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In Vivo Structure–Function Analyses of *Caenorhabditis elegans* MEC-4, a Candidate Mechanosensory Ion Channel Subunit

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Mechanosensory signaling mediated by mechanically gated ion channels constitutes the basis for the senses of touch and hearing and contributes fundamentally to the development and homeostasis of all organisms. Despite this profound importance in biology, little is known of the molecular identities or functional requirements of mechanically gated ion channels. We report a genetically based structure–function analysis of the candidate mechanotransducing channel subunit MEC-4, a core component of a touch-sensing complex in *Caenorhabditis elegans* and a member of the DEG/ENaC superfamily. We identify molecular lesions in 40 EMS-induced *mec-4* alleles and further probe residue and domain function using site-directed approaches. Our analysis highlights residues and subdomains critical for MEC-4 activity and suggests possible roles of these in channel assembly and/or function. We describe a class of substitutions that disrupt normal channel activity in touch transduction but remain permissive for neurotoxic channel hyperactivation, and we show that expression of an N-terminal MEC-4 fragment interferes with *in vivo* channel function. These data advance working models for the MEC-4 mechanotransducing channel and identify residues, unique to MEC-4 or the MEC-4 degenerin subfamily, that might be specifically required for mechanotransducing function. Because many other substitutions identified by our study affect residues conserved within the DEG/ENaC channel superfamily, this work also provides a broad view of structure–function relations in the superfamily as a whole. Because the *C. elegans* genome encodes representatives of a large number of eukaryotic channel classes, we suggest that similar genetic-based structure–activity studies might be generally applied to generate insight into the *in vivo* function of diverse channel types.

Key words: MEC-4; touch sensation; mechanosensation; mechanotransduction; neurodegeneration; degenerin; Na^+ channel; ENaC; mutagenesis

The molecular mechanisms by which mechanical signals direct biological responses remain a largely uncharted area in the field of signal transduction. Electrophysiological studies indicate that mechanotransduction can be mediated by ion channels that open or close in response to force (French, 1992; García-Añoveros and Corey, 1997; Sukharev et al., 1997; Ghazi et al., 1998). Such channels play essential roles in a diverse range of activities including cell volume control, development, morphogenesis, and the neuronal signaling underlying touch sensation, hearing, proprioception, and balance. Until recently, eukaryotic mechanosensitive ion channels have eluded cloning efforts, and thus little is understood of their structures and functions.

The nematode *Caenorhabditis elegans* has proved a facile model system for the identification of molecules involved in touch transduction. Extensive genetic mutant screens have yielded hundreds of mutations that specifically disrupt gentle body touch sensation mediated by six specialized mechanosensory neurons (Chalfie and Thomson, 1979; Chalfie and Sulston, 1981; Chalfie and Au, 1989). These mutations define at least nine structural genes (designated *mec* genes for the mechanosensory abnormal phenotype of the mutants) that encode proteins hypothesized to participate in a touch-transducing molecular complex (for review, see Chalfie, 1993; Tavernarakis and Driscoll, 1997). The core molecules in the complex, MEC-4 (Driscoll and Chalfie, 1991; Lai et al., 1996) and the homologous MEC-10 (Huang and Chalfie, 1994), are postulated to be subunits of a mechanically gated touch-transducing channel. Gating tension is thought to be exerted on the channel via attachments to the touch neuron-specific extracellular matrix and a specialized cytoskeleton. Several *mec* genes encode molecules that might associate with extracellular or intracellular MEC-4 domains to deliver the channel-gating force (for review, see Chalfie, 1993; Tavernarakis and Driscoll, 1997).

mec-4 and *mec-10* are members of the *C. elegans* degenerin family, composed of ~20 members (Mano and Driscoll, 1999). Two additional degenerins, *unc-8* and *unc-105*, have been implicated in mechanical signaling (Liu et al., 1996; Tavernarakis et al., 1997; García-Añoveros et al., 1998). Degenerins belong to the DEG/ENaC superfamily (named for the *C. elegans* degenerins and the vertebrate epithelial Na^+ channel) that includes subunits of the amiloride-sensitive epithelial Na^+ channel (Rossier et al., 1994) [some data suggest ENaC subunits may be mechanically sensitive (Awayda et al., 1995; Drummond et al., 1998; but see Awayda and Subramanyam, 1998; Rossier, 1998)], acid-sensitive ion channels that may contribute to pain perception (ASICs) (Waldmann and Lazdunski, 1998), snail FMRF-amide gated channel FaNaC (Lingueglia et al., 1995), *Drosophila* ripped

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pocket and pickpocket (Adams et al., 1998a; Darboux et al., 1998a,b) (the latter of which has been implicated in mechanosensation), and *C. elegans flr-1* (Take-Uchi et al., 1998). All DEG/ENaCs have two transmembrane domains and a single large extracellular region (see Fig. 1*A* for MEC-4 transmembrane topology). Highly conserved regions include short amino acid stretches both before and after the first membrane-spanning domain (MSDI), an extracellular Cys-rich domain (CRD) corresponding to MEC-4 CRDIII, a short region before predicted transmembrane domain II (MSDII) [which may be functionally analogous to the H5 loop of Shaker-type K^+ channels, although no primary sequence homology is apparent (Jan and Jan, 1994; Schild et al., 1997)], and the amphipathic MSDII. Conserved residues before and within MSDII contribute to the channel pore (Hong and Driscoll, 1994; Waldmann et al., 1995; Schild et al., 1997; Adams et al., 1998a,b; Kellenberger et al., 1999a,b; Snyder et al., 1999). Included in the MSDII region is a key residue that influences channel activity; large-side chain amino acid substitutions for a conserved small residue situated close to MSDII cause channel hyperactivation (MEC-4 position 713) (Driscoll and Chalfie, 1991; Hong and Driscoll, 1994; Huang and Chalfie, 1994; García-Añoveros et al., 1995, 1998; Waldmann et al., 1996; Adams et al., 1998a,b; Champigny et al., 1998). In *C. elegans,* this genetically induced channel hyperactivation can induce necrotic-like cell death (Chalfie and Wolinsky, 1990; Driscoll and Chalfie, 1991; Hall et al., 1997).

Deciphering structure–activity relationships in mechanically gated channels is essential for elaborating molecular mechanisms of mechanotransduction. However, functional analysis of specialized mechanosensitive channels is far from straightforward. Study in heterologous expression systems is complicated by the fact that tension-conferring contacts of accessory proteins and channel subunits are expected to be required for gating. Another challenge is that prediction of target residues for site-directed mutagenesis is difficult because of the paucity of information on the structure and function of mechanically gated channels. Here we report results of a genetic approach to structure–function studies based on the large-scale analysis of *mec-4* mutations shown to confer behavioral consequences *in vivo*. We further probe domains that exhibited particularly high or low distributions of channel-disrupting substitutions among EMS-induced *mec-4* mutants by testing for effects of site-directed mutations on normal channel function, aberrant toxicity, and complex assembly. Our data significantly extend the understanding of structure–activity relations for the MEC-4 mechanotransducing channel. Because many substitutions we analyzed affect residues conserved within the DEG/ENaC superfamily, our findings hold important implications for DEG/ENaC superfamily regulation and function.

MATERIALS AND METHODS

Strains and genetic analysis. C. elegans strains were grown at 20°C and maintained as described by Brenner (1974). *mec-4* alleles used in this work were described by Chalfie and Sulston (1981) and Chalfie and Au (1989). The dominant death-inducing *mec-4(d)* allele encoding the A713V substitution was *u231*.

Molecular methods. For direct PCR product sequencing, *mec-4* alleles of genomic DNA of 1 γ-ray-induced and 49 EMS-induced *mec-4(r)* mutants were isolated as described in Emmons et al. (1979). Four sets of primers (sense and antisense) were used to amplify genomic DNAs from nucleotide 420 to 4877 that cover all the coding regions of MEC-4. The following sequencing primers were used: for exon 1, antisense $^{5'}$GGGAACAAAATACAATTGCATAC$^{3'}$; for exon 2, sense $^{5'}$AAGTCGCAGC TGAGTAATCTAAC ATTT$^{3'}$; for exon 3, sense $^{5'}$AATCATGTGCTCCTTACTGAGCTT^{3} and sense $^{5'}$CGAAGTTGTCACC GAACCACCCACCA$^{3'}$; for exon 4, sense $^{5'}$TAATATTAATGGTGAGTGTTGCAT$^{3'}$; for exon 5, sense $^{5'}$CAGAAGTTCATATGAGACGTTT$^{3'}$; for exon 6, sense $^{5'}$CTAGCTTCACTTGTTTGATTTTAC$^{3'}$; for exon 7, sense $^{5'}$ATTTCAGGTAACAA-TCACAAATA$^{3'}$; for exon 8, sense $^{5'}$CGTGTGATATCGAAGCGTTA G$^{3'}$; for exon 9, sense $^{5'}$TGAAGTCCGTTATGTATAAACC$^{3'}$; for exon 10, sense $^{5'}$CATTTGGATTACGA TTCGTATTA$^{3'}$; for exon 11, sense $^{5'}$GTTAAAATAGCAATTAAATATAGAACTTA$^{3'}$; for exon 12, sense $^{5'}$ATTGCGATGCAGCAGACCCTATTG$^{3'}$; for exon 13, antisense $^{5'}$GTTCTCTCAAATAGGCCCA$^{3'}$; for exon 14, antisense $^{5'}$CGCTAGTAGTAATTCGGCATTT$^{3'}$; and for exon 15, antisense $^{5'}$TTATTTTAAGA CACAACATTGCAAT$^{3'}$. Oligonucleotides were purchased from Keystone Laboratories and Genosys Biotechnologies. DNA-sequencing reactions were performed according to the manufacturers' specifications (United States Biochemicals, Cleveland, OH, or Life Technologies, Gaithersburg, MD) (Hong and Driscoll, 1994). All mutations were confirmed on both strands.

