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Nodule development and biogenesis of the peribacteroid membrane compartment

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The Ohio State University, 1993
NODULE DEVELOPMENT AND BIOGENESIS OF THE PERIBACTEROID MEMBRANE COMPARTMENT

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree of Doctor of Philosophy in the Graduate
School of the Ohio State University

by

Choong-Ill Cheon, B.S., M.S.

* * * * *

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To my wife, Hae-Ran, and my parents
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CHAPTER I

NODULE DEVELOPMENT AND BIOGENESIS OF THE PERIBACTEROID MEMBRANE COMPARTMENT

1.1 Introduction

Interactions between plants and microbes often involve pathogens that are harmful to the plant. However, bacteria of the genus *Rhizobium* and leguminous plants interact symbiotically to form specialized organs, root nodules, in which the rhizobia inhabit and fix nitrogen. Nitrogen fixation by bacteria is one of the most important contributors to the nitrogen cycle and it is significant in agriculture because it makes legume plants auxotrophic for external nitrogen, one of the most limiting factors for plant growth. This unique association between *Rhizobium* and its legume host is attained by a series of cellular and molecular interactions involving formation of nodule meristems, invasion of *Rhizobium* into hair cells and cortical cells, release of bacteria into host cytoplasm, nitrogen fixation and assimilation and ultimately senescence of the nodule (Verma and Delauney, 1988; Fisher and Long, 1992). Specific signals are exchanged during these interactions (Peters *et al.*, 1986; Verma, 1992).
As nodule development advances, sequential induction of both bacteria-encoded (bacteroidins) and host-encoded (nodulins) nodule-specific proteins occur in a temporal manner. Several bacterial genes involved in nodulation and fixation of nitrogen, and host genes encoding nodulins that are essential for development of root nodules, have been characterized (Long, 1992; Delauney and Verma, 1988). Attention is currently focused on the successful biogenesis of the subcellular compartment that harbors the bacteria. The failure to form this compartment may bring about pathogenic reactions in the host in all endosymbioses (Nester and Verma, 1992).

1.2 Nodule organogenesis

1.2.1 Early signal exchange between *Rhizobium* and legumes

The early interaction between two symbiotic partners can be characterized as a two-way molecular conversation. Leguminous plants synthesize specific flavonoids, which are products of the phenylpropanoid pathway and a few other phenolic compounds and release these compounds into the rhizosphere (see Peters and Verma, 1990). These compounds interact with a common nodulation gene product (Nod D) of *Rhizobium*. Nod D binds to promoter regions (*nod* boxes) of *nod* operons, activating the gene expression of *nod* genes. The *nod* genes encode enzymes involved in the synthesis of Nod factor, a substituted oligosaccaride which acts as a return signal to legumes. This molecule alone can induce nodule morphogenesis, completing the early round of molecular conversation between the two symbiotic partners (Fisher and Long, 1992), which is schematically presented in Fig. 1.
Figure 1. Early interactions and signal exchange(s) between the legume plants and Rhizobia leading to endocytosis and establishment of the symbiotic association.
The Nod factors from bacteria are all oligomers of N-acetyl glucosamine (Lerouge et al., 1990). Precursor sugars of Nod factors are polymerized by several enzymes including the product of nod C, one of the common nod genes (Bulawa and Wasco, 1991). In the case of NodRm-IV (C16:2), a Nod factor from Rhizobium meliloti, the nod H and nod Q genes were shown to determine changes in host range. Their products participate in the addition of sulfur to the Nod factor. Host range is specified by specific fatty acyl chains at the other end of the Nod factor, which require the action of nod F and nod E products. The functions and properties of the other nod genes have been studied actively (Fisher and Long, 1992). The signal molecule from Bradyrhizobium japonicum, which nodulates tropical legumes, is very similar to NodRm-IV (16:2) with some modifications.

The signal molecules elicit several plant responses including cortical cell division, root hair curling, infection thread initiation and early nodulin expression. In the case of NodRm-IV (16:2) and its acylated form, NodRm-IV (Ac, 16:2), the purified molecule at concentrations in the micromolar to nanomolar range can induce cortical cell divisions in asceptically grown seedlings of alfalfa (Truchet et al., 1991). Sometimes it has been observed that individual bacterial strains produce a family of factors that have slightly modified side chains (Spaink et al., 1991; Roche et al., 1991). When non-specific Nod factors were tested on Vicia sativa, they caused only hair deformation, while fully host-specific factors caused both root hair deformation and induction of nodule meristems. The comparison of the effects of different Nod metabolites with slight chemical modifications will be important for understanding the mechanism of nodule morphogenesis by Nod factors.
1.2.2 Induction of nodule meristem

The most conspicuous change in the root after Rhizobium attachment is the appearance of numerous centers of cortical cell division. The first hypodermal cell division in soybean was observed by 12 h after inoculation (Calvert et al., 1984). The first few divisions are always anticlinal (perpendicular to the longitudinal axis of the root) and subsequent divisions are both anticlinal and periclinal to form more organized meristems. Mitotic cell division starts at the same time as, or earlier than, root-hair deformation.

Nodule initiation occurs by two different modes depending on the host (Fig. 2). In the case of indeterminate nodules, cell divisions occur in the inner cortical cells adjacent to the endodermis which is close to the xylem pole. These cells, when stimulated to divide, have enlarged nucleoli within large nuclei, while unstimulated cortical cells have much smaller nucleoli and nuclei (Newcomb et al., 1979). The newly divided cells by 24 h after inoculation have multiple cytoplasmic strands across the cell and brightly staining membranes bordering new cell walls (Dudley et al., 1987). Two days after inoculation, the nodule primordium extends into the vascular stele. Some cells in the activated region cease dividing and become invaded by infection threads containing rhizobia. On the proximal side of the primordium, small cells rich in cytoplasm constitute the apical meristem of the incipient nodule. After endocytosis of rhizobia into inner cortical cells, the invaded cells cease mitotic activity. Mitosis continues in the middle cortex which forms the nodule meristem. The proximal side of the meristem produces a mass of infected cells and some uninfected cells. In the distal side of nodule meristem, cells become the nodule cortex. The infected cells are enlarged and thus the nodule meristem are shifted outward radially, forming the
Figure 2. The initiation of nodule development on legume roots following infection with *Rhizobium*: (1) root tip, (2,3) successive stages in the initiation of a pea nodule, and (4,5) successive stages in the initiation of a soybean nodule (from Brewin, 1991). ep, epidermis; v, vascular bundle; c, cortex; rhc, root hair curling; it, infection thread; np, nodule primordium; en, endodermis; per, pericycle.
cylindrical shape of indeterminate nodules (Newcomb et al., 1979). Mature nodules in temperate legumes have several zones with different stages of development; a proximal green zone just above the nodular vascular bundles consists of senescencing infected cells. A pink zone at the center of nodule contains cells with high nitrogen fixation activity. A distal white zone consists of the nodule meristem and newly invaded cells.

The other type of nodule initiation can be found in determinate nodules such as soybean (Glycine max). A hypodermal cell anticlinally divides twice or three times. This mitotic activity occurs only in the outermost layer of the nodule; there are no observable changes in inner cortical cells (Calvert et al., 1984; Mathews et al., 1989). Two days after inoculation, a second center of cell division is observed in the inner cortical cells close to the endodermis and pericycle. The progeny of the outer cortical cells have enlarged nuclei and darkly-staining cytoplasm with numerous small vacuoles. In contrast, the progeny of the inner cortical cells contain a small amount of cytoplasm and large vacuoles. Continued cell divisions result in cytoplasm-rich outer cell layers and highly vacuolated inner zones (Newcomb, 1981). The cytoplasm-rich cells are invaded by rhizobia and form a defined nodule meristem. The infected cells in the nodule meristematic zone continue to divide, spreading rhizobia (Brewin, 1991). As development advances, nodules which emerge from the root consist of central infected zones and surrounding vacuolated cells. The increase in volume of infected cells causes the nodule bulge radially, forming a spherical structure varying in size depending on the host. The mature determinate nodule consists of a pink central zone containing uninfected cells and enlarged infected cells, and white cortical layers including vacuolated parenchymal cells and several vascular
bundles. Thus, the determinate nodule has a transient meristematic activity while indeterminate nodule has a persistent meristem.

Nodule initiation shares many similarities with lateral root initiation, but also has some distinct differences. The primordium of a lateral root is initiated in the pericycle or its neighboring structures and emerges by secreting hydrolytic enzymes and lysing adjacent cortical cells (Charlton, 1991). In contrast, the nodule meristem arises in cortical cells and shows high mitotic activity until rhizobia invade the divided cells. However, de-differentiation of cortical cell layers, shown by induction of the primordium and subsequent cell divisions, occurs in both types of initiation.

Nod factors may induce cell divisions in the root cortex during nodule organogenesis by activating an intracellular signaling pathway. Higher plants share a close resemblance in mitosis with other eucaryotic cells. The activation mechanism may stimulate cortical cells, which are otherwise arrested in either G1 or G2 stage of the cell cycle to re-enter cell division. Although the receptors and regulatory factors involved in cycling plant cells are unknown, at least one of the genes involved in cell cycle control machinery, cdc2/CDC28 protein kinase, is conserved in plant cells (Doonan, 1991). It is expected that cell cycle control by cdc2 kinase in legume plants will prove to be similar to other organisms (Verma, 1992; Feiler and Jacobs, 1990). Cell cycle from G2 to M is progressed by MPF (mitosis promotion factor) activation and MPF activity is controlled by dephosphorylation of p34cdc2 protein kinase (Nurse, 1990). Miao et al. (1993) showed that two functional homologs of cdc2 exist in soybean nodules and one of the cdc2 genes is induced upon Bradyrhizobium infection, while the other cdc2 gene is not and instead responds to auxin treatment. These results strongly
suggest that the cell-cycle-control mechanisms contributes to nodule organogenesis.

1.2.3 Root hair deformation and infection thread development

The surface polysaccharides of rhizobia and the lectins of legume roots are thought to be involved in the attachment of rhizobia to the root surface (Dazzo et al., 1984). Bacterial attachment at the emerging root hair commonly leads to root hair curling which can be observed within 12 h of inoculation in soybean (Turgeon and Bauer, 1981). In general, only curled root hairs become infected, which can be visualized by autofluorescence in the presence of blue light (Callaham and Torrey, 1980). Infection thread formation in soybean is visible about 24 h after inoculation (Turgeon and Bauer, 1982). During this process, a localized degradation of the mucigel layer and the folded hair cell wall seem to occur (Turgeon and Bauer, 1985). In the case of soybean, the infection thread arrives at the base of the root hair cell by 48 h after inoculation and soon advances into the cortical cell layers. An increase in cortical cell division and in mitosis occurs in advance of the penetrating infection thread. Endoplasmic reticulum, Golgi, microtubules and small vesicles are abundant in host cytoplasm adjacent to the infection thread (Turgeon and Bauer, 1985). The cytoskeleton in the cortical cells may be reorganized to sort the small vesicles in the cytoplasm into the growing point of infection thread (Brewin, 1990). Mitotic centers in root cortex lacking association with curled root hairs fail to form nodules. This suggests that induction of cortical cell divisions and root hair deformation are independent processes. Similar observations were made when Agrobacterium tumefaciens or
E. coli containing nodulation genes from Rhizobium meliloti formed pseudonodules on alfalfa (Truchet et al., 1984).

1.2.4 The induction of early nodulin gene expression

Many early nodulin genes have been shown to be expressed sequentially during nodule organogenesis (Scheres et al., 1990a, b; van de Weil et al., 1990a). Their induction coincides with morphological changes in root cortical cells which are brought about by rhizobial Nod signals. Moreover, it was shown that nod gene-related factors of Rhizobium leguminosarum bv. viciae induced the gene expression of ENOD12, one of the early nodulins in pea. These results suggest that the induction of early nodulin genes is elicited by Nod factors along with nodule morphogenesis.

The induction of RH-42 and RH-44 genes was found in the root hair cells of pea upon rhizobial inoculation (Gloudemans et al., 1989). In nodules and uninoculated roots, there were either very low or no expression of these genes. The products of these genes seem to be involved in curling and deformation of the root hair. PsENOD12 encodes a proline-rich protein with a putative signal sequence (Scheres et al., 1990a). Transcripts of PsENOD12 were present in root hairs, but not in uninoculated root hairs. At later stages, PsENOD12 transcripts were found in both infected and uninfected cells of the invasion zone, but not in the meristematic cells. PsENOD12 may be involved in the formation of a nodule primordium as well as the infection process. Expression of PsENOD12 can be induced by naphthylptalamic acid (NPA), an auxin transport inhibitor. The latter can cause induction of nodule primordia. Transcripts of PsENOD5, another early nodulin expressed during the infection process, were present only in cells
containing the growing infection thread (Scheres et al., 1990b). This nodulin is also a proline-rich, but lacks a repeated motif, unlike PsENOD12. The differential expression pattern between PsENOD12 and PsENOD5 clearly shows molecular changes in the infected zone and nodule primordium.

PsENOD3 and PsENOD14 genes were expressed only in infected cells during early stages of nodule development (Scheres et al., 1990b). Both early nodulins contain a putative signal sequence at the amino terminal end and four properly-spaced cysteine residues. This suggests that PsENOD3 and PsENOD14 may be metal-binding proteins. Expression of ENOD2, an early nodulin with two repeating pentapeptides, was detected in the nodule inner cortex of pea, soybean and alfalfa (van de Weil et al., 1990b). Based on the presence of ENOD2 mRNA in the nodule parenchyma, it has been suggested that this nodulin may contribute to the formation of the oxygen diffusion barrier in nodules.

