A Novel Protein Phosphatase is a Binding Partner for the Protein Kinase Domains of UNC-89 (Obscurein) in Caenorhabditis elegans

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Submitted January 22, 2008; Revised March 3, 2008; Accepted March 5, 2008

Monitoring Editor: Erika Holzbaur

Mutation of the Caenorhabditis elegans gene unc-89 results in disorganization of muscle A-bands. unc-89 encodes a giant polypeptide (900 kDa) containing two protein kinase domains, PK1 and PK2. Yeast two-hybrid screening using a portion of UNC-89 including PK2, yielded SCPL-1 (small CTD phosphatase-like-1), which contains a C terminal domain (CTD) phosphatase type domain. In addition to the PK2 domain, interaction with SCPL-1 required the putative autoinhibitory sequence, and immunoglobulin (Ig) and fibronectin type 3 (Fn3) domains lying N-terminal of the kinase domain. SCPL-1 also interacts with PK1, and it similarly requires the kinase domain and upstream Fn3 and Ig domains. Analogous regions from the two other giant kinases of C. elegans, twitchin and TTN-1, failed to interact with SCPL-1. The interaction between SCPL-1 and either Ig-Fn3-PK2 or Fn3-Ig-PK1 was confirmed by biochemical methods. The sclp-1b promoter is expressed in the same set of muscles as unc-89. Antibodies to SCPL-1 localize to the M-line and a portion of the I-band. Bacterially expressed SCPL-1 proteins have phosphatase activity in vitro with properties similar to previously characterized members of the CTD phosphatase family. RNA interference knockdown results in a defect in the function of egg-laying muscles. These studies suggest a new role for the CTD phosphatase family, that is, in muscle giant kinase signaling.

INTRODUCTION

The sarcomere is the molecular machine that performs the work of contraction in striated muscle. It is comprised of overlapping, interacting thin and thick filaments and their attachment structures, the M-lines and Z-disks. The assembly and maintenance of the sarcomere involves specific interactions between hundreds of different proteins. Sarcomeres are unusually enriched for very large polypeptides, generally >700,000 Da, including nebulin and titin. These giant proteins are primarily composed of multiple inexact copies of small domains, typically 35–100 residues. From the now extensive knowledge about the many binding partners of the 3-MDa polypeptide titin in vertebrate muscle, several themes have emerged (Lange et al., 2006). First, depending on its location within the titin polypeptide chain, the same type of domain, the immunoglobulin domain, can interact with many different partners. Second, some titin interactors are located only at the sarcome, whereas others are located at the sarcomere and the nucleus. One of the newest muscle giant modular proteins to be identified is UNC-89 in Caenorhabditis elegans (Ferrara et al., 2005).

The nematode C. elegans is an excellent system in which to study the organization, assembly, and function of striated muscle in a whole organism (Waterston, 1988; Moerman and Fire, 1997; Moerman and Williams, 2006). The gene unc-89 was originally identified in genetic screens for worms that are both slow moving or paralyzed and that have disorganization of their myofilament lattice (Waterston et al., 1980), and then molecularly cloned (Benian et al., 1996). Obscurein was originally identified through a two-hybrid screen by using portions of titin as bait (Bang et al., 2001; Young et al., 2001), and identified as a homologue of UNC-89 (Young et al., 2001). The body wall muscle of unc-89 mutants shows an especial disorganization of the A-band, and for most mutant alleles, lacks M-lines, the structures in the middle of the A-band at which thick filaments are cross-linked (Waterston et al., 1980; Benian et al., 1999). Similarly, obscurein has been shown to have a role in the assembly or maintenance of A-bands: overexpression of a C-terminal portion of obscurein in primary skeletal myotubes results in a disorganization of sarcomeric myosin (Kontrogianni-Kostantopoulous et al., 2004).

