

# Differential Expression of a Glycosyl Inositol Phospholipid Antigen on the Peribacteroid Membrane during Pea Nodule Development

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**Monoclonal antibody JIM18 recognizes a glycosylated form of an inositol-containing phospholipid. In pea (*Pisum sativum*), this epitope is associated with the plasma membrane of both infected and uninfected cells in root nodules induced by *Rhizobium leguminosarum* bv. *viciae*. On the plant-derived peribacteroid membrane that surrounds endosymbiotic bacteria in infected host cells, expression of the JIM18 epitope was found to be developmentally regulated. In situ immunolocalization studies revealed that the JIM18 epitope was present on the peribacteroid membrane surrounding actively dividing bacteria (juvenile bacteroids), but reactivity with the JIM18 antibody probe was abruptly lost from this membrane as differentiation of the symbiotic compartment progressed. This loss of reactivity was concomitant with accumulation of starch granules in infected host cells. This stage in host cell differentiation occurs earlier than the accumulation of nodule-specific proteins, such as leghemoglobin and nitrogenase. The biochemical nature of the JIM18 antigen in pea and carrot was investigated by thin-layer chromatography and by analysis of the effects of various chemical modifications. In carrot cells, the incorporation of radiolabeled precursors was investigated.**

*Additional keyword:* phosphoinositide.

Development of the legume root nodule is associated with the production of a new form of plant membrane, termed the peribacteroid membrane (PBM). This membrane has a specialized composition and functions to enclose the endosymbiotic *Rhizobium* bacteria (bacteroids) which develop the capacity for symbiotic nitrogen fixation (Brewin 1990; Verma et al. 1992). The PBM in pea nodules is an interesting model system for the study of membrane biogenesis and vesicle targeting within the plant endomembrane system. It originates during the internalization of symbiotic bacteria, through a process involving the invagination of the plasma membrane (Robertson et al. 1985; Kannenberg and Brewin 1994). The cell compartment enclosed by PBM is unusual because endocytosis is a very uncommon phenomenon in higher plants.

Plant cell wall components have not been found associated with the PBM, suggesting that some of the functions typical of the plasma membrane are lost during endocytosis (Rae et al. 1992). During the symbiotic interaction, the structure and function of the PBM gradually differentiate from those of the plasma membrane. Specific transport systems on the PBM play a primary role in regulating the exchange of metabolites between the host cell and the bacteroids (Day and Copeland 1991; Rosendahl et al. 1992; Day and Udvardi 1993). The cytoplasmic compartment enclosed by the PBM has been termed the symbiosome (Roth and Stacey 1989) because it develops as a semiautonomous organellelike compartment within the infected nodule cells (Verma et al. 1992).

Differentiation of pea nodule tissues can be followed in situ by examining longitudinal sections that show an axis of development from the apical nodule meristem (Nap and Bisseling 1990; Scheres et al. 1990). The differentiation of all symbiosomes within each host cell appears to be closely synchronized (Kannenberg et al. 1994; de Maagd et al. 1994), so that the progress of differentiation for bacteroid and PBM can be studied by comparing host cells in different zones of the nodule. Individual host cells in the central tissue of a nodule contain several thousand endosymbiotic bacteroids, so that the total surface area of the PBM is 30- to 100-fold greater than the surface area of plasma membrane (Brewin 1990; Verma et al. 1992). This increase in membrane surface requires an active and massive biosynthesis of all structural components of the PBM.

In addition to some nodule-specific proteins (nodulins) targeted to the PBM (Miao et al. 1992), other components of the PBM are characteristic also of the plasma membrane, endoplasmic reticulum, and tonoplast (reviewed in Brewin 1995). This suggests a hybrid origin for the components of the PBM (Roth and Stacey 1989), but the mechanisms that target lipid and protein components to the symbiosome are still unclear (Verma et al. 1994). It has been suggested (Mellor and Werner 1987) that the biogenesis and the differentiation of the symbiosome in nodule cells may share the same vesicle targeting mechanisms as the endosome of mammalian cells. Recent work by Cheon et al. (1993) has identified cDNAs encoding a legume homologue of the mammalian Rab7 protein: This small GTP-binding protein was localized on late endosomes (Chavrier et al. 1990) and is involved in vesicle trafficking. In root nodules, the level of mRNA encoding for Rab7p protein is 12 fold higher than in the root meristem (Cheon et al. 1993).

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We have examined the composition of the PBM using monoclonal antibodies as analytical probes, and our previous studies have revealed several epitopes on glycoproteins and glycolipids that are common to both PBM and plasma membranes (Brewin et al. 1985; Bradley et al. 1988; Perotto et al. 1991). We now report the identification of a glycolipid antigen, recognized by monoclonal antibody JIM18, which is consistently present on the plasma membrane, but is developmentally regulated on the PBM of pea nodule cells. In situ immunolocalization studies using this antibody have revealed the occurrence of sudden changes in composition of the PBM during the early stages in the differentiation of the symbiosome. The JIM18 glycolipid antigen was also found in nonlegume plants, and biochemical analysis on the antigen isolated from carrot membranes suggests that this compound is an inositol-containing glycosyl phospholipid.