Site-directed mutagenesis (Kunkel, 1985) was performed on plasmid pTU#12 [a rescuing 6.1 kb genomic *mec-4(+)* clone that contains ~1.9 kb upstream of the predicted *mec-4* initiation codon including the final coding exons of the gene 5′ to *mec-4*, all introns, and ~0.5 kb of 3′-flanking sequence] or pTU#14, the equivalent *mec-4* clone with the A713V substitution (Driscoll and Chalfie, 1991; Lai et al., 1996), using the Muta-Gene phagemid *in vitro* mutagenesis kit protocol (Bio-Rad, Hercules, CA). All mutations were confirmed by sequence analysis. *mec-4* expression from these plasmids appears specific to the touch sensory neurons because (1) *lacZ* and green fluorescent protein reporter fusions to the intact *mec-4* gene in pTU#12 consistently label only the six touch sensory neurons (Mitani et al., 1993) (M. Driscoll, unpublished observations), (2) anti-MEC-4 antibody staining of transgenic lines harboring pTU#12 visualizes only the six touch sensory neurons, and (3) pTU#14 induces the exclusive degeneration of the touch receptor neurons.

Generation and scoring of transgenic animals. Germ line transformation was performed as described (Driscoll, 1995; Mello and Fire, 1995). Plasmid DNAs (50 μg/ml) were coinjected with cotransformation marker DNA (50 μg/ml) for all the samples tested. *mec-4* alleles encoding single amino acid substitutions or deletions were introduced into wild-type N2 or recessive *mec-4(u253)* animals. *mec-4(u253)* has a partial deletion of *mec-4*-coding sequences (see Table 1) and is a likely functional null allele. Plasmid pRF4, which carries the dominant marker *rol-6(su1006)* (Kramer et al., 1990), was coinjected with *mec-4* alleles to facilitate the identification of roller transformants. *rol-6* is expressed in the hypodermal lineage, which does not segregate early from the neuronal lineages that produce the touch cells, but a small number of transgenic Rol animals are expected to lack the transgene array in the touch neurons. To ensure that transforming DNA (which exists as an extrachromosomal array that can be lost during cell division) would be present in as many cells as possible, we scored only rollers from lines in which transforming DNA was passed on to a minimum of 30%, but on average >50%, of the progeny for touch sensitivity as described (Chalfie and Sulston, 1981). For each *mec-4* allele tested, at least 100 rollers from three independent transgenic lines were assayed for touch sensitivity; the percent scores listed are the average values for these three lines. For cell death assays, transgenic lines were examined during the first larval stage (L1) and the fourth larval stage (L4) for swollen degenerating PLM neurons in the tail. Observations were performed at 400× magnification using Nomarski differential interference contrast optics. Animals that had at least one swollen PLM cell were scored as positive. Death in the L1 stage was scored by examining a population of ~200 L1 animals from each of the three transgenic lines derived for each construct. In the L1 stage, Rol animals harboring introduced DNA cannot be distinguished from those that have lost transforming DNA, so only a fraction of the L1 population is expected to exhibit degenerations. Degeneration in the L4 stage was scored by examining 50 Rol animals from each of the three lines. Scores listed are the average percent degeneration from three independent lines. N2 animals do not exhibit degenerations in L1 or L4 animals ($n > 200$); in *mec-4(u231)*, which has two genomic copies of the allele encoding the A713V substitution, 99% of the animals exhibit degenerations in the L1 stage, but no animals exhibit degenerations in the L4 stage because the dead cells are eliminated ($n > 200$).

Whole-mount histochemistry. Transgenic animals were stained using the biotin–avidin system (Molecular Probes, Eugene, OR) with antibody AbM4(1–69), which recognizes the MEC-4 N terminal (N-terminal mutations and CRD region deletions), or AbM4(746–760), which recog-

nizes the C terminal (all single amino acid substitutions), as described (Driscoll, 1995; Lai et al., 1996). Secondary biotinylated FITC-conjugated anti-rabbit antibodies were added at a 1:200 dilution and incubated 4 hr at room temperature. After washing 2 hr at room temperature, fluorescein–avidin D antibodies (1:500 dilution) were added, and animals were mounted in fluorescein–avidin D buffer for observation. Note that low endogenous levels of MEC-4(+) protein are not detectable (Lai et al., 1996). A positive score for staining indicates readily detectable immunoreactivity of touch neurons over background (see Fig. 2); minus indicates no apparent staining over background.

RESULTS

EMS-induced mutations highlight four regions that are critical for *mec-4* function *in vivo*

Fifty-three independently isolated recessive *mec-4* alleles *(r)*, most of which act genetically as loss-of-function alleles, have been isolated in screens for touch-insensitive *C. elegans* mutants (Chalfie and Sulston, 1981; Chalfie and Au, 1989). To identify amino acids essential for MEC-4 function *in vivo*, we sequenced coding regions of 50 of these *mec-4* alleles, 49 generated by EMS mutagenesis and 1 generated by γ-ray mutagenesis.

We identified single nucleotide changes within the coding sequence of 32 *mec-4(r)* alleles (Table 1). Of these, 22 are missense mutations that specify single amino acid substitutions, 7 are nonsense mutations, and 3 disrupt splice junction consensus sites. Three *mec-4(r)* alleles (*u45, u51,* and *u56)* encode two independent nucleotide substitutions that affect amino acids encoded by different exons. Two EMS-induced alleles harbor identical 4 bp insertions 11 nucleotides in front of the predicted *mec-4* initiation codon, which introduce a potential initiation codon out of frame with the *mec-4*-coding sequences (*u229* and *u308*), and four alleles harbor rearrangements that affect coding regions (*e1497, u85, u253,* and *u423*). We did not detect nucleotide changes within the *mec-4*-coding sequence in nine alleles. These alleles are likely to harbor mutations outside of the sequenced coding region, e.g., upstream of position −30, within large introns or within the 3′-untranslated region.

The distribution of EMS-induced mutations relative to the *mec-4*-coding sequence is depicted in Figure 1*B*. Nonsense mutations are generally dispersed along the length of the coding sequence. By contrast, there are four regions where single amino acid substitutions that disrupt MEC-4 function (specified by missense mutations) are clustered: (1) a short stretch of conserved intracellular amino acids that precede the first membrane-spanning domain (designated In91–95 for intracellular residues 91–95), (2) a small region that precedes the third Cys-rich domain in the extracellular domain (Ex533–542), (3) a nearby short stretch within the third Cys-rich domain (ExCRDIII595–601), and (4) a region including the second membrane-spanning domain (MSDII713–739). EMS, which causes C to T and G to A transitions (Coulondre and Miller, 1977), has the potential to alter 548 of the 768 MEC-4 amino acids (see the more detailed description of susceptible codons at http://touchworms.rutgers.edu/posted). Because mutations are selected in our screen only when they exert a functional consequence on behavior, the clustering of channel-disrupting substitutions suggests that these regions are particularly critical for MEC-4 function.

Interestingly, many affected amino acids are highly conserved among members of the DEG/ENaC superfamily, consistent with important roles in the function of this channel class. We therefore performed further tests to deduce more about the activity of the highlighted residues and domains.

Extended study of domains in which channel-inactivating substitutions cluster or are absent: strategies for *in vivo* analysis of site-directed mutations

Routine electrophysiological analysis from small *C. elegans* neurons is not yet technically feasible. Moreover, isolation of mechanically gated channels from their normal physiological contexts that occurs in heterologous expression systems is unlikely to reconstitute channels with normal gating [see Tavernarakis and Driscoll (1997), their discussion]. An alternative approach toward elaborating MEC-4 structure–function relationships in *C. elegans* that confers the benefit of examining mutational effects in a whole-animal model is to generate transgenic nematodes harboring site-directed mutations and to assay cellular and behavioral consequences *in vivo*. Such an *in vivo* approach enabled us previously to collect data suggesting that residues within MEC-4 MSDII contribute to a channel pore-lining domain (Hong and Driscoll, 1994), a hypothesis later supported by electrophysiological analysis of related mutant channels (Waldmann et al., 1995, 1996; Adams et al., 1998a; Champigny et al., 1998; García-Añoveros et al., 1998).

