The genetics of ivermectin resistance in *Caenorhabditis elegans*

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Communicated by H. Boyd Woodruff, Soil Microbiology Associates, Watchung, NJ, December 27, 1999 (received for review November 11, 1999)

The ability of organisms to evolve resistance threatens the effectiveness of every antibiotic drug. We show that in the nematode *Caenorhabditis elegans,* simultaneous mutation of three genes, *avr-14, avr-15,* and *glc-1,* encoding glutamate-gated chloride channel (GluCl) α-type subunits confers high-level resistance to the antiparasitic drug ivermectin. In contrast, mutating any two channel genes confers modest or no resistance. We propose a model in which ivermectin sensitivity in *C. elegans* is mediated by genes affecting parallel genetic pathways defined by the family of GluCl genes. The sensitivity of these pathways is further modulated by *unc-7*, *unc-9*, and the Dyf (dye filling defective) genes, which alter the structure of the nervous system. Our results suggest that the evolution of drug resistance can be slowed by targeting antibiotic drugs to several members of a multigene family.

Ivermectin is used to treat numerous parasitic infections of humans, pets, and livestock (1). Treatment with ivermectin is the cornerstone of efforts to eradicate river blindness (onchocerciasis). However, reports of resistance to ivermectin in nematodes are increasingly common (2–4). Ivermectin also kills the nematode *Caenorhabditis elegans* at therapeutic concentrations, making *C. elegans* a useful model system in which to examine mechanisms of ivermectin toxicity and resistance. Ivermectin activates glutamate-gated chloride channels (GluCls) that contain α-type channel subunits (5–7). In *C. elegans,* α-type subunits are encoded by a family of genes including: *glc-1* (encoding GLC-1/GluClα1), *avr-15* (encoding AVR-15/GluClα2), and possibly other uncharacterized genes found in the genome sequence (5–8). Severe loss-of-function mutations in *glc-1* or *avr-15* do not make worms resistant to ivermectin (6, 7), either because GluCls are not physiologically important targets of ivermectin, or because multiple GluCl genes contribute independently to ivermectin sensitivity. To clarify the role of the GluCls in the nematocidal effects of ivermectin, we had screened for ivermectin-resistant mutants (6). Here we analyze the effects of these and other, previously characterized mutations on ivermectin sensitivity. We show that simultaneous mutation of three genes encoding GluCl α-type subunits confers high-level resistance to ivermectin. Our results suggest that the ability of ivermectin to target several members of a multigene family may decrease the rate at which resistance evolves.

Methods

Genetics. Unless otherwise indicated the mutant alleles used were: *avr-14(ad1302)*, *avr-15(ad1051)*, *gcl-1(pk54::Tc1)*, *unc-7(e5)*, *unc-9(e101)*, *osm-1(ad1307)*, *osm-5(ad1308)*, *dyf-11(ad1303)*, and *che-3(ad1306). avr-15(ad1051), glc-1(pk54::Tc1), unc-7(e5)*, and *unc-9(e101)* appear to be molecular nulls (refs. 6, 7, and 18; T. Starich, personal communication). Ivermectin-resistant mutants were isolated in a screen for ivermectin resistance in an *avr-15(ad1051)* background by using the mutagen ethyl methanesulfonate as described (6). All strains were outcrossed twice with N2 and reisolated by selecting for ivermectin-resistant progeny in the F_2 generation.

avr-14 was mapped to chromosome I by crossing DA1302 *avr-14(ad1302)*; *avr-15(ad1051)* males into *DA438 bli-4(e937); rol-6(e187); daf-2(e1368) vab-7(e1562); unc-31(e928); dpy-11(e224); lon-2(e678)*, selecting the F_2 progeny on ivermectin and scoring for markers in the survivors. *avr-14* was three-point mapped by crossing DA1302 to CB4776 *bli-3 unc-13* or DA568 *dpy-5 eat-5 unc-29*. F_2 progeny were selected for ivermectin resistance, singled, and scored for the presence of markers in the progeny. Of 89 resistant progeny of the DA568 cross, we found three whose progeny contained Unc non-Eat non-Dpy animals and one with Dpy non-Eat non-Unc progeny. Of 98 ivermectin-resistant progeny of the CB4776 cross, there were 27 whose progeny contained Bli animals and seven with Unc progeny.

The alleles of Dyf genes were mapped to a chromosome by using the mapping strain DA438; ivermectin-resistant progeny were cloned from heterozygotes and markers were scored among the progeny. Noncomplementation tests then were performed between the mutants we isolated and candidate Dyf genes on that chromosome by scoring the Dyf phenotype.

Strains containing multiple mutations were constructed by using standard genetic techniques. *avr-15* was scored by electropharyngeogram (6), *glc-1* was scored by PCR (7), and *avr-14* was scored by using test crosses to score ivermectin sensitivity in an *avr-15* background (details available from the corresponding author on request).

