BIOCHEMISTRY
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Biochemistry **2009,** *48,* 12329–12336 12329
DOI: 10.1021/bi901234a

Receptor and Subunit Specific Interactions of RIC-3 with Nicotinic Acetylcholine Receptors[†]

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Received July 20, 2009; Revised Manuscript Received November 9, 2009

ABSTRACT: RIC-3 belongs to a conserved family of proteins influencing maturation of nicotinic acetylcholine receptors (nAChRs). RIC-3 homologues were shown to differently affect different nAChRs. Here we show that coexpression with RIC-3 increases the level of surface expression of DEG-3 while slightly reducing the level of surface expression of DES-2, both subunits of the DEG-3/DES-2 nAChRs. Those different effects are a likely explanation for the previously demonstrated effects of RIC-3, an endoplasmic reticulum resident protein, on properties of this receptor. To understand how RIC-3 interacts with different nAChR subunits, we identified and characterized domains and residues enabling this interaction. This analysis shows that conserved residues in the second RIC-3 transmembrane domain are needed for its interactions with two different *Caenorhabditis elegans* nAChRs, DEG-3/DES-2 and ACR-16. These conserved residues do not, however, function alone; rather, we show that additional domains also enable RIC-3's interactions with these receptors. Interestingly, the relative importance of these residues or of other domains mediating interactions of RIC-3 with nAChRs differs for the two different receptors. Differences in the way that RIC-3, predicted to be an intrinsically disordered protein, interacts with different receptors and receptor subunits suggest that it may adopt different conformations to enable these interactions. Such differences may explain both the effects of RIC-3 on receptor properties and the differences in its effects on different receptors.

Nicotinic acetylcholine receptors (nAChRs)[1] are members of the Cys-loop ligand-gated ion channel superfamily. The nAChRs form a large family of receptors mediating both excitatory and modulatory roles (*1*). The nAChRs are pentamers, usually heteromers, and each nAChR subunit traverses the membrane four times and is modified by glycosylation and disulfide bond formation (*2*). Maturation of nAChRs into functional ion channels at the surface membrane is both a complex and a time-consuming process (*3*). Hence, nAChR maturation is likely to require assistance from cellular chaperones. Indeed, a number of chaperones were shown to affect nAChR maturation (*4*−*7*).

ric-3 was first identified in *Caenorhabditis elegans* as a gene needed specifically for the maturation of nAChRs (*7*). *C. elegans* RIC-3 is a member of a conserved family of proteins similar in sequence and function (*8*). In addition to effects of RIC-3 on surface expression of nAChRs, *C. elegans* RIC-3 was also shown to affect properties (desensitization efficiency and agonist affinity) of the *C. elegans* DEG-3/DES-2 nAChR (*9*). Similarly, human RIC-3 does not simply enhance surface expression of nAChRs, as it was also shown to reduce the level of expression of some receptors depending on the experimental system (*8*, *10*−*12*). Effects of RIC-3 homologues on receptor surface expression or properties are subtype specific and are thus likely to enable regulation both of the strength and of the properties of cholinergic signaling.

Here we use biochemistry and electrophysiology in *Xenopus* oocytes expressing two different *C. elegans* nAChRS, DEG-3/DES-2 and ACR-16, together with RIC-3 or RIC-3 mutants. DEG-3/DES-2 is an obligatory heteromeric receptor; expression of either the DEG-3 or the DES-2 subunit alone does not produce a functional channel (*13*). In *C. elegans*, RIC-3 is required for the proper functioning and maturation of the DEG-3/DES-2 receptor, and in *Xenopus* oocytes, RIC-3 both enhances whole cell current amplitudes and influences properties (desensitization efficiency and agonist affinity) of the DEG-3/DES-2 receptor (*7*, *9*). The similarity of the effects of RIC-3 coexpression to the effects of increasing the DEG-3 to DES-2 ratio suggested that it influences receptor stoichiometry (*9*). Results presented here support this suggestion, showing that RIC-3 increases the level of DEG-3 surface expression while reducing the level of DES-2 surface expression. We also show that effects of RIC-3 on DEG-3/DES-2 are separable, as mutation of conserved residues in the second RIC-3 transmembrane weakens the effects of RIC-3 on current amplitude but not on the desensitization efficiency of the DEG-3/DES-2 receptor. Mutating these conserved residues also weakens the effects of RIC-3 on the homomeric ACR-16 nAChR. Our work shows that effects of RIC-3 on each receptor require more than one region. We previously showed that the DEG-3/DES-2 receptor requires only the region spanning the two transmembrane domains (*9*); here we show that within this region it requires both conserved residues within the second transmembrane domain and yet unidentified residues. ACR-16, however, also requires additional regions outside the

[†]This work was supported by the Israel Science Foundation (Grant 379/06).

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[1]Abbreviations: nAChRs, nicotinic acetylcholine receptors; TM, transmembrane; CC, coiled coil; ER, endoplasmic reticulum.

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Published on Web 11/09/2009

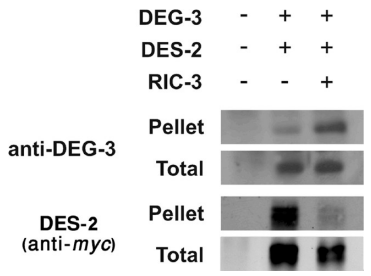

FIGURE 1: RIC-3 enhances surface expression of DEG-3 but not DES-2. Representative Western analysis showing total protein extracts and pellets from surface biotinylation experiments. Samples are from uninjected oocytes (controls), oocytes expressing DEG-3 and DES-2, or oocytes expressing DEG-3, DES-2, and RIC-3; top panels show samples hybridized with anti-DEG-3 antibodies to detect DEG-3 (55 kDa), and bottom panels show samples hybridized with anti-*myc* antibodies to detect *myc*-tagged DES-2 (75 kDa). Ten oocytes were used per experiment.

transmembrane domain-spanning region (*14*). Interestingly, all mutations and deletions weakening the effects of RIC-3 on nAChR maturation also weaken its interactions with the receptors. Thus, interaction strength and effects of RIC-3 on nAChRs appear to be intimately linked.

