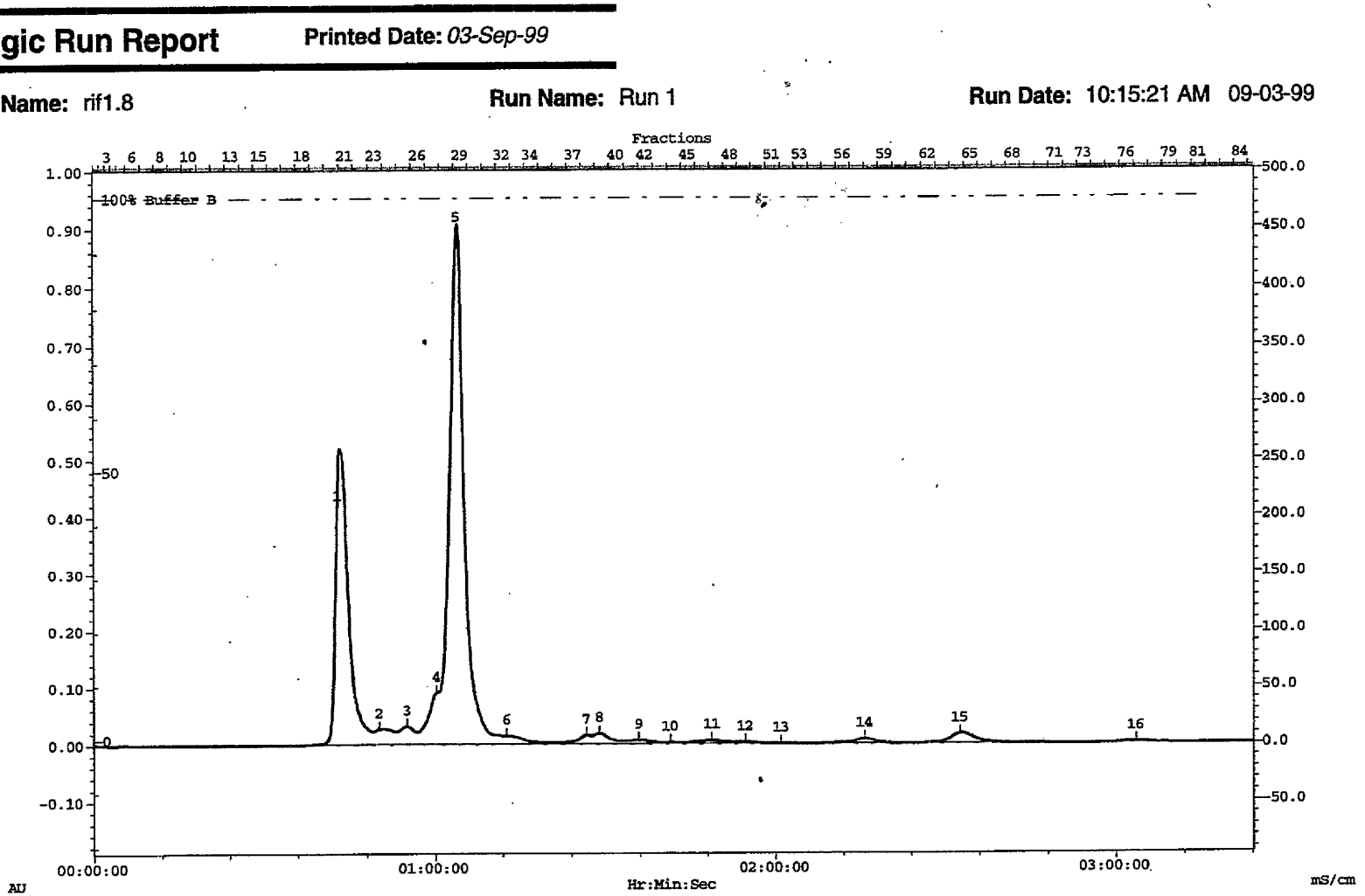

Figure 6.7. FPLC trace of *A. suum* body fluid after passage down a Superdex 75 size exclusion column.

