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CRIP homologues maintain apical cytoskeleton to regulate tubule size in *C. elegans*

Xiangyan Tong [1], Matthew Buechner *

Dept. of Molecular Biosciences, 1200 Sunnyside Drive, 8035 Haworth Hall, University of Kansas, Lawrence, KS 66045-7534, USA

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ABSTRACT

Maintenance of the shape and diameter of biological tubules is a critical task in the development and physiology of all metazoan organisms. We have cloned the *exc-9* gene of *Caenorhabditis elegans*, which regulates the diameter of the single-cell excretory canal tubules. *exc-9* encodes a homologue of the highly expressed mammalian intestinal LIM-domain protein CRIP, whose function has not previously been determined. A second well-conserved CRIP homologue functions in multiple valves of *C. elegans*. EXC-9 shows genetic interactions with other EXC proteins, including the EXC-5 guanine exchange factor that regulates CDC-42 activity. EXC-9 and its nematode homologue act in polarized epithelial cells that must maintain great flexibility at their apical surface; our results suggest that CRIPs function to maintain cytoskeletal flexibility at the apical surface.

Introduction

Tubular structures play important biological roles in all metazoan organisms. Maintaining the structure of tubules such as vasculature, lung sacs, and kidney nephrons is critical for many biological functions (Lubarsky and Krasnow, 2003). Most tubules are constructed from a series of linked epithelial cells. Tubules such as the *Caenorhabditis elegans* excretory canal and the single-cell tips of branches of the *Drosophila* trachea provide simplified models in which a single epithelial cell forms a long tubular structure (Buechner, 2002; Casanova, 2007; Ghabrial and Krasnow, 2006; Kerman et al., 2006; Tonning et al., 2005).

The *C. elegans* excretory canal regulates the osmolarity of the nematode (Nelson and Riddle, 1984). The excretory canal cell body forms and extends a tubule from each side of the cell body on the ventral surface of the nematode; the two tubules bifurcate when they reach the lateral side, to form an H-shaped structure that extends the entire length of the animal (Nelson et al., 1983; White, 1987). Guidance and full extension of the canals to the anterior and posterior ends of the animal are compromised in mutants affecting basolateral receptors and basement membrane proteins such as the UNC-6 netrin, EPI-1 laminin, and UNC-52 heparan sulfate proteoglycan (Buechner et al., 1999; Hedgecock et al., 1987; Huang et al., 2003). In contrast, apically expressed proteins affect the morphology of the lumen of the canals. These proteins include ion channels and pumps (Berry et al., 2003; Liegeois et al., 2007), cytoskeletal proteins (Gao et al., 2001; Gobel et al., 2004; McKeown et al., 1998; Praitis et al., 2005; Suzuki et al., 2001), an apically secreted protein (Jones and Baillie, 1995), and proteins that affect trafficking of apical cytoskeletal components (Fujita et al., 2003). The functions of a large number of other genes that alter the apical cytoskeleton to affect excretory canal shape remain to be identified (Buechner et al., 1999). A single cell must closely regulate the relative growth of the apical and basal surfaces in order to create a lumen of uniform diameter that extends to the full length of the tubule (Buechner, 2002). In addition, outgrowth of the excretory canals occurs while the animal is growing and bending, so the cytoskeleton at both the apical and basal surfaces of these narrow tubules must exhibit significant flexibility during and after growth.

We have identified the EXC-9 protein as a small LIM-domain protein homologous to the mammalian Cysteine-Rich Intestinal Protein (CRIP) (Blackshaw et al., 2004; Davis et al., 1998; Lanningham-Foster et al., 2002). A second CRIP homologue found in multiple valve tissues of *C. elegans* can substitute for EXC-9 if expressed in the excretory canal, and its absence inhibits the function of these valves. The function of CRIP proteins in mammals is presently not known. Overexpression of *exc-9* in other *exc* backgrounds indicates that EXC-9/CRIP acts upstream of the EXC-5 FGD-like guanine exchange factor (GEF). We suggest that CRIPs modulate stability of the actin cytoskeleton to affect cell morphology.

Materials and methods

Nematode genetics

All mutants were derived from the N2 Bristol strain background. The *exc-9*(*gk395*) deletion allele was generously supplied by the *C. elegans* Knockout Consortium (Vancouver, http://ko.cigenomics.bc.ca). Other strains were generously supplied by the *Caenorhabditis* Genetics Center (Minneapolis; http://biosci.umn.edu/CGC). All strains were maintained as described (Brenner, 1974).

We refined the map position of *exc-9* on linkage group IV (Buechner et al., 1999) by use of snip-SNP mapping. *bli-6(sc16) exc-9(n2669)* and *exc-9(n2669) fem-3(q20)* double mutants were crossed to the Hawaiian strain CB4856. From the heterozygous F1 progeny, Bli non-Exc and Exc non-Fem recombinant F2 progeny were picked and

* Corresponding author. Fax: +1 785 864 5294.
E-mail addresses: tongx@uci.edu (X. Tong), buechner@ku.edu (M. Buechner).
[1] Current address: School of Medicine, University of California at Irvine, 328 Sprague Hall, ZC7700, Irvine, CA 92697, USA.

