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Small GTP-Binding Proteins and Membrane Biogenesis in Plants[1]

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One of the amazing features of the cellular machinery is that all organeller and membrane proteins, as well as those destined for secretion, have an attached address label for targeting to a specific site. Following synthesis, these proteins are folded, shipped, delivered, and received at the right compartment. Their assigned functions are performed only when they are properly placed at a designated site in the cell. Membrane vesicles play an essential role in protein transport as carriers of specific proteins to intracellular compartments. This process begins immediately after perception of specific signals and involves membrane ruffling, budding and transport of ER vesicles, fusion and passage through the Golgi, and release of vesicles from trans-Golgi cisternae to target to the vacuoles and plasma membrane. The components of the vesicle-mediated protein trafficking system are not, however, well defined. It is not known what kind of biochemical principles are operative for unidirectional transport of vesicles. How are vesicles fused to the target compartment? Although much remains to be understood, many studies from yeast and mammalian systems have identified some key players in this pathway. Isolation of plant homologs of some of these proteins has confirmed that these steps are conserved in evolution and must involve well-defined reactions. We focus here on the relevance of small GTP-binding proteins in vesicle-mediated protein transport (for earlier reviews, see Balch, 1990; Bednarek and Raikhel, 1992; Pryer et al., 1992; Terryn et al., 1993; Zerial and Stenmark, 1993).

THE DIVERSITY OF THE SMALL GTP-BINDING PROTEIN FAMILY

A group of GTP-binding proteins ranging in molecular mass from 20 to 30 kD (referred to as small GTP-binding proteins) is found in all eukaryotic cells. These proteins share high amino acid sequence identity and overall structure, suggesting that they evolved from a common ancestral gene. These proteins also share a common mechanism to function as a molecular switch that can be turned on by binding to GTP and turned off by hydrolyzing GTP to GDP. This switch enables transduction of signals across membranes, controlling cell proliferation and directing transport of vesicles to their destinations (Bourne et al., 1991). Based on the similarity in amino acids and the presumed function, small GTP-binding proteins can be grouped into five subfamilies: Ras/Ras-like, Ran/TC4, Rho/Rac, Rab/Ypt, and Arf/Sar.

Ras is the best-characterized small GTP-binding protein. Ras and Ras-like (including Rap, Ral, and Rras) proteins are believed to be involved in signal transduction and regulation of cell growth and differentiation (Hall, 1990). Ran/TC4 (including human TC4 and yeast GSP and SPI) proteins are localized in the nucleus and are required for DNA synthesis and protein import into the nucleus (Moore and Blobel, 1993; Lounsbury et al., 1994). The Rho/Rac family has been implicated in cytoskeletal organization and regulation of growth factor-induced membrane ruffling (Ridley et al., 1992).

Members of the Rab/Ypt subfamily have been shown to be involved in vesicular transport. Identification of Rab proteins began with cloning of *YPT1*, a *ras*-like gene in yeast, followed by identification and isolation of *SEC4* (see Balch [1990] and refs. therein). Disruption of the *SEC4* gene results in accumulation of membrane vesicles in yeast, whereas duplication of *SEC4* suppresses *sec* mutants of post-Golgi events, implicating its product in vesicular transport at the post-Golgi level. Sec4p (the protein encoded by *SEC4*) is localized on the plasma membrane and on secretory vesicles. A mutation in *YPT1* causes defects in early secretion and membrane proliferation, and the function of Ypt1p has been suggested to be in the transport of vesicles from ER to Golgi. Further studies on Ypt1p and Sec4p led to the discovery of their homologs, Rab proteins, in both mammalian and plant cells. A number of genes encoding Rab proteins have been isolated. Different Rab proteins are localized on distinct compartments in the secretory and endocytic pathways (see Zerial and Stenmark, 1993).

