Temperature-sensitive Mutant of the Caenorhabditis elegans Neurotoxic MEC-4(d) DEG/ENaC Channel Identifies a Site Required for Trafficking or Surface Maintenance*[^5]

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DEG/ENaC channel subunits are two transmembrane domain proteins that assemble into heteromeric complexes to perform diverse biological functions that include sensory perception, electrolyte balance, and synaptic plasticity. Hyperactivation of neurally expressed DEG/ENaCs that conduct both Na\(^+\) and Ca\(^{2+}\), however, can potently induce necrotic neuronal death in vivo. For example, Caenorhabditis elegans MEC-4/ENaC comprises the core subunit of a touch-transducing ion channel critical for mechanosensation that when hyperactivated by a mec-4(d) mutation induces necrosis of the sensory neurons in which it is expressed. Thus, studies of the MEC-4 channel have provided insight into both normal channel biology and neurotoxicity mechanisms. Here we report on intragenic mec-4 mutations identified in a screen for suppressors of mec-4(d)-induced necrosis, with a focus on detailed characterization of allele bz2 that has the distinctive phenotype of inducing dramatic neuronal swelling without being fully penetrant for toxicity. The bz2 mutation encodes substitution A745T, which is situated in the intracellular C-terminal domain of MEC-4. We show that this substitution renders both MEC-4 and MEC-4(d) activity strongly temperature sensitive. In addition, we show that both in Xenopus oocytes and in vivo, substitution A745T disrupts channel trafficking or maintenance of the MEC-4 subunit at the cell surface. This is the first demonstration of a C-terminal domain that affects trafficking of a neurally expressed DEG/ENaC. Moreover, this study reveals that neuronal swelling occurs prior to commitment to necrotic death and defines a powerful new tool for inducible necrosis initiation.

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[^9]: The abbreviations used are: ENaCs, epithelial amiloride-sensitive Na\(^+\) channel; GFP, green fluorescent protein; PBS, phosphate-buffered saline; EMS, ethane methyl sulfonate; ts, temperature sensitive; ASIC, acid-sensing ion channel; PLM, posterior lateral microtubule cell.
sive disorder Liddle’s syndrome (34–36). Taken together, these findings underscore the profound therapeutic importance of understanding the molecular nature of both the normal and hyperactivated functions of DEG/ENaC channels.

One advantage of studying the nematode DEG/ENaCs is the capacity to conduct extensive genetic characterization of these channels within a physiological context. To identify molecular requirements for mec-4(d)-induced necrosis, we screened for genetic suppressors of neuronal death. One major suppressor class we isolated includes intragenic second site changes that maintain the channel hyperactivating substitution (A713V) but have a second site substitution (A745T) that influences channel trafficking or stability. Moreover, this substitution (MEC-4(A745T)) renders necrosis strongly temperature-inducible. We discuss implications for structure/function/organization of intracellular C-terminal residue that influences channel trafficking or stability. Furthermore, this substitution (MEC-4(A745T)) renders necrosis strongly temperature-inducible. We discuss implications for structure/function/organization of intracellular C-terminal residue that influences channel trafficking or stability.

**EXPERIMENTAL PROCEDURES**

Genetic Screen for Suppressors of mec-4(d)-induced Cell Death—Strain ZB164, harboring the mec-4(d) mutation and expressing a GFP transgene exclusively in touch neurons ([pMec-4::GFP](#H11001)), was used to generate mutagenesis strain ZB1081, harboring the mec-4(u231) X[TU231]; mec-4(u231) = mec-4(d) (26). Strain ZB164 was constructed by site-directed mutagenesis (QuikChange site-directed mutagenesis kit, Stratagene) using PMEC-4::GFP as a template.

Our screen used nematode strain ZB1081, harboring the mec-4(d) mutation and expressing a GFP transgene exclusively in touch neurons ([pMec-4::GFP](#H11001)). Our screen used nematode strain ZB1081, harboring the mec-4(d) mutation and expressing a GFP transgene exclusively in touch neurons ([pMec-4::GFP](#H11001)).

C. elegans Strains, Growth, and Touch Assay—Nematode strains were maintained at 20 °C unless otherwise stated on NGM seeded with Escherichia coli strain OP50 as food source (38). For b2 GFP expression studies, plasmids were injected into Bristol (N2) and mec-4(u253) (mec-4 null) strains. We performed gentle touch tests by stroking the body at anterior and posterior positions with an eyelash as described (40).

Molecular Biology—The P_mec-4::GFP vector was created by introducing a HindIII/BamHI fragment including the mec-4 promoter into vector pPD95.77, which includes enhanced GFP (constructed by Scott Clark, NYU). The BamHI fragment was introduced by site-directed mutagenesis at the mec-4 initiation codon. P_mec-4::GFP was constructed by subcloning a 4.7-kb HindIII-BamHI fragment from plasmid TU44 (41), which includes mec-4 promoter and coding sequences except for the last 7 amino acids, into pPD95.77 (Fire lab vector kit, Ref. 42). The P_mec-4::GFP and P_mec-4::GFP were constructed by site-directed mutagenesis (QuickChange site-directed mutagenesis kit, Stratagene) using P_mec-4::GFP as template. mec-2, mec-4(d), and mec-10(d) cDNAs subcloned into pGEM-HE or pSGEM, a gift from the Chalfie laboratory (30), were amplified using the SMC4 bacterial strain (30). The A745T mutation was introduced by site-directed mutagenesis (QuickChange site-directed mutagenesis kit).

