Rice SCAMP1 Defines Clathrin-Coated, trans-Golgi–Located Tubular-Vesicular Structures as an Early Endosome in Tobacco BY-2 Cells

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We recently identified multivesicular bodies (MVBs) as prevacuolar compartments (PVCs) in the secretory and endocytic pathways to the lytic vacuole in tobacco (Nicotiana tabacum) BY-2 cells. Secretory carrier membrane proteins (SCAMPs) are post-Golgi, integral membrane proteins mediating endocytosis in animal cells. To define the endocytic pathway in plants, we cloned the rice (Oryza sativa) homolog of animal SCAMP1 and generated transgenic tobacco BY-2 cells expressing yellow fluorescent protein (YFP)–SCAMP1 or SCAMP1-YFP fusions. Confocal immunofluorescence and immunogold electron microscopy studies demonstrated that YFP-SCAMP1 fusions and native SCAMP1 localize to the plasma membrane and mobile structures in the cytoplasm of transgenic BY-2 cells. Drug treatments and confocal immunofluorescence studies demonstrated that the punctate cytosolic organelles labeled by YFP-SCAMP1 or SCAMP1 were distinct from the Golgi apparatus and PVCs. SCAMP1-labeled organelles may represent an early endosome because the internalized endocytic markers FM4-64 and AM4-64 reached these organelles before PVCs. In addition, wortmannin caused the redistribution of SCAMP1 from the early endosomes to PVCs, probably as a result of fusions between the two compartments. Immunogold electron microscopy with high-pressure frozen/freeze-substituted samples identified the SCAMP1-positive organelles as tubular-vesicular structures at the trans-Golgi with clathrin coats. These early endosomal compartments resemble the previously described partially coated reticulum and trans-Golgi network in plant cells.

INTRODUCTION

Endocytosis is a well-established process, operating as clathrin-dependent, receptor-mediated, and lipid raft (caveolin) pathways in mammalian cells (Le Roy and Wrana, 2005; Verma and Hong, 2005). In clathrin-dependent endocytosis, receptor–ligand complexes internalized at coated pits in the plasma membrane (PM) travel through several endosomal compartments before the ligand reaches the lysosome/vacuole. Receptors often separate from their ligands in the early endosome and are recycled back to the PM via clathrin-coated vesicles from a specialized domain of this compartment (Gruenberg, 2001). Early endosomes mature into late endosomes, which represent a junction between the endocytic pathway and the biosynthetic route for acid hydrolases to the lysosome/vacuole. As a consequence, the mannosyl 6-phosphate receptor cycles between the trans-Golgi network (TGN) and the late endosome: the anterograde transport step being accomplished via clathrin-coated vesicles, and the retrograde via retromer-coated vesicles (Arighi et al., 2004; Seaman, 2004).

Endosomal compartments in mammalian cells characteristically have internal vesicles and hence are termed multivesicular bodies (MVBs). Formation of the internal vesicles occurs by invagination of the boundary membrane, a process involving ubiquitinylation (Hicke and Dunn, 2003) and the ESCRT complex (Babst, 2005; Bowers and Stevens, 2005), both of which serve to selectively internalize membrane proteins, including receptors destined for degradation. The internal vesicles and the soluble contents of MVBs are finally delivered into the lumen of the lysosome/vacuole via direct fusion of the MVB with the lysosome/vacuole (Luzio et al., 2000; Katzmann et al., 2001).

Considerable evidence for endocytosis in plants has accumulated during recent years (Battey et al., 1999; Samaj et al., 2004; Lam et al., 2005). Some of the components of the clathrin-based internalization machinery have been identified (Holstein, 2005; Murphy et al., 2005), and data for the uptake of cell surface receptor–ligand complexes is accumulating (Russinova et al., 2004; Robatzek et al., 2006). In addition, the fluorescent dyes FM4-64 and FM1-43 have been successfully used to study the endocytic pathway in plant cells. After their insertion in the PM,
these styryl dyes pass through endosomal compartments on their way to the tonoplast (Vida and Emr, 1995, Kim et al., 2001; Ueda et al., 2001; Emans et al., 2002; Tse et al., 2004; Dettmer et al., 2006). Therefore, colocalization of proteins with internalized FM4-64 may be used to judge whether a particular compartment labeled by a specific protein is a putative endosomal compartment in plant cells. Proteins localized to plant endosomes in this way include the Arabidopsis thaliana Rab GTPases AAA6 and AAA7 (Ueda et al., 2001, 2004), Arabidopsis PRA2 (SYP111) (Inaba et al., 2002), the small GTPase ARF1 in maize (Zea mays) (Baluska et al., 2002, 2004), the Arabidopsis GNOM protein and the auxin-efflux carrier PIN1 (Geldner et al., 2003), and vacuolar H⁺-ATPase (Dettmer et al., 2006). However, in the great majority of these cases, the ultrastructural morphology of the compartments bearing these proteins was not given. As a result, the identity of early and recycling endosomal compartments in plants has remained somewhat equivocal.

On the other hand, a prevacuolar/late endosomal compartment (PVC) in plants has been identified and partially characterized. Recent investigations in tobacco (Nicotiana tabacum) BY-2 cells and Arabidopsis protoplasts have shown that PVCs are enriched in VSR proteins and are also characterized by the presence of Rha1, a Rab5 homolog, the t-SNARE Pep12p, and plant retromer homologs (Li et al., 2002; Sohn et al., 2003; Tse et al., 2004; Oliiviusson et al., 2006). These organelles have a typical multivesiculate morphology (Tse et al., 2004; Mo et al., 2006; Oliiviusson et al., 2006). On the basis of uptake studies using electron-dense tracers, such MVBs have long been recognized as lying on the endocytic pathway in plant cells (Hillmer et al., 1986, 1988; Tanchak and Fowke, 1987; Galway et al., 1993). Moreover, recent FM4-64 uptake studies have confirmed their dual role in endocytosis and vacuolar protein transport by showing that the internalized dye reaches a VSR-enriched compartment (Sohn et al., 2003; Tse et al., 2004).

To identify early endosomal compartments in tobacco BY-2 cells, we have expressed and localized a class of membrane proteins not hitherto investigated in plant cell biology; secretory carrier membrane proteins (SCAMPs). These proteins were initially identified as secretory vesicle components in mammalian exocrine glands and later found to be ubiquitous proteins in eukaryotes (Fernandez-Chacon and Sudhof, 2000). SCAMPs are also found in the PM and vesicles that internalize from and shuttle back to the PM (Brand and Castle, 1993). SCAMPs are found in both the TGN and the endosomal recycling compartment in NRK cells, and they appear to be concentrated within the motile population of early and recycling endosomes (Castile and Castle, 2005). Thus, SCAMPs appear to be reliable indicators for post-Golgi endocytic and exocytic trafficking in animal cells (Fernandez-Chacon and Sudhof, 2000; Castle and Castle, 2005; Liu et al., 2005).

Plant SCAMP homologs have been found in rice (Oryza sativa), Arabidopsis, and pea (Pisum sativum) and are thought to be present in many other plant species (Fernandez-Chacon and Sudhof, 2000). We hypothesized that plant SCAMPs might also play a role in mediating endocytosis in plant cells. Therefore, in this study, we used plant SCAMPs as probes to study the endocytic compartments in tobacco BY-2 cells. To this end, we cloned the full-length rice SCAMP1 cDNA and generated transgenic tobacco BY-2 cell lines expressing yellow fluorescent protein (YFP)-SCAMP1 or SCAMP1-YFP fusions. Confocal immunofluorescence and immunogold electron microscopy (EM) studies demonstrated that both YFP-SCAMP1 fusions and the native SCAMP1 localize to both PM and mobile cytosolic organelles. Drug treatments and confocal immunofluorescence studies demonstrated that the YFP-SCAMP1-labeled organelles are distinct from the Golgi apparatus and the PVC/MVB. SCAMP1-bearing organelles may represent an early endosome because the internalized endosomal tracers FM4-64 and AM4-64 reached these organelles before reaching the VSR-labeled PVC/MVB. Further confocal immunofluorescence and immunoelectron microscopy studies suggested that wortmannin causes SCAMP1 to redistribute from the putative early endosomes to the PVC/MVB. Immunogold EM identified the SCAMP1-labeled early endosome as tubular-vesicular structures with clathrin coats and enriched in vacuolar (V)-ATPase. These structures were often found at the trans face of the Golgi apparatus and have the appearance of the previously described partially coated reticulum (PCR) (Hillmer et al., 1986, 1988). Therefore, our results confirm the recent observations of Dettmer et al. (2006) and firmly establish the TGN as a compartment upstream of the PVC/MVB on the plant endocytic pathway.