unc-89 is a complex gene, which through the use of three different promoters expressed in different sets of muscles, and alternative splicing, generates at least six major protein isoforms UNC-89-A–F (Benian et al., 1996; Small et al., 2004; Ferrara et al., 2005). The largest of these isoforms, UNC-89-B and UNC-89-F, which are each ~900,000 Da, contain 52 immunoglobulin (Ig) domains; two fibronectin type 3 (Fn3) domains; a triplet of Src homology 3 (SH3), Dbl homology (DH), and pleckstrin homology (PH) domains near their N termini; and two protein kinase domains near their C termini. Two somewhat smaller isoforms, UNC-89-A and UNC-89-E, lack the kinase domains at their C termini. The smallest isoforms, UNC-89-C and UNC-89-D, begin with partial first kinase domains, and each is directed by its own tissue-specific promoter. The human obscurein gene similarly...
has 3′-most exons encoding two protein kinase domains, with differential splicing producing obscurin A, which lacks the kinase domains (like UNC-89-A and -E); obscurin B (like UNC-89-B and -F), which contains the kinase domains; and two smaller obscurins, one obscurin of which contains intact first and second kinase domains (Bang et al., 2001; Russell et al., 2002; Fukuzawa et al., 2005). Although obscurin contains all the same domains as nematode UNC-89, a difference is that the SH3, DH, PH trio is located near the C terminus rather than near the N terminus as it is in UNC-89. For C. elegans UNC-89, homology modeling indicates that the first kinase domain (PK1) is inactive, but that the second kinase domain (PK2) may have kinase activity (Small et al., 2004). So far, however, neither substrates nor phosphotransferase activity has been demonstrated. Antibodies generated to three distinct regions of UNC-89 localize the proteins exclusively to the M-lines by immunofluorescence microscopy, and more recently, by immuno-electron microscopy (A. Reedy, G. Benian, and P. Hoppe, unpublished data). Vertebrate obscurin is located at the peripheries of both M-lines and Z-disks (Kontrogianni-Konstantopoulos et al., 2003). However, the situation is more complex, with obscurin A (lacking kinase domains) located at the M-line, obscurin B located at the A/I junction and additional isoforms located at the Z-disk and Z/I junction (Bowman et al., 2007).

To help understand how UNC-89 performs its functions, we wanted to identify its binding partners. To begin, we have focused on the C-terminal protein kinase domains. We expected that binding partners of the protein kinase do.

## MATERIALS AND METHODS

### Worm Strains, Culture, and RNA Interference (RNAi)

Nematodes were grown at 20°C on NGM plates with Escherichia coli strain OP50 as food source (Brenner, 1974). Bristol N2 was the wild-type strain, and mutants included unc-89 (su75) (Small et al., 2004), unc-89 (tm752) (Ferrar et al., 2005), and two intragenic deletions from the C. elegans Gene Knockout Consortium, scpl-1(hk1080) and scpl-1(gk283). RNAi for scpl-1 was performed on wild-type and unc-89 mutants by using a feeding procedure essentially as described in Kamath and Ahringer (2003). Full-length cDNAs for scpl-1a and scpl-1b were cloned into pPD129.36, and these plasmids or the empty vector were transformed into HT115 (DE3) bacteria before feeding the worms.

### Plasmid Construction

For the screening of the two-hybrid library with Ig-Fn3-PK2 kinase, this region of UNC-89 was cloned into the bait plasmid pGBDU-C1 by using BamHI and SalI sites of the vector. This region of UNC-89, called 15/K/14 in Figure 1A, was polymerase chain reaction (PCR) amplified from the RB2 random primed cDNA library (a gift from Robert Barstead, Oklahoma Medical Research Foundation), by using primers NTSY-15 and NTSY-14 (see Supplemental Table 1 for all primer sequences).

To test for interaction between SCPL-1a or -b prey and various segments of UNC-89 surrounding the PK2 kinase, we first made a series of three baits in the pGBDU-C1 vector, with the same N terminus lying just after the end of the F3 domain and varying amounts of sequence C-terminal of the PK2 kinase catalytic core called 11/12, 11/13, and 11/14 (Figure 1A). PCR was used to amplify the corresponding coding sequences from the RB2 cDNA library, by using the same 5′ primer, NTSY-11, with added BamHI site, and three different 3′ primers with added SalI site, NTSY-12, NTSY-11, or NTSY-14. Two additional PK2 region baits were also made similarly, called 15/12 and 15/13 (Figure 1A), by using the primers NTSY-15, -12, and -13.

To construct two-hybrid plasmids expressing various fragments of SCPL-1, PCR-amplified fragments of scpl-1 were cloned into the plasmid pGAD-C1 by using EcoRI and SalI sites of the vector. The primers used for amplification are as follows: SCPL-1a (amino acids 303-491 of SCPL-1b). The prey plasmid harboring amino acids 245-491 of SCPL-1b was isolated from the library during two-hybrid screening.

To test whether the Ig-Fn3-kinase regions from twitchin and TTN-1 interact with SCPL-1a, -b, -c, -d, or -e, these regions of twitchin and TTN-1 were first cloned into the bait plasmid pGBDU-C1 by using the BamHI and SalI sites of the vector. The relevant regions of twitchin were PCR amplified from the RB2 cDNA library by using primers TWI-F and TWI-R, and for TTN-1, the primers were TTN-F and TTN-R.