Oocyte Expression and Electrophysiology—Capped RNAs were synthesized using T7 mMESSAGE mMACHINE kit (Ambion), purified (Qiagen RNAeasy columns), and run on denaturing agarose gels to check for size and cRNA integrity. cRNA quantification was then performed spectrophotoscopically. Stage V-VI oocytes were manually defolliculated after selecting them among multistaged oocytes dissected by a 2-h collagenase treatment (2 mg/ml in CaCl2-free OR2 solution) from Xenopus laevis ovaries (NASCO). Oocytes were incubated in OR2 media, which consists of 82.5 mM NaCl, 2.5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 1 mM Na2HPO4, 0.5 mM HEPES (pH 7.2), supplemented with penicillin and streptomycin (0.1 mg/ml) and 2 mM sodium pyruvate. Oocytes were then injected with 50 nl of cRNA mix for a final amount of 5 ng/oocyte of each cRNA except for MEC-6, which was injected at the concentration of 1 ng/oocyte. Oocytes were incubated in OR2 at 20 °C for 4 days before recording.

Currents were measured 4–10 days after cRNA injection using a two-electrode voltage clamp amplifier (GeneClamp 500B, Axon Instruments) at room temperature. Electrodes (0.3–1 mm) were filled with 3 M KCl, and oocytes were perfused with a NaCl solution containing (in mM): NaCl (100), KCl (2), CaCl2 (1), MgCl2 (2), HEPES (10), pH 7.2 or...
with a CaCl₂ solution containing CaCl₂ (73), KCl (2), HEPES (10), pH 7.2. Chemicals were obtained from Sigma and Calbiochem. We used the pCLAMP suite of programs (Axon Instruments) for data acquisition and analysis. Currents were filtered at 200 Hz and sampled at 1 kHz.

**Immunocytochemistry**—Staining of oocytes was performed following previously reported procedures (31, 43). Briefly, 5 days after injection, oocytes were fixed at 4°C overnight with 4% paraformaldehyde. The next day, oocytes were washed four times for 5 min each in PBS, imbedded in low melting point agarose (3% in PBS), and cut in 50-μm thick slices using a vibrotome. Slices were incubated for 2 h at room temperature in 0.2% bovine serum albumin in PBS plus 0.1% Tween 20 and subsequently incubated with anti-MEC-4 antibody directed against amino acids 527–539 in the extracellular loop ((44)1:50 in 1% bovine serum albumin dissolved in PBS and 0.1% Tween 20) overnight at 4°C. Slices were washed three times for 5 min with PBS and incubated with Cy2-conjugated goat anti-rabbit antibody (1:2000; Jackson ImmunoResearch) for 1 h at room temperature. After slices were washed three times for 5 min in PBS, they were mounted with VECTOREX medium (Vector) and photographed using a Zeiss Axiplan 2 microscope equipped with digital camera. Images were analyzed and mounted with Adobe Photoshop.

**RESULTS**

Suppressors of mec-4(d)-induced Neurodegeneration Include Intragenic Mutations That Disrupt MEC-4 Function.—With a goal of defining genes required for mec-4(d)-induced necrosis, we screened for novel mutations that block or delay the death of the touch receptor neurons in a mec-4(d) mutant background. We expressed GFP exclusively in the six touch neurons using the mec-4 promoter (reporter bzi8[p_mec-4GFP]) (Fig. 1A). We then introduced mec-4(d) (allele mec-4(u231)) into the bzi8 background (Fig. 1B) and compared neuronal survival in the L4/young adult stage by counting fluorescent touch neurons. mec-4(d) induces necrosis efficiently in the bzi8[p_mec-4GFP] line such that 94% (n = 200) of animals lack any detectable fluorescent touch neurons and the remaining 6% have only one fluorescent touch cell (nearly always the PVM neuron that functionally differs from the other touch cells (45)) (Fig. 1C). We confirmed that the extent of touch neuron degeneration, as scored by the presence of swollen PLM touch neurons in the L1 larval stage, was tightly correlated with the lack of fluorescent touch neurons in L4 or adult stage animals as we had previously documented (31, 46) (TABLE ONE).

We reasoned that touch neurons genetically spared from death (but still able to express mec-4) would be easily identified by their
restored fluorescence in the 

