



UNC-31/CAPS docks and primes dense core vesicles in *C. elegans* neurons

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ABSTRACT

UNC-31 or its mammalian homologue, Ca^{2+} -dependent activator protein for secretion (CAPS), is indispensable for exocytosis of dense core vesicle (DCV) and synaptic vesicle (SV). From N- to the C-terminus, UNC-31 contains putative functional domains, including dynactin 1 binding domain (DBD), C2, PH, (M)UNC-13 homology domain (MHD) and DCV binding domain (DCVBD), the last four we examined in this study. We employed UNC-31 null mutant *C. elegans* worms to examine whether UNC-31 functions could be rescued by ectopic expression of full length UNC-31 vs each of these four domain-deleted mutants. Full length UNC-31 cDNA rescued the phenotypes of *C. elegans* null mutants in response to Ca^{2+} -elevation in ALA neurons. Surprisingly, MHD deletion also rescued UNC-31 exocytotic function in part because the relatively high Ca^{2+} level (pre-flash Ca^{2+} was 450 nM) used in the capacitance study could bypass the MHD defect. Nonetheless, the three other domain-truncation cDNAs had almost no rescue on Ca^{2+} evoked secretion. Importantly, this genetic null mutant rescue strategy enabled physiological studies at levels of whole organism to single cells, such as locomotion assay, pharmacological study of neurotransmission at neuromuscular junction, *in vivo* neuropeptide release measurement and analysis of vesicular docking. Our results suggest that each of these UNC-31 domains support distinct sequential molecular actions of UNC-31 in vesicular exocytosis, including steps in vesicle tethering and docking that bridge vesicle with plasma membrane, and subsequently priming vesicle by initiating the formation of soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) core complex.

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1. Introduction

UNC-31 or its mammalian homologue Ca^{2+} -dependent activator protein for secretion (CAPS) is an essential factor in Ca^{2+} -dependent secretion [1–4]. It is predicted to contain 5 conserved function domains, from N-terminus to C-terminus: dynactin 1 binding domain (DBD), C2, pleckstrin homology (PH), (M)UNC-13 homology domain (MHD) and DCV binding domain (DCVBD) [5]. The DBD is required for UNC-31 sorting [6]. Next is the C2 domain, which is reported to be a Ca^{2+} sensor that mediates Ca^{2+} -dependent binding to phospholipids and translocation of the protein to plasma-membrane [7]. The following PH domain interacts with acidic phospholipids and binds with plasma membranes [8]. It displays

an ability to bind with phosphatidylinositol4,5-bisphosphate [9]. Next to the PH domain is MHD. MHD domain directly interacts with syntaxin, a regulatable SNARE protein, transforms syntaxin from closed conformation into open form which confers competence of syntaxin to form the SNARE core complex [10–14]. The C-terminal DCV binding domain (DCVBD) abutting with MHD mediates UNC-31 targeting to and associating with dense core vesicles [9]. As DBD domain is required for sorting of UNC-31, it may not be required for its functions in docking and priming process, so we focused on last four domains and retained the DBD domain in all mutant constructions in the following study.

UNC-31 may tether and dock dense core vesicles at plasma membrane by binding with plasmalemma via both C2 and PH domains and by binding with DCVs via DCVBD; the MHD initiates the formation of SNARE core complex and thus may further dock and prime vesicles. Therefore, all domains are required for the exocytosis to proceed. To test such hypothesis, we adopted genetic manipulation such as *unc-31* null mutation and introduction of full length *unc-31* cDNA or domain-deletion sequences, and used high temporal membrane capacitance measurement, *in vivo* record of DCVs' neuropeptide release, pharmacological and behavioral assays and

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observation of vesicular docking process by TIRFM to analyze the functions of each of these four domains of UNC-31. Our results demonstrated that all four domains are indispensable for the function of UNC-31. Besides, we found that high Ca^{2+} level (450 nM) could bypass the requirement of MHD for the function of UNC-31 in electrophysiology experiments on cultured neuron cells.

2. Materials and methods

2.1. Worm culture and strains

Worms were maintained at 20 °C in nematode growth medium agar (NGM) plates, seeded with *Escherichia coli* OP50. Strains used in this study: wild-type N2 Bristol, *ida-1::gfp* transgene KM246 [15], *unc-31(e928)*. We crossed *unc-31(e928)* with EG3680(*oxIs206*) and KM246 to obtain *unc-31(e928);EG3680(oxIs206)* and *unc-31(e928);KM246*.