EXPERIMENTAL PROCEDURES

Heterologous Expression and Electrophysiology. DEG-3-, *myc*-tagged DES-2-, GFP-tagged RIC-3-, and GFP-tagged RIC-3 TM-expressing clones were described previously (*9*), and ACR-16- and *myc*-tagged ACR-16-expressing clones were also described (*14*). *myc*-tagged DEG-3 has 6×*myc* fused to the C-terminus of DEG-3 instead of the last amino acid, like the previously described *myc*-tagged DES-2. In vitro transcribed and capped cRNAs were injected at final concentrations of 0.1 μg/μL each in electrophysiology experiments and 0.3 μg/μL each in experiments that included biochemical analysis. In stoichiometry experiments, the total quantity of injected cRNA was kept constant. For experiments described in the legend of Figure 5, the DEG-3 to DES-2 ratio was reduced to enable better resolution of the desensitization value; RNAs were injected at a concentration of 0.075 μg/μL for DEG-3 and 0.15 μg/μL for DES-2. The effects of coexpressing RIC-3 or its derivatives on DEG-3/DES-2 or ACR-16 were assayed between the first and second day following injections.

Data Analysis. Data analysis was conducted using Origin version 7.0. Results are presented as the means ± the standard error of the mean [N is the number of frogs (independent experiments), and n is the number of oocytes]. To reduce noise due to variability in expression levels between different experiments, we normalized the current amplitudes, in the experiments described in the legend of Figure 5, to the average response for oocytes expressing only the receptor without RIC-3. Desensitization efficacy was calculated as follows: (peak amplitude − steady state amplitude)/peak amplitude.

Biochemical Analysis. In all experiments, RIC-3 is tagged with GFP. DES-2, ACR-16, and (in Figure 2) DEG-3 are tagged with 6×*myc* (*9*, *14*). Surface biotinylation (Figure 1) was conducted on the day following injection in which oocytes showed a clear effect of RIC-3, i.e., enhancement of current amplitudes by at least 4 fold. To identify surface-expressed proteins, oocytes were incubated with sulfo-NHS-LC-biotin, and following homogenization, biotinylated proteins were precipitated using straptavidin as described by Stern-Bach et al. (*15*). Pellets of surface-expressed proteins represent 10 oocytes, and total represents 5% of homogenized extracts from the same experiments. Co-immunoprecipitations and Western analysis were conducted as described previously; all co-immunoprecipitations were conducted using anti-GFP antibodies to precipitate RIC-3 complexes, and the Western blots were hybridized with anti-DEG-3 antibodies to detect DEG-3, anti-*myc* antibodies to detect *myc*-tagged DES-2 or *myc*-tagged ACR-16, or GFP to detect GFP-tagged RIC-3 (*9*, *14*). To analyze levels of different proteins from the same experiments, Western blots were first hybridized with antibodies against one protein and then stripped and rehybridized. Stripping was done as follows. The membrane was submerged in stripping buffer [100 nM β-mercaptoethanol, 2% sodium dodecyl sulfate, and 62.5 mM Tris-HCl (pH 6.7)] and incubated at 50 °C for 30 min with occasional agitation. Following stripping, the membrane was washed twice in TBS+tween before blocking and hybridization were conducted. Images of Western blots were obtained using Fuji Film LAS-3000 and quantified using TINA 2.10 g (Raytest, Straubenhardt, Germany).

Generation of RIC-3 Mutants Used in This Study. All point mutations were generated using overlap PCR mutagenesis using an external primer and an appropriate mutant internal primer to generate two overlapping PCR fragments that were then combined for a second round of PCR. The resulting mutant PCR fragments were inserted by ligation into different RIC-3 deletion mutants as described previously (*14*).

RESULTS

Effects of RIC-3 on Surface Expression of DEG-3/DES-2 Receptor Subunits. We have previously shown that effects of RIC-3 on the properties of the DEG-3/DES-2 receptor (increased desensitization efficiency and increased agonist affinity) mimic effects of an increasing DEG-3 to DES-2 ratio. This suggested that RIC-3 preferentially enhances the maturation of DEG-3 rich receptors (*9*). To study this suggestion, we examined surface expression of the two subunits using cell surface biotinylation assays on oocytes expressing the DEG-3 and DES-2 receptor subunits with or without co-expression of RIC-3. To reduce noise due to expression level or to exposure level differences between experiments, effects of RIC-3 on each subunit are given as the ratio of subunit expression in the presence of ric-3 relative to its expression in the absence of ric-3 within individual and independent experiments. This analysis shows that co-expression with RIC-3 leads to a 2.64 ± 0.91-fold increase in the quantity of surface-expressed DEG-3 [N = 3 (Figure 1)]. Similar analysis of the effects of RIC-3 on surface-expressed DES-2 shows no increase. Rather, co-expression with RIC-3 leads to a reduction in the level of DES-2 surface expression [0.68 ± 0.1; N = 4 (Figure 1)]. These effects are not a result of changes in steady state levels of the subunits since quantities of surface-expressed subunits are normalized to relative whole cell expression levels of the same subunit in the same experiments. Moreover, we did not see consistent effects of RIC-3 on steady state levels of DEG-3 or DES-2 in these experiments.

Together, our results show that RIC-3 increases the level of surface expression of the DEG-3 subunit while reducing the level of surface expression of the DES-2 subunit. Such changes may increase or decrease the overall level of surface expression of the DEG-3/DES-2 receptor, depending on subunit stoichiometry in surface-expressed DEG-3/DES-2 receptors in the absence of

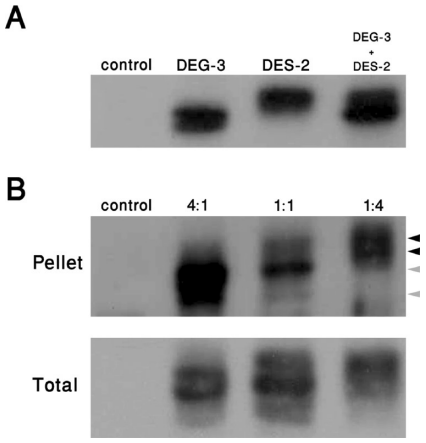

FIGURE 2: Effects of DEG-3 to DES-2 ratio on surface expression of DEG-3/DES-2 receptor subunits. (A) Western analysis using anti-*myc* antibodies to detect both subunits expressed alone or together. This analysis shows slightly different migration of *myc*-tagged DEG-3 relative to *myc*-tagged DES-2. The two subunits run at 70−75 kDa. (B) Surface biotinylation experiments using anti-*myc* antibodies to detect both *myc*-tagged DEG-3 and *myc*-tagged DES-2. Samples represent uninjected oocytes (controls), oocytes injected with a 4:1 DEG-3:DES-2 ratio, oocytes injected with a 1:1 DEG-3:DES-2 ratio, and oocytes injected with a 1:4 DEG-3:DES-2 ratio. Pellets above and total protein extracts below. We note that DEG-3 is seen as two bands (gray arrows) running below two DES-2 bands (black arrows). Ten oocytes were used per experiment.

