Mutations in the Extracellular Domain and in the Membrane-Spanning Domains Interfere with Nicotinic Acetylcholine Receptor Maturation†

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Received March 11, 2002; Revised Manuscript Received August 15, 2002

ABSTRACT: The *deg-3*(*u662*) mutation is a degeneration-causing mutation in a *Caenorhabditis elegans* nicotinic acetylcholine receptor. In a large screen for mutations that suppress the deleterious effects of this mutation we identified 32 mutations in the *deg-3* gene. Among these, 11 are missense mutations, affecting seven residues within the extracellular domain or the membrane-spanning domains. All of these mutations greatly reduce the degeneration-causing activity of *deg-3*(*u662*). All but one of these mutations cause defective localization of the DEG-3 protein, as seen in immunohistochemical analysis. Thus our screen identifies multiple residues within the nicotinic acetylcholine receptor needed for normal folding, assembly, or trafficking of this receptor. Interestingly, these mutations lead to distinct localization defects suggesting differences in their effect on DEG-3's maturation process. Specifically, mutations in the extracellular domain lead to a phenotype more severe than mutations in the membrane-spanning domains. Differences in the effects of the mutations are also predicted by homology-based modeling, showing that some mutations in the extracellular domain are likely to disrupt the native fold of the protein, while others are likely to disrupt trafficking.

Nicotinic acetylcholine receptors are members of the ligand-gated ion channel superfamily. These receptors are pentamers, usually heteromers, composed of specific α and non-α subunits. The subunits have a large amino-terminal extracellular domain, four membrane-spanning domains, and a large intracellular loop between transmembrane domains III and IV (*1*). The receptor subunits fold and assemble into pentamers in the endoplasmic reticulum and are then exported through the Golgi apparatus to the plasma membrane. An initial step in this folding and assembly process requires intersubunit contacts mediated by the amino-terminal extracellular domain, a process that leads to formation of the ligand binding site. This initial assembly step is followed by protein folding and additional intersubunit contacts likely to be governed by residues outside the amino-terminal extracellular domain (*2*−*4*). The identity of specific residues that mediate either the initial or the later steps of receptor assembly or folding is largely unknown. The process of folding and pentamer assembly is a prerequisite for ER[1] exit (*5*−*7*), and unassembled or misfolded receptors are targeted for degradation (*8*).

To identify genes and residues needed for nAChR activity in *Caenorhabditis elegans*, we screened for suppressors of a dominant mutation in the nAChR subunit DEG-3 (*9*). The *deg-3*(*u662*) mutation is a substitution of an amino acid in the pore-forming domain (transmembrane domain II) of the DEG-3 protein, a mutation leading to deregulated channel activity and consequently to cell death (*9*). This screen led to the identification of mutations in *des-2*, a subunit of the DEG-3 receptor (*10*), and in *ric-3*, a gene needed for nAChR maturation (*11*). In addition, this screen identified many mutations in DEG-3 itself. Analysis of these mutations identified seven residues (11 alleles) that when mutated suppress the degeneration-causing activity of *deg-3*(*u662*). Mutations affecting all but one of these residues lead to mislocalization of DEG-3. Thus, our analysis identified specific residues within the amino-terminal extracellular domain and within the second and third membrane-spanning domains that are necessary for receptor folding, assembly, or trafficking.

EXPERIMENTAL PROCEDURES

Strain Maintenance and Genetics. The wild type was N2 Bristol, and all strains were grown as previously described (*12*). Screens for suppressors of *deg-3*(*u662*) were described previously (*9*). In short, more than 30000 EMS mutagenized haploid genomes were screened for suppression of the uncoordinated and mechanosensory defective phenotypes associated with *deg-3*(*u662*). Mutations in *deg-3* were identified following outcross of the mutant strains identified in these screens with N2 males. Mutations in *deg-3* itself suppress the appearance of the dominant *deg-3*(*u662*) phenotype in all F1 and F2 progeny of these crosses (at least 1000 F2 progeny were examined). The *des-2*(*hm71*) mutation was identified in a noncomplementation screen for *des-2*

† This research was supported by the Israel Science Foundation (Grant 493/01-16.6). A.O.S. is supported by U.S.−Israel Binational Science Foundation Grant 98-328 to Jacob Anglister.

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[1] Abbreviations: nAChR, nicotinic acetylcholine receptor; AChBP, acetylcholine binding protein; EMS, ethyl methanesulfonate; ER, endoplasmic reticulum.

10.1021/bi020193y CCC: $22.00
Published on Web 09/20/2002

alleles. Specifically EMS mutagenized N2 males were mated with *des-2*(*u695*)*deg-3*(*u662*) hermaphrodites, and their progeny were screened for suppression of the behavioral defects seen in *des-2*(*u695*)*deg-3*(*u662*)/++ animals.