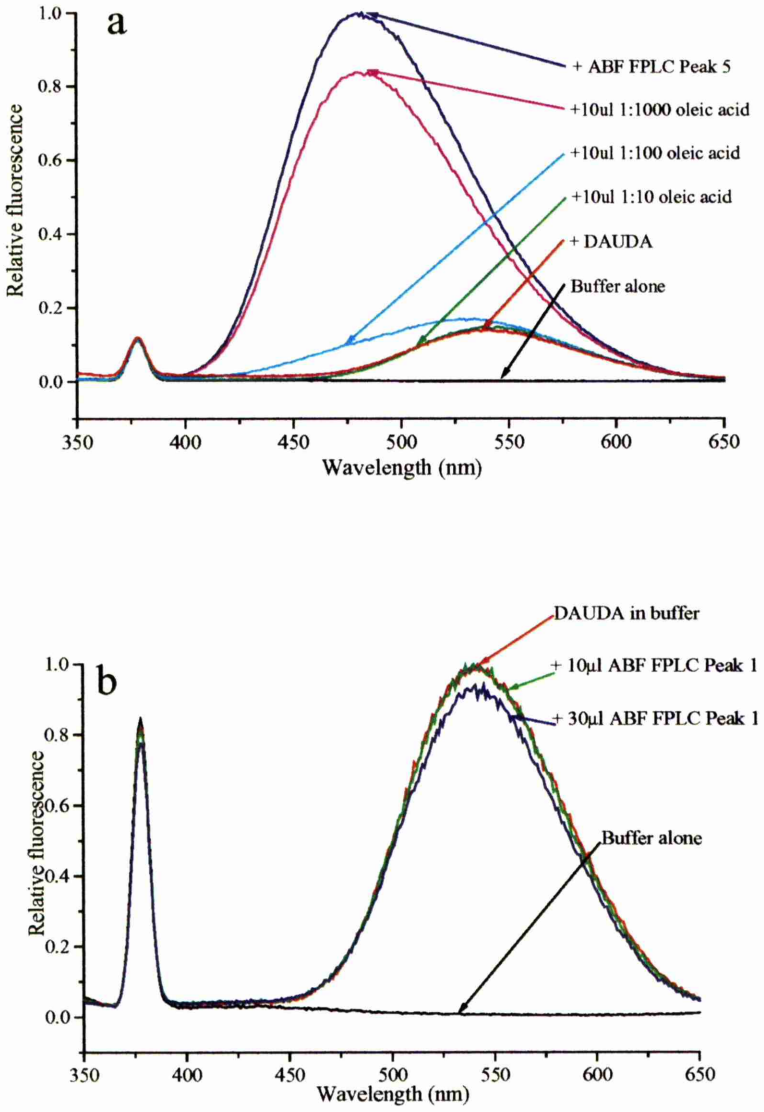

Figure 6.8. Fatty acid-binding activity in *A.suum* body fluid FPLC fractions. **a**. Fluorescence emission spectrum of buffer alone (black), in the presence of DAUDA (red) with characteristic maximum emission a 543nm and upon addition of 10µl of ABF FPLC Peak 5 with maximum emission at 482nm (blue). Gradual reversal of wavelength shift upon addition of 10µl of 1:1000 (magenta), 1:100 (cyan) and 1:10 (green) 1µM oleic acid indicating fatty acid-binding activity in this fraction. **b.** Emission spectrum of buffer alone (black), in the presence of DAUDA (red) with characteristic maximum emission a 543nm and upon addition of 10µl (green) and 30µl (blue) of ABF FPLC Peak 1 showing no wavelength shift or increase in emission intensity indicating no fatty acid-binding activity in this fraction.

Fractions containing protein were then concentrated and dialysed into assay buffer as described in Chapter 2 for use in the assays. To confirm the presence of active protein in the fractions, each were tested for binding to DAUDA using spectrofluorimetry as described in Chapter 4 (Figure 6.8). Fractions exhibiting fatty acid-binding activity were then concentrated and used in the assays, both alone and in combination with the other fractions to test the hypothesis that more than one nematode component is required. However, none of the fractions showing fatty acid-binding activity as described above caused any inhibition of aggregation alone or in combination with the other fractions. It is possible, however, that this occurred because the fractions used did not contain enough protein to see an effect or that the nematode lipoproteins were inactivated due to freezing as is known to occur with vertebrate LDL. It is also possible that NPAs in the body fluid and nematode homogenates localised in the lipid layer on the surface of the liquid or in the insoluble pellets, both of which were removed prior to the commencement of the experiments and thus were not applied to the column. The fatty acid-binding activity seen in the fractions would appear to negate this but could have been due to other lipid-binding proteins such as FABPs which are of a similar size to the NPAs and thus could have appeared in the NPA-containing fractions.

6.4 Discussion

Attempts to test Gp-FAR-1 for binding to larger lipids such as phospholipids proved unsuccessful, as discussed previously, due to limitations of the technique used. Vesicle interaction assays have been widely used to investigate the lipid-binding activities of plant and mammalian lipid transfer proteins (e.g., Tassin *et al.*, 1998, Record *et al.*,1995, Van Paridon *et al.*, 1988). In fluorescent assays, donor vesicles consist of self-quenching vesicles of pyrene-labelled phosphatidylcholine (pyrPC) and

acceptor vesicles contain unlabelled lipids. When these lipids are transferred from the donor membrane to the acceptor membrane, the fluorescence emission of the transferred molecule increases because of a decrease in quenching from other pyrPC molecules in the donor membrane. When a donor lipid molecule is transferred to the acceptor membrane, a lipid molecule from the acceptor membrane is exchanged. This procedure is the classical method for observing phosholipid transfer activity and could be used to assay for phosholipid binding by Gp-FAR-1 and ABA-1.