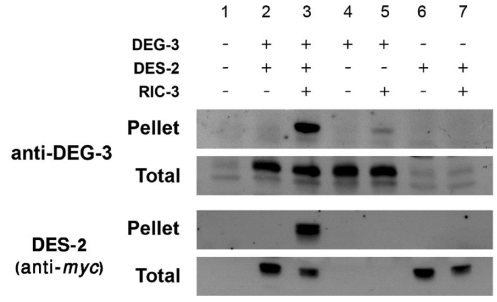

FIGURE 3: Interactions of RIC-3 with DEG-3/DES-2 receptor assembly intermediates depend on their subunit composition. Representative Western analysis from uninjected oocytes and oocytes expressing different combinations of DEG-3, *myc*-tagged DES-2, and GFP-tagged RIC-3. The top two panels show Western blots hybridized with anti-DEG-3 antibodies to detect DEG-3 (55 kDa) in the pellet precipitated with anti-GFP antibodies above and in the total below. The bottom two panels are hybridized with anti-*myc* antibodies, used to detect *myc*-tagged DES-2 (75 kD) in the pellet precipitated with anti-GFP antibodies above and in the total below. Ten oocytes were used per experiment.

RIC-3. Given the results described in Figure 2, showing a similar or slightly higher quantity of DEG-3 relative to DES-2 in the absence of RIC-3, we suggest that these changes will lead to an increase in the overall level of surface expression of DEG-3/DES-2 receptor subunits. Assuming that surface-expressed subunits are mostly incorporated into functional receptors, this analysis implies that the presence of RIC-3 leads both to an increase in the number of functional receptors and to an increased level of surface representation of DEG-3 rich receptors, i.e., receptors having high DEG-3 to DES-2 ratios.

Assuming that DEG-3 to DES-2 ratios are 3:2 or 2:3 in the absence of RIC-3, the results described above suggest that RIC-3 leads to a 1.8- or 1.46-fold increase, respectively, in the overall level of surface expression of the DEG-3/DES-2 receptor. This increase, however, is smaller than the observed increase in current amplitudes [6.40 ± 1.10-fold effect on current amplitudes in the same experiments ($N = 3$; $n = 14-16$)]. Thus, we examined the possibility that effects of RIC-3 on subunit ratio at the surface may also modify the conductance of individual receptors. For this purpose, we compared current amplitudes in oocytes treated with a 4:1 DEG-3:DES-2 ratio relative to oocytes injected with a 1:4 DEG-3:DES-2 ratio. This analysis shows that a higher DEG-3 to DES-2 ratio indeed leads to higher current amplitudes (3.2 ± 0.6-fold higher for 4:1 relative to 1:4 ratios; $N = 6$; $n = 49$; $p < 0.05$). However, this effect of subunit ratio on current amplitudes could be a result of differences in the amount of current conducted by receptors having different subunit ratios, or differences in the maturation efficiency of receptors having different subunit ratios. To examine effects of ratio manipulation on surface expression of the two subunits, we used cell surface biotinylation on oocytes expressing DEG-3 tagged with the same 6×*myc* tag used to tag DES-2. Tagged DEG-3 behaves in a manner similar to that of untagged DEG-3 in this analysis, having similar effects on desensitization value and current amplitudes. Unfortunately, migration of this tagged protein is only slightly different than migration of tagged DES-2 (Figure 2A), making accurate quantification of the subunit ratio difficult. Nonetheless, this analysis shows that manipulation of injected subunit ratios has the expected effect on surface expression of the two subunits (Figure 2B). This analysis also shows a higher total level of surface expression of DEG-3/DES-2 subunits at a 4:1 relative to a 1:4 DEG-3 to DES-2 subunit ratio [1.6-fold increase (Figure 2B)]. Thus, the maturation process appears to favor surface expression of DEG-3 rich receptors, providing an explanation for the differences in current amplitude seen when expressing a 4:1 relative to a 1:4 DEG-3:DES-2 ratio. Hence, these results do not explain the discrepancy between the small effects of RIC-3 on surface expression of the DEG-3/DES-2 receptor relative to its larger effects on current amplitudes. Other possible explanations for this discrepancy are that the assays used to measure surface expression versus functional expression may have different sensitivities or that RIC-3 may have an additional mechanistically distinct effect on receptor function

Differential Interactions of RIC-3 with DEG-3/DES-2 Receptor Subunits. RIC-3 was previously shown to precipitate with the DES-2 subunit when both DEG-3 and DES-2 were expressed in *Xenopus* oocytes (*9*). This analysis could not discriminate between direct and indirect interactions of RIC-3 with DES-2, nor could it determine the assembly state of subunits interacting with RIC-3. To address these questions, we examined interactions of RIC-3 with DEG-3 and DES-2 when expressed together or when each is expressed alone (Figure 3). For this purpose, we used anti-GFP antibodies to precipitate GFP-tagged RIC-3 and then examined the precipitate for the presence of DEG-3 or DES-2 (anti-*myc* antibodies are used to detect *myc*-tagged DES-2) (*9*).