1.3 Endocytosis of *Rhizobium*

1.3.1 Internalization of bacteria into host cytoplasm

Successful infection requires internalization of rhizobia into the cytoplasm of host cells. Rhizobia in the infection thread are still outside of the host cells. Entry of the rhizobia occurs from unwalled regions of the infection thread by a poorly understood process resembling endocytosis. In the infected cell, "unwalled droplets" are found at the tip or side of the infection thread (Verma and Long, 1983; Newcomb, 1976). The unwalled droplets appear to be formed by degradation of the infection thread wall mediated by cellulase and pectinase (Verma et al., 1978a). Robertson and Lyttleton (1982) observed that the number of coated
vesicles associated with the infection thread membrane near the tip of the thread was about 20 fold greater than that associated with the membranes towards the base of the thread.

Recent studies on the interaction of pathogenic bacteria with their animal hosts has revealed components of bacterial invasion and a possible mechanism of signal transduction. (Falkow, 1991; Bliska et al., 1993). The enteropathogenic Yersinia pseudotuberculosis in its interaction with mammalian cells uses invasin and YadA (an adhesin) as bacterial attachment and entry factors. Yad A protein is encoded on the Yersinia virulence plasmid and may form a fibrillar surface structure for bacterial attachment (Kapperud et al., 1987). Invasin is an outer membrane protein on Yersinia and is solely responsible for the accumulation of actin and actin-associated proteins around the entering bacterium (Young et al., 1992). Latex beads coated with invasin becomes internalized, suggesting that invasin is sufficient for the entry process. Mammalian cell receptors are known to be integrins, which constitute a large family of α/β heterodimemric transmembrane proteins. Multiple members of the β1 chain integrin family (α3β1, α4β1, α5β1, and α6β1) are bound by invasin. These proteins are involved in cell to cell interactions and cellular adhesions to Extracellular matrix (ECM) proteins (e.g. collagen, fibronectin and laminin) (Hynes, 1992). However, the signal transduction mechanism mediated by integrins is not known. Several outer membrane proteins of Yersinia (Yop) have been shown to have homologies to eukaryotic signal transduction proteins; YopH (protein tyrosine phosphatase; PTPase), YopM (glycoprotein Iba receptor), YpkA (Ser/Thr protein kinase) (Bliska et al., 1993). Studies on the interaction of these proteins may reveal the signal transduction mechanism for Yersinia internalization.
In the case of *Salmonella*-mammalian cell interactions, membrane ruffling occurs in the infected cells, accompanied by profound cytoskeletal rearrangements at bacteria-host cell contact point. A number of cytoskeletal proteins, including actin, α-actinin, talin, tubulin, tropomyosin, and ezrin, accumulate at these sites (Finlay *et al.*, 1991). Membrane ruffling, cytoskeletal rearrangements and [Ca++] influx caused by *Salmonella* attachment to the cell seems to occur as a consequence of the activation of a number of host cell surface receptors including the epidermal growth factor receptor (EGFR) (Kadowaki *et al.*, 1986; Moolenar *et al.*, 1984; Rijken *et al.*, 1991). Rac and Rho, small GTP-binding proteins, are also known to be involved in growth factor-induced membrane ruffling and cytoskeletal rearrangements in cultured cells (Ridley and Hall, 1992; Ridley *et al.*, 1992).

A study with ineffective rhizobial mutants (Morrison and Verma, 1987) provided insight into rhizobial internalization legumes. When the *Tn5* mutant of *B. japonicum* were used to infect host plants, infection and nodule differentiation proceeded normally. However, bacteria were not released from the infection thread, and bacterial invasion was stopped. Nodules formed by the mutant were devoid of bacteria. These results suggest that the internalization process requires the involvement of both plant and rhizobial genes.

1.3.2 Endocytic pathway

1.3.2.1 Coated-vesicle formation

The process of coated pit/vesicle formation involves the recruitment and assembly of clathrin and specific clathrin-associated molecules at the plasma membrane (Pearse, 1987; Schmid, 1993). Coat proteins assemble from
cytoplasmic pools onto the membrane to form a planar protein. The major subunits of coat proteins are clathrin triskelions, which consist of three heavy chains and three tightly associated light chains, and AP2 complexes, which are heterotetramers, consisting of two larger and two smaller subunits (α and β) (Keen, 1990). Four stages in coated-vesicle formation can now be biochemically delineated: initiation/priming, coat assembly/receptor recruitment, coated-pit invagination/sequestration and coated-vesicle budding/internalization (Schmid, 1993). Early events leading to ligand sequestration into deeply invaginated coated pits specifically require direct interactions with receptor tails, AP2 complexes and clathrin. Both sequestration and internalization of bound ligand requires incubation at 37°C, ATP hydrolysis and multiple cytosolic factors (Carter et al., 1993). ATP and GTP hydrolysis are required at multiple stages of coated-vesicle formation including clathrin assembly into functionally active coated pits, and the invagination of pits. Vesicular transport by nonclathrin- or COP-coated vesicles requires multiple GTPases including heterotrimeric G proteins, members of Rab (proteins from rat brain) family and Adenosine ribosylation factor (ARF) of small GTP-binding proteins. COP-coated pit assembly appears to be regulated through interactions of ARF with heterotrimeric G proteins, while Rab proteins appear to participate in later events (Rothman and Orci, 1992). Studies of the shibire mutation in Drosophila has suggested that dynamin (the mammalian homologue of the shibire gene product) might be one GTPase required for endocytosis (Kosaka and Ikeda, 1983; van der Bliek and Meyerowitz, 1991). Dynamin might play a role in governing the decision to close a coated pit by triggering invagination or vesicle budding (Schmid, 1993).
1.3.2.2 Early and late endosomes

The endosomes constitute the next station in the endocytic pathway. These relatively recently discovered prelysosomal organelles represents a heterogeneous complex set of vacuoles located in the peripheral and perinuclear cytoplasm (Mellman et al., 1986; Kornfeld and Mellman, 1989; Gruenberg and Howell, 1989). They consist of complex vacuolar and tubular elements, and many have the appearance of multivesicular bodies. They constitute the main site for sorting of endocytosed material and membrane recycling in the cell.

After internalization, the ligand-mannose 6-phosphate receptor (MPR) complexes are delivered first to the early endosomes (Schmid et al., 1988, 1989), a distinct subpopulation of endosomes that appear to be distributed largely in the peripheral cytoplasm (Gruenberg et al., 1989). Early endosomes have an internal pH of about 6 to 6.3 (Schmid et al., 1989; Sipe and Murphy, 1987) that is sufficiently acidic to cause the dissociation of ligands from receptors, which rapidly recycle to the cell surface. Thus early endosomes are likely to be the primary site from which this class of plasma membrane receptors return to the surface (Schmid et al., 1988). The individual elements of the early endosome appear to be highly dynamic, continuously exchanging membrane and content. Gruenberg et al. (1989) have proposed that these interactions may pool the endocytosed material destined to be degraded from more than one early endosomal element, prior to the transfer of these materials to putative microtubule-dependent carrier vesicles. Since late endosomes may be concentrated more in the perinuclear cytoplasm (Gruenberg et al., 1989), translocation of early endosomes (or transport vesicles derived from early endosomes) from the peripheral to the perinuclear cytoplasm may be required. A variety of considerations predict that such vesicle
translocations indeed occur and are mediated by microtubules perhaps in conjunction with cytoplasmic dynein.

In addition to their microtubule-dependence, late endosomes (pH = 5.5-6.0) also differ from early endosomes in their general appearance (Fig. 3). Late endosomes, particularly in the perinuclear region, are often larger and exhibit a more complex organization of internal membranes than early endosomes, and they have often been described as multivesicular structures (Dunn et al., 1986). Late endosomes containing the internalized hydrolases and any remaining MPR may have several fates. First, they may fuse directly with dense, hydrolase-rich lysosomes, as suggested by light microscopic analysis. This would result in the degradation of digestible contents and endosomal membrane components that fail to recycle rapidly after fusion. In addition, some of the late endosomes may fuse with Golgi-derived vesicles carrying newly synthesized lysosomal enzymes. Finally, late endosomes may fuse with each other. Lysosomes (vacuoles), then, would be recognized as the final population of vesicles to which endocytic tracers are delivered, and from which all r.on-lysosomal components have either recycled or been degraded.

1.3.2.3 Lysosomes (vacuoles)

Lysosomes (vacuoles) are generally considered as the end station of the endocytic pathway. In addition to their full complement of mature hydrolases and absence of cation-independent receptor for lysosomal hydrolases (CI-MPR), lysosomes can be discriminated from late endosomes by their morphological appearance, although this may be difficult in some cell types in the absence of
Figure 3. The involvement of small GTP-binding proteins in the endocytic and exocytic pathways (from Gruenberg and Clague, 1992). ER, endoplasmic reticulum; TGN, trans-Golgi network; EE, early endosome; LE, late endosome; ECV, endosomal carrier vesicles.
specific markers. Their lumenal pH is about 4.5, which corresponds to the optimal pH of many hydrolases and is lower than the late endosomes (Mellman et al., 1986). Lysosomes are highly dynamic organelles. Interspecies cell fusion experiments have shown that two populations of lysosomes rapidly exchange both content and membrane in vivo, in a temperature- and microtubule-dependent manner (Ferris et al., 1987; Deng and Storrie, 1988). These experiments also have monitored transfer from late endosomes to lysosomes; despite a 1-2 h chase, some internalized tracer still have been present in late endosomes. Further, the lysosomal membrane glycoproteins used as markers appear to label late endosomes as well as lysosomes (Brown et al., 1986). These data suggest that lysosomes may interact with each other via fusion events.

1.3.2.4 Roles of small GTP-binding proteins in endocytic membrane traffic

It has been well established from in vitro studies that early endosomal elements can fuse with each other (Gruenberg and Howell, 1989). Relatively little is known, however, about the molecules controlling this fusion event, except that it requires an N-ethylmaleimide sensitive factor (NSF) (Diaz et al., 1989) and that it is sensitive to low concentrations of GTPyS (Mayorga et al., 1989), suggesting that GTP-binding proteins are involved.

The Rab proteins are the mammalian counterparts of two small GTP-binding proteins, Ypt1p and Sec4p, of yeast. Rab proteins were localized on endocytic and exocytic membranous compartments, suggesting that they are involved in the regulation of membrane traffic (Chavrier et al., 1990). Gorvel et
al. (1991) showed that fusion of early endosomes could be inhibited by cytosol containing the overexpressing mutant Rab5p Ile^{133} and by antibodies against Rab5p, but not against Rab2p or Rab7p. The inhibition mediated by anti-Rab5p antibodies could be overcome by complementing the assay with the cytosol containing wild-type Rab5p, but not with the same cytosol depleted of Rab5p, nor with cytosol containing the Rab5p mutants or Rab2p. These data strongly suggest that Rab5p is involved in the process of early endosome fusion.

Bucci et al. (1992) and van der Sluijs et al. (1992) investigated the functions of Rab4p and Rab5p in hamster cell lines. Both of them used overexpression of wild-type proteins and also mutant proteins in transgenic cells to examine the effects on membrane traffic. Overexpression of Rab5p (Bucci et al., 1992) was found to increase the rate and extent of fluid-phase endocytosis, as well as the endocytosis and recycling of the transferrin receptor. The overexpression also decreased the number of cell-surface coated pits. These results suggest that higher Rab5p levels increase the rate of endocytic vesicle formation. In contrast, expression of the defective Rab5p mutant, Rab5p^{I-133}, reduces fluid-phase endocytosis and the rate of transferrin uptake by about 50%. Rab4p overexpression induces a redistribution of the transferrin receptor so that at steady state 80% is on the cell surface, compared with 20% in normal cells (van der Sluijs et al., 1992). Although the rates of transferrin uptake were not affected by Rab4p overexpression, the release of apo-transferrin in early endosomes is much reduced and these cells fail to accumulate iron from transferrin. Furthermore, in these cells the intracellular transferrin is located with narrow tubules, which do not exhibit significant acidification in vitro and which resemble a previously identified transferrin-recycling subcompartment of the endosome. Rab4p may thus direct
transferrin receptor-transferrin complexes to an endosome compartment that is involved in recycling. These results demonstrate that two different Rab proteins with overlapping subcellular distributions regulate different transport events. It is possible that each Rab family member mediates equivalent functions but at different membrane transport steps (Marsh and Cutler, 1993).

Rab7p, another small GTP-binding protein involved in the endocytic pathway, was localized to late endosomes (Chavrier et al., 1990) in mammalian cells and its yeast homolog, Ypt7p, has been isolated (Wichmann et al., 1992). Yeast ypt7 null mutants are characterized by highly fragmented vacuoles and differential defects of vacuolar protein transport and maturation. The uptake of α factor in the mutant was not affected, but degradation of the endocytosed pheromone was severely inhibited. They argued that Ypt7p might function in transport of proteins from early to late endosome-like compartments.

