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MEC-2 Is Recruited to the Putative Mechanosensory Complex in *C. elegans* Touch Receptor Neurons through Its Stomatin-like Domain

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Summary

Background: The response to gentle body touch in *C. elegans* requires a degenerin channel complex containing four proteins (MEC-2, MEC-4, MEC-6, and MEC-10). The central portion of the integral membrane protein MEC-2 contains a stomatin-like region that is highly conserved from bacteria to mammals. The molecular function of this domain in MEC-2, however, is unknown.
Results: Here, we show that MEC-2 colocalizes with the degenerin MEC-4 in regular puncta along touch receptor neuron processes. This punctate localization requires the other channel complex proteins. The stomatin-like region of MEC-2 interacts with the intracellular cytoplasmic portion of MEC-4. Missense mutations in this region that destroy the interaction also disrupt the punctate localization and degenerin-regulating function of MEC-2. Missense mutations outside this region apparently have no effect on the punctate localization but significantly reduce the regulatory effect of MEC-2 on the MEC-4 degenerin channel. A second stomatin-like protein, UNC-24, colocalizes with MEC-2 in vivo and coimmunoprecipitates with MEC-2 and MEC-4 in *Xenopus* oocytes; *unc-24* enhances the touch insensitivity of temperature-sensitive alleles of *mec-4* and *mec-6*.
Conclusion: Two stomatin homologs, MEC-2 and UNC-24, interact with the MEC-4 degenerin through their stomatin-like regions, which act as protein binding domains. At least in the case of MEC-2, this binding allows its nonstomatin domains to regulate channel activity. Stomatin-like regions in other proteins may serve a similar protein binding function.

Introduction

Mechanosensation underlies such diverse senses as touch, hearing, balance, and proprioception [1]. Compared with what is known for other senses, we know relatively little about the molecular machinery that transduces mechanical stimuli: what molecules constitute the mechanosensory machinery, how they are organized, and how they function. Genetic studies of mechanosensory transduction in *C. elegans*, *Drosophila*, zebrafish, and mouse, however, have begun to identify molecules that are either the components of such mechanosensory machinery or necessary for developing specialized mechanosensory cells and circuits. These studies suggest that ion channels formed from either degenerin/epithelial sodium channel (DEG/ENaC) or transient receptor potential (TRP) proteins are essential for mechanosensation [2–8]. Most models of mechanosensation suggest that mechanosensitive channels are activated by mechanical tension either through alteration of membrane properties or through coupling with cellular and/or extracellular components [1, 9].

In *C. elegans*, six touch receptor neurons sense gentle body touch [10]. These cells, whose sensory processes are closely attached to the body wall, are distinguished from other *C. elegans* neurons because they are packed with 15-protofilament microtubules and have prominent extracellular matrix. Saturation genetic screens for touch-insensitive mutants have identified several genes needed for the function of these cells. Four genes encode membrane-associated proteins that interact with each other and form an amiloride-sensitive sodium channel complex in heterologous cells [2, 3, 11, 12]. These proteins are the two degenerin channel subunits MEC-4 and MEC-10, the stomatin-like protein MEC-2, and the paraoxonase-like protein MEC-6. Two other proteins needed for touch neuron function, the α-tubulin MEC-12 and β-tubulin MEC-7, are needed to form touch neuron-specific 15-filament microtubules [13, 14]. Three other proteins, a collagen (MEC-5) and two Kunitz and EGF domain-containing proteins (MEC-1 and MEC-9), are components of the extracellular matrix (L. Emtage et al., in press) [15].

Here, we report on the roles that different domains of MEC-2 have on its localization and function. The *mec-2* gene encodes a 481 amino acid polypeptide needed for touch sensitivity [16]. The sequence from amino acid 114 to 363 is 64% identical to human stomatin, a protein that has been implicated in regulating red blood cell conductance [17]; the sequences that lie N- and C-terminal to the stomatin-like region are unique to MEC-2. MEC-2 activates the MEC-4 degenerin channel in *Xenopus* oocytes and coimmunoprecipitates with the other members of the touch receptor degenerin complex [11]. Previous research has suggested that the MEC-2-specific N terminus is needed for the distribution of MEC-2 along the touch receptor processes [16]. Here we show that MEC-2 interacts in vitro and colocalizes in vivo with MEC-4 through its stomatin-like domain. The MEC-2-specific C-terminal domain is required for self association. A second protein, UNC-24, which has an N-terminal stomatin-like region and C-terminal domain similar to nonspecific lipid transfer proteins [18], also colocalizes and coprecipitates with components of the complex. Functionally, mutation of *unc-24* enhances the touch insensitive phenotype produced by temperature-sensitive *mec-4* and *mec-6* alleles.