The amino acid sequence of the Arf/Sar proteins suggests that they are distantly related to the Rab/Ypt family. Arf and Sar1 subgroups share high homology (60%) with each other

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Abbreviations: azaC, 5-azacytidine; GAP, GTPase-activating protein; GDI, GDP dissociation inhibitor; GEF, guanine-nucleotide-exchange factor; NSF, *N*-ethylmaleimide-sensitive fusion protein; PBF, peribacteroid fluid; PBM, peribacteroid membrane; SNAP, soluble NSF attachment proteins; SNARE, SNAP receptors; TGN, trans-Golgi network; t-SNARE, SNAP receptors that exist on the target membrane; v-SNARE, SNAP receptors that exist on the vesicular membrane.

but have distinct functions. Arf (ADP ribosylation factor) was originally identified and purified as a protein cofactor required for ADP ribosylation of the α subunit of heterotrimeric G proteins, proteins that function in signal transduction. Arf is associated with Golgi membranes and is essential in forming coatomers, the protein complexes that coat Golgi-derived vesicles. Arf and other coat proteins of vesicles were suggested to be involved in the vesicle budding process, which is regulated by heterotrimeric G proteins (Bauerfeind and Huttner, 1993). *SAR1* was discovered as a multicopy suppressor of a *sec12* temperature-sensitive strain. It interacts with Sec12p (a GEF specific for Sar1p), Sec23p (a GAP specific for Sar1p), and Sec24p. Formation of this complex is required for vesicle budding from the ER (Nakano and Muramatsu, 1989; Barlowe and Schekman, 1993).

SMALL GTP-BINDING PROTEINS ARE ASSOCIATED WITH DIFFERENT MEMBRANE COMPARTMENTS

Despite sharing a high level of sequence similarity, small GTP-binding proteins appear to associate with distinct membrane compartments and perform different biological functions. Most of the Ras/Ras-like, Rho/Rac, and Rab/Ypt proteins possess a variable sequence at the C terminus that contains a CAAX, CXC, or CC motif. This motif is a signal for addition of a farnesyl or geranylgeranyl lipid moiety to the proteins, a process known as prenylation by which these proteins are attached to membranes. Many Ras/Ras-like proteins have been localized on the plasma membrane (Hall, 1990). Unlike other small GTP-binding proteins, Ran/TC4 proteins are localized in the nucleus. These proteins lack the consensus CAAX motif for prenylation at the C terminus, but they have an acidic C-terminal tail that has been implicated in reactions with other nuclear proteins (Moore and Blobel, 1993; Lounsbury et al., 1994). Arf and Arl (Arf-like) proteins also lack the CAAX motif at the C terminus, but they have a Gly at position 2 of the N terminus that serves as a site for N myristoylation (Bauerfeind and Huttner, 1993). Sar1 proteins do not contain motifs for potential membrane modification. These proteins are associated with the ER membranes by forming a complex with Sec12p, an integral ER membrane protein (Nakano and Muramatsu, 1989; Barlowe and Schekman, 1993).

The Rab/Ypt subfamily comprises a large number of proteins (at least 30) with distinct subcellular locations (Table I; see also Pryer et al., 1992; Zerial and Stenmark, 1993). Rab1a and Rab2 are shown to be involved in the ER-Golgi transport and Rab3 is shown to be involved in transport of synaptic vesicles. Rab4, Rab5, Rab7, Rab9, Rab22, and Rab24 are engaged in the endocytic pathway, whereas both Rab4 and Rab5 are associated with the early endosomes and have different roles in endocytosis (Wichmann et al., 1992; Olkkonen et al., 1993). Rab5 appears to function in endosome-endosome fusion and Rab4 appears to function in a recycling pathway from early endosomes to the cell surface. Vps21, a Rab5 homolog from yeast, is required for the sorting of vacuolar proteins (Horazdovsky et al., 1994). Rab6p is localized in the Golgi and plays a role at an early step in the biogenesis of synaptic vesicles (Tixier-Vidal et al., 1993). Rab7p is located on late endosomes and may be required for transport from early to late endosomes, and Rab9 is involved in transport from late endosomes to the trans-Golgi network.

