Caenorhabditis elegans wsp-1 Regulation of Synaptic Function at the Neuromuscular Junction*

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The regulation of actin cytoskeleton plays important roles in the proper execution of a number of dynamic processes, such as cell motility, endocytosis, cell division, and transport (1). Assembly and disassembly of specific actin structures are under the control of phosphoinositides and the Rho GTPase family of small GTP-binding proteins (2). For example, the activation of Rac and Cdc-42 play roles in promoting the formation of lamellipodia and filopodia, whereas Rho activation causes stress fiber formation (3). In the case of Cdc-42, filopodia formation is stimulated upon activation of this GTPase through the effector protein Wiskott-Aldrich syndrome protein (WASP).2

The human WASP family consists of WASP, N-WASP, and three Scar/WAVE molecules where WASP is expressed solely in hematopoietic cells, whereas N-WASP and Scar/WAVE are expressed more generally (4). Members of the WASP family are multidomain actin nucleating-promoting factors and activate the actin-related protein 2 and 3 (Arp2/3) complex at the correct time and place (5). The ability for proteins such as WASP and WAVE to activate Arp2/3 is tightly controlled by a combination of many signaling complexes such as binding of activated Cdc42 to the Cdc42/Rac interactive binding (CRIB) domain of WASP, phospholipid interactions, and Src homology 3 domain binding to proline-rich regions. Once activated, the Arp2/3 proteins enhance the cellular assembly of actin and modulate actin dynamics (5).

We were interested in determining the role of Caenorhabditis elegans WSP-1 at the neuromuscular junction. C. elegans has proven to be a useful model organism to study the genetics of synaptic function (6). To evaluate synaptic transmission in C. elegans, we used the acetylcholinesterase inhibitor aldicarb complemented with transgenic and mutational analysis, as done previously (7). In this report, we demonstrated that wsp-1 mutants rapidly became paralyzed in the presence of the aldicarb. Transgenic experiments showed that the activity of WSP-1 was required in neurons to rescue this phenotype, and mutational analysis showed that a functional CRIB domain in WSP-1 was required. We also show that wsp-1 demonstrates genetic interactions with other genes involved in synaptic transmission at the neuromuscular junction as the wsp-1(gm324) mutation suppressed the aldicarb resistance seen in unc-13(e51), unc-11(e47), and snt-1(md290) mutants. Together, these results show that WSP-1 plays a role in neuromuscular synaptic regulation by maintaining the actin cytoskeletal network at the neuromuscular junction of the adult nematode to prevent aberrant release of synaptic vesicles.

EXPERIMENTAL PROCEDURES

C. elegans Strains—Routine growth and maintenance of C. elegans were done as described previously (8). Mutants and alleles used in this study were: tom-1(ok285) I, unc-13(e51) I, unc-11(e47) I, snt-1(md290) II, and wsp-1(gm324) IV. Double mutants of wsp-1 animals were generated using standard techniques. Strains generated for this study: YF78 [unc-13(e51); wsp-1(gm324)], YF79 [unc-11(e47); wsp-1(gm324)], YF80 [snt-1(md290); wsp-1(gm324)], YF81 [tom-1(ok285); wsp-1(gm324)], and YF82 [wsp-1(gm324); jls219[pSY3[sgn-1::GFP], pRF4[rol-6(su1006)]]]. The transgenic worms created were: YF74 [wsp-1(gm324); qtxEx36], YF75 [wsp-1(gm324); qtxEx39], YF78 [wsp-1(gm324); qtxEx37], YF83 [wsp-1(gm324); qtxEx38], YF84 [wsp-1(gm324); qtxEx41], and YF85 [qtxEx42]. All mutants were identified by either their visible phenotypes (e.g. unc) or by single worm PCR for the deletion mutants.

