Volume 15 Number 2 1987 Nucleic Acids Research

Nodulin-26, a peribacteroid membrane nodulin is expressed independently of the development of the peribacteroid compartment

Marc G.Fortin, Nigel A.Morrison and Desh Pal S.Verma*

Centre for Plant Molecular Biology, Department of Biology, McGill University, 1205 Docteur Penfield Avenue, Montreal H3A 1B1, Canada

Received September 3, 1986; Revised and Accepted December 11, 1986

ABSTRACT

The peribacteroid membrane (pbm) of root nodules is derived from the plant cell plasma membrane but contains in addition several nodule-specific host proteins (nodulins). Antibodies raised against purified pbm of soybean were used to immunoprecipitate polysomes to isolate an RNA fraction that served as a template for the synthesis of a cDNA probe for screening a nodule-specific cDNA library. Clone p1B1 was found to encode a 26.5 kDa polypeptide (nodulin-26) which is immunoprecipitable specifically with the anti-pbm serum. Nodulin-26 has features of a transmembrane protein and its structure differs from that of nodulin-24 which appears to be a surface protein of pbm. The expression of these two pbm nodulins was examined in nodules induced by *Bradyrhizobium japonicum* Tn*5* mutants that arrest nodule development at different stages of pbm biosynthesis. Nodules that do not show release of bacteria from the infection thread express nodulin-24 at a very low level. In contrast, the expression of nodulin-26 occurs fully in nodules that form infection threads only and is not affected by the release of bacteria from the threads.

INTRODUCTION

The development of the endosymbiosis between a legume and *Rhizobium* that leads to nitrogen fixation requires the expression of new sets of genes in both the host and microsymbiont. The products of these genes are responsible for forming proper nodule structure and for creating conditions favorable for the reduction and assimilation of atmospheric nitrogen. In all legume root nodules examined so far, the bacteroids (nitrogen-fixing form of *Rhizobium*) are segregated within the host cytoplasm in an "organelle-like" structure delimited by the peribacteroid membrane (pbm) (see ref. 1 for review). Failure of the host to maintain this barrier results in direct contact of the bacteria with the host cell cytoplasm and in some cases, with the induction of host-defense responses (2,3). Such nodules are unable to reduce nitrogen.

Pbm originates from the plant cell plasma membrane that surrounds the infection thread at the time of release of bacteria from the thread into the nodule cell (4,5). A large amount of membrane material has to be synthesized

© IRL Press Limited, Oxford, England.

and directed to the pbm, since there is 20 to 40 times more pbm than plasma membrane in the infected cells of soybean nodules (4). Not only does the pbm increase coordinately with bacteroid proliferation (6), it also selectively acquires new proteins (nodulins) that are required by pbm to perform its specific roles. Nodulins have been identified in the pbm of soybean nodules and have been shown to be inserted specifically in this membrane (7). No pbm nodulins could be detected in the plasma membrane of nodule cells. For this targetting, the biosynthetic machinery must distinguish pbm from the plasma membrane despite the great similarity between these two membranes. This can be achieved by the differential properties of the target and/or of the proteins to be directed. We identified a transmembrane pbm nodulin, nodulin-26 and compared its structure with nodulin-24, another pbm nodulin (7,8), to determine primary and secondary structural features that could facilitate such targetting.

Using Tn*5* mutants of *Bradyrhizobium japonicum* that block nodule development at different stages of pbm biosynthesis, we show that regulation of pbm nodulin gene expression occurs at different stages of the release of *Rhizobium* from the infection thread. Nodulin-24 is virtually absent in nodules that do not form a pbm compartment, indicating that the release of bacteria from the infection thread is a prerequisite for its efficient expression; or conversely, that failure to induce the expression of nodulin-24 prevents the formation of pbm. The induction of nodulin-26 in the absence of bacterial release from the infection thread suggests the presence of at least two levels of regulation of pbm nodulins.

MATERIALS AND METHODS

Plant Material

Soybean seeds (*Glycine max L.* cv. Prize) were inoculated with *Bradyrhizobium japonicum* strain 61A76 or Tn*5* mutants T5-95 and T8-1 (9) grown in YM medium. Plants were grown in vermiculite as described (10).