**RESULTS**

**Highly Conserved Plant SCAMPs**

A total of 39 cDNAs encoding SCAMPs can be found in the National Center for Biotechnology Information protein database. Among them, 19 cDNA clones were identified from Arabidopsis, 17 from rice, two from Schistosoma japonicum, and one from pea. However, these numbers of cDNAs identified at the National Center for Biotechnology Information database may be an overrepresentation of SCAMPs in Arabidopsis and rice, because there are only five Arabidopsis SCAMP genes and eight rice SCAMP genes.

As a first step to study plant SCAMPs, we cloned a full-length SCAMP cDNA from rice via nested PCR amplification of a rice cDNA library with a SCAMP EST sequence (gi 7332504). This full-length rice SCAMP cDNA contains 918 nucleotides with a predicted molecular mass of ~35 kDa. The SCAMP cDNA (Figure 1A) used in this study is almost identical to a rice cDNA clone from the database (Os34899754). In addition, this rice SCAMP has high similarity (>80% at the amino acid level) to all known plant SCAMPs, including Arabidopsis and pea (Krajinski et al., 1998) (Figure 1A, At15220305 and Ps3941289) and the animal SCAMP1 (Rn3914958), except that additional sequences are present at the N terminus of the rice SCAMP (Figure 1A). Because of its high similarity to the animal SCAMP1, we thus named this particular rice SCAMP rice SCAMP1 in this study.

Using TMHMM server version 2.0, the rice SCAMP1 was predicted to have four transmembrane domains (amino acids 145 to 167, 172 to 194, 207 to 229, and 255 to 277) with two NPF (Asn-Pro-Phe) motifs at its cytosolic N terminus and a short cytosolic C terminus (Figure 1B). The NPF motif is believed to interact with proteins containing an epsin-homology domain (Fernandez-Chacon and Sudhof, 2000). The overall structure of...
this rice SCAMP1 is similar to that of animal SCAMPs. The N-terminal NPF repeats are conserved in both plant and animal SCAMPs (Guo et al., 2002). The transmembrane region of SCAMPs is also conserved in plants and animals, especially the cytoplasmic peptide loop between the second and third transmembrane domains. However, the C-terminal sequence is only present among plant SCAMPs. In addition, as with their mammalian counterparts, there was no hydrophobic signal peptide at the N terminus in plant SCAMPs. In a study of SCAMP37, which is ubiquitous in mammalian cells, Brand and Castle (1993) proposed that the first transmembrane domain serves as an internal uncleaved signal sequence and in in vitro translation experiments showed that the yield increased considerably in the presence of pancreatic rough microsomes, with no change in molecular mass. Presumably, plant SCAMPs also have an internal sequence for insertion into the endoplasmic reticulum (Brand and Castle, 1993).

Generation of Transgenic SCAMP1-YFP BY-2 Cells and Characterization of SCAMP1 Antibodies

As a first step to illustrate the functional roles of SCAMPs in plants, we studied the subcellular localization of the rice SCAMP1 using two approaches. First, a fusion protein approach in transgenic tobacco BY-2 cells was used. Because the rice SCAMP1 did not contain a predicted signal peptide (Figure 1), we made both N-terminal and C-terminal fusions of SCAMP1 to YFP under the control of the 35S constitutive promoter and 3’ NOS terminator (Figure 2A). The resulting chimeric constructs, SCAMP1-YFP and YFP-SCAMP1, were then inserted into tobacco BY-2 cells via Agrobacterium tumefaciens–mediated transformation. More than 100 transgenic BY-2 cell lines were generated from each construct. Protein gel blot analysis was performed to confirm the expression of SCAMP1-YFP and YFP-SCAMP1 fusions in these newly generated transgenic BY-2 cell lines. As shown in
Figure 2A. GFP antibodies detected a band at 65 kD, the predicted molecular mass of the full-length SCAMP1-YFP or YFP-SCAMP1 fusion proteins, mainly in the membrane fractions of transgenic BY-2 cells expressing either YFP-SCAMP1 (lanes 3 and 4) or SCAMP-YFP1 (lanes 5 and 6). No such band was detected in wild-type cells (lanes 1 and 2). Thus, both SCAMP1-YFP and YFP-SCAMP1 fusions were successfully expressed in tobacco BY-2 cells with the correct molecular mass and membrane insertion.

As the second approach, we generated two SCAMP1 antibodies (termed SCAMP1a and SCAMP1b) by injecting rabbits with synthetic peptides corresponding to the NPF repeats and the second conserved loop region (Figure 1A), as described in Methods. These two peptides were chosen for antibody
generation because they represent the conserved regions in both plant and animal SCAMPs. The specificity of these SCAMP1 antibodies was first tested via protein gel blot analysis. As shown in Figure 2B, affinity-purified SCAMP1a antibodies detected a single band at ~37 kD, the expected size of SCAMP1, mainly in the cell membrane fraction of wild-type BY-2 cells (Figure 2B). Similarly, SCAMP1b antibodies also specifically detected the 37-kD SCAMP1 in wild-type BY-2 cells (Figure 2C, lane 1). In five of six BY-2 cell lines overexpressing SCAMP1 (Figure 2C), a major band at ~37 kD was also detected by SCAMP1b antibodies, but the band was significantly stronger (Figure 2C, lanes 2, 3, and 5 to 7) than that in wild-type cells (lane 1). One overexpressing cell line (lane 4) showed little SCAMP1, probably as a result of gene silencing.

The specificity of these SCAMP antibodies was further confirmed via confocal immunofluorescence with SCAMP1 antibodies in fixed wild-type and overexpressing SCAMP1 BY-2 cells. As shown in Figure 2D, both SCAMP1 antibodies detected punctate structures in the cytoplasm and along the PM (Figure 2D, panels 1 and 3; arrowheads indicate examples of PM localization of punctate signals), but the signals along the PM were increased dramatically in transgenic BY-2 cells overexpressing SCAMP1 (Figure 2D, panels 2 and 4), indicating the specific detection of overexpressed SCAMP1 by the SCAMP1 antibodies. Such a PM localization for SCAMP1 in wild-type and overexpressing BY-2 cells is easily recognizable when confocal images are compared with the corresponding differential interference contrast images (see Supplemental Figure 1 online). Together, these results demonstrated that both SCAMP1a and SCAMP1b antibodies specifically detected both endogenous tobacco SCAMP1 and the overexpressed rice SCAMP1 in tobacco BY-2 cells.

Subcellular Localization of the SCAMP1-YFP Fusion Construct and SCAMP1 in BY-2 Cells

Confocal immunofluorescence microscopy was first performed to determine the subcellular localization of both SCAMP1-YFP and YFP-SCAMP1 fusions in tobacco BY-2 cells. As shown in Figure 3, strong fluorescent signals localizing to the cell surface were readily detected in cells expressing either YFP-SCAMP1 (Figure 3, panel 1) or SCAMP1-YFP (see Supplemental Figure 2 online, panel 1), but no such signals were detected in wild-type BY-2 cells (data not shown). To determine whether these fluorescent signals were localized to the PM, the periplasmic space, or the cell wall, two treatments were performed on these transgenic BY-2 cells before confocal image collection. First, protoplasts were generated. Second, transgenic BY-2 cells were plasmolyzed by treating with NaCl2 to cause the withdrawal of

![Figure 3](image-url)  
**Figure 3.** Subcellular Localization of YFP-SCAMP1 Fusion Constructs in Transgenic Tobacco BY-2 Cells. Confocal images of YFP signals in cells expressing either YFP-SCAMP1 fusion construct were collected from untreated cells (panel 1), protoplasts (panel 2), and cells treated with 1.5 M NaCl2 (panel 3). DIC, differential interference contrast. Bar = 50 μm.
the PM from the cell wall. As shown in Figure 3, a strong fluorescent signal remained in the PM of protoplasts (Figure 3, panel 2; see Supplemental Figure 2 online, panel 2). In addition, in plasmolyzed cells, fluorescent signals remained in the shrunken PM with little detectable signal in the cell wall of both transgenic cell lines (Figure 3, panel 3; see Supplemental Figure 2 online, panel 3). These results demonstrated that both YFP-SCAMP1 and SCAMP1-YFP fusions localized to the PM of transgenic BY-2 cells and demonstrate that neither N-terminal nor C-terminal fusion affects this localization.

