

# Mammalian Late Vacuole Protein Sorting Orthologues Participate in Early Endosomal Fusion and Interact with the Cytoskeleton

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Submitted June 4, 2003; Revised October 27, 2003; Accepted October 27, 2003

Monitoring Editor: Juan Bonifacino

In *Saccharomyces cerevisiae*, the class C vacuole protein sorting (Vps) proteins, together with Vam2p/Vps41p and Vam6p/Vps39p, form a complex that interacts with soluble N-ethylmaleimide-sensitive factor attachment protein receptor and Rab proteins to “tether” vacuolar membranes before fusion. To determine a role for the corresponding mammalian orthologues, we examined the function, localization, and protein interactions of endogenous mVps11, mVps16, mVps18, mVam2p, and mVam6. We found a significant proportion of these proteins localized to early endosome antigen-1 and transferrin receptor-positive early endosomes in Vero, normal rat kidney, and Chinese hamster ovary cells. Immunoprecipitation experiments showed that mVps18 not only interacted with Syntaxin (Syn)7, vesicle-associated membrane protein 8, and Vti1-b but also with Syn13, Syn6, and the Sec1/Munc18 protein mVps45, which catalyze early endosomal fusion events. Moreover, anti-mVps18 antibodies inhibited early endosome fusion in vitro. Mammalian mVps18 also associated with mVam2 and mVam6 as well as with the microtubule-associated Hook1 protein, an orthologue of the *Drosophila* Hook protein involved in endosome biogenesis. Using in vitro binding and immunofluorescence experiments, we found that mVam2 and mVam6 also associated with microtubules, whereas mVps18, mVps16, and mVps11 associated with actin filaments. These data indicate that the late Vps proteins function during multiple soluble N-ethylmaleimide-sensitive factor attachment protein receptor-mediated fusion events throughout the endocytic pathway and that their activity may be coordinated with cytoskeletal function.

## INTRODUCTION

Soluble N-ethylmaleimide sensitive factor attachment protein receptor (SNARE) proteins are central to membrane fusion, forming tight 4 helix bundles that pull opposing membranes together (Hay, 2001). Although much is known about how SNARE complexes form, less is known about the upstream processes that regulate SNARE assembly. Some specificity is contributed by the SNARE proteins themselves, which catalyze fusion only in particular combinations due to the conformation of both the SNARE domain and N-terminal regulatory domain (Sollner, 2003). However, this intrinsic specificity is unlikely to suffice in vivo, and many other SNARE-interacting proteins have been proposed to contribute to the specificity of membrane fusion (Wickner and

Haas, 2000; Chen and Scheller, 2001; Hey, 2001; Wendler and Tooze, 2001; Sollner, 2003).

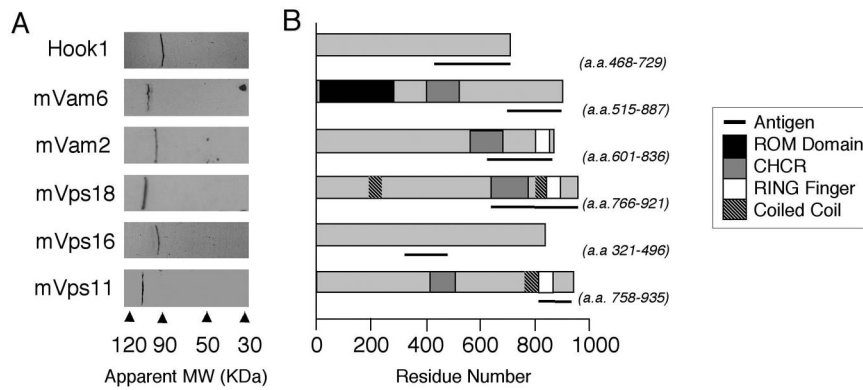
One important function that controls fusion specificity is the aggregation of compartments before the formation of *trans*-SNARE pairs or SNARE-pins. So-called “tethering” factors provide this function and are typically comprised of oligomeric complexes that associate directly or indirectly with SNARE proteins, Rab proteins, and Sec1/Munc-like (S/M) proteins (Waters and Pfeffer, 1999; Whyte and Munro, 2002). Only limited sequence or mechanistic conservation has been observed between tethering complexes, implying that each complex is tailored to provide and integrate a number of events specific to each fusion step (Waters and Pfeffer, 1999; Waters and Hughson, 2000).

Some of the yeast late vacuole protein sorting (Vps) proteins act as membrane tethers during fusion to yeast endosomes and vacuoles (Peterson and Emr, 2001; Price *et al.*, 2000; Sato *et al.*, 2000; Srivastava *et al.*, 2000). These proteins form a hetero-oligomeric complex comprised of the class C Vps proteins (Vps18p, Vps16p, Vps11p, and the S/M protein Vps33p; Rieder and Emr, 1997; Seals *et al.*, 2000) as well as a subcomplex consisting of the class B Vps proteins Vam6p and Vam2p (Price *et al.*, 2000). The resulting homotypic vacuolar protein sorting (HOPS) complex localizes to subdomains of the vacuole where fusion occurs (Price *et al.*, 2000; Seals *et al.*, 2000; Wurmser *et al.*, 2000; Eitzen *et al.*, 2002; Wang *et al.*, 2003). The HOPS complex also interacts with both SNAREs (such as Vam3p, Nyv1p, and Vti1p) and the

Article published online ahead of print. Mol. Biol. Cell 10.1091/mbc.E03-06-0358. Article and publication date are available at [www.molbiolcell.org/cgi/doi/10.1091/mbc.E03-06-0358](http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E03-06-0358).

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Abbreviations used: Av-ALP, avidin-alkaline phosphatase conjugate; EEA1, early endosome antigen-1; GST, glutathione S-transferase; HOPS, homotypic protein sorting and vacuolar fusion complex; MBP, maltose binding protein; PNS, postnuclear supernatant; S/M, Sec1/Munc like protein; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor(s); TfR, transferrin receptor; Vps, vacuole protein sorting.



**Figure 1.** Antibody detection and domain structure of mammalian late Vps proteins. (A) Affinity-purified polyclonal antibodies to the following proteins were used to immunoblot 50  $\mu$ g of postnuclear supernatant from B16 melanoma cells: Hook1 (~85 kDa), mVam6 (~101 kDa), mVam2 (~98.5 kDa), mVps18 (~110 kDa), mVps16 (~95 kDa), and mVps11 (~108 kDa). (B) Schematic of each protein's predicted domain structure and the region to which antibodies were generated (—).

Rab GTPase Ypt7p (Price *et al.*, 2000; Sato *et al.*, 2000; Seals *et al.*, 2000; Wurmser *et al.*, 2000; Eitzen *et al.*, 2002; Wang *et al.*, 2003). Mammalian orthologues of the HOPS proteins have been described and seem to also function in late endosomal fusion events. Overexpression of components such as mVps18 or mVam6 clusters late endocytic compartments and immunoprecipitation experiments show that Syntaxin (Syn)7, which catalyzes late endosomal fusion events, associates with the mammalian class C Vps proteins (Mullock *et al.*, 2000; Caplan *et al.*, 2001; Kim *et al.*, 2001; Poupon *et al.*, 2003).

The *Drosophila* orthologue of Vps18 (Deep Orange, DOR) also interacts with the Hook protein, and mammalian isoforms of Hook associate with the cytoskeleton (Lloyd *et al.*, 1998; Kramer and Phistry, 1999; Sevrioukov *et al.*, 1999; Sunio *et al.*, 1999; Narayanan *et al.*, 2000; Walenta *et al.*, 2001). This latter association is interesting because fusion events within the endocytic pathway are also controlled spatially by the cytoskeleton, which serves to transport and anchor compartments at the right place to undergo fusion (Qualmann *et al.*, 2000). Moreover, other putative tethering complexes have been found to associate with the cytoskeleton, suggesting that cytoskeletal functions are coordinated with the SNARE-based fusion apparatus.

This study expands the role of the mammalian HOPS proteins in the endocytic system. We find that the endogenous HOPS proteins significantly colocalize with early endosomal compartments. We also find that mVps18 associates not only with late but also early endosomal SNAREs such as Syn13 and Syn6 as well as the S/M protein mVps45. Additionally, antibodies specific to mVps18 inhibit the fusion of early endosomes *in vitro*. Interestingly, the mammalian HOPS proteins differentially interact with the actin and microtubule cytoskeleton, suggesting they may coordinate cytoskeletal attachment and movement of endosomes with their SNARE-dependent fusion.

## MATERIALS AND METHODS

Unless otherwise stated all reagents were from Fisher Scientific (Pittsburgh, PA) or Sigma-Aldrich (St. Louis, MO).

### Cloning of cDNAs

Using standard BLAST algorithms, mouse cDNAs encoding mouse (m)Vps18, mVps16, and mVps11, and mVam2 and mVam6 were identified by searching the mouse expressed sequence tag (EST) database for contiguous sequences that showed significant homology to known yeast open reading frames. PCR primers designed to amplify a ~300-base pair fragment of each coding region were used to screen several mouse cDNA libraries arrayed in microtiter plates (Origene, Rockville, MD). Sequencing cDNAs encoding the full-length protein showed complete identity with recently described sequences in GenBank. The sequences we used were mVps18 (BC039176), mVps16 (AB056721) and

mVps11 (AK090030), mVam2 (XM\_127209), and mVam6 (XM\_031720). Mouse homologues of *Drosophila* Hook were identified by BLAST searching the mouse EST database. A C-terminal fragment of one contiguous sequence was amplified by PCR and sequenced. This fragment corresponds to the mouse homologue of the human Hook1 isoform (GenBank NM\_015888). The eGFP-Rab5 expressing plasmid was a kind gift from Prof. Phillip Stahl (Washington University, St. Louis, MO) (Roberts *et al.*, 1999).