Figure 1. (A) Yeast two-hybrid assays demonstrate the specificity of the interaction of UNC-89 PK2 with SCPL-1. Left, schematic representation of baits used to test full-length SCPL-1a and -b prey. Right, images of yeast growth on – Ade plates. Note that for UNC-89 interaction requires the catalytic core (PK2) plus the N-terminal Ig and Fn3 domains, and the C-terminal autoinhibitory domain. Note that comparable regions from the two other giant kinases in the worm, twitchin and TTN-1, fail to interact with SCPL-1. (B) The PK1 region of UNC-89 also interacts with SCPL-1 in the yeast two-hybrid assay. Domain mapping indicates that interaction of the PK1 region with SCPL-1 requires, in addition to the catalytic core (PK1), the Ig and Fn3 domains, and the C-terminal autoinhibitory domain. Note that comparable regions from the other two giant kinases in the worm, twitchin and TTN-1, fail to interact with SCPL-1. (C) The PK1 region of UNC-89 also interacts with SCPL-1 in the yeast two-hybrid assay. Domain mapping indicates that interaction of the PK1 region with SCPL-1 requires, in addition to the catalytic core (PK1), the Ig and Fn3 domains, and the C-terminal autoinhibitory domain. Note that comparable regions from the other two giant kinases in the worm, twitchin and TTN-1, fail to interact with SCPL-1.
To determine whether SCPL-1a or -1b preys interact with PK1 region baits, baits called AB, EB, and AG (Figure 1B), were made in pGBDU-C1 by using the Smal and Sali sites, by insertion of PCR-amplified fragments by using primers U89-PK1-A, U89-PK1-B, U89-PK1-E, U89-PK1-F, and U89-PK1-G.

To test whether UNC-89 Ig-Fn3-PK2 of UNC-89 or comparable regions of twitchin or TTN-1 interact with SCPL-2, -3a, and -4, full-length cDNAs for these other phosphatases were PCR amplified by using the following primers, and then these were inserted into the prey plasmid pGAD-C1 by using BamH and Sali: for SCPL-2, SCPL-2-F and -R; for SCPL-3a, SCPL-3-F and -R; and for SCPL-4, SCPL-4-F and -R.

To express hemagglutinin (HA)-tagged UNC-89 fragments in yeast, we prepared the following plasmids. The PCR-amplified fragments of the relevant region of UNC-89 PK2 by using the primers U89-PK2-1 and -2 for fragment 11/12; U89-PK2-1 and -3 for fragment 11/13; U89-PK2-1 and -4 for 11/14; U89-PK2-5 and -2 for 15/12; U89-PK2-5 and -3 for 15/13; and U89-PK2-4 for 15/14 were cloned into pKS-HA8(XbaI) (three HA-tagged vectors) by using the EcoRV site of the vector. The PCR-amplified fragments from the relevant region of UNC-89 PK1 by using the primers as described above were cloned into pKS-HA8(Nhel) (three HA-tagged vectors) by using EcoRV and Sall sites. HA-tagged UNC-89 11/12, 11/13, 11/14, 15/12, 15/13, and 15/14, AB were cut out with XbaI or Nhel from HA-tagged vectors and cloned into pGAP-C-Nhe (yeast expression vector, TRP1 marker) by using the Nhel site of the vector.

To express myc-tagged SCPL-1 in yeast, we prepared the following plasmids. The PCR-amplified fragments of full-length SCPL-1a and SCPL-1b by using the primers as described above, were cloned into pMAL-KK-1 (myc-tagged vector) by using EcoRI and XhoI sites of the vector. Myc-tagged SCPL-1a and myc-tagged SCPL-1b fragments were cut out with SpeI from myc-tagged vector and cloned into pGAPU-C-Nhe (yeast expression vector, URA3 marker) by using the Nhel site of the vector.

To express glutathione-S transferase (GST) or maltose-binding protein (MBP) fusions of SCPL-1 in bacteria, we prepared the following. The full-length fragments of SCPL-1a and SCPL-1b were PCR amplified by using primers as described above and cloned into pMAL-KK-1 by using EcoRI and XhoI sites of the vector. The fragments of SCPL-1 (1-156 aa of SCPL-1a, 1-302 aa of SCPL-1b, and 1-146 aa of SCPL-1b) were PCR amplified by using the primers as described above. Amplified fragments were cloned into pGEX-KK-1 and pMAL-KK-1 by using EcoRI and XhoI sites of the vector. The plasmids derived from pGEX-KK-1 were used for production of GST fusion proteins, whereas the plasmids derived from pMAL-KK-1 were used for production of MBP fusion proteins.