To further confirm the PM localization of SCAMP1 in BY-2 cells, affinity-purified SCAMP1 antibodies were used in both confocal immunofluorescence and immunogold EM studies of fixed BY-2 cells. As shown in Figure 2D, both SCAMP1a and SCAMP1b antibodies detected punctate structures in the cytoplasm and along the PM of wild-type and overexpressing SCAMP1 BY-2 cells, and the signals from these two antibodies colocalized in double labeling experiments (Figure 4, panel 1), indicating that these two antibodies detected the same proteins and thus the same organelles in BY-2 cells. In addition, both

![Figure 4](image-url)
SCAMP1a and SCAMP1b antibodies colocalized with SCAMP1-YFP fusions on the PM in BY-2 cells expressing the YFP fusions (Figure 4, panels 2 and 3). The PM localization of SCAMP1 in BY-2 cells was further confirmed by immunogold EM studies with SCAMP1 antibodies, in which gold particles were found mainly at the cytosolic face of the PM in both SCAMP1-YFP–transformed and wild-type BY-2 cells (Figure 4, panels 4 and 5). Therefore, the PM localization of SCAMP1 was not a result of its overexpression. Together, these results demonstrated that both SCAMP1 and the YFP-SCAMP1 fusion localized to the PM and unidentified punctate organelles in the cytosol in BY-2 cells and that overexpression of SCAMP1 and the YFP-SCAMP1 fusion most likely did not lead to a missorting of SCAMP1 in transgenic BY-2 cells. However, we cannot exclude the possibility that the YFP tag might interfere with the recycling or internalization of SCAMP1, causing its retention in the PM.

**Dynamics of YFP-SCAMP–Labeled Organelles in the Cytoplasm**

In addition to the fluorescent signals detected in the PM, punctate signals were also found within the cytoplasm of the transgenic BY-2 cells expressing either the YFP-SCAMP1 or SCAMP1-YFP fusion (Figure 3; see Supplemental Figure 2 online). The cytoplasmic organelles labeled by the SCAMP1-YFP or YFP-SCAMP1 fusion were highly mobile. As shown in Figure 5 (and in Supplemental Movie 1 online), these organelles moved to, moved along, and moved away from the PM in transgenic BY-2 cells. This finding indicates that the organelles labeled by SCAMP1-YFP may represent endosomal compartments in BY-2 cells (see below).

**YFP-SCAMP1–Labeled Cytoplasmic Organelles Are Distinct from the Golgi Apparatus and the PVC**

We previously demonstrated that the drug brefeldin A (BFA) at low concentrations (5 to 10 μg/mL) caused the aggregation of Golgi stacks in transgenic BY-2 cells but that PVCs remained unaffected. Conversely, the drug wortmannin was without effect on the Golgi apparatus but caused the PVCs to vacuolate (Tse et al., 2004). Thus, these differential drug treatments serve as reliable tools to identify and distinguish the Golgi from the PVC in transgenic BY-2 cells.

Because mobile punctate signals were detected in transgenic BY-2 cells expressing either YFP-SCAMP1 or SCAMP1-YFP, we wanted to determine whether these YFP-labeled organelles in the cytosol were Golgi or PVC and therefore applied BFA and wortmannin. As shown in Figure 6A, BFA at 10 μg/mL induced the GONST1-YFP–labeled Golgi to form aggregates, but the GFP-BP-80–labeled PVC remained unchanged (Figure 6A, left panels). However, when transgenic BY-2 cells expressing either SCAM1P-YFP or YFP-SCAMP1 were treated with BFA at the same concentration, the YFP-SCAMP1–labeled organelles also formed aggregates (Figure 6A, right panels). Similar results were obtained when the cells were treated with BFA at 5 μg/mL (data not shown). These results indicate that SCAMP1-YFP– or YFP-SCAMP1–labeled organelles are BFA-sensitive and thus are not PVCs. However, because they formed aggregates in response to BFA, they could be part of the Golgi apparatus.

To determine whether the labeled YFP-SCAMP1 or SCAMP1-YFP structure belonged to the Golgi apparatus, we treated transgenic BY-2 cells with wortmannin at 16.5 μM. As shown in Figure 6B, wortmannin at 16.5 μM did not cause visible changes in the size or number of Golgi stacks in BY-2 cells expressing the Golgi marker GONST1-YFP (Figure 6B, left panels). However, wortmannin did induce the PVCs to form small vacuoles in the transgenic BY-2 cell line expressing the PVC marker GFP-BP-80 (Figure 6B, left panels). Similarly, wortmannin caused the YFP-labeled cytosolic organelles to dilate in the transgenic BY-2 cell lines expressing SCAMP1-YFP or YFP-SCAMP1 (Figure 6B, right panels). Similar results were obtained when the cells were treated with wortmannin at 8.25 μM (data not shown). Therefore,
because they were sensitive to wortmannin, the SCAMP-YFP– or YFP-SCAMP1–labeled structures were not identical to the BFA-sensitive portion of the Golgi apparatus. Together, these results demonstrated that the SCAMP1-YFP– or YFP-SCAMP1–labeled structures did not behave as typical Golgi or PVC in transgenic BY-2 cells.

**YFP-SCAMP1–Labeled Organelles Are Early Endosomes**

As demonstrated in Figure 6, on the basis of differential BFA and wortmannin treatments, the novel organelles in BY-2 cells labeled by SCAMP1-YFP or YFP-SCAMP1 were neither Golgi nor PVC. To further confirm this and to positively identify these organelles, we first performed confocal immunofluorescence microscopy in transgenic BY-2 cells with SCAMP1 antibodies in which the SCAMP1-labeled structures were compared with established Golgi or PVC markers. Tobacco BY-2 cell lines expressing the cis-Golgi marker Man1-GFP (Nebenführ et al., 1999), the trans-Golgi marker GONST1-YFP (Baldwin et al., 2001; Tse et al., 2004), and the PVC marker GFP-BP-80 (Tse et al., 2004) were used. As shown in Figure 7, SCAMP1a-positive organelles were mostly separate from the Golgi markers.

**Figure 6.** Drug Effects on YFP-SCAMP1–Labeled Organelles in Transgenic BY-2 Cells.

(A) BFA-induced aggregation of YFP-SCAMP1–labeled organelles. Tobacco BY-2 cells expressing the Golgi marker GONST1-YFP, the PVC marker GFP-BP-80, and the SCAMP1-YFP or YFP-SCAMP1 fusion construct were treated with BFA at concentrations of 0 and 10 μg/mL as indicated for 1 h before confocal images were collected. Bar = 50 μm.

(B) Wortmannin-induced YFP-SCAMP1–labeled organelles form dilated structures. Tobacco BY-2 cells expressing the Golgi marker GONST1-YFP, the PVC marker GFP-BP-80, and the SCAMP1-YFP or YFP-SCAMP1 fusion construct were treated with wortmannin at concentrations of 0 and 16.5 μM as indicated for 1 h before confocal image collection. Bar = 50 μm.
Man1-GFP (Figure 7, panel 1) and GONST1-YFP (Figure 7, panel 2) as well as from the PVC marker GFP-BP-80 (Figure 7, panel 3). Similarly, organelles labeled by SCAMP1b antibodies were also separate from Golgi or PVC markers in transgenic BY-2 cells (see Supplemental Figure 3 online, panels 1 to 3). These results again demonstrate that the SCAMP1-positive organelles were neither Golgi nor PVC.