## RESULTS

### Immunolocalization of the JIM18 antigen.

JIM18 was identified during an extensive search for monoclonal antibodies reacting with pea membranes (Knox et al. 1994). These antibodies were routinely tested against isolated nodule membrane components following SDS-polyacrylamide gel electrophoresis and immunoblotting. Preliminary results indicated that the JIM18 recognized a membrane lipid component, similar to other monoclonal antibodies previously isolated as reacting with PBM, like MAC 206 (Perotto et al. 1991). However, following immunostaining of longitudinal sections of pea root nodules, it was found that expression of the JIM18 epitope was developmentally regulated (Fig. 1B), whereas expression of MAC 206 was not (Fig. 1A). High power light micrographs revealed that the JIM18 antibody reacted with both the PBM and the plasma membranes of infected cells near to the nodule meristem, corresponding to the zone of host cell invasion and the early symbiotic zone (Fig. 2A and B). In the region of early symbiotic differentiation, reactivity of the JIM18 antibody with PBM was abruptly lost (Fig. 2B), although the presence of antigen could still be recognized on the plasma membrane of these host cells (Figs. 2C and 3D). The abrupt decrease of immunostaining with JIM18 antibody occurred in the nodule region that corresponds to the beginning of interzone II-III (Franssen et al. 1992), which is characterized by the accumulation of peripheral starch (Figs. 4 and 5). Ultrastructural immunocytochemical analysis of infected pea nodule cells (Fig. 3) confirmed the membrane localization of the JIM18 antigen and also showed that no significant labeling was found on other cell compartments such as endoplasmic reticulum, mitochondria, plastids, and nuclei (Fig. 3B). The decrease in the expression of the JIM18 epitope on the PBM occurred at a stage where bacteria were already partially developed into Y-shaped bacteroids. In infected cells where the JIM18 epitope was no longer detectable on the PBM, vesicles associated with Golgi bodies were still commonly found to express the JIM18 epitope (Fig. 3E).

### Correlation with other markers for symbiotic differentiation.

The observed loss of reactivity of the JIM18 antibody with the PBM was correlated with the expression of three other

developmentally regulated protein antigens for which specific antisera were available: starch synthase, leghemoglobin, and nitrogenase (Fig. 4). Loss of reactivity of the JIM18 antibody to the PBM (Fig. 4A) occurred in a region of the nodule tissue where starch synthase was present on the starch granules forming at the cell margins (Fig. 4B), but where leghemoglobin and nitrogenase were not yet detectable (Figs. 4C and 4D). Phase-contrast microscopy showed that the abrupt decrease of JIM18 immunostaining on the PBM could be closely correlated with the appearance of starch granules at the margins of infected cells (Fig. 5), which marks the transition between the prefixation zone II and the interzone II-III, according to the nomenclature proposed for pea by Franssen et al. (1992). In nodules formed by *nifA* (1MA137) and *nifH* (1MA78) bacterial mutants (Ma et al., 1982), it was observed that maturing PBM lost its reactivity with the JIM18 antibody even though no nitrogenase was produced, showing that loss of JIM18 epitope expression from the PBM takes place before, or independently from, expression of nitrogenase.

### Biochemical characterization of the JIM18 antigen.

Because the general behavior of the JIM18 antigen was similar to that previously reported for plant glycolipid anti-

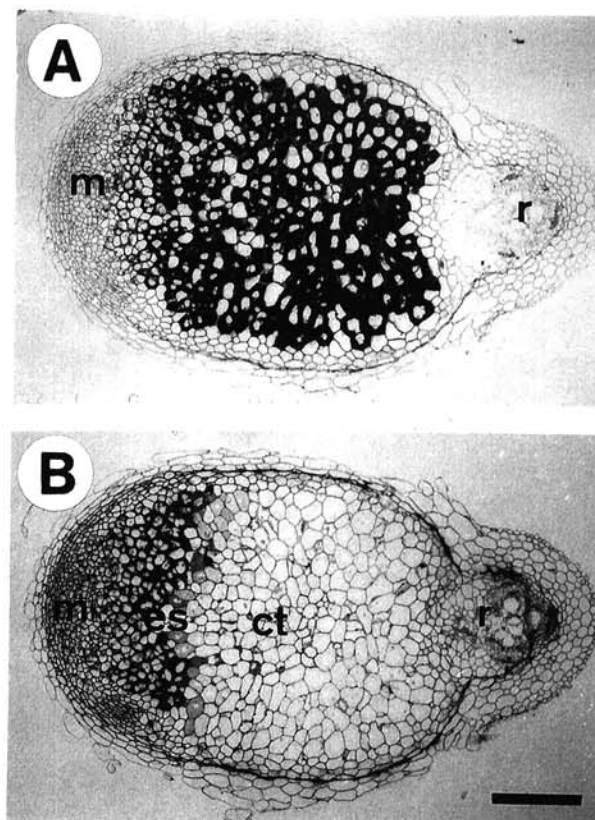


Fig. 1. Light micrographs of longitudinal sections of a pea root nodule labeled with the immunogold/silver enhancement technique. **A**, Monoclonal antibody MAC 206 recognizes a glycolipid antigen expressed throughout the pea nodule. **B**, The epitope recognized by monoclonal antibody JIM18 is strongly expressed only in the apical part of the nodule. These sections were not counterstained, and the negative control in which the primary antibody was omitted resulted in a completely blank image. m, nodule meristem; es, early symbiotic zone; ct, central tissue (nitrogen-fixing zone); r, root. The bar is 0.5 mm.

gens (Perotto et al. 1991), the lipid fraction was extracted from pea nodule membranes by partitioning into acidified organic solvents. After thin-layer chromatography (TLC), it was revealed by immunostaining that the JIM18 antigen had chromatographic properties similar to those of polar phospholipids such as polyphosphoinositides (Fig. 6A). Use of chromogenic spray reagents and iodine vapor indicated that the JIM18 antigen was a minor constituent of the lipid fractions (Fig. 6A). The JIM18 antigen was identified in lipid extracts from peribacteroid or nodule membranes, as well as from other pea tissues such as uninfected roots, leaves, or stems (Fig. 6B). When membrane lipid extracts from two other plant species (French bean nodules and a carrot cell line) were compared with pea membranes, it was found that the more prominent and faster migrating antigen band was conserved among all the species tested, whereas the slower migrating band was present in French bean but absent from the carrot cell line (Fig. 6B). The small difference in mobility observed for the two antigen bands may reflect differences in the fatty acid composition. Cultured carrot cells were used for further analysis of the JIM18 antigen.

