Vacuolar Protein Sorting Protein 13A, TtVPS13A, Localizes to the *Tetrahymena thermophila* Phagosome Membrane and Is Required for Efficient Phagocytosis

Haresha S. Samaranayake,‡ Ann E. Cowan, and Lawrence A. Klobutcher*

Department of Molecular, Microbial, and Structural Biology, University of Connecticut Health Center, Farmington, Connecticut 06032

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Phagocytosis is the cellular process responsible for the ingestion of particles more than 0.5 μm in diameter (reviewed in references 1, 43, and 47). In vertebrates, phagocytosis is well developed in cells of the immune system, such as macrophages and neutrophils, that utilize the process to engulf and degrade invading pathogens. Many unicellular, eukaryotic microbes (e.g., ciliates and amoebae) are also capable of phagocytosis, using it to ingest food microorganisms (9, 51). In each case, once formed, the phagosome undergoes a series of vesicular fusion and fission events that serve to acidify the phagosome, deliver hydrolytic enzymes required for the digestion of the phagosome contents, and recycle components of the process.

The subject of this study, the ciliate *Tetrahymena thermophila*, carries out phagocytosis but with some specialized features compared to those of other organisms (reviewed in reference 13). For example, there is a specialized structure on the cell surface, termed the oral apparatus, that serves to gather food organisms and functions as the sole site for phagosome formation. In addition, the mature phagosome/phagolysosome ultimately fuses with a specialized structure at the posterior end of the cell, termed the cytoproct, expelling its residual contents (3, 49). *Tetrahymena* is an attractive system for studying this process, as phagocytosis is conditionally essential (32), facilitating genetic analyses. We previously carried out a mass spectrometry-based analysis of the *T. thermophila* phagosome proteome and identified 73 proteins that are candidates for involvement in phagocytosis (19). Many of the proteins identified were known to be involved in phagocytosis in other organisms, but a number of the identified proteins had not been implicated in this process. This included the *T. thermophila* vacuolar protein sorting 13 protein (TtVPS13A). More recent proteome analyses of mouse phagosomes have also identified a VPS13 protein (36, 40, 45). VPS13 proteins are large (e.g., the human VPS13A protein is 3,174 amino acids in length [48]) and conserved across eukaryotes. Most organisms possess a family of genes that encode VPS13 proteins, although yeast is a notable exception in having a single VPS13 gene (5). In addition, alternative forms of *VPS13* mRNAs have been observed for a number of organisms (e.g., see reference 48), further increasing the potential types of VPS13 proteins produced.

Mutations of *VPS13* genes have been identified and studied in several organisms. The gene was initially identified in *Saccharomyces cerevisiae* (the *SOI* or *VPS13* gene), where it has been implicated in membrane protein trafficking between the trans-Golgi and prevacuolar compartments (5, 35). More recently, deletions of the yeast *VPS13* gene also have been shown to result in defects in prosome membrane formation during sporulation (30), a process that involves Golgi-derived vesicles. In *Dictostelium*, a *VPS13* homologue (*tipC*) was identified in a screen for mutations affecting the multicellular patterning process that leads to sporulation (41). Humans possess four *VPS13* genes (48), two of which have been implicated in genetic diseases. Mutations in the *VPS13A* gene result in a neurological disorder called chorea-acanthocytosis (ChAc) (34, 46), which is characterized by progressive hyperkinetic movements (chorea) and the presence of misshapen red blood cells (RBCs) (acanthocytosis). The main pathological feature is the degeneration of the striatum of the brain, manifested by cognitive impairment.

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* Corresponding author. Mailing address: Department of MMS Biology, University of Connecticut Health Center, Farmington, CT 06032. Phone: (860) 679-2816. Fax: (860) 679-3408. E-mail: klobutcher@nso2.uchc.edu.
† Supplemental material for this article may be found at http://ec.asm.org/.
‡ Present address: Yale University, Department of Internal Medicine, Section of Rheumatology, New Haven, CT 06520-8031.
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ment plus behavioral and personality changes, ultimately leading to premature death (reviewed in reference 11). Mutations in the human VPS13B gene also result in an autosomal recessive disorder, Cohen syndrome, with neurological symptoms (22). Finally, mice with a mutation in the homologue of human VPS13A have been generated, mimicking some features of ChAc, particularly in old age (44).

The function of the VPS13 protein is not entirely clear, but a number of studies have examined its expression and localization. In mouse, Western blot analyses indicated that VPS13A was expressed in most tissues, with particularly high expression levels in the testis, brain, kidney, spleen, and muscle (24). In addition, immunohistochemical analyses indicated that it was ubiquitously expressed in all brain regions, including the striatum, which is the region impacted by ChAc. Similarly, all four human VPS13 genes were found to be expressed in the heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas (48), and VPS13A has been shown to be expressed in numerous regions of the brain (34). While the exact subcellular location of the VPS13A protein is unknown, two studies indicated that it is associated with cellular membranes. Using a VPS13A antibody, Dobson-Stone et al. (10) reported that VPS13A was present in membrane fractions derived from erythrocytes. In yeast, Bricker and Fuller (5) found that a hemagglutinin (HA)-tagged version of VPS13/STO1p sedimented with a cellular fraction containing the Golgi membrane and transport and secretory vesicles and obtained evidence that the protein is part of a complex that is peripherally associated with membranes.