**Yeast Two-Hybrid Screens and Assays**

Two-hybrid screening was performed as described in Miller et al. (2006). Use of the two-hybrid method to study protein–protein interaction was performed as described in Mackinnon et al. (2002).

**Purification of Bacterially Expressed Proteins**

GST or MBP fusions proteins were prepared as described in Mercer et al. (2006).

**Assays to Confirm Interaction between SCPL-1 and UNC-89 PK2 or PK1**

To confirm an interaction between SCPL-1 and the PK2 region of UNC-89, we expressed in the same yeast cell, myc-tagged SCPL-1b and individual HA-tagged derivatives of the UNC-89 PK2 region were expressed. 11/12, 11/13 . . . refer to the derivatives presented in Figure 1A. Total protein lysates were incubated with agarose beads coated with antibodies to the HA tag, washed, and eluted, and portions were run on two gels and blotted. One blot was reacted with anti-HA to detect the presence of the PK2 derivatives (top). As shown above, appropriately sized proteins were detected from each PK2 derivative. The other blot was reacted with anti-myC to detect the presence of SCPL-1 that might have been brought down with the PK2 protein (bottom). Only derivative 15/14 (Ig-Fn3-PK2-autoinhibitory region) is immunoprecipitated. An arrow designates the position on the blot of myc-SCPL-1b from the lysate.

(B) Bacterially expressed MBP-SCPL-1a interacts with yeast-expressed HA-PK1 or PK2. Total protein extracts were prepared from yeast expressing either HA-PK2 15/14 (see Figure 1A) or HA-PK1 AB (see Figure 1B), incubated with agarose beads coated with antibodies to HA, washed, incubated with purified, bacterially expressed MBP or MBP-SCPL-1a, and washed. The proteins were eluted, and portions of each sample were run on two gels and blotted. One blot was reacted with anti-HA to detect the presence of the yeast expressed protein, the other blot was reacted with anti-MBP to detect possible binding with MBP or MBP-SCPL-1a. (C) Coomassie stained gel of purified MBP-SCPL-1a and MBP-SCPL-1b. In each case, the top band is likely to be the full-length fusion protein; the second band may have resulted from degradation.
centrifuged for 5 min to pellet the beads. The supernatant was then carefully transferred to a fresh tube. Two 12% SDS-polyacrylamide gels were run containing either 10 or 40 µl of supernatant, and they were blotted to nitrocellulose. The blot that had 10 µl of supernatant was reacted with anti-HA (rabbit antibodies, affinity purified, 1:1000; H6908, Sigma-Aldrich), and the blot that had 40 µl of supernatant was reacted with anti-myc (mouse monoclonal purified immunoglobulin, 1:400; M4439, Sigma-Aldrich), reacted with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies, and detected by enhanced chemiluminescence (ECL). Controls included no added yeast lysate, and 10 µg of total protein from any of the yeast to verify the expression of the myc-tagged SCPL-1b.

To confirm an interaction between SCPL-1 and the PK1 region of UNC-89 and to further verify an interaction with the PK2 region of UNC-89, the following procedure was used. Yeast expressing either an HA-tagged PK1 region (segment AB in Figure 1B) or HA-tagged PK2 region (15/14 in Figure 1A) were grown; yeast lysates were prepared; and beginning with 300 µg of total protein, the HA-tagged UNC-89 segments were immunoprecipitated by using anti-HA agarose beads, and washed, as described above. A binding reaction was set up in which the HA-tagged UNC-89 proteins attached to beads were incubated at 4°C for 1 h with shaking in a total volume of 500 µl with either bacterially expressed and purified MBP (10 µg) or MBP-SCPL-1a (10 µg). The beads were washed three times with IP buffer, transferred to clean Eppendorf tubes, centrifuged for 5 min, and as much supernatant was recovered. Green fluorescent protein (GFP) fluorescence images of adults mid was injected into wild-type strain N2 worms, and one transgenic line was SCPL-1b-promo-F and SCPL-1b-promo-R. The promoter-gfp expression plasmid was incubated with HRP-conjugated anti-MBP (1:5000; New England Biolabs, Beverly, MA) and detected by ECL.

