Endocytosis, Actin Cytoskeleton, and Signaling[1]

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Endocytosis is the internalization of plasma membrane proteins and lipids, extracellular molecules, fluids, particles, exosomes, viruses, and bacteria. Endocytic internalization is a conserved process for all eukaryotic cells that is required for diverse cellular functions. These include turnover and degradation of plasma membrane proteins and receptors, transduction and dispersal of signals within the cell and between cells of an organized tissue, spread of morphogens, cell-to-cell communication at synapses, elimination of pathogenic microorganisms, establishment of symbiosis with microorganisms, and nutrient uptake. Endocytosis has played a crucial role in endosymbiosis during eukaryotic evolution and laid the foundation for the emergence of specialized organelles, such as mitochondria and plastids. Several basic forms of endocytosis have been defined according to the type of cargo and molecular machinery driving its internalization. The endocytic pathways include clathrin-mediated, caveolae/lipid raft-mediated, clathrin-, and caveolae-independent endocytosis, fluid-phase endocytosis, and phagocytosis. Among them, clathrin-dependent endocytosis represents the best studied form of endocytic internalization. During the last decade, a significant number of studies revealed that clathrin-mediated endocytosis is highly regulated by structural, adaptor, regulatory, and signaling proteins involved in the formation (budding) of endocytic vesicles, their pinching off the plasma membrane, trafficking and selective fusion with endosomal/lysosomal compartments (for review, see Brodsky et al., 2001). For example, the structural protein clathrin and several adaptor proteins build up the macromolecular lattice on the surface of endocytic vesicles (known as clathrin coat) that interacts with the large GTPase dynamin, as well as with cytoskeletal and signaling protein complexes (for review, see Engqvist-Goldstein and Drubin, 2003). Importantly, endocytosis is required not only for retrieval and desensitization of receptors, but also for efficient signal dispersal within the cell (for review, see Sorkin and von Zastrow, 2002; Piddini and Vincent, 2003).

In this update, we highlight recent progress in plant endocytosis. We also discuss data revealing inherent interactions between endocytosis, the actin cytoskeleton, and mitogen-activated protein kinases (MAPKs) in mammalian models with possible implications for plant cells. Finally, we outline a perspective of future research in this emerging field of plant cell biology. We do not deal here with biosynthetic and vacuolar pathways merging eventually with endosomes since these have been covered elsewhere recently (Surpin and Raikhel, 2004).

SHORT OVERVIEW OF ENDOCYTOSIS IN MAMMALS

Endocytic pathways in mammals, such as clathrin-mediated, caveolae/lipid raft-mediated, clathrin- and caveolae-independent endocytosis, fluid-phase endocytosis, and phagocytosis, differ with regard to the nature of internalized cargo, the size of vesicles, the associated molecular machinery, and the type of regulation (for review, see Conner and Schmid, 2003). These considerable differences are highlighted in Tables I and II. Interestingly, in cultured mammalian cells, as much as one-half of the endocytic uptake can be by non-clathrin mechanisms (for review, see Maxfield and McGraw, 2004).

Endocytic pathways employ morphologically diverse membranous tubulo-vesicular compartments encompassing sorting endosomes (also called early endosomes), recycling endosomes, multivesicular bodies, late endosomes, and lysosomes. These endocytic compartments differ in their internal pH, enrichment in specific membrane lipids, and in membrane-associated small GTPases of the Rab family (Table III).

Clathrin-Mediated Endocytosis

Based on structural studies, clathrin-mediated endocytosis in mammals was subdivided into distinct stages: (1) clathrin coat assembly on membranes, (2) vesicle invagination, (3) fission, (4) movement of vesicles into the cell interior, (5) vesicle uncoating, and (6) fusion with early endosomes. Adaptor proteins such as AP2, AP180, and epsins are interacting with plasma membrane phospholipids, cytoplasmic domains of receptors, and with synaptotagmin. When adaptor proteins are bound

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Table I. *Overview of endocytosis in animals*

Pathway	Size of Vesicles	Internalization of	Coat Proteins	Adaptor Proteins	Type of Regulation
Clathrin	120 nm	Ligand/receptor, toxin, nutrients	Clathrins[a]	AP1, AP2[a], AP3, AP4, AP180[a], β-arrestin	Phosphorylation, ubiquitination
Caveolae/lipid raft	50–80 nm	Albumin, virus, toxin, IgE, glycoprotein, folic acid GPI-anchored receptors	Caveolins	Not known	Phosphorylation
Phagocytosis	300 nm–few μm	Bacteria, IgG, receptor, particle	No	CBL, NCK, GRB2, CRKL, CED, DOCK180	Phosphorylation
Pinocytosis/fluid phase	0.5–5 μm	Fluids, solutes	No	Not known	Not known
Clathrin/caveolae-independent	80–100 nm	Virus, toxin, Interleukin-2 receptor	No	Not known	Not known

[a]Homologous proteins found in plants.

to the plasma membrane, they recruit clathrin and promote the assembly of the clathrin triskelion into a clathrin coat on the inner surface of the plasma membrane. Other proteins, such as β-arrestins, interact with receptors and the clathrin lattice. Additionally, β-arrestins are involved in signal transduction since they serve as scaffolds recruiting MAPK cascades onto endosomal vesicles (see below). Endophilin, a lysophosphatidic acid transferase, is involved in invagination of coated plasma membrane domains resulting in the formation of clathrin-coated pits (CCPs). The large GTPase dynamin is essential for fission of the clathrin-coated vesicles (CCVs) from the plasma membrane. Auxilin, Hsc70, and synaptojanin are likely involved in disassembly of the clathrin coat before endocytic vesicles fuse with early endosomes (for review, see Brodsky et al., 2001; Holstein, 2002 for plant cells).

Caveolae/Lipid Raft-Mediated Endocytosis

Caveolae/lipid raft-dependent endocytosis is characterized by its clathrin independence. Caveolae are membrane invaginations enriched in structural sterols, which might but do not need to be coated with caveolins, serving the internalization of some plasma membrane glycosphingolipids, glycosylphosphatidylinositol (GPI)-anchored proteins, extracellular ligands such as albumin and folic acid, bacterial toxins including tetanus and cholera, as well as uncoated Polyoma or Simian viruses (for review, see Parton and Richards, 2003). Interestingly, both caveolae and plasma membrane lipid rafts are enriched with cholesterol and sphingolipids, and they are involved in signaling events at the plasma membrane. In comparison to clathrin-dependent endocytosis, little is known about different stages of caveolae formation. Nevertheless, the study by Pelkmans et al. (2002) revealed that dynamin is essential for the fission step of caveolae from the plasma membrane.

