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DIRECT INTERACTIONS BETWEEN *C. ELEGANS* RAB-3 AND RIM PROVIDE A MECHANISM TO TARGET VESICLES TO THE PRESYNAPTIC DENSITY

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Abstract

Rim is a multi-domain, active zone protein that regulates exocytosis and is implicated in vesicle priming and presynaptic plasticity. We recently demonstrated that synaptic defects associated with loss of *C. elegans* Rim (termed UNC-10) are accompanied by a reduction in docked vesicles adjacent to the presynaptic density. Since Rim is known to interact with the vesicle-associated GTPase Rab3A, here we asked whether UNC-10-dependent recruitment of synaptic vesicles to the presynaptic density was through an UNC-10/Rab-3 interaction. We first established that *C. elegans* Rab-3 (termed RAB-3) in its GTP but not GDP-bound state interacts with UNC-10. We then demonstrated by EM analysis that *rab-3* mutant synapses exhibit the same vesicle-targeting defect as *unc-10* mutants. Furthermore, *unc-10;rab-3* double mutants phenocopy the targeting defects of the single mutants, suggesting UNC-10 and RAB-3 act in the same pathway to target vesicles at the presynaptic density. Endogenous release of *unc-10;rab-3* double mutants was similar to that of *unc-10* single mutants, but more severe than *rab-3* mutants, suggesting the common targeting defects are reflected by the milder *rab-3* release defect. Rim has recently been shown to positively regulate calcium influx through direct interactions with calcium channels. Consistent with this notion we found UNC-10 colocalized with the calcium channel, UNC-2 at *C. elegans* presynaptic densities and synaptic release in *unc-10* and *rab-3* mutants exhibit reduced calcium-sensitivity. Together these results suggest that vesicles targeted to the presynaptic density by RAB-3/UNC-10 interactions are ideally positioned for efficient calcium-dependent release.

Keywords

Caenorhabditis elegans; Rim; Rab3; synaptic transmission; calcium channel; docking

Introduction

Rim is a component of the presynaptic density (PD), first identified as a Rab3a interacting molecule [24,26]. Subsequently, Rim has been shown to interact with several other presynaptic

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proteins including liprin, Munc-13 and calcium channels [11,21]. Genetic disruption of *C. elegans* Rim(*unc-10*) causes reduced neuromuscular transmission [12]. Similarly, mouse Rim1 knockouts exhibit release defects attributable to reduced synaptic vesicle (SV) priming and impaired long-term synaptic plasticity [2,3,14,21]. In both organisms, the actions of Rim were originally thought to occur downstream of SV docking, based on EM analysis using conventional chemical fixation [12,21]. However, re-examination of *C. elegans unc-10*(Rim) mutants using high-pressure freeze fixation [25], revealed a specific loss of docked SVs adjacent to the PD [26]. This result suggests one function of UNC-10(Rim) is to target SVs to this specialized region of the synaptic terminal [26]. Given that vertebrate Rim interacts with Rab3A [24], we postulated that UNC-10(Rim) recruits SVs to the active zone via RAB-3 interactions.

Rab3 is a monomeric G-protein that associates with SVs in its GTP-bound state and dissociates upon GTP hydrolysis [7,9,17]. Both *C. elegans rab-3* mutants and mouse Rab3A knockouts exhibit moderate synaptic defects [9,17]. To test whether these functional defects result from SV mistargeting, several studies have examined synaptic ultrastructure following Rab3 perturbation, with mixed findings. *C. elegans rab-3* mutants exhibited a redistribution of SVs to intersynaptic regions following conventional chemical fixation [17], whereas, synapses from Rab3 (A,B,C,D) knockouts appeared normal [20]. Synaptosomes from mouse Rab3A knockouts also exhibited normal SV distributions, but showed a reduction in activity-dependent SV recruitment to the active zone [13]. In contrast, neuromuscular junctions (NMJs) of Rab3A mouse mutants exhibited reduced docked SVs even under resting conditions, although vesicle distribution relative to the active zone was not examined [4]. Several studies suggest that Rab3 also regulates dense core vesicle (DCV) docking. For example, docking of DCVs in PC12 cells following Rab3A RNA interference was impaired and resulted in a concomitant decrease in exocytic event frequency [22]. Conversely, overexpression of Rab3A in PC12 and chromaffin cells increased the number of docked DCVs [16,23].