Expression Pattern of scpl-1b Promoter
A 7.5-kb genomic segment that includes the initiator ATG, 5′-untranslated region and putative promoter sequence upstream of scpl-1b was produced by PCR and cloned into pPD95.77 (a gift from A. Fire, Stanford University, Stanford, CA), by using the Splh and BamII sites. The primers used were SCPL-1b-promo-F and SCPL-1b-promo-R. The promoter-gfp expression plasmid was injected into wild-type strain N2 worms, and one transgenic line was recovered. Green fluorescent protein (GFP) fluorescence images of adults were obtained as described in Qadota et al., 2007.

SCPL-1 Antibodies
For production of anti-SCPL-1b and anti-SCPL-1a, MBP fusion of residues 1-302 of SCPL-1b and MBP fusion of residues 1-156 of SCPL-1a were used as immunogens. For affinity purification of anti-SCPL-1b and anti-SCPL-1a, GST fusion of residues 1-146 of SCPL-1b and GST fusion of residues 1-156 of SCPL-1a were used, respectively. Affinity purification was carried out as described in Mercer et al. (2006).

Western Blot and Immunofluorescence Microscopy
Procedures for preparing worm protein lysates and Western blots were described previously (Mercer et al., 2006). The following worm strains were used to make lysates: wild type (N2), scpl-1(ok1080) and scpl-1(qk281). The blot also contained lysates from yeast expressing myc tagged SCPL-1a and -1b. Yeast lysates were prepared by suspending a yeast pellet from a 2 ml overnight culture in Laemmli buffer and heating at 95° for 5 min. Affinity-purified anti-SCPL-1a was used at 1:200 dilution, and affinity purified anti-SCPL-1b was used at 1:200 dilution. Wild type adult worms were immunostained after fixation by the method described by Nonet et al. (1993). Anti-SCPL-1b was used at 1:100 dilution, and anti-UNC-89 (MH42) was used at 1:200 dilution. Secondary antibodies and confocal microscopy were as described in Qadota et al. (2007).

SCPL-1 Phosphatase Activity Assays
Hydrolysis of phosphate from p-nitrophenyl phosphate (pNPP) was assayed in a 1-ml reaction mixture containing 50 mM Tris acetate, pH 5.0, 10 mM MgCl2, 20 mM pNPP, and MBP or MBP-SCPL-1b (typically 10 µg) as described in Zheng et al. (2005). The amount of protein, the pH, and inclusion of different divalent cations was as described in Zheng et al. (2005), and the use of various phosphatase inhibitors was as described in Kamenski et al. (2004).

Egg-Laying Assays
Examination of drug response to egg laying was performed as described in Trent et al. (1983). Briefly, 96-well plates with 100 µl of M9 containing 5 mg/ml serotonin or 0.75 mg/ml imipramine in each well were prepared. Single adult worms were soaked in each well for 1 h (serotonin) or 1.5 h (imipramine) at room temperature. After incubation, the numbers of laid eggs were counted.

RESULTS
With the goal of identifying binding partners or possible substrates for the protein kinase domains of UNC-89, a portion of the protein containing PK2 was used to screen a yeast two-hybrid library. The bait contained the domains Ig-Fn3-PK2 (Figure 1A, 15/14), including the putative auto-

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Figure 3. The SCPL family of proteins in C. elegans and demonstration that UNC-89 PK2 region specifically interacts with SCPL-1. (A) Schematic representation of domain organization of proteins containing CTD type (or FCPH) phosphatase domains. C. elegans has five genes that encode proteins with FCPH domains: one domain called CeFCP-1 is more closely related to FCP proteins in that it has both phosphatase and BRCT domains. The others, including SCPL-1, which interacts with UNC-89, are more closely related to small CTD phosphatases (called SCPs), and they are designated SCPL. The percentages indicate the percentage of identical amino acids in the ~200-residue phosphatase domains, compared with the phosphatase in CeB0379.4a (SCPL-1). (B) By yeast two-hybrid assays, the UNC-89 PK2 region interacts with SCPL-1, but not SCPL-2, -3 or -4. The comparable regions of twitchin and TTN-1 fail to interact with any of the SCPL proteins. +, growth and --, no growth on – Ade plates.
inhibitory domain. After screening $8.1 \times 10^6$ colonies, 42 positives were confirmed after retransformation, and all of them represented portions of a single gene, B0379.4. As predicted on WormBase and confirmed by sequencing cDNAs, this gene encodes two transcripts, B0379.4a and B0379.4b, by alternative splicing in the 5′ half of the gene. Our prey clones included representatives of both splice isoforms. The proteins encoded by B0379.4a and 4b are 345 and 491 residues, respectively. The only recognizable domain found in these proteins is a protein phosphatase domain (~170–200 aa) in the C-terminal half of each isoform (Figures 1C and 3A). For reasons explained below, we have renamed B0379.4 as SCPL-1.

