A surface-associated retinol- and fatty acid-binding protein (Gp-FAR-1) from the potato cyst nematode *Globodera pallida*: lipid binding activities, structural analysis and expression pattern

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Parasitic nematodes produce at least two structurally novel classes of small helix-rich retinol- and fatty-acid-binding proteins that have no counterparts in their plant or animal hosts and thus represent potential targets for new nematicides. Here we describe a protein (Gp-FAR-1) from the plant-parasitic nematode *Globodera pallida*, which is a member of the nematode-specific fatty-acid- and retinol-binding (FAR) family of proteins but localizes to the surface of this species, placing it in a strategic position for interaction with the host. Recombinant Gp-FAR-1 was found to bind retinol, *cis*-parinaric acid and the fluorophore-tagged lipids 11-(dansylamino)undecanoic acid and dansyl-D,L-α-amino-octanoic acid. The fluorescence emission characteristics of the dansylated analogues indicated that the entire ligand enters the binding cavity. Fluorescence competition experiments showed that Gp-FAR-1 binds fatty acids in the range C_{11} to C_{24}, with optimal binding at C_{15}. Intrinsic fluorescence analysis of a mutant protein into which a tryptophan residue had been inserted supported computer-based predictions of the position of this residue at the protein's interior and possibly also at the binding site. Of direct relevance to plant defence systems was the observation that Gp-FAR-1 binds two lipids (linolenic and linoleic acids) that are precursors of plant defence compounds and the jasmonic acid signalling pathway. Moreover, Gp-FAR-1 was found to inhibit the lipoxygenase-mediated modification of these substrates *in vitro*. Thus not only does Gp-FAR-1 function as a broad-spectrum retinol- and fatty-acid-binding protein, the results are consistent with the idea that Gp-FAR-1 is involved in the evasion of primary host plant defence systems.

Key words: host–parasite interaction, lipoxygenase, plant defence, secretions.

INTRODUCTION

Plant parasitic nematodes destroy up to 10% of the world's agricultural output, costing as much as $\$77 \times 10^9$ each year in yield losses and control costs [1]. Of this figure, the greatest proportion by far is caused by the sedentary endoparasitic nematodes such as the root knot nematodes (*Meloidogyne* spp.) and the cyst nematodes including the potato cyst nematodes *Globodera rostochiensis* and *Globodera pallida*. These biotrophic parasites spend most of their life cycles buried within the roots of their host plants. Emergence from the environmentally robust cysts is stimulated by host root diffusates; second-stage juveniles (J2s) of potato cyst nematodes invade the root tip in the zone of elongation and migrate intracellularly to the cortex surrounding the vascular tissue. Here they induce a series of specific changes in the cells of their host, which become enlarged, multinucleate and highly metabolically active, forming a syncytium, which provides the resources for the parasite to develop through a further three moults to the adult stage. After establishment of this feeding site, the locomotory musculature of the parasites degenerates and they become entirely dependent on the modifications of the host tissue that they have induced.

Secreted proteins of plant parasitic nematodes have been implicated in many aspects of the host–parasite interaction. For example, secreted cell-wall-degrading enzymes are thought to be important in migration of the nematode through the root before the induction of the feeding site [2,3]. Secretions might be involved in the induction and maintenance of the syncytium, although direct evidence for this is still lacking (reviewed in [4]). The nematodes spend most of their development embedded within an organism that possesses a battery of chemical and physical defences; the parasites consequently release substances capable of masking them from their host or of down-regulating host defence responses [5].

With the completion of genomic sequence of the nematode *Caenorhabditis elegans* it has become clear that approx. 58% of the encoded proteins have no known counterparts. Of these new protein types, only two have been ascribed biochemical activities. One of these is a recently described family of fatty-acid- and retinol-binding (FAR) proteins [6], which are structurally distinct from proteins of similar biochemical activity and size from other groups of organisms and might perform special functions in obligate parasites. Until now the biochemistry of FAR-type proteins has been investigated only in animal-parasitic nematodes; here we describe the properties of one (Gp-FAR-1) from the plant-parasitic nematode *G. pallida*. We demonstrate that this retinol- and fatty-acid-binding protein is capable of inhibiting a major plant defence pathway *in vitro*; we argue that it is

Abbreviations used: DACA, dansyl-D,L-α-amino-octanoic acid; DAUDA, 11-(dansylamino)undecanoic acid; FAR, fatty-acid- and retinol-binding protein; Gp-FAR-1, FAR from *Globodera pallida*; J2, second-stage juvenile; rGp-FAR-1, bacterial recombinant Gp-FAR-1 protein.

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strategically located, and possesses relevant biochemical properties, for it to have a crucial role in the host–parasite interaction.

MATERIALS AND METHODS

cDNA cloning

Cysts, J2s, virgin females and adult females of *G. pallida* were obtained by methods described previously [7]. An antiserum (SCRI-5835) was raised against whole J2s of *G. pallida* by using a method described previously [8]. Immunocytochemical studies showed that this antiserum recognized a range of structures of potato cyst nematode J2s including the parasite surface. To identify surface components, this antiserum was then used to screen approx. 25000 plaques of a mixed-stage *G. pallida* cDNA library with the use of methods described previously [7]. Inserts from plaques identified as positives after secondary screening were subcloned into a plasmid vector (pCR-Script; Stratagene) for further analysis. Sequence analysis of all clones was performed on an ABI 373 Stretch automatic sequencer with an Applied Biosystems PRISM Ready Reaction DyeDeoxy Terminator cycle sequencing kit. Sequence comparisons were made with a range of databases by using BLAST searches through the EBI World-Wide Web pages (http://www.ebi.ac.uk/ebi-home.html). SignalP and PSORT were used to predict the position of the signal/leader sequence and protein localization signals. Structural predictions were performed with programs available through the ExPASy molecular biology server (http://expasy.hcuge.ch/www/tools.html). Confirmation that the 5′ end of the mRNA had been isolated was obtained by generating a PCR product with an internal primer (GPFAR5′r) in combination with a primer designed to bind to the SL1 *trans*-spliced leader sequence of nematodes. This PCR product was then cloned into pCRScript and sequenced as above.