Isolation of pbm nodulin cDNA clones

Polysomes were isolated from three-week-old nodules induced by *B. japonicum* 61A76 essentially as described (10). Polysomal pellet was resuspended (6.6 mg RNA per ml) in 4 ml of buffer (300 mM NaCl, 50 mM Tris-Cl pH 7.5, 5 mM $MgCl_2$, 0.1 % (v/v) NP-40, 1 mM β-mercaptoethanol) (11) and was incubated with 1.5 ml of purified pbm and peribacteroid fluid (pbf) antisera (7) at 4° C for two hours in the presence of 300 units of

RNasin (Promega Biotec, Madison). The polysome-antisera mixture was passed over a Protein A-Sepharose column (Pharmacia Fine Chemicals, Montréal) at $4^{o}C$ and washed with 20 ml of the above buffer. RNA was eluted with 25 ml of 25 mM Tris-HCl, pH 7.5, 25 mM EDTA and polyA^{+} RNA was selected by oligo-dT-cellulose chromatography as described (10). cDNA was synthesized using [^{32}P]-dCTP and oligo-dT priming (12). The cDNA probe was hybridized to a nodule-specific cDNA library (approximately 650 clones) depleted of known nodulin sequences (12). Filters (GeneScreen, New England Nuclear, Boston) were washed at $65^{o}C$ in 150 mM NaCl, 15 mM sodium citrate (pH 7.0) and exposed to X-ray films for 48 hr.

Hybrid selection and *in vitro* translation of mRNA

PolyA^{+} RNA was isolated from total polysomes (10) and hybridized to electrophoretically-purified insert DNA from eight randomly-selected recombinant clones bound to GeneScreen (13). Released RNA was translated *in vitro* in rabbit reticulocyte lysate (Bethesda Research Laboratories, Bethesda) in the presence of [^{35}S]-methionine, and the products were immunoprecipitated by incubation with antisera raised against pbm, pbf and nodule cytoplasmic fractions followed by Protein A-Sepharose binding (12). Translation products were separated by $NaDodSO_4$-PAGE (14). After electrophoresis, the gel was treated with EnHance (New England Nuclear), dried and autofluorographed.

DNA sequence determination

Subclones were constructed in M13 mp18 and mp19 vectors (15) and sequenced using the dideoxynucleotide chain termination procedure (16). Restriction maps were determined by digesting a *Sph*I cut, 3'-end labelled (13) *Pst*I subclone in pUC19 with various enzymes (15).

Protein structure analysis

Secondary structure prediction were made using the algorithm described (17). Hydrophobicity values of the amino acids were those of the normalized consensus scale of Eisenberg (18). Hydrophobic moment was calculated as in (18).

RNA dot blots

Polysomal RNA purification, dot blots and hybridization conditions were as described (19). Filters were washed under high stringency conditions, exposed for different times and the X-ray film was scanned by densitometer coupled with an integrator.

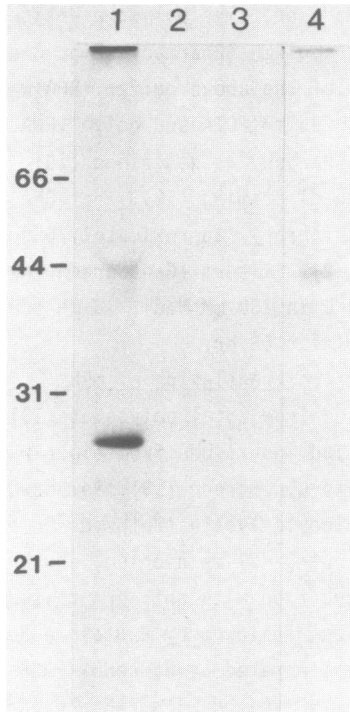

Figure 1. Immunoprecipitation of [^{35}S]methionine-labeled *in vitro* translation products of mRNA hybrid-released from membranes (0.15 cm^2, GeneScreen) on which was bound gel-purified insert DNA from the p1B1 cDNA clone (lanes 1,2,3) or pBR322 vector (lane 4). After *in vitro* translation in rabbit reticulocyte lysate, labelled proteins were incubated with sera raised against purified pbm (lane 1), pbf (lane 2), nodule cytoplasmic fraction (lane 3), or all three sera combined (lane 4). Antigen-antibody complexes were precipitated with Protein A-Sepharose and separated by $NaDodSO_4$-PAGE. Protein markers are shown as $M_r \times 10^{-3}$.

