Multidrug resistance-associated protein MRP-1 regulates dauer diapause by its export activity in *Caenorhabditis elegans*

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Summary

Multidrug resistance-associated proteins (MRPs), when overexpressed, confer drug resistance to cancer cells by exporting anti-cancer agents through the cell membrane, but their role in animal development has not been elucidated. Here we show that an MRP homolog regulates larval development in the nematode *Caenorhabditis elegans*. *C. elegans* forms a special third-stage larva called a dauer larva under conditions inappropriate for growth. By contrast, we found that mutants in *mrp-1*, an MRP homolog gene, form dauer larvae even under conditions appropriate for growth, in the background of certain mutations that partially block the insulin signaling pathway. A functional *mrp-1*::GFP gene was shown to be expressed in many tissues, and the wild-type *mrp-1* gene must be expressed in multiple tissues for a wild-type phenotype. Human MRP1 could substitute for *C. elegans* MRP-1 in dauer larva regulation, and an inhibitor of the human MRP1 transport activity impaired this function, showing that export activity is required for normal dauer larva regulation. Epistasis studies revealed that MRP-1 acts in neither the TGF-β nor the cGMP signaling pathway. *mrp-1* mutations enhanced the dauer-constitutive phenotype of mutants in the insulin signaling pathway more strongly than that in other pathways. Thus, MRP-1, through its export activity, supports the induction of the normal (non-dauer) life cycle by the insulin signaling pathway.

Key words: *C. elegans*, Multidrug resistance-associated protein, *mrp-1*, ABC transporter, Dauer larva

Introduction

When living organisms encounter an environment inappropriate for their survival and growth, they often adapt to the environment by physiological changes. The dormant state is a representative example of such adaptation and is observed in various organisms; for example, hibernation of Mammalia, Reptilia and Amphibia, diapause of insects, dormant buds of plants, and sporulation of fungi and bacteria (Denlinger, 2002; Carey et al., 2003). The dormancy of these organisms has been studied mostly by physiological examinations, ecological observations and biochemical analyses. However, the molecular mechanisms of the regulation of dormancy remain largely unstudied.

The nematode *C. elegans* also has a diapause stage, called a dauer larva. In the normal life cycle, *C. elegans* grows to adulthood through four larval stages (L1 to L4) in 2-3 days at 20°C. But under inadequate conditions for growth, that is, under reduced food availability, crowding or high temperature, the animal arrests development and forms the dauer larva, corresponding to the third larval stage (Cassada and Russell, 1975; Golden and Riddle, 1984). When the environmental conditions are improved, the dauer larva molts to a normal L4 larva and resumes the life cycle. The dauer larva has a characteristic shape and common features of dormant animals, i.e. low metabolism, no feeding, no aging, accumulation of fat and resistance to stress (Cassada and Russell, 1975; Klass and Hirsh, 1976; Riddle, 1988; Wadsworth and Riddle, 1989). Because many molecular biological and genetic techniques are available, *C. elegans* is a good model organism for studying the regulation of transition into the diapause stage at both molecular and cellular levels.

Genes that regulate dauer larva formation have been studied by the isolation and characterization of mutants that show abnormality in this function. These mutants, called *daf* (dauer larva formation abnormal), consist of two groups: dauer-constitutive (*daf-c*) mutants, which form dauer larvae even under non-crowding and well-fed conditions, and dauer-defective (*daf-d*) mutants, which do not form dauer larvae, even under conditions of crowding and starvation. The genetic pathways of dauer larvae formation have been revealed by epistasis tests between *daf-c* and *daf-d* mutations, and by molecular cloning of the mutant genes. At least four signal transduction pathways control dauer larva formation: the cGMP, TGF-β, insulin and steroid hormone signaling

pathways (Riddle and Albert, 1997; Gerisch et al., 2001; Jia et al., 2002).

In addition to mutations that show an abnormality in dauer larva formation by themselves, mutations that show Daf-c phenotypes only in the background of another mutation have been discovered and called synthetic Daf-c mutations. A great majority of them were isolated by other phenotypes, mostly those in neuronal functions, and were later found to show this phenotype when double mutants of these mutations were constructed. For example, *unc-3*, *unc-31* and *aex-3* single mutants produce no or few dauer larvae under favorable conditions, but the double mutants *unc-31;unc-3* and *unc-31;aex-3* produce many dauer larvae (Bargmann et al., 1990; Iwasaki et al., 1997). The *unc-3* gene encodes an OLF-1/EBF (O/E) family transcription factor (Prasad et al., 1998). The *unc-31* gene encodes a homolog of CAPS (calcium-activated protein for secretion) (D. Livingstone, PhD thesis, University of Cambridge, 1991) (Ann et al., 1997), which is required for the exocytosis of dense core vesicles (Berwin et al., 1998). *unc-31* mutants show slow locomotion (Brenner, 1974) and a Daf-c phenotype in the wild-type background at a very high temperature (27°C), at which *C. elegans* cannot reproduce (Ailion and Thomas, 2000). The *aex-3* gene encodes a guanine nucleotide-exchange factor for Rab3 GTPase, which is required for intraneuronal transport (Iwasaki et al., 1997). Although *daf-c* mutations have already been saturated (Malone and Thomas, 1994), many synthetic Daf-c mutations remain to be identified.

To identify new genes regulating dauer larva formation and to discover new mechanisms, we isolated 44 synthetic Daf-c mutants in the *unc-31(e169)* background and named them *sdf* (synthetic abnormality in dauer larva formation) mutants (N.S., T.I. and I.K., unpublished). Of these mutants, the genes for *sdf-9* and *sdf-13* have been cloned. The *sdf-9* gene encodes a protein tyrosine phosphatase-like molecule. It is expressed in a pair of neuron-associated cells called XXXL/R, and regulates dauer larva formation in the steroid hormone signaling pathway (Ohkura et al., 2003). *sdf-13* encodes a homolog of the transcription factor Tbx2/Tbx3, and is expressed in AWB, AWC and ASJ sensory neurons, and in many pharyngeal neurons. It controls olfactory adaptation in AWC and dauer larva formation in cells other than AWC (possibly ASJ) (Miyahara et al., 2004).

In this report, we describe the cloning, expression and functional analyses of another *sdf* gene, *sdf-14*, in which three mutant alleles, *ut151*, *ut153* and *ut155*, were isolated. *sdf-14* is allelic to *mrp-1*, which encodes an MRP (multidrug resistance-associated protein) homolog. It acts in multiple tissues to regulate dauer larva formation. Human MRP1 can substitute for *C. elegans* MRP-1 in dauer larvae regulation, for which the transport activity of MRP1 is required. We discuss the mechanism of dauer larva regulation by MRP-1.

Materials and methods

Worm culture and strains
Worms were grown by standard methods (Brenner, 1974) except for CB4856, a burrowing strain, for which 3% NGM agar plates were used. The strains used in this study were N2 (wild type), CB4856, RW7000, and the following mutants derived from N2: *sdf-14/mrp-1 (ut151, ut153, ut155, pk89)* X, *unc-31(e169)* IV, *daf-1(m40)* IV, *daf-2(e1370)* III, *daf-5(e1386)* II, *daf-11(m47)* V, *daf-14(m77)* IV, *daf-16(m26, m27, mu86)* I, *che-3(e1124)* I.