Results

We generated separate rabbit polyclonal antibodies against the MEC-2-specific N terminus and the remain-

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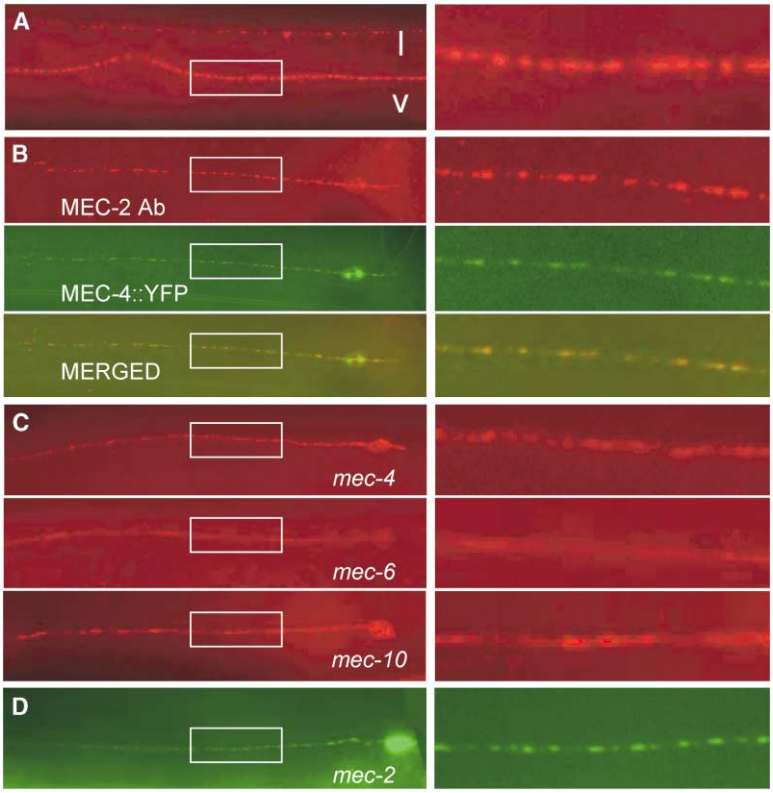

Figure 1. MEC-2 Localization

The right panel shows the enlarged portion corresponding to each boxed area on the left.
(A) MEC-2 puncta in PLM (lateral, l) and PVM (ventral, v) processes. The average distances between puncta in different touch receptor neuron processes were ALM: 3.4 ± 0.2 μm (mean ± SEM, n = 17), PLM: 4.3 ± 0.2 μm (n = 16), AVM: 3.2 ± 0.2 μm (n = 9), and PVM: 2.9 ± 0.1 μm (n = 10).
(B) MEC-2 (red) and MEC-4::YFP (green) puncta colocalize in an ALM touch receptor neuron in a young adult.
(C) The regular punctate pattern of MEC-2 in ALM processes is disrupted in *mec-4(u253)*, *mec-6(u450)*, and *mec-10(u20)* young adults.
(D) MEC-4::YFP puncta in an ALM process are not affected in a young *mec-2(u37)* adult.

der of the protein and purified them by preabsorption to extracts from animals with the *mec-2* null mutation *u37*. These antibodies bound exclusively to the six touch receptor neurons in a punctate pattern that depended on the presence of MEC-2 (Figure 1). Previously, *mec-2* β-galactosidase and GFP reporters driven by 2.5 kb of upstream promoter sequence were expressed in these cells plus additional neurons in the head and tail [16]. We confirmed this additional expression with a full-length GFP protein fusion by using 16.5 kb of *mec-2* genomic DNA (including the same 2.5 kb of 5′ upstream sequence). In contrast a CFP promoter fusion with just the 5′ upstream sequence was only expressed in the touch receptor neurons (data not shown). Together, these data suggest that the additional gene expression is controlled by sequences outside this 5′ upstream region (perhaps in one or more of the large *mec-2* introns), but this expression does not lead to detectable amounts of protein. The exclusive production of MEC-2 in six touch cells is consistent with the touch insensitive phenotype, the only known defect, of *mec-2* mutants.

The MEC-2 puncta are regularly spaced along the touch receptor processes with the average spacing between puncta being larger in the posterior lateral processes than in the ventral processes (Figure 1A). The MEC-2 puncta colocalized with those from a full-length MEC-4::YFP fusion (Figure 1B), supporting the hypothesis that MEC-2 and MEC-4, which functionally interact and coimmunoprecipitate in *Xenopus* oocytes [11], form a complex in vivo along the touch processes.

All three other components of the putative mechanosensory channel complex (MEC-4, MEC-6, MEC-10) are needed for the localization of MEC-2 in puncta; dispersed rather than regular punctate staining was seen in *mec-4 (u253)*, *mec-6 (u450)*, and *mec-10 (u20)* animals (Figure 1C). In contrast, the null *mec-2 (u37)* mutation did not disrupt the punctate localization of a full-length MEC-4::YFP fusion protein (Figure 1D). Thus, MEC-2 appears to be recruited to the degenerin channel complex by the other components. Although MEC-2 binding to MEC-6 may be important in this recruitment, the effect of the *mec-6* mutation is also likely to be indirect because *mec-6* is needed for MEC-4 localization [12].

We have identified mutations in 35 additional *mec-2* alleles (twelve had been identified previously [16, 19], Figure 2A and Table 1). Twenty-three of the 47 sequenced alleles are missense mutations; 16 are nonsense mutations; six are splice junction mutations; and two are transposon insertions. Twenty-one of the missense mutations are located within the stomatin-like domain (Figure 2A). In contrast to the wild-type protein or the product from the remaining two missense mutations, the MEC-2 protein from missense alleles gave a more-dispersed immunostaining with the MEC-2 puncta being reduced or eliminated (Figure 2B). The touch-insensitive phenotype of *mec-2* mutants ranges from weak to complete touch insensitivity [10, 20]. For the missense mutations within the stomatin-like region, the severity of the touch insensitive phenotype correlated with the disruption of the punctate pattern (Figure 2C) but not with the position of the mutation within the domain (data not shown). Taken together this correlation, the presence of MEC-2 puncta in animals with *mec-2* mutations outside the stomatin-like region, and the presence of MEC-4 puncta in the absence of MEC-2 suggest that association of MEC-2 with the degenerin channel complex occurs through the stomatin-like region.