ACCESSORY FACTORS ASSOCIATED WITH SMALL GTP-BINDING PROTEINS

Similar to other GTP-binding proteins, Rab proteins undergo GTP-bound and GDP-bound states. This switch is regulated by several accessory proteins. GAP accelerates GTP hydrolysis by enhancing the intrinsic GTPase activity associated with GTP-binding proteins; otherwise, the GTP hydrolysis rate is very low. GEF mediates the replacement of GDP with GTP, causing GTPase to become active (Fig. 1). GDI inhibits the dissociation of GDP and prevents the GDP-bound form of Rab proteins from binding to membranes. These accessory proteins were first identified for Ras and heterotrimeric G proteins (Boguski and McCormick, 1993). Recently, Rab-specific accessory proteins have been identified. The GAP protein of Ypt6p stimulates the GTPase activity of Ypt6p but not that of Rab proteins (Strom et al., 1993). Yeast Dss4 protein was found to have GEF activity for Sec4 and its mammalian homolog. Mss4 protein has GEF activity for Ypt1p and Rab3a as well as for Sec4p (Burton et al., 1993; Moya et al., 1993). A newly identified membrane component, termed GDI-dissociation factor, has been implicated in recruitment of specific Rab proteins into the vesicles (Soldati et al., 1994). This protein causes dissociation of the Rab-rabGDI complex and thus promotes binding of the Rab protein to the membrane.

In addition to Rab proteins and their accessory protein factors, several other essential proteins of the transport machinery have been identified. Both mammalian and yeast cells require NSF and SNAPs for vesicle fusion (Rothman and Orci, 1992). Recently, membrane proteins that bind to SNAPs were isolated from brain extract and suggested to mediate the fusion of synaptic vesicles (Söllner et al., 1993). These findings have led to a general model of vesicle transport and fusion, termed the SNARE hypothesis (Fig. 2; see also Novick and Brennwald, 1993; Takizawa and Malhotra, 1993; Zerial and Stenmark, 1993). This model presumes that the SNAP-binding proteins are SNAP receptors that exist on vesicular membrane (v-SNARE) and the target membrane (t-SNARE). According to the model, v-SNARE on vesicles may bind specifically to t-SNARE on the target membrane as a first step of fusion and then interact with SNAP. NSF and other unidentified proteins then drive the fusion process. In the context of the SNARE hypothesis, Rab proteins may regulate v-SNARE in a way that v-SNARE would be active only when it is bound to GTP-Rab proteins (Novick and Brennwald, 1993). It remains to be answered whether the SNARE hypothesis can be generally true for the various steps of intracellular transport and is applicable to plant cells.

SMALL GTP-BINDING PROTEINS IN PLANTS

Early experiments on mammalian systems have demonstrated that GTP-binding proteins, when resolved by SDS-PAGE and transferred to nitrocellulose filters, retain the ability to bind to GTP. GTPγS, a nonhydrolyzable GTP analog, binds irreversibly to GTP-binding proteins. This

Table I. *Localization and possible function of small GTP-binding proteins in different membrane compartments*

Protein	Localization	Possible Function
Ras	Plasma membrane	Signal transduction
Ran	Nucleus	Nuclear protein import, DNA synthesis
Rac	?	Phagocytosis, membrane ruffling
Rho	Golgi, post-Golgi vesicles	Actin cytoskeleton
Arf	Golgi	Regulate budding from the ER and fusion at the Golgi stacks, endosomes, and nuclear vesicles
Sar	ER	Vesicular budding from the ER
Rab		
Rab1a	?	ER-Golgi transport
Rab1b	ER, Golgi	ER-*cis*-Golgi transport
Rab2	ER-Golgi intermediate compartment	ER-Golgi transport
Rab3a	Synaptic vesicles, chromaffin granules	Regulated exocytosis
Rab3b	Mainly in cytosol	Regulated exocytosis
Rab3c	Synaptic vesicles	Regulated exocytosis
Rab4	Early endosomes	Early endosome-plasma membrane recycling pathway
Rab5	Early endosomes, plasma membrane	Plasma membrane-early endosome transport, fusion of early endosomes
Rab6	TGN, post-Golgi transport vesicles	Budding from TGN
Rab7	Late endosomes	Transport in endocytic pathway
Rab8	Post-Golgi basolateral secretory vesicles	Golgi-plasma membrane transport
Rab9	Late endosomes, TGN	Late endosome-TGN transport
Rab11	TGN, secretory granules, synaptic vesicles	?
Rab12	Golgi	?
Rab13	Tight junction	Polarized transport, assembly of tight junction
Rab17	Basolateral plasma membrane	Transcellular transport
Rab22	Plasma membrane, endosomes	Endocytic pathway
Rab24	ER, *cis*-Golgi, late endosomes	Endocytic pathway