Construction of Transgenic Constructs—For somatic expression of WSP-1, full-length wsp-1 cDNA was amplified from
expressed sequence tag yk576d2, and the PCR product was introduced into vector pPD103.05 at the Xmal site to generate the plasmid pJZ2. This plasmid was mixed at a concentration of 25 \( \mu \)g/ml with 25 \( \mu \)g/ml pTG96_1 plasmid (9) and microinjected into N2 worms to make tqEx36. For muscle-specific expression of WSP-1, the full-length wsp-1 cDNA was amplified, and the PCR product was introduced into vector pPD95.86 at the Sall site to generate the plasmid pJZ3. This plasmid was mixed at a concentration of 25 \( \mu \)g/ml with 25 \( \mu \)g/ml GFP containing co-injection marker pAC12 (10) to select for lines with the array expressed in body muscles (tqEx37). For neuronal expression of WSP-1, the full-length wsp-1 cDNA was amplified and introduced into the vector pH1045 at the BamHI and KpnI sites to generate the plasmid pJZ4. This plasmid was mixed at a concentration of 25 \( \mu \)g/ml with 25 \( \mu \)g/ml Psur-5::mCherry plasmid to select for lines with the array expressed in neurons (tqEx39). For hypodermal expression of WSP-1, the full-length wsp-1 cDNA was introduced into vector containing a synthetic intron and the promoter of WSP-1, the full-length cDNA was amplified and introduced into the vector pPD103.05 at the Xmal site to generate the plasmid pJZ5. This plasmid was mixed at a concentration of 25 \( \mu \)g/ml with 25 \( \mu \)g/ml GFP containing co-injection of a pdpy-7::Venus construct to select for lines with the array expressed in hypodermal cells (tqEx40). For the transgene containing the CRIB H266D mutation (tqEx41), site-directed mutagenesis was carried out using the QuikChange method, and the mutated wsp-1 cDNA was amplified and introduced into the vector pPD103.05 at the Xmal site to generate the plasmid pJZ6. This plasmid was mixed at a concentration of 25 \( \mu \)g/ml with 25 \( \mu \)g/ml Pf25B3.3::GFP plasmid to select for lines with the array expressed in neurons. The In-Fusion Dry down PCR cloning kit from Clontech was used for subcloning. To visualize co-localized expression of WSP-1, the full-length cDNA was amplified and introduced into the vector pPD103.05 at the Xmal site to generate the plasmid pJZ7. This plasmid was mixed at a concentration of 25 \( \mu \)g/ml with 25 \( \mu \)g/ml Psur-5::mCherry plasmid to select for lines with the array expressed in neurons (tqEx39). For hypodermal expression of WSP-1, the full-length wsp-1 cDNA was introduced into vector containing a synthetic intron and the promoter of dpy-7 (11) in pSL301 at the BamHI site to generate the plasmid pJZ5. This plasmid was mixed at a concentration of 25 \( \mu \)g/ml with 25 \( \mu \)g/ml GFP containing co-injection of a pdpy-7::Venus construct to select for lines with the array expressed in hypodermal cells (tqEx40). For the transgene containing the CRIB H266D mutation (tqEx41), site-directed mutagenesis was carried out using the QuikChange method, and the mutated wsp-1 cDNA was amplified and introduced into the vector pPD103.05 at the Xmal site to generate the plasmid pJZ6. This plasmid was mixed at a concentration of 25 \( \mu \)g/ml with 25 \( \mu \)g/ml Pf25B3.3::GFP plasmid to select for lines with the array expressed in neurons. The In-Fusion Dry down PCR cloning kit from Clontech was used for subcloning. To visualize co-localized expression of WSP-1 with the synaptic protein RAB-3, the tqEx42 transgene was made by mixing 25 \( \mu \)g/ml plasmid pJZ4 and 25 \( \mu \)g/ml Pmig-13::mCherry::rab-3 (12).

**Drug Sensitivity Assays**—Aldicarb was purchased from CHEM Service. Fifteen to 30 synchronized adult worms were put to the aldicarb plates and left at room temperature for 2–3 h depending on the sensitivity of the test strain. The worms were assayed for paralysis, which was defined as absence of movement when prodded three times with a platinum wire on the head (13). For transgenic rescue experiments, one plate was used for all the strains being assayed at one time to minimize the variation that may occur between aldicarb-containing plates.