2.2. Plasmid construction

The 3780-bp *unc-31* cDNA [16] under the control of the *rab-3* promoter was constructed for genetic rescue. The 1.2 kb *rab-3* promoter was amplified by PCR and subcloned to pPD49.26 (1997 Fire Lab Vector Kit) to gain pPD49.26-Prab-3. Deletions of the conserved UNC-31 domains (C2, PH, MHD and DCVBD) were generated by overlap extension PCR and subcloned to pPD49.26-Prab-3.

2.3. Germline transformation

Germline transformation was performed by use of a standard microinjection method [17,18]. 70 ng/ μl of full length or domain-truncated *unc-31* cDNA and 30 ng/ μl P_{myo-3} TDimer2 were co-injected into *unc-31(e928);EG3680(oxIs206)* for assay of ANF-GFP release; 70 ng/ μl of full length or domain-truncated *unc-31* cDNA plus 30 ng/ μl P_{ida-1} IDA-1::GFP were injected into *unc-31(e928)* for electrophysiological experiments, locomotion assay, pharmacological study and TIRFM imaging.

2.4. Cell culture and electrophysiology

Embryonic cells were isolated from nematode eggs and cultured as described [19,20]. The plasma membrane capacitance (C_m) of the *C. elegans* primary culture neuron was measured as described previously [21] using an EPC10 amplifier (Heka Electronics, Lambrrecht, Germany) in whole-cell patch clamp configuration. The cells were voltage clamped at a holding potential of -50 mV and a sine wave voltage command with peak-to-peak of 50 mV and frequency of 5 kHz was applied. Currents were filtered at first step 30 kHz and second step 10.5 kHz, sampled at 62.5 ksamples/s. The pressure polished and paraffinum coated pipette with resistance 2–4 M Ω and approximately 1 μm tip opening was used, and the leak current in whole-cell recording was restricted to <20 pA.

2.5. Ca^{2+} -uncaging and $[\text{Ca}^{2+}]_i$ measurement

A homogenous global $[\text{Ca}^{2+}]_i$ elevation was generated by photolysis of the Ca^{2+} -caging compound, NP-EGTA, (Molecular Probes, Carlsbad, CA, USA), with UV light generated by JML-C2 flash lamp system (Rapp Optoelektronik, Hamburg, Germany) and coupled into Olympus IX 70 microscope. $[\text{Ca}^{2+}]_i$ was measured by use of dual wavelength excitation alternating between 350 and 385 nm and mixed dyes of 0.4 M fura-4f and 0.4 M furaptra as described [22]. The external bath solution consisted of (in mM): 145 NaCl, 5 KCl, 1 CaCl_2 , 5 MgCl_2 , 10 HEPES, 20 D-glucose, adjusted to pH 7.2 using NaOH or HCl, and to osmolarity 340 mOsm. The NP-EGTA

containing pipette solution contained (in mM): 110 Cs-glutamate, 8 NaCl, 35 HEPES, 2 MgATP, 0.3 GTP, 10 nitrophenyl-EGTA, 0.4 fura-4f, 0.4 mag-fura-2, adjust to pH 7.2, to osmolarity 330 mOsm and to the free Ca^{2+} level about 450 nM. The data were analyzed by using Igor Pro (Version 5.0.3.0, WaveMetrics, Lake Oswego, Oregon, USA).

2.6. TIRFM imaging

Our TIRFM setup was constructed on Olympus IX70 microscope equipped with an alpha Plan-Fluar oil-immersion 100 \times objective lens (NA = 1.45, Carl Zeiss MicroImaging Inc., Göttingen, Germany) based on the prism-less and through-the-lens configuration by using of TILL TIRF condenser (TILL Photonics GmbH, Lochhamer Schlag 21, Gräfelfing, Germany). A 488 nm argon laser was used for fluorescence excitation. 14-bit digital images at 40 Hz and 25 ms exposure time were acquired by iXon^{EM} + DU 885 EMCCD camera (Andor Technology plc, Springvale Business Park, Belfast BT12 7AL, United Kingdom). Imaging was controlled by Solis 4.9.3 (Andor Technology plc). Stacks of TIRF images were analyzed by a self-written program in Matlab R2008a (Mathworks, Natick, MA, USA). Single vesicle detection was performed by use of an à trous wavelet transformation with the level $k = 3$ and detection level $ld = 1.0$ [23], producing a binary mask image. Only spots with areas larger than 4 (2×2) pixels were accepted as vesicles for further analysis and those of less than four pixel-wide were rejected. The fluorescence intensity and positions of the vesicles were determined using a 2D Gaussian fitting with a background offset. For tracking the vesicles, we used a robust single particle tracking algorithm provided by Khuloud Jaqaman [24]. For dwell time analysis, vesicles dwelling in the evanescent field less than three frames were excluded. Only when vesicles moved less than one pixel laterally between adjacent frames for at least three consecutive frames with a concurrent stabilized increase in fluorescence, were they checked manually and finally accepted as dwelling events.

