The EMBO Journal vol.14 no.9 pp.1858–1866, 1995

A P-glycoprotein protects *Caenorhabditis elegans* against natural toxins

Annegien Broeks, Hans W.R.M.Janssen[1], Jero Calafat[1] and Ronald H.A.Plasterk[2]

Division of Molecular Biology and [1]Division of Cell Biology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

[2]Corresponding author

Communicated by P.Borst

P-glycoproteins can cause resistance of mammalian tumor cells to chemotherapeutic drugs. They belong to an evolutionarily well-conserved family of ATP binding membrane transporters. Four P-glycoprotein gene homologs have been found in the nematode *Caenorhabditis elegans*; this report describes the functional analysis of two. We found that PGP-3 is expressed in both the apical membrane of the excretory cell and in the apical membrane of intestinal cells, whereas PGP-1 is expressed only in the apical membrane of the intestinal cells and the intestinal valve. By transposon-mediated deletion mutagenesis we generated nematode strains with deleted P-glycoprotein genes and found that the *pgp-3* deletion mutant, but not the *pgp-1* mutant, is sensitive to both colchicine and chloroquine. Our results suggest that soil nematodes have P-glycoproteins to protect themselves against toxic compounds made by plants and microbes in the rhizosphere.

Key words: *Caenorhabditis elegans*/drug resistance/excretory cell/P-glycoproteins

Introduction

P-glycoproteins are evolutionarily well-conserved membrane-bound proteins which belong to the family of ATP binding cassette (ABC) transporters (Higgins, 1992). These proteins were first identified in mammalian tumor cells as causing multidrug resistance (MDR) against anti-cancer drugs by active drug export (Juliano and Ling, 1976). The P-glycoproteins are located in the plasma membrane and consist of two similar halves, each with six transmembrane domains and an intracellular ATP binding site (Endicott and Ling, 1989; Gottesman and Pastan, 1993). Mammalian P-glycoproteins are mainly expressed in the apical membrane of epithelia of excretory organs and endothelia of blood tissue barriers (Thiebaut *et al.*, 1987; Georges *et al.*, 1990; Ueda *et al.*, 1992; Cordon-Cardo *et al.*, 1989; van der Valk *et al.*, 1990). It is thought that they provide a protective mechanism against exogenous toxins present in the diet and environment (Ames *et al.*, 1990). By extruding structurally and functionally unrelated agents they can rescue the cell, and the organism, from toxic drug effects. The drugs that are generally transported are large hydrophobic molecules of plant, fungal or microbial origin.

P-glycoproteins are encoded by small gene families: in humans there are two members (*MDR*1 and *MDR*3, also known as *MDR*2) and in mice three (*mdr*1a, *mdr*1b and *mdr*2). Not all P-glycoproteins are involved in drug resistance; human *MDR*1 and mouse *mdr*1a and *mdr*1b can confer resistance, but *MDR*3 and *mdr*2 cannot (Gros *et al.*, 1986, 1988; Ueda *et al.*, 1987; Schinkel *et al.*, 1991; Devault and Gros, 1990). The mouse *mdr*2 gene (and its human *MDR*3 homolog) is involved in secretion of phosphatidylcholine into bile and is probably a phospholipid translocator (Smit *et al.*, 1993; Ruetz and Gros, 1994; Smith *et al.*, 1995). P-glycoproteins are also found in many other organisms, e.g. invertebrates (Wu *et al.*, 1991; Lincke *et al.*, 1992; Gerrard *et al.*, 1993) and parasitic protozoa (Foote *et al.*, 1989; Wilson *et al.*, 1989; Ouellette *et al.*, 1990; Samuelson *et al.*, 1990; Legare *et al.*, 1995). In *Drosophila melanogaster* three P-glycoprotein genes have been identified and at least one of them (*Mdr49*) has been suggested to be implicated in drug resistance (Wu *et al.*, 1991).

As in cancer chemotherapy, drug resistance is also a major problem in the treatment of many parasitic diseases. P-glycoproteins are sometimes involved in this resistance. The parasite *Leishmania* has a P-glycoprotein gene family of at least six members, some of which are involved in drug resistance (Ouellette *et al.*, 1990, 1991; Grondin *et al.*, 1993; Hendrickson *et al.*, 1993; Légaré *et al.*, 1995), e.g. *ltpgpA* is involved in low level oxyanion resistance, which includes resistance to antimony, the drug of choice in treatment of leishmaniasis (Callahan and Beverley, 1991), and *ldmdr1* is involved in resistance to several compounds included in the mammalian MDR spectrum (Hendrickson *et al.*, 1993). In some resistant malaria parasites, *Plasmodium falciparum*, amplification of a P-glycoprotein gene (*pfmdr1*) is observed (Foote and Cowman, 1994; Pussard and Verdier, 1994). For long it was thought that resistance to chloroquine, the drug of choice for treating malaria, was caused by a P-glycoprotein-mediated mechanism (Foote *et al.*, 1989; Wilson *et al.*, 1989). Recent work has shown this is not the case; the copy number of the *pfmdr1* gene does not correlate with the level of resistance to chloroquine (Wellems *et al.*, 1990; Wilson *et al.*, 1993), although the copy number of the *pfmdr1* gene does correlate with the level of resistance in mefloquine- and halofantrine-resistant *Plasmodium* (Wilson *et al.*, 1993; Cowman *et al.*, 1994). In yeast the P-glycoprotein homolog STE6 has a well-defined physiological function. The STE6 protein is involved in pheromone transport, transporting the **a** mating factor (McGrath and Varshavsky, 1989).

In the nematode *Caenorhabditis elegans* four P-glycoprotein homologs have been identified (*pgp-1*, *pgp-2*,

pgp-3 and *pgp-4*) and three have been analyzed in detail. They share similarities with mammalian P-glycoproteins in their predicted protein structure (Lincke *et al.*, 1992; A.Broeks, unpublished results). *pgp-1* and *pgp-3* are expressed throughout the life cycle and promoter−*LacZ* fusion experiments indicated that they are exclusively expressed in the intestinal cells (Lincke *et al.*, 1993).

Caenorhabditis elegans is a free-living soil nematode. Natural toxins are produced by plants and microbes as a defence against invaders and predators and these toxins will be present in the environment and, therefore, also in the food of the nematodes. Wild-type nematodes are extremely resistant to toxins. We have determined whether nematode P-glycoproteins are involved in this resistance.

We have used the *Tc1*-induced deletion mutagenesis method, recently developed in our laboratory for isolation of mutants of genes of known sequence (Zwaal *et al.*, 1993). P-glycoprotein mutants were analyzed for phenotypic alterations, including altered sensitivity to toxins. To determine which organs are involved in protection of the nematode against toxins, we analyzed the tissue distribution of PGP-1 and PGP-3 by immunohistochemistry.

Results

Generation and analysis of P-glycoprotein-deficient and P-glycoprotein-over-expressing nematode strains

In order to isolate null-alleles of both *pgp-1* and *pgp-3* we have used the method described previously for isolation of *Tc1* insertion mutants and their deletion derivatives (Zwaal *et al.*, 1993). From the *pgp-1* intron insertion mutant *pgp-1*(*pk28*), a unidirectional deletion derivative was isolated, *pgp-1*(*pk17*) (Figure 1A and B). In this mutant the putative transmembrane domains 3–6 and half of the first nucleotide binding domain are deleted. The deletion causes a frame-shift which results in the creation of a stop codon in exon 8. The wild-type protein consists of 1321, the putative deletion mutant protein of 207 amino acids. It is not clear if this truncated protein is actually made. From the *pgp-3* exon insertion mutant *pgp-3*(*pk30*) a bidirectional deletion derivative was isolated, *pgp-3*(*pk18*) (Figure 1A and B). This deletion mutant lacks the complete first nucleotide binding domain and the putative transmembrane domains 7 and part of 8. This deletion also causes a frame-shift and the introduction of a stop codon in exon 10. The wild-type protein consists of 1254, the putative deletion mutant protein of 369 amino acids. Based on deletion mutagenesis studies of other *pgp* genes (Azzaria *et al.*, 1989; Pastan *et al.*, 1991), it is very likely that both deletions result in complete loss of gene function. The double mutant strain *pgp-1*(*pk17*) *pgp-3*(*pk18*), in which both *pgp-1* and *pgp-3* are deleted, was made by crossing the single mutant strains. Under standard laboratory conditions all mutant strains are viable and fertile and they are genetically stable, because the mutator allele is no longer present.