Together these observations suggest that Rim and Rab3 both regulate synaptic function and vesicle docking, although definitive evidence that they act together to target synaptic vesicles to the PD has yet to be obtained. To test whether Rim and Rab3 act in the same targeting pathway we examined interactions between *C. elegans* UNC-10(Rim) and RAB-3.

Materials and Methods

Yeast-2-hybrid

C. elegans RAB-1, -3, -5, -8, -11, -27, and -37 were amplified from first strand cDNA, and cloned into the yeast LexA binding domain vector pBHA (see supplemental methods for details). GTP-bound, GDP-bound and Δ CxC geranyl-geranylation motif lesions were introduced by site-directed mutagenesis (see supplemental methods for details). The N-terminal domain of Rim was introduced into the GAL4 activation domain vector pACT II. UNC-10 clones containing a.a. 1-143, 1-187, and 1-331, all showed activity. Tom Sudhof provided pLexNRAB3A(T36N), pLexNRAB3A(Q81L), and pREYRim-100 [24]. Plasmids were co-transformed into the yeast strain L40 (MATa, ade2, trp1 Δ 1, leu2-2,113, his3 Δ 200, LYS2::lexAop_(x4)-HIS3, URA3::lexAop_(x8)-LacZ) using a LiAC/ssDNA/PEG method. Interactions were visualized using an X-Gal filter lift assay and quantified using ONPG assays (see supplemental methods for details).

High-pressure freeze electron microscopy (EM)

Wild-type (N2 Bristol), *unc-10(md1117)*, *rab-3(js49)* and *unc-10(md1117);rab-3(js49)* animals were prepared for EM by high-pressure freeze fixation as described previously [26].

Morphometric analysis

40–50 nm thick serial sections spanning complete NMJs, defined as contiguous synaptic profiles with a PD were analyzed. For each synaptic profile, the number of SVs and distance from SV membranes perpendicular to the plasma membrane, and circumferential distance along the membrane to the center of the closest PD, were measured using ImageJ with the scorer blinded to genotype identity.

ImmunoEM

Animals were prepared for immuno EM analysis as previously described [26]. UNC-2 goat primary antibody (Santa Cruz Biotechnologies) was diluted 1:10 and anti-goat-15nm gold bead conjugated antibodies were diluted 1:80. The distance from each bead to the plasma membrane, and to the center of the closest PD was measured.

Electrophysiology

Electrophysiological methods were as previously described [10].

Results

RAB-3 and UNC-10/Rim interactions

Vertebrate Rim is known to bind selectively to activated Rab3 through an Zinc finger containing N-terminal domain [6,24]. To determine whether the Zinc finger domain of UNC-10 (Rim) interacts with *C. elegans* RAB-3, we performed yeast two hybrid assays using the zinc finger region of both *C. elegans* and vertebrate Rim. In the two hybrid assay, *C. elegans* and vertebrate Rim both interacted with the activated *C. elegans* GTP-bound RAB-3(Q81L) mutant, but not the GDP-bound RAB-3(T36N) mutant (Fig. 1A). This interaction was independent of the C-terminal CxC geranyl-geranylation motif that mediates membrane-association of RAB-3 (Fig. 1A). Furthermore, the *C. elegans* Rim-RAB-3 interaction was selective for RAB-3 as Rim failed to interact with wild-type or GTP-bound mutant forms of *C. elegans* RAB-1, -5, -8, -11, -27 and -37 (Fig. 1B). Vertebrate and *C. elegans* Rim showed very similar RAB-3 specificity (data not shown), suggesting the Rim/RAB-3 interaction has been highly conserved during evolution. To confirm the interaction under more native conditions we prepared Triton-X100 solubilized membrane extracts from WT *C. elegans*. However, neither UNC-10(Rim) nor the active zone-associated protein ELKS-1 was solubilized in Triton-X100 complicating the immunoprecipitation protocol. Instead, we spiked a Triton-X100 solubilized membrane extract with a Rim Zinc finger fusion protein at 0.5ng/ul and performed immunoprecipitations from the extract. Antibodies directed against synaptobrevin (SNB-1) efficiently immunoprecipitated SNB-1, but did not pull down either Rim or RAB-3 indicating that the membranes were efficiently solubilized. By contrast, immunoprecipitation of Rim pulled down RAB-3, but not SNB-1. Pre-immune sera failed to immunoprecipitate any of the three proteins.