2.7. Assay of ANF-GFP release in neurons

The ectopically expressed neuropeptide ANF-GFP was measured as the intensity of fluorescence in coelomocytes as described [25]. Confocal imaging was performed on an Olympus FV500 laser-scanning confocal imaging system with a 60 \times objective lens (NA = 1.40, Olympus). The laser power and the imaging setting were held constant in each group of experiments. The worms of mutants and controls were imaged alternately. Only the coelomocytes that were not masked by other tissues (gut and gonads) and did not abut with body wall were captured. Images were displayed and analyzed by Image-J 1.43b (Wayne Rasband, National Institute of Health, USA).

2.8. Assay of body bend and aldicarb resistance

For body bend assay, worms were tracked for 10 min at 20 °C on NGM plates spread with a thin layer of freshly grown OP50 bacteria as described [26]. Aldicarb resistance assay was performed with the method described by Lackner [27]. Worms that failed to respond at all to the harsh touch were identified as paralyzed. Each experiment was repeated at least three times.

2.9. Statistic analysis

Data analysis was conducted by use of SigmaStat (Systat Software, Inc., Suite E Point Richmond, CA, USA) or Igor Pro (WaveMetrics, Lake Oswego, OR, USA). The data were presented as the mean value \pm S.E.M. with the indicated number of experiments (n). Statistical significance was evaluated using Students t' test or Mann-Whitney rank sum test according to the normality of data distribu-

tion. Asterisks denote statistical significance as compared with control, with a P value less than 0.05 (*), 0.01 (**), and 0.001 (***).

3. Results and discussion

3.1. The function of the domains of UNC-31 in DCV exocytosis

The change of plasma membrane area measured by high-temporal capacitance recording is a direct readout of cellular exocytosis and endocytosis. As shown in Fig. 1A, flash photolysis evoked a burst C_m increase of 12.7 ± 1.7 fF ($n = 19$, black trace) in IDA-1::GFP-labeled ALA neurons of KM246 (*ida-1::gfp* transgene). Under similar step-like $[Ca^{2+}]_i$ elevations, the burst of exocytosis in the cells of *unc-31(e928)*;KM246 was significantly reduced to 6.1 ± 1.1 fF ($n = 17$, light gray trace), a 48% reduction. The exocytotic defect was fully rescued by reintroduction of full length *unc-31* cDNA (13.1 ± 1.5 fF, $n = 12$, heavy gray trace). These results confirm that the UNC-31 protein is essential for the secretion of DCVs [25].

To elucidate the UNC-31 structure–function, we employed the strategy of rescue with expression of specific UNC-31 domain-deletion, and then subject the neurons to functional analysis. Four domain-truncated *unc-31* cDNAs were constructed and used to

germinally transfect *unc-31(e928)* worms, P_{ida-1} ::GFP injection was used to label the ALA neurons. As shown in Fig. 1B and 1D, reintroduction of UNC-31 deleted of C2- (8.8 ± 1.1 fF, $n = 17$, dark gray trace Fig. 1B), PH- (8.4 ± 1.2 fF, $n = 15$, light gray trace Fig. 1B) and DCVBD- (6.2 ± 0.7 fF, $n = 15$, gray trace Fig. 1D) domain did not fully rescue the exocytotic phenotype in ALA neuron of *unc-31* null mutant.