In order to increase the level of expression of individual P-glycoprotein genes, we introduced the wild-type genes plus several kilobase pairs of flanking DNA, together with a dominant selectable marker, into the gonads of wild-type nematodes by microinjection (Mello *et al.*, 1991).

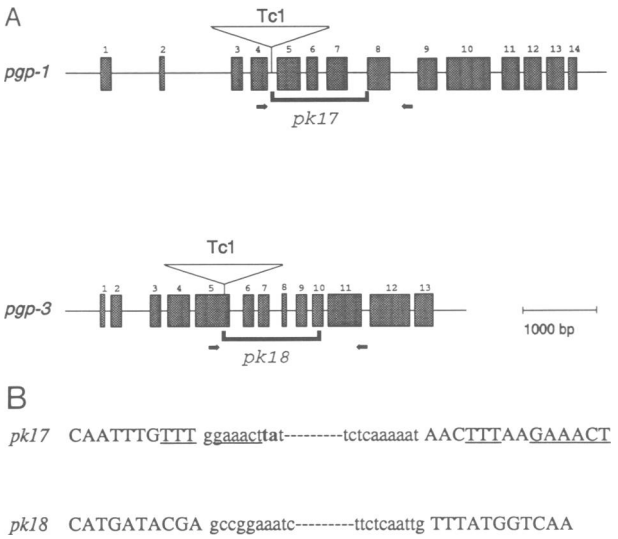

Fig. 1. *Tc1* insertion and deletion mutants of *pgp-1* and *pgp-3*. (**A**) Structure of the *pgp-1* and *pgp-3* gene. The numbered boxes indicate the exons, the *Tc1* element is indicated with a triangle and the bar underneath the gene indicates for each gene the deleted region. The *pgp-1* insertion mutant NL111[*pgp-1*(*pk28*)] has a *Tc1* element insertion in the intron between exons 4 and 5. This insertion mutant was used to isolate the deletion mutant NL132[*pgp-1*(*pk17*)]. The *pgp-3* insertion mutant NL110[*pgp-3*(*pk30*)] has a *Tc1* transposon insertion in exon 5 and this insertion mutant was used to isolate the deletion derivative NL131[*pgp-3*(*pk18*)]. The arrows indicate the primer sites used for detection of the deletion mutants. (**B**) Sequences of the deletion mutants derived from *Tc1* insertion alleles. Sequences around the junction sites are shown; the nucleotides that remained after the deletion are capitalized. For *pk17* short direct repeats near the end points of the deletion are underlined and the *Tc1* insertion site, prior to deletion, is in bold. In the case of *pk18*, a bidirectional deletion, the *Tc1* insertion site is too far removed from the deletion end-points and therefore not indicated.

Semi-stable transgenic lines were obtained with *pgp-1* or *pgp-3* as part of a large extra-chromosomal array (Stinchcomb *et al.*, 1985).

P-glycoprotein levels in wild-type, deletion mutant and transgenic nematode strains

In order to investigate whether the partially deleted genes could still encode detectable protein and to determine the level of increased expression in the transgenic strains in comparison with the wild-type, we isolated protein from membrane fractions of mixed stage populations and analyzed the fractions on a protein immunoblot (Figure 2). The presence of P-glycoprotein was analyzed using the mouse monoclonal antibody (MAb) C219. This antibody recognizes a highly conserved epitope found in all Pgp isoforms known so far (Georges *et al.*, 1990). The epitope is present in the first and second nucleotide binding domain of both PGP-1 and PGP-3 (the epitope is also present in PGP-4, our unpublished sequence data). Therefore, it is not possible to distinguish between the different P-glycoprotein members using this antibody. In both the *pgp-1* and *pgp-3* mutants, the region that codes for the first epitope has been deleted.

We have found that the total amount of P-glycoprotein in the wild-type strain (N2) is low (Figure 2, lane 2). Several bands are visible, the product of ~150 kDa (indicated by the arrow) probably being P-glycoprotein. In the double and single deletion mutant strains the

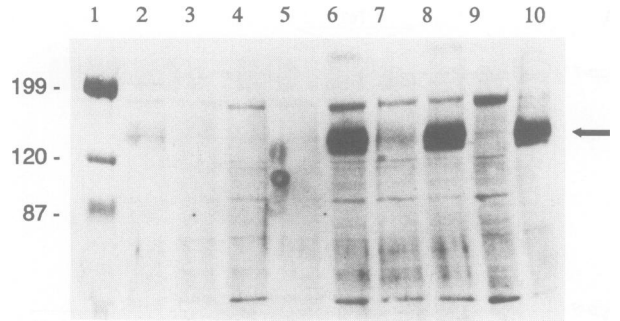

Fig. 2. P-glycoprotein levels in wild-type, deletion mutant and transgenic strains. Protein immunoblot of membrane fractions of mixed stage nematode populations. Lane 1, BioRad pre-stained high molecular weight marker. Lane 2, wild-type N2. Lane 3, *pgp-1 pgp-3* double mutant NL130[*pgp-1(pk17) pgp-3(pk18)*]. Lane 4, *pgp-3* deletion mutant NL131[*pgp-3(pk18)*]. Lane 5, *pgp-1* deletion mutant NL132[*pgp-1(pk17)*]. Lane 6, *pgp-3* transgenic strain NL135[*pgp-3(pkEx580)*]. Lane 7, *pgp-1* transgenic strain NL136 [*pgp-1(pkEx581)*], lane 8, NL137[*pgp-3(pk18) pgp-3(pkEx580)*], the *pgp-3* transgene in the *pgp-3* mutant strain. Lane 9, NL140[*pgp-1(pkEx581) pgp-3(pk18)*], the *pgp-1* transgene in the *pgp-3* mutant strain. Lane 10, NL143[*pgp-1(pk17) pgp-3(pkEx580)*], the *pgp-3* transgene in the *pgp-1* mutant strain. The protein was visualized with the MAb C219. This MAb recognizes a highly conserved epitope found in all P-glycoprotein isoforms known so far (Georges *et al.*, 1990). P-glycoprotein is indicated with an arrow and the marker sizes are indicated. The amount of protein was approximately the same in each lane (5 μg), as determined by the BioRad protein assay. The blot was over-exposed to demonstrate the presence of the protein in the wild-type strain.

P-glycoprotein could barely be detected, the band of ~150 kDa seeming to be decreased compared with the wild-type level. If we compare the intensities of the 150 kDa protein bands present in the deletion mutants, it seems that PGP-3 is responsible for the largest fraction of the total amount of P-glycoprotein present in wild-type animals. In the *pgp-3* transgenic strain there is a large increase in the amount of P-glycoprotein, due to the presence of multiple *pgp-3* gene copies in the extra-chromosomal array (Figure 2, lane 6). In the *pgp-1* transgenic strain there is no detectable increase in the amount of protein, in comparison with the wild-type level (Figure 2, lane 7). Based on staining results (see below), we know that the *pgp-1* construct present as a transgene is expressed, since we could detect a slight increase in expression, apparently not detectable on the immunoblot.