Although this work did not conclusively show that NPAs function as exchangeable apolipoproteins, it seems obvious that some form of mass lipid transport system must be present in nematodes. The absence of sequences with significant similarity to known insect or vertebrate exchangeable apolipoproteins in the *C. elegans* genome indicates that nematodes have a distinct system for mass lipid transport. This could be because nematodes have a sterol auxotrophy unlike vertebrates and insects and so all sterols will be of a dietary origin. This could perhaps explain why the NPAs are expressed as polyproteins because upon feeding large amounts of NPA would be required quickly to sequester as much lipid as possible whereas in insects and vertebrates both of which can synthesise sterols *de novo* this would not be so important.

If the NPAs are indeed exchangeable apolipoproteins with analogous functions to the insect and vertebrate proteins, then nematodes may also express a non-exchangeable apolipoprotein. As mentioned earlier, searches of the *C. elegans* genome databases indicate that no homologs of apolipoprotein B or apolipophorin I & II are present in nematodes. Thus, it is possible that a distinct, unrelated form of non-exchangeable apolipoprotein is present in nematodes. Alternatively, it is possible that a nematode vitellogenin acts as a non-exchangeable apolipoprotein. Vitellogenins are present in nematodes, vertebrates and insects, and function in storage of lipids in

oocytes. These proteins are also synthesised in the gut, where upon expression they become loaded with dietary lipids before travelling to the developing oocytes. Although nematodes are more primitive than other oviparous organisms, their Vgs appear to be more complex (Spieth & Blumenthal, 1985) perhaps indicating greater functional diversity in nematode vitellogenins. Nematodes express two distinct types of Vg particle, with one type encoded by five closely related vit genes, vit1-5 while the other is encoded by a distantly related vit gene, vit-6 which is immunologically and physically distinct from the other 5 vit genes (Spieth & Blumenthal, 1985). Interestingly, Nakamura *et al.*, 1999, found that carbonylated vit-6 protein was present in aged, non-reproducing nematodes indicating that this protein may serve other functions, in addition to lipid storage in oocytes. Unlike vit1-5, the protein encoded by vit-6 is cleaved into two precursors, which are roughly the same sizes as the insect non-exchangeable apolipoproteins ApoLpI and II. Insect lipophorin has been shown to localise in developing oocytes indicating that they can act as yolk protein precursors like vitellogenins (Sun *et al.*,2000). Thus, the overall structural similarity between vit-6 protein and the insect non-exchangeable apolipophorins I & II and the presence of vit-6 in aged, non-reproducing nematodes could therefore indicate that vit-6 acts as a non-exchangeable apolipoprotein in nematodes.

Future work, therefore could include an Lp substitution assay using lipid-loaded vit-6 in place of LDL to test this protein for non-exchangeable apolipoprotein activity with the NPAs. Also, if the NPAs do function as exchangeable apolipoproteins, they may interact with the LDL receptor (Bennet & Caulfield, 1991) and LDL receptor-related proteins (Yochem & Greenwald, 1993) known to be present in nematodes, therefore future work should also include experiments to test this hypothesis. Further experiments could include using the insect lipophorin system for the substitution assay

as this may be more similar to a nematode system, although it has been shown that nematode vitellogenins are more closely related to vertebrate vitellogenins than insect vitellogenins. Other work could include, ligand blotting of ABA-1 with radioactively-labelled LDL to investigate whether ABA-1 binds to the surface of the LDL. Crystallographic studies are now underway on both the NPAs and FAR-1 proteins. When this work is completed more SDM should be undertaken to further explore the residues and structural features required for normal functioning of these proteins and compare this with crystallographic data for the insect and vertebrate exchangeable apolipoproteins. This could include engineering disulphide bridges to lock adjacent helices together and investigate whether this affects the function, as was shown to be the case with insect apoLpIII (Narayanaswami *et al.*,1996). DMPC disc assays could also be used, where protein is added to a solution of dimyristoyl-phosphatidylcholine (DMPC) vesicles (Wientzek *et al.*, 1994). If exchangeable apolipoprotein activity is present the formation of disc-shaped lipid:protein complexes are observed.