MHD domain interacts with syntaxin, initiating the formation of SNARE complex and functions in vesicle docking and priming [10,13,14]. Thus it is natural to deduce that MHD is essential for the function of UNC-31. To our surprise, expression of *unc-31* cDNA with MHD-deletion did however fully rescue the exocytotic defect in ALA of *unc-31(e928)* strain (13.1 ± 1.2 fF, $n = 22$, gray trace Fig. 1C). The most possible explanation is that the non-physiological and relatively high pre-flash Ca^{2+} level (about 450 nM) used in the Ca^{2+} -uncaging experiments could bypass the requirement of MHD for the UNC-31's function of priming in vesicular exocytosis. We tried to examine the effect of lower basal Ca^{2+} levels in the intracellular solutions (100, 200 and 300 nM). Unfortunately, the exocytotic responses to Ca^{2+} -uncaging we recorded in ALA neurons in low pre-flash $[Ca^{2+}]_i$ was totally distorted (data not shown). We then adopted other assays to answer the question.

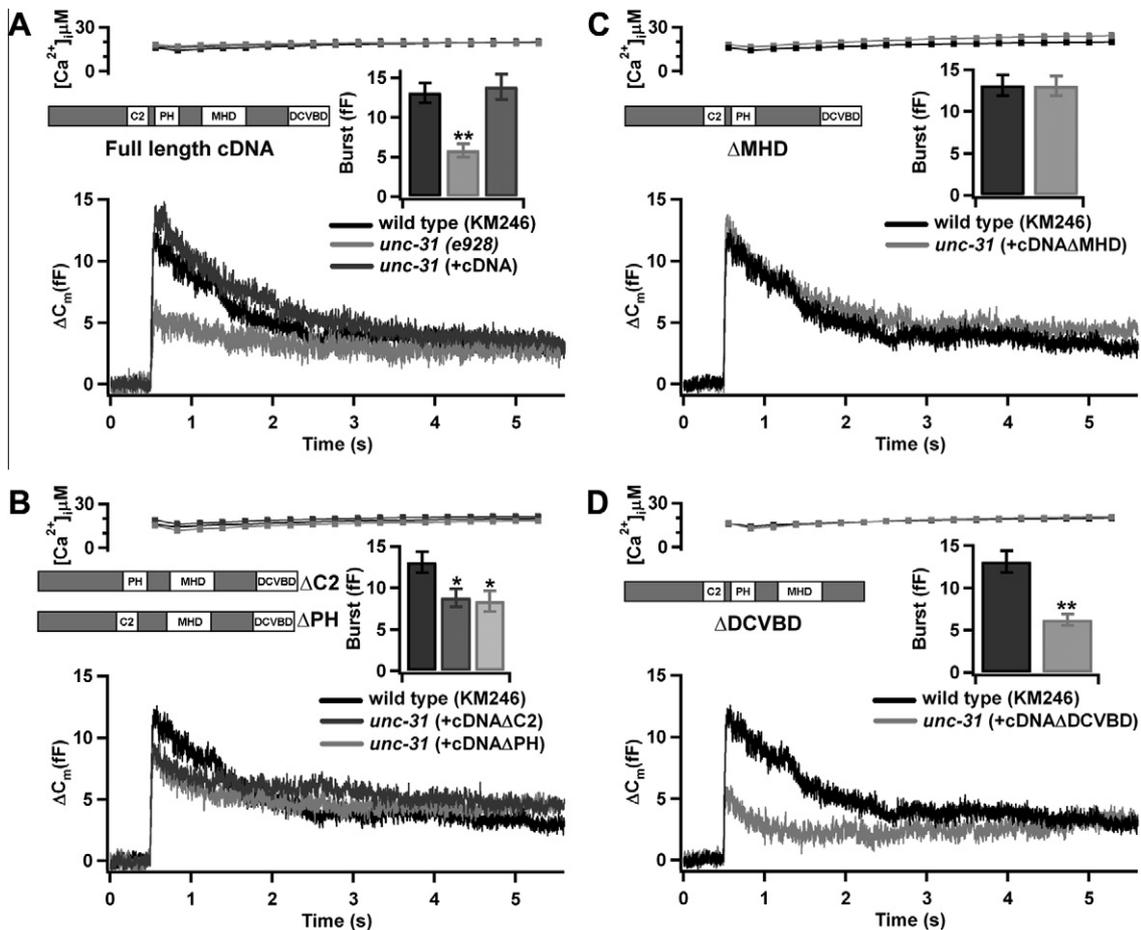


Fig. 1. Domain-truncation *unc-31* c-DNA sequences did not rescue the exocytosis elicited by $[Ca^{2+}]_i$ elevation in IDA-1::GFP-labeled ALA neuron, except the one of MHD deletion. The schematic diagrams of full length and the domain-truncation *unc-31* cDNA and statistic analyses of the exocytotic bursts are shown in the middle panels. Exocytotic burst was quantified as the amplitude of an exponential fit to the C_m trace after Ca^{2+} -uncaging. (A) Comparison of C_m increases (bottom) induced by similar $[Ca^{2+}]_i$ elevations (top) in the ALA cells of wild-type (N2) (black trace), *unc-31(e928)* mutant (light gray trace), *unc-31* mutant expressing full length *unc-31* cDNA (dark gray trace). (B) Averaged C_m responses (bottom) triggered by similar $[Ca^{2+}]_i$ elevations (top) in wild-type (black trace), *unc-31* mutant expressing P_{rab-3} *unc-31* Δ C2 (dark black trace) and P_{rab-3} *unc-31* Δ PH. (C) Comparison of averaged C_m responses (bottom) induced by similar $[Ca^{2+}]_i$ elevations (top) in wild-type (black trace) and *unc-31* mutant expressing P_{rab-3} *unc-31* Δ MHD are shown. (D) Averaged C_m responses (bottom) induced by similar $[Ca^{2+}]_i$ elevations (top) from wild-type (black trace) and *unc-31* mutant expressing P_{rab-3} *unc-31* Δ DCVBD. Error bars indicate SEM.

3.2. UNC-31 needs all functional domains to act in worm's locomotion and neurotransmission

Locomotion assay is a generally used method to examine neurotransmission in *C. elegans*. In order to answer the question of physiological function of these domains in live animals, we performed locomotion assay in worms of wild-type N2, *unc-31* null mutant and the mutant expressing the full length and domain-truncated UNC-31. The worms of *unc-31(e928)* mutant displayed a low body bend rate, which can be rescued by the full length UNC-31. But the domain-truncated *unc-31* c-DNA sequence are invalid (Supplementary Fig. 1).