Fluid-Phase Endocytosis

Pinosomes are large vesicles (0.5–5 μm in diameter) that internalize extracellular fluid. This extracellular fluid can be labeled by fluid phase markers such as Lucifer Yellow and rhodamin-labeled dextran. Several proteins, including phosphoinositol-dependent kinase and small GTPases of the Ras and Rho families,

Table II. *Links between endocytosis, actin cytoskeleton, and signaling*

Pathway	Endocytosis/Cytoskeleton Interface	Cytoskeletal Proteins	Signaling Proteins
Clathrin	Dynamin[a], Hip1R, ankyrin, intersectin, ACK1[a], ACK2, epsin[a], Auxilin[a], synaptojanin[a], Hsc70[a], Eps15[a], synaptotagmin[a], amphiphysin[a], annexin[a], GGA, syndapin, endophilin, pascilin	Actin[a], ARP2/3[a], cortactin, WASP, cofilin, ABP1, myosin I, myosin VI, fimbrin[a], talin, alpha-actinin	GAK, BIK, AAK1, PLD[a], P13-K[a], casein kinase[a], PIP5K, PDK[a], MAPKs[a], PKC[a], ARF6, Sar1, phosphatases[a]
Caveolae lipid raft	Dynamin[a]	Actin[a], ARP2/3[a], WASP	Src, Abl, Fyn, Ret, Lyn, Syk tyrosine kinases, PKC[a], NO synthase[a], Rac[a], Rho A[a], PLC[a], Ras[a], Raf[a], MAPks[a]
Phagocytosis	Dynamin[a], annexin[a], ERM Protein family	Actin[a], ARP2/3[a], WASP, coronin, cofilin, ABP120, alpha-actinin, myosin I, mysoin II, myosin VII	Src tyrosine kinases, SYK, casein kinase[a], PKC[a], PLD[a], PI3-K[a], PIP5K I, Rho[a], Cdc42, ARF6, Rac[a], POR1, Rap1
Pinocytosis	Dynamin[a]	Actin[a], ARP2/3[a], WASP, cortactin	PDK[a], Ras[a], Rho[a], ARF6, PI3-K[a], p21-activated kinase

[a]Homologous proteins found in plants.

Table III. *Classification of endosomes*

Type	Lipid Markers	Rab Markers	pH	Other Markers
Sorting (early)	Structural sterols, PI-3-P	Rab5, Rab4	5,9–6,0	Annexin II
Recycling	Structural sterols	Rab4, Rab11	6,4–6,5	
Multivesicular body	PI-3-P		5,0–6,0	ESCRT, Hrs, Alix
Late	Lysobisphosphatidic acid	Rab7, Rab9	5,0–6,0	Alix
Lysosome/vacuole		Rab27A	5,0–5,5	

together with their effectors, including p21-activated kinase and ADP-ribosylation factor (ARF6), were shown to have a regulatory function during macropinocytosis (for review, see Cardelli, 2001). Besides the fission step, dynamin was also implicated in the formation of actin comet tails, which are necessary for the intracellular movement of macropinosomes (Orth et al., 2002).

Phagocytosis

Phagocytosis is a special type of endocytosis occurring in free living unicellular organisms or specialized cells of higher organisms such as neutrophils and macrophages, when an entire foreign particle or microorganism is engulfed by the formation of phagocytic cups. Distinct stages have been identified during phagocytosis encompassing attachment of the particles to cell surface receptors, engulfment of the particle by dynamic shape changes dependent on the polymerization of actin and membrane exocytosis, and, finally, the formation of phago-lysosomes (for review, see Cardelli, 2001). All these phagocytic processes are dependent on rearrangements of the actin cytoskeleton. As highlighted by May and Machesky (2001), phosphoinositide lipids and multicomponent signaling complexes are important for signal transduction from phagocytic receptors to the actin cytoskeleton.

ENDOCYTOSIS IN PLANTS

Initially, the existence of endocytosis in plant cells was drawn into question because of their turgor pressure and rigid cell walls (Oparka et al., 1993; Hawes et al., 1995). However, numerous subsequent reports that used endocytic markers (for review, see Low and Chandra, 1994; Bahaji et al., 2001; Battey et al., 1999), as well as a number of recent localization and functional studies employing membrane-associated styryl FM dyes, filipin-labeled plant sterols, the fluid-phase marker Lucifer Yellow, green fluorescent protein (GFP)/yellow fluorescent protein (YFP)-tagged Rab GTPases, and BP-80 (a prevacuolar compartment/multivesicular body marker) and the fungal inhibitor of vesicular traffic, brefeldin A (BFA), clarified the existence and extraordinary dynamics of endocytic activity in plant cells (Geldner et al., 2001, 2003; Ueda et al., 2001; Baluška et al., 2002, 2004; Emans et al., 2002; Inaba et al., 2002; Grebe et al., 2003; Tse et al., 2004).

In the light of recent published work on endocytosis, it is expected that at least four basic forms of endocytosis, including clathrin-dependent, lipid raft-dependent, phagocytosis, and fluid-phase endocytosis, operate in plants. Here, we briefly summarize the supporting evidence.

Plants possess clathrin and they are equipped with most proteins necessary for clathrin-dependent endocytosis, including adaptor proteins involved in the formation of the clathrin coat on the surface of plasma membrane and endocytic vesicles (for review, see Holstein, 2002). In their recent work, Barth and Holstein (2004) biochemically and functionally characterized two of these adaptor proteins, AP180 and αC-adaptin, in Arabidopsis. Plant AP180 functions as a clathrin assembly protein while αC-adaptin binds AP180 and mammalian endocytic proteins, including amphiphysin, Eps15, and dynamin. Interestingly, plant CCVs have smaller sizes (70–90 nm; Barth and Holstein, 2004) in comparison to their mammalian counterparts (average size 120 nm; Conner and Schmid, 2003), which might be a consequence of endocytic internalization against higher turgor pressure in some plant cell types. Direct involvement of CCVs in regulated (ligand/receptor-mediated) endocytosis has not been demonstrated yet.