Immunohistochemistry and lacZ Stainings. Visualization of DEG-3 localization was done as described previously using an antibody directed against a peptide identical to the C-terminus of DEG-3 (*13*). Quantification of the DEG-3 staining patterns was done in the Hebrew University−Hadassah Medical School Interdepartmental Equipment Department on pictures acquired using a Zeiss Axiovert 200 microscope equipped with a Sensicam PCO camera under nonsaturating conditions and analyzed using Image Pro Plus (version 4.1). Cells were assigned to each class of staining patterns according to the staining distribution within the cell and morphology of the cell. Intensity of staining is the mean density of pixels within the cell body. A measurement for the distribution of staining is provided by measuring the area occupied by pixels whose density is above the median density relative to the entire area of the stained cell body (the nuclei which do not stain were excluded from this analysis). The significance of these results is measured using the paired *t*-test. Survival of *deg-3 lacZ* expressing PVDs was examined as in ref *10*.

Sequencing of Mutations and Sequence Analysis. Sequencing of *deg-3* alleles was done directly from PCR-amplified DNA in the Life Science Sequencing Facilities (Hebrew University). Primers used for amplification were CCTATCTTGACCTTCTTGACCAAC and GCATTCCTTCAGCCTCCAAC, leading to amplification of the entire DEG-3 open reading frame. Sequencing was done using the same primers and internal primers CGCGTGTTGTGCCGAGCC and CCATTAACATCAAGATAGAC. Sequence alignments were done using ClustalW analysis [McVector (*14*)].

Homology Modeling of deg-3. The structural template that served for the *deg-3* modeling was AChBP (*15*). The sequence alignment of *deg-3* and AChBP presented 19% sequence identity (and a sequence similarity of over 33%). The *deg-3* model was delimited to residues 54−266 of the *deg-3* subunit, because they spanned the AChBP sequence and presented no insertion or deletion exceeding five amino acids. The sequence alignment obtained from ClustalW displayed six minor insertions and two single amino acid deletions. The segments connecting these insertions and deletions were considered structurally conserved regions, in which the conformation of the polypeptide chain is unchanged. Random loops were generated where the insertion or deletions had occurred using the Homology module in the Accelrys package. No backbone−backbone clashes were observed. Side chains exhibiting steric clashes with other side chain or backbone atoms were manually assigned with an alternative rotamer conformation. Finally, the loops' conformations were refined using molecular dynamics calculations.

RESULTS

deg-3(*u662*) is a missense mutation in the pore-forming domain of the acetylcholine receptor subunit DEG-3. This mutation (I314N) causes deregulated receptor activity leading to degeneration of neurons expressing this receptor and concomitantly to uncoordinated movement and mechanosensory defects (*9*, *10*). We screened for mutations which suppressed the behavioral defects caused by the *deg-3*(*u662*) mutation. For this purpose we mutated *deg-3*(*u662*) homozygous animals and screened for F2 progeny that regained normal behavior. Presumably, such suppressor mutations would identify genes and residues required for DEG-3 receptor function. This screen produced 51 mutations that suppressed the behavioral defects as well as neuronal degeneration. These mutations fall into three groups: mutations in *des-2*, a second subunit of the DEG-3 receptor [11 mutations (*10*)], mutations in *ric-3*, a gene needed for nAChR maturation [4 mutations (*11*)], and mutations in *deg-3* itself (36 mutations). DEG-3 is a nonessential receptor; thus either reduction in the activity of DEG-3 or specific suppression of the *u662* gating defect will suppress the deleterious effects of *deg-3*(*u662*).

FIGURE 1: Genomic map of *deg-3* showing positions of mutations identified in this study: black boxes, coding region; empty boxes, membrane-spanning domain; lines, introns; circles, missense mutations; triangles, nonsense mutations. The results of sequence analysis of the mutations are as follows, including the position and nature of the amino acid substitution, the position and nature of the base change (using the first A of the first ATG in the cDNA as reference), and the number of identical alleles (when more than one): missense mutations, E112K:G334A, P137S:C409T, P137L:C410T, G170R:G508A, G170E:G509A (two alleles), S205N:G614A, G305E:G914A (two alleles), L309F:C925T, Y337D:T1009G; nonsense mutations, W104:G315A (five alleles), W109:G326A, W135:G405A (three alleles), R196:C586T (two alleles), W253:G758A, Q323:C967A, W395:G1184A, Q449:C1345T (three alleles), W479:G1436A (two alleles), W517:G1551A (two alleles).