### Antibodies

PCR fragments encoding regions of mVps11, mVps16, mVps18, mVam2, mVam6, and Hook1 (Figure 1, A and B) were cloned into pGEX (Amersham Biosciences, Piscataway, NJ) or pMAL (New England Biolabs, Beverly, MA) vectors to produce glutathione S-transferase (GST) or maltose binding protein (MBP) fusion proteins (Table 1) according to manufacturers' instructions. When truncated proteins were produced, BL21RIL codon plus cells were used (Stratagene, La Jolla, CA) (Shin *et al.*, 1997) and led to the production of full-length soluble proteins in every instance.

Serum collected from immunized rabbits was exhaustively subtracted by passage over a column made by conjugating a soluble protein fraction derived from bacteria expressing both GST and MBP to Affi-gel10 and Affi-gel 15 Sepharose (Bio-Rad, Hercules CA). Subtracted serum was then passed over a column containing covalently bound recombinant antigen (Table 1), eluted with 100 mM glycine pH 2.5, and then exhaustively dialyzed against phosphate-buffered saline (PBS). Antibodies specific for mVps11, mVps16, and mVam2 were further immunodepleted using saponin-extracted, aldehyde-fixed nuclei.

Antibody specificity was characterized using a series of immunoblotting and immunofluorescent experiments against cultured cell types and tissues. Figure 1 shows the results of immunoblotting mouse B16F10 postnuclear supernatant with each antibody.

Previous studies have defined two splice variants of mVam2, one of which lacks the C-terminal Zn finger domain (McVey Ward *et al.*, 2001). This variant occurs by insertion of an extra exon near the C terminus that introduces a premature stop codon. Although the intron/exon structures of the mouse and human genes are similar, the extra human exon is not conserved in the mouse genome. We did not observe any splice variants (in this region) in the mouse EST database, consistent with previous studies in human cells, showing this is not an abundant transcript (McVey Ward *et al.*, 2001). Our anti-mVam2 antibodies recognized a single band corresponding to full-length mVam2 (Figure 1A). In agreement with Kramer and colleagues (Walenta *et al.*, 2001) our antibodies to mouse Hook1 recognized a single band of ~85 kDa. No cross reactivity was observed with the other smaller Hook isoforms, Hook2 and Hook3 (Walenta *et al.*, 2001).

All other antibodies were characterized previously or were from commercial sources (Table 2) except rabbit polyclonal antibodies to mouse Vti1-b

**Table 1.** Plasmids used to isolate antibodies

Gene	Immunized vector	Plasmid	Purification vector	Plasmid
mVPS18	pGEX	pPL1180	pMAL	pPL1407
mVPS16	pMAL	pPL1244	pMAL	pPL1244
mVPS11	pMAL	pPL1241	pMAL	pPL1699
mVam2	pGEX	pPL1091	pMAL	pPL1479
mVam6	pMAL	pPL1654	pMAL	pPL1654
Hook1	pMAL	pPL1553	pMAL	pPL1553

**Table 2.** Antibodies used

Antigen	Clone/Catalog no.	Antibody	Source
EEA1	(E41120)	MAb	Transduction Laboratories (Lexington, KY)
Syn6	(S5542)	MAb	Transduction Laboratories
hTfR	(H68.4)	MAb	Zymed Laboratories (South San Francisco, CA)
CD63	(RFAC4)	MAb	Biodesign (Saco, ME)
Actin	(JLA20)	MAb	DSHB <sup>a</sup>
$\beta$ -Tubulin	(E7)	MAb	DSHB <sup>a</sup>
LAMP1	(H4A3)	MAb	DSHB <sup>a</sup>
LAMP2	(H4B4)	Mab	DSHB <sup>a</sup>
Lactate dehydrogenase	(HH17)	Mab	Sigma-Aldrich
Syn13	(110 132)	PAb (rabbit)	Synaptic Systems (Goettingen, Germany)
Texas Red anti-rabbit	(T-2767)	PAb (goat)	Molecular Probes (Eugene, OR)
Texas Red anti-mouse	(T-862)	PAb (goat)	Molecular Probes
Alexa 488 anti-rabbit	(A11008)	PAb (goat)	Molecular Probes
Alexa 488 anti-mouse	(A11001)	PAb (goat)	Molecular Probes
Cy5 anti-rabbit	(611 110122)	PAb (goat)	Rockland (Gilbertsville, PA)
Cy5.5 anti-mouse	(610 713124)	PAb (goat)	Rockland

<sup>a</sup> DSHB, Developmental Studies Hybridoma Bank, (University of Iowa, Iowa City, IA).

(amino acids 1–207), Vamp7 (amino acids 1–180), which were raised against GST fusion proteins and affinity purified essentially as described for antibodies to mouse Vps proteins above. Plasmids encoding mouse Vti1-b were a kind gift from Dr. Gabrielle Fischer Von-Mollard (University of Oregon, Eugene, OR). Antibodies to Syn7 (rabbit and goat) and mVps45 were described previously (Tellam *et al.*, 1997; Mullock *et al.*, 2000).

**Cell culture** Vero, CHO and NRK cells (ATCC number CCL-81, CCL-61 and CRL-6509) were cultured using standard conditions. For transient transfections Vero cells were seeded at  $5 \times 10^5$  cells/well into 6 well plates (PGC Scientifics, Frederick, MD) containing a sterile glass coverslip and allowed to grow for a further 24 h before transfection. Transfection was performed in 1 ml of serum-free media containing 5  $\mu$ g of plasmid DNA/well as detailed (in Poupon *et al.*, 2003) and fixed 24 h after transfection.

### Immunofluorescence Localization

Cells grown on coverslips were fixed using immersion in methanol ( $-20^\circ\text{C}$  for 5 min) or 2% (wt/wt) paraformaldehyde (20 min at  $25^\circ\text{C}$ ). Aldehyde-fixed cells were quenched and permeabilized (0.2% [vol/vol] Triton X-100 and 50 mM glycine in PBS pH 7.2 [5 min at  $25^\circ\text{C}$ ]). Saponin extraction before fixation was performed as follows. After washing three times in PBS, cells were immersed in 0.05% (wt/vol) saponin, 80 mM PIPES pH 6.5, 5 mM EGTA, and 1 mM  $\text{MgCl}_2$  (60 s at  $25^\circ\text{C}$ ), washed once quickly with PBS, and fixed. Cells were then incubated in 2% (vol/vol) goat serum in PBS (60 min at  $25^\circ\text{C}$ ) and then with primary and secondary antibodies.

### Quantitation

Quantitation was performed using the program "CoolLocalizer" (version 1.0.7; CytoLite, Stockholm, Sweden). Tagged image files depicting confocal micrographs were analyzed by setting the region of interest to include whole, saponin-extracted, individual cells immunostained with the stated markers. Cells that met the above criteria were then selected for analysis at random. The colocalization of markers (percentage) was defined by multiplying the Pearson's coefficient by 100. During each analysis  $n = 5$  Vero cells.

### Cell Fractions

Preconfluent (95%) cultures in 150-mm<sup>2</sup> dishes were washed three times with PBS and scraped into HES buffer (20 mM HEPES pH 7.2, 1 mM EDTA, 250 mM sucrose, 100 mM NaCl containing 1 mM phenylmethylsulfonyl fluoride and 5 $\times$  Complete EDTA free protease inhibitors cocktail; Roche Diagnostics, Mannheim, Germany) prechilled to  $4^\circ\text{C}$ . Postnuclear supernatant (PNS) was made by centrifuging cell lysates (4000  $\times g$  for 5 min) prepared by passage (10 $\times$ ) through a 21-gauge needle. The supernatant was used to make crude cell membrane and crude cytosolic fractions by centrifuging the PNS at 100,000  $\times g$  (60 min at  $4^\circ\text{C}$ ). Detergent-solubilized membranes were made by resuspending crude membrane fractions in HES containing 1% 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate (CHAPS) and 100 mM NaCl (5 min at  $25^\circ\text{C}$ ) followed by incubation for (30 min  $4^\circ\text{C}$ ) before centrifugation at 17,000  $\times g$  (10 min at  $4^\circ\text{C}$ ) (Wade *et al.*, 2001). Nuclei isolated from the initial low-speed pellet were layered on top of a 2.2 M sucrose cushion and subjected to 30,000 rpm in an SW60 rotor for 60 min at  $4^\circ\text{C}$ . The pellet was saponin extracted, paraformaldehyde fixed, and blocked in serum as described pre-

viously. Bovine brain cytosol was prepared as described previously (Wattenberg and Rothman, 1986).

