Endocytotic Cycling of PM Proteins

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Abstract

Plasma membrane protein internalization and recycling mechanisms in plants share many features with other eukaryotic organisms. However, functional and structural differences at the cellular and organismal level mandate specialized mechanisms for uptake, sorting, trafficking, and recycling in plants. Recent evidence of plasma membrane cycling of members of the PIN auxin efflux facilitator family and the KAT1 inwardly rectifying potassium channel demonstrates that endocytotic cycling of some form occurs in plants. However, the mechanisms underlying protein internalization and the signals that stimulate endocytosis of proteins from the cell-environment interface are poorly understood. Here we summarize what is known of endocytotic cycling in animals and compare those mechanisms with what is known in plants. We discuss plant orthologs of mammalian-trafficking proteins involved in endocytotic cycling. The use of the styryl dye FM4-64 to define the course of endocytotic uptake and the fungal toxin brefeldin A to dissect the internalization pathways are particularly emphasized. Additionally, we discuss progress in identifying distinct endosomal populations marked by the small GTPases Ara6 and Ara7 as well as recently described examples of apparent cycling of plasma membrane proteins.

Contents

INTRODUCTION

Eukaryotic cells respond to external signals by altering transcriptional output and regulating the abundance and distribution of PM proteins that comprise the functional interface with the external environment. In animals and yeast, PM protein removal and recompartmentalization is regulated by endocytosis and endocytotic cycling. As the mechanisms underlying endocytotic cycling have been elucidated, the expectation that similar mechanisms would be found in plants has increased. Recent evidence that the PM localization of the PIN1 auxin efflux carrier complex and the KAT1 potassium channel are dynamically regulated (44, 102) has intensified efforts to conclusively demonstrate endocytotic recycling in plants. In both cases, the proteins involved have been shown to respond to both signaling molecules and external environmental cues (12, 72, 123) although no apparent cell surface receptor is involved. Many efforts are now underway to fit plant vesicular cycling mechanisms into the context of mammalian models. However, as **Figure 1** shows, developmental and structural differences at both the cellular and organismal level preclude the wholesale application of animal models to plants. In this review, we attempt to summarize the current understanding of this area of plant cell biology.

PM: Plasma Membrane

Vectorial redirection: redistribution of a protein from the apical or basal plasma membrane to a lateral membrane

ENDOSOMAL CYCLING IN ANIMALS: AN OVERVIEW

Endocytosis is an essential eukaryotic phenomenon whereby cells take up extracellular substances and/or internalize PM proteins for transport to endosomes. Eukaryotic cellular membranes are highly dynamic structures; in a typical cell the entire surface area of the PM turns over completely on an hourly basis (171). Endocytotic cycling helps maintain homeostatic regulation primarily by increasing or decreasing the surface content of mediating molecules in response to extracellular cues. From the endosomes, PM proteins are either targeted into the lysosome/vacuole for degradation or recycled back to cell surface (116). Mammalian endocytosis regulates multiple physiological processes such as nutrient uptake, retrieval of exocytosed vesicle components, downregulation of signaling receptors, and the localization/abundance of membrane transporters (116).

In animals, endosomal cycling is an essential component of endocrine and cell-to-cell signaling. Although the cycling of membrane transport proteins in response to internal signals has been documented (93), the endocytotic cycling of PM receptors in a process known as RME has been more intensively studied. In RME, the binding of an extracellular ligand to a PM

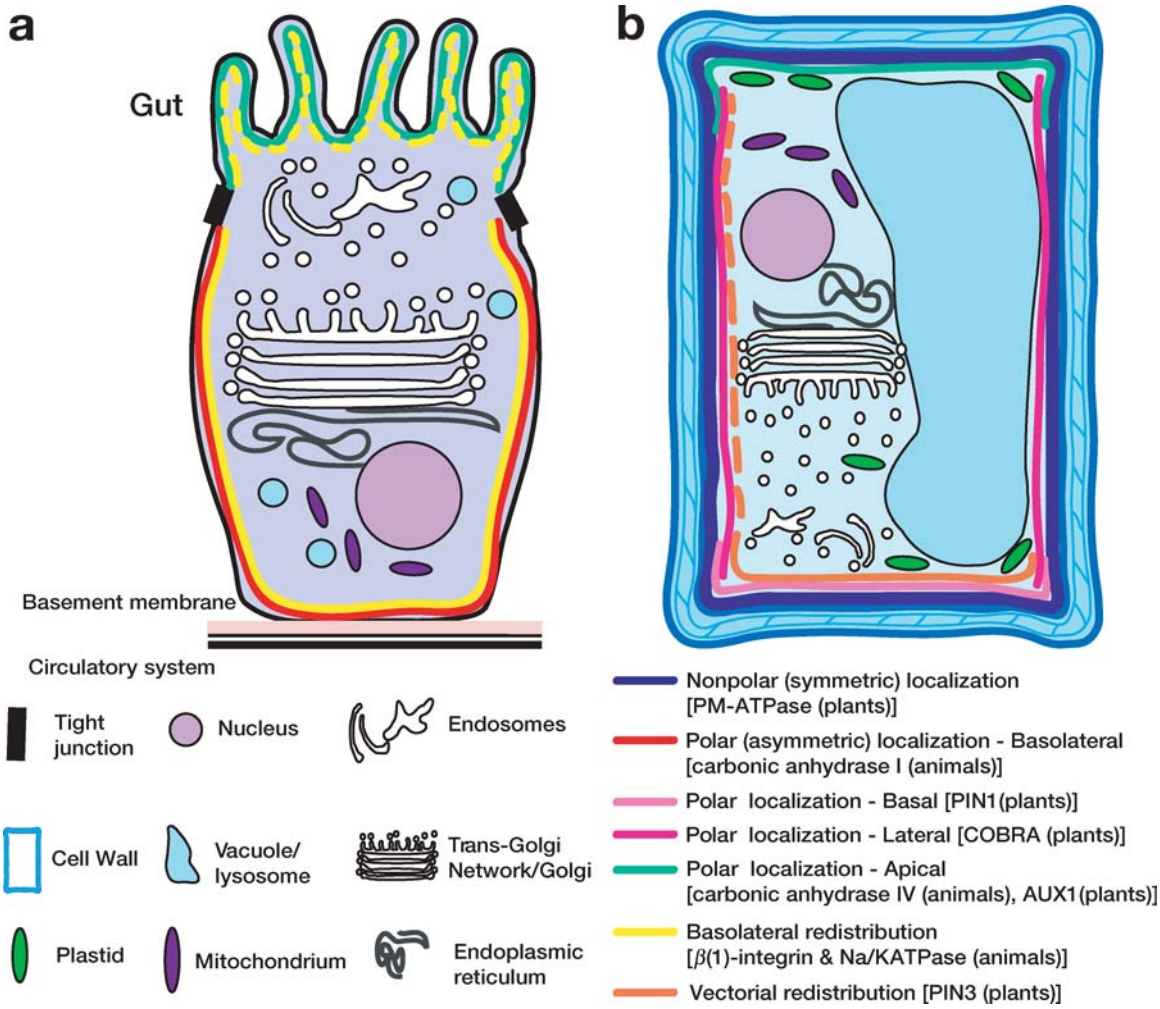


Figure 1

A comparison of plasma membrane (PM) localization in polarized animal and plant cells. Polarized animal cells have tight junctions that delineate the cell into basolateral and apical regions. As a result, animal cells exhibit targeting of PM proteins to basolateral or apical membranes (36, 85), basolateral redistribution of mistargeted proteins (186), and symmetric redistribution of proteins to all sides of the PM following loss of polarity, as seen when pathogenic *E. coli* infection disrupts tight junctions (115). Plant cells have cell walls and lack tight junctions to define the polarity of the cell. To date, four types of PM targeting have been documented in plants (basal, lateral, apical, and nonpolar) (28, 39, 145, 163, 165). Vectorial redistribution of PM proteins following a stimulus has been shown, as with the PIN3 auxin efflux facilitator that is redistributed from the basal to the lateral side of cells after tropic stimulus (39). (*A*) Generalized animal intestinal epithelial cell showing basolateral localization of carbonic anhydrase XII, apical localization of carbonic anhydrase IV, and basolateral redistribution of β(1)-integrin or Na^+/K^+ ATPase after a pathogen-induced change in cellular polarity. Dashed lines denote redistributed proteins. (*B*) Generalized plant cell showing nonpolar localization of the PM ATPase, basal localization of PIN1, lateral localization of COBRA, apical localization of AUX1, and vectorial redistribution of PIN3 after a gravitropic stimulus.

receptor initiates the uptake of proteins or protein complexes from the cell surface. Of the various animal cell surface protein uptake mechanisms, clathrin-dependent endocytosis is by far the best investigated. Less is known about clathrin-independent processes, which are more difficult to study because they are not concentrative and often lack specific markers. Clathrin-independent pathways include caveolae/lipid raft-mediated endocytosis, fluid-phase endocytosis, and phagocytosis. PM lipid microdomains enriched in cholesterol and sphingolipids sequester membrane proteins and play a role in trafficking (59). Studies with recycling Shiga toxin and Glycosylphosphatidyl inositol (GPI)-anchored proteins such as the folate receptor suggest a role for lipid microdomains in sorting in the ERC. Fluid-phase endocytosis and phagocytosis have been documented in animal cells as molecular internalization mechanisms but it has not been demonstrated that molecules internalized by these processes are recycled to the PM.