Other studies reported endocytic uptake of plant- or pathogen-derived elicitors, such as oligogalacturonic acid, glycoproteins, and exopolysaccharides, which are produced during plant defense (Romanenko et al., 2002). While molecular links between receptor-mediated endocytosis and signaling cascades are still missing in plants, receptor-like kinases (RLKs) were favored by Holstein (2002) as candidates for internalization via clathrin-mediated endocytosis. Interestingly in this respect, Shah et al. (2002) ele gantly demonstrated that the kinase-associated protein phosphatase KAPP regulates endocytosis of AtSERK1, a Leu-rich repeat Ser/Thr RLK. Another closely related RLK named BAK1 (AtSERK3) can likely form a brassinosteroid receptor complex together with BRI1 (Li et al., 2002). In the future, it will be interesting to study whether brassinosteroids can trigger receptor internalization and how this signal is transduced and coupled to cellular responses.

Plants contain a large family of regulatory GTPases, called Rab, which have been implicated in vesicle budding and fusion events (for review, see Rutherford

and Moore, 2002; Ueda and Nakano, 2002; Vernoud et al., 2003). As highlighted by Ueda and Nakano (2002), more than 30 putative endosomal Rab GTPases were found in the Arabidopsis genome, indicating an essential role of endocytosis in plant cells. Plant Rab GTPases Ara6 and Ara7, related to the mammalian endosomal protein Rab5, as well as Pra2, related to mammalian Rab11, have been localized to endosomes or putative endosomes, respectively (Ueda et al., 2001; Inaba et al., 2002). In animal cells, the endosome-binding domain encompassing the FYVE motif is a conserved signaling module that localizes PI(3)P-binding proteins to the early endosomes (Gillooly et al., 2000). We have found that a double FYVE construct binds selectively to plant endosomes because it colocalizes with bona fide endosomal markers such as RabF2a (Fig. 1) or Ara6. Therefore, this tandem FYVE construct tagged with fluorescent proteins can be considered a useful marker for plant endosomes.

Little is known about clathrin-independent endocytosis in plants. There are no published data on caveolin, and this protein is missing in plant databases altogether. Nevertheless, existence of lipid rafts and their association with GPI-anchored proteins was discussed in plants (Borner et al., 2003; Lalanne et al., 2004). Interestingly in this respect, plants are equipped with the structural sterols stigmasterol and sitosterol that are even more potent in organizing lipid rafts in vitro than cholesterol (Xu et al., 2001). Recently, Grebe et al. (2003) reported that plant structural sterols of the plasma membrane labeled with fluorescent filipin were internalized and colocalized with GFP-tagged Ara6 on endosomes. Moreover, structural sterols were implicated to play a role in polar localization of the putative auxin efflux carrier PIN1 (Willemsen et al., 2003). Additionally, it has been discussed that recycling of some proteins may be dependent on structural sterols and lipid rafts (Grebe et al., 2003; Willemsen et al., 2003). Together, these results clearly indicate that membrane sterols play a role in plant endocytosis.

Rhizobia are soil bacteria internalized into plant cells via phagocytosis during symbiotic interaction with roots of legume plants. These bacteria first enter the root hairs by means of a tubular invagination initiated from the tip of the hair, the so-called infection thread, and are then passed to the inner cortex cells. They are finally completely internalized into cells of the infection zone of the developing nodule, in which nitrogen fixation takes place (for review, see Schultze and Kondorosi, 1998). In a recent report, Son et al. (2003) found that the small GTPase Rab7 is essential for phagocytosis of rhizobia. Nod factors, bacterial lipochitooligosaccharides that trigger symbiotic events, were reported to be internalized in legumes (Timmers et al., 1998). Two research groups (Limpens et al., 2003; Radutoiu et al., 2003) recently identified Nod factor receptors in legumes and proposed their participation in the internalization of Nod factors in root hairs. In the future, it would be interesting to study whether these receptors undergo receptor-mediated endocytosis upon specifically binding to Nod factor ligands. The availability of a large spectrum of both plant and bacterial mutants affected in all stages of the interaction between *Medicago truncatula* and *Rhizobium meliloti*, efficient transformation protocols, as well as the many cell biological tools applicable to this interaction, will be very useful in elucidating mechanisms involved in microbial entry into plant cells.

While root cortical cells are responsible for development of nodules in legumes, these cells also perform fluid-phase endocytosis as revealed by internalization of the fluid phase marker Lucifer Yellow in maize (*Zea mays*) root apices (Baluška et al., 2004). Such fluid-phase endocytosis takes place at myosin VIII/actin-enriched plasmodesmata/pit-fields, located near unloading phloem elements, and obviously serves nutritional purposes in maize root apices. These data indicate that endocytic events in plant cells such as phagocytosis and fluid-phase endocytosis are important for symbiotic interactions with bacteria and for nutritional demands of some plant cells and tissues.

Brefeldin A: A Useful Tool to Study Endocytosis in Plants

BFA is a fungal metabolite that inhibits exocytosis but allows first steps of endocytosis to proceed in eukaryotic cells (Lippincott-Schwartz et al., 1991; Baluška et al., 2002; Nebenführ et al., 2002; Geldner et al., 2003). In plant cells treated with BFA, rapidly recycling plasma membrane proteins like putative auxin efflux carriers (members of the PIN family), including PIN1 (Geldner et al., 2001, 2003; Baluška et al., 2002) and PIN2 (Boonsirichai et al., 2003; Grebe et al., 2003), plasma membrane H^+-ATPase (Geldner et al.,

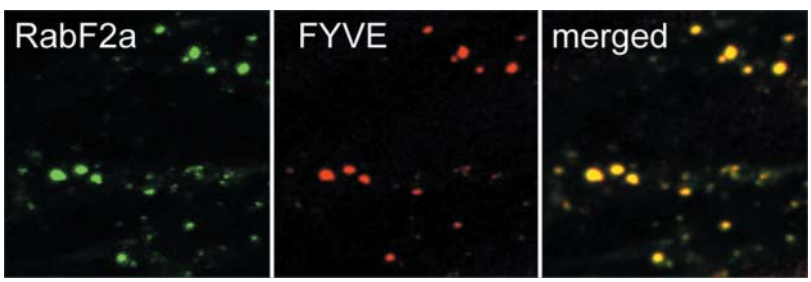

Figure 1. Onion cells were cotransformed with two constructs: YFP-tagged RabF2a (shown in artificial green color) and DsRed-tagged double FYVE (shown in red). Pronounced colocalization of both constructs on endosomes (yellow) is shown in the merged image indicating that the double FYVE construct is a reliable endosomal marker in plant cells.