RESULTS

Selection of cDNA clones forming a pbm and pbf specific library

Antibodies raised against purified pbm and pbf were used to selectively immunoprecipitate polysomes synthesizing pbm and pbf nodulins to make a cDNA probe. In a nodule-specific cDNA library that was depleted of previously characterized nod A, B, C (nodulin-24), and D sequences (12), 196 clones were found to hybridize with this probe. It has previously been shown that nodulin-24, a pbm nodulin (7,8), represents an additional 61 clones (see ref. 12). Thus, pbm nodulin sequences constitute approximately 20% of the soybean nodule-specific cDNA library (excluding leghemoglobin).

```
                    20                  40                  60
GAAGAACACCTCCGAAACAATCCAACGCTCAGACTCCCTTGTCTCTGTTCCTTTCTTGCAGAAGTTGGTAGCTGGAAGC

                   100                 120                 140
GGTGGAAACATATTTCCTGATATTTGCAGGGTGTGCTTCATTGGTGGTGAACGAGAACTACTACAAC ATG ATA ACA
                                                                    Met Ile Thr
   160                     180                     200
TTT CCT GGG ATA GCA ATT GTT TGG GGT CTG GTT CTG ACG GTG TTG GTT TAC ACT GTC GGT
Phe Pro Gly Ile Ala Ile Val Trp Gly Leu Val Leu Thr Val Leu Val Tyr Thr Val Gly

   220                     240                     260
CAC ATC TCT GGT GGC CAT TTC AAT CCT GCT GTC ACC ATT GCT TTT GCC TCC ACC AGA AGA
His Ile Ser Gly Gly His Phe Asn Pro Ala Val Thr Ile Ala Phe Ala Ser Thr Arg Arg

   280                     300                     320
TTC CCC TTG ATC CAG GTA CCA GCT TAT GTA GTA GCT CAA CTC CTA GGA AGC ATA CTT GCA
Phe Pro Leu Ile Gln Val Pro Ala Tyr Val Val Ala Gln Leu Leu Gly Ser Ile Leu Ala

   340                     360                     380
AGT GGA ACT CTG AGA CTA TTA TTT ATG GGG AAT CAT GAC CAG TTT TCA GGA ACA GTC CCA
Ser Gly Thr Leu Arg Leu Leu Phe Met Gly Asn His Asp Gln Phe Ser Gly Thr Val Pro

   400                     420                     440
AAT GGG ACT AAC CTG CAG GCT TTT GTG TTT GAA TTC ATA ATG ACC TTT TTC CTC ATG TTC
Asn Gly Thr Asn Leu Gln Ala Phe Val Phe Glu Phe Ile Met Thr Phe Phe Leu Met Phe

   460                     480                     500
GTC ATA TGC GGG GTT GCC ACC GAT AAC AGA GCG GTT GGT GAG TTG GCT GGG ATT GCA ATT
Val Ile Cys Gly Val Ala Thr Asp Asn Arg Ala Val Gly Glu Leu Ala Gly Ile Ala Ile

   520                     540                     560
GGG TCG ACA TTA CTG CTG AAT GTG ATT ATT GGA GGG CCA GTG ACA GGA GCA TCA ATG AAC
Gly Ser Thr Leu Leu Leu Asn Val Ile Ile Gly Gly Pro Val Thr Gly Ala Ser Met Asn

   580                     600                     620
CCA GCT AGA AGC CTA GGA CCT GCT TTT GTA CAC GGT GAA TAC GAA GGA ATA TGG ATA TAT
Pro Ala Arg Ser Leu Gly Pro Ala Phe Val His Gly Glu Tyr Glu Gly Ile Trp Ile Tyr

   640                     660                     680
TTG TTG GCA CCT GTT GTG GGG GCC ATA GCT GGA GCA TGG GTA TAC AAC ATC GTT AGG TAC
Leu Leu Ala Pro Val Val Gly Ala Ile Ala Gly Ala Trp Val Tyr Asn Ile Val Arg Tyr

   700                     720                     740
ACA GAC AAG CCA TTG AGT GAG ATC ACC AAG AGT GCT TCT TTC CTC AAA GGC CGT GCT GCC
Thr Asp Lys Pro Leu Ser Glu Ile Thr Lys Ser Ala Ser Phe Leu Lys Gly Arg Ala Ala

   760                     780                     800                     820
TCC AAA TAA TTTATATGAGCAAATGGAGACACTCAATAAATTTTTCCTTGTAATAGTAATTAGAAAAAAATAAATA
Ser Lys ***

      840                     860                     880                     900
TGAATGAAAGAAATCACTCAAAGAAACTACGCTATGCTGTTTCATTTCCTTGTTTTATGAAAGGTCATGCACTTGAATA

      920                     940                     960
TCATCTCCCTCTTTTTCCTCTCTATTTGGATTAAAAATAAATGATTTTTGA
```