MEC-2 and MEC-4 produced in *Xenopus* oocytes or

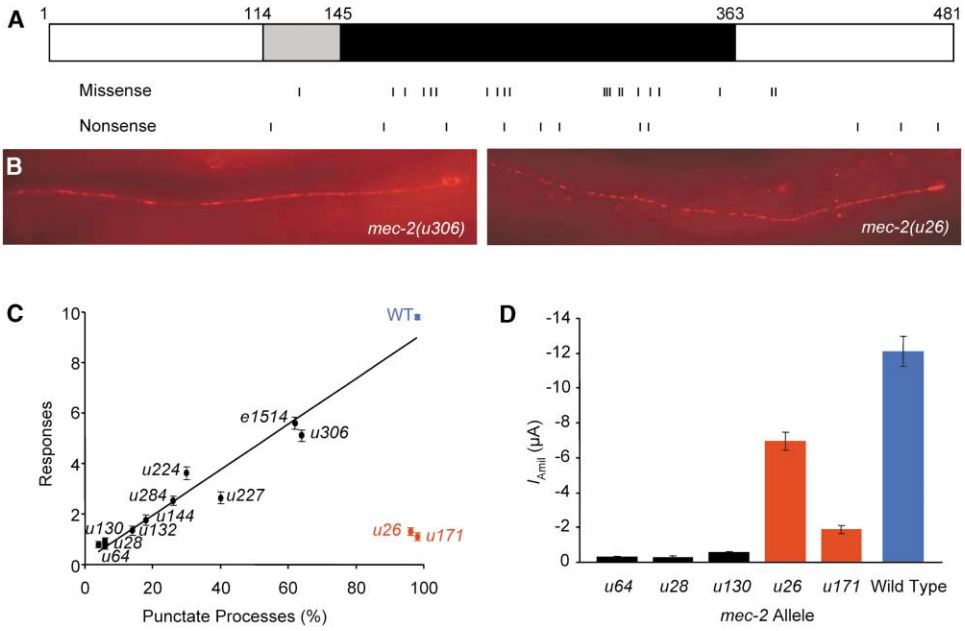

Figure 2. Effect of *mec-2* Mutations on the Subcellular Localization of MEC-2 and Touch Sensitivity

(A) Distribution of missense and nonsense mutations in MEC-2. The filled box indicates the highly conserved stomatin-like region in MEC-2 (the gray area is the hydrophobic region) and the vertical bar indicates the position where a mutation was found in the MEC-2 sequence.

(B) The *mec-2(u306)* mutation in the stomatin-like region affects the punctate pattern of MEC-2 (left panel), whereas the *mec-2(u26)* in the C-terminal region does not (right panel).

(C) The loss of puncta correlates the loss of touch sensitivity for mutations in the stomatin-like region (black). Only 12 missense alleles were characterized because the remaining missense strains were lost. Correlation coefficient R = 0.95. The values for wild-type (WT, blue) and two missense mutations in the C terminus (red) are also shown. Each mutant point is labeled by the corresponding allele name.

(D) Effect of *mec-2* missense mutations on the amiloride-sensitive current (I_{Amil}) of the MEC-4d channel in *Xenopus* oocytes. Mean currents (μA at −85 mV) ± SEM (n = 10 – 14 oocytes from at least two frogs for each) for wild-type MEC-2 (blue) and mutant proteins defective in the stomatin-like region (black) and the C terminus (red) are presented. Further support for the importance of the stomatin-like region for MEC-2 activation comes from our finding that the MEC-2 (114–481) construct we used previously [11] had a Q145R mutation in the stomatin-like region. The mutated construct produced a current of only 0.85 ± 0.1 μA [11]. Without the mutation, the construct yields a current of 4.8 ± 0.6 μA.

CHO cells coimmunoprecipitate [11, 12]. We found that myc-tagged MEC-4 immunoprecipitated the stomatin-like region of MEC-2 (amino acids 114–363, tagged with HA) in *Xenopus* oocytes (Figure 3A). Because MEC-2 and stomatin are thought to be anchored in but not cross the plasma membrane [16, 21], MEC-2 is likely to interact with the cytoplasmic and/or membrane spanning regions of MEC-4. Both structural modeling and in vivo functional analysis of the N-terminal portion of MEC-4 (amino acids 1–108) suggest that this region is the possible site for cytoplasmic interaction with other proteins [16, 22–24]. GST-MEC-4(1–108), but not GST alone, pulled down full-length MEC-2 and the MEC-2(88–375) stomatin-like region when these proteins were expressed in bacteria (Figures 3B and 3C). This region no longer bound GST-MEC-4(1–108) if the MEC-2 sequence contained any of the changes (R184C, A207T, and P357L) found in the three missense mutations that most severely disrupted the MEC-2 punctate pattern in vivo (Figure 3D). This result suggests that the loss of interaction with the degenerin channel causes the mutant MEC-2 proteins to become distributed in a more diffuse pattern in vivo. These same mutations abolish at least 95% of MEC-2-increased MEC-4d current in *Xenopus* oocytes (Figure 2D), indicating that the interaction mediated by stomatin-like region is also essential for the regulatory function of MEC-2.