Ivermectin Sensitivity Assay. The whole-worm ivermectin sensitivity assay was performed as described (6). Survival at various concentrations was normalized to the average survival in the absence of ivermectin. For each curve two separate experiments were performed for a total of $n = 6$ at each point. To calculate the EC_{37}, the dose-response curves were fit to the equation:

$$normalized\ survival = xe^{(-[\mathrm{ivm}]/y)},$$

by using IGOR PRO (WaveMetrics, Lake Oswego, OR) with x and y as free parameters. The y parameter is the EC_{37}.

Ivermectin Binding. Membranes (about 0.3–3 μg protein), prepared from *C. elegans* wild type (N2) and mutants by standard techniques, were incubated with [^{35}S]sulfonamide-ivermectin (Drug Metabolism Group, Merck) from 60 fM to above 100 pM, for 1 day, by using techniques similar to those described (9). Bound radioactivity was separated from free by filtration; nonspecific retention of the ligand was measured by inclusion of 100 nM unlabeled ivermectin. Specific binding was modeled as binding to a single site or populations of sites with the same affinity for the ligand. The K_d was modeled at 3 pM for N2 and most mutants. The B_{max} values for the different mutants are

Abbreviations: GluCl, glutamate-gated chloride channel; GFP, green fluorescent protein.

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Table 1. Ivermectin EC_{37}s for mutant strains

Wild-type ivermectin receptors present			Wild-type innexins present		EC_{37}, ng/ml	Error SEM
The GluCls AVR-14, AVR-15, and GLC-1 each independently make worms sensitive to ivermectin						
AVR-14	AVR-15	GLC-1	UNC-7	UNC-9	1.1	0.1
AVR-14			UNC-7	UNC-9	1.1	0.1
	AVR-15		UNC-7	UNC-9	1.4	0.3
		GLC-1	UNC-7	UNC-9	13.8	1.0
Together, the three GluCls account for all sensitivity to >1,000 ng/ml						
None			UNC-7	UNC-9	4,264	1,750
UNC-7 and UNC-9 gap junctions appear to act in parallel to the AVR-15 pathway						
AVR-14	AVR-15	GLC-1	UNC-7		0.6	0.1
AVR-14	AVR-15	GLC-1		UNC-9	1.3	0.1
	AVR-15	GLC-1	UNC-7		0.9	0.1
	AVR-15	GLC-1		UNC-9	0.5	0.1
AVR-14		GLC-1	UNC-7		10.3	1.2
AVR-14		GLC-1		UNC-9	733	136
AVR-14 and UNC-9 appear to act via the same pathway						
		GLC-1	UNC-7	UNC-9	13.8	1.0
AVR-14		GLC-1	UNC-7		10.3	1.2
		GLC-1	UNC-7		14.0	0.5
UNC-7 appears to contribute to ivermectin sensitivity via the AVR-14 and GLC-1 pathways						
		GLC-1	UNC-7	UNC-9	13.8	1.0
AVR-14				UNC-9	483	81.2
AVR-14		GLC-1		UNC-9	733	136
		GLC-1		UNC-9	2,110	595
Evidence for a fourth ivermectin-sensitive channel that activates at very high concentrations and acts via UNC-7						
None			UNC-7	UNC-9	4,264	1,750
None				UNC-9	>10,000	NA

NA, not applicable.

given relative to the N2 B_{max}, which was 0.6 ± 0.08 pmol/mg protein (see Table 2).

Cloning. Cosmids were isolated and injected according to standard protocols (10). *avr-14* was sequenced by designing sets of primers that spanned groups of exons and used to PCR from the mutant strains. After gel purification, the PCR products were sequenced by Applied Biosystems sequencers using the amplification primers for dye terminator reactions. *avr-14/gbr-1* cDNAs were kindly provided by Adrian Wolstenholme (University of Bath, Bath, U.K.). These were cloned into the pT7 vector by using PCR to incorporate appropriate restriction sites. RNA transcription, oocyte manipulation, and electrophysiology have been described (6). PCR was used to amplify the 5′ end of *avr-14* and add a *Pst*I site in the sixth exon that could be used to clone into the green fluorescent protein (GFP) fusion vector, pPD95.77 (gift from A. Fire, J Ahn, G. Seydoux, and S. Xu, the Carnegie Institution of Washington, Washington, D.C.). This plasmid, pPD95.77.TM.avr-14.5′, was used to analyze GFP expression. Overlap extension PCR was used to generate a rescuing minigene construct, pBSaly.gbr3, consisting of the 5′ end of the *avr-14* genomic fused in-frame at the introduced *Pst*I site (which was removed in the process) to the *avr-14* cDNA with the *unc-54* 3′ untranslated region from pPD30.69. Sequences of primers are available on request.