### Sedimentation Experiments

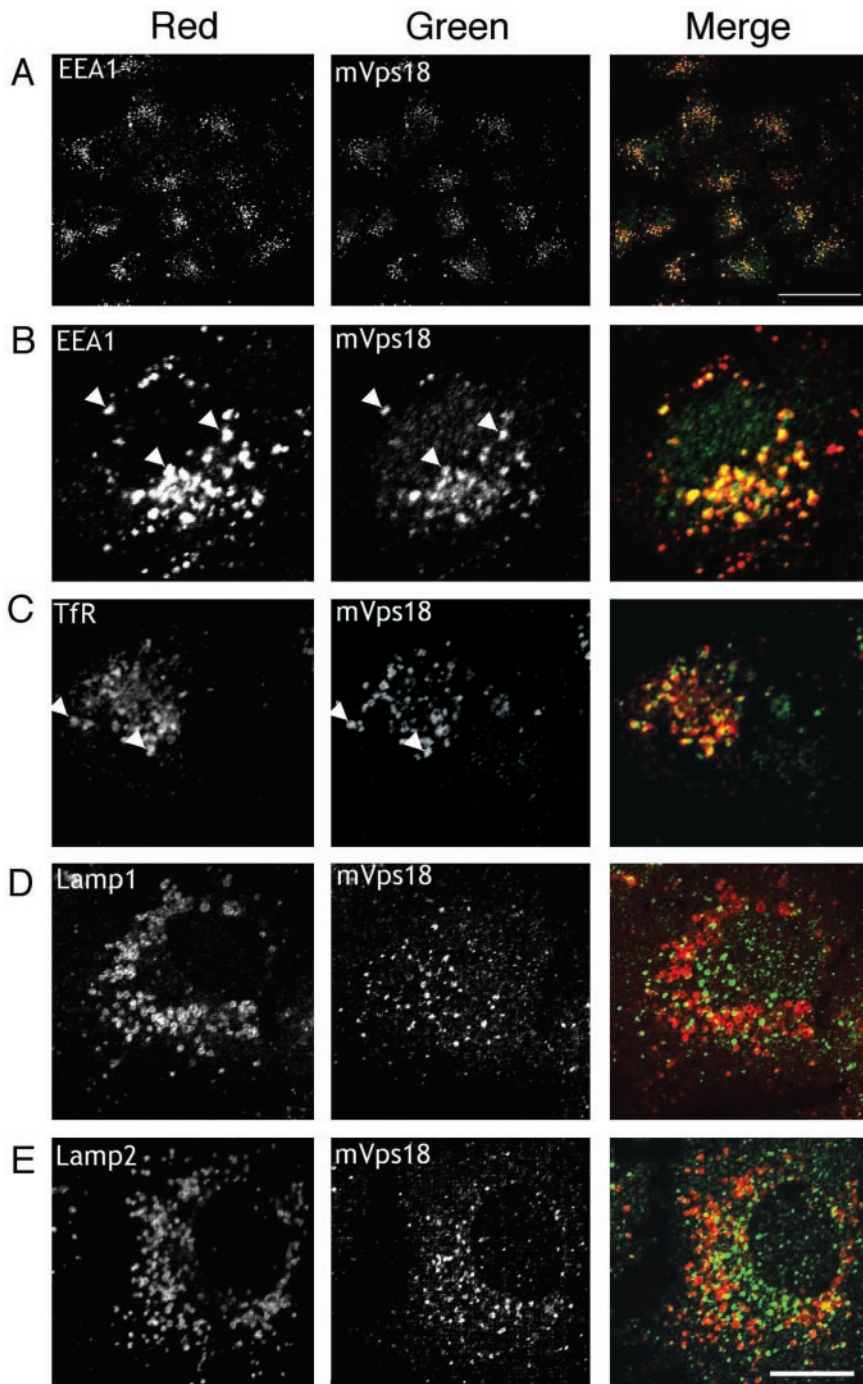
Microtubules were assembled using the CytoDYNAMIX kit according to manufacturer's instructions (Cytoskeleton, Denver, CO). Bovine brain cytosol (2 mg) was incubated with 5  $\mu$ g of taxol-stabilized microtubules (30 min at  $37^\circ\text{C}$ ). The microtubule fraction was then collected after centrifugation through a sucrose cushion as described previously (Walenta *et al.*, 2001) and subjected to immunoblot analysis. Actin polymerization was performed using 2 mg of bovine brain cytosol, 2 mM ATP, 20 nM phalloidin, and 100  $\mu\text{M}$  nocodazole (all from Sigma-Aldrich) as described previously (Fucini *et al.*, 2000). Polymerized actin was isolated and washed after centrifugation as described previously (Fucini *et al.*, 2000) and subjected to immunoblot analysis.

### Immunoprecipitation

Polyclonal antibody-saturated protein A agarose (Invitrogen, Carlsbad, CA) was cross-linked using dimethyl pimelimidate (Accoceberry *et al.*, 2001). Beads (50  $\mu$ l) were added to detergent-solubilized (CHAPS) membrane fractions (1.2 mg) containing 100 mM NaCl in a volume of 400  $\mu$ l. After incubation at  $4^\circ\text{C}$  for 3 h on a shaking platform, beads were collected by centrifugation (21,000  $\times g$  for 2 min at  $25^\circ\text{C}$ ) and washed three times in 0.5 $\times$  HES (containing 100 mM NaCl) and 0.5 $\times$  PBS. Beads were eluted twice (sequentially) with 25  $\mu$ l of 100 mM glycine pH 2.5. Eluate was combined with Laemmli sample buffer, the pH neutralized (10  $\mu$ l of 1 M Tris pH 7.0), and analyzed by immunoblotting. Where appropriate, 2-mercaptoethanol (10% vol/vol) was used. Human IgG (Sigma-Aldrich) was added to the blocking reagent (5% [wt/vol] nonfat dried milk in PBS containing 0.01% [vol/vol] Tween 20) at a final concentration of 1 mg/ml for immunoblotting.

### Endosome Fusion Assay

Two 150-mm<sup>2</sup> dishes of CHO cells (80% confluent) were washed with PBS (containing 1 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , and 10 mM glucose) and were allowed to endocytose either biotinylated rabbit IgG or avidin-conjugated alkaline phosphatase (Av-ALP) for 5 min at  $37^\circ\text{C}$ . Cells were washed twice in cold PBS pH 5.0 and then three times in cold PBS. Cell were lysed (as described above) in 250 mM sucrose, 3 mM imidazole pH 7.0, 5 $\times$  Complete EDTA free protease inhibitors cocktail (Roche Diagnostics). PNS preparations were then incubated on ice in the presence or absence of antibodies for 20 min. Reactions were then initiated by mixing both treated PNS fractions with bovine brain cytosol and an ATP-regenerating system (10 mM ATP, 1 M creatine phosphate, and 3.7 mg/ml creatine phosphokinase, 20 mM PIPES pH 6.8) in triplicate. Fusion reactions also contained 60  $\mu$ g/ml biocytin to prevent nonspecific formation of IgG-enzyme complexes. Fusion was allowed to proceed at  $37^\circ\text{C}$  and then stopped with the addition of 0.1 volume 10% (vol/vol) Triton X-100 in PBS. Immune complexes were captured on 50  $\mu$ l of protein A agarose (Invitrogen, Carlsbad, CA). Enzyme activity was measured kinetically by conversion of paranitrophenol at 405 nm (Gorvel *et al.*, 1991).



**Figure 2.** Immunolocalization of mVps18 in Vero cells. Vero cells were fixed, permeabilized, and labeled with polyclonal anti-mVps18 antibodies together with Texas Red-conjugated goat anti-rabbit (labeling anti-mVps18) secondary and double labeled with monoclonal antibodies to EEA1 (A and B), TfR (C), LAMP1 (D), or LAMP2 (E) together with Alexa488-conjugated secondary antibody and visualized by confocal microscopy. Cells labeled for EEA1 and TfR (A–C) were fixed in aldehyde, whereas cells labeled for LAMPs were fixed with methanol (D and E). Arrowheads denote examples of colocalization between vesicles positive for mVps18 and either EEA1 or TfR (B and C). Bar, 5  $\mu\text{m}$  (top); 10  $\mu\text{m}$  (other).

## RESULTS

### *Endogenous mVps18 Localizes to Early Endosomes*

Polyclonal antibodies raised to recombinant protein fragments of mouse isoforms of mammalian (m)Vps18, mVps16, mVps11, mVam2, mVam6, and Hook1 (Figure 1A) were used in a series of immunolocalization experiments (Figures 2–6). The yeast class C Vps proteins Vps18/Pep3p and Vps11/Pep5p localize and function on the vacuolar membrane (Rieder and Emr, 1997; Wang *et al.*, 2003). This suggests that the mammalian counterparts might localize to and function in late-endocytic compartments. Previous studies indicate that mVps18 colocalizes with several endocytic

markers (Kim *et al.*, 2001). Surprisingly, we found that anti-mVps18 clearly labeled compartments positive for the early sorting endosomal marker early endosome antigen-1 (EEA1) in Vero cells (Gaulhier *et al.*, 1999) (Figure 2, A and B). Localization of mVps18 to EEA1-positive compartments was observed in cells fixed with either aldehyde or methanol (Figures 2, A and B, and 3F, respectively), and anti-mVps18 antibody labeling was abolished when the recombinant antigen, but not GST or MBP alone, was included during the labeling procedure (our unpublished data). Morphometric analysis of several micrographs ( $n = 5$ ) showed that  $87 \pm 4\%$  of pixels positive for mVps18 were also positive for EEA1. Interestingly, there was a subset of EEA1-positive compart-

ments that did not have appreciable mVps18 labeling. There was some colocalization with transferrin receptor (TfR) that labeled a broader pattern of endosomal compartments (Figure 2C). However, there was little immunolocalization of mVps18 upon late endocytic structures (Figure 2, D and E) positive for LAMP1, LAMP2 (Gough *et al.*, 1999), or CD63 (Metzelaar *et al.*, 1991) (our unpublished data). We found  $32 \pm 12\%$  were positive for TfR labeling, whereas only  $6 \pm 2.9\%$  of pixels positive for mVps18 were also positive for CD63.

We performed additional experiments on normal rat kidney (NRK) cells and again found colocalization between mVps18 and EEA1 (Figure 3A). mVps18 also localized diffusely through the cell and likely reflects the larger cytosolic pool of protein in this cell type ( $\sim 60\%$  total by fractionation; our unpublished data). We also found that mVps18 showed little colocalization with endocytosed dextran (1-h pulse/4-h chase) that was used to label Lgp120-positive late endosomal structures (Figure 3, B and C). To better assess the relative distribution of mVps18 between early and late endosomes, we performed triple labeling experiments using anti-EEA1 and anti-mVps18 antibodies in Vero cells that had internalized Texas Red-conjugated bovine serum albumin (BSA) to late endosomes (1-h pulse/4-h chase) (Figure 3E). Antibodies to mVps18 showed high levels of colocalization with EEA1 but not with late endosomal BSA.

Although we used polyclonal antibodies raised to a large region of mVps18, we were not certain whether these antibodies recognized a small subpopulation of mVps18 proteins by immunofluorescence. Therefore, we analyzed the distribution of a functional eGFP-mVps18 fusion protein characterized previously (Poupon *et al.*, 2003) (Figure 3F). In transient transfection experiments, eGFP-mVps18 reaches very high levels after 48 h whereupon significant clustering of late endosomal compartments is observed (Poupon *et al.*, 2003). After 24 h (posttransfection) before major clustering is observed, we found eGFP-mVps18 on EEA1-positive vesicles in NRK cells. In previous studies, the ability of mVps18 or mVam6 overexpression to cause clustering of late endosomal compartments supported the idea that these proteins function in part to tether late endosomal compartments together (Caplan *et al.*, 2001; Poupon *et al.*, 2003). Although those studies observed minor clustering of early endosomal compartments in NRK cells, we found that overexpression of eGFP-mVps18 in Vero cells did result in clusters of endosomes that contained both EEA1-positive and LAMP1-positive compartments (Figure 3F). Interestingly, the structures that contained each of these markers were still discrete but clustered within the same region of the cell, suggesting that under these conditions, mVps18 overexpression could cluster both early and late endosomes (Figure 3F).