Dauer larva formation assay
Three to eight adult hermaphrodites were transferred onto 35-mm plates seeded with *Escherichia coli* OP50, and allowed to lay eggs for 3-8 hours at 18, 20, 23.5, 25.5, 26.5 or 27°C. The parents were then removed, and the progeny were cultured at the same temperatures. The numbers of dauer larvae and non-dauer animals (L4/adults) among the progeny were scored after 4-5 days at 18°C and 20°C, 3-4 days at 23.5°C to 26.5°C, and 3-5 days at 27°C. At 27°C, the growth rates were variable from animal to animal.

In the experiments for testing the effect of drugs, PAK-104P, agosterol A (AG-A), MK571 and sodium arsenite were dissolved in dimethyl sulfoxide (PAK-104P), ethanol (AG-A) or H$_2$O (MK571, sodium arsenite), and added to the NGM agar medium for plates, when it was cooled to 60°C, to give appropriate concentrations. Dauer larva formation was assayed with these plates after *E. coli* OP50 was grown. PAK-104P, AG-A, MK571 and sodium arsenite were obtained from Nissan Chemical Industries (Chiba, Japan), Dr Shunji Aoki (Graduate School of Pharmaceutical Sciences, Osaka University), Cayman Chemical Company (Ann Arbor, Michigan, USA), and Wako Pure Chemical Industries (Osaka, Japan), respectively.

Mapping of *sdf-14* mutations
sdf-14 mutations were mapped near the left end of the X chromosome by STS mapping with the strain RW7000 (Williams et al., 1992). The map position was determined more precisely by using single-nucleotide polymorphisms (SNPs) (Wicks et al., 2001). For SNP mapping, CB4856 males were crossed to *unc-31(e169);sdf-14(ut153)* double mutant hermaphrodites at 20°C. Then, individual F2 animals that showed the Unc-31 phenotype were picked and cultured separately at 25.5°C for the test of dauer larva formation. Genomic DNA was prepared from bulk of F3 self-progeny, and SNPs were detected by RFLPs or DNA sequencing. In addition to the SNP data provided by Dr Wicks, we also found some SNPs by sequencing the CB4856 genomic DNA and used them for mapping. The results showed that *sdf-14* mutations are located between the cosmid clones M02E1 and F02G3.

Rescue of *sdf-14* mutations for positional cloning
The cosmid clones between M02E1 and F02G3, either separately or as combinations of two adjacent clones, were injected into the germline of the *unc-31(e169);sdf-14(ut153)* double mutant (Mello et al., 1991). Of all the injected clones, only the combination of F57C12 and F55H6 rescued the Daf-c phenotype, which suggested that the *sdf-14* gene was identical to F57C12.5. We confirmed this by injecting various genomic DNA clones that contained only the F57C12.5 gene. One of these clones, pSDF14, was made by the integration of the following three fragments into the *Hinc*II-*Xma*I site of pBluescript II KS(–): (1) the *Sph*I-*Cla*I fragment of the cosmid F57C12; (2) the *Cla*I-*Aor*51HI fragment of the cosmid F57C12; and (3) the *Xma*I-*Sph*I fragment corresponding to the promoter and N terminus of F57C12.5 gene. Fragment 3 was made by PCR amplification of genomic DNA using the F57C12.5-4 primer GCT GGA TGA TTT GCA CTT CGA GTA GTT GGC and the F57C12.5-35 primer GCC GAA CAT CAA TTT GAC GG, cloning of the PCR fragment into the *Xba*I-*Sph*I site of pUC119, and excision of the *Xma*I-*Sph*I fragment from the clone.

In the rescue experiments, the genomic DNA clones were injected into *unc-31(e169);sdf-14(ut153)* animals at concentrations of 5-10 ng/μl, together with injection marker DNA (*gcy-10*::GFP; 25-30 ng/μl). Dauer larva formation of the transformants was assayed at 25.5°C.

Rescue of the dauer-constitutive phenotype with *mrp-1* isoforms
The DNA constructs of b-, c- or e-type isoforms for transformation experiments were made from cDNA clones, yk1067b09(b),

yk46e1(c), yk494b1(e) and yk831b09(e), kindly provided by Y. Kohara. The cDNA sequences were confirmed by sequencing. Then, the exon 3-19 region of pSDF14::GFP was replaced by the corresponding part of each cDNA, flanked by *Bst*EII and *Sap*I sites. The resulting isoform constructs (pSDF14b::GFP, pSDF14c::GFP and pSDF14e::GFP) were injected at a concentration of 10 ng/μl together with the *gcy-10*::GFP injection marker (25-30 ng/μl) into *unc-31(e169);mrp-1(ut153)* animals, respectively. Dauer larva formation of the transformants was assayed at 25.5°C.

Analysis of expression pattern

To examine the expression pattern of the *mrp-1* gene, we made a GFP-tagged *mrp-1* gene (pSDF14::GFP) as follows. The region containing the GFP-coding sequence and the *unc-54* 3′ UTR sequence of pPD95.79 (gift from A. Fire) was amplified by PCR with the pPD95.75-*Age*I primer GAG GGT ACC GGT AGA AAA ATG AGT AAA GGA GAA GAA CTT TTC ACT GGA G and the AMP3 primer CTC AAC CAA GTC ATT CTG AGA ATA GTG. Then, the *Age*I-*Apa*I fragment of the PCR product was inserted at the end of the *mrp-1* coding sequence of pSDF14 at which point an *Age*I site was made in advance.

pSDF14::GFP and the *rol-6(dom)* marker DNA were co-injected into wild-type animals at concentrations of 40 ng/μl and 50 ng/μl, respectively. The expression pattern of the transformants was observed under a fluorescence microscope. To check whether pSDF14::GFP was functional, pSDF14::GFP was injected into *unc-31(e169);mrp-1(ut153)* animals together with the *gcy-10*::GFP marker at concentrations of 10 ng/μl and 12 ng/μl, respectively. Dauer larva formation of the transformants was assayed at 25.5°C.

Rescue by tissue-specific expression with extrinsic promoters

The construct for pharyngeal muscle expression (pMyo2p::SDF14b::GFP) was made by inserting the following two fragments of pSDF14b::GFP into the *Kpn*I-*Apa*I site of pPD30.69 (gift from A. Fire, containing the *myo-2* promoter): (1) the *Kpn*I-*Sph*I fragment of the PCR product amplified from pSDF14b::GFP with the KpnI/5up primer CGG GGT ACC AAT TAA GAA ATG TTC CCG TTA G and the F57C12.5-36-1 primer CGT TCA ACC TTC GTC AAC TGC; and (2) the *Sph*I-*Apa*I fragment of pSDF14b::GFP. For intestinal and neuronal expression, the constructs were made in the same way except that the *myo-2* promoter in pPD30.69 was replaced by the *ges-1* promoter or the H20 promoter, respectively (pGes1p::SDF14b::GFP and pH20p::SDF14b::GFP). These constructs were injected either separately or in combination into *unc-31(e169);mrp-1(ut153)* animals together with the *gcy-10*::GFP injection marker. The concentration of *gcy-10*::GFP was 25-30 ng/μl, and that of the tissue-specific expression constructs was 10 ng/μl. Dauer larva formation of the transformants was assayed at 25.5°C.