Chapter 7

General Discussion

7.1. Summary

The work presented in this thesis provides the first data on the FAR and NPA proteins in plant-parasitic nematodes. In summary, this work showed:

- that like its counterpart in *O. volvulus*, Gp-FAR-1 binds fatty acids (of chain lengths greater than C11) and retinol with affinities similar to those reported for FABP-type proteins
- Gp-FAR-1 mRNA is present in the hypodermis and the protein is present on the surface of nematodes and in material shed from it.
- Gp-FAR-1 inhibits the lipoxygenase-mediated peroxidation of fatty acids *in vitro* and therefore may play a role in countering plant defence reactions.
- two highly conserved lysine residues in Gp-FAR-1 are not essential for ligand-binding but substitution of these does alter the ligand-binding affinities.
- that computer predictions for secondary structure in Gp-FAR-1 were supported by the SDM results.
- in PCN, mRNA encoding the NPAs is present in the gut and hypodermis.
- ABA-1 can inhibit the PLC-mediated aggregation of mammalian low density lipoprotein *in vitro* and therefore could function in the disruption of host lipid transport or may be involved in a previously undescribed mass lipid transport system in nematodes.

7.2. General Metabolic Functions

The presence of FAR-1 and NPA proteins in a range of free-living and plant- and animal-parasitic nematodes indicates common roles for these fatty acid and retinol-binding

proteins in the general metabolism of nematodes by sequestering oxidation-sensitive hydrophobic ligands. In higher animals, fatty acids and retinol are generally bound by distinct proteins i.e., intracellularly by FABP and CRBP and extracellularly by serum albumin and retinol-binding protein (RBP) (Bass, 1993). However, it appears that in nematodes, individual lipid-binding proteins such as the FAR and NPA proteins bind both fatty acids and retinol competitively (e.g., this work, Kennedy *et al.*, 1997, Kennedy *et al.*, 1995a, b). This indicates that fatty acids and retinol must compete with one another for binding to the FAR or NPA proteins *in vivo* and, as these molecules are relatively sensitive to oxidation, those not bound would presumably be in danger of degradation. This may indicate a role for the NPAs or FAR proteins in modulation of the proportions of retinol and free fatty acids in their extracellular environments. However, to understand the significance of this, information on the relative amounts of fatty acids and retinol and these proteins at their sites of occurrence would be required. Both fatty acids and retinol are precursors of molecules which play central roles in signalling and regulation (i.e., eicosanoid- and retinoid-based signalling). It is tempting to speculate that, by binding these precursors, the FAR and/or NPA proteins could modulate such signalling and regulatory pathways.

It is important to remember, however, that, the ligand-binding studies carried out in Chapter 4 were not exhaustive and Gp-FAR-1 may bind other ligands, as is the case with the NPAs (Kennedy, 2000). Also, since fatty acid- and retinol-binding was not ablated upon substitution of the most highly conserved residues in Gp-FAR-1 (Chapter 5), or ABA-1 (McDermott, 2000), it is possible that this is not the true, or sole, function of these proteins. Interestingly, a glycoprotein isolated from *Drosophila melanogaster* was shown to bind fatty acids and retinol with K_d values of 0.35 and 0.29μM respectively (Kutty *et al.*,

1996). However, this protein was then identified as proapolipohorin, a precursor of the two proteins which constitute the basis of lipoprotein particles in insects. So, it is possible that retinol and fatty acid-binding by FAR and NPA proteins may not be the only function of these proteins.

7.3. General Surface Function/ Cuticle

The epicuticle of nematodes is composed of rigid non-diffusing domains of lipids (Proudfoot *et al.*, 1991) and is thought to act as a permeability barrier. Its structural integrity must, therefore, be vital to the survival of the nematode. The surface localisation of Gp-FAR-1 suggests that it may interact with the lipid-rich epicuticle and could therefore play a role in maintenance of this structure by delivery and insertion of fatty acids and/or larger lipids into the epicuticle to ensure its continued integrity. It is also possible that Gp-FAR-1 can bind other hydrophobic cuticle components and function in synthesis and maintenance of the cuticle as a whole. Indeed, a lipid-binding protein from insects, lipophorin, has previously been implicated in cuticle synthesis (Takuchi & Chino, 1993).

Future work could include more detailed temporal expression studies since, although RT-PCR showed the presence of FAR-1 mRNA in all lifecycle stages tested, this technique gives no information on where the mRNA or proteins are expressed. Also, since three larval stages (J3, J4 and males) were not included in the RT-PCR experiments it is possible that Gp-FAR-1 is not expressed in these stages. The small size and endoparasitic lifecycle of these nematodes makes it difficult to obtain J3 and J4 stage PCN as these are exclusively endoparasitic and so are difficult to isolate in high enough quantities for analysis. However, this problem could be overcome by carrying out ISH or

immunolabelling of sections of potato roots infected with J3 or J4 PCN and subjecting these to electron microscopic analysis. Alternatively, the use of a co-ordinated approach to the study of these proteins could be employed as proposed by Plenefisch *et al.*, (2000) i.e., the co-ordinate use of a genetically amenable species such as *C. elegans* in tandem with a physically amenable species such as *A. suum* to study the biological function(s) of the homologous proteins. However, the morphological and genetic diversity among nematodes makes direct comparison and generalisation of their genes and their encoded proteins difficult and the localisation and function of orthologous proteins may differ between species. This is especially true for comparisons of free-living and parasitic species, as ancestral internal proteins from free-living species may have evolved functions as secreted proteins involved in host defence evasion in parasitic species (e.g., Robertson *et al.*, 2000; Cookson *et al.*, 1992).