2001; Baluška et al., 2002), as well as peripheral membrane protein ARG1 (altered response to gravity 1; Boonsirichai et al., 2003) undergo endocytic internalization and accumulate within BFA-induced compartments (Fig. 2A). These data clearly demonstrated that BFA inhibits endocytic recycling of plasma membrane proteins. Such BFA-induced compartments are also enriched with other molecules, including cytokinesis-specific syntaxin KNOLLE (Geldner et al., 2001, 2003) and its interacting protein AtSNAP33 (Heese et al., 2001), as well as with structural sterols (Grebe et al., 2003), small GTPase ARF1 (Baluška et al., 2002; Ritzenthaler et al., 2002; Couchy et al., 2003), and small GTPase Pra2 (Inaba et al., 2002; sterols and ARF1 are depicted in Fig. 2A). Additionally, protophloem cells of Arabidopsis root accumulate putative auxin-influx carrier AUX1 in BFA compartments (Grebe et al., 2002; Fig. 2A). As far as the cargo of endocytic vesicles is concerned, a study on maize roots treated with BFA revealed that cell wall polymers such as pectins cross-linked with boron or calcium are also internalized via the same recycling pathway used for the turnover of above-mentioned plasma membrane proteins (Baluška et al., 2002; Nebenführ, 2002). As shown in Figure 2C, cell wall pectins labeled with JIM5 antibody colocalize with PIN1 at the endosomal BFA compartment. Thus, recycling of both plasma membrane proteins as well as cell wall pectins is inhibited by BFA in roots of intact plants and therefore can substantially contribute to the dwarfed phenotypes of roots in BFA-treated seedlings (Geldner et al., 2001). Interestingly, endocytosis of cell wall pectins in maize root cells can be effectively inhibited by short-term deprivation of boron (Yu et al., 2002), but clarification of the molecular mechanism behind this inhibition requires further study.

The effects of BFA are dependent on the time and amount used, as well as the tissue investigated. For Arabidopsis roots, Geldner et al. (2001) reported that

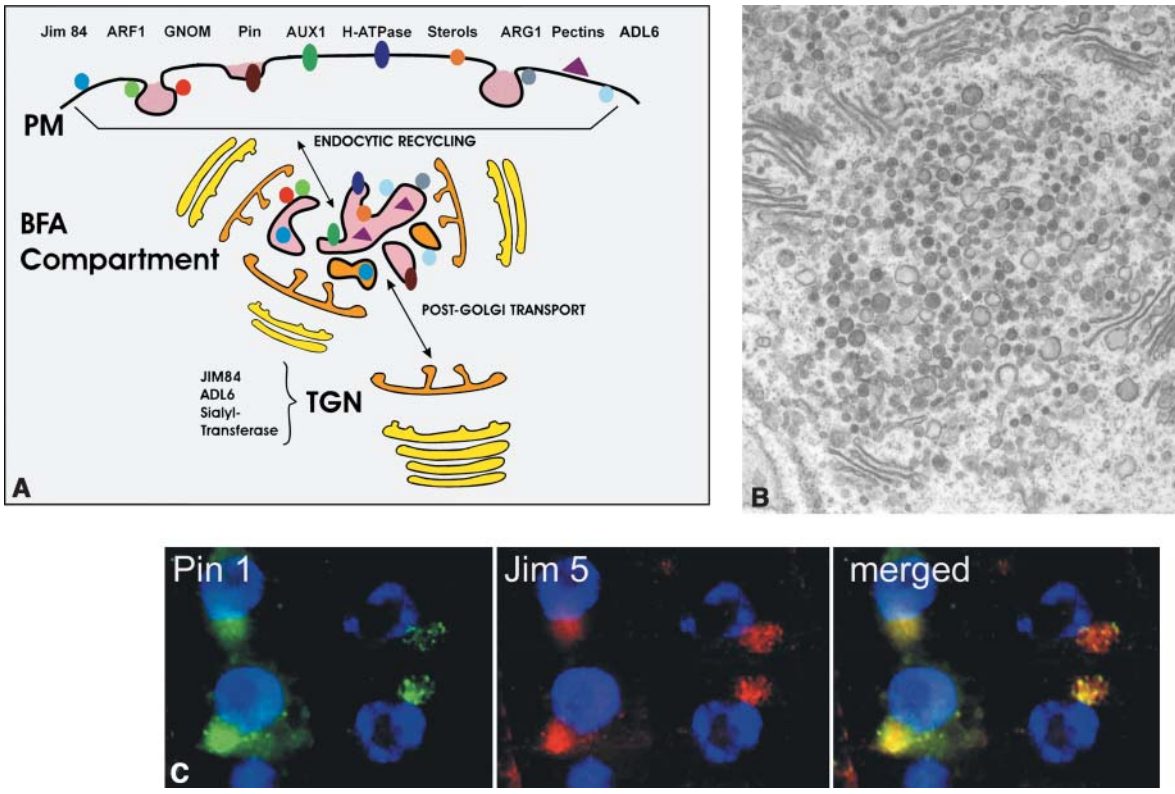

Figure 2. A, Endocytosis in plants—insights from BFA compartments. Upon BFA treatment, the following plasma membrane and plasma membrane-associated molecules accumulate in BFA compartments: PINs (putative auxin efflux carriers), AUX1 (putative auxin influx carrier), plasma membrane H-ATPase, plasma membrane structural sterols, and peripheral membrane protein ARG1 (altered response to gravity). Except this, cell wall pectins cross-linked by boron, small GTPase ARF1, and ARF activator GNOM (ARF-GEF) accumulate within BFA compartments. The JIM84 carbohydrate epitope and dynamin ADL6 associate both with plasma membrane and TGN, and can be eventually transported to the BFA compartment from both locations. Internalization of cell wall pectins could be inhibited by short-term boron deprivation. AUX1 accumulation to BFA compartments is restricted to protophloem cells. B, Ultrastructure of BFA compartment after 30-min incubation of root epidermal cell with 25 μM BFA (reproduced with permission from Grebe et al., 2003). C, Immunofluorescence colocalization of putative auxin efflux carrier PIN1 (second antibody coupled to FITC; green) and cell wall pectins recognized by monoclonal antibody JIM5 (secondary antibody coupled to TRITC; red) on BFA compartments (yellow) in maize root cells treated with 100 μM BFA for 2 h. Nuclei (blue) are counterstained with DAPI.