property has allowed researchers to detect the presence of small GTP-binding proteins in plant extracts and in thylakoid and microsomal membranes (Hasunuma and Funadera, 1987; Zbell et al., 1990). Two major proteins from soybean plasma membranes (24 and 28 kD, Zbell et al., 1990) and from root nodule peribacteroid membranes (26 and 28 kD, Z. Hong and D.P.S. Verma, unpublished data) have been shown to bind GTPγS. In a preliminary study on rice, it was shown that the binding of GTPγS to vesicles in vitro was increased by the growth hormone IAA, suggesting the role of GTP-binding proteins in cell elongation stimulated by auxin (Zaina et al., 1990). It was reported that a substantial amount of a 28-kD GTP-binding protein was translocated from the ER and Golgi fractions to the plasma membrane and chloroplasts when cells of the green alga *Dunaliella salina* were subjected to hypoosmotic swelling (Memon et al., 1993).

A variety of approaches have been taken for the isolation of a number of cDNAs encoding small GTP-binding proteins from plants. Many of these cDNAs were isolated by using degenerate oligonucleotides corresponding to one of the consensus sequences in the GTPase superfamily. Complementation of yeast mutants with a plant cDNA library was successful in isolating a *SAR1* homolog cDNA from *Arabidopsis* (d'Enfert et al., 1992). A subtraction screening strategy has been used in cloning a rab11 homolog cDNA (*rgp1*) from rice (Sano and Youssefian, 1991). Low-stringency screening using heterologous probes has allowed isolation of homologous cDNAs like *Rab7* from *Vigna aconitifolia* (Cheon et al., 1993) and *rgp2* from rice (Youssefian et al., 1993). By sequencing 130 randomly selected clones from a maize leaf cDNA library, a Rab5 homolog clone was identified (Keith et al., 1993).

From *Arabidopsis thaliana*, at least seven different clones encoding small GTP-binding proteins have been isolated (Matsui et al., 1989; Anai et al., 1991; Anuntalabhochai et al., 1991; Bednarek et al., 1994). Although their specific functions are not known, Rha1, one of the seven *Arabidopsis* clones, was highly homologous to Rab5, which is localized

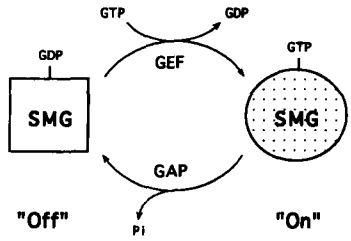

Figure 1. Small GTP-binding proteins as molecular switches. The switch is turned on when a small GTP-binding protein (SMG) binds to GTP with the help of GEF. GAP promotes GTP hydrolysis, which turns the switch off.

to early endosomes. Its expression, revealed by *rha1* promoter-driven β-glucuronidase activity, was found mainly in the guard cells and also in the stipules, root tips, and young leaves, and in the receptacles of flowering *Arabidopsis* plants (Terryn et al., 1993). It was hypothesized that Rha1 may be involved in cell-plate formation or cell-wall thickening, which requires vesicle-mediated processes. *A.t.RAB6*, one of the *Arabidopsis* genes, encodes a Rab6 homolog that is localized to medial and trans-Golgi (Goud et al., 1990; Antony et al., 1992). It complemented a yeast *ypt6* mutant (Bednarek et al., 1994), demonstrating its functional conservation. However, dominant expression of a mutated Rab6p failed to inhibit vesicular transport through mammalian Golgi (Tisdale et al., 1992). Furthermore, Ypt6 is not essential for cell viability. Overexpression of mutated *A.t.RAB6* gene or antisense regulation by the wild-type gene may reveal interesting information concerning the soluble protein sorting process at the trans-Golgi network.

