C. elegans Rab GTPase 2 is required for the degradation of apoptotic cells

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During apoptosis, the dying cell activates an intrinsic mechanism that quickly dismantles itself. The apoptotic cell corpses are then recognized and removed by neighboring cells or professional phagocytes. How dying cells are degraded after internalization is poorly understood. Here, we report the identification and characterization of unc-108, the Caenorhabditis elegans homolog of the human Rab GTPase 2, as a novel component involved in the degradation of apoptotic cells. unc-108 is expressed and functions in the engulfing cells and is likely to affect the degradation rather than the internalization of cell corpses. Similar to other Rab GTPases, unc-108 also affects endocytosis, acting in the endosomal trafficking from early to late endosome and late endosome to lysosome. UNC-108 co-localizes with RAB-5, RAB-7 and LMP-1 to the phagosome and promotes cell corpse degradation, possibly by mediating phagosome maturation.

KEY WORDS: C. elegans, Apoptotic cell, Degradation, unc-108, Rab GTPase 2, Endocytosis, Phagosome

INTRODUCTION

Apoptotic cells generated by programmed cell death are recognized and cleared by neighboring cells or professional phagocytes. Efficient clearance of apoptotic cell is crucial for tissue homeostasis and the regulation of immune responses. Defects in this process contribute to persistent inflammatory diseases and autoimmune disorders (Savill et al., 2002; Savill and Fadok, 2000). Phagocytosis of apoptotic cell is regulated by mechanisms that are highly conserved from the nematode C. elegans to humans (Fadeel, 2003). For example, cells that undergo programmed cell death in C. elegans are quickly removed by neighboring cells (Sulston and Horvitz, 1977). Two partially redundant pathways have been identified in C. elegans that result in internalization of foreign particles or apoptotic cells. The internalized vesicle, the phagosome, matures through a process. Although phagosome composition and maturation have been extensively studied in mammalian cells using latex-bead-containing phagosomes (Garin et al., 2001; Stuart et al., 2007), the maturation of these genes involved in cell corpse engulfment, many crucial components for this process are still missing, including genes involved in the degradation of cell corpses. In addition, how phagosomes form and mature, and how internalized cell corpses are degraded remain unclear.

Phagocytosis is a receptor-mediated, actin-dependent process that results in internalization of foreign particles or apoptotic cells. The internalized vesicle, the phagosome, matures through interaction with organelles of the endocytic pathway to generate the phagolysosome, which is capable of degrading particles or apoptotic cells (Desjardins et al., 1994; Henry et al., 2004; Vieira et al., 2002). In C. elegans, internalized cell corpses are enclosed by the phagosome, which may undergo a similar maturation process. Although phagosome composition and maturation have been extensively studied in mammalian cells using latex-bead-containing phagosomes (Garin et al., 2001; Stuart et al., 2007), the formation and maturation of the phagosome that lead to the degradation of apoptotic cells in vivo, remain poorly understood.

In the present study, we have identified C. elegans UNC-108 as a novel component involved in the degradation of apoptotic cells. Both loss-of-function by RNA interference (RNAi) and a gain-of-function mutant of unc-108, sm237, resulted in accumulation of cell corpses. Furthermore, we showed that cell corpses persisting in the unc-108(sm237) mutant or unc-108(RNAi) animal are internalized, but not degraded. UNC-108 co-localizes with the endolysosomal markers RAB-5, RAB-7 and LMP-1 to the phagosome in C. elegans embryos. We also present evidence that unc-108 is required for endosomal trafficking, affecting the transition from the early to the late endosome, the recycling endosome and the maturation of lysosome. Our results suggest that UNC-108 promotes cell corpse degradation, possibly by

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Accepted 18 January 2008

mediating phagosome maturation, and is a novel component crucial for the post-engulfment/cell corpse degradation process in *C. elegans*.

**MATERIALS AND METHODS**

**C. elegans strains**

Strains of *C. elegans* were cultured at 20°C using standard procedures (Brenner, 1974). The N2 Bristol strain was used as the wild-type strain, except for polymorphism mapping that used Hawaiian strain CB4856.

Mutations used are described in *C. elegans* II (Riddle et al., 1997) unless otherwise indicated. Linkage group I (LGI): dpv-5(e61), unc-29(e403), unc-11(e477), cdIs141(e1735), unc-12(n3261) (Zhou et al., 2001), n301, n777, hT2(bli-4(e937)let-7;+ q782)qas48/CED-1(e2406) and ok1426 (Wormbase: www.wormbase.org), sm237 (this study). LGII: cd-6(n2095), ced-7(n2094), LGIII: ced-3(n717), ced-10(n1162). LGIV: ced-1(n1994), ced-5(n812), ced-10(n3246).

The following strains carrying integrated transgenes were kindly provided by Dr Hanna Fares: bls34 (RME-8::GFP) (Zhang et al., 2001); cdi373 (RME-mrFP) (Treich et al., 2004); cdi540 (pcc1::GFP; pcc5 I) (Treich et al., 2004); cdi597 (pcc1::mCHERRY::CUP-5; cdi 839 [pcc1::RME-1(271a1)] (Poteryaev et al., 2007); bks06 (GFP::RME-1::P-rf4) (Grant et al., 2001); cdi141 (pcc1::mCHERRY::RAB-7); and cdi113 (pcc::mCHERRY::RAB-5).