SV-docking defects in *unc-10*, *rab-3* and *unc-10;rab-3*

Since UNC-10(Rim) is an integral active zone protein [26] that interacts with RAB-3::GTP, predicted to be SV-associated, we hypothesized that UNC-10(Rim) recruitment of SVs to the PD requires RAB-3. To test this model we used high-pressure freeze fixation to resolve the ultrastructural phenotype of *rab-3* mutants. *rab-3(js49)* mutant synapses contained normal numbers of SVs (23.78 ± 0.45 SVs per profile, $n=79$ for WT vs. 23.08 ± 0.81 SVs per profile, $n=39$ profiles for *rab-3* (ns $p>0.4$)) (Fig. 2A,B). However, there was a specific reduction in SVs contacting the plasma membrane near the presynaptic density, relative to the wild type ($p=0.0052$ in the 30nm bin adjacent to the PD) (Fig. 2A, 2C). This *rab-3* mutant docking defect phenocopied *unc-10(md1117)* mutants (Fig. 2A, 2D) in which docked SVs within 30nm of the

PD were also significantly reduced ($p < 0.0001$) [26]. This correlation suggests RAB-3 and UNC-10(Rim) function together to localize SVs at the PD. If UNC-10 and RAB-3 act in the same docking pathway we would expect the SV targeting defect of *unc-10;rab-3* double mutants to phenocopy those of *unc-10* and *rab-3* single mutants. Consistent with this model, the proportion of plasma membrane-contacting SVs adjacent to the PD in *unc-10;rab-3* mutants was reduced to the same extent as *unc-10* and *rab-3* single mutants ($p = 0.042$ for 30 nm, Fig. 2A, 2E). In addition to the SV targeting defect, *unc-10;rab-3* synaptic terminals contained significantly more SVs than wild-type (28.75 ± 0.85 SVs per profile, $n = 28$ profiles for *unc-10;rab-3*; $p < 0.0001$ Vs. 23.78 ± 0.45 SVs per profile, $n = 79$ for WT), similar to the accumulation observed in *unc-10* mutants (26.65 ± 0.66 SVs per profile $n = 84$ profiles, $p = 0.0001$) (Fig. 2A, 2B). This SV accumulation was not observed in *rab-3* single mutants, suggesting that in the absence of UNC-10(Rim) exocytic events are more severely impaired. This conclusion is consistent with previous behavioral analyses showing *unc-10* and *unc-10;rab-3* double mutants have more pronounced locomotory defects than *rab-3* single mutants [12].

Release defects in *unc-10*, *rab-3* and *unc-10;rab-3* mutants

To compare the functional consequences of *rab-3* and *unc-10* vs. *unc-10;rab-3* double mutants we performed electrophysiological analyses. The evoked responses were significantly reduced in all mutants, the *unc-10;rab-3* double mutant exhibiting the most pronounced defect (2157 ± 87.13 pA, $n = 16$, for WT vs. 1233 ± 161.3 pA, $n = 10$, for *rab-3*, $p < 0.0001$; 1074 ± 103.1 pA, $n = 17$, for *unc-10*, $p < 0.0001$ and 577.3 ± 96.49 pA, $n = 11$, for *unc-10;rab-3*, $p < 0.0001$) (Fig. 3A, B). Endogenous event frequency was also impaired in all three genotypes, *unc-10* and *unc-10;rab-3* mutants exhibiting a more severe decrease (20% of wild type) than *rab-3* mutants (65% of wild type) (105.9 ± 8.6 Hz, $n = 19$ for WT Vs. 68.40 ± 7.5 Hz, $n = 12$ for *rab-3*, $p = 0.0052$; 21.45 ± 2.3 Hz, $n = 19$ for *unc-10*, $p < 0.0001$ and 20.53 ± 2.5 Hz, $n = 11$ for *unc-10;rab-3*, $p < 0.0001$) (Fig. 3C,D). The average miniature event amplitudes were unaltered in the three mutant genotypes (27.67 ± 1.6 pA, $n = 17$ for WT; 27.42 ± 1.2 pA, $n = 12$ for *rab-3*; 23.91 ± 1.3 pA, $n = 19$ for *unc-10* and 25.47 ± 1.9 pA, $n = 11$ for *unc-10;rab-3*) (Fig. 3C,E), suggesting that the postsynaptic receptor field was unaffected. Together, these data indicate that UNC-10 and RAB-3 have partially overlapping presynaptic functions that likely reflect a common SV docking role, loss of UNC-10 producing a more severe functional defect.