In clathrin-mediated RME, ligands bound to their respective receptors and destined for degradation in lysosomes become internalized via clustering in clathrin-coated pits (103). As a result of recycling to the PM, some receptors are reused up to several hundred times. Endocytotic and biosynthetic recycling also maintain the identity of the respective organelles by retrieving specific sets of functional proteins (organelle homeostasis). RME is also essential to the basolateral redirection that maintains the specificity of apical and basolateral membranes in polarized cells (186). Because clathrin-mediated internalization is the most well understood endocytotic process, this overview focuses on recycling events in this pathway (**Figure 2**).

The first step in clathrin-mediated endocytosis is the recruitment of adaptor protein (AP) complexes and clathrin to the PM. Phosphoinositides initiate coat assembly by directly binding to endocytotic proteins such as the AP2 adaptor complex and AP-180 (88, 140). An ARF6 GTPase was also implicated in this initial recruitment step. APs link specific cargo to sites of coat assembly. The pinching off of the coated vesicle requires additional proteins such as dynamins, amphiphysin, and Eps15. The vesicles rapidly shed the coat proteins to undergo fusion with target endosomes and to allow recycling of the coat components. This uncoating process requires auxilin and the molecular chaperone Hsc70 (66, 175). The newly formed endosomes fuse with one another and with pre-existing sorting endosomes in a process that requires *N*-ethylmaleimide-sensitive factor adaptor protein receptor (SNARE) and Rab proteins.

The first stop for transmembrane proteins internalized by clathrin-mediated endocytosis is the early endosomes, which include the peripherally localized tubular-vesicular structures of the sorting endosomes as well as the ERC. Due to their intracellular localization, the sorting endosomes are the first organelles to receive PM-derived cargo such as the low-density lipoprotein (LDL) and transferrin (Tf). In the acidic lumen of the sorting endosome (pH 6.0) (75), the dissociation of ligands from their receptors occurs. The sorting endosome is also the first main junction in the RME pathway because two main destinations can be reached from there: the PM and the late endosomes. Extensive studies of Tf and its receptor (Tf-R) show that recycling to the PM can either occur directly from the sorting endosome or via the ERC (55, 154). For recycling the Tf-R and some fluorescent lipid analogs (e.g., C6-NBD-sphingomyelin) from the sorting endosome, the kinetics are fast, with a half-life ($t_{1/2}$) of about two minutes and with an efficiency of more than 95%.

The ERC is associated with microtubules and exhibits a variable distribution, although more specific distributions in the vicinity of the nucleus and microtubule-organizing centers have been reported (50). In contrast to the sorting endosome, the ERC does not contain ligands or receptors that are destined for degradation, such as lysosomal enzymes and LDL, but instead is marked by the presence of LDL-R and iron-free Tf bound to the

RME: receptor-mediated endocytosis, the process by which a ligand-bound receptor is internalized and sorted

ERC: endosomal recycling compartment, a network of tubules marked by the presence of LDL-R and iron-free Tf bound to the Tf-R. Proteins from the sorting endosomes are directed to the ERC for recycling to the plasma membrane or for sorting to the degradation pathway.

Rab: small GTPases implicated in several steps of endocytotis. Of the plant orthologs, Ara6 is unique to the kingdom.

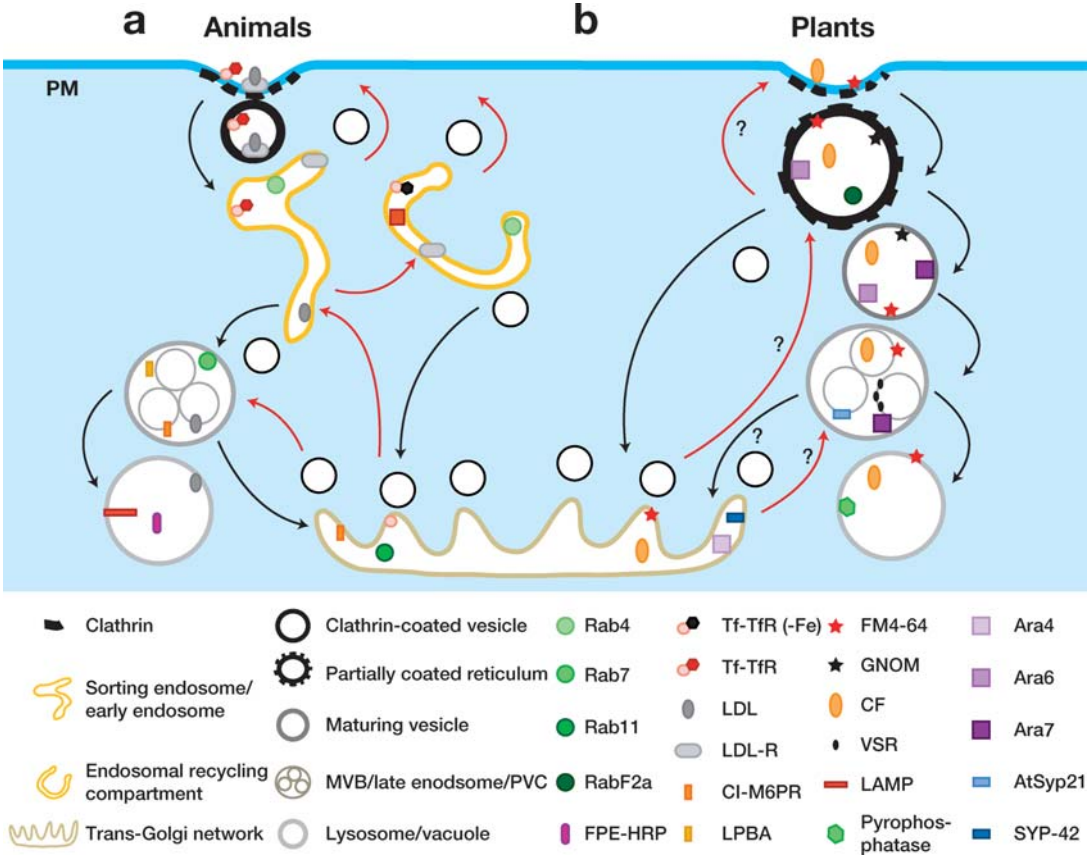


Figure 2

Endocytotic cycling in animals and plants. Endocytotic cycling is well studied in animal cells with several types of cycling known: clathrin-mediated, clathrin-coated receptor-mediated, nonclathrin-mediated (caveolae/lipid raft–mediated, fluid-phase endocytosis, phagocytosis). In contrast, little is known about endocytosis in plants and much has been extrapolated from animal literature. Two discrete endosomal compartments have been identified in plants, as well as a gradation of maturation from one to the other. Redirection of endocytosed PM proteins back to the PM has not been directly demonstrated but is strongly supported by indirect evidence (43, 44). Black arrows indicate the endocytotic pathway; red arrows indicate the secretory pathway. (*A*) In animals, clathrin-coated and receptor-mediated endocytosed vesicles from the plasma membrane (PM) are directed to the sorting endosome/early endosome [Rab4, LDL-receptor (LDL-R) and transferrin receptor (TfR) markers]. From there, cargo is transported to the PM, endosomal recycling compartment [Rab4, Rab11, LDL-R, transferrin bound to transferrin receptor (Tf-TfR)], or multivesicular body (MVB)/late endosome [Rab7, M6PR, lysobisphosphatidic acid (LPBA) (87)]. From the endosomal recycling compartment, cargo can traffic back to the PM or the trans-Golgi network (TGN) (Rab11). From the MVB/late endosome, cargo can travel to the TGN [cationic-independent mannose-6-phosphate receptor (CI-M6PR)] or the lysosome [fluid-phase endocytosed HRP (FPE-HRP), lysosome-associated membrane protein (LAMP) (24)]. Cargo can also travel from the TGN to the sorting endosome/early endosome and MVB/late endosome. (*B*) In plants, cargo and PM proteins/markers are endocytosed into the partially coated reticulum (PCR) [catonized ferritin (CF), GNOM, Ara6, FM4-64, RabF2a]. From the PCR, vesicle maturation results in direction of cargo to MVBs [CF, FM4-64, Ara7, AtSyp21, VSR proteins] through vesicle maturation with overlapping compartment markers [Ara6 and Ara7] or the TGN [CF, FM4-64, SYP-42, Ara4]. From the MVB/late endosome/prevacuolar compartment (PVC), cargo is trafficked to the vacuole [CF, FM4-64, pyrophosphatase (139)]; trafficking to the TGN is hypothesized. Trafficking from the TGN to the PCR or MVB has not been demonstrated.

IRAP: insulin-responsive aminopeptidase, a type II integral membrane protein that colocalizes with GLUT4 in specialized insulin-responsive compartments and exhibits similar trafficking dynamics as GLUT4

TGN: trans-golgi network

EGF: Epidermal Growth Factor

Tf-R (99, 111). Two kinetics have been described for ERC-to-PM recycling: a fast rate used by the Tf-R and C6-NBD-sphingomyelin, which recycle almost completely back to the PM ($t_{1/2}$ = 10 minutes), and a slower rate used by the GPI-anchored folate receptor ($t_{1/2}$ = 30 minutes). This latter rate can be increased up to threefold when the cholesterol level in CHO cells is reduced by about 40%, indicating that lipid microdomains may modulate sorting in the ERC (111). The ERC membrane is the main intracellular cholesterol repository (54).