| Allele | Mutation site | Base change | Alteration | Location |
|--------|---------------|-------------|------------|----------|
| bz149  | 2956          | C-T         | R601C      | Extracellular |
| bz183  | 3055          | G-A         | R619K      | Extracellular |
| bz195  | 3103          | ΔC          | S635F frameshift | Extracellular |
| bz133  | 3116          | G-A         | 5’-AGGTRAG | Extracellular |
| bz134  | 3116          | G-A         | 5’-AGGTRAG | Extracellular |
| bz143  | 3116          | G-A         | 5’-AGGTRAG | Extracellular |
| bz94   | 3194          | C-T         | P643L      | Extracellular |
| bz104  | 3220          | C-T         | P655L      | Extracellular |
| bz112  | 3230          | G-A         | W658Stop   | Extracellular |
| bz150  | 3246          | C-T         | Q664Stop   | Extracellular |
| bz122  | 3349          | G-A         | 3’-TTTCAGR | Extracellular |
| bz159  | 3406          | C-T         | T701I      | Extracellular |
| bz173  | 3590          | G-A         | G716S      | Pore |
| bz11   | 3593          | G-A         | G717E      | Pore |
| bz165  | 3602          | G-A         | G720D      | Pore |
| bz139  | 3611          | G-A         | C723Y      | Pore |
| bz45   | 3620          | C-T         | S726F      | Pore |
| bz101  | 3625          | C-T         | L728F      | Pore |
| bz184  | 3629          | C-T         | T729I      | Pore |
| bz8    | 3658          | G-A         | L739E      | Pore |
| bz179  | 3748          | T-A         | stop769R, 7 AA extension | C terminus |
| bz2    | 3766          | G-A         | A745T      | C terminus |

* The underlined nucleotide is changed to A causing loss of the splice site.

EMS-induced intragenic suppressor mec-4(d) mutations that alter the genome sequence in the vicinity of the MEC-4 pore-encoding region

Listed are nucleotide and amino acid changes (numbering according to Ref. 47) associated with intragenic mec-4(d) suppressor mutations that affect the 3’-half of the gene (nucleotides corresponding to 103–2996–3872, which encodes from amino acids 610 to the 3’-end untranslated region). All alleles include the mec-4(d) mutation u231 encoding A713V in addition to the change indicated. Bold indicates mutations not reported in previous screens. Genomic sequence encoding the N terminus was not determined for most alleles, and thus although unlikely, the alleles listed could include additional mutations. The mec-4(d)u231bz2 strain was fully sequenced from 221 bp prior the start codon to 316 bp after the stop codon.

The underlined nucleotide is changed to A causing loss of the splice site.

Intragenic Death Suppressor Mutations Alter Multiple Amino Acids within and near the Channel Pore—We expected that second site intragenic mutations in mec-4(d) that disrupt function of the MEC-4 channel subunit would constitute one death suppressor class and that intragenic mutations should have genetic properties of X-linked recessive loss-of-function mec-4 alleles (i.e. mec-4(+)/mec-4(u231bz2) trans-heterozygotes should be touch-sensitive, where x indicates the new intragenic mutation). In addition, we anticipated that most intragenic mec-4 loss-of-function alleles should express GFP strongly in all six touch receptor neurons. 80 necrosis suppressor alleles had these genetic properties, and we therefore considered them strong candidates for second site mutations in mec-4(u231).

To determine if amino acid changes near or within the MEC-4 channel pore were encoded by any intragenic mec-4 alleles, we sequenced the mec-4 genomic sequence over an interval that included coding regions of part of the third extracellular Cys-rich domain, the transmembrane channel pore, and the cytosolic C-terminus (see Ref. 47 for details of domain structure). We identified single nucleotide changes in 22 mec-4(d) suppressor alleles, 14 of which encode single amino acid substitutions, 2 of which encode frame-shifts, 2 of which encode stop codons, and 4 of which alter splicing sites (TABLE TWO). Our data confirm that intragenic null mutations and other likely loss-of-function mutations constitute a significant death suppressor class isolated in our screen. In addition, we identify several residues previously unknown to be critical...
for function of the hyperactivated mec-4(d) channel, including the first EMS-induced mutations affecting the MEC-4 C terminus. We note implications of specific changes for channel structure/function in greater detail in the "Discussion."

In mec-4(u231bz2), Neurons Swell and Appear Necrotic but Some Can Then Recover—One X-linked death suppressor allele, mec-4(u231bz2), has distinctive properties. We noted that this mutation partially uncouples necrotic swelling and cell death. In the mec-4(d) background, swollen PLM neurons are apparent in young L1 larvae and disappear by the L4 stage (46, 48) (Fig. 2 D). In mec-4(u231bz2) at 20 °C, however, while 24% of PLM tail neurons still swell in the L1 larval stage, at the L4 stage only 11% appear to have died (TABLE ONE). These data suggested that some touch neurons might swell significantly but then recover. To address this possibility, we scored posterior touch neurons in individual animals at the L1 and then again at the L4 stage (Fig. 2). Indeed, when we followed 21 individuals (42 PLM neurons) from L1 to L4 we found that ~one-fourth of PLMs became swollen but then recovered to appear as fluorescent neurons with normal morphology in the L4 stage; ~one-third of PLM neurons appeared healthy at the L1 stage and then either remained healthy (n = 9) or died (n = 5). We conclude that in the mec-4(u231bz2) background, the toxic insult is sufficient to induce dramatic swelling but not strong enough to efficiently kill neurons. This uncoupling of swelling and death suggests that the swelling stage of necrosis occurs prior to the point of death commitment.