1.4 Maturation of PBM compartment

1.4.1 Targeting of PBM nodulins

1.4.1.1 Protein targeting in the early secretory pathway (ER and Golgi)

The secretory pathway of eukaryotic cells shares routes with the targeting of newly synthesized proteins, the generation and maintenance of subcellular compartments and organelles (Rothman and Orci, 1992). Many of the components of the secretory pathway appear to be universal: the same machinery operates in yeast as in animals. A key enzyme for fusion of a vesicle with the
Golgi is also needed for fusion of endocytic vesicles. The coats that pinch off endocytic and Golgi vesicles have underlying similarities not evident from their morphology. Members of gene families like small GTP-binding proteins are modular units that perform the same tasks in different places, combining a general mechanism with compartmental specificity (Vemer and Schatz, 1988; Mellman and Simons, 1992; Gruenberg and Clague, 1992). The organelles of the secretory system include the ER, Golgi, endosomes, secretory vesicles, vacuoles (lysosomes), and plasma membranes. Each independent organelle system in the cell appears to have a unique site of entry for proteins from the cytosol. Targeting sequences have been defined in proteins destined for the ER, mitochondria, chloroplasts, nuclei, vacuoles and peroxisomes (Vemer and Schatz, 1988; Chrispeels and Raikhel, 1992).

The first step in the secretory process is the sequestration of proteins into the lumen of the ER or their incorporation into ER membranes. Proteins initially synthesized on ribosomes in the cytosol of the cell are selectively targeted to the ER. Targeting to the ER occurs by a signal sequence which resides at the amino terminus of the protein. Signal sequences are 15-35 amino acid residues long and consist of 3 domains: a basic amino-terminal region, a hydrophobic stretch of 7 or 8 amino acid residues and a more polar carboxyl terminal region (von Heijne, 1983, 1986; Perlman and Havorlsen, 1983). The signal sequences are bound by signal recognition particle (SRP), resulting in an arrest in the elongation of the nascent polypeptide (Nunnari et al., 1991). The complex of the ribosome-nascent chain-SRP is dissociated when the SRP binds to its receptor on the ER membrane. Translocation machinery on the ER membrane enables the nascent chain to insert into and become translocated across the ER membrane. While the signal sequence
is cleaved by signal peptidase, a resident enzyme in ER membrane, the polypeptide chain is translocated into the lumen of the ER and interacts with BiP, a member of the heat-shock protein 70 family, for proper folding and assembly. The signal sequence of a potato tuber vacuolar protein was shown to have ER targeting function (Iturriaga et al., 1989) using β-glucuronidase (GUS) as a reporter, demonstrating that targeting information is also present in signal sequences of plant proteins as well. Munro and Pelham (1987) identified a retention signal (KDEL or HDEL) for ER resident proteins. Addition of this tetrapeptide to the carboxyl terminus of an otherwise secreted protein strongly supported the proposed role of this sequence as a retention signal. The same information has been obtained for the ER-associated auxin-binding protein of maize (Hesse et al., 1989).

Vesicle-mediated protein transport from the ER to the Golgi has been divided into two stages, vesicle budding and targeting or fusion, based on the phenotype of sec mutants of yeast (Schekman, 1992; Kaiser and Schekman, 1990). In vitro studies have shown that the formation of transport vesicles during transfer from the ER to Golgi in yeast depends on Sar1p, a small GTP-binding protein (Fig. 3) and Sec12p, a membrane protein (Nakano and Muramatsu, 1989; Rexach and Schekman, 1991). Sec12p appears to facilitate nucleotide exchange for Sar1p and its business end is toward the cytoplasmic face on the ER membrane. Sec23p has a GTPase-activating protein (GAP) activity and may interact with Sec12p to increase the rate of GTP hydrolysis by Sar1p (Hicke and Schekman, 1989). Attachment of transport vesicles to their acceptor membranes (cis Golgi) requires both Ypt1p, a prototype member of the Sec4/YPT1/Rab family (Fig. 3), and Sec18p, the yeast homologue of NSF (Segev, 1991; Rexach and
Schekman, 1991). Although Sar1p and Ypt1p belong to different subfamilies of small GTP-binding proteins, both are required for vesicle formation and targeting/fusion between the ER and the Golgi.

The Golgi is a polarized and dynamic organelle, consisting of three functionally distinct, adjacent subcompartments (Mellman and Simon, 1992): the cis-Golgi network (CGN), the Golgi stack (containing several distinct subcompartments), and the trans-Golgi network (TGN). The cis- and trans-Golgi networks are the entry and exit faces of the stack, respectively, and are mainly sorting centers. The TGN is where proteins with differing final destinations, such as vacuoles (lysosomes), secretory storage vesicles, and plasma membrane domains diverge (Rothman and Orci, 1992). The Golgi rapidly disintegrates and reforms during mitosis or brefeldin A treatment (Kreis, 1992). Vesicular carriers and tubular connections mediate membrane transport both within the Golgi complex and from this organelle to adjacent compartments. This membrane traffic is tightly regulated and cargo is delivered to its proper destination in the cell. Several classes of vesicular carriers and probably different types of transient tubular connections have been implicated in these transport processes. Coat proteins associated with the cytoplasmic face of vesicular carriers of the Golgi complex have been identified (Pearse and Robinson, 1990). Some of these proteins of the clathrin and non-clathrin coats are homologous (Duden et al., 1991). This homology may suggest a common principle for their putative regulatory role in Golgi membrane traffic. The mechanism of this targeting and binding to the corresponding membrane domains are as yet elusive, but increasing evidence suggests the involvement of GTP-binding proteins (ARF) (Donaldson et al., 1991).
1.4.1.2 Protein secretion and vacuolar targeting in plants

Plant cells secrete proteins via the constitutive pathway, meaning that newly synthesized proteins continuously exit the cell (Chrispeels, 1991). Each of the steps along the secretory pathway (ER--Golgi--secretory vesicle--plasma membrane) is a default step, defining overall the bulk-flow pathway through the secretory system (Kelly, 1985). To be retained in an organelle along the secretory pathway (e.g., the ER, the Golgi), a protein needs additional signals. Wieland et al. (1987) used a tripeptide (NYT) to study the rate of bulk flow in the secretory pathway. The peptide was secreted in 10-20 min. This defines the bulk-flow rate of ER to cell surface movement and thereby the default pathway. There are many examples demonstrating that secretion occurs by a default pathway in plants. The nonsecretory enzymes phosphinothricin acetyltransferase (PAT), neomycin phosphotransferase II (NPT II), and β-glucuronidase (GUS) were secreted when targeted to the lumen of the ER by signal peptide-mediated translocation (Denecke et al., 1990). When a chimeric gene consisting of the signal sequence of phytohemagglutinin and the coding sequence of cytosolic seed protein (PA2) was expressed in tobacco, the fusion protein entered the secretory system and was efficiently secreted in suspension culture (Dorel et al., 1989; Hunt and Chrispeels, 1991).

The sorting signal for lysosomes, the mammalian counterpart to the vacuole, resides on the glycans of proteins (Kornfeld and Mellman, 1989). But in plants and yeast, the sorting signal for the vacuole is contained in polypeptide domains. Several short polypeptide domains may act as vacuolar sorting signals in plants: an N-terminal propeptide (e.g., sporamin: Matsuoka and Nakamura, 1991), a C-terminal extension (e.g., cucumber chitinase: Bednarek and Raikhel,
1991), or an internal portion of a mature protein (e.g., phytohemagglutinin: Tague et al., 1990). There is no one targeting signal for all plant vacuolar proteins. The sorting determinants for each protein or group of proteins may be tailored to the specific structure of the protein (Bednarek and Raihkel, 1992; Chrispeels and Raikhel, 1992).

1.4.1.3 Targeting of nodulins to the PBM compartment

Release of bacteria from the infection thread requires proliferation of PBM during early stages of the infection process and redirecting some of the plasma membrane proteins and all of the PBM nodulins to this de novo formed subcellular compartment. Many differences were found in the protein profiles between the PBM and the plasma membrane (Fortin et al., 1985), supporting the idea that the PBM harbors many new proteins and lacks some of the plasma membrane proteins to meet the requirements of symbiosis. According to Robertson and Lyttleton (1982), 20 times more vesicles fuse with the tip of the infection thread than with its base or with the plasma membrane. After the release of bacteria and their enclosure within the PBM, five times more vesicles fuse with the PBM than with the plasma membrane. Extensive membrane proliferation may be achieved by nodule-specific stimulation of many enzymes, including a new form of choline kinase (Mellor et al., 1986). Evidently the presence of bacteria redirects the transport of vesicles to their vicinity. In the same context, the subcellular localization of nodulin-24, nodulin-26 and other PBM nodulins indicates that they are targeted to the PBM following synthesis and are not incorporated in the plasma membranes (Fortin et al., 1985, 1987).
However, the sequence comparison of the three PBM nodulins (nodulin-23, nodulin-24 and nodulin-26) has not identified any common features, either at the primary or at the secondary structure levels. Nodulin-23 and nodulin-24 have a cleavable amino-terminal signal sequence, while nodulin-26 does not (Cheon and Verma, unpublished results; Miao et al., 1992), although both nodulin-24 and nodulin-26 were shown to be co-translationally translocated. Based on a hydropathy plot, nodulin-24 lacks a putative membrane-spanning region while nodulin-26 has six membrane-spanning domains. In addition, nodulin-26 is induced prior to the formation of the PBM compartment, whereas nodulin-24 is induced after the release of rhizobia (Fortin et al., 1987). Quite possibly, the mechanism of targeting of these nodulins to the PBM may differ. Nodulin-26 may have a sorting mechanism that is similar to other vacuolar membrane proteins, such as vacuolar H\textsuperscript{+}-ATPase, or tonoplast intrinsic protein (TIP; Ludevid et al., 1992). In contrast, the targeting mechanism for nodulin-24 may be connected to its post-translational modification which is responsible for the size increase from 20 kD to 33 kD and its membrane attachment. The PBM acquires strongly acidic phospholipids that are not found in other endomembrane systems (Mellor et al., 1985). Interaction of PBM nodulins with these PBM specific phospholipids at the TGN may be responsible for their sorting to the PBM.

1.4.2 The PBM compartment and symbiosis

The formation of the PBM is essential for maintaining bacteria inside the host cell, segregating the metabolism of the host and microsymbiont. The PBM acts as an O\textsubscript{2}-barrier for nitrogenase in rhizobia, as the nitrogenase is sensitive to oxygen. Tricarboxylic acid (TCA) cycle intermediates are provided by the plant to
the bacteria as a carbon source. In addition to the carbon substrates required for bacteroid metabolism, the flow of many other compounds essential for nodule function is controlled by the PBM, suggesting the presence of several transporters. For example, a dicarboxylic acid transporter (DCT) has been characterized in soybean PBM (Uvardi et al., 1988). The DCT is capable of mediating a rapid flux of dicarboxylate anions, such as malate and succinate, to the bacteroids inside the nodule. Malate transporters have been identified in the tonoplast membrane although their properties are different from PBM transporter. The H⁺-ATPase activity of the PBM from infected roots was compared with that of the plasma membrane from uninfected roots (Blumwald et al., 1985). The enzymes from both sources are quite similar, but the enzyme from the PBM is more sensitive to stimulation by NH₄⁺. This difference may be relevant to the transport of NH₄⁺ through the PBM. It was suggested that acidification of the peribacteroid space by this enzyme may contribute to the conversion of NH₃ fixed by bacteroids into NH₄⁺. The PBM compartment shares many properties with the vacuole, a lytic compartment in the plant cell. In addition to the transport of dicarboxylates by DCT and acidification of the peribacteroid space by H⁺-ATPase, the peribacteroid space contains many enzymes that are present in the vacuole including acid phosphatase, α-mannosidase II, proteases, and protease inhibitors (Kinnback et al., 1987; Manen et al., 1991; Brewin, 1990). Nodulin-26, an intrinsic membrane protein of the PBM, resembles an integral tonoplast membrane protein (TIP) (Pao et al., 1991). The central vacuole disappears in infected cells of determinate nodules after the endocytosis of Rhizobium. Bacterial lysis occurs inside the PBM compartment in some incompatible associations (Werner et al., 1984) as well as in senescencing nodules. It is possible that the PBM compartment has some vacuolar
functions with limited activities until senescence ensues. However, it is not known whether the vacuolar activities in the PBM compartment are present from the beginning of symbiosis or appear following an incompatible association of the symbiotic partners.

1.4.3 Bacteroid differentiation

The bacteroid form of *Rhizobium* is different from the free-living form in many respects, such as extracellular polysaccaride (EPS) formation, DNA content and synthesis, cell wall thickness etc. (Verma and Long, 1983; Werner, 1992). Reduction in bacterial wall thickness and rigidity, and changes in the outer membrane were often observed in the bacteroid state of *Rhizobium* (Robertson et al., 1978). β-polyhydroxybutyrate (PHB) can be found in some species of bacteria. The DNA content per bacteroid increases by several fold (Sutton et al., 1977). Bacteroids appear to have specific components in electron transport pathway such as bacteroid-specific cytochromes (Thöny-Meyer et al., 1989; Appleby, 1984) although their specific roles are not known.

