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Nodulin-24 Follows a Novel Pathway for Integration into the Peribacteroid Membrane in Soybean Root Nodules*

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Nodulin-24 is a nodule-specific protein of the peribacteroid membrane (PBM) in soybean. It has an apparent molecular mass of 33 kDa while its full-length cDNA encodes a polypeptide of only 24 kDa. *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 microsequencing of the [^{3}H]leucine-labeled processed peptide. Fusion of the signal sequence of nodulin-24 with the β-glucuronidase peptide prevented co-translational cleavage of the signal sequence although the translocation of the fused protein into microsomes occurred co-translationally. Trypsin treatment of membrane-translocated nodulin-24 did not result in any alteration in size suggesting that the newly synthesized peptide is fully protected in the membrane vesicle. Fusion of nodulin-24 with β-glucuronidase also showed no change in size following trypsin treatment, suggesting that nodulin-24 has no membrane-spanning region. In addition, *in vitro* synthesized nodulin-24 was present in the supernatant fraction after sonication of microsomal membranes. Mature nodulin-24, on the other hand, is not solubilized from PBM by sodium carbonate (pH 11) or EGTA and is soluble only in detergent. These data suggest that nodulin-24 is synthesized as a lumenal protein in the endoplasmic reticulum and post-translationally attached to the membranes *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 as this protein shares characteristics with membrane lipoproteins of many pathogenic bacteria.

Successful invasion of legume roots by rhizobia, followed by endocytosis of the bacteria into the host cytoplasm, results in the development of root nodules effective in nitrogen fixation (Verma, 1992; Brewin, 1991). Rhizobia are segregated inside the host cell into a subcellular compartment surrounded by a membrane, the peribacteroid membrane (PBM),[1] of host origin (Verma *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).

Many nodule-specific host proteins (nodulins) have been isolated (Delauney and Verma, 1988) and some of them, *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). We have demonstrated that both amino and carboxyl ends of nodulin-26 face the host cell cytoplasm (Miao *et al.*, 1992). On the other hand, nodulin-24 appears to be associated with the PBM and was suggested to be located on the surface facing the bacteroids (Fortin *et al.*, 1987). The nodulin-24 gene contains five exons, three of which (exons 2–4) 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-kDa 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 kDa, 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 COOH-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 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 or not. 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. The machinary to cleave the signal sequence is conserved between animals and plants (Chrispeels, 1991).

In this study, we examined 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 microsequencing of [^{3}H]leucine-labeled *in vitro* translation product. The requirements for signal peptide cleavage were tested by protease-protection assay of nodulin-24 and β-glucuronidase fusion proteins. The results suggest that nodulin-24 is synthesized on the ER-bound ribosomes and is released into the lumen of the ER. Further post-translational processing, presumably in Golgi, attaches this protein to the membrane. Its overall hydrophobic and amphipathic 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.

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[1] The abbreviations used are: PBM, peribacteroid membrane; TX-100, Triton X-100; ConA, concanavalin A; PAGE, polyacrylamide gel electrophoresis; ER, endoplasmic reticulum; PCR, polymerase chain reaction.

EXPERIMENTAL PROCEDURES

Materials—Soybean (*Glycine max* L. cv. Prize) root nodules were obtained as described (Cheon *et al.*, 1993). *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.

Plasmid Constructions—For *in vitro* transcription, a series of fusion constructs of nodulin-24 and β-glucuronidase were made as follows: a *Hin*dIII-*Bam*HI 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). Constructs T1 and T2 were made by cloning cDNA fragments containing sequences corresponding to exon 1, and exons 1–2, respectively, of the nodulin-24 gene (see sequence accession no. M10595) into *Xba*I and *Sma*I-cut pBI221. The fragment containing exon 1 was obtained by digestion of pN24 with *Xba*I and *Dra*I, 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 *Pst*I and mungbean nuclease followed by digestion with *Xba*I. The entire length of the nodulin-24 cDNA, except the termination codon, was amplified by polymerase chain reaction and fused with the β-glucuronidase gene, resulting in the formation of construct T3. All the above constructs were subcloned into pGEM2 for *in vitro* transcription.

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.

In Vitro Translation and Processing of the Products—*In vitro* synthesized transcripts were translated in a rabbit reticulocyte lysate using either [^{35}S]methionine or [^{3}H]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 β-glucuronidase 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 (0.25 M sucrose, 10 mM Tris-HCl, 150 mM NaCl). Protease digestion with either L-1-tosylamide-2-phenylethyl chloromethyl ketone treated trypsin or proteinase-K (final concentration of 100 μg/ml) was carried out on ice for 1 h in the absence or presence of Triton X-100 (TX-100) at a final concentration of 1%. ConA-Sepharose 4B (Pharmacia LKB Biotechnology Inc.) binding was performed following immunoprecipitation protocol and replacing protein A with ConA-Sepharose. *In vitro* translated products were subjected to ten 5-s pulses of sonication (Sonifier 450, Branson Sonic Power Co., Danbury, CT) at maximum output and then centrifuged at 356,000 × *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 EnH^{3}ance (DuPont-New England Nuclear).