Tissue distribution of Pgp-1 and Pgp-3

Previously, expression of *pgp-1* and *pgp-3* in the nematode was studied using promoter−*LacZ* fusion constructs. These studies indicated that both *pgp-1* and *pgp-3* promoters were active in the intestinal cells. In order to investigate the tissue distribution at the protein level we performed immunohistochemistry on mixed stage populations using the MAb C219 and a fluorescein-conjugated sheep anti-mouse IgG (Figure 3). Because the total amount of protein in wild-type nematodes is very low (Figure 2, lane 2 and Figure 3E), we used the transgenic strains, which should over-express PGP-1 or PGP-3, to determine the tissue distribution. Since the transgenes contain the complete genes plus 5′- and 3′-flanking sequences (see Materials and methods), we assume that they faithfully reflect the expression pattern of the chromosomal gene. Figure 3A and B show that in the *pgp-3* transgenic strain PGP-3 is mainly expressed in the apical membrane of the large H-shaped excretory cell. Staining was also found in the apical membrane of the intestinal cells and in the membrane of the most anterior region of the pharynx (the membrane of the buccal cavitity, the procorpus and the metacorpus). The H-shaped excretory cell is the largest mononucleated cell in *C.elegans*, with the cell body located ventrally to the terminal pharyngeal bulb. The cell body forms a bridge between two lateral canals that extend both anteriorly and posteriorly for almost the entire length of the animal (Nelson *et al.*, 1983; Nelson and Riddle, 1984). Staining was localized in the membrane towards the lumenal side of the canals (the channels) and in the sinus in the cell body (Figure 3B and see below). Figure 3C shows that in the *pgp-1* transgenic strain PGP-1 was expressed in the apical membrane of the intestinal cells. An increase in staining, in comparison with staining in wild-type animals, was detected in the first part of the intestine, the intestinal valve. Again there was staining in the membrane of the anterior region of the pharynx, but there was no staining in the excretory cell (Figure 3C). In the double deletion mutant strain there was no staining detectable except for the anterior region of the pharynx (Figure 3D). In the wild-type (Figure 3E) and single deletion mutant strains (data not shown) the level of expression was too low for specific localization of the protein. As an additional control for specificity of staining we crossed the transgenes into deletion strains. Over-expression of *pgp-3* in a *pgp-1* mutant background resulted in staining in both the apical membrane of the excretory cell and the apical membrane of the intestinal cells. Over-expression of *pgp-1* in a *pgp-3* mutant background resulted in staining only in the apical membrane of the intestinal cells, however, no staining was found in the excretory cell (data not shown).

Localization of the protein in the apical membrane

The excretory cell is polarized, with a basal and an apical face. The apical face is adjacent to the lumen (channel) that runs inside the canals and the cross-bridge; the basal face is at the outside of the cell. The lumen of the excretory cell is lined with canaliculi (small dead-end channels), which usually appear as small circles in transverse section (Nelson and Riddle, 1984). In order to investigate subcellular localization of the protein in the apical membrane of the excretory cell, we used electron microscopy. To visualize the protein in the transgenic strain over-expressing PGP-3, MAb C219 was used in combination with a peroxidase-conjugated rabbit anti-mouse IgG. Membrane morphology is well-conserved if the animals are fixed immediately with glutaraldehyde (Figure 4A and B), the canaliculi of the apical membrane towards the lumen at the inside of the arms of the excretory cell processes being clearly visible (Figure 4A and B). Glutaraldehyde or paraformaldehyde fixation in combination with specific staining with the MAb C219 is not possible (the epitope apparently becomes cross-linked). Therefore, we used methanol/acetone fixation before incubation with C219 and, although membrane structure is not well conserved, it is clear that peroxidase staining is on the inside of the excretory cell canals (Figure 4C), at the apical membrane, towards the channel. Assuming