Given that none of those previous studies suggested a role of VPS13A in phagocytosis, we sought to further analyze the function of this gene in T. thermophila. The T. thermophila VPS13A (TtVPS13A) gene structure has been defined, and, taking advantage of the genetic tools available for Tetrahymena, strains carrying either green fluorescent protein (GFP)-tagged or knockout alleles of TtVPS13A have been constructed and analyzed. Our results indicate that TtVPS13Ap is localized to the phagosome membrane throughout phagolysosome biogenesis and that the protein is necessary for efficient phagocytosis. The possible implications of these findings in relation to the function of the protein as well as how human VPS13 gene mutations might result in pathology are discussed.

MATERIALS AND METHODS

Cells and cell culture. Tetrahymena thermophila strain CU428.2 cells (kindly provided by Donna Cassidy-Hanley, Cornell University, Ithaca, NY) were grown at room temperature or at 30°C with agitation in SPPA medium containing 250 μg/ml penicillin G, 250 μg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B (Sigma-Aldrich) (14). This strain was used to construct the TtVPS13A::GFP and TtVPS13A deletion mutant strains. For the CU522-derived strain overexpressing GFP-tagged cathespin B (19), pataclitase (20 μg/ml) and CdCl2 (1 to 2 μg/ml) were also included.

To analyze the growth of Tetrahymena strains under conditions where phagocytosis was required, cells were grown in 10 mM Tris (pH 7.4) supplemented with bacteria (Klebsiella pneumoniae), strains carrying either green fluorescent protein (GFP)-tagged or knockout alleles of TtVPS13A have been constructed and analyzed. Our results indicate that TtVPS13Ap is localized to the phagosome membrane throughout phagolysosome biogenesis and that the protein is necessary for efficient phagocytosis.

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maintained at 0.1 μg/ml. As cells did not grow well at 43.3 mg/ml paromomycin, the cultures were subsequently maintained at 21.6 mg/ml paromomycin.

Total genomic DNA extracted before and after phenotypic assortment was analyzed by either Southern hybridization, PCR, or RT-PCR to determine the copy number ratio between endogenous and mutated gene copies. The primers used for the PCR analysis were CHIN2F and CHIN2R, to detect the wild-type (WT) copies, and pNEO4MTT1R and CHIN2F, to detect the deleted gene copies. For RT-PCR, forward primer CH1617L and reverse primer CHEX3R were used to amplify wild-type transcripts. Forward primer EXSL and reverse primer rEX2 were also included in the RT-PCR mixtures to amplify transcripts of the GRL1 gene, which served as a loading control. Primer concentrations of EXSL and rEX2 were half the concentrations of primers CH1617L and CHEX3R.

Western blot analysis. Whole-cell protein extracts were prepared by collecting log-phase cells by centrifugation at 2,400 × g in the presence of 10% Complete EDTA-free protease inhibitor cocktail (Roche, Mannheim, Germany). Cell pellets were resuspended in NuPAGE LDS sample buffer (Invitrogen), frozen in a dry ice-ethanol bath, and stored at −80°C until use. When needed, samples were thawed at room temperature and heated at 70°C for 10 min in the presence of the NuPAGE sample-reducing agent. Protein samples were separated by electrophoresis from 3 to 8% NuPAGE Novex Tris-acetate minigels (Invitrogen) (ζ = 35 M (for 3 μL of cell equivalents of protein per well were typically used)). Protein transfer onto polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA), blocking of the membranes, and incubation with antibodies were done as previously described (19). Following incubation with antibodies, membranes were washed twice with Tris-buffered saline (TBS) containing 0.1% Tween 20 (Sigma-Aldrich) for 20 min, and alkaline phosphatase-conjugated antibody to rabbit immunoglobulin G (IgG, Sigma-Aldrich) or mouse IgG at a dilution of 1:10,000 in combination with 5-bromo-4-chloro-3-indolyl phosphate–Nitro Blue Tetrazolium liquid substrate (Sigma-Aldrich) was used to detect binding.

The anti-GFP polyclonal antibody was purchased from Invitrogen, affinity purified against soluble GFP (Clontech Laboratories Inc., Mountain View, CA) as described previously by Sambrook et al. (38), and used at a 1:2,000 dilution.

Molecular biological techniques. Plasmid DNAs and whole-cell genomic DNA were isolated by using Wizard Plus Miniprep or MidiPrep DNA purification systems (Promega Corp., Madison, WI) and a Wizard Genetic DNA purification kit (Promega Corp., respectively). Either KlenTaq DNA polymerase (Sigma-Aldrich) or Pfu Ultra High Fidelity DNA polymerase was used for PCRs. Oligonucleotide primers were purchased from IDT Inc. (Coralville, IA). PCRs were carried out with a volume of 50 μL containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 50 mM MgCl₂, 10 mM each deoxynucleoside triphosphate (dNTP), 50 pmol each primer, 100 ng of DNA substrate, and 2.5 units of enzyme. Thirty cycles of amplification were carried out by using a model PTC-150 Minicycler (MJ Research Cambridge, MA). PCR products were purified directly by using the QIAquick PCR purification system (Qiagen Inc., Valencia, CA) or purified from low-melting-point agarose gels by use of the Wizard SV gel and PCR purification kit (Promega Corp.) according to manufacturers' instructions. DNA sequencing of PCR fragments and sequence alignments were completed as described by the enzyme suppliers (Invitrogen and New England BioLabs, Beverly, MA). One Shot Top 10 cells (chemically competent; Invitrogen) were used for bacterial transformation according to the manufacturer's instructions. For Southern hybridization, probes were labeled with [α-32P]dATP using either DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, Mannheim, Germany) or the Decaprim II Random Primed DNA labeling kit (Ambion), Hybridization was carried out according to methods described previously by Sambrook et al. (38). Sequencing was carried out by the University of Connecticut Health Center Molecular Core Facility (Farmington, CT) or Genewiz Inc. (South Plainfield, NJ).