We have experimental tools to address four basic questions concerning engineered MEC-4 variants. (1) Is the mutant protein produced, (2) does the mutant protein function, (3) does a given substitution affect MEC-4 A713V neurotoxicity, and (4) can the mutant protein participate in channel complex assembly? We test protein production by antibody staining, which indicates whether the protein is made, although this does not offer the resolution to establish that the protein reaches its appropriate subcellular localization. We test mutant protein function by the transgene rescue of a *mec-4* mutation. *mec-4* alleles encoding an engineered substitution are introduced into a *mec-4* deletion mutant background, and alleles that fail to restore touch sensitivity in transgenic animals are scored as nonfunctional. To ask whether specific amino acid residues are essential *in cis* for the activity of the constitutively open A713V channel, we construct *mec-4* alleles that encode both the toxic A713V substitution and a substitution for the residue in question. The doubly mutant *mec-4* allele is assayed for the ability to induce touch cell degeneration *in vivo*. A substitution that disrupts normal channel function but does not affect the toxic phenotype suggests that the amino acid identified may be needed for normal channel opening and closing but is dispensable after the channel is hyperactivated; we consider such residues candidate protein contacts by which mechanical gating force might be administered. Finally, we address the ability of a mutant protein to assemble with some components of the channel complex on the basis of previous observations regarding the wild-type subunit expression in transgenic animals. Introduction of wild-type *mec-4* transgenes into a wild-type background [i.e., *mec-4(+)*] can partially disrupt touch sensitivity, a phenomenon likely to result because excess introduced subunits (which are produced at a few-fold higher level than the endogenous protein because of transgene dosage) sequester other components required for synthesis, assembly, or function of the mature channel (Hong and Driscoll, 1994). This interference phenomenon does not result from competition for transcriptional regulatory factors between endogenous and plasmid *mec-4* genes because plasmids that contain promoter sequences but do not produce any protein (because they encode a termination codon immediately after the *mec-4* initiation codon) do not interfere with wild-type gene function (Hong and Driscoll, 1994) (Fig. 2*B*, *middle plot*). To extend our understanding of mutant engineered *mec-4* alleles that

Table 1. EMS-induced *mec-4(r)* mutations

mec-4(r) allele[a]	Site of mutation[b]	Base change[c]	Amino acid change	Type of mutation	Exon
u167	601	A –T	K72Stop	Nonsense	2
u25	659	C –T	T91I	Missense	2
u339	662	C –T	S92F	Missense	2
e1818	671	G –A	G95E	Missense	2
u335	709	C –T	R108Stop	Nonsense	2
u45(ts)	737	G –A	G117E	Missense	2
u316	1123	G –A	G230E	Missense	3
u175	1304	G –A	W273Stop	Nonsense	4
u19	1661	G –A	A321T	Missense	5
u46	1715	G –A	W338Stop	Nonsense	5
u424	1778	G –A		Splicing	5/6
u51	1983	C –T	P395L	Missense	6
u45(ts)	1988	G –A	E397K	Missense	6
u126	2070	G –A		Splicing	6/7
u219	2105	C –T	A420V	Missense	7
u89	2179	G –A	E445K	Missense	7
u51	2452	G –A		Splicing	8/9
u342	2578	G –A	G533S	Missense	9
u242(ts)	2597	G –A	G539E	Missense	9
u209	2606	C –T	S542F	Missense	9
e1601	2939	G –A	C595Y	Missense	11
u273	2939	G –A	C595Y	Missense	11
u128	2939	G –A	C595Y	Missense	11
u340	2939	G –A	C595Y	Missense	11
u221	2948	G –A	G598E	Missense	11
u315	2950	G –A	D599N	Missense	11
u56[d]	2956	C –T	R601C	Missense	11
u140	3054	G –A		Splicing	11/12
u72	3246	C –T	Q664Stop	Nonsense	14
u441[e]	3246	C –T	Q664Stop	Nonsense	14
u56	3581	C –T	A713D	Missense	15
u2	3590	G –A	G716D	Missense	15
u260	3608	G –A	W722Stop	Nonsense	15
e1789[f]	3620	C –T	S726F	Missense	15
u35	3620	C –T	S726F	Missense	15
u246	3629	C –T	T729I	Missense	15
u238	3629	C –T	T729I	Missense	15
u29	3637	G –A	E732K	Missense	15
Rearrangements					
u229, u308	insertion of 4 nucleotides (GCTT) at position 6, 11 bases before initiation codon which creates an out-of-frame initiation codon				
u85	deletion of 7 nucleotides at starting position 1157 creating frame shift in exon 3				
u253, u423	deletion in exon 3				
e1497	deletion and insertion in exon 13 which creates a termination codon				
Alleles that do not include coding region mutations:					
u32, u230, u330, u331, u207, u133, u183, u302, e1851					

[a] In a touch assay, all alleles except ts u45 and u242 confer the same touch-insensitive phenotype.

[b] Nucleotide and protein sequences are numbered as in Lai et al. (1996).

[c] All but one point mutation were GC to AT transitions as expected for EMS mutagenesis (Coulondre and Miller, 1977).

[d] u56 encodes substitution A713V, which by itself should induce neurodegeneration (Driscoll and Chalfie, 1991). However, u56 also encodes substitution R601C, which blocks toxicity.

[e] Allele u441 was generated by γ ray mutagenesis.

[f] Mutations encoded by alleles e1789, u35, u246, u238, and u29 were reported in Hong and Driscoll (1994).

are nonfunctional in complementation assays, we introduce them into a *mec-4(+)* background. If the nonfunctional channel protein partially disrupts touch sensitivity when introduced into a wild-type background, we infer that channel activity is disrupted in one or both of two ways: (1) mutant subunits may form mature but defective channel complexes, or (2) as is true for wild-type subunits, mutant subunits may associate with and sequester other essential proteins of the channel complex, disrupting the overall

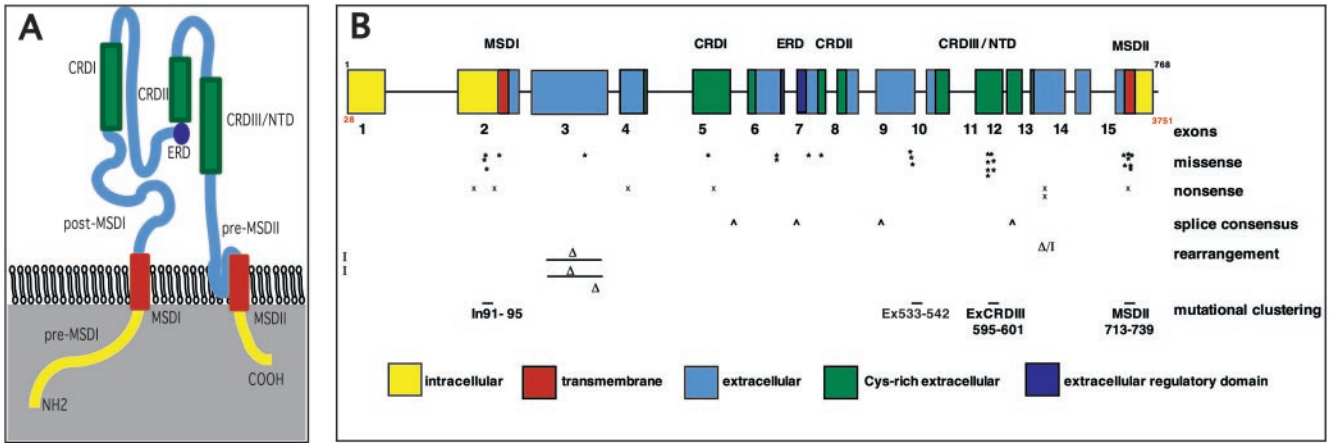

Figure 1. Channel-inactivating substitutions cluster to four regions within MEC-4. *A*, Transmembrane topology and relative positioning of MEC-4 domains. N and C terminals are intracellular; CRDs are extracellular. Domains are not drawn to scale. *B*, Distribution of identified *mec-4(r)* mutations relative to the *mec-4*-coding sequence. Exons 1–15 are represented by *boxes* filled to indicate domain identity and transmembrane position. Amino acids (*top left* and *right* of the *row* of exon *boxes*) and nucleotides (*bottom left* and *right* of the *row* of exon *boxes*; *red*) are numbered as in Lai et al. (1996). *Symbols* indicate the following: *, position of individual missense mutation; ×, nonsense; ^, splice site; *I*, insertion; and Δ, deletion mutation. Four "hot spots" where substitutions of MEC-4-inactivating substitutions cluster are indicated: In91–95, intracellular amino acids 91–95; Ex533–542, extracellular amino acids 533–542; ExCRDIII595–601, extracellular residues 595–601 within the Cys-rich domain III; and MSDII713–739, membrane-spanning domain II residues 713–739. *ERD*, extracellular regulatory domain; *NTD*, neurotoxin-related domain.

assembly of functional channels. In either case, we can infer that the mutant MEC-4 protein is produced and participates in some aspect of the multistep process of channel complex assembly.

Probing structure and activity of the intracellular N-terminal MEC-4 domain

Transgenic mutant strains exhibit behavioral defects identical to those in EMS-induced genomic mutants

The In91–95 genetic "hot spot" for N-terminal MEC-4 channel-inactivating substitutions falls within a short region near MSDI that is highly conserved among DEG/ENaC superfamily members (corresponding to MEC-4 amino acids 87–95; Fig. 2*A*). To investigate further the functional importance of residues within In91–95, we constructed *mec-4* point mutations and deletions and assayed mutant alleles for function *in vivo*.

We first tested the validity of *in vivo* transgenic channel assays by reconstructing mutant *mec-4* alleles encoding the T91I, S92F, and G95E substitutions known to disrupt MEC-4 function when encoded by genomic mutations (refer to Table 1). The analogous engineered substitution constructs were tested for function in transgenic animals by assaying for complementation of the *mec-4*(*u253*) deletion allele, hereafter referred to as *mec-4(−)*. Mutant T91I, S92F, and G95E *mec-4* constructs fail to rescue the touch insensitivity defect in the *mec-4(−)* background (Fig. 2*B*, *top plot*). We confirmed that mutant proteins are expressed *in vivo* by demonstrating that constructs encoding *mec-4*(T91I), *mec-4*(S92F), and *mec-4*(G95E) produce proteins detectable by antibody staining that interfere with mechanosensation when introduced into a wild-type background (Fig. 2*B*, *middle plot*). The observed transgene interference also indicates that at least some aspects of channel assembly can occur when the In91–95 region is disrupted. Note that not all substitutions in the conserved region disrupt MEC-4 function; for example, the Y89R mutant behaves as does the wild type in all assays.