Figure 2. Nucleotide sequence of nodulin-26 of soybean. The amino acid sequence of the only open-reading frame is shown under the DNA sequence. The 5' 125 nucleotides in the non-coding region were determined from another clone p1F11 which was obtained by screening the cDNA library with the 5' *Pst*1 fragment of p1B1. p1F11 clone was sequenced up to the *Kpn*I site at position 294 and was found to be identical to p1B1. The putative signal sequence is underlined by a solid line. The cleavage site for the signal peptidase was determined using the rules of Heijne (29). The three polyadenylation signals (AATAAA) are underlined by dashed lines. *** : stop codon.

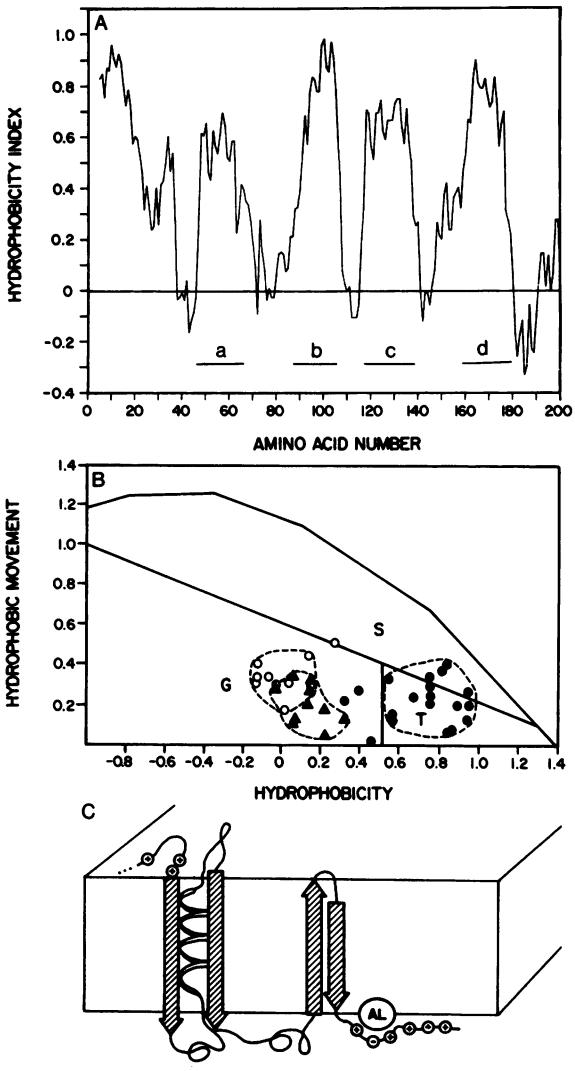

Figure 3. (A) Hydrophobicity analysis of the amino acid sequence of nodulin-26. Hydrophobicity values for individual amino acids were those of the normalized consensus hydrophobicity scale of Eisenberg (18). Values were averaged over 10 amino acids and the result recorded at the fifth position of that window. The window was moved by increments of 1. Domains a, b, c and d represent hydrophobic regions of sufficient hydrophobicity (>0.42) and length to traverse a membrane. (B) Hydrophobic moment plot of the putative transmembrane α-helix of nodulin-26. Each data point represent the average over amino acids n-4 to n+5. The window of ten residues was moved by increments of 1. Filled circles represent residues 88 to 107 (Domain b); open circles residues 108 to 117; and triangles residues 77 to 88. Amino acids 89 to 107 can be folded into an α-helix configuration and are proposed