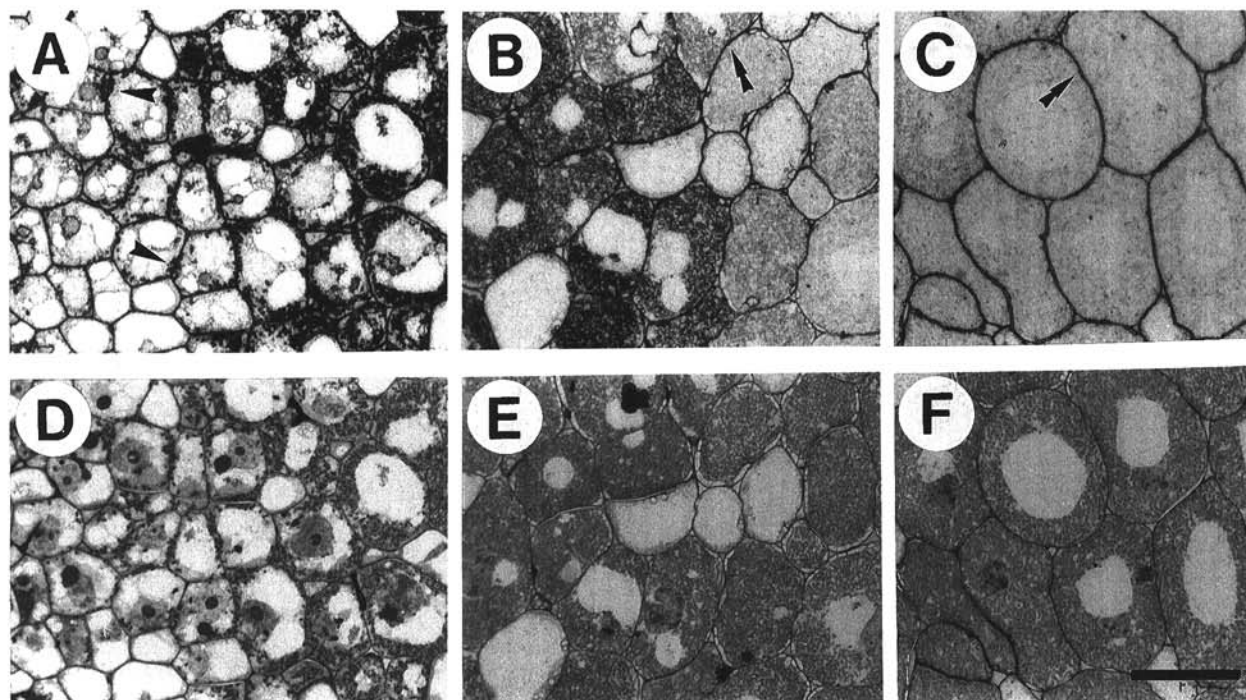
#### Radioisotope studies.

When carrot lipids radiolabeled with [<sup>33</sup>P]-orthophosphate were separated by TLC, the JIM18 antigen was found to co-migrate with a faint radioactive component (Fig. 7A). Co-migration was maintained following elution of the antigen band and rechromatography in a different solvent system: A

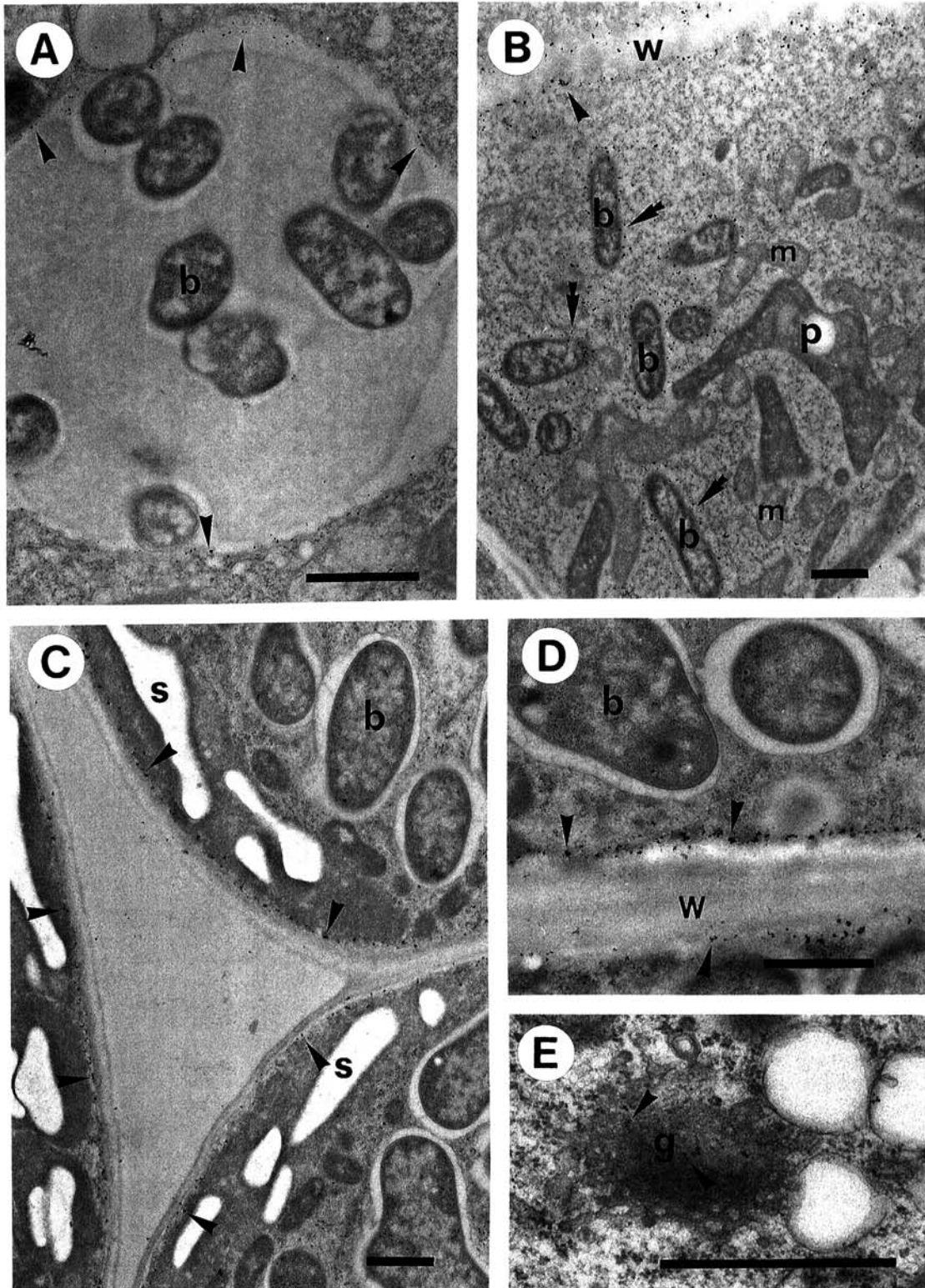
major radioactive band was found, which corresponded to the JIM18 antigen when immunostained on the same plate (Fig. 7B).