Lack of whole-body coordination can be caused by defects of pre-synaptic neurotransmitter release or/and postsynaptic muscular function, which we delineated in the following studies. Previous works showed the null mutation of *unc-31* is hypersensitive to levamisole [28], an agonist of nicotinic acetylcholine receptor, or as sensitive as wild-type strain [16], which suggests that *unc-31* null mutant would not have defect in post synaptic response. Therefore, we used only aldicarb, a cholinesterase inhibitor, to detect UNC-31 function in pre-synaptic neurotransmitter release. Consistent with the results of locomotion assays, ectopic expression of domain-truncated *unc-31* c-DNA sequences in *unc-31(e928)* mutant did not rescue the aldicarb-resistance phenotype (Supplementary Fig. 2).

3.3. Four UNC-31 domains are required to maintain the release of ectopically expressed ANF-GFP in neurons

The ectopically expressed neuropeptide ANF-GFP targets to the DCVs in PC12 cells [29], and in the neurons of *C. elegans* under the drive of *aex-3* promoter [25]. The released ANF-GFP in pseudoco-

lom is endocytosed by the coelomocytes, which are professional scavenger cells that function to cleaning up fluid from the pseudocoelom by phagocytosis in *C. elegans* [30–33]. The fluorescence intensity of the ANF-GFP in coelomocytes is suitable to assay the DCV exocytosis in neurons in *C. elegans in vivo* under physiological condition [25].

To further validate our above observations, we measured the fluorescence in coelomocytes in worms of EG3680(*oxIs206*), *unc-31(e928)*;EG3680(*oxIs206*) and *unc-31(e928)*;EG3680(*oxIs206*) mutant germinal transfected with full length UNC-31 cDNA and domain-deletion sequences which was also co-transfected with *P_{myo-3}*TDimer2 as a screening marker. We observed a remarkable reduction of fluorescence intensities in coelomocytes in worms of *unc-31(e928)*;EG3680(*oxIs206*) (light gray column, *n* = 22, Fig. 2) compared with controls of EG3680(*oxIs206*) (black column, *n* = 22, Fig. 2). Ectopically expressed full length of UNC-31 (dark gray column, *n* = 21, Fig. 2), but none of all the four domain-deletion proteins (other columns, Fig. 2) fully reestablished the exocytotic ability of DCVs in neurons of *unc-31(e928)*;EG3680(*oxIs206*) worms. All data from *in vivo* studies support our explanation of that high pre-flash Ca^{2+} level could bypass the requirement of MHD domain for UNC-31 function in DCV exocytosis.