This analysis shows that RIC-3 interacts with DEG-3 when expressed alone or when co-expressed with DES-2 (Figure 3, lanes 3 and 5). We note that a weak nonspecific band is sometimes seen in the position of the DEG-3 protein; however, the band seen in lane 5 is clearly above background and is reproducible. Quantification of DEG-3 staining shows that more DEG-3 is precipitated by RIC-3 in the presence of DES-2 [4.73 ± 1.18-fold; $N = 2$ (in Figure 3, compare lane 3 to lane 5)]; a similar reduction in the interaction strength with DEG-3 in the absence of DES-2 is also seen for RIC-3 TM (Figure 6),

a functional RIC-3 deletion mutant (*9*). This difference in the quantity of precipitated DEG-3 is not due to effects of subunit expression on steady state levels of RIC-3, as steady state levels of RIC-3 are unaffected by the presence or absence of DEG-3 and/or DES-2 (results not shown and Figure 6). Thus, our results show that while interaction of RIC-3 with DEG-3 does not require assembly of DEG-3 with DES-2, it is enhanced by the presence of DES-2, suggesting that RIC-3 prefers DEG-3/DES-2 heteromers to DEG-3 monomers or homomers. Similar analysis of the interactions of RIC-3 with DES-2 shows that interaction of RIC-3 with DES-2 requires assembly of DES-2 with DEG-3; no interaction is detected in the absence of DEG-3 [$N = 4$ (Figure 3, lane 7)]. Although we cannot rule out a very weak and thus undetectable interaction of RIC-3 with DES-2 in the absence of DEG-3, our results suggest that RIC-3 interacts with DES-2 via DEG-3 or that the interaction between RIC-3 and DES-2 depends on a conformation change within DES-2 that follows assembly with DEG-3. Thus, our results demonstrate a difference in the way that RIC-3 interacts with DEG-3 versus its interaction with DES-2. This difference may serve as an explanation for the subunit specific effects of RIC-3 on surface expression of these two subunits.

Conserved Residues in the Second Transmembrane Domain of RIC-3 Are Needed for Its Interaction with DEG-3/DES-2 Receptors. We have previously shown that a region spanning the two transmembrane domains of RIC-3 (RIC-3 TM) is sufficient for its effects on quantity and properties of the DEG-3/DES-2 receptor (*9*). To improve our understanding of how the RIC-3 TM protein affects receptor quantity and quality, we decided to identify functional residues within it. For this purpose, we focused on residues within the second transmembrane domain, as this domain is the most highly conserved (*8*).

Six residues within the second transmembrane domain are conserved among all RIC-3 homologues (Figure 4A). These conserved residues can be divided into two clusters, an N-terminal cluster [proline, tyrosine, and glycine at positions 5, 7, and, 10, respectively (Figure 4A)] and a C-terminal cluster [tyrosine, leucine, and lysine at positions 17, 19, and 21, respectively (Figure 4A)]; conserved residues within each cluster are separated by no more than two residues, while the distance between the two clusters is six residues. Mutating residues at positions 5, 7, 10, and 17 individually to alanine had little effect on RIC-3 TM function as measured by its effects on current amplitudes and on receptor desensitization. Only the G to A mutation, affecting position 10 within the N-terminal cluster, weakened the effects of the RIC-3 TM on current amplitudes in some but not all experiments; thus, effects of this mutation were not considered significant (results not shown). These results suggested that function of RIC-3 TM is resilient to mutation of single conserved residues. Thus, we decided to mutate individual clusters (Figure 4B). Analysis of the resulting mutants demonstrated a role for the N-terminal cluster in RIC-3 TM function, as mutating the three N-terminal residues (RIC-3 TM-N*) but not the three C-terminal residues (RIC-3 TM-C*), to alanine, weakened the effects of RIC-3 TM on current amplitudes (Figure 5A). This reduction is not due to the reduced stability of this mutant protein as seen in Western blot analysis (Figure 5D). We note that this N-terminal cluster includes the residue at position 10 that when mutated reduced RIC-3 function in a nonconsistent manner. Since mutating all three C-terminal cluster residues to alanine led to no discernible effect, we did not separately analyze mutations of residues 19 and 21.

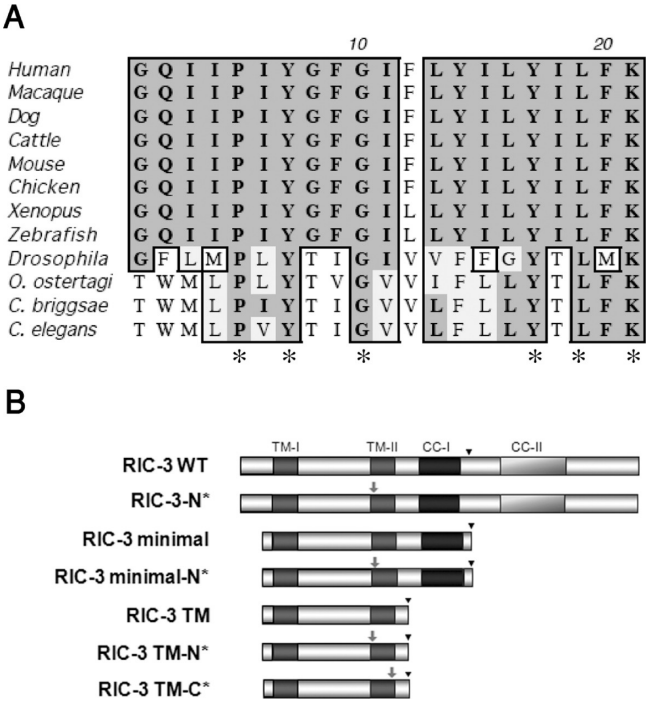

FIGURE 4: Conserved residues in the second transmembrane domain of RIC-3 and constructs used in this study. (A) Alignment of the second transmembrane domain from known RIC-3 homologues (ClustalW). Conserved residues are shaded, and asterisks denote residues conserved in all the sequences. (B) Constructs used in this study. The top panel is a schematic diagram of wild-type (WT) RIC-3 domain organization; indicated are the positions of transmembrane domains (TM-I and TM-II) and coiled-coil domains (CC-I and CC-II). Below are schematic diagrams of RIC-3 constructs used in this study, in which arrows point to positions of mutations in each construct; N* denotes that the first three conserved residues in the second transmembrane domain (see panel A) were mutated to alanines and C* a similar mutation of the last three conserved residues. Arrowheads point to the position of the GFP tag.

Interestingly, RIC-3 TM-N* had weakened effects on DEG-3/DES-2 current amplitudes relative to RIC-3 TM while retaining full effects on its desensitization value (Figure 5B,C). Thus, we can dissect the effects of RIC-3 TM on the DEG-3/DES-2 receptor into two separable effects. (1) Increasing the desensitization value, probably a result of an increased level of expression of DEG-3 rich receptors, is not affected by mutation of conserved residues in the second transmembrane domain. (2) Enhancing functional expression while having no effect on receptor desensitization requires conserved residues within the N-terminus of the second transmembrane domain.