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allowed to self-fertilize. Homozygous F3 progeny were tested for the presence of CB4856 SNP markers on LG IV. SNP site C06A6:2004 was tested on *Bli non-Exc* recombinants, and SNP site B0218:15870 was tested on *Exc non-Fem* recombinants; the results indicated that these two sites are the boundary of *exc-9*. Cosmids in this region, provided by the Sanger Center, Cambridge, UK, were prepared and microinjected into worms as described (Mello and Fire, 1995). The plasmid or cosmid DNA was microinjected together with pRF4, which contains the dominant mutation *rol-6(su1006)* as marker (Kramer et al., 1990), into the gonadal syncytia of hermaphrodites. 100 ng/μl was the concentration used for injection unless otherwise specified.

DNA

Transcriptional *exc-9* construct: Primers specific to the 2.2 kb upstream of predicted gene F20D12.5 (5′-CTGCAGTGTTGGCTCTCTGAAATGGA-3′ and 5′-AAGCTTCAACTTCGGGTCCTGGCACGA-3′) were used to amplify the *exc-9* promoter from N2 genomic DNA. Translational construct: The same upstream primer was used in combination with a primer specific to the last predicted exon of *exc-9* (5′-AAGCTTCAACTTCGGGTCCTGGCACGA-3′). For both constructs, amplified DNA was first ligated into pCR®-XL-TOPO® vector (Invitrogen), then digested with *Pst*I and *Hin*dIII and cloned into the multi-cloning site of pPD95.75 (gift of A. Fire), upstream and in-frame with the *gfp* gene.

Construction of *Pvalv-1*::*gfp*, in which the *valv-1* (B0496.7) promoter drives *gfp* expression, was done in a similar way as was the *exc-9* transcriptional vector. The primers used are: 5′-CTGCAGGATTATTACGATGGTTTTG-3′; 5′-AAGCTTATCGTAATATCGTTCATTTT-3′.

The P*vha-1*::*exc-9*::*gfp* construct, incorporating the *exc-9*::*gfp* translational fusion under the control of the *vha-1* promoter, was made by ligating the *exc-9* coding region into the *Ms*cI and *Sac*I restriction sites of the vector pCV01 (Oka et al., 2001). The primers used are:

exc-9 upstream: 5′-TGGCCACATTTCAGAGAGCCAACA-3′
exc-9 downstream: 5′-GAGCTCGGAACCTGAAAATGTTGAGAAT-3′
valv-1 upstream: 5′-TGGCCAATGCCAAACTGTCCAAAT-3′
valv-1 downstream: 5′-GAGCTCGGATTTCCAGTAGTTCCTTGAA-3′

RNAi

Double-stranded RNA was synthesized by use of the MEGAscript T7 kit (Ambion) with an *exc-9* PCR product (primers: 5′-TAATACGACTCACTATAGGAAGTCATTCGGAGGTTTGTTTAG-3′ and 5′-TAATACGACTCACTATAGGTCTTACCTGTCCAGTTTGTC-3′) from N2 genomic DNA as template. For *valv-1*, primers 5′-TAATACCACTCACTATAGGGTAAGTTGTTTTTTCAAGTT-3′ and 5′-TAATACCACTCACTATAGGTTTCCAGTAGTTCCTTGA-3′ were used. The dsRNA was microinjected directly into N2 animals without further treatment (Fire et al., 1998), along with plasmid pRF4 containing the *rol-6*(*su1006*) dominant allele as marker.

Sequence analysis

The *exc-9 (n2669)* amber mutation was identified via PCR amplification of the F20D12.5 sequence from DNA purified from *exc-9 (n2669)* mutant animals. The amplified fragment was ligated into pCR®2.1-TOPO® vector (Invitrogen). Five positive colonies with the insert were sequenced to make sure the error was not introduced randomly by the PCR. DNA sequencing of the amplified region was performed on both strands.

Alignment of the *exc-9* gene and homologues was performed using ClustalW analysis on Vector NTI (Invitrogen), followed by the use of TreeView to draw the phylogenetic tree. Homologous sequences used for analysis (and their GenBank accession numbers) include: Nematode proteins EXC-9 (NP_501326), VALV-1 (NP_501187), CBG17716 (CAE70917), and CBG05866 (CAE61880); vertebrate proteins CRIP and CRP1 (AAP36964, XP_001333107, AAI35403, NP_031789), CRP2 (NP_001005968, NP_001303, NP_077185), and CRP3 (NP_996805, Q6Q6R3, XP_696083), insect CRP1 (XP_967808, XP_001122454); leech CRP1 (AAN73075); and distant CRP homologues from insect (XP_001120788, NP_651126, EAT43197, XP_310992, XP_969829) and mushroom (EAU87616). Additional vertebrate proteins (accession numbers XP_001501981, XP_001094052, XP_001137689, NP_001102773, XP_855529, Q5R7Y1, AAI35603, CAF93748, CAG13149, XP_001507066, XP_001364171, NP_001020520, EDL18557, EDL93791, XP_001171359, XP_001507066, NP_001087303, NP_001087271) mapped to the same branches in Fig. 3 as did the included genes, but were omitted for clarity. A full tree (including homologues from echinoderm and trematode, accession numbers XP_001188306, AAX30636) is included as Supplementary Fig. S1.

