The SM protein VPS-45 is required for RAB-5-dependent endocytic transport in Caenorhabditis elegans

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Rab5, a small guanosine triphosphatase, is known to regulate the tethering and docking reaction leading to SNARE (soluble NSF attachment protein receptor)-mediated fusion between endosomes. However, it is uncertain how the signal of the activated Rab5 protein is transduced by its downstream effectors during endosome fusion. Here, we show that the Sec1/Munc18 gene vps-45 is essential for not only viability and development but also receptor-mediated and fluid-phase endocytosis pathways in Caenorhabditis elegans. We found that VPS-45 interacts with a Rab5 effector, Rabenosyn-5 (RAB5-S), and the mutants of both vps-45 and rabs-5 show similar endocytic phenotypes. In the macrophage-like cells of vps-45 and rabs-5 mutants, aberrantly small endosomes were accumulated, and the endosome fusion stimulated by the mutant RAB-5 (Q78L) is suppressed by these mutations. Our results indicate that VPS-45 is a key molecule that functions downstream from RAB-5, cooperating with RAB5-S, to regulate the dynamics of the endocytic system in multicellular organisms.

Keywords: vps-45; rabenosyn-5; Sec1/Munc-18; endocytosis; Caenorhabditis elegans

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INTRODUCTION

Endocytosis is an important cellular process in all eukaryotic cells. In endocytic pathways, endosomes undergo continuous fusion and fission for sorting internalized materials (Zerial & McBride, 2001). Membrane fusion is controlled by three main protein families: Rab [GTPase] (guanosine triphosphatase), SNARE (soluble NSF attachment protein receptors) and SM (Sec1/Munc18; Jahn et al, 2003). It is widely accepted that Rab GTPase proteins regulate specific tethering and docking reactions (initial recognition between two membranes) and SNARE proteins execute fusion reactions as the core of the fusion machinery (Jahn et al, 2003). However, the functions of SM proteins have not yet been clarified.

An SM family is a group of hydrophilic proteins of 60–80 kDa, which consist of three domains (Misu et al, 2000). The loss of activity of each SM member leads to severe impairment of specific transport pathways in various species (Toonen & Verhage, 2003). For example, loss of Sly1 blocks the ER-to-Golgi transport, loss of Vps45 and Vps33 blocks the Golgi-to-vacuole transport and loss of Munc18-1 blocks the neurotransmitter release in neuronal cells (Toonen & Verhage, 2003). Intensive studies have shown that SM proteins interact with some syntaxin proteins, which are classified as Qa-SNAREs (Fasshauer et al, 1998; Toonen & Verhage, 2003). However, it is unknown whether SM proteins function primarily by their interaction with the cognate syntaxins, as the mechanisms underlying the interactions are divergent (Dulubova et al, 2003; Gallwitz & Jahn, 2003).

Previous studies using yeast have demonstrated that the SM genes interact genetically with the Rab/Ypt GTPase genes in various transport pathways (Dascher et al, 1991; Tall et al, 1999; Toonen & Verhage, 2003). Although no physical interactions between these families have been found so far, several SM proteins bind directly to Rab effectors (Tall et al, 1999; Coppola et al, 2002). A recent report has suggested that the human SM protein (VPS45)—whose orthologous protein in yeast (Vps45)—is required for a transport pathway from the Golgi to vacuoles (biosynthetic pathway)—binds to the Rab5 effector Rabenosyn-5.
We found that a translational fusion gene (vps-45) is highly homologous to yeast Vps45 and human VPS45 (Bock et al., 2001). We found that a translational fusion gene (vps-45; EGFP) is expressed in all main tissues from the early embryonic stage to adulthood (supplementary Fig 2 online), indicating that the vps-45 exon and is probably a null allele. Homozygous progeny of the vps-45 mutant resemble those of wild-type (WT) worms grown on cholesterol-depleted medium. As cholesterol is endocytosed with yolk proteins into oocytes (Matyash et al., 2001), we tested whether receptor-mediated endocytosis of yolk proteins is defective in vps-45 mutants.