1.5 Proposed research

The primary objectives of my dissertation project were to understand how small GTP-binding proteins contribute to the biogenesis of the PBM compartment in soybean-*Bradyrhizobium* symbiosis, and to understand the topology and membrane-anchoring process of nodulin-24, a soybean nodulin. The Rab family of small GTP-binding proteins is involved in vesicular transport. Since membrane proliferation and vesicular transport are very active in nodule tissue, I proposed to clone legume homologs of the Rab family and to study their roles in the biogenesis
of the PBM compartment using antisense-mediated repression. Nodulin-24, an abundant PBM protein, was shown to be cotranslationally processed to a 20 kD polypeptide and was localized to the PBM, but as a 33 kD-protein. I proposed to determine the cleavage site of signal sequence in nodulin-24 and to study how the processed form of nodulin-24 becomes membrane-bound using \emph{in vitro} translated nodulin-24 and mature nodulin-24 from PBM. These studies contributed to our understanding of the biogenesis of the PBM compartment.
CHAPTER II

ROLE OF PLANT HOMOLOGS OF RAB1P AND RAB7P IN THE BIOGENESIS OF PERIBACTEROID MEMBRANE, A SUBCELLULAR COMPARTMENT FORMED DE NOVO DURING ROOT NODULE SYMBIOSIS

2.1 Abstract

The peribacteroid membrane (PBM) in legume root nodules is derived from plasma membrane following endocytosis of *Rhizobium* by fusion of newly synthesized vesicles. We studied the roles of plant Rab1p and Rab7p homologs, the small GTP-binding proteins involved in vesicular transport, in the biogenesis of the PBM. Three cDNA's encoding legume homologs of mammalian Rab1p and Rab7p were isolated from soybean (sRab1p, sRab7p) and *Vigna aconitifolia* (vRab7p). sRab1p was confirmed to be a functional counterpart of yeast Ypt1p (Rab1p) by complementation of a yeast ypt1-1 mutant. Both *srab1* and *vrab7* genes are induced during nodulation with the level of *vrab7* mRNA being twelve times higher than that in root meristem and leaves. This induction directly correlates with membrane proliferation in nodules. Antisense constructs of *srab1*
and *vrab7*, under a nodule-specific promoter (leghemoglobin, Lbc3), were made in a binary vector and transgenic nodules were developed on soybean hairy roots obtained through *Agrobacterium rhizogenes*-mediated transformation. Both antisense *srab1* and *vrab7* nodules were smaller in size and showed lower nitrogenase activity than controls. The antisense-*srab1* nodules showed lack of expansion of infected cells, fewer bacteroids per cell and their frequent release into vacuoles. In contrast, antisense-*vrab7* expressing nodules showed accumulation of late endosomal structures and multivesicular bodies in the perinuclear region. These data suggest that both Rab1p and Rab7p facilitate the development of the PBM compartment in effective symbiosis.

### 2.2 Introduction

Development of root nodules on legume plants following interaction with *Rhizobium* involves a series of unique cellular and molecular events (see Verma and Long, 1983; Nap and Bisseling, 1990; Verma, 1992). Nod factors from bacteria cause the induction of meristematic activity in the root cortex (Truchet *et al.*, 1991), followed by the bacterial invasion of newly divided cells through the infection thread (Fisher and Long, 1992). "Endocytosis" of bacteria occurs by budding-off of plasma membrane from the infection thread and fusion of the new membrane vesicles with the peribacteroid membrane (PBM) (Robertson and Lyttleton, 1982). In an infected cell of a soybean nodule, there is almost 30 times more membrane as PBM in comparison to the plasma membrane (Verma *et al.*, 1978; Verma and Fortin, 1989). This high rate of PBM biogenesis enables the host to house the microsymbiont in a new subcellular compartment. It is not
known how membrane proliferation is initiated or how vesicle transport to the tip of the infection thread or the PBM occurs. The nature of this newly formed subcellular compartment and the targeting mechanism of the PBM nodulins to this structure are not known.

A number of GTP-binding proteins have been shown to play important roles in vesicular transport of proteins (Hall, 1990; Bourne et al., 1991). One of these, Ypt1p, isolated from yeast (Gallwitz et al., 1983), shows high homology to the Ras family of proteins. A mutation in the yeast YPT1 gene resulted in accumulation of unfused vesicles, aberrant structure of the ER and Golgi apparatus, and synthesis of incompletely glycosylated secretory proteins (Segev et al., 1988). Ypt1p has been shown in a cell-free system to mediate vesicular transport from the ER to Golgi (Segev, 1991). Another GTP-binding protein in the same class, Sec4p, was shown to function in protein transport between the Golgi and the plasma membrane (Salimen and Novick, 1987).

Mammalian homologs of Ypt1p and Sec4p (Rab proteins) have been isolated and localized to endocytic and exocytic compartments (Chavrier et al., 1990). Especially, Rab4p and Rab5p were localized on early endosomes and Rab7p on late endosomes, suggesting the requirement for GTP hydrolysis in endocytosis (Mayorga et al., 1989). Expression of mutated Rab4p and Rab5p reduced the rate of endocytosis and led to abnormal endosome formation in both cases (Bucci et al., 1992; van der Sluijs et al., 1992). Recently, plant cDNAs encoding small GTP-binding proteins (Rha1, YPTm1, YPTm2) with high homology to members of the Ras family were isolated (Anuntalabhochai et al., 1991; Palme et al., 1992). However, the functions of these proteins in plant vesicular transport are not understood.
The infection process as well as establishment of the endosymbiotic association in root nodules requires extensive vesicular transport in the host cell to form the PBM compartment. Proper synthesis and maintenance of this subcellular compartment are essential for it to function as a symbiotic organelle, or "symbiosome" (Roth et al., 1988). In order to dissect the process of PBM biogenesis, we initiated studies on small GTP-binding proteins which may be involved in endocytosis of bacteria and the development of PBM compartment. Homologs of Ypt1p and Sec4p were isolated from soybean (sRab1p and sRab7p), and Vigna (vRab7p). Reducing the expression of these proteins by antisense cDNAs under the control of a tissue-specific promoter drastically affected PBM biogenesis and nodule development, leading to inefficient nodules.

\section*{2.3 Materials and Methods}

\subsection*{2.3.1 Plant tissues, bacteria and yeast strains}
Soybean (\textit{Glycine max} L. cv. Prize) was infected with \textit{Bradyrhizobium japonicum} (strain 61A76) and grown on vermiculite at 28°C, 16 hr photoperiod, and 70% humidity in a growth chamber. Leaves, roots and nodules were harvested as described previously (Miao et al., 1993). The \textit{Saccharomyces cerevisiae ypt1-1} mutant (DBY1083) used for complementation analysis was provided by David Botstein.

\subsection*{2.3.2 PCR amplification and screening of nodule cDNA library}
A soybean nodule cDNA library in the λ Zap vector (Stratgene, La Jolla, CA) was pooled and plasmids were excised \textit{in vivo} (Delauney and Verma, 1990).
Cesium chloride-purified plasmid DNA was used as the template for PCR. The primers used for cloning of srabl were a degenerate oligonucleotide (41-mer; GTAAAGCICAGATTTGGGACACICAGGACA (G/A)GA(G/A)CGnT) corresponding to the G3 region of the GTPase superfamily, and either of the sequencing primers (reverse primer or M13-20 primer). PCR was performed using the GeneAmp PCR Kit (Perkin-Elmer Cetus, Norwalk, CT) according to the manufacturer's instructions. The concentration of Mg++ in the reaction was 2 mM and 0.5 mg of template DNA was used. The PCR cycles were 94°C for 1 min, 52°C for 1.5 min, 72°C for 1 min and the final cycle had a 10 min extension at 72°C. PCR products were resolved on a 1.2% agarose gel. DNA bands were excised, purified, cloned into pBluescript SK(-) phagemid, and sequenced. The DNA fragment homologous to rabl from the PCR was used to screen the soybean cDNA library and a Vigna cDNA library (constructed in pcDNA II; Invitrogen, San Diego, CA) as previously described (Lee et al., 1993).

2.3.3 Yeast complementation

srabl cDNA was cloned in the yeast expression vector pYEUra3 (Clontech, Palo Alto, CA). A yptl-1 mutant strain (DBY1083) was transformed by the method of Treco and Lundblad (1989) and plated on minimal media without uracil. The growing colonies were further selected at 14°C. pRB320, a 2 μm plasmid containing the yeast YPT1 gene (Segev and Botstein, 1987) was used as a positive control. For the cosegregation test, the method of Rose and Broach (1991) was followed.
2.3.4 Development of transgenic root nodules

Binary plasmids containing antisense constructs were transferred to *Agrobacterium rhizogenes* (K599) by the freeze-thaw method (Höfgen and Willmitzer, 1989). The transformants were used to form hairy roots on soybean. Four to five days after germination of soybean seeds, hypocotyl regions of seedlings were wounded with an 18-G needle loaded with *A. rhizogenes* (K599). The plants were grown for 10-14 days, the wounded sites being covered with vermiculite. Hairy roots, formed at the wounded sites, were tested for GUS activity (Jefferson, 1987). At this stage, the main roots were removed and only GUS-positive hairy roots (one or two) were kept. For some of the antisense-*srab1* plants, one GUS-negative (wild type) hairy root was left together with one GUS-positive hairy root, which was tagged. On the same day, roots were inoculated with *Bradyrhizobium japonicum* (61A76) for nodulation.

2.3.5 Northern blot analysis

Total RNA was extracted from 5 g of tissue according to Verwoerd *et al.* (1989). 30 mg of total RNA were loaded on a 1.2% agarose gel containing formaldehyde and transferred to a nitrocellulose filter. Hybridization was performed according to Mahmoudi and Lin (1989). The level of hybridization was quantified by a Phospho-Imager (Molecular Dynamics, Sunnyvale, CA).

2.3.6 Construction of antisense-*srab1* and -*vrab7* expression cassettes

Full length cDNA's of *srab1* and *vrab7* were inserted in antisense orientation between a Lbc3 promoter and NOS 3' terminator into a pUC vector.
(pMLB3) containing the 2.7 kb Lbc3 promoter (Brisson and Verma, 1982).

EcoRI fragments containing the entire cassettes were inserted at the EcoRI site of pBI121 (Jefferson, 1987). The final constructs are shown in Fig. 8.

2.3.7 RNA-PCR amplification of antisense transcripts

Isolation of total RNA and RNA-PCR were performed as before (Lee et al., 1993). The primers used for RNA-PCR are shown below.

<table>
<thead>
<tr>
<th>gene transcripts</th>
<th>upstream/downstream</th>
<th>primer sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>sense</td>
<td>downstream*</td>
<td>5'-GATCTAGAATAGAACCTAACCACAGTTGCT-3'</td>
</tr>
<tr>
<td>rabi</td>
<td>upstream</td>
<td>41-mer (same as above)</td>
</tr>
<tr>
<td>antisense</td>
<td>downstream*</td>
<td>5'-AAGCCATCAGGGGATTCAGAAT-3'</td>
</tr>
<tr>
<td>sense</td>
<td>downstream*</td>
<td>5'-TCCCTGCTAAAAACAGGAATCAATAA-3'</td>
</tr>
<tr>
<td>vrab7</td>
<td>upstream</td>
<td>5'-GGAAATCTGCTGTTTCTGAGAA-3'</td>
</tr>
<tr>
<td>antisense</td>
<td>downstream*</td>
<td>upstream primer for vrab7 sense transcript</td>
</tr>
</tbody>
</table>

*primer used in the reverse transcription reaction.

RNA-PCR was performed according to the manufacturer's instructions using the GeneAmp RNA-PCR Kit (Perkin Elmer Cetus, Norwalk, CT). The PCR cycles were 94°C for 0.5 min, 55°C for 1 min, 72°C for 1 min and the final cycle had a 10 min extension at 72°C. Twenty cycles of amplification were carried out. PCR products were resolved on a 1.2% agarose gel and confirmed by Southern hybridization with appropriate probes.

2.3.8 Acetylene reduction assay

Plants were placed in a 500 ml canning jar with a lid. Fifty ml of acetylene was added to the jar from which 50 ml of air had been removed. Sampling was
performed at 10, 20 and 30 min after the injection of acetylene using a vacutainer (4 ml). One ml of gas from the vacutainer was analyzed using a Varian model 3700 gas chromatograph. The amount of ethylene produced was converted from nmoles per ml to nmoles per plant or to nmoles per fresh weight (gFW) nodules.

2.3.9 Electron Microscopy

Mature nodules were processed for electron microscopy according to Newcomb et al. (1985). In brief, nodules were fixed in 3% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) and postfixed in 1% OsO$_4$. Fixed samples were dehydrated in a graded acetone series and then gradually embedded in Spurr's resin. Sections were cut on a Sorvall MT-2 Ultramicrotome, stained with 2% uranyl acetate and lead citrate, and examined with a Philips 300 transmission electron microscope.

2.4 Results

2.4.1 Isolation of cDNAs encoding plant homologs of Rab1p and Rab7p from soybean and Vigna

We isolated several cDNAs encoding small GTP-binding proteins from nodule cDNA libraries of soybean and Vigna aconitifolia using PCR. The soybean cDNA library was constructed in λ Zap vector so that a pool of plasmids (pBluescript SK phagemids) could be obtained through a mass excision process (Short et al., 1988). Also, the pBluescript SK plasmid possesses M13 reverse and -20 primer sequences, so that a single oligonucleotide specific to a gene of interest can be used in combination with either of the sequencing primers for PCR.
Accordingly, a degenerate oligonucleotide (41-mer; see Materials and Methods) was made based on a GTP-binding domain consensus sequence of the Ras superfamily, including yeast Ypt1p and its maize homolog, YPTm1 (Palme et al., 1992). One of the PCR fragments was found to be homologous to rab1 (srabl) as confirmed by cloning and sequencing. This fragment was used to isolate full-length clones from the soybean and Vigna nodule cDNA libraries.

Two kinds of clones were isolated: srabl and srab7 from soybean and vrab7 from a Vigna cDNA library. The deduced amino acid sequence of srabl showed extensive identity (74%) to Rab1p (Fig. 4A) and is designated as sRab1p. The homology not only included all five sequence motifs (G1-G5) conserved in the GTPase superfamily (Bourne et al., 1991), but also Cys-Cys residues at the carboxyl end, known to be a site of post-translational modification for membrane attachment of this group of proteins (Molenaar et al., 1988). Similarly, sequences encoded by srab7 and vrab7 (sRab7p and vRab7p) showed high identity (65%) to Rab7p (Fig. 4B) and are more similar to each other as compared to mammalian Rab7p. These sequences also have all the conserved domains of the GTPase superfamily and two cysteine residues near their carboxy termini. These data suggest that sRab1p, sRab7p and vRab7p may have analogous functions in plants compared to their homologs in yeast and mammalian systems.