To determine which portions of the UNC-89 bait are minimally required to interact with SCPL-1, deletion derivatives of the segment Ig-Fn3-PK2 were tested by two-hybrid against SCPL-1a and -1b full-length prey. As shown in Figure 1A, all the domains are required for interaction, including the C-terminal autoinhibitory region. Because the pattern of domains, Ig-Fn3-kinase, is conserved among the giant protein kinases, we tested the comparable regions from the two other giant kinases in worm muscle, twitchin and TTN-1. As can be seen in Figure 1A, these giant kinases failed to interact with SCPL-1a or -1b.

Given that UNC-89 has a second protein kinase domain, PK1, which is predicted to be catalytically silent (Small et al., 2004), we wondered whether it, too, might interact with SCPL-1. Therefore, fragment Fn3-Ig-PK1 was used as bait to check for interaction with SCPL-1 by two-hybrid analysis. As indicated in Figure 1B, interaction was found, and all the domains, Fn3, Ig, and PK1 need to be present to obtain interaction of the PK1 region with either SCPL-1a or -1b. To determine which portion of SCPL-1a or -1b is required for interaction with either PK1 or PK2 full-length regions, deletion derivatives of SCPL-1a and -1b were tested by two-hybrid analysis. As shown in Figure 1C, the minimal region required is the phosphatase domain.

To provide additional evidence that SCPL-1 interacts with the PK2 and PK1 regions of UNC-89, further experiments were performed. Although each isoform of SCPL-1 could be expressed in bacteria as MBP or GST fusions, segments of UNC-89, either Ig-Fn3-PK2, or Fn3-Ig-PK1 as GST or His-tagged fusions, could not be expressed. In fact, the PK1 or PK2 proteins seemed to significantly reduce the growth rate of *E. coli*. Therefore, in the same yeast cell, myc-tagged SCPL-1b and individual HA-tagged derivatives of the PK2 region corresponding to those depicted in Figure 1A were expressed. Total protein lysates were prepared and incubated with agarose beads coated with antibodies to the HA tag, washed, and eluted, and portions were run on two gels and blotted. One blot was reacted with anti-HA to detect the presence of the PK2 derivatives. As shown in Figure 2A, appropriately sized proteins were detected from each PK2 derivative. The second blot was reacted with anti-myc to purified using the same regions. Below are Western blots reacted with the designated antibodies. In each case, the left-most three lanes are worm extracts, and the right-most lanes are yeast extracts. WT, wild type; *scpl-1(ok1080)* and *scpl-1(gk283)* are intragenic deletions of the *scpl-1* gene. Vector, Myc-SCPL-1a, and Myc-SCPL-1b are yeast harboring either the empty vector or myc-tagged SCPL-1 isoforms. (C) Anti-SCPL-1 localize to M-lines and I-bands in body wall muscle. Anti-SCPL-1b and anti-UNC-89 were coincubated with wild-type worms, and the muscle was imaged by immunofluorescence microscopy. The images show a portion of one body wall muscle cell. Weak labeling of the M-line and a portion of the I-band is seen. UNC-89 is a marker of the M-line. Bar, 10 μm.

Figure 4. Expression pattern of the SCPL-1b promoter and characterization of antibodies to SCPL-1. (A) SCPL-1b is expressed in the same muscle cells as UNC-89. The exon/intron structure of the SCPL-1b gene is shown, as predicted on WormBase. 7.5 kb of DNA sequence upstream of the initiator methionine together with codons for a few amino acids of the first exon of SCPL-1b were fused in-frame with GFP, and used to create transgenic animals, and the sites of GFP expression were recorded. This promoter is expressed in pharyngeal (left), vulval (center), and body wall muscle (right). (B) Anti-SCPL-1b and anti-SCPL-1a antibodies specifically recognize SCPL-1b and SCPL-1a, respectively. Above is shown immunogens used to generate rabbit antibodies that were later affinity
detect the presence of any SCPL-1b that might have been brought down with the PK2 protein. As shown in Figure 2A (bottom), only derivative 15/14 (Ig-Fn3-PK2-autoinhibitory region) was communoprecipitated, consistent with the domain mapping two-hybrid experiment shown in Figure 1A.