Because the JIM18 antigen was found in the same region of the TLC plate as very polar phosphoinositides, the possible presence of inositol in the JIM18 antigen molecule was investigated by feeding carrot cultured cells with *myo*-[2-<sup>3</sup>H]-inositol. When separated in solvent 1, the JIM18 antigen co-migrated with a [<sup>3</sup>H]-radiolabeled lipid, and this correspondence was maintained following rechromatography in solvent 2 (results not shown). Furthermore, when the JIM18 antigen band was eluted from the second TLC plate, subjected to acid hydrolysis and fractionated by isocratic HPLC, a broad <sup>3</sup>H-labeled peak eluted in the region where inositol-monophosphates are known to chromatograph (Drøbak et al. 1991).

Carrot lipids radiolabeled with [<sup>3</sup>H]-inositol and [<sup>33</sup>P]-Pi were mixed, and a radioactive band corresponding to the JIM18 antigen was purified from TLC plates after double separation in solvents 1 and 2. This radiolabeled antigen was used to investigate the nature of the chemical linkage between the polar head and the lipid tail in the JIM18 antigen. After transacylation and phase-partitioning between butanol and water, about 90% of the [<sup>3</sup>H]-label was found in the butanol phase. When the same butanol and water phases were tested by dot blot immunoassay, the JIM18 antigen was also found exclusively in the butanol phase, demonstrating that the antigen was not cleaved after treatment. No shift in antigen mo-