3.4. Anatomization of the functions of UNC-31 domains in vesicular docking and priming processes by TIRFM

Total internal fluorescence microscopy (TIRFM) has been employed to observe the docking and priming processes of vesicles in live cells under relatively physiological condition. When a vesicle docks at the plasma membrane, it will be caged in a relatively

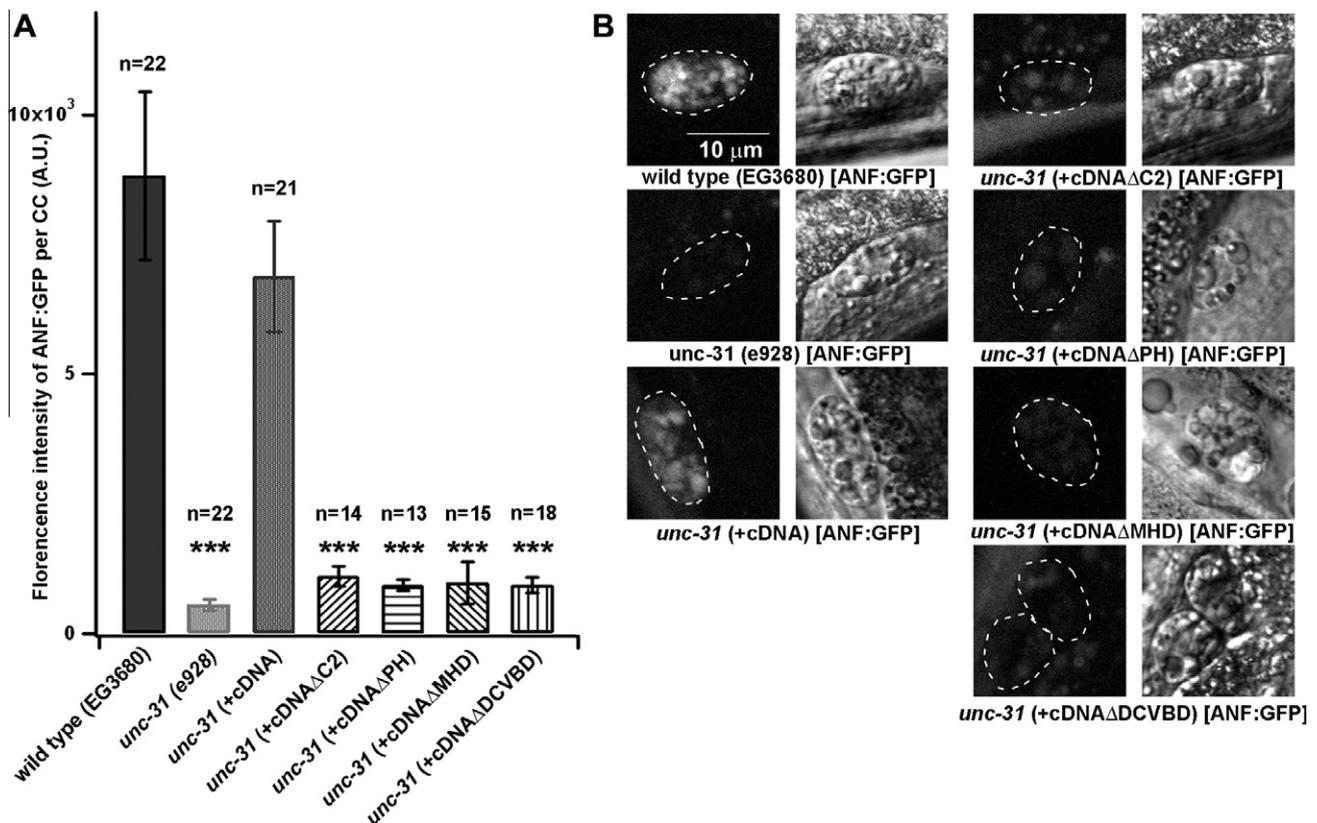


Fig. 2. *In vivo* assays of the ANF-GFP secretion in wild-type N2, *unc-31(e928)* mutant and worms expressing full length and domain-truncated *unc-31* cDNA. (A) Histograms show the averaged total fluorescence intensity of ANF-GFP in coelomocytes in worms of EG3680, *unc-31(e928)*;EG3680 and *unc-31(e928)*;EG3680 harboring full length (+cDNA) and domain-truncated *unc-31* cDNA (+cDNA Δ C2,+cDNA Δ PH,+cDNA Δ MHD and +cDNA Δ DCVBD). (B) Representative examples shown are fluorescent and bright field images of coelomocytes in worms of indicated strains. Coelomocytes are outlined with dashed lines. Fluorescence outside the coelomocytes is gut autofluorescence or the leak fluorescence of expressed TDimer2 in body wall muscle, which was used as a marker of genetic transfection. Error bars indicate SEM.