Other endodorsks markers used were: arl37 (P_mnuC,800::GFP) (Fares and Greenwald, 2001a); pwi50 (LMP-1::GFP) (Treich et al., 2004); and bls1 (VIT-2::GFP) (Grant and Hirsh, 1999).

**Mapping and cloning of unc-108**

sm237 was mapped very close to unc-11 on the left arm of linkage group I. From unc-11 dpv-5/sm237 mothers, 60 of 77 Dpy non-unc recombinants contained sm237, whereas 0 of 44 Unc non-Dpy recombinants contained sm237. We then performed single nucleotide polymorphism (SNP) mapping to locate sm237 between SNP markers Ytk1476A (−3.21) and smp-R12E2 (−1.64). Transformation rescue experiments showed that one fosmid in this region, WRMO636aD05, rescued the persistent cell corpse phenotype of the sm237 mutant. Several deletion clones of WRM0636aD05 were generated and one subclone that contains the Mhul-SaclI fragment of WRM0636aD05 possessed the rescue activity. Only one intact open reading frame, F54F10.4, was found in this region, which corresponds to a previously identified gene, unc-108. We determined the sequence of unc-108 in the sm237 mutant and identified a missense mutation that caused the substitution of Gly18 with a residue.

**Quantification of cell corpses**

The number of somatic cell corpses in the head region of living embryos or L1 larvae and the number of germ cell corpses in one gonad arm from animals at various adult ages were scored using Nomarski optics as described (Gumieny et al., 1999; Wang et al., 2002).

**unc-108 RNAi**

Sense and antisense RNA were in vitro transcribed from the T7- and SP6-flanked PCR template (unc-108 cDNA nucleotides 5-599) using RiboMAX Large Scale RNA Production System (Promega, USA). Double-stranded RNA (dsRNA) was generated by annealing the sense and antisense RNA for 10 minutes at 65°C, followed by incubating at 37°C for 15 minutes. dsRNA of unc-108 (550 ng/ml) was injected into the gonad or the body cavity of wild-type animals at various adult ages were scored using Nomarski optics as described (Hersh et al., 2002) with a few modifications. Briefly, embryos were collected from the gravid adults treated with 1.6 M NaOH/12% hyochlorite until dissolved. Embryos were washed several times in M9 buffer and then incubated in 50 µg/ml AO in M9 buffer for 1 hour before observation by epifluorescence microscopy. For AO staining in germ cells, aged adults were soaked in 50 µg/ml AO in M9 buffer for 2 hours and recovered on OP50-seeded plates for 3 hours before observation.

**Endocytosis assay**

In vivo pulse-chase experiments were performed as described (Zhang et al., 2001). Briefly, Texas Red-conjugated BSA (TR-BSA; Sigma, USA) was injected at 1 mg/ml into the body cavity in the pharyngeal region. Injected worms were transferred to a seeded NGM plate at room temperature and the coelomocyte uptake was monitored at different time points (5, 10, 15, 20, 30 and 60 minutes; 6, 12 and 24 hours). At each time point, the injected worms were transferred to an ice-cold NGM plate to stop the intracellular trafficking of endocytosed molecules before examination by epifluorescence microscopy.

The apical uptake of fluid-phase material in the intestine was analyzed by soaking L4/young adults in 1 mg/ml TR-BSA in M9 buffer for 8 hours in the dark at room temperature. Animals were recovered on a seeded NGM plate for 2 hours before observation. For examining the apical uptake of lipophilic dye in the intestine, L4/young adults were soaked in 40 µM FM4-64 (Invitrogen, USA) for 30 minutes in the dark at room temperature and recovered on a seeded NGM plate for 30 minutes before observation. The basolateral uptake in the intestine was analyzed by injecting 1 mg/ml TR-BSA or 40 µM FM4-64 into the body cavity. The injected worms were transferred to a seeded NGM plate and recovered at room temperature for 30 minutes before observation.

**Plasmid construction**

To construct Punc-108::gfp and Punc-108::mcherry, we inserted a 4 kb fragment containing the genomic sequence of the unc-108 gene including 2 kb promoter region into the pPD95.77 or pPD95.77-mcherry vector (generated from pPD95.77 by replacing the gfp fragment with mcherry) via its Sphl BamHI sites. To construct Punc-108::unc-108 and Pegl::unc-108, the full-length unc-108 cDNA was amplified from a *C. elegans* cDNA library (Invitrogen, USA) and cloned into the Pcd-7 vector via its KpnI site or into Pegl through its Newf-NcoI sites. To generate Punc-108::unc-108, we first amplified a fragment containing the 2 kb DNA region upstream of the start codon of the unc-108 gene and cloned it into a pPD49.26 vector via its Sphl BamHI sites to create the construct punc-108. The full-length cDNA of unc-108 was then cloned to Punc-108 at the Newf-EcoRV sites to generate Punc-108::unc-108. To construct the endosomal and lysosomal markers driven by the ced-1 promoter, the mcherry fragment was amplified from plasmid PPD95.77-mcherry and cloned into the pPD49.26 vector through its Newf-KpnI sites to yield pPD49.26-mcherry. The full-length genomic sequence of the *rab-5* and *rab-7* genes were then amplified using N2 genomic DNA as a template.
unc-108 promotes cell corpse engulfment