Substitutions T91I, S92F, and G95E block channel hyperactivation caused by the A713V substitution

In the well studied Shaker-type K^+ channel, a cytoplasmic inhibitory domain associates with the channel pore to inhibit ion transport (Hoshi et al., 1990; Zagotta et al., 1990). Because a common ancestor has been suggested for DEG/ENaCs and Shaker-type K^+ channels, we considered the possibility that the highly conserved domain in DEG/ENaCs, which includes the In91–95 region, has a similar role in channel regulation. We reasoned that if the In91–95 region were required for negative regulation of channel activity, substitutions that disrupt this region of MEC-4 might confer the channel-hyperactivated phenotype of degeneration. However, examination of the T91I, S92F, and G95E mutants throughout their life span and into senescence failed to reveal any signs of neurodegeneration (data not shown). In a second test for channel-activating effects of these substitutions, we asked how alterations within the In91–95 domain affect channel activity when situated *in cis* to the channel-hyperactivating substitution A713V. We reasoned that if the In91–95 region were solely required for negative regulation of the channel as in the ball-and-chain model of the K^+ channel, disruption of this inhibitory domain should increase channel activity (or leave it unaffected) and thus would not block the toxicity of the hyperactivated A713V substitution. We generated double-mutant alleles that harbor the disrupting substitutions T91I, S92F, or G95E or the deletion Δ89–92 *in cis* to the toxic A713V substitution, introduced the double-mutant alleles into the *mec-4(−)* background, and tested transgenic lines for cell death (Fig. 2*B*, *bottom plot*). We find that the N-terminal substitutions T91I, S92F, and G95E and Δ89–92 fully block channel toxicity when situated *in cis* to the A713V substitution. Taken together, our data argue against an exclusive negative regulatory role for the conserved In91–95 domain.

Overexpression of the N-terminal domain interferes with endogenous channel function via a mechanism that requires residues S91, T92, and G95

To learn more about the function of the conserved In91–95 region, we sought to distinguish between (1) a potential role in protein interactions required for channel assembly or function and (2) an exclusive role for the In91–95 domain in channel activation. We reasoned that if the conserved In91–95 region

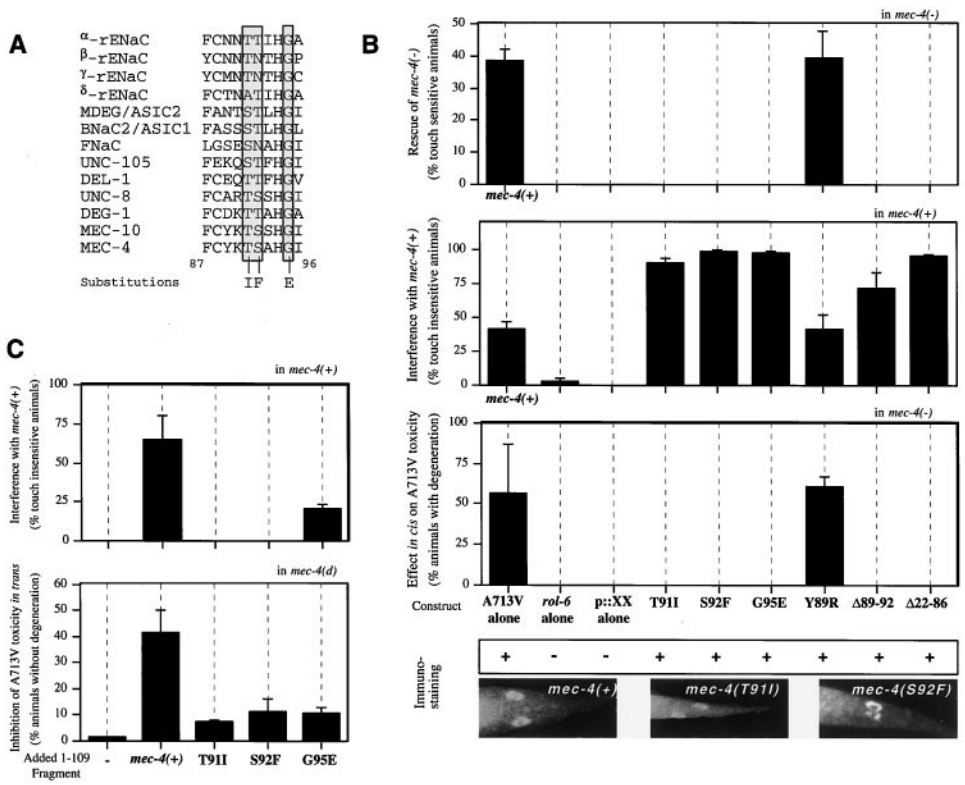

Figure 2. The MEC-4 N terminal: channel-inactivating substitutions cluster to a conserved region implicated in human ENaC disease. *A*, N-terminal substitutions that inactivate MEC-4 affect conserved residues. MEC-4-inactivating amino acid substitutions in the N terminal cluster to a region conserved in all DEG/ENaC superfamily members. *Gray boxes* highlight corresponding residues in the family members listed; *numbering* indicates the MEC-4 amino acid position. MEC-4 G95 corresponds to human βENaC G37, which when substituted causes human pseudohypoaldosteronism type I, a loss-of function disorder (Chang et al., 1996). *B*, *In vivo* assays of mutant *mec-4* transgenes are shown. *Top Plot*, Transgenes were introduced into the *mec-4(−)* mutant (deletion allele *u253*) to indicate rescue as shown by the percent of touch-sensitive animals. *Middle Plot*, Transgenes were introduced into a wild-type *mec-4(+)* background to test for the degree of interference (scored as the percent of touch-insensitive animals). *Bottom Plot*, Effects of double substitutions of the amino acid (AA) change indicated situated *in cis* to the A713V channel-activating substitution as assayed in the *mec-4(−)* background are shown. The score is the percent of animals that harbor at least one degenerating tail touch neuron as assayed at the L1 stage; results were identical in examination at the L4 larval stage. Genotypes of transformation host strains are indicated in the *top right corner* of *each plot*. Control constructs and specific amino acid substitutions tested are indicated *below* the *bottom plot*. *rol-6(su1006)* is a cotransformation marker that causes animals to roll; this marker was included in all transgenic lines to facilitate identification of transformed animals. *p:: XX* harbors the *mec-4* promoter and the coding region with two stop codons inserted after the *mec-4* initiation codon. For all *panels*, scores for transgenic lines were the average of at least 100 animals scored for three independently derived lines. Error bars indicate SD. *Bottom Images*, The immunoreactivity in transgenic animals that harbor the introduced gene in the *mec-4(−)* background is shown. Antibody staining of PLM tail cells is shown for *mec-4(−)* mutants that harbor transgenes encoding the indicated MEC-4 substitutions. The antibody used was polyclonal AbM4(746–760), which recognizes the C terminal. Touch cell positions appear different because they are photographed from different angles; scales also differ slightly. *C*, Effects of overexpression of wild-type and mutant MEC-4 N-terminal fragments (AA1–109) are shown. *Top*, The ability of N-terminal fragments to inhibit endogenous MEC-4 channel function was scored as the percent of touch-insensitive animals; transgenic lines were constructed in the *mec-4(+)* background. *Bottom,* The ability of N-terminal fragments to disrupt the toxicity of endogenous MEC-4 A713V channels was scored as the percent of animals lacking degeneration; transgenic lines were constructed in the dominant *mec-4(u231)* background in which PLM touch cells degenerate in >90% of animals.

mediates a critical association with other channel subunits or other components of the mechanotransducing complex, overexpression of this domain might disrupt endogenous channel activity by competing with wild-type subunits for functional contacts. Alternatively, if the In91–95 domain is needed for opening but does not require any specific protein interaction to do so, overexpression of this domain is not expected to interfere with endogenous channel function. We overexpressed wild type and mutant variants of the MEC-4 N-terminal 1–109 amino acid fragment to begin to distinguish between these two possibilities. Unlike overexpression of the complete MEC-4 subunit, these experiments focus on the ability of the N terminal to form a functional domain that can interact with the expression machinery or components of the functional complex.

We first created transgenic lines expressing MEC-4(1–109) and assayed for interference with endogenous MEC-4 channel activity. We find that expression of the wild-type N-terminal fragment causes strong interference with normal MEC-4 channel function in the wild-type background; transgenic lines expressing this fragment exhibit defective touch sensitivity (Fig. 2*C*, *top*). Moreover, expression of the MEC-4(1–109) N-terminal fragment in the *mec-4(d)* background (encoding the hyperactivating A713V substitution) causes a significant reduction in the number of degenerating touch neurons (Fig. 2*C*, *bottom*). Taken together, our data indicate that expression of the N-terminal domain interferes *in trans* with the assembly or function of the MEC-4 channel.

We then tested whether the amino acid residues identified as essential for *in vivo* MEC-4 function are important for N-terminal 1–109 fragment-mediated channel interference by expressing N-terminal fragments containing either the T91I, the S92F, or the G95E substitutions (Fig. 2*C*). We find that both interference with normal channel function and interference with *mec-4(d)*-induced degeneration are eliminated by the T91I and the S92F substitutions. The G95E substitution also significantly disrupts interference *in trans*, although to a slightly lesser degree. We conclude that residues T91, S92, and G95 are essential both for normal channel function and for the inhibitory interaction mediated by the transgenically expressed N-terminal 1–109 domain.