Microscopy

Worms and embryos were observed at high magnification on 2% agarose pads in M9 buffer, with 1% of 1-phenoxy-2-propanol as anaesthetic added as needed (Sulston and Hodgkin, 1988). DIC and/or fluorescence microscopy (Sulston and Hodgkin, 1988) images were taken on a Zeiss Axioskop Microscope with a MagnaFire Electronic Camera (Optronics), and images merged with Corel PhotoPaint software. GFP-positive cells were identified according to their position and morphology (www.wormatlas.org) (Sulston and Horvitz, 1977).

Canal measurements

Excretory canals of living animals expressing a GFP canal marker were observed through a Zeiss Axioskop Microscope. Shortening of the lumen due to cyst formation was graded as either: completely shortened with no lumen visible past cysts at the cell body (0); significantly shortened to less than half-length (1); lumen reaching only midlength to the area around the vulva (2); lumen slightly shortened, reaching to between the vulva and tail (3); or no shortening/full length to or past the anus (4). While many animals had 1 or 2 large cysts, we instead graded the overall general canal cyst size either as: large — over half the width of the animal (0); medium — 1/4 to 1/2 the width of the animal (1.3); small — less than 1/4 the width of the animal (2.7); or no cysts (4). Wild-type canals were graded as (4) for both canal length and cyst size, while highly affected cystic mutants have lower scores. “Convoluted canals,” in which canal extension fails because the basolateral surface is affected (Suzuki et al., 2001) rather than due to shortening via cyst formation and failure of the apical surface, were graded as full-length (4) for length, and 4 (no cysts) for cyst size. An overall score was calculated by averaging all scores together. Statistical analysis of canal length was conducted by binning similar categories: numbers of canals with poor growth (no or short outgrowth, scores 0 or 1); numbers of canals with partial growth (halfway or 3/4 length, scores 2 or 3); and number of canals with full outgrowth (score 4). The binned results were then analysed via a 3×2 Fisher's Exact Test to measure the presence of any full or partial rescue. Canal cyst lumen size was similarly evaluated with a 3×2 Fisher's Exact Test by binning numbers of canals with large or very large cysts (score 0 or 1.3); vs. number of canals with small cysts (score 2.7); and number of canals with no cysts (score 4). A *p*-value of 0.0001% or lower was regarded as strong statistical significance of rescue; *p*-values between 0.0001% and 0.01% or less were regarded as partial rescue.

Yeast two-hybrid assay

A cDNA clone of *exc-9* was purified and sent to the Molecular Interaction Facility of the University of Wisconsin, Madison, where the clone was ligated into a bait vector (encoding GAL4 upstream of EXC-9) and tested via two-hybrid assay at low stringency with the Facility's library of 36 million *C. elegans* RNAs derived from mixed-stage hermaphrodites. Five putative interactors were isolated; one was eliminated as a false positive because it also interacted with empty bait vector. The four validated prey clones all encoded CSN-5, the nematode homologue to subunit 5 of the COP9 signalosome.

Results

exc-9 mutation alters epithelial structure

Mutation in *exc-9* was first identified through changes in the shape and size of the lumen of the excretory canal. Normally, the canal tubules extend the length of the animal and have a lumen that is only several microns wide (Buechner et al., 1999) (Figs. 1A, B). In contrast, both *exc-9* alleles (*n2669* and *gk335*) exhibit penetrant defects in canal morphology; the canal lumen is variably wide and short, often with septations between cystic areas (Fig. 1C). In *n2669*, almost half of the animals exhibit at least one very large fluid-filled cyst that is greater than half the diameter of the animal (Fig. 1D), and all animals have multiple smaller cysts (Table 1). In addition to canal defects, mutations in *exc-9* affect the fine structure of the thin tail whip of hermaphrodite worms (Fig. 1E); 40% of hermaphrodite *exc-9* mutants show tail defects (Fig. 1F). Finally, male *exc-9* mutants show defects in genital development. Wild-type males form 9 rays on each side of the tail (Fig. 1G); each ray is composed of a single epithelial cell surrounding a pair of neurons (Emmons, 2005). In 40% of *exc-9* mutant male animals, ray development is abnormal and leads to abnormally shaped or fused rays (Fig. 1H). As a result, *exc-9* male worms show impaired mating efficiency, but overall are capable of mating.

EXC-9 is homologous to a mammalian intestinal protein with a conserved LIM domain

We used snip-SNP mapping to narrow the position of *exc-9* to a region of 13 cosmids. Injection of cosmid F20D12 rescued the canal and tail whip phenotypes of *exc-9*(*n2669*) animals (Fig. 2A). Injections of dsRNA corresponding to the predicted gene F20D12.5 encoded on this cosmid caused defects both in the canals (Fig. 2B) and in the tail whip (Fig. 2C) identical to those of *exc-9* mutation.