7.4. Role in Defence Evasion

The presence of FAR proteins in free-living and parasitic nematodes indicates a general function in all nematodes, such as cuticle maintenance or delivery of fatty acids or retinol to sites of utilisation as discussed above. However, this work has shown that Gp-FAR-1 can inhibit plant lipoxygenase *in vitro* and, given the presence of this protein on the nematode surface, it may also function in the inhibition of host plant octadecanoid signalling pathways as discussed in Chapter 4. This inhibition is presumably brought about by the sequestration of linolenic acid (released from membranes by wounding or defence-related phospholipases) which prevents its peroxidation by lipoxygenase. By inhibiting the jasmonate synthesis pathway at the first step (i.e., the lipoxygenase-mediated peroxidation

of linolenic acid), Gp-FAR-1 could also inhibit the formation of other defence compounds produced by this pathway, such as, volatile aldehydes, traumatic acid and cutin monomers. However, localised wounding can also cause the systemic accumulation of JA (Creelman *et al.*, 1992) so a systemic defence response may still be mounted. Binding of Gp-FAR-1 to nematode-derived arachidonic acid (C20:4), a fatty acid known to elicit resistance-related responses in potato (Altamura *et al.*, 1994) and found in abundance in PCN (Holz *et al.*, 1997, 1998a,b), is another mechanism by which Gp-FAR-1 could inhibit plant defence responses.

The work undertaken in this study was not quantitative and did not address the issue of whether the relative quantities Gp-FAR-1, fatty acids and lipoxygenase *in vivo* would be sufficient to effectively inhibit host defences. However, given the presence of other putative host-defence evading or inhibiting proteins on the nematode surface such as, peroxiredoxin (Tpx) (Robertson *et al.*, 2000), glutathione peroxidase (Cookson *et al.*, 1992) and possibly NPAs (Chapter 3), the cumulative effect may be sufficient to damp down the overall localised defence response. It would be interesting to examine whether the NPAs also have the ability to inhibit lipoxygenase and thus contribute to defence evasion or inhibition. Since BSA was also shown to inhibit lipoxygenase in this work (Chapter 4) and L-FABP has also been reported to do this, it seems likely that this is a general feature of fatty acid-binding proteins. Indeed, these results support the hypothesis that fatty acid and retinol binding proteins main function is in protection of these labile molecules from oxidation. Thus, it would be interesting to examine the ability of these proteins to inhibit other enzymes which use fatty acid or retinoid substrates. As discussed in detail in Chapter 4, the jasmonate signalling pathway in plants is analogous to the eicosanoid signalling

pathway in animals (Bergey *et al.*, 1996) and as a result, the role in plant defence inhibition proposed here for *G. pallida* could also apply for animal-parasitic nematodes. Unfortunately, a comparison with free-living species is not possible because all information to date on the FAR and NPA proteins has been gained from parasitic nematodes and little is known about these proteins in non-parasitic species. Searches of the *C.elegans* genome database indicate the presence of at least seven FAR genes in this species whereas all parasitic species studied to date appear to contain only one FAR gene. In the absence of any detailed information on the localisation and function of the *C. elegans* FAR proteins it is difficult to draw any conclusions on the significance of this. Parasitic species may contain more than one FAR gene like *C. elegans* but they may be sufficiently diverged that they are not recognised as homologous or, perhaps more likely, parasitic nematodes may need to devote more of their genetic and metabolic capacity to parasitism at the expense of FAR genes. Alternatively, the presence of seven FAR genes in *C. elegans* may be a specific adaptation of this nematode to a free-living, bacterivorous lifestyle and other non-parasitic nematodes may contain only one FAR gene. It would also be interesting to look at FAR genes in ectoparasitic plant nematodes. As these nematodes are free-living would they encode multiple *C. elegans*-type FAR proteins, or given that they parasitise plants would they contain only one? Screening of EST datasets indicates that in PCN, FAR-encoding transcripts are present in abundance, whereas in ESTs from the plant ectoparasitic nematode, *Xiphinema index* no FAR-encoding transcripts were identified from over 500 ESTs (J. Jones, pers. comm.). The increasing number of parasitic nematode EST and genome sequence databases should help provide the starting material for such studies. Future work should therefore include a genetic analysis of the FAR genes in other free-

living nematode species to address this issue. Future work could also include gene knockout (dsRNA inhibition) experiments. However, this would be difficult to interpret in the genetically amenable *C. elegans* due to the presence of at least seven FAR genes in this species and so this experiment would be dependent on multiple gene knockouts in this species or the development of gene knockout technology in parasitic nematodes.