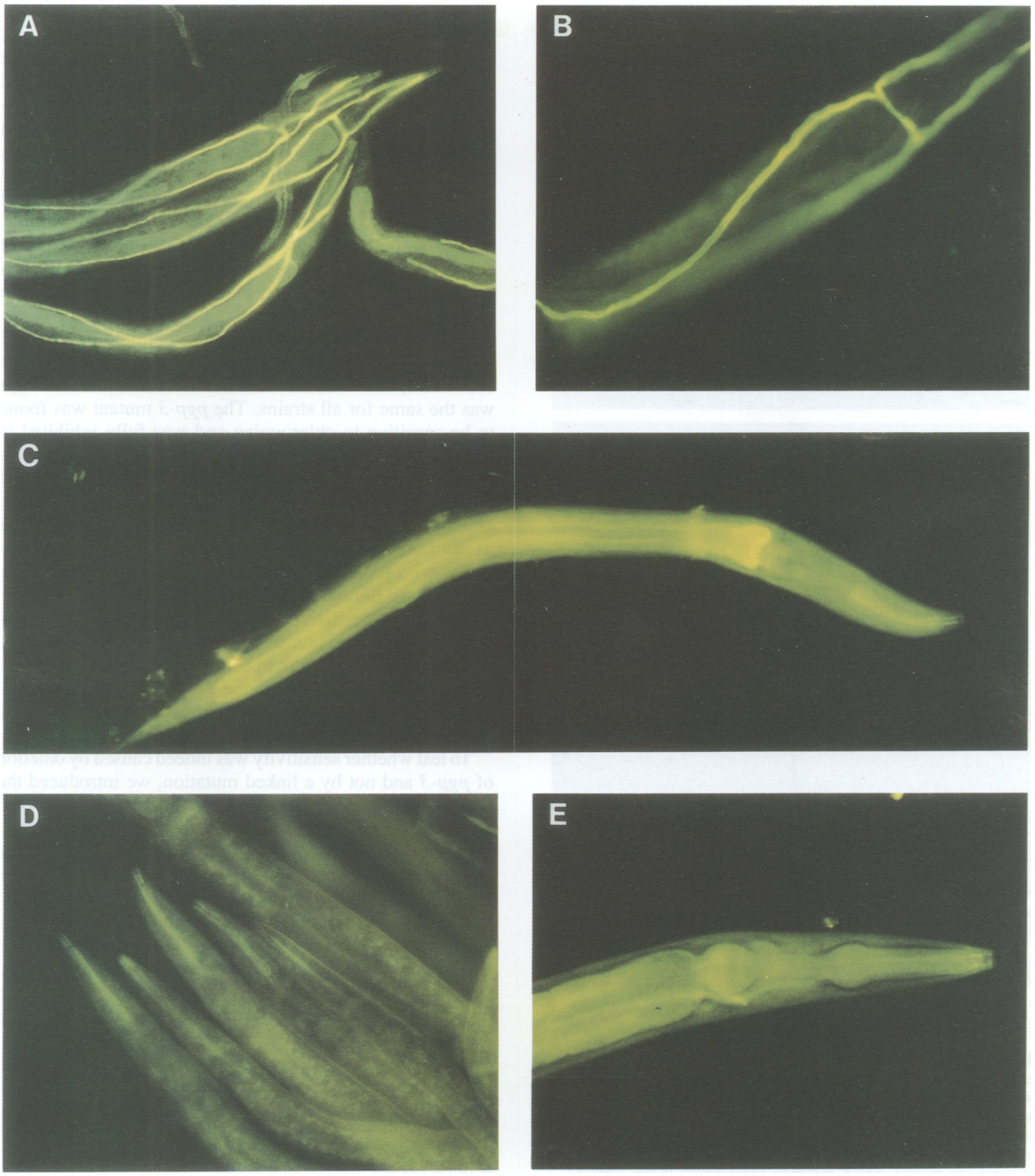

Fig. 3. Tissue distribution of PGP-1 and PGP-3. *Caenorhabditis elegans* mixed stages were fixed with acetone and methanol and stained with MAb C219. The bound antibody was visualized by incubating with a fluorescein-conjugated anti-mouse IgG antiserum and viewed under a fluorescence microscope. (**A** and **B**) In strain NL135, overexpressing PGP-3 in a wild-type background, staining is found in the membrane of the large H-shaped excretory cell, in the membrane of the intestinal cells and in the anterior region of the pharynx. (**C**) Strain NL136, over-expressing PGP-1 in a wild-type background. Most intense staining is localized in the membrane of the intestinal valve cells, with further staining in the membranes of the intestinal cells. There is also staining in the anterior region of the pharynx. (**D**) In strain NL130, a *pgp-1 pgp-3* double mutant, no specific staining could be seen, except for the anterior region of the pharynx. (**E**) In the wild-type strain there is weak staining. Staining can be seen in the excretory cell (one canal visible in this picture), the anterior region of the pharynx and in the membrane of the intestinal cells (less clear in this picture).

that the polarity of the pump is similar to that of all P-glycoproteins analyzed to date, we expect that the protein pumps compounds out of the excretory cell into the lumen, thus clearing 'waste' from the body.

The pgp-3 mutant is sensitive to chloroquine and colchicine

Under standard laboratory conditions the mutant strains were phenotypically wild-type. We have determined

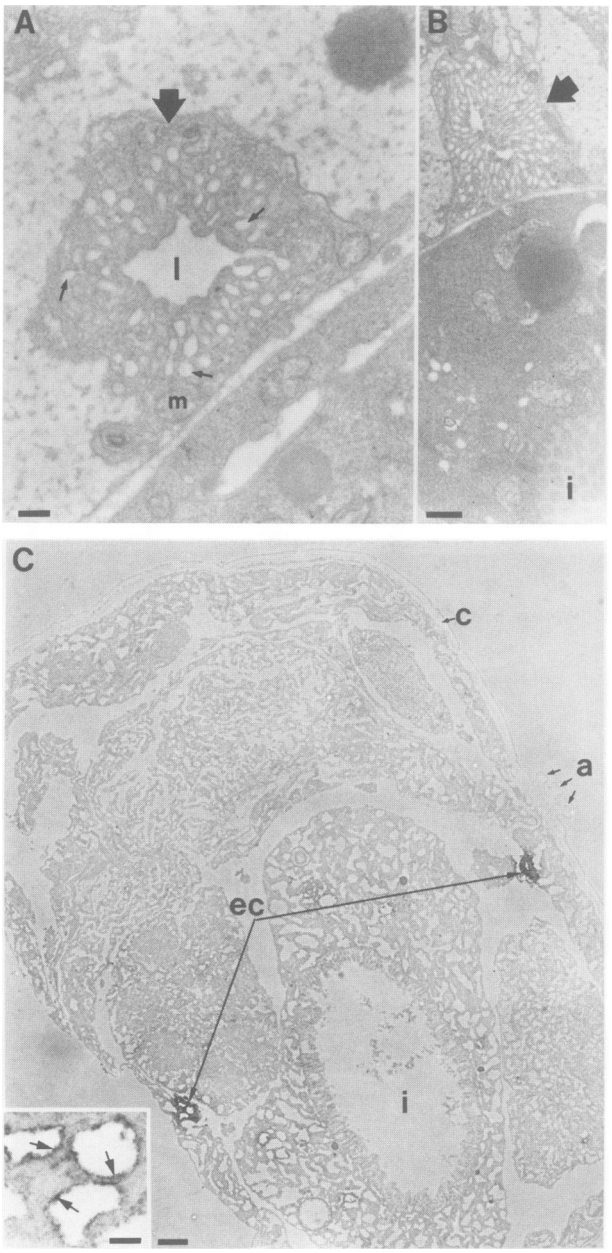

Fig. 4. Localization of PGP-3 in the apical membrane of the excretory cell, determined using electron microscopy. (A and B) Glutaraldehyde fixation of wild-type *C.elegans*. Thin cross-section through the worm showing the two opposite excretory canals (thick arrows). (**A**) Higher magnification showing the open channel lumen (l) surrounded by the cytoplasm of the excretory cell process. In the cytoplasm numerous canaliculi are present, which appear as small circles in this section (small arrows). Mitochondria (m) are a prominent feature of the canal cytoplasm. (**B**) Lower magnification showing localization of an excretory canal (arrow) in relation to the intestine (i).
Bars: (A) 200 nm; (B) 500 nm. (**C**) *Caenorhabditis elegans* strain NL135 over-expressing the PGP-3 protein fixed with acetone and methanol and incubated with the MAb C219 followed by incubation with peroxidase-conjugated anti-mouse IgG antiserum. Thin cross-section through the worm showing a positive reaction in the bilateral canals of the H-shaped excretory cell (ec). Although the worms have been frozen and thawed, the general organization of the body is well preserved: the cuticle (c) with the alae (a) and the intestine (i) can be easily recognized. The membrane structure is not well conserved. Inset, high magnification of the canal on the left-hand side showing that the DAB reaction product is on the membrane surrounding the lumina of the canals (arrows). Bars: (C) 2000 nm; inset 200 nm.

whether disruption of the P-glycoprotein genes results in increased sensitivity and over-expression in increased resistance to specific compounds. We determined the effect of the drugs emetine, ivermectin, chloroquine and colchicine on nematode development. Most of these compounds are known substrates for specific P-glycoproteins in other organisms (Gerlach *et al.*, 1986; Samuelson *et al.*, 1990; Schinkel *et al.*, 1994; van Es *et al.*, 1994).

Deletion of the *pgp-3* gene resulted in increased sensitivity to two drugs: colchicine and chloroquine. The drug colchicine had no effect on brood size, but development of the *pgp-3* mutants was completely inhibited at a concentration of 3–4 mM colchicine. At the same concentration all other strains used in this assay, the double mutant excepted, were only inhibited by 20–40% (Figure 5A). The drug chloroquine did have an effect on brood size, however, the reduction in total number of eggs was the same for all strains. The *pgp-3* mutant was found to be sensitive to chloroquine and was fully inhibited in development at a concentration of 4 mM. The other strains, the double mutant excepted, were inhibited by 40–50% at this concentration (Figure 5B). Deletion of *pgp-1* had no effect on sensitivity of the nematodes to any of the drugs used in the test. These mutant animals were as resistant as the wild-type control animals (Figure 5A and B). The *pgp-1 pgp-3* double mutant was as sensitive as the *pgp-3* mutant alone; deletion of the *pgp-1* gene had no additional effect. Over-expression of *pgp-1* or *pgp-3* in a wild-type background did not result in a further increase in resistance of the nematodes to any of the drugs used in this test. It should be noted that wild-type animals are already extremely resistant to high doses of these drugs.

To test whether sensitivity was indeed caused by deletion of *pgp-3* and not by a linked mutation, we introduced the wild-type *pgp-3* gene, under the control of its own promoter, into the deletion mutant by microinjection. The wild-type gene present as a transgene rescued the phenotype of the deletion mutant; the resistance to the drugs colchicine and chloroquine was restored (Figure 5C and D). The resistance of the transgenic strain was not as high as that of the wild-type strain. The transgenic strains used in these assays are semi-stable transgenic strains. The exogenous DNA is not integrated into the genome, but remains as an extra-chromosomal array. This extra-chromosomal array is heritable for many generations, but is also frequently lost, a certain percentage of the offspring no longer having the transgenic DNA (Stinchcomb *et al.*, 1985).