A variation of this experiment was conducted in which yeast was used to express the PK2 or PK1 segments, and E. coli was used to express SCPL-1. Protein extracts were prepared from yeast expressing either HA-PK2 15/14 (Figure 1A) or HA-PK1 AB (Figure 1B), and these UNC-89 segments were immunoprecipitated with anti-HA antibody-conjugated agarose beads. After washing, these beads were incubated with purified, bacterially expressed MBP or MBP-SCPL-1a (Figure 2C), washed, and the proteins were eluted. Portions of each sample were run on two gels and blotted. One blot was reacted with anti-HA to detect the presence of the yeast expressed protein, the other blot was reacted with anti-MBP to detect possible binding with MBP or MBP-SCPL-1a. As shown in Figure 2B, this method demonstrates interaction between MBP-SCPL-1a and either Ig-Fn3-PK2, or Fn3-Ig-PK1. Using MBP-SCPL-1b, we obtained similar results (data not shown).

Application of BLAST and Pfam reveals that the phosphatase domain of B0379.4 (SCPL-1) is most closely related to the protein phosphatase domains of the CTD phosphatase family. The founding member of this family is FCP1 (TFIIIF-associated component of CTD phosphatase), which is conserved from yeast to humans, and it is known to dephosphorylate the C-terminal domain (CTD) of the largest subunit of RNA polymerase II. This phosphatase family has no sequence similarity to other phosphatases, except for the motif DXDX(T/V) that is common to both phosphotransferases and phosphohydrolases (Collet et al., 1998). In addition to a phosphatase domain, FCP proteins have a Brcal C-terminal (BRCT) domain. Other members of this family are generally smaller proteins that lack the BRCT domain, and they are called small CTD phosphatases (SCPs). The function of the SCPs is still being established (see Discussion). Our analysis has revealed that C. elegans has five genes that encode proteins with FCP1 homology (FCPH) or CTD-type phosphatase domains (Figure 3A). One of them is more closely related to FCP (in sequence and the presence of both phosphatase and BRCT domains); thus, it is called CeFCP-1. The others, including B0379.4 (SCPL-1), are more closely related to SCPs; therefore, we call these proteins SCP-like (SCPL). It seems noteworthy that the phosphatase domain of worm SCPL-1 is more closely related to the phosphatase domains of human SCPL1, SCPL2, and SCPL3 (64–67% identical amino acids over a span of ~200 amino acids), than it is to the other SCPs in C. elegans (ranging from 23 to 38% identity). It is also clear that the phosphatase domain of SCPL-1 is least similar to the phosphatase domains from worm, yeast, and human FCPs (16–19% identity).

To determine whether the PK2 and PK1 regions of UNC-89 interact specifically with SCPL-1, prey plasmids were generated specifying the complete coding sequences for nematode SCPL-2, SCPL-3, and SCPL-4, and they were tested by two-hybrid for interaction. As shown in Figure 3B, when the Ig-Fn3-PK2 segment was tested against all the SCPL proteins, interaction was found only with SCPL-1. In addition, as shown in Figure 3B, when the comparable kinase-containing regions of twitchin and TTN-1 were tested, they failed to interact with any of the SCPL proteins.

Because the two-hybrid library contains cDNAs made from mRNA prepared from whole worms (including all tissues and cell types), it was important to determine whether scpl-1 is expressed in muscle. To do this, we fused in-frame with GFP, 7.5 kb of DNA sequence upstream of the predicted initiator methionine together with codons for a few amino acids of the first exon of scpl-1b. This DNA was used to create transgenic animals, and the sites of GFP expression were recorded. As shown in Figure 4A, scpl-1b is expressed in pharyngeal, vulval, and body wall muscle, the same muscle cells that express unc-89 (Small et al., 2004). This result bolsters the likelihood that SCPL-1 and UNC-89 interact in vivo.

To determine where the SCPL-1 proteins are expressed and localized, we generated affinity-purified rabbit antibodies that specifically recognize SCPL-1a or SCPL-1b. As shown in Figure 4B, by Western blot, anti-SCPL-1b recognizes yeast-expressed myc-tagged SCPL-1b, but not SCPL-1a, and anti-SCPL-1a recognizes yeast-expressed myc-tagged SCPL-1a but not SCPL-1b. These antibodies recognize the expected sized proteins from C. elegans. Anti-SCPL-1b reacts with an ~70-kDa protein from wild-type worms likely to be SCPL-1b, and with truncated proteins from the intragenic deletion mutants, ok1080 and gk283. Similarly, anti-SCPL-1a reacts with an ~50-kDa protein

<table>
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<th>Strain</th>
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<th>Imipramine</th>
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</table>

+, more than 8 eggs; (+), 4–7 eggs; (−), 1–3 eggs; and −, 0 eggs.