**RESULTS AND DISCUSSION**

**vps-45 mutation causes severe endocytosis defects**

The *C. elegans* genome has a single gene (vps-45) that is highly homologous to yeast Vps45 and human VPS45 (Bock et al., 2001). We found that the receptor-mediated endocytosis-defective (Rme) phenotype—that is, YP170/EGFP is not endocytosed by oocytes at 25°C (Fig 1A). This phenotype was rescued by expression of vps-45 (+) complementary DNA (Fig 1A). To investigate whether defective endocytosis is also observed in other tissues of vps-45 mutant worms, we checked the fluid-phase endocytosis by scavenger cells called coelomocytes. We used Pmyo-3::ssGFP transgenic worms in which GFP expressed in body wall muscles is secreted into the pseudocoelom (Fares & Greenwald, 2001). In the WT background, pseudocoelomic GFP was efficiently endocytosed by coelomocytes and bright fluorescent vesicles were seen in these cells (Fig 1B). Secreted GFP accumulated at a higher temperature at any stage results in developmental arrest. The arrested larvae show intestinal phenotypes such as an enlarged lumen and accumulation of large refractile granules, which are thought to be lysosome-related organelles in the intestine (supplementary Fig 2 online). Electron microscopy showed the presence of multilamellar bodies (MLBs) in the mutant intestine, which are typical structures observed in the cells that accumulate that cholesterol in lysosomes (Lajoie et al., 2005; supplementary Fig 2 online). In addition, the arrested larvae are often encased in the old cuticle (supplementary Fig 2 online). The genetic properties and the molting-defective phenotype in the vps-45 mutant resemble those of wild-type (WT) worms grown on cholesterol-depleted medium. As cholesterol is endocytosed with yolk proteins into oocytes (Matyash et al., 2001), we tested whether receptor-mediated endocytosis of yolk proteins is defective in vps-45 mutants.

Oocyte uptake of yolk proteins was visualized by the vitellogenin::EGFP (YP170/EGFP; EGFP for enhanced green fluorescent protein) reporter (Grant & Hirsh, 1999). We found that the vps-45 mutant exhibits the receptor-mediated endocytosis-defective (Rme) phenotype—that is, YP170/EGFP is not endocytosed by oocytes at 25°C (Fig 1A). This phenotype was rescued by expression of vps-45 (+) complementary DNA (Fig 1A). To investigate whether defective endocytosis is also observed in other tissues of vps-45 mutant worms, we checked the fluid-phase endocytosis by scavenger cells called coelomocytes. We used Pmyo-3::ssGFP transgenic worms in which GFP expressed in body wall muscles is secreted into the pseudocoelom (Fares & Greenwald, 2001). In the WT background, pseudocoelomic GFP was efficiently endocytosed by coelomocytes and bright fluorescent vesicles were seen in these cells (Fig 1B). Secreted GFP accumulated at a higher temperature at any stage results in developmental arrest. The arrested larvae show intestinal phenotypes such as an enlarged lumen and accumulation of large refractile granules, which are thought to be lysosome-related organelles in the intestine (supplementary Fig 2 online). Electron microscopy showed the presence of multilamellar bodies (MLBs) in the mutant intestine, which are typical structures observed in the cells that accumulate that cholesterol in lysosomes (Lajoie et al., 2005; supplementary Fig 2 online). In addition, the arrested larvae are often encased in the old cuticle (supplementary Fig 2 online). The genetic properties and the molting-defective phenotype in the vps-45 mutant resemble those of wild-type (WT) worms grown on cholesterol-depleted medium. As cholesterol is endocytosed with yolk proteins into oocytes (Matyash et al., 2001), we tested whether receptor-mediated endocytosis of yolk proteins is defective in vps-45 mutants.
in the pseudocoelom at 25 °C in the vps-45 mutant (Fig 1B), which is consistent with its phenotype showing defects in coelomocyte uptake (Cup). The Cup phenotype was rescued when vps-45 (+) cDNA was expressed in the vps-45 mutant coelomocytes, indicating a cell-autonomous function of VPS-45 protein (data not shown).