The role of the ERC in insulin-regulated trafficking of the glucose-transporter GLUT4 was also recently described (18). At low insulin concentrations, GLUT4 is equally distributed between the ERC and another distinct compartment, characterized by the presence of the insulin-responsive aminopeptidase (IRAP) and the lack of Tf-R (18, 195) (**Figure 3**). In the absence of insulin stimulation, GLUT4 is constitutively transported from the internal compartments and cycles to the PM in a manner similar to Tf-R and other cell-surface proteins (141). However, in response to increased insulin concentrations, a rapid net translocation of GLUT4 from the IRAP internal storage compartments to the PM occurs, resulting in higher uptake of glucose in fat and muscle cells (195).

Unlike the sorting endosomes, which mature into late endosomes within 10 minutes, the ERC is a relatively long-lived organelle that requires vesicle coat proteins for recycling. The ERC uses clathrin-dynamin-coated vesicles for transport to the PM in nonpolarized and polarized cells (164, 177, 178). As such, the clathrin coat does not play a prominent role in sorting or recycling steps (11). It is also not required for concentrating the Tf-R because a truncated version of this protein that does not interact with APs recycles at the same rate as the wild-type receptor in nonpolarized cells (98). Rab11 and an Eps15-homology protein containing an EH domain (EHD1/Rme1) also specifically regulate transport from the ERC to the TGN and PM (92, 187). In addition, COPI coat proteins (2), sorting nexins, (191) and ARF6 (125) have been implicated in ERC sorting or ERC-mediated sorting of receptors. In nonpolarized cells, no sorting signal is required for trafficking from the sorting endosome to the ERC and from the ERC to the PM. However, in polarized cells, sorting motifs are required for targeting to the apical or basolateral PM, as demonstrated by the recycling of Tf-R and LDL-R to the basolateral PM via AP1/μ1B-containing vesicles (41).

Because sorting endosomes mature into late endosomes, no vesicular carriers or concentration mechanisms are needed to transport soluble ligands. However, before sorting endosomes mature into late endosomes, most transmembrane proteins destined to be recycled are removed rapidly and efficiently in a process described as "geometry-based" sorting. In this process, up to 80% of the membrane of sorting endosomes separates into narrow-diameter–tubules, which have a greater surface-area-to-volume ratio; removing these tubules results in separation of recycling receptors from soluble cargo (98, 111). Although this recycling step requires no sorting motifs, further sorting of the internalized transmembrane proteins into the degradation pathway (receptor downregulation) does.

Signaling receptors such as the G-protein-coupled receptors and the receptor–protein kinases contain an ubiquitin moiety at their cytoplasmic tails. Ubiquitination serves as a sorting signal for targeting into the invaginated membranes of the late endosome, a sorting process that simultaneously terminates their signaling function (81). Subsequently, an hepatocyte-growth-factor-regulated tyrosine kinase substrate (HRS) serves as a linker between the ubiquitinated receptors and the endosomal flat clathrin lattice, resulting in a bilayered clathrin coat that contains the mammalian equivalents to the yeast ESCRT (endosomal sorting complex required for transport) complexes (134, 143). For the EGF receptor protein kinase, the bulk of EGF receptors are bound to EGF and follow the process of receptor downregulation, but are also recycled from either the sorting

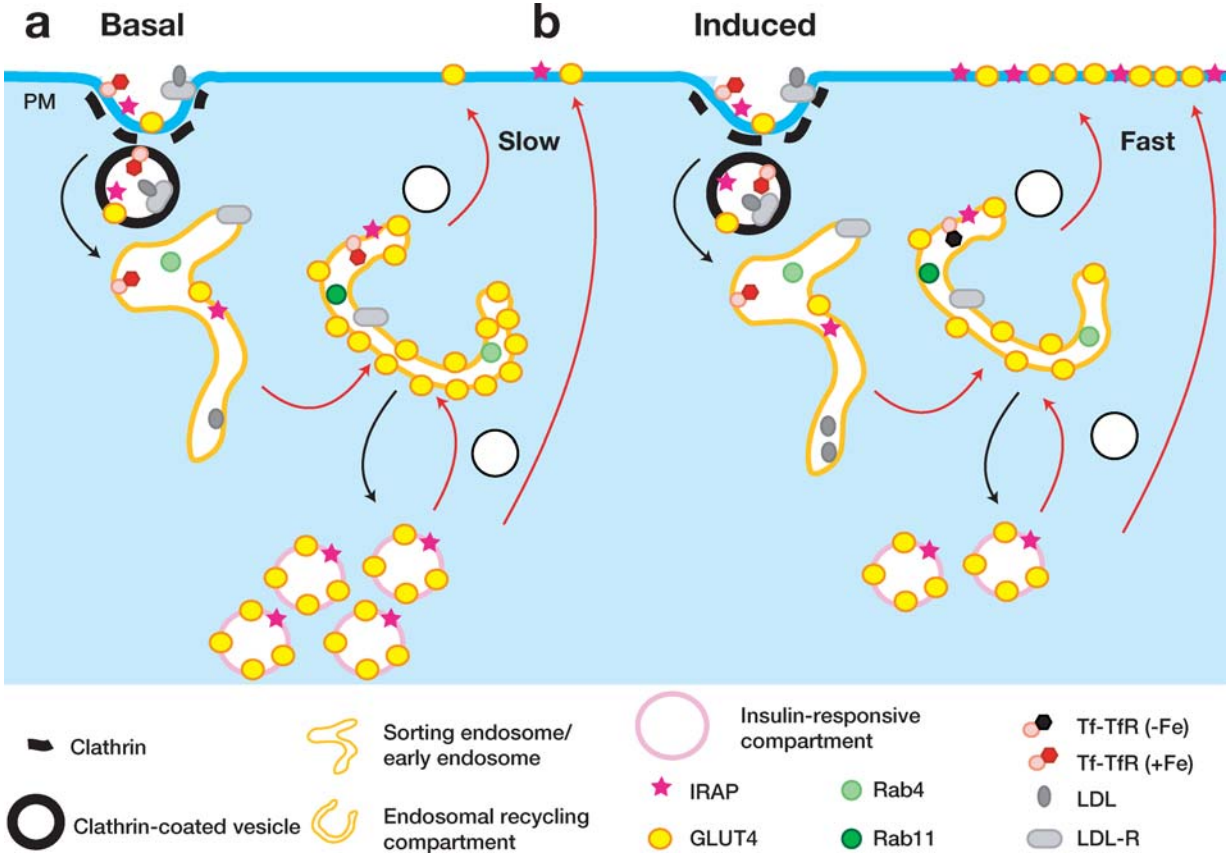


Figure 3

GLUT4/IRAP, a special case of endocytotic recycling in animals. The insulin-responsive compartment (IRC) is characterized by the presence of the GLUT4 glucose transporter, the insulin-responsive aminopeptidase (IRAP), and an absence of Tf-R. The IRC originates from the endosomal recycling compartment. Black arrows indicate the endocytotic pathway; red arrows indicate the secretory pathway. (*A*) Under basal insulin conditions, the trafficking of GLUT4/IRAP to the PM is constitutive. The presence of IRAP in the IRC has not been conclusively demonstrated. (*B*) Under high insulin conditions, induction occurs and trafficking of GLUT4/IRAP to the PM is ~sevenfold greater than that of the basal rate. When insulin levels return to the basal state, so does the trafficking rate.

endosome as free receptors or from the ERC after internalization of its ligand TGFα (161, 162). G-protein-coupled receptors follow the same recycling routes described for receptor protein kinases. The recycling of dephosphorylated G-protein-coupled receptors from the ERC via clathrin-coated vesicles containing βarrestin results in their rapid resensitization at the PM (46, 162). Because no direct input of PM vesicles to late endosomes has been demonstrated, late endosomes appear to function primarily as interfaces between endocytotic and biosynthetic pathways.

The cation-independent mannose 6-phosphate receptor (CI-M6PR) is an example of a more complex recycling pathway. Its early itinerary resembles that of TGN38 (45) but then resembles the itinerary of the transmembrane endoprotease furin in that it passes through the TGN and late endosomes en route to the PM. CI-M6PR is internalized via clathrin-coated pits from the PM and is

delivered via sorting endosomes to the ERC. From there it cycles several times to the PM. During each recycling round, a fraction returns to the late endosome via the TGN. Because the CI-M6PR is also the sorting receptor for acid hydrolases from biosynthetic pathways, it is transported between the TGN and late endosomes, but it is clearly absent from lysosomes. TGN to late endosomal sorting of CI-M6PR is mediated via an acidic-cluster-dileucine motif in its carboxy terminal and a monomeric Golgi-localized, γ-ear-containing, ARF-binding adaptor protein (GGA2). Return to TGN from the late endosome involves TIP47 (tail-interacting protein) and Rab9, whereas return of CI-M6PR from the early endosome involves AP1 and the cytosolic PACS-1 APs. Recently, an additional retromer that is also implicated in the maintenance of cellular polarity (183) was identified in mammalian CI-M6PR endosomal retrieval to the Golgi (4, 149).

The small Rab GTPases are master regulators in vesicle targeting and fusion events and thus also regulate recycling (196). Although Rab5 regulates fusion of homotypic sorting endosomes, it is also required for heterotypic fusion of internalized clathrin-coated vesicles with sorting endosomes. This fusion requires an effector known as the early endosome antigen 1 (EEA1) (155). Rab4 and Rab11 are also implicated in Tf-R recycling because Tf-R sequentially moves through Rab4- (sorting endosome and ERC) and Rab11- (ERC, TGN) positive structures (196). Rab function is also regulated by interactions between proteins containing specific phospholipid interaction domains and localized endosomal membrane phospholipid concentrations (27). Because of this, Rab5, Rab4, and Rab11 are often distributed into distinct domains within the same endosome (160, 169). Proteins interacting with Rabs in this category are EEA1 and its associated endosomal SNARE protein, syntaxin13 (100), other PI3P-binding proteins such as the sorting nexin3 (193), and Rabip4′, which functions in endosome-to-PM recycling (37).

