Topology and Phosphorylation of Soybean Nodulin-26, an Intrinsic Protein of the Peribacteroid Membrane

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Abstract. Soybean nodulin-26, a homologue of bovine eye lens major intrinsic protein (MIP-26), is an integral protein of the peribacteroid membrane in symbiotic root nodules. It comprises 271 amino acids with six potential transmembrane domains and lacks an amino-terminal signal sequence. A full-length nodulin-26 cDNA and its various deletion derivatives were transcribed in vitro after linking them to bacteriophage T_3 promoter. In vitro translation of these transcripts in a rabbit reticulocyte lysate, in the presence or absence of canine pancreatic microsomal membranes, suggested that nodulin-26 is cotranslationally inserted into the microsomes without a cleavable signal peptide. The first two transmembrane domains (103 amino acids) of the protein are sufficient for microsomal membrane insertion. Membrane-translocated nodulin-26 binds to Con-A and is sensitive to endoglycosidase-H treatment, suggesting that it is glycosylated. Native nodulin-26 from root nodules retains its sugar moiety as it, too, binds to Con-A. Chemical cleavage mapping at cysteine residues, a trypsin protection assay, and the Con-A binding affinity of nodulin-26 suggested that both the NH_2 and COOH termini of this protein are on the cytoplasmic surface of the peribacteroid membrane, while the glycosidic residue is on the surface of the membrane facing the bacteroids. In vitro phosphorylation experiments showed that nodulin-26 is a major phosphorylated protein in the peribacteroid membrane. This phosphorylation is mediated by a Ca^{2+}-dependent, calmodulin-independent protein kinase located in the peribacteroid membrane. Externally supplied acid phosphatase dephosphorylates this protein, but alkaline phosphatase does not. Based on its homology with several eukaryotic and prokaryotic channel-type membrane proteins, nodulin-26 may form a channel translocating specific molecules to the bacteroids during endosymbiosis in legume plants.

EFFECTIVE interaction between *Rhizobium* and legume plants gives rise to the root nodule, a unique organ where nitrogen is fixed symbiotically. While nodule morphogenesis and the induction of some of the early nodule-specific host genes encoding nodulins can be triggered by signals produced by *Rhizobium* in response to its host (Truchet et al., 1991; see Verma, 1992), the release of *Rhizobium* from the infection thread is essential in order to induce most late nodulin genes (Verma et al., 1988). The bacteria are enveloped in a host-derived plasma membrane, the peribacteroid membrane (PBM),[1] and have no direct contact with the host-cell cytoplasm (Verma et al., 1978; Robertson et al., 1978). Formation of the PBM is essential for maintenance and function of rhizobia inside the host cells. Failure to develop PBM may trigger host defense responses and cause degeneration of the invading bacteria, rendering nodules ineffective in nitrogen fixation (Werner et al., 1985).

To sustain the symbiotic state, constant metabolic flow of carbon and nitrogen as well as other metabolites must be maintained between the plant and the microsymbiont. Biochemical and genetic evidence has shown that dicarboxylic acids are the primary carbon source supplied to the bacteroids by the plant (see Dilworth and Glenn, 1984). The flow of these compounds is controlled by the PBM. Several carbon and amino acid transport systems have been identified in the PBM using isolated peribacteroid units (Day et al., 1990). None of these putative transporters has been isolated and characterized at the molecular level. Since these transport functions are unique to the nodule, nodule-specific proteins (nodulins) in the PBM most likely fulfill these transport functions.

Although the PBM is derived from plasma membrane, it acquires a number of features which differ from the host plasma membrane (Verma et al., 1978; Fortin et al., 1985; Verma and Fortin, 1989). Several PBM nodulins have been identified in soybean (Fortin et al., 1985). The induction of different PBM nodulins requires different signals; blocking nodule development at specific stages by bacterial mutants results in two distinct patterns of PBM nodulin gene expression (Morrison and Verma, 1987).

As one of the PBM nodulins, nodulin-26 (Fortin et al., 1987; Sandal and Marcker, 1987) shares significant amino

1. *Abbreviations used in this paper*: PBM, peribacteroid membrane; PCR, polymerase chain reaction.

acid sequence homology with a number of intrinsic channel-type proteins from *Escherichia coli* (Muramatsu and Mizuno, 1989; Sweet et al., 1990), yeast (Van Aelst et al., 1991), *Drosophila* (Rao et al., 1990), plants (Guerrero et al., 1990; Johnson et al., 1990; Yamamoto et al., 1990), and mammals (Gorin et al., 1984; Kent and Shiels, 1990). Each of these proteins seems to consist of six putative membrane-spanning domains. The striking conservation of these proteins from *E. coli* to mammals has led to the proposal that these proteins may have derived from a common ancestor to play similar roles in diverse organisms (Baker and Saier, 1990); however, the function of only the *E. coli* channel protein has been established so far.