To improve our understanding of the effects of mutating the N-terminal cluster, we also examined their influence on the interaction of RIC-3 mutants with DEG-3/DES-2 receptor subunits. This analysis shows that these mutations greatly weaken the interactions of RIC-3 TM with DEG-3/DES-2 receptor subunits, as seen by the very weak interaction of this mutant with DES-2 or DEG-3, in the presence or absence of DES-2 (Figure 6). Interestingly, the remaining interaction with DES-2, following normalization to DES-2 expression levels (Figure 6, total) in the same experiments, is much weaker than the remaining effects on current amplitudes [0.19 ± 0.1 of interaction with wild-type RIC-3 TM; $N = 3$ (Figures 5A and 6)], suggesting that the interaction responsible for half of the effects of the RIC-3 TM on functional expression, but not for its effects on desensitization efficiency, is stronger or less transient

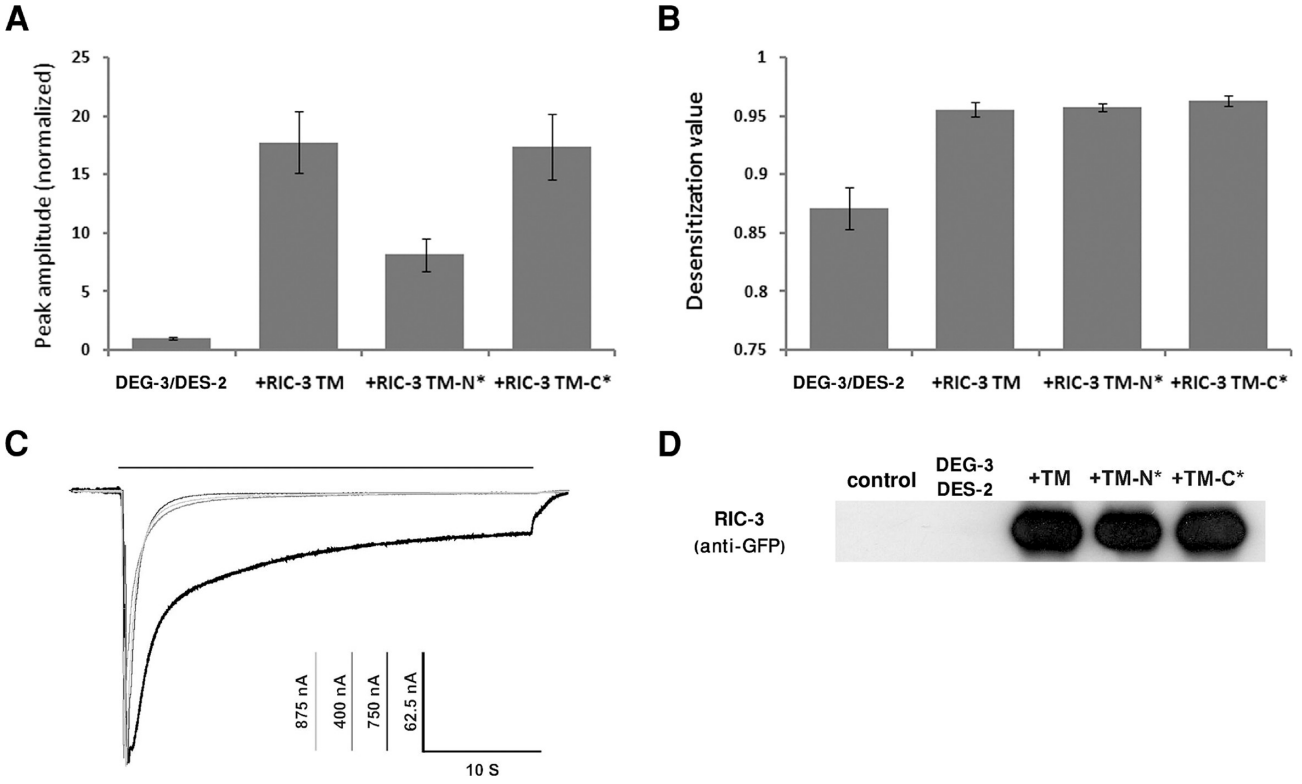

FIGURE 5: Specific effects of mutating residues in the second RIC-3 transmembrane domain. (A) Average normalized current amplitudes, elicited by 3.2 mM choline, in oocytes expressing DEG-3 and DES-2 without RIC-3 or with different RIC-3 TM derivatives: RIC-3 TM, RIC-3 TM-N* (RIC-3 TM in which the N-terminal cluster is mutated to alanines), and RIC-3 TM-C* (RIC-3 TM in which the C-terminal cluster is mutated to alanines). Currents are normalized to the average current amplitudes of oocytes expressing the DEG-3/DES-2 receptor without RIC-3 in the same experiment. Effects of mutating the N-terminal cluster on DEG-3/DES-2 expression are smaller relative to those of wild-type RIC-3 TM ($N = 3$; $n = 27-34$; $p < 0.05$; ANOVA). (B) Average desensitization value (D value) from the same experiments, in oocytes expressing DEG-3 and DES-2 without RIC-3 or with different RIC-3 TM derivatives ($N = 3$; $n = 26-32$). (C) Superimposed representative current traces for oocytes expressing DEG-3 and DES-2 without RIC-3 or with different RIC-3 TM derivatives. Current amplitude scales differ between each response to better visualize the change in desensitization efficiency (D value). The trace for DEG-3/DES-2 alone is colored black, and traces for DEG-3/DES-2 with RIC-3 TM, RIC-3 TM-N*, or RIC-3 TM-C* are colored in successively lighter shades of gray. (D) Representative Western analysis showing levels of the different RIC-3 TM derivatives expressed in oocytes. Western blots are hybridized with anti-GFP antibodies to detect the GFP-tagged RIC-3 TM derivatives (37 kDa).