PROTEIN COMPONENTS OF ENDOCYTOTIC CYCLING MECHANISMS

Adaptins and Adaptor Complexes

Adaptors mediate the selection of cargo molecules for inclusion into coated vesicles in the late secretory and endocytotic pathways. One group of cargo adaptors are monomeric proteins such as AP180, the β-arrestins, the GGAs (Golgi-localized γ-adaptin-ear-homology-ARF-binding proteins), or the stonins. AP180 is the only plant monomeric adaptor ortholog identified to date (7). Another group consists of heterotetrameric AP complexes composed of adaptin subunits. Each complex contains two large ~100-kDa subunits consisting of one clathrin interactor (β_{1-4}) and another large subunit that is complex specific (α-adaptin/AP-2, γ-adaptin/AP-1, δadaptin/AP-3 and ε-adaptin/AP-4). A small ~20-kDa adaptin subunit (σ_{1-4}) and a ~50-kDa medium subunit t(μ_{1-4}) complete the complex (13, 84). In mammals, there are four AP complexes, but only the TGN-localized AP-1 and the endocytic AP-2 are connected with clathrin. *Saccharomyces cerevisiae*, *C. elegans*, and *Drosophila* all lack the AP-4 complex, but the total number of adaptin orthologs in *Arabidopsis* points to the existence of four AP complexes (13). Like mammals, the *Arabidopsis* genome contains several isoforms of some adaptins, namely two α-adaptin (7, 67), five β-adaptin (13), three γ-adaptin (13, 146), five μ-adaptin (56), and five σ-adaptin genes. In addition, δ- and ε-adaptins are coded for by single genes (13). Like mammalian β1- and β2-adaptins, the βB- and βC-adaptins from *Arabidopsis* are highly similar. Some plant adaptin homologs are referred to by letters because the composition of plant AP complexes has not been elucidated and assignment to particular complexes cannot be made on sequence similarity alone.

Adaptins have been identified in the rice genome and have been reported in other plant species, particularly a σ1-adaptin from the

Chinese medicinal tree (95), a σ2-adaptin from maize (138), and a zucchini CCV β-adaptin that was recognized by bovine β1/β2-specific antibodies (65) and tentatively localized at the PM (31).

Little is known about adaptin functions in plants. Only two adaptins and one monomeric adaptor homolog from *Arabidopsis* have been functionally characterized on the molecular level. The μA-adaptin AtμA-Ad was identified as a receptor-binding partner (56), and a plant ortholog of mammalian α-adaptins, which play a crucial role in endocytosis, was identified as a binding partner for network proteins (AtαC-Ad). AtμA-Ad and AtαC-Ad are found in high molecular weight AP-like complexes (7, 56). Consistent with a role in endocytosis, At-AP180 promoted the formation of clathrin cages of nearly uniform size, thus functioning as a plant clathrin-assembly protein (7). Thereby, the assembly of clathrin triskelia depends on a single-DLL clathrin-binding motif, a feature that has not been described for mammalian AP180 (7). AtμA-Ad is strongly implicated in clathrin-dependent vacuolar-trafficking events, and its receptor-binding domain exhibited tyrosine-dependent interactions with the tyrosine-based sorting motif (YXXØ) of the mammalian TGN38 protein and the (VSR-PS1) vacuolar sorting receptor from pea (56). Based on their conserved features, the other four plant μ-adaptins are also expected to function as receptor-binding partners of plant AP complexes because the YXXØ motif functions in endocytosis as well (15) and some plant receptor-like kinases contain this motif. At-αC-Ad functions as a network-protein-binding partner because its carboxy-terminal ear region interacted not only with the mammalian network proteins Eps15, AP180, and amphiphysin, but also with the plant monomeric At-AP180. Little is known about plant β- and γ-adaptins. β-adaptins were identified as interactors with PM complexes containing *Arabidopsis* auxin transport proteins and the *Arabidopsis* gluzincin membrane aminopeptidase and AtAPM1 (114), and, more recently, γ-adaptins were identified as interactors with ADL6 (DRP2) and the same complexes derived from microsomal membranes (A. Murphy, unpublished data). Yeast–two-hybrid studies confirm that an *Arabidopsis* γ-adaptin interacts with the dynamin-like protein ADL6 (DRP2), probably involved in Golgi-originating vesicle trafficking (90). Although adaptin function appears to be largely conserved between animals and plants, there are cases where plant function differs, as when plant adaptins other than the β subunit interacted with clathrin (S. Holstein, unpublished data).

ARFs/ARF-GEFs

ARFs are a ubiquitous group of 20-kDa, ras-related GTP-binding proteins that maintain organellar integrity and regulate intracellular transport (21). Originally identified by their ability to stimulate the ADP-ribosyltransferase activity of cholera toxin (110), the six types of ARFs recruit vesicle coats crucial for vesicle budding and cargo selection (30). Under normal conditions, cytosolic GDP-bound ARFs are inactive (29). A vesicle-associated GTPase-activating protein (GAP) hydrolyzes GTP and releases the ARF protein, resulting in coat dissociation prior to vesicle fusion. GDP release requires an interaction with GEF proteins that contain a 200 amino acid sec domain required for GDP exchange activity. GEFs that contain sec7 are generally sensitive to Brefeldin A (BFA), a noncompetitive inhibitor that stabilizes an abortive ARF-GDP complex (126) and inhibits activation of ARF GTPases by blocking recruitment of vesicle coat components to membranes. However, a subset of this group contains a modified sec7 domain and is BFA resistant.

ARF6, the least conserved member of this family, appears to regulate endosome recycling and cortical actin reorganization (121). Studies in mammalian systems using Madin-Darby Canine Kidney cells indicate that ARF6 is present only on the apical surface of polarized epithelial cells (1). Overexpression of ARF6-Q67L, a GTP hydrolysis–deficient mutant, stimulates endocytosis at this surface. However, the

ARF: ADP-ribosylation factor

GEF: guanine nucleotide exchange factor

ARF/GEF: ADP-ribosylation factor. Guanine nucleotide exchange factors are a family of small GTPases that regulate endocytotic cycling.

ARL: ARF-like

PH: pleckstrin homology domain

PRD: proline-rich domain

DRP: dynamin-related proteins in plants, large GTPases implicated in cycling and cell wall synthesis

ARF6-T27N that retains bound GDP also stimulates apical endocytosis to a lesser extent. A dominant negative form of dynamin or clathrin can inhibit this endocytosis. Overexpression of these mutant forms of ARF6 also leads to an increase in the number of clathrin-coated pits in the PM (1). This study indicates that ARF6 is an important modulator of clathrin-mediated endocytosis at the apical surface of the PM. In a similar study, when ARF6 and its mutant forms were expressed in Chinese hamster ovary cells, the protein either colocalized to a perinuclear recycling compartment or accumulated at the PM, depending on its nucleotide status. This suggests that the protein cycles between the PM and recycling endosomes and may be involved in the outward flow of recycling membranes (32). Franco et al. (38) showed that EFA6, a protein exchange factor for ARF6, regulates endosomal membrane recycling and promotes the redistribution of transferrin receptors to the cell surface. EFA6 stimulates ARF6 guanine nucleotide exchange and localizes to a dense matrix on the cytoplasmic face of PM invaginations. ARF6 is also required for cortical actin rearrangements (133) and may link membrane traffic with cytoskeleton organization.

The *Arabidopsis* genome contains 21 ARF GTPase family members genomes, with isoforms present in both ARF and ARL GTPase subfamilies. ARF6 localizes to the periphery of the cell where it has an essential role in endocytotic pathways (104). There are eight *Arabidopsis* proteins with sec7 domains, including GNOM, which is essential for recycling PM proteins from endosomes and is required for polarized distribution of the auxin efflux facilitator PIN1 (43, 44). However, the ARF substrate involved in GNOM-regulated endosomal trafficking has not been determined.

Dynamins

Dynamins constitute a family of large (100-kDa) GTP phosphohydrolases that carry out diverse roles in eukaryotic membrane cycling (61, 78). These proteins have a pH, which allows them to bind to phosphoinositides, and a PRD, which mediates binding to proteins with Src homology 3 (SH3) domains. A coiled-coil region (CC domain) serves as the GTPase effector domain (152). Instead of functioning as classic switch GTPases, dynamins participate in membrane scission events by self-assembling into multimeric ring structures (64). In vivo studies show interactions of dynamins with proteins such as endophilin1 that alter the geometry of lipid structures, resulting in the formation of vesicles (147). Association of dynamins with actin or actin-binding and actin-depolymerizing proteins suggests that dynamin-actin interactions may also contribute to vesicle formation (131, 190). Studies in mammals suggest that dynamins link cellular membranes to the actin cytoskeleton and thereby regulate endocytosis (120).