#### **Endogenous mVps11 and mVps16 Localize to Early Endosomes in Permeabilized Cells**

The data mentioned above indicate that mVps18 may control aspects of early endosomal traffic and are consistent with recent genetic studies in yeast indicating that Vps18p, Vps11p, and Vps16p may have additional roles earlier in the endocytic pathway besides fusion to the vacuole (Srivastava *et al.*, 2000; Peterson and Emr, 2001). Furthermore, fractionation studies in yeast indicate that the class C proteins fractionate more like the endosomal-localized Pep12 marker protein rather than completely as a vacuolar protein (Rieder and Emr, 1997). To further test this hypothesis, we next used antibodies to mVps16 and mVps11 to determine whether these proteins also localized to early endosomes. Preliminary experiments showed the localization of these proteins

to EEA1-positive compartments; however, a large cytosolic pool of these proteins obscured visualization of the membrane associated pool. Therefore, saponin permeabilization before fixation was used to deplete the cytosolic pool of each protein. In saponin-permeabilized cells, mVps18 was still localized to EEA1-positive compartments (Figure 4A) and verified that cell morphology as well as the localization of mVps18 was not altered by saponin treatment. Using this method of fixation, we found that both mVps16 (Figure 4B) and mVps11 (Figure 4C) also localized predominantly to EEA1-positive early endosomal structures that were distinct from late endosomal and lysosomal compartments positive for LAMP1 or CD63 (Figure 4, D and E). We also observed a nonvesicular, filamentous pattern for mVps18, mVps16, and mVps11 antibodies toward the periphery of the cell (Figure 4, A and B, open arrows), which was further analyzed in subsequent experiments (see below).

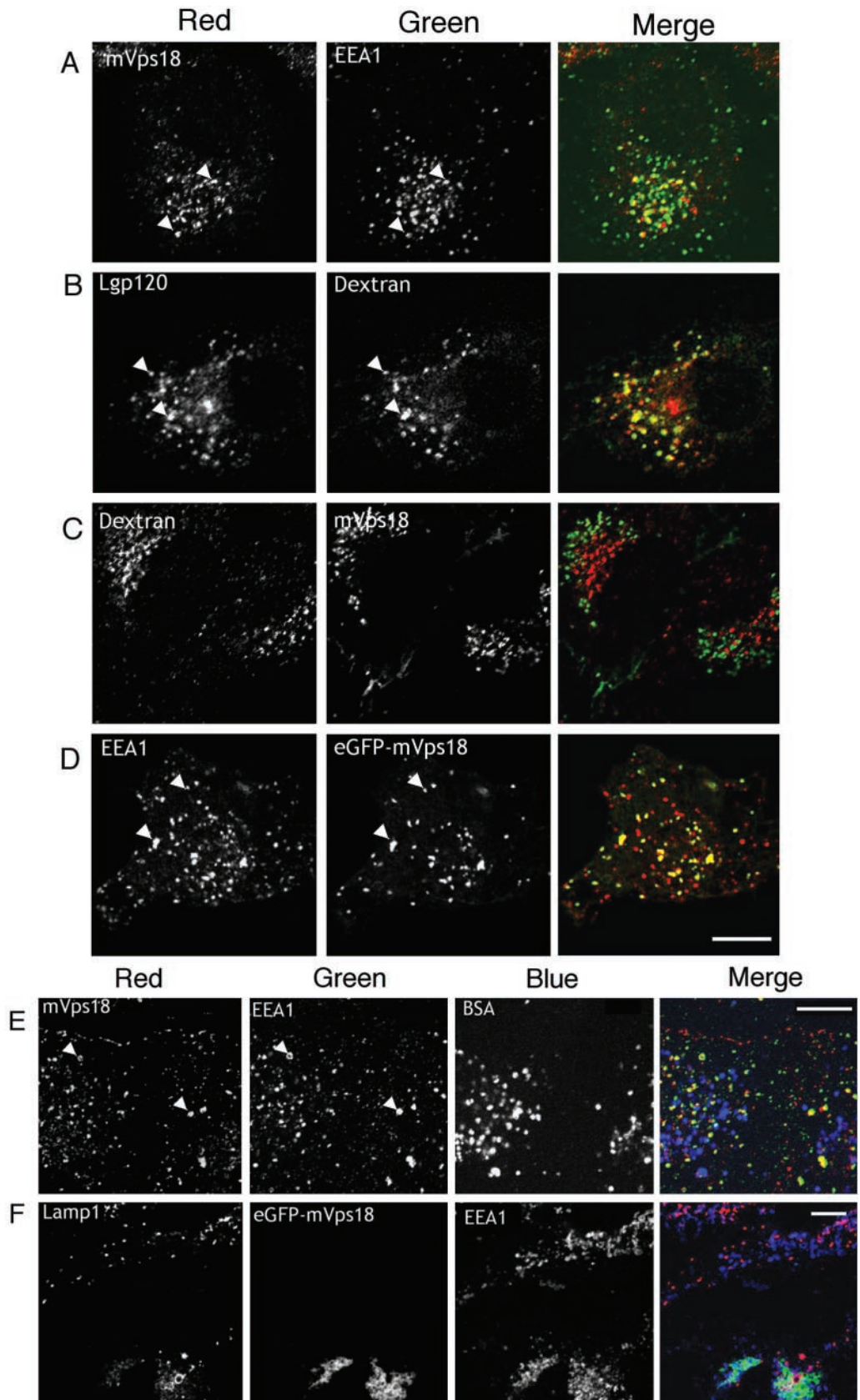
#### **Immunolocalization of mVam2**

Having localized the mammalian class C Vps proteins to early endocytic structures, we were interested in the intracellular distribution of mVam2, because its yeast counterpart, Vam2p/Vps41p, physically interacts with the yeast class C Vps proteins (Price *et al.*, 2000; Seals *et al.*, 2000).

Figure 5 shows saponin-permeabilized cells labeled with antibodies specific to mVam2 and counterlabeled with monoclonal antibodies specific for LAMP1 (Figure 5A) and EEA1 (Figure 5B). Here again, although we found only low levels of mVam2 localizing with late endosomal markers, we found clear instances of localization with EEA1. Additionally, we found that mVam6 antibodies also labeled numerous filamentous structures (Figure 5, A and B). These were much more prominent when we used aldehyde fixation (e.g., EEA1; Figure 5B) than when we used methanol fixation (e.g., labeling with LAMP1). Because the predominantly vesicular pattern of mVam2 immunolabeling in methanol-fixed cells could not be compared with that of aldehyde-fixed cells, we performed additional localization experiments between mVam2 and GFP-Rab5 introduced by transient transfection (Figure 5, C and D). As expected, both mVam2 (Figure 5C) and mVps18 (Figure 5D) colocalized with GFP-Rab5. Although, a significant amount of the GFP-Rab5 did not localize with mVam2- or mVps18-positive compartments, the level we observed is consistent with the localization of the latter proteins with EEA1, which also localizes with only a portion of Rab5-positive structures (Roberts *et al.*, 1999). Together, these results indicate that at least a portion of mVam2 also localizes with early endosomal compartments.

#### **Mammalian HOPS Proteins Associate with the Cytoskeleton**

Although the filamentous structures for mVam2 observed after aldehyde fixation were prominent, they were also observed in methanol-fixed cells. In addition, inspection of either intact or saponin-permeabilized cells showed filamentous patterns for mVps18, mVps16, and mVps11 labeling, especially toward the basal surface of the cells (Figure 4, A and B, open arrows; our unpublished data), and could clearly be seen when fluorescence signals from areas relatively devoid of vesicular structures were amplified (Figure 6, A and B). To determine whether these filamentous structures corresponded to particular cytoskeletal elements, we performed a series of double labeling experiments by using anti-mVps18, anti-mVps16, anti-Vps11, and anti-mVam2 antibodies in concert with the fungal toxin phalloidin (conjugated with Alexa Fluor448) to label actin filaments, or with



**Figure 3.** Localization of mVps18 to early endosomes in NRK and Vero cells. NRK cells were aldehyde fixed and immunolabeled for mVps18 (red) and EEA1 (green) together with anti-rabbit Texas Red- and Alexa488-conjugated anti-mouse. Arrowheads highlight examples of EEA1-mVps18 colocalization (A). To label late endosomal/lysosomal compartments, NRK cells were allowed to endocytose Texas

a monoclonal anti- $\alpha$  tubulin antibody to label microtubules (Figure 6, C–F). We then focused on cellular regions where the filamentous pattern was more discernible. These data showed that apart from their vesicular localization, the class C Vps protein mVps16 (Figure 6C) (and mVps18 and mVps11; our unpublished data) colocalized with actin filaments. No colocalization was observed between mVps16 (as well as mVps18 and mVps11; our unpublished data) and microtubules (Figure 6D). In contrast, mVam2 did not colocalize with phalloidin labeled actin filaments (Figure 6E). Rather, mVam2 was localized to microtubules (Figure 6F).

We then used an *in vitro* binding assay to verify that the class C Vps proteins (mVps16 and mVps18) associated specifically with phalloidin polymerized actin filaments (Figure 6H), whereas mVam2 and mVam6 associated with taxol-stabilized microtubules (Figure 6G). Microtubules were polymerized *in vitro*, incubated in bovine brain cytosol, and collected by centrifugation before analyzing protein recruitment from cytosol by immunoblotting. Actin filaments were generated by adding phalloidin and ATP to bovine brain cytosol and collected by centrifugation. Consistent with previous results, we found that mammalian Hook1 also associated with microtubules and interestingly also associated with actin filaments. The cytosolic lactate dehydrogenase enzyme was included to show that the association was specific and not due to contamination by cytosolic proteins. Thus, by both immunolocalization experiments and *in vitro* binding experiments we find that components of the mammalian HOPS complex differentially associated with both the actin and microtubule cytoskeleton.