Additional N-terminal residues, not highlighted by EMS-induced substitutions, are required for MEC-4 function

The identified clustering of MEC-4-inactivating amino acid substitutions to In91–95 suggested that the rest of the N terminal, which is not conserved among DEG/ENaC family members,

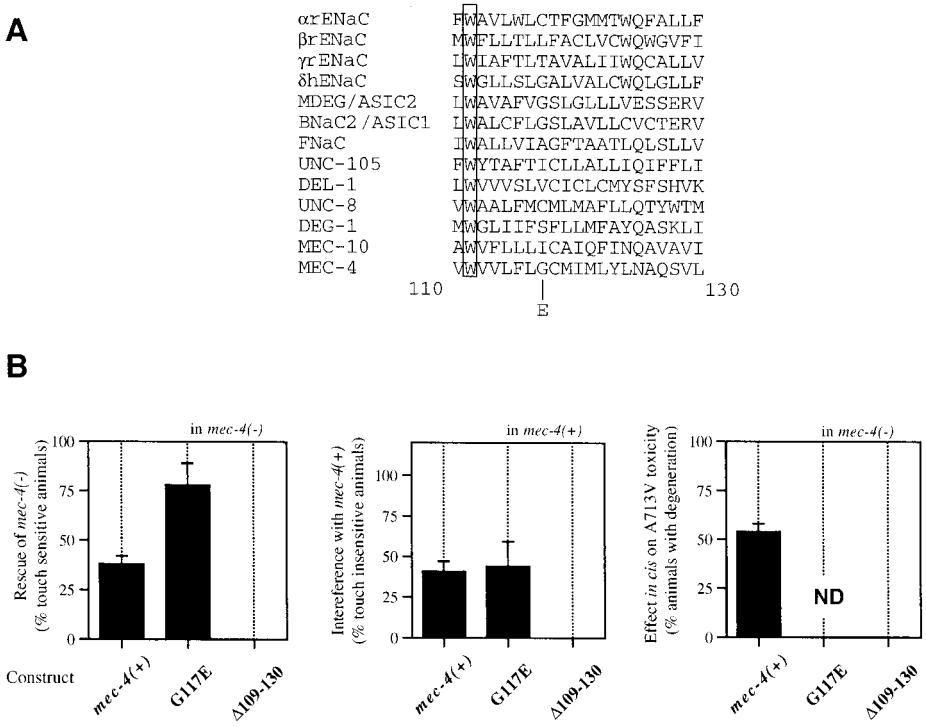

Figure 3. MEC-4 MSDI: single amino acid substitutions that disrupt channel function are conspicuously absent, although the domain is required for channel assembly. *A*, Sequence alignment of predicted MSDI regions from representative DEG/ENaC family members is shown. Note that apart from an invariant Trp residue highlighted by a *gray box*, the domain is not highly conserved in primary sequences. The position of the G117E substitution is indicated. *Numbers* indicate positions of MEC-4 amino acids. *B*, *In vivo* assays of mutant *mec-4* transgenes are shown. *Left*, Transgenes were introduced into the *mec-4(−)* mutant (deletion allele *u253*) to indicate rescue as scored by the percent of touch-sensitive animals. *Middle*, Transgenes were introduced into a wild-type *mec-4(+)* background to test for the degree of interference (scored as the percent of touch-insensitive animals). *Right,* The effects of double substitutions of the AA change indicated situated *in cis* to the A713V channel-activating substitution as assayed in the *mec-4(−)* background are shown. The score is the percent of animals that harbor at least one degenerating tail touch neuron. Control constructs and specific amino acid substitutions tested are indicated *below each panel*. *ND*, Not determined.

might be dispensable for MEC-4 function. To determine whether this might be the case, we assayed a deletion mutation that affected most of the nonconserved portion of the N-terminal region (Δ22–86; Fig. 2*B*). Although *in vivo* interference assays and immunocytochemistry indicate that mutant MEC-4 protein is produced and is able to initiate channel assembly, *mec-4*(Δ22–86) fails to complement the *mec-4(−)* mutation. Thus, sequences outside the highly conserved N-terminal domain are required for MEC-4 function. Nonconserved regions of ENaCs have also been shown to be important for channel function (Chalfant et al., 1999).

Extending models for the function of the conserved In91–95 region in DEG/ENaC channels

Our data hold implications for models of MEC-4 function that are of particular interest in the context of recent work on mammalian DEG/ENaC family members. Corresponding residues in ENaC subunits modulate channel-gating kinetics (Gründer et al., 1997, 1999). Residues in the conserved region of the ASIC2 (also named BNC1 and MDEG) N-terminal domain have been shown to influence ion selectivity, indicating that the conserved domain may loop back into the membrane to contribute to the channel pore (Coscoy et al., 1999). The finding that MEC-4 substitutions T91I, S92F, and G95E disrupt both normal channel function and channel hyperactivation is consistent with contributions of residues within the In91–95 region to pore function and/or formation; we showed previously that residues in the MSDII pore are essential for toxicity of the hyperactivating A713V substitution (Hong and Driscoll, 1994). However, inhibitory effects of the expressed N-terminal domain suggest that if this is the case, either (1) the domain can insinuate itself into the pore without being physically tethered to the rest of the channel, or (2) the conserved In91–95 subdomain serves additional functions.

The fact that overexpression of the MEC-4 N-terminal 109 amino acids interferes with channel activity is also consistent with a model in which the fragment associates with endogenous intact channel subunits or with other proteins in the channel complex; introduction of a domain that competes for required interacting proteins should disrupt overall channel activity, possibly by sequestering essential proteins or by initiating the assembly of aberrant complexes that are targeted for degradation. In this regard, it is interesting that the N-terminal fragment of γENaC interacts with αENaC and interferes with ENaC activity in the oocyte expression system by reducing ENaC protein levels (Adams et al., 1997). Our studies expand observations on N-terminal inhibition by DEG/ENaC family members by demonstrating that inhibition can occur *in vivo* and by defining three conserved amino acid residues critical for N-terminal fragment-mediated interference. MEC-4 residues T91, S92, and G95 may normally promote intersubunit association during channel assembly. Alternatively, these residues might be critical for fragment stability or the folding of an interaction domain elsewhere on the N-terminal fragment.

Although EMS-induced mutations do not cluster to membrane-spanning domain I (MSDI), the domain is critical for channel function and assembly

The first transmembrane domain of DEG/ENaC superfamily members includes a few highly conserved amino acids (Fig. 3*A*). Substitution of MEC-4 MSDI for ASIC2 MSDI has a modest effect on channel conductance [by contrast, a similar swap of MSDII causes dramatic changes in channel properties (Waldmann et al., 1995)], but little is understood of MSDI contributions to channel function. Interestingly, among 50 sequenced *mec-4* alleles, we did not identify any single mutation that disrupted channel activity and mapped to MSDI, despite the fact that 17 of the 21 codons in this domain have the potential to change amino acid specification consequent to EMS mutagenesis (see http://touchworms.rutgers.edu/posted). Allele *u45* encodes MSDI substitution G117E as well as a second substitution, E397K (see Table 1), but the weak ts phenotype of this allele is not conferred by the G117E substitution alone because an engineered *mec-4*