In animals, there is extensive evidence that dynamins are involved in clathrin-mediated endocytosis, internalization of caveolae, synaptic vesicle recycling, and vesicular trafficking to and from the Golgi complex (60, 118, 119, 137, 176). In cultured mammalian cells, mutations in the GTP-binding domain of dynamin resulted in markedly reduced endocytotic uptake of transferrin receptors (62). An analysis of intermediates in coated vesicle formation in these mutants indicated that endocytosis was blocked after the initiation of coat assembly and before the sequestration of ligands into deeply invaginated coated pits. ER-to-Golgi transport was unaffected in these mutants, suggesting that dynamins function in early endocytotic events (180). However, subsequent confocal imaging studies showed association of dynamin with the TGN and perinuclear late endosomes containing the CI-mannose-6-P receptor (118). GFP-dynamin chimeras localized in the clathrin-coated vesicles at the cell surface also localize to the TGN (76).

Six dynamin-like protein subfamilies have been identified in plants that include the 16 *Arabidopsis* dynamin-related proteins (DRPs) (70). Of these, only members of the DRP2 subgroup, which are implicated in TGN to vacuolar trafficking (74), contain all of the signature domains

of classical mammalian dynamins (71). Members of DRP families 1 and 3 lack both pleckstrin and Pro-rich domains and are unique to plants (179). The DRP1 phragmoplastins constitute the largest plant-specific dynamin subfamily. In *Arabidopsis*, five dynamin-like protein gene families have been identified, with partially overlapping functions (78). DRP1-mediated membrane trafficking is essential for PM formation and recycling in plants (78, 79). A probable role in endocytotic events was suggested when DRP1 and several other proteins involved in endocytosis and vesicular trafficking copurified with auxin efflux protein complexes (114). More recently, the same complexes isolated from microsomal rather than PM fractions were found to be enriched in DRP2A bound to γ adaptin (A. Murphy, unpublished data; 114). Consistent with findings in animals, cellular localization of ADL1A (DRP1A) was disrupted by an actin antagonist, suggesting involvement of actin-dynamin interactions in endosomal regulation in *Arabidopsis* (78). Another yeast dynamin-like protein, VPS1p, is involved in protein transport from Golgi to an endosomal compartment (189), suggesting that the plant dynamin-like proteins could function in intracellular trafficking events other than endocytosis.

Rab GTPases

Rabs comprise a small family of ubiquitous proteins that are essential components of the membrane-trafficking machinery. Rabs are implicated in vesicular formation, loading, transport along cytoskeletal elements, and docking/fusion at target membranes (105, 109, 150, 167). Recent evidence shows that cargo proteins act as Rab effectors and regulate their own trafficking by direct interactions with the transport machinery (157). Like most GTPases, Rab proteins cycle between an inactive GDP-bound state and an active GTP-bound state. In the GTP-bound state, Rabs are associated with membranes and are attached to the cytoplasmic surface of compartments (106). The mechanism by which Rabs regulate endocytosis has not been elucidated, but it is hypothesized that the GTP-GDP cycle of Rabs might act as a timer switch to regulate the functionality of a membrane domain. It is also possible that Rabs act as GTP-activated switches that simply stabilize protein complexes required for transition events. However, the ability of Rabs to link membranes to cytoskeletal motor proteins (148) suggests that they can generate uniquely functional membrane subdomains.

In eukaryotes, specific Rab GTPases have been associated with the regulation of membrane-trafficking events in distinct compartments (184). In mammals Rab4, Rab5, Rab7, Rab9, and Rab11, are involved in endocytotic vesicular trafficking (127, 171). Rab4, Rab5, and Rab11 localize to early endocytic organelles where they regulate distinct events in the transferrin receptor pathway (106). Rab5 regulates docking and fusion of early endosomes and mediates interactions with multiple effectors. In its GTP-bound state, Rab5 builds an effector domain on the membrane, a process that requires the recruitment of PI3 kinases and PI3P-binding proteins that contain the FYVE motif (196). Rab4 and Rab11 are also involved in regulating protein recycling back to the PM. Rab4 regulates formation of endosomal recycling vesicles in ongoing cycles of association and dissociation from early endosomes (105). In mammals, human Rab11A and Rab11B isoforms have been localized in recycling endosomes (174), and in epithelial cells mammalian Rab11A is crucial for the exit of internalized proteins from apical recycling endosomes (33). Rab6, a mammalian homologue of yeast Ypt6p, initially thought to function only in *intra*-Golgi transport, is required for recycling proteins between endosomes and the TGN (96).

A large number of highly conserved Rab GTPases have been identified in plants, and their localization generally coincides with that of their eukaryotic homologs, particularly those functioning in the exocytotic pathway (129). However, unique isoforms of endocytosis-related Rabs have been identified in *Arabidopsis*, including two Rab5 orthologs, seven Rab7

PVC: prevacuolar compartment

orthologs, and Ara6, which is structurally dissimilar to all known GTPases (173). Orthologs of Rab4 and Rab9 are missing altogether in *Arabidopsis*. AtAra-4 and Pea Pra3 are similar to the mammalian Rab11 and localize to or function within the TGN (73, 172). A Rab6 ortholog in *Arabidopsis* complements a mutation in the yeast Ypt6 protein that was implicated in protein recycling from endosomes to the Golgi and from late to early Golgi (10, 94). AtRabA4b, which is similar to Rab11, labels a novel compartment that accumulates at the tips of root hair cells (129). The tobacco ortholog of the mammalian Rab2 that regulates membrane flow in Golgi intermediates localizes to Golgi bodies in pollen tubes (22, 167, 168). However, Rha1, which is associated with trafficking of cargo molecules to the vacuole through late endosomal/PVCs in *Arabidopsis*, is an ortholog of Rab5 (158), which regulates fusion of endosomal compartments in mammals. It is still unknown how Rabs are delivered to distinct subcellular compartments or what other effectors are involved.

SNAREs

N-ethylmaleimide-sensitive factor adaptor protein receptors (SNAREs) are essential components of vesicle-trafficking machinery where they play a central role in membrane fusion events. SNAREs comprise a family of small proteins that are associated with different intracellular membranes and, as such, define the interactions and dynamic structures involved in endocytotic cycling. The role of SNARES in the endosomal system has been studied using endosomal fusion in both cell-free and intact cell assays. In mammals, SNARES are involved in two distinct homotypic fusion steps involving early and late endosomes (52). The SNAREs VAMP-7, VAMP-8, and syntaxins 7, 8, and 13 have been implicated in both fusion events, whereas complexation of VAMP-8 with the syntaxins 7 and 8 and Vti1b are involved in the homotypic fusion of late endosomes (3). The v-SNARE cellubrevin has been implicated in the recycling of endocytic receptors back to the PM (101) and the t-SNARE Tlg2p in early stages of endocytotic internalization (151). More recently, direct transport from early/recycling endosomes to the TGN (bypassing the late endosome) was shown to be mediated by a complex formed between the TGN-localized syntaxin 6, syntaxin 16, and Vti1a and the EE/RE-localized v-SNARES VAMP 3 and VAMP4 (96). In a recent study, polarized recycling of T cell antigen receptors to the PM and their eventual fusion was shown to involve the t-SNARES syntaxin-4 and SNAP-23 (26).

In *Arabidopsis*, where studies of SNAREs have focused primarily on secretory and vacuolar targeting, two SNARES (AtVTI1a and AtVTI1b) are involved in trafficking to the PVC (198). In addition, the syntaxins SYP21, SYP22, SYP4, SYP5, SYP42, and SYP61 are associated with various endosomal compartments, including the PVC and TGN.

Other Cytoskeletal Interactions

All endocytotic pathways of membrane protein internalization depend on interaction with the cytoskeleton. Direct participation of F-actin in internalization events has been documented in animals and yeast (34). Actin dynamics are also crucial for lipid raft-mediated endocytosis (124). However, the molecular details of the interaction are not known and the specific steps of endocytosis where the interactions are involved have not been identified. Studies in *Arabidopsis* show that disrupting the actin cytoskeleton by latrunculin B or cytochalasin D inhibits the formation of BFA-induced endocytotic compartments involved in recycling PM proteins (44).

A number of proteins play an essential role in mediating interactions between vesicular structural components and the actin cytoskeleton in animals (34). Some of these molecular linkers have also been identified in plants. Myosin, molecular motors that traverse along actin filaments, have been implicated in the distribution of endocytotic vesicles and membrane-bound molecules in *Arabidopsis* (68). Components of the actin-related protein (ARP) 2/3 complex have also been implicated in the internalization step and in the organization of the

actin cytoskeleton (144). These functions of the ARP2/3 proteins are mediated by their interactions with calmodulin. The ARP2/3 complex in *Arabidopsis* has a similar function (91).

Other Proteins Contributing to PM Protein Cycling

Rho GTPases are members of a small but diverse protein family (135, 199) involved in signaling and regulation of endocytotic traffic (130). Although Rho-GTPases are not found in plants, plant Rho-related GTPases (ROPs) comprise a plant-specific subfamily (194). ROPs have been localized to the tips of root hairs, have been implicated in polar growth (107), and play a key role in fusing docked vesicles and endocytosis in these growing tips (19). Immunophilins were originally discovered as receptors for a family of immunosuppressive drugs including cyclosporin A, FK506, and rapamycin. Later, when immunophilins were found in bacteria, yeast, animals, and higher plants, it became evident that immunophilin-like proteins were likely involved in fundamental cellular processes. Most immunophilins possess peptidyl prolyl cis/trans-isomerase activity required for protein folding and modification. The *Arabidopsis* genome contains 52 genes encoding putative immunophilins, including 23 putative FK506- and rapamycin-binding proteins (FKBPs) and 29 putative Cyclosporin As (CYPs) (57), many of which are localized to the chloroplast. Recent evidence suggests that a cyclophilin interacts with the ARF-GEF GNOM (49) and that an FKBP immunophilin, TWD1, may be involved in trafficking PM transport proteins (42). Fatty acyl CoA has been identified in cycling GLUT4 vesicles and has been implicated in membrane budding and fusion events (156). In another study, acyl CoA dehydrogenase (ACD) was found to mediate intracellular retention of GLUT4 vesicles via association with IRAP (80). A membrane-associated acyl CoA-binding protein that functions in intracellular lipid transfer from the ER and maintains a membrane-associated acyl pool at the PM was identified in *Arabidopsis* (47). AtAPM1, the IRAP homolog in *Arabidopsis*, was recently found to interact with ACDs (O. Lee & A. Murphy, unpublished data), suggesting a trafficking role for these proteins in plants.