RESULTS

sm237 is a new allele of unc-108 that contains many persistent cell corpses

The sm237 mutant was isolated from a psr-1 enhancer screen (X.W. and D. Xue, unpublished), but its phenotype is not dependent on or enhanced by the psr-1 deletion mutant tm469 (data not shown). The sm237 animal contains many persistent cell corpses at late embryonic stages that would normally have very few cell corpses (Fig. 1A). The appearance of cell corpses in the sm237 mutant is totally blocked by strong loss-of-function mutations in the ced-3 and ced-4 genes that are required for almost all apoptosis in C. elegans, indicating that the persistent cell corpses observed in sm237 are indeed apoptotic cells (data not shown). We cloned the gene affected by sm237 and found that it corresponds to a previously identified gene, unc-108 (see Materials and methods; see Fig. S1A in the supplementary material). unc-108 encodes a small GTPase that shares high sequence homology with human RAB2 (RAB2A) (87% sequence identity and 93% similarity; see Fig. S1B in the supplementary material), a member of the Ras GTPase superfamily (see Fig. S1B in the supplementary material) (Pereira-Leal and Seabra, 2000). We determined the sequence of unc-108 in the sm237 mutant and identified a missense mutation that results in substitution of Gly18 with Glu (G18E), which affects a conserved nucleotide-binding motif (PM1) present in all members of the Ras GTPase superfamily (see Fig. S1B in the supplementary material) (Pereira-Leal and Seabra, 2000). Expression of the full-length unc-108 cDNA under the control of its own promoter (Punc-108::unc-108) efficiently rescued the persistent cell corpse phenotype of the sm237 mutant (see Fig. S1A in the supplementary material). Expression of mouse Rab2 cDNA driven by heat-shock promoters (Psupmrab2) or the ced-1 promoter (Pced::mrab2) also rescued the corpse phenotype of the sm237 mutant (see Fig. S1A in the supplementary material), indicating that mouse Rab2 can substitute for UNC-108 in removing apoptotic cells in C. elegans.

The unc-108(sm237) mutant is defective in cell corpse removal

To determine whether accumulation of cell corpses in sm237 animals is due to a defect in cell corpse clearance, we performed a time-course analysis of cell corpse appearance during development.
(Wang et al., 2003). In both somatic and germ cells, significantly higher numbers of cell corpses were observed in the sm237 mutant than in wild-type animals at all developmental stages (Fig. 1A,B). To confirm that the increase in cell corpses in sm237 is caused by a defect in cell corpse removal, we performed 4D microscopy analysis to measure the duration of embryonic cell corpses in sm237 animals (Wang et al., 2003). In wild-type animals, the majority of the cell corpses persisted from 10 to 50 minutes, whereas in the unc-108(sm237) mutant most cell corpses persisted from 30 to 110 minutes (Fig. 1C). On average, the duration of cell corpses in unc-108(sm237) embryos was 93% longer than in wild-type embryos (Fig. 1C), indicating that the removal of apoptotic cells is defective in the unc-108(sm237) mutant.

### sm237 represents a gain-of-function allele of unc-108

sm237 animals are viable but display a dominant Unc (uncoordinated) phenotype, which is consistent with the previous characterization of the unc-108 gene (Park and Horvitz, 1986). Different from its dominant Unc phenotype, we found that the persistent cell corpse phenotype of sm237 is semi-dominant and shows a maternal effect: sm237 homozygous embryos produced by sm237/+ heterozygous mothers showed a weak Ced (cell death abnormal) phenotype equivalent to that of the mother (sm237/+), which was weaker than that of the sm237/sm237 embryos produced by the homozygous mothers (Table 1). The weak Ced phenotype observed in sm237/+ embryos from the heterozygous mother could be explained by the gain-of-function nature of sm237 or haploid insufficiency of sm237. To distinguish between these two possibilities, we examined an unc-108 deletion mutant (ok1246), which contains a 2198 bp deletion that removes the whole gene locus and represents a null allele of unc-108 (Wormbase: www.wormbase.org; see Fig. S2A in the supplementary material).

Most homozygous ok1246 embryos from the heterozygous mother (hT2/ok1246) appeared to develop normally during embryogenesis, but failed to hatch or were arrested at early larval stage, indicating that UNC-108 is essential for *C. elegans* development. However, no obvious Ced phenotype was observed either in hT2/ok1246 animals or their ok1246 progeny, suggesting that sm237 is likely to be a gain-of-function allele (Table 1). Furthermore, the Ced phenotype in sm237/ok1246 embryos was weaker than that of sm237/sm237 embryos, but stronger than that of sm237/+ embryos (Table 1), indicating that sm237 indeed represents a gain-of-function allele of unc-108 and that the wild-type unc-108 activity antagonizes the unc-108(sm237) allele. This result is also consistent with the finding that overexpression of wild-type unc-108 was able to rescue the persistent cell corpse phenotype of sm237 animals and that wild-type gene product contributed maternally was able to partially rescue the Ced phenotype of the homozygous sm237 progeny (see Fig. S1A in the supplementary material; Table 1).