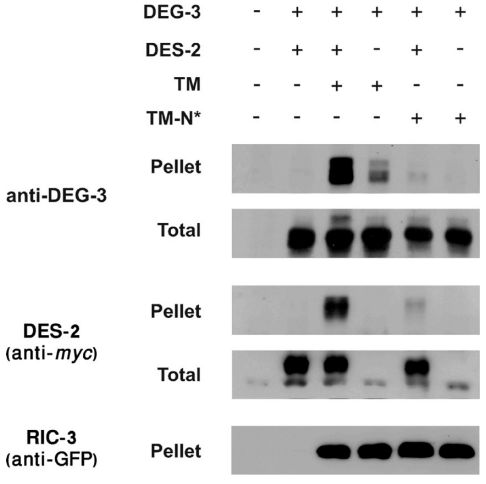

FIGURE 6: Mutation of the N-terminal cluster weakens interaction with DEG-3/DES-2 subunits. Representative Western analysis from uninjected oocytes and oocytes expressing different combinations of DEG-3, DES-2, RIC-3 TM, and RIC-3 TM-N*. Pellets precipitated using GFP antibodies (all RIC-3 TM derivatives are tagged with GFP), and Western blots are hybridized with anti-DEG-3 antibodies (pellets above total below), anti-*myc* antibodies to detect *myc*-tagged DES-2 (pellets above total below), or anti-GFP antibodies to detect GFP-tagged RIC-3 in pellets (55, 75, and 37 kDa, respectively).

than the interaction responsible for the remaining effects on current amplitudes and for the effects on desensitization efficiency.

Conserved Residues in the Second Transmembrane Domain of RIC-3 Are Required for Its Effects on the Homomeric ACR-16 nAChR. Results presented in Figure 6 show that conserved residues in the N-terminus of the second RIC-3 transmembrane domain, when mutated, weaken RIC-3's interaction with the DEG-3/DES-2 nAChR. To improve our understanding of the importance of these residues, we decided to examine their influence on a homomeric *C. elegans* nAChR, ACR-16, also known to require RIC-3 for its maturation (*8*, *16*, *17*). Expression of ACR-16 with RIC-3 TM leads to a large increase in ACR-16-dependent current amplitudes (55 ± 5.3 and 2270 ± 394 nA for ACR-16 alone and for ACR-16 with RIC-3 TM, respectively; $N = 8$ or 4; $n = 86$ or 33). This increase is greatly reduced when ACR-16 is expressed with RIC-3 TM-N* [493 ± 112; $N = 5$; $n = 47$ (Figures 4B and 7A,B)]. Mutating the same residues in the context of full-length RIC-3 or RIC-3 minimal, lacking nonconserved N- and C-domains [longer RIC-3 constructs have stronger effects on ACR-16 expression (*14*)], also reduces their effects on ACR-16 expression, although to a lesser extent (Figures 4B and 7A,B; 6222 ± 355, 4933 ± 420, 6175 ± 391, and 2315 ± 356 for ACR-16 and RIC-3, ACR-16 and

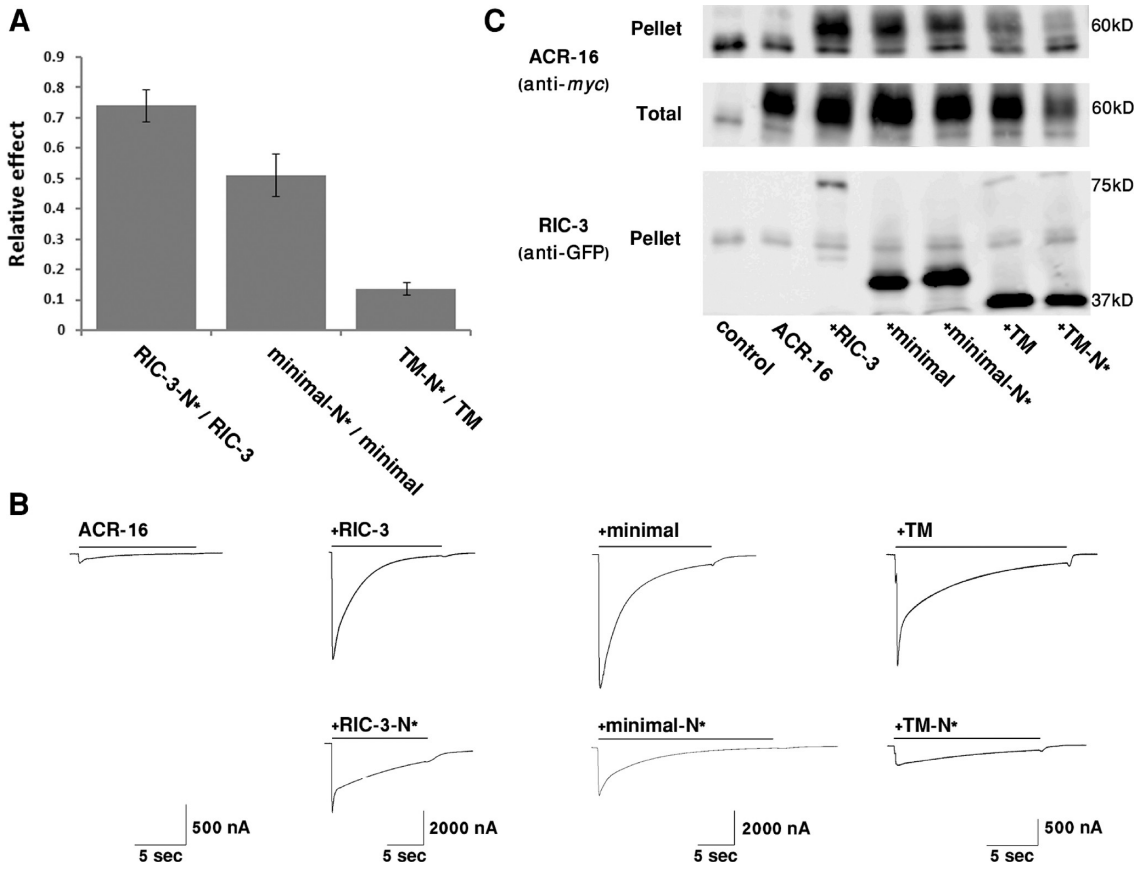

FIGURE 7: Residues and regions enabling the effects of RIC-3 on the ACR-16 nAChR. (A) Effects of mutating the N-cluster residues on wild-type RIC-3, RIC-3 minimal, or RIC-3 TM deletion mutants as seen by their effects on ACR-16 currents elicited by 1 mM acetylcholine. Results are given as the ratio between effects on current amplitudes of RIC-3-N* to wild-type RIC-3, RIC-3 minimal-N* to RIC-3 minimal, and of RIC-3 TM-N* to RIC-3 TM. Differences in the effects of the N-cluster mutations on the different RIC-3 derivatives are significant ($n = 28-63$; $N = 4-9$; $p < 0.05$; ANOVA). (B) Representative current traces elicited by 1 mM acetylcholine, in oocytes expressing ACR-16 alone or with the different RIC-3 derivatives. Scale bars apply to the traces above them. (C) Co-immunoprecipitation from control (uninjected) oocytes, oocytes expressing ACR-16, or oocytes expressing ACR-16 with a different RIC-3 derivatives (10 oocytes each). Precipitation was achieved using anti-GFP antibodies directed at RIC-3 derivatives, and precipitates were analyzed using anti-*myc* antibodies, to detect *myc*-tagged ACR-16 or anti-GFP to quantify levels of RIC-3 and RIC-3 mutants in the precipitate (pellet). Also shown is the level of ACR-16 expression in the same oocytes (total). Numbers at the right indicate protein size in kilodaltons.