Analysis of gene expression patterns

Temporal expression patterns were examined by reverse-transcriptase-mediated PCR by using the primers shown below with cDNA extracted from J2s, virgin females and adult females. Negative control reactions contained mRNA from the appropriate nematode stage that had not been subjected to a reverse transcriptase reaction. PCR products were separated on 1.5% (w/v) agarose gels and stained in ethidium bromide by using standard protocols [9].

The spatial localization of the mRNA was examined by using *in situ* hybridization performed essentially as described by De Boer et al. [10]. In brief, PCR products were generated with the primers below, and the sense or anti-sense strand was labelled with digoxigenin (Roche Diagnostics, Lewes, East Sussex, U.K.). These probes were hybridized to fragments of *G. pallida* J2s that had previously been fixed in 2% (w/v) paraformaldehyde and permeabilized with proteinase K. After extensive washing, bound probe was detected with anti-digoxigenin antibody coupled to alkaline phosphatase (Roche Diagnostics), which was revealed with Nitro Blue Tetrazolium and X-Phosphate (Roche Diagnostics, Lewes, East Sussex, U.K.) as described previously [9].

The fate of the protein encoded by the cDNA isolated from library screening was investigated with an antibody raised against recombinant protein (see below). This antiserum was raised in a rabbit that was injected with four 100 μg aliquots of recombinant Gp-FAR-1 (rGp-FAR-1) at 2-week intervals. The antiserum was tested by Western blotting. Nematodes were processed for these observations with methods described previously [11]. Negative controls (specimens incubated in preimmune serum or secondary antibody alone) were examined throughout. Specimens were viewed under an Olympus fluorescence microscope.

Expression of recombinant Gp-FAR-1

A fragment of *gp-far*-1 corresponding to the entire coding region except for the predicted signal sequence was amplified from plasmid stocks with primers (GPFARf and GPFARr), then cloned into pGEX2T (Pharmacia) by standard protocols. Plasmid stocks were initially made with DH5 alpha cells (Life Technologies), but purified plasmid was then transformed into BL21 cells for the expression of fusion protein. Protein was expressed by following the kit manufacturer's instructions; rGp-FAR-1 was released from the glutathione S-transferase moiety by cleavage with thrombin. The purity of the protein was confirmed by standard SDS/PAGE analysis on Tris/glycine Mini-protean 10–20% (w/v) polyacrylamide gels (Bio-Rad). Samples were passed down a 2 ml Extracti-gel D column (Pierce) to remove residual detergent. Protein yield was quantified by spectrophotometry, with a predicted molar absorption coefficient at 280 nm (ϵ_{280}) of 2560 $M^{-1} \cdot cm^{-1}$ based on the amino acid composition of the recombinant protein.

Spectrofluorimetry

All fluorescence experiments were performed with a SPEX FluorMax spectrofluorimeter (Spex Industries, Edison, NJ, U.S.A.) with 2 ml samples in silica cuvettes as described previously [12,13]. PBS blanks were used to correct for Raman and background scattering where necessary. The ligand binding capacity of rGp-FAR-1 was investigated with the fluorescent ligands 11-(dansylamino)undecanoic acid (DAUDA), dansyl-D,L-α-amino-octanoic acid (DACA), *cis*-parinaric acid (Molecular Probes), dihydroergosterol and retinol (Sigma). Interaction of the dansyl fluorophore reporter group in isolation was investigated with 5-(dimethylamino)naphthalene-1-sulphonamide (dansylamide; Sigma). Binding of non-fluorescent ligands was detected by a reversal of the wavelength shift and a decrease in fluorescence emission intensity on successive additions of test ligand to a DAUDA–rGp-FAR-1 complex.

The fluorescent compounds dansylamide, DAUDA, DACA, *cis*-parinaric acid and dihydroergosterol were prepared as 10 mM stock solutions in ethanol. DAUDA, DACA, dansylamide and dihydroergosterol were diluted 1:1000 in PBS for use in the assays; *cis*-parinaric acid was diluted 1:2000 in PBS. Retinol solution was freshly prepared at 10 mM in ethanol and was further diluted in ethanol and added directly to the protein solutions to avoid the degradation of retinol in water. Stock solutions of all the non-fluorescent competitors were made to approx. 10 mM in ethanol, then diluted 1:10, 1:100 and 1:1000 in PBS for use in the assays.

Dissociation constants were estimated in fluorescence titration experiments as described previously [12,13]: protein solution was added incrementally to 2 ml of buffer containing approx. 1 μM DAUDA in a fluorescence cuvette. For non-fluorescent ligands, apparent dissociation constants [K_d (app)] were obtained by incremental additions of test ligand to a preformed DAUDA–Gp-FAR-1 complex. Because of the lability of retinol in water, it was added as a solution in ethanol.