Human *MRP1* cDNA experiments

The construct of wild-type human *MRP1* cDNA with the *C. elegans mrp-1* promoter (pSDF14p::hMRP1) was made by inserting the *mrp-1* promoter into the *Xba*I-*Xma*I site of MCSI (multi-cloning site I), and human *MRP1* cDNA into the *Kpn*I-*Sac*I site of MCSII in pPD49.26 (gift from A. Fire). The *mrp-1* promoter (about 3.7 kb) was amplified by PCR, by which an *Xba*I site and an *Xma*I site were made at the 5′ and 3′ end, respectively. The full-length human *MRP1* cDNA was manipulated as the 5′ half (*Sal*I-*Eco*RI fragment) and the 3′ half (*Eco*RI-*Not*I fragment). A *Kpn*I site was made by PCR upstream of the initiation codon in the 5′ fragment, and a *Sac*I site downstream of the stop codon in the 3′ fragment. The construct of the dmL_0MRP1 mutant cDNA driven by the *C. elegans mrp-1* promoter (pSDF14p::mhMRP1) was made by replacing the *Dra*III-*Eco*RI region of pSDF14p::hMRP1 with that of

dmL_0MRP1 cDNA. The PCR primers used were as follows. For the addition of *Xba*I and *Xma*I sites at the ends of the *mrp-1* promoter fragment, –3707/XbaI-FW primer GCT CTA GAA TTA TAT CAC TTT TCG and –3707/XmaI-RV primer TCC CCC CGG GTT CTT AAT TGG CTC GGT TCG G. For introducing a *Kpn*I site upstream of the initiation codon of human *MRP1* cDNA, hmrp1/KpnI-1FW primer CGG GGT ACC AAT TAA GAA ATG GCG CTC CGG GGC TTC TG and hmrp1-121RV CCC ACA CGA GGA CCG TG. For introducing a *Sac*I site downstream of the stop codon of human *MRP1* cDNA, hmrp1/3881FW primer GCT GGT TCG GAT GTC ATC TG and hmrp1/SacI-4739RV primer GAT GCG GAG CTC TAT CAC ACC AAG CCG GCG TC.

To make transformants, pSDF14p::hMRP1 (40 ng/μl) and pSDF14p::mhMRP1 (100 ng/μl) were injected into *unc-31(e169);mrp-1(ut153)* animals together with the *gcy-10*::GFP (25-30 ng/μl) marker. Dauer larva formation of the transformants was assayed at 25.5°C.

Results

sdf-14 encodes a multidrug resistance-associated protein (MRP) homolog

To elucidate the regulatory mechanism of dauer larva formation, we first investigated the phenotypes of *sdf-14* mutants. All the three mutants, *ut151*, *ut153* and *ut155*, showed strong dauer-constitutive (Daf-c) phenotypes in the *unc-31(e169)* background at 25.5°C (Fig. 1). Namely, although these mutants and the *unc-31(e169)* mutant produced less than 1% dauer larvae in the wild-type background at 25.5°C, the double mutants *unc-31(e169);sdf-14(ut151, ut153* or *ut155)* produced more than 60% dauer larvae at the same temperature.

We then cloned the *sdf-14* gene by positional cloning, using the synthetic Daf-c phenotype (see Materials and methods for details). The mutant phenotype was rescued by a genomic DNA
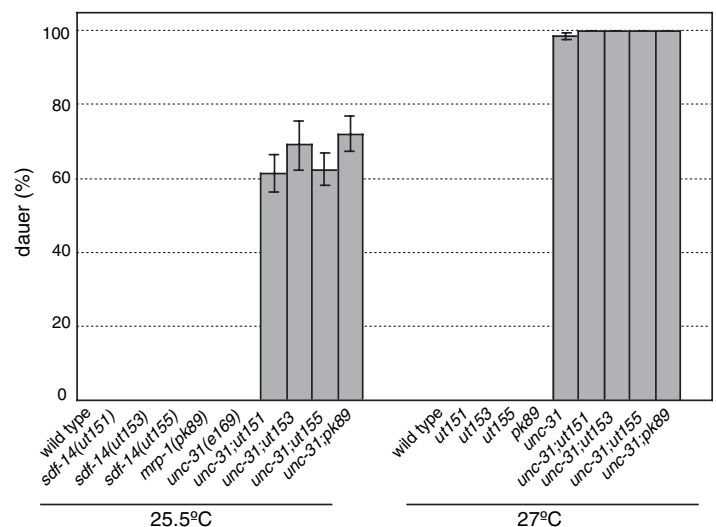

Fig. 1. Dauer larva formation of *unc-31(e169);sdf-14* double mutants. All the *unc-31(e169);sdf-14* double mutants formed dauer larvae at high percentages at 25.5°C, whereas the *unc-31(e169)* single mutant formed dauer larvae only at 27°C and not at 25.5°C. *sdf-14* mutants and wild-type animals did not form dauer larvae at either 25.5°C or 27°C. The results of the deletion mutant *mrp-1(pk89)* and *unc-31(e169);mrp-1(pk89)* are also shown, because *sdf-14* was found to be allelic to *mrp-1* in this study. The means of three plates are shown (33-199 animals/plate). The error bars indicate s.e.m. in all the figures of this paper.

fragment containing only the F57C12.5 gene (pSDF14; Fig. 2). The *C. elegans* database WormBase (http://www.wormbase.org/; Release WS136) indicated that F57C12.5 encodes a homolog of the multidrug resistance-