Another striking property of mec-4(u231bz2) that we noted is that this allele is strongly temperature sensitive for neurotoxicity (TABLE ONE and Fig. 3, A and B). mec-4(u231) is a potent inducer of necrosis at either 15 °C or 25 °C. By contrast, in the mec-4(u231bz2) background, necrosis is potent at 15 °C, intermediate at 20 °C, and essentially absent at 25 °C. The strong ts phenotype associated with this allele enables necrosis induction in specific cells late in development, a feature with highly advantageous potential for future necrosis analysis (see "Discussion").

mec-4(u231bz2) Encodes an A745T Change in the MEC-4 C Termi-
nus That Causes Temperature-dependent Disruption of Both the MEC-
4(d) and MEC-4(+) Channels—To determine the molecular basis of the mec-4(u231bz2) phenotype, we sequenced mec-4 genomic sequence throughout the coding region. We found that mec-4(u231bz2) specifies amino acid change A745T in the intracellular MEC-4 C terminus, the first identified EMS-induced mec-4 mutation to affect this domain. Although the C-terminal domains of DEG/ENaC family members are not highly conserved, ClustalW alignment of C termini indicates that
The position corresponding to MEC-4(745) is often a nonpolar residue in family members (supplemental Fig. 1).

We confirmed the A745T substitution to be causative for the mec-4(u231) phenotype by engineering this second change into a mec-4(u231) transgene (which encodes substitution A713V), introducing it into wild-type animals, and assaying for toxicity. Pmec-4(u231bz2) induces necrosis efficiently at 15°C, but exerts only weak effects at 25°C, similar to the genomically encoded mec-4(u231) (Fig. 3, A and B).

Substitution of A745T affects the otherwise wild-type MEC-4 channel similar to the MEC-4(d) channel. When we engineered the A745T substitution alone into a mec-4(d) transgenic line, we found that the Pmec-4(d) transgene was able to complement the mec-4-null mutation u253 for touch sensitivity at the permissive temperature (15°C), but this complementation was not effective at 25°C (Fig. 3, C and D). We conclude that Ala745 is critical for MEC-4 channel activity in vivo both for the wild-type protein and the hyperactivated MEC-4(d) mutant.

In Xenopus Oocytes, the MEC-4(A713V/A745T) Subunit Maintains Basic MEC-4(d) Electrophysiological Properties yet Whole Cell Currents Are Reduced—To establish how mec-4(d) toxicity function is impaired by second site substitution A745T, we heterologously expressed the double mutant channel in Xenopus oocytes and performed electrophysiological analysis. The best characterized MEC-4(d) channel includes DEG/ENaC subunits MEC-4(d) and MEC-10(d), stomatin-related protein MEC-2, and paraoxonase-like protein MEC-6 (29–31). (We designate this channel arrangement as the MEC-4(d) channel complex.) We therefore co-expressed MEC-4(A713V/A745T) with MEC-10(d), MEC-2, and MEC-6 and compared current amplitude and properties...
with the MEC-4(d) channel complex. Consistent with previous observations (29–31), expression of the MEC-4(d) channel complex induced a large voltage-independent Na\(^+\)/H\(^+\) current (Fig. 4A) that was blocked by amiloride (not shown). By contrast, expression of the MEC-4(A713V/A745T) channel complex was associated with amiloride-sensitive Na\(^+\) currents that were reduced in amplitude, conducting ~30% of MEC-4(d) (Fig. 4, B–D).

We previously documented that MEC-4(d) is permeable to Ca\(^{2+}\) ions (31), a Ca\(^{2+}\) influx that may trigger catastrophic ER Ca\(^{2+}\) release necessary for the completion of the necrotic program. In *Xenopus* oocytes