To understand how the mutations in DEG-3 suppress its toxic activity, we amplified and sequenced the genomic region spanning the entire DEG-3 open reading frame from each *deg-3* mutant strain. This analysis identified mutations in 32 of 36 mutant strains. The remaining 4 strains probably contain mutations in yet uncharacterized regulatory regions. The 32 mutations affecting *deg-3* include 21 nonsense mutations that truncate the DEG-3 protein in 10 different sites and 11 missense mutations affecting seven different amino acids in the extracellular domain and in the second and third membrane-spanning domains (Figure 1 and Table 1). The clustering of several mutations to single sites, 9 of 17 sites were mutated several times, shows that this mutagenesis is close to saturation. In addition, this clustering is evidence for nonrandomness of the mutagenic process. Specifically, 7 of the missense mutations cluster within 93 amino acids of the 565 amino acids DEG-3 protein. The reasons for this nonrandom distribution are sequence specificity of ethyl methanesulfonate (EMS; the mutagen used in the screen) and differing sensitivity of receptor domains to mutation. Specifically, amino acids that when mutated lead to significant suppression of toxic *deg-3*(*u662*) activity may be rare. Indeed, the clustering of missense mutations, to a region spanning less than half of the mature protein, is even more pronounced than the clustering of nonsense mutations, suggesting that this second reason is an important determinant for the observed clustering.

All of the missense mutations are strong suppressors of the degeneration process, as seen by their effect on the number of swollen cells, the hallmark of the degeneration

Table 1: Summary of Missense Mutations

allele	mutation	domain[a]	no. of swollen cells[b]	monomer stability[c]	staining pattern[d]
hm52	E112K	extracellular	0.26 ± 0.4	no effect	B
hm24	P137S	extracellular	0.08 ± 0.27	reduced	B
hm60	P137L	extracellular	ND	reduced	A
hm39, hm40	G170E	extracellular	ND	no effect	B
hm30	G170R	extracellular	ND	no effect	A
hm16	S205N	extracellular	ND	reduced	B
hm10, hm12	G305E	TMDII	ND	NA	WT
hm61	L309F	TMDII	ND	NA	C
hm59	Y337D	TMDIII	0.02 ± 0.14	NA	C

[a] TMD = transmembrane domain. [b] The number of swollen cells was examined in 50 newly hatched larvae 0−3 h after hatching for each strain. When the same mutation was found in more than one allele, a strain carrying the underlined allele was examined. Compare to 2.3 ± 1.2 swollen cells in newly hatched *deg-3*(*u662*) larva. [c] Monomer stability is predicted for mutations affecting the N-terminus according to their effects on intermolecular interactions likely to stabilize the DEG-3 monomer, as seen in the homology-based model. [d] This provides the dominant staining pattern according to data summarized in Figure 4.

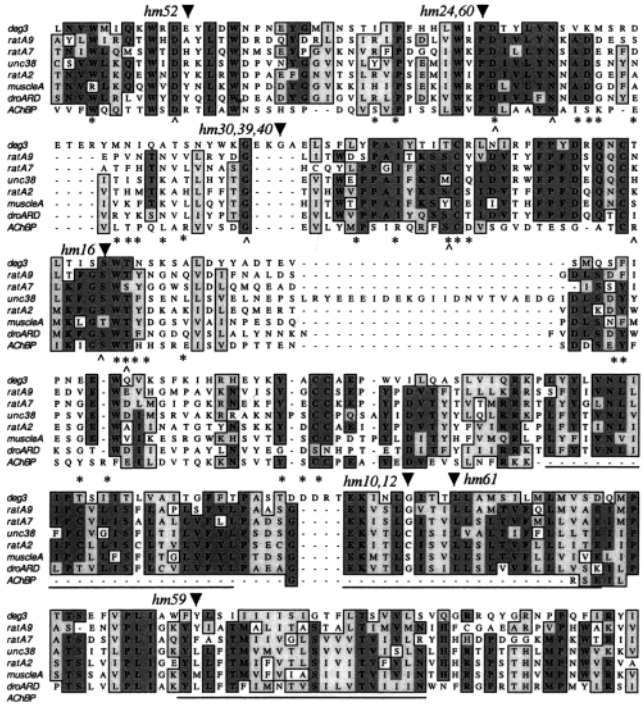

FIGURE 2: Alignment of representative nAChR α subunits and AChBP (ClustalW analysis). Shown is the region spanning the mutations found in this study. Filled arrows indicate the mutated residues, asterisks appear below residues at the interface between AChBP subunits, ∧ appear below residues implicated in folding and stabilization of AChBP, and lines appear below the membrane-spanning domains.

process (Table 1; relative to 2.3 ± 1.2 swollen cells in newly hatched *deg-3*(*u662*) larva, $n = 50$). Such mutations are likely to exert their suppressing effect through a strong reduction in receptor activity or specific suppression of the degeneration-causing activity. To gain further insights into the role played by these residues in nAChR function, we aligned the sequence of DEG-3 with representatives of the nAChR family (*16*) and with the recently characterized acetylcholine binding protein [AChBP (*15*); Figure 2]. In addition, we used a homology-based model of DEG-3 in order to identify mutations leading to destabilization of protein structure or loss of intermolecular interaction needed for protein stabilization (Figure 3). The results of this analysis are provided in the next paragraphs.