ROP: Rho-related GTPases specific to plants, involved in endocytosis and fusion of docked vesicles

WHAT DO WE KNOW ABOUT ENDOCYTOTIC CYCLING IN PLANTS?

Endocytosis

Most of what is known about endocytosis comes from studies in yeast and mammalian cells. Until recently, endocytosis in plants was a questionable phenomenon because of the unfavorable energetics inherent in the endocytosis of vesicles in turgid cells with a rigid cell wall. Even though clathrin-coated pits were detected in the plant PM decades ago and polyvalent electron-dense tracers were subsequently shown to be internalized by protoplasts (136), this important phenomenon received little attention from plant biologists. However, recent studies utilizing styryl dyes, filipin-labeled plant sterols, reporter-tagged marker proteins characterizing endocytotic compartments, and the fluid-phase marker LuciferYellow indicate that endocytosis mediates the internalization and recycling of PM molecules, including membrane proteins and sterols in plants (5, 43, 44, 49, 72). Furthermore, because vigorous, constitutive endocytosis and recycling of the potassium channel KAT1 in turgid guard cells was recently demonstrated (102), turgidity can be ruled out as an inhibitory factor for endocytosis.

At least four forms of endocytosis operate in plants: clathrin independent and dependent, phagocytotic, fluid-phase and lipid raft-mediated endocytosis. The clathrin-independent pathways are mediated by actin polymerization and require small GTPases (20, 23). Rhizobia are internalized into root cells by a Rab 7-dependent phagocytotic process (159) and Baluska et al. (6) recently confirmed the occurrence of fluid-phase endocytosis in plants using the fluid-phase marker Lucifer Yellow. Mongrand (108) and collegues recently demonstrated the existence of lipid raft microdomains

PCR: Partially coated reticulum, an apparent plant analog of mammalian early endosomes.

MVBs: multivesicular bodies, also known as late endosomes or prevacuolar compartments

in tobacco PM. Many GPI-anchored proteins in animals cluster into sphingolipid- and sterol-enriched lipid raft microdomains (112). The structural sterols of plants, stigmasterol and sitosterol, form lipid rafts much more readily compared to cholesterol (192), suggesting the possibility of raft-mediated endocytosis in plants. Recent studies identified GPI-anchored proteins associated with lipid rafts in plants (17, 89). Other studies suggest that PINFORMED (PIN) auxin efflux proteins may depend on structural sterols and lipid rafts for their recycling in plants (49, 188). A GPI-anchored immunophilin (TWD1) and a fasciclin-like arabinogalactan protein (FAGP2) were recently shown to associate with auxin efflux proteins in *Arabidopsis* (42, 114). Although the direct involvement of clathrin-coated vesicles in regulated endocytosis has not yet been demonstrated in plants, clathrin-coated vesicles and associated APs mediating clathrin-dependent endocytosis have been found in *Arabidopsis* (7). To a large extent, studies of clathrin-mediated endocytosis are still predominantly exercises in comparison to mammalian processes (see Overview, above).

At present, molecular mechanisms underlying reversible membrane protein internalization in plants are unknown. However, polypeptide signals and receptors for plant defense, growth, and development have been identified (142), kinase-associated protein phosphatase (KAPP)-mediated endocytosis of At-SERK1 was recently documented (153), and many other plant membrane receptor kinases with phosphorylation- and tyrosine-based internalization motifs have been identified (67).

Endocytotic Compartments

Endosomal compartments have been characterized largely by studies tracing the course of endocytosed molecules through dynamically changing compartments. This makes the compartments difficult to define and identify because no molecule permanently resides in any compartment. Molecules may be transported into or out of the compartment through transport vesicles or, alternatively, the compartment itself may be modified and mature over time (98), as diagrammed in **Figure 2**.

Plant endocytotic compartments are not well characterized and the term endosome is often used for any compartment containing an endocytosed material. Styryl dyes such as FM4-64 are incorporated into the plant PM, subsequently endocytosed, and transported to vacuoles via transport intermediates (185) (**Figure 2**). These dyes are widely used in colocalization studies to ascertain the nature of intracellular vesicles that are putative endosomal compartments (14, 49, 173). Ultrastructural studies indicate that the PCR, a structure that was originally thought to arise from the TGN (63), is analogous to early/recycling endosomes of animals. Time-course studies tracing the path followed by cationized ferritin in soybean and white spruce protoplasts first labeled these compartments (40, 166). Molecule sorting occurs in the early endosomes, from where they are either recycled back to the PM or are transported to the Golgi apparatus or MVBs (8, 77). From the MVBs they are targeted to vacuoles for degradation. In a recent study using *N. tabacum* BY-2 cells, MVBs were identified as PVCs that are common to both secretory and endocytotic pathways (170).

In animals, proteins destined to be recycled back to the cell surface can be directly transported to the PM by the fission of tubules from sorting endosomes or can be transported via the ERCs. In plants there is still no clear evidence of sorting and recycling endosomes, but two distinct classes of early endosomes were identified in *Arabidopsis* using double-labeling experiments (173). One class of endosomes is characterized by Ara6, a unique plant Rab GTPase, whereas another is characterized by Ara7, the *Arabidopsis* homolog of mammalian Rab5. A small population of endosomes also contains both Ara6 and Ara7, suggesting endosomal maturation (**Figure 2**). However, although Ara6 and Ara7 both drive early endosomal fusion, it is unclear whether they mediate fusion of different endosomal populations. These two distinct early endosomal compartments could

participate in the sorting events that target endocytosed proteins to the recycling or degradation pathways.

A number of plant proteins cycle between the PM and intracellular compartments. Pharmacological studies with the fungal toxin BFA, which targets the catalytic domains of ARF-GEFs (126), demonstrate that the PIN1 PM auxin efflux facilitator can be reversibly retained in intracellular compartments (44), diagrammed in **Figure 4**. The compartments are characterized by the presence of the endocytotic markers Ara6 and FM4-64. The ARF-GEF GNOM is a specific target of BFA (43), suggesting that similar compartments function in the normal cycling of PIN1 and other proteins. These results also suggest that GNOM is functionally distinct from similar ARF-GEFs in animals that mediate ER-Golgi or intra-Golgi trafficking (197).

Distinct compartments that are not labeled by endocytotic markers have also been identified both in animals and plants. In animals, the most striking of these is the compartments that house the GLUT4 glucose transporter in the absence of an insulin signal. These compartments lack transferrin receptors and are characterized by the presence of IRAP, which exhibits trafficking dynamics similar to GLUT4 (80). Comparable mechanisms in plants are suggested by the PM and intracellular localization of AtAPM1, an IRAP homolog in *Arabidopsis* (114).

Endosomal Sorting and Redirection

In animals the first branch point for endocytosed proteins is the sorting endosomes. Proteins in the sorting endosomes can have three distinct fates: they can be directly recycled back to the PM, transported to the ERC for eventual return to the PM, or they can end up in the late endosomes. The low luminal pH of the sorting endosomes underlies the first recycling step, dissociation of ligands from the receptors (128). In contrast to animals ligand-receptor binding in plants occurs in an acidic (pH ~5.5) extracellular environment. As such, it is likely that ligand dissociation in the early endosomes of plants requires either greater acidification than is seen in animal cells or a different sorting mechanism altogether. Studies with the vacuolar proton pump inhibitor bafilomycin A1 indicate that endosomal V-ATPase activity is essential for transporting proteins from late endosomes to lysosomes because, in its absence, receptor-ligand complexes continuously recycle between the PM and the early endosome (181). In *Arabidopsis*, acidification is also required for sorting, but may involve PM ATPase activity as well (9).