Phagocytosis assays. To assess the formation of phagosomes, 1-mL *Tetrahymena* cultures were prepared in 15-mL conical tubes at a cell density of 8 × 10⁷ cells/ml and incubated at 30°C for 1 h with gentle agitation. Red fluorescent latex beads (2-μm or 1.1-μm diameter; Sigma-Aldrich) were then added to a final dilution of 1:1,000. After various times, 10-μL samples were removed and diluted in 5% formaldehyde prepared in 10 mM Tris. The number of phagosomes in a total of 25 cells at each time point was then determined by fluorescence microscopy.

To monitor the acidification of phagosomes, *Tetrahymena* cultures were pre-incubated with LysoTracker Red DND-99 dye (Molecular Probes) at a concentration of 5 μM/ml for 10 min. Pulse-labeling of phagosomes was then carried out by the addition of 1:1-μm nonfluorescent latex beads (1:1,000 dilution; Sigma) for 2 min, followed by filtration using an 8-μm (pore diameter) Nitex membrane (Tetko Inc., Briarcliff Manor, NY) to remove noningested beads. The recovered cells were resuspended in culture medium with 5 μg/ml LysoTracker Red DND-99 dye and incubated at room temperature with agitation. Samples of 500 μL were drawn every 15 min and collected by centrifugation at 2,000 × g for 30 s. The cell pellets were then resuspended in 20 μL of culture medium containing LysoTracker dye and used for imaging.

A spore uptake/digestion assay utilized decocted *Bacillus subtilis* strain PS5518 spores (8), which carry the GFP gene under the control of the *spol* gene promoter. *Tetrahymena* cultures in log phase were washed with EPP medium to remove antibiotics, paromomycin, and/or CdCl₂; resuspended in EPP medium; and incubated at 30°C for 1 h. The *Tetrahymena* cells were then incubated with the GFP-tagged spores (final OD₀₅₀ of 0.5) for 10 min, and the *Tetrahymena* cells were recovered by filtration as described above. Cell samples were then removed every 15 min and centrifuged at 2,000 × g for 30 s, and the cell pellets were resuspended in 20 μL of culture medium for analysis by fluorescence microscopy.

Microscopy. *Tetrahymena* cells were fixed according to a procedure described previously by Stuart and Cole (42). The final pellet was resuspended in 20 μL of SlowFade Antifade solution (Molecular Probes, Eugene, OR) and stored at 4°C until use. For live-cell imaging, 4 μL of cells was mixed with 3 μL of methyl cellulose (10% methyl cellulose; Connecticut Valley Biological Supply, Southampton, MA), and the mixture was applied onto a slide along with a coverslip for observation.

All images were collected by using a Zeiss LSM510 inverted microscope (Carl Zeiss Inc.) equipped with a Plan-Apochromat 63×, 1.4-numerical aperture, differential interference contrast (DIC) oil immersion lens. The 488-nm line of an argon laser and the 543-nm line of an HeNe laser were used to excite the green and red fluorochromes, respectively. The sizes of phagosomes and other intracellular vesicles were measured by using Zeiss AIM software.

For the imaging of the TtVPS13A::GFP- and TtVPS13A::GFP-expressing cells, nonfluorescent 1:1-μm polystyrene latex beads (10% solid; Sigma-Aldrich) were added to cultures at a final dilution of 1:1,000. After 10 min, excess beads were removed via filtration using an 8-μm Nitex membrane. Samples were drawn at 0, 1, 5, and 10 min (i.e., before filtering of the beads) and at 30, 60, and 120 min, and the cells were fixed for analysis.

Computational analysis. The *Institute for Genomic Research* (TIGR) database was used to identify DNA sequences and preliminary protein predictions of *TtVPS13A* and other *TtVPS13* genes in *Tetrahymena*. Database searches for *TtVPS13* gene and protein homologues were performed by using BLAST (www.ncbi.nlm.nih.gov/BLAST) or TBLASTN (www.ncbi.nlm.nih.gov/TBLASTN) or the Decaprime II Random Primed DNA labeling kit (Ambion). The final pellet was resuspended in 20 μL of SlowFade Antifade solution (Molecular Probes, Eugene, OR) and stored at 4°C until use. For live-cell imaging, 4 μL of cells was mixed with 3 μL of methyl cellulose (10% methyl cellulose; Connecticut Valley Biological Supply, Southampton, MA), and the mixture was applied onto a slide along with a coverslip for observation.

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Nucleotide sequence accession number. The sequence of the *TtVPS13A* mRNA has been deposited in the GenBank database under accession number HQ011927.