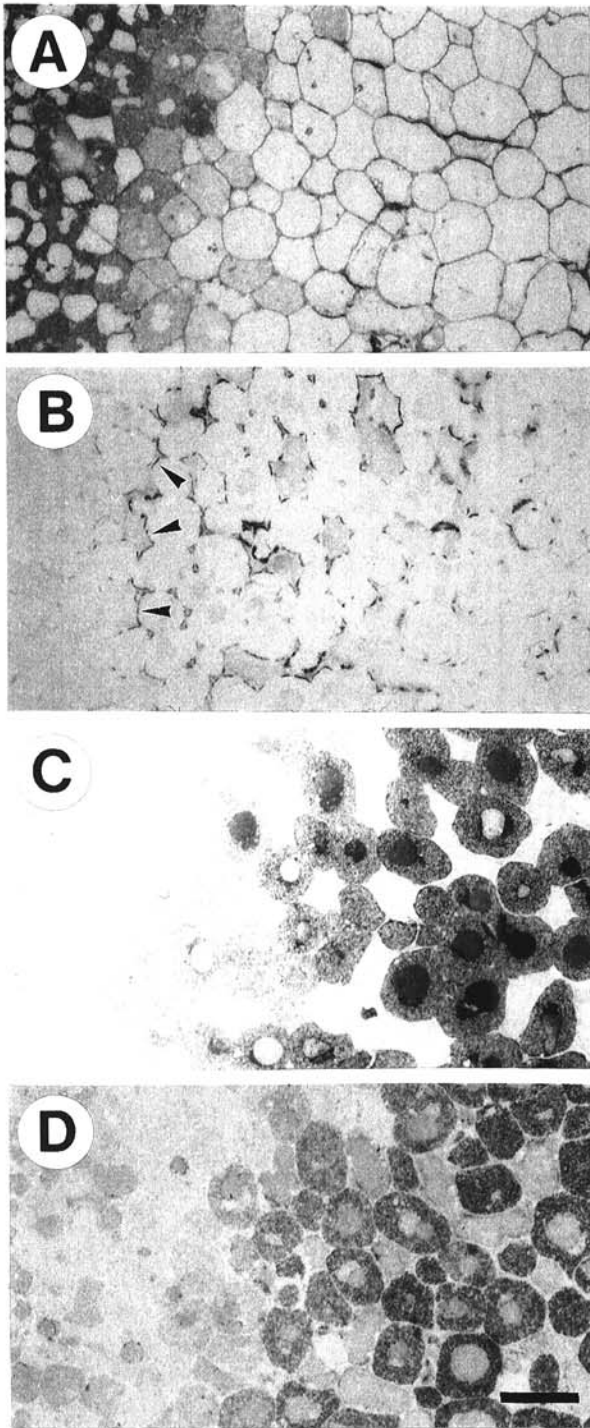


**Fig. 2.** Light micrographs of longitudinal sections from a 3-week-old pea nodule, showing (from left to right) successive stages of symbiosome development. Sections were: (A–C) immunogold labeled with JIM18 antibody, followed by silver enhancement; (D–F) contiguous sections stained with toluidine blue to show morphological features of nodule cells. A and D, Nearest to the apical region, plant cells derived from the nodule meristem have been infected by rhizobia, which divide in the host cytoplasm. The JIM18 antibody immunolabeled both the cell margins and the cell area containing bacteria (arrowheads). B and E, Proceeding from the invasion zone across the early symbiotic zone, infected cells have become filled with bacteria: reactivity of PBMs with JIM18 antibody declined rapidly across this zone, although reactivity of the plasma membrane was maintained (double arrowhead). C and F, In the central nodule region, the JIM18 epitope is still detected on the plasma membrane of infected and uninfected cells (double arrowhead), whereas only traces are found elsewhere in the cell. The bar is 100  $\mu$ m.



**Fig.3.** Electron micrographs of infected pea nodule cells, immunogold labeled with JIM18 antibody followed by silver enhancement. **A**, In the invasion zone, bacteria are released into the plant cell from an infection droplet which is bounded by the plant plasma membrane: JIM18 antigen was immunocalized on this membrane (arrowheads). **B**, In host cells of the early symbiotic zone JIM18 antigen is expressed on the plasma membrane (arrowheads) and around intracellular bacteria (double arrowheads). No significant JIM18 immunolabeling was found on other plant organelles such as mitochondria and starch granules. **C**, In the central tissue, JIM18 antigen is not expressed on the PBM but is only found on the plasma membrane (arrowheads). Peripheral starch granules are found at the margin in these cells. **D**, Higher magnification showing JIM18 immunolabeling restricted to the plasma membrane (arrowheads). **E**, Golgi body immunolabeled by JIM18 antibody. b, bacteria; g, Golgi body; m, mitochondrion; p, plastid; s, starch granule; w, cell wall. The bar is 1  $\mu$ m.

bility was found when treated and untreated samples were separated by TLC and immunostained with JIM18 antibody. In the same experiment, a diacylglycerol lipid control was completely cleaved after treatment, whereas a sphingolipid control was not affected. An alternative hydrolysis procedure,



**Fig. 4.** Contiguous longitudinal sections taken from the early symbiotic zone of a 3-week-old pea nodule, with the axis of symbiosome differentiation proceeding from left to right. Sections were immunogold labeled/silver enhanced following treatment with: **A**, JIM18; **B–D**, polyclonal antibodies directed to: **B**, starch synthase (arrowheads); **C**, leghaemoglobin and **D**, nitrogenase. The bar is 200  $\mu\text{m}$ .

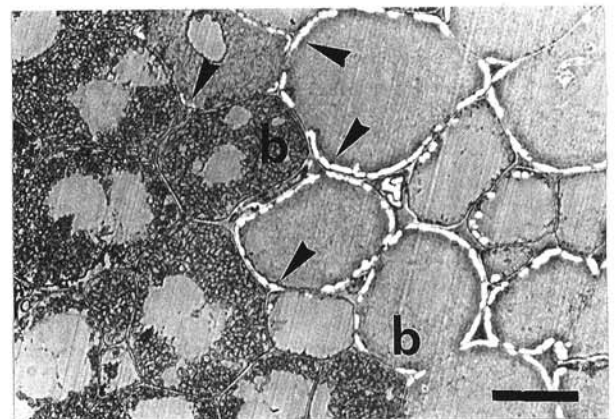
in which carrot and pea nodule membrane lipids were treated with 6 N ammonia (McConville et al. 1993), gave identical results. Both experiments indicated that the JIM18 antigen is not a diacylglycerol lipid.

The presence of glucosamine in the JIM18 antigen was investigated in a separate experiment. Following incorporation of [ $^{14}\text{C}$ ]-glucosamine, a radioactive lipid component was found to comigrate with the JIM18 antigen (Fig. 8A). Complete loss of reactivity of the JIM18 antibody on the corresponding antigen after nitrous acid treatment of carrot and pea nodule membrane lipids (Fig. 8B) further indicated that the JIM18 epitope contains an amino-sugar in the non-acetylated form.

## DISCUSSION

The monoclonal antibody JIM18 identifies an antigen found on the plasma membrane of both legume and non-legume plants. In pea nodules, this antigenic component is also targeted for some time to the PBM, an endomembrane which originates by invagination of the plasma membrane and encloses symbiotic bacteria. There is subsequently a massive amount of membrane biogenesis during the differentiation of the endosymbiotic compartment, the symbiosome. Whereas other components common to PBM and plasma membrane are present on the PBM throughout symbiosome differentiation (Perotto et al. 1991), the antigen recognized by the JIM18 antibody was not found on the mature form of PBM.