Discussion

We found that at least one member of the P-glycoprotein gene family of the nematode *C.elegans* is involved in drug resistance. Animals whose *pgp-3* gene has been deleted are found to be sensitive to the cytotoxic agent colchicine and the antimalarial/antiprotozoal agent chloroquine. Sensitivity to drugs is indeed caused by the *Tc1*-induced deletion of *pgp-3*, since introduction of the wild-type gene into the mutant restored resistance. This is not a trivial control, because the mutagenesis method (Zwaal *et al.*, 1993) utilizes a strain of high transposon copy number and it is not *a priori* impossible that phenotypes result from other mutations elsewhere in the genome.

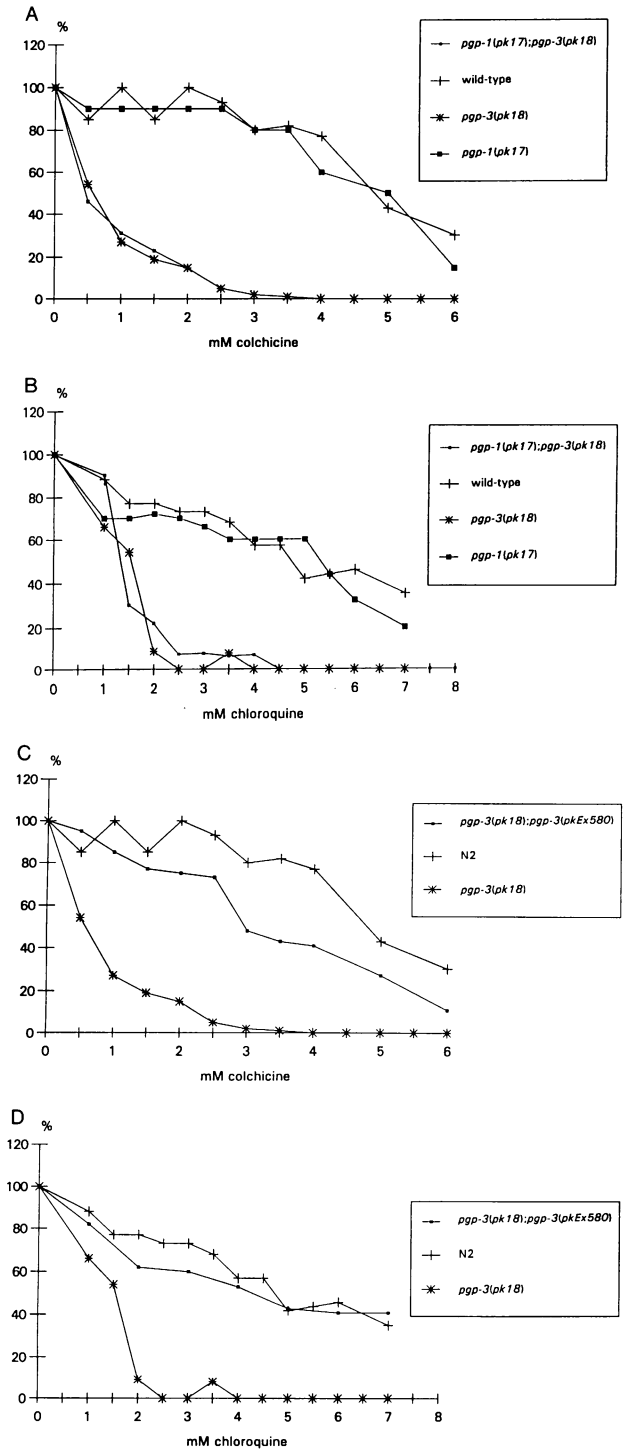

Fig. 5. Drug sensitivity assay. The percentage of developing animals in relation to increasing concentrations of the drugs colchicine and chloroquine (as described in Materials and methods). (**A**) Sensitivity to colchicine. (**B**) Sensitivity to chloroquine. (**C** and **D**) Rescue of the sensitive phenotype; the *pgp-3* transgene was introduced into the *pgp-3* mutant strain by microinjection. The strains used are indicated by the symbols explained in the legend.

Colchicine and chloroquine are structurally unrelated agents with dissimilar mechanisms of action. Colchicine, a major alkaloid derived from *Colchicum autumnale* L., acts on microtubuli and inhibits mitotic spindle formation and the secretion of glycoproteins (Gerlach *et al.*, 1986; Budavari, 1989). This drug is included in the MDR spectrum of mammalian cells. Chloroquine, a 4-amino-quinoline derivative, is related to the arylaminoalcohols such as quinine, an alkaloid derived from the bark of the cinchona tree. Chloroquine is the drug of choice in malaria treatment. It kills the asexual forms of *Plasmodium* and *Amoeba histolytica* and it also has anti-inflammatory properties. The working mechanism is probably based on its inhibition of heme polymerase, which is necessary for detoxification of degraded hemoglobin (WHO, 1990; Slater and Cerami, 1992; van Es *et al.*, 1994). PGP-3 possibly provides the nematode with protection from a whole range of toxins, including colchicine- and chloroquine-related compounds, present in the diet and environment.

Our results have shown that *pgp-1* is not involved in resistance to any of the drugs tested. Based on these results it cannot be concluded that PGP-1 is not involved in drug resistance. PGP-1 and PGP-3 are only 40% identical at the amino acid level, which could result in a difference in substrate specificity. From the Western blot analysis we know that PGP-3 is responsible for the major fraction of the total amount of P-glycoprotein. This is in agreement with previous studies (Lincke *et al.*, 1993), *pgp-3* mRNA levels being considerably higher than the mRNA levels of *pgp-1*. So it is possible that the low expression level of *pgp-1* explains the fact that it plays no significant role in resistance to the drugs colchicine and chloroquine. The cytotoxic agent emetine and the anti-helminthic ivermectin do not seem to be substrates for either PGP-1 or PGP-3. Recently it has been found that ivermectin, a semi-synthetic derivative of avermectin (a fermentation product of *Streptomyces avermitilis*), is a P-glycoprotein substrate. Mice homozygous for a disruption of the *mdr*1a gene are sensitive to ivermectin (Schinkel *et al.*, 1994). Wild-type nematodes are normally sensitive to this drug (Cully *et al.*, 1994). We have shown that resistance to this drug cannot be obtained by over-expression of either PGP-1 or PGP-3.

In studies with mammalian cells it has been shown that drug resistance is associated with increased expression of *mdr* genes (Croop *et al.*, 1988; Endicott and Ling, 1989; Gottesman and Pastan, 1993). We found no clear increase in resistance upon over-expression of one of the P-glycoprotein genes, however, it is possible that the wild-type levels are already set for maximal possible resistance to these compounds. This can be understood in the light of the fact that *C.elegans* lives in the fluid phase of the soil and has no capacity to avoid direct encounters with whatever compounds happen to be present there.

The mouse MAb C219 recognizes a highly conserved epitope present in all P-glycoprotein isoforms. This makes it less useful for analysis of tissue distribution of the individual P-glycoproteins. We solved this, however, using transgenic strains that over-express only one family member from their transgene. Transgenic *pgp-3* is over-expressed in the apical membrane of the large H-shaped excretory cell and in the apical membrane of the intestinal cells. The excretory cell is the largest mononucleated cell and together with three other cells (the duct cell, the A-shaped gland cell and the pore cell) it forms the excretory/secretory system of the nematode. The four cells together connect the lumina of the four excretory canals to

the pore cell, the pore opening to the outside environment (Nelson *et al.*, 1983; Nelson and Riddle, 1984).