Laser Ablation. I1 pharyngeal neurons were ablated as described (11). Operated worms were allowed to recover in the absence of ivermectin for 1 day. They then were transferred to plates containing 10 ng/ml of ivermectin and allowed to grow for 21 days. Statistical significance was tested by using a two-tailed Mann–Whitney *U* test.

Results

Isolation of Ivermectin-Resistant Mutants. In a previously reported genetic screen for resistance to 10 ng/ml of ivermectin in an *avr-15(ad1051)* mutant background, we identified two classes of genes: alleles of four Dyf genes (*osm-1*, *osm-5*, *dyf-11*, and *che-3*), and two alleles of the GluCl subunit gene *avr-14* (6). The Dyf class of genes includes more than 20 genes sharing defects in amphid sensory endings that result in the decreased ability of the sensory neurons to absorb dye from the environment. Analysis of the four Dyf mutants we isolated as well as a random sample of previously identified Dyf strains indicated that Dyf mutations generally confer low level (2- to 5-fold) resistance to ivermectin as single homozygous recessives, consistent with the reports of C. Johnson and Rand (12) and as cited in ref. 13; for example, see Table 4. In contrast, single homozygous *avr-14* mutations confer no resistance to ivermectin in a wild-type background; resistance was synthetic, i.e., only a homozygous *avr-14; avr-15* double mutant was resistant (6, 12, 14) (see Table 1).

Molecular and Electrophysiological Characterization of *avr-14*. To better understand the mechanism of synthetic ivermectin resistance, we cloned *avr-14* by a combination of genetic mapping and identification of a candidate GluCl gene, *gbr-2*. *gbr-2* encodes two alternatively spliced transcripts whose ORFs predict proteins that belong to the GluClα class of channel subunits (8). Two overlapping cosmids containing the *gbr-2* gene, K07D2 and F56E2, as well as a minigene construct (see below) restored the