Microsequencing—The [^{3}H]leucine-labeled nodulin-24, co-translationally processed in the presence of microsomal membranes, was resolved in a 10% SDS-polyacrylamide gel, and eluted by soaking in 0.1% SDS. The sample was adsorbed on a glass fiber filter that had been precoated with polybrene 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.

Western Blotting—PBM was prepared as described (Fortin *et al.*, 1985). The isolated PBM proteins were either extracted with 10 mM sodium carbonate, pH 11, 10 mM EGTA, or 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 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.).

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.

RESULTS

Cleavage of Signal Sequence and Topology of Nodulin-24—Nodulin-24 has been shown to be co-translationally processed into a 20-kDa 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 [^{35}S]methionine in the absence (Fig. 1, *lane 1*) or presence (Fig. 1, *lane 2*) of microsomal membranes, and immunoprecipitated with polyclonal antibody against nodulin-24 (see "Experimental Procedures"). As expected, nodulin-24 was processed into a 20-kDa polypeptide in the presence of microsomal membranes. Tryptic digestion of the translation mixture did not change the size of this polypeptide (Fig. 1, *lane 3*), although trypsin together with a nonionic detergent digested the processed nodulin-24 completely (Fig. 1, *lane 4*). This suggests that no part of the processed nodulin-24 protrudes from the ER into the cytoplasm.

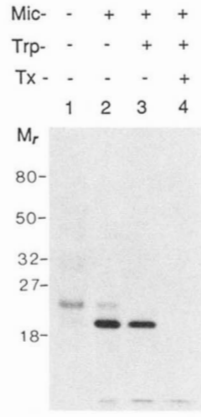

FIG. 1. ***In vitro* translation and processing of nodulin-24.** Nodulin-24 cDNA was transcribed *in vitro* using bacteriophage T7 polymerase, and the resulting transcript was translated *in vitro* using reticulocyte lysate and [^{35}S]methionine in the absence or presence of microsomal membranes. The translation product was immunoprecipitated with antibody against a synthetic peptide corresponding to one of the repeated domains of nodulin-24. Translation products were assayed by tryptic digestion with and without the addition of TX-100. *Mic*, canine pancreatic microsomal membranes; *Trp*, trypsin; *Tx*, Triton X-100.

In order to 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. 2*A* 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 [^{3}H]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 in the deduced sequence of nodulin-24, if the cleavage occurred as predicted above. The data suggest that nodulin-24 is co-translationally processed into a mature polypeptide of 122 residues with arginine at the amino terminus.

The Entire Nodulin-24 Sequence Is Required for Signal 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 gene (Fig. 3*A*). Construct T1 contained the exon-1 sequence encoding the putative signal peptide plus 5 more residues fused with the β-glucuronidase gene; T2 had, in addition to the T1 sequence, the exon-2 sequence encoding 18 residues (repetitive domains in nodulin-24, Katanikis and Verma, 1985) while T3 had the whole nodulin-24 coding region fused with β-glucuronidase. The *in vitro* translation product of

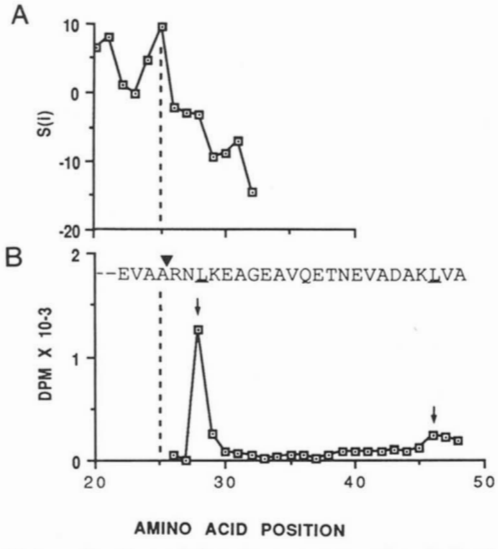

FIG. 2. **Determination of the cleavage site of nodulin-24 signal sequence.** *Panel A* shows the prediction of the cleavage site of signal sequence based on the method of von Heijne (1983). *S(i)* values are obtained from calculations at the indicated amino acid positions. The most probable cleavage site with the highest *S(i)* 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. [^{3}H]Leucine-labeled translation product of nodulin-24 mRNA was resolved by SDS-PAGE, and processed nodulin-24 peptide was eluted and subjected to microsequencing. Radioactivity released at each cycle of Edman degradation was determined. The *arrows* indicate the peaks of radioactivity corresponding to leucine residues present in the native sequence.