2.4.2 Complementation of a yeast ypt1 mutant with sRab1p

GTP-binding proteins perform diverse functions in the cell (Chavrier et al., 1990; Gruenberg and Clague, 1992). In order to confirm that sRab1p is an authentic Ypt1p (Rab1p) homolog (Haubruck et al., 1989), the srabl cDNA was
Figure 4. Amino acid comparison of sRab1p with Rab1p (A), and amino acid comparison of sRab7p and vRab7p with Rab7p (B). Identical amino acids are shown as dashes (-). Consensus sequences in the GTPase superfamily are boxed and numbered according to Bourne et al. (1991). The nucleotide sequences have been submitted to the EMBL and GenBank Databases with the accession numbers L14929 (srabl), L14930 (srab7), and L14928 (vrab7).
inserted downstream from the \textit{GAL1} promoter in pYEUra3. A yeast \textit{yptl} mutant (Segev and Botstein, 1987) was transformed with the resulting vector (pSR1). The complemented colonies growing on uracil-deficient medium were further selected by growth on YP-Gal medium at 14\degree C, as this \textit{yptl} mutant is cold-sensitive. A cosegregation test was used to check if the ability to grow at 14\degree C was conferred by the presence of pSR1 (Rose and Broach, 1991). After 24 h growth, replica plates were made on two different media, which selected the plasmid marker and the mutant phenotype, respectively. The transformants with pSR1 were found to cosegregate both phenotypes, indicating that the \textit{srab1} sequence could rescue the yeast \textit{yptl} mutant as shown in Fig. 5, and confirming that sRab1p is functionally equivalent to Ypt1p and Rab1p. Since sRab7p and vRab7p were very similar to Rab7p, we did not attempt to carry out any functional test of these sequences and they were presumed to be homologs of Rab7p (see below).

\textbf{2.4.3 Induction of \textit{rabl} and \textit{rab7} genes during nodulation}

In order to determine whether plant Rab1p and Rab7p are involved in membrane biogenesis, particularly proliferation of membranes in nodules, RNAs from leaves and roots as well as nodules of different developmental stages were used for northern blot analysis. As shown in Fig. 6, \textit{srab1} is expressed at almost the same level in most soybean tissues, including young nodules. However, its level is enhanced as the nodule enlarges, with mature nodules having about 3 times more transcript than root meristem and young nodules, and 5 times more transcript than leaves. \textit{Vigna} nodule RNAs from different stages of development (Fig. 7, lanes 1-3) contained about 10 times more \textit{vrab7} transcript than root meristem.
Figure 5. Functional complementation of *Saccharomyces cerevisiae* yptl-1 mutant by soybean homolog of Rab1p. *srabl* was cloned downstream of the *GAL1* promoter in pYEUra3 (Clontech). Yeast mutant was transformed with the resulting vector (pSR1), and selected at the nonpermissive temperature, 14°C. A cosegregation test of the transformants was performed to ensure that they are complementing colonies. The media and temperature used are (A) uracil-deficient minimal plate at 28°C, (B) YPD plate at 14°C. 1, *yptl*-1 cells only; 2, *yptl*-1 with pYEUra3; 3, *yptl*-1 with pRB320 (a vector containing yeast *YPT1* gene); 4, *yptl*-1 with pSR1.
Figure 6. Northern blot analysis of *srab1* gene expression. Total soybean RNAs from leaf (1), root elongation zone (2), root meristem (3), 13 day-old nodules (4), and 21-day old nodules (5) were resolved on an agarose gel and hybridized with *srab1*. The transcript levels were quantified using a Phospho-Imager (Molecular Dynamics) and normalized based on 28S rRNA hybridization with soybean genomic rDNA.
Figure 7. Northern blot analysis of vrab7. Total RNA's from 13 day-old nodules (1), 17 day-old nodules (2), 21 day-old nodules (3), root meristem (4), and leaf (5) of Vigna aconitifolia were used for Northern analysis.
Thirteen-day old nodules have the highest level of \textit{vrab7} transcript, representing a stage when release and proliferation of bacteria in the plant cell cytoplasm are most active (Robertson and Lyttleton, 1982). These results suggest that Rab1p and Rab7p may be important in membrane proliferation during endocytosis of bacteria. To test whether nodule development is coordinated with the endocytic event, we performed the following series of experiments.

\textbf{2.4.4 Development of transgenic soybean nodules expressing antisense-\textit{srab1} and antisense-\textit{vrab7} transcripts}

Antisense-mediated inhibition of gene expression has proven to be extremely powerful in obtaining dominant mutations in higher eukaryotes which are recalcitrant to the application of classical genetics, and we have demonstrated that this approach works in root nodules (Lee \textit{et al.}, 1993). We used this strategy to examine the roles of Rab1p and Rab7p homologs in peribacteroid membrane biogenesis during nodule development.

To permit normal expression of Rab1p and Rab7p in transgenic roots while reducing the expression of these proteins in transgenic nodules, we used a nodule-specific promoter. The \textit{srab1} and \textit{vrab7} cDNA’s were inserted in antisense orientation downstream of the leghemoglobin (Lbc3) promoter. This promoter is highly active following the release of bacteria in host cells (Jensen \textit{et al.}, 1986) and is expressed primarily in infected cells (Nguyen \textit{et al.}, 1985). Because \textit{vrab7} and \textit{srab7} sequences were very similar even in the 5’ untranslated region, we used \textit{vrab7} due to convenience in cloning. Hairy roots were induced on soybean by \textit{Agrobacterium rhizogenes} (K599) transformed with pBI121 containing the
antisense constructs or with the vector only (used as control; Fig. 8). These constructs contained, in addition to antisense-srabl or -vrab7, the CaMV 35S promoter driving expression of the GUS reporter gene. The latter was used as a reporter to select transgenic hairy roots. The main root as well as all the hairy roots were excised except for one or two GUS-positive (transgenic) hairy root(s) (Fig. 10A). For some of the antisense-srabl experiments, one GUS-negative (wild type) hairy root was also kept together with one GUS-positive hairy root (Fig. 10B), because the wild type hairy root may rescue the plant in case antisense-srabl is too deleterious. Roots were inoculated with Bradyrhizobium japonicum (61A76) and nodules were allowed to form following transplantation of the infected plants in nitrogen-free medium.

Nodules formed on individual transgenic soybean roots were harvested and used to detect the presence of antisense-srabl and -vrab7 transcripts. Due to limiting amounts of transgenic nodule tissue, the presence of antisense-srabl and -vrab7 transcripts was observed using PCR as described (Lee et al., 1993). Primers used in RNA-PCR are shown in Materials and Methods. Fig. 9 shows that both srabl and vrab7 antisense transcripts were expressed in transgenic soybean nodules although the exact levels of the transcripts could not be determined in this assay.

2.4.5 Nodules expressing antisense-srabl were retarded in early development

About ten days after excision of main roots, both control plants and plants bearing nodules that expressed antisense-srabl began to show nitrogen deficiency
Figure 8. Schematic representation of constructs used for producing transgenic nodules. Antisense cassettes of *srab1* (B) and *vrab7* (C), and a sense cassette of *vrab7* (D) were cloned at the EcoRI site of the binary vector pBI121 (A). β-glucuronidase (GUS) was used as a reporter for transformation. Roots transformed with vector alone were used as control. P-35S, 35S promoter of cauliflower mosaic virus (CaMV); NOS3', 3'-flanking region of nopaline synthase gene; P-Lbc3, promoter of a leghemoglobin gene; H, *Hind* III; R, *Eco*RI.
Figure 9. Expression of antisense-\textit{srabl} (A), and antisense-\textit{vrab7} (B) in transgenic nodules. RNA-PCR was performed using total RNA extracted from transgenic nodules. Primers used in each reaction are shown in Materials and Methods. The PCR products in lane 3 of (A) and lane 1 of (B) show the sense transcript and lanes 4 and 5 of (A) and lanes 2 and 3 of (B) show the antisense transcript. (A) lanes 1 and 2, no-primer and no-RNA controls; lanes 3 and 4, RNA from pBI121 nodules; lanes 5, RNA from antisense \textit{srabl} nodules. (B) lanes 1 and 2, RNA from pBI121 nodules; lane 3, RNA from antisense-\textit{vrab7} nodules.
Figure 10. The phenotypes of nodules containing the antisense-srab1 construct. (A) Plant on the left has nodules on hairy roots formed with pBI121 (used as control), and the plant on the right with antisense-srab1 construct. (B) The hairy root tagged with a yellow tape is GUS-positive (transgenic) and the other hairy root is GUS-negative (wild type; used as control).
(yellowish leaves). As development proceeded, the leaves on control plants recovered and became green, indicating that nitrogen fixation commenced. However, leaves on antisense-\textit{sra} \textit{b} \textit{l} plants turned more yellow in color. At 3.5 weeks post-inoculation, nodules on each plant were examined morphologically, anatomically and functionally. Nodule growth was severely inhibited as a result of antisense-\textit{sra} \textit{b} \textit{l} expression with concomitant reduction in nitrogen fixation (Table 1). Nodules formed on antisense-\textit{sra} \textit{b} \textit{l} hairy roots (Fig. 10A) were 5 to 10-fold reduced in weight relative to controls (nodules on pBI121 hairy roots) (Table 2). Also, in the group of plants having one transgenic and one wild type (control) hairy root on the same plant (Fig. 10B), nodules on control hairy roots were 3 to 5 times heavier than nodules on antisense-\textit{sra} \textit{b} \textit{l} hairy roots (Table 2). In addition, nodules on wild type hairy roots on the same plant (Fig. 10B) were smaller than nodules on pBI121-containing hairy roots on independent plants (Fig. 10A), perhaps as a result of systemic control on the size of nodules (see Rolfe and Gresshoff, 1988).

Plants with antisense-\textit{sra} \textit{b} \textit{l} nodules showed 5-fold less nitrogenase activity than controls (Table 1), which correlates with differences in nodule weight (Table 2). When the enzyme activity of nodules was calculated per g fresh weight (FW), the difference was only about 2 fold, as the antisense-\textit{sra} \textit{b} \textit{l} nodules were very small in size and the ratio of symbiotic tissue in control and transgenic nodules is not the same. These results suggest that antisense-\textit{sra} \textit{b} \textit{l} nodules were arrested at an early stage of development (see Discussion).
<table>
<thead>
<tr>
<th>Plants</th>
<th>Ethylene produced per plant*</th>
<th>Ethylene produced per gFW nodules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>64 ± 12</td>
<td>147 ± 27</td>
</tr>
<tr>
<td>Antisense-\textit{srrbl}</td>
<td>13 ± 7</td>
<td>86 ± 16</td>
</tr>
</tbody>
</table>

*Four plants containing transgenic nodules were used for assay.
Table 2. Effect of *srab1* antisense expression on nodule weight

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Tissue type</th>
<th>Plant #</th>
<th>Nodule weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ac 4</td>
<td>4.0 ± 0.5c</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ac 6</td>
<td>4.9 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>Ac 7</td>
<td>5.3 ± 0.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ac 9</td>
<td>5.4 ± 0.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ac13</td>
<td>5.4 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>Aa</td>
<td>Ay 1</td>
<td>0.5 ± 0.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ay 3</td>
<td>0.6 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Antisense</td>
<td>Ay 6</td>
<td>0.4 ± 0.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ay 8</td>
<td>0.9 ± 0.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ay12</td>
<td>0.7 ± 0.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Be 3</td>
<td>2.8 ± 0.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Be 4</td>
<td>4.0 ± 1.9</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>Be 5</td>
<td>3.9 ± 0.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Be 9</td>
<td>3.1 ± 1.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Be12</td>
<td>3.3 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>Bb</td>
<td>By 3</td>
<td>0.4 ± 0.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>By 4</td>
<td>0.6 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Antisense</td>
<td>By 5</td>
<td>0.7 ± 0.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>By 9</td>
<td>0.3 ± 0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>By12</td>
<td>1.0 ± 0.2</td>
<td></td>
</tr>
</tbody>
</table>

a Plants which had only one GUS-positive hairy root.
b Plants which had a GUS-positive and a GUS-negative hairy root.
c The weights of 5 individual nodules from each plant were measured and shown as mean ± SE.
2.4.6 The possible involvement of *srabbit* in membrane proliferation and the release of bacteroids into the vacuole in antisense-*srabl* nodules

Retardation of nodule development in antisense-*srabl* nodules suggested that as the nodule enlarged (around 10 days), the expression of antisense-*srabl* under the control of *Lbc3* promoter arrested further development of this tissue. Light and electron microscopic examination of the nodules expressing antisense-*srabl* provided further insights regarding this phenomenon.

As nodule development proceeds, the volume of bacteroid-containing cells normally increases to house the increasing number of bacteroids (up to 20,000 per cell; Bergersen, 1982). Infected cells of nodules expressing antisense-*srabl* showed several abnormal phenotypes. Their size appeared almost the same as that of interstitial cells, whereas in control nodules, infected cells were several fold larger than uninfected cells (Fig. 11A and B). In addition, infected cells of antisense-*srabl* nodules had fewer bacteria than those of controls as judged by toluidine blue staining. Normally, the central vacuole in infected cells of soybean nodules disappears upon endocytosis of bacteria, but most infected cells in antisense-*srabl* nodules showed persistence of the central vacuole (Fig. 11B). This defect may result from inhibition of the general vesicular transport from the ER. It was also observed that cells surrounding the central tissue had more starch granules in antisense-*srabl* nodules than did controls, a phenomenon characteristic of ineffective or inefficient nodules (see Stanley *et al.*, 1986; Forrest *et al.*, 1990).