**FIGURE 4.** MEC-4(A713V/A745T) channels produce reduced whole cell currents in *Xenopus* oocytes. Oocytes were maintained and assayed at 20 °C. When oocytes were maintained at 25 or 15 °C they were either leaky or unhealthy. *A*, example of sodium currents elicited by voltage steps from −160 to +60 mV from a holding potential of −30 mV in an oocyte injected with *mec-4*(d), *mec-10*(d), *mec-2*, and *mec-6* and exposed to a NaCl solution. *B*, same as in *A* for an oocyte injected with *mec-4*(u231bz2), *mec-10*(d), *mec-2*, and *mec-6*. Note the significant reduction in whole cell currents despite the injection of the same amount of RNA in both *A* and *B* (see “Experimental Procedures”). *C*, enlarged scale for current shown in *B* demonstrates that despite being strongly reduced, currents associated with expression of MEC-4(A713V/A745T) are still present. *D*, average Na\(^+\) current at −100 mV recorded from oocytes injected with *mec-10*(d), *mec-2*, and *mec-6* + *mec-4*(d) (*n* = 14), or *mec-4*(u231bz2) (encoding MEC-4(A713V/A745T)) (*n* = 8). *E*, Ca\(^{2+}\)-activated currents induced by mutant MEC-4 channels when extracellular Ca\(^{2+}\) is present. The oocyte shown in *A* was exposed to a solution in which Na\(^+\) was substituted with Ca\(^{2+}\). This activates an endogenous *Xenopus* oocyte Ca\(^{2+}\)-activated Cl\(^-\) current, which we have previously shown to be activated by entry of Ca\(^{2+}\) through the MEC-4(d) channel (31). *F*, MEC-4(A713V/A745T) oocyte shown in *B* was exposed to the Ca\(^{2+}\) solution resulting in the activation of the endogenous Ca\(^{2+}\)-activated Cl\(^-\) current. MEC-4(A713V/A745T) double mutant channels retain the Ca\(^{2+}\) permeability property typical of toxic MEC-4(d), but the current is greatly reduced. *G*, same current in *F* is shown enlarged. *H*, average Ca\(^{2+}\)-activated Cl\(^-\) current at −160 mV. Note that for MEC-4(A713V/A745T), both Na\(^+\) currents and Ca\(^{2+}\)-activated Cl\(^-\) currents, which are a measure of the amount of Ca\(^{2+}\) permeating through the degenerin channel, are reduced to ~30% of MEC-4(A713V). Thus, Ca\(^{2+}\) permeability is not grossly affected by the second site mutation. MEC-4(A713V) *n* = 6; MEC-4(A713V/A745T) *n* = 8. Data are expressed as mean ± S.E. ** indicates *p* < 0.01 by comparison with MEC-4(d)-expressing oocytes, by Student’s *t* test.
FIGURE 5. MEC-4(A713V/A745T) mutant channels do not reach the oocyte plasma membrane efficiently. A, fluorescent micrograph of a non-injected oocyte stained with anti-MEC-4 antibodies, establishing the absence of background plasma membrane staining. B and C, pictorial comparison of plasma membrane expression for mutant MEC-4 channels. Oocytes expressing MEC-4(d) + MEC-10(d) + MEC-2 + MEC-6 and MEC-4(A713V/A745T) + MEC-10(d) + MEC-2 + MEC-6, respectively, stained with anti-MEC-4 antibodies. (The antibody was produced by immunization against the synthetic peptide SRLPAPYGDC located extracellularly in MEC-4 and corresponding to amino acids 552–561, Ref. 44.) MEC-4(d) staining is clearly visible at the plasma membrane (white arrow), but the MEC-4(A713V/A745T) mutant subunit cannot be readily detected, suggesting a problem in stability or trafficking of the double mutant subunit. D, quantitation of fluorescence at the plasma membrane. We used AdobePhotoshop (two pictures/oocyte, 6 oocytes/sample) to quantify fluorescence at the plasma membrane by isolating pixels corresponding to the plasma membrane and determining average luminosity. Because of slightly different luminosity levels of the background, we subtracted background fluorescence from each measurement. Note that if we artificially manipulate photographs of MEC-4(d) expressing oocytes to reduce their luminosity level to 30% of its value (MEC-4(A713V/A745T)) currents are 30% of MEC-4(d), we no longer detect a membrane-associated fluorescent signal. This suggests that lack of fluorescent signal in MEC-4(A713V/A745T) oocytes is because of reduction below detectable level. Data are expressed as mean ± S.E. n is 12 for all samples. Pictures were taken with a Zeiss Axio-Plan 2 microscope equipped with a digital camera, 0.7 s exposure time for all photographs.

bathed with CaCl2 entry of Ca2+ through MEC-4(d) activates an endogenous Ca2+-activated Cl− current that we monitor as a measure of MEC-4(d) Ca2+ permeability (31, 49) (Fig. 4E). In oocytes expressing the MEC-4(A713V/A745T) channel complex, we detected activation of the endogenous Ca2+-activated Cl− current, but the amplitude of the current was reduced to ~30% of the MEC-4(d) channel complex (Fig. 4, F–H). We conclude that although amiloride sensitivity, ion selectivity, and voltage/time characteristics are maintained in the mutant MEC-4(A713V/A745T) channel, a significant reduction in the amount of whole cell current is introduced by the A745T substitution.

The Number of Surface-expressed MEC-4(A713V/A745T) Subunits Is Reduced in Oocytes, Suggesting a Trafficking or Stability Defect—Reduced whole cell currents associated with MEC-4(A713V/A745T) could result from changes in single channel conductance and/or open probability or from reduced numbers of channels at the cell surface. To measure MEC-4(A713V/A745T) at the cell surface, we used anti-MEC-4 antibodies (polyclonal against epitope corresponding to extraacellular amino acids 552–561 (44)) to visualize MEC-4 subunits in sectioned oocytes. Non-injected stained oocytes had no fluorescent signal at the plasma membrane, confirming specificity of the MEC-4 antibody that we employed (Fig. 5A). Oocytes expressing the MEC-4(d) subunit displayed clear membrane staining (Fig. 5B) (31). By contrast, oocytes expressing MEC-4(A713V/A745T) did not exhibit significant fluorescent signal at the plasma membrane (Fig. 5C), and quantitation of plasma membrane fluorescence intensity derived from several oocyte sections indicated that the signal in MEC-4(A713V/A745T) oocytes did not rise above background levels (Fig. 5D). These results suggest that a significantly decreased number of MEC-4(A713V/A745T) channel subunits are present at the oocyte plasma membrane under conditions of equal cRNA injection for MEC-4(A713V) and MEC-4(A713V/A745T). We conclude that reduced ion fluxes associated with expression of MEC-4(A713V/A745T) in Xenopus oocytes result from diminished numbers of channel subunits at the cell surface.