7.5. Mass Lipid Transport in Nematodes

Lipids are the major energy reserve in nematodes and are the most widely studied macromolecules in these organisms (Chitwood, 1998) but very little is known about the acquisition, transport, storage and utilisation of lipids in nematodes. However, several lines of evidence suggest that nematodes may have a mass lipid transport system similar to those of insects and mammals.

The mass lipid transport systems of insects and mammals have been widely studied and have proved to be highly complicated, hormonally regulated systems involving a range of interchangeable proteins and lipoprotein particles. These systems function in the receptor-mediated transport of a range of hydrophobic compounds (including phospholipids, di- and/or tri-acylglycerols, free cholesterol, cholesterol esters, carotenoids and other hydrocarbons) in highly ordered, soluble lipoprotein particles, from their sites of acquisition or synthesis to their sites of storage, utilisation or excretion.

The main functions of the insect lipophorin system are proposed to be the mobilisation of lipid reserves to fuel prolonged flight activity in adult insects, and lipid acquisition and storage in larval stages (Ryan & Van der Horst, 2000). In mammals, the main functions of the lipoprotein system are in the acquisition of dietary lipids and the forward and reverse

transport of cholesterol between the liver (site of synthesis and excretion) and peripheral tissues (site of utilisation). Since nematodes have a sterol auxotrophy, it is likely that they have evolved highly efficient systems for the uptake, transport, storage and utilisation of these molecules and this may involve a lipoprotein-like system similar to the functionally conserved systems of insects and mammals. The advantage of these systems is that a few protein molecules can bind multiple molecules of a variety of hydrophobic compounds and permits a high cholesterol (and TAGs etc) acquisition per molecule of protein expressed.

Also, as discussed in Chapter 6, FAR and NPA amino acid sequences have stretches of low levels of amino acid similarity with mammalian and insect exchangeable apolipoproteins and ABA-1 was shown in the present study to inhibit aggregation of human LDL *in vitro*. A low density lipoprotein (LDL) receptor-related protein was identified in *C. elegans* (Yochem & Greenwald, 1993) which has homologues in both mammals and insects (Ryan & van der Horst, 2000). This protein is expressed in and apically secreted from the hypodermis in *C. elegans* and mutants lacking this gene are defective in cuticle moulting, a phenotype which is also observed upon sterol starvation (Yochem *et al.*, 1999), again indicating the possible involvement of a lipoprotein-like system in nematodes. This could indicate that the putative nematode lipoprotein system is also involved in cuticle synthesis and moulting. Transport of hydrocarbons and carotenes to sites of cuticle synthesis has also been proposed as a function of the insect lipophorin system (Takuchi & Chino, 1993). So, several lines of evidence exist which could indicate the presence of a previously undescribed nematode lipoprotein system.

The presence of NPA mRNA in the gut of PCN reported in this work supports previous proposals of a role for the NPAs in lipid nutrient acquisition and transport. Work

in animal-parasitic nematodes indicates that the NPA proteins are present in abundance in pseudocoloemic fluid (Kennedy *et al.*, 1988). It is possible then that NPA genes are expressed in the gut where the NPAs become loaded with dietary-acquired or stored lipid and then transport to the pseudocoelomic fluid for delivery to their sites of utilisation. Mobilisation of lipid reserves from sites of storage to their sites of utilisation is the best understood aspect of mass lipid transport in insects and mammals and is used here to illustrate how the NPAs could be involved in mass lipid transport in nematodes. The gut is the main site of storage for lipids in the form of droplets of triacylglycerols, which are the main energy reserve in nematodes (Barrett & Wright, 1998). This is analogous to adipose tissue and the fat body which are the main sites of triacylglycerol storage in mammals and insects respectively. Mobilisation of these stored lipid reserves in mammals and insects share similarities and striking differences which could give clues as to how this is achieved in nematodes. In mammals, during prolonged exercise, a hormone-sensitive lipase (HSL) is activated in adipose tissue which moves from the cytosol to the fat droplet and catalyses the hydrolysis of TAG to DAG, MAG and finally, free fatty acids. These fatty acids are bound by serum albumin in plasma and transported to muscle cells where they are released and undergo β-oxidation to provide energy (Ryan & Van der Horst, 2000). In insects, in response to flight activity, adipokinetic hormone is released which activates fat body TAG lipase leading to the hydrolysis of TAG to DAG. The DAG then binds to HDLp in the haemolymph and the exchangeable apolipoprotein, ApoLpIII binds to and stabilises the DAG-enriched LDLp particle. LDLp then moves through the haemolymph to the flight muscles where the DAG is hydrolysed to free fatty acids which undergo β-oxidation to provide energy (Ryan & Van der Horst, 2000). Thus, the similarities between mammalian