### Proteins Interacting with mVps18

Previous studies have established that mVps18 can be coimmunoprecipitated in a complex with mVps16 and mVps11 (Kim *et al.*, 2001). To confirm this interaction and to investigate further protein interactions, antibodies specific to either mVps18, Hook1, or GST were used to perform immunoprecipitation experiments from a rat liver membrane fraction solubilized in 1% CHAPS. From this fraction, we were able to isolate between 20 and 30% of the total mVps18 in this fraction, which was sufficient for us to detect at least some of the potential interactions of mVps18. Consistent with previous results (Kim *et al.*, 2001) mVps16 coimmunoprecipitated with mVps18 as did Syn7 (Figure 7) and mVps11 (our unpublished data). We also found that similar to the yeast HOPS proteins (Price *et al.*, 2000), mVps18 also associated with mVam2 and mVam6. This is consistent with previous

results showing that over expressed eGFP-mVps18 also associated with mVam6 and mVps11 (Poupon *et al.*, 2003).

Based on previous studies showing the interaction of *Drosophila* Vps18 (Deep Orange) with the protein Hook (Kramer and Phistry, 1999; Sevrioukov *et al.*, 1999; Narayanan *et al.*, 2000), we also examined whether mVps18 could associate with the mammalian Hook1 isoform, which has a similar localization pattern as mVam2. Figure 7 shows that Hook1 was specifically coimmunoprecipitated with anti-mVps18 antibodies and that mVps18 was coprecipitated with anti-Hook1 antibodies.

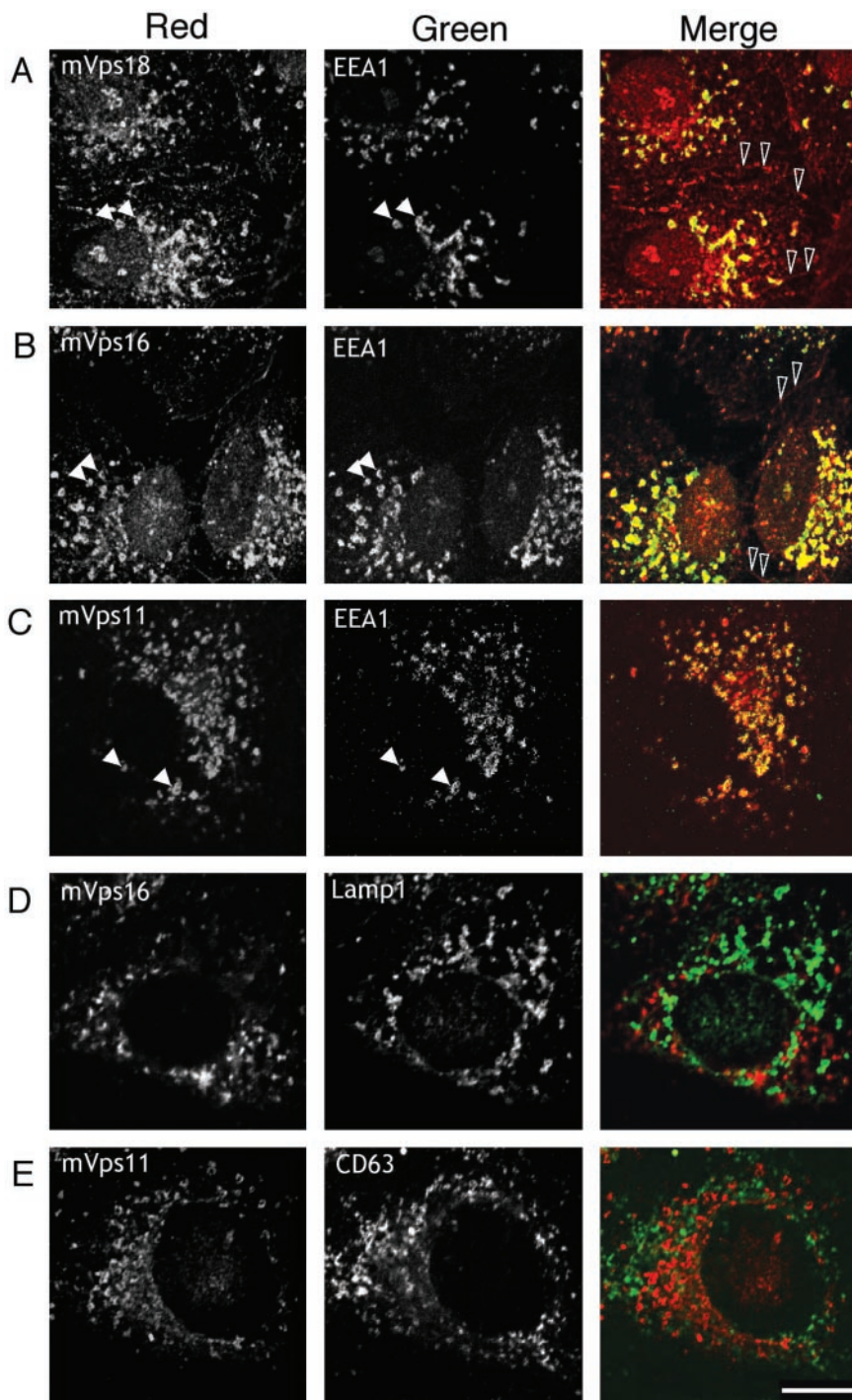
We also found that aside from its interaction with Syn7, mVps18 also interacted with Vti1-b, another t-SNARE light chain that functions with Syn7 to effect late endosomal fusion (Mullock *et al.*, 2000; Wade *et al.*, 2001; Antonin *et al.*, 2002). In addition, we found that mVps18 also associated with Syn13, a heavy chain t-SNARE, and the t-SNARE light chain Syn6, both of which are involved in early endosome fusion (Mills *et al.*, 2001; Zerial and McBride, 2001). Separate blotting experiments were performed to show that the Syn13 antibodies did not cross react with Syn7, which migrated slightly faster during SDS-PAGE (our unpublished data). These results are consistent with the localization of mVps18 to EEA1/Rab5-positive endosomes. Interestingly, mVps18 did not coimmunoprecipitate with EEA1, another putative tethering factor that associates with Syn13 and Rab5 (Christoforidis *et al.*, 1999). We also found that mVps18 associated with the S/M protein mVps45. Thus, in addition to its association with another S/M protein mVps33a (Kim *et al.*, 2001), mVps18 seems to be able to form a complex with more than one S/M protein. The interaction of mVps18 with mVps45 is consistent with a function in early endosomes because mVps45 binds to Syn6, Syn13, and Rabenosyn 5, all effectors of early endosome fusion (Tellam *et al.*, 1997; Nielsen *et al.*, 2000).

We also found that mVps18 also associated with Vamp8, a v-SNARE that participates in both early endosomal and late endosomal homotypic fusion events (Prekeris *et al.*, 1999; Wade *et al.*, 2001; Antonin *et al.*, 2002). Interestingly, Vamp7 did not coimmunoprecipitate with mVps18, indicating that mVps18 may not participate directly in Vamp7-dependent fusion events (McVey Ward *et al.*, 2000; Bogdanovic *et al.*, 2002). These combined data suggest that an mVps18-containing complex of proteins can bind to multiple SNAREs, most likely a *cis*-SNARE complex, which stands in contrast to studies in yeast that indicate Vps18 binds exclusively to uncomplexed Syntaxin (Vam3p) (Sato *et al.*, 2000). However, yeast Vam2 and Vam6 have been found to associate with SNARE complexes, indicating that mVps18 might associate with SNAREs in the context of its larger HOPS complex (Price *et al.*, 2000).

### A Function for mVps18 in Early Endosomal Fusion

Having immunolocalized mVps11, mVps16, and mVps11 to early endosomes and coimmunoprecipitated mVps18 with early endosomal fusion machinery, we assessed whether mVps18 could play a role in early endosome fusion by using an *in vitro* assay (Gorvel *et al.*, 1991). We loaded endosomes from two populations of CHO cells with a 5-min pulse of either IgG-Biotin or Av-ALP. We then assayed the effects of specific antibodies upon content mixing that was allowed to proceed for 30 min at 37°C *in vitro* in the presence of cytosol and ATP before isolation and quantitation of IgG-ALP complexes. We confirmed that mVps18 localized to EEA1-positive compartments in this cells type (Figure 8A). Immunolocalization experiments showed that Av-ALP complexes were resident on mVps18-positive compartments after a

**Figure 3 (cont).** Red-conjugated dextran for 60 min followed by a 4-h chase in the presence of Leupeptin (200  $\mu$ m) before methanol fixation and labeling with anti-Lgp120 (B) or anti-Vps18 (C). NRK cells expressing eGFP-mVps18 at 24 h posttransfection were counterimmunolabeled with anti-EEA1 (D) monoclonal antibodies together with Texas Red-conjugated anti-mouse secondary antibodies. Bar, 10  $\mu$ m. (E) Vero cells were allowed to endocytose Cy5-conjugated BSA for 60 min followed by a 4-h chase in the presence of leupeptin (200  $\mu$ m) (E). Cells were aldehyde fixed and immunolabeled with anti-mVps18 and Texas Red-conjugated anti-rabbit antibodies and with anti-EEA1 and Alexa488-conjugated anti-mouse secondary antibodies. The merged image shows lysosomal BSA in blue, EEA1 in green, and mVps18 in red. Yellow areas depict colocalization between mVps18 and EEA1 (examples are also highlighted with arrowheads). Bar, 10  $\mu$ m. (F) Vero cells were transiently transfected with an eGFP-mVps18 expressing plasmid and methanol-fixed and labeled with monoclonal anti-LAMP1 (Texas Red) and polyclonal anti-EEA1 (Cy5). Bar, 10  $\mu$ m.