associated proteins (MRPs) belonging to the ATP-binding cassette (ABC) transporter superfamily. Sheps et al. (Sheps et al., 2004) reported that F57C12.5 belongs to the same group as human MRP1, MPR2, MPR3 and possibly MRP6. The amino
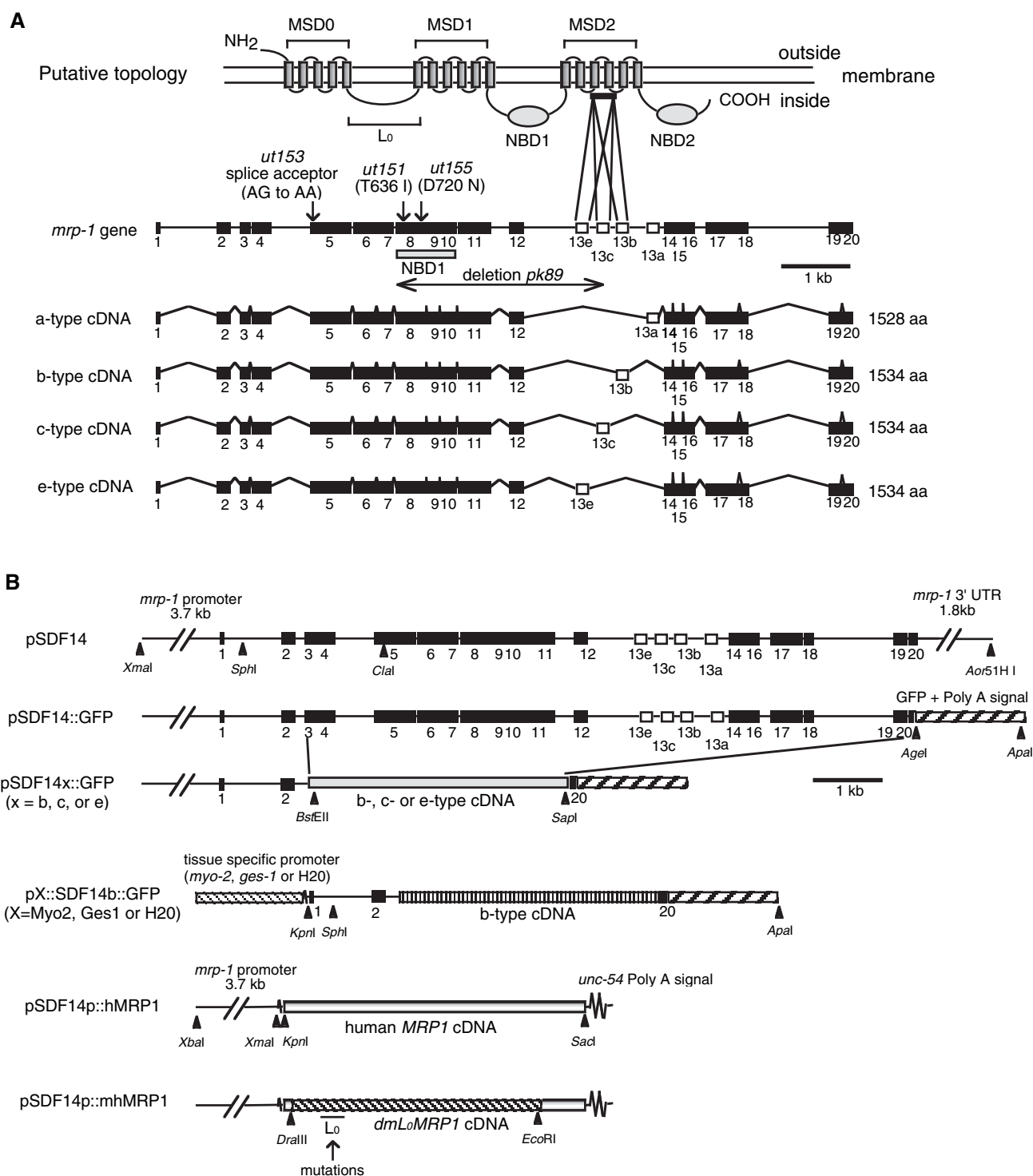
Fig. 2. *C. elegans* MRP-1. (A) Putative topology of the *C. elegans* MRP-1 protein (top); structure of the *mrp-1* gene and its cDNAs (bottom). Like human MRP1, *C. elegans* MRP-1 seems to have two nucleotide-binding domains (NBDs) and three membrane-spanning domains (MSDs). The *mrp-1* gene has 20 exons, of which the thirteenth exon is variable. The mutation sites of *ut151*, *ut153* and *ut155*, as well as the region deleted in *pk89* are shown in the gene structure. The *C. elegans* database WormBase listed the *mrp-1* cDNA isoforms, a-, b- and c-types, as well as d.1-, d.2- and d.3-types. The latter three cDNAs are not shown in this figure because they encode only short polypeptides. We found a fifth isoform (e-type), but could not confirm the existence of the a-type. (B) Constructs used for transgene experiments.
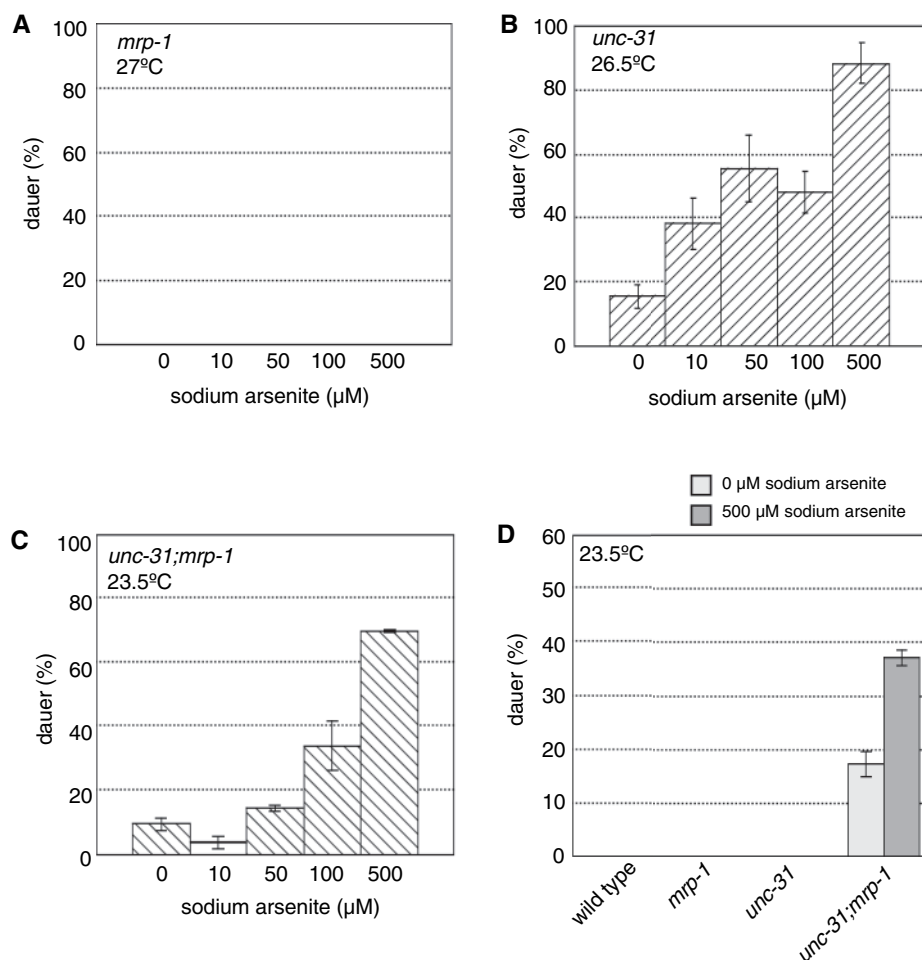
Fig. 3. Effect of sodium arsenite on the dauer larva formation of various strains. (A-C) Sodium arsenite did not enhance the dauer larva formation of *mrp-1(ut153)* (A), but enhanced that of *unc-31(e169)* (B) and *unc-31(e169);mrp-1(ut153)* (C). (D) The effects of sodium arsenite on *unc-31(e169)* and *unc-31(e169);mrp-1(ut153)* were measured at the same temperature for comparison. The means of three plates are shown (53-229 animals/plate).

Kast and Gros, 1998), SDF-14 also seemed to have 17 transmembrane regions, based on the homology.

To identify the mutation sites of *ut151*, *ut153* and *ut155*, we sequenced the genomic DNA of these mutants. *ut151* and *ut155* had missense mutations in NBD1 (T636I and D720N; amino acids that are conserved in human MRP1), whereas *ut153* had a mutation at the splice acceptor site of the fourth intron (AG to AA) (Fig. 2A).
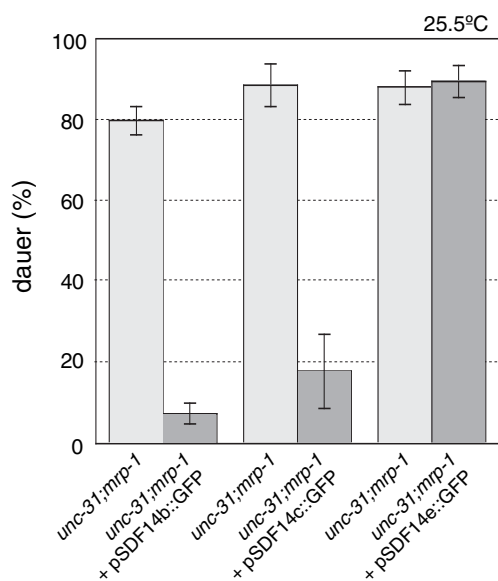
sdf-14 was allelic to *mrp-1*, which was investigated by Broeks et al. (Broeks et al., 1996). We therefore use *mrp-1* as the gene name from now on. They identified *mrp-1* as an MRP homolog gene, examined its expression pattern using a non-functional *mrp-1::lacZ* fusion gene, isolated a deletion mutant (*pk89*), and showed that the mutant is hypersensitive to sodium arsenite and cadmium chloride. However, they did not survey phenotypes concerning the dauer larva. We therefore examined the dauer-related phenotypes of *pk89* and found that they are essentially the same as those of *ut151*, *ut153* and *ut155* (Fig. 1). Because the *pk89* mutation showed phenotypes both in dauer larva regulation and arsenite sensitivity, we tested the influence of sodium arsenite on dauer larva formation. As shown in Fig. 3, sodium arsenite enhanced the dauer larva formation of *unc-31(e169)* animals (Fig. 3B), and the *mrp-1(ut153)* mutation further enhanced it (Fig. 3D).