The JIM18 antigen is a protease-insensitive plant lipid component soluble in organic solvents. The purified JIM18 antigen from carrot was shown to incorporate [ $^{33}\text{P}$ ]-orthophosphate, [ $^3\text{H}$ ]-inositol, and [ $^{14}\text{C}$ ]-glucosamine in labeling experiments. The two known groups of glycosylated inositol-containing phospholipids comprise the glycosyl phosphosphingolipids (GPS) (Laine and Hsieh 1987) and the glycosyl phosphatidylinositides (GPI) (McConville and Ferguson 1993). A characteristic common to both groups is the occurrence of glucosamine in the nonacetylated form, and this was demonstrated to be present in the JIM18 antigen of both pea and carrot by nitrous acid treatment. Because of its resistance to mild alkaline hydrolysis, the JIM18 antigen may

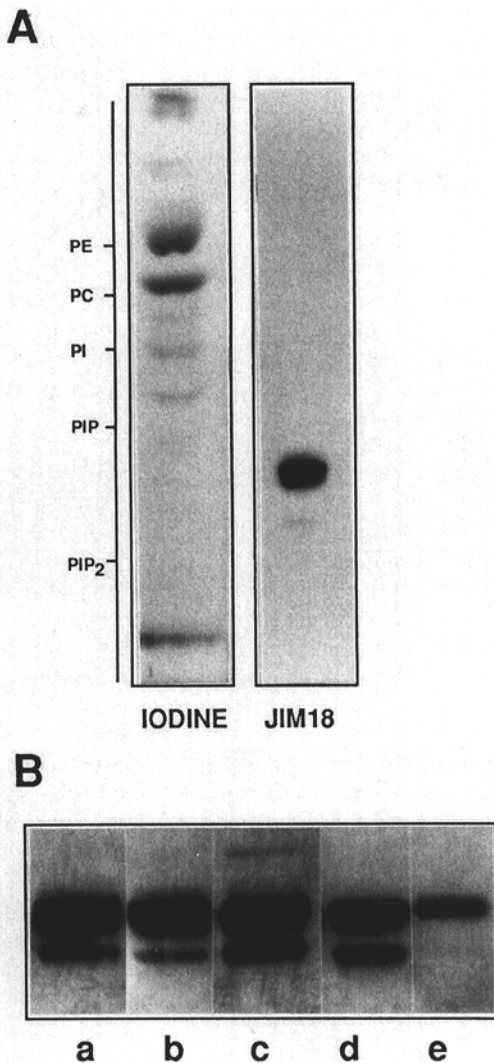


**Fig. 5.** Phase contrast micrograph of a nodule section immunostained with JIM18 followed by silver enhancement. Starch granules (arrowheads) are visible as birefringent structures at the margin of cells infected by bacteria (**b**). The bar is 50  $\mu\text{m}$ .

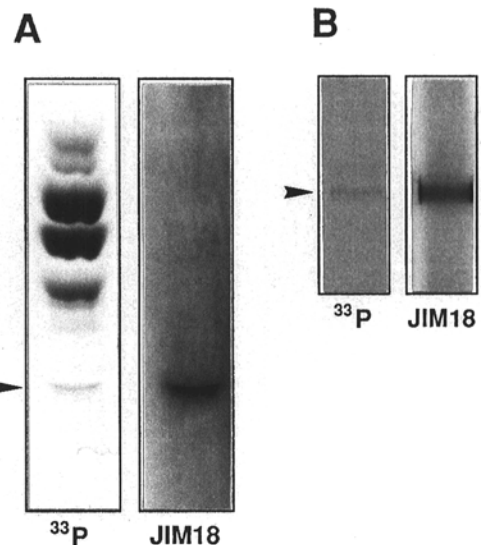
be more closely related to the group of glycosyl phosphosphingolipids, which seem to be exclusively found in walled organisms such as plants and fungi, and whose function remains mostly unknown (Laine and Hsieh 1987). Glycosylated phosphosphingolipids that function as protein-anchors have been identified in *Dictyostelium discoideum* (Stadler et al. 1989) and in walled organisms like yeasts (Conzelmann et al. 1992). The protein-anchor function had previously been as-

cribed to glycosyl phosphatidylinositide molecules in animal cells (Simons and Wandiger-Ness 1990) and so perhaps these two classes of glycolipids may have overlapping functions. In plants, however, glycosyl phosphatidylinositides have not yet been identified. Further studies are now needed to identify the precise molecular structure of the JIM18 antigen both in pea and in carrot, in order to confirm their identity and to verify the relationship between fast and slow migrating forms of the pea antigen. This analysis should also help to understand whether the sudden loss of reactivity of the JIM18 antibody with the PBM reflects the disappearance of the JIM18 antigen from the PBM following changes in vesicle targeting pathways, or whether it is caused by structural modifications of the PBM antigen.

Expression of the JIM18 epitope on both PBM and plasma membrane indicates the relatedness of these two membranes, and confirms previous immunological results involving protein and glycolipid antigens (Brewin et al. 1985; Bradley et al. 1988; Perotto et al. 1991). However, the distribution of the JIM18 epitope in nodule sections was unique because it was undetectable on mature forms of PBM, although it was maintained on the plasma membrane of the same cells. Loss of detectability of the JIM18 antigen on the PBM occurs in those infected nodule cells where accumulation of starch is just about to take place. Accumulation of peripheral starch presumably reflects a change in carbon metabolism in infected cells and their enclosed bacteria. In pea nodules, this developmental marker identifies a region where major changes in bacterial and plant gene expression have been identified. Expression of a bacterial outer membrane protein is abruptly down-regulated (de Maagd et al. 1994) in the same nodule region where the JIM18 epitope is lost from the PBM,



**Fig. 6.** TLC plates showing fractionation of membrane lipids. **A**, Total pea nodule membrane lipids separated by TLC and stained with iodine vapour or with JIM18 antibody followed by immunoperoxidase treatment and enhanced chemiluminescence. The mobilities of lipid standards are indicated: PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidyl inositol; PIP, phosphatidylinositol-4-monophosphate; PIP<sub>2</sub>, phosphatidylinositol-4,5-biphosphate. (Note that with a reduced level of immunostaining, the broad JIM18 antigen band identified could be resolved into the doublet band presented in tracks B a-e). **B**, Chromatographic plate immunostained with JIM18 antibody, showing the presence of JIM18 antigen in lipids extracted from: a, pea PBM; b, pea nodule membranes; c, uninfected pea root membranes; d, *Phaseolus* bean nodule membranes; e, cultured carrot membranes. A minor band of higher mobility (lane c) was visible in heavily loaded samples. Negative controls, where JIM18 antibody was omitted or a different antibody was used showed no bands and therefore confirmed that this immunostaining pattern was specific to JIM18.