RESULTS

Characterization of the *TtVPS13A* gene structure. Our previous analysis identified a *VPS13A*-like protein (TtVPS13A) (TGD accession number 8.m00549) as a component of the *T. thermophila* phagosome proteome (19). The *TtVPS13A* preliminary gene prediction present in the *Tetrahymena* Genome Database (www.jcvi.org) consisted of 29 exons spanning a 16.5-kb region of the genome (Fig. 1A), but sequence data on the mRNA (e.g., expressed sequence tags [ESTs]) were not available to support this gene prediction. As a first step to study gene function, the structure of the *TtVPS13A* gene was deduced by the amplification of the entire mRNA using 5‘ rapid amplification of cDNA ends (RACE), 3‘ RACE, and RT-PCR, followed by sequencing of the overlapping fragments (see Table S1 in the supplemental material for a list of oligonucleotide primers used). These analyses revealed that the first
11 predicted exons were not present in the *TtVPS13A* mRNA, as well as identified the true boundaries for a number of the internal predicted exons and introns. The true gene occupies 12.45 kb in the macronuclear genome (Fig. 1B) and consists of 17 exons. The *TtVPS13A* gene generates an mRNA of 10,637 nucleotides that encodes a putative protein of 3,475 aa (Fig. 1).

No evidence for the alternative splicing of *TtVPS13A* transcripts was observed in the above-described RT-PCR-based analyses. However, alternative splicing has been observed for transcripts of *VPS13* genes in other organisms, including humans (e.g., see reference 48). As alternative splicing could complicate further studies of the gene, we carried out additional analyses. By use of RT-PCR, each internal exon was amplified by using primers designed for flanking exons (see Fig. S1 in the supplemental material). In each case, the major PCR products observed were of the sizes expected for no exon skipping. Overall, while it remains formally possible that some form of alternative processing may exist (e.g., the skipping of multiple adjacent exons), we obtained no evidence of alternatively spliced *TtVPS13A* transcripts.

When the predicted *TtVPS13A* protein sequence was used in BLAST searches of the GenBank database, *VPS13* proteins from a wide range of eukaryotes were detected, ranging from single-cell organisms (e.g., *Entamoeba histolytica*, with a BLAST E value of 1e^-44) to mammals (e.g., *VPS13A* in *Homo sapiens*, with an E value of 6e^-41). The regions of shared similarity between *TtVPS13A* and the *VPS13* proteins from other organisms spanned the majority of the protein but were concentrated at the N and C termini, with the N-terminal 179 aa of *TtVPS13A* showing 26 to 35% sequence identity and 48 to 62% sequence similarity and the C-terminal 273 aa of *TtVPS13A* sharing 32 to 36% sequence identity and 55 to 58% sequence similarity with the *VPS13* proteins from other organisms (Fig. 1B and see Fig. S2 in the supplemental material). *TtVPS13A* was also found to contain a DUF1162 (domain of unknown function) domain (probability = 2e^-11) between amino acids 2425 and 2751 (Fig. 1B), which is conserved in other *VPS13* proteins, as well as a region with weak similarity to a pleckstrin homology (PH) domain (probability = 0.007). In addition, a possible vacuole-targeting motif (VTM) was found at position 2771 (ILPI) (Fig. 1B). No conclusive evidence was found for membrane-spanning domains within the *TtVPS13A* protein.

A BLAST search of the *Tetrahymena* preliminary protein prediction database using the human *VPS13A* protein sequence identified at least 8 *Tetrahymena* genes encoding similar proteins (*TtVPS13A*, -B, -C, -E, -D, -F, -G, and -H), with E values ranging from 6.4 × 10^-16 to 1.3 × 10^-31 (see Table S2 in the supplemental material). In reciprocal BLAST analyses, the *TtVPS13A* and human *VPS13A* genes were the top matches in searches of the respective genomes. Among the other *Tetrahymena* *VPS13* paralogues, preliminary analyses indicated that at least the *TtVPS13B*, *TtVPS13C*, and *TtVPS13D* proteins are expressed (data not shown). It is noteworthy that one of these proteins, *TtVPS13C*, might also be a component of the *Tetrahymena* phagosome based on previous mass spectrometry analyses (19); 2 out of 13 peptide sequences that were identified for the *TtVPS13A* protein are identical to predicted peptides of the *TtVPS13C* protein. Moreover, *TtVPS13C* is quite similar to *TtVPS13A*, with 70% sequence identity over a 1,033-aa-long N-terminal region and 66% sequence identity in the C-terminal region corresponding to amino acids 1854 to 3475 of *TtVPS13A*.

**Construction of a *Tetrahymena* strain expressing a *TtVPS13A*::GFP fusion protein.** A *T. thermophila* strain expressing a *TtVPS13A::GFP* fusion protein was constructed to examine the localization and expression of the protein. This involved the preparation of a construct in which the *TtVPS13A::GFP* fusion protein was introduced into *T. thermophila* strain CU428.2 cells by biolistic particle bombardment (7). All transformations in *Tetrahymena* occur by homologous recombination, but only a subset of the ~45 copies of each of the *TtVPS13A* genes in the macronucleus are expected to be replaced initially by the *TtVPS13A::GFP* construct. Therefore, a phenotypic assortment protocol (see Materials and Methods) (20) was carried out on *TtVPS13A::GFP* transformants to determine if it was
possible to obtain cells exclusively containing the GFP fusion allele. Following growth with successively increasing concentrations of paromomycin, clonal cell lines were isolated, DNA was extracted, and a PCR analysis was carried out to assess the amounts of wild-type and TtVPS13A::GFP alleles. Untransformed cells produced the 246-bp PCR product expected from the endogenous TtVPS13A locus, while four assorted subclones derived from two independent transformants showed primarily the 322-bp PCR product indicative of the GFP-tagged gene (Fig. 2B and data not shown). While trace amounts of the 246-bp product were detectable from the assorted subclones (Fig. 2B), these minor products are likely derived from the two untagged copies of the TtVPS13A gene present in the micronucleus.