FM4-64, a fluorescent styryl dye that is internalized via endocytosis from the PM to the lytic vacuole, serves as a marker for the endocytic pathway in plant cells (Emans et al., 2002; Tse et al., 2004; Lo and Jiang, 2006). We previously demonstrated that FM4-64 colocalizes with the PVC marker YFP-BP-80 but not the Golgi marker GONST1-YFP in transgenic BY-2 cells (Tse et al., 2004), indicating that the secretory and endocytic pathways merge in PVCs. To determine whether the YFP-SCAMP1– or SCAMP1-YFP–labeled organelles were on the endocytic pathway, we performed uptake studies with FM4-64 on transgenic BY-2 cells expressing various reporters. As shown in Figure 8, the Golgi marker GONST1-YFP showed little colocalization with the internalized FM4-64 at 30 min after uptake (Figure 8, panel 1). Similarly, in cells expressing the PVC marker GFP-BP-80, the internalized FM4-64 did not colocalize with GFP-BP-80 during the same 30-min uptake (Figure 8, panel 2). However, in cells expressing either SCAMP1-YFP or YFP-SCAMP1, internalized FM4-64 colocalized mostly with the SCAMP1 constructs during the same 30-min uptake study (Figure 8, panel 3). Therefore, we conclude that the internalized FM4-64 reached the SCAMP1-YFP–labeled compartments before the GFP-BP-80–labeled PVCs. Thus, SCAMP1-YFP–containing organelles represent either an early endosomal compartment or a recycling endosome in tobacco BY-2 cells.

However, we were aware that this conclusion is based on an indirect comparison between observations made on different transgenic cell lines expressing either GFP-BP-80 or YFP-SCAMP1. In addition, it is also possible that overexpression of SCAMP1-YFP might lead to an acceleration of FM4-64 uptake in cells expressing SCAMP1-YFP compared with BY-2 cells expressing the PVC marker. To rule out these possibilities, we performed uptake studies on wild-type BY-2 cells using the fixable form of the styryl dye, AM4-64, to compare the time course of dye arrival into VSR-labeled PVCs against SCAMP-labeled organelles. AM4-64 shares the same molecular structure as FM4-64.

Figure 7. SCAMP1–Labeled Organelles Are Distinct from the Golgi and PVC Markers in Transgenic BY-2 Cells.
Tobacco BY-2 cells expressing the Golgi marker Man1-GFP (panel 1) or GONST1-YFP (panel 2) or the PVC marker GFP-BP-80 (panel 3) were fixed and then labeled with SCAMP1a antibodies. Bar = 50 μm.
as FM4-64 with an additional chemically fixable amide group. Therefore, wild-type BY-2 cells were collected at both early and late stages during dye internalization for chemical fixation, followed by extensive washing to remove the noninternalized dye, before the fixed cells were labeled with either VSR or SCAMP1 antibodies. As shown in Figure 9A, in cells labeled with VSR antibodies, very little colocalization between the VSR-marked PVCs (green) and the internalized AM4-64 (red) was detected in fixed cells at 15 min, whereas some colocalization (20%) and almost full colocalization (80%) were observed at 30 and 60 min, respectively (Figure 9A). By contrast, when the cells were labeled with SCAMP1 antibodies, an increasing percentage of colocalization between the SCAMP1-positive organelles (green) and AM4-64 (red) was detected as internalization proceeded, with partial colocalization (30 to 40%) detected at 15 min and almost full colocalization (80 and 90%) observed at 30 and 60 min (Figure 9B). These results are consistent with those (Figure 8) obtained using transgenic cells expressing various markers. Therefore, detection of internalized FM4-64 in SCAMP1-labeled organelles earlier than in GFP-BP-80–labeled PVCs is attributable neither to a difference in transgenic cell lines nor to the overexpression of YFP-SCAMP1. Therefore, the organelles labeled by either SCAMP1 or YFP-SCAMP1 must represent an endocytic compartment upstream from the PVCs. In consequence, we will refer hereafter to SCAMP1-labeled organelles as early endosomes in BY-2 cells. However, because AM4-64 did not colocalize with SCAMP1-labeled organelles during the very early stages of uptake, these AM4-64–labeled organelles might also represent a different population of early endosomes distinct from those labeled by SCAMP1.

**SCAMP1 Colocalizes with Internalized FM4-64 to BFA Compartments in BY-2 Cells**

BFA inhibits ARF1 GTPase and thus blocks the COPI-mediated recycling pathway from the Golgi to the endoplasmic reticulum (Nebenführ et al., 2002). In tobacco BY-2 cells, BFA treatment leads to the formation of both endoplasmic reticulum–Golgi hybrids and to so-called BFA compartments (Ritzenthaler et al., 2002). BFA compartments contain endosomal proteins and
Figure 9. The Internalized Endosomal Marker AM4-64 Colocalizes with SCAMP1 Earlier Than VSRs in Fixed Wild-Type BY-2 Cells.

Wild-type tobacco BY-2 cells were incubated with AM4-64 and washed, followed by sample collection for chemical fixation at the times indicated at left. Fixed cells were then labeled with either VSR antibodies (A) to detect PVCs or SCAMP1 antibodies (B) to detect SCAMP1-marked organelles. Numbers at right indicate the percentage colocalization based on calculation using >20 cells, as described previously (Jiang and Rogers, 1998), either between VSR and AM4-64 (A) or between SCAMP1 and AM4-64 (B). Bar = 50 μm.
colocalize with the internalized endosomal marker FM4-64 (Geldner et al., 2001, 2003; Samaj et al., 2004). Therefore, if the cytosolic organelles labeled by SCAMP1-YFP or YFP-SCAMP1 were endocytic compartments, they should localize to the BFA compartments and colocalize with the internalized FM4-64 in BFA-treated BY-2 cells. However, as shown in Figure 10, when BY-2 cells expressing the Golgi marker GONST1-YFP were treated with BFA at 10 μg/mL for 30 min to induce the formation of BFA compartments, and then pulsed with FM4-64, the Golgi-derived aggregates (green) showed a close association but remained separate from the internalized FM4-64 aggregates (red) throughout the 45-min uptake period (Figure 10A). By contrast, when the same treatment was performed in BY-2 cells expressing YFP-SCAMP1 (Figure 10B) or SCAMP1-YFP (Figure

**Figure 10.** YFP-SCAMP1–Labeled Early Endosomes Colocalize with Internalized FM4-64 in BFA Compartments in Transgenic Tobacco BY-2 Cells. Tobacco BY-2 cells expressing the Golgi marker GONST1-YFP (A) or YFP-SCAMP1 (B) or the SCAMP1-YFP fusion construct (C) were treated with 10 μg/mL BFA for 1 h to induce the formation of aggregates, followed by FM4-64 internalization. Samples were removed at the times indicated for confocal image collection. Yellow color indicates colocalization of the two proteins. DIC, differential interference contrast. Bar = 50 μm.
10C), the BFA-induced aggregates derived from YFP-SCAMP1– or SCAMP1-YFP–labeled organelles showed good colocalization with the internalized FM4-64 at the end of the 45-min uptake period (Figures 10B and 10C). Therefore, in terms of endocytic trafficking, organelles labeled with SCAMP1-YFP or possessing SCAMP1 represent a compartment that is upstream and distinct from the Golgi apparatus and PVCs in BY-2 cells.

**Wortmannin Induces the Redistribution of SCAMP1 from Early Endosomes to the PVC**

We previously demonstrated that wortmannin causes GFP-BP-80–labeled PVCs/MVBs to dilate in transgenic tobacco BY-2 cells (Tse et al., 2004; Miao et al., 2006). The vacuolation of the PVCs might be the result of a homotypical PVC fusion or be derived from fusions between early endosomes and PVCs. To test for the latter possibility, we compared the time-course response of PVCs and SCAMP1-labeled early endosomes to wortmannin in BY-2 cells. Therefore, BY-2 cells expressing the PVC marker GFP-BP-80 were first treated with wortmannin at 8.25 μM and cells were collected at 0-, 15-, 30-, and 60-min intervals for chemical fixation. The fixed transgenic BY-2 cells were then labeled with SCAMP1 antibodies so that the GFP-labeled PVCs could be compared with SCAMP1-labeled early endosomes directly in the same cells. As shown in Figure 11, in transgenic BY-2 cells treated with wortmannin at 8.25 μM, SCAMP1-labeled organelles (red) were separated from GFP-labeled PVCs (green) before wortmannin treatment (Figure 11, 10C).