**Fig. 7.** Incorporation of [<sup>33</sup>P]-orthophosphate into material that co-chromatographed with the JIM18 antigen. **A**, Total [<sup>33</sup>P]-radiolabeled carrot lipids separated on a chromatographic plate with solvent 1. After autoradiography and subsequent immunostaining of the TLC plate, a faint radioactive band (arrowhead) corresponded in mobility to the JIM18 antigen. **B**, This radioactive component was eluted from the TLC plate, loaded on a new TLC plate and separated in solvent 2. After autoradiography and immunostaining of the plate, a major [<sup>33</sup>P]-radioactive band (arrowhead) was still found to correspond to the JIM18 antigen.

although a possible correlation between these two events remains to be elucidated. Loss of the JIM18 epitope from the PBM just precedes the expression of plant and bacterial genes associated with the maturation of the symbiosome into a nitrogen-fixing organelle, for example the expression of leghemoglobin and nitrogenase genes (deBilly et al. 1991). The loss of JIM18 epitope is therefore the earliest known cytological marker in the process of differentiation of the PBM. It is possible that the disappearance of the JIM18 epitope from the PBM signals the advent of a specific targeting pathway for PBM biogenesis, and marks the point where this membrane becomes differentiated from the plasma membrane to perform its specialized functions in the nodule.

## MATERIALS AND METHODS

### Antibodies.

Monoclonal antibody JIM18 (class IgM) was originally isolated as reacting with the microsomal fraction of guard cell protoplasts (Knox et al. 1994). About 20 different hybridomas from two separate fusion experiments produced monoclonal antibodies that gave similar immunostaining patterns on Western blot and on nodule sections after immunogold/silver enhancement, and JIM18 was taken as a representative of this class. AFRC MAC 206 rat monoclonal antibody recognizes an epitope present on membrane glycolipids abundant on the PBM (Perotto et al. 1991). Polyclonal antibodies directed to pea leghemoglobin, nitrogenase, and starch synthase were kind gifts of T. Bisseling (Wageningen, NL), P. Ludden (Madison, Wisconsin) and A. M. Smith (Norwich, UK) (Smith 1990), respectively.

### Microscopy.

Immunostaining of semithin pea nodule sections followed by immunogold/silver enhancement was performed as de-

scribed by VandenBosch et al. (1989). Immunogold labeling with JIM18 (cell culture supernatant diluted 1:50) used a secondary antibody conjugated to 5 nm colloidal gold particles followed by silver enhancement for 2 to 6 minutes, because the use of larger gold particles (10 to 15 nm) dramatically reduced the level of immunostaining that could be visualized by electron microscopy. No significant deposition of silver precipitate was observed on the tissue when the primary antibody was omitted.

### Dot immunoassay.

Samples were immobilized onto PVDF (immobilon) membrane. The membrane sheet was blocked in 2% bovine serum albumin in Tris-buffered saline, immunolabeled with JIM18 and incubated with a secondary antibody conjugated to alkaline phosphatase. Binding was visualized with a chromogenic substrate as described by Bradley et al. (1988).

### Membrane fractionation.

Membranes from 3-week-old pea and bean nodules, from different plant tissues, and from carrot cell cultures were fractionated following the protocol of Perotto et al. (1991). An additional fraction containing endomembranes and plasma membranes (nodule membranes) was obtained from nodule homogenates after removal of symbiosomes and cell wall debris, and ultracentrifugation at  $100,000 \times g$  for 40 min in a Beckmann TLA-100 ultracentrifuge.

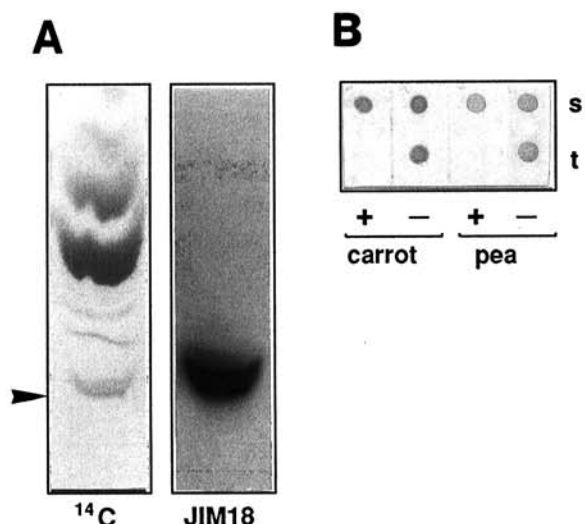
### Extraction of lipids and separation by thin-layer chromatography.

Samples (500  $\mu$ l) of membrane suspensions were transferred to a dounce homogenizer on ice and lipids were extracted under acidic conditions as described by Drøbak and Roberts (1992). After phase partitioning, the upper aqueous phase was removed and the lower organic phase was re-extracted twice with 2.5 ml of fresh upper phase. These upper-phase fractions were pooled and back-extracted 4 to 5 times with fresh lower phase from the partitioned solvent. Fractions were dried under a stream of nitrogen and resuspended in appropriate solvent before further analysis.