So far, the only known function of the excretory system is osmoregulation (Nelson and Riddle, 1984). Although PGP-3 is localized in the apical membrane of the excretory cell, this protein does not seem to be required for osmoregulation. We have tested survival and rescue of all wild-type, mutant and transgenic strains described in this report on NGM plates containing varying salt concentrations (0–2 M NaCl). No differences in osmosensitivity, survival or rescue were found between the different strains used in the tests (data not shown).

A suggested function for the excretory cell is transport of compounds. This transport can be in two directions, to excrete wastes into the canals or to resorb substances from the lumina. The action of PGP-3 in this cell is probably to transport compounds across the apical membrane into the lumen. The energy required for active transport across canal membranes could be made available by the abundant mitochondria within the canal cytoplasm. The substances secreted in the excretory canals are presumably transported from the organism's body via the excretory sinus through the duct to the excretory pore (Romanowski *et al.*, 1971). PGP-3 is also expressed in the membrane of the intestinal cells, although there is hardly any over-expression detected in the staining pattern when the gene is over-expressed as a transgene in comparison with the wild-type staining level in the intestine. The intestine consists of a tube of 20 cells, each bearing microvilli on its apical surface. The function of the intestine is probably to secrete digestive enzymes into the lumen and to absorb processed nutrients. The cells also function as a storage organ, containing granules filled with lipid protein or carbohydrate, and they produce yolk protein to nurture the germ cells (White, 1988).

It is likely that toxins will enter the nematode's body in the food through the intestine, the main opening to the outside. The first level of protection could take place in the intestinal cells: drugs could be extruded from the intestinal cells if they are substrates for membrane-bound P-glycoproteins. Drugs that are taken up and transported through the body might be cleared from the body via the excretory system, by the PGP-3 protein present in the apical membrane of the excretory cell. The nematode has no liver or kidney and we would like to suggest that the function of the excretory system can be compared with these organs. It is, of course, possible that there are additional functions for the PGP-3 protein, such as transport of a specific peptide or hormone. However, deletion of the gene did not result in any obvious developmental or behavioral defect that might have resulted from such a function being impaired.

In previous experiments, using *pgp* promoter–*LacZ* fusion constructs (Lincke *et al.*, 1993), β-galactosidase staining was only found in the nuclei of intestinal cells. This nuclear localization was due to the presence of a nuclear localization signal (NLS). In those experiments only 5′ regulatory sequences were used in front of the marker gene. The constructs described in this article contain the complete coding region, the 5′ promoter region (identical to regulatory sequences used in the *LacZ* fusion construct) and 2 kb 3′ sequences. Therefore, we assume that the immunohistochemical data are the more faithful reflection of the tissue specificity. Since we had to use amplified transgenic constructs to visualize the expression pattern, it is theoretically possible that the results are not identical to expression of the endogenous gene, but this seems highly unlikely, since there are no indications of long distance regulation effects on gene expression in *C.elegans*. The incomplete result of the *pgp-3*–LacZ fusion constructs (there was no staining found in the nucleus of the excretory cell) might be due to regulatory elements within the gene or at the 3′-end.

PGP-1 was found to be expressed only in the membrane of the intestinal cells. When it was over-expressed as a transgene there was no increase detectable in the total amount of protein on an immunoblot. Using immunohistochemistry with MAb C219 there was a slight increase in staining found in the membrane of the intestinal valve cells, in comparison with wild-type. This intestinal valve is made up of six cells and connects the pharynx to the intestine. For preparation of the membrane fractions we made use of semi-stable, non-integrated transgenic lines. A certain percentage of the animals will lose the transgene every generation. If the percentage of animals carrying the transgene is low and the number of cells expressing the extra-chromosomal *pgp-1* gene is limited, this could be the reason why we could not detect an increase in PGP-1 expression on the immunoblot. The function of PGP-1 in the intestine could be the same as that suggested for PGP-3, only with different or partially overlapping substrate specificity.

In the wild-type nematode strain the staining was very weak. There was staining visible in the membrane of the excretory cell, in the membrane of the intestinal cells and in the anterior region of the pharynx, although all these patterns were difficult to detect. In the double mutant strain there was no staining detectable in the intestinal cells and in the excretory cell, so the staining obtained after over-expression of one of the genes as a transgene is specific for that gene. The only staining that could be seen in the double mutant was that in the anterior region of the pharynx. This could be specific for one of the other members of the P-glycoprotein gene family, PGP-2 or PGP-4.

The most studied substrate/protein combination of MDR drugs is that of cancer chemotherapeutics and P-glycoproteins causing acquired resistance. These drugs were not made by plants to be used in chemotherapy and mammalian cells do not make P-glycoproteins to interfere with effective chemotherapy. We have described a 'natural' combination of drugs and mdr pumps: plants make them to fight off invaders (*C.elegans* itself is not parasitic, but other nematodes are) and the nematodes make P-glycoproteins to protect themselves against these compounds.

Materials and methods

Generating insertion and deletion mutants

All strains were cultured as described by Sulston and Hodgkin (1988). The strains used were: for microinjection the Bristol N2 strain; the CB14789[*him-8*(*e1489*)] strain as a source of males for crosses; for *Tc1* mutagenesis the MT3126[*mut-2*(r459)] strain. Isolation of the insertion mutants NL111[*pgp-1*(*pk28*)] and NL110[*pgp-3*(*pk30*)] and their deletion derivatives NL132[*pgp-1*(*pk17*)] and NL131[*pgp-3*(*pk18*)] has been described previously (Zwaal *et al.*, 1993). The primers (Pharmacia, The

Netherlands) used in the PCR to identify the *Tc1* insertions are: for *pgp-1::Tc1*, two gene-specific primers 2979 (5'-acatcccgatgatccgacaag) and 2978 (5'-tgcgacagtttgacctgcatt) and the *Tc1*-specific L1 and L2 primers (Zwaal *et al.*, 1993); for *pgp-3::Tc1*: two gene-specific primers 1926 (5'-ttttaccttggagtcgcact) and 1925 (5'-gtgcttattggaactagacgtctcgga) and the *Tc1*-specific primers R1 and R2 (Zwaal *et al.*, 1993). The PCR primers used (Amersham) to identify the deletions were: for *pgp-1*, 2.5 + 1.6 kb apart, 2981 (5'-ctgagacaggagatttcatgg) and 2980 (5'-tgacaccaac-cattcgggaac), both located upstream of the *Tc1* insertion site, and 2976 (5'-ttgctaaagtcacgcgccatc) and 2975 (5'-gactcttaatctggttgtcgc), both located downstream of the insertion site; for *pgp-3*, 2.1 + 1.6 kb apart, 1926 and 1925, upstream of the insertion site, and 3373 (5'-agcatcgtacatatattcttg) and 3372 (5'-gagcttgcacagtcttccagt), downstream of the insertion site.

The nucleotide sequences of the deletion junctions were determined (Zwaal *et al.*, 1993) using the gene-specific primers 1925 and 2980. The isolated deletion mutant strains were back-crossed twice with *him-8* males to improve their health and to remove the mutator locus to make them genetically stable. After each cross the presence of the deletion allele was checked by PCR. The double deletion mutant strain NL130[*pgp-1(pk17) pgp-3(pk18)*] was constructed by first crossing NL131 with *him-8* males and then crossing the F1 males with NL132. Single F2 cross progeny was put on separate plates and allowed to produce progeny and these were checked for the presence of the deletion alleles. From worms containing both alleles, single animals were picked and using PCR homozygous cultures were selected by screening for the presence of the deletion allele and the absence of the wild-type gene.