The interaction of the stomatin-like region of MEC-2 with MEC-4, however, is necessary but not sufficient for MEC-2 function because previous experiments showed that the stomatin-like region of MEC-2 or human stomatin had little effect on the MEC-4d current in *Xenopus* oocytes [11]. In addition, we found that two missense mutations outside the stomatin-like region, R385H (*u26*) and C387Y (*u171*), which virtually abolish touch sensitivity in vivo, reduced but did not abolish the activation of MEC-4d by MEC-2 in oocytes (Figure 2D).

Human stomatin has been reported to oligomerize in red blood cells [25], and interallelic complementation within *mec-2* suggested that MEC-2 also forms oligomers in vivo [10, 20]. We expected, therefore, that the stomatin-like region of MEC-2 would be sufficient to associate with MEC-2 when both proteins were expressed (without MEC-4) in *Xenopus* oocytes. We found, however, that the stomatin-like region was insufficient; the MEC-2-specific C terminus was also needed to immunoprecipitate full-length MEC-2 (Figure 3E). (We could not test whether the C terminus was sufficient for oligomerization because we could not express it in oocytes.)

MEC-2 is not the only stomatin-like protein that is expressed in the touch receptor neurons. UNC-24 is also expressed in these and other cells (T. Barnes and S. Hekimi, personal communication) [26]. If the stomatin-like region of UNC-24 also localizes the protein to the

Table 1. Point Mutations in *mec-2*

Allele	Nucleotide Change	Amino Acid Change	Type of Mutation	Reference
u96	G2754A	–	splice junction	
u226	G2758A	–	splice junction	
u37	G9567A	W119*	nonsense	
u90	G9567A	W119*	nonsense	
u274	C9610T	P134S	missense	
u153	G9762A	–	splice junction	
u232	C10324T	R179*	nonsense	Huang et al., 1995
u64	C10339T	R184C	missense	
u224	G10357A	V190M	missense	
u306	C10388T	S200F	missense	
e75	G10399A	A204T	missense	Gu et al., 1996
u284	G10399A	A204T	missense	Gu et al., 1996
u28	G10408A	A207T	missense	
u16	C10423T	R212*	nonsense	
u277	C10423T	R212*	nonsense	
u1	G10447A	–	splice junction	
u227	C10540T	A234V	missense	Huang et al., 1995
e1608	G10555A	R239H	missense	Huang et al., 1995
u236	G10567A, A10572T	G243E, K245*	missense, nonsense	
u7	G10567A	G243E	missense	Huang et al., 1995
u750	C10576T	T246I	missense	Huang et al., 1995
e1524	C10623T	Q262*	nonsense	Huang et al., 1995
u206	G10718A	W272*	nonsense	
u144	C10789T	A296V	missense	
u318	G10791A	E297K	missense	
e1514	G10797A	E299K	missense	Huang et al., 1995
u243	G10812A	A304T	missense	
u24	G10818A	A306T	missense	
u217	G10888A	E314K	missense	
u233	C10891T	Q315*	nonsense	
e1084	C10903T	R319*	nonsense	Huang et al., 1995
u157	C10903T	R319*	nonsense	
u328	C10903T	R319*	nonsense	
u311	C10907T	A320V	missense	
u43	C10922T	A325T	missense	
u130	C11067T	P357L	missense	
u132	C11066T	P357S	missense	
u26	G12856A	R385H	missense	Gu et al., 1996
u171	G12862A	C387Y	missense	
u272	C13066T	Q430*	nonsense	
u79	G14284A	–	splice junction	
u169	C14298T	Q453*	nonsense	
u733	C14298T	Q453*	nonsense	Gu et al., 1996
u8	C14358T	Q473*	nonsense	

Mutation positions are numbered according to the *mec-2* genomic sequence (GenBank Accession U26736) and the full-length MEC-2 amino acid sequence (GenBank Accession Q27433, Huang et al., 1995). The asterisk indicates a stop codon.

degenerin complex, we would expect a punctate pattern for an UNC-24::GFP fusion in the touch receptor neurons. The fusion is indeed distributed in puncta that colocalize with those of MEC-2 (Figure 4A). *unc-24* mutants are touch sensitive, but we could uncover a role for *unc-24* in touch sensitivity using sensitized backgrounds [19]. Specifically, *unc-24(e138)* enhanced the touch insensitive phenotype of *mec-6(u247)* animals at 15°C and *mec-4(u45)* at 21°C (Figure 4B). *unc-24* mutants also displayed a slight harsh touch phenotype in the head (Figure 4C), but this was not enhanced in the *mec* double mutants.

Expression of UNC-24 in *Xenopus* oocytes did not replace MEC-2 with regard to the increase of the MEC-4d current (Figure 5A). Moreover, addition of UNC-24 to MEC-4d and MEC-2 caused a 30.6% reduction in the current at −85 mV. The significance of this partial reduction is unclear. Nonetheless, UNC-24 interacts with both of these proteins in *Xenopus* oocytes. HA-tagged UNC-24, the stomatin-like region of UNC-24, but not the C-terminal lipid transfer domain immunoprecipitated MEC-2 (Figure 5B) and Myc-tagged MEC-4d (Figure 5C). Neither MEC-2 nor MEC-4 was needed for or interfered with the binding of the other protein to UNC-24 (data not shown). These results suggest that all three proteins can form a complex and that the reduction in the MEC-4/MEC-2 current does not result from competition for binding of MEC-2 and UNC-24.