and insect systems of lipid mobilisation indicate functional conservation of these systems, however distinct differences are also apparent. Future work should therefore be directed at identifying the components involved in nematode lipid mobilisation pathways. Indeed, *C. elegans* sequence database searches identified one protein with strong similarity to mammalian HSLs. Since this family of lipases is distinct from other lipase classes (i.e., has little amino acid similarity) (Osterlund *et al.*,1996), it is likely that the *C. elegans* protein plays a similar role in lipid mobilisation as the mammalian HSLs. If so, what hormone (if any) is responsible for its activation? Does it catalyse hydrolysis of TAG to free fatty acids (like mammals) or to DAG (like insects)? The presence of NPA mRNA in gut cells, and the reported ability of these proteins to bind fatty acids and larger lipids (Xia *et al.*, 2000, Kennedy *et al.,* 1995c) indicate that the NPAs could function in either or both of these pathways and so could have both albumin-like and apolipophorin-like properties.

Little is known about the composition of lipid storage droplets in nematodes, and future work could be undertaken to investigate whether any proteins are involved in their makeup. In mammals, for example, lipid droplets in adipose tissue are bounded by unique proteins called perilipins, which stabilise the droplet. These proteins also play a role in regulation of lipid mobilisation by allowing HSL access to the droplet upon lipolytic stimulation (cited in Ryan & van der Horst, 2000). Searches of *C. elegans* sequence databases found no evidence of perilipin-like sequences in *C. elegans* so future work could be aimed at identifying nematode proteins which play this role. Indeed, it is possible that the NPAs are involved in this so future work should be undertaken to investigate this.

Future work in this area could also include attempts to isolate lipoprotein-like particles from pseudocoloemic fluid or nematode homogenates using techniques described

for purification of mammalian lipoprotein particles and/or insect lipophorin particles. If found, these could be tested in the lipoprotein substitution assay described in Chapter 6 to see if they are interchangeable with the mammalian and insect systems. SDS-PAGE analysis could then be used to estimate the size(s) of the proteins involved. Some workers report the secretion of NPAs into host tissues and/or culture medium (e.g., Tweedie *et al.*, 1993, Culpepper *et al.,* 1992, Poole *et al.*, 1992) and these are in agreement with the hypodermal localisation of NPA mRNA observed in this study (Chapter3). However, in other species, such as *T. canis*, secretion does not appear to occur (Maizels *et al.*, 1984). So, given the results in Chapter 6, it is possible that these proteins have adapted from an internal role in mass lipid transport in nematodes and, in animal parasitic nematodes at least, could be involved in disruption of, and acquisition of lipids from the host mass lipid transport system although this was not studied in the present work. Indeed, it was reported that the trematode *S. mansoni* can bind host lipoprotein particles on their surface and then internalise them (Bennet & Caulfield, 1991) so this would support the above hypothesis.

In future experiments on the effect of NPAs on mass lipid transport it would be interesting to investigate the effects of adding fatty acids or retinol to the mixture. As ABA-1 has been shown to bind fatty acids it is possible that this could act as a regulatory mechanism in lipid metabolism by preventing binding of larger lipids destined for catabolic breakdown when concentrations of fatty acid are high (and less lipid metabolism is therefore required). Indeed this could be the function of retinoid- and fatty acid-binding observed in the *D. melanogaster* proapolipophorin discussed above.

The NPA monomers from the *A. suum* polyprotein, such as the ABA-1A monomer used in this work, are relatively monomorphic and so probably fulfil the same function as

one another. However, in other nematodes, such as *C. elegans* and *D. viviparus*, the NPA monomers have been shown to be highly polymorphic and therefore may fulfil distinct functions. Indeed, two diverged Dva-1 NPA monomers have been shown to bind haem and cations such as Ni^{2+}, Cu^{2+}, Zn^{2+} (Kennedy, 2000). It would therefore be useful to clone and express sequence-divergent NPA monomers from polymorphic species and subject these to functional studies to determine whether these also bind fatty acids and retinol. Localisation studies using probes or antibodies specific to the different monomers should also be carried out as distinct expression patterns may also point to distinct functions. Interestingly, NPAs are translated as a long polyprotein containing all the NPA monomers separated by tetrabasic endoproteinase cleavage sites. The insect apolipophorins I & II are also translated as one long polypeptide, proapolipophorin which contains a dibasic endoproteinase cleavage site between the two proteins (Kutty *et al.,* 1996). It is proposed that this form of expression ensures a 1:1 stoichiometry for the apolipophorins. It is possible then that the NPA polyproteins are expressed in this way to ensure the proteins are present in the correct stoichiometry.