We are interested in the biogenesis of the peribacteroid membrane compartment in symbiotic nitrogen-fixing root nodules and the function of specific nodulins in the PBM. Because PBM nodulins are targeted to the PBM and not to the plasma membrane from which the PBM is derived, a specific mechanism appears to target these proteins to this de novo formed subcellular compartment. In this report, we show that nodulin-26, a major PBM protein, is cotranslationally inserted into membranes without cleavage of any signal sequence. The first two membrane-spanning domains of this protein are sufficient for membrane insertion of the peptide. Furthermore, we have demonstrated that nodulin-26 is cotranslationally glycosylated at a site in the loop that connects the third and fourth membrane-spanning domains facing the bacteroid. Both the NH_2 and the COOH terminals of this protein face the cytoplasm of the host cell, a topology similar to that proposed for MIP-26 (Gorin et al., 1984). Nodulin-26 is phosphorylated near COOH terminus by a kinase located in the PBM; this phosphorylation may be directly related to the regulation of nodulin-26 function in root nodules.

Materials and Methods

Plant Tissue

Soybean seeds (*Glycine max* L. cv. prize) were inoculated with *Bradyrhizobium japonicum* strain 61A76 and grown in vermiculite. Nodules were harvested 3 wk after inoculation. Control roots and leaf tissues were obtained from uninoculated 5- and 10-d-old seedlings, respectively.

Preparation of Plasma Membrane and PBM

PBM from nodules was isolated as described previously (Fortin et al., 1985). The PBM recovered from the interface of 34/45% sucrose gradient was washed once in cold TB (50 mM Tris-Cl, pH 8.0, 5 mM EDTA, 5 mM β-mercaptoethanol, 0.5 mM PMSF, once in 10 mM Na_2CO_3 (pH 10.5), and once again in cold TB, each washing followed by vortex mixing and centrifugation at 100,000 *g*. Plasma membrane from roots and leaves of control plants was purified essentially as described (Verma et al., 1978), using the same TB buffer and washing regimen.

Antibody Preparation and Western Blotting

Antibodies against PBM-specific proteins were obtained as described (Fortin et al., 1985). A 15-amino acid peptide corresponding to the highly antigenic NH_2-terminus of nodulin-26 between amino acid residue 16 to 28 (NVTKNTSETIQRSDS; Sandal and Marcker, 1987) was synthesized (Multiple Peptide Systems, San Diego, CA). This was further coupled to a carrier protein (keyhole hemocyanin), and injected into rabbits for antibody production. Antibodies against native nodulin-26 were prepared by injecting the rabbits with SDS-gel purified nodulin-26. The preimmune rabbit serum was used as control serum. Western blot analysis was done as described by Burnette (1981). Proteins were resolved on 15% SDS-PAGE and transferred onto nitrocellulose membrane. Nodulin-26 was detected using ECL Western blotting detection system (Amersham Corp., Arlington Heights, IL) with rabbit antibody (1:5,000 dilution) raised against purified soybean nodulin-26.

Isolation of Full-length Nodulin-26 cDNA Clone and DNA Sequencing

A soybean nodule cDNA library made in the λ Zap II vector (Delauney and Verma, 1990) was screened to isolate a full-length nodulin-26 cDNA clone (pNod-26) using a partial cDNA sequence for nodulin-26 (Fortin et al., 1987). The cDNA insert in pNod-26 was sequenced directly in the in vivo excised pBlueScript plasmid by the dideoxy nucleotide sequencing method (Sanger et al., 1977) using Sequenase version II (United States Biochemicals, Cleveland, OH). The full-length sequence in pNod-26 is longer than that previously reported by Sandal and Marcker (1987) and has been submitted to the EMBL/GENE BANK nucleotide sequence data base (Accession X04782).

Deletions and Mutations of Nodulin-26 cDNA by Polymerase Chain Reaction

Specific oligonucleotides used as primers at 3'-end of each construct to generate deletions and mutations corresponding to D-1 to D-7 (see Fig. 4 A) were: (D-1) 5'-TTAGTAGTTCTCGTTCACCACC-3', (D-2) 5'-TTAAGCAATGGTGACAGCAGG-3', (D-3) 5'-TTACTGGTCATGATTCCCCATAAA-3', (D-4) 5'-TTACTCACCAA-CCGCTCTGTT-3', (D-5) 5'-TGATGCTCCTGTCACTGGCCC-3', (D-6) 5'-TTATACCCAT-GCTCCAGC-3', and (D-7) 5'-TTATTTGGCGGCAGCACGGCCTTTGAGGAAAGCAGCACTCT-3'. The 5' primer used to amplify D-1 to D-7 was the M13 reverse primer. Therefore, all the fragments amplified from the pBlueScript-SK vector using these primers contained the T3 promoter at the 5' end of the coding sequences. The underlined sequences in D-7 primer denote the changed codons for alanine (GCU and GCC) where these were for serine in the native sequence (UCU and UCC). Polymerase chain reaction (PCR) was carried out according to the manufacturer's instructions (Perkin Elmer Cetus). 5 ng of pnod-26 was used as a template. A total of 30 cycles were run using an annealing temperature of 50°C. The amplified DNA products were separated on an agarose gel, extracted, and used as a template for in vitro transcription.