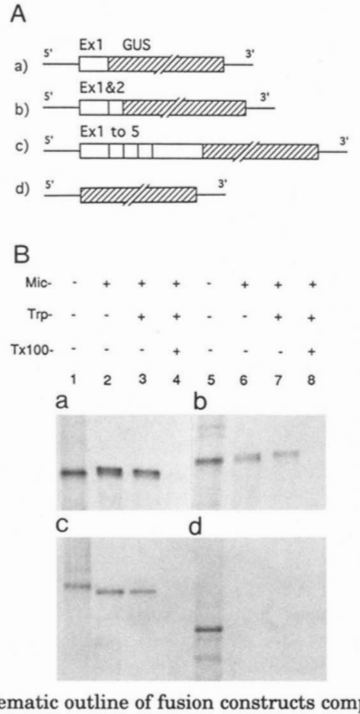

FIG. 3. *A*, schematic outline of fusion constructs comprising different domains of nodulin-24 and β-glucuronidase. Different lengths of nodulin-24 cDNA (*open boxes*) were fused in-frame with β-glucuronidase gene (*hatched boxes*) to obtain constructs T1 to T3 (*a–c*). *Ex*, exon; *GUS*, β-glucuronidase gene. *B*, *in vitro* co-translational translocations of the fusion proteins. *In vitro* transcription and translation were carried out as in Fig. 1. Translation products were immunoprecipitated with antibody against β-glucuronidase.

β-glucuronidase mRNA alone could not be translocated into microsomal membranes as expected (Fig. 3*B*, *panel d*) and served as a control. When T1 was transcribed and translated *in vitro*, cleavage of the signal sequence was not observed. In-

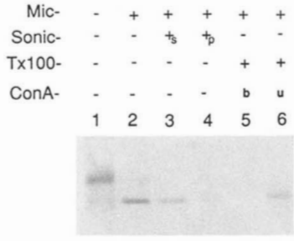

FIG. 4. **Presence of nodulin-24 in the lumen of microsomes and its lack of ConA binding.** After *in vitro* translation, the reaction mixtures were centrifuged, and half of the pellets were sonicated and then centrifuged to separate membranes (*lane 4*) and lumenal proteins (*lane 3*), while the other half were processed for ConA binding. *Lane 5*, ConA-bound fraction; *lane 6*, ConA-unbound fraction. *Sonic*, sonication; *ConA*, concanavalin A.

stead, the size of the band increased due to co-translational glycosylation of β-glucuronidase (see Fig. 4; 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 trypsin-protection assay showed complete digestion of the products. However, cleavage occurred only 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 almost full-length of nodulin-24 flanking the cleavage site.

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. 3*A*) should have a stop transfer domain which would be detectable in the protease-protection assay. In that event, the entire β-glucuronidase protein should be located outside the membrane vesicles and thus become accessible to trypsin. However, no digestion of T3 product was observed (Fig. 3*B*). 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 membrane or in the lumen of the ER. To test its location, we subjected microsomal membranes following *in vitro* translation, to sonication and centrifugation, and separated the microvesicles from the supernatant (Fig. 4, *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 but is present in the lumen. However, mature nodulin-24 isolated from root nodules is tightly attached to the 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 (Fig. 5), while TX-100 solubilized it along with the PBM (data not shown). 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.

No co-translational glycosylation of nodulin-24 was detected using ConA (Fig. 4, *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. 6). 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 homolo-

gous domain to the lipid attachment site of prokaryotic membrane lipoproteins at carboxyl-terminal region (Table I; 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.

DISCUSSION

Nodule development involves extensive membrane proliferation in the infected cells to enclose invading bacteria. 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. The expression of nodulin-26 and nodulin-24 is altered in ineffective nodules or nodules devoid of bacteria (Morrison and Verma, 1987). If the vesicular transport of PBM proteins is retarding using antisense expression of *rab7* gene, the late endosomes accumulate in the perinuclear region and these compartments become lytic, degrading endocytosed bacteria (Cheon *et al.*, 1993).

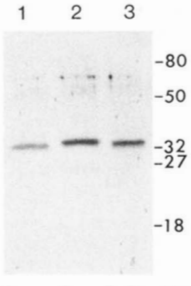

FIG. 5. **Tight association of nodulin-24 with the PBM.** *Lane 1*, PBM without any treatment; *lane 2*, PBM washed with sodium carbonate (pH 11); *lane 3*, PBM washed with EGTA. Each pellet from different treatments was resolved by SDS-PAGE, blotted, and probed with nodulin-24 antibody.