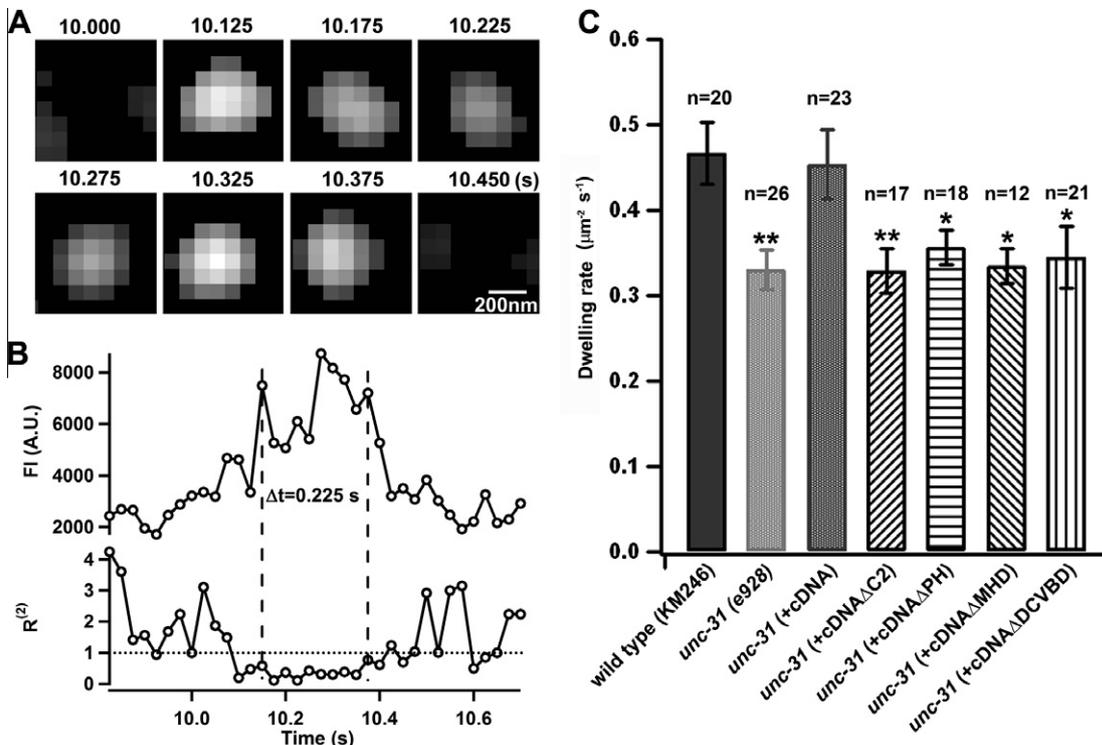


Fig. 3. Dwell rate in evanescent field of dens core vesicles (DCVs) in ALA neurons in worms of KM246 transgene and *unc-31(e928)* mutant expressing full length and domain-deleted UNC-31 proteins under resting condition. (A) Example of sequential images of a single IDA-1::GFP-labeled DCVs in a live neuron. (B) Time course of the fluorescence intensity in arbitrary units (A.U.) and the 2D displacement ($R^{(2)}$) of the docked vesicle shown in A. The horizontal dotted line marks $R^{(2)}$ level of 1 pixel (80 nm), which was used as a threshold to identify immobilization. The duration of vesicular $R^{(2)}$ below this threshold combined with fluorescence intensity above threshold indicated by two vertical dashed lines measured the dwell time, 0.225s in this case. (C) The dwell indexes (dwelling events of DCVs per μm^2 per second) were used for the analyses of roles of full length UNC-31 and domain-truncated proteins in the docking and priming processes of DCVs in neurons. The worms of *ida-1::gfp* transgene KM246 was used as controls. *P_{ida-1}*IDA-1::GFP germlinal transfected *unc-31(e928)* mutant (*unc-31(e928)*, light gray bar) exhibited reduced dwell rate and was fully rescued by expression of full length *unc-31* cDNA (*unc-31(+cDNA)* dark gray bar). All the *P_{ida-1}*IDA-1::GFP and domain-deleted *unc-31* cDNA transgene worms (*unc-31(+cDNAΔC2)*, *unc-31(+cDNAΔPH)* and *unc-31(+cDNAΔMHD)*, *unc-31(+cDNAΔDCVBD)*) displayed significant reduced dwell rates (patterned bars). Error bars indicate SEM.