In Vitro Transcription

Nodulin-26 mRNA for in vitro translation was transcribed from the T_3 promoter in pBlueScript-SK in an in vitro transcription system. The full-length nodulin-26 cDNA was linearized by complete digestion with ApaI and used as a template. Different deleted and mutated derivatives generated by PCR were directly used as templates for transcription. The transcription and 5' capping reactions were carried out according to instructions provided by the supplier (Stratagene, La Jolla, CA), except that 50 U of RNasin were included in the reaction. Transcription was carried out at 37°C for 2 h. The DNA template was removed by the addition of 10 U of RNase-free DNase I for 10 min. The reaction was stopped by extraction with phenol/chloroform (1:1) followed by chloroform, and mRNA was precipitated by the addition of 0.1 vol of 3 M sodium acetate (pH 5.0) and 2.5 vol of ethanol at −20°C for 2 h. The mRNA was pelleted by centrifugation for 20 min at 4°C in a microfuge, washed with 75% ethanol, and dried under vacuum. The mRNA was redissolved in 25 μl of diethylpyrocarbonate-treated water, quantified by UV absorption, sized by formamide-agarose gel electrophoresis, and used for in vitro translation.

In Vitro Translation and Processing of Nodulin-26

In vitro synthesized RNA was translated in a micrococcal nuclease–treated rabbit reticulocytic lysate cell-free translation system (Promega Biotec, Madison, WI) at 30°C for 90 min. Nodulin-26 mRNA was included at a concentration of 15 μg/ml. For cotranslational processing of nodulin-26, canine pancreatic microsomal membranes (New England Nuclear, Boston, MA or Promega Biotec, Madison, WI) were included at the beginning of translation. [^{35}S]methionine was used as labeled amino acid in all translation reactions. In the case of posttranslational processing, the microsomal membranes were added after the completion of translation and treatment with RNase A (1 μg/ml), and the reaction was allowed to proceed for an additional 90 min. The membrane-associated translation products were pelleted by centrifugation at 48,000 *g* (Beckman TL-100 ultracentrifuge) for

20 min at 4°C. The membrane pellet was resuspended and washed once in 10 mM Na_2CO_3 (pH 10.5) and once in 20 mM Hepes buffer (pH 7.5). The resulting membrane pellets were either analyzed directly by SDS-PAGE and fluorography or resuspended in an appropriate buffer for further analysis.

For trypsin digestion, 1 μg of TPCK-treated trypsin (Sigma Chemical Co., St. Louis, MO) was added and the samples were incubated at room temperature for 30 min. Where indicated, Triton X-100 was included to a final concentration of 1%. To stop the digestion, soybean trypsin inhibitor (20 μg) was added to the reaction. Membrane-associated nodulin-26 translation products were deglycosylated with endoglycosidase-H. The membrane pellet was resuspended in 20 μl of 20 mM Hepes buffer (pH 7.5). Membranes were solubilized by the addition of 0.5 μl of 3 M sodium acetate (pH 5.2), 2 μl of 10% SDS, and incubation at 100°C for 5 min. Endoglycosidase-H (20 U; Boehringer Mannheim Biochemicals, Indianapolis, IN) was added to the solubilized sample for 3 h at 37°C.

Immunoprecipitation

Immunoprecipitation with antibody against PBM (Fortin et al., 1987) was carried out by adding 10 μl of antibody to the translation sample or solubilized membrane fraction as described by Anderson and Blobel (1983). The immunocomplexes were adsorbed to 25 μl of protein A–Sepharose 4B beads (Pharmacia-Biotech) at room temperature for 2 h. The beads were washed four times with 0.1% Triton X-100, 0.02% SDS, 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), and 5 mM EDTA at room temperature. After the final wash, 40 μl of SDS-gel electrophoresis sample buffer containing 50 mM DTT was added to the beads. The beads were centrifuged out and the supernatant was applied to an SDS-polyacrylamide gel. Con-A binding was performed under similar conditions. 10 μl of Con-A Sepharose 4B (Pharmacia LKB-Biotech, Piscataway, NJ) was incubated with the solubilized membrane fraction for 3 h at room temperature. After washing the Con-A bound proteins were eluted by boiling in SDS-gel sample buffer and subjected to SDS-PAGE. Electrophoresis was carried out on 12, 15, or 17.5% SDS-polyacrylamide gels. The gels were dried after fluorography with EnH^{3}ance (DuPont/NEN, Boston, MA) and exposed to Kodak XAR-5 film.

Peptide Cleavage at Cysteine Residues

Cleavage of nodulin-26 at cysteine residues was performed as described by Stark (1977). Nodulin-26 or trypsinized nodulin-26 was dissolved in 100 μl of 6.5 M guanidine chloride, 1 mM EDTA, 0.2 M Tris-acetate buffer, pH 8.0, and incubated for 30 min at 37°C. A 10-fold M excess of 2-nitro-5-thiocyanobenzoic acid over the total thiol groups in the protein was added and incubated for another 30 min at 37°C. The cyanylated protein was dialyzed against 50% acetic acid and lyophilized. Cleavage of the cyanylated protein was carried out in 6 M guanidium chloride, 0.1 M sodium borate buffer, pH 9.0, at 37°C for 16 h. The cleaved peptides were recovered by passing the reaction mixture through a spin column (BioRad Laboratories, Cambridge, MA) in the presence of 0.1 M NH_4 OH and dried by lyophilization. The peptides were resuspended in 1 mM Tris-HCl, pH 9.0, and 1 mM EDTA, and the samples were subjected to SDS-PAGE (Christy et al., 1989) followed by fluorography.