Generating transgenic nematodes

Transgenic animals were obtained after co-injection of 150 μg/ml *rol-6*^{D} (pRF4) (Kramer *et al.*, 1990) and 50 μg/ml *pgp-1* or *pgp-3* wild-type gene construct into the gonads of the Bristol N2 strain (Mello *et al.*, 1991). The constructs are subclones derived from bacterial phage λ clones containing genomic DNA made by Lincke *et al.* (1992). The *pgp-1* gene construct is a 12 kb *Xho*I fragment cloned from the λ9 clone into the *Xho*I site of pGEM-3Zf(−) (pRP581). The *pgp-3* gene construct was made by a three-step cloning. A 4.2 kb *Xba*I fragment (from λ13) plus a 1.3 kb *Xba*I−*Eco*RI fragment (λ4) plus a 2.4 kb *Eco*RI fragment (λ4) were together cloned into the *Xba*I/*Eco*RI sites of pGEM-3Zf(−) (pRP580). Semi-stable transgenic animals were selected by their roller phenotype. Several transgenic strains were made: NL135[*pgp-3(pkEx580)*], a wild-type strain over-expressing *pgp-3* as a transgene, NL136[*pgp-1(pkEx581)*], a wild-type strain over-expressing *pgp-1* as a transgene, NL137[*pgp-3(pk18) pgp-3(pkEx580)*], with the *pgp-3* gene as a transgene in a *pgp-3* deletion mutant background, NL140[*pgp-3(pk18) pgp-1(pkEx581)*], containing *pgp-1* as a transgene in a *pgp-3* mutant background, and NL143[*pgp-1(pk17) pgp-3(pkEx580)*], a strain with *pgp-3* as a transgene in a *pgp-1* mutant background.

Membrane isolation and protein immunoblot analysis

Nematodes were washed off one large (9 cm) NGM plate with M9, pelleted and resuspended in 1 ml isolation buffer (IB; 50 mM Tris−HCl, pH 7.4, 20 mM KCl, 0.5 mM EDTA, 1 mM β-mercaptoethanol) with 1 μl/ml leupeptin (1 mg/ml), 1 μl/ml aprotinin (1 mg/ml), 1 μl/ml pepstatin (1 mg/ml) and 10 μl/ml phenylmethylsulfonyl flouride (100 mM) freshly added. The worm solution was ground in liquid nitrogen to a fine white powder using a pestle and ceramic mortar. Again, 1 ml IB was added and the solution was homogenized with a Dounce Potter homogenizer (40 strokes). The solution was centrifuged for 10 min at 5000 *g* to remove carcasses and nuclei. The supernatant was then centrifuged in a SW50.1 rotor in an ultracentrifuge for 1 h at 40 000 *g* (4°C) to pellet the membranes. The membrane pellet was resuspended in 40 μl IB and the amount of protein was determined using the BioRad protein assay. Laemmli sample buffer (40 μl) was added to the sample and the sample was incubated for 15 min at room temperature before loading. The protein samples were size fractionated on a 7.5% polyacrylamide gel containing 0.1% SDS (2 h at 100 V; BioRad) and transferred onto nitrocellulose using an electroblotter (12 h at 30 V; BioRad). The blot was stained with Ponceau S to determine the amount of protein in each lane. The blots were pre-incubated in phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBST) and 5% non-fat milk for 2 h at room temperature and then incubated with MAb C219 (4 μg/ml) for 16 h at 4°C. All subsequent washing and incubation steps were in PBST and at room temperature. After washing (3×10 min), the bound primary antibody was visualized by incubation with a 1:2500 dilution of peroxidase-conjugated rabbit anti-mouse IgG antiserum (Dako, Glostrup, Denmark) for 2 h at room temperature, followed by enhanced chemiluminescence detection (Amersham, Little Chalfont, UK).

Immunohistochemistry

Animals were fixed with methanol and acetone on poly-L-lysine-coated slides (Sulston and Hodgkin, 1988). After fixation the slides were stored at −20°C or used immediately. The slides were washed twice with Ab buffer (1× PBS, 1% BSA, 0.5% Triton X-100, 1 mM EDTA, 0.05% sodium azide) dried and incubated with MAb C219 (10 μg/ml) in Ab buffer (300 μl/slide) in a humidity chamber for 3 h at room temperature. After three washes with Ab buffer (0.1% BSA), the first bound antibody was visualized by incubation with a 1:50 dilution of fluorescein-conjugated sheep anti-mouse antibody (Boehringer Mannheim, Mannheim, Germany) for 30 min at room temperature (300 μl/slide). The slides were washed twice with Ab buffer (0.1% BSA), dried and mounted in DABCO solution (80% glycerol, 0.2 M Tris−HCl, pH 8.0, 2.5% 1,4-diazobicyclo-[2.2.2]-octane) to prevent photobleaching. Staining was examined using fluorescence microscopy and photographs taken on Kodak extrachrome Panther p1600 color reversal film.

Electron microscopy

Animals were fixed and stained as described in the previous section. For this staining we used the strain NL135[*pgp-3(pkEx580)*]. After incubation with the second antibody the worms were fixed in 2.5% glutaraldehyde for 10 min and processed for peroxidase cytochemistry in acetate buffer (pH 6.0) containing 0.5 mg 3,3'-diaminobenzidine (DAB), 1% $NiSO_4$, 0.068% imidazole, 0.8% NaCl, 1 μl 30% H_2O_2 (1 μl/10 ml buffer) for 10 min. The reaction was enhanced using 0.5% $CoCl_2$ in 0.1 M Tris−HCl, pH 7.4, for 20 min. The stained animals were cut in half and the anterior halves post-fixed for 1 h in 1% osmium tetroxide. The halves were all placed in one direction and flat-embedded in a mixture of LX112/Araldite. As a control for morphology, N2 animals were fixed following the same protocol without the first fixation or antibody incubation. From all preparations, transverse thin sections were examined with a Philips CM10 electron microscope.

Drug sensitivity assay

The drugs used in the sensitivity assay were: ivermectin (Ivomec; Merck, Sharp and Dohme AgVet), emetine (E-2375; Sigma), colchicine (C-9754; Sigma) and chloroquine (C-6628; Sigma). Drugs were added to the NGM medium before pouring the plates; 0.1–10 ng/ml ivermectin, 0.1–10 mM emetine, 0.1–6 mM colchicine and 1–20 mM chloroquine. Each test was performed twice 6-fold. Single young adult hermaphrodites were put on the plates and allowed to lay eggs for 24 h. The parents were removed and the eggs were counted. Viability was calculated as the total number of adults divided by the total number of eggs. Scores were normalized to those obtained in the absence of drugs and expressed as percentages.

Acknowledgements

We thank Dr J.Kramer for the plasmid pRF4, Dr C.R.Lincke for the phage λ clones λ4, λ9 and λ13, David P.E.Satijn for making the construct pRP580 and Nico Ong for preparing the electron micrographs. We also thank our colleagues Jonathan Pettitt, Jan Chris Vos, Alfred Schinkel and Piet Borst for critical comments and advice on the manuscript. This work was supported by a Pionier grant from the Netherlands Organization for Scientific Research (NWO) to R.H.A.P.

References

Ames,B.N., Profet,M. and Gold,L.S. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 7782–7786.

Azzaria,M., Schurr,E. and Gros,P. (1989) *Mol. Cell. Biol.*, **9**, 5289–5297.

Budavari,S. (1989) *The Merck Index*. Merck & Co. Inc, Rahway, NJ.

Callahan,H.L. and Beverley,S.M. (1991) *J. Biol. Chem.*, **266**, 18427–18430.