to span the peribacteroid membrane. Data points in the G region of the graph represent globular domains of the protein, whereas the T region contains transmembrane domains and the S region surface associated domains (see Ref. 18). (C) Proposed secondary structure and topology of nodulin-26 in the pbm. AL, acidic lipids.

To confirm that the hybridizing clones were indeed coding for pbm nodulins, eight clones containing different size inserts in the *Pst*I site of pBR322 were chosen at random and used to hybrid-select polyA$^+$ RNA from three-week-old nodules. Hybrid-released RNAs were translated and their products immunoprecipitated by antisera against purified pbm, pbf or nodule cytoplasmic fractions. The corresponding translation products of six clones (including p1B1) were immunoprecipitated only by pbm antiserum (results not shown). One showed no reaction with any of the antisera, and one was precipitated by all sera. Clone p1B1 selects an mRNA encoding a protein of apparent molecular mass of 26.5 kDa (nodulin-26) (Figure 1). Purified pbm contains several nodulins in the 26 to 29 kDa range (7).

The nucleotide sequence of p1B1 (Figure 2) contains an open-reading frame capable of coding for a 22.5 kDa protein. The amino acid sequence of nodulin-26 is extremely hydrophobic (see Figure 3A) and may account for its lower migration rate on $NaDodSO_4$-PAGE as in the case of nodulin-24 (8). The methionine codon located at position 147 has all the attributes of an eucaryotic initiator codon (20). It is flanked by the sequence ANNATGR (where R = purine) which is found around 62% of the eucaryotic initiator methionines (20). Nodulin-26 possesses at its amino terminus a segment that fulfills all the requirements of a signal sequence (21), namely the presence of at least one charged residue followed by an hydrophobic segment and finally by a helix-breaking residue(s) and a cleavage site (see Figure 2). In the case of nodulin-24, the signal can be cleaved *in vitro* co-translationally (8). No attempts were made to cleave the signal peptide of nodulin-26.

Clone p1B1 was verified to be nodule-specific by Northern blot analysis with RNA purified from nodule and root tissues (results not shown).

Pbm nodulin-26 is a transmembrane protein

Secondary structure analysis of nodulin-26 suggests that it is a transmembrane protein. Nodulin-26 displays four regions of sufficient hydrophobicity (hydrophobicity index >0.42, see ref. 18) and length to span the membrane (Figure 3A). However, only domain B can be folded in a stable α-helix. It is defined as a transmembrane region from the hydrophobic moment