To investigate further which step is defective when endocytosed materials are transported through different endosomal compartments in a vps-45 mutant, we carried out a pulse–chase analysis by using Texas-Red-conjugated BSA (TR–BSA; Zhang et al, 2001). TR–BSA was injected into the pseudocoelom of WT and vps-45 worms expressing an endosomal marker RME-8/GFP (Zhang et al, 2001). TR–BSA was initially present in RME-8/GFP–labelled endosomes in both WT and vps-45 coelomocytes (Fig 2). In the WT, TR–BSA subsequently accumulated into discrete foci, which might represent endocytic intermediates, and finally reached RME-8-negative compartments by 30 min (Zhang et al, 2001; Fig 2A). The RME-8-negative compartments are probably lysosomes, because TR–BSA reached lysosomal-associated membrane protein orthologue (LMP-1)/GFP-labelled compartments at 45 min after injection (supplementary Fig 3 online). By contrast, TR–BSA remained in RME-8-positive endosomes in vps-45 mutant coelomocytes at 30 min after TR–BSA injection, and thus delivery of endocytic cargoes from endosomes to lysosomes is retarded (13 out of 21 coelomocytes; Fig 2B). In some cases (8 out of 21 coelomocytes), we could see little or no uptake of TR–BSA (data not shown; supplementary Fig 3 online). Thus, Rme and Cup phenotypes of the vps-45 mutant and the impairments in uptake of TR–BSA in the vps-45 mutant coelomocytes indicate that VPS-45 might affect a common molecular step in both receptor-mediated endocytosis and fluid-phase endocytosis.

**rabs-5 causes the same endocytic phenotypes as vps-45**

To explain the mechanism of the endocytic function of vps-45, we first analysed the cognate syntaxin genes for vps-45. Vps45 interacts with several syntaxins: Pep12 and Tlg2 in yeast (Burd et al, 1997; Nichols et al, 1998), and Pep12-like syntaxin 13, Tlg2-like syntaxin 16 and syntaxin 4 in mammals (Nielsen et al, 2000; Yamaguchi et al, 2002). We found that C. elegans VPS-45 interacts specifically with SYN-13 (orthologue of syntaxin 13) and SYN-16 (orthologue of syntaxin 16) among all nine syntaxins on the C. elegans genome (Bock et al, 2001; supplementary Fig 4 online). The syn-13(tm2037), syn-16(tm1560) and syn-13(tm2037);syn-16(tm1560) deletion mutants showed no obvious endocytic defects, including Cup and Rme (data not shown; supplementary Fig 5 online).

We next investigated whether vps-45 functions with C. elegans Rabenosyn-5 (rabs-5) in vivo. We found that VPS-45 interacts physically with RABS-5, as reported previously in mammals (Nielsen et al, 2000; Fig 3A). Neither RAB-5 nor early endosomal antigen 1, which is another Rab5 effector with the FYVE domain, interacted with VPS-45 (Fig 3A), suggesting the specificity of interaction between VPS-45 and RABS-5. The rabs-5 mutant worms (tm2036 and ok1513) were indistinguishable from the vps-45 mutant worms in both the ts lethality (data not shown) and the ts endocytic defects (Fig 3B). In addition, defects in rabs-5; vps-45 double mutants were not more severe than the single mutants (data not shown). These results indicate that the VPS-45 and RABS-5 proteins might form a complex and function in the same endocytic pathway.