The ability to obtain cells that contain primarily the GFP-tagged TtVPS13A allele indicates that the expression of the fusion protein from the endogenous promoter is not toxic to the cell. We also assessed the growth rates of assorted TtVPS13A::GFP transformants both on a rich synthetic medium where phagocytosis is nonessential (EPP medium) (32) as well as in 10 mM Tris-HCl supplemented with the bacterium Klebsiella pneumoniae, where phagocytosis is required for growth. No significant differences in growth between the assorted transformants and control strain CU428.2 cells were observed under either growth condition (see Fig. S3 in the supplemental material), indicating that the TtVPS13A::GFP fusion protein does not impair growth.

Western blot analyses using affinity-purified anti-GFP antibodies were performed to confirm the expression of the fusion protein. A protein species of the size expected for the TtVPS13A::GFP fusion protein (~424 kDa) as well as a series of likely degradation products were weakly but consistently detected in the assorted TtVPS13A::GFP transformants (Fig. 2C). The yeast VPS13/SOL1 protein was also reported previously to be sensitive to proteolytic digestion (5). We suspect that the TtVPS13A::GFP protein is expressed at low levels, as the protein level was near the detection limit in the Western blotting analysis as well as by fluorescence microscopy (see below). The lack of expressed sequence tags in the Tetrahymena genome database that are derived from the endogenous VPS13A gene also suggests a low level of expression.

**TtVPS13A::GFP associates with phagosomes.** The assorted TtVPS13A::GFP transformants were examined by using confocal microscopy to determine the subcellular localization of the TtVPS13A::GFP protein. To unambiguously identify phagosomes, cells were fed with latex beads for 30 min prior to analysis. In TtVPS13A::GFP transformants, fluorescence was observed mainly as a ring around the bead-filled phagosomes, implying that the protein is associated with the phagosome membrane (Fig. 3A). Transformants also showed weak cytoplasmic fluorescence, while essentially no fluorescence was seen in the untransformed, parental cell line (Fig. 3A). Non-bead-filled vesicular structures with a diameter of 3 to 4 μm with fluorescent rings were also present in TtVPS13A::GFP cells. These smaller vesicles were also observed in cells that had not been fed latex beads (Fig. 3A), and a number of analyses suggested that these structures are also phagosomes that form either by the ingestion of particulate matter from the culture medium or by the engulfment of the medium itself (see reference 37 for details). Confocal microscopy of live TtVPS13A::GFP cells was also performed. These analyses were hampered by the autofluorescence from latex beads, which was also observed for a number of other types of particles that we attempted to feed to Tetrahymena cells. Nonetheless, even in such live cells, we observed fluorescent rings around phago-
somes that were not seen in control cells (data not shown), consistent with our observations of fixed-cell preparations.

A time course analysis was carried out to determine at what point during phagocytosis TtVPS13A::GFP associates with the phagosome. Cultures of cells were fed with latex beads for 10 min, and the excess beads were then removed by filtration (time zero = the addition of beads). Even at 1 min after the addition of beads, at least 50% of the phagosomes were outlined with a faint signal of green fluorescence (Fig. 3B and 4C). By 60 min, fluorescent rings were observed around at least 80 to 90% of the bead-filled phagosomes, and this fluorescence was maintained through 120 min (Fig. 3B and 4C). A complete cycle of phagocytosis (ingestion through egestion) occurs over a period of about 2 h in Tetrahymena (31), indicating that TtVPS13A is associated with the phagosomes throughout phagocytosis.

There are currently few molecular markers in Tetrahymena that allow the various stages of phagosome biogenesis to be distinguished. However, we were able to monitor both phagosome acidification, using LysoTracker Red dye, and the delivery of cathepsin B, using a previously constructed cell line that overexpresses a cathepsin B::GFP fusion protein (19). Acidified phagosomes were observed as early as 20 min after phagosome formation, but the bulk of the phagosomes were not acidified until ~50 to 60 min after the addition of beads (Fig. 4A and C). In these analyses, small acidic punctate structures (0.6 to 1.0 μm in size) were also observed for the cells throughout the analysis (Fig. 4A), and these presumably represent the