Four residues, identified through seven *deg-3*(*u662*) suppressing mutations, affect the N-terminal extracellular domain. These residues cluster to a region of 93 amino acids which overlaps with regions required for intersubunit contacts during nAChR and AChBP assembly (*3*, *15*).

Three of the mutations affecting the N-terminal domain (*hm16*, *hm24*, *hm60*) affect the highly conserved residues Ser 205 and Pro 137, conservation that is consistent with the importance of these residues in receptor function. Indeed, substituting the highly conserved hydrophobic Pro 137, a deeply buried core residue, by the polar amino acid serine is likely to cause significant destabilization of the DEG-3 monomer (Figure 3). Changing Pro 137 into leucine is also likely to perturb the backbone of the protein. Similarly, the hydroxyl group of Ser 205 forms a hydrogen bond with the carboxylate of the deeply buried Asp 138 and is directly involved in the maintenance of native DEG-3 folding (*15*; Figure 3). This hydrogen bond is disrupted by the elongated side chain of the *hm16* mutant residue Asn 205, thus destabilizing the N-terminal domain.

However, other N-terminal domain mutations (*hm52*, *hm30*, *hm39*, *hm40*) affect nonconserved residues Glu 112 and Gly 170 (Gly 170 is found within a small sequence of amino acids that is unique to DEG-3). Such residues may be needed for DEG-3 specific functions. *hm52* (E112K), a mutation that substitutes positive charge instead of the original negative charge, affects a residue that is exposed on the surface of DEG-3 whose AChBP analogue is also a positively charged arginine (Figures 2 and 3). As *hm52* affects a surface residue that is not at the subunit interface, it is unlikely to influence protein structure or subunit assembly. Similarly, substitution of Gly 170 by Glu or Arg (*hm39*, *hm40*, and *hm30*, respectively), leading to insertion of bulky charged residues instead of the small glycine, is unlikely to destabilize DEG-3 structure or affect its assembly, since Gly 170 is located close to the surface of DEG-3. Gly 170 is in the vicinity of Glu 112 on the ternary structure (Figure 3). Thus, the region that contains Glu 112 and Gly 170 may mediate interaction with yet unknown factors needed for maturation or activity of DEG-3.

Two residues in transmembrane domain II mutate to suppress *deg-3*(*u662*); both residues face away from the channel pore on a hydrophobic surface of the helix (*17*; homology-based modeling not shown). G305E (*hm10*, *hm12*) inserts a large negatively charged residue, instead of the small glycine residue, that is probably neutralized by a salt bridge formed with Lys 301. This mutation therefore is unlikely to affect the overall conformation of DEG-3; however. it may affect channel properties. L309F (*hm61*) affects a highly conserved aliphatic residue that may be needed for protein−protein interactions between the membrane helices (Figure 2).

Last, one mutation affecting transmembrane domain III also suppresses *deg-3*(*u662*); Y337D (*hm59*) affects a residue likely to face away from the lipid interface (*18*). While the role of this residue is unknown, insertion of a negative charge into a mostly hydrophobic region may interfere with protein−