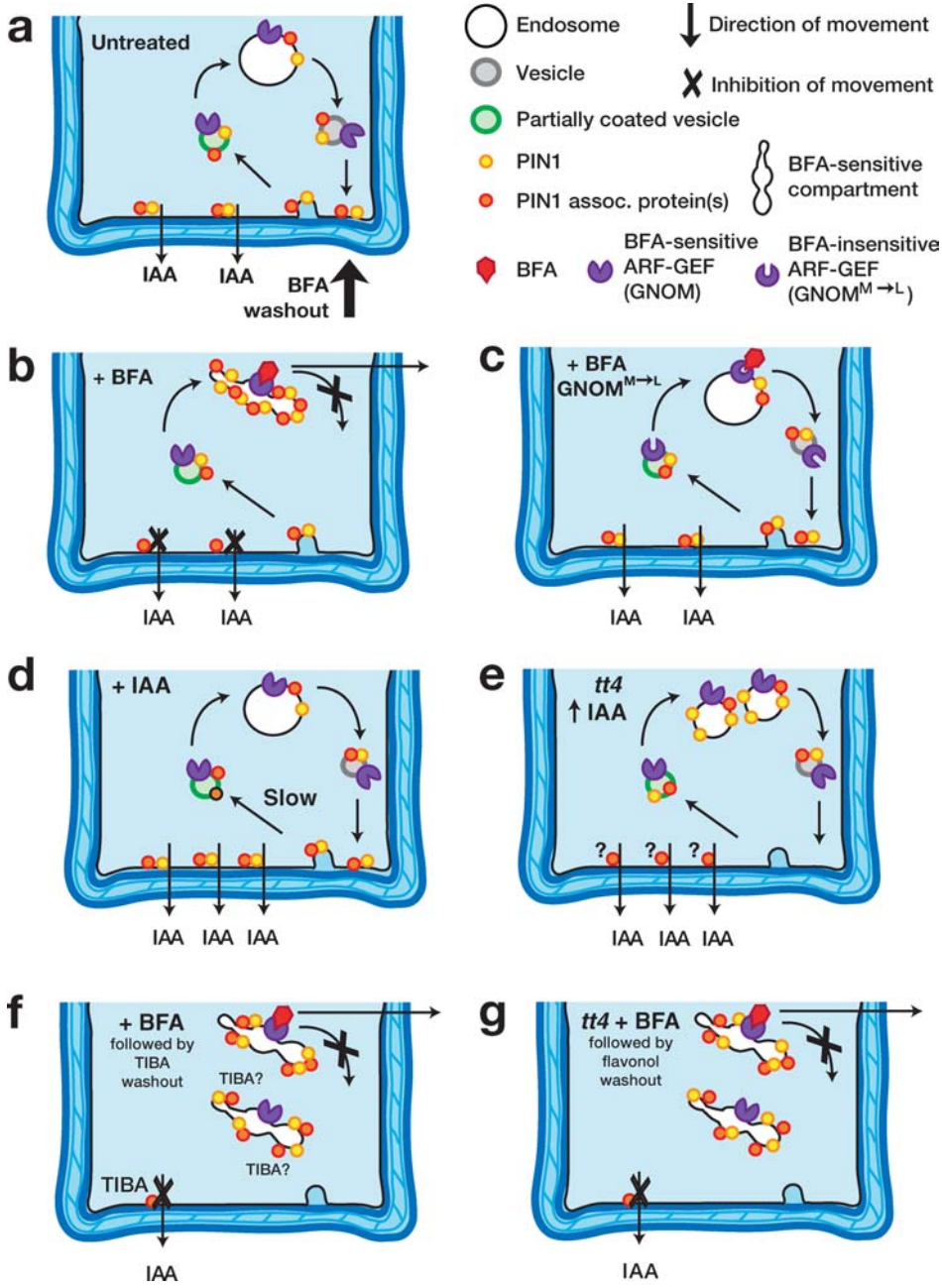
In mammals, phosphoinositol 3-phosphate (PI3P) and Rab5 recruit microtubule motors to endosomes, thereby establishing specific membrane domains (160). PI3P is required for two critical functions in early endosomes (51). It regulates the dynamics of the compartment and, subsequently, participates in the retention of proteins in domains that mature into late endosomes. PI3P fuses to FYVE domain from human EEA and tags to GFP colocalized with BP-80 in the PVC when transiently expressed. Overexpression of the chimeric protein inhibits targeting of Sporamin to the vacuole (83).

In mammals and yeast, ubiquitination of receptors and transporters serves as a signal for their internalization. Studies with the yeast endocytic cargo uracil permease (Fur4p) indicated that ubiquitination was required for its internalization but deubiquitination was not required for its recycling.Ubiquitinated early endosomal proteins are directed by the ESCRT I, II, and III complexes to the MVB. Upon fusion with the lysosome, the internal vesicles of the MVBs and their cargo are accessible to luminal lytic enzymes. Studies in *S. cerevisiae* lacking a functional ESCRT complex resulted in mistargeting of endocytotic and biosynthetic ubiquitinated cargoes. Deficiencies in this complex also lead to the accumulation of receptors and transporters at the PM in yeast and higher eukaryotes. Predicted proteins with sequence similarity to the Vps23 component of the ESCRT-I complex are present in the *Arabidopsis* genome, suggesting that a similar mechanism may exist in plants.

EVIDENCE FOR ENDOCYTOTIC RECYCLING IN PLANTS

Auxin Transport Proteins and GNOM

Establishing plant polarity depends on the polar transport of the growth hormone auxin. The directionality of auxin transport is maintained by a polar transport apparatus that requires asymmetrically localized transporters and regulators. The PIN proteins are the best characterized of such asymmetrically localized auxin efflux regulators. Pharmacological, physiological,



and molecular genetic studies suggest that activity, membrane localization, and vectorial realignment of PIN proteins is regulated by dynamic cycling between the PM and endosomal compartments (12, 39, 43, 44, 123).

PIN1 undergoes rapid actin-dependent cycling. When treated with BFA, PIN1 is still internalized but accumulates in unidentified, juxtanuclear compartments rather than returning to the PM (44). Although initially proposed to result from the fusion of endosomal and post-Golgi membranes (117), the BFA-induced bodies are surrounded by remnants of Golgi stacks and are actually aggregated endosomal compartments that accumulate the endocytotic tracer FM4-64 (43). Similar BFA-induced internalization occurs for other PM-ATPases (44), the PM marker Lti6a (49), the peripheral membrane protein ARG1 (16), and the putative auxin influx carrier AUX1 (48). Cell wall polymers such as pectins cross-linked with boron or calcium are also recycled by the same BFA-sensitive pathway in maize roots (5).

The BFA-induced compartments also accumulate other molecules involved in vesicular trafficking such as the cytokinesis-specific syntaxin KNOLLE and its interactor AtSNAP33, the small GTPase ARF1 and small GTPase Pra2 (25, 43, 44, 58, 73). This suggests that internalization and recycling mechanisms similar to those found in animals exist in plants. This cycling is regulated by extracellular signals such as hormones and flavonoids (123), as is the case with the mammalian glucose transporter GLUT4.

As diagrammed in **Figure 4**, the inhibition of PIN1 cycling by BFA is mediated by an ARF-GEF (43). In *gnom* mutant embryos, PIN1 localization is largely randomized, suggesting that GNOM is required for proper PIN1 localization on the PM (163). When plants are transformed with an engineered BFA-resistant version of GNOM, PIN1 is localized on the

Figure 4

PINFORMED (PIN) protein endocytosis and auxin transport in root tips. Indirect evidence suggests that the PIN components of the auxin efflux carrier complex undergo endocytosis and redistribution to the plasma membrane (PM). This was demonstrated with pharmacological studies utilizing the fungal exocytosis inhibitor brefeldin A (BFA), exogenous application of the transported hormone auxin (IAA), studies of mutants exhibiting altered auxin flux, and analysis of the effects of exogenous and endogenous auxin transport regulators (43, 44, 123). (*A*) In untreated WT root tip cells with normal auxin levels, PIN1 protein is detected on the PM. (*B*) Indirect evidence suggests that PIN1 undergoes endocytosis and redistribution to the PM. When root tips are treated with BFA, PIN1 is endocytosed into BFA-sensitive compartments and is no longer observed on the membrane. Following BFA washout, PIN1 is redistributed to the PM, as in (*A*). (*C*) BFA activity affecting PIN1 localization depends on binding to an ARF-GEF, GNOM. When GNOM is mutated ($GNOM^{M\rightarrow L}$) so that it can no longer bind BFA, then PIN1 is observed on the PM in BFA-treated cells. (*D*) When roots are treated with IAA, the rate of uptake of FM4-64 from the membrane is reduced (A. Murphy, unpublished data) and more PIN1 is observed on the PM. IAA also decreases the transcription of *PIN1*; therefore, no nascent PIN1 is delivered to the PM. If the cells are treated with the artificial auxin NAA plus BFA, then PIN1 is observed on the PM and not in BFA compartments (T. Paciorek & J. Friml, personal communication). (*E*) Flavonols are endogenous compounds that can modulate auxin efflux at the apices. In the flavonoid-deficient mutant *tt4*, auxin delivery to the root tip is greater than in WT, and PIN1 exhibits predominantly intracellular localization and is not found on the PM. *PIN1* transcription is also reduced in the mutant, apparently reflecting the effect of long-term IAA exposure on PIN1 localization at the root tip. (*F*) The competitive auxin efflux inhibitor triodobenzoic acid (TIBA) binds at the efflux site. When BFA is washed out with TIBA, PIN1 localization remains in BFA compartments and does not return to the PM. (*G*) When *tt4* is treated with BFA, PIN1 is observed in BFA compartments (as in BFA-treated WT, unlike untreated *tt4*). When BFA is washed out, then PIN1 is observed on the membrane, as in WT. In *tt4*, when BFA is washed out with flavonols, then PIN1 remains in BFA compartments; however, in WT, PIN1 returns to the membrane. This suggests that BFA has pleiotropic effects on cycling and does not inhibit exocytosis via ARF-GEFs only. This also suggests that flavonols interact with a protein that is required for PIN1 retention before sequestration in BFA compartments.

VDAC: voltage-dependent anion channel

PM, but is not internalized in response to BFA treatment. Therefore, GNOM appears to be required for recycling of PIN1 from endosomes to the PM. However, cycling of other PM proteins such as PIN2, a PM-ATPase, and the syntaxin KNOLLE are not affected by GNOM, suggesting that specific ARF-GEFs might regulate the endocytotic recycling of discrete groups of PM proteins.