Discussion

Electrophysiological studies and genetic interactions [19] have suggested that a degenerin channel complex formed with MEC-2, MEC-4, MEC-6, and MEC-10 underlies mechanosensation in the touch receptor neurons. Our results further support this model by showing that MEC-2 colocalizes with MEC-4 in vivo. Because MEC-4 puncta form in the absence of MEC-2, MEC-2 does not

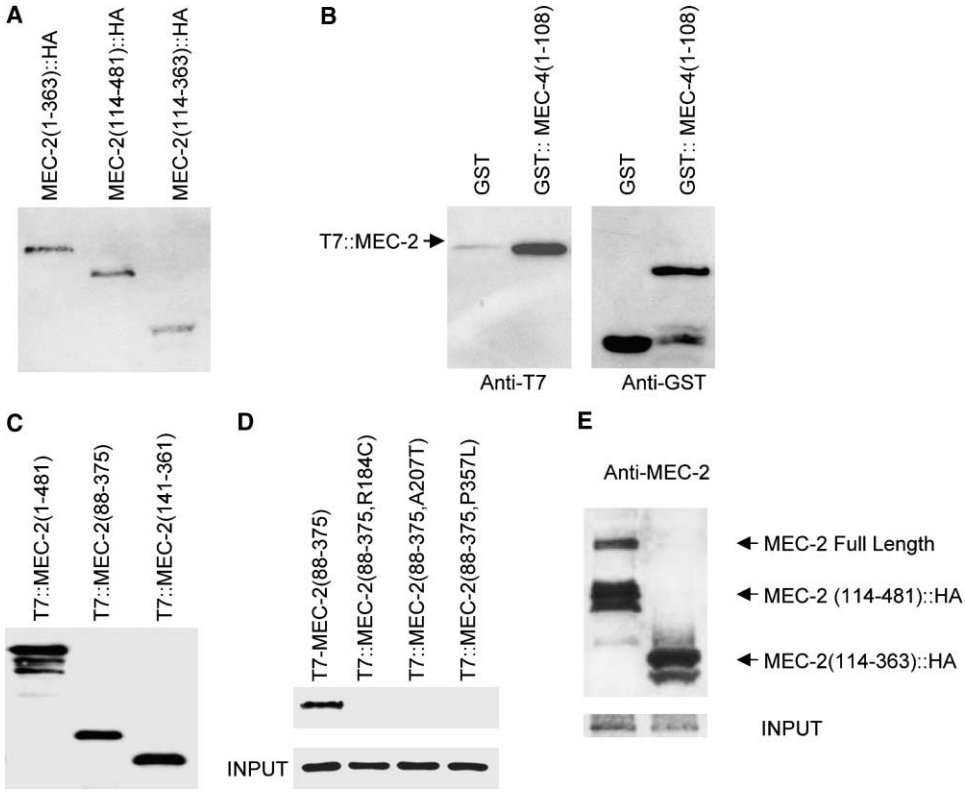

Figure 3. The Stomatin-Like Domain of MEC-2 Interacts with MEC-4

Recombinant proteins were expressed in *Xenopus* oocytes (A and E) and in bacteria (B–D).

(A) Immunoblot of HA-tagged MEC-2 deletion fragments with anti-HA antibody after immunoprecipitation of Myc-MEC-4d with an agarose-conjugated anti-Myc antibody. The MEC-2 fragments used here and in other panels are indicated.

(B) Immunoblot of the T7-tagged MEC-2 with anti-T7 antibody (left panel) and anti-GST antibody (right panel) after GST pull-down with GST::MEC-4(1–108) or GST alone. The blot was probed with anti-T7 antibody first, then stripped and reprobed with anti-GST antibody.

(C) Immunoblot of T7-tagged MEC-2 deletion fragments with anti-T7 antibody after GST pull-down with GST::MEC-4(1–108).

(D) Immunoblot of T7-tagged MEC-2(88–375) containing indicated missense mutations with anti-T7 antibody after GST pull-down with GST::MEC-4(1–108). Bottom panel shows the input of the indicated T7-tagged MEC-2 fragment for each lane. Alleles for the missense mutations are *u64* (R184C), *u28* (A207T), and *u130* (P357L).

(E) Immunoblot of full-length MEC-2 with anti-MEC-2 antibody after immunoprecipitation of HA-tagged MEC-2 fragments with an agarose-conjugated anti-HA antibody. HA-tagged MEC-2 fragments were also recognized by the anti-MEC-2 antibodies. The bottom panel shows the input of the full-length MEC-2.

initiate complex formation. The production of the MEC-2 punctate pattern, however, does require the other proteins of the degenerin complex, suggesting that it may be recruited late in the formation of the complex. In contrast, the distribution or maintenance of MEC-4 along the process requires MEC-6 [12], a difference that may explain why touch cell degeneration caused by dominant mutations in MEC-4 requires MEC-6 but not MEC-2 [27].