Figure 12 shows electron micrographs of the transgenic nodules expressing antisense-*srabl*. Infected cells in these nodules contained fewer bacteroids and accumulated unfused vesicles and small vacuoles (Fig. 12C).
Figure 11. Effects of antisense expression of *srab1* and *vrab7* on nodule structure. Light micrographs of nodules containing (A) pBI121 (control), (B) antisense construct of *srab1*, (C) antisense construct of *vrab7*, and (D) sense construct of *vrab7*. i, infected cell; u, uninfected cell; v, vascular bundle.
Figure 12. Ultrastructure of transgenic nodules expressing srab1 antisense RNA. Electron micrographs of nodules of (A) control, (B and C) infected cells of srab1-antisense nodules, and (D) uninfected cells of srab1-antisense nodules. n, nucleus; b, bacteroid; v, vacuole; s, starch granule; G, the Golgi; arrowhead, PBM; cv, central vacuole; rb, released bacteroid; p, peroxisome; m, mitochondria. Thick arrows indicate where PBM is broken and the thin arrow points out released bacteroids in the central vacuole. Bar in panel B corresponds to 2.0 μm and bar in panel C to 1.4 μm. Panel A and B are of the same magnification while panel C and D are the same.
Infected cells contained a central vacuole, and some bacteroids were liberated into the vacuole (Fig. 12B and 12C), apparently by fusion of the PBM compartment with the vacuolar membrane. However, uninfected cells showed intact membrane structures (peroxisomes, central vacuoles, and amyloplasts), confirming that the abnormalities in infected cells were due to antisense-\textit{sra}W expression under the control of the Lbc3 promoter (Fig. 12D). The \textit{Lb} gene is not expressed in uninfected cells of nodules (Nguyen \textit{et al.}, 1985). It seems that the infection process in nodules expressing antisense-\textit{sra}W occurred normally, but steps leading to continuous proliferation of PBM compartment (expansion of PBM and transport of PBM nodulins) may have been impaired.

\textbf{2.4.7 Nodules expressing antisense-\textit{vra}b7 revealed large numbers of small unfused vesicles and multivesicular bodies}

In mammalian cells, Rab7p was localized on the late endosomes, which are transitional structures from early endosomes to lysosomes (Gruenberg and Howell, 1989; Chavrier \textit{et al.}, 1990). Accordingly, we hypothesized that vRab7p may play a role in the process that allows the newly formed PBM compartment to become a symbiotic organelle (symbiosome). In antisense-\textit{vra}b7 nodules, the number of bacteroids was reduced in the infected cells and both cortical and interstitial cells contained amyloplasts (Fig. 11C). However, in nodules expressing sense-\textit{vra}b7 and control nodules, the infected cells were densely packed with bacteroids (Fig. 11D). The size of the infected cells in antisense-\textit{vra}b7 nodules was not significantly affected and the central vacuole was absent.

Many small unfused vesicles and some large multivesicular bodies (MVB) were observed around the nuclear region of the infected cells in these nodules.
Most of these vesicles contained some electron dense material which appeared to be degraded products of bacteroids and membranes (arrows in Fig. 13). The structure of nodules formed on sense-vrab7 roots was identical to that from control nodules (data not shown). Hence, Fig. 13 may be compared with Fig. 12A. Similar to antisense-srab1, the uninfected cells in antisense-srab7 nodules showed normal organellar structures (data not shown), indicating that the abnormalities observed in Fig. 13 are due to antisense-vrab7 expression. Taken together, expression of antisense-vrab7 appears to have affected maturation of the PBM, resulting in a smaller number of PBM compartments and accumulation of late endosome structures in infected cells.

2.5 Discussion

We have shown that biogenesis of the PBM compartment in soybean nodules involves homologs of the small GTP-binding proteins, Rab1p and Rab7p. Reducing the expression of these proteins has pleiotropic effects on nodule development, affecting the size of the nodules, compartmentalization of bacteria, and the efficiency of root nodules in fixing nitrogen. While the level of srab1 transcript is only 3 fold higher, the level of vrab7 is almost 12 times higher in root nodules than in root and shoot meristematic tissues. This suggests an important role of Rab7p in the formation of the PBM in root nodules. The integrity of this membrane is vital for the symbiotic interaction between the host plant and Rhizobium.
Figure 13. Ultrastructure of transgenic nodules expressing \textit{vrab7} antisense RNA. n, nucleus; b, bacteroid; mv, multivesicular body; le, late endosome. Thick arrows indicate bacteroids inside enlarged late endosomes/vacuoles which are apparently degraded. Bar corresponds to 2.0 \textmu m.
2.5.1 Nodule development and induction of small GTP-binding proteins

Root nodule organogenesis involves new meristematic activity in the root cortex that gives rise to a group of cells which are infected by *Rhizobium* (see Verma, 1992). While normal meristem is refractive to bacterial infections, the newly divided cells formed as a result of *Rhizobium* interaction are receptive to invasion by these bacteria. In determinate nodules, such as those formed on soybean and *Vigna*, cell division ceases after the release of bacteria in the infected cell and development of the nodule takes place largely by cell enlargement, however, cell division has been observed in infected cells of some determinate nodules (see Brewin, 1991).

The level of expression of *srabl* increases about 3 fold in 21 day nodules suggesting a possible involvement of Rab1p in membrane proliferation. In yeast and mammalian systems, several small GTP binding proteins have been demonstrated to be involved in membrane trafficking. Rab1p has been localized on *cis* Golgi (Segev *et al.*, 1988) and Rab7p on late endosomes (Chavrier *et al.*, 1990). A null mutation in yeast *YPT1* (*rab1*) blocks transport and modification of secretory proteins and accumulates unfused vesicles (Segev *et al.*, 1988). Overexpression of a mutant Rab1p in mammalian cells created *trans*-dominant inhibition of transport of vesicular stomatitis virus glycoprotein to plasma membrane (Tisdale *et al.*, 1992). Reducing the expression of *rab1* in soybean nodules by antisense-*srabl* demonstrated that endocytosis of *Rhizobium* proceeds normally but proliferation of the membrane system is inhibited, with a loss of integrity of the peribacteroid membrane that is initially formed. As a result, many bacteroids were found to be released in the vacuoles. Moreover, most infected
cells retained their central vacuole, which normally disappears concomitantly with the release of bacteria from the infection thread (see Morrison and Verma, 1987).

Antisense-srabi nodules were very small in size, having reductions in both infected and uninfected cell volumes. This suggests that the development of nodules is arrested at an early stage due to reduction in Rablp expression because the accumulation of antisense rab1 under Lbc3 promoter control is expected to begin 5-6 days after infection (Fuller and Verma, 1984; Jensen et al., 1986). Nitrogen fixation ability of these nodules was severely impaired.

In mammalian cells Rab7p was localized to late endosomes, a transitional organelle in the endocytic pathway (Gruenberg and Howell, 1989; Chavrier et al., 1990). In contrast to rabl, a high level expression of rab7 was observed in early nodules and this level was maintained in mature nodules (Fig. 7). The difference in the expression of rab7 between nodule and root meristem is consistent with the role of this protein in the late endocytic pathway. Enhancement of vrab7 expression during nodule development correlated with the proliferation of bacteria in infected cells. Since determinate mature nodules have no meristematic activity (Brewin, 1991), persistent expression of rab7 in mature nodules was quite unexpected. This suggests that high level expression of rab7 is not due to meristematic activity in nodules. This protein may function in post-endocytosis step(s), e.g. maturation of the PBM compartment and its presence may be required to maintain constant turnover of the PBM in mature nodules.

2.5.2 Biogenesis, proliferation and the nature of PBM compartment

The PBM compartment is formed by endocytosis of Rhizobium and although it is derived from the plasma membrane, it eventually becomes an
independent organelle. Accordingly, a number of nodulins, including nodulin-24 and nodulin-26, that are targeted to this compartment may perform specific functions. A Tn5 mutant (T8-1) of *B. japonicum* (61A76) developed normal size of nodules but failed in endocytosis and hence no PBM compartment was formed (Morrison and Verma, 1987). This suggests that the enlargement of infected cells and bacterial proliferation are two independent phenomena.

Extensive membrane proliferation in nodules requires ER to Golgi transport, in which Rab1p has been suggested to be involved (Haubruck *et al.*, 1989). Many PBM nodulins are synthesized in the ER, targeted to the PBM, and believed to be essential for PBM biogenesis (Cheon and Verma, unpublished data; Miao *et al.*, 1992). A lack of supply of new PBM vesicles may result in premature degradation of this subcellular compartment essential for symbiosis. In nodules expressing antisense-*srabl*, fusion of PBM with vacuolar membrane or disruption of PBM was observed. Although the infected cells in these nodules contained some bacteroids liberated into large vacuoles, they still showed many intact PBM compartments. Apparently, as the concentration of antisense-*srabl* transcripts is increased under the control of the Lbc3 promoter, the block in sRab1p function may inhibit transport of some nodulins essential for maturation of the PBM compartment.

The period of copious membrane synthesis in nodules correlates with the induction of Rab7p, suggesting the possible involvement of this GTP-binding protein in PBM biogenesis. Antisense inhibition of vrab7 impaired biogenesis of the PBM compartment. The release of *Rhizobium* from the infection thread (endocytosis) appears to have occurred normally in these nodules but subsequent maturation of the PBM compartment was affected. In the case of the yeast Rab7p
homolog (Ypt7p), a null mutation caused defects in vacuolar protein transport as well as a factor degradation (Wichmann *et al.*, 1992). These authors suggested that Ypt7p is involved in transport of membrane-bound vacuolar proteins in yeast. The PBM compartment has properties common to vacuoles; *e.g.* peribacteroid fluid contains many host derived vacuolar proteins including α-mannosidase and protease inhibitor (Mellor, 1989; Manen *et al.*, 1991; Kinnback *et al.*, 1987). An alteration in the PBM may turn it into a "lysosomal" compartment where bacteria would be killed and degraded. In fact, many vesicles in antisense-vrab7 nodules are found to contain degraded bacteria (Fig. 13). These data suggest that one of the major steps in developing symbiosis is to establish a suitable environment inside the host cell where *Rhizobium* is not recognized as a pathogen.

Considering the extensive membrane trafficking that occurs in infected cells of root nodules and the importance of proper membrane biogenesis in symbiotic interactions, the basic machinery involved in transport of vesicles to various subcellular compartments needs to be studied including the role of specific proteins (*e.g.* heterotrimeric G-proteins, dynamin and small GTP-binding proteins) in the formation of the PBM.
CHAPTER III

NODULIN-24, A PERIBACTEROID MEMBRANE PROTEIN IN SOYBEAN ROOT NODULE, IS SYNTHESIZED AS A LUMENAL PROTEIN AND POST-TRANSLATIONALLY ATTACHED TO THE MEMBRANE

3.1 Abstract

Nodulin-24 is a nodule-specific protein of the peribacteroid membrane (PBM) in soybean. It has an apparent molecular mass of 33 kD while its full-length cDNA encodes a polypeptide of only 24 kD. In vitro transcription of nodulin-24 cDNA followed by translation resulted in a peptide translocated into microsomal membranes with cleavage of a signal sequence. The cleavage site of the signal sequence in nodulin-24 was determined to be between Ala (A25) and Arg (R26) by radiosequencing of the [3H]leucine-labeled processed peptide. Fusion of the signal sequence of nodulin-24 with the β-glucuronidase (GUS) gene prevented co-translational cleavage of the signal sequence although translocation of the fused protein into microsomes occurred co-translationally. Membrane-translocated nodulin-24 did not show any alteration in size after trypsin treatment. A GUS fusion with the entire coding region of the nodulin-24 also showed no
change in size in trypsin protection assay, suggesting that nodulin-24 has no membrane-spanning region. In addition, \textit{in vitro} synthesized nodulin-24 was present in the supernatant fraction after sonication of microsomal membranes. Mature nodulin-24, however, was not solubilized from PBM by sodium carbonate (pH 11) or EGTA, and was soluble only in detergent. These data suggest that nodulin-24 is synthesized as a lumenal protein in the ER and post-translationally attached to the membranes \textit{en route} to the PBM. This processing results in a significant increase in the apparent molecular mass of nodulin-24, which may be due to the attachment of membrane lipids. This protein shares characteristics with membrane lipoproteins of many pathogenic bacteria.

### 3.2 Introduction

Successful invasion of legume roots by rhizobia, followed by endocytosis of the bacteria into the host cytoplasm, results in the development of effective root nodules (Verma, 1992; Brewin, 1990). Rhizobia are segregated inside the host cell into a subcellular compartment surrounded by a membrane, the peribacteroid membrane (PBM), of host origin (Verma \textit{et al.}, 1978). The PBM plays critical roles in symbiosis as all metabolic exchanges between the two partners occur through this membrane (Verma and Fortin, 1989). Moreover, compartmentalization of bacteria by the PBM facilitates control of the host defense mechanism and ensures the effectiveness of symbiosis.