after treatment with 50 mM BFA for 30 min, Golgi stacks are still intact, whereas BFA compartments are already formed from aggregating and enlarging endosomes. After 20 min of BFA treatment with similar concentrations (35,7 mM), most of the trans-cisternae of Golgi stacks are lost in tobacco (*Nicotiana tabacum*) BY2 cells (Ritzenthaler et al., 2002) and could eventually associate with peripheries of the growing BFA compartment. After 1 h, the BFA compartment itself is composed of a mixture of vesicles and membranous compartments of different sizes, shapes, and contents originating preferentially from the endocytic pathway(s). Previously, it was proposed that the BFA compartment represents a membranous vesicular organelle generated by both endosomal and post-Golgi endomembrane flow (Baluška et al., 2002; Nebenführ, 2002; Nebenführ et al., 2002; see also Fig. 2A). A similar trans-Golgi network (TGN)-endosome hybrid organelle was reported for BFA-treated animal cells (Lippincott-Schwartz et al., 1991; Wood and Brown, 1992). In roots of intact plants, the BFA compartment is surrounded by remnants of Golgi stacks in a polarized fashion with most trans-cisternae facing the BFA compartment (Fig. 2B; see also Grebe et al., 2003). Importantly, some TGN and Golgi markers, such as α-2,6-sialyl transferase and Arabidopsis *N*-acetylglucosaminyl transferase I, accumulate preferentially in these peripheral remnants of Golgi stacks surrounding the BFA compartment (see figure 3, G and H, in Grebe et al., 2003). On the other hand, both endocytic vesicles containing recycled plasma membrane molecules and/or pectins (Geldner et al., 2001, 2003; Baluška et al., 2002), as well as plasma membrane sterols (Grebe et al., 2003), accumulate in core vesicles of the BFA compartment. It is a mystery how this transient assembly of diverse vesicles can hold together in the BFA compartment and disassemble again after BFA removal. One possibility could be that some matrix proteins similar to the Golgi matrix proteins are involved in maintaining its integrity. It seems that one of the main criteria for the accumulation of vesicles in the core of BFA compartments is the type of internalized membrane, for example, its enrichment in structural sterols. Another important criterion can be the type of membrane coating since membranes coated by clathrin (a typical coat present exclusively on plasma membrane and TGN-derived vesicles but not on ER or Golgi-derived vesicles) will end up in BFA compartments, whereas Golgi-derived vesicles coated with COP proteins will accumulate at Golgi remnants surrounding the periphery of BFA compartments. This was reported by Geldner et al. (2003) for γCOP recently.

ARF-guanosine exchange factors (ARF-GEFs) are the target for BFA action in mammalian cells. The plant ADP-ribosylation factor ARF1, which is a small GTPase of the Ras family regulated via ARF-GEF, was localized to punctate structures on the plasma membrane in control cells and to the BFA compartments in BFA-treated maize root cells (Baluška et al., 2002; Fig. 2A). This work provided the first evidence that a protein closely associated with the BFA target is localized on endosomal compartments. The localization of ARF1 to BFA bodies was confirmed by Couchy et al. (2003). A recent study by Geldner et al. (2003) revealed that the endosomal GNOM protein, an ARF-GEF, represents a target of BFA action in plant cells. Upon BFA treatment, GNOM became trapped at BFA-induced endosomal compartments, which also accumulated putative auxin-efflux carrier PIN1 (Fig. 2A). A BFA-resistant GNOM mutant protein was engineered, and transformed *gnom* plants were rescued. The rescued *gnom* plants showed no redistribution of PIN1 protein to BFA bodies in response to BFA treatment. Additionally, both shape and size of endosomes labeled with GFP-tagged Ara7 were altered in protoplasts isolated from these plants. However, recycling of other proteins, such as PIN2, PM-ATPase, and syntaxin KNOLLE (SYP111), seems to be partially or completely independent of GNOM, suggesting that there are other ARF-GEFs or different molecular mechanisms involved in their endocytic recycling. Altogether, these data provide convincing evidence that endosome-resident GNOM can control recycling of PIN1 via its targeting to endosomes (Geldner et al., 2003).

Endocytosis and Tip Growth of Plant Cells

Tip-growing cells such as pollen tubes and root hairs must recycle an immense membrane surplus resulting from the massive vesicle delivery to their tip regions by compensatory endocytosis (Fig. 3). CCPs and vesicles have been visualized in the subapical regions of growing pollen tubes and root hairs, and clathrin was immunolocalized to the tips of pollen tubes, thus suggesting a role for clathrin-dependent endocytosis in tip growth (for review, see Holstein, 2002). Several recent studies have revealed that the FM styryl dyes FM4-64 and FM1-43 represent reliable markers for endocytosis in plants (Ueda et al., 2001; Emans et al., 2002; Inaba et al., 2002; Shope et al., 2003; Geldner et al., 2003). Results on isolated protoplasts and suspension cells were corroborated by experiments with FM4-64 and FM1-43 in tip-growing cells revealing internalization of these styryl dyes preferentially at apical regions of growing pollen tubes (Parton et al., 2001; Camacho and Malho, 2003) and root hairs (Ketelaar et al., 2003).

