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C. elegans NIMA-related kinases NEKL-2 and NEKL-3 are required for the completion of molting

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ABSTRACT

Caenorhabditis elegans molting is a process during which the apical extracellular matrix of the epidermis, the cuticle, is remodeled through a process of degradation and re-synthesis. Using a genetic approach, we identified *nekl-3* as essential for the completion of molting. NEKL-3 is highly similar to the mammalian NEK kinase family members NEK6 and NEK7. Animals homozygous for a hypomorphic mutation in *nekl-3*, *sv3*, had a novel molting defect in which the central body region, but not the head or tail, was unable to shed the old cuticle. In contrast, a null mutation in *nekl-3*, *gk506*, led to complete enclosure within the old cuticle. *nekl-2*, which is most similar to mammalian NEK8, was also essential for molting. Mosaic analyses demonstrated that NEKL-2 and NEKL-3 were specifically required within the large epidermal syncytium, hyp7, to facilitate molting. Consistent with this, NEKL-2 and NEKL-3 were expressed at the apical surface of hyp7 where they localized to small spheres or tubular structures. Inhibition of *nekl-2*, but not *nekl-3*, led to the mislocalization of LRP-1/megalin, a cell surface receptor for low-density lipoprotein (LDL)-binding proteins. In addition, *nekl-2* inhibition led to the mislocalization of several other endosome-associated proteins. Notably, LRP-1 acts within hyp7 to facilitate completion of molting, suggesting at least one mechanism by which NEKL-2 may influence molting. Notably, our studies failed to reveal a requirement for NEKL-2 or NEKL-3 in cell division, a function reported for several mammalian NEKs including NEK6 and NEK7. Our findings provide the first genetic and in vivo evidence for a role of NEK family members in endocytosis, which may be evolutionarily conserved.

Introduction

Studies on cell cycle regulation in the fungus *Aspergillus nidulans* identified Never in Mitosis A (NimA), a serine–threonine protein kinase involved in multiple aspects of mitosis (Oakley and Morris, 1983; Osmani et al., 1988). Subsequently, NimA family members, referred to as NEKs, have been identified in a wide range of organisms (Supplementary material Fig. S1) (Fry et al., 2012; Moniz et al., 2011). The nematode *Caenorhabditis elegans* has four NIMA-related kinase genes. The product of *nekl-1* (*NE*ver in mitosis *K*inase *L*ike) resembles mammalian NEK9 and, to a lesser extent, NEK8, both of which have regulator of chromosome condensation (RCC1) repeats carboxyl-terminal to their kinase domain. NEKL-4 is an ortholog of Nek10, which has Armadillo/β-catenin-like repeats amino-terminal to the kinase domain. *nekl-2* and *nekl-3* both encode small proteins that consist mainly of a kinase domain.

NEKL-3 is a compelling ortholog of mammalian NEK6 and NEK7, which are 82% identical to one another and, like NEKL-3, contain little more than a protein kinase domain. Studies in tissue culture systems have implicated NEK6 and NEK7 in mitotic spindle formation, cytokinesis, centrosome separation, centriole duplication, and the control of microtubule dynamics (Bertran et al., 2011; Kim and Rhee, 2011; Lee et al., 2008; Motose et al., 2011, 2012; O'Regan et al., 2007; O'Regan and Fry, 2009; Rapley et al., 2008; Sdelci et al., 2011; Yin et al., 2003; Yissachar et al., 2006). In addition, overexpression of NEK6 and NEK7 is associated with cancer progression (Nassirpour et al., 2010; Wang et al., 2013) and NEK6 may inhibit cellular senescence (Jee et al., 2010, 2013). Mice homozygous for a deletion mutation in NEK7 typically arrest in late embryogenesis or within a month after birth, and mouse embryonic fibroblasts derived from NEK7 mutants show increased aneuploidy (Salem et al., 2010). NEK6 and NEK7 are stimulated by NEK9-mediated phosphorylation (Belham et al., 2003; Bertran et al., 2011; O'Regan et al., 2007; O'Regan and Fry, 2009; Richards et al., 2009), but the substrates of Nek6 and Nek7, with

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the possible exception of the kinesin Eg5 (Bertran et al., 2011; Rapley et al., 2008), have not been clearly defined (Belham et al., 2001; Lizcano et al., 2002). Interestingly, NEK6 and NEK7 were identified in a non-biased high-throughput screen as positive regulators of clathrin-mediated endocytosis, suggesting that these NEK family members have functions unrelated to cell division (Pelkmans et al., 2005).