Many nodule-specific host proteins (nodulins) have been isolated (Delauney and Verma, 1988) and some of them, \textit{e.g.} nodulin-24 and nodulin-26, have been localized to the PBM. Nodulin-26 is an intrinsic membrane protein and
lacks a cleavable signal sequence (Miao et al., 1992). The topology of this protein suggests that both the amino- and carboxy-ends face the host cell cytoplasm (Miao et al., 1992). On the other hand, nodulin-24 may be located on the surface facing the bacteroids. The nodulin-24 gene contains five exons, three of which (exons 2 to 4) encode a repeated amphipathic domain (Fortin et al., 1985; Katinakis and Verma, 1985). In vitro translation of nodulin-24 mRNA in the presence of microsomal membranes suggested that nodulin-24 is processed co-translationally into a 20 kD polypeptide (Katinakis and Verma, 1985). However, when PBM proteins were reacted with antibody against nodulin-24, the size of native nodulin-24 was found to be about 33 kD, suggesting that this nodulin undergoes a significant post-translational modification (Fortin et al., 1985). Nodulin-24 has homologies to nodulin-16 and a few glycine-rich plant proteins in signal peptide and C-terminal regions (Nirunsuksiri and Sengupta-Gopalan, 1990; Sandal et al., 1992).

The nature of the PBM is unique as it possesses properties common to both plasma membrane and tonoplast (Verma et al., 1978; Miao et al., 1992). The biogenesis of the PBM compartment requires extensive vesicular transport (Cheon et al., 1993) and specific targeting of PBM and peribacteroid fluid (PBF) nodulins. It is not known how PBM nodulins are specifically targeted to this de novo formed subcellular compartment. All PBM nodulins are synthesized on membrane-bound polysomes (Jacob et al., 1987) and co-translationally inserted into the membrane irrespective of whether they carry an amino-terminal signal sequence. Co-translationally cleavable signal sequences of proteins contain a positively charged region and a hydrophobic core (von Heijne, 1983, 1986) but the downstream domain flanking the cleavage site is not well characterized.
In this study, we examined the synthesis, processing and topology of nodulin-24 using *in vitro* translated nodulin-24 peptides and isolated PBM. In addition, the cleavage site of the signal sequence in nodulin-24 was determined by radiosequencing of \[^{3}H\]leucine-labeled *in vitro* translation product. The requirements for signal peptide cleavage were tested by protease protection assay of β-glucuronidase (GUS) fusion proteins. The results suggest that nodulin-24 is synthesized as a lumenal protein in the ER and is post-translationally attached to the PBM. Its overall hydrophobic character and the presence of a characteristic lipid-binding domain may allow this nodulin to be buried in the lipid of the PBM surface facing the bacteroids.

3.3 Materials and Methods

3.3.1 Materials

Soybean (*Glycine max* L. cv. Prize) was inoculated with *Bradyrhizobium japonicum* strain 61A76 and grown in vermiculite for three weeks as described (Cheon *et al.*, 1993) before harvesting root nodules. *In vitro* transcription and translation systems were from Promega Corp. (Madison, WI). \[^{3}H\]leucine (142 Ci/mmol) and \[^{35}S\]methionine (1000 Ci/mmol) were from Amersham Corp. (Arlington Heights, IL).

3.3.2 Plasmid construction

For *in vitro* transcription, a series of fusion constructs of nodulin-24 and GUS were made as follows: A HindIII-BamHI restriction fragment from pN24, a pUC19 derivative containing the nodulin-24 cDNA insert from pNod20 (Katinakis
and Verma, 1985), was cloned downstream of the bacteriophage T7 promoter in pGEM2 (Promega Corp., Madison, WI) and used for in vitro transcription.

Constructs T1 and T2 were made by cloning exon 1, and exons 1-2, respectively of the nodulin-24 gene into XbaI and SmaI-cut pBI221. The exon 1 fragment was obtained by digestion of pN24 with XbaI and Dral, followed by elution of a 0.1 kb-band from an agarose gel, while the fragment containing exons 1-2 was obtained by digestion of pN24 with PstI and Mungbean nuclease followed by digestion with XbaI. The entire length of the nodulin-24 cDNA, except the termination codon, was amplified by PCR and fused with the GUS gene, resulting in the formation of construct T3. All the above constructs were subcloned into pGEM2 for in vitro transcription with T7 RNA polymerase.

3.3.3 In vitro transcription

Plasmids were linearized by cutting with appropriate restriction enzymes downstream of the gene of interest and transcribed with T7 RNA polymerase according to the manufacturer’s instructions. The integrity of all RNA transcripts was confirmed by electrophoresis in 5% acrylamide/7M urea gels before further use.

3.3.4 In vitro translation and processing of the products

In vitro synthesized transcripts were translated in a rabbit reticulocyte lysate using either [35S]methionine or [3H]leucine and canine microsomal membranes (Promega Corp., Madison, WI). The protease protection assay was carried out as described (Spiess and Lodish, 1986) and the labeled peptides were immunoprecipitated (Anderson and Blobel, 1983) with nodulin-24 or GUS
antibody. After in vitro translation, the reaction mixture was centrifuged at 356,000 x g (Beckman TL-100) for 20 min at 4°C. The pellet was dissolved in STBS buffer (0.25M sucrose, 10 mM Tris-HCl, 150 mM NaCl). Protease digestion with either TPCK-treated trypsin or proteinase-K (final concentration of 100 µg/ml) was carried out at 4°C for 1 h in the absence or presence of Triton X-100 (TX-100) at a final concentration of 1%. Concanavalin-A (Con-A) binding was performed with Con-A Sepharose 4B (Pharmacia LKB-Biotech, Piscataway, NJ) following immunoprecipitation protocol and replacing protein-A with Con-A Sepharose. In vitro translated products were subjected to ten five-second pulses of sonication (Sonifier 450, Branson Sonic Power Co., Danbury, CT) at maximum output and then centrifuged at 356,000 x g for 30 min at 4°C. Samples were resolved in 10% or 14% SDS-polyacrylamide gels. Following electrophoresis, labeled products were analyzed by fluorography using EnHance (DuPont/NEN, Boston, MA).

### 3.3.5 Radiosequencing

The [³H]leucine-labeled product of nodulin-24, co-translationally processed in the presence of microsomal membranes, was resolved in a 10% SDS-polyacrylamide gel, eluted by soaking in 0.1% SDS, and subjected to Edman degradation on a protein sequenator (Model 470A/900A, Applied Biosystems Inc., Foster City, CA). The radioactivity of fractions released in each cycle was measured by scintillation counting.
3.3.6 Western blotting

PBM proteins were prepared as described (Fortin et al., 1985). The isolated PBM proteins were either extracted with 10 mM sodium carbonate (pH 11) and 10 mM EGTA, or mixed with different concentrations of proteinase-K and subjected to sonication followed by incubation on ice for 15 min. The proteinase-K digestion was terminated by 1 mM phenylmethylsulfonyl fluoride (PMSF) before adding SDS-sample buffer. Proteins were resolved on a 14% SDS-polyacrylamide gel, electroblotted to nitrocellulose membrane (Burnette, 1981), and detected using the ECL-Western blotting system (Amersham Corp., Arlington Heights, IL).

3.3.7 Sequence analysis

The amino acid sequence of nodulin-24 was analyzed using Motifs program (Bairoch, 1991) of the GCG (Genetics Computer Group, Inc., Madison, WI) package.

3.4 Results

3.4.1 Cleavage of signal sequence and topology of nodulin-24

Nodulin-24 has been shown to be co-translationally processed into a 20 kD polypeptide (Katinakis and Verma, 1985), but it is not known how it is associated with the membrane. We determined the topology of nodulin-24 in microsomal membranes. In vitro transcribed nodulin-24 transcripts were translated using rabbit reticulocyte lysate and [35S]methionine in the absence (Fig. 14, lane 1) or presence (Fig. 14, lane 2) of microsomal membranes, and immunoprecipitated
Figure 14. *In vitro* translation and processing of nodulin-24. Nodulin-24 cDNA was transcribed *in vitro* using bacteriophage T7 polymerase and the resulting transcript was translated *in vitro* using reticulocyte lysate and [*35S*]methionine in the absence or presence of microsomal membranes. The translation product was immunoprecipitated with antibody against a synthetic peptide corresponding to one of the repeated domains of nodulin-24. Translation was assayed by tryptic digestion and/or addition of TX-100. Mic, canine pancreatic microsomal membranes; Trp, trypsin; Tx, Triton X-100.
with polyclonal antibody against nodulin-24 (see Materials and Methods). As expected, nodulin-24 was processed into a 20 kD polypeptide in the presence of microsomal membranes. Tryptic digestion of the translation mixture did not change the size of the polypeptide (Fig. 14, lane 3), although trypsin together with a nonionic detergent digested the processed nodulin-24 completely (Fig. 14, lane 4). This suggests that no part of the processed nodulin-24 protrudes from the ER into the cytoplasm.

In order to unequivocally define the cleavage site of the signal sequence, the most probable cleavage site in nodulin-24 was first identified using a procedure described by von Heijne (von Heijne, 1986). Fig. 15A shows S(i) values at each potential cleavage site which were obtained by summing the weights of residues including positions −13 and +2 relative to each site. The highest S(i) value assigned the cleavage site between Ala (A25) and Arg (R26). This site also satisfies the criteria of the (−3, −1)−rule for cleavage (von Heijne, 1983; Perlman and Halvorson, 1983) with Val in the relative position −3 and Ala in the relative position −1. For experimental determination of the cleavage site, the [3H]leucine-labeled co-translationally processed product of nodulin-24 was subjected to Edman degradation. Radioactive peaks were found at the 3rd and the 21st cycles of sequential degradation which correspond to the positions of leucine residues if the cleavage occurred as predicted above. These data confirm the prediction of the cleavage site. Therefore, we conclude that nodulin-24 is co-translationally processed into a mature polypeptide of 122 residues with arginine at the amino terminus.
Figure 15. Determination of the cleavage site of nodulin-24 signal sequence. Panel A shows the predicted cleavage site of the signal sequence based on the method of von Heijne (1983). S-values are obtained from calculations at the indicated amino acid positions. The most probable cleavage site with the highest S-value is denoted by the broken line. Panel B is the radiosequencing profile of the in vitro translation product in the presence of microsomal membranes. The translation product of nodulin-24 using $[^{3}\text{H}]$leucine was resolved by SDS-PAGE. Processed nodulin-24 protein was eluted and subjected to Edman degradation. Radioactivity released at each cycle was determined. The arrows indicate the peaks of radioactivity corresponding to leucine residues present in the native sequence.
3.4.2 The amino terminal segment of processed nodulin-24 is required for signal sequence cleavage

The putative signal peptide of nodulin-24 identified above was tested for its role in targeting a protein to the ER. Different lengths of nodulin-24 cDNA were fused in-frame with the β-glucuronidase (GUS) gene (Fig. 16A). Construct T1 contains the exon-1 sequence encoding the putative signal peptide plus 5 additional residues; T2 contains, in addition to the T1 sequence, the exon-2 sequence encoding 18 residues (repetitive domains in nodulin-24), while T3 contains the whole nodulin-24 coding region except the termination codon. The in vitro translated product of GUS alone could not be translocated into microsomal membranes as expected (Fig. 16B, panel d) and served as a control. When T1 was transcribed and translated in vitro, cleavage of the signal sequence was not observed. Instead, the size of the band increased due to co-translational glycosylation of GUS (see Fig. 17; Iturriaga et al., 1989). The same results were obtained in the case of T2 which had 23 additional amino acids after the signal peptide. When a longer segment of nodulin-24 (containing exons-1, -2 and -3) was used, similar results were obtained (data not shown). Translocation of the fusion products occurred in all cases as detergent addition to the trypsin protection assay showed complete digestion of the products. However, cleavage did occur in the case of the T3 translation product. These results indicate that although the signal peptide is sufficient for translocation across microsomal membranes, the co-translational processing of nodulin-24 requires additional sequences flanking the cleavage site of signal peptide.
Figure 16. (A) Schematic outline of fusion constructs comprising different domains of nodulin-24 and GUS. Different lengths of nodulin-24 (open boxes) were fused in-frame with GUS (hatched boxes) to obtain constructs T1 to T3 (a-c). Ex, exon; GUS, β-glucuronidase gene. (B) In vitro co-translational translocations of the fusion proteins. In vitro transcription and translation were carried out in the same manner as in Fig. 1. Translation products were immunoprecipitated with antibody against GUS.
3.4.3 Nodulin-24 is a lumenal protein in the ER and is modified post-translationally to become membrane-associated