Phosphorylation and Dephosphorylation of PBM Proteins

Purified PBM was suspended in kinase buffer (20 mM Hepes, pH 7.9, 20 mM KCl, 1 mM $CaCl_2$, 10 mM $MgCl_2$, 0.1 mM EDTA, 2 mM DTT, 0.5 mM PMSF) at a protein concentration of 0.1 μg/μl. Phosphorylation of PBM proteins was started by the addition of 1 μCi of γ-^{32}P-ATP (specific activity 3,000 Ci/mmol; DuPont/NEN) to 20 μl of the PBM suspension and the reaction was allowed to proceed for 20 min at room temperature (Suzuki and Verma, 1989). The phosphorylated PBM was pelleted in a microcentrifuge and washed with distilled water. The washed membrane pellet was used for further dephosphorylation tests or was directly solubilized in 30 μl of 1× SDS sample loading buffer of which 10 μl was loaded on to a 15% SDS-polyacrylamide gel. After electrophoresis, the gel was dried and autoradiographed. For the protein kinase inhibition assay, 1 mM of EGTA or 1 mM of trifluoperazine (TFP) was TFP included in the reaction.

The dephosphorylation of PBM was performed by treatment of the phosphorylated PBM with exogenous acid phosphatase and alkaline phosphatase. The phosphorylated PBM was washed in ACP buffer (200 mM Na acetate, pH 5.0), pelleted by microcentrifugation, and resuspended in 20 μl of ACP buffer. 1 U of acid phosphatase from potato (Boehringer Mannheim Biochemicals) was added for 1 h at 30°C followed by pelleting of the membrane and solubilization in 30 μl of 1× SDS sample buffer. For alkaline phosphatase treatment, 1 U of calf intestine alkaline phosphatase (Boehringer Mannheim Biochemicals) in ALP buffer (500 mM diethanolamine, 0.5 mM $MgCl_2$, pH 9.0) was used.

Results

Nodulin-26 Is a Major Intrinsic Peribacteroid Membrane Protein in Soybean Root Nodules

A cDNA clone encoding nodulin-26 was initially isolated by reverse transcription of RNA enriched through immunoprecipitation of nodule polysomes by antibodies against soybean PBM (Forin et al., 1987). The in vitro translation product of RNA hybrid selected by this cDNA corresponded to a PBM protein of 26 kD and was recognized by PBM-specific antibodies (Fortin et al., 1985, 1987). The PBM, however, is derived from plasma membrane of the host cell and contains several protein bands common to the plasma membrane. A 26-kD peptide is also present in the plasma membrane of uninfected soybean root (Fortin et al., 1985). To determine its identity, antibodies were raised against a synthetic peptide corresponding to the most antigenic region of nodulin-26 (NH_2 terminal, see Materials and Methods) and were used to purify the native nodulin-26 from the PBM. An antiserum was then raised against the purified native nodulin-26 protein. Using this antibody, we have demonstrated (Fig. 1, lanes *1–3*) that nodulin-26 is indeed a PBM-specific protein, since no antigenically related proteins were detected in the plasma membrane fraction of soybean leaf or uninfected roots by Western blot analysis. The same results were obtained using antibody against the NH_2-terminal synthetic peptide (data not shown).

Solubility of nodulin-26 in various nonionic detergents showed (Fig. 1, lanes *4* and *5*) that virtually all the protein partitioned into the Triton-X114 detergent-rich phase at 30°C, indicating that nodulin-26 is a highly hydrophobic protein. The solubility of nodulin-26 and its ability to bind to a hydroxyapatite column (data not shown) were useful in purification. Denaturing of nodulin-26 in SDS sample buffer for 5 min at 100°C, a common step in the preparation of protein samples for SDS-PAGE, caused significant dimerization

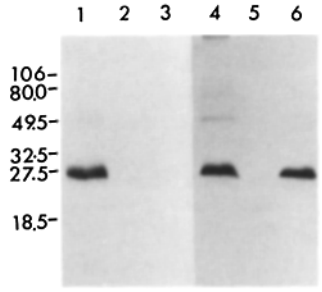

Figure 1. Western blot of different membrane protein fractions probed with antibody against soybean native nodulin-26. Soybean PBM (lane *1*), uninfected root plasma membrane (lane *2*), and leaf plasma membrane (lane *3*) were resolved on SDS-PAGE, transferred onto nitrocellulose membrane, and reacted with the antibody against nodulin-26; PBM was solubilized in 2% Triton X-114, and the solution was separated into aqueous and detergent-rich phases at 30°C. Samples from the detergent-rich phase (lane *4*) and aqueous phase (lane *5*) were heated at 100°C for 5 min in SDS sample buffer before being subjected to SDS-PAGE. Treatment of the sample in lane *6* is identical to that in lane *4*, except that the sample in lane *6* was incubated at 37°C for 15 min.