To investigate the preference of Gp-FAR-1 for fatty acid chain length, the same molarity of test fatty acid was added to a DAUDA–Gp-FAR-1 complex and the percentage decrease in DAUDA fluorescence was recorded at the peak fluorescence

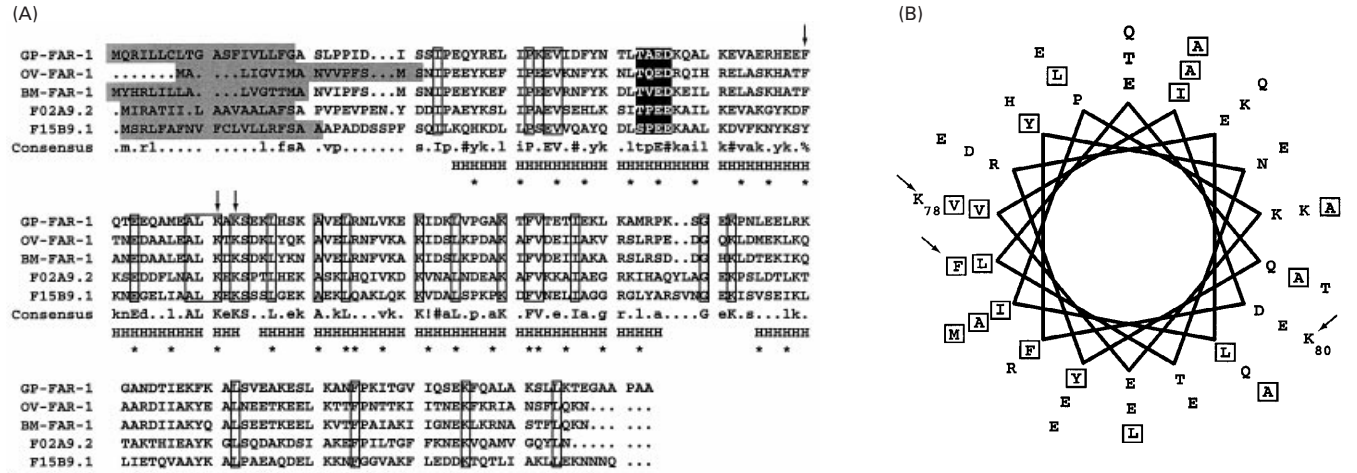

Figure 1 Sequence analysis of the FAR family of proteins

(**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. A larger alignment is available at http://nema.cap.ed.ac.uk/genes-of-interest/LBP-html/LBP-20.html. 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 [17], and the black boxes indicate a potential casein kinase II site, the position of which is conserved in all counterparts studied so far [13]. The consensus line indicates absolutely conserved amino acid positions as well as amino acids considered to be functionally conserved with the Dayhoff substitution 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 α-helix and gaps indicate regions for which no structural prediction emerged. No β-structure was predicted by this or other programs. Amino acids selected for site-directed mutagenesis are indicated by arrows. Ov-FAR-1, *Onchocerca volvulus* homologue [35]; Bm-FAR-1, *Brugia malayi* homologue; 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 arrows. This region is also a region of strongly predicted coiled coil (see the text).

emission wavelength of DAUDA in the protein (488 nm). All fatty acids used in this experiment were dissolved in ethanol as 10 mM stock solutions, then diluted 1:10, 1:100 and 1:1000 in PBS or ethanol for use in these assays.

Site-directed mutagenesis

The GeneEditor site-directed mutagenesis (SDM) kit (Promega) was used with mutagenic oligonucleotides to introduce mutations at selected residues of Gp-FAR-1 (see Figure 1). The binding properties of the mutant proteins were investigated with the use of fluorimetric techniques as described above. K_d values for *cis*-parinaric acid were obtained for each mutant. The intrinsic fluorescence of the tryptophan residue introduced in the mutant Phe-67 → Trp (F67W) was used to investigate the surface accessibility of this residue and its distance from the binding site of Gp-FAR-1. Surface accessibility was determined by succinimide quenching by using the Stern–Volmer equation as previously described [14,15]. Quenching of L-tryptophan was measured to represent fully exposed tryptophan; quenching of tryptophan in Gp-FAR-1 and in Gp-FAR-1 denatured by exposure to 6 M guanidine hydrochloride was compared.

Lipoxygenase assays

Lipoxygenase-mediated breakdown of fatty acids to their hydroperoxides can be quantified spectrophotometrically by measuring A_{234}, an increase in which indicates the formation of double bonds. Under the conditions used this indicates the 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 the absence of rGp-FAR-1. Soybean lipoxygenase (Sigma) was diluted in ice-cold 0.2 M borate buffer and used at a concentration of 100 units per reaction. rGp-FAR-1, BSA (Northumbria Biologicals Ltd) and ribonuclease A (Boehringer Mannheim) stock solutions were made to 100 μM in PBS for use in the assays. Linoleic acid (Sigma) was emulsified in 95% (v/v) ethanol and dissolved in sterile distilled water, then diluted in filter-sterilized 0.2 M borate buffer, pH 9.0, to a final concentration of 134 μM for use in these assays. A 600 μl sample of the above linoleic acid solution was added to the cuvette, and A_{234} was measured every 5 s for 5 min in an Ultrospec 2000 UV/visible spectrophotometer (Pharmacia) and used as reference. Lipoxygenase (100 units) was added quickly, and the absorbance was measured as above; the blank was 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 reference. Lipoxygenase was then added quickly and A_{234} was measured as above. To quantify any dilution effect, another reaction was set up where PBS was added in place of protein and A_{234} was measured as before.