Several molecules potentially important for endocytosis were recently localized within tips of growing root hairs, including dynamin (Kang et al., 2003b), actin (Baluška et al., 2000; Ketelaar et al., 2003), profilin (Baluška et al., 2000), and actin-related protein 3 (ARP3)-like protein (van Gestel et al., 2003). The actin cytoskeleton has been implicated in calcium-dependent vesicular traffic in tip-growing cells (Hepler et al., 2001). In root hairs, BFA inhibits tip growth. The inhibition of tip growth correlates with the disappearance of fine actin microfilaments at the tip (Šamaj et al., 2002). Small GTPases of the ROP (Rho of plants) family play a role in regulating both F-actin meshworks and

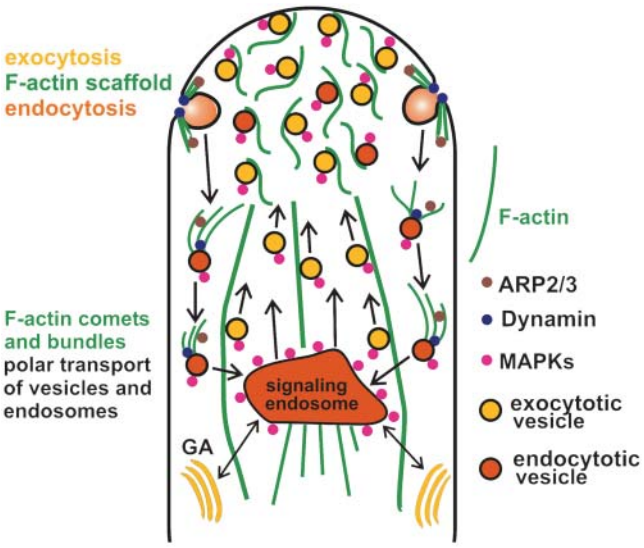

Figure 3. Working model depicting endosomal/vesicular trafficking and possible roles of the actin filaments in an idealized tip-growing root hair. Local actin polymerization together with accumulation of dynamin could facilitate endocytic recycling of receptors, ion channels, and cell wall molecules (e.g. pectins and AGPs) by assisting the pinching off the endocytic vesicles and by forming actin comets on these vesicles dependent on ARPs. Signaling molecules such as MAPKs associate both with endosomal vesicles and the actin cytoskeleton. Additionally, dense meshworks of actin filaments regulated by profilins and ARPs are suggested to act as a structural scaffold in order to sequester and maintain signaling and regulatory molecules, including MAPKs within the apical vesicle pool (clear zone). GA, Golgi apparatus. Arrows indicate polar trafficking of exo- and endocytic vesicles/endosomes, as well as putative transport between TGN and endosomes.

the tip-focused calcium ion gradient in root hairs and pollen tubes (Yang, 2002). We propose that actin polymerization-driven processes may play an important role during vesicle budding and movement of endosomes (Fig. 3). This is consistent with our recent in vivo data showing F-actin-dependent movement of early endosomes labeled with GFP-tagged Rab GTPases Ara6 and RabF2a or with FYVE domain-fusion constructs in tip-growing root hairs of Arabidopsis and Medicago (B. Voigt, A. Timmers, J. Šamaj, A. Hlavacka, T. Ueda, M. Preuss, E. Nielsen, J. Mathur, N. Emans, H. Stenmark, A. Nakano, F. Baluška, and D. Menzel, unpublished data). In addition to the function of actin in endocytosis, tip-focused fine actin filaments and meshworks may serve as molecular scaffold for vesicles and associated regulatory and signaling molecules (Fig. 3), including MAPKs (Šamaj et al., 2002, 2004).

Endocytosis and Stomata Movements

Guard cells accomplish dramatic changes in their surface area—up to 40% was recorded by Shope et al. (2003). As elastic stretching of the plasma membrane is only about 2% of surface area (Wolfe and Steponkus, 1983), it is inevitable that abundant exocytosis and endocytosis events are tightly interlinked processes in the turgor-related movements of guard cells (Blatt, 2000). Previous patch-clamp experiments of Homann and Thiel (1999) revealed that osmotic swelling and shrinking of guard-cell protoplasts was associated with fusion and fission of vesicles at the plasma membrane. More recently, Shope et al. (2003) have shown that internalization of the endocytic tracer FM4-64 from the cell surface in closing stomata was tightly correlated with the loss of surface area followed by reutilization of these vesicles during the opening of stomata. Additionally, recent important work by Meckel et al. (2004) reports about constitutive endocytosis of plasma membrane and GFP-tagged potassium channel in intact guard cells. These data indicate that endocytosed material is recycled during the stomatal opening phase. It is likely that these recycling vesicles are packed with cell wall pectins as shown here (Fig. 2C) and reported previously for maize root apices (Baluška et al., 2002) or other stomata cell wall components.

Inhibition of PI-3-kinase by wortmannin abolished stomata closing and the same effect on stomata was induced by overexpression of the endosomal FYVE construct (Jung et al., 2002). Endocytosis is inhibited by wortmannin in plant cells (Emans et al., 2002). Thus, several independent lines of evidence suggest that endocytosis is essential for closing of stomata.

Endocytosis and Dynamin in Plants

Dynamin is a large GTPase implicated to play an essential role in almost all forms of endocytosis in higher eukaryotes, including fission of clathrin- and caveolin-coated vesicles and movement of CCVs, macropinosomes, and phagosomes (Pelkmans et al., 2002; for review, see Orth and McNiven, 2003). Dynamin is a multidomain protein that interacts with a plethora of other proteins, including those that associate with the actin cytoskeleton (for review, see Orth and McNiven, 2003). For example, dynamin interacts through its Pro-rich domain with profilins and with proteins containing SH3 domains, while its PH (pleckstrin homology) domain serves as PIP2 binding module. Proteins containing SH3 domains were identified in Arabidopsis, and one of them, AtSH3P1, colocalized with clathrin and was able to bind to actin (Lam et al., 2001).

Plants contain a large family of dynamin-related proteins (Hong et al., 2003a). Recent work has provided evidence for a role of the Arabidopsis dynamin-like protein1 (ADL1) in plasma membrane vesiculation (Kang et al., 2003a, 2003b). In mutant ADL1 plants, the plasma membrane was studded with supernumerary invaginations (Kang et al., 2003a, 2003b), clearly indicating defective fission of vesicles. Moreover, another plant dynamin, ADL6, associates with CCVs and with the plasma membrane (Lam et al., 2002; Hong et al., 2003b). All this strongly suggests that at least some dynamins take part in endocytosis in plant cells.

ENDOCYTOSIS AND THE ACTIN CYTOSKELETON

There are many correlative data suggesting multiple interactions between endocytosis and the actin cytoskeleton in yeast and mammals (for review, see May and Machesky, 2001; Engqvist-Goldstein and Drubin, 2003). In animal cells, an intact actin cytoskeleton is necessary for all forms of endocytosis. Additionally, intact filamentous actin is required in order to transport caveolin from the invaginated plasma membrane domains (caveolae) into early endosomes and for activation of the MAPK signaling pathway (Pol et al., 2000; see below for discussion on signaling endosomes).