Vol. 19, June 2008 2429
from wild-type worms likely to be SCPL-1a, and this protein is missing from the intragenic deletion mutants.

Anti-SCPL-1b and anti-UNC-89 were coincubated with wild-type worms, and the muscle was imaged by immunofluorescence microscopy. The image presented in Figure 4C shows a portion of one body wall muscle cell. Labeling of the M-line (marked by UNC-89) and a portion of the I-band is seen. In experiments not shown, the I-band location was confirmed by use of known I-band or dense body markers. Similar results were obtained using anti-SCPL-1a (data not shown). Thus, the partial colocalization of SCPL-1 with UNC-89 at the M-line adds further credibility to a functional interaction between SCPL-1 and UNC-89.

Genome-wide RNAi screening (on WormBase) has reported that \textit{scpl-1(RNAi)} results in a reduction of egg laying or Egl phenotype. We had a difficult time discerning this subtle effect. Therefore, we chose to assay the response to drugs that induce egg laying as described in Trent et al. (1983) and shown in Table 1. Compared with wild type, \textit{scpl-1(RNAi)} shows a weak response to serotonin, suggesting a defect in function of egg laying muscles. \textit{unc-89} mutants showed normal responses to serotonin, \textit{unc-89; scpl-1(RNAi)} double showed the same defect as \textit{scpl-1(RNAi)}, suggesting that \textit{scpl-1} is epistatic to \textit{unc-89}. Thus, \textit{scpl-1} may function downstream of \textit{unc-89} in egg laying muscles.

To determine whether SCPL-1 has phosphatase catalytic activity, bacterially expressed MBP fusion proteins of SCPL-1a and SCPL-1b (Figure 2C) were tested in vitro for ability to remove the phosphate from the nonspecific substrate \(p\)-nitrophenyl phosphate. Results are shown in Figure 5A for SCPL-1b; similar results were obtained for SCPL-1a; MBP itself showed no activity. As shown in Figure 5A, the amount of product generated was proportional to the amount of added protein. The enzymatic properties are very similar to those of previously characterized FCPs and SCPs (Kamenski et al. (2004); Zheng et al. (2005)), including a pH optimum of \(\sim 5.0\), a preference for Mg\(^{2+}\) as divalent cation, and unusual response to small molecule inhibitors. Figure 5D shows that SCPL-1 is not inhibited by the typical phosphatase inhibitors NaF or Na\(_3\)VO\(_4\), but it is inhibited by BeF\(_3\) and AlF\(_4\).

**DISCUSSION**

We have shown that both the putatively catalytically active (PK2) and catalytically inactive (PK1) kinase domains of UNC-89 interact with a novel protein phosphatase, SCPL-1. For UNC-89, this requires the upstream Ig and Fn3 domains, as well as the autoinhibitory region at least in PK2. For SCPL-1, only the phosphatase domain itself is required for interaction with UNC-89. There are two indications of the specificity of this interaction: 1) SCPL-1 does not interact with similar regions of the two other giant titin-like kinases in the worm, twitchin and TTN-1. 2) The UNC-89 PK2 region does not interact with three other related proteins in \textit{C. elegans}: SCPL-2, -3, and -4, which SAGE data available on WormBase suggest express in body wall muscle. The plausibility of the UNC-89–SCPL-1 interaction is indicated by finding the following: 1) The \textit{scpl-1b} promoter is expressed in the same sets of muscles (body wall, pharyngeal, and vulval) as \textit{unc-89}. 2) By antibody staining of body wall striated

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**Figure 5.** Biochemical properties of SCPL-1. SCPL-1b was expressed as an MBP fusion protein in \textit{E. coli}, and they are shown to have phosphatase activity toward a model substrate, \(p\)-nitrophenyl phosphate (A). Enzymatic properties were very similar to previously characterized FCPs and SCPs, including a pH optimum of \(\sim 5.0\) (B), preference for Mg\(^{2+}\) (C), and unusual response to small-molecule inhibitors (not inhibited by NaF or Na\(_3\)VO\(_4\) but inhibited by BeF\(_3\) and AlF\(_4\)) shown in D).
Novel Phosphatase Binds Giant Protein Kinase

ACKNOWLEDGMENTS

REFERENCES

Vol. 19, June 2008 2431
et al.