By differential screening of cDNA libraries made from wild-type and dwarfing plants induced by azaC, a DNA methylation inhibitor, Sano and Youssefian (1991) isolated a *rab11* homolog cDNA (*rgp1*) from rice. Expression of this gene is reduced in azaC-treated rice dwarfing plants. Interestingly, the expression level of a second *rab11* homolog (*rgp2*, which has 53% amino acid identity with *rgp1*) is not affected by azaC treatment (Youssefian et al., 1993). Expression of sense and antisense *rgp1* in transgenic tobacco showed reduction in apical dominance and increased tillering (Kamada et al., 1992). Two *rab* homologs, *Np-ypt3* (a *rab11* homolog) and *Nt-rab5* (a *rab5* homolog), have been identified in tobacco (Dallmann et al., 1992). The expression patterns of these genes are similar; expression is highest in flowers and undetectable in leaves.

SAR1 homolog cDNAs have been recently identified from *Arabidopsis*, tomato, and soybean (d'Enfert et al., 1992; Davies, 1994; Z. Hong and D.P.S. Verma, unpublished data). Although the tomato *SAR1* gene is expressed in all tissues tested (Davies, 1994), expression patterns of four soybean *SAR1* genes are very different, one being preferentially expressed during nodule organogenesis (Z. Hong and D.P.S. Verma, unpublished data). *Rho1*, a gene implicated in the control of microfilament organization, and a *rab7* homolog cDNA have also been isolated recently from pea (Drew et al., 1993; Yang and Watson, 1993).

A Ypt1/Rab1 homolog was identified in maize coleoptile cells having all the consensus sequences and the C-terminal Cys motif (Palme et al., 1992). We have isolated cDNA clones encoding Rab1 and Rab7 homologs from soybean and *V. aconitifolia* root nodules (Cheon et al., 1993). Functionally, the plant Rab1p is able to complement the yeast *ypt1* mutant. The expression of *rab7* is enhanced significantly during nodulation, with the level of *rab7* mRNA being 12 times higher than that in root meristem and leaves. This coincides with the membrane proliferation and endocytosis of *Rhizobium* in root nodules. Reducing the expression of these proteins by antisense cDNAs under the control of nodule-specific (leghemoglobin) promoter drastically affected the biogenesis of symbiotic organelle and nodule development (Cheon et al., 1993). Considering the extensive membrane trafficking that occurs in infected cells of root nodules and the importance of proper membrane biogenesis in symbiotic interaction, the root nodules provide an excellent system with which to study the basic machinery involved in vesicle-mediated transport in plants. Temporal and spatial control of expression of these genes using tissue-specific promoters and antisense or negative complementation approaches may allow dissection of the roles of these proteins in the vesicular transport system.

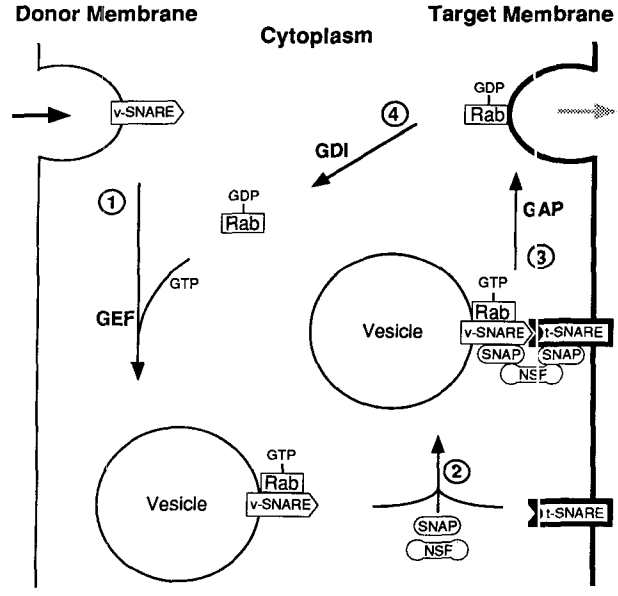

Figure 2. The SNARE hypothesis for vesicle fusion with the target membrane. According to this hypothesis, the v-SNARE protein located on the vesicles interacts with the t-SNARE found on the target membrane in the presence of NSF, SNAPs, small GTP-binding proteins, and other uncharacterized components. This interaction leads to the eventual fusion of the transport vesicles with the target membrane. In step 1, the small GTP-binding protein Rab is recruited to the vesicle in the presence of GEF and GTP. The recognition and interaction between v-SNARE and t-SNARE mediated by NSF and SNAP bring the vesicle to the target membrane (step 2). Hydrolysis of GTP by Rab with the help of GAP triggers the fusion of two distinct lipid bilayers (step 3). The GDP-bound Rab is released by GDI from the membrane to the cytosol for recycling (step 4). Fusion of the vesicle to the target membrane also releases the cargo of the vesicle for secretion into or outside of the membrane compartments (shaded arrow). Solid arrow indicates the formation of vesicles.