**Figure 11.** Wortmannin-Induced Redistribution of SCAMP1 from Early Endosomes to Vacuolated PVCs in BY-2 Cells.

BY-2 cells expressing the PVC marker GFP-BP-80 were incubated with wortmannin at 8.25 μM, followed by sample removal at the times indicated for chemical fixation. The fixed cells were then labeled with SCAMP1 antibodies. Arrowheads indicate examples of colocalization between the GFP-BP-80–marked PVCs (green) and SCAMP1-labeled early endosomes (red). Bar = 50 μm.
Identification of Tubular-Vesicular Structures as Early Endosomal Compartment in BY-2 Cells

To morphologically identify the early endosomal compartments that were labeled by SCAMP1 and YFP-SCAMP1 fusion constructs in tobacco BY-2 cells, we performed an immunogold EM study with SCAMP1 and GFP antibodies. Both wild-type and BY-2 cells expressing YFP-SCAMP1 (untreated and treated with wortmannin) were subjected to high-pressure freezing/freeze substitution, and thin sections were then processed for immunogold staining. As shown in Figure 12, SCAMP1 antibodies specifically labeled tubular-vesicular structures that were usually found (in >80% of the observed EM images) close to the trans face of Golgi stacks and sometimes in the adjacent cytoplasm (Figure 12, panels 1 to 3). The labeling was specific for these structures because little labeling was observed over the Golgi apparatus (Figure 12, panels 1 to 3; indicated by g) and MVBs (data not shown). When GFP antibodies were used to detect YFP-SCAMP1-labeled organelles in sections prepared from transgenic YFP-SCAMP1 BY-2 cells, similar results were obtained (data not shown). In addition, GFP antibodies did not label MVBs in ultrathin sections prepared from high-pressure frozen/freeze-substituted transgenic BY-2 cells expressing the YFP-SCAMP1 fusion (see Supplemental Figure 5 online), indicating that the YFP-SCAMP1 fusion did not locate to MVBs.

Morphological studies on these tubular-vesicular structures were also performed on sections prepared from untreated and wortmannin-treated tobacco BY-2 cells. As shown in Figure 13, the tubular structures labeled by SCAMP1 antibodies (Figure 12) seemed to bear coats on their surface (Figure 13, panel 1, arrowheads) in untreated BY-2 cells. In addition, in sections prepared from wortmannin-treated (at 16.5 μM for 1 h) BY-2 cells, enlarged MVBs/PVCs (Figure 13, panel 2, asterisk) were often found closely associated with vesicular structures (Figure 13, panel 3, arrowheads), suggesting possible fusions between early endosomes and MVBs. These results are consistent with the immunofluorescence data, in which SCAMP1 was redistributed from early endosomes to PVCs in the presence of wortmannin in a time-dependent manner (Figure 11), and support the idea that SCAMP1 redistribution is likely caused by the fusion of the early endosome to the PVC/MVB rather than by transport of proteins between the two compartments.

Early Endosomal Compartments in BY-2 Cells Equate with the PCR and/or TGN

SCAMP1-labeled early endosomes also have a tubular-vesicular morphology not unlike the previously described PCR (Pesacreta and Lucas, 1985). A further common feature between these two structures is that both have domains that are coated with clathrin. To determine whether SCAMP1-labeled early endosomes bear clathrin coats, we performed confocal immunofluorescence studies using a monoclonal antibody against mammalian clathrin heavy chain (CHC) in BY-2 cells. As shown in Figure 14, CHC antibodies labeled punctate structures that were mostly colocalized with organelles detected by SCAMP1 antibodies in BY-2 cells (Figure 14, panel 2), indicating that the tubular-vesicular endosomes have clathrin coats. Little colocalization was observed between CHC-labeled organelles and the GONST1-YFP-labeled Golgi apparatus or YFP-BP-80-labeled PVCs (Figure 14, panels 1 and 3). Furthermore, CHC antibodies specifically labeled the same structures in ultrathin sections prepared from high-pressure frozen/freeze-substituted samples of BY-2 cells (Figure 14, panel 4, arrows and arrowheads). These results strongly suggest that the SCAMP1-containing early endosomes bear clathrin coats and that the PCR and TGN are probably one and the same structure in BY-2 cells.

V-ATPase was recently shown to locate to the TGN in Arabidopsis root tip cells (Dettmer et al., 2006). To determine whether V-ATPase was also present in SCAMP1-labeled organelles in BY-2 cells, we performed confocal immunofluorescence and immunogold EM studies with a vacuolar ATPase antibody raised against the total V-ATPase from the tonoplast of Kalanchoe daigremontiana (Haschke et al., 1989; Sikora et al., 1998). As shown in Figure 15, the punctate organelles in BY-2 cells labeled by the V-ATPase antibody were completely separate from the GONST1-YFP-labeled Golgi organelles (Figure 15, panel 1). By contrast, V-ATPase immunogold staining showed a high degree of colocalization with SCAMP in both untreated (Figure 15, panel 2) and wortmannin-treated (at 8.25 μM for 1 h) (Figure 15, panel 3) BY-2 cells, in which the latter was found to colocalize to vacuolated PVCs. These results are consistent with the possible redistribution of SCAMP1 from early endosomes to wortmannin-induced vacuolated PVCs, as shown in Figure 11. In addition, the V-ATPase antibodies in immunogold EM also labeled similar trans-Golgi-located tubular-vesicular structures (data not shown). Therefore, the trans-Golgi structures labeled by SCAMP1, clathrin, and V-ATPase are likely to be one and the same organelle.

Two Arabidopsis t-SNAREs, At TLG2a/SYP41 and At TLG2b/SYP42, have been shown to localize to the TGN in Arabidopsis roots (Bassham et al., 2000). Therefore, we performed confocal immunofluorescence studies using TLG2a antibodies (currently the only TLG antibodies available) in BY-2 cells. TLG2a showed some colocalization with GONST1-YFP but showed no colocalization with the PVC reporter GFP-BP-80. After wortmannin treatment, TLG2a labeling, unlike that for SCAMP1, did not overlap with vacuolated PVCs (data not shown). This finding indicates that TLG2a and SCAMP1 label different structures. However, the value of these observations is questionable because of the low specificity of these antibodies on protein gel blots of BY-2 cell extracts (data not shown).
SCAMP1 Localizes to the PM and Early Endosomes of Plant Cells

With four membrane-spanning domains, SCAMPs belong to the group of tetraspan proteins (Maeker et al., 1997). As noted by Liu et al. (2005), a characteristic feature of these proteins is that they are concentrated on intracellular membranes but exercise a function at the PM. This seems to be borne out in the case of SCAMP1 to SCAMP4, which are found predominantly on the membranes of exocrine and endocrine storage granules and post-Golgi recycling carriers (Guo et al., 2002). Only in the case of SCAMP2 is a higher proportion detectable at the PM (~25% of total cell SCAMPs). In PC12 cells, a neuroendocrine cell line, SCAMP2 proteins are localized to specific foci randomly distributed

DISCUSSION

SCAMP1 Localizes to the PM and Early Endosomes of Plant Cells

Ultrathin sections prepared from high-pressure frozen/freeze-substituted samples of BY-2 cells expressing SCAMP1-YFP were immunogold-labeled with SCAMP1 antibodies. Arrows point to examples of labeled tubular-vesicular structures, and arrowheads indicate examples of budding vesicles in these structures. g, Golgi apparatus. Panels 1 to 3 represent three different examples of similar SCAMP1-labeled endosomes. Bars = 200 nm.

Figure 12. SCAMP1 Identifies Tubular-Vesicular Structures as Early Endosomes in Transgenic Tobacco BY-2 Cells.
throughout the cell surface, and these represent part of a fusion pore complex for dense core vesicles (Liu et al., 2005). This function of SCAMP2 involves the E peptide, a highly conserved segment lying at the cytosolic surface of the molecule and linking the second and third transmembrane domains. Previously, the major function ascribed to SCAMP1 to SCAMP3 has been to facilitate clathrin-mediated endocytosis (Fernandez-Chacon and Südhof, 2000). This was predicted on the basis of NPF repeats in the N-terminal cytoplasmic extension, which are capable of recruiting scaffolding proteins having epsin-homology domains (e.g., Eps15). It was confirmed in cells expressing an N-terminally truncated SCAMP1, which acts as a dominant-negative mutant.

**Figure 13.** Effects of Wortmannin on the Morphology of the PVC and Early Endosome in Tobacco BY-2 Cells.

Untreated BY-2 cells (panel 1) or BY-2 cells treated with wortmannin at 16.5 μM for 1 h (panel 2) were subjected to high-pressure freezing/freeze substitution before ultrathin sections were prepared for structural studies. Panel 1 shows an example of typical tubular-vesicular structures as early endosomes or TGN and PVC/MVB (asterisks) in untreated BY-2 cells. Panel 2 shows an example of possible fusions between early endosomes (arrowheads) and the vacuolated PVC/MVB (asterisk) in wortmannin-treated BY-2 cells. g, Golgi apparatus. Bars = 200 nm.
for clathrin-mediated endocytosis (Fernandez-Chacon et al., 2000).