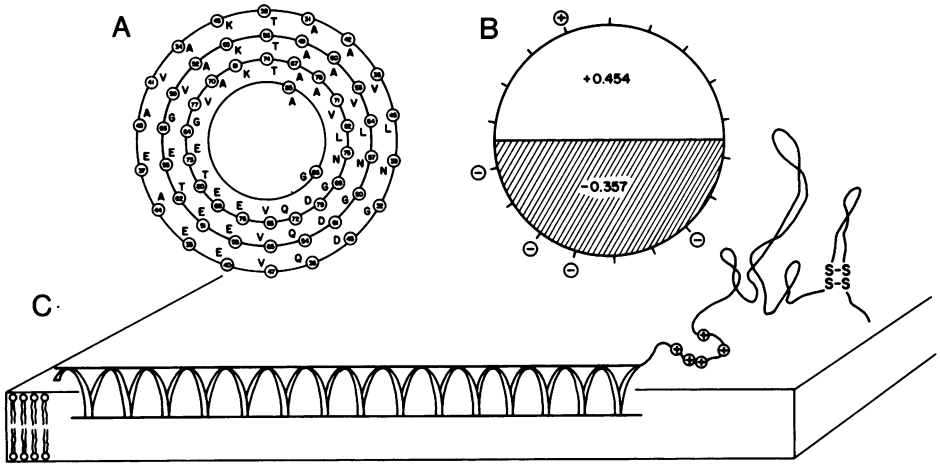

Figure 4. Analysis of the membrane associated domain of nodulin-24. (A) Helical wheel diagram of the α-helix from amino acid 31 to 86. The amino terminus is located on the outermost ring on the diagram, and the carboxyl terminus on the innermost one. Numbers indicate amino acid position from the amino terminus of the unprocessed protein. Values in each half of the helical wheel projection of the helix in (B) represent the average hydrophobicity of their respective half-helix. (-) and (+) indicate negatively and positively charged residues. (C) Proposed topology of nodulin-24 at the surface of pbm. The upper surface of the membrane faces the peribacteroid fluid whereas the lower surface faces the cytoplasm.

plot (18) (Figure 3B). Hydrophobic domains A, C and D have predominantly β-sheet structures, the length of which is sufficient to traverse the membrane. The existence of transmembrane β-sheets has recently been demonstrated in other systems (22,23). The above features allow us to propose a transmembrane structure of this protein as shown in Figure 3C. The relative orientation of nodulin-26 in pbm cannot be determined since it is not known whether the signal sequence is cleaved or not.

Comparison with the structure of nodulin-24

We analyzed the secondary structure of nodulin-24 in order to find any common features among pbm nodulins that may be involved in their specific targetting. The primary structure of nodulin-24 (8) can be folded into an α-helix of 55 amino acids (from residue 31 to 86) encompassing three 18 amino acid repeats (Figure 4). When viewed down the longitudinal axis, the helix has a remarkable property of exposing the same amino acids at exactly the same angle over the entire length of the helix (Figure 4). Only two amino acids substitutions have occurred: Ala has replaced Thr at position 44

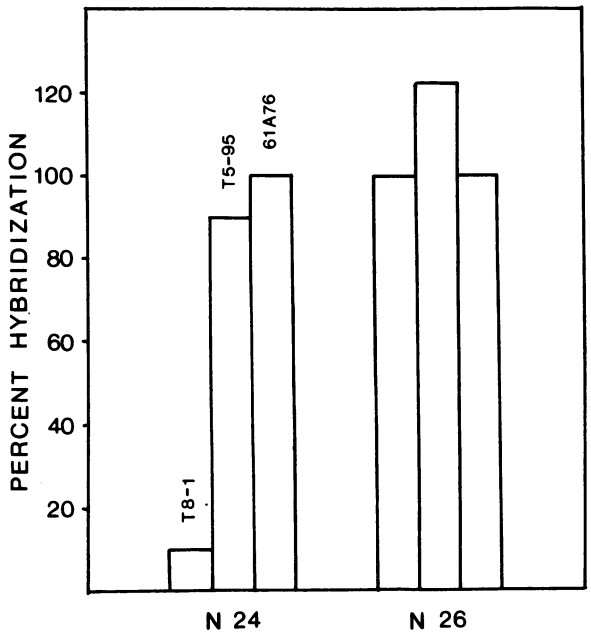

Figure 5. Hybridization of nodulin-24 and -26 cDNA probes with RNA dot blots of total polysomal RNA extracted from nodules formed by Tn5 generated mutants of *B. japonicum*. Mutant T8-1 forms nodules that contain infection threads but no pbm compartment, and mutant T5-95 nodules that form pbm but that do not reduce nitrogen. Radioactivity retained on the filter by RNA extracted from the T8-1 and T5-95 mutant-induced nodules is indicated as percent of wild-type (*B. japonicum* 61A76).

and Gly at position 48. These substitutions conserve the properties of the replaced amino acids. This unique distribution of amino acids confers the helix a strong amphiphilic character. A 0-180° cross-section of the helical wheel displays an average hydrophobicity of + 0.454 (Figure 4B), whereas the 180-360° section has an hydrophobicity of - 0.357 and comprises nine negative charges over the 55 amino acids (with no positive charge). Amino acids 87 to 147 of nodulin-24 are mainly hydrophilic and show no striking secondary structure features.