If nodulin-24 had any membrane-spanning region, construct T3 (Fig. 16A) should have a stop transfer domain which would be detectable in the protease protection assay. In that event, the entire GUS protein should be located outside the membrane vesicles and thus become accessible to trypsin. When trypsin was added to membrane fraction after in vitro translation of T3, no digestion of the fusion protein was observed (Fig. 16B). However, when microsomal membranes were solubilized by TX-100, the fusion protein was completely digested. Similar results were obtained using proteinase-K in place of trypsin (data not shown). These results suggest that nodulin-24 may be located inside the microsomal membranes. To test this possibility, we subjected microsomal membranes following in vitro translation to sonication and centrifugation, and separated the microvesicles from the supernatant (Fig. 17, lanes 3 and 4). Most of the nodulin-24 was found in the supernatant, indicating that nodulin-24 is not associated with the ER membranes. However, mature nodulin-24 isolated from root nodules is tightly attached to membrane (Fortin et al., 1985). Sodium carbonate (pH 11) (Fujiki et al., 1982) or EGTA treatment (van Renswoude and Kempf, 1984) of soybean PBM did not remove nodulin-24 from the membrane, while TX-100 solubilized it along with the PBM (Fig. 18). When PBM was sonicated and subsequently centrifuged, nodulin-24 was found in the pellet fraction (data not shown). Taken together, these data suggest that nodulin-24 is a lumenal protein in the ER, but becomes modified post-translationally and attached to the membrane during its passage to the PBM.
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Figure 17. Presence of nodulin-24 in the lumen of microsomes and its lack of glycosylation. After *in vitro* translation, reaction mixtures were centrifuged and the pellets were subjected to two different treatments. Half of the fractions were sonicated and then centrifuged to separate membranes (lane 4) and lumenal proteins (lane 3). The other half was processed for Con-A precipitation. Lane 5, Con-A-bound fraction; lane 6, Con-A-unbound fraction. Sonic, sonication; Con-A, Concanavalin-A; +s, the supernatant of sonicated sample; +p, the pellet of sonicated sample; b, bound fraction to Con-A; u, unbound fraction to Con-A.
Figure 18. Tight association of nodulin-24 with the PBM. Lane 1, PBM without any treatment; lane 2, PBM washed with sodium carbonate (pH 11); lane 3, PBM washed with EGTA. Each pellet from different treatments was resolved by SDS-PAGE, blotted, and probed with nodulin-24 antibody.
Figure 19. Proteinase-K digestion of PBM. Panel A is a SDS-PAGE of PBM after proteinase-K digestion and panel B is a western blot of the gel in the panel A reacted with nodulin-24 antibody. Nodulin-26 bands were also identified by western blotting (data not shown). Proteinase-K was added to the PBM fraction, and the mixture was sonicated and incubated as described in Materials and Methods. M, molecular markers in kD; lane 1, PBM; lane 2, PBM with 0.3 μg/ml of proteinase-K; lane 3, PBM with 3 μg/ml of proteinase-K. N24, nodulin-24; N26, nodulin-26; arrowhead, partially digested nodulin-26.
No co-translational glycosylation of nodulin-24 occurs as shown by the lack of binding with Con-A (Fig. 17, lanes 5 and 6). Treatment of intact or sonicated PBM proteins with proteinase-K did not alter the size of nodulin-24 while nodulin-26 was cleaved (Fig. 19). This suggests that nodulin-24 is buried in the lipid layer and is not accessible to protease digestion. Addition of TX-100 to the reaction mixture resulted in complete digestion of nodulin-24 (data not shown), confirming that the protein is masked by lipids. Furthermore, nodulin-24 was found to have a domain homologous to the lipid-attachment site of prokaryotic membrane lipoproteins at carboxyl-terminal region (Fig. 20; Bairoch, 1991; Sankaran and Wu, 1993). These data indicate that nodulin-24 may be post-translationally anchored to lipid, possibly through this lipid-attachment site, and become a membrane protein (see below) although no direct evidence is available for this phenomenon in eukaryotes.

3.5 Discussion

During nodule development, extensive membrane proliferation occurs in the infected cell to produce 30 times more PBM than plasma membrane. Although the PBM is derived from the plasma membrane, progressive changes in composition of the PBM have been observed (Verma et al., 1978; Fortin et al., 1985) including the acquisition of several new proteins such as nodulin-26 and nodulin-24. These changes are essential for an effective symbiosis as the expression of nodulin-26 and nodulin-24 is altered in ineffective nodules or nodules devoid of bacteria (Morrison and Verma, 1987). Unless significant changes occur in the PBM compartment, the endocytosed rhizobia would be
Figure 20. Presence of a lipid-binding domain in nodulin-24 analogous to bacterial lipoproteins. X's in the consensus sequence (from the relative position -9 to -4) denote the negation of amino acids D, E, R and K which is followed in nodulin-24. The underlined sequences denote matched amino acid residues between nodulin-24 and the lipid-binding consensus sequence (Bairoch, 1991).
degraded, which happens in certain incompatible associations. By retarding the vesicular transport of certain PBM proteins using the antisense expression of the \textit{rab7} gene, it was demonstrated that the late endosomes accumulated in the perinuclear region and these compartments became lytic, degrading endocytosed bacteria (Cheon \textit{et al.}, 1993).

We determined the topology of nodulin-24. Unlike nodulin-26, which does not have a cleavable signal sequence (Miao \textit{et al.}, 1992), nodulin-24 was shown to have a cleavable signal sequence indicating that these two PBM proteins are targeted to this newly formed compartment through different routes. The amino acid sequences of nodulin-23 (Mauro \textit{et al.}, 1985) and nodulin-24 were analyzed using a weight-matrix approach (von Heijne, 1986) and potential cleavage sites were identified between amino acid residues A20 and E21 for nodulin-23, and between A25 and R26 for nodulin-24. This suggests that nodulin-23 and nodulin-24 may be directed to the PBM by a similar sorting mechanism.

We addressed the question of how nodulin-24 becomes attached to the PBM and is increased in molecular mass from 20 kD to 33 kD (Fortin \textit{et al.}, 1985). Based on a hydropathy plot, the repeated domains at amino terminal region of processed nodulin-24 are highly amphipathic while the carboxyl-half of this protein is hydrophobic. No membrane-spanning region, which in most cases is a hydrophobic stretch of about 20 amino acid residues (Jennings, 1989), was observed in this protein (Katinakis and Verma, 1985). To test for the presence of a transmembrane domain using a protease protection assay (Garoff, 1985), various regions of the nodulin-24 gene were fused with the GUS reporter gene. When the entire cDNA of nodulin-24 was fused with the GUS gene and the membrane-
translocated product was treated with trypsin or proteinase-K (Fig. 16B (d)),
neither nodulin-24 nor GUS was digested by the protease. This result suggested
either that nodulin-24 is a peripheral membrane protein which has hydrophobic
interactions with membrane lipids or that it is a lumenal protein in the ER and is
subsequently modified to become associated with the membrane. The
experimental data suggest the latter possibility. Nodulin-24 was found in the
supernatant fraction when the in vitro translation product, obtained in the presence
of microsomal membranes, was sonicated and centrifuged (Fig. 17). However, as
in the case of nodulin-26, a polytopic integral membrane protein, mature nodulin-
24 from PBM was found in the pellet fraction after the same treatment. Neither
sodium carbonate (pH 11) nor EGTA treatment dissociated nodulin-24 from the
PBM (Fig. 18), while TX-100 completely solubilized this protein along with the
PBM. These data suggest that nodulin-24 in the ER is not membrane-bound but
becomes modified post-translationally along the sorting route to the PBM.
Proteinase-K treatment of PBM vesicles after sonication (Fig. 19) resulted in
partial digestion of nodulin-26, while nodulin-24 was not attacked. Nodulin-26
has six membrane-spanning regions, so that its membrane-integrated portion may
be protected from proteinase-K (Miao et al., 1992). On the other hand, nodulin-24
does not appear to have a membrane-spanning region, but may be covalently
bound to and embedded in membrane lipids, which may account for the increase of
its molecular mass.

There are a few examples of lipids that act as membrane anchors. The
variant surface glycoprotein (VSG) of the parasitic protozoan, Trypanosoma
brucei, is linked to the membrane via the glycosyl-phosphatidylinositol (GPI)
moiety (Low, 1989). Many proteins in the GTPase-superfamily are isoprenylated
or myristoylated or palmitoylated and become membrane-bound (Hancock et al., 1989; Spiegel et al., 1991; Magee and Newman, 1992). Nodulin-24 does not carry consensus sequences for these modifications. However, it has a region homologous to the lipid-binding domain (Fig. 20) of bacterial lipoproteins (Bairoch, 1992; Sankaran and Wu, 1993). Diglyceride is attached to a cysteine residue in the lipid-binding domain before processing of prolipoprotein by a signal peptidase II followed by N-acylation at the cysteine residue. This modification of membrane proteins with diacylglycerol and palmitate is unique to prokaryotes. It is interesting to find this characteristic lipid-binding domain for prokaryotic membrane proteins in nodulin-24. Furthermore, Coleman et al. (1985) showed that lipid-binding still occurs in the absence of propeptide processing, raising the possibility that nodulin-24 may have the same mode of lipid-binding even though no cleavage occurs at the carboxyl-terminal region which contains the lipid-binding domain.

The role of a signal sequence in targeting protein to the ER has been demonstrated by constructing fusion proteins with reporters (Garoff, 1985). Fusion of the nodulin-24 signal sequence with GUS (construct T2) did not allow cleavage of the signal sequence following in vitro translation with microsomal membranes, although the fusion product was targeted to the ER and translocated across the membranes. Even when an additional fragment of 18 amino acids of nodulin-24 was added to the construct, cleavage still did not occur (data not shown). Studies on co-translational translocation and signal peptidase processing using human preproapolipoprotein-A (Folz and Gordon, 1987; Nothwehr et al., 1989) suggested that the NH2-terminal propeptide may affect the cleavage site of signal peptidase and there may exist structural characteristics for recognition and
cleavage. In the case of the F$_1$-ATPase $\beta$-subunit, a mitochondrial protein, deletion of 17 residues distal to the cleavage site of the targeting sequence resulted in mitochondrial import without the cleavage of targeting sequence (Vassarotti et al., 1987). It was concluded that the deleted protein may not have a common structure to other mitochondrial precursors that are recognized by the matrix protease of mitochondria. Likewise, fusion constructs of nodulin-24 with the GUS may lack a structure recognized by signal peptidase.

Nodulin-24 appears to follow a unique path to become associated with the inner surface of the PBM. Despite the lack of any transmembrane domain, this protein is still protected from protease digestion, suggesting that it is buried in the lipid facing the bacteroids. The intriguing presence of a lipid-binding domain analogous to the membrane proteins of many pathogenic bacteria raises the possibility that a gene encoding this protein may have been transferred from bacteria into the plant genome during recent evolution.
CHAPTER IV

SUMMARY AND CONCLUSION

*Rhizobium* enters the root cells of legume plants via an endocytic process. To reach appropriate host cells and to avoid any pathogenic reaction, both organisms have an elaborate mechanism involving many signals that induce specific genes leading to the formation of the infection thread, eventual release of bacteria into host cytoplasm and maturation of the PBM compartments. The PBM is derived from the plasma membrane that surrounds the infection thread at the time of bacterial release into the host cell. The synthesis of the PBM appears to occur via the fusion of cytoplasmic membrane vesicles, both smooth and coated, with the infection thread membrane. These vesicles have been proposed to be derived from the Golgi. A high rate of vesicular fusions appears to contribute to the formation of the PBM and maturation of the PBM compartment as a symbiotic organelle. For these activities, extensive membrane proliferation occurs inside the infected cells. As a result, the PBM comprises approximately 20 times more membrane than the plasma membrane. Root nodules, therefore, provide an ideal system to study membrane biogenesis and vesicular transport in plants.
The aim of my studies was to understand the roles of the legume homologs of Rab1p and Rab7p in nodules and to examine the topology and membrane-anchoring process of nodulin-24. I was interested in dissecting the formation and modification of the PBM compartment. Since the PBM compartment is formed via endocytic events and active vesicular transport occurs in infected cells (Robertson and Lyttleton, 1982; Verma, 1992), the Rab family of proteins which are involved in vesicular transport (Hall, 1990) were chosen for my study. On the other hand, nodulin-24, one of the abundant host-specific proteins in nodules, has previously been localized to the PBM and shown to be induced after the endocytosis of *Rhizobium* (Fortin *et al.*, 1985, 1987), suggesting that it is specifically targeted to the PBM and may play a role in maturation of the PBM compartment. Therefore, nodulin-24 was also chosen for this study.

Three cDNA's encoding legume homologs of mammalian Rab1p and Rab7p were isolated from soybean and *Vigna* (moth bean). A Rab1p homolog was shown to complement a yeast ypt1-1 mutant, confirming that it is a functional counterpart of yeast Ypt1p (Rab1p). Induction of *rab1* and *rab7* genes was examined by northern analysis. *rab7*, in particular, was found to be highly induced in young nodules. Full length cDNA's of both small GTP-binding proteins were used to construct antisense cassettes for repression of each gene in nodules. Nodules containing antisense-*rab1* were smaller in size and showed lower nitrogenase activity than controls. The antisense-*rab1* nodules had structurally unstable PBM compartments, resulting in frequent release of rhizobia into vacuoles. In contrast, nodules expressing antisense-*rab7* contained fewer PBM compartments and many late endosomal structures in the perinuclear region. Both Rab1p and Rab7p were clearly shown to be essential for the development of
the PBM compartment. The induction mechanism of Rab proteins in nodules which might be connected to the burst of cortical cell division may be an interesting subject of further study.

Soybean nodulin-24 is a 33 kD protein, whereas the in vitro translated product of full-length nodulin-24 cDNA is 20 kD after cleavage of the signal sequence. The cleavage site of the signal sequence in nodulin-24 was determined by radiosequencing of the [3H]leucine-labeled processed peptide. A fusion construct of full-length nodulin-24 cDNA and the GUS gene was made and used for in vitro transcription and translation. Nodulin-24 was shown to have no membrane-spanning domain in protease protection assays using the fusion protein. In addition, in vitro synthesized nodulin-24 was present in the supernatant fraction after sonication of the microsomal membranes and subsequent centrifugation. However, mature nodulin-24 from the PBM has properties of an integral membrane protein, as shown by its inability to be solubilized following sodium carbonate (pH 11) or EGTA treatment. Nodulin-24 was found to have a region homologous to the consensus sequence of the lipid attachment site of bacterial lipoproteins, suggesting a possible mechanism of its attachment to membrane. This mechanism might be also relevant to increase in apparent MW of nodulin-24 from 20 kD in the ER to 33 kD en route to the PBM. Understanding the topology of nodulin-24 in the PBM may give a clue to its function in symbiosis.
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