**Protein Mixing Protocol**—pMal-C2 and pMal-CEWS-1 CRIB domain wild-type and H266D mutation were transformed into Escherichia coli BL21(DE3) and induced with isopropyl 1-thio-\( \beta \)-d-galactopyranoside. After a 3-h induction, pelleted cells were resuspended in lysis buffer consisting of 20 mM Tris, 150 mM NaCl, 10% glycerol, 1 mM dithiothreitol, 0.1% Nonidet P-40, and protease inhibitors mixture. The cells were lysed by sonication, and 20 \( \mu \)l of amyllose resin (New England Biolabs) was added to the lysate followed by a 1-h incubation at 4 °C, and the beads were then washed three times with the lysis buffer. For CDC-42 production, 2 \( \mu \)g of vector for mammalian expression of Myc-CDC-42 was transfected into HEK293T cells using polyethyleneimine (Sigma). After 72 h, the cells were harvested and lysed in 0.75 ml of HNTG lysis buffer (20 mM Hepes (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 5% glycerol, 2 mM MgCl\(_2\), proteinase inhibitor mixture, and GTPγS). The supernatant was then incubated for 1 h with the beads containing malse-binding protein (MBP), MBP-WASP CRIB domain wild-type, and H266D mutation at 4 °C, washed for five times, and then boiled in 2 \( \times \) SDS-polyacrylamide gel loading buffer. SDS-PAGE and membrane transfer was performed with the Bio-Rad mini gel system and semidry apparatus. After transfer, the membrane was blocked for 1 h in 5% milk in TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20), probed with antibody 9E10 or anti-MBP antibody (New England Biolabs) followed by goat anti-mouse horseradish peroxidase, and was developed with Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific).

**RESULTS**

**wsp-1 Mutant Displays Aldicarb Sensitivity**—We set out to investigate the postdevelopmental signaling function of WSP-1 in *C. elegans*. The N-WASP homolog in *C. elegans* (wsp-1) was previously identified and corresponds to transcripts C07G1.4a and C07G1.4b. It generates two splicing variants that produce transcripts of 2.1 kb (isoform A) and 3.5 kb (isoform B). Isoform A is predicted to produce a protein of 608 amino acids and contains the WASP homology 1 domain, IQ motif, CRIB binding motif, and two WASP homology 2 (WH2) domains. Isoform B is a protein consisting of 781 amino acids that has the same C-terminal region of isoform A but differs in its N-terminal region such that the WASP homology 1 domain and IQ motifs are replaced with amino acids that have no homology to any known protein. We obtained from the CGC a strain that contains a homozygous deletion that removes 1892 bp from the genomic sequence of *wsp-1*. This deletion targets the second and third exons of the gene, removing the WH1 and IQ motifs, and would be predicted to affect only the major isoform A (Fig. 1A).

We used aldicarb-induced paralysis to evaluate the synaptic transmission in *C. elegans* in *wsp-1* mutant worms. Aldicarb is a chemical analog of the neurotransmitter acetylcholine and prevents efficient breakdown of acetylcholine, leading to acetylcholine accumulation at synaptic clefts, overactivation of muscarinic acetylcholine receptors, muscle hypercontraction, and paralysis (15). A mutant with a defect in acetylcholine synaptic transmission demonstrates either resistance or sensitivity to aldicarb compared with wild type. We placed 1-day-old adult homozygous *wsp-1* (gm324) mutants and N2 wild-type worms on plates containing 1 mM aldicarb and assayed for paralysis over time. As shown in Fig. 1B, *wsp-1* mutant worms demonstrated significant sensitivity (\( p = 0.0023 \)) to aldicarb because 50% were paralyzed at 37.1 min compared with 78.0 min for the wild-type worms. Therefore, *wsp-1* mutation results in higher sensitivity of this strain to aldicarb, suggesting that WSP-1 plays a role in acetylcholine synaptic transmission.
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CRIB Domain of wsp-1 Is Required to Rescue the Aldicarb Hypersensitivity Phenotype of wsp-1(gm324) Mutant—To demonstrate that the aldicarb sensitivity phenotype of wsp-1 mutant worms was due to the deletion in the wsp-1 gene and not due to a secondary, unidentified mutation in the genomic background, we carried out rescue experiments using transgenic constructs of wsp-1. A transgene capable of expressing in all somatic cells was made, injected, and crossed into wsp-1 mutant animals. Transgenic wsp-1 mutant worms that expressed WSP-1 in somatic cells were significantly (p = 0.02) more resistant to aldicarb than the mutant worms, as 50% of the transgenic strain was paralyzed at 52.5 min compared with 50% of the wsp-1 mutant worms (Fig. 2B). Therefore, the CRIB domain on WSP-1 capable of interacting with CDC-42 was required for proper function of WSP-1 at the neuromuscular junction.