Primers

For experiments examining gene expression throughout the parasite life cycle, the same pair of primers (GPFARf and GPFARr), designed to amplify part of the coding region for subcloning into an expression vector, were used. The GPFARf primer was used in combination with the GPFARISHr primer to generate PCR products for *in situ* hybridization. The sequences of these primers and the others used in this study were

as follows: GPFARf, 5′-agctggatccTCGCTGCCGCCCATTG-AT-3′; GPFARr, 5′-agctgaattcGGCTGCCGGCGCGGCG-CC-3′; GPFARISHr, 5′-TGCTGTGCAGCTTCTCGCTG-3′; GPFAR5′r, 5′-TTCCATTGCCTGTTCTTC-3′; SL1, 5′-GGT-TTAATTACCCAAGTTTGAG-3′.

RESULTS AND DISCUSSION

Gp-FAR-1 protein

Antibody screening of a mixed-stage *G. pallida* cDNA library and sequencing of cDNA species obtained from several independent positively reacting plaques provided a full-length cDNA sequence of 743 nt. This value is commensurate with the size of the mRNA to which a probe derived from the cDNA hybridized on Northern blots (results not shown). Reverse-transcriptase-mediated PCR experiments indicated that the nematode SL1 leader sequence is *trans* spliced to the mRNA *in vivo*. At the 3′ end of the mRNA a putative polyadenylation signal (AATAAA) precedes the start of the poly(A) tail by 12 nt, a similar spacing to that observed on many *C. elegans* mRNA species [16]. The cDNA predicts a 20922.32 Da protein with close similarity to the nematode-specific FAR proteins [6,13]. The encoding gene was designated *gp-far*-1 and the protein Gp-FAR-1, in keeping with the terminology previously suggested for this group of proteins [6]. The predicted amino acid sequence includes a hydrophobic leader peptide that is predicted by the SignalP program [17] set for eukaryotic sequences to be cleaved between Gly-19 and Ala-20 to give a mature polypeptide of 18844.71 Da. The PSORT program predicts that the leader is involved in directing the protein to a secretory compartment (Figure 1). This leader is presumably removed post-translationally; the putative cleavage site corresponded to the N-terminus of a homologue from another nematode from which sufficient protein was obtained for direct sequencing [13]. A predicted N-linked glycosylation site (Asn-Asp-Thr) is present at residues 138–141 and, in common with all other counterparts of this protein [13], a conserved consensus casein kinase II phosphorylation site is present at residues 50–53 (Figure 1). Only minor, if any, post-translational modifications are likely, because Western blots with an antiserum raised against rGp-FAR-1 revealed an entity of approx. 20 kDa in homogenates of the parasites (Figure 2), a figure similar to the calculated molecular mass of the predicted mature protein. In common with FARs from animal parasites [13], secondary structure analysis of Gp-FAR-1 predicts a structure rich in α-helix, with several strongly amphipathic helical stretches (Figure 1). A further structural property shared with the counterparts from animal parasites is that a coiled coil structure is strongly predicted for Gp-FAR-1 by the Coils algorithm [18], with the prediction being strongest between positions 60–100 and 130–168 (results not shown).

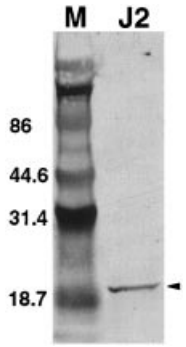

Figure 2 Presence of Gp-FAR-1 in infective J2s of *G. pallida*

Western blot analysis of proteins extracted from *G. pallida* J2s probed with an antiserum raised against rGp-FAR-1. A single band of molecular mass approx. 20 kDa is detected. Lane M, molecular mass standard proteins (Kaleidoscope markers; Bio-Rad), whose molecular masses are as indicated (in kDa) at the left.

Expression site and protein localization

Immunolocalization studies with antiserum raised against recombinant Gp-FAR-1 (rGp-FAR-1) showed that the protein was present on the surface of the infective J2 after hatching (Figures 3a and 3b). The target antigen was also shed from the surface of J2s (Figure 3c). *In situ* hybridization showed that the mRNA derived from *gp-far*-1 was present in the hypodermis (which immediately underlies the cuticle) of *G. pallida* J2s (Figure 3d); it remains to be established whether the protein is transported across the acellular matrix of the cuticle or released via secretory glands. Experiments with reverse-transcriptase-mediated PCR indicated that the *gp-far*-1 mRNA was present in all life-cycle stages tested (Figure 4).

Ligand binding

In fluorescence-based binding assays, rGp-FAR-1 was found to bind the fluorophore-tagged fatty acid DAUDA (Figure 5a) and the intrinsically fluorescent *cis*-parinaric acid (Figure 5c). Dihydroergosterol was not found to bind to rGp-FAR-1 (results not shown). The degree of blue shift in fluorescence emission by DAUDA (from 543 nm in buffer to 485 nm) indicated that rGp-FAR-1 has a highly apolar binding site (see Figure 5) [19]. The blue shift in emission was much greater than occurs when DAUDA enters the binding site of members of the FABP/P2/CRBP/CRABP family of β-structure-rich fatty-acid-binding proteins such as rat liver FABP and rat intestinal FABP, which produce shifts in DAUDA emission to 496 and 492 nm respectively [20,21] and is much closer to that produced with the polyprotein lipid-binding proteins of nematodes (approx. 475 nm [12]). DAUDA and DACA (which have the dansyl fluorescent reporter group attached to the methyl and α-carbon atoms respectively) both bound to rGp-FAR-1 with similar wavelengths of peak fluorescence emission. The fact that the blue shifts were similar regardless of which end of a fatty acid bore the dansyl fluorophore can be taken to indicate that the ligand is held entirely within the binding site and away from polar solvent. Dansylamide did not bind to rGp-FAR-1, so the dansyl fluorophore does not itself seem to contribute to the binding of DAUDA and DACA.