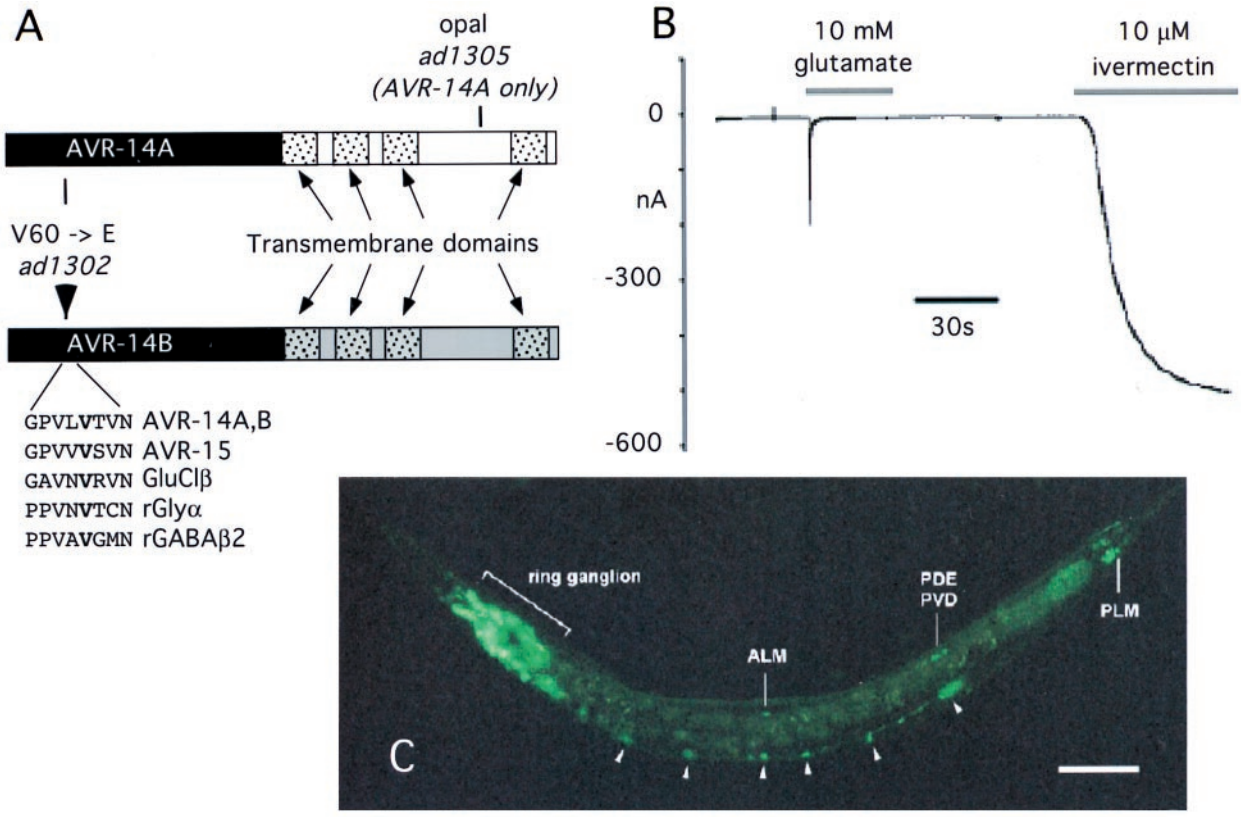

Fig. 1. *avr-14* encodes a subunit of an ivermectin-sensitive GluCl. (*A*) The mutations in alternatively spliced transcripts of *avr-14*. The exons encoding the extracellular domain (black) are shared by both transcripts. The exons encoding the transmembrane domains (stippled) are produced by alternatively spliced exons (white in AVR-14A and gray in AVR-14B). The region surrounding the conserved valine (bold) that is mutated to a glutamate in the *ad1302* allele (resulting from a T to A mutation in the second base of the V60 codon) is shown lined up with the corresponding region in other GluCl subunits and in the rat glycine and γ-aminobutyric acid (GABA) type A-β channel subunits (29, 30). The *ad1305* mutation results from an A to T change in the first base of the R387 codon. (*B*) Electrophysiology of oocytes expressing AVR-14B/GluClα3B. Oocytes were injected with RNA transcribed from AVR-14B/GluClα3B and were voltage clamped; 10 mM glutamate or 10 μM ivermectin (in 1% DMSO) were applied during the periods of time indicated by the bars. (*C*) Expression of an *avr-14* reporter construct. A GFP reporter construct was generated by fusing GFP in-frame to the sixth exon of *avr-14*. GFP fluorescence of an L4 worm is shown. The identified neuronal cell bodies (arrows) are indicated by their three-letter code. Arrowheads indicate ventral cord neurons. Multiple fluorescent neurons in the ring ganglia are indicated by the bracket. (Scale bar indicates 50 μm).

ivermectin-sensitive phenotype when transformed into the *avr-14; avr-15* double mutant (data not shown). We sequenced two alleles of *avr-14* and found that one allele, *ad1302*, resulted from a missense V60E mutation in an exon common to both transcripts (Fig. 1*A*). This allele may be null for channel function; the mutated valine is conserved, even among vertebrate γ-aminobutyric acid type A and glycine receptors, and may play a role in subunit association (15). The second allele, *ad1305*, was a premature termination (nonsense) mutation that eliminates the last transmembrane domain of the channel encoded by transcript A and is likely to be null for the protein encoded by that transcript. We expressed RNAs corresponding to each of the two proteins encoded by *gbr-2* in *Xenopus* oocytes. Oocytes injected with RNA encoding transcript A did not respond to ivermectin or glutamate. However, oocytes expressing transcript B gave a robust response to both ivermectin and glutamate (Fig. 1*B*) but did not respond to either of the chloride channel agonists γ-aminobutyric acid or glycine at 10 mM. As with the other characterized α-type subunits, the ivermectin response was only weakly desensitizing and was irreversible (5–7). We propose that the subunits encoded by *avr-14*/*gbr-2* be called AVR-14A/GluClα3A and AVR-14B/GluClα3B.

avr-14 appears to act in the nervous system. We generated a reporter fusion between the *avr-14* promoter and GFP that includes ≈3 kb of DNA upstream from the start ATG as well as the first six exons, which are common to both transcripts of *avr-14*. The *avr-14*::GFP fusion was expressed exclusively in a subset of ≈40 extrapharyngeal neurons (Fig. 1*C*). Most of the neurons were in the ring ganglia of the head, but some motor neurons in the ventral cord and mechanosensory neurons also expressed GFP. Because GFP expression may not always accurately represent expression of the native protein, we wanted to confirm that the pattern of GFP we saw with our reporter construct could account for the ivermectin sensitivity conferred by *avr-14*. Therefore, we also constructed a minigene that consists of the same *avr-14* promoter region fused to the *avr-14*B cDNA. When used to transform an *avr-14; avr-15; gcl-1* triple mutant (see below), the combined minigene restored ivermectin sensitivity (data not shown).