**VPS-45 is required for endosomal dynamics**

Previous work using in vitro fusion assay has suggested that Rabenosyn-5 might regulate homotypic fusion between endosomes in mammalian cells (Nielsen et al, 2000). To test whether VPS-45 and RABS-5 are involved in regulating endosomal dynamics, we investigated the size of endosomes using the RME-8/GFP endosomal marker in the vps-45 and rabs-5 mutant coelomocytes. We constructed transgenic mosaic strains, which have vps-45 (+) or rabs-5 (+) cDNA on the extrachromosomal (Ex) array in the respective mutant backgrounds. Both vps-45 and rabs-5 mutant coelomocytes, in which Ex arrays are lost (vps-45;Ex--; rabs-5;Ex--), contained aberrantly small RME-8/GFP.
vesicles accumulated in the peripheral regions (Fig 3C,D). These endosomal phenotypes were restored in mutant coelomocytes with Ex arrays (vps-45;Ex+; rabs-5;Ex+; Fig 3C,D). We found that endosomes labelled with another endosomal marker containing 2/C2 FYVE domain (Dang et al., 2004) are also small in the vps-45 mutant coelomocytes (data not shown). However, there was no obvious reduction in the size of LMP-1/GFP-labelled lysosomes (supplementary Fig 3 online), suggesting that the defects of endosomes are specific in the endocytic compartments of vps-45 mutants. The size of endosomes in syn-13, syn-16 and the syn-13;syn-16 mutant coelomocytes was in the normal range (Fig 3C,D; data not shown).

Taken together, the above data suggest that VPS-45 might function in Rab5-dependent fusion between endosomes, probably with RABS-5. To ascertain this, we expressed a GTP-locked, dominant active form of RAB-5 (Q78L), which promotes homotypic endosome fusion in C. elegans (Patton et al., 2005). RAB-5 (Q78L) expression induced enlarged endosomes filled with GFP in WT coelomocytes (Fig 4). However, we rarely detected these enlarged endosomes in both vps-45 and rabs-5 mutant coelomocytes (Fig 4). These results indicate that VPS-45 and RABS-5 proteins are required for Rab5-mediated endosome fusion. By contrast, the endosomal enlargement was not suppressed by the syn-13, syn-16 and syn-13;syn-16 mutations (Fig 4; supplementary Fig 6 online).

Our results provide strong evidence that VPS-45 has a crucial role in the endocytic system of C. elegans, cooperating with RAB-5 effector RABS-5. Specific interactions of VPS-45 with its cognate syntaxins (supplementary Fig 4 online) suggest that these SNAREs might also act in endocytic trafficking. However, unlike severe endocytic phenotypes of both vps-45 and rabs-5 mutants, endocytic events in syn-13;syn-16 double mutants were not affected. To examine whether the other syntaxins are necessary for RAB-5-dependent endocytic transport, we carried out a large endosome assay for all C. elegans syntaxins. We did not detect any depletions in syntaxins that resulted in defects in this assay (supplementary Fig 6 online). The lack of syntaxins might be compensated by the redundant functions of other syntaxins and/or non-syntaxin SNAREs in mutant cells. syn-13 and syn-16 are not essential in endocytic pathways, indicating that the binding of VPS-45 to them might not necessarily be required for the proper function of VPS-45. Consistent with our results, the mutant forms of SM proteins, which abolish the
Monocellular organisms. An additional uncoordinated movement gain the endocytic function in multicellular organisms, as their VPS-45, carrying the EMBO reports. We found that the murine orthologue (VPS45) is functionally functional in yeast (Peng & Gallwitz, 2004; Carpp high-affinity interactions with their cognate syntaxins, are fully mediated by binding to RABS-5 and/or SNAREs other than syntaxins in endocytic pathways. By contrast, studies using mammalian cells also indicated that SM proteins bind directly not only to cognate t-SNAREs and v-SNAREs (Peng & Gallwitz, 2004; Carpp et al, 2006). Further studies with the C elegans, 2003; Williams et al, 2004). They have suggested that the interaction between SM and syntaxin is mediated by binding to RABS-5 and/or carrying only the Pmyo-3::ssGFP array (−RAB-5 (Q78L)). RAB-5 (Q78L)-induced large endosomes accumulating GFP were detectable in WT (arrow) but not in the vps-45 mutant background. Scale bar, 5 μm. (B) Quantitative analysis of (A). In coelomocytes with red fluorescence, percentages of the coelomocyte with large endosomes per total coelomocyte were calculated. Error bars indicate s.e.m. *P < 0.001. GFP, green fluorescent protein; RAB-5, Rab5 homologue; RABS-5, Rabenosyn-5; SYN-13 and SYN-16, Syntaxin; WT, wild type.

