Proteolytic Processing of the Extracellular Scaffolding Protein LEV-9 Is Required for Clustering Acetylcholine Receptors

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Background: LEV-9 is an extracellular scaffolding protein required for synaptic clustering of acetylcholine receptors in C. elegans.

Results: C-terminal cleavage of LEV-9 is necessary for its synaptic function.

Conclusion: LEV-9 is activated by proteolytic cleavage following the final tandem complement control protein (CCP) modules.

Significance: Many LEV-9-related factors are likely cleaved after CCP domains, suggesting that cleavage location can be conserved in distant architecturally related proteins.

Correct positioning of neurotransmitter-gated receptors at postsynapses is essential for synaptic transmission. At Caenorhabditis elegans neuromuscular junctions, clustering of levamisole-sensitive acetylcholine receptors (L-AChRs) requires the muscle-secreted scaffolding protein LEV-9, a multidomain factor containing complement control protein (CCP) modules. Here we show that LEV-9 needs to be cleaved at its C terminus to exert its function. LEV-9 cleavage is not required for trafficking nor secretion but directly controls scaffolding activity. The cleavage site is evolutionarily conserved, and post-translational cleavage ensures the structural and functional decoupling between different isoforms encoded by the lev-9 gene. Data mining indicates that most human CCP-containing factors are likely cleaved C-terminally from CCP tandems, suggesting that not only domain architectures but also cleavage location can be conserved in distant architecturally related proteins.

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Scaffold-mediated receptor clustering at postsynapses is a key element of synaptic transmission. The activity of scaffold proteins can be regulated by several post-translational mechanisms including phosphorylation and degradation (1, 2), but also by limited proteolysis. For example, the rapid insertion of excitatory glutamate-gated AMPA receptors is regulated by cleaving the scaffolding protein GRIP (glutamate receptor-interacting protein) by Ca2+-dependent calpains (3). Calpains are also thought to regulate glutamate-gated NMDA receptor levels by acting upon the canonical cytoplasmic scaffolding protein PSD-95 (4). Extracellular proteolysis of scaffolding factors can also regulate synaptic receptor quantities. For instance, cleavage of the neuronal pentraxin receptor NRP by the extracellular metalloprotease TNF-converting enzyme (TACE) accelerates AMPA receptor endocytosis, resulting in a decreased content of AMPA receptors at synapses, a process necessary for mGlur1/5-dependent long-term depression in hippocampal and cerebellar synapses (5).

At excitatory neuromuscular junctions (NMJs) in Caenorhabditis elegans, an extracellular scaffold containing the LEV-9 and LEV-10 proteins is required for L-AChR clustering (6, 7). LEV-9 is a muscle-secreted factor composed by a WAP domain (whey acidic protein) followed by a tandem of eight consecutive CCP modules (also known as short consensus repeats or SUSHI repeats) (see Fig. 1A). It is architecturally related to several plasma proteins in vertebrates, including regulators of the complement system (8). LEV-10 is composed chiefly by CUB ectodomains (for complement C1r/C1s, Uegf, Bmp1) anchored to the muscle membrane via a transmembrane domain. LEV-10 belongs to a superfamilly of ancillary proteins that regulate glutamate-gated receptors in vertebrates and invertebrates (8). Interaction between LEV-9, LEV-10, and L-AChRs is stabilized by OIG-4, a small secreted protein made of one immunoglobulin domain (9). In the absence of any of its components, including the L-AChRs themselves, the LEV-9-LEV-10 complex disassembles entirely. In lev-9 or lev-10 mutants, L-AChRs are declustered, but the total amount of functional receptors in the muscle membrane remains unchanged. However, these mutants survive chronic exposure to concentrations of the L-AChR agonist levamisole that are lethal to wild-type animals (6, 7, 9). Therefore, the L-AChR-associated complex is an attractive model for synaptic scaffolding by virtue of its domain architecture, constitutive activity, and involvement in downstream regulation of synaptic transmission.