Three GluCls Mediate Ivermectin Sensitivity. Because simultaneous mutation of two GluCl subunits, *avr-14* and *avr-15*, caused moderate ivermectin resistance (6, 14), we investigated the possible contribution of the third characterized GluCl subunit encoded by *glc-1*. By constructing double and triple GluClα-subunit mutants, we demonstrated that each of the GluCl genes represents a parallel genetic pathway that confers ivermectin sensitivity on *C. elegans*. Of the three double mutant strains in which only one of the three GluCls is wild type, only the strain expressing wild-type GLC-1 and lacking AVR-14 and AVR-15 was significantly resistant (Table 1). However, the *avr-14; avr-15; glc-1* triple mutant, a strain in which all three GluClα subunit

Table 2. High affinity ivermectin binding sites in worm membranes relative to wild type

Wild-type GluCls Present			B_{max}*	SEM
AVR-14	AVR-15	GLC-1	1.00	
AVR-14		GLC-1	0.37	0.15
AVR-14	AVR-15		0.55	0.11
None			0†	N.A.

N.A., not available.
*Relative to N2, which had 0.6 pmol/mg pro.
†No high affinity binding site detected; if a site exists, its affinity is lower than 3 nM or constitutes less than 3% of the wild-type B_{max}.

proteins are mutant, was ≈4,000-fold resistant (Table 1). In spite of having mutations in three GluCl*α* subunits, these worms show surprisingly subtle behavioral changes including slightly inefficient eating and a tendency to reverse direction often (J.A.D., unpublished observation).

Innexins Contribute to Ivermectin Sensitivity by Altering the Structure of the Nervous System. *unc-1, unc-7*, and *unc-9* had been proposed to affect sensitivity to ivermectin and volatile anesthetics (16, 17). We did not find any resistance in the canonical loss-of-function *unc-1(e719)* allele either alone or in combination with *avr-15* (data not shown). However, both *unc-7* and *unc-9*, two genes encoding innexins (subunits of invertebrate gap junctions), contribute to ivermectin sensitivity (18–20). UNC-7 and UNC-9 act in parallel to AVR-15 because wild-type AVR-15 is sufficient to render worms sensitive to ivermectin regardless of the presence or absence of UNC-7 or UNC-9 (Table 1). However, strains with mutations in AVR-15 and UNC-7 or AVR-15 and UNC-9 showed increased resistance to ivermectin (Table 1). The UNC-9 gene product appears to be necessary for AVR-14-mediated sensitivity because: (*i*) when AVR-15 is mutated, mutation of either AVR-14 or UNC-9 resulted in strains with similar levels of resistance (Table 1), and (*ii*) when both AVR-14 and AVR-15 are mutated, the presence or absence of wild-type UNC-9 did not alter resistance substantially (Table 1), indicating that UNC-9 does not confer additional sensitivity in the absence of AVR-14. UNC-7 appears to mediate the effects of ivermectin acting via AVR-14 and GLC-1 and perhaps via an additional GluCl subunit. A strain in which UNC-7 and AVR-15 are mutated was more resistant than the strain with AVR-14 and AVR-15 mutated (Table 1), suggesting that mutating UNC-7 reduces sensitivity through a target in addition to AVR-14. However, mutating UNC-7 does not completely eliminate sensitivity mediated by AVR-14 because the strain with mutations in AVR-14, AVR-15, and UNC-7 was more resistant than the strain with mutated AVR-15 and UNC-7 (Table 1). On the other hand, the strain lacking all three GluCl subunits and UNC-7 was substantially more resistant than the strain lacking only the GluCl subunits (Table 1).

To determine whether the gap-junctional channels formed by UNC-7 and UNC-9 also might be targets of ivermectin, we performed ivermectin-binding studies on some of our mutant strains. As expected, membranes from strains lacking AVR-15 or GLC-1 had reduced ivermectin binding although they retain a fraction of the high affinity ivermectin binding found in wild type (Table 2). However, the strain lacking all three GluCl subunits had no detectable specific ivermectin binding. Lack of UNC-7 or UNC-9 had no significant effect on ivermectin binding (data not shown). Thus, the three genes encoding GluCl subunits appear to account for all high affinity ivermectin binding up to 100 pM (30 times the K_d of wild type). Although a small amount of ivermectin binding by the UNC-7 or UNC-9 proteins might have been missed, the amount they bind is not commensurate with their effects on ivermectin resistance. Rather than UNC-7 and UNC-9 being targets of ivermectin, their absence likely changes the connectivity of the nervous system to make it less susceptible to the effects of ivermectin on the GluCls.