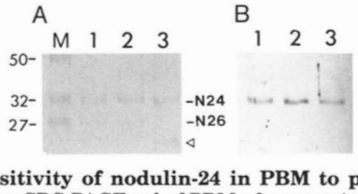

FIG. 6. **Insensitivity of nodulin-24 in PBM to protease digestion.** *Panel A* is a SDS-PAGE gel of PBM after proteinase-K digestion, and *panel B* is a Western blot of the gel in *panel A* reacted with nodulin-24 antibody. Nodulin-26 was also identified by Western blotting (data not shown). Proteinase-K was added to the PBM fraction, and the mixture was sonicated and incubated as described under "Experimental Procedures." *M*, molecular markers in kDa; *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.

Nodulin-24 and nodulin-26 are targeted to the PBM via different mechanisms since nodulin-26 has no cleavable signal sequence (Miao *et al.*, 1992) while nodulin-24 was shown to have a cleavable signal sequence. The amino acid sequences of nodulin-23 (Mauro *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 Ala^{20} and Glu^{21} for nodulin-23, and between Ala^{25} and Arg^{26} 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 increases its molecular mass from 20 to 33 kDa (Fortin *et al.*, 1985). Based on a hydropathy plot, the repeated domains at the 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 cDNA were fused with the β-glucuronidase reporter gene. When the entire cDNA of nodulin-24 was fused with the β-glucuronidase gene and the membrane-translocated product was treated with trypsin or proteinase-K (Fig. 3*B* (*d*)), neither nodulin-24 nor β-glucuronidase was digested by the protease. This result suggested that either nodulin-24 has hydrophobic interactions with membrane lipids and is attached to the membrane 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. 4). These data suggest that nodulin-24 in the ER is not membrane bound but becomes attached to the membrane post-translationally along the sorting route to PBM. Proteinase-K treatment of PBM vesicles after sonication (Fig. 6) resulted in partial digestion of nodulin-26, while nodulin-24 was unaffected. Since nodulin-24 does not have any membrane spanning region, it may be covalently bound to and embedded in the membrane lipids which may be also responsible for the increase of its molecular mass. These lipids may also protect it from proteolytic digestion.

There are a few examples of lipids that act as membrane anchors. The variant surface glycoprotein of the parasitic protozoan, *Trypanosoma brucei*, is linked to the membrane via the glycosyl-phosphatidylinositol (GPI) moiety (Low, 1989). Many

TABLE I

Presence of a consensus lipid attachment site in nodulin-24

A prokaroytic membrane lipoprotein lipid attachment site was found in nodulin-24 sequence with no mismatch. In this motif (Bairoch, 1991) the first 6 residues can be any amino acid except D, E, R, or K. The following 2 residues can be any 2 amino acids of L, I, V, M, F, S, T, A, and G. The 9th residue should be one of the following: I, V, M, S, T, A, G, or Q. The 10th position can be any of A, G, or S. A cysteine residue in the last position of this motif is invariant and serves as the lipid attachment site.

Accession no.	Membrane lipoproteins	Lipid attachment motif
M12163	*E. coli* lipoproteins-28	LLLAGILLAGC
X57402	*E. coli* lipoprotein-34	GVSLVLLLAAC
M22859	*E. coli* osmB lipoprotein	AITLAMSLSAC
X05123	*E. coli* pal lipoprotein	IALPVMAIAAC
X51393	*Pseudomonas* lppL lipopeptide	LALLAGSIAAC
M84922	*Pseudomonas* endoglycanase	ASVAALMLAGC
X17337	*Streptococcus* amiA protein	VLLAAGVLAAC
M10595	*Glycine max* nodulin-24	FPSSLGGSVSC

Consensus: ~(D, E, R, K)6(L, I, V, M, F, S, T, A, G,)2(I, V, M, S, T, A, G, Q)(A, G, S)C

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 any consensus sequence for these modifications. However, it has a region homologous to the lipid-binding domain (Table I) of bacterial lipoproteins (Bairoch, 1991; 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 a 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; see for review, Chrispeels, 1991). Fusion of the nodulin-24 signal sequence with β-glucuronidase (construct T1) did not allow cleavage of the signal sequence in *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, the cleavage still did not occur (Fig. 3*B*). Studies on co-translational translocation and signal peptidase processing using human preproapolipoprotein-A (Folz and Gordon, 1987; Nothwehr *et al.*, 1989) suggested that the NH_2-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 β-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 β-glucuronidase may lack a structure recognized by signal peptidase.

Thus, 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.

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