These characteristics of nodulin-24 define a protein which resides at the surface of pbm, with the hydrophobic half of the helix embedded in the lipophilic section of the membrane, while the charged hydrophilic half faces the peribacteroid fluid. No transmembrane segment could be identified. This orientation of nodulin-24, with respect to the pbm, results from the presence and cleavage of the signal sequence at the amino terminus of the

protein (see ref. 24). In order for this protein to be on the cytoplasmic face of pbm, a transmembrane domain would be required at the amino terminus. No amino acid sequence homology exists between nodulin-24 and -26. Their secondary structures do not share significant similarities either (see Figures 3 and 4). Despite these differences, these proteins are targetted to the same subcellular compartment. Either the targetting uses features that are not detected in these analyses, or different targetting mechanisms are involved for each of these two pbm nodulins. The latter is a likely possibility since they are expressed at different stages of nodule development.

Pbm nodulins are induced at two different stages of peribacteroid membrane development

We addressed the question of whether different mechanisms are involved in the induction of pbm nodulins by determining the levels of expression of nodulin-24 and -26 in nodules arrested at different stages of pbm biosynthesis. Mutant T5-95 induces the formation of nodules of normal ultrastructure with abundant pbm-enclosed bacteroids. However, due to the lack of nitrogenase induction, these nodules do not fix nitrogen (9). Mutant T8-1 induces normal size nodules that contain infection threads but do not have intracellular pbm compartments with bacteroids due to a block in release and proliferation of the invading bacteria (N. Morrison and D.P.S. Verma, manuscript in preparation). Nodulin-24 mRNA levels were severely depressed in T8-1-induced nodules while normal levels were detected in T5-95 nodules, suggesting that expression of nodulin-24 is coordinated with the synthesis of pbm. Nodulin-26 was expressed at almost full level irrespective to whether bacteria were released or not, indicating that its induction occurs independantly from that of nodulin-24, and possibly, by a different mechanism. This data suggests the presence of at least two developmental stages in the induction of pbm nodulins during internalization of *Rhizobium* leading to symbiosis. Moreover, the distinct structural features observed in these two pbm nodulins indicates that they may be targetted independently (probably by different mechanisms) to the pbm compartment.

DISCUSSION

A large amount of membrane is synthesized during endosymbiosis of *Rhizobium* since up to 5,000 pbm compartments per cell are formed in soybean nodules (25). Furthermore, this membrane acquires new properties with the incorporation of pbm nodulins (4,7). Once membrane biogenesis is

induced, how is the intracellular flow of vesicles directed to allow discrimination between the pbm and the plasma membrane since targetting of pbm nodulins is pbm specific (7)? N-linked glycosylation and amino acid sequence features are not involved in this process since the glycosylation signal Asn-X-Thr/Ser is absent from nodulin-24 and since these two nodulins do not share significant primary or secondary structure homologies.

Important changes occur in the pathways of membrane/organelle biosynthesis following infection by *Rhizobium*. Vacuoles and peroxisomes disintegrate following the release of bacteria into root cells (26). The pbm also acquires strongly acidic phospholipids that are not found in endoplasmic reticulum, mitochondrial or tonoplast membranes of nodules (27). Clusters containing three or four basic residues in a group of 10 to 12 amino acids are found adjacent to the membrane associated domain in both nodulins 24 and 26. Interaction of pbm nodulins with these pbm specific phopholipids may provide one of the bases for their segregation into the pbm.

The developmental blocks produced by Tn*5* mutants allowed us to determine at which stages the different pbm nodulins are expressed. Accumulation of mRNAs coding for nodulin-24 clearly depends on *Rhizobium* release and differentiation. Since the peribacteroid compartment may have acquired distinctive characteristics from the plasma membrane by the time nodulin-24 is synthesized, specific targetting of this nodulin to pbm may not require it to be recognized as pbm-destined by some particular feature. Robertson and Lyttleton (28) have observed that large numbers (2.3 coated vesicles/um infection thread) of vesicles fuse with the membrane near the tip of the infection thread prior to the release of bacteria. At the same developmental stage, only 0.1 coated vesicles/um were observed to fuse with the plasma membrane at the base of the infection thread and in the surrounding regions. Evidently, the presence of bacteria directs the transfer of vesicles, and consequently of pbm nodulins, to their vicinity. The exact role of these pbm nodulins in symbiotic nitrogen fixation remains to be elucidated.