In the present work, we show that LEV-9 is cleaved both in a heterologous expression system and in vivo. The cleaving site maps to a highly conserved set of residues present at the C-terminal end of LEV-9 that are predicted to be recognized by proprotein convertases. LEV-9 cleavage is required for its clustering activity.

The abbreviations used are: NMJ, neuromuscular junction; L-AChR, levamisole-sensitive acetylcholine receptor; CCP, complement control protein; PC, subtilisin/kexin2p-like proprotein convertase(s); NdK, nardilysin(s); Ct, C-terminal; NS, not significant.
but not for secretion nor traffic. We also show that the lev-9 locus encodes multiple isoforms, including a long polypeptide containing 17 CCP domains that is found in other species. We suggest that the post-translational cleavage of LEV-9 might serve to ensure its functional and structural decoupling from any C-terminal polypeptide attachments, as we provide some evidence suggesting that other CCP-containing proteins found in humans might undergo similar post-translational processing.
EXPERIMENTAL PROCEDURES

Genetics—Strains were maintained at 15 or 20 °C on regular Nematode Growth Medium plates. OP50 Escherichia coli was used as food source. Transgenic lines were obtained injecting construct at 10–20 ng/μL DNA and co-injection marker pEK15 (lin-15+) at 80 ng/μL. Injections were done in the unc-29 (kr208::tagRFPt); lev-9 (ox177) lin-15 (n765ts) strain, except for dominant-negative experiments that were done in unc-29 (kr208::tagRFPt); lin-15 (n765ts) strain. The strains used are compiled in the supplemental material.

Cell Culture—S2 Drosophila cells were maintained following the supplier recommendations (Invitrogen, catalog number R690-07) as described previously (7) and transiently transfected using calcium phosphate. Recombinant protein expression was driven by a metallothionein promoter and induced by copper sulfate.

Biochemistry—Media of transfected S2 cells were recovered and cleared by spinning. Cells were recovered, washed, and lysed with Triton X-100 and sonication. Worms were recovered from mixed staged plates, washed, and resuspended in β-mercaptoethanol-containing loading buffer and boiled. SDS-PAGE and immunoblots were done following standard procedures. The antibodies used were anti-GFP monoclonal JL-8 (Clontech, catalog number 632380, dilution 1:2500), anti-MYC (GeneTex, catalog number GTX29106, dilution 1:1000), and anti-T7 (Novagen catalog number 69522, dilution 1/2000). The horseradish peroxidase-conjugated goat anti-rabbit (DAKO, catalog number K4002) and goat anti-mouse (DAKO, catalog number K4000) were used as secondary antibodies.

Fluorescent Microscopy and Quantification—Young adult worms were immobilized on agarose pads containing M9 medium/sodium azide. Images were acquired using a Leica DM5000B microscope, a CSU10 (Yokogawa) spinning disk head, a CoolSNAP HQ2 (Photometrics) CCD camera, and the software MetaMorph (v7.6.1.0, Molecular Devices). A cross-profile was obtained from z-projection of the most anterior 30 μm of the dorsal nerve cord using ImageJ. A background line was interpolated using the “tail” values, and the corresponding background values were subtracted from each point of the curve. The total intensity was obtained by summing the latter normalized values.

Bioinformatics—LEV-9 orthologs were identified with PSI-Blast, using C. elegans and Caenorhabditis briggsae orthologues as probes. The National Center for Biotechnology Information (NCBI) (ncbi.nlm.nih.gov), Joint Genome Institute (JGI), ENSEMBL, and Wellcome Trust Sanger Institute databases were analyzed. A subset of sequences was selected considering phylogenetic distribution; alternative splice variants and redundant sequences were removed. Multiple sequence alignment was done using M-Coffee (10) and curated using BioEdit (v7.1.3, Ibis Biosciences). The Eukaryotic Linear Motif (ELM) resource (11) and ProP 1.0 Server (12) were used to identify protease sites. European Molecular Biology Laboratory (EMBL) SMART (13) was used to identify protein domains. WebLogo was used to create sequence logos. Secondary structure and disorder regions were predicted with Jpred3 (14) and PrDOS (15), respectively.