C. elegans MRP-1 has multiple isoforms

WormBase suggested that the *mrp-1* gene (F57C12.5) produces three types of cDNAs encoding apparently functional isoforms (a-, b- and c-type; Fig. 2A), as well as those encoding short polypeptides (d.1, d.2 and d.3-type; not shown). Because
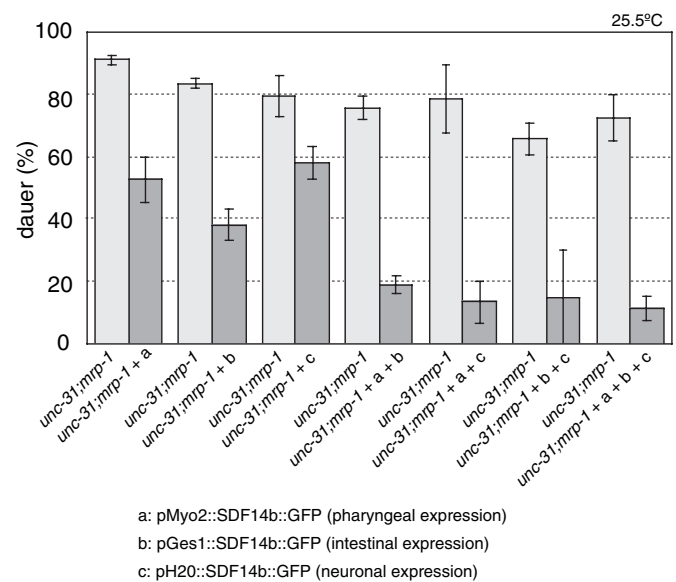
acid sequence of SDF-14 showed about 60% homology to that of human MRP1, and the homology extended all over the primary structure. Like human MRP, SDF-14 seemed to have three membrane-spanning domains (MSDs) and two nucleotide-binding domains (NBDs). As human MRP1 was shown to have 17 transmembrane regions by experiments (Bakos et al., 1996;

Fig. 4. Functional difference among the *mrp-1* isoforms. The b- and c-type isoforms, but not the e-type isoform, can rescue of the Daf-c phenotype of *unc-31(e169);mrp-1(ut153)*. The means of multiple lines are shown (two lines of b-type, eight lines of c-type, and four lines of e-type). The assays were carried out with 13-128 animals/plate at 25.5°C. Animals carrying an extrachromosomal array of transgenes segregated those that had lost the extrachromosomal array. Dauer formation of these animals was also examined and is shown as a control on the left of each data set.

Development
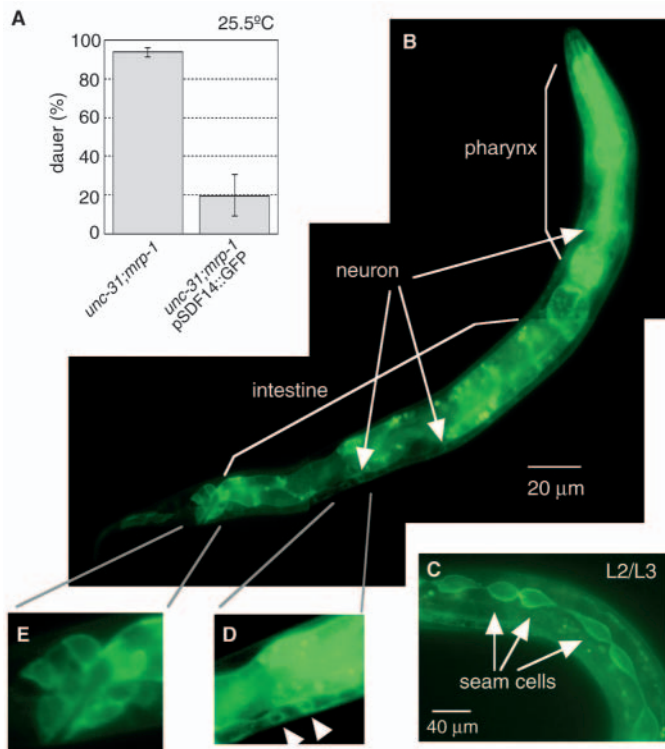
Fig. 5. Expression pattern of MRP-1. (A) Dauer larva formation of *unc-31(e169);mrp-1(ut153)* was rescued by the *mrp-1*::GFP fusion gene, showing that it was functional. The means of four plates are shown (21-94 animals/plate). (B) The *mrp-1*::GFP fusion gene was expressed in various tissues (L1 larva). (C) Expression in seam cells (L2 or L3 larva). (D) Neuronal expression; neurons are indicated by arrowheads. (E) Localization of GFP at the cell membrane. The expression patterns were observed with wild-type animals.

a: pMyo2::SDF14b::GFP (pharyngeal expression)
b: pGes1::SDF14b::GFP (intestinal expression)
c: pH20::SDF14b::GFP (neuronal expression)

Fig. 6. MRP-1 needs to be expressed in multiple tissues for the wild-type phenotype. Functional *mrp-1*::GFP fusion genes driven by various tissue-specific promoters (*myo-2* promoter for pharyngeal muscles, *ges-1* promoter for intestinal cells, and H20 promoter for neurons) were introduced either separately or in combination into *unc-31(e169);mrp-1(ut153)* double mutant animals. The results show that expression in multiple tissues is necessary to rescue the abnormality of dauer larva formation efficiently. The means of two to eight lines are shown (19-92 animals/line). Because the expression in neurons and intestinal cells looked weaker than in pharyngeal cells, we increased the concentration of the former two DNA constructs by 4-fold (40 ng/µl). However, expression in one tissue still resulted in partial rescue. Animals carrying an extrachromosomal array of transgenes segregated those that had lost the extrachromosomal array. Dauer formation of these animals was also examined and is shown as a control on the left of each data set.

the diversity of the former isoforms originates from differences in exon 13, we sequenced the exon 13 region of all of the nine cDNA clones kindly donated by Y. Kohara. The results showed that four clones (yk15b10, yk131e9, yk892h09 and yk1067b09) belonged to the b-type, two (yk46e1 and yk1289f01) belonged to the c-type, and two (yk494b1 and yk831b09) belonged to a new type, which we call the e-type. The remaining one clone (yk1240d1), a partial-length cDNA, had the a-type sequence in exon 13, but retained intron 13, which caused a frame shift that resulted in a stop codon in exon 14. Thus, we could not confirm the presence of the a-type cDNA. One of the e-type clones, yk831b09, which contained full-length cDNA, was sequenced completely (DDBJ/EMBL/GenBank Accession Number AB199793).