To confirm this result, we overexpressed the UNC-108(G18E) mutant product in wild-type animals using *C. elegans* heat-shock promoters [P~het:UNC-108::G18E] and found that UNC-108(G18E) resulted in a similar, albeit slightly weaker, corpse phenotype to that of the sm237 mutant (see Fig. S2C in the supplementary material). Since ok1246 larvae do not survive, we could not examine their progeny for the Ced phenotype and therefore cannot rule out the possibility that the maternal contribution of the wild-type allele is sufficient to mediate the normal clearance of cell corpses that we observed in ok1246 embryos. To determine whether a loss-of-function mutation in the unc-108 gene affects cell corpse removal, we treated wild-type animals with unc-108 RNAi and examined the persistent cell corpse phenotype in the progeny. Indeed, we found that unc-108 RNAi caused similar phenotypes to that of the sm237 mutant in both somatic and germ cells, indicating that the wild-type unc-108 functions to promote cell corpse clearance (Fig. 1A-C). Two other alleles of unc-108 (n501 and n777) isolated previously by dominant Unc phenotype also displayed a weak Ced phenotype (see Fig. S2B in the supplementary material) (Park and Horvitz, 1986).

### UNC-108 is expressed and functions in the engulfing cells to promote cell corpse removal

To examine the expression pattern of unc-108, we generated UNC-108 translational GFP fusions under the control of its own promoter [P~unc-108::gfp] [UNC-108::GFP] and P~unc-108::gfp::unc-108 (GFP::UNC-108)], which partially rescued the persistent cell corpse phenotype of sm237 animals (see Fig. S1A in the supplementary material). The expression of unc-108::gfp was ubiquitously expressed in the embryo, starting from the very early stage of 50 to 100 cells and throughout the larval and adult stages. The expression of unc-108::gfp was observed in engulfing cells, such as hypodermal cells, intestine cells and gonadal sheath cells (see Fig. S3A in the supplementary material; data not shown). unc-108::gfp was also seen in many head and tail neurons as well as ventral cord neurons (see Fig. S3A in the supplementary material). Interestingly, unc-108 is also expressed in the coelomocytes, the scavengers in *C. elegans* that constantly uptake macromolecules from the body cavity (see Fig. S3A in the supplementary material). This expression pattern is consistent with the function of UNC-108 in endosomal trafficking (see below). Similar expression patterns with more-vesicular localizations were observed in animals expressing GFP:UNC-108 fusion protein (see Fig. S3B in the supplementary material).

To determine whether UNC-108 activity is required in the engulfing cells or dying cells for cell corpse removal, we expressed unc-108 under the control of the ced-1 promoter (P~ced-1::) or egl-1 promoter (P~egl-1::), which drives gene expression specifically in the engulfing cells or dying cells, respectively (Conradt and Horvitz, 1998; Zhou et al., 2001b), and examined whether expression of unc-108 in these cells rescued the persistent cell corpse phenotype of the sm237 mutant. Expression of unc-108 in engulfing cells (P~ced-1::unc-108), but not in dying cells (P~egl-1::unc-108), rescued the cell corpse's viability.
unc-108 promotes cell corpse engulfment

**Abnormal endosomal compartments in unc-108(sm237) mutant coelomocytes**

The involvement of human RAB2 in vesicular trafficking (Tisdale, 1999; Tisdale and Balch, 1996; Tisdale et al., 1992) and the coelomocyte localization of UNC-108 prompted us to investigate whether unc-108 affects endocytosis in *C. elegans*. In adult hermaphrodites, there are six coelomocytes acting as scavenger cells to take up macromolecules from pseudocoelom. We first examined whether coelomocyte uptake is affected in the unc-108(sm237) mutant using the Cup assay (coelomocyte uptake) (Fares and Greenwald, 2001a). We introduced arIs37 [pmyo-3::ssGFP] into the unc-108(sm237) mutant and examined the uptake of secreted soluble GFP (ssGFP) by the coelomocytes. Compared with efficient uptake of ssGFP by coelomocytes of wild-type animals, the initial uptake of ssGFP by the coelomocytes of unc-108(sm237) animals decreased and GFP accumulated in the body cavity (Fig. 2A). Moreover, unlike the GFP pattern in wild-type coelomocytes, internalized GFP was present in the enlarged vacuoles in unc-108(sm237) animals (Fig. 2A). To further investigate the possible cause of this defect, we examined whether the coelomocytes in unc-108(sm237) animals contained normal endosomal and lysosomal compartments using different markers fused with GFP. RME-8::GFP marks endosome membrane, displaying many ring-like structures representative of endosomes in wild-type coelomocyte (Zhang et al., 2001). We observed three different patterns of RME-8::GFP in the coelomocytes of sm237 animals. Twenty percent of coelomocytes in unc-108(sm237) animals contained normal endosomes that showed a similar RME-8::GFP pattern to that in wild type. Forty percent of coelomocytes contained enlarged vacuoles that were labeled by RME-8::GFP, half of which were so large that they almost occupied the whole coelomocyte. Another 40% of coelomocytes showed a punctate pattern of RME-8::GFP instead of the normal ring structure, and this did not correlate with the age of hermaphrodites (Fig. 2B; data not shown). Moreover, we saw very few endosomes of normal morphology in this type of coelomocyte (Fig. 2B). These data suggest that sm237 animals contain both damaged and enlarged endosomes.