RIC-3-N*, ACR-16 and RIC-3 minimal, and ACR and RIC-3 minimal-N*, respectively; $N = 4-9$; $n = 28-63$). This context-dependent effect of mutating the second transmembrane domain "N-terminal cluster" on ACR-16 expression suggests that these residues may function together with other RIC-3 domains to enhance ACR-16 maturation.

Previously, we have shown that interaction of RIC-3 with ACR-16 requires the conserved RIC-3 coiled-coil domain (CC-I) together with downstream sequences (likely to be the second coiled-coil domain, CC-II) (*14*). Thus, we have examined interaction of the different RIC-3 mutants with ACR-16 using co-immunoprecipitations. For this purpose, we used anti-GFP antibodies to precipitate GFP-tagged RIC-3 or RIC-3 mutants and then examined the precipitate for the presence of *myc*-tagged ACR-16 using anti-*myc* antibodies (Figure 7C). To better quantify this interaction and to reduce noise due to differences in expression level and precipitation efficiency, quantities of *myc*-tagged ACR-16 in the precipitate (pellet) were normalized both to the expression level of *myc*-tagged ACR-16 as seen in the total protein extract from the same experiment and to the quantity of the GFP-tagged RIC-3 derivative used for the precipitation within the precipitate (pellet). This analysis shows that each deletion or mutation weakens the interaction of ACR-16 with RIC-3. Specifically, the interaction strength of ACR-16 with RIC-3 mutants decreases in the following order: wild-type RIC-3, RIC-3 minimal, RIC-3 minimal-N*, RIC-3 TM, and RIC-3 TM-N* (the strongest interaction is observed with wild-type RIC-3 given here as 100% interaction, while the levels of interaction with RIC-3 minimal, RIC-3 minimal-N*, RIC-3 TM, and RIC-3 TM-N* are 31.8, 14.4, 7.9, and 5.5%, respectively). Thus, the different RIC-3 domains and residues analyzed here appear to additively enhance the interaction of RIC-3 with ACR-16, and the strength of this interaction, for each mutant, correlates with the extent of its effect on current amplitude.

RIC-3 Is Likely To Be an Intrinsically Disordered Protein. The results described in Figure 7 show that multiple dispersed regions enable the interaction of RIC-3 with ACR-16. Our results cannot distinguish between direct and indirect roles of these regions in the interaction with ACR-16. However, the additive nature of the interaction enhancement by these regions makes direct interaction likely. Support for this suggestion comes from analysis predicting that RIC-3 is an intrinsically disordered protein (Figure 8); this prediction, using IUPred (*18*) or VLH3 (*19*), shows that >80% of RIC-3 residues are likely to be disordered. Specifically, both predictions suggest that only two regions encompassing the first and second transmembrane domains are ordered (Figure 8). Disordered proteins are known to have larger (per residue) surface area and protein−protein

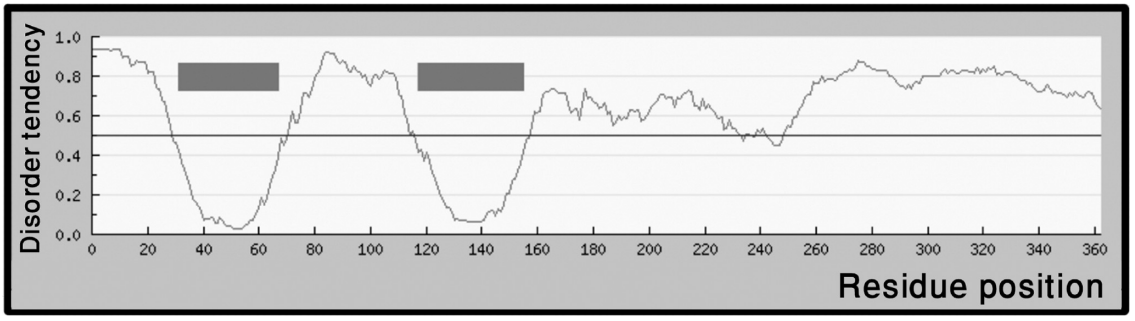

FIGURE 8: RIC-3 is likely to be an intrinsically disordered protein. Analysis was done using the IUPred analysis (http://iupred.enzim.hu/index.html). Only residues 32−68 and 118−156 encompassing each of the transmembrane domains (bars) are predicted to be ordered.

interaction mediating interface areas (*20*). In addition, intrinsically disordered proteins are likely to fold upon interaction with their binding partner (*21*). Thus, our results suggesting that RIC-3 uses different, although partly overlapping, regions to interact with different nAChRs also imply that RIC-3 will adopt different conformations upon interaction with the two different receptors.

DISCUSSION

In *C. elegans*, RIC-3 is required for the maturation of multiple nAChRs (*7*). RIC-3 belongs to a conserved gene family. Both the *C. elegans* RIC-3 and its human homologue affect surface expression of nAChRs in heterologous expression systems (*7*, *8*). Interestingly, human RIC-3 enhances expression of some receptors while inhibiting or enhancing expression of other receptors depending on the receptor subtype and on the experimental system (*8*, *10*−*12*). Here we show that RIC-3 enhances surface expression of one DEG-3/DES-2 receptor subunit, DEG-3, while having a negative effect on expression of the other subunit, DES-2. We have previously shown a similarity between the effects of RIC-3 and of a high DEG-3 to DES-2 ratio on properties (desensitization efficiency and agonist affinity) of the DEG-3/DES-2 receptor (*9*); thus, the differential effects of RIC-3 on surface expression of the two DEG-3/DES-2 subunits provide a likely explanation for its effects on these properties of the mature receptor, supporting the suggestion that RIC-3 affects subunit stoichiometry of surface-expressed DEG-3/DES-2 receptors.