Cordon-Cardo,C., O'Brien,J.P., Casals,D., Rittman-Grauer,L., Biedler, J.L., Melamed,M.R. and Bertino,J.R. (1989) *Proc. Natl Acad. Sci. USA*, **86**, 695–698.

Cowman,A.F., Galatis,D. and Thompson,J.K. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 1143–1147.

Croop,J.M., Gros,P. and Housman,D.E. (1988) *J. Clin. Invest.*, **81**, 1303–1309.

Cully,D.F., Vassilatis,D.K., Liu,K.K., Paress,S., van der Ploeg,L.H.T., Schaeffer,J.M. and Arena,J.P. (1994) *Nature*, **371**, 707–711.
Devault,A. and Gros,P. (1990) *Mol. Cell. Biol.*, **10**, 1652–1663.
Endicott,J.A. and Ling,V. (1989) *Annu. Rev. Biochem*, **58**, 137–171.
Foote,S.J. and Cowman,A.F. (1994) *Acta Trop.*, **56**, 157–171.
Foote,S.J., Thompson,J.K., Cowman,A.F. and Kemp,D.J. (1989) *Cell*, **57**, 921–930.
Georges,E., Bradley,G., Gariepy,J. and Ling,V. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 152–156.
Gerlach,J., Endicott,J., Juranka,P.F., Henderson,G. and Sarangi,F. (1986) *Nature*, **324**, 585–489.
Gerrard,B., Stewart,C. and Dean,M. (1993) *Genomics*, **17**, 83–88.
Gottesman,M.M. and Pastan,I. (1993) *Annu. Rev. Biochem.*, **62**, 385–427.
Grondin,K., Papadopoulou,B. and Ouellette,M. (1993) *Nucleic Acids Res*, **21**, 1895–1901.
Gros,P., Ben-Neriah,Y., Croop,J. and Housman,D.E. (1986) *Nature*, **323**, 728–731.
Gros,P., Raymond,M., Bell,J. and Housman,D. (1988) *Mol. Cell. Biol.*, **8**, 2770–2778.
Hendrickson,N., Sifri,D.C., Henderson,D.M., Allen,T., Wirth,D.F. and Ullman,B. (1993) *Mol. Biochem. Parasitol.*, **60**, 53–64.
Higgins,C.F. (1992) *Annu. Rev. Cell Biol.*, **8**, 67–113.
Juliano,R.L. and Ling,V. (1976) *Biochim. Biophys. Acta*, **455**, 152–162.
Kramer,J.M., French,R.P., Park,E.C. and Johnson,J.J. (1990) *Mol. Cell. Biol.*, **10**, 2081–2089.
Légaré,D., Hettema,E. and Ouellette,M. (1994) *Mol. Biochem. Parasitol.*, **68**, 81–91.
Lincke,C.R., The,I., van Groeningen,M. and Borst,P. (1992) *J. Mol. Biol.*, **228**, 701–711.
Lincke,C.R., Broeks,A., The,I., Plasterk,R.H.A. and Borst,P. (1993) *EMBO J.*, **12**, 1615–1620.
McGrath,J.P. and Varshavsky,A. (1989) *Nature*, **340**, 400–404.
Mello,C.C., Kramer,J.M., Stinchcomb,C. and Ambros,V. (1991) *EMBO J.*, **10**, 3959–3970.
Nelson,F.K. and Riddle,D. (1984) *J. Exp. Zool.*, **231**, 45–56.
Nelson F.K., Albert,P.S. and Riddle,D.L. (1983) *J. Ultrastruct. Res.*, **82**, 156–171.
Ouellette,M., Fase-Fowler,F. and Borst,P. (1990) *EMBO J.*, **9**, 1027–1033.
Ouellette,M., Hettema,E., Wust,D., Fase-Fowler,F. and Borst,P. (1991) *EMBO J.*, **10**, 1009–1061.
Pastan,I., Willingham,M.C. and Gottesman,M.M. (1991) *FASEB J.*, **5**, 2523–2528.
Pussard,E. and Verdier,F. (1994) *Fundam. Clin. Pharmacol.*, **8**, 1–17.
Romanowski,R.D., Thompson,D.E. and Madden,P.A. (1971) *Proc. Helminthol. Soc. Wash.*, **38**, 143–149.
Ruetz,S. and Gros,P. (1994) *Cell*, **77**, 1–20.
Samuelson,J., Ayala,P., Orozco,E. and Wirth,D. (1990) *Mol. Biochem. Parasitol.*, **38**, 281–290.
Schinkel,A.H., Roelofs,M.E.M. and Borst,P. (1991) *Cancer Res.*, **51**, 2628–2635.
Schinkel,A.H. *et al.* (1994) *Cell*, **77**, 491–502.
Slater,A.F.G. and Cerami,A. (1992) *Nature*, **355**, 167–169.
Smit,J.J.M. *et al.* (1993) *Cell*, **75**, 451–462.
Smith,A.J., Timmermans-Hereijgers,J.L.P.M., Roelofsen,B., Wirtz,K.W.A., Blitterswijk,W.J., Smit,J.J.M., Schinkel,A.H. and Borst,P. (1994) *FEBS Lett.*, **354**, 263–266.
Stinchcomb,D., Shaw,J., Carr,S. and Hirsch,D. (1985) *Mol. Cell. Biol.*, **5**, 3484–3496.
Sulston,J. and Hodgkin,J. (1988) In Wood,W.B. (ed.), *The Nematode Caenorhabditis elegans*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 587–606.
Thiebaut,F., Tsuruo,T., Hamada,H., Gottesman,M.M., Pastan,I. and Willingham,M.C. (1987) *Proc. Natl Acad. Sci. USA*, **84**, 7735–7738.
Ueda,K., Cardarelli,C., Gottesman,M.M. and Pastan,I. (1987) *Proc. Natl Acad. Sci. USA*, **84**, 3004–3008.
Ueda,K., Okamura,N., Hirai,M., Tanigawara,Y., Saeki,T., Kioka,N., Komano,T. and Hori,R. (1992) *J. Biol. Chem.*, **267**, 24248–24252.
van der Valk, P. *et al.* (1990) *Ann. Oncol.*, **1**, 56–64.
van Es,H.G., Karcz,S., Chu,F., Cowman,A.F., Vidal,S., Gros,P. and Schurr,E. (1994) *Mol. Cell. Biol.*, **14**, 2419–2428.
Wellems,T.E., Panton,L.J., Gluzman,I.Y., do Rosario,V.E., Gwadz,R.W., Walker-Jonah,A. and Krogstad,D.J. (1990) *Nature*, **345**, 253–255.
White,J. (1988) In Wood,W.B. (ed.), *The Nematode Caenorhabditis elegans*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 81–122.
WHO (1990) *Model Prescribing Information: Drugs Used in Parasitic Diseases*. World Health Organization, Geneva, Switzerland.
Wilson,C.W., Serrano,A.E., Wasley,A., Bogenschutz,M.P., Shankar,A.H. and Wirth,D.F. (1989) *Science*, **244**, 1184–1186.
Wilson,C.W., Volkman,S.K., Thaithong,S., Martin,R.K., Kyle,D.E., Milhous,W.K. and Wirth,D.F. (1993) *Mol. Biochem. Parasitol.*, **57**, 151–160.
Wu,C.T., Budding,M., Griffin,M.S. and Croop,J.M. (1991) *Mol. Cell. Biol.*, **11**, 3940–3948.
Zwaal,R.R., Broeks,A., van Meurs,J., Groenen,J.T. and Plasterk,R.H.A. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 7431–7435.

Received on December 9, 1994; revised on February 6, 1995