Table 3. Effects of I1 ablation on sensitivity to 10 ng/ml of ivermectin

	Number of worms growing to each stage		
	Larva	Young adult	Fertile adult
Control (*avr-15 glc-1*)	18	1	0
I1 ablated (*avr-15 glc-1*)	9	3	7

One explanation for the effects of UNC-7- and UNC-9-containing gap junctions on ivermectin sensitivity is that they allow ivermectin-induced hyperpolarization of nonessential, GluCl-expressing cells to spread to other, essential excitable cells (19). Thus, in the absence of UNC-7 and UNC-9, ivermectin's effects would be restricted to cells expressing GluCls, inhibition of which does not cause severe toxicity. If so, what then might the essential cells be? One possibility is that the essential cells are in the pharynx. We observed that when the *avr-15* mutant is exposed to ivermectin, it stops pumping. But when its pharynx is dissected away from the rest of the worm, pumping resumes. We explain these observations by supposing that ivermectin-induced hyperpolarization of extrapharyngeal neurons (through its action on AVR-14) flows via gap junctions back to the pharynx and inhibits pharyngeal function. If so, then ablating the neurons that connect the pharynx to the extrapharyngeal nervous system should break this link and decrease the ivermectin sensitivity of the *avr-15* mutant. The pharynx is connected to the extrapharyngeal nervous system via gap junctions between the bilaterally symmetric I1 pharyngeal neurons and the extrapharyngeal RIP neurons. When we ablated the I1 neurons in an *avr-15 glc-1* background, the worms were more resistant than mock-ablated worms ($P = 0.0036$; Table 3).

Mutations in OSM-1 Act Additively with Other Genes to Increase Resistance. OSM-1, the product of the Dyf gene *osm-1,* appears to act by a different mechanism than any of the other genes because worms lacking OSM-1 showed some resistance and all strains with

Table 4. EC_{37}s for mutant strains without and with the Dyf mutation *osm-1(ad1307)*

Wild-type GluCls present			Innexin present	Wild-type OSM-1		Mutant OSM-1	
				EC_{37}, ng/ml	Error	EC_{37}, ng/ml	Error
AVR-14	AVR-15	GLC-1	UNC-9	1.1	0.1	2.6	0.2
	AVR-15	GLC-1	UNC-9	0.6	0.1	2.5	0.1
AVR-14		GLC-1	UNC-9	1.0	0.1	8.2	0.4
AVR-14			UNC-9	1.1	0.1	10.6	1.3
		GLC-1	UNC-9	13.8	1.0	59	8.4
AVR-14		GLC-1		10.3	1.2	32.8	1.2
			UNC-9	4,264	1,750	5,490	2,270

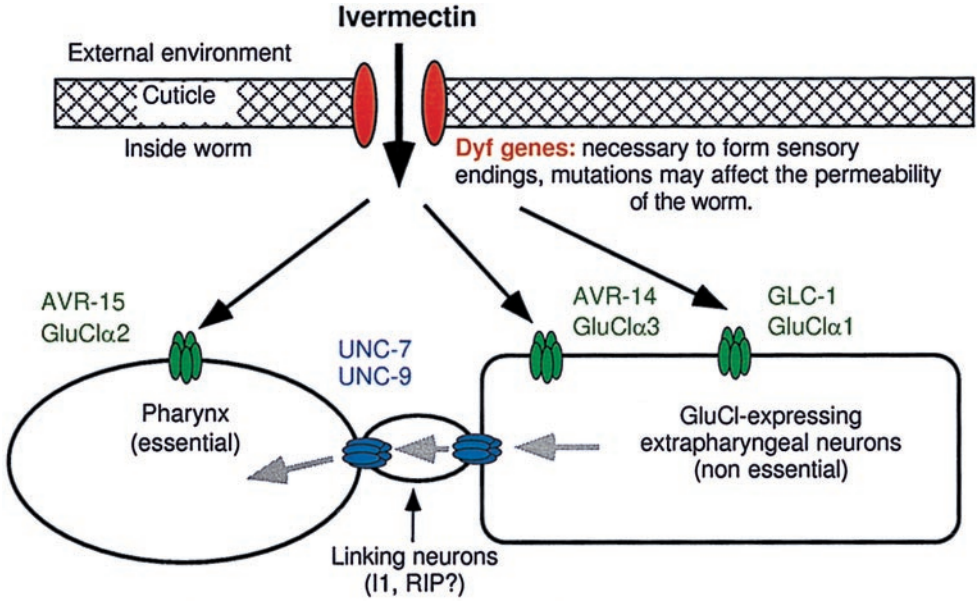

Fig. 2. Preposed model to describe mechanisms of ivermectin sensitivity. Black arrows indicate the diffusion of ivermectin and the gray arrows indicate the flow of ivermectin-induced hyperpolarizing potential. Dyf gene mutations, which may reduce ivermectin permeability, act additively with all combinations of receptor mutants in the tier below. Ivermectin acts independently on each of the GluCl subunits to hyperpolarize cells: GluClα2 (AVR-15) acts in pharyngeal muscle, GluClα3 (AVR-14) acts in neurons. GluClα1 (GLC-1) is presumed to be neuronal but, because we do not know where *gcl-1* is expressed, our model is not meant to imply that GluClα1 (GLC-1) and GluClα3 (AVR-14) are necessarily coexpressed or that they do or do not associate to form a channel. The effect of ivermectin on neurons expressing GluCls is not sufficient to kill the worms at low concentrations but requires that the ivermectin-induced hyperpolarization spread via gap junctions encoded by *unc-7* and *unc-9* to other excitable cells that are essential to the function of the worm. Our results indicate that the spread of hyperpolarization from the extrapharyngeal nervous system back to the pharynx is an important component of the gap-junction-mediated ivermectin sensitivity conferred by GluClα3 (AVR-14) and GluClα1 (GLC-1). The flow of hyperpolarizing potential from the extrapharyngeal neurons to the pharynx occurs via linking neurons such as I1 and RIP that may not themselves express GluCls.