MEC-4(A745T) Has a Temperature-sensitive Trafficking Defect in Vivo—A critical question regarding the mechanism of necrosis suppression for mec-4(u231bz2) is whether the MEC-4(A745T) subunit is disrupted for trafficking or protein stability in vivo in nematode neurons. To assess this possibility, we monitored full-length MEC-4::GFP fusion proteins in vivo. Wild-type MEC-4::GFP can functionally complement a mec-4 null mutation, suggesting that this protein traffics correctly in vivo (47). MEC-4::GFP is evident in the cell body and in puncta in the process that are thought to be sites of the MEC-4 channel complex (29, 50, 51). When we compared MEC-4(A745T)::GFP in transgenic animals at 15 and 25 °C, we found that puncta and cell body fluorescence are present at low temperature (Fig. 6, A–C) when the protein is functional, but the GFP distribution to the process is markedly diminished at 25 °C when the protein is non-functional (Fig. 6, D–F). We conclude that the MEC-4(A745T) substitution disrupts channel trafficking or stability in a temperature-dependent manner. Our data implicate a residue in the MEC-4 C terminus in the functional assembly of the channel complex. Necrosis suppression in the mec-4(u231bz2) mutant is most likely conferred by a significant reduction in channel activity that has a basis in defective trafficking and/or stability.

DISCUSSION

We exploited C. elegans genetics to identify molecular changes that can block toxicity of the hyperactivated MEC-4(d) channel. Here we report several new amino acid changes within MEC-4 itself that disrupt...
C. elegans DEG/ENaC Channel MEC-4(d) Trafficking

![Diagram](image_url)

FIGURE 6. MEC-4(A745T) is impaired for trafficking. A and B, fluorescent micrographs of PLM touch neurons expressing MEC-4::GFP (A) and MEC-4(A745T)::GFP (B) proteins under the control of the mec-4 promoter (MEC-4::GFP and MEC-4(A745T)::GFP) in a mec-4(null) genetic background, in adult worms reared at 15 °C. Expression of the MEC-4::GFP fusion protein (which lacks only the last 7 C-terminal amino acids) can complement a mec-4 deletion allele (47). Evident are the puncta structures along the neuronal processes that are thought to be sites of the MEC-4 channel (29, 50, 51). C, number of puncta from the cell body up to ten times the cell body length (~20 μm) in the two strains reared at 15 °C. The number of neuronal processes scored was 29 and 25, respectively. D and E, same as in A and B, but the worms were reared at 25 °C. Note the dramatically reduced number of puncta in PMEC-4(A745T)::GFP neuron. Puncta become less evident and are substituted by a more diffuse staining about halfway through the scored length. F, scoring of the number of puncta in the two strains reared at 25 °C confirms the strong reduction in PMEC-4(A745T)::GFP neurons. Note the slight reduction in the number of puncta for PMEC-4::GFP worms when reared at 25 °C (compare first bar on the left in C and F, p < 0.05 by Student’s t test) suggesting that the wild-type channel trafficking has a moderate temperature sensitivity. Neuronal processes scored were 12 and 14, respectively. ** indicates p < 0.01 by comparison with PMEC-4::GFP neurons, by Student’s t test.

channel function in vivo, including one that is strongly temperature sensitive for channel assembly but normal in channel functional properties. We examine “borderline” conditions that induce neuronal necrosis and probe the relationship of cell swelling to necrotic death. Our data provide novel information on MEC-4 structure/function and MEC channel complex formation at the same time they reveal mechanistic insight into necrosis.

New Insight into MEC-4 Structure/Function

Of the 80 mec-4 intragenic mutations identified in our large scale screen of 76,000 mutagenized genomes for suppressors of mec-4(d)-induced death, five specify amino acid substitutions in the third extracellular cysteine-rich domain of MEC-4 (the cysteine-rich domain common to other DEG/ENaC family members), underscoring that this domain is critical for normal and toxic DEG/ENaC function (47). 14 mec-4 alleles encode single amino acid substitutions in and around MSDII (11 of these mutations are novel). Eight of these mutations affect residues in the pore region and highlight amino acids that have been implicated in mammalian DEG/ENaC amiloride blockage and ion selectivity (53–57), emphasizing the importance of pore integrity and ion transport for toxicity in vivo and supporting conservation of structure/function throughout the channel class. Three novel sites in the region (Leu279, Thr279, and Leu279) have not previously been implicated in channel function and might affect only the hyperactive configuration of the channel.

Functions of the MEC-4 C-terminal Domain

Genetic screens for touch-insensitive mutants (40, 58), and our screen for specific suppressors of mec-4(d)-induced neuronal death have been extensive and yet a striking paucity of point mutations affecting the intracellular C-terminal MEC-4 domain (amino acids 740–768) has been identified (47). In fact, of the 136 (50 (47) + 86 (this study)) sequenced mutant mec-4 alleles, only two (both newly reported here) affect the C-terminal domain. One implication of this finding is that the intracellular C-terminal domain may not provide functions essential for channel activity. However, since genetically engineered mutations that eliminate the C terminus or that substitute Ala for a stretch of four lysines (amino acids 753–756) disrupt both normal MEC-4 and hyperactive MEC-4(d) activity (47), it appears more likely that the C terminus is critical to MEC-4 function but that multiple changes may be often required to disrupt biological activity.