Our data reveal both a PM and an endosome localization for the rice SCAMP1 in wild-type BY-2 cells and transgenic BY-2 cells overexpressing SCAMP1, even though more PM labeling was seen in BY-2 cells overexpressing SCAMP1. Similarly, both YFP-SCAMP1 and SCAMP1-YFP fusions in transgenic BY-2 cells were also found to localize to both PM and cytosolic organelles identified as early endosomes. Although a function at the molecular level for plant SCAMPs remains to be found, a recent preliminary report has shown that *dab5-1* (for delayed abscission) is a SCAMP mutant that, in addition to causing a delay in abscission, also produces irregular cell elongation proximal to the leaving organ. DAB5-GFP fusions also locate to the PM (S.E.

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**Figure 14.** Tubular-Vesicular Endosomes in Tobacco BY-2 Cells Have Clathrin Coats.

Panels 1 to 3, confocal images showing a high degree of colocalization of CHC with SCAMP1 (panel 2) but separation from the Golgi marker GONST1-YFP (panel 1) and the PVC marker GFP-BP-80 (panel 3). Bar = 50 μm. Panel 4, immunogold EM localization of CHC to tubular-vesicular endosomes in ultrathin sections prepared from high-pressure frozen/freeze-substituted samples of BY-2 cells. Arrows point to gold particles, and arrowheads indicate examples of vesicles in the labeled early endosome. g, Golgi apparatus. Bar = 200 nm.
Patterson, H. Rao, K. Kusner, M. Butenko, W. Wang, J. Lindsey, and A. Robertson, unpublished data).

**SCAMP1 Allows the Identification of an Early Endosome in BY-2 Cells**

When expressed in tobacco BY-2 cells, YFP-tagged SCAMP1 from rice localizes to the PM and also to small cytoplasmic organelles. Because SCAMPs seem to have an internal signal sequence permitting their insertion into the endoplasmic reticulum (Brand and Castle, 1993), some of these labeled structures may represent organelles lying on the exocytic pathway transporting newly synthesized SCAMP1 to the PM. However, our immunogold EM studies revealed little if any labeling of the endoplasmic reticulum and Golgi apparatus, except for the TGN. This suggests that at any one time only small numbers of SCAMP1 molecules are in transit in the early secretory pathway and that the presence of SCAMP1 at the PM is a consequence of their gradual accumulation in this membrane. The SCAMP1-labeled structures in the cytoplasm, therefore, must represent organelles on the endocytic/recycling pathways. We believe that the evidence from this study described below support this notion and help in identifying an early or recycling endosomal compartment in tobacco BY-2 cells.

First, confocal immunofluorescence studies with SCAMP1 antibodies on fixed BY-2 cells demonstrated that organelles labeled by SCAMP1 were separate from both the Golgi apparatus and the PVCs. Second, the effects of BFA and wortmannin on SCAMP1-labeled organelles are different from those on the Golgi apparatus and PVC, indicating again that SCAMP1-labeled organelles in BY-2 cells are neither Golgi nor PVC. Third, treatment with BFA caused SCAMP1 to enter aggregated structures similar to the endosomal BFA compartment described for other plants. Fourth, wortmannin caused a redistribution of SCAMP1 from the SCAMP1-labeled structures to the PVC, suggesting a possible fusion between the two compartments. Fifth, FM4-64 and AM4-64 uptake studies demonstrated that these dyes reached the SCAMP1-labeled organelles before the PVC, indicating that the SCAMP1-positive compartments lie upstream of the PVC on the endocytic pathway. Finally, immunogold EM positively and unequivocally identified the SCAMP1-labeled organelles as clathrin-coated, trans-Golgi–located, tubular-vesicular structures that are morphologically distinct from Golgi cisternae and PVC/MVB. Thus, SCAMP1 has proved to be a most useful

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**Figure 15. SCAMP1-Labeled Tubular-Vesicular Endosomes Colocalize with V-ATPase in Transgenic Tobacco BY-2 Cells.**

Confocal images showing complete separation of V-ATPase from GFP-BP-80–labeled PVCs in untreated BY-2 cells (panel 1) but colocalization with SCAMP1 in untreated (panel 2) and wortmannin-treated (at 16.5 μM for 1 h) BY-2 cells (panel 3). Bar = 50 μm.
mediated by the retromer complex.

Traffic involves clathrin-coated vesicles and the backward traffic is TGN/PCR (dotted arrows) before reaching the LV, where the forward traffic involves clathrin-coated vesicles and the backward traffic is mediated by the retromer complex.

The early endosomes in tobacco BY-2 cells that have been identified on the basis of rapid styryl dye labeling and the presence of SCAMP1 have the morphological appearance of tubular-vesicular structures in the EM. These structures, which are situated at the trans face of the Golgi stacks, also have clathrin coats, as demonstrated by both confocal immunofluorescence and immunogold labeling. Therefore, they are very similar to an organelle previously termed the PCR (Pesacreta and Lucas, 1985). Tracer uptake studies have shown that this organelle lies on the endocytic pathway (Tanchak et al., 1988), and its relationship to the TGN has also been demonstrated (Hillmer et al., 1985). However, in our experience, the PCR as an entity lying separately and distant from Golgi stacks is rarely if ever seen in tobacco BY-2 cells.

Therefore, our observations point in the same direction as those recently published by Dettmer et al. (2006), who found that the first endocytic compartment labeled with internalized FM4-64 in Arabidopsis root tip cells was enriched in V-ATPase. Our own immunogold labeling studies with a V-ATPase subunit antiserum confirm these observations. Dettmer et al. (2006) also found that the V-ATPase–positive TGN in Arabidopsis did not clearly colocalize with either ARA6- or ARA7–labeled compartments. We have not been able to test for these two endosomal markers because antibodies against Arabidopsis ARA6/7 are not available at present, and even if they were available, their specificity for tobacco BY-2 cells would have to be proven. It will be interesting to determine whether the corresponding tobacco homologs to these GTPases colocalize instead with VSRs to the MVB/PVC or whether additional endosomal populations exist in tobacco BY-2 cells.

**Wortmannin Targets Both Early and Late Endosomes (PVC) in Tobacco BY-2 Cells**

Wortmannin is a specific inhibitor of Vps34p, phosphatidylinositol 3-kinase (PI-3K) (Vanhaesebroeck et al., 1997), a protein that is essential for vacuolar protein sorting in yeast (Schu et al., 1993). In plant cells, wortmannin also blocks the trafficking of some vacuolar proteins (Matsuoka et al., 1995; Di Sansebastiano et al., 1998) and prevents the recycling of VSR proteins from the PVC to the Golgi apparatus (daSilva et al., 2005, 2006).

Morphologically, wortmannin induces the vacuolation of BP-80–labeled PVCs/MVBs in BY-2 cells (Tse et al., 2004; Lo and Jiang, 2006), and it seems that such a vacuolation is a general response to wortmannin treatment in various plant cell types and species (Miao et al., 2006; Tse et al., 2006). Wortmannin inhibits the formation of the internal vesicles in multivesicular endosomes in both mammalian and plant cells and causes these organelles to enlarge significantly (Fernandez-Borja et al., 1999; Bright et al., 2001; Tse et al., 2004). The source of the extra membrane required for this dilation has not been identified, but it has been calculated that it exceeds the amount predicted to arise from a lack of internal vesicle production (Futter et al., 2001).

A considerable body of literature indicates that wortmannin also targets early endosomes in mammalian cells. It would seem that whereas the internalization of PM receptors continues, or may even be increased, in the presence of wortmannin, receptor recycling or the further transport of receptors down the degradative pathway is inhibited (Roth et al., 2004). In addition to being present on multivesicular late endosomes, the substrate for PI-3K, PI3P, is also found on early endosomes (Rubino et al., 2000). PI-3K activity is required for the fusion of endocytic vesicles with the early endosome as well as for homotypic endosome fusion, and wortmannin has been shown to prevent this fusion (Jones and Clague, 1995; Jones et al., 1998). However, there is no indication in the animal literature than wortmannin leads to the fusion of early and late endosomes. Our data showing a relocalization of both SCAMP1 and V-ATPase to the BP-80/VSR–labeled PVC strongly suggest that wortmannin also targets the early endosome in plants, but unlike the situation in mammalian cells, in...
plants it seems to lead to the fusion of early and late endosomes and may provide the extra membrane required for the wortmannin-induced vacuolation of the PVC. Indeed, the drug BFA also targets both Golgi and PVC in plant cells (Tse et al., 2006).