To learn whether there are functional differences among these isoforms, we made DNA constructs with b-, c- and e-type cDNA (Fig. 2B), and examined whether they could rescue the Daf-c phenotype of *unc-31(e169);mrp-1(ut153)*. We neglected the a-type, which we could not find, and the d-types, which encode short polypeptides. The results showed that the Daf-c phenotype of the *unc-31(e169);mrp-1(ut153)* double mutant was rescued by the b- and c-type constructs, but not by the e-type construct (Fig. 4), although all of them showed normal expression, as judged by GFP fluorescence.
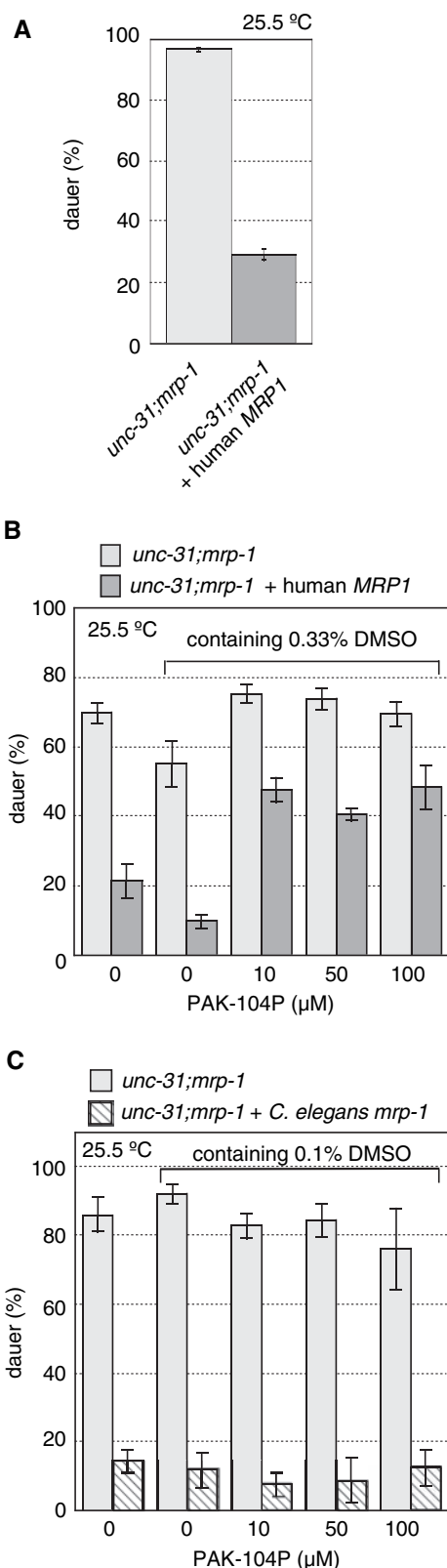
mrp-1 is expressed in many tissues and acts in dauer larva regulation in multiple tissues

To investigate the expression pattern of MRP-1, we examined wild-type animals that expressed a GFP fusion gene (pSDF14::GFP; Fig. 2B). This construct was functional and rescued the Daf-c phenotype of *unc-31(e169);mrp-1(ut153)* (Fig. 5A). The results were similar to those obtained by Broeks et al. (Broeks, et al., 1996), who examined expression with a non-functional *mrp-1::lacZ* gene and reported that the *lacZ* fusion transgene was expressed in the pharynx, pharynx-intestinal valve cells, anterior intestinal cells, intestinal-rectum valve cells and epithelial cells of the vulva. We found that the functional *mrp-1*::GFP gene was expressed not only in these cells but also in some neurons, as well as other intestinal cells and hypodermal seam cells, and that it was localized in cell membrane. We did not examine the time course of expression in each tissue in detail, but expression was detected already in the pharynx, intestine and neurons in L1 larvae (Fig. 5). The expression of *C. elegans* MRP-1 in many tissues may be similar to that of human MRP1, which is also expressed in many tissues (Flens et al., 1996).

To learn in which tissue MRP-1 acts in the regulation of dauer larva formation, we investigated the phenotype of *unc-31(e169);mrp-1(ut153)* in which MRP-1 was expressed in

A

B

C

(Fig. 6). The results show that there is no specific tissue in which MRP-1 acts to prevent dauer larva formation, and that MRP-1 molecules in neurons, intestinal cells and pharyngeal muscles act together for this function.

Human MRP1 can substitute for *C. elegans* MRP-1 in dauer larva regulation

If human MRP1 can substitute for *C. elegans* MRP-1 in dauer larva regulation, it will provide a good experimental system for elucidating the mechanism of this function, because human MRP1 has been characterized extensively. We therefore introduced human *MRP1* cDNA (GenBank Accession Number L05628) connected to the *C. elegans mrp-1* promoter (pSDF14p::hMRP1; Fig. 2B) into *unc-31(e169);mrp-1(ut153)* animals. The human *MRP1* construct rescued the Daf-c phenotype of the double mutant (Fig. 7A), i.e. human MRP1 substituted for *C. elegans* MRP-1 in dauer larva regulation.

We then carried out two experiments to investigate whether the transport activity of human MRP1 is required for this function. We first examined whether dmL_0MRP1 mutant cDNA, driven by the *C. elegans mrp-1* promoter, can rescue the Daf-c phenotype of *unc-31(e169);mrp-1(ut153)*. The dmL_0MRP1 protein has multiple amino acid substitutions in the L_0 region and cannot transport the known substrates leukotriene C_4 (LTC_4) and 17β-estradiol 17β-(D-glucuronide) ($E_2$17βG) (T. Noguchi, X.-Q. Ren and T.F., unpublished). Although as many as 19 transformant lines were examined, the dauer larva formation abnormality of *unc-31(e169);mrp-1(ut153)* was not rescued in any of them (data not shown). Thus, the amino acid residues of human MRP1 that are essential for its function as a transporter in human cells are also essential for dauer larva regulation in *C. elegans*.

Next, we investigated the effects of human MRP1 inhibitors on the dauer larva formation of *unc-31(e169);mrp-1(ut153)* mutants carrying the extrachromosomal array of human *MRP1* cDNA connected to the *C. elegans mrp-1* promoter. We used three inhibitors, PAK-104P (Shudo et al., 1990), agosterol A (AG-A) (Aoki et al., 1998) and MK571 (Jones et al., 1989), each of which was added to the agar plates for the assay of dauer larva formation. The results showed that PAK-104P, but not AG-A (0-200 μM) or MK571 (0-200 μM), enhanced the dauer larva formation of the strain expressing human MRP1 (Fig. 7B and data not shown). None of the inhibitors had any effect on *unc-31(e169);mrp-1(ut153)* carrying the

various tissues by using extrinsic promoters. Expression of MRP-1 in only one tissue (neurons, intestinal cells or pharyngeal muscles) rescued the dauer larva formation abnormality only weakly. By contrast, the abnormality was rescued efficiently by expressing MRP-1 in two or three tissues

extrachromosomal array of the *C. elegans mrp-1* gene (Fig. 7C). These experiments indicate that PAK-104P acts specifically on human MRP1 and that the transporter activity of human MRP1 is required for the suppression of dauer larva formation.

Position of the *mrp-1* gene in the genetic pathways of dauer larva formation

To obtain information on the mechanism of dauer larva regulation by MRP-1, we investigated the position of the *mrp-1* gene in the regulatory pathway. It is known that four pathways are involved in dauer larva regulation: the cGMP, TGF-β, insulin and steroid hormone signaling pathways (Riddle and Albert, 1997; Gerisch et al., 2001; Jia et al., 2002). The following two sets of experiments indicate that *mrp-1* does not act in the cGMP or TGF-β signaling pathway.

First, *mrp-1* mutations did not enhance the Daf-c phenotype of *daf-11* or *daf-1* specifically and strongly. Thomas et al. (Thomas et al., 1993) reported that *daf-c*(cGMP);*daf-c*(TGF-β) double mutants display strong synthetic enhancement of their Daf-c phenotype, but that *daf-c*(cGMP);*daf-c*(cGMP) and *daf-*c(TGF-β);*daf-*c(TGF-β) do not. We constructed *daf-11(m47);mrp-1(pk89)* and *daf-1(m40);mrp-1(pk89)*, where *daf-11* and *daf-1* are members of the cGMP and TGF-β signaling pathways, respectively. Assays of dauer larva formation showed that the *mrp-1(pk89)* mutation enhanced the Daf-c phenotype of both *daf-11(m47)* and *daf-1(m40)* only to small and similar extents (Fig. 8A). These results do not fit with the reported properties of mutations in the cGMP or TGF-β signaling pathway.