One of the substitutions at the C terminus (encoded by bz179) eliminates the MEC-4 stop codon and would extend the C terminus by 7 amino acids if translated. Because the last 12 MEC-4 amino acids appear dispensable (as determined by analysis of genetically engineered mec-4 mutants capacity for functional complementation) and because GFP can be added at the end of MEC-4 to create a MEC-4 protein fusion that can restore function in a null mutant background (47), it may be that the mutation bz179 disrupts transcription termination sites or confers a novel function to disrupt channel activity.

A Mutation That Confers a Temperature-sensitive Defect in Channel Surface Expression

Another change in the MEC-4 C terminus, the A745T substitution, confers several interesting properties to the channel subunit. The A745T substitution introduces additional amino acid volume and polarity at this site in the MEC-4 C-terminal region. If the amphipathic transmembrane pore α-helix extends into the C-terminal domain, res-
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This possibility could easily be tested for mammalian family members using site-directed mutagenesis techniques.

It is interesting that the low efficiency surface expression phenotype of the A745T subunit is common to both Xenopus oocytes and nematode neurons. One possibility is that a change in subunit structure is causative for the defect; alternatively, the trafficking/maintenance machinery might be conserved between nematodes and vertebrates. Note that it is not unusual for a mutation that interferes with efficient channel transport to the cell surface to be temperature-sensitive (ts); ts mutants have been characterized for other channel types including CFTR and cardiac K⁺ channel KCNH2 (also known as HERG) (59, 60). In these cases, the channel subunits also function normally but are less efficiently inserted into the plasma membrane and are massively retained in the endoplasmic reticulum (60).

Implications for the in Vivo Study of Necrosis Mechanisms

Necrotic Swelling Can Occur without Death—We initially identified mec-4(u231bz2) as a mutation that limited the extent of neuronal necrosis as compared with the mec-4(u231) background. We later found a strong temperature-dependence for necrosis induction in the mec-4(u231bz2) mutant: at 15 °C death is extensive, at 20 °C death is reduced, and at 25 °C is nearly eliminated. Necrosis suppression appears likely attributed to limited ion influx as fewer functional channels are at the cell surface as the temperature rises. Such an explanation is consistent with the working hypothesis that a critical threshold level of Na⁺ and/or Ca²⁺ influx is required for necrosis initiation.

In mec-4(d) mutants, touch receptor neurons swell to several times their normal cell diameter before they disappear from the animals (46, 48). At the ultrastructural level, swelling is associated with expansion of intracellular electron dense whorls that appear membranous in nature. In mec-4(u231bz2) mutants, we observed that some touch receptor neurons swell and appear necrotic, but these same cells then recover to survive and adopt a normal morphology later in adulthood. This finding indicates that dramatic neuronal swelling can occur prior to the commitment to death. In addition, this result suggests that neurons have the capacity to recover after extensive swelling to restore ion homeostasis. The point of commitment to necrosis must occur consequent to swelling.

New Capacity for Inducible Necrosis Induction—The strong temperature dependence of necrosis induction in the mec-4(u231bz2) mutant constitutes a long sought breakthrough for the in vivo analysis of necrosis mechanisms by enabling necrosis to be induced by temperature shift. For example, in C. elegans screens for pharmacological reagents that disrupt ion channel toxicity using this model have been thus far hampered by the fact that MEC-4(d) toxicity is induced in late embryogenesis when embryos are still protected by the egg shell from drugs in the external environment. Delaying necrosis until adulthood should for the first time render high throughput screens for novel necrosis inhibitors feasible and efficient. Moreover, options for temperature-sensitive necrosis induction will facilitate elaboration of the temporal sequence of events that transpire during necrosis.