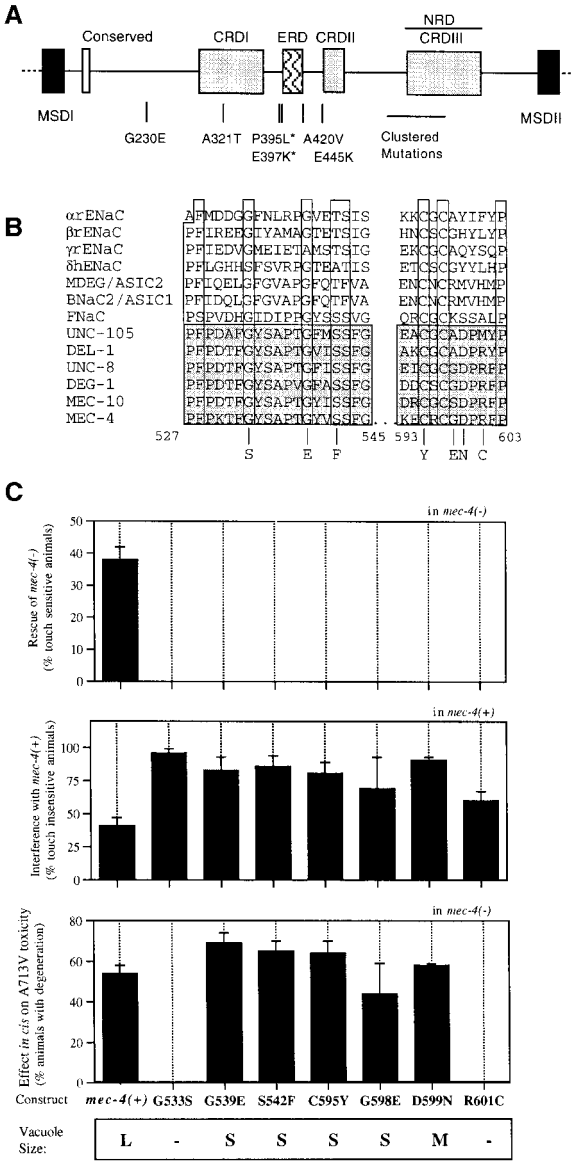

Figure 4. The MEC-4 extracellular region: substitutions identify residues that may be critical for mechanotransduction; some substitutions disrupt mechanosensation without preventing neurotoxicity. *A*, Schematic drawing of MEC-4 extracellular domains and relative positions of amino acid substitutions disrupting channel activity is shown. *Black boxes* indicate membrane-spanning domains, *gray boxes* indicate Cys-rich domains, the *hatched box* is an extracellular regulatory domain, and the *NRD-labeled box* is an neurotoxin-related domain. Domains are not drawn exactly to scale. *Vertical lines* indicate sites of nonconserved amino acid changes that disrupt MEC-4 function outside the CRDIII region; the *horizontal line* indicates the region where changes are clustered. *B*, Sequence alignment of amino acids near and within CRDIII from representative DEG/ENaC family members is shown. *Unfilled boxes* highlight residues highly conserved throughout the superfamily; *gray boxes* highlight degenerin family members that are especially well conserved in these regions. *Numbers* indicate positions of MEC-4 amino acids. *C*, *In vivo* assays of mutant *mec-4* transgenes are shown. *Top*, Transgenes were introduced into the *mec-4(−)* mutant (deletion allele *u253*) to indicate rescue as scored by the percent of touch-sensitive animals. *Middle*, Transgenes were introduced into a wild-type *mec-4(+)* background to test for the degree of interference (scored as the percent of touch-insensitive animals). *Bottom*, The effects of double substitutions of the AA change indicated situated *in cis* to the A713V channel-activating substitution as assayed in the *mec-4(−)* background are shown. The score is the percent of animals that harbor at least one degenerating tail touch neuron. Control constructs and specific amino acid substitutions tested are indicated *below* the *bottom panel*. Also indicated *below* this *panel* are scores for the size of degenerating neurons: *L*, large, neurons similar to those occurring in the *mec-4(u231)* A713V substitution background; *M*, medium, degenerating neurons that swell to approximately one-half the size noted for *mec-4(u231)* and vacuolar degenerations that are visible at 100× magnification; *S,* small vacuoles only apparent at 400× magnification; and −, no detectable degeneration.

allele, encoding only the G117E change, behaves similar to the wild type in all assays (Fig. 3*B*). Although we cannot exclude that the lack of *mec-4* mutations affecting MSDI is caused by chance, the paucity of MSDI mutations suggests that individual residues in this domain may not be critically important to MEC-4 activity. MSDI may serve as a hydrophobic structural domain that directs required transmembrane topology and might stabilize the channel complex by surrounding the pore-lining MSDII in the membrane.

Analysis of a mutant allele in which MSDI is deleted, *mec-4*(Δ109–130), suggests that MSDI functions in directing appropriate transmembrane topology are essential for channel assembly. Transgenic animals bearing the *mec-4*(Δ109–130) allele are touch insensitive, and animals harboring the double substitution Δ109–130,A713V do not exhibit neurodegeneration (Fig. 3*B*, *left*, *right*). However, it is remarkable that MEC-4(Δ109–130) is the only defective channel subunit tested in our large survey that completely fails to interfere with endogenous channel function when introduced into the wild-type *mec-4(+)* background (Fig. 3*B*, *middle*). Because antibody staining confirms that the MEC-4(Δ109–130) protein is produced, the inability of the mutant protein to interfere with endogenous channel activity suggests that MEC-4(Δ109–130), which could adopt an inverted transmembrane topology, never encounters other channel subunits and is defective in an early process essential for channel assembly. Note that the N-terminal domain, which itself can interfere with assembly, is intact in the MEC-4(Δ109–130) protein, suggesting that the N terminal participates in an aspect of assembly that is not encountered by the ΔMSDI mutant.

Channel-inactivating substitutions in the MEC-4 extracellular domain cluster around CRDIII; some substitutions uncouple channel function and neurotoxicity

The MEC-4 extracellular region includes a domain close to MSDI that is strikingly conserved among all superfamily members (FPAITLCNLNPYK, MEC-4 AA151–163), three Cys-rich domains (with CRDI unique to degenerins), and a small degenerin-specific domain that influences channel gating [extracellular regulatory domain (ERD) (García-Añoveros et al., 1995)]. Interestingly, CRDIII includes a region of low but significant sequence similarity to scorpion venom neurotoxins (NTD AA572–646) that is conserved in DEG/ENaC family members (Tavernarakis and Driscoll, 2000). Venom neurotoxins are known to associate with voltage-gated Na^+ channel domains at high affinity (Catterall, 1980; Rogers et al., 1996; Cestèle et al., 1998), and thus related structures are candidate interaction domains.

EMS-induced single amino acid substitutions in the extracellular domain that disrupt MEC-4 function affect several residues, many of which cluster before and within CRDIII (AA533–542 and AA595–601, respectively) and thus distinguish this region as critical for channel function (Fig. 4*A,B*). Within this region, channel-inactivating substitutions alter residues that are highly

conserved either among the DEG/ENaC superfamily or among the degenerin subfamily. By constructing transgenic lines, we confirmed that the mutant alleles identified (encoding G533S, G539E, S542F, C595Y, G598E, D599N, and R601C) produce protein but fail to complement the *mec-4(−)* mutant (Fig. 4*C*, *top*, *middle*). Although all these substitutions fully abolish touch sensitivity on their own, they have three distinct effects on channel toxicity when situated *in cis* to A713V (Fig. 4*C*, *bottom*). Substitutions G533S and R601C block channel activity, even in the context of the hyperactivating A713V substitution, suggesting these residues are critical to a channel that can assemble and open. In contrast, other substitutions in this region uncouple channel function in touch transduction from the ability to induce neurodegeneration when the channel is hyperactivated by A713V. The D599N,A713V double mutant has a moderate effect such that the average size of a degenerating touch receptor cell body is approximately one-half the size of that in the MEC-4 A713V transgenic animals. Substitutions G539E, S542F, C595Y, and G598E allow degeneration when situated *in cis* to A713V, but the size of detectable degenerating cell bodies is markedly reduced compared with those in animals harboring the A713V substitution alone. Because we noted previously an approximate correlation of the level of toxic channel expression with the degree of swelling (Hall et al., 1997), the smaller cells might reflect an overall decrease in the ion influx into touch neurons (compared with the A713V mutant). If so, conductance in the D599N,A713V channel may be more than that of G539E,A713V; S542F,A713V; C595Y,A713V; and G598E,A713V channels, which may be more than that of G533S,A713V and R601C,A713V channels.

CRDIII substitutions G539E, S542F, C595Y, G598E, and D599N fully disrupt touch sensitivity yet confer intermediate effects on A713V-mediated toxicity. Two explanations, not necessarily mutually exclusive, could be suggested. One possibility is that the channel activity required for touch sensation must be precisely controlled for neuronal signaling, but the ion transport activity required for neurotoxicity is less constrained; accumulated influx over time can kill the neuron, and the rate of influx may vary within limits. Another possibility is that the G539E, S542F, C595Y, G598E, and D599N substitutions alter residues that are partially dispensable when the channel is chronically open. Thus, this region may be involved in appropriate gating, a process that is less important in the constitutively open channel than in the wild-type channel.

It is striking that four independently isolated *mec-4* alleles alter MEC-4 C595, a residue conserved in DEG/ENaC channels and venom neurotoxins. Also of interest is that the corresponding Cys in αrENaC is not critical for ENaC channel function under the conditions of assay (Firsov et al., 1999). In the ENaC channel, this Cys might be needed only in β and γ ENaC subunits, or a small-side chain amino acid at this position may suffice for ENaC function. An alternative possibility is that MEC-4 C595 might have been co-opted to serve a role in mechanical gating that is not relevant to the ENaC channels. In this regard, it is noteworthy that nearby MEC-4 residues highlighted by EMS-induced channel-inactivating mutations (G598, D599, and R601) are highly conserved only in the degenerin subfamily. This hot spot falls within the venom neurotoxin-related domain, which, by analogy, is predicted to mediate high-affinity interactions with specific targets. Also interesting is that channel modulation by endogenous venom toxin-related proteins has been demonstrated recently in mammals (Miwa et al., 1999). The subdomain highlighted by CRDIII mutations might interact with the specialized extracellular matrix proteins needed for appropriate gating [for example, matrix protein MEC-9, which itself includes many venom toxin-related Kunitz domains (Du et al., 1996)].