NEKL-2, although also composed almost exclusively of a kinase domain, does not closely resemble NEKL-3 (Supplementary material Figs. S1 and S2). Its closest mammalian relative is NEK8, but NEK8 has RCC1 repeats, which are lacking in NEKL-2. In vertebrates, NEK8/NPHP9 has been implicated primarily in ciliogenesis (Fry et al., 2012; Mahjoub et al., 2004, 2005) and kidney function (Liu et al., 2002; Mahjoub et al., 2005; Otto et al., 2008; Smith et al., 2006; Trapp et al., 2008). More recent studies have implicated NEK8 in the response to DNA-replication stress and maintenance of genomic stability (Choi et al., 2013; Jackson, 2013). NEK8 may negatively regulate caveolae/raft-mediated endocytosis but function as a positive regulator of clathrin-mediated endocytosis (Pelkmans et al., 2005), suggesting, as for NEK6 and NEK7, that individual NEKs may carry out a broad range of biological functions, including roles in intracellular trafficking.

As described below, we have shown that *C. elegans nekl-2* and *nekl-3* are required for the completion of molting. Nematodes typically undergo four juvenile or larval molts of their exoskeleton, termed the cuticle. The cuticle is composed of cross-linked collagen and other components and is largely derived from the apical surface of hyp7, a large syncytium that makes up most of the epidermis (termed hypodermis). As one of the larval molts commences and a new cuticle is synthesized under the old, a series of movements ensue, followed by rupture and escape from the old cuticle (Page and Johnstone, 2007; Singh and Sulston, 1978). In *C. elegans*, inactivation of many individual genes by mutation or by RNA interference (RNAi) results in a frequent failure to shed the old cuticle completely (Frand et al., 2005; Kang et al., 2013). Gene products required for proper molting include nuclear hormone receptors (Gissendanner and Sluder, 2000; Hayes et al., 2006; Kostrouchova et al., 1998, 2001; Monsalve and Frand, 2012), matrix metalloproteases (Altincicek et al., 2010; Davis et al., 2004; Hashmi et al., 2004; Kim et al., 2011; Stepek et al., 2011; Suzuki et al., 2004), selenoproteins (Stenvall et al., 2011), enzymes controlling sterol and fatty acid synthesis (Entchev and Kurzchalia, 2005; Jia et al., 2002; Kuervers et al., 2003; Li and Paik, 2011), hedgehog-related proteins (Hao et al., 2006; Zugasti et al., 2005), and LRP-1, an ortholog of megalin, a large member of the LDL receptor family (Yochem et al., 1999). A number of molting genes are associated with secretion, endocytosis, and vesicle trafficking (Frand et al., 2005; Kang et al., 2013; Liegeois et al., 2007), and several of these, including the Disabled adapter ortholog *dab-1*, the *Saccharomyces cerevisiae* Vps27p ortholog *hgrs-1*, and the *S. cerevisiae* Sec23p ortholog *sec-23*, affect trafficking and endocytosis of LRP-1 from the apical membrane of hyp7 (Holmes et al., 2007; Kamikura and Cooper, 2006; Kang et al., 2013; Roberts et al., 2003; Roudier et al., 2005).

The genetic studies described herein strengthen the notion that NEK kinases have functions not linked to cell division or to ciliogenesis. The requirement of *nekl-2* and *nekl-3* for proper completion of molting, instead, suggests their involvement in extracellular matrix remodeling and vesicular trafficking. Data consistent with this possibility are presented.