The association of MEC-2 with the complex (and of MEC-2 with the N terminus of MEC-4 in vitro) depends on the stomatin-like region of MEC-2. The distribution of MEC-2 along neuronal processes does not require this region [16] or the other members of the degenerin complex; instead the MEC-2-specific N-terminal region is sufficient for the dispersal of MEC-2 [16]. MEC-2-specific termini appear to be important for the activation of the channel complex [11] and for the oligomerization of MEC-2 (this paper). We propose that the stomatin-like domain of MEC-2, by interacting with the degenerin channel, positions the N and C termini of MEC-2 in the complex so they can activate channel activity. Several stomatin-like proteins in *C. elegans* (STL-1 and STO-5; S.Z. and M.C., unpublished observations), bacteria (HflK and HflC), yeast (Phb2p), and mammals (podocin) have additional domains that could be similarly localized through the binding of their stomatin-like regions. Roselli et al. [28] recently reported that many mutations in the stomatin-like region of podocin prevented its localization to the plasma membrane. The colocalization of UNC-24 with MEC-2 in the touch receptor neurons, the enhancement of the mechanosensitive defect in *mec-4ts* and *mec-6ts* animals, and the immunoprecipitation of MEC-2 and MEC-4 by UNC-24 suggest that UNC-24 is also a member of the degenerin complex. Our data suggest that the stomatin-like domain of this protein brings it to the complex. Other eukaryotic stomatin proteins may also interact with DEG/ENaC proteins.

Both prokaryotes and eukaryotes have stomatin-like genes [29]. The *C. elegans* genome has ten stomatin-

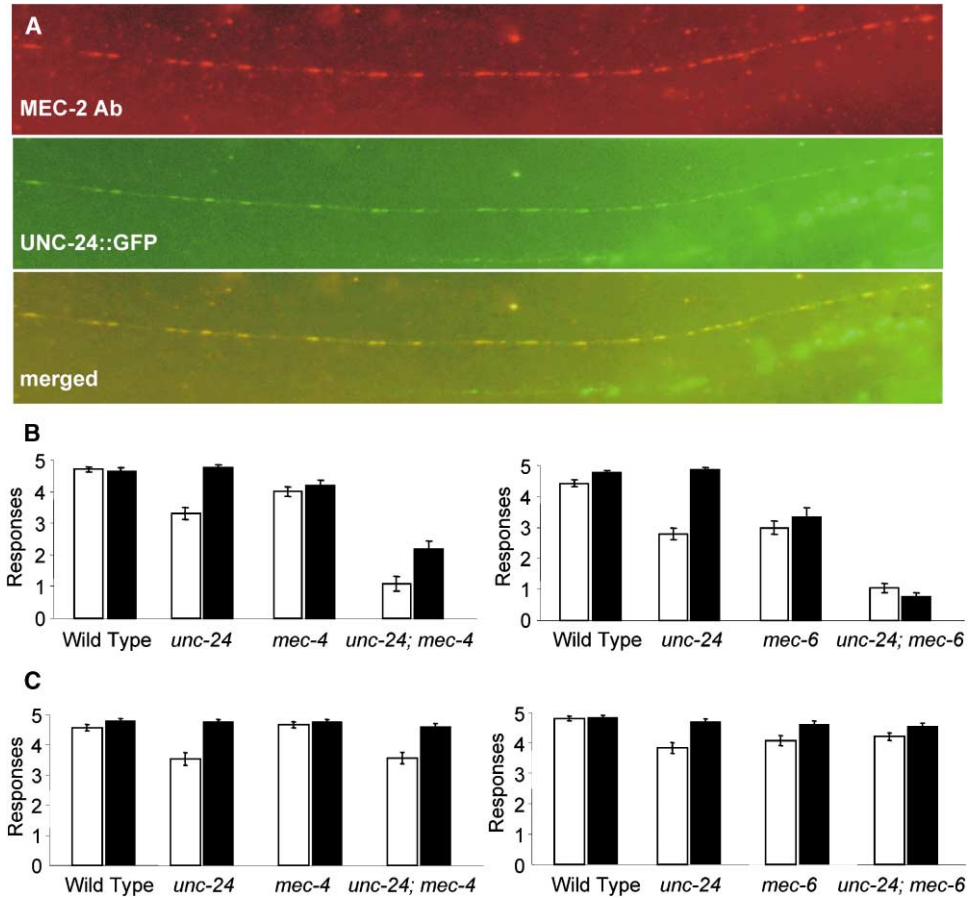

Figure 4. UNC-24 Localizes with MEC-2 and Is Needed for Touch Sensitivity

(A) MEC-2 and UNC-24::GFP puncta colocalize in a PLM process.

(B) *unc-24(e138)* enhances the touch insensitive phenotype of *mec-4(u45)ts* at 21°C and *mec-6(u247)ts* at 15°C. Animals were touched five times in the head (white bars) and five times in the tail (black bars) alternately and the total number of responses for each area was recorded (mean ± SEM, n = 29–30 for each).

(C) *unc-24* animals have a somewhat temperature-dependent insensitivity to harsh touch that is not enhanced in double mutants with *mec-4(u45)* and *mec-6(u247)*. Animals were treated as in (B) except that they were touched with a platinum wire probe instead of an eyebrow hair.

like genes [30] of which only *mec-2*, *unc-24*, and *unc-1* have been characterized. The *unc-1* gene is widely expressed in the nervous system, and it has been implicated in anesthetic sensitivity [31, 32]. Promoter-containing transcriptional GFP reporters for the six of the other stomatin-like genes express predominantly in neurons (S.Z. and M.C., unpublished data). One possibility is that these proteins interact with many other degenerin proteins in *C. elegans*.