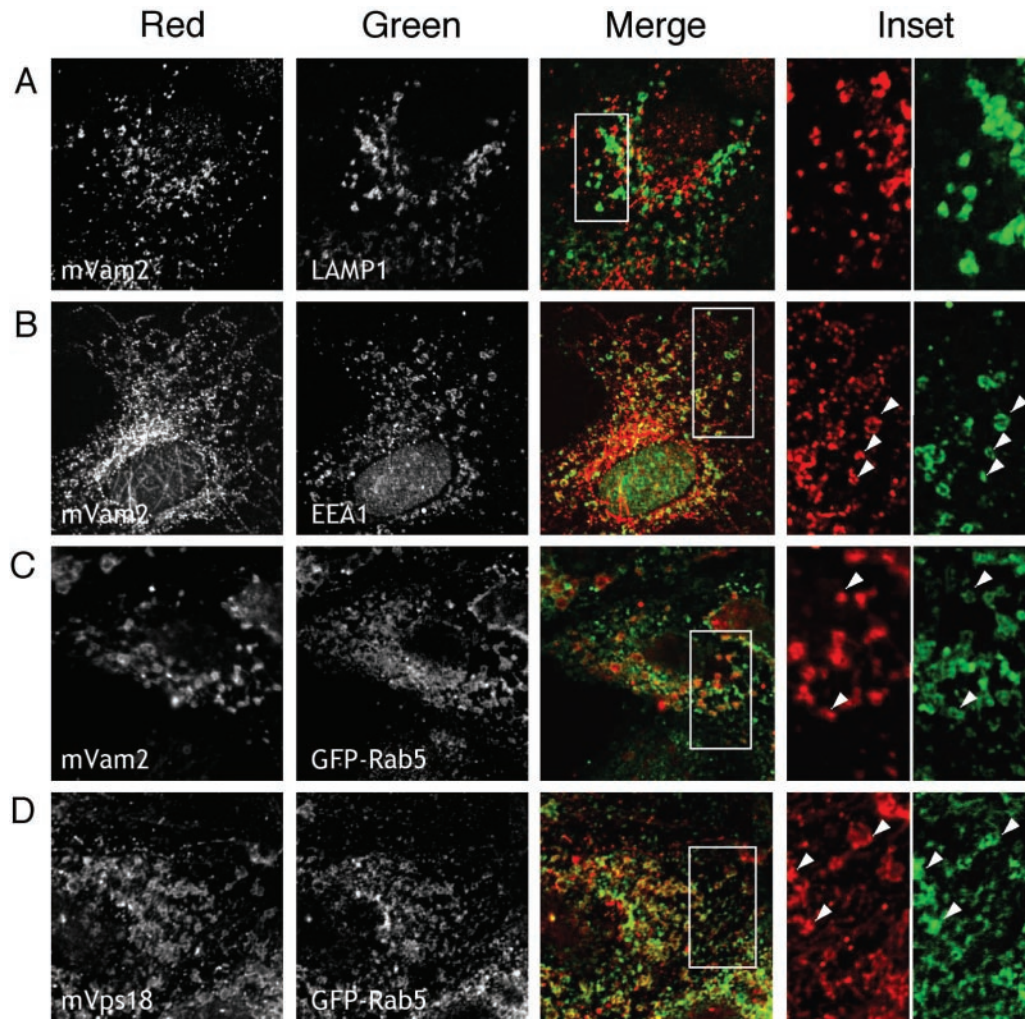
**Figure 4.** Immunolocalization of mammalian Vps11 and Vps16 in permeabilized cells. Vero cells were permeabilized with saponin and fixed before immunolabeling with antibodies to mVps18 (A), mVps16 (B), or mVps11 (C) together with Texas Red-conjugated goat anti-rabbit secondary antibodies. Cells were colabeled with antibodies to EEA1 (A-C), LAMP1 (D), and CD63 (E), as indicated together with Alexa488-conjugated goat anti-mouse secondary antibodies. Filled arrowheads (A–C) denote examples of colocalization between mVps18, mVps16, mVps11, and EEA1. Some of the filamentous pattern observed for the class C Vps proteins is indicated by open arrowheads (A and B). Bar, 10  $\mu\text{m}$ .

5-min chase (Figure 8B). Figure 8, C and D, also confirmed that a 5-min pulse of endocytosed biotinylated IgG colocalized with EEA1 and TfR (closed arrows).

After the fusion reaction was complete,  $\sim 30\%$  of the total ALP in endosomes was found within the IgG–ALP complex indicating that  $\sim 30\%$  of the endosomes had fused. The reaction was temperature and ATP dependent because reactions maintained at  $0^\circ\text{C}$ , or that contained EDTA or that did not contain the ATP-regenerating system had little content mixing (Figure 8E). Antibodies specific to mVps18 caused a significant inhibition of content mixing (Figure 8E),

whereas the addition of anti-GST antibodies, or nonspecific antibodies to  $600 \mu\text{g/ml}$ , had no effect on the fusion assay. Inclusion of polyclonal antibodies to Syn6 also gave significant inhibition, consistent with previous studies showing a role for the t-SNARE complex of Syn13 and Syn6 in catalyzing early endosomal fusion events in vitro (McBride *et al.*, 1999, Mills *et al.*, 2001). These same anti-Syn6 antibodies did not inhibit a late endosome heterotypic fusion assay with lysosomes (B. Mullock, P. Pryor, and J.P. Luzio, unpublished data), further indicating that the assay we used was specific for early endosome fusion. Separate experiments demon-





**Figure 5.** Immunolocalization of mVam2. Saponin-permeabilized Vero cells were methanol fixed and immunolabeled with anti-mVam2 (A and B) and anti-LAMP1 (A) or anti-EEA1 (B) antibodies, together with Texas Red-conjugated anti-rabbit and Alexa488 anti-mouse secondary antibodies. Both the red and green channels from the inset designated in the merged image are shown on the far right. Note the filamentous pattern of mVam2 labeling present in aldehyde-fixed cells (B) but less prominent in methanol-fixed cells (A). Rab5 colocalization with both mVam2 (C) and mVps18 (D) was done by transiently expressing eGFP-Rab5. Bar, 10  $\mu\text{m}$  (except for micrographs from inset).

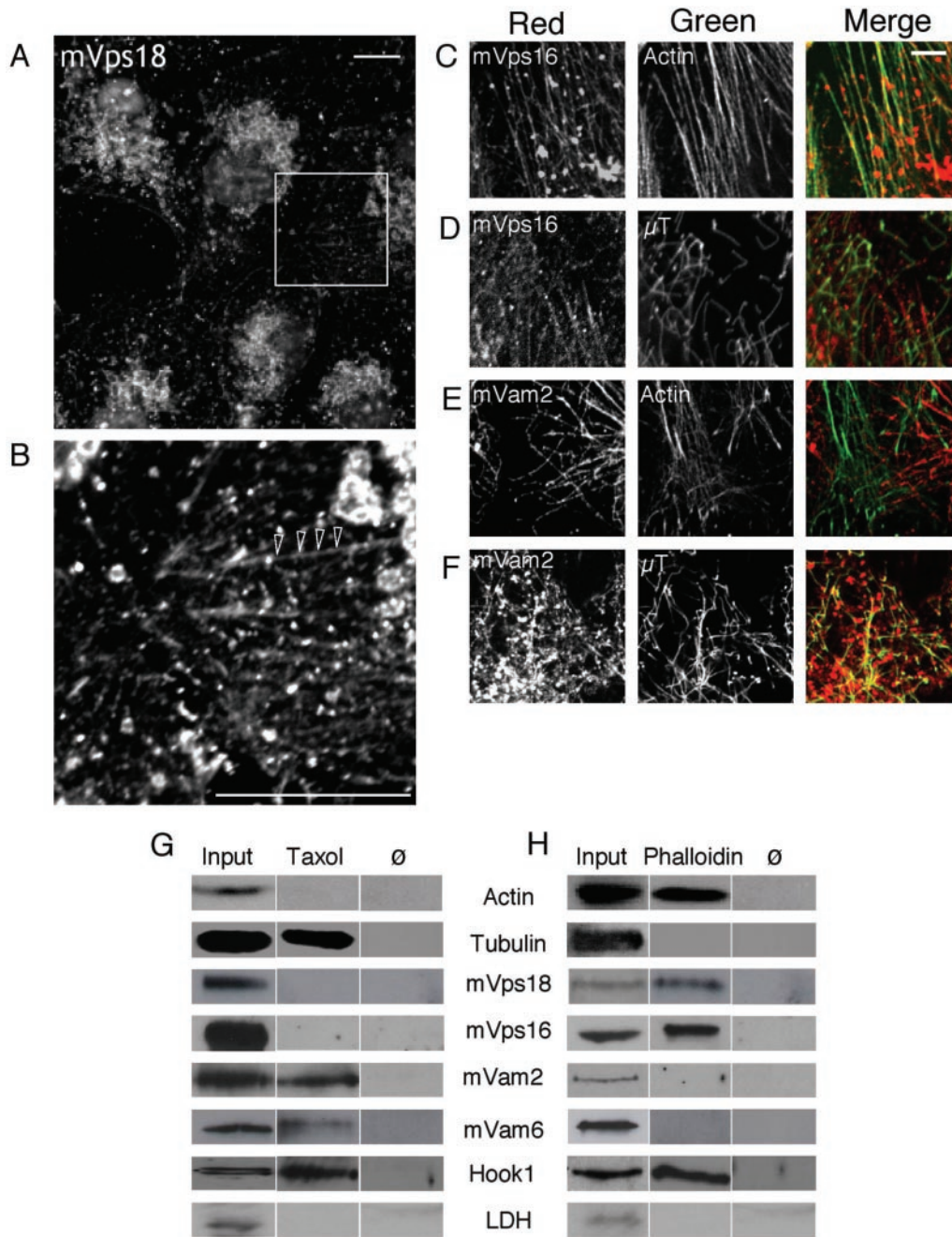
strated that this early endosome fusion assay was resistant to a batch of polyclonal anti-Syn7 antibodies previously shown to inhibit a late endosome/lysosome fusion assay (Mullock *et al.*, 2000; our unpublished data). These data show that antibodies specific to mVps18 specifically inhibit content mixing between early endosomes in a Syn6-dependent and Syn7-independent insensitive *in vitro* fusion assay.