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Next, we asked whether the rescue of wsp-1 aldicarb sensitivity phenotype required an intact CRIB domain. We made a mutation in the WSP-1 CRIB domain (H266D) and expressed the mutated CRIB domain in bacteria and carried out a protein-mixing experiment with lysate of HEK293T cells transfected with wild-type myc-tagged CDC-42 as described above. We found that the H266D mutation in the CRIB domain abolished its interaction with CDC-42 in vitro (Fig. 2B).

Subsequently, we made a WSP-1 transgene that contained the H266D mutation. The transgene was designed for expression in somatic cells of nematodes, for which the wild-type WSP-1 expression in the wsp-1 mutant background was capable of rescuing. We generated transgenic animals and performed the aldicarb assay with this strain. Expression of the WSP-1 protein carrying the H266D mutation was unable to rescue the aldicarb sensitivity of the wsp-1 mutant worms because 50% of the transgenic worms were paralyzed at 43.2 min, similar to 43.4 min of the wsp-1 mutant worms (Fig. 2C). Therefore, the CRIB domain on WSP-1 capable of interacting with CDC-42 was required for proper function of WSP-1 at the neuromuscular junction.

Tissue-specific Rescue of the wsp-1 Mutant—To determine the site of function within the nematode that is responsible for the aldicarb sensitivity phenotype, we created transgenes that expressed WSP-1 in specific tissues. We considered the possibility that there could be different tissues responsible for the aldicarb sensitivity phenotype. The first could be that wsp-1 is required in nerve cells and has a presynaptic role in neuromuscular function. The second alternative could be that WSP-1 is required in muscle cells, and perhaps the aldicarb hypersensitivity phenotype was due to the absence of wsp-1 in postsynaptic tissues. Finally, we also considered the possibility that wsp-1 plays an important role in hypodermal cells, which are responsible for forming the cuticle surrounding the worm. It is possible that if the wsp-1 worms have a weaker cuticle than normal, it would result in higher concentrations of aldicarb at the neuromuscular junction, resulting in the aldicarb sensitivity phenotype.

For specific expression of wsp-1 in these three tissues, the wsp-1 cDNA was subcloned into vectors downstream of established tissue specific promoters. These constructs were co-injected with plasmids coding for specific GFP expression patterns to ensure that the transgene was expressed in the desired tissues. The transgenes were crossed into wsp-1 mutant worms,
and they were tested for rescue of the aldicarb sensitivity phenotype.

We found that only the neuronal specific expression of WSP-1 was capable of rescuing the aldicarb-sensitive phenotype, as 50% of these worms were paralyzed at 57.7 min compared with 44.6 min of the wsp-1 mutant worms ($p = 0.004$), whereas the transgenic expression of WSP-1 in muscle or in hypodermal cells individually was not able to rescue the aldicarb phenotype (Fig. 3). Therefore, neuronal expression of WSP-1 is required to rescue (at least partially) the defect at the neuromuscular junction present in wsp-1 mutant worms, although these experiments do not rule out that WSP-1 may play a role postsynaptically in muscle or in hypodermal cells that could contribute to the aldicarb sensitivity phenotype.