Colocalization of calcium channels and Rim at PDs

Vertebrate Rim has been shown to suppress voltage-dependent inactivation of calcium channels through direct interactions [11]. Therefore, the more severe evoked defect of *unc-10* compared to *rab-3* mutants could in part, reflect a reduction in calcium influx at PDs where UNC-10 is localized [26]. The calcium channel alpha subunit, UNC-2 is required for evoked responses at the *C. elegans* NMJ [18], however, the subcellular localization of this calcium channel is unknown. To test whether UNC-2 co-localizes with UNC-10 at NMJ PDs we performed immunoEM. In a total of 44 synaptic profiles, the majority of gold beads recognizing UNC-2 labeled the membrane (59%, 41 of 70 gold beads total) and 37% of membrane-associated beads (15 of 41 gold beads) were specifically within 90nm of the PD, overlapping the subcellular distribution of UNC-10(Rim) Fig 4A,B).

If UNC-10 regulates local calcium entry, we would expect to see a reduction in the calcium-sensitivity of release in both *unc-10* and *rab-3* mutants. Consistent with this prediction, the evoked defects of *unc-10* and *rab-3* and single and double mutants were exacerbated when calcium was lowered from 5mM to 1mM (Fig 4C,D), loss of UNC-10 causing a larger decline than RAB-3 alone.

Together these results suggest that Rim may target a subset of SVs via RAB-3 interactions to regions in which calcium influx is enhanced, thereby ensuring efficient SV release.

Discussion

Given that Rim is an integral component of the PD and Rab3 is associated with SVs, interactions between these two proteins are predicted to target SVs to the active zone *in vivo*. To test of this model we first established that *C. elegans* RAB-3::GTP interacts with UNC-10(Rim). These results suggest that contrary to a previous report [8], the interaction between vertebrate Rim and Rab3A, extends to *C. elegans* homologs. This conclusion is further supported by the observation that vertebrate Rab3A and *C. elegans* RAB-3 are sufficiently conserved to allow cross species interactions with UNC-10(Rim) and vertebrate Rim.

The similarity in the SV docking phenotype observed in *unc-10* and *rab-3* mutants and the lack of additivity in the docking defect of the double mutant, further supports the notion that *C. elegans* Rim and RAB-3 functionally interact *in vivo*. A similar defect in active zone targeting of SVs has been observed in isolated synaptosomes from Rab3A knockout mice following synaptic activity [23], conditions which are likely to be comparable to the physiologically active state under which *C. elegans* synapses were fixed in the present study.

In order to understand how vesicle targeting to the PD influences synaptic transmission we compared the electrophysiological phenotypes of *C. elegans* *rab-3* and *unc-10* single mutants with that of *unc-10;rab-3* double mutants. As previously demonstrated, synaptic release was moderately impaired in *rab-3* mutants, whereas *unc-10* mutants and *unc-10;rab-3* double mutants exhibited similar, more severe defects particularly in endogenous release rates [12, 15,17]. It is therefore likely that the targeting defects common to both *rab-3* and *unc-10* mutants are reflected by the milder synaptic defects of *rab-3* mutants, where as the accumulation of synaptic vesicles in *unc-10* single and double mutants, reflects the more severe exocytic defects associated with loss of UNC-10. Vertebrate Rab3 knockouts also exhibit modest synaptic defects [20][4], which have been attributed to a reduction in calcium-sensitivity and release probability of a subset of the primed vesicle pool [19]. Thus, Rab3 is proposed to boost the efficiency of calcium-triggered release of this subset, elevating these vesicles to a “superprimed” state. Two mechanisms have been proposed to explain Rab3-dependent superpriming: Rab3 may target SVs to sites with higher-release probabilities and/or Rab3 may recruit additional proteins that enhance SV release efficiency [19]. Our analyses provide support for both mechanisms. The Rab3 binding partner, Rim has been shown to directly interact with voltage-gated calcium channels [5],[11], interactions with the beta subunit reducing channel inactivation and thus, enhancing calcium influx [11]. We have demonstrated by immunoEM that *C. elegans* UNC-10(Rim) [26] and UNC-2-containing calcium channels colocalize at the PD, in the region where RAB-3-dependent docking occurs. Therefore, the interaction between RAB-3 and UNC-10(Rim), is predicted to promote the targeting of vesicles adjacent to regions of high calcium entry [26]. This targeting mechanism provides an explanation for the reduced calcium-sensitivity of release observed in both cultured hippocampal neurons [19] and NMJs of Rab3A knockout mice [4] as well as the *C. elegans* *rab-3* and *unc-10* mutants, shown here.