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REFERENCES

1. Kellenberger, S., and Schild, L. (2002) Physiol. Rev. 82, 735–767
2. Hummler, E., Barker, P., Gaty, J., Beeramann, F., Verdueno, C., Schmidt, A., Boucher, R., and Rossier, B. C. (1996) Nat. Genet. 12, 325–328
3. Barker, P. M., Nguyen, M. S., Gaty, J. T., Grubb, B., Norman, H., Hummler, E., Rossier, B., Boucher, R. C., and Koller, B. (1998) J. Clin. Investig. 102, 1634–1640
4. McDonald, F. J., Yang, B., Hrstka, R. F., Drummond, H. A., Tarr, D. E., McCray, P. B., Jr., Stokes, J. B., Welsh, M. J., and Williamson, R. A. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 1727–1731
5. Garty, H., and Palmer, L. G. (1997) Physiol. Rev. 77, 359–396
6. Kristal, O. (2003) Trends Neurosci. 26, 477–483
7. Price, M. P., Lewin, G. R., McIlwrath, S. L., Cheng, C., Xie, J., Heppenstall, P. A., McIlwrath, S. L., Cheng, C., Xie, J., Heppenstall, P. A., and Welsh, M. J. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 4170–4175
8. Price, M. P., Ainsley, J. A., Pettus, J. M., Bosenko, D., Gerstein, C. E., Zinkevich, N., Anderson, C. C., Lamani, E., Cassell, M. D., Freeman, J. H., Jr., and Welsh, M. J. (2003) Nature 423, 5496–5502
9. Wemmie, J. A., Askwith, C. C., Cassell, M. D., Freeman, J. H., Jr., and Welsh, M. J. (2003) J. Neurosci. 23, 463–477
10. Wemmie, J. A., Askwith, C. C., Cassell, M. D., Freeman, J. H., Jr., and Welsh, M. J. (2003) J. Neurosci. 23, 463–477
11. Liu, L., Johnson, W. A., and Welsh, M. J. (2000) Brain Res. 884, 1–12
12. Askwith, C. C., Benson, C. J., Welch, M. J., and Snyder, P. M. (2001) Proc. Natl. Acad. Sci. U. S. A. 100, 2128–2133
13. Kellenberger, S., Auberson, M., and Welsh, M. J. (2000) Brain Res. 884, 1–12
14. Peng, B. G., Ahmad, S., Chen, S., Chen, P., Price, M. P., and Lin, X. (2004) J. Neurosci. 24, 10167–10175
15. Kretz, O., Barbry, P., Bock, R., and Lindemann, B. (1999) J. Histochim. Cytochem. 47, 51–64
16. Liu, L., Leonard, A. S., Motto, D. G., Feller, M. A., Price, M. P., Johnson, W. A., and Welsh, M. J. (2000) Neurosci 188, 14745–14754
17. Rosenbluth, R. E., Cuddeford, C., and Ballie, D. L. (1985) Genetics 109, 493–511
18. Breuer, H. J., and Pusch, H. (1996) Am. J. Physiol. 271, C161–C175
19. Emtage, L., Gu, G., Hartwig, E., and Chalfie, M. (2004) Neuron 44, 795–807
20. Shim, J., Umemura, T., Noethstoin, E., and Rongo, C. (2004) Mol. Biol. Cell 15, 4818–4828
21. Bocker, S., and Bender, J. (1992) J. Cell Biol. 119, 803–814
22. Chalfie, M., and Mo, S. (1989) Science 243, 1027–1033
23. Driscoll, M., and Schafer, W. R. (2001) Neuron 29, 795–807
24. Chalfie, M., and Mo, S. (1989) Science 243, 1027–1033
25. Chalfie, M., and Mo, S. (1989) Science 243, 1027–1033
26. Chalfie, M., and Mo, S. (1989) Science 243, 1027–1033
27. Chalfie, M., and Mo, S. (1989) Science 243, 1027–1033
28. Chalfie, M., and Mo, S. (1989) Science 243, 1027–1033
29. Chalfie, M., and Mo, S. (1989) Science 243, 1027–1033
30. Chalfie, M., and Mo, S. (1989) Science 243, 1027–1033
31. Chalfie, M., and Mo, S. (1989) Science 243, 1027–1033
32. Chalfie, M., and Mo, S. (1989) Science 243, 1027–1033
33. Chalfie, M., and Mo, S. (1989) Science 243, 1027–1033
34. Chalfie, M., and Mo, S. (1989) Science 243, 1027–1033
35. Chalfie, M., and Mo, S. (1989) Science 243, 1027–1033
36. Chalfie, M., and Mo, S. (1989) Science 243, 1027–1033
37. Chalfie, M., and Mo, S. (1989) Science 243, 1027–1033
38. Chalfie, M., and Mo, S. (1989) Science 243, 1027–1033
39. Chalfie, M., and Mo, S. (1989) Science 243, 1027–1033
40. Chalfie, M., and Mo, S. (1989) Science 243, 1027–1033
41. Chalfie, M., and Mo, S. (1989) Science 243, 1027–1033
42. Chalfie, M., and Mo, S. (1989) Science 243, 1027–1033
43. Chalfie, M., and Mo, S. (1989) Science 243, 1027–1033
44. Chalfie, M., and Mo, S. (1989) Science 243, 1027–1033
45. Chalfie, M., and Mo, S. (1989) Science 243, 1027–1033
46. Chalfie, M., and Mo, S. (1989) Science 243, 1027–1033
47. Chalfie, M., and Mo, S. (1989) Science 243, 1027–1033
48. Chalfie, M., and Mo, S. (1989) Science 243, 1027–1033
49. Chalfie, M., and Mo, S. (1989) Science 243, 1027–1033
50. Chalfie, M., and Mo, S. (1989) Science 243, 1027–1033
51. Chalfie, M., and Mo, S. (1989) Science 243, 1027–1033
52. Chalfie, M., and Mo, S. (1989) Science 243, 1027–1033
53. Chalfie, M., and Mo, S. (1989) Science 243, 1027–1033
54. Chalfie, M., and Mo, S. (1989) Science 243, 1027–1033
55. Chalfie, M., and Mo, S. (1989) Science 243, 1027–1033
56. Chalfie, M., and Mo, S. (1989) Science 243, 1027–1033
57. Chalfie, M., and Mo, S. (1989) Science 243, 1027–1033
58. Chalfie, M., and Mo, S. (1989) Science 243, 1027–1033
59. Chalfie, M., and Mo, S. (1989) Science 243, 1027–1033
60. Chalfie, M., and Mo, S. (1989) Science 243, 1027–1033
61. Chalfie, M., and Mo, S. (1989) Science 243, 1027–1033