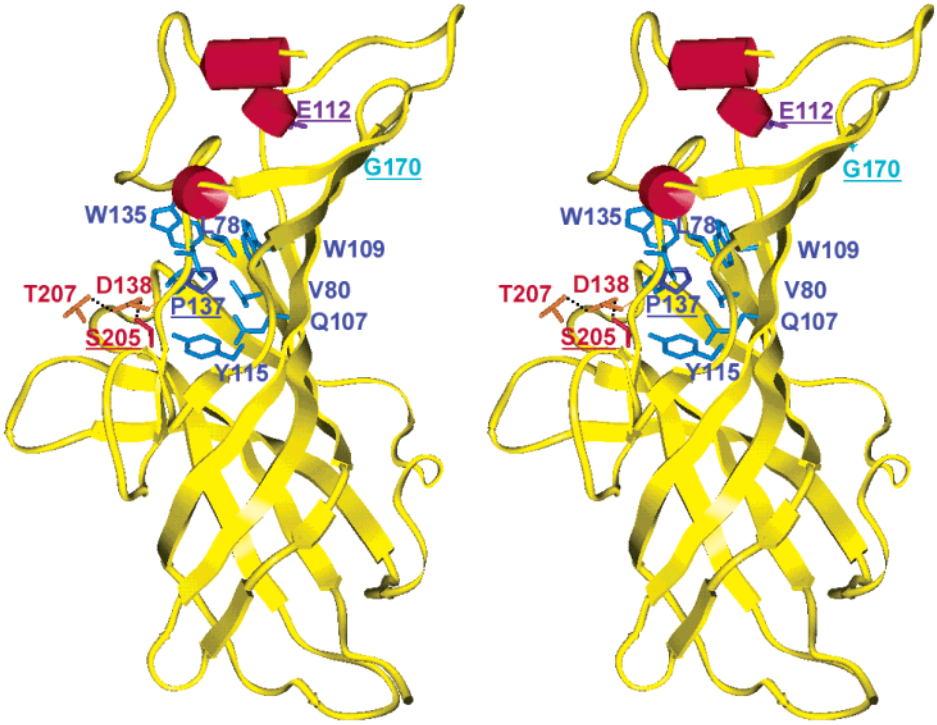

FIGURE 3: Stereoview of the DEG-3 extracellular N-terminal domain. Shown is a ribbon diagram representation of the model, the mutations located on the extracellular domain, and their interactions. Underlined are residues affected by missense mutations: E112 in magenta, P137 in dark blue, G170 in cyan, and S205 in red. Residues in blue are in close contact (<4 Å) and interact with P137. Residues in orange form a network of hydrogen bonds with S205 through their side chain.

protein interactions needed for receptor folding, assembly, or stability (Figure 2).

The sequence analysis suggests that some of the missense mutations identified in this study are likely to affect receptor folding, assembly, or trafficking. Folding and pentamer assembly are a prerequisite for ER exit (*5*−*7*), and unassembled or misfolded receptors are probably targeted for degradation (*8*). Thus mutations that reduce receptor assembly, folding, or trafficking are likely to result in both reduction of receptor quantity and receptor mislocalization. To examine this possibility, we used antibodies directed against the C-terminus of DEG-3 for immunohistochemical analysis (*13*). As expected, strains carrying nonsense mutations did not stain for DEG-3, in agreement with the fact that all nonsense mutations truncate DEG-3 before the C-terminus (data not shown). On the other hand, all missense mutants stain for DEG-3, but the distribution and intensity of staining are affected in all but one (G305E in TMDII) of these mutations (Figures 4−6).

To further characterize the localization defects seen in the various mutants, we focused on the PVD sensory neurons, a pair of neurons that express high levels of DEG-3 and that are easily identified (*13*). In wild-type (N2) animals DEG-3 antibodies stain the PVD's cell body (in the midbody): two long processes, one extending to the head and one to the tail, and additional lateral processes (Figure 4D). In all but one (G305E) of the missense mutations we found significant alterations in this staining pattern. Careful analysis of DEG-3's distribution in the mutants led to the identification of three distinct mutant staining patterns in addition to the wild-type staining pattern (Figures 4 and 6). The A-type staining pattern is defined by intense staining of the cell body while staining in the processes cannot be detected (Figure 4A).

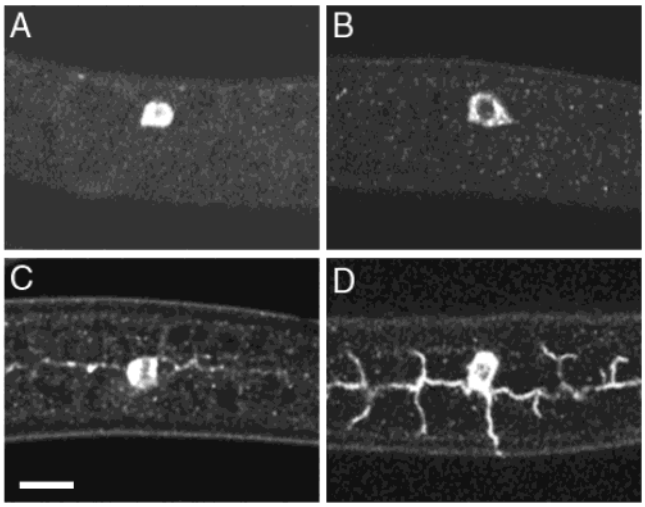

FIGURE 4: Representative staining types: A, B, and C as in the text; D, wild-type staining. Pictures are confocal thin sections. Scale bar is 10 μm.