To confirm the identity of the large vacuole, we introduced LMP-1::GFP, an early lysosome maker into sm237 animals (Treusch et al., 2004). To our surprise, these large vacuoles were also marked by LMP-1::GFP, suggesting that they might represent aberrant hybrids of endosome and lysosome (Fig. 2C). In addition, only a few normal lysosomes with LMP-1::GFP were found in the coelomocytes of sm237 mutant (Fig. 2C; Fig. 4D). Other endosomal and lysosomal...
markers, such as RAB-7, which associates with late endosome and lysosome, and CUP-5, a lysosomal component, were also found to be associated with the large vacuole (Poteryaev et al., 2007; Treusch et al., 2004) (see Fig. S4A,B in the supplementary material). To further confirm this result, we introduced RME-8:eGFP and LMP-1::GFP or RME-8::GFP and mCherry::CUP-5 simultaneously into the sm237 mutant and found that these markers co-localized to the enlarged vacuoles, rather than localizing separately to endosomes or lysosomes as in the wild-type coelomocytes (see Fig. S4C,D in the supplementary material), indicating that the enlarged vacuoles in the sm237 mutant represent hybrids of endosome and lysosome.

Taken together, our data showed that sm237 animals contained damaged endosomes and enlarged vacuoles with both endosomal and lysosomal components, suggesting that unc-108 is involved in both an early step of endosomal trafficking and in lysosome formation from late endosome. To further investigate whether sm237 affects an early step of endosomal transport, we examined the localization of GFP::RME-1, an EH-domain-containing ATPase associated with recycling endosomes (Lin et al., 2001). In wild-type coelomocytes, RME-1 was mostly found in close proximity to the plasma membrane (Fig. 2D). By contrast, this pattern was disrupted in the coelomocytes of sm237 animals as GFP::RME-1 was often found around the endosomes (Fig. 2D, arrow). We also checked the pattern of early endosome-associated RAB-5 (Pfeffer and Aivazian, 2004; Poteryaev et al., 2007). Many coelomocytes of sm237 animals showed a normal GFP::RAB-5 pattern, except for those that contained no other compartments but one large vacuole that was labeled by GFP::RAB-5 (data not shown). Therefore, we conclude that unc-108 functions in both early and late steps of endosomal trafficking, affecting the transition from early to late endosome, the recycling endosomes and the late endosome to lysosome transition. Consistent with this result, using early endosome marker mCherry::RAB-5 and lysosome-associated mCherry::CUP-5, we found that GFP::UNC-108 localized to both endosomes and lysosomes (Fig. 3).

### Lysosome maturation is affected in the unc-108(sm237) mutant

In order to examine the endosomal trafficking defect of sm237 animals with higher temporal resolution, we performed in vivo pulse-chase analysis of endocytosis by injecting TR-BSA (Texas Red-conjugated BSA) into the body cavity of adult hermaphrodites and examined the uptake of TR-BSA into the coelomocytes in both wild-type and sm237 animals carrying different endosomal/lysosomal markers. In wild-type animals, 5 minutes after injection, TR-BSA started to appear in the endosomes labeled by RME-8::GFP. After 15 minutes, a significant amount of TR-BSA left the RME-8::GFP ring, and after 30 minutes most of the TR-BSA was present in the lysosomes lacking RME-8::GFP (Fig. 4A). In the sm237 mutant, however, TR-BSA appeared in the RME-8::GFP-labeled compartment 5 minutes after injection and stayed there throughout the time-course of the experiment (Fig. 4B; see Materials and methods; data not shown). We also monitored the uptake of TR-BSA using the early lysosomal marker LMP-1::GFP, and found that TR-BSA started to accumulate in the compartments lacking LMP-1::GFP 5 minutes after injection. After 15 minutes, TR-BSA appeared in the lysosomes marked by LMP-1::GFP (Fig. 4C). By contrast, 5 minutes after injection, TR-BSA accumulated in the vacuole marked by LMP-1::GFP in the sm237 mutant (Fig. 4D). During the remainder of the time points, most TR-BSA stayed within the vacuole or enlarged endosomes that were labeled by LMP-1::GFP and failed to move out even at 24 hours post-injection (Fig. 4D; data not shown). Therefore, our pulse-chase experiments showed that lysosome biogenesis was severely affected in the sm237 mutant, suggesting that UNC-108 is required for the formation of lysosome from late endosome.

### Yolk protein trafficking and apical uptake in the intestine are blocked in unc-108(sm237) animals

In C. elegans, yolk uptake by growing oocytes presents a typical example of receptor-mediated endocytosis (Grant and Hirsh, 1999). Using a VIT-2::GFP reporter (Grant and Hirsh, 1999), we examined whether sm237 affects yolk uptake by oocytes. We did not observe any defect of initial uptake of yolk protein in unc-108(sm237) oocytes (Fig. 5A). Consistently, the localization of GFP::RME-1 was also normal in the oocytes of sm237 animals (data not shown). However, the redistribution of yolk protein to gut primordium in the embryo or to the intestine in larva was blocked in the mutant (Fig. 5B,C; data not shown). These results indicate that UNC-108 is not required for the initial uptake step of receptor-mediated endocytosis in developing oocytes, but is involved in the resecretion and trafficking of the yolk protein. A similar yolk redistribution defect has been observed previously in rab-7(RNAi) animals and in the sand-1 mutant, which might suggest that the yolk needs to reach the late endosomal compartment for its later resecretion (Grant and Hirsh, 1999; Poteryaev et al., 2007). Therefore, the yolk redistribution defect that we observed in sm237 animals could be due to the disruption of UNC-108 function in the late step of endosomal trafficking. To test whether UNC-108 is required for endocytosis in the intestine, animals were fed with TR-BSA (fluid-phase material) or with the lipophilic dye FM4-64, and the apical...
(luminal) uptake of the dyes was assayed. Both TR-BSA and FM4-64 were quickly taken up from the lumen by the intestinal cells in wild-type animals (see Fig. S5 in the supplementary material). However, in sm237 animals, most of the TR-BSA or FM4-64 accumulated in the intestinal lumen, indicating that the apical uptake was mostly blocked (see Fig. S5 in the supplementary material). We did not observe any obvious defect in sm237 animals when both markers were delivered basolaterally (data not shown).