Second, the Daf-c phenotype of the *daf-2(e1370);mrp-1(pk89)* double mutant was not suppressed by *daf-d* mutations in the cGMP or TGF-β signaling pathway. It is known that the *daf-2(e1370)* mutant, which affects the insulin signaling pathway, shows a strong Daf-c phenotype at 25°C, but not at 20°C (Gems et al., 1998). Because the *daf-2(e1370);mrp-1(pk89)* double mutant showed a strong Daf-c phenotype at 20°C (Fig. 8A), we examined whether this phenotype was suppressed by *che-3(e1124)* or *daf-5(e1386)*, *daf-d* mutations in the cGMP and TGF-β signaling pathway, respectively. As shown in Fig. 8B,C, the Daf-c phenotype of *daf-2;mrp-1* was suppressed neither by *che-3* nor by *daf-5*. It is known that *che-3* and *daf-5* suppress all the *daf-c* mutations in the cGMP and TGF-β signaling pathway, respectively, but not *daf-2(e1370)* (Vowels and Thomas, 1992; Thomas et al., 1993). Hence, we concluded that *mrp-1* acts in neither the cGMP nor the TGF-β signaling pathway. As expected, the Daf-c phenotype of *daf-2(e1370);mrp-1(pk89)* was suppressed by *daf-16(mu86)* (Fig. 8D), a *daf-d* mutation in the insulin signaling pathway.

As the *mrp-1* gene acts in neither the cGMP nor the TGF-β signaling pathway, it may act through either the insulin or steroid hormone signaling pathway, which interact each other (Ohkura et al., 2003; Matyash et al., 2004). In fact, the enhancement of dauer larva formation by
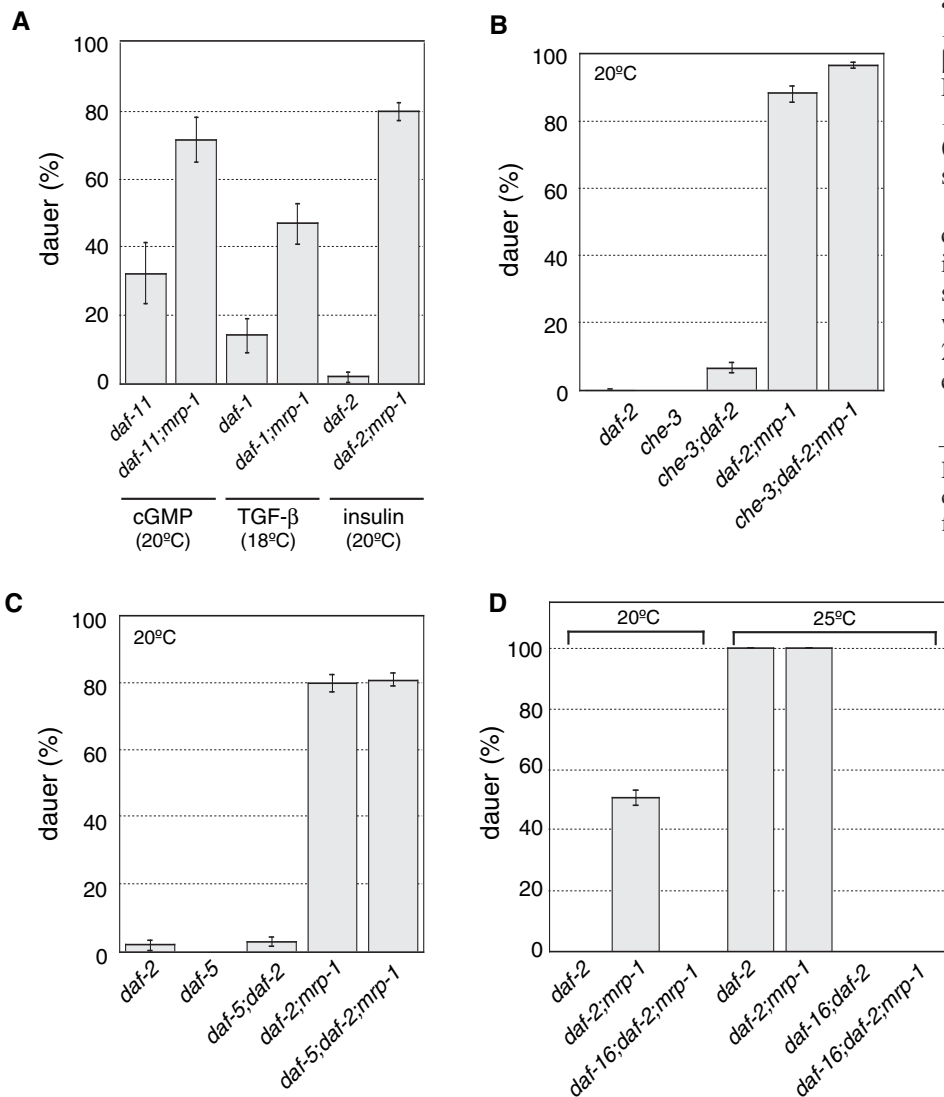
Fig. 8. The *mrp-1* gene acts neither in the cGMP nor in the TGF-β signaling pathway for dauer larva regulation. (A) The *mrp-1(pk89)* mutation enhanced the dauer larva formation of *daf-11(m47)* (cGMP signaling pathway) and *daf-1(m40)* (TGF-β signaling pathway) to a small and similar extent. By contrast, it enhanced that of *daf-2(e1370ts)* (insulin signaling pathway) to a large extent. (B) The dauer larva formation of *daf-2(e1370);mrp-1(pk89)* was not suppressed by *che-3(e1124)*, a *daf-d* mutation in the cGMP signaling pathway. (C) The dauer larva formation of *daf-2(e1370);mrp-1(pk89)* was not suppressed by *daf-5(e1386)*, a *daf-d* mutation in the TGF-β signaling pathway. (D) As expected, the dauer larva formation of *daf-2(e1370);mrp-1(pk89)* was suppressed by *daf-16(mu86)*, a *daf-d* mutation in the insulin signaling pathway, which suppresses *daf-2(e1370)*.

mrp-1 mutations is especially strong in the background of *daf-2(e1370ts)* at 20°C or *unc-31(e169)* at 25.5°C, i.e. if the insulin signaling pathway is partially blocked (Kimura et al., 1997; Ailion and Thomas, 2000). Our attempts to prove that *mrp-1* acts in the insulin signaling pathway have failed so far. We planned to test whether *daf-16* mutations, which suppress *daf-c* mutations in the insulin signaling pathway, suppress the Daf-c phenotype of *daf-1(m40);mrp-1*, *daf-14(m77);mrp-1* or *daf-11(m47);mrp-1*, where *daf-1* and *daf-14* genes belong to the TGF-β signaling pathway and *daf-11* belongs to the cGMP signaling pathway. However, these experiments turned out to be inappropriate, because *daf-16* partially suppressed *daf-1*, *daf-14* and *daf-11* at the temperatures at which *mrp-1* mutations enhanced the Daf-c phenotype of these mutations (T.Y., T.I. and I.K., unpublished) (Vowels and Thomas, 1992). Furthermore, we could not find conditions under which *mrp-1* mutants produce the dauer-like larva, which is characteristic of mutants in the steroid hormone signaling pathway (Gerisch et al., 2001; Jia et al., 2002; Ohkura et al., 2003).