Natural, non-fluorescent fatty acids were found to bind to rGp-FAR-1 in competition with DAUDA (Figure 5a); a change in the fluorescence emission of retinol when added to the protein indicated that it, too, bound (Figure 5b). Fatty acids were found to displace retinol from its binding site, indicating that the retinol- and fatty-acid-binding sites are either congruent or interactive (Figure 5b). Titrations were performed to determine the binding affinities of DAUDA, retinol and *cis*-parinaric acid, yielding K_d values of 1.16 μM for DAUDA, 0.14 μM for retinol and 0.08 μM for *cis*-parinaric acid. The results were consistent with a 1:1 ligand-to-protein stoichiometry. Binding affinities for linoleic and linolenic acids were measured indirectly by incremental additions of the competitors to preformed DAUDA–Gp-FAR-1 complexes; values of 5.2 and 2.9 μM respectively were obtained. All of these values fall within the range of dissociation constants (approx. 0.1–10 μM) reported for

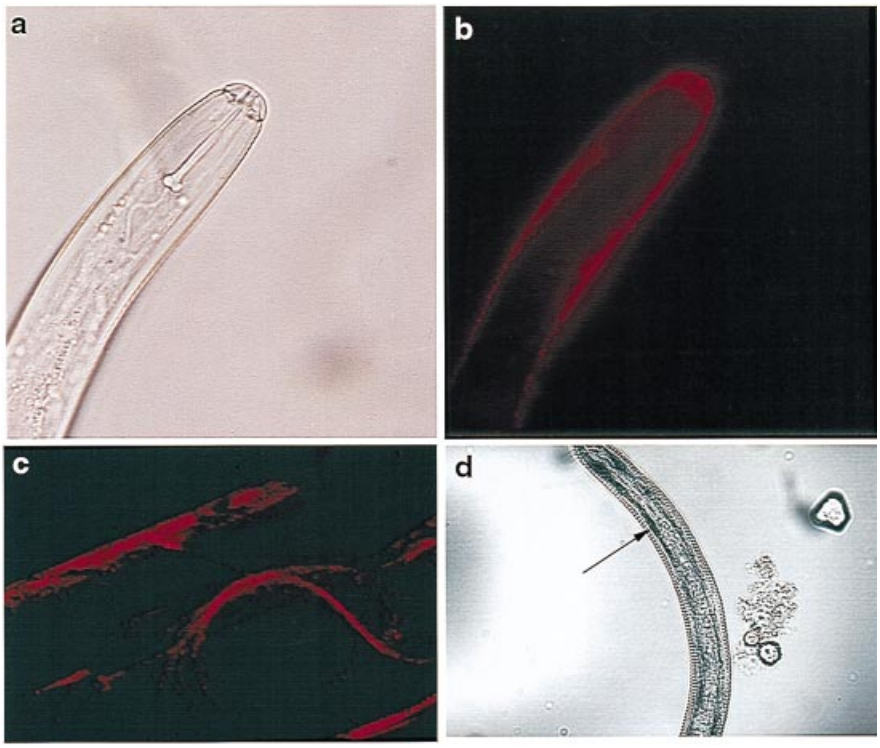

Figure 3 Localization of the Gp-FAR-1 protein and site of transcription

Immunofluorescence localization with an antiserum raised against rGp-FAR-1, showing that the protein is present at the surface of the J2. (**a**) Bright-field image. (**b**) Same specimen viewed under fluorescence optics. (**c**) Presence of target antigen in material shed from the surface of the J2. Controls using preimmune serum or secondary antibody alone showed no such staining (results not shown). (**d**) Localization of mRNA encoding Gp-FAR-1 to the hypodermis (arrow) of the nematode; the hypodermis is the cell layer beneath the cuticle of the worm; it produces the cuticle and associated structures.

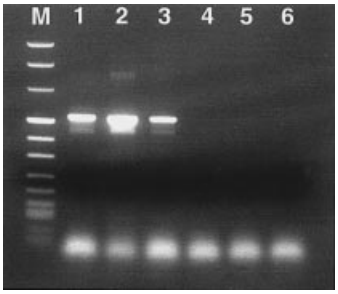

Figure 4 Timing of transcription in the life cycle of *G. pallida*

Reverse-transcriptase-mediated PCR analysis showing that the mRNA encoding Gp-FAR-1 is present in all life-cycle stages of the parasite examined. The expected size of the PCR product is 520 bp. Lane M, DNA size markers (Marker 8; Roche Diagnostics) [marker sizes in descending order are 1114, 900, 692, 501 and 489 (these two migrating as one band), 320, 242, 190 and 147 bp, with several smaller bands]; lane 1, J2 cDNA; lane 2, virgin female cDNA; lane 3, adult (gravid) female cDNA; lanes 4–6, negative controls (mRNA, no reverse transcriptase reaction) for each life-cycle stage.

transporter/shuttle proteins carrying fatty acids and retinoids [22–24].