Fig. 4. Endocytic trafficking in the coelomocytes of the unc-108(sm237) mutant is blocked from late endosome to lysosome. (A-D) TR-BSA was injected into the body cavity and its transport through endocytic compartments is shown over time in wild-type (A,C) and unc-108(sm237) mutant (B,D) animals with endosomal marker RME-8::GFP (A,B) or lysosomal marker LMP-1::GFP (C,D). White arrows point to the compartments that contain TR-BSA. The blue arrow in D indicates the normally sized lysosome that lacks TR-BSA. Scale bars: 2.5 μm.
Loss-of-function of unc-108 causes similar endocytosis defects to those of sm237

The data shown above indicate that sm237 affects the transition from early to late endosome, recycling endosomes as well as lysosome biogenesis in coelomocytes, and yolk protein trafficking and apical uptake in the intestine. Since sm237 represents a gain-of-function allele of unc-108, we determined whether loss-of-function of unc-108 caused by RNAi affects endocytosis. We found that treatment with unc-108 RNAi caused similar endocytosis defects to those of the sm237 mutant. First, in 83% of animals treated with unc-108 RNAi, the uptake of ssGFP was affected, among which 75% failed to uptake any ssGFP, a more severe phenotype than that of the sm237 mutant (Fig. 2A). Second, the abnormal endosomal compartments were observed in 76% of coelomocytes after RNAi treatment, including enlarged vacuoles containing both endosome and lysosome components as revealed by labeling with endolysosomal markers RME-8, LMP-1 and CUP-5 (60%), and damaged endosomes as indicated by the punctate pattern of RME-8::GFP (16%) (Fig. 2B,C; see Fig. S4C,D in the supplementary material). These distorted endosomal compartments were also found in the coelomocytes of sm237 mutants, but at slightly different frequency (40% each). Third, we found similar mislocalization of RME-1::GFP around endosomes in animals treated with unc-108 RNAi (Fig. 2D). Fourth, the endosomal transport was also carefully examined in pulse-chase experiments after RNAi treatment. We found that most TR-BSA was trapped within the endosomes for up to 12 hours after injection, whereas in the wild-type coelomocytes it was transported to lysosomes within 15 to 30 minutes post-injection (see Fig. S6 in the supplementary material; Fig. 4C; data not shown). However, this blockage was not as complete as that in the sm237 mutant in which TR-BSA stayed inside the endosomes even at 24 hours post-injection. Fifth, the resecretion and trafficking of yolk protein was totally blocked in the embryos or larvae after RNAi treatment, whereas the uptake of yolk protein was not affected (Fig. 5D; data not shown). Finally, the unc-108 RNAi-treated animals showed similar apical uptake defects in the intestine to the sm237 mutants (data not shown). Taken together, our data showed that loss of unc-108 function caused various endocytosis defects that were similar to those of the gain-of-function allele, sm237, demonstrating that the wild-type unc-108 activity is required for endocytosis and is likely to act in both early and late steps of endosomal trafficking.

unc-108 affects the degradation of cell corpses

Our data indicate that unc-108 plays an important role in endocytosis. We next examined the role of UNC-108 in cell corpse clearance. We first examined whether cell corpses accumulating in the unc-108(sm237) mutant or in animals treated with unc-108 RNAi were internalized, using Acridine Orange (AO), which preferentially stains engulfed apoptotic cells (Gumienny et al., 1999; Lettre et al., 2004). Similar to that in wild-type animals, both persistent somatic cell corpses and germ cell corpses in the sm237 mutant or animals treated with unc-108 RNAi could be labeled by AO (Fig. 6A-C; data not shown). By contrast, the persistent cell corpses in the ced-1(e1735) or ced-12(n3261) mutant failed to be internalized and were not stained (Gumienny et al., 1999; Lettre et al., 2004; Zhou et al., 2001a; Zhou et al., 2001b) (Fig. 6D; data not shown). These data suggest that the persistent cell corpses in sm237 mutant or unc-108(RNAi) animals were internalized but not degraded. Thus, unc-108 is likely to affect the degradation rather than the internalization of cell corpses.

unc-108 functions downstream of the engulfment pathway to promote cell corpse degradation

The cell corpse degradation process is compromised in the sm237 mutant and in unc-108(RNAi) animals. Several genes have been described previously that act in two partially redundant pathways to regulate cell corpse engulfment in C. elegans (Reddien and Horvitz, 2004; Wang et al., 2003; Yu et al., 2006). We analyzed double mutants between sm237 and strong loss-of-function
mutations in several other genes acting in the two cell-corpse engulfment pathways (ced-1, ced-6, ced-7 in one pathway, and ced-2, ced-5, ced-10 and ced-12 in the other) and found that sm237 does not significantly affect or enhance the engulfment defect of mutants in either pathway (data not shown). Similar results were obtained with unc-108RNAi treatment (data not shown), suggesting that unc-108 does not act in a specific pathway and might function downstream of both engulfment pathways to promote cell corpse degradation.