FIG. 3. Subcellular localization of the TtVPS13A::GFPA5:10 fusion protein. (A) Confocal images of fixed Tetrahymena TtVPS13A::GFPA5:10 and CU428.2 cells that had been either fed with nonfluorescent latex beads for 30 min or grown in the absence of latex beads. For slide preparation, cells were collected by centrifugation and resuspended in SlowFade antifade agent. Fluorescent and bright-field (BF) images of cells are shown. Thin arrows indicate selected bead-filled phagosomes, and arrowheads indicate selected small vesicles. (B) Time course analysis of the TtVPS13A::GFP association with phagosomes. Cells were fed latex beads for 10 min and examined by confocal fluorescence (GFP) and bright-field microscopy over the next 120 min. These results were reproducible in multiple, independent time course analyses.
acidosomes or primary lysosomes (12) that fuse with the phagosomes, triggering their acidification. In contrast, cathepsin B was delivered to phagosomes much earlier (Fig. 4B and C). *Tetrahymena* cells carrying the cathepsin B::GFP fusion construct displayed fluorescent phagosomes as early as 1 min after the addition of latex beads to cultures and reached a peak at 5 min after the addition of beads. In this case, patchy fluorescence around the periphery of the phagosomes was observed (Fig. 4B), which likely represents the fusion of vesicles containing cathepsin B with phagosomes, as well as more diffuse fluorescence within the phagosomes. In addition, small fluorescent punctate bodies were observed in the cells throughout the analysis, which presumably represent the vesicular compartment responsible for the delivery of cathepsin B to the phagosome. Overall, the results indicated that cathepsin B is delivered to phagosomes very early and in a time frame similar to that observed for TiVPS13A::GFP and that both of these events precede phagosome acidification.

**Generation and analysis of a TiVPS13A gene knockout.** To generate a TiVPS13A knockout strain, a construct was generated in which neomycin resistance gene 4 (*NEO4*) under the control of the Cd-inducible *MTT1* promoter (28) was flanked by sequences from the upstream region of the TiVPS13A gene. When integrated, the region of the construct containing the neomycin resistance gene would be expected to replace a 728-bp region in the TiVPS13A gene beginning within the...
second intron and extending into the third exon (Fig. 5A). Tetrahymena CU428.2 vegetative cells were transformed with the disruption construct by biolistic particle bombardment (7), and four independent transformants were selected randomly for further analysis. The initial transformants showed a partial replacement of the endogenous wild-type TtVPS13A gene (Fig. 5B), so cells were again carried through phenotypic assortment to determine if complete gene replacement was possible. As before, this involved the gradual increase of the paromomycin concentration in the culture medium (from 180 μg/ml to 43.2 mg/ml), but in this case the cells were also cultured in EPP medium throughout the process, as this medium allows Tetrahymena cells to grow in the presence of a defect in phagocytosis (32). One subclone was selected from each assorted transformant, and two of these were used for further analyses (TtVPS13AA4PA and TtVPS13AB2PA).

A PCR assay was initially used to determine if there was a complete replacement of the endogenous macronuclear copies of the TtVPS13A gene in the two independent assorted subclones. The results indicated that the assorted subclones contained predominantly disrupted copies of the TtVPS13A gene (Fig. 5B). However, weak PCR products of the size expected for the wild-type gene were also seen, which likely represent the TtVPS13A gene copies in the micronucleus. To definitively determine if complete macronuclear gene replacement had occurred, an RT-PCR analysis of total cellular RNA was performed to directly look for TtVPS13A transcripts, as there is no transcription from the micronucleus during vegetative growth. While an RT-PCR product derived from TtVPS13A transcripts was readily detected in control CU428.2 cells, no such product was observed for the assorted transformants, indicating that there was indeed complete gene replacement and that a TtVPS13A gene knockout was generated (Fig. 5C).

Growth assays were next performed to determine if the loss of a functional TtVPS13A gene impaired cellular function. When growth was assessed with EPP medium, where phagocytosis is not required, the TtVPS13AA4PA and TtVPS13AB2PA knockouts displayed significantly different population doubling times that did not differ significantly from those of wild-type CU428.2 cells (Fig. 6A). In contrast, when cells were grown under conditions requiring phagocytosis (i.e., with Klebsiella bacteria as the food source), the knockout strains grew significantly slower than wild-type cells (Fig. 6B). Wild-type CU428.2 cells had a population doubling time of 3.60 ± 0.15 h (mean ± standard error, based on three experiments) under conditions requiring phagocytosis, whereas the TtVPS13AA4PA and TtVPS13AB2PA knockouts displayed significantly different population doubling times of 6.93 ± 0.88 h and 6.10 ± 0.46 h, respectively. These results indicate that the TtVPS13A gene is not essential for growth in either EPP or Klebsiella-supplemented medium but that it is necessary for optimal growth when phagocytosis is required.

Additional analyses were carried out in an attempt to identify which stage of phagocytosis might have been impaired in the TtVPS13A knockout mutants. First, a latex bead uptake assay was employed to investigate whether the TtVPS13A knockouts had a defect in phagosome formation. Mutant cells were incubated in EPP medium containing 2-μm red fluorescent latex beads, and cell samples were collected and analyzed by fluorescence microscopy over a 2-h time period to determine the number of phagosomes formed per cell. Wild-type cells were able to form 8 to 10 phagosomes on average during this analysis, while the two assorted TtVPS13A knockout strains formed only 4 to 6 phagosomes per cell (Fig. 6C). Similar results were obtained when cells were fed latex beads with a diameter of 1.1 μm (5 to 7 phagosomes per wild-type cell versus 1 to 3 phagosomes per each of the knockout strains [two separate experiments]). These results indicate that phagosome formation is partially impaired in the TtVPS13A deletion mutant.