Molecular Biology—The primers used for the amplification of intron X:6286117–6286504 were: PrimerA (5’-AGTCTTT-TAAAGCTGCCAATTTGGC-3’), PrimerB (5’-GAGTTGTC-CGGATGAGTTGA-3’), and PrimerC (5’-CAGGTTTCCAT-TCCCGTTA-3’).

Behavioral and Pharmacological Assays—Chronic (>16 h) adaptation and acute response tests to levamisole (Sigma-Aldrich, catalog number L9756) were done as described previously (6). To analyze thrashing defects, single worms were transferred to wells of 48-well plates containing M9 medium and incubated there for 1 min; head swings were counted three times per animal for 15 s. Results were reported as swings per second.

Statistics—Levamisole adaptation tests were analyzed using a nonparametric Kolmogorov-Smirnov test. Receptor clustering quantification, which fitted a normal distribution (p < 0.05, normal distribution Kolmogorov-Smirnov test), was analyzed using an unpaired non-equal variance two-sample t test.

RESULTS AND DISCUSSION

The C-terminal Region of LEV-9 Is Required for AChR Clustering—LEV-9 orthologs are readily identified in ecdysozoans including nematodes and insects. Amino acid conservation is high in the region coding the WAP and the eight CCP domains but suddenly drops 33 residues after the last CCP domain. No protein domains are predicted to be fully contained within this 66-residue C-terminal (Ct) region of LEV-9. However, the Eukaryotic Linear Motif (ELM) on-line resource (11) predicted two adjacent recognition sites for convertases: one motif for a dibasic nardilysin convertase (residues Asp-571/Arg-572/Lys-573) and a PC motif for a dibasic convertase (residues Lys-574/Arg-575/Ser-576). LEV-9 was confirmed by the PC-specific prediction software ProP (score 0.819; false-negative prediction <0.9% (12)), which fitted a normal distribution (p < 0.05, normal distribution Kolmogorov-Smirnov test). Nardilysins (NdK) are zinc proteases of the M16 peptidase family that have been recently involved in axon maturation and synaptic clustering of L-AChRs 

FIGURE 1. LEV-9 regulates the adaptation to the L-AChR agonist levamisole and is required to localize L-AChRs at NMJs in C. elegans. A, LEV-9 domain composition: signal peptide (sp), a WAP domain, CCP domains, and a Ct region devoid of predicted tertiary structure. The Ct region contains predicted cleavage sites for NdK (residues Asp-571/Arg-572/Lys-573) and PC (residues Lys-574/Arg-575/Ser-576). B, multiple sequence alignment at the Ct region of LEV-9 orthologs. Ce, C. elegans; Cn, Caenorhabditis brenneri; Cr, Caenorhabditis remanei; Cb, C. briggsae; Dm, D. melanogaster; Dy, Drosophila yakuba; De, Drosophila erecta; Ag, Anopheles gambiae; Aa; Ae; Ae; Cip, C. pipiens; Ct, Capitella teleta; L9+, lev-9(T07H6.4 fusion transcript). C, sequence logo of the PC predicted site (Arg-575 shown in position P1). D, detection of GFP endocytosed by coelomocytes after expression of GFP-tagged LEV-9 constructs in muscle cells and secretion into the extracellular space. Scale bars represent 10 μm. E, examples of NMJs used for quantification in lev-9 mutants rescued with different versions of LEV-9. Scale bars represent 5 μm. F, sensitivity to 18-h exposure to levamisole and synaptic clustering of L-AChR-tagRFPt in lev-9 mutants rescued with different versions of LEV-9 expressed under the control of the muscle specific promoter Pmyo-3. Columns and error bars represent averages and S.E., respectively; n numbers of worms (more than three lines) are shown at the base of columns. *** and NS equal p < 0.001 and p > 0.05 in a t test, respectively. x x x and NS’ equal p < 0.001 and p ≥ 0.05 in a nonparametric Kolmogorov-Smirnov test, respectively.
FIGURE 2. LEV-9 is cleaved within the predicted proprotein convertase site (residues Lys-574/Arg-575/Ser-576), and cleavage is required for clustering L-AChR at synapses. A, LEV-9 constructs transfected into Drosophila S2 cells. sp, signal peptide (sp); AP, alkaline phosphatase. B, immunoblots (IB) using anti-MYC and anti-T7 on samples of cell extracts and extracellular conditioned media recovered from transient transfections of S2 cells. C, sequence of constructs where the predicted nardilysin (NdK) and PC (PC) sites were removed. D and E, immunoblots of whole-cell extracts and extracellular media recovered from S2 cells expressing constructs lacking either of the predicted convertase sites. In D, the white arrow indicates the size shift between cell extracts (black arrow) and media. In E, the black arrow points to small cleaved C-terminal fragment. F, immunoblots using anti-GFP using whole protein extracts of C. elegans expressing different versions of LEV-9 from transgenes. The black arrowhead points to the proteolytic fragment found in LEV-9-GFP but not L9PC-GFP. G, detection of GFP endocytosed by coelomocytes after expression of GFP-tagged LEV-9 constructs in muscle cells and secretion into the extracellular space. H, quantification of L-AChR-tagRFP clustering in lev-9 worms rescued with different versions of LEV-9 expressed from transgenes. I, quantification of L-AChRs clustering in wild-type worms showing dominant-negative effect of wild-type and cleavage-resistant LEV-9 (ΔPC). J, locomotion in liquid reported as thrashing cycles per second (hertz). In all panels, columns and error bars represent averages and S.E., respectively; n numbers are shown at the base of columns. ***, **, and NS equal p < 0.001, p < 0.01, and p ≥ 0.05 in a t-Test, respectively.
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**A**