In mammals, the TGN is a separate entity from the early endosome, and the mannosyl-6-phosphate receptor is known to cycle between these two compartments (Kundra and Kornfeld, 1998). Moreover, wortmannin does not appear to change causes in the morphology of the mammalian TGN (Reaves et al., 1996). However, as discussed above, there is now good evidence in plants that the TGN, or its immediate derivative the PCR, assumes the function of an early endosome. Thus, the difference in modes of action of wortmannin in mammals and plants may relate to the more complicated role that the TGN/PCR plays in plant cells. Despite the many caveats in working with wortmannin (Roth et al., 2004), which also probably apply to plants, it will be interesting to determine whether the effects of wortmannin as described here in tobacco BY-2 cells can be confirmed in other plant cells. Indeed, an endosome as defined by labeling with At SNX1-GFP in Arabidopsis cells was recently shown to form small vacuoles in response to wortmannin treatment (Jaillais et al., 2006).

The PCR/TGN as an Plant Early Endosome: Consequences

Based on our knowledge of the endocytic pathway in mammalian cells, we can make predictions and draw some conclusions from the identification of the PCR/TGN as the early endosome in plant cells. In mammalian cells, the first recipient of internalized receptor–ligand complexes is the sorting endosome, in which, as a result of an acidic lumenal pH, many ligands dissociate from their receptors (Mukherjee et al., 1997). These receptors and up to 80% of the membrane proteins in the sorting endosome are rapidly sequestered into tubular extensions, which separate from the early endosome and are called recycling endosomes (Sheff et al., 1999; Maxfield and McGraw, 2004). The residual sorting endosome quickly matures into the late endosome, whereas the receptors in the relatively stable recycling endosome collect into a specific class of clathrin-coated vesicles characterized by an m1 β adaptin-containing API adaptor complex (Stoorvogel et al., 1996; Gan et al., 2002).

In mammalian cells, those PM proteins that are destined for degradation in the lytic compartment (e.g., the constitutively downregulated epidermal growth factor receptor) become ubiquitinylated in their cytosolic tails (Reggiori and Pelham, 2001). This signal is recognized by elements of the ESCRT complex, leading to the specific internalization in vesicles within the endosome (Katzmann et al., 2002). It is not clear from the literature whether this segregation event is restricted to late endosomes or is initiated in early, sorting endosomes (Raiborg et al., 2002). However, the existence of a double-layered clathrin plaque at the surface of the endosome in question is a structural indicator of this sorting process (Sachse et al., 2002, 2004). Structurally similar plaques are characteristic of the MVB/PVC in BY-2 cells (Tse et al., 2004), but these structures appear to be spherical, without any indication of the tubular extensions characteristic of the early endosomes of mammalian cells.

Although it is not clear whether plants have separate sorting and recycling endosomal compartments (Murphy et al., 2005), the recognition of the PCR/TGN as the early/recycling endosome means that this organelle is the true junction of the biosynthetic and endocytic routes to the vacuole in plants, rather than the MVB/PVC, as maintained previously (Tse et al., 2004; Lam et al., 2005). Because clathrin-coated vesicles are presumably also responsible for receptor recycling to the PM in plants, this means that two different clathrin-mediated sorting events occur in this organelle: one for the recycling of ligand-free PM receptors, the other for the capture and packaging of acid hydrolases and other proteins destined for the vacuole (Oliviusson et al., 2006, and references cited therein; Pimpi et al., 2006). Another consequence is that the sorting of PM proteins that are to be degraded in the vacuole must occur very late in the endocytic pathway of plants, because only the MVBs/PVCs have the clathrin/ESCRT plaque-like structure that is associated with the formation of internal vesicles. A working model for protein trafficking in the secretory and endocytic pathways of plants, in which the SCAMP1-labeled TGN/endosome serves as the junctional structure for these two transport routes, is presented in Figure 16.

METHODS

General methods for the construction and characterization of recombinant plasmids, the maintenance of suspension cultured tobacco (Nicotiana tabacum) BY-2 cells, and the preparation and characterization of antibodies are described previously (Jiang and Rogers, 1998; Jiang et al., 2000, 2001; Tse et al., 2004).

Plasmid Construction

For the construction of YFPSCAMP1K and SCAMP1YFPK (K = kanamycin-resistant), the full-length rice (Oryza sativa) SCAMP1 (Os SCAMP1) cDNA sequences were amplified by PCR using pGA2791 as a template. pGA2791 is a plasmid containing the full-length rice SCAMP1 cDNA (Jang, 2002). Two-step cloning was performed to make the final constructs of YFPSCAMP1K and SCAMP1YFPK to be used in Agrobacterium tumefaciens-mediated transformation of tobacco BY-2 cells. First, full-length SCAMP1 cDNA sequences were cloned into pBI121. Two primers (forward, 5′-GGGGGATCCGGGGGCGCTACGACAGCACC-3′; reverse, 5′-GGGGAGCTCAAAAGCTGCCCGCATAGCAC3′) were used to amplify the full-length rice SCAMP1 cDNA sequences for YFPSCAMP1K, whereas two other primers (forward, 5′-GGGGGATCCATGGCGGGGCCTAGACGACC-3′; reverse, 5′-GGGGGATCCCTATTACGAAAGGTTCATCCATGCC-3′) were used to amplify the full-length rice SCAMP1 cDNA sequences for SCAMP1YFPK. The PCR-amplified fragments were digested with BamHI/SacI and cloned into Lj491 (Jiang and Rogers, 1998) via the same restriction sites.

Second, the YFP sequence was amplified by PCR using pGONST1-YFP (Baldwin et al., 2001) as a template. Again, two sets of primers were used. For YFPSCAMP1K, two primers (forward, 5′-GGGGGATCCATGAGTAGAAAGGAGGAAATTTC-3′; reverse, 5′-GGGGGATCCCTATTTCGACTTATTGTTTACCGCC-3′) were used, whereas two other primers (forward, 5′-GGGGGATCCCTATTTCGACTTATTGTTTACCGCC-3′; reverse, 5′-GGGGGATCCCTATTTCGACTTATTGTTTACCGCC-3′) were used for SCAMP1YFPK. The amplified PCR fragments were digested with BamHI and SacI, respectively, and subcloned into pBluescript SK+ via the same restriction sites. The BamHI/SacI-digested YFP fragment was finally subcloned into Os SCAMP1-containing binary vector pBI121 from step one. All constructs were checked and verified by both restriction mapping and DNA sequencing.
Transformation of BY-2 Cells

For Agrobacterium-mediated transformation, plasmids YFPSCAMP1K and SCAMP1YFPK were first introduced into Agrobacterium (strain LBA4404) by electroporation before they were used to transfect wild-type tobacco BY-2 cells as described previously (Tse et al., 2004). BY-2 cells were maintained in Murashige and Skoog (MS) medium by subculturing twice per week at room temperature in a shaker set at 125 rpm. Transfected BY-2 cells were transferred onto MS medium (Sigma-Aldrich) containing kanamycin (50 µg/mL) and cefotaxin (300 µg/mL) and incubated at room temperature for 3 to 4 weeks until transformed colonies were visible. Resistant cell colonies (~50 to 100 from each construct) were subjected to preliminary screening for YFP signals and patterns via confocal immunofluorescence. Selected transgenic cell lines (5 to 10 per construct) were further transferred into MS liquid medium containing kanamycin to initiate suspension culture and used for subsequent analysis. Transgenic BY-2 cell lines were maintained in both liquid and solid culture via subculturing (twice per week for suspension cultures and twice per month for calli on agar plates).