This defective distribution correlates with a change in the morphology of the cells; the cells appear round, and an intense uniform staining obscures the nucleus. This is unlike wild-type cells that are triangular or oval and where the DEG-3 distribution is more circumferential. The second staining pattern, type B, like type A, is characterized by staining of the cell body with no detectable staining in processes (Figure 4B); unlike type A staining, the intensity of type B staining is normal and cell morphology appears normal. Last, we find cells, defined as type C, in which staining is seen in both cell body and processes (Figure 4C). However, the stained processes are short and have no lateral processes. Using these definitions for the three mutant

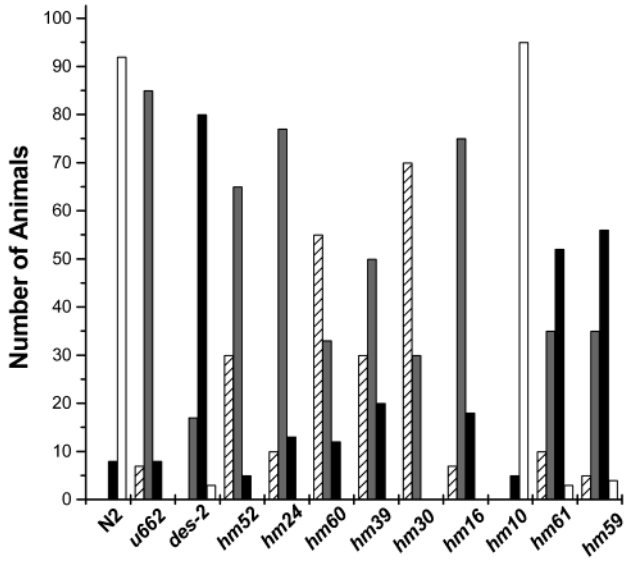

FIGURE 5: Distribution of staining patterns in mutants. PVD cells from different strains were examined for their staining pattern: type A staining, striped; type B staining, gray; type C staining, black; wild-type staining, white. One hundred animals were characterized in each strain.

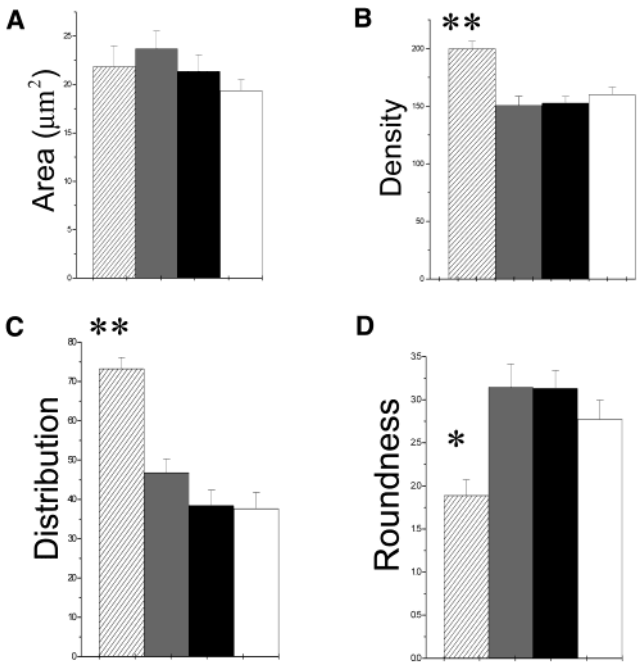

FIGURE 6: Analysis of staining patterns: (A) average area of stained cells (μm^2); (B) mean density (intensity) of pixels within the cell body; (C) distribution of staining within the cells; (D) deviation of cell shape from being round (1, no deviation). The analysis was done on 20 cells from each staining pattern (except for the analysis of staining intensity where only 10 cells where examined for wild-type and type C stainings). Staining patterns: type A staining, striped; type B staining, gray; type C staining, black; wild-type staining, white. Significance relative to wild type was examined using a paired *t*-test: one asterisk, 95% confidence; two asterisks, 99% confidence.

staining patterns, we analyzed the staining patterns in different mutants (Figure 5). This analysis shows a mixture of staining phenotypes for most mutants, suggesting that maturation defects manifest themselves in more than one defective localization pattern. However, each mutation is associated with one dominant staining pattern that appears in half or more of the animals.