![Diagram showing primers T07H06.5 and T07H06.4 and their positions in the sequence.](image)

**B**

![Image of an electrophoresis gel showing primer pairs A + B and A + C with their respective bands.](image)

**C**

- **splice donor:**
  - isoform A: G
  - isoform B: V
  - isoform C: S

- **splice acceptor:**
  - isoform A: G
  - isoform B: V
  - isoform C: S

- 361 bases

**D**

- **LEV-9 isoform A:**
  - C. elegans

- **LEV-9 isoform B:**
  - C. elegans

- **A8WZ207:**
  - C. briggsae

- **Q8INW2:**
  - D. melanogaster

**E**

- **lev-9 locus**
  - T07H06.5
  - T07H06.4

- **Intron 12**

- **Embryos**
  - Single transcript

- **Adults**
  - Translation & Secretion

- **LEV-9B1**
  - (L-AChR scaffolding)

- **LEV-9B2**
  - (unknown function)

- **LEV-9A**
  - (L-AChR scaffolding)

- **Propeptide**
  - (no function)

**F**

**SOLUBLE CCP-CONTAINING PROTEINS with known cleavage sites**

- **Beta-2-glycoprotein 1** (Human)
  - Refs: PMID:17872974/20435405/19625706
  - Lys317

- **Factor B** (Human)
  - PMID:21862585
  - Arg234

- **Factor D** (human)
  - Cr1-like protease

- **Complement Factor C2** (Human)
  - PMID:19237749
  - Xc3

**G**

**CCP-CONTAINING PROTEINS with predicted PC sites**

- **SVEP1** (Human; Uniprot #Q4LD5)
  - ProP score = 0.954
  - Arg405

- **SRPX2** (Human; Uniprot #O60687)
  - ProP score = 0.504
  - Arg461
myelination (16). PCs are related to subtilisin and yeast kexin2p protease families that play a major role in the proteolytic processing of both neuropeptides and peptide hormone precursors (17, 18). Both motifs were found present in most, if not all, identified LEV-9 orthologs (Fig. 1, B and C).