A number of proteins have been implicated as functional components at the interface between the endocytic internalization and the actin cytoskeleton. In mammals, some of these proteins, like dynamin, myosin VI, ankyrin, amphiphysin, HIP1 and HIP1R, WASP, ARP2/3 complex, ACK1, profilin, and synaptojanin, were designated as molecular linkers between endocytosis and the actin cytoskeleton (Table II). Other potential molecular linkers between dynamin and actin are represented by syndapin (dynamin-interacting protein), ABP1, cortactin, and intersectin (for review, see Qualmann et al., 2000; Engqvist-Goldstein and Drubin, 2003; Table II). Several endocytic steps require local actin polymerization. It was shown that actin polymerization at the plasma membrane controls both the alignment and mobility of CCPs, facilitates the internalization step, and drives rapid transport of early endosomes away from the plasma membrane into the cytosol (for review, see Engqvist-Goldstein and Drubin, 2003).

Nevertheless, the function of actin in endocytosis is far from being fully understood. For instance, it is not known for which steps of endocytosis the actin cytoskeleton is essential. Several roles have been proposed for the actin cytoskeleton such as trapping endocytosis into restricted plasma membrane sites, deformation and invagination of plasma membrane, inhibition of vesicle formation as a rigid barrier, vesicle fission and detachment from the plasma membrane, vesicle motility through the cytoplasm, and vesicle fusion (for review, see Qualmann et al., 2000). In two recent studies, a role of filamentous actin in compressing compensatory endocytic vesicles was proposed by Sokac et al. (2003), and actin patches were identified as sites of endocytosis in yeast cells (Kaksonen et al., 2003). A more recent study revealed that both the endocytic and the actin assembly machineries work in concert; in particular, endocytic hot-spots act as actin filament organizing centers in analogy to microtubule organizing centers of the microtubular cytoskeleton (Engqvist-Goldstein et al., 2004). This recent evidence suggests inherent and very dynamic interactions between endocytosis and the actin cytoskeleton.

Importantly, evidence is strengthening that such endocytic functions of actin are related to localized and dynamic actin polymerization. In the case of the actin patches in yeast, the nature of the internalized cargo is not yet known. However, it should be considered that, in analogy to the situation in plant cells (Baluška et al., 2002; Yu et al., 2002), matrix polysaccharides of the yeast cell wall are internalized and recycled via actin patch-mediated endocytosis.

Plants possess some of the molecular linkers between endocytic components (i.e. clathrin) and actin, including dynamin-related proteins, profilins, and the ARP2/3 complex, whereas other important players known from other eukaryotic systems, such as WASP proteins and their direct activators cortactin and ABP1, were not found in plants. In addition, other WASP and dynamin interacting proteins, including syndapin and intersectin, are also missing from the plant gene databases (for review, see Holstein, 2002; Hussey et al., 2002). Nevertheless, several recent studies reported that the actin cytoskeleton is required for endocytosis in plant cells based mostly on pharmacological evidence. It was shown that actin disruption by latrunculin B or cytochalasin D, two actin-depolymerizing drugs, inhibited the formation of large endocytic BFA compartments, which accumulate recycling plasma membrane molecules and adhesive cell wall pectins (Geldner et al., 2001; Baluška et al., 2002; Nebenführ et al., 2002; Yu et al., 2002). Recently, we reported that latrunculin B and 2,3-butanedione monoxime, a general myosin inhibitor, abolished or inhibited fluid phase endocytosis of Lucifer Yellow into maize root cells (Baluška et al., 2004). Endocytic transport of sterols is also dependent on the intact actin cytoskeleton as revealed by experiments with cytochalasin D and studies of the *actin2* mutant (Grebe et al., 2003). All these results support an essential role of actin in endocytic internalization. The exact function of the actin cytoskeleton in plant endocytosis, however, remains to be established, and exciting new findings should be expected from further studies on molecular, cell biological, and genetic levels.

ENDOSOMES AS MOTILE SIGNALING PLATFORMS IN MAMMALS

During the last 5 years, a growing body of evidence has emerged from the study on mammalian cell systems showing that components of MAPK signaling pathways associate in their activated (phosphorylated) states with signaling endosomes (Pol et al., 2000; Howe et al., 2001; for review, see Sorkin and von Zastrow, 2002). Additionally, several members of MAPK signaling modules interact directly or indirectly with the actin cytoskeleton and they are involved in actin dynamics (for review, see Šamaj et al., 2004). It was reported that dynamin-regulated endocytosis is required for MAPK signaling mediated via extracellularly regulated kinase 1 (ERK1) because dominant negative dynamin mutants were inhibited in both the formation of endocytic vesicles and the activation of ERK1 and its upstream activator MEK1 (Daaka et al., 1998; Kranenburg et al., 1999). Moreover, phosphorylated MEK was localized exclusively at the

plasma membrane and endocytic vesicles but not within the cytosol (Kranenburg et al., 1999). Scaffold proteins such as β-arrestins, which target G-protein-coupled receptors (e.g. epidermal growth factor receptor) for endocytosis, are also necessary for ERK1 activation (Daaka et al., 1998; DeFea et al., 2000). Recently, it was revealed that β-arrestins represent scaffold proteins that not only enhance MEK1-mediated activation of ERK1 but also target both activated ERK1 and JNK (C-Jun kinase) to endosomes (McDonald et al., 2000; Luttrell et al., 2001). Besides the vesicular signaling of ERKs dependent on the motility of signaling endosomes, Cavalli et al. (2001) nicely demonstrated that another mammalian MAPK, namely p38, accelerates endocytosis by stimulating complex formation between guanosyl-nucleotide dissociation inhibitor and Rab5, a small GTPase functioning on early endosomes. The above-mentioned data suggest that signaling endosomes in animal cells serve as motile assembly platforms for diverse signaling pathways recruiting multiprotein complexes composed of scaffold proteins, MAPK modules, as well as their upstream activators and interacting partners. These results also indicate that endocytosis plays a crucial role in MAPK-dependent signal dispersal and proper targeting within the cell (for review, see Sorkin and von Zastrow, 2002). Additionally, MAPK-mediated signaling is required for bacterial invasion and phagocytosis. For example, ERK and MEK1 activation is necessary for invasion of cells by Listeria (Tang et al., 1998).