All mutations characterized in this study were obtained in the background of the *u662* mutation. Thus, we needed to rule out the possibility that this mutation is sufficient for the observed localization defects. Characterization of DEG-3 staining in *u662* animals is difficult, as cells die soon after their first appearance. Specifically, survival of the PVDs, a pair of neurons that appears late in development (L3-L4), is very low as seen using *deg-3 lacZ* expression. In 50 L4 adults no *deg-3 lacZ* is seen in PVDs, unlike wild type where 27 of 28 animals showed PVD *deg-3 lacZ* expression. However, among many DEG-3 stained animals we did find a few animals with staining in the PVDs; these showed a predominantly type B staining pattern. Thus, we cannot rule out that type B localization defects are caused by the *u662* mutations. However, a more likely explanation for this staining pattern is that we are identifying a transient intermediate stage in the maturation of the PVD, shortly after the first expression of DEG-3 in these cells. This usually transient stage predominates in the population of *deg-3*(*u662*) stained PVDs since additional progress in the maturation of the DEG-3 receptor leads to immediate cell death. Alternatively, *deg-3*(*u662*)-induced degenerations are similar to what is seen in degenerin [*mec-4d* and *deg-3*(*u38*)] induced degenerations (*9*); thus it is also possible that the *deg-3*(*u662*)-induced degeneration process interferes with extension of axons, as was seen in *mec-4d*-induced degenerations, leading to type B like staining (*19*). Indeed, the staining patterns seen in *deg-3*(*u662*) differ from what is seen in the double mutants; in *deg-3*(*u662*) 85% of PVDs show type B staining unlike other mutants (except *hm24*) where significant numbers of other defective staining patterns are found in addition to the typeB staining pattern (Figure 5).

The immunohistochemical analysis clearly shows that the newly identified DEG-3 mutant proteins are not found in their wild-type position. Thus, it is of interest to find out the fate of the mislocalized proteins. For this purpose it is necessary to quantify DEG-3 in the wild-type and in the mutant strains. Because the length and complexity of PVD processes make quantification of DEG-3 in the processes difficult, we quantified DEG-3 within the cell body. Measurement of the area occupied by the cell body in cells belonging to each type of staining pattern showed no significant difference (Figure 6). Thus, it is possible to use the average intensity of staining within each cell as a measurement for the relative quantity of DEG-3 found within that cell. This analysis shows that all mutant cells have wild-type or greater than wild-type staining intensity. Specifically, type B and C cells show wild-type staining intensity while type A staining is 36% higher. In contrast, staining in the processes of the mutants is either eliminated (type A and B staining) or greatly reduced (type C staining); thus the overall quantity of DEG-3 in all of these cell types is lower than in the wild type. These findings can be interpreted if we assume that most of DEG-3 staining within the cell body is caused by immature receptor intermediates. These maturation intermediates are sequestered within the cell body pending maturation or degradation. Thus, the quantity of DEG-3 within the cell body represents an equilibrium between synthesis, degradation, and maturation followed by transport out of the cell body. According to this model, DEG-3 staining patterns in all mutant cell types suggest a defect in maturation that interferes with transport of DEG-3 out of the cell body,

leading instead to degradation of the nonmaturing receptors. In type A cells, the increase in the intensity of DEG-3 staining suggests inefficiency of this degradation process. Interestingly, in type A staining increased quantities of DEG-3 in the cell body correlate with changes in staining distribution and morphology (Figures 4 and 6), suggesting ER accumulation of stable unfolded or unassembled proteins similar to what is found in some dominant retinal degeneration causing mutations in *Drosophila* rhodopsin (*20*).

All but one of the mutations characterized in this study are likely to affect DEG-3 maturation. Thus it is interesting to compare their effects to these other mutations likely to affect DEG-3 maturation. Previously, we have shown that in *ric-3* mutants DEG-3 staining is seen in the processes but with greatly reduced intensity (*11*). This staining pattern is clearly different from the mutant staining patterns described here, in all of which stained processes are either very short or absent, suggesting a difference in the effects of *ric-3* mutations relative to the effects of mutations characterized here on DEG-3 maturation. Mutations in *des-2*, a subunit of the DEG-3 channel (*10*), are likely to affect assembly of the DEG-3 receptor. Here we show that in a *des-2*(*hm71*) mutant most PVD cells show type C staining (Figure 5). Thus a mutation in *des-2* likely to affect receptor assembly leads to a phenotype that is similar to what is seen in *deg-3*(*hm61*) and *deg-3*(*hm59*) mutations in transmembrane domains II and III, respectively.

DISCUSSION

In a large screen for mutations that suppress the cytotoxic effects of deregulated DEG-3 activity we identified seven residues that when mutated interfere with DEG-3 activity. This screen is close to saturation as evidenced by the identification of multiple mutations affecting the same residue. Thus, the identification of only seven residues that when mutated suppress *deg-3*(*u662*) is surprising. Two reasons combine to explain this small number: First, EMS (a chemical mutagen) preferentially causes G/C to A/T base transitions; thus some amino acids will mutate to other amino acids at very low frequencies (*21*). Indeed the mutations identified in this study demonstrate this preference with 69% G to A base transitions, 25% C to T, and 3% T to G and C to A (one mutation each). Second, our screen identified mutations that either greatly reduce DEG-3 activity or specifically suppress the *u662* mutations, mutations that may be rare. Thus, while we saturated for EMS-generated mutations that suppress *deg-3*(*u662*), we may have not saturated for all possible mutations that suppress *deg-3*(*u662*).