To improve our understanding of the different effects of RIC-3 on the two DEG-3/DES-2 subunits, we examined its interactions with them when expressed alone or together. This analysis shows that RIC-3 can interact with the DEG-3 subunit in the absence of its obligatory partner, the DES-2 subunit, although more DEG-3- and RIC-3-containing complexes are detected in the presence of DES-2. In contrast, a detectable interaction of RIC-3 with DES-2 is only seen in the presence of DEG-3. The different interaction of RIC-3 with DEG-3 versus its interactions with DES-2 may explain the different effects of RIC-3 on surface expression of the two subunits and hence on properties (desensitization efficiency and agonist affinity) of the DEG-3/DES-2 receptor.

Understanding the mechanisms enabling effects of RIC-3 requires knowledge of its functional localization. RIC-3 homologues were shown to reside in the ER (*7*, *10*, *11*, *22*) and are likely to interact with receptor subunits within the ER, since they were shown to interact with subunits that are unable to exit the ER and with unassembled or partly assembled subunits that are also likely to be retained in the ER (*9*, *10*, *12*, *22*). These findings, however, cannot rule out the possibility that RIC-3 is also found at the plasma membrane where it also interacts with mature receptors. To examine this possibility, several studies analyzed RIC-3 surface expression. Such studies showed no RIC-3 in the *C. elegans* neuromuscular junction, a site of mature nAChRs, or at the surface of mammalian cell lines (*7*, *10*, *23*). However, another study using mammalian cell lines did show surface expression of RIC-3 (*24*). Thus, we also examined this question using cell surface biotinylation in *Xenopus* oocytes. Results of this analysis are mixed showing surface expression of RIC-3 in one of four experiments (H. Farah, not shown). We conclude that RIC-3 can be found at the surface rarely or at low (hard to detect) levels. This surface expression may be functional or an artifact of RIC-3 overexpression following heterologous expression.

Effects on receptor properties are usually attributed to interactions with functional receptors at the surface membrane. RIC-3 is likely to reside and function in the ER (see above). Thus, we need to identify a mechanism enabling effects of an ER resident protein on receptor properties. Previously, we have shown that increasing the DEG-3 to DES-2 subunit ratio mimics the effects of RIC-3 on receptor desensitization and agonist affinity (*9*). This analysis together with the results obtained here for the different effects of RIC-3 on surface expression of the two DEG-3/DES-2 receptor subunits suggests a mechanism enabling effects of ER-resident proteins on receptor properties. In addition, our results for interactions of RIC-3 with the DEG-3 and DES-2 receptor subunits when expressed alone suggest preferential interaction of RIC-3 with DEG-3. Thus, we suggest that differential interactions of an ER-resident protein with different receptor subunits may bias the maturation process toward production of receptors having specific subunit compositions and properties. Similarly, NRA-2 and NRA-4 are also ER-resident proteins that were shown to affect properties of a *C. elegans* nAChR, and these effects correlated with differential effects on surface expression of different receptor subunits (*25*).

In addition to results described here showing different effects and interactions of RIC-3 with the two subunits of the DEG-3/DES-2 receptor, previous results have shown different effects of RIC-3 on different nAChRs (*8*, *11*). To improve our understanding of the different effects of RIC-3 on different receptors, we identified and analyzed domains and residues enabling the effects of RIC-3 on two different *C. elegans* nAChRs. Results described here together with previously described results (*14*) show that effects of RIC-3 on the ACR-16 nAChR require at least four different regions: the first conserved coiled-coil domain (CC-I), sequences downstream from it (probably the second coiled-coil domain, CC-II), conserved residues in the N-terminus of the second transmembrane domains (N-cluster), and other yet unidentified residues within RIC-3 TM; these unidentified

residues are responsible for the residual effects seen following mutation of the N-cluster in the RIC-3 TM deletion mutant. In contrast, the DEG-3/DES-2 nAChR requires residues only within RIC-3 TM (*9*). In addition, while effects of RIC-3 on both receptors are weakened via mutation of the N-cluster residues, the relative effect of these mutations differs for the two receptors; N-cluster mutations greatly weaken the effects of RIC-3 TM on ACR-16 but lead to a much smaller reduction in its effects on DEG-3/DES-2. Moreover, the N-cluster mutations do not weaken the effects of RIC-3 TM on DEG-3/DES-2 desensitization efficiency. The residues within RIC-3 TM-N* that enable its effects on DEG-3/DES-2 desensitization efficiency and whether these residues are the same as the residues enabling the residual effects of RIC-3 TM-N* on ACR-16 are not known.

Overall, we show (1) multiple regions and/or residues are needed for the effects of RIC-3 on nAChRs, (2) different nAChRs require different subsets of these regions and/or residues, and (3) the relative importance of each region or residue differs for the different receptors. Thus, RIC-3 interacts differently with different receptors. Interestingly, RIC-3 is predicted to be an intrinsically disordered protein. Intrinsically disordered proteins are likely to fold upon binding to interaction partner (*21*). Thus, the differences in the sequences and domains needed for interaction of RIC-3 with different receptors suggest that RIC-3 is likely to adopt different conformations upon interactions with different receptors. Such differences may underlie the different effects of RIC-3 on different receptors and receptor subunits. Specifically, RIC-3 both in *C. elegans* and in *Xenopus* oocytes has a much stronger effect on ACR-16 expression relative to its effects on DEG-3/DES-2 expression (*7*, *14*). We suggest that this difference may be a result of the more extensive interactions, i.e., requiring more regions, of RIC-3 with ACR-16. Indeed, our analysis shows that strengthening the interaction of RIC-3 with ACR-16 via addition of regions or residues enabling this interaction also increases the effects of RIC-3 on ACR-16 expression. Interestingly, intrinsically disordered proteins may also adopt different conformations upon interactions with the same protein, differences leading to different outcomes (*26*). This property of intrinsically disordered proteins may also explain the different outcomes resulting from interactions of RIC-3 with specific nAChRs (*8*, *10*, *12*).

ACKNOWLEDGMENT

We thank David Sattelle for the ACR-16 clone.

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