In animals, endocytotic recycling also plays an essential role in maintaining the correct membrane protein composition in apical and basolateral membranes of polarized cells. However, in plants, although delocalization of PIN proteins from the PM (12) and vectorial relocalization of PIN3 in response to gravity (39) were recently demonstrated, no basolateral redirection has been observed. Recent evidence of polar reorientation of PIN proteins in *PID* overexpression transformants (39a) suggests that a PID-dependent regulatory mechanism controls the polarity of PIN proteins.

The KAT1 Inward-Rectifying Potassium Channel

The dogma that the high turgor of plant cells precludes endocytosis was recently challenged by evidence that the KAT1 K^+ inward-rectifying channel is constitutively endocytosed from the guard cell PMs against high turgor pressure (72) (**Figure 5**). In WT and KAT1-GFP transformed protoplasts, increases or decreases in PM surface area resulting from a change in cellular pressure led to the incorporation and withdrawal of vesicular membranes carrying an active K^+_{in} rectifier. The density of KAT1 was higher in vesicular membranes than in the PM, suggesting that the channels remained clustered while trafficking to and from the PM (72). The endocytosis process starts immediately following pressure stimulation, ruling out channel concentration prior to internalization. It is suggested that KAT1 channels form stable clusters and that membrane areas containing these clusters are retrieved preferentially during pressure-driven endocytosis. The KAT1::GFP chimera was later shown to be internalized and accumulated in small vesicles in the cortical regions of the cells. These bodies were also labeled with the styryl dye FM4-64 and were thus confirmed to be endocytosed vesicles (102).

Other Examples

Reports of environmentally responsive changes in the subcellular compartmentation of membrane proteins suggest that membrane protein internalization and recycling are part of the regulatory repertoire of plants. In mammals, hormonal signals trigger the cycling of aquaporins between the PM and internal vesicles (53, 86). Similar mechanisms may be at work in plants because the *Arabidopsis* aquaporins (PIPs) can localize both to the PM and vacuoles (132), and the *Mesembryanthemum crystallinum* aquaporins, *Mc*TIP1 and 2, are internalized from the PM and redistributed to the tonoplast and other membrane fractions in response to mannitol-induced water stress (182). *Mc*TIP1 and 2 were also localized to some unique multivesicular endosomal compartments and the observed redistribution was arrested when treated with BFA and other inhibitors of vesicular trafficking. The VDACs have a dual localization in plants as in animals, being localized to the mitochondrial outer membrane and PM (35, 97), which suggests cycling of these proteins between the PM and internal organelles. The recent finding that AtAPM1 interacts with VDACs (O. Lee & A. Murphy, unpublished data) suggests that VDACs may be trafficked by this mechanism. In another study focusing on membrane protein endocytosis, BFA induced internalization of the low-temperature-inducible protein Lti6a and resulted in its colocalization with FM4-64 in discrete subcellular compartments (49). A PM H^+-ATPase also accumulated within BFA compartments in an F-actin-dependent manner, but the redistribution did not require intact microtubules (49). In another recent study Kim et al. (82) reported that the metal tolerance protein (TgMTP1) can be localized to the PM and the

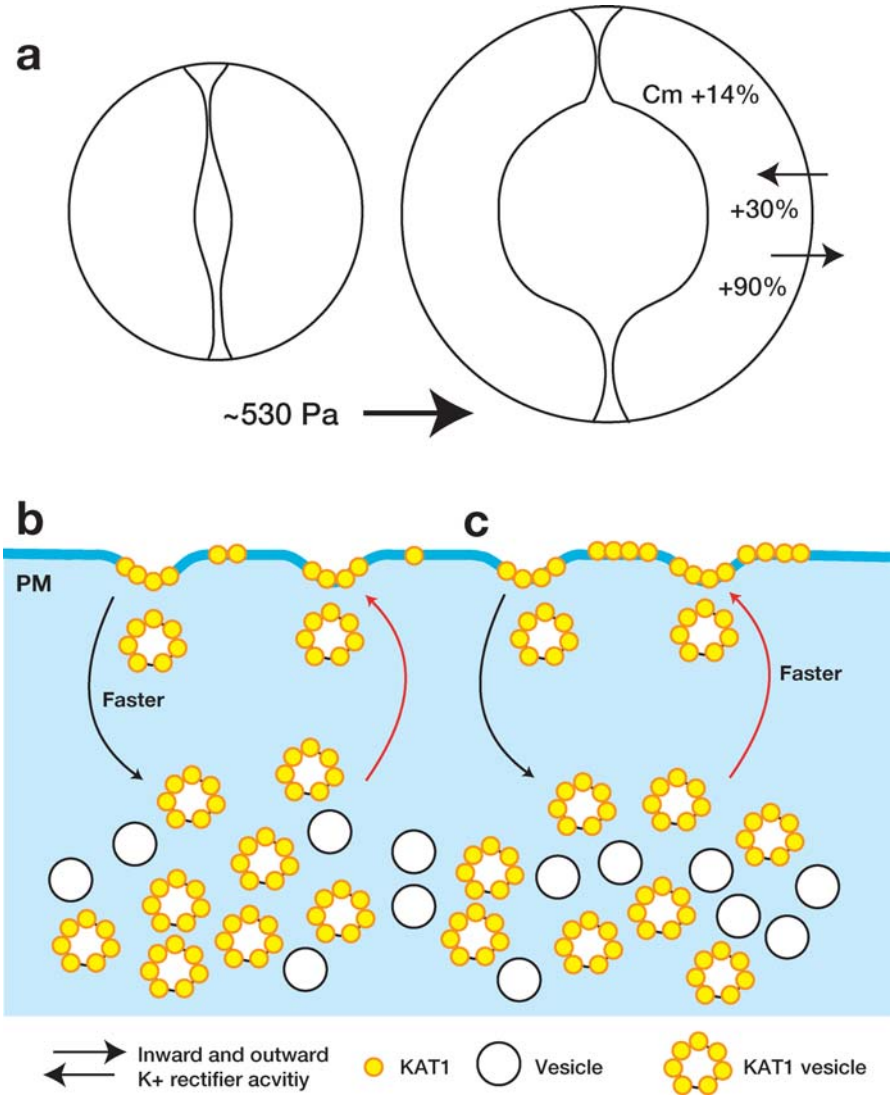


Figure 5

KAT1 recycling and a technique to quantitate endocytosis in plants. Endocytosis was thought not to occur in plants due to turgid cells and rigid cell walls. However, the variable surface area and capacitance of stomatal guard cells provide a method to quantitate endocytosis in plants (69, 72, 102). Black arrows indicate the endocytotic pathway; red arrows indicate the secretory pathway. (*A*) Guard cells swell and shrink to open and close the stomatal pores, thereby regulating the amount of gas exchanged with the environment. The activity of potassium channels, as indicated by membrane capacitance (Cm), correlates with the surface area of the guard cell protoplast. The surface area and the capacitance increase with hydrostatic pressure. The surface area increases up to 40%, and only a 3% to 4% increase of area can be accounted for by stretching the plasma membrane (PM); the capacitance increases by 14%. Therefore, recruitment of additional PM material and ion channels occurs during stomatal opening and retrieval of PM material and ion channels occurs as stomata are closing. (*B*) KAT1, an inward potassium channel rectifier, is found in greater abundance in the endomembrane system than on the PM. Negative hydrostatic pressure results in reduced cell area, lower capacitance, and faster and selective endocytotic retrieval of KAT1 from the PM. (*C*) Positive pressure results in greater cell surface area, higher capacitance, and faster and selective insertion of KAT1 into the PM.

vacuole, suggesting that its dynamic relocalization between the PM and various endomembrane systems could have a role in regulating metal hyperaccumulation. Similar endocytosis-mediated trafficking of metal transporters has been documented in mammals. The PM zinc transporter Dri 27/ZnT4 has been colocalized with the transferrin receptor and the clathrin adaptor complexes AP-1 and AP-2 in endosomal vesicles, suggesting that they are internalized in a clathrin-dependent manner from the PM (113).

CONCLUSION

Recent experimental evidence suggests that plant cells respond to developmental programming and environmental conditions by regulating the endocytosis of PM proteins. This evidence also suggests that some of the mechanisms underlying endocytotic cycling are conserved between plants and animals. However, as plant endosomal compartments and the proteins that characterize them are still poorly defined, the extent of that conservation has yet to be determined. Until recently, studies in plant cell biology focused primarily on secretory mechanisms at the expense of internalization pathways. Because of this, researchers should be cautious about overextrapolation of mammalian models to plant systems. The proliferation of molecular tools in *Arabidopsis*, the advances in imaging techniques, and the growing availability of organellar markers, dyes, and reporter proteins are expected to accelerate our understanding of endocytotic cycling in plants and animals as well.

SUMMARY POINTS

1. Occurrence of endocytosis in plants is evident from recent reports.
2. Plant endocytosis processes and players share common features with animals, but there are features unique to plants.
3. Clathrin and adaptor complexes have been identified in plants, but evidence for receptor-mediated endocytosis is lacking.
4. FM4-64 and BFA studies have made a significant contribution to the understanding of the endocytotic pathway in plants.
5. Endosomes are not well characterized in plants, but two early endosomal populations have been identified by double-labeling experiments; Ara6 is targeted to only one of them.
6. There is indirect evidence for cycling of PIN auxin efflux facilitators. The ARF-GEF GNOM is required for the observed cycling.
7. The KAT1 K^+ inward-rectifying channel is endocytosed against high-turgor pressure of guard cells, suggesting turgor is not an inhibitory factor for endocytosis.
8. Several other membrane transporters and channels are regulated by endocytotic recycling.

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