UNC-108 co-localizes to phagosomes with RAB-5, RAB-7 and LMP-1

UNC-108 is expressed and required in engulfing cells to promote cell corpse removal. To further investigate its function in cell corpse degradation, we examined whether UNC-108 associates with phagosomes that contain internalized cell corpses. CED-1 is a phagocytic receptor and CED-1::GFP localizes to the extending pseudopods and nascent phagosomes (Yu et al., 2006; Zhou et al., 2001b). In unc-108(sm237) mutant or unc-108(RNAi) animals, the clustering of CED-1::GFP around the cell corpse was not affected (data not shown), which is consistent with our finding that UNC-108 is not required for the internalization of cell corpses. To find out whether UNC-108 associates with phagosomes, we first checked if it clusters around cell corpses and co-localizes with CED-1::GFP to the extending pseudopods or nascent phagosomes. In wild-type embryos carrying P_{unc-108}::unc-108::gfp, strong GFP signal was seen surrounding the cell corpses (Fig. 7A), indicating that UNC-108 might associate with phagosomes. Similar phagosome localization was observed in embryos expressing N-terminally GFP-tagged UNC-108 (GFP::UNC-108) (see Fig. S7A in the supplementary material).

We next examined embryos expressing both P_{unc-108}::unc-108::mcherry and P_{ced-1}::ced-1::gfp and found that both UNC-108::mCHERRY and CED-1::GFP clustered around cell corpses, but we could barely detect any co-localization of these two proteins around dying cells. As a phagocytic receptor, the localization of CED-1 on phagosomes is transient and it disappears long before the complete degradation of cell corpses (Yu et al., 2006). Since UNC-108 is likely to be involved in the degradation of cell corpses, one possible explanation is that UNC-108 is recruited to phagosomes after CED-1 completes its task and disappears. To test this hypothesis, we followed the recruitment of CED-1 and UNC-108 to phagosomes in embryos expressing both P_{unc-108}::unc-108::mcherry and P_{ced-1}::ced-1::gfp by time-lapse recording. Consistent with our hypothesis, we found that CED-1::GFP and UNC-108::mCHERRY were recruited to the phagosomes at different times during the engulfment process. We set the time point as 0 min when a clear CED-1::GFP ring was seen. At +5 minutes, CED-1::GFP formed a bright ring around the cell corpse, whereas UNC-108::mCHERRY was not seen (Fig. 7Ba-c). At +8 minutes, CED-1::GFP became weaker and the UNC-108::mCHERRY signal started to appear (Fig. 7Bd-f). At +11 minutes, almost no CED-1::GFP could be detected whereas the UNC-108::mCHERRY formed a clear circle around the cell corpse (Fig. 7Bj-l). The UNC-108::mCHERRY signal could still be detected at +26 minutes when the ‘button-like’ morphology of the cell corpse was lost (Fig. 7Bm-o). UNC-108::mCHERRY eventually disappeared at +29 minutes (data not shown). These data indicate that UNC-108 is recruited to the same engulfment site as CED-1 and its association with the phagosome is preceded by that of CED-1 and lasts until the degradation of cell corpses. Similar phagosome recruitment kinetics were observed with the N-terminally tagged UNC-108 (mCHERRY::UNC-108) (see Fig. S7B in the supplementary material).

To investigate the potential function of UNC-108 in phagosome maturation, we examined whether UNC-108 co-localizes with several other phagosome-associated proteins that function at different phagosome maturation stages in mammals. Rab5 is an early endosome marker and has been shown to be associated with the phagosome and to play an important role in phagosome maturation in mammals and fruit flies (Desjardins et al., 1994; Henry et al., 2004; Stuart et al., 2007; Vieira et al., 2002). Rab7, a late endosome component, is recruited to the phagosome by Rab5 and mediates the fusion of phagosome with lysosome (Henry et al., 2004; Vieira et al., 2003). Lysosomal protein LAMP1 (vertebrate ortholog of C. elegans LMP-1) was also found to be associated with the phagosome and functions in mediating phagosome maturation (Garin et al., 2001). In wild-type embryos transgenic for P_{ced-1}::mcherry::rab-5 and P_{unc-108}::unc-108::gfp, we found that mCHERRY::RAB-5 and UNC-108::GFP co-localized to the phagosome, forming a ring-like structure around the cell corpse (Fig. 7Ca-d). Similar phagosome co-localization was observed in embryos expressing P_{ced-1}::mcherry::rab-7 and P_{unc-108}::unc-108::gfp or P_{ced-1}::mcherry::rab-5 and P_{unc-108}::unc-108::gfp, as well as in
animals expressing N-terminally GFP-tagged UNC-108 (GFP::UNC-108) (Fig. 7Ce-h,i-l; see Fig. S7C in the supplementary material). Since Rab5 is recruited to the phagosome at a very early stage and LAMP1 is likely to be involved in the late step of generating the phagolysosome in mammals (Vieira et al., 2002), the co-localization of UNC-108 with both of these markers on the phagosome suggests that UNC-108 might function in both early and late stages of phagosome maturation.