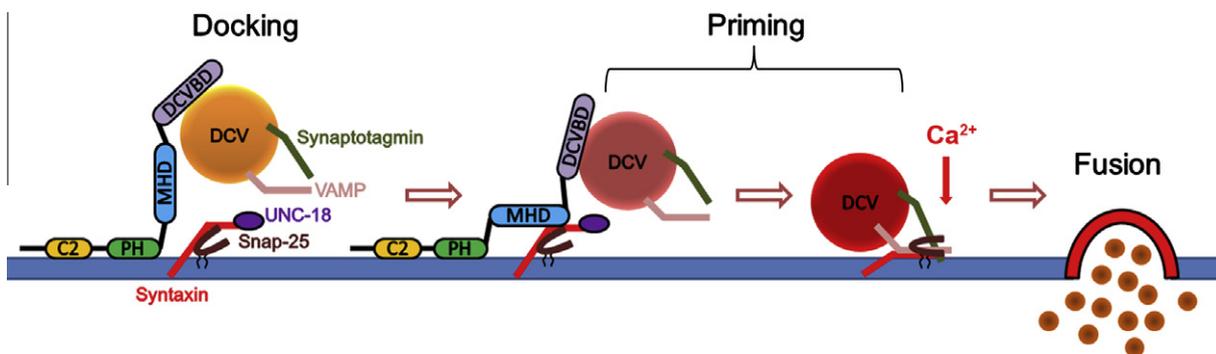


Fig. 4. The hypothesized model of the mechanism for UNC-31 function in vesicle's docking and priming. By binding with plasmalemma at its N-terminal via both C2 and PH domains and by binding with DCVs at its C-terminal via DCVBD, UNC-31 tethers and docks dense core vesicles at the plasma membrane. The MHD initiates the formation of molecular machinery consisted of SNARE complex and synaptotagmin and thus further docks and primes vesicles via its interaction with syntaxin. The SNARE complex is formed by zippering among the SNARE motif of syntaxin, SNAP-25 (synaptosome-associated protein of 25 kDa) and VAMP (vesicle associated membrane proteins, or synaptobrevin).

“moveless” stage, with a small caged diameter, a restricted x-y-z movement, a low fluorescent fluctuation and bright fluorescence, as shown in Fig. 3A and Fig. 3B. The priming process will prolong this stage. The diameter of DCVs in *C. elegans* neurons is much smaller than that in mammalian cells, which move much faster than those in mammalian cells (data not shown). In our observation, about half of vesicles dwelled in evanescent field for only 75–100 ms. It is hard to set a temporal threshold for vesicular docking. We used dwell rate in resting condition (dwelling events per μm^2 per second) instead of docking time and set the threshold

of time to 75 ms to analyze the functions of the domains of UNC-31 in the DCVs' docking and priming.

As shown in Fig. 3C, *unc-31* null mutation significantly reduced the dwell rate of DCVs in ALA neurons in worms of *unc-31(e928)* (0.330 ± 0.023 , $n = 26$) in comparison with that of control worms (0.466 ± 0.036 , $n = 26$). The full phenotypic rescue by reintroduction of full length of UNC-31 (*unc-31(+cDNA)*, 0.453 ± 0.041 , $n = 23$) demonstrates that the effect in *unc-31(e928)* is caused by loss of the gene *per se*. None of the ectopic expression of domain-deletion *unc-31* cDNAs restored the normal vesicular docking and

priming processes (Fig. 3C). This anatomization of function of UNC-31 domains in vesicular docking and priming processes further confirmed the results of the *in vivo* assays.

Taking all the data together, we proved that UNC-31 needs all domains for its role in vesicular docking and priming. We suggest a hypothesized model as shown in Fig. 4 of the mechanism for UNC-31 function, basing on the existed knowledge and our present results.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.05.148.

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