**FIG. 5.** Generation of a TtVPS13A gene knockout. (A) Diagrams of the knockout construct (KO), which contains a neomycin resistance gene (NEO4R) under the control of the cadmium-inducible MTti promoter (open boxes), and the 5’ end of the endogenous TtVPS13A gene (exons are shown as black boxes). The integration of the construct by homologous recombination results in the deletion of parts of exon 2 and exon 3. Arrows denote the primers used in PCR and RT-PCR analyses to confirm that the transformants carried the expected deletion. The TtVPS13A transfectants are expected to have the NEO4 gene between exon 2 and exon 3. The arrows indicate the locations of the primers used in the PCR and RT-PCR analyses. (B) PCR analysis to determine the ratio of wild-type (WT) and deleted (knockout) gene copies in the transformants before and after pheno- typic assortment. The 1% agarose gel shown contains the PCR mix- tures from the TtVPS13AA4 (A4) and TtVPS13AB2 (B2) transformants, wild-type (WT) CU428.2 cells, and the assorted subclones TtVPS13AA4PA (A4PA) and TtVPS13AB2PA (B2PA). PCR was carried out on total genomic DNA from transformants and the WT by using the pNEO4MTT1 and CHIN2R reverse primers, along with forward primer CH1617L (see above). The positions of the expected WT (0.22 kb) and knockout mutant (0.40 kb) PCR products are indicated. (C) Gel with RT-PCR products representing TtVPS13A and GRL1 gene transcripts in wild-type cells and the TtVPS13AA4PA and TtVPS13AB2PA knockout subclones. RT-PCR was carried out by using forward primer CH1617L and reverse primer CHEX3R to amplify TtVPS13A transcripts, and forward primer EX5L and reverse primer CH1617L were included to amplify GRL1 transcripts, which served as an internal control. The sizes of the expected PCR products for TtVPS13A and GRL1 are indicated in kilobase pairs.
We also assessed the ability of the TtVPS13A knockout mutant cells to ingest and digest prey microorganisms by feeding them decoated Bacillus subtilis spores that express the GFP protein (strain PS3518) (8). Bacillus spores with coat defects were previously shown to be digested by Tetrahymena following phagocytosis (Tris buffer supplemented with Klebsiella bacteria) (21). When the GFP-labeled, decoated spores were fed to wild-type Tetrahymena cells, tightly packed, fluorescent spores were initially seen within phagosomes, but over time the fluorescence diminished, and distinct spores were no longer distinguishable (Fig. 7A). These changes in fluorescence and morphology provide a measure of the timing of digestion of the phagosomal contents. For the analysis, the TtVPS13AA4PA knockout strain and wild-type CU428.2 cells were pulsed with spores for 10 min to form phagosomes, unengulfed spores were removed by filtration, and the Tetrahymena cells were then monitored by fluorescence microscopy for 2 h to assess the percentage of phagosomes with intact spores. At the earliest time points (20 and 30 min), essentially all phagosomes in the knockout mutant cells were intact, while 20% and 30% of the phagosomes in wild-type cells displayed degraded spores at 20 and 30 min, respectively (Fig. 7B). The number of phagosomes with intact spores
in wild-type cells rapidly decreased and reached 0% after 60 to 70 min. In contrast, it was not until 100 min that phagosomes with intact spores were no longer seen in the TtVPS13A knockout cells (Fig. 7B), indicating that the degradation of phagosomal contents was partially impaired.

Finally, we note that during this analysis, mutant cells often contained phagosomes with two separate clumps of tightly packed spores (see Fig. S4 in the supplemental material), which suggests phagosome fusion. The percentage of TtVPS13A mutant cells containing such structures was 17% in these analyses, compared to only 0.7% of wild-type cells. The significance of this observation is not entirely clear, but it may be indicative of an alteration in the phagosome membrane that enhances fusion. Finally, this phagosome fusion may, in part, contribute to the decreased number of phagosomes observed for the knockout mutant.

**DISCUSSION**

The *Tetrahymena* TtVPS13A gene spans 12.45 kb in the macronuclear genome and produces a transcript of 10,637 bases. As opposed to other organisms (e.g., see reference 48), we have found no evidence of an alternative splicing of *TtVPS13A* gene transcripts, which should simplify further experimental analyses. The predicted 3,475-amino-acid TtVPS13A protein shares sequence similarity with the VPS13 proteins of a variety of organisms, with the strongest sequence conservation focused in regions corresponding to the N-terminal 196 aa of TtVPS13A and a block of 273 aa near the C terminus (Fig. 1). As has been the case with VPS13 proteins of other organisms, the TtVPS13A sequence provides few clues as to its function.

TtVPS13A is a member of a family of at least eight *VPS13*-like genes in *Tetrahymena*. In this regard, *Tetrahymena* is more similar to multicellular eukaryotes, which typically possess multiple *VPS13* genes (e.g., see reference 48), than to yeast, which contain a single gene (5). The presence of multiple genes suggests that there may be a functional redundancy of some of the encoded proteins and/or that family members may have diversified in function, perhaps serving similar functions but at different cellular sites. The presence of multiple genes in *Tetrahymena* presents a unicellular model to further investigate these possibilities.