More plant genes coding for small GTP-binding proteins will continue to be isolated by means of PCR, heterologous screening, and complementation of yeast mutations. It is a much more challenging task to figure out the function of each gene product in membrane biogenesis. Although direct complementation of yeast mutants will continue to prove useful for testing the biological functions of some plant genes, many plant small GTP-binding proteins may not have counterparts in yeast or their respective mutants may not have been isolated. Although many small GTP-binding proteins have been localized on different subcellular compartments in mammalian cells (Table I), there is a conspicuous lack of information about their subcellular distribution in plant cells. Even if the basic machinery for membrane biosynthesis is conserved between mammalian and plant cells, it remains to be answered by plant cell biologists what are the small GTP-binding proteins that are responsible for biogenesis of the membrane compartments unique to plant cells, e.g. vacuoles and the peribacteroid membranes in root nodules (Fig. 3). It is apparent that these additional subcellular compartments would require specific SNAREs to be recognized as distinct target membranes. The fusion of these vesicles with respective membranes also facilitates unloading the "cargo" through the exocytic (secretory) pathway. Such cargo in the case of plasma membrane constitutes extracellular proteins, soluble vacuolar proteins for vacuoles, and PBF proteins secreted by fusion of the vesicles with PBM. Although the sorting of some of the secretory proteins to vacuolar and plasma membrane vesicles has been worked out (see Bednarek and Raikhel, 1992; Chrispeels and Raikhel, 1992), the targeting of PBF proteins is not clear. Moreover, the mechanism of targeting of the PBM proteins seems to vary (cf. nodulin-26 versus nodulin-24; Miao et al., 1992; Cheon et al., 1994). This may be due to the fact that PBM is a mosaic membrane having properties common to both plasma membrane and vacuoles. Therefore, involvement of various GTP-binding proteins in PBM biogenesis may be more complex. Membrane biogenesis of the organelles such as plastids, mitochondria, and peroxisomes follows different routes.

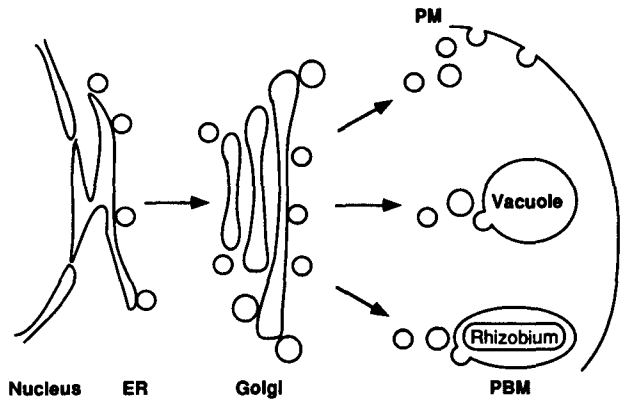

Figure 3. Three major subcellular compartments in plant cells for vesicular targeting of membrane proteins and exocytic flow. The latter constitutes extracellular proteins from the plasma membrane, soluble vacuolar proteins for vacuoles, and proteins of the PBF in legume root nodules. Fusion of vesicles from Golgi with the plasma membrane, tonoplast membrane, or the peribacteroid membrane would require specific SNAREs uniquely associated with the respective compartment.

Overexpression of a sense, antisense, or dominant-mutated form of a specific gene in transgenic plants should provide some clues about the role of small GTP-binding proteins in membrane biogenesis, as have been used in studies of *rgp1* in tobacco (Kamada et al., 1992) and Rab1 and Rab7 in soybean and *Vigna* (Cheon et al., 1993). Eventually, development of an in vitro vesicle fusion system in plants, as has been established from yeast and mammalian cells, although difficult due to the presence of cell walls and the abundance of proteases in the central vacuole, will be essential to decipher the detailed reactions involved in specific steps of vesicle traffic and membrane biogenesis.

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