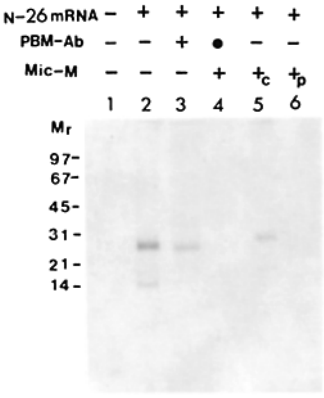

Figure 2. Synthesis and cotranslational insertion of nodulin-26 in microsomes. The nodulin-26 (*N-26*) cDNA, cloned downstream for the T_3 promoter in pBlueScript SK, was transcribed in vitro and translated in a rabbit reticulocyte lysate using [^{35}S]methionine in the presence or absence of microsomal membranes. The translation products were analyzed on SDS-PAGE and fluorographed. $^{+}$c, microsomal membranes added cotranslationally; $^{+}$p, microsomal membranes added posttranslationally; ●, control serum; Mic-M, microsomal membranes from dog pancreas; N-26 mRNA, nodulin-26 mRNA; PBM-Ab, antibodies against total peribacteroid membrane (PBM; Fortin et al., 1985). Molecular mass markers are indicated in kilodaltons.

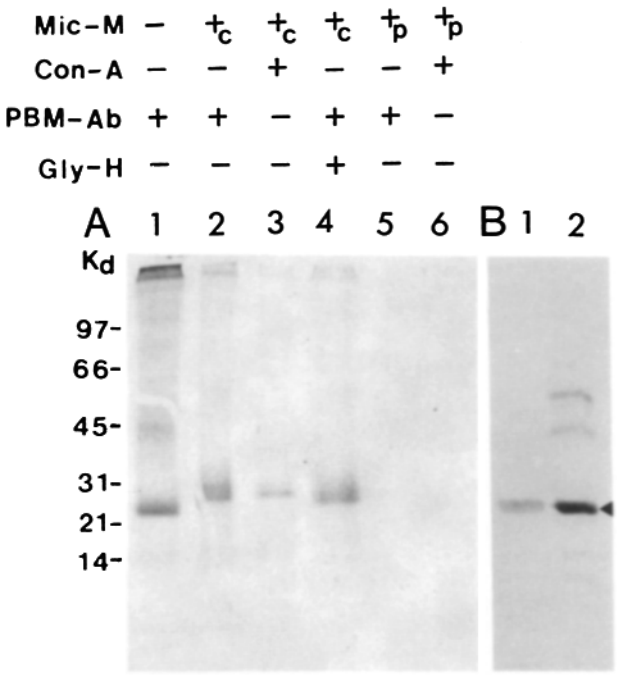

Figure 3. Cotranslational glycosylation of N-26: (*A*) in vitro. In vitro translation was carried out as in Fig. 2. Cotranslational (^{+}c) and posttranslational (^{+}p) addition of microsomal membranes. (*B*) Western blot of proteins resolved on SDS-PAGE from PBM (lane *1*) and Con-A precipitated proteins from PBM (lane *2*). Antibody against soybean native nodulin-26 was used. Gly-H, endoglycosydase H (see also Fig. 2).

of nodulin-26 as compared with the 37°C treatment of the sample (Fig. 1, lanes *4* and *6*). Longer heating of the sample at 100°C led to further oligomerization and eventually, polymers that failed to enter the separating gel were formed (Fig. 1, lane *4*). Oligomerization of MIP-26 (Wong et al., 1978) and TIP-26 (Johnson et al., 1990), homologues of nodulin-26, has also been reported.

Insertion of Nodulin-26 into Microsomal Membranes Occurs Cotranslationally without Detectable Cleavage of a Signal Peptide

Using a partial sequence of nodulin-26 (Fortin et al., 1987) as a probe, full-length cDNA of nodulin-26 was obtained by screening a soybean nodule cDNA library constructed in the λ Zap II vector (Delauney and Verma, 1990). This sequence was longer at both the 5′- and 3′-nontranslated ends than the sequence reported by Sandal and Marcker (1987). The encoded polypeptide of 271 amino acids has the potential to form six membrane-spanning domains and lacks an apparent amino-terminal signal sequence. To define the mode of membrane insertion and the potential signal and/or structural requirements for this event, nodulin-26 cDNA was ligated downstream of T_3 polymerase promoter in the pBlueScript vector. In vitro transcription carried out with linearized recombinant plasmids in the presence of the 5′ mRNA capping analogue ($7MeG_{ppp}G$) resulted in RNA capable of translation in a cell-free system. The translation product from nodulin-26 mRNA in reticulocyte lysate consisted of a major peptide with an apparent M_r of 26,000 and some smaller minor bands (Fig. 2, lane *2*) which appear to result from internal initiation during translation (see below and Discussion). The major translation product (26 kD) was immunoprecipitated by the antibody, whereas the fast moving peptides did not bind to the antibody (Fig. 2, lane *3*). Addition of dog pancreatic microsomal membranes at the start of translation resulted in the synthesis of a peptide with an apparent M_r of 27,000 (Fig. 2, lane *5*). This product was associated with the membranes and was not removed by washing with Na_2CO_3 (see also Fig. 5). An increase in the size of the translation product in the presence of microsomes (Fig. 2, lane 5), rather than the decrease in size that would result from signal cleavage (Katinakis and Verma, 1985), suggested that nodulin-26 does not contain a cleavable signal sequence. These data are consistent with the lack of a hydrophobic amino acid sequence and potential cleavage site in this peptide based on the general rules of signal cleavage (von Heijne, 1986). When microsomes were added after the translation was complete and the reaction was terminated with RNase A treatment, no insertion of nodulin-26 into the membrane was observed (Fig. 2, lane *6*). This demonstrated that insertion of nodulin-26 into the microsomes does not occur posttranslationally.