The preference of Gp-FAR-1 for fatty acid chain length was investigated by competition for binding with DAUDA with saturated and unsaturated fatty acids of different chain lengths (Figure 6). Gp-FAR-1 showed maximal binding to saturated fatty acids in the range $C_{12:0}$ to $C_{17:0}$; DAUDA displacement was greatest with $C_{15:0}$ (Figure 6). The longer-chain-length fatty acids are sparingly soluble in water, so the experiments were repeated with ethanol as the solvent for the competitors; the ethanolic solutions, added directly to the fluorescence cuvettes. The results were essentially unchanged, with maximal binding occurring in the range $C_{12:0}$ to $C_{19:0}$ and the greatest DAUDA displacement with $C_{15:0}$ fatty acids (results not shown). Experiments with unsaturated fatty acids gave similar results; no preference was shown for saturated or unsaturated chains, although the longer, highly unsaturated arachidonic ($C_{20:4}$) and docosahexaenoic ($C_{22:6}$) acids competed well. The preference of Gp-FAR-1 for fatty acid chain length therefore correlates favourably with the most abundant fatty acids found in the organism [25].

Although retinol and a broad range of fatty acids bound to Gp-FAR-1, no binding was observed with a range of hydrophobic compounds of relevance to plant defence responses, including the plant defence glycoalkaloids α-chaconine and α-solanine and several phenylpropanoids (caffeic, chlorogenic and ferulic acids) (results not shown).

Inhibition of lipoxygenase

The binding specificity of Gp-FAR-1 encompasses lipids (such as linoleic and linolenic acids) involved in plant defence responses, either as effectors or signalling molecules or as their precursors. The location of the protein at the surface of the invasive stage of the parasite could also be taken to indicate a crucial role for Gp-FAR-1 in countering plant defence reactions. The binding of lipids such as linoleic or linolenic acids by the protein, for

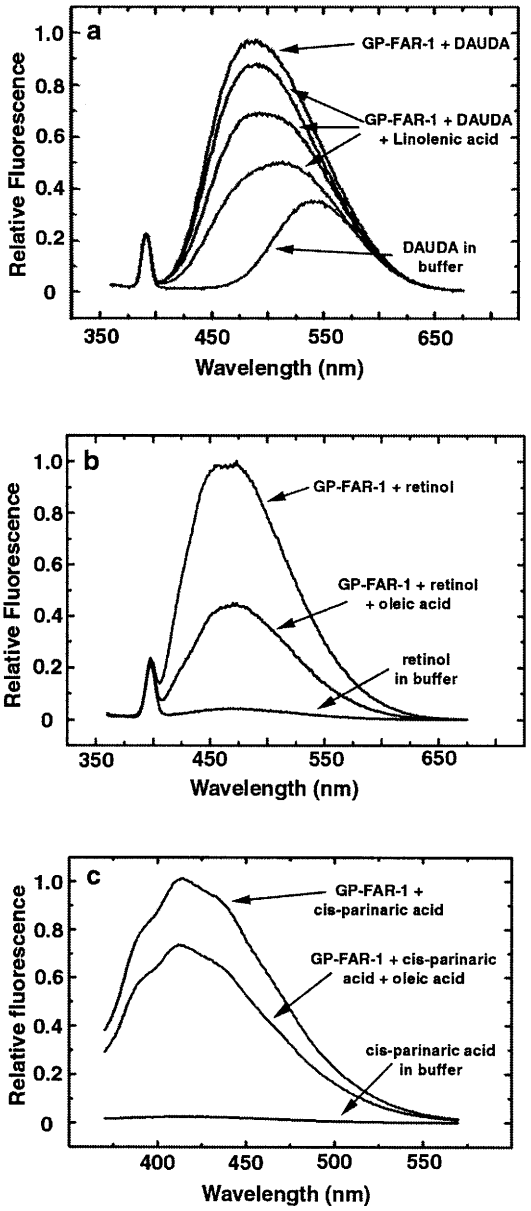

Figure 5 Fatty acid and retinol binding by Gp-FAR-1

(**a**) Fluorescence emission spectra (excitation at 345 nm) of 0.1 μM DAUDA in buffer or on the addition of 1.5 μM rGp-FAR-1. Subtraction of these two spectra to provide the wavelength of peak emission of DAUDA when in the protein binding site gave a value of 482 nm (results not shown). Also shown is the competitive displacement of DAUDA from rGp-FAR-1 by the progressive addition of linolenic acid: 10 μl of 1:10, 1:100 and 1:1000 dilutions of a 10 mM stock solution of linolenic acid added directly to the fluorescence cuvette. (**b**) Binding of retinol by rGp-FAR-1: 5 μl of a 1 mM solution of retinol alone or when added to 2 ml of 1.5 μM rGp-FAR-1 in a fluorescence cuvette. The competitive effect of adding 10 μl of a 1:10 dilution of 10 mM oleic acid to the retinol–Gp-FAR-1 solution is also shown. Excitation was at 350 nm. (**c**) The effect of addition of 50 μl of 60 μM protein to 0.1 μM *cis*-parinaric acid in buffer, and the effect of the subsequent addition of 10 μl of 0.1 mM oleic acid solution to the mixture. Excitation was at 319 nm.