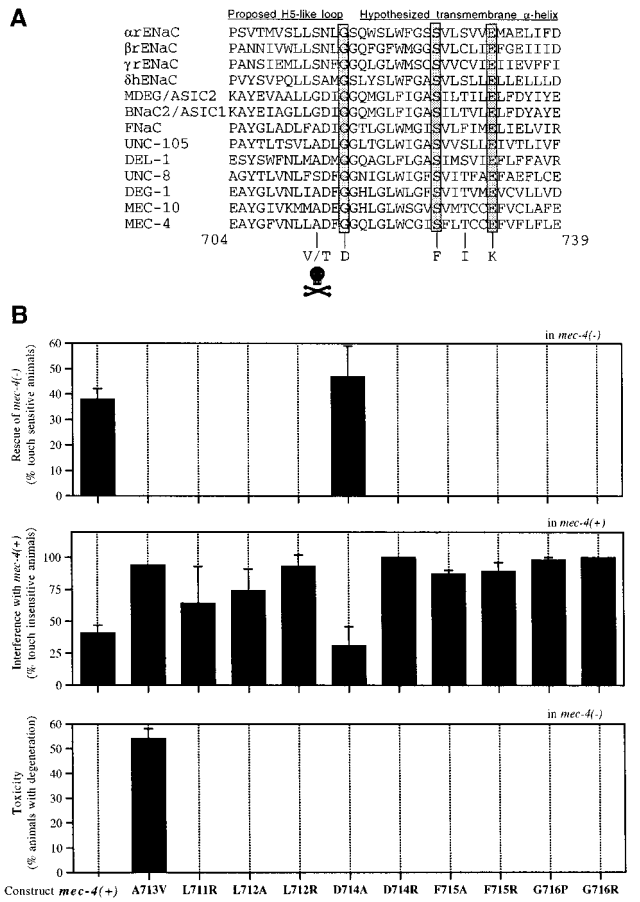

Figure 5. The MEC-4 pore region: primary sequence constraints within the vicinity of the channel pore. *A*, Sequence alignment of amino acids near and within MSDII from representative DEG/ENaC family members is shown. *Gray boxes* highlight strictly conserved residues. *Numbers* indicate positions of MEC-4 amino acids; boundaries of the pre-MSDII loop and the transmembrane α-helix are not known. The *skull* and *crossbones* indicates MEC-4 A713; substitutions of large-side chain amino acids at this position induce neurodegeneration (Driscoll and Chalfie, 1991). Substitutions within predicted MSDII were reported in Hong and Driscoll (1994). *B*, *In vivo* assays of mutant *mec-4* transgenes are shown. *Top*, Transgenes were introduced into the *mec-4(−)* mutant (deletion allele *u253*) to indicate rescue in terms of the percent of touch-sensitive animals. *Middle*, Transgenes were introduced into a wild-type *mec-4(+)* background to test for the degree of interference (scored as the percent of touch-insensitive animals). The A713V value in this panel is from Driscoll and Chalfie (1991). *Bottom*, The effects of double substitutions of the AA change indicated situated *in cis* to the A713V channel-activating substitution as assayed in the *mec-4(−)* background are shown. The score is the percent of animals that harbor at least one degenerating tail touch neuron. Control constructs and specific amino acid substitutions tested are indicated *below* the *bottom panel*.

Additional residues implicated in mechanotransduction are positioned throughout the extracellular domain

Additional substitutions in the extracellular domain highlight certain amino acids as candidate participants in a critical function of mechanically gated channels or the degenerin subfamily (Fig. 4*A*). For example, A321T affects a residue common only to MEC-4 and MEC-10 and could identify a residue essential to the action of the touch-transducing channel. Somewhat unexpectedly,

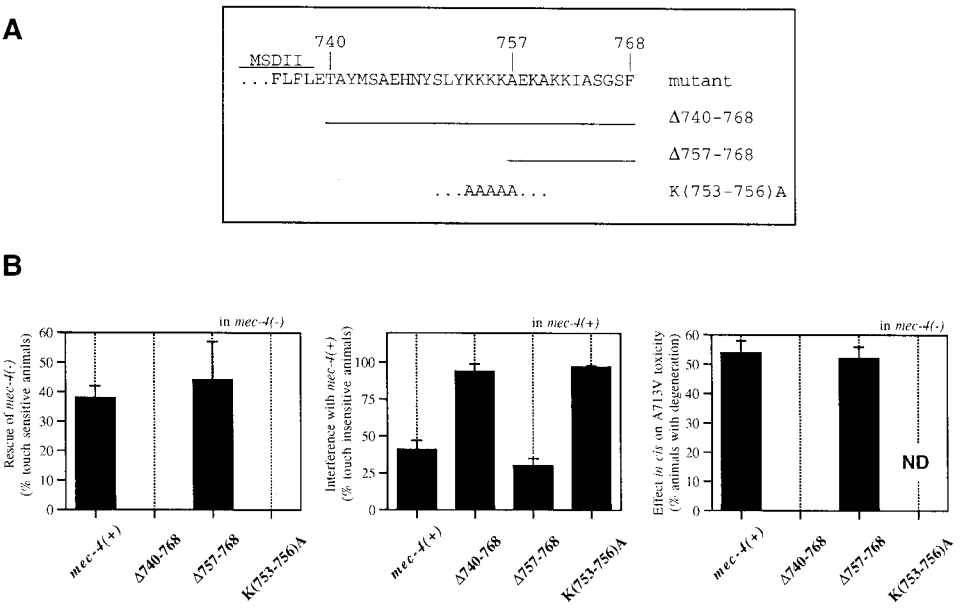

Figure 6. The MEC-4 C terminal: a basic region in the MEC-4 C terminal is essential for channel function. *A*, C-terminal-coding sequence of MEC-4 and deletions and substitutions assayed are shown. *Numbers* indicate MEC-4 amino acid positions. *Black horizontal lines* indicate regions deleted in mutant constructs; in K(753–756)A, Ala residues replace all Lys residues, and the remainder of the C-terminal sequence is intact. *B*, *In vivo* assays of mutant *mec-4* transgenes are shown. *Left*, Transgenes were introduced into the *mec-4(−)* mutant (deletion allele *u253*) to indicate rescue in terms of the percent of touch-sensitive animals. *Middle*, Transgenes were introduced into a wild-type *mec-4(+)* background to test for the degree of interference (scored as the percent of touch-insensitive animals). *Right,* The effects of double substitutions of the AA change indicated situated *in cis* to the A713V channel-activating substitution as assayed in the *mec-4(−)* background are reported. The score is the percent of animals that harbor at least one degenerating tail touch neuron. Control constructs and specific amino acid substitutions tested are indicated *below each panel.*

the single amino acid substitutions affecting nonconserved or degenerin-specific residues (G230, A321, A420, E445, P395, and E397; the latter two are each present in combination with a second substitution and thus may not inactivate the channel on their own) do not cluster to the degenerin-specific CRDI or ERD or to any other domain, suggesting that sites distant in the primary sequence might play most important roles in MEC-4/degenerin channel function. This observation underscores the value of genetic approaches for identification of residues critical for *in vivo* function that are not apparent from sequence alignment.

MSDII and surrounding regions: constraints in and near the channel pore

Genetic and electrophysiological evidence indicates that certain residues within MSDII participate in the conduction pore of DEG/ENaC channels (Hong and Driscoll, 1994; Waldmann et al., 1995; Schild et al., 1997; Adams et al., 1998a,b; Kellenberger et al., 1999a,b; Snyder et al., 1999). The conserved region preceding MSDII is thought to loop back into the membrane to contribute also to the pore, similar to the H5 loop of K^+ channels (Jan and Jan, 1994; Renard et al., 1994) (see Figs. 1*A*, 5*A*), although recent experimental data suggest that the ENaC pore structure may differ in several ways from the K^+ channel (Snyder et al., 1999).

A substitution in the predicted pore loop can interfere with hyperactive channel function in trans

In addition to previously reported EMS-induced amino acid substitutions implicated in the formation of the channel pore [S726, T729, and E739 (Hong and Driscoll, 1994)], we identified one substitution in the pre-MSDII region, G716D, that affects a site highly conserved among superfamily members. This site is of particular interest because substitutions at the corresponding site in degenerins MEC-10 (Huang and Chalfie, 1994) and UNC-8 (Shreffler et al., 1995) can suppress the effects of toxic hyperactivated channels in trans-heterozygotes although these substitutions do not exert dominant effects on wild-type subunits. This property extends to MEC-4; when MEC-4 G716D and MEC-4 A713V are cosynthesized in *mec-4* trans-heterozygotes, touch cell death is diminished (27% cells viable) compared with that when MEC-4(+) and MEC-4 A713V are cosynthesized (10% cells viable). One possible explanation for the apparent restriction to interference with hyperactivated channels is that the structural constraints introduced by the G716D subunit may be relevant only to the aberrant open conformation induced by the A713V residue.

Local substitutions that hyperactivate the channel appear restricted to AA position 713

A well characterized substitution in the MEC-4 pre-MSDII region is the toxic A713V change (Driscoll and Chalfie, 1991; Lai et al., 1996). Initial studies in *C. elegans* indicated that steric hindrance plays a role in the degeneration mechanism because large-side chain amino acid substitutions at this position are toxic, whereas small-side chain amino acids are not (Driscoll and Chalfie, 1991). Elegant studies on the corresponding position in the ASIC2 (also named BNaC1 and BNC1) subunit suggest a model in which the transmembrane helices rotate when the channel is activated, exposing the Ala residue to reagents in the extracellular environment (Adams et al., 1998b). According to the working model, steric constraints provided by large-side chain amino acids at position 713 prevent the rotation back to the inactive conformation, effectively locking the channel open.

A713 is the only residue in the pore region known to induce cell death when substituted. To test whether steric disruption elsewhere in the local region might also lock the channel open and to learn more about structure–function relationships in this area, we systematically introduced the large positively charged residue Arg and, for comparison, the small residue Ala, into positions flanking 713. (Arg substitutions were selected in part because in the genetic screen for *mec* mutations Arg substitutions would not have been generated because of the specificity of the EMS mutagen.) In addition to our standard test for rescue of *mec-4(−)* and interference with *mec-4(+)* (Fig. 5*B*, *top, middle*), we assayed engineered substitutions *in vivo* for the ability to initiate neurodegeneration on their own (Fig. 5*B*, *bottom*). We conclude that most substitutions in this region disrupt normal channel function,

underscoring that resides in this region play a critical role in channel biology. It is noteworthy, however, that substitution D714A, affecting a highly conserved amino acid, does not disrupt channel activity. Introduction of the large-side chain amino acid Arg into positions immediately flanking A713 does not induce degeneration.