Nodulin-26 Is Glycosylated Cotranslationally

The increase in apparent M_r of the translation product from ~26 to 27 kD with the cotranslational addition of microsomes (Fig. 2, lane *5* and Fig. 3 *A*, lane *2*) suggests that the newly synthesized peptide is cotranslationally modified by the addition of a sugar moiety. This was confirmed by binding of the translation product to Con-A–coupled Sepharose-4B (Fig. 3 *A*, lane *3*). When digested with endoglycosidase-H, an enzyme that cleaves asparagine-linked core mannose-type oligosaccharides, the 27-kD band was reduced to ~26 kD (Fig. 3 *A*, lane *4*), similar to the mass of the initial translation product in the absence of microsomes. These results show that nodulin-26 is glycosylated cotranslationally in rough microsomes and further support that no signal cleavage occurs during the targeting of this protein.

To determine if mature nodulin-26 in the PBM is also glycosylated, soybean PBM was solubilized in 1% SDS and diluted with a nonionic detergent (TX-100), then mixed with

Con-A–coupled Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) beads. Con-A-bound material was eluted with SDS sample buffer, resolved on SDS-PAGE, blotted onto a nitrocellulose membrane, and probed with nodulin-26 antibody. Fig. 3 *B* shows binding of nodulin-26 to Con-A, demonstrating that nodulin-26 is indeed a glycoprotein in the PBM with the same mannose-type sugar residues as in the initial translation product. No extensive posttranslational glycosylation appears to occur after the synthesis of nodulin-26 in the ER. To eliminate the possibility that nodulin-26 might form a heterologous complex with any other glycoprotein(s) that bind(s) to Con-A and coprecipitates with this protein, PBM proteins resolved on SDS-PAGE were transferred to a nitrocellulose membrane and probed with biotinylated Con-A (Sigma Chemical Co.), followed by reaction with HRP-labeled Streptavidin. Detection of HRP using the ECL-Western blotting system (Amersham Corp.) showed that nodulin-26 binds directly to Con-A (data not shown).

The First Two Membrane-spanning Domains of Nodulin-26 Are Sufficient for Membrane Insertion

To be translocated across membranes, secreted proteins generally require a cleavable signal peptide, while transmembrane proteins usually lack a cleavable signal sequence. To decipher the signal and structural requirements for nodulin-26 insertion into the membrane, a series of deletions of nodulin-26 from the COOH-terminal were constructed using PCR (Fig. 4 *A*). Specific primers corresponding to different trans-membrane segments (see Materials and Methods) were synthesized and used as 3′-end primers in PCR. The M13 reverse primer pairing with the corresponding site in the pBlueScript vector was used as a 5′-end primer. Thus, each amplified fragment contained a T_3 polymerase promoter adjacent to the translation start codon. PCR-generated deletion fragments were purified on agarose gel and used directly as templates in a cell-free transcription system.

In vitro translation of RNA from different deletion constructs (encoding different membrane spanning domains) of nodulin-26 (Fig. 4 *A*) was carried out in the presence of microsomal membranes. As shown in Fig. 4 *B*, deletions containing the fifth (D-5) and sixth (D-6) transmembrane segments insert into the membrane with approximately the same efficiency (Fig. 4 *B*, lanes *3* and *4*) as intact nodulin-26 (Fig. 4 *B*, lane *1*). Shortening the peptide from the COOH terminal or mutation of potential phosphorylation sites (serine to alanine) at this end had no effect (Fig. 4 *B*, lane *2*) on the efficiency of membrane insertion. Further deletions from the COOH terminus seemed to slightly decrease the efficiency of membrane insertion (Fig. 4 *B*, lanes *5–7*). The peptide that contains only the first two trans-membrane domains from the amino terminus was still detectable in the microsomal fraction, indicating that the first two membrane-spanning domains are sufficient for insertion of nodulin-26 into the microsomal membrane. This suggests that the signal required for membrane insertion is contained within the first 103 amino acids of nodulin-26. We observed that several smaller distinct peptides were synthesized and inserted into the microsomal membrane (Fig. 4 *B*); these peptides are most likely generated by internal initiation rather than specific peptide cleavage, as the sizes of these peptides decrease in proportion to the step deletions from the COOH terminus. Furthermore, the sizes of fast-moving peptides