## DISCUSSION

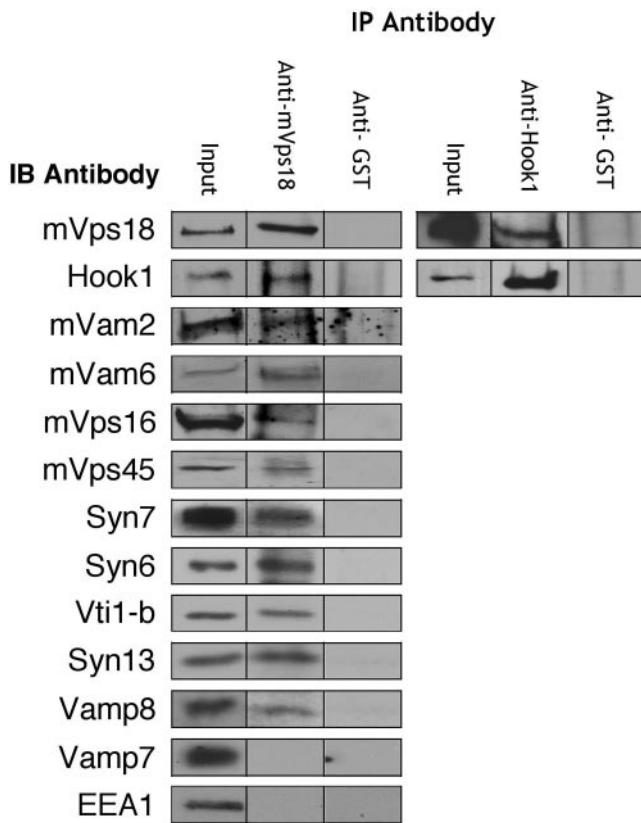
In addition to its proposed role during late endosomal fusion (Poupon *et al.*, 2003), our data indicate a role for mVps18 in early endosomal biogenesis. We found that a significant proportion of the class C Vps proteins, as well as mVam2, localize to EEA1- and Rab5-positive endosomes under a variety of fixation conditions and within a variety of cell types. This localization need not exclude a direct role for these factors in late endosome fusion. Indeed, the localization of these proteins to earlier compartments in the endosomal system could indicate where these factors are initially loaded onto membranes. These proteins would then pro-

gram regions of the early endosome for fusion with late endosomes after the separation of this subdomain by endosome maturation or the formation of endosomal carrier vesicles (Gruenberg *et al.*, 1989; Stoorvogel *et al.*, 1993). The SNARE requirements for this fusion event are not yet defined but would be predicted to include many of those that we found associated with mVps18.

The model that the HOPS proteins are involved in early endosome fusion events is not only supported by their localization but also the association of mVps18 with known effectors of early endosome fusion that include Syn6 and Syn13 (Prekeris *et al.*, 1998; Simonsen *et al.*, 1999). Furthermore, mVps18 also associated with the S/M protein mVps45, which has also been implicated in control of early endosome fusion (Tellam *et al.*, 1997; Nielsen *et al.*, 2000) and represents the second S/M protein (besides Vps33a) to which mVps18 can associate (Kim *et al.*, 2001). In addition, we find that under certain conditions, overexpression of eGFP-mVps18 induces clustering of not only late endosomal compartments but also early endosomes. Previous studies have implicated a role for mVps18 in late endosome fusion



**Figure 6.** Cytoskeletal localization of the mammalian late Vps proteins. Vero cells were methanol fixed and labeled with anti-mVps18. A field at low magnification (A) and a field at high magnification (B), designated by the box is shown. The fluorescence signal was intensified in the high-power field to visualize the filamentous pattern that could be discerned in addition to the vesicular-like pattern (open arrowheads). Bar, 10  $\mu$ m (top); 10  $\mu$ m (bottom). Saponin-permeabilized Vero cells were also aldehyde fixed and labeled with polyclonal antibodies to mVps16 (C and D), or mVam2 (E and F). Cells were counterlabeled for actin filaments with Alexa488-conjugated phalloidin (C and E) or microtubules (D and F) by using monoclonal anti-tubulin antibodies together with Alexa488-conjugated goat anti-mouse secondary antibodies. Anti-mVps16 colocalized to actin filaments, whereas mVam2 colocalized with microtubules. Bar, 5  $\mu$ m. Micrographs were taken of cellular subregions where the filamentous pattern could be clearly discerned. (G) Bovine brain cytosol (2 mg) was mixed with taxol-stabilized microtubules (Taxol) or unpolymerized tubulin ( $\emptyset$ ). Samples were incubated at 37°C for 30 min. Pellets obtained after centrifugation at 100,000  $\times$  g were immunoblotted along with an 20% equivalent sample of the total input protein before centrifugation (input). (H) Bovine brain cytosol (2 mg) was incubated in the absence ( $\emptyset$ ) or presence (phalloidin) of 20  $\mu$ M phalloidin and 100  $\mu$ M nocodazole for 30 min at 37°C. Pellets obtained after centrifugation at 100,000  $\times$  g were immunoblotted along with an equivalent sample of the total input protein before centrifugation (input). Lactate dehydrogenase was used as a negative control for cytoskeletal association.



**Figure 7.** Coimmunoprecipitation of proteins associated with mVps18. A detergent-soluble crude membrane fraction was subjected to immunoprecipitation with antibodies to mVps18, GST or Hook1 as indicated. Immunoprecipitated complexes were analyzed by immunoblotting with the antibodies to the indicated proteins. For comparison a 10% equivalent of input protein is shown except in the case of mVps16, Syn13, Vam2m, and mVam6 where a 20% equivalent is shown.

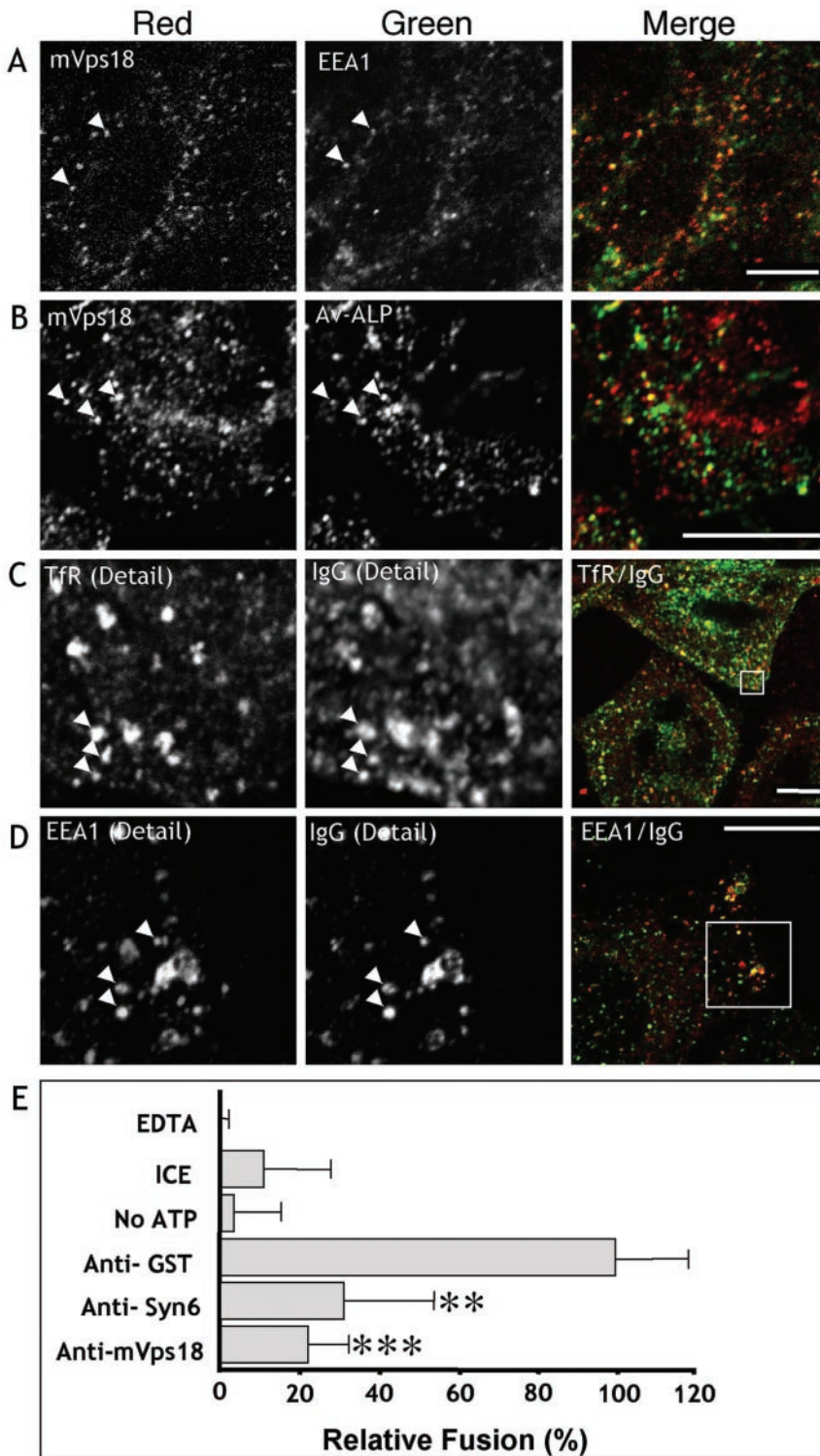
based on its ability to tightly cluster late endosomes (Poupon *et al.*, 2003). Although those studies showed only low levels of clustering EEA1-positive compartments, we found significant clustering of early endosomal compartments (Figure 3F), which is likely explained by our use of different cell types. Finally, we observe that mVps18 specific antibodies inhibit early endosome fusion *in vitro*. Like other early endosome fusion assays (Mills *et al.*, 2001), the assay we used was also sensitive to antibodies to Syn6 but not Syn7 demonstrating its specificity. Further studies are required to establish whether the HOPS proteins are involved in the same rab5-mediated homotypic fusion reaction that has previously been characterized (Gorvel *et al.*, 1991), or whether this represents a different type of early endosomal fusion with different requirements. Interestingly, although we find that mVps18 does localize to Rab5-positive compartments, mVps18 is concentrated differently than Rab5, indicating that each of these proteins might occupy different endosomal subdomains (Figure 5D).