The ability to bind and regulate other proteins appears to be a conserved feature of stomatin-like proteins. As with the degenerin channel complex in the *C. elegans* touch receptor neurons, some complexes contain more that one stomatin-like protein. The bacterial stomatin-like proteins HflK and HflC complex with a metalloprotease, FtsH, and regulate its activities [33]. The yeast stomatin-like proteins Phb1p and Phb2p complex with the m-AAA protease and negatively regulate its activity [34]. In *C. elegans* the wild-type *unc-24* gene is needed for the proper localization of UNC-1 [35]. In rat, stomatin associates with the GLUT1 glucose transporter and appears to regulate glucose transport in red blood cells [36, 37].

Mammalian stomatin proteins are associated with a cholesterol-rich, Triton X-100-insoluble fraction from the plasma membrane (lipid rafts). Human stomatin is abundant in erythrocyte lipid rafts [38]; it is thought to act as a protein scaffold at the cytoplasmic surface [39]. In mouse, the Stomatin Related Olfactory protein (SRO) of olfactory sensory neurons is associated with caveolin-1, a protein associated with lipid rafts [40]. Finally, human podocin, a stomatin-like protein expressed in kidney podocytes, is also associated with lipid rafts [41]. Building on these observations, we suggest that MEC-2, UNC-24, and by extension the mechanosensory channel complex, may be associated with a specialized lipid environment that may influence channel activity.

Experimental Procedures

Strain Growth and Characterization

C. elegans strains were cultured at 20°C unless otherwise indicated [42]. Wild-type (N2) [42], *mec* mutants [10, 43], and *unc-24(e138)*

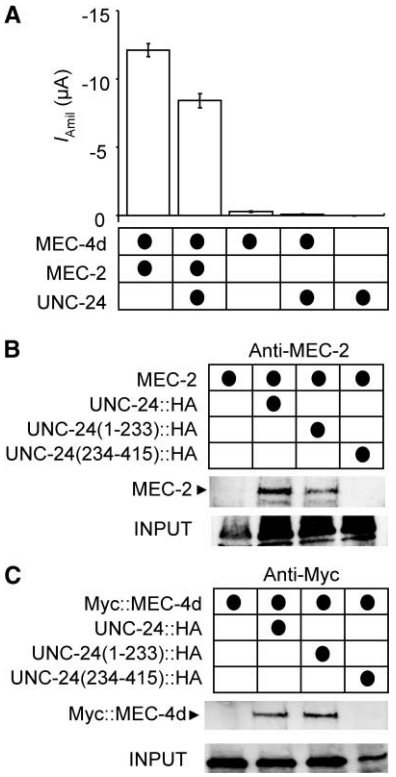

Figure 5. Interaction of UNC-24 with MEC-2 and MEC-4d in *Xenopus* Oocytes

(A) Amiloride-sensitive current (I_{Amil}) at −85 mV of the MEC-4d channel in the presence and absence of MEC-2 and/or UNC-24 in *Xenopus* oocytes. Mean currents ± SEM (n = 12 – 16 oocytes from at least two frogs for each combination except for UNC-24 alone for which we examined 5 oocytes from two frogs).

(B) Immunoprecipitation of MEC-2 by HA-tagged UNC-24, the stomatin-like region (1–233 aa) of UNC-24 but not the lipid transfer domain (234–415 aa) of UNC-24. The anti-HA immunoprecipitates were immunoblotted with the anti-N-terminal MEC-2 antibody. Bottom panels show the input for MEC-2.

(C) Immunoprecipitation of Myc-tagged MEC-4d by HA-tagged UNC-24, the stomatin-like region (1–233 aa) of UNC-24, but not the lipid transfer domain (234–415 aa) of UNC-24. The anti-HA immunoprecipitates were immunoblotted with anti-Myc antibody. Bottom panels show the input for Myc-MEC-4d.

[18] have been described previously. We grew strains for at least three consecutive generations without starvation before carrying out these experiments.

The *mec-2* mutations were determined using PCR-based sequencing [16]. All the protein-coding sequences involved in this work were verified by sequencing (GeneWiz, Inc., North Brunswick, NJ).

Touch Assays

We assayed gentle and harsh touch sensitivity in blind tests by stimulating with an eyebrow hair or prodding with a platinum wire, respectively, as described before [10, 27]. To quantify the response, we recorded the number of responses in about 30 animals to 10 touches delivered alternately near the head and tail. For experiments involving *mec-2* mutants, we combined the head and tail responses; for the experiments involving *unc-24* the responses were scored separately because we found that *unc-24* animals showed considerable insensitivity to both gentle and harsh touch to the head.