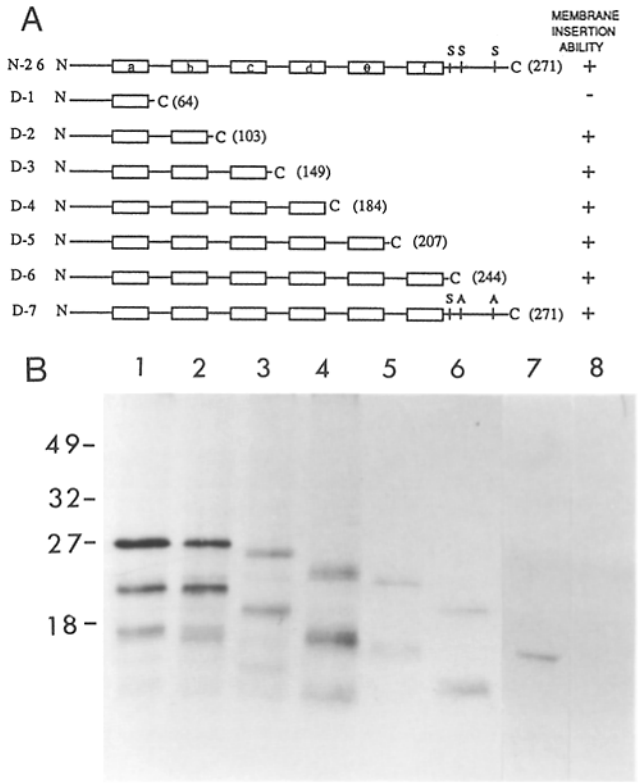

Figure 4. Requirements of specific sequences for membrane insertion. RNA transcripts made in vitro from various deleted and mutated fragments of nodulin-26 (*A*) were translated in vitro in a rabbit reticulocyte lysate in the presence of microsomal membranes (*B*) using [^{35}S]methionine as described in Materials and Methods. A nodulin-26 (N-26) full-length cDNA (Fortin et al., 1987; Sandal and Marcker, 1987) having six membrane-spanning domains (see also Fig. 8) was deleted/mutated using PCR and different oligonucleotides as 3′-primers (see Materials and Methods) to generate DNA fragments D1–D7 using PCR. Of the three potential (serine) phosphorylation sites in nodulin-26, two were mutated to alanine as shown in D-7. The RNA transcribed from various deleted/mutated fragments was translated in vitro in the presence of microsomal membranes. The membrane-associated peptides were obtained after centrifugation and washing and were analyzed on SDS-PAGE. (Lane *1*) N-26; (lanes *2–8*) translation products of transcripts from D-7 to D1. *S*, serine; *A*, alanine.

translated from the intact nodulin-26 mRNA approximately match the predicted internal methionine positions in the protein (Fortin et al., 1987; Sandal and Marcker, 1987). Because these peptides are detected in the membrane fraction, specific amino acid sequences may not be essential for membrane insertion of these proteins. Rather, the overall hydrophobic structure of the peptide appears to be important for membrane insertion and anchoring. Because 14- and 22-kD bands, presumably devoid of the NH_2 terminus, are still inserted in the membrane (Fig. 4 *B*), other regions in the hydrophobic domains could also act as membrane insertion signal(s).

Topology of Nodulin-26 in the Peribacteroid Membrane

Treatment of the microsomal fraction with a limited amount of trypsin revealed an 18-kD peptide that resisted trypsin digestion (Fig. 5, lane *3*). Solubilization of the microsomal

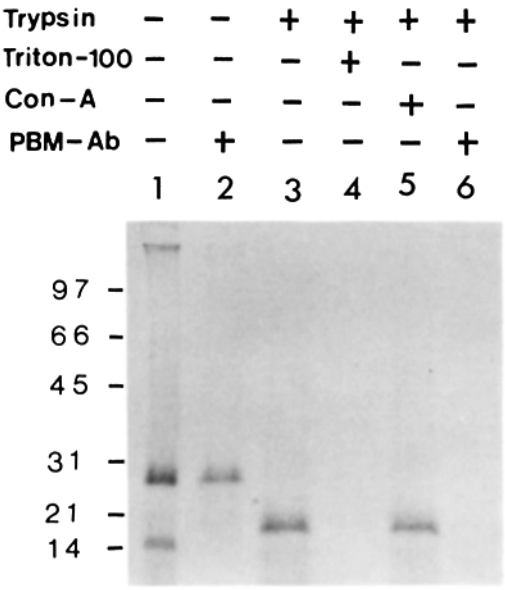

Figure 5. Sensitivity of membrane-inserted nodulin-26 to trypsin. In vitro transcribed nodulin-26 mRNA was translated in a rabbit reticulocyte lysate in the presence of dog pancreatic microsomal membranes, and the products were analyzed by SDS-PAGE (see Materials and Methods) after treatments as indicated over each lane (see Figs. 2 and 3 for symbols). Both Con-A binding and immunoprecipitation were carried out after solubilization of the trypsin-treated microsomal fraction.