Discussion

sdf-14 encodes a homolog of MRPs, members of the ABC transporter superfamily

sdf-14 mutations were isolated by their dauer-constitutive phenotype in the *unc-31(e169)* background. In this study, we identified the *sdf-14* gene by positional cloning, and found that it is allelic to *mrp-1*, which encodes a homolog of the multidrug resistance-associated proteins (MRPs) belonging to the ATP-binding cassette (ABC) transporter superfamily. The *mrp-1* gene has been studied already by Broeks et al. (Broeks et al., 1996), as a homolog of mammalian MRP1 that confers resistance to arsenite and cadmium ions. By contrast, this study revealed the involvement of *mrp-1* in the regulation of dauer larva formation. The Daf-c phenotype of *mrp-1* mutations is most evident in the background of weak mutations in the insulin signaling pathway. We discuss below that dauer larva formation is regulated by the export activity of MRP-1 in multiple tissues, based on the results of this study and the present knowledge on MRPs and ABC transporters, which is summarized below.

ABC transporters form a large superfamily of proteins that are found in all kingdoms (Higgins, 1992; Dassa and Bouige, 2001). The function of most ABC transporters is the export of a wide variety of substrates, such as the extrusion of noxious substances, secretion of extracellular toxins, and targeting of membrane components, although some prokaryotic members are involved in the import of essential nutrients. Functional ABC transporters have four essential domains: two membrane-spanning domains (MSDs) and two nucleotide-binding domains (NBDs), which are contained in a single polypeptide in many cases, but in more than one polypeptide in some cases. The MSD consists of several transmembrane α-helices connected by polypeptides, whereas the NBD contains three consensus motifs: Walker A motif, Walker B motif and Linker peptide (Schneider and Hunke, 1998).

The human ABC transporters are classified into eight subfamilies (subfamily A to H) (http://www.gene.ucl.ac.uk/nomenclature/genefamily/abc.html), of which the MRPs belong to subfamily C. All MRPs have the structure of (MSD-NBD)×2, while some of them, including MRP1, have an additional MSD at the N terminus (MSD0) (Bakos et al., 1996). MSD0 is not essential for the transport function, but the intracellular linker domain (L_0) connecting MSD0 and MSD1 is required (Bakos et al., 1998).

The human *MRP1/ABCC1* gene was first identified as a gene similar to multidrug resistance protein 1/P-glycoprotein 1 (*MDR1/PGY1/ABCB1*). MDR1 and MRP1 contribute to the multidrug resistance of various cancer cell lines. Multidrug resistance, by which tumor cells become resistant to multiple structurally and functionally unrelated drugs, is due to the extrusion of drug compounds from inside cells to outside. Besides anti-cancer drugs, human MRP1 transports various organic anions and nonanionic compounds conjugated by glutathione, glucuronide or sulfate, and also co-transports nonanionic compounds with glutathione without conjugation (Russel et al., 2002). Most of these substrates are unnecessary compounds for the organism (conjugates, xenobiotics and detoxification products) except for Leukotriene C_4 (LTC_4) (Renes et al., 2000), a glutathione-conjugated organic anion that acts as an inflammatory mediator.

Structure and function of *C. elegans* MRP-1

Phylogenetic tree analysis showed that *C. elegans* MRP-1 belongs to the same group as human MRP1, MRP2, MRP3 and MRP6 in the ABCC subfamily (Sheps et al., 2004). It has homology to these human MRPs throughout the amino acid sequence, including in the MSDs, NBDs, Walker A and B motifs, and Linker peptide. The structural similarity and conservation of the motifs suggests that the structure-function relationship of *C. elegans* MRP-1 is similar to that of these human MRPs. The results of this study are consistent with this idea.

First, NBD1 seems to be important in *C. elegans* MRP-1, because the two missense mutations in this domain, *ut151* and *ut155*, showed phenotypes nearly as strong as the null mutation *pk89*. This is similar to the case in human MRP1, in which NBD1 mutations markedly decrease transport activity if not completely suppress it (Gao et al., 2000). The two NBDs of human MRP1 possess different properties: NBD1 binds ATP with high affinity, whereas NBD2 is hydrolytically more active and binds ADP with high affinity (Gao et al., 2000).

Second, our result agrees with the importance of the fourteenth transmembrane region for substrate specificity in human and mouse MRP1 (Zhang et al., 2003). The isoform rescue experiments showed that both the b-type and c-type, but not the e-type, can rescue the Daf-c phenotype of *unc-31(e169);mrp-1(ut153)*, whereas exon 13, which is different in these isoforms, roughly corresponds to the fourteenth and fifteenth transmembrane regions (Fig. 2). This may be due to a difference in substrate specificity between b/c-type and e-type, although this remains to be proved. It is intriguing that the *Drosophila* CG6214 gene, an MRP1 homolog, has two variant copies of exon 4 and seven variant copies of exon 8 (Grailles et al., 2003), where exon 8 partially overlaps with the sequence encoding the fourteenth transmembrane region.

The transport activity of MRP1 is required for the regulation of dauer larva formation

We found that wild-type human MRP1 can substitute for *C. elegans* MRP-1 in dauer larva regulation. Because human MRP1 had been studied intensively, we took advantage of this

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and investigated the requirements for successful substitution. The results showed that human MRP1 must retain its export activity. First, dmL$_0$MRP1, which is defective in the transport of LTC$_4$ and E$_2$17βG, could not rescue the Daf-c phenotype of *unc-31;mrp-1*. Second, PAK-104P, a competitive inhibitor of human MRP1 (Sumizawa et al., 1997), antagonized the function of human MRP1 but not *C. elegans* MRP-1 in dauer larva regulation. The specific effect of PAK-104P indicates that its target is human MRP1 and not other molecules inherent in *C. elegans* cells. These results also strongly suggest that *C. elegans* MRP-1 acts as an exporter in the regulation of dauer larva formation.

These data indicate that there is a dauer-regulatory substance(s) among the substrates of MRP-1. The substance would have to be a dauer-inducer, if it acts inside the cell, whereas it would have to be a dauer-inhibitor, if it acts outside the cell. The identification of the regulatory substance would be a crucial next step for elucidating the mechanism of dauer larva regulation by MRP-1.

Possible involvement of stress response in dauer larva regulation by MRP-1

mrp-1 mutations strongly enhance the Daf-c phenotype of insulin signaling mutants, when compared with that of cGMP or TGF-β signaling mutants. This regulation may involve a stress response, as suggested by the following indirect evidence. (1) Insulin signaling genes are closely related to stress response in their control of expression (Murphy et al., 2003; McElwee et al., 2004) and their mutant phenotypes (Honda and Honda, 1999; Baryste et al., 2001). (2) RNAi of the heat shock factor (*hsf-1*) gene, which is essential for stress response, suppresses the Daf-c phenotype of insulin signaling mutants (Walker et al., 2003; Morley and Morimoto, 2004). (3) Both high temperature and sodium arsenite, which induce stress response, enhance the Daf-c phenotype of insulin signaling mutants efficiently, as do *mrp-1* mutations (Ailion and Thomas, 2003) (this study). (4) Human MRP1 transports the arsenite ion as a complex with glutathione (Zaman et al., 1995), and probably the same is true for *C. elegans* MRP-1, because its mutants are hypersensitive to arsenite ions (Broeks et al., 1996).

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