Lipids were separated by thin layer chromatography (TLC) on silica gel 150 Å LK5 plates (Whatman, UK) impregnated with 1% potassium oxalate, 1.5 mM EDTA and activated for 2 h at 120°C before use. Two solvent systems were employed. Solvent 1 contained chloroform:methanol:ammonia:water (90:70:4:16, v/v/v/v). For further purification, the antigen was removed from the TLC plate, eluted with solvent 1, loaded onto a new TLC plate and developed with solvent 2, ethanol:water (65 : 35, v/v).

### In vivo radiolabeling of cell glycolipids.

Feeding experiments with radiolabeled precursors were performed on carrot cell cultures growing in log-phase at 25°C. Cell components were radiolabeled by incubating 60 ml carrot cell cultures for 24 h with 55  $\mu$ Ci of [ $^{33}$ P]-orthophosphate (Du Pont NEN, UK; 1.48 TBq/mg), or for 48 h with either 30  $\mu$ Ci of *myo*-[2- $^3$ H] inositol or 15  $\mu$ Ci of D-[1- $^{14}$ C] glucosamine hydrochloride (Du Pont NEN, UK; specific activities 868 GBq/mmol and 1.67 GBq/mmol respectively). To extract the radiolabeled lipid fraction, the growth medium was removed by filtration and the cell pellet was immediately



**Fig. 8.** Incorporation of [ $^{14}$ C]-glucosamine and sensitivity to nitrous acid treatment. **A.** The mobility of [ $^{14}$ C]-radiolabeled carrot lipids in solvent 1 is revealed by autoradiography and compared with the mobility of the JIM18 antigen after immunostaining; a radioactive band comigrates with the JIM18 antigen band (arrowhead). **B.** Dot immunoassay of lipid fractions from carrot membranes and pea nodules immunostained with JIM18 after nitrous acid deamination. The same amount of starting material (s) was treated (t) with buffer containing (+) or not containing (-) the reagent sodium nitrite.

homogenized in 15 ml ice-cold quenching cocktail (chloroform:methanol:conc. HCl, 10:10:0.07, v/v/v) as described by Drøbak and Roberts (1992).

#### Immunolabeling on chromatographic plates.

After separation of lipids by TLC, the silica gel plate was allowed to dry and processed for immunolabeling with monoclonal antibody JIM18 following a protocol modified from Strömberg et al. (1988). The silica gel plate was soaked in a solution of 0.5% poly(isobutyl)-methacrylate (Polysciences, Pennsylvania) in diethyl ether for 1 min. The dried plate was briefly soaked in cold TBS buffer containing 0.2% Tween 20 (TBST) and then transferred to a blocking solution (5% dried milk in TBST) for 2 to 3 h. Overnight incubation with JIM18 antibody (hybridoma cell culture supernatant diluted 1:100 in blocking solution) at 4°C was followed by 4-5 rinses with TBST. The plate was then incubated for 3 to 4 h at room temperature with a secondary anti-rat antibody conjugated to horseradish peroxidase (Sigma, UK). Labelling was visualized by enhanced chemiluminescence (Amersham, UK). Alternatively, contiguous 0.5 cm lane segments of the TLC plates were individually removed and the corresponding lipid fractions eluted in solvent 1 were tested by dot immunoassay. Immunolabeling of whole TLC plates and eluted lipid fractions gave similar results. Control experiments were carried out in which either an alternative primary antibody or no primary antibody was used.

#### Acid hydrolysis of purified JIM18 antigen.

To confirm the presence of inositol in the JIM18 antigen, carrot lipids that had been radiolabeled with *myo*-[2-<sup>3</sup>H] inositol were separated by TLC with solvent 1, and the band corresponding to the JIM18 antigen was eluted from the plate and re-chromatographed in solvent 2. The lipid component found in the position corresponding to the JIM18 antigen was eluted from the TLC plate and dried. After addition of 0.3 ml from a glacial acetic acid: 0.15 M H<sub>2</sub>SO<sub>4</sub> mixture (9:12, v/v), the sample was incubated for 4 h at 80°C. Acid was removed by evaporation and the sample was reconstituted in water and analyzed by isocratic HPLC on a Partisil 10-SAX column (Drøbak et al. 1991).

#### Mild alkaline hydrolysis.

Sensitivity of the JIM18 antigen to mild alkaline hydrolysis was investigated using two different protocols. The first was performed on pea and carrot membrane lipids and used 6 N ammonia in 20% 1-propanol (McConville et al. 1993). After hydrolysis, fatty acids, and nonhydrolyzed lipids were partitioned into water and water-saturated butanol, and subsequently separated on TLC plates which were then immunolabeled with JIM18 antibody. Alternatively, the presence of the JIM18 antigen in the two phases was tested by dot blot immunoassay. A band containing [<sup>33</sup>P] and [<sup>3</sup>H]-labeled carrot antigen purified by double chromatography was also tested for its susceptibility to an alkaline hydrolysis using monomethylamine (Clarke and Dawson 1981). The products of the reaction were fractionated in butanol/water as described above. This method permits quantitative estimates of the partitioning of radioactivity between different fractions after chemical treatment.

#### Nitrous acid deamination.

Chemical cleavage with nitrous acid was used to determine whether the amino-sugar identified in the JIM18 antigen was present in the acetylated form. Both a microsomal fraction and a lipid extract from carrot and pea samples were treated as described by Güther et al. (1992). Glucosamine and N-acetyl glucosamine were treated in the same conditions as controls.

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