To test whether the Ct region was required for LEV-9 function, we expressed in muscle a LEV-9 version truncated after the eighth CCP domain and tagged with GFP. Like LEV-9-GFP, L9ΔCt-GFP was detected in coelomocytes, six scavenger cells that filter the fluid of the C. elegans pseudocoelom cavity, arguing for correct folding and secretion into the extracellular medium (Fig. 1D). L-AChR clustering was assessed and quantified using a knock-in strain engineered by homologous recombination in which the subunit UNC-29 is tagged with tagRFPt (19) (Fig. 1F). As opposed to full-length LEV-9, L9ΔCt-GFP did not rescue L-AChR clustering defects of lev-9 null mutant. Surprisingly, worms expressing L9ΔCt-GFP showed a wild-type like sensitivity to 1 mm levamisole (p > 0.5, Kolmogorov–Smirnov test) despite considerable and significant (p < 0.001, t test) lack of L-AChRs at NMJs when compared with the wild type (Fig. 1, E and F).

These data indicated for the first time that it was possible to dissociate the levamisole effect, which kills the worms and requires the N-terminal part of the LEV-9 protein, and the clustering of the levamisole receptor, which requires the C-terminal region of LEV-9. There are two main models to account for this result. First, the N-terminal part of LEV-9 might couple L-AChRs to an actuator such as a channel or a kinase that would amplify L-AChR signaling and make levamisole lethal. Second, the N-terminal part of LEV-9 might stabilize L-AChRs in the plasma membrane independently from synaptic determinants. In fact, prolonged exposure to levamisole causes partial removal of L-AChRs from the muscle cell surface. The absence of LEV-9 in lev-9 mutants might enhance L-AChR removal, thus decreasing levamisole toxicity. In this model, L9ΔCt would be able to stabilize the levamisole receptors in the membrane and rescue levamisole sensitivity independently from L-AChR synaptic clustering.

A LEV-9 Predicted Cleavage Site Is Biochemically Active—To test whether the cleavage sites predicted in the Ct region are functional, we used S2 cells to express a LEV-9 construct tagged with a T7 epitope at its N terminus and with a MYC tag at its C terminus (T7-ΔCt-MYC) (Fig. 2A). Whole protein extracts were analyzed by Western blot using anti-T7 antibodies, whereas it was only detected by anti-MYC antibodies, suggesting that the C-terminal MYC tag was cleaved off the secreted protein (Fig. 2, A and B). Similar results were obtained after removing the CCP-7 or the CCP-8 domain (T7-ΔCCP7-MYC and T7-ΔCCP8-MYC, respectively, in Fig. 2, A and B). However, the MYC tag remained detected in the medium of cells expressing a tagged version with a deletion of the Ct region, suggesting that the cleavage site was contained within this region.

To identify which of the predicted cleavage sites might be processed, we synthesized constructs lacking either the nardi (T7-ΔNdK-MYC) or the proprotein convertase (T7-ΔPC-MYC) sites (Fig. 2, C–E). In both ΔNdK and ΔPC, intracellular and secreted LEV-9 molecules could be detected through their N-terminal T7 epitope, but the apparent size of wild-type- and ΔNdK variants decreased after secretion (Fig. 2D). No size shift could be found with ΔPC constructs (Fig. 2D). When the samples were run in a high percentage acrylamide gel (15%) and probed with the C-terminal MYC epitope, small proteolytic fragments could be found in the extracellular medium of wild-type and ΔNdK transfected cells, but not in ΔPC (Fig. 2E). The sizes of these fragments corresponded to the size expected if the constructs were cut in the Ct region. These results showed that in S2 cells LEV-9 was proteolytically processed at the predicted PC site, but processing of LEV-9 was not required for its secretion.

To verify that a similar post-translational maturation was also occurring in C. elegans, we expressed GFP-tagged LEV-9 with wild-type sequence or deletion of the protease predicted sites (L9ΔNdK-GFP and L9ΔPC-GFP in Fig. 2F). Whole protein extracts were analyzed by Western blot using anti-GFP antibodies (Fig. 2F). Partial proteolytic cleavage of the GFP was observed when LEV-9 was tagged at its C terminus, and the observed fragment corresponded to the size expected if the constructs would have been cut approximately at their Ct region. N-terminally GFP-tagged LEV-9 is also very likely cleaved at its C terminus; however, the little size difference (5 kDa) between the cleaved and uncleaved forms could not be resolved. Clipping off the C-end GFP tag was suppressed by removing either the whole Ct region or the predicted PC site, but not by removing the predicted NdK site (Fig. 2F and data not shown). In the construct where the whole Ct region was removed, an additional band of ~50 kDa was observed; one possible explanation would be the presence of a cryptic cleaving site that could be favored when the Ct region is removed. Prevention of the cleavage did not impair secretion based on GFP detection in coelomocytes of all constructs (Fig. 2G).