Speculations

We found that the murine orthologue (VPS45) is functionally interchangeable with vps-45, but the yeast orthologue is not (supplementary Fig 7 online). vps-45/Vps45 might have evolved to gain the endocytic function in multicellular organisms, as their transport pathways are required to be more complex than monocellular organisms. An additional uncoordinated movement (Unc) phenotype of the vps-45 mutant (data not shown) might imply the presence of vesicle recycling defects in neuronal synapses. Future investigations into the roles of VPS-45 in integrating upstream RAB-5-dependent regulation and execution of membrane fusion by SNARE will allow us to understand not only the regulatory mechanism of endosome fusion but also human diseases associated with the defects of endocytic transport in various tissues.

METHODS

C. elegans strains and culture conditions. C. elegans strains were cultured using standard techniques. All strains were derived from the WT Bristol strain N2. Strains carrying vps-45(tm246), rabs-5(ok1513) and rabs-5(tm2036) were grown at the permissive temperature 15 °C. Various phenotypes of these strains were examined when worms were shifted to the restrictive temperature 25 °C. For Rme phenotypes of vps-45 and rabs-5 mutants, homozygous animals (m+ z+) derived from heterozygous mothers were examined.

Genetics. Genetic crosses were carried out by standard genetic protocols. The deletion alleles were identified by PCR amplification with primers spanning the deleted region.

Pulse–chase analysis in coelomocytes. Texas-Red-conjugated BSA (TR-BSA, Sigma) (1 mg/ml) was injected into the pseudo-coelom of adult worms, incubated at 25 °C for the indicated time period and assayed as described previously (Zhang et al, 2001). vps-45(tm246);bs34 and vps-45(tm246);pws50 were shifted to the restrictive temperature (25 °C) for 48 h before this assay.

Analysis of the RME-8/GFP-positive endosomes. bs34, vps-45(tm246);bs34;tmEx1315, rabs-5(ok1513);bs34;tmEx1387 and syn-13(tm2037);syn-16(tm1560);bs34 were used for analysing the size of endosomes in coelomocytes. tmEx1315 has an extrachromosomal array that rescues the tm246 mutation, and the tmEx1387 has an extrachromosomal array that rescues the ok1513 mutation. Confocal images of the coelomocytes expressing RME-8/GFP from adult hermaphrodites were taken by z-sectioning at 1 μm intervals. The average number of RME-8/GFP-positive endosomes with a diameter of 2 μm or more was counted by analysing a series of z-sectioning images with IP-lab software. All strains were grown at 25 °C. In the vps-45(tm246) and rabs-5 (ok1513) mutations, two types of coelomocyte were analysed: the coelomocytes that carry the array (DsRed positive, depicted as vps-45;Ex+ or rabs-5;Ex+ in Fig 3) and the coelomocytes that have lost the array (DsRed negative, depicted as vps-45;Ex– or rabs-5;Ex– in Fig 3). At least 20 coelomocytes were analysed for each strain.

Statistics. Statistical analysis was carried out using analysis of variance followed by Tukey–Kramer test to localize the significant difference. P < 0.001 was considered significant. All statistics were run with R (R project http://www.r-project.org/).

Supplementary information is available at EMBO reports online (http://www.emboreports.org).

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REFERENCES