Rab3 is also known to form a tripartite complex with Rim and the priming factor Munc-13 [6] and Rim has been shown to recruit Munc13-1 to mammalian synapses [1]. We have demonstrated by immunoEM, that the *C. elegans* homolog, UNC-13 is highly enriched at the PD [26]. Therefore, through the formation of this tripartate complex it is likely that RAB-3-dependent interactions lead to the placement of a subset of the primed vesicle pool at calcium hotspots, promoting efficient calcium-evoked release.

In conclusion, the biochemical and *in vivo* characterization of RAB-3 and UNC-10(Rim) presented here, provide evidence for a molecular interaction resulting in vesicle targeting to the PD that may explain the phenomenon of Rab3-dependent superpriming.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. UNC-10 interacts with RAB-3^{GTP}

A. The Rim-RAB-3 interaction is conserved across species boundaries. X-gal filter lift two-hybrid assays reveal interactions between worm UNC-10 Rim₁₋₁₈₇ and rat Rim₁₋₃₄₅ and both rat and worm GTP-bound mimic mutant RAB-3(Q81L), but not the GDP-bound mimic RAB-3(T36N). **B.** Interactions between worm UNC-10 Rim₁₋₁₈₇ were highly selective for RAB-3 compared to other RABs. **C.** Rim interacts with RAB-3 in worm extracts. Whole worm solubilized extracts spiked with purified His6Rim Zinc Finger fusion protein were immunopurified using anti-vSNARE SNB-1, anti-Rim, and preimmune sera, separated by SDS-PAGE and analyzed by Western blot using antibodies against SNB-1, Rim and RAB-3. 2% of the input and 10% of each immunoprecipitate were loaded on the gels.

Figure 2. Ultrastructural analysis of *rab-3*, *unc-10* and *unc-10;rab3* mutant synapses
A, Representative micrographs for wild type, *rab-3*, *unc-10* and *unc-10;rab-3* animals. The PD (arrow) and closest membrane-contacting vesicle to the PD is indicated (arrowhead). Scale bar 200 nm. **B**, The number of SVs per profile was significantly increased in *unc-10* and *unc-10;rab-3* relative to the wild-type. Bar shades for each genotype are also used in subsequent graphs. **C,D,E** Distribution of membrane-contacting vesicles relative to the PD in *rab-3* vs. wild type animals (**C**) and *unc-10* vs. wild type (**D**) and *unc-10;rab-3* vs. wild type (**E**). All three mutant genotypes exhibit a decrease in the ratio of SVs contacting the plasma membrane adjacent to presynaptic density. Data expressed as mean $\pm$ SEM; data in C–E are grouped into 30 nm bins.

Figure 3. Electrophysiological phenotypes of *rab-3*, *unc-10* and *unc-10;rab3*

A. Representative evoked synaptic recordings for wild type, *rab-3*, *unc-10* and *unc-10;rab-3*. **B.** Average evoked amplitudes were reduced in *rab-3*, *unc-10* and *unc-10;rab-3* compared to wild-type animals. **C.** Representative endogenous miniature postsynaptic event recordings from wild type, *rab-3*, *unc-10* and *unc-10;rab-3*. **D.** Average frequencies of miniature postsynaptic currents were reduced in *rab-3*, *unc-10* and *unc-10;rab-3* compared to wild-type animals. **E.** Average miniature event amplitudes were not altered in *rab-3*, *unc-10* and *unc-10;rab3* mutants compared to the wild type. Data expressed as mean $\pm$ SEM. All recordings obtained in 5mM extracellular calcium.

Figure 4. Distribution of the membrane-associated calcium channel UNC-2 at the NMJ
A. Micrographs of NMJs prepared for immunoEM, 15nm gold beads (arrow) labeling UNC-2. Scale bar 200nm. **B.** Bar graph showing the spatial distribution of membrane-associated gold beads relative to the PD (position 0 on the x-axis). **C.** Average evoked amplitudes of *rab-3*, *unc-10* and *unc-10;rab-3* and wild-type animals in 1mM extracellular calcium. **D.** Plots of the % decrease in average evoked responses obtained in 1mM Vs 5mM calcium for *rab-3*, *unc-10* and *unc-10;rab-3* and wild-type animals.