Fluorescent Protein Fusions

A *mec-2* promoter fusion was made by PCR amplification of the 2.5 kb 5′ to the start codon of *mec-2*; a *Bam*HI site was generated right after *mec-2* start codon during PCR. This 2.5 kb PCR product was digested with *Pst*I and *Bam*HI and cloned into Fire vector pPD95.77 (www.ciwemb.edu/pages/firelab.html) mutated to express CFP rather than GFP. A full-length *mec-2::gfp* fusion was constructed based on the 20.5 kb *Pst*I rescuing *mec-2* genomic fragment [16]. The first 15 kb *Pst*I-*Xba*I fragment was cloned into Fire vector pPD95.77. The 1.5 kb sequence 3′ of the *Xba*I site had a *Bam*HI site created immediately before the stop codon and was amplified by PCR, digested with *Xba*I and *Bam*HI, and then added to the construct. A full-length *unc-24::gfp* construct was created based on 3.6 kb *Hin*dIII rescuing fragment [18] similarly by cloning a 2.1 kb *Hin*dIII-*Xba*I fragment into pPD95.77 and adding the remaining 1 kb DNA (the fragment to the stop codon) as an *Xba*I and *Bam*HI fragment. The *mec-4::yfp* fusion has been described previously [12].

Transgenic animals were generated by microinjection usually using the pRF4 dominant roller plasmid as a transformation marker [44]. The *unc-24::gfp* fusion fully rescued an *unc-24; lin-15* double mutant by using *lin-15* rescuing plasmid as an injection marker [45].

Antibody Generation and Immunochemistry

cDNAs encoding MEC-2 (1–112 aa) and MEC-2 (145–481 aa) were cloned into pET24a (Novagen, Madison, WI) with C-terminal $(His)_6$ tags. These two fragments were expressed in *E. coli* strain BL21 (DE3), and the $(His)_6$-tagged proteins were purified according to the pET system manual. Antibodies were generated and affinity purified (Pocono Rabbit Farm & Laboratory, Canadensis, PA). Western blots were initially used to monitor the presence of these two antibodies. The antibodies were further purified by adsorption on acetone powders [46] from *mec-2(u37)* null mutants.

Whole-mount immunochemistry was carried out essentially as before [46] using acetone powder-purified MEC-2 antibody (1:1000) and rhodamine-conjugated goat anti-rabbit IgG (H+L) antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA; 1:1000). Both MEC-2 antibodies were used in parallel in all immunostaining experiments and gave the same results. MEC-2 antibody-staining pattern was scored in blind tests. Fifty randomly selected touch cell processes for each allele were scored. Only processes with regular puncta throughout the entire length were scored as punctate although processes with mixed punctate and dispersed localization were also observed but difficult to quantify. Average distance between MEC-2 puncta was measured on at least nine processes in a region with at least 25 puncta for each touch-cell type. The average distance was calculated by dividing the length of the region over the number of puncta in it.

GST Pull-down and Western Blotting

A GST fusion with the first 108 amino acids of MEC-4 was generated by adding the appropriate *mec-4* cDNA fragment to pGEX-6P-1 (Amersham Biosciences, Piscataway, NJ); an N-terminal T7 tag was added to full-length and truncated MEC-2 proteins by cloning appropriate cDNA fragments into pET24a (Novagen, Madison, WI). Missense mutations were introduced with the Stratagene Quikchange kit (Stratagene, La Jolla, CA). All the proteins were expressed in BL21 (DE3) for 3 hr at 37°C following induction with 0.4 mM IPTG. Bacteria pellets were sonicated in cold lysis buffer (20 mM HEPES, [pH 7.5], 150 mM NaCl, 5% glycerol, 0.5% Triton-X-100 and 2 mM EDTA supplemented with a protease inhibitor cocktail tablet from Roche Molecular Biochemicals, Mannheim, Germany) and the lysates collected after centrifugation at 14,000 rpm for 15 min at 4°C.

Equilibrated glutathione Sepharose beads were incubated with lysates containing GST or GST-MEC-4(1–108 aa) for 1 hr at 4°C. The beads were washed three times with cold lysis buffer, incubated overnight with one of the MEC-2 lysates at 4°C, and washed as before. Bound protein was eluted in SDS-PAGE sample buffer by heating at 90°C for 3 min. Horseradish peroxidase (HRP)-conjugated anti-T7 antibody (Novagen, Madison, WI) and anti-GST antibody (Amersham Bioscience, Piscataway, NJ) were used at 1:200,000 and 1:2000, respectively. HRP was visualized by ECL Western Blotting Detection Reagents (Amersham Bioscience, Piscataway, NJ).

Oocyte Expression and Electrophysiology

Oocyte expression, electrophysiology, and MEC-4d and MEC-2 oocyte expression constructs were essentially as described [11].

To confirm the specificity of the immunoprecipitation, we probed the immuno-complexes with a monoclonal antibody (8C8, Developmental Studies Hybridoma Bank, University of Iowa, IA) for β-integrin, an endogenous *Xenopus* oocyte membrane protein [47]. No β-integrin was detected (data not shown). In the UNC-24 experiments, oocytes were cultured in ND96 medium instead of L15. Similar pGEM-HE constructs were made for UNC-24 and UNC-24::HA expression. *mec-2* mutations were introduced using the Quikchange mutagenesis kit from Stratagene (La Jolla, CA). 300 μM amiloride was always included in the oocyte culture medium. Expression of mutant MEC-2 proteins was verified by Western blotting. All mutant MEC-2 proteins were expressed at comparable levels to wild-type MEC-2 (data not shown). Agarose-conjugated anti-Myc and anti-hemagglutinin (HA), HRP-conjugated anti-HA, and HRP-conjugated anti-Myc antibodies were purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA.

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