a mutated OSM-1 were more resistant than strains of the same genetic background but with wild-type OSM-1 (with the possible exception of strains in which all three GluCls were lacking; Table 4). However, lack of OSM-1 seemed to preferentially affect the pathway defined by AVR-14 because worms lacking both OSM-1 and AVR-15 were more resistant than worms with only OSM-1 mutated whereas worms lacking AVR-14 and OSM-1 were just as sensitive as worms with only OSM-1 mutated.

Discussion

***avr-14* Encodes a GluClα Subunit.** We have shown that the *avr-14* ivermectin resistance locus corresponds to a GluCl gene cloned previously and known as *gbr-2*. Furthermore, we have demonstrated that this subunit forms a channel that is ivermectin- and glutamate-gated when expressed in *Xenopus* oocytes. However, we have only been able to observe a response to ligands in the *avr-14B* splice variant. This is odd because a mutation that specifically affects the *avr-14A* variant, *avr-14(ad1305),* confers ivermectin resistance to roughly the same extent as a mutation that affects both splice variants (J.A.D., unpublished observation). Furthermore, expressing only the *avr-14B* variant via our minigene construct is sufficient to confer ivermectin sensitivity on the triple GluCl mutant containing the *avr-14(ad1302)* allele. These results can be explained if these two splice variants associate *in vivo*. Although AVR-14B/GluClα3B appears sufficient to form a channel in oocytes, perhaps *in vivo* it must associate with the mutant *ad1302* AVR-14A/GluClα3A subunits to form a channel.

Ivermectin Targets Three Members of the Family of Ivermectin-Sensitive GluCl Subunits. Ivermectin has been shown to activate GluCls in several organisms including *C. elegans*. However, previous attempts to directly demonstrate the role of GluCls in ivermectin sensitivity by mutating individual GluCl genes were unsuccessful (6, 7), calling into question the role of GluCls in mediating physiologically relevant ivermectin toxicity. Our results clearly show that the GluCls are the physiologically relevant mediators of ivermectin toxicity and that the reason mutations in individual GluCls are not resistant is that ivermectin resistance is synthetic. Each of the three GluCls genes, *avr-14, avr-15*, and *glc-1*, constitutes a parallel genetic pathway that independently confers a high level of ivermectin sensitivity on worms. Consequently, mutations that reduce the sensitivity of any one pathway will not confer resistance (Table 1). A triple GluCl mutant, which reduces the sensitivity of all three pathways, is required before 4,000-fold resistance is achieved (Table 1).

The presence of a fourth ivermectin receptor is suggested by the increased resistance of the triple GluCl mutant when combined with an *unc-7* mutation (Table 1). This observation could be explained if: (*i*) the mutations in AVR-14 and GCL-1 were not null for ivermectin sensitivity and mutating UNC-7 reduced the residual sensitivity of mutant AVR-14 or GCL-1 channels, or (*ii*) UNC-7 is necessary to confer sensitivity via yet another pathway, possibly one defined by one of the other GluCl-like subunits identified by the genome project. The first hypothesis predicts that mutating UNC-9 would have the same affect as mutating UNC-7, it would further reduce the sensitivity of worms with mutations in AVR-14 and AVR-15. This is not the case (Table 1) so we favor the second hypothesis. Because AVR-14 appears to contribute very little to ivermectin binding, it would not be surprising if a fourth, lower-affinity receptor were undetectable in our binding assay.

Ivermectin Can Inhibit Pharyngeal Pumping Via Two Pathways. One way worms die from ivermectin is as a result of starvation caused by the inhibition of pharyngeal pumping (21–24). In light of this and previous studies it is clear that one of the genetic pathways leading

to the inhibition of pharyngeal pumping is defined by the *avr-15*-encoded GluClα2 expressed in the pharyngeal muscle (6). A second pathway appears to consist of *avr-14, unc-7*, and *unc-9* expressed in the extrapharyngeal nervous system. Three lines of evidence indicate that the extrapharyngeal nervous system is a separate target for ivermectin: (*i*) AVR-14 is not necessary for glutamatergic neurotransmission by the pharyngeal motor neuron M3, (*ii*) *avr-14*::*GFP* is expressed only in extrapharyngeal neurons, and (*iii*) in the triple GluCl mutant, expression of AVR-14B in the nervous system is sufficient to confer ivermectin sensitivity. GluClα1, possibly in conjunction with UNC-7, appears to constitute a third genetic pathway in a set of as-yet-unidentified excitable cells.