WSP-1 Localizes to the Neuromuscular Junction—Because rescue of the wsp-1 aldicarb sensitivity occurs with neuronal expression of WSP-1, we were interested in determining the subcellular localization of WSP-1. For this, we used the neuronal wsp-1-expressing transgene that has GFP fused to the coding region of WSP-1. When this strain was examined, GFP
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expression was observed diffused in the nerve cords but was also enriched in discrete punctate structures along the ventral nerve cord (Fig. 4A). We compared the localization of WSP-1-GFP with the synaptic vesicle protein RAB-3 tagged with the mCherry fluorescent protein (17). We found that WSP-1 localized to regions immediately adjacent to RAB-3 puncta (Fig. 4, A–C). This region of WSP-1 localization corresponds to periactive zone localization, which is the F-actin-rich region that regulates synaptic growth and plasticity (18). Therefore, WSP-1-GFP fusion protein localizes to the presynaptic region of the neuromuscular junction.

One possibility for the aldicarb sensitivity of the wsp-1 mutant nematodes is that phenotype may be due to improper synaptogenesis. One commonly used synaptic marker is the C. elegans ortholog of synaptotagmin, SNG-1. We examined the synapse of wsp-1 mutants by introducing the SNG-1-GFP transgene into wsp-1(gm324) and checked the number and strength of puncta. We observed that there were no gross abnormalities detected between wild-type worms carrying the SNG-1-GFP fusion marker and the wsp-1 mutants in terms of number of synapses, shape, or intensity of puncta (Fig. 4, D and E). Therefore, the wsp-1 mutants worms are not defective in synaptogenesis.

wsp-1 Mutant Genetically Interacts with Known C. elegans Genes Involved in Synaptic Vesicle Release and Recycling—There are many C. elegans proteins involved with synaptic function, such as unc-13, unc-11, snt-1, and tom-1 (6, 7). The proteins produced from these genes are involved in different steps of the synaptic vesicle cycle. UNC-13 regulates neurotransmitter release by altering the conformation of synaptotagmin, which is needed for the priming of synaptic vesicles (19). UNC-11 encodes for multiple isoforms of the clathrin adaptor protein AP180 and functions in clathrin-mediated endocytosis (20), whereas SNT-1 encodes for the ortholog of the synaptic vesicle protein synaptotagmin, which regulate both synaptic vesicle endocytosis and exocytosis (21). TOM-1 is a syntaxin-binding par-}

FIGURE 4. WSP-1 is localized at the presynaptic terminal in neuronal cells, and wsp-1 mutants exhibit normal synaptogenesis. A, representative image of the ventral cord of wild-type animals containing the qEx42 transgene mounted on agarose pads in 2 mx levamisole and viewed with an Olympus BX61 confocal microscope. WSP-1-GFP localization is in the ventral nerve cord. B, RAB-3::mCherry localization in the same worm. C, localization of both GFP and mCherry fusion proteins shows that WSP-1 resides immediately adjacent to RAB-3 containing puncta. D and E, wsp-1(gm324) display normal synaptogenesis. For visualizing the synaptic specializations of wsp-1 mutant, we used the expression of SNG-1-GFP in wild-type (E) and wsp-1(gm324) (D) nematodes. The wsp-1 mutant carrying the SNG-1-GFP marker has the similar synapses pattern compared with the wild-type worm carrying the SNG-GFP marker.
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We also examined the genetic interactions between wsp-1 and known regulators of synaptic function, such as unc-13, unc-11, snt-1, and tom-1, and showed that wsp-1 rescued the aldicarb resistance phenotype of unc-13, unc-11, and snt-1 and further enhanced the aldicarb sensitivity of tom-1, indicating that wsp-1 is epistatic to C. elegans genes involved in multiple steps of synaptic transmission, such as vesicle priming, exocytosis, and endocytosis (6, 7). These results suggest that WSP-1 plays a general role at the neuromuscular junction and not a specific role in any of these individual cellular processes.