As ApoB, MTP, Vgs & ApoLps are all derived from a common ancestor (Babin *et al*, 1999), it is likely that apolipoprotein molecules from nematodes would also be related. Thus, screening of *C. elegans* genome with these sequences could identify such proteins.

Interestingly, *C. elegans* is known to encode six vitellogenin genes, *vit-1-vit6*, which function in transport of lipids to, and storage in oocytes. Five of these proteins have been shown to be closely related to one another, however, *vit-6* encodes a distantly related protein (Spieth & Blumenthal, 1985). This diverged vitellogenin is also present in aged non-reproducing nematodes indicating functions other than lipid storage in eggs (Nakamura

et al., 1999). It is possible then that vitellogenin-6 could act as a non-exchangeable apolipoprotein in nematodes. Alternatively, the nematode lipid transport system in nematodes may be diverged from those of mammals and insects and it is possible that NPAs alone are involved in lipoprotein particle. This theory is supported by the recent discovery of atypical lipophorin particles in *Dactylopius confusus* and *Musca domestica* which contained small apolipophorins between 20 and 26 kDa, which were postulated to function in cuticle formation in insects (cited in Ryan & van der Horst, 2000).

As discussed previously, the morphological and genetic diversity of nematodes makes generalisation and comparison of their genes difficult. It is important to note that in this study *A. suum* NPA protein was used because no recombinant PCN NPA was available. Thus, it is possible that PCN NPA may not have inhibited lipoprotein aggregation and that this ability is an adaptation of animal-parasitic nematodes to their host environment. Also, the morphological diversity of PCN and *Ascaris spp.* could lead to divergence in the lipid transport systems of these species. For example, *Ascaris spp.* have a large pseudocoloemic space while those of plant-parasitic nematodes are generally very small and so lipoprotein particles from *Ascaris* would require to travel further throughout the body of this nematode. Future work, therefore should include the expression and functional characterisation of NPAs from free-living, entomopathogenic and plant-parasitic species to allow comparison with the NPAs from animal-parasitic species.

7.6. Control and Diagnosis

The FAR and NPA proteins have several features which make them ideal targets for diagnosis and control strategies. Both the FAR and NPA proteins appear to be specific to

nematodes, they are relatively sequence-diverse and some are secreted onto the nematode surface and therefore easily accessible (to inhibitors etc). Also, it has been reported that a decline in nematode infectivity is associated with a decline in lipid reserves in PCN and that slower hatching of *G. pallida* (compared to *G. rostochiensis*) correlates with the slower lipid utilisation seen in *G.pallida* J2s (reviewed by Barrett & Wright, 1998). As both the NPAs and FAR proteins are involved in lipid-binding , and the NPAs have been implicated in nutrient acquisition, it is possible that inhibiting these proteins could slow lipid utilisation enough to prevent infection.. Attempts were made to screen phage-displayed peptide libraries with Gp-FAR-1 to isolate peptides which would bind to and inhibit the function of this protein. It was hoped that any peptides isolated in this way could be inserted into potato roots by either transformation or microinjection and then exposed to PCN infection. As well as yielding information on the importance of Gp-FAR-1 in nematode pathogenesis this could also have identified potential control candidates. As Gp-FAR-1 is expressed in all life-cycle stages of *G.pallida*, control strategies could also be targeted to the free-living infective stage juveniles. This could allow development of soil applications avoiding the politically contentious issue of genetic modification of food crops and, given the diversity of FAR sequences across species, would be unlikely to cross-react with any non-pathogenic nematodes in the soil. However, screening of five constrained and one linear phage-displayed peptide libraries and an antibody display library with Gp-FAR-1 yielded no binding peptides. It is unclear why no binding peptides were isolated especially since in subsequent experiments using another surface-secreted PCN protein, peroxiredoxin (Tpx), two high affinity binding peptides were isolated after only two rounds of selection and preliminary experiments indicate that one of these peptide inhibits the function of Tpx.

The specificity of these proteins for nematodes and the sequence diversity of these genes across species also make these proteins and their genes ideal candidates for the development of diagnostic tools. Indeed, preliminary work on the gene sequences of plant-parasitic nematodes has shown that differences may exist which can be used to differentiate between plant-parasitic nematode species and also between different pathotypes within the same species (M. Phillips, pers. comm.), although further work is required to confirm this. It is likely that this would also apply for animal-parasitic and free-living species (as these are equally sequence-diverse) and so these genes may represent ideal candidates for the development of diagnostic tools which can detect interspecific (PPN) and intraspecific (different pathotypes) variation and therefore be used for nematode identification.

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