The screen for suppressors of *deg-3*(*u662*) behavioral defects has identified *deg-3* mutations that show strong suppression of the degeneration process. This suppression is caused by truncation of the protein or by disrupting its maturation. One mutant alone shows normal DEG-3 localization; this mutant, G305E (*hm10*, *hm12*), may lead to production of a properly localized but nonfunctional receptor or to production of a functional receptor in which the toxic effects of *deg-3*(*u662*) are specifically suppressed. Indeed, molecular modeling suggests that the G305E mutation stabilizes DEG-3-folding while perturbing pore structure. Likewise, the other missense mutations may affect receptor function in addition to their effect on receptor maturation, as E112K (*hm52*), a mutation that eliminates DEG-3 from the processes, still causes some cell swelling, demonstrating that localization defects alone, although very severe, may not explain the elimination of *deg-3*(*u662*)-induced cell swelling seen in most missense mutants.

We have previously shown that DEG-3 staining is seen in both the cell body and cell processes of DEG-3 expressing neurons. We have also suggested that staining within the cell body is a result of receptor maturation intermediates that have not yet reached the membrane (*13*). Indeed, most of the missense mutations identified in this study, mutations that are likely to interfere with receptor maturation, show normal DEG-3 staining in the cell body while staining in the processes is eliminated or greatly reduced. Such a finding is consistent with the suggestion that most staining in the cell body represents nonfunctional intermediates in the maturation of the DEG-3 receptor. This analysis also supports the suggestion that receptors that do not proceed to maturity are degraded (*8*). However, two mutations, G170R (*hm30*) and P137L (*hm60*), lead to increased DEG-3 staining within the cell body. This increase can be explained if these mutations stabilize receptor intermediates. Interestingly, these two mutations affect the same residues as the G170E (*hm39*, *hm40*) and P137S (*hm24*) mutations that do not stabilize the intermediates and which, in the case of P137S, are likely to destabilize receptor folding leading to receptor degradation, as seen in the homology-based modeling. Thus the identity of residues within these sites may lead to stabilization of alternative folding intermediates or affect the efficiency of the machinery that targets unassembled or unfolded receptors for degradation. For G170R, affecting a residue close to the surface of the protein, the second possibility appears more likely.

Our immunohistochemical analysis identified three types of localization defects. The first two, type A and type B, show no detectable DEG-3 in the processes and the third, type C, shows some process staining. Interestingly, all mutations affecting the N-terminal extracellular domain eliminate staining of the processes. Previous studies suggested a role for the N-terminal domain in cotranslational, rapid association of receptor subunits, an association, occurring in the rough endoplasmic reticulum, that is a prerequisite for further maturation (*4*). In addition, studies in mammalian neurons have suggested that the rough endoplasmic reticulum is confined to the cell body (*22*). Three N-terminal domain mutations identified in this study (P137S, P137L, and S205N) are likely to disturb folding of the N-terminus, thus interfering with formation of intersubunit interfaces and with the initial assembly of the DEG-3 receptor. The resulting unfolded and unassembled intermediates remain confined to the rough endoplasmic reticulum leading to DEG-3 staining that is confined to the cell body. Interestingly, three other N-terminal domain mutations (E112K, G170E, and G170R) are unlikely to cause folding or assembly problems but lead to similar DEG-3 mislocalization defects. These mutations may affect interactions with yet unknown chaperones needed for trafficking of properly folded proteins out of the rough endoplasmic reticulum. Here we note that in the folded monomer Glu 112 and Gly 170 are neighbors on the outside surface of the protein. Membrane-spanning domain mutants, unlike N-terminal domain mutants, show staining in the processes similar to what is seen in a

des-2 mutant. This finding is consistent with a role for the membrane-spanning domains at later stages of receptor maturation, assembly, or stabilization of receptors. Thus, our analysis of the in vivo effects of mutations on nAChR maturation detects distinct effects of N-terminal domain mutations vs membrane-spanning domain mutations, a difference predicted by structure−function studies in heterologous expression systems (*4*).

ACKNOWLEDGMENT

We thank Reem Younis for technical assistance, Arieh Weiss for help in image analysis, and Yael Stern Bach for careful reading.

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BI020193Y