The excess in cholinergic transmission seen with wsp-1 mutants is similar to a number of previous studies that demonstrated that treatment of actin-depolymerizing agents on neuronal cell culture promotes exocytosis of synaptic vesicles (26, 27, 28). Actin is present as a dense network that requires remodeling during exocytosis, most notably a local disassembly of actin that permits synaptic vesicles to gain access to the plasma membrane for docking and fusion, leading to the hypothesis that the cortical actin network is a physical barrier that prevents neurotransmitter release (29). However, actin is also believed to play a positive role in propulsion of exocytotic vesicles, with a number of studies demonstrating decreased secretion in presence of high concentrations of actin-depolymerizing agents, indicating that at least a minimal amount of actin structure is required (30).

Mammalian N-WASP is a well established regulator of actin structure, and upon activation by Cdc42, it activates the Arp2/3 complex to nucleate actin polymerization (18). Previous reports showed that overexpression of N-WASP in PC12 cells produced a stimulatory effect on secretion and that the stimulatory effect was dependent on its ability to induce actin polymerization (31). Therefore, based on previous results that double mutants, the wsp-1 hypersensitivity phenotype is expressed with respect to the phenotypes of other mutations, regardless of which step of the synaptic cycling pathway was otherwise affected.

DISCUSSION

In C. elegans, previous studies of the N-WASP homolog (WSP-1) homozygous mutant worm NG324 show slight neuronal migration and axon outgrowth defects (22, 23). In this study, we demonstrated that the C. elegans wsp-1 mutant showed strong aldicarb sensitivity that was probably due to excess cholinergic neurotransmission. This mutation was rescued with transgenic expression of WSP-1 in neuronal cells, indicating that the altered synaptic transmission in wsp-1 mutant worms originates from a defect in the nervous system. Furthermore, WSP-1 containing a mutation in its CRIB domain did not bind to activated CDC-42, whereas the wild-type CRIB domain could. This experiment was fashioned after a series of experiments that has been used extensively in the studies of CDC-42 regulation of WASP in mammalian cells and Drosophila (16, 24, 25) and demonstrated that the nature of the physical association between activated CDC-42 and WASP is conserved in C. elegans. Furthermore, we observed that the transgene to express this mutant form of WSP-1 did not rescue the aldicarb sensitivity of the wsp-1(gm324) mutant, indicating that an intact CRIB domain of WSP-1 is required to maintain the normal synaptic function.

When we looked at the tom-1(ok285); wsp-1(gm324) double mutant, 50% of the worms were paralyzed at 21.1 min whereas 50% of the tom-1 single mutant worms were paralyzed at 42.0 min (p = 0.0062; Fig. 5C), showing that the wsp-1 mutation enhances the aldicarb sensitivity of tom-1. Therefore, in the
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WASP is an actin cytoskeleton modulator and our genetic and pharmacological evidence presented here, we propose that C. elegans WSP-1 is required for the proper formation of the actin cytoskeleton at the neuromuscular junction that allows it to restrain fusion of synaptic vesicles at the active zone. The model we suggest is that the absence of WSP-1 causes the depolymerization of actin, probably through the disruption of the actin-nucleating Arp2/3 complex, to the extent that it relieves the actin “barrier” resulting in an increase in synaptic transmission, thus causing the wsp-1 mutant to be epistatic to a number of aldicarb resistant genes. Although WSP-1 appears to have a role in maintaining the actin cytoskeleton to prevent synaptic vesicle cycling pathway. These results show that wsp-1 is epistatic to other genes involved in multiple steps of the synaptic vesicle cycling pathway. These results show that WSP-1 plays an essential role regulating neuromuscular activity by stabilizing the actin cytoskeleton at the nerve ending in the adult nematode. Further studies are required to determine in more detail how WSP-1 regulates actin dynamics to coordinate vesicle targeting and exocytosis.

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