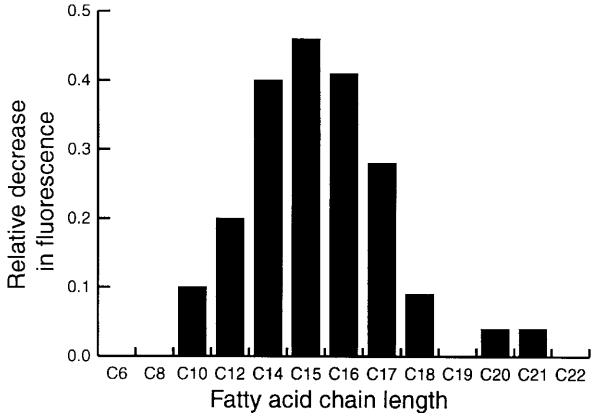

Figure 6 Preference of Gp-FAR-1 for fatty acid chain length

Solutions (10 μl; 1 mM) of each of the (saturated) fatty acids (chain length C_6 to C_{22} as shown) were added to 2 ml of 1.5 μM Gp-FAR-1 solution in buffer containing 1 μM DAUDA in the fluorescence cuvette; the decrease in fluorescence of the DAUDA–protein complex was measured at 488 nm. Excitation was at 345 nm.

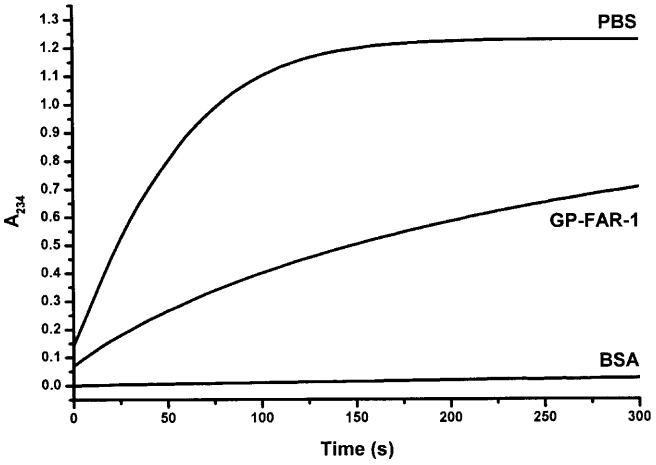

Figure 7 Inhibition of lipoxygenase activity by Gp-FAR-1

Assay tracking lipoxygenase-mediated breakdown of linoleic acid in the presence of PBS, rGp-FAR-1 or BSA. Lipoxygenase (100 units) was added to 80 μM linoleic acid in 0.2 M borate buffer. The breakdown of linoleic acid was measured by the change in A_{234} in the presence of 10 μM rGp-FAR-1, BSA or RNase A.

instance, could interfere with signalling events either by sequestering signalling molecules or making them unavailable for lipoxygenase activity in the generation of other mediators. 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 [26], although jasmonic acid itself was found not to bind (results not shown). Furthermore, peroxidation of fatty acids containing 1,4-*cis*,*cis*-pentadienyl moieties by lipoxygenase results in the formation of toxic lipid hydroperoxides and free radicals, which are the first line of plant defence that nematodes encounter on invasion of the plant [27]. We therefore examined the effect of rGp-FAR-1 on plant root lipoxygenase activity *in vitro*.

A pronounced inhibition of lipoxygenase-mediated breakdown of linoleic acid was observed in the presence of rGp-FAR-1 (Figure 7). Serum albumin showed an even greater ability to inhibit the breakdown of linoleic acid on a molar basis, which was expected given that this protein possesses multiple fatty-acid-binding sites per molecule [28], as opposed to the single binding site apparently present in Gp-FAR-1. However, a control

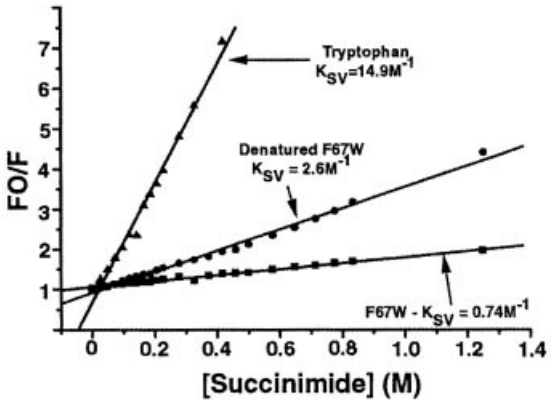

Figure 8 Exposure of the single tryptophan side chain to solvent in the F67W mutant protein

Stern–Volmer plot of native or denatured F67W mutant in the absence and in the presence of 6 M guanidinium chloride, where F_0 is the fluorescence emission of protein alone and F is the fluorescence emission of protein in the presence of succinimide. Increasing quantities of succinimide were added to 5 μM F67W protein in buffer or in 6 M guanidinium chloride. The intensity of fluorescence emission was measured at the peak wavelength of the protein in buffer (335 nm). Excitation was at 290 nm. Stern–Volmer coefficients (K_{sv}) were calculated from the Stern–Volmer equation [14,15] and were 0.74 M^{-1} for the native protein and 2.64 M^{-1} for the unfolded state.

protein (ribonuclease A) with no lipid binding activity had no effect on hydroperoxide formation (results not shown).