Materials and methods

Genetic strains and maintenance

All strains were cultured at 20 °C (unless otherwise stated) on nematode growth medium (NGM) supplemented with *Escherichia coli* OP50 as a food source according to standard protocols (Stiernagle, 2006). Preexisting strains used in theses studies are as follows: N2, var. Bristol, designated the wild-type strain (Brenner, 1974); LH191 (*eqIs1* [*lrp-1::gfp*]; *rrf-3*(*pk1426*)II) (Kang et al., 2013); VC1733 (*nekl-2*(*gk839*) I/hT2[*bli-4*(*e937*) *let-?*(*q782*) *qIs48*](I;III)); VC1774 (*nekl-2*(*gk841*) I/hT2 [*bli-4*(*e937*) *let-?*(*q782*) *qIs48*](I;III)); and VC2632 (*nekl-2*(*ok3240*) I/hT2 [*bli-4*(*e937*) *let-?*(*q782*) *qIs48*](I;III)). LH243 (*nekl-3*(*sv3*)X; eqIs3 [*nekl-3::gfp*; pRF4]), LH373 (*nekl-3*(*gk506*)X; *mnEx174*[*F19H6; pTG96*]), SP2735 (*nekl-3*(*sv3*)/*lin-2*(*e1309*) *unc-9*(*e101*) X), SP2736 (*nekl-3*(*sv3*)X; *mnEx174*[*F19H6; pTG96*]), WY967 (*eqIs1*[*lrp-1::gfp*]; *rrf-3*(*pk1426*)II; *nekl-3*(*sv3*)X; *mnEx174*[*F19H6; pTG96*]), WY977 (*eqIs1*[*lrp-1::gfp*]; *rrf-3*(*pk1426*)II; *nekl-3*(*gk506*)X; *mnEx174*[*F19H6; pTG96*]), WY1061 (*nekl-2*(*gk839*); *fdEx257*), WY1070 (*fbn-1*(*tm290*), *fdEx250*, eqIs1[*lrp-1::gfp*]; *rrf-3*(pK1426)II), WY1073 (*nekl-2*(*gk839*); *fdEx261*), WY1077 (*nekl-2*(*gk839*); fdEx261; *nekl-3*(*sv3*)X; *eqIs3* [*nekl-3::gfp*; *pRF4*]); WY976 (*eqIs1*[*lrp-1::gfp*]; *dab-1*(*gk291*)II). RT424 (*pwIs126*; GFP::EEA-1), RT1113 (*pwIs439*; GFP::RAB-5, RT1378 (*pwIs528*; GFP::CHC-1), RT225 (*pwIs94*; GFP::SNX-1), WY1095 (*lin-35*(*n745*); *pwIs94*). The *gk506* mutation was outcrossed eight times prior to strain construction.

Isolation of sv3

The *sv3* allele was identified in a screen for recessive mutations that affect the completion of molting. L4 larvae of the N2 strain were treated with 50 mM ethyl methanesulfonate (Brenner, 1974). F1 animals from the mutagenesis were placed on many NGM plates (three animals per plate). After growth at 25 °C, the plates were examined for the presence of F2 progeny exhibiting defects in molting, including a failure to shed all of the old cuticle. Because mutants exhibiting defective molting are often incapable of reaching adulthood, non-mutant siblings were individually isolated from candidate plates for the identification of heterozygotes that could be used to maintain the mutations until they were mapped and balanced. Because none of the mutations proved to be temperature sensitive, a temperature of 20° was used for their maintenance and analysis. In all, 3600 haploid genomes were examined, and 12 mutations identified. Consistent with its large size (Yochem et al., 1999), *lrp-1* proved a large target, accounting for six of the mutations. Standard genetic mapping placed *sv3* ~0.04 cM to the left of *lin-2* on LGX. After the *sv3* isolate was outcrossed eight times, the stock SP2735 [*sv3/lin-2*(*e1309*) *unc-9* (*e101*) X] was established. Stocks cannot be maintained if *sv3* is homozygous.

Plasmid construction and site-directed mutagenesis

A functional fusion between *nekl-3* coding sequences and GFP sequences was constructed from DNA generated by PCR (Pfu Turbo, Stratagene) using a rescuing cosmid clone, F19H6, as template. A DNA fragment for the 5′ portion of the gene, comprising ~5 kb from the *Bgl*II through the *Eco*RI sites of the genomic sequence, was generated using LRB-504 and LRB-505 (Supplementary material Table S1) and cloned into BlueScript. The 3′ portion of the gene was generated in two fragments, to add a unique *Pst*I site immediately before the stop codon of the open reading frame. Three alanine codons were also added immediately preceding the *Pst*I site. First, LRB-506 and LRB-507 were used to generate a fragment containing an *Eco*RI site from within the gene and the new *Pst*I site. Then, LRB-508 and LRB-509 were used to generate a fragment containing the new *Pst*I site and a genomic *Xho*I site and including the stop codon and 3′UTR. These two PCR products were combined in the proper order into Bluescript. The *Eco*RI to *Xho*I region was then excised and subsequently ligated into the plasmid containing the 5′ *Bgl*II to *Eco*RI fragment, thereby creating a complete gene with three alanine codons and a *Pst*I site immediately before the stop codon. Successful