In plants, apices of tip-growing root hairs and pollen tubes are well known as sites of balanced exo- and endocytosis (for review, see Hepler et al., 2001). Recently, we reported that cross-talk between stress-induced MAPK (SIMK), the actin cytoskeleton, and vesicular trafficking is involved in regulation of polarized tip growth (Šamaj et al., 2002). SIMK was accumulated in vesicle-rich tip regions of root hairs. In root hairs treated with BFA, both SIMK and F-actin disappeared from growing tips, while SIMK redistributed into enlarged patches attached to the remaining cytoplasmic actin filaments. Inhibition of the MAPK pathway with UO 126 (a MEK inhibitor in mammalian cells) abolished tip growth and induced pronounced changes in vacuolar morphology, vesicular traffic, and general cyto-architecture of root hairs as revealed by video-enhanced microscopy. Importantly, vesicular trafficking and behavior of vacuoles were not affected when plants overexpressing a gain-of-function (constitutively active) construct of SIMK were treated with UO 126 (Šamaj et al., 2002), thus implicating a potential role of SIMK in vesicular traffic and vacuole dynamics. MAPKs were immunolocalized to spot-like structures in the cytoplasm of plant cells (Šamaj et al., 2002). The nature of these spots remains unknown. Nevertheless, it was recently reported that phorbol ester, an inducer of endocytosis and MAPKs in animal cells (Park et al., 2003), is able to activate tobacco MAPK, which is immunologically related to wound-induced protein kinase WIPK (Baudouin et al., 2002). Unfortunately, these biochemical results were not accompanied with cytological analysis.

DENN domain proteins link together Rab-based endocytic trafficking and MAPK-based signaling (Levivier et al., 2001). SCD1 is a plant DENN domain protein required for polarized cell expansion, notably of root hairs, as well as for cell plate formation during cytokinesis of plant cells, which involves large-scale membrane recycling (Falbel et al., 2003). Further studies on SCD1 will surely shed more light on the interactions between actin-dependent endocytosis, polarized growth, and MAPK signaling.

CONCLUSIONS AND OUTLOOK

Endocytosis is an essential cellular process occurring in all eukaryotic cells. Recent progress revealed that endocytosis in plants, in analogy to mammals, is involved in the internalization and recycling of plasma membrane molecules including membrane proteins and sterols (Geldner et al., 2001, 2003; Baluška et al., 2002; Grebe et al., 2003), in the uptake of extracellular fluids (Baluška et al., 2004), and phagocytosis of soil bacteria (Son et al., 2003). Moreover, plant cells are able to internalize cell wall components such as pectins cross-linked with boron and calcium (Baluška et al., 2002; Yu et al., 2002). These findings are consistent with the attractive concept that endocytosis not only regulates the abundance of plasma membrane proteins but also modulates the mechanical properties of cell walls. It would be interesting to study similar endocytosis-dependent cell wall remodeling in yeast cells, where actin patches emerge as functionally linked components in this process.

In mammals and yeast, the actin cytoskeleton is required for endocytosis (for review, see Qualmann et al., 2000; Engqvist-Goldstein and Drubin, 2003; Kaksonen et al., 2003; Sokac et al., 2003) and is also involved in endocytic internalization in plants (Geldner et al., 2001; Baluška et al., 2002, 2004; Grebe et al., 2003). During the last few years, it was demonstrated that endocytosis in mammals is coupled to signaling cascades regulated by MAPKs which, in turn, are recruited to signaling endosomes and rapidly transported to proper cellular destinations (for review, see Sorkin and von Zastrow, 2002). In future studies, it will be important to test this scenario of signal transduction and amplification via endocytosis and signaling endosomes in plants. Moreover, cross-talk between actin and MAPK signaling were documented that involves signal-dependent rearrangements of the actin cytoskeleton and activation of MAPK signal transduction cascades and their downstream effectors (for review, see Šamaj et al., 2004).

It must be noted that there appear to be some basic differences in endocytosis between yeast and higher eukaryotes at the molecular level. Yeast, for instance, does not contain synaptotagmins, annexins, and dy-

namins (Craxton, 2000; for review, see Holstein, 2002; Engqvist-Goldstein and Drubin, 2003; Gruenberg and Stenmark, 2004), and clathrin and AP2 do not play significant roles in yeast endocytosis either (Yarar, 2003), suggesting that a constitutive (unregulated) type of endocytosis is more important for yeast than a regulated one. Moreover, yeast also seems to lack slow and rapid routes of protein recycling between endosomes and plasma membrane known from mammals (for review, see Gruenberg and Stenmark, 2004) and plants (for example PIN1). Synaptotagmin, a component of the regulated type of endocytosis, is missing in yeast but does indeed occur in Arabidopsis (Craxton, 2000). It not only acts as a sensor for calcium-regulated exocytosis but is also necessary for compensatory endocytosis that requires clathrin and AP2 in mammals (Poskanzer et al., 2003). Additionally, some animal and plant cell types, but not yeast, are able to accomplish phagocytosis of pathogenic or symbiotic bacteria. Animals and plants certainly differ in the composition of their molecular links between endocytosis and the actin cytoskeleton, since no WASP, WASP-activating proteins such as cortactin and ABP1, or WASP-interacting proteins such as syndapin and intersectin were found in the plant gene databases yet (for review, see Holstein, 2002; Hussey et al., 2002). On the other hand, dynamins and proteins constituting the Arp2/3 complex, which are required for vesicle fission and endosomal motility, seem to be conserved in plants (for review, see Deeks and Hussey, 2003; Hong et al., 2003a).

Recently, it was elegantly demonstrated in mammalian cells that two different endocytic pathways determine the fate of a receptor. It was shown that clathrin-mediated endocytosis promotes signaling of the TGF receptor, whereas the same receptor was internalized and turned over via caveolae (Di Guglielmo et al., 2003). Whether this holds true for fate determination of plant receptors is not yet known. As rapid progress has been made and is likely to continue in this field, one can expect that new crucial endocytic molecules will be identified and functionally characterized at a fast pace. The recently discovered GNOM (ARF-GEF) as an endosome-resident protein (Geldner et al., 2003) is a good example. Further cell biological, functional, and genetic study will help to clarify the complex interactions among endocytosis, the actin cytoskeleton, and signaling cascades in plant cells.

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