However, ivermectin's interaction with any one of these three pathways is sufficient to inhibit pharyngeal pumping. How then does the interaction of ivermectin with GluCls in the extrapharyngeal nervous system inhibit pharyngeal function? Our results indicate that ivermectin-induced hyperpolarization spreads from *avr-14*-expressing extrapharyngeal neurons to the pharynx (Fig. 2). Breaking the connection between the pharynx and the extrapharyngeal nervous system of an *avr-15* or an *avr-15 glc-1* mutant either by: (*i*) dissecting the pharynx away from the rest of the worm, or (*ii*) laser ablation of the neurons that connect the pharynx to the extrapharyngeal nervous system, decreases ivermectin sensitivity.

Because *unc-7* and *unc-9* code for innexins (subunits of gap junctional channels), presumably making the *avr-15; unc-7* or *avr-15; unc-9* double mutant is yet another way of breaking the connection between the pharynx and the extrapharyngeal nervous system. This would explain why these double mutants, but not the *avr-14; unc-7* or *avr-14; unc-9* double mutants, show some resistance to ivermectin in our growth assay. We do not know where *unc-7* and *unc-9* are expressed and, in our model, they could be expressed anywhere in a chain of electrically coupled neurons linking the *avr-14*-expressing cells to the pharynx. Electron microscopic reconstruction of *C. elegans* indicates that there are gap junctions between the I1 and RIP neurons that form the connection between the pharyngeal and extrapharyngeal nervous systems (25). These gap junctional channels may require UNC-7 and UNC-9.

Dyf Mutations Appear to Affect Cuticle Permeability. The Dyf mutants are so-named because of their inability to take up fluorescent dyes from the environment via the amphid sensory endings. It is therefore reasonable to think that the Dyfs confer resistance to ivermectin because they render the worm less permeable to the drug. Our results support this idea. All genetic backgrounds we tested (including wild type) have a higher EC_{37} when the Dyf gene *osm-1* is mutated. This is the expected result if the equilibrium ivermectin concentration inside the worm as a fraction of the externally applied drug is reduced by the *osm-1* mutation. Although not all Dyf mutants that are ivermectin resistant completely exclude dye [e.g., *osm-5(ad1308)*], it is possible that dye filling is reduced though not absent in these mutants or that the Dyf mutation differentially affects the permeabilities of the drug and the dye. Interestingly, the *avr-15; osm-1* double mutant is significantly more resistant than the *osm-1* single mutant whereas the *avr-14; osm-1* double is not, suggesting that the *osm-1* mutation preferentially reduces exposure of the extrapharyngeal nervous system to ivermectin. This makes sense in that the neurons with sensory endings at the amphid and that fill with dye are extrapharyngeal neurons.

What Can We Learn from the Genetics of Ivermectin Resistance in *C. elegans*? The evolution of resistance in parasitic worms exposed to ivermectin is increasingly a problem. To better manage the use of ivermectin, it would be useful to be able to track this resistance by molecular means (26). Our study identifies several candidate genes that could be markers of resistance in parasites. Furthermore, the availability of clones from *C. elegans* will make it easier to identify homologs of these genes in parasitic worms. Interestingly, evidence suggests that ivermectin resistance in *Haemonchus contortus* is linked to GluCl genes (27). However, ivermectin-resistant strains of *H. contortus* with autosomal dominant resistance loci or wild-type ivermectin binding also have been observed (3).

Finally, as our ability to design drugs becomes more sophisticated and the pool of potential antibiotic drug targets grows, the choice of drug target becomes more important. Our results suggest principles to guide the design of antibiotic drugs. First, even proteins that have subtle loss-of-function phenotypes can be effective drug targets if the drug activates the target. Second, targeting a multigene family can help delay the evolution of resistance if it means that many drug targets must be mutated simultaneously for resistance to occur. If no single resistance allele can confer resistance on a strain, then the evolution of resistance requires the less probable simultaneous occurrence of two or more resistance alleles in different genes. This may account in part for the relative longevity of the avermectins and milbemycins as effective antiparasitic drugs (6, 28).

We thank Carl Johnson for sharing strains and unpublished data, Wes Shoop and Ann Smith for critical reading of the manuscript, Brande Thomas and Vivien Warren for assistance with the binding assay, Stan Zborovski for help with sequencing, and the *Caenorhabditis* Genetics Center for providing strains. L.A. was supported by an Established Investigator award from the American Heart Association. J.A.D. was supported by the American Cancer Society and the Haberecht Wild-Hare Idea program.

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