Site-directed mutagenesis

No empirical structural information yet exists for the FAR proteins, apart from CD analysis which showed that one member of the class at least is rich in α-helix [13]. Secondary-structure prediction algorithms also predicted extensive helicity, and helical wheel projections indicated the presence of amphipathic α-helices (Figure 1). The hydrophobic faces of these helical stretches probably fold towards the interior of the protein and might also comprise the sides of a binding pocket or groove for hydrophobic ligands. Confirmation that these regions are indeed internally orientated would enhance the value (see below) of a mutational analysis to examine the side chains that are crucial to the mechanism of ligand binding and to the structure of the binding site. As a first step towards this, we took a region of the protein's sequence for which amphipathicity was strongly predicted (Figure 1) and in which there was strong conservation of amino acid type, and replaced Phe-67 with a tryptophan residue. This conservative substitution ought not to disrupt the structure and function of the protein, but provides a probe for the predicted interior of the protein by using the fluorescence emission characteristics of the introduced (and solitary) tryptophan. The F76W mutation had no effect on the ligand binding characteristics of the protein (results not shown), so the mutant protein was taken to be functionally and structurally intact. Furthermore, the quality of the linear fit obtained for the Stern–Volmer plot of the F67W mutant (see Figure 8) suggests that the protein folded homogeneously (see below).

The wild-type Gp-FAR-1 contains no tryptophan, so the disposition of the inserted tryptophan residue can be examined in isolation. The emission maximum of the F67W mutant when excited at 290 nm was 335 nm (results not shown), indicating that the introduced tryptophan is not exposed to solvent water but is buried to a significant extent within the protein [14] as predicted. Moreover, the relatively low value of the Stern–Volmer constant (K_{sv}) for quenching by succinimide (0.74 M^{-1}) is consistent with an internal location for the side chain of the introduced tryptophan (Figure 8). To place these results in context, the K_{sv} (for succinimide quenching) of various proteins containing a single tryptophan residue range from 0.26 M^{-1} for type I dehydroquinase from *Salmonella typhi* (buried tryptophan [29]), to 4.8 and 3.2 M^{-1} for shikimate kinase of *Erwinia chrysanthemi* and type II dehydroquinase from *Streptomyces coelicolor* respectively (exposed tryptophans [30,31]). Unfolding of the F67W protein in 6 M guanidinium chloride led to a red shift in the emission peak to 351 nm and resulted in a K_{sv} for succinimide quenching of 2.6 M^{-1} (Figure 8), which is of a similar order to that observed for other single-tryptophan-containing proteins when unfolded [30–32]. In some lipid binding proteins the fluorescence emission spectrum of tryptophan residues within or near the binding site can be altered by ligand binding [33,34], but in F67W no such changes were detectable on the addition of oleic or linoleic acid (results not shown). This means either that the inserted tryptophan is not in the binding site or that it is in the binding site but its immediate environment is not affected by ligand binding.

The above results indicate that the Phe-67 position is, as predicted, interior to the protein. This position lies in the region of strongest amino acid conservation among this highly sequence-diverse family of proteins (Figure 1). Two residues close to Phe-67, which are either absolutely conserved (Lys-78) or conserved in all except one sequence (Lys-80, conservatively replaced by an arginine residue [6]), were also targeted for further site-directed mutagenesis experiments. When Lys-78 was replaced with the similarly sized but charge-neutral residue glutamine, there was a slight increase in binding strength in titration experiments with *cis*-parinaric acid, changing the K_d from 0.077 μM for the wild-type protein to 0.047 μM. Replacement of Lys-78 instead with the larger residue arginine resulted in a higher K_d (0.2 μM), indicating that ligand binding is weakened in this mutant. One interpretation of these results is that the bulkier arginine side-chain prevents efficient entry of ligand into the binding site or in some way disrupts the binding of ligand within the binding site. Lys-80 is conserved in all except one of the the FAR family of proteins, and a Lys-80 → Gln substitution resulted in an increased K_d (0.226 μM), suggesting that a positive charge might be required for ligand binding in this position or for maintaining the structure of the protein.

None of the substitutions produced a marked change in ligand binding, which is interesting given the absolute or strong conservation of the positions in an otherwise sequence-diverse set of proteins. It can therefore reasonably be deduced that these residues are essential for some purpose other than direct and essential involvement in ligand binding.

The free-living nematode *C. elegans* is known to possess genes encoding six FAR-like proteins [6]. In contrast, parasitic nematodes of plants (*G. pallida*) or animals (such as the river-blindness parasite, *Onchocerca volvulus*, or the agent of lymphatic filariaisis, *Brugia malayi*) have been found to possess only one [6,35]. In all these cases the proteins have been found to be important antigens and/or abundantly produced secreted products, leading to the suggestion that their ligand-binding propensities might relate to the modulation of host defence mechanisms and other modifications of the tissue environment to the advantage of the parasite [13]. It is interesting to speculate why *C. elegans* requires six members of the family, whereas parasitic species from a broad range of nematode clades possess only one. There is as yet insufficient information on parasitic nematodes to be certain that they do not possess more than one form of FAR, although the

extensive expressed sequence tag data sets available have revealed only one FAR gene per species. However, if we can assume that the production of only one type is the norm for nematodes, then it might be that *C. elegans* is atypical and that the possession of six counterparts represents a particular adaptation of this organism. Alternatively, the evolution of parasitism in plants and animals might have involved the loss of expression of all except one type. However, in view of the number of independent occasions on which parasitism is thought to have evolved in nematodes [36], it seems unlikely that the same radical modification occurred in phylogenetically diverse nematode groups, having a corresponding diversity in life histories. However, in terms of a role in parasitism, the FAR proteins might once have been internal lipid transport proteins of free-living nematode antecedents that have been adapted as secretory/surface products for external purposes. The modulation of lipid-based signalling by these proteins in both plant and animal host tissues is now, from the present findings on Gp-FAR-1, a possibility worthy of investigation.

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