membrane with TX100 resulted in complete digestion of translated product (Fig. 5, lane *4*), confirming that it is indeed translocated and inserted into the microsomal membrane. The protected 18-kD peptide bands to Con-A (Fig. 5, lane *5*), suggesting that the asparagine at position 150 of the protein is probably glycosylated and both NH_2 and COOH terminals are digested by trypsin. Since the NH_2 terminus is in the cytoplasm, the putative glycosylation sites at this end (see Fig. 8) are not glycosylated. The 18-kD fragment is not recognized by antibody against PBM proteins (Fig. 5, lane *6*), suggesting that the antigenic domains face the outside of the membrane and are sensitive to trypsin digestion. This is consistent with antigenicity analysis which predicted the NH_2-end of nodulin-26 to be the only antigenic domain in this protein. Accordingly, the fast-moving peptides (resulting from internal initiation) are also not recognized by the PBM antibody (Fig. 1, lanes *2* and *3*). However, antibodies raised to a synthetic peptide from the carboxy end are able to recognize native nodulin-26 (Weaver et al., 1990).

To confirm the sidedness of nodulin-26 in the membrane, we carried out topological mapping of the protein by chemical cleavage at cysteine residues with and without pretreatment with trypsin. As represented diagrammatically in Fig. 6 *A*, we expected that the two internal cysteine residues at positions 55 and 172 of nodulin-26 would generate distinct peptides after trypsin digestion and cysteine cleavage. Chemical cleavage at cysteine residues of in vitro translated nodulin-26 in the presence of microsomal membranes generated three peptides with apparent M_r of ~11, 10, and 6 kD (Fig. 6 *B*, lane *1*). The same experiment after trypsin treatment exhibited two peptides with apparent M_r of ~11 and 8 kD (Fig. 6 *B*, lane *2*). Generated from the middle of the protein, the 11-kD peptide remained unchanged by trypsin treatment before cysteine cleavage. The 6- and 10-kD peptides located at the NH_2 and COOH terminals are apparently sensitive to trypsin treatment; the COOH-terminal 10-kD fragment was converted to a smaller fragment of ~8 kD and the NH_2-terminal 6-kD fragment was not detected (presumably due to the presence of several potential trypsin-digestible sites). These data suggest that both NH_2 and COOH terminals of the nodulin-26 are outside of the microsomes and accessible to trypsin, whereas the sugar moiety (added cotranslationally at asparagine residue 150) faces the lumen of the microsomes.

Nodulin-26 Is Phosphorylated by a Protein Kinase Located in the PBM

A protein kinase capable of phosphorylating a synthetic peptide that corresponds to the last 15 amino acids of the carboxy-terminal sequence of soybean nodulin-26 was reported to exist in both soluble and particulate fractions of the nodule, as well as in root and leaf extracts of soybean (Weaver et al., 1991). To determine the subcellular location of the protein kinase which phosphorylates native nodulin-26, we extracted soluble fractions from different tissues of soybean and used them to phosphorylate highly purified PBM proteins. Results in Fig. 7 showed that a purified PBM fraction, washed with Na_2CO_3 and thus free of any absorbed proteins, still contained the protein kinase activity that efficiently phosphorylated native nodulin-26. The addition of leaf, root, and even nodule soluble extracts did not change the degree of nodulin-26 phosphorylation as judged by band intensity, suggesting that either the kinase activity in the PBM is high enough to phosphorylate nodulin-26 without kinase activity from other soluble fractions, or that soluble extracts might not contain protein kinase activity capable of phosphorylating native nodulin-26. To determine whether or not our soluble extracts contained any protein kinase activity, we incubated the nodule soluble fraction with γ-^{32}P-ATP and found that the nodule soluble extract phosphorylated two proteins of different molecular weights, but had no activity in phosphorylating nodulin-26 (Fig. 7, lane *1*). To test for possible contamination of the PBM fraction by soluble proteins, we blotted PBM proteins onto a nitrocellulose membrane and probed with antibody against uricase, one of the major proteins in the soluble nodule extract (Bergmann et al., 1983); no trace of uricase protein was detected in the PBM fraction (data not shown). With extensive washing of the PBM fraction with Na_2CO_3, contamination with soluble proteins is also very unlikely. Using the protocol we developed for PBM isolation, the PBM is free of other endomembrane and soluble protein contaminations (Verma et al., 1978). Therefore, we propose that nodulin-26 is phosphorylated in planta by a protein kinase located in the PBM.

To confirm that the major phosphorylated protein of PBM of M_r of 26,000 is nodulin-26, half of the phosphorylated PBM sample was subjected to SDS-PAGE and Western blot assay. The size of the band reacting with nodulin-26 antibody was identical to that shown by autoradiography after phosphorylation (Fig. 7, *A* and *B*), as confirmed by immunoprecipitation of phosphorylated proteins using antibody against nodulin-26 (data not shown).