ACKNOWLEDGEMENTS

We wish to thank Dr. Meng-Kang Fan for his help at various stages of this work and Miss Yvette Mark for her excellent secretarial assistance. We also thank Dr. Patrick Argos who independently analyzed the nodulin-24 structure for possible transmembrane features. This work was supported by

an Operating and a Strategic research grant from the Natural Sciences and Engineering Research Council of Canada. MGF was supported by an NSERC fellowship.

Abbreviations: pbm, peribacteroid membrane; pbf, peribacteroid fluid

* To whom correspondence should be addressed

REFERENCES

1 Verma, D.P.S., Long, S. (1983) in: International Review of Cytology, ed K. Jeon, Academic Press, Supp 14, pp 211-245
2 Werner, D., Mellor, R.B., Hahn, M.G., Grisebach, H. (1985) Z. Naturforsch. 40c : 179-181
3 Werner, D., Morschel, E., Kort, R., Mellor, R.B., Bassarab, S. (1984) Planta 162 : 80-16
4 Verma, D.P.S., Kazazian, V., Zogbi, V., Bal, A.K. (1978) J. Cell Biol. 78 : 919-936
5 Robertson, J.G., Warburton, M.P., Lyttleton, P., Fordyce, A.M., Bullivant, S. (1978) J. Cell Sci. 30 : 151-174
6 Robertson, J.G., Lyttleton, P. (1984) J. Cell Sci. 69 : 147-157
7 Fortin, M.G., Zelechowska, M., Verma, D.P.S. (1985) EMBO J. 4 : 3041-3046
8 Katinakis, P., Verma, D.P.S. (1985) Proc. Natl. Acad. Sci. USA 82 : 4157-4161
9 Rostas, K., Sista, P.R., Stanley, J., Verma, D.P.S. (1984) Mol. Gen. Gen. 197: 230-235
10 Verma, D.P.S., Nash, D.T., Schulman, H.M. (1974) Nature (London) 251 : 74-77
11 Korman, A.J., Knudsen, P.J., Kaufman, J.F., Strominger, J.L. (1982) Proc. Natl. Acad. Sci. USA 79: 1844-1848.
12 Fuller, F., Kunstner, P.W., Nguyen, T., Verma, D.P.S. (1983) Proc. Natl. Acad. Sci. USA 80 : 2594-2598
13 Maniatis, T., Fritsch, E.F., Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual (Cold Spring Harbor)
14 Laemmli, U.K. (1970) Nature New Biol. 227 : 680-685
15 Yannisch-Perron, C., Vieira, J., Messing, J. (1985) Gene 33 : 103-119
16 Sanger, F., Nicklen, S., Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74 : 5463-5467
17 Argos, P., Narayana, S.V.L., Nielsen, N.C. (1985) EMBO J. 4: 1111-1117
18 Eisenberg, D. (1984) Ann. Rev. Biochem. 53 : 595-623
19 Fuller, F., Verma, D.P.S. (1984) Plant Mol. Biol. 3: 21-28
20 Kozak, M. (1983) Microbiol. Rev. 47 : 1-45
21 Watson, M.E.E. (1984) Nucleic Acids Res. 12 : 5145-5164
22 Paul, C., Rosenbusch, J.P. (1986) EMBO J. 4 : 1593-1597
23 Kleffel, B., Garavito, R.M., Banmeister, W., Rosenbusch, J.P. EMBO J. 4 : 1589-1592
24 Wickner, W.T., Lodish, H.F. (1985) Science 230 : 400-407
25 Bergersen, F.J., Goodchild, D.J. (1972) Aust. J. Biol. Sci. 26 : 741-756.
26 Kijne, J.W. (1975) Physiol. Plant Pathol. 5 : 75-79
27 Mellor, R.B., Christensen, J.M.E., Bassarab, S., Werner, D. (1985) Z. Naturforsch. 40c : 73-79
28 Robertson, J.G., Lyttleton, P. (1982) J. Cell Sci. 58: 63-78
29 von Heijne, G. (1986) Nucl. Acids Res. 14: 4683-4690