The possibility that the class C Vps proteins mediate some type of Syn6-/Syn13-dependent early endosome fusion event *in vivo* is also consistent with recent studies in yeast. The severe phenotype of *vps11*, *vps16*, *vps18* and *vps33* mutants cannot be explained by loss of vacuole fusion events alone, implying that fusion events earlier in the endocytic

pathway also rely on these proteins (Srivastava *et al.*, 2000; Peterson and Emr, 2001). Indeed, the class C Vps phenotype is mimicked when fusion to the vacuole and endosomal compartments are both blocked by mutation of *VAM3* and *PEP12* SNARE genes (Peterson and Emr, 2001). The localization of the yeast class C Vps proteins is also consistent with a broader function in the endosomal system. Although GFP fusion proteins to the various HOPS complex components do localize to the vacuole, fractionation experiments show that Vps18, Vps11, and Vps16 fractionates with Pep12p, a SNARE localized to endosomal compartments (Rieder and Emr, 1997). Additionally, the class C complex interacts genetically and physically with Vps8p, which is involved in transport steps before vacuole fusion (Horazdovsky *et al.*, 1996; Woolford *et al.*, 1998) and, in turn, interacts with Vps21p, the yeast orthologue of Rab5 (Horazdovsky *et al.*, 1996; Woolford *et al.*, 1998). Finally, both yeast Vps11p and Vps18p interact with Vac1p/Pep7p (Peterson *et al.*, 1999), which is also involved in fusion to the yeast endosome (Peterson and Emr, 1999; Srivastava *et al.*, 2000). The closest mammalian Vac1p homologue is Rabenosyn-5, which binds Rab5 and Rab4 (de Renzis *et al.*, 2002) and interacts with the S/M protein mVps45 (Nielsen *et al.*, 2000). Like the proposed dual role for the mammalian class C Vps complex, Rabenosyn-5 is not only required for early endosome fusion but also its overexpression slows the delivery of cathepsin D to late endosomes (Nielsen *et al.*, 2000). Together, these observations support the idea that the HOPS proteins in both yeast and animal cells participate in more than one fusion event.

Much more work is required to decipher the array of protein-protein interactions among the mammalian HOPS proteins that occur while they execute their function. Our coimmunoprecipitation experiments confirm that mVps18 interacts with Syntaxin heavy chains, yet we also coimmunoprecipitated a number of other t-SNAREs, including Syn6 and Vti1-b as well as the v-SNARE Vamp8. These results contrast with previous data in yeast where Vps18p associated exclusively with the uncomplexed Syntaxin Vam3p (Sato *et al.*, 2000). Instead, our results indicate that mVps18 associates with *cis*-SNARE complexes. These data are, however, consistent with other studies in yeast showing that HOPS components (Vam6p-Vam2p) associate with *cis*-SNARE complexes (Price *et al.*, 2000). It is likely that variation in the methods used between these different studies in yeast account for their discrepancy. However, further experimentation with recombinant proteins from both yeast and animal systems should better resolve this issue.

We also find that mVam2 and mVam6 interact with mVps18 and also show significant localization to EEA1- and Rab5-positive endosomes. Yeast Vam6p and Vam2p are thought to form a subcomplex, and Vam6p has been proposed to act as a GTP exchange factor for the Ypt7p Rab protein (Wurmser *et al.*, 2000). Although Ypt7p is homologous both in sequence and proposed function to Rab7, we have not yet been able to see interaction of Rab7 with the mammalian HOPS components (our unpublished data). Furthermore, we were unable to find significant colocalization of mVps18 with either eGFP-Rab7 or eGFP fusions with activated forms of Rab7 (Q67L) (our unpublished data). Interestingly, the lysosomal clustering evoked by overexpression of mVam6 that has been documented previously is independent of Rab7 (Caplan *et al.*, 2001). Thus, it remains to be determined whether the mVam2-mVam6 subcomplex provides a GTP exchange function in animal cells and which GTPase(s) it controls.



**Figure 8.** Antibodies to mVps18 inhibit a Syn6-dependent early endosome in vitro fusion assay. CHO cells were aldehyde fixed and labeled for mVps18 and EEA1 (A) as indicated and closed arrows denote colocalization (A). CHO cells allowed to endocytose avidin-conjugated alkaline phosphatase (B) for 5 min before fixation and immunolabeling for mVps18 and Av-ALP by using biotinylated Alexa488-conjugated antibodies. Bar, 5 μm. CHO cells allowed to endocytose biotin conjugated rabbit IgG (C and D) for 5 min before fixation and immunolabeling with monoclonal anti-Tfr (C) or anti-EEA1 (D) in combination with biotinylated antibody conjugated to avidin. Images at right show low power merged images. The designated inset is shown in detail at left. Bar, 5 μm. (E) Membranes from CHO cells allowed to endocytose either Biotin-IgG or Av-ALP for 5 min were incubated in the presence or absence of the indicated antibodies for 20 min on ice and then mixed with bovine brain cytosol and incubated at 37°C for 30 min. Immunocomplexes formed upon fusion were isolated on protein A agarose beads and quantitated by alkaline phosphatase activity (mean ± SD). Omission of the ATP-regenerating system, inclusion of EDTA (to chelate Mg<sup>2+</sup> ions), or reactions kept on ice showed significantly less IgG-Biotin-Av-ALP activity, and the latter condition was used as the background. Fusion reactions containing anti-GST antibodies (50 μg/ml) were taken as 100% relative fusion, which represented ~30% of the total enzyme activity within the PNS fractions. Significant inhibition was observed when anti-mVps18 polyclonal antibodies were included (150 μg/ml; \*\*\*p = 0.0012) and when anti-Syn6 polyclonal antibodies were included (225 μg/ml; \*\*p = 0.0072).

All of the HOPS proteins we analyzed associated with the cytoskeleton. Furthermore, we were also able to demonstrate interaction between mVps 18 and the microtubule-associated Hook1 protein that is also implicated in endosomal biogenesis by virtue of its homology with *Drosophila* Hook protein (Lloyd *et al.*, 1998; Sevrioukov *et al.*, 1999). We believe that the cytoskeletal association of these components likely represents an important aspect of their function. In-

terestingly, the clustering of endosomal compartments induced by overexpression of eGFP-mVps18 is also accompanied by dramatic changes in the organization of actin that forms a network of actin polymers that surround the clusters of endosomes (Poupon *et al.*, 2003). Biochemical analysis revealed that the class C Vps proteins associated with actin filaments, whereas mVam6 and mVam2 associated with microtubules. Despite the ability of the mVam6-mVam2

complex to associate with class C mVps18 protein, our cytoskeletal fractionation experiments showed that only Hook1 was capable of associating with both filaments and microtubules generated *in vitro*. These data imply that the association of the class C Vps proteins with the mVam6–mVam2 proteins is precluded by association with the cytoskeleton.

Further analysis will be devoted toward determining the function of the association between the mHOPS proteins and the cytoskeleton. Microtubules and actin filaments provide a network important for the movement and positioning of both early and late endosomes as well as lysosomes (Rogers and Gelfand, 2000; Cordonnier *et al.*, 2001). Membrane traffic to and through late endosomes is not only sensitive to microtubule and actin filament depolymerization but is controlled by many proteins that interact with actin and microtubules such as Rho family GTPases, myosins, kinesins, and the dynein/dynactin complex (Allen, 1995; Murphy *et al.*, 1996; Chimini and Chavrier, 2000; Ellis and Mellor, 2000; Rogers and Gelfand, 2000; Apodacia, 2001; Muller *et al.*, 2001; Eitzen, 2003). If vesicle movement as well as immobilization is an important factor in determining the efficiency of cargo transport (Kelly, 1990; Qualmann *et al.*, 2000), then there should exist mechanisms to integrate cytoskeletal interactions with downstream fusion events. Perhaps, the members of the mammalian HOPS complex could function at this interface by orchestrating a switch between vesicle movement and vesicle fusion. Thus, the HOPS proteins would join a growing collection of proteins that interact with both the cytoskeleton and effectors of fusion such as RILP, Rab6, Rab27a, BLOC-1, and the Exocyst complex (Rogers and Gelfand, 2000; Schroer, 2000; Falcon-Perez *et al.*, 2002; Langford, 2002; Lipschutz and Mostov, 2002; Short *et al.*, 2002; Yi *et al.*, 2002). Alternatively, the cytoskeleton and particularly actin could play a more direct role in the fusion process, a function distinct from its role in vesicle movement or positioning. Two different *in vitro* late endosome fusion assays show dependence on actin polymerization under situations where the spatial distribution of compartments might not be a critical factor (Jahraus *et al.*, 2001; Eitzen *et al.*, 2002). The yeast vacuolar homotypic fusion assay not only requires proper actin polymerization and depolymerization but also the activity of Cdc42p (Eitzen *et al.*, 2001; Muller *et al.*, 2001). Kinetic analysis of vacuole fusion shows that Cdc42p and actin are required at the Ypt7p-dependent step of vesicle tethering (Eitzen *et al.*, 2001). Actin itself is also coconcentrated with HOPS components along vertices of tethered vacuoles where fusion occurs (Eitzen *et al.*, 2002; Wang *et al.*, 2003). Thus, actin could be an integral part of the glue that tethers the fusion apparatus of opposing membranes together.

## ACKNOWLEDGMENTS

This work was supported by a grant from the Human Frontiers Science Program to R.C.P., J.P.L., and David E. James and by a grant to R.C.P. from the AHA (9730275N). S.C.W.R. was supported by AHA postdoctoral fellowship grants 0120475Z and 0325605Z. V.P. was supported by a Wellcome Trust travelling fellowship awarded to J.P.L.

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