The restriction of apparent steric constraints for closing to a single position could reflect the action of A713 as a critical but small flexible swivel joint that allows the conformational change between active and inactive channel states. Alternatively, residues in the conserved pore region flanking position 713 could be essential for channel opening such that substitutions for most residues in this region fully disrupt channel function and thus eliminate the option of defective closing. Our *in vivo* findings are different from a recent study using MTSEA modification of residues in the region of γENaC, which reports channel activation by modification of residues near, and corresponding to, MEC-4 A713 (Snyder et al., 1999). It is not clear whether such differences indicate a fundamental distinction between degenerin and ENaC subunits or reflect differences in the assay in the experimental system, i.e., comparison of engineered amino acid substitutions in a native environment with chemically induced modifications of a functional channel expressed in a heterologous system.

Basic residues in the C-terminal domain are critical for MEC-4 channel function

The intracellular C terminals of mammalian ENaC family members include Pro-rich sequences that mediate channel localization and subunit recycling (Shimkets et al., 1994; Hansson et al., 1995a,b; Schild et al., 1995, 1996; Snyder et al., 1995; Firsov et al., 1996; Goulet et al., 1998; Prince and Welsh, 1999). The intracellular C terminals of *C. elegans* superfamily members do not have Pro-rich regions or other striking sequence similarities to their mammalian counterparts. Moreover, C terminals of *C. elegans* family members are highly divergent. Our sequence analysis of EMS-induced *mec-4* alleles failed to identify any mutations affecting the MEC-4 C-terminal domain, prompting us to question whether this domain is dispensable for MEC-4 function. To test this possibility, we constructed a truncated subunit that lacked MEC-4 amino acids 740–768 and tested for *in vivo* function. *mec-4*(Δ740–768) does not rescue the touch-insensitive phenotype in the *mec-4(−)* background although the truncated protein is immunologically detectable and interferes with *mec-4(+)* function in transgenic animals (Fig. 6*B*, *left, middle*). Thus, the MEC-4 C-terminal domain is not dispensable for channel function. Because in the mammalian ENaC channel C-terminal deletions in some subunits cause a net increase in Na^+ uptake, we tested the truncated MEC-4 subunit for the capacity to induce the channel-hyperactivated neurodegeneration phenotype. We find *mec-4*(Δ740–768) alone does not induce touch cell death (data not shown). Moreover, the double substitution mutant protein MEC-4(Δ740–768,A713V) does not induce degeneration, indicating that the MEC-4 C-terminal domain must be intact to make a hyperactive channel (Fig. 6*B*, *right*).

To define C-terminal residues required for channel function better, we constructed a partial deletion of the MEC-4 C terminal by introducing a termination codon after K756 (Fig. 6*A*). *mec-4*(Δ756–768) can complement the *mec-4(−)* mutant and, in conjunction with the A713V substitution, can induce neurodegeneration (Fig. 6*B*, *right*). Thus, amino acids residues after amino acid 756 are dispensable for both normal and aberrant MEC-4 function. One sequence feature that stands out in the remaining C-terminal region required for function is a highly Lys-rich region (K753–K762). To test the importance of this basic region in function, we substituted Ala for Lys residues (MEC-4 K(753–756)A). This substitution disrupts channel function (Fig. 6*B*) but does not by itself generate a hyperactive channel that is toxic to touch neurons (data not shown). The basic residues essential for MEC-4 function could mediate an interaction with other channel subunits or a cytoplasmic component of the mechanotransducing complex, participate in assembly, or influence folding and/or channel stability. If the basic residues do affect subunit stability, this process is likely mechanistically distinct from that operative for the Pro-rich regions of ENaC channels. In the case of MEC-4, disruption of this region lessens channel activity, whereas in mammalian ENaC channels deletion of the Pro-rich SH3-binding domains causes channels to become more stable. Although our results appear to underscore differences in channel functions mediated via C-terminal sequences in MEC-4 and ENaCs, it should be noted that C terminals of ENaC family members do include clusters of basic residues that, like those in MEC-4, might mediate essential channel functions.

DISCUSSION

In vivo genetic approaches in the dissection of molecular mechanisms of mechanotransducing channel function

The elaboration of structure–function relationships in mechanotransducing channels presents a unique challenge in channel biology because gating is thought to require tethering of the channels in their normal physiological context. We have exploited powerful *C. elegans* genetic approaches to define critical domains or amino acids in the *C. elegans* MEC-4 touch-transducing channel. A key advantage of the genetic approach to channel structure and function is that no assumptions need be made a priori regarding the potential functional significance of any residues. Moreover, only substitutions that alter channel function in the normal cellular context are identified. Our analysis has both suggested new roles for specific conserved domains and has highlighted unsuspected residues as candidate participants in mechanotransduction-specific processes.

Interpreting data from EMS-induced mutagenesis: a survey of *mec-4*-inactivating mutations

We deduced the molecular identities of a large number of EMS-induced MEC-4 mutations to initiate a broad-based structure–function survey of a candidate mechanotransducing channel. A few points regarding our data set should be noted. First, the specificity of the EMS mutagen restricts the number of codons that can be altered to specify amino acid changes [EMS induces primarily AT-to-CG transitions (Coulondre and Miller, 1977; Anderson, 1995); see http://touchworms.rutgers.edu/posted. for detailed information on EMS-susceptible MEC-4 codons], and thus a complete spectrum of channel-inactivating substitutions cannot be generated using EMS. Second, even among susceptible codons, a Poisson analysis of existing mutations indicates that it is unlikely that all substitutions capable of channel disruption have been identified among the collection of EMS-induced *mec-4* alleles; the screen is not saturated for all possible *mec-4*-inactivating mutations. Third, although generally indicative of relative importance, hot-spot regions of locally concentrated substitutions or "cold-spot" regions lacking channel-inactivating substitutions do not necessarily always reflect the regions of greatest or least (respectively) functional importance. Mutational hot and

cold spots might sometimes reflect a bias of the mutagen for specific sequences or chromatin configurations. Finally, it should be emphasized that the *mec-4* mutant collection is biased toward single residue changes that disrupt channel function. In some instances, single amino acid substitutions may not disrupt activity, but more substantial alterations would reveal functional requirements, for example, as might be the case for MSDI or the MEC-4 C-terminal domain. Moreover, some MEC-4 substitutions might not disrupt function because MEC-4 is assembled *in vivo* in the context of other subunits in the channel complex. For example, we can envisage a scenario in which both MEC-4 and MEC-10 provide a functionally vital interaction with the extracellular protein MEC-9. If a substitution renders MEC-4 no longer able to bind MEC-9, the remaining MEC-10 interaction with MEC-9 may still be sufficient for channel function, and thus the MEC-4 substitution will have no behavioral consequence. In summary, the absence of a channel-inactivating substitution at a given site cannot be interpreted to mean that that amino acid does not play an important role in channel function.

Genetic modeling of channel structure and activity: extending models for mechanotransduction and DEG/ENaC function

Our analysis of mutagen-induced and site-directed *mec-4* mutations provides novel insight into touch channel function and holds several implications for members of the DEG/ENaC channel class. Our key findings include (1) identification of residues in the conserved intracellular N-terminal region required for channel activation and demonstration of sequence-dependent inhibitory effects for N-terminal fragment expression, (2) demonstration that MSDI must be present for early steps of channel assembly to occur, (3) highlighting of extracellular residues that might function specifically in mechanotransduction and demonstration of the functional significance of residues in the neurotoxin-related domain, (4) identification of substitutions that uncouple normal channel activity in mechanosensation and abnormal activity in neurodegeneration, (5) demonstration that features in the microenvironment of amino acid residue 713, which cause channel hyperactivation, do not extend to flanking residues, and (6) definition of a basic region in the short MEC-4 C terminal needed for channel activity. Because many substitutions affect conserved sites, our findings are generally relevant to deciphering DEG/ENaC function and set the stage for biochemical and electrophysiological analysis of analogous substitutions in mammalian counterparts.

The finding that *in vivo* expression of the MEC-4 N-terminal cytoplasmic domain can interfere with endogenous channel function is consistent with a model in which this region interacts with other components of the channel complex. The observations that N-terminal fragments of both nematode and human DEG/ENaCs negatively influence channel function suggest that N-terminal fragment transinhibition is a common property of the channel class and thus suggest a possible strategy for dominant-negative interference with channel activity. Tissue- or temporal-specific expression of N-terminal domains could be used for comparison of the knock-down of channel activity in flies or mice, enabling tests of working hypotheses of ENaC function in restricted tissues [for example, as the proposed role of ENaC channels in baroreceptor mechanotransduction (Drummond et al., 1998) or the role of ASIC channels in pain sensation], or small peptides including the N-terminal domain might be used for therapeutic *in vivo* modulation of blood pressure.

Although many eukaryotic channel types have been identified, few have been exhaustively dissected by genetic approaches. With the exciting accomplishment of the complete *C. elegans* genome sequence and the relative ease of generation of deletion mutations in defined genes, it becomes feasible to apply broad-scale genetic-based approaches, similar to that described here, to channel structure and function in the nematode. Such *in vivo* studies are highly likely to reveal new insights into the mechanisms of channel function in their native contexts.

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