**Function of the TtVPS13A protein.** In a *Tetrahymena* strain expressing a TtVPS13A::GFP fusion protein, the TtVPS13A protein was found to be associated with the membrane of phagosomes during essentially their entire transit through the cell. It should be noted that while the time course data shown in Fig. 4C indicate that some phagosomes lack TtVPS13A::GFP at early time points, the fluorescence signal is typically weaker at early stages, and we sought to be conservative in scoring phagosomes as positive. As a result, it is quite possible that TtVPS13A is present at the earliest stages of phagosome formation. These data support previous proteomic analyses indicating that VPS13 proteins are associated with phagosomes (19, 36, 40, 45). Moreover, the observed TtVPS13A membrane localization is somewhat consistent with data from studies in yeast indicating that its VPS13 protein is a peripherally associated intracellular membrane protein (5) as well as data from studies in mouse indicating that a VPS13 protein is present in cellular fractions containing microsomes (24). While yeast does not perform phagocytosis, genetic data indicate that its single VPS13 protein is involved in the vesicular trafficking of proteins between the trans-Golgi and prevacuolar (a late endosome analogue) compartments (5). How TtVPS13A becomes associated with the phagosome is currently unclear. We have not observed any small vesicles containing TtVPS13A::GFP, suggesting that the protein is not delivered via vesicular trafficking. Weak cytoplasmic GFP fluorescence was observed (Fig. 3), suggesting that TtVPS13A might be recruited directly to the phagosome membrane.

TtVPS13A is nonessential, as it was possible to obtain strains where there was a complete replacement of endogenous gene copies with a knockout construct. However, the knockout strains displayed a number of phenotypes consistent with a partial defect in phagocytosis. When grown in nutrient broth, the knockout strains grew with division times similar to those of wild-type cells but displayed elevated division times when grown in bacterized medium where phagocytosis is essential. The numbers of phagosomes formed by the mutants were also reduced compared to the numbers of phagosomes formed by the wild-type strain, and the digestion of food microorganisms was delayed. Also note that while TtVPS13A is not essential for phagocytosis, it is possible that one of the other VPS13 proteins in *Tetrahymena* (e.g., TtVPS13C) might serve a similar or identical function in phagocytosis so that the knockout of the TtVPS13A gene results in only a partial impairment.

While the data support a role of TtVPS13A in phagocytosis, the biochemical function of the protein remains unclear. Any proposed model of the role of TtVPS13A needs to take into account its association with phagosomes throughout essentially their entire transit through the cell and the observed mutant defects in both the formation of phagosomes and the processing of the phagosomal contents. We suggest that TtVPS13A, along with a second VPS13 family member, such as TtVPS13C, are peripheral phagosomal membrane proteins that help identify the phagosomal membrane compartment and facilitate the fusion of multiple vesicle types during phagosome formation and maturation. In ciliates, phagosome formation involves the fusion of “discoidal vesicles” to the nascent phagosome at the oral apparatus (reviewed in reference 2). The discoidal vesicles are derived in part from phagosomes that are fusing at the cytopyroct, essentially recycling the phagosome membrane. Under the proposed scenario, the loss of TtVPS13A might both decrease the phagosomal membrane pool in the cell and hinder the fusion of the discoidal vesicles to the nascent phagosome, resulting in the reduced number of phagosomes seen for the knockout. The model would also explain the delay in the processing of food microorganisms, as one would expect an impaired fusion of either acidosomes or lysosomes during phagosome maturation. While this model is clearly speculative, further characterization of the defect in the TtVPS13A knockout, as well as the proteins that normally interact with TtVPS13A, should provide additional insights into the function of TtVPS13A.

**Implications for chorea-acanthocytosis.** Based on reciprocal BLAST analyses, the TtVP13A gene and the human VPS13A (CHAC) gene, which is defective in chorea-acanthocytosis, are likely orthologues. While the evolutionary distance between ciliates and humans is large, and some gene functions have not been conserved between these groups (e.g., see reference 6),
the current results may provide some insight into the pathology of chorea-acanthocytosis and help to determine the direction of further research. The two primary manifestations of chorea-acanthocytosis (11) are the appearance of abnormally shaped red blood cells (RBCs), termed acanthocytes, and choreic involuntary movement associated with the degeneration of the basal ganglia. A partial defect in phagocytosis might underlie both of these disease features. RBCs are removed from the circulation by phagocytosis in multiple tissues, including by the Kupffer cells of the liver (reviewed in reference 15). A partial defect in RBC phagocytosis might lead to older cells with membrane defects in the circulation. In the brain, microglia are the major phagocytic cells, but neurons have also been reported to carry out phagocytosis (4), While the mechanism is not yet entirely clear, microglia have been implicated in neuronal loss in a number of neurodegenerative disorders (reviewed in reference 33), raising the possibility that a partial defect in microglial phagocytosis might be responsible for the brain pathology observed for chorea-acanthocytosis.

It is also possible that one or more VPS13 proteins are involved in autophagys, as some proteins appear to be shared by the phagocytosis and autophagy pathways (40), and both processes ultimately converge with the lysosome. During RBC development, autophagy is involved in the degradation of cellular organelles, including mitochondria (27). The impairment of autophagy could affect RBC maturation, giving rise to the acanthocyte phenotype. Similarly, autophagy has been implicated in the pathology of a number of neurodegenerative disorders (reviewed in reference 52). We suggest that determining the subcellular localization of the human chorein protein and assessing whether cells of individuals with chorea-acanthocytosis have defects in either phagocytosis or autophagy would be important in further defining the basis of the disease and whether the Tetrahymena system might prove a useful model for further studies.

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