**DISCUSSION**

**UNC-108 may promote phagosome maturation required for cell corpse degradation**

Phagosome maturation is a dynamic process that involves a series of interactions among endocytic compartments, which eventually fuse with lysosomes to generate phagolysosomes that possess degradative properties (Henry et al., 2004; Vieira et al., 2002; Vieira et al., 2003). In many ways, this maturation process resembles the
progression of endocytic compartments, which undergo a series of fissions and fusions to modify membrane composition and acquire new contents (Vieira et al., 2002). Our data indicate that unc-108 is required for both endocytosis and cell corpse degradation in C. elegans, suggesting that there might be an intrinsic connection between these two processes. UNC-108 affects the degradation of cell corpses, associates with the phagosomes containing internalized cell corpses, and co-localizes with early endosome protein RAB-5, late endosomal component RAB-7, and lysosomal protein LMP-1. Since UNC-108 localizes to both endosomes and lysosomes and functions in both early and late steps of endosomal trafficking, it is possible that UNC-108 is recruited to the phagosomes during its fusion with early endosome and regulates phagosome maturation.

The gain-of-function allele, sm237, has a missense mutation that changes Gly18 to Glu (G18E) within the PM1 motif (GxxxxGKs, mutation underlined) that is required for the binding of phosphate and Mg2+ and is conserved in all Ras small GTPase superfamily members (Valencia et al., 1991). Structural and biochemical studies indicate that mutations in this motif may affect the catalytic activity of GTPase (Pai et al., 1989; Reinstein et al., 1990). Therefore, G18E mutant protein might possess less GTPase activity and stay in the active GTP-bound form that binds to the effector protein. The persistent interaction of UNC-108(G18E) with downstream effectors might block phagosome maturation at a certain intermediate stage and affect the degradation of cell corpses. Overexpression of wild-type UNC-108 might increase the chance of interaction between wild-type UNC-108 and its effectors, which would promote normal degradation of apoptotic cells. This competition between wild-type UNC-108 and G18E mutant in binding to effector proteins might explain the variable rescuing activities we observed with different unc-108 transgenes, which are likely to carry different copy numbers of wild-type unc-108. In line with this competition model, we found that overexpression of the UNC-108(G18E) mutant in wild-type embryos indeed resulted in a similar persistent cell corpse phenotype to that of the sm237 mutant. Further experiments need to be undertaken to understand the biochemical features of the UNC-108(G18E) protein and test the above competition hypothesis.

**UNC-108 regulates endosomal trafficking at different steps in C. elegans**

Human RAB2 has been implicated in Golgi-ER retrograde transport (Stenmark and Olkkonen, 2001), but the mechanism by which RAB2 controls this transport is unknown. In addition, it is not clear whether RAB2 is involved in other aspects of endocytosis or vesicle trafficking. In the present study, we identified a gain-of-function allele of unc-108, sm237, that affects the uptake of ssGFP by coelomocytes, transition from early to late endosomes, recycling endosomes, lysosome formation, yolk protein trafficking and the apical uptake in the intestine. These data demonstrate that the wild-type unc-108 activity is required for endocytosis and it is likely to regulate endosomal trafficking at different steps, including the progression from early to late endosome, cargo recycling and lysosome maturation. Identification of the downstream effector(s) or the regulatory proteins that act together with UNC-108 is needed to understand its exact function at these different steps of endocytosis.

**Rab GTPases function as important regulators in removing apoptotic cells**

Rab proteins are small GTPases that constitute the largest branch of the Ras GTPase superfamily. Rabs have been implicated in almost all types of membrane trafficking and have emerged as central regulators of vesicle budding, docking and fusion with specific target organelles (Mukherjee et al., 1997; Vieira et al., 2002). Several Rabs have been found to associate with phagosomes containing latex beads in mice and fruit flies including Rab1, Rab2, Rab3, Rab4, Rab5, Rab7, Rab11 and Rab14 (Garin et al., 2001; Stuart et al., 2007). However, the phagocytosis of foreign particles and of apoptotic cells involve different phagocytic receptors and elicit different immune responses. Therefore, the involvement of Rabs in regulating apoptotic cell clearance was not firmly established. Our identification and characterization of UNC-108 in mediating cell corpse degradation and the finding that mouse Rab2 can substitute for its function in removing apoptotic cells indicate that Rab proteins are potential regulators of apoptotic cell clearance in vivo and that this function is likely to be conserved in mammals as well. In addition to UNC-108/Rab2, C. elegans Rab5 and Rab7 also localize to the phagosome and an increased number of cell corpses was observed in rab-5(RNAi) or rab-7(RNAi) animals, suggesting that these two Rab GTPases might also be involved in the clearance of apoptotic cells (Fig. 7C; data not shown). Consistent with our findings, recent studies showed that overexpression of Rab5 in NIH3T3 fibroblast cells or bone marrow-derived macrophages promoted the uptake of apoptotic thymocytes, whereas the dominant-negative constructs inhibited it (Nakaya et al., 2006).

We thank Drs Hanna Fares for providing endocytosis markers and A. Fire for vectors; Dr Ding Xue for his critical reading of the manuscript and Drs Chonglin Yang, Hong Zhang and members in our laboratory for helpful discussion and suggestions. The sm237 mutant was isolated in Dr Ding Xue’s laboratory. Some strains used in this work were obtained from the Caenorhabditis Genetic Center (CGC), which is supported by a grant from the NIH. This work was supported by the National High Technology Project 863 from the Ministry of Science and Technology.

**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/6/1069/DC1

**References**