Antibodies

Two synthetic peptides corresponding to the Os SCAMP1 cytosolic N-terminal NPF domains (CMAGRYDSNPFEEEDVNPFSQARG) and the second (middle) cytosolic loop sequences (CWIYRLPYRPQYNVTR-TDSALK) were synthesized (GeneScript). Each synthetic peptide was conjugated with keyhole limpet hemocyanin and used to immunize two rabbits at the animal house of the Chinese University of Hong Kong (CUHK). The generated antibodies (anti-SCAMP1a and anti-SCAMP1b) were further affinity-purified with a CnBr-activated Sepharose (Sigma-Aldrich; catalog No. C9142-15G) column conjugated with synthetic peptides as described previously (Rogers et al., 1997; Tse et al., 2004). The production of a polyclonal antibody specific for VSRAt-1 was described previously (Tse et al., 2004). GFP antibodies were purchased from Molecular Probes (catalog No. A-11122) or generated using recombinant GFP purchased from Roche Applied Science (catalog No. 1,814,524) as antigens to inject rabbits at the CUHK and then affinity-purified using a CnBr-activated Sepharose (Sigma-Aldrich) column conjugated with recombinant GFP. A monoclonal antibody against CHC (at 250 µg/mL) was purchased from BD Bioscience (catalog No. C610500). The V-ATPase antibodies used in this study were raised in rabbits against total V-ATPase from the tonoplast of Kalanchoe daigremontiana as described previously (Haschke et al., 1989; Sikora et al., 1998). The Arabidopsis thaliana TGL2a antibodies (Basham et al., 2000) were kindly provided by Natasha Raikhel (University of California, Riverside). Secondary rhodamine and fluorescein isothiocyanate–conjugated affinity-purified anti-rabbit antibody bodies were purchased from Jackson ImmunoResearch Laboratories (catalog Nos. 711-295-152 and 711-095-152). For protein gel blot analysis, GFP antibodies and SCAMP1 antibodies were used at 4 µg/mL.

Confocal Immunofluorescence Studies

Fixation and preparation of tobacco BY2 cells and their labeling and analysis by epifluorescence and confocal immunofluorescence have been described previously (Jiang and Rogers, 1998; Jiang et al., 2000; Li et al., 2002; Tse et al., 2004). The settings for collecting confocal images within the linear range were as described (Jiang and Rogers, 1998). For single or double immunolabeling, polyclonal or monoclonal antibodies were incubated at 4°C overnight at a 1:200 dilution. All confocal fluorescence images were collected using the Bio-Rad Radiance 2100 system. Images were processed using Adobe Photoshop software as described previously (Jiang and Rogers, 1998).

The extent of colocalization of two antibodies or fluorescent signals in confocal immunofluorescence images from BY2 cells was quantitated as described previously (Jiang and Rogers, 1998; Jiang et al., 2000; Tse et al., 2004). Control assays to ascertain the specificity of double labeling experiments were performed as described previously (Jauh et al., 1999; Jiang et al., 2000). Briefly, for labeling experiments with two polyclonal antibodies, primary antibodies were incubated at 4°C overnight (in PBS supplemented with 0.1% Triton X-100 [PBST] + 1% BSA) followed by washing in PBST. A rhodamine-conjugated Fab fragment then was added and incubated at room temperature for 4 h before a second wash, followed by the addition of the second primary antibody. Additionally, we confirmed that the labeling pattern for an antibody used individually matched the pattern obtained with the same antibody when used in double labeling experiments (Jiang et al., 2000; Li et al., 2002). Secondary antibodies alone were also used as an additional control.

FM4-64 and AM4-64 Uptake Study

Transgenic tobacco BY-2 cell lines expressing various reporters were used in the FM4-64 uptake study. The suspension cultured BY-2 cells were first washed with MS liquid medium, followed by the addition of FM4-64 (from a 12 mM stock and diluted to working solution at 12 µM with MS liquid medium just before use) to 500 µL of cultured cells to reach the final concentration and incubated on ice for 10 min. The FM4-64–treated cells were then washed with ice-cold MS medium several times and transferred onto a slide with medium for time-course observation and image collection using a 60× objective oil lens in the Bio-Rad Radiance 2100 system. The filter sets used were as follows: for YFP, excitation wavelength of 514 nm, dichroic mirror 560DCLPIXR, and emission filter HQ545/40; for FM4-64, excitation wavelength of 543 nm and emission filter HQ660LP. Images were processed using Adobe Photoshop software as described previously (Jiang and Rogers, 1998).

Wild-type tobacco BY2 cells were used in the AM4-64 uptake studies. The cells were first washed with MS liquid medium, followed by the addition of AM4-64 (from a 12 mM stock solution and diluted to working solution at 12 µM) to each 5 mL of cells to reach the final concentrations, and incubated on ice for 10 min. The AM4-64–treated cells were then washed with ice-cold MS medium several times and transferred to room temperature for uptake. During the incubation period, cells were collected at the indicated time intervals for chemical fixation and confocal immunofluorescence with antibodies against VSR and SCAMP according to procedures described previously (Jiang and Rogers, 1998; Jiang et al., 2000; Li et al., 2002; Tse et al., 2004).

Drug Treatments

For BFA experiments, aliquots of BFA (Sigma-Aldrich; B-6542; stock solution at 2.5 mg/mL in DMSO) solution were added to 2- to 3-d-old suspension cultures at log phase to give the proper final concentrations and incubated for 30 min, after the addition of FM4-64 to start the uptake and cell collection at the indicated times for direct confocal imaging, or fixed for confocal immunofluorescence and EM. For wortmannin experiments, aliquots of the wortmannin (stock solution at 2.5 mg/mL in DMSO) solution were added to BY-2 cells to give the proper final concentrations, followed by incubation and cell collections at the indicated times for direct confocal imaging, or chemically fixed for confocal immunofluorescence and EM as described previously (Tse et al., 2004). Each drug treatment for confocal imaging and confocal immunofluorescence was repeated at least twice with similar results.

EM of Resin-Embedded Cells

The general procedures for transmission electron microscopy sample preparation and thin sectioning of samples of BY-2 cells were performed.
essentially as described previously (Ritzenthaler et al., 2002; Tse et al., 2004). For high-pressure freezing, BY-2 cells were harvested by filtering and immediately frozen in a high-pressure freezing apparatus (HPF010; Bal-Tec; Balzers). For subsequent freeze substitution, the frozen samples were first kept at ~85°C for 60 h, then gradually warmed up to 0°C over 18 h. Substitution was performed in an AFS freeze-substitution unit (Leica). The substitution medium (dry acetone) was supplemented with 0.1% (w/v) uranyl acetate. When the samples reached 0°C, the medium was replaced with 100% ethanol, which was again changed to fresh 100% ethanol 10 min later. The cells were then infiltrated stepwise with HM20 at ~20°C, embedded, and polymerized under UV light. Immunolabeling on HM20 sections was done using standard procedures as described previously (Hohl et al., 1996; Tse et al., 2004) with SCAMP1 or GFP antibodies at 40 μg/mL, CHC antibodies (50 μg/mL) at 1:5 dilution, and gold-coupled secondary antibodies at 1:50. Aqueous uranyl acetate/lead citrate poststained sections were examined with a JOEL JEM-1200 EXII transmission electron microscope operating at 100 kV or a Phillips CM10 transmission electron microscope operating at 80 kV, as described previously (Tse et al., 2004).

Accession Number
Sequence data for the rice SCAMP1 cDNA can be found in the GenBank/EMBL data libraries under accession number gi 7332504.

Supplemental Data
The following materials are available in the online version of this article.

Supplemental Figure 1. Subcellular Localization of SCAMP1.

Supplemental Figure 2. Subcellular Localization of the SCAMP1-YFP Fusion Construct in Tobacco BY-2 Cells.

Supplemental Figure 3. SCAMP1-Labeled Organelles Are Distinct from Golgi and PVC Markers in Transgenic BY-2 Cells.

Supplemental Figure 4. Wortmannin-Induced Redistribution of SCAMP1 from Early Endosomes to Vaccumol PVCs in BY-2 Cells.

Supplemental Figure 5. The SCAMP1-YFP Fusion Does Not Localize to MVB in Transgenic Tobacco BY-2 Cells.

Supplemental Movie 1. Dynamics of YFP-SCAMP1–Marked Organelles (Early or Recycling Endosomes) in Transgenic Tobacco BY-2 Cells.

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Rice SCAMP1 Defines Clathrin-Coated, \textit{trans}-Golgi–Located Tubular-Vesicular Structures as an Early Endosome in Tobacco BY-2 Cells
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