LEV-9 Cleavage Is Required for L-AChR Synaptic Clustering—To test the functional significance of LEV-9 cleavage at the PC site, we tested the activity of L9ΔPC-GFP and demonstrated that it was no longer rescuing L-AChR clustering, whereas it restored levamisole sensitivity, similar to the truncated LEV-9.

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version lacking the entire Ct region (Figs. 1A and 2H and data not shown). Worms expressing LEV-9 lacking the nonfunctional NDK site (residues Asp-571/Arg-572/Lys-573 removed) partially rescued L-AChR clustering, but not as efficiently as wild-type LEV-9. This suggested that the last residues present just before the C terminus generated by cleavage at position Arg-575 were important for LEV-9 clustering function. Interestingly, a CCP7-CCP8-Ct construct only partially rescues AChR clustering, suggesting that the Ct functionally interacts with other LEV-9 regions. Alternatively, the short Ct terminus peptide release by cleavage might also have some biological activity. Expression of a truncated LEV-9 version ending at position Arg-575 (L9ΔProP) was as efficient as WT LEV-9 (Fig. 2H). Further removal of 5 residues reduced the clustering activity. Adding a GFP at the end of this truncated version completely abolished the activity of the protein. These data suggested that the C-terminal propeptide had no intrinsic activity and that unmasking the C-terminal residues before position Arg-575 is required to activate L-AChR clustering activity. Because LEV-9 is part of multimolecular scaffold, we tested whether the processing-resistant LEV-9 variant would behave as a dominant-negative. In fact, overexpression of the processing-resistant LEV-9 variant in a wild-type background completely abrogated the clustering of L-AChRs ($p < 0.001$, $t$ test) (Fig. 2I). Overexpression of wild-type LEV-9 partially reduced the number of L-AChRs at NMJs ($p < 0.001$, $t$ test), suggesting that LEV-9 expression or activity is tightly regulated to achieve proper L-AChR clustering.

LEV-9 Cleavage Is Required for Proper Function of the Neuromuscular Junction—Because LEV-9 cleavage is required for proper L-AChR positioning but can be inhibited without affecting the sensitivity to levamisole, we wondered whether LEV-9 processing modulated synaptic activity. We therefore quantified the coordinated movement of worms in liquid, commonly referred to as thrashing. Animals lacking L-AChRs, such as unc-29 mutants, or lacking LEV-9 have a significant thrashing frequency decrease as compared with wild-type worms (Fig. 2J). Thrashing rescue of lev-9 mutants was significantly more efficient with wild-type LEV-9 than with a cleavage-resistant LEV-9 version, indicating that LEV-9 processing is required for proper function of the neuromuscular junction. Altogether, these results suggest that cleaving LEV-9 at its C-end region is a limiting factor for L-AChR clustering and synaptic function.

Proteolytic Processing Generates a Constant LEV-9 Product out of Multiple lev-9 Transcripts—We previously demonstrated that lev-9 is encoded by the T07H6.5 gene because this genomic region rescues all synaptic defects of lev-9 mutants (7). A second gene, T07H6.4, located 300 bp downstream to T07H6.5, is predicted to code for a CCP-containing polypeptide. However, it lacks an identifiable signal peptide in contrast to LEV-9, and most CCP-containing proteins are usually extracellular. Disruption of T07H6.4 by a Tcl transposon insertion causes no synaptic phenotype (7). Recent data from the mod-ENCODE consortium annotated the genomic region between T07H6.5 and T07H6.4 as the intron of a putative T07H6.5-4

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