Requirement of the *Caenorhabditis elegans* RapGEF *pxf-1* and *rap-1* for Epithelial Integrity

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The Rap-pathway has been implicated in various cellular processes but its exact physiological function remains poorly defined. Here we show that the *Caenorhabditis elegans* homologue of the mammalian guanine nucleotide exchange factors PDZ-GEFs, PXF-1, specifically activates Rap1 and Rap2. Green fluorescent protein (GFP) reporter constructs demonstrate that sites of *pxf-1* expression include the hypodermis and gut. Particularly striking is the oscillating expression of *pxf-1* in the pharynx during the four larval molts. Deletion of the catalytic domain from *pxf-1* leads to hypodermal defects, resulting in lethality. The cuticle secreted by *pxf-1* mutants is disorganized and can often not be shed during molting. At later stages, hypodermal degeneration is seen and animals that reach adulthood frequently die with a burst vulva phenotype. Importantly, disruption of *rap-1* leads to a similar, but less severe phenotype, which is enhanced by the simultaneous removal of *rap-2*. In addition, the lethal phenotype of *pxf-1* can be rescued by expression of an activated version of *rap-1*. Together these results demonstrate that the *pxf-1/rap* pathway in *C. elegans* is required for maintenance of epithelial integrity, in which it probably functions in polarized secretion.

INTRODUCTION

Rap1 is a member of the family of Ras-like GTPases that function in signal transduction processes by relaying signals received by transmembrane receptors to downstream effector molecules. Ras-like GTPases switch between an active GTP-bound state and an inactive GDP-bound state. Activation of Ras-like GTPases is mediated by guanine nucleotide exchange factors (GEFs), which induce the dissociation of GDP to allow binding of the more abundant GTP. Four different types of GEFs specific for Rap1 have been described in vertebrates, namely C3G, CD-GEF, Epac, and PDZ-GEF (also named RA-GEF [Liao *et al.*, 1999], Nrap-GEF [Ohtsuka *et al.*, 1999], or CNrasGEF [Pham *et al.*, 2000]; reviewed in Bos *et al.*, 2001). These Rap1-GEFs share a highly homologous catalytic domain including a so-called REM or LTE domain. In addition, each GEF has its own characteristic domains, which regulate GEF activity and/or determine its subcellular location. Together these GEFs may account for

the various modes of Rap1 activation seen after ligand occupation of distinct growth factor receptors (Altschuler *et al.*, 1995; Franke *et al.*, 1997; Vossler *et al.*, 1997; Posern *et al.*, 1998; Zwartkruis *et al.*, 1998; reviewed in Bos *et al.*, 2001). For example, EPAC directly binds cAMP, which leads to stimulation of its exchange activity (de Rooij *et al.*, 1998; Kawasaki *et al.*, 1998). Regulation of PDZ-GEFs is less well understood. The N-terminal part of PDZ-GEF resembles a cAMP-binding domain, but because the absence of certain conserved residues prohibits binding of cAMP (Liao *et al.*, 1999; Ohtsuka *et al.*, 1999; Kuiperij *et al.*, 2003) an as yet unidentified second messenger has been suggested to regulate PDZ-GEFs (de Rooij *et al.*, 1999). However, it has also been reported that cAMP can induce Ras-GEF activity of PDZ-GEF, under conditions where its PDZ-domain is bound to the β 1-adrenergic receptor (Pak *et al.*, 2002). Apart from interacting with GTPases via its catalytic domain, PDZ-GEF1 can stably interact with GTP-bound Rap1 via its Ras-associating (RA) domain in vitro (Liao *et al.*, 1999), whereas PDZ-GEF2 interacts with M-Ras (Gao *et al.*, 2001). The C-terminal VSAV sequence of PDZ-GEF1 can bind to the second PDZ-domain of the neural scaffolding protein S-SCAM, MAGI-1 or the related product of the KIAA0705 gene (Ohtsuka *et al.*, 1999; Kawajiri *et al.*, 2000; Mino *et al.*, 2000), suggesting that PDZ-GEF functions in a signaling complex with scaffolding proteins at sites of cell-cell contact.

Although cloning of the different Rap1-specific GEFs has opened the way to study the precise mechanisms of Rap1 activation, the physiological function of these GEFs and their target Rap1 is still enigmatic. Experiments in tissue culture cells have pointed toward a role for Rap1 in modulation of the Ras-signaling pathway (Kitayama *et al.*, 1989;

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Cook *et al.*, 1993; Vossler *et al.*, 1997) and cell cycle control (Altschuler and Ribeiro-Neto, 1998; reviewed in Bos *et al.*, 2001). More recently Rap1 was shown to be involved in activation of integrins in T-cells and macrophages (Caron *et al.*, 2000; Katagiri *et al.*, 2000; Reedquist *et al.*, 2000) and overexpression of the Rap1-specific GAP Spal was found to abolish adhesion of various cell types to extracellular matrix proteins (Tsukamoto *et al.*, 1999). The mechanism by which Rap1 affects integrin function is largely unknown, but may depend on the polarizing activity of Rap1 (Shimonaka *et al.*, 2003). In addition, a role for Epac2 in cAMP-regulated insulin secretion has been reported (Ozaki *et al.*, 2000) and Epac1 has been implicated in the regulation of intracellular calcium levels via phospholipase ϵ (Schmidt *et al.*, 2001) or the ryanodine receptor (Kang *et al.*, 2003).

Genetic studies in *Drosophila* have revealed that Rap1 is an essential gene required during embryogenesis. In the absence of maternally supplied Rap1, mutants display morphogenetic defects including mesodermal cell migration, dorsal closure, and head involution (Asha *et al.*, 1999). These defects have been attributed to a diminished activity of the AF-6 homologue Canoe, which directly can bind to GTP-bound Rap1 and colocalizes in adherens junctions (Boettner *et al.*, 2003). Interestingly, generation of Rap1^{-/-} clones in the wing results in defective adherens junction formation (Knox and Brown, 2002), but it is unclear if Canoe is involved in this as well. Apart from Canoe, the *Drosophila* RalGEF RGL has been proposed to act as a Rap1 effector on the basis of genetic experiments (Mirey *et al.*, 2003). Finally, in the same organism loss of PDZ-GEF leads to larval lethality. Escapees and animals overexpressing PDZ-GEF show defects that suggest a role for PDZ-GEF and Rap1 in ERK activation (Lee *et al.*, 2002).

We have turned to *Caenorhabditis elegans* as a model system to define its biological function and dissect the Rap1 signaling pathway by genetic approaches. Here we show that the *C. elegans* homologue of PDZ-GEF, *pxf-1*, acts upstream of *rap-1* and *rap-2*. Animals, mutant for either *pxf-1* or double mutant for *rap-1* and *rap-2* remain scrawny, have a disorganized cuticle, and display molting defects. Furthermore, a progressive degeneration of the hypodermis is seen, and adults frequently die with a burst vulva phenotype. From this and the fact that an activated version of RAP-1, RAP-1^{V12}, rescues the lethal phenotype of *pxf-1*, we conclude that the *pxf-1/rap* pathway is essential for maintenance of epithelial integrity in *C. elegans*.

MATERIALS AND METHODS

Worms

General methods for culturing and manipulating worms used in these studies were as described (Lewis and Fleming, 1995). Worms were cultured on NGM plates at 20°C. Strains and mutations used were Bristol N2, *dpy-20* (e1362), *mut-7* (pk204), ML652 [*mcEx204*: pML902 + pRF4], PS3352 *pkEx246* [pMH86 + JP#38 (*cdh-3::GFP*)], JR667 [*unc-119*(e2498::Tc1) III; *wls51*], *velS13* [*col-19::GFP* + pRF4], BC3106 (*let-53* (s43) *unc-22*(s7)/nT1 IV; +/nT1 V), BC2895 (*let-73* (s685) *unc-22*(s7)/nT1 IV; +/nT1 V), BC2049 (*let-658* (s1149) *unc-22* (s7 *unc-331* (e169)/nT1 IV; +/nT1 V), and BC2067 (*let-659* (s1152) *unc-22* (s7 *unc-331* (e169)/nT1 IV; +/nT1 V). Transgenic animals were obtained by injection of plasmid DNA into the gonad of worms (Mello *et al.*, 1991). Transgenic arrays were integrated by irradiating animals with 40 Gy of gamma radiation from a 137 Cs source (Way *et al.*, 1991). Mutant animals isolated from frozen libraries were out-crossed at least five times to wild-type N2 animals before phenotypic analysis.

Constructs

cDNAs from the T14G10.2 gene were obtained from Dr. Kohara (est yk222e6) or directly isolated by PCR on cDNA and cloned into pGEM-T (Promega, Madison, WI). Nucleotide and protein sequences of the different isoforms have been deposited at GenBank under accession numbers AF308447 (PXF-

1-A), AF308448 (PXF-1-B), and AF308449 (PXF-1-C). To determine the specificity of the catalytic domain of *pxf-1* the region encompassing the REM-, the PDZ-, and the catalytic domain of PXF-1-A (amino acids 467-1173) was amplified using the primers CTCGAGTTCAGAAAGTTGAACGAAGGAC and GCGGCCGCTGTTCATTGCTGAAAAAAGCTG and cloned as an *XhoI/NotI* fragment into pMT2HA.

For reporter studies genomic fragments were obtained by PCR and inserted into the *SphI/BamHI* site of the pPD95.75 GFP vector (Fire, personal communication). Primers used for pT14G10.2::GFP-I were TAAGATCTTCCTGTC-GAGGTTTCCGAGG and ATAGCATGCCTTGACCATTTCCTATCTGTG; those for pT14G10.2::GFP-II were ATAGCATGCGATGGAGATGTCGCAC-GAAG and TAGGATCCCATTCTCGACAGAATCTCG.

Isolation and Rescue of *pxf-1* and *rap-1* Mutant Animals

Worms mutant for *pxf-1* (NL2808 (pk1331)) were isolated from a frozen library as described in Jansen *et al.* (1997). The first round of the nested PCR reactions was done with the primers AATTGATGCTCTGGTTTGCC and GGTGGTA-AACCTCGTGAGAGA, followed by a reaction with TCGAGACGAACGAT-GAGATG and AGGAAGAACACCCGGAAGAT. To detect the wild-type *pxf-1* allele, the primers CGATCTGAACCTCTGTACCTG and CCAAATTT-TCCATTGTGTGG were used.

For rescue experiments worms with the genotype *dpy-20* (e1362), *pxf-1* (pk1331)/+, + IV (NL2817) were generated. This line was then crossed with *dpy-20* (e1362)/*dpy-20* (e1362) IV pkEx10[T14G10 + pMH86] (NL2850). Rescue of the lethal phenotype by the cosmid T14G10 was evident from the appearance of F2 animals that segregated fertile, phenotypically wild-type animals, and animals that combined the *pxf-1* mutant phenotype with a dumpy appearance. In addition, genotyping by PCR confirmed that these latter animals were homozygous *pxf-1* mutants. Because cosmids K04D7 or K08F4 did not rescue, only mutations in T14G10.1 and 2 could be causative of the mutant phenotype. Therefore, double-stranded RNA for either gene was fed to N2 and ML652 worms. For T14G10.2 this resulted in a delayed progression through larval stages. In addition, microscopic analysis of *dgl-1::GFP* showed loss of a restricted number of seam cells in 7% (n = 100) of the animals. Complementation tests with *let-53*, *let-73*, *let-658*, and *let-659* mutants was done by first placing these mutations in trans to a *dpy-20* (e1362) allele, followed by mating to *pxf-1* +/+ *dpy-20* (e1362) animals. In all cases complementation was observed.

Mutant *rap-1* animals (FZ0181; pk2082) were isolated from a frozen library by means of target selected mutagenesis. To this end an 800-base pair fragment was amplified using the primers GACGAGAGTTTATTGTTACAG and GTGAGTTTCAAAAAATGTGTG, followed by a nested reaction using TGTA-AAACGACGGCCAGTCGAAAATGTATCATATCGAGAC and AGGAAA-CAGCTATGACCATGTTAGCTCTCTTTTACATTGAG. Sequencing of heteroduplex products revealed the presence of a C to T mutation, resulting in a premature stop at position 130 of RAP-1. Rescue was performed by expression of heat shock promoter driven *rap-1*, which restored alae formation and restored brood size, to the level seen for wild-type animals, carrying the *hsp::rap-1* construct.

Analysis of GFP-expression Patterns and Immunofluorescence

Expression of *pxf-1* was done by generating worms carrying pT14G10.2::GFP-I (FZ0116; *bjEx40*[pT14G10.2::GFP-I/pMH86] and FZ0117; *bjEx40*[pT14G10.2::GFP-I/pMH86]); and pT14G10.2::GFP-II (NL2815 or NL2821). NL2815 and NL2821 are independent lines showing an identical expression pattern. These and other GFP lines described were mounted on agarose pads and observed under a Nikon (Garden City, NY) or Zeiss (Thornwood, NY) immunofluorescence microscope equipped with Nomarski optics. For analysis of pharyngeal expression in NL2815 and NL2821 eggs were collected from bleached gravid adults and viewed at various time-points with a dissecting microscope under UV light. DPY-7 localization was done using the DPY7-5A antibody and procedures described in Roberts *et al.* (2003) using a Cy3-conjugated donkey anti-mouse antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) for detection.

Electron Microscopy

Homozygous *pxf-1* larvae that failed to molt to L3, L2, and L3 larvae from N2 were fixed in 2.5% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.2) at 4°C for 4 h, followed by postfixation in 2% OsO₄ at 4°C for 2 h. Samples were dehydrated by acetone series and embedded in Epon resin. Ultrathin sections were double-stained with uranyl acetate and lead citrate and viewed with JOEL 1010 (Tokyo, Japan) transmission microscope.

In Vivo Activation of Small GTPases

The specificity of PXF-1 was determined essentially as described in de Rooij *et al.* (1999). Briefly, Cos-7 cells were transfected with HA-tagged versions of human Rap1, Ral, or Ras and increasing amounts of PXF-1. After 24 h of serum starvation, cells were lysed and GTP-bound GTPases were isolated using activation-specific probes (GST-RalGDS-RBD for Rap1, GST-RalBP for Ral, and GST-Raf1-RBD for Ras). Isolated GTPases were visualized by means

region deleted in *pxf-1* mutants (Δ) are indicated as thin lines (see also *Materials and Methods*). (C) Schematic representation of the three splice variants of *pxf-1*. RCBDS, related to cNMP binding domain; REM, Ras exchange motif; RA, ras association domain; GEF, guanine nucleotide exchange factor; VSRV, amino acid sequence of the PDZ-binding motif. Numbers at the right indicate the number of amino acids for each isoform.

of Western blotting using the 12CA5 monoclonal antibody (de Rooij *et al.*, 1999). Rap2 activation was measured in [32 P]orthophosphate-labeled cells as described in de Rooij *et al.* (1999).

RESULTS

Genomic Organization of *pxf-1* and Analysis of cDNAs

Analysis of the complete genome of *C. elegans* (Greenstein *et al.*, 1998) reveals the presence of a single homologue for each of the four types of Rap1-GEFs found in vertebrates (Figure 1A). Previously, we had already reported the existence of a *C. elegans* homologue of the human PDZ-GEF1, which we named CelPDZ-GEF at the time (de Rooij *et al.*, 1999). However, in order to comply with the international nomenclature, we renamed this gene *pxf-1* for PDZ-domain containing exchange factor. *pxf-1* is located on LG IV and the complete coding sequence is covered by cosmid T14G10 (gene T14G10.2). We cloned three different SL1-spliced messengers from *pxf-1* by PCR using primers designed on the basis of predictions made by GENEFINDER, ACeDB as well as sequence information from ESTs of this gene. The protein isoforms encoded by these mRNAs were named PXF-1A to C (Figure 1, B and C). PXF-1-A is the longest isoform (1470 amino acids) and has also been published under the name RA-GEF (Liao *et al.*, 1999). A profile-scan shows that it encodes two cNMP-binding motifs (RCBDs), followed by a Ras exchange motif (REM), a PDZ-domain, a Ras-association (RA) domain and a catalytic domain (GEF). PXF-1-B is encoded by an alternatively spliced messenger and lacks the consensus PDZ-binding motif VSRV at the C-terminus. PXF-1-C resembles PXF-1-A, but does not contain the N-terminal cNMP-binding motif. It is encoded by a transcript in which SL1 is transspliced to exon four. This transcript is derived from an intragenic promoter (see below).

PXF-1 Is a Rap-specific Exchange Factor

To determine the specificity of PXF-1 for the various Ras-like GTPases cotransfection studies were performed in Cos7 cells. A HA-tagged version of PXF-1, encompassing amino acids 467-1173 of PXF-1-A, was coexpressed with HA-tagged human Rap1, Ral, or Ras. GTP-bound GTPases were isolated by virtue of their high-affinity binding to activation-specific probes (see *Materials and Methods*) and detected by Western blotting. Increasing the amount of PXF-1 specifically activated Rap1 but not Ras or RalA (Figure 2A). Because Rap2 has a high basal level of GTP, the activation-

specific probe assay is less sensitive for Rap2. Therefore, the effect of PXF-1 on Rap2 was tested in [32 P]orthophosphate-labeled cells, from which HA-tagged Rap2 was immunopre-

Figure 2. (A) In vivo activation of Rap1 by PXF-1. HA-Rap1, HA-RalA, and HA-Ras were cotransfected with increasing amounts of *pxf-1* (0, 1, and 3 μ g) into Cos-7 cells. As positive controls Epac, CalDAGGEFIII, and Rlf were used as positive controls (lanes labeled +) for Rap, Ras, and Ral activation, respectively. GTP-bound Rap1, Ral, or Ras was isolated using activation specific probes and visualized with 12CA5 antibodies after Western blotting (top panels). Total lysates were blotted and probed with 12CA5 to show equal levels of the transfected HA-tagged GTPases (middle panels) or increasing PXF-1 expression (bottom panels). (B) TLC of nucleotides bound to HA-Rap2A, isolated from [32 P]orthophosphate-labeled Cos-7 cells, which had been transfected with HA-Rap2A in the presence or absence of 3 μ g *pxf-1*. GTP/GDP ratios were measured using a phospho-imager. Activation of HA-Rap2A by human EPAC1 is shown as a positive control.

cipitated. Separation of nucleotides bound to Rap2 by TLC showed that coexpression of PXF-1 with HA-tagged Rap2A resulted in a clear increase in the GTP/GDP ratio of Rap2A (Figure 2B). Together, these results show that PXF-1 is a Rap-specific exchange factor.

Expression of *pxf-1* as Determined by GFP Reporter Constructs

Expression from the upstream *pxf-1* promoter was studied by means of a GFP-reporter construct, containing genomic sequences from nucleotide -2394 to +26, relative to the translational start codon in exon I (pT14G10.2::GFP-I; Figure 1B). Expression was first seen at the comma stage in endodermal precursor and slightly later in the hypodermal cells of the embryo (Figure 3, A and B, and unpublished data). During elongation and larval stages many cells of the embryo express GFP, but particularly strong staining was seen in the hypodermis (Figure 3, C and D) and gut. In addition, several neurons in the head were brightly labeled. In general, expression in the lateral seam cells (Figure 3, E and F) and *hyp10* was most prominent. However, expression could also clearly be seen in the other hypodermal cells, like the syncytial *hyp7* and the Pn.p cells during and after the time of vulval induction (Figure 3, G and H, and unpublished data). In the adult new sites of strong expression included the hermaphrodite-specific neurons (HSNs) and the oviduct sheath cells. The lateral seam cells, which are fused in the adult and other hypodermal cells were also GFP positive (unpublished data).

To investigate expression from the downstream promoter a reporter construct was used that contains sequences from nucleotide +990 relative to the translation start of exon I to +4500 located in exon VI (plasmid pT14G10.2::GFP-II; Figure 1B). Expression was detected first in neuronal precursor cells at the comma stage. Slightly later strong expression was found in the hypodermal cells. In newly hatched L1 larvae this construct continued to be expressed in neuronal cells in the head and tail, in cells of the ventral nerve cord and of the pharynx. During the early L1 stage hypodermal expression disappeared. The GFP-positive neurons in animals carrying pT14G10.2::GFP-II were identified as the RMDD, RMD, RMDV, SMDD, and SMDV, which are ring motoneurons. Also the more posteriorly located BDU cells and the ALN, PVR, and PVT cells in the tail expressed GFP. In the course of our studies we noticed a striking difference in GFP expression levels in the pharynx. Analysis of synchronized worms showed that GFP is present around the time of molting, but is barely detectable during part of the intermolts (Figure 3, I and J) or in adults. During the outgrowth of the gonadal primordium expression was seen in the distal tip cell and oviduct sheath cells (unpublished data). It is possible that the two reporter constructs used here contain enhancer elements, which act on both promoters. However, the results clearly show that *pxf-1* is widely and dynamically expressed in the main epithelia during all stages.

Isolation of Animals Mutant for *pxf-1*

To investigate the function of *pxf-1*, a deletion library of mutant worms was screened by PCR (Jansen *et al.*, 1997). A single mutant allele of *pxf-1* was isolated, in which a 3.4-kb genomic region (encoding amino acids 613-1306 of PXF-1-A; see Figure 1B) was deleted. PXF-1 protein derived from this allele lacks most of the PDZ domain as well as the complete catalytic domain, and therefore is likely to be nonfunctional, at least with respect to Rap activation. No homozygous line could be established because deletion of *pxf-1* results in lethality during late larval stages or early adulthood. Timed

Figure 3. Expression of *pxf-1*. DIC (A, C, E, and G) and epifluorescence (B, D, F, H, I, and J) micrographs of embryos and larvae carrying pT14G10.2::GFP-I as an extrachromosomal array (A–H) or an integrated pT14G10.2::GFP-II construct (I and J). In each case the bar indicates 10 μ m. (A and B) Expression in endodermal cells of a comma stage embryo. (C and D) Expression in the *hyp7* syncytium (anterior edge marked by arrow) and the annular *hyp4* (anterior and posterior margins marked by arrowheads) of a L2 larva. GFP-expressing gut cells are outside the focal plane. (E and F) Expression in lateral seam cells in early L4 larva. (G and H) Expression in hypodermis during vulval formation. (I) Expression in head neurons, but not in the pharynx of a L3 larva during intermolt (see text for details). (J) Expression in head neurons and in the pharynx of a molting L3 larva.

egglays showed that mutant animals progressed slower through larval stages than wild-type animals. A fraction of the mutant animals could be identified after 24 h by their uncoordinated movement and their slightly smaller size. Inspection by Nomarski optics at 48 h after egg lay showed the appearance of vacuoles just underneath the cuticle in a small fraction of mutant animals. Most commonly these

Figure 4. Phenotype of *pxf-1* (*pk1331*) mutant animal as seen by DIC microscopy (A–H) or immunofluorescence microscopy (I and J). In each case the bar indicates 10 μ m. (A) Small vacuoles (white arrows) underneath the cuticle in the pharynx region of a L4 larva. P, the pharynx. (B) L4 larva encased in its old cuticle. Note that the pharyngeal cuticle has been expelled. (C) Unshed cuticle at the posterior part of a L4 larva. The unshed cuticle is indicated with an arrowhead, the new cuticle with an arrow. (D) Adult animal in which the gonads are protruding from the body cavity. (E) Alae of an adult wild-type animal. (F) Disrupted alae of *pxf-1* mutant. (G) Sensory rays of wild-type male. (H) Lack of sensory rays in *pxf-1* mutant male. (I and J) Localization of DPY-7 in the anterior part of wild-type (I) and *pxf-1* (J) L3 larvae, using a monoclonal anti-DPY-7-5A antibody. (K) Percentage of survival to the indicated developmental stage of wild-type animals (♦; n = 75), *pxf-1*^{-/-} mutants from heterozygous hermaphrodites (□; n = 82), and from homozygous mutants (▲; n = 76).

vacuoles were found in the tail region or around the anterior part of the pharynx (Figure 4A). These vacuoles appear to be signs of degeneration of the hypodermis, which at later time points can become more pronounced. From the L2 stage onward, mutant animals displayed molting defects: frequently animals were enclosed within their old cuticle or part of the anterior cuticle remained attached to the pharynx, obstructing it (Figure 4, B and C, Table 1). In some instances animals that were encased in their old cuticle escaped from it and resumed feeding. It is not uncommon for such animals to have trails of old cuticle attached to their bodies or encircling it. The lumen of the gut was enlarged in most mutants and contained undigested bacteria. About 25% of homozygous mutant animals reached adulthood (Table 2). Almost all of them moved in an uncoordinated manner and their body size was strongly reduced as compared with wild-type animals (see Figure 9). Homozygous animals had developed a fairly normal gonad and were able to lay a restricted number of fertilized eggs (see Table 2). Hereafter,

the proximal gonad started to degenerate and egg-laying stopped. Mutants then became immotile and died as a “bag of worms.” Alternatively, worms died as a result of a burst vulva (Figure 4D).

Given the high expression of *pxf-1* in seam cells, we inspected the alae, which are cuticular structures secreted by lateral hypodermal (seam) cells after fusion. In virtually all mutant adults alae were less distinct or interrupted (Figure 4, E and F). In males, the lateral hypodermal cells V5, V6, and T form sensory rays in the tail. In *pxf-1* mutant males these structures are malformed and barely visible (n = 9; Figure 4, G and H and unpublished data). Formation of spicules on the other hand appeared normal. Although clear cuticle defects in *pxf-1* adults were noticed, the earliest defects in *pxf-1* mutants were observed at the L2 stage. At this stage the cuticle does not contain any alae, but only circumferential ridges (the annuli), which appeared to be present. To obtain a more detailed view of the cuticle, we immunostained wild-type and *pxf-1* mutant larvae with an antibody against the collagen DPY-7 (kindly provided by Dr. I. Johnstone). In wild-type animals this antibody detects DPY-7 in very regular circumferential bands (Roberts *et al.*, 2003). In contrast, in *pxf-1* animals DPY-7 was present in bands, which not always ran parallel and were wider and often interrupted (Figure 4, I and J). This demonstrates that also at earlier stages the hypodermis is affected in secretion of the cuticle. Taken together, these results indicate a requirement for PXF-1 in the hypodermis, especially in the seam cells.

Adult homozygous *pxf-1* hermaphrodites produce a small brood. Only a minority of the offspring from these mutants develops beyond the L3 stage (Figure 4K). They display the same, albeit more severe phenotypic traits. In addition, a low level of embryonic lethality is seen (7%). This strongly suggests that there is maternal contribution of PXF-1 protein to embryos. Yet it also demonstrates that significant development in the absence of PXF-1 is possible. This is in striking

Table 1. *pxf-1*^{-/-}[p] animals analyzed in three separate experiments

Hours after hatching	unc (%)	vac (%)	mol (%)
24	34	4	0
48	34	12	2
72	62	32	32
96	86	70	62
120	92	84	62

Values are cumulative percentage of 62 animals.

unc, uncoordinated movement; vac, presence of vacuoles; mol, molting defect.

contrast with the situation in *Drosophila*, where PDZ-GEF mutant animals from heterozygous mothers show high level of embryonic lethality (Lee *et al.*, 2002).

Transformation of mutant animals with the cosmid T14G10, carrying the complete *pxf-1* gene, as an extrachromosomal array led to complete rescue of all aspects of the above described phenotype (see Table 2). No rescue was seen with the overlapping cosmids K04D7 or K08F4. Because RNAi with T14G10.2 (see also *Materials and Methods*) resulted in a delayed growth and a low frequency (7%) of hypodermal abnormalities described below we conclude that the phenotype described is caused by disruption of *pxf-1*.

Hypodermal Defects in *pxf-1* Mutant Animals

To analyze the hypodermal defects in more detail, various GFP-reporter constructs were introduced into a *pxf-1* mutant background. The seam cells are known to be important for cuticle secretion, and their cell division is tightly coupled to molting events (Sulston and Horvitz, 1977; Austin and Kenyon, 1994). When the seam cells of mutant L1 larvae were marked using either the *cdh-3::GFP* (Pettitt *et al.*, 1996) or the SCM::GFP construct (Koh and Rothman, 2001), it was clear that the correct number of seam cells had been specified. The SCM::GFP marker marks the nuclei of seam cells, which are very regularly spaced in wild-type animals (Figure 5, A and B). Using the SCM::GFP marker, no abnormalities were seen in the division patterns of these seam cells during the L1 to L2 molt (Sulston and Horvitz, 1977; Austin and Kenyon, 1994). However, at later stages mutant animals were seen in which expression of the SCM::GFP was extremely weak or absent at various positions along the body. The seam cell nuclei were often irregularly spaced in more affected animals (Figure 5, C–H). Weakly staining nuclei often had a shrunken appearance. Nomarski microscopy confirmed that the lack of SCM::GFP expression resulted from a loss of seam cells (Figure 5, G and H). Importantly, however, animals that could not complete molting frequently had normal appearing seam cells, demonstrating that hypodermal degeneration does not necessarily precede molting defects. We also noted a number of mutant animals in which seam cell expression was weak in the midbody and in which the nuclei of the seam cells still could be seen by Nomarski optics. This most likely resulted from the inability of mutants to feed, because starvation of wild-type animals resulted in a similar decrease of SCM::GFP expression in the midbody.

Given the fact that lack of Rap1 in *Drosophila* perturbs adherens junction formation (Knox and Brown, 2002), we used *dlg-1::GFP* to mark the adherens junctions of seam and

other cells (Figure 6; McMahon *et al.*, 2001). Adherens junctions in *pxf-1* mutant L1 larvae had a regular, uninterrupted appearance and showed that also the shape of seam cells was normal. As expected, also with this marker we noticed a loss of seam cells from the early L2 stage onward. Interestingly, in a restricted number of animals this marker revealed seam cells with an abnormal morphology (Figure 6, C–F), suggesting that such cells were affected in their ability to reorganize cell-cell contacts or the cytoskeleton. *dlg-1::GFP* staining in other parts of the body like the pharynx and the Pn.p cells was normal. If present, the seam cells fused normally at the L4 to adult molt. Analysis of *pxf-1* mutants with various other GFP constructs, including pT14G10.2::GFP-II, did not reveal any other abnormalities (Figure 6, G and H).

As a complementary approach in the analysis of *pxf-1* mutants, electron microscopy was used to investigate L2 and L3 larvae (Figure 7). In contrast to wild-type worms, which have a characteristic round shape, *pxf-1* mutants were deformed (Figure 7, A and B). The lumen of the gut was clearly enlarged, consistent with images from light microscopy. Microvilli are present but have a shortened appearance. The most striking difference was found in the cuticle of mutants, which was irregular around the entire circumference, varied in thickness and was folded. Furthermore, the overall organization appears much more loose than in wild-type siblings. Importantly, both the underlying lateral hypodermis and hyp7 had a fairly normal appearance, with a basal lamina and fibrous organelles being present (Figure 7, C, D, G, and H). Muscles of *pxf-1* mutants were less regularly spaced compared with those of wild-type animals, slightly disorganized and their dense bodies were less prominent (Figure 7, E and F). The defects in muscle organization were unexpected given the fact that no muscle expression of *pxf-1* was seen using GFP-reporters. However, it is well established that the hypodermis is of crucial importance for muscle architecture and mechanical coupling of muscle cells with the cuticle (for a review, see Michaux *et al.*, 2001). Therefore, it is a realistic option that disorganization of muscle cells may result from hypodermal defects. Other cells like the ventral cord neurons appeared normal (unpublished data). In summary, ultrastructural analysis demonstrates that *pxf-1* is required in the hypodermis for proper formation of the cuticle and that hypodermal degeneration does not precede cuticle defects.

pxf-1 Acts Upstream of *rap-1* and *rap-2*

To obtain genetic evidence for *pxf-1* as a rap-specific GEF, *rap* mutants were investigated. The *C. elegans* genome contains three genes with high homology to vertebrate Rap

Table 2. Survival and proliferation rates of *pxf-1* mutant animals

Genotype	% survival until adult stage	No. of progeny ^a
Wild type	100	165 ± 20 (5)
<i>pxf-1</i> ^{-/+}	100	155 ± 36 (5)
<i>pxf-1</i> ^{-/-}	25 (54/212)	5 ± 3 (14)
<i>pxf-1</i> ^{-/-} pkEx10[<i>dpy-20</i> (+) T14G10]	71	54 ± 22 (14)
<i>pxf-1</i> ^{-/-} bjl520[<i>Rol-6</i> ;hsp::rap-1 ^{V12} ;rap-1::GFP]	66	43 ± 17 (14)

Larvae (L1 or L2) from heterozygous hermaphrodites were singled and scored for their survival to the adult stage. For *pxf-1*^{-/-} bjl520[*Rol-6*;hsp::rap-1^{V12};rap-1::GFP], counting of animals surviving to adulthood and number of offspring was done on animals given a daily heat shock. Genotyping of animals was done by PCR.

^aValues in parentheses are number of animals.

Figure 5. *pxf-1* mutants display loss of seam cells. DIC (A, C, E, and G) and epifluorescence (B, D, F, and H) micrographs of wild-type (A and B) and *pxf-1* mutant animals (C–H) transgenic for *SCM::GFP*. Scale bars, 100 μ m (A and C); 10 μ m (E and G). (A and B) Late wild-type L2 larva, showing the regular spacing of seam cells. Descendants of the V1 and V2 cells are indicated. (C and D) *pxf-1* mutant L2 larva, in which one of the V1 and V4 descendants are missing. Descendants of the V1 and V2 cells are indicated. (E and F) *pxf-1* mutant L2 larva, in which V3 descendants have degenerated. (G and H) Enlargement of the region indicated in F. The black and white arrow indicates the place in which a V3 descendant is expected, but where no nucleus is seen. The brightly fluorescent dots may represent remnants of this nucleus. Other seam cell nuclei are indicated with white arrows.

Figure 6. Morphology of seam cells in *pxf-1* visualized by *dlg-1::GFP*. Epifluorescence (A, B, D, F, and H) and DIC (C, E, and G) micrographs of wild-type (A) and *pxf-1* mutant animals (B–H) transgenic for *dlg-1::GFP* (A–F) or pT14G10.2::GFP-II (G and H). In each case the bar indicates 10 μ m. (A) Wild-type L2 larva, showing the regular organization of seam cells. (B) *pxf-1* mutant L2 larva, in which anterior seam cells are missing (indicated by a black line above their expected location). A white arrow indicates the array of Pn.p cells. (C–F) *pxf-1* mutant in which seam cells have undergone their first round of division during L2. (E and F) A higher magnification of the inset from C. Seam cells have formed processes extending in anterior and posterior directions from their cell body. The descendant of H2 (H2pp) has a dumb-bell shape with a very thin cytoplasmic bridge connecting the anterior part with a nucleus (arrow) and the posterior part (arrowhead), which is contacting the descendant of V1 (V1pa). (G and H) Normal appearance of neurons labeled by pT14G10.2::GFP-II in a *pxf-1* mutant adult that displayed uncoordinated movement and had a molting defect.

Figure 7. Electron microscopic analysis of wild-type (A, C, E, and G) and *pxf-1* mutants (B, D, F, and H). Pictures are taken from the midbody of L3 larvae. (A and B) Cross sections, showing the overall shape of wild-type (A) and *pxf-1* mutant (B). Note the widened gut lumen in the *pxf-1* mutant. The gut cells (labeled G) contain fewer electron dense vesicles as compared with wild-type cells. Hypodermal cells are labeled h and in the *pxf-1* mutant underlie a cuticle, which is thin and irregular around the entire circumference. (C and D) Lateral hypodermis (h). Abnormal accumulation of cuticle structures in *pxf-1* mutant is indicated by an arrow. (E) Wild-type animal, showing the regular organization of muscle cells. (F) Muscle quadrant of *pxf-1* mutant. White arrow indicates dense body. (G and H) Hyp7 (h) and cuticle of wild-type and *pxf-1* mutant animals. The cuticle has a striated and regular organization in the wild-type animal (G), whereas those of the *pxf-1* mutant display extra folds and a looser organization (H). The basal lamina underneath the hypodermis is indicated with white arrowheads; fibrous organelles are marked with black arrowheads. Bar, 5 μ m (A and B), 1 μ m (C–F), or 200 nm (G and H).

proteins. Of these, *rap-1* (C27B7.8) and *rap-2* (C25D7.7) are clear homologues of Rap1 and Rap2, respectively, whereas *rap-3* (C08F8.7) encodes a more distinct member. *rap-2* null

Figure 8. DIC micrographs of *rap-1* (A–C) and *rap-1*; *rap-2* mutant animals (D). Scale bars, 10 μ m (A, C, and D); 100 μ m (B). (A) Disrupted alae in *rap-1* mutant. (B) Adult with burst vulva. (C) *rap-1* male, lacking rays posterior to ray 6 (numbered as in Sulston and Horvitz, 1977). (D) Widened gut with undigested material in a L4 larva, double mutant for *rap-1* and -2.

mutants (VC14; kindly provided by Dr. R. Barstead) did not display any detectable phenotype. We therefore used target-selected mutagenesis and isolated a *rap-1* mutant carrying a premature stop-codon at position 130 (FZ0181, *pk2082*). Truncation of RAP-1 at this position leads to a loss of the G5-loop, known to be required for GTP binding and of the C-terminal CAAX-box motif, involved in membrane targeting. We therefore believe that this mutant represents a null allele. Homozygous *rap-1* mutants are viable and fertile, but show striking similarities to *pxf-1* mutants. First, they develop slower than wild-type and especially the molting process appears retarded. Alae of adults are almost always (94%) absent or interrupted (Figure 8A), and loss of seam cells is seen during late larval stages using the SCM::GFP and *dlg-1*::GFP markers (unpublished data). Adult hermaphrodites die with a burst vulva phenotype at a low frequency (~5%, Figure 8B) or die as a bag of worms after degeneration of the proximal gonad. Consequently, *rap-1* mutants have a reduced brood-size (36% of that of wild-type animals). In males, sensory rays are formed, but especially the most posterior rays are frequently lost (Figure 8C). To see if RAP-2 would function redundantly with RAP-1, double mutants were generated. Although removal of a single *rap-2* allele did not affect the *rap-1* phenotype, removal of both alleles largely reproduced the *pxf-1* phenotype: it caused lethality at a late larval or early adult stage, and animals had an enlarged gut lumen with undigested bacteria (Figure 8D). Furthermore, animals remained scrawny (Figure 9E), they showed molting defects, and adults almost invariably died with a burst vulva phenotype or as a bag of worms as seen for *pxf-1*.

Given the fact that the *rap-1* phenotype was most similar to that of *pxf-1*, we tried to rescue *pxf-1* mutants with an activated version of RAP-1, RAP-1^{V12}. When placed under the control of the heat shock promoter (*hsp*), expression of RAP-1^{V12} by a daily heat shock in wild-type animals slowed progress through larval stages, but did not affect viability. In *pxf-1* mutants expression of RAP-1^{V12} significantly increased the number of mutants that reached adulthood as well as the number of progeny (Table 2). Furthermore, when grown continuously at 20°C, lines homozygous for *pxf-1* could be propagated. However, even though RAP-1^{V12} largely res-

Figure 9. Overview micrographs of adult wild-type and mutant worms. Scale bar, 100 μ m. Pictures are taken 2 d after the animals had reached the L4 stage. (A) Wild-type N2; (B) *pxf-1* (*pk1331*); (C) *pxf-1* (*pk1331*) *pkEx10[dpy-20 (+) T14G10]*; (D) *rap-1* (*pk2082*); (E) *rap-1* (*pk2082*); *rap-2* (*gk11*); and (F) *pxf-1* (*pk1331*) *bjIs20[Rol-6;hsp::rap-1^{V12}]*.

cued the lethality of the *pxf-1* mutation, *pxf-1^{-/-}*; *hsp::rap-1^{V12}* animals develop slower and remain somewhat smaller than wild-type worms (Figure 9F).

DISCUSSION

PXF-1 Is an Exchange Factor for RAP1 and RAP2

In this report we have characterized the *pxf-1* gene (T14G10.2) of *C. elegans*, which encodes three protein isoforms. When compared with human PDZ-GEF proteins, it is clear that PXF-1-C is most similar to hPDZ-GEF1 because it contains a single RCBD. The other isoforms contain two of these motifs and consequently resemble hPDZ-GEF2 (de Rooij *et al.*, 1999; Liao *et al.*, 1999; Kuiperij *et al.*, 2003). Strikingly, alternative splicing of mRNA from this latter gene also yields proteins that either contain a PDZ-binding motif at the C-terminus, resembling PXF-1-A, or lack it as seen for PXF-1-B (Kuiperij *et al.*, 2003). Like its mammalian homologues, PXF-1 displays guanine nucleotide exchange activity toward Rap1 and Rap2. Our transfection studies confirm the findings of Liao *et al.* (1999), who showed that in tissue culture cells PXF-1 activates Rap1. However, these authors also demonstrated Ras-exchange activity *in vitro*, which we could not detect in our cotransfection experiments. Ras activation by human PDZ-GEF1 in tissue culture cells has also been reported (Pak *et al.*, 2002), and we have currently no explanation for this discrepancy. Importantly, our genetic experiments provide evidence that PXF-1 functions as a RAP-exchange factor *in vivo*. First, disruption of *pxf-1* or *rap-1* shows that they both function in the hypodermis and in particular the lateral hypodermal cells. Clearly, the *pxf-1* phenotype is more severe than that of *rap-1* mutants, which appears to result from functional redundancy of *rap-1* with *rap-2*. The presence of a single functional *rap-1* or *rap-2* allele is sufficient for viability, but double mutants display a lethal phenotype, which is virtually identical to that of *pxf-1* mutants. Moreover, overexpression of an activated version of RAP-1 (RAP-1^{V12}) rescues the lethal phenotype of *pxf-1*, which strongly supports the notion that *pxf-1* acts upstream of *rap-1* *in vivo*. *pxf-1^{-/-}*; *hsp::rap-1^{V12}* animals still develop more slowly and remain smaller than wild-type animals. It is possible that this is due to the fact that PXF-1 has functions other than RAP-1 activation. However, we think it is more likely that heat-shock driven overexpression of RAP-1^{V12} does not mimic the spatio-temporal control of RAP-1 activation by PXF-1. Furthermore, despite being less sensitive to GTPase activating proteins, RAP-1^{V12} may still require PXF-1 activity to become highly GTP-bound. Indeed, in an

analogous situation signaling by a gain-of-function allele of *let-60/ras* was found to be diminished by lowering the activity of its upstream activator SOS-1 (Chang *et al.*, 2000). So far, analysis of *pxf-1* mutants did not reveal a role for PXF-1 as a LET-60/RAS-GEF: vulval development in hermaphrodites and spicule formation in males, both known to be dependent on LET-60 RAS, appear normal. This is consistent with a genetic analysis of PDZ-GEF in *Drosophila*, which led to the conclusion that it functions as a Rap1-GEF rather than a Ras-GEF (Lee *et al.*, 2002).

pxf-1 Is Required in the Hypodermis

GFP-reporter studies show that *pxf-1* is widely expressed in a specific spatio-temporal pattern, suggesting a function for *pxf-1* in multiple tissues. In line with this, *pxf-1* mutants have a pleiotropic phenotype, which includes uncoordinated movement, an enlarged gut lumen, and a strongly reduced fertility. Many aspects of the phenotype however, point to a critical function for *pxf-1* in seam cells, where it is highly expressed. For example, the alae, which are secreted by the seam cells, are faint and interrupted in almost all mutant adults. Second, the sensory rays in males, formed by the seam cells V5, V6, and T are malformed. Also the "burst vulva" phenotype hints to a compromised seam cell function, because it is indicative of a loss of contact between seam cells and the utse or VulE cells, which are connected to the uterus (Sharma-Kishore *et al.*, 1999; Newman *et al.*, 2000).

The molting defect seen in *pxf-1* mutants provides the most direct evidence for a hypodermal function of *pxf-1*. Molting is a complex process in which first a stage-specific, collagenous cuticle is synthesized underneath the old one by cells of the pharynx and hypodermis. During this period high secretory activity is seen in the hypodermis, including the lateral seam cells (Singh, 1978). Simultaneously, the cortical actin cytoskeleton in the hypodermis undergoes a dramatic reorganization into circumferential-oriented actin bundles, which disappear again during the final stages of cuticle formation (Costa *et al.*, 1997). Two observations suggest that the molting defect in *pxf-1* mutants is not simply the result of the observed loss of seam cells, but instead suggest that *pxf-1* plays an active role in the process of molting. First, in many young animals, which could not complete molting, all seam cells were present and no signs of degeneration of the hypodermis were seen. Also ultrastructural analysis shows the presence of hypodermal cells underneath an abnormally organized cuticle in *pxf-1* mutants. Second, pharyngeal expression of *pxf-1* precisely coincides with molting and is barely detectable during inter-

molts. Similar oscillating expression patterns have been reported for genes encoding structural proteins of the cuticle like collagen genes (Johnstone, 2000; McMahon *et al.*, 2003), but also for the *lin-42* gene, which regulates the timing of molting events (Jeon *et al.*, 1999) and the nuclear hormone receptor *nhr-23*, disruption of which also causes molting defects (Kostrouchova *et al.*, 1998). Finally, loss of seam cells has also been described for worms, mutant for *plx-1*, which encodes a plexin-type of transmembrane receptor for semaphorins (Fujii *et al.*, 2002). However, for *plx-1* no molting defects have been reported. Loss of seam cells in *plx-1* mutants is caused by a reduced number of cell divisions during larval stages. After each division the anterior seam cell daughter fuses with *hyp-7* in wild-type worms, whereas the posterior cell will undergo further divisions. These divisions require that the posterior cell reestablishes contacts with its anterior and posterior neighbors (Austin and Kenyon, 1994). In *plx-1* mutants this does not occur, most likely because of changes in adhesive properties. Given the fact that the Rap1 pathway is involved in cell-matrix and cell-cell adhesion, we considered this an attractive scenario for our *pxf-1* mutant. Although seam cells sporadically display an abnormal morphology in *pxf-1* mutants, analysis of seam cells with the SCM::GFP marker show that the majority of cell divisions occurs normally and that seam cells are lost by degeneration. The precise cause of this degeneration is currently unknown, but it is possible that the disorganized cuticle provides insufficient protection against mechanical stress.

The hypodermal degeneration and rupture of the vulval region distinguishes *pxf-1* mutants from previously described molting mutants like those carrying mutations in *lrp-1*, *nhr-23*, or *nhr-25* (Kostrouchova *et al.*, 1998; Yochem *et al.*, 1999; Asahina *et al.*, 2000; Gissendanner and Sluder, 2000). Instead, the *pxf-1* phenotype much more resembles that of worms, in which *sec-23* functioning is reduced by RNAi during larval stages (Roberts *et al.*, 2003). Apart from the molting defect and burst vulva phenotype, these animals also grow slowly and have an enlarged gut lumen filled with undigested bacteria. In addition, both have a disorganized cuticle, as determined by detection of DPY-7 collagen using immunofluorescence. Null mutants of *sec-23* were isolated on the basis of their inability to secrete cuticle components. Although *sec-23* mutants are embryonic lethal and thus have a more severe phenotype, the RNAi result shows that compromised secretion of cuticle components can lead to molting defects. Likewise, loss of *pxf-1* or *rap-1* and *rap-2* may reduce the (polarized) secretory activity of the hypodermis to a level below that required for molting. A role for *pxf-1* in secretion is in line with the expression observed in tissues involved in polarized secretion, such as the hypodermis and gut. Furthermore, also the Rap-specific exchange factor, Epac2, has been shown to be involved in enhancing secretion, namely of insulin from pancreatic beta-cells (Ozaki *et al.*, 2000). The Rap1 pathway appears not to be absolutely required for secretion, but may increase the efficiency indirectly by organizing some spatial feature. Intriguingly, in yeast the RAP-1 homologue RSR1 is essential for proper bud site selection, but not for bud formation per se. Bud site selection relies on polarization of the cytoskeleton to target secretory vesicles to a specific site in the cell. Also the well-documented stimulatory effect of Rap1 on integrin function in lymphocytes may depend on the polarizing activity of Rap1 in these cells (Shimonaka *et al.*, 2003), possibly via its effector RapL (Katagiri *et al.*, 2003). Definite proof for a role of *pxf-1* and *rap-1/2* in polarized secretion awaits further characterization of these mutants. In any case, the described hypodermal defect offers a good starting point for

identification of additional components of the Rap1 pathway in *C. elegans* and opens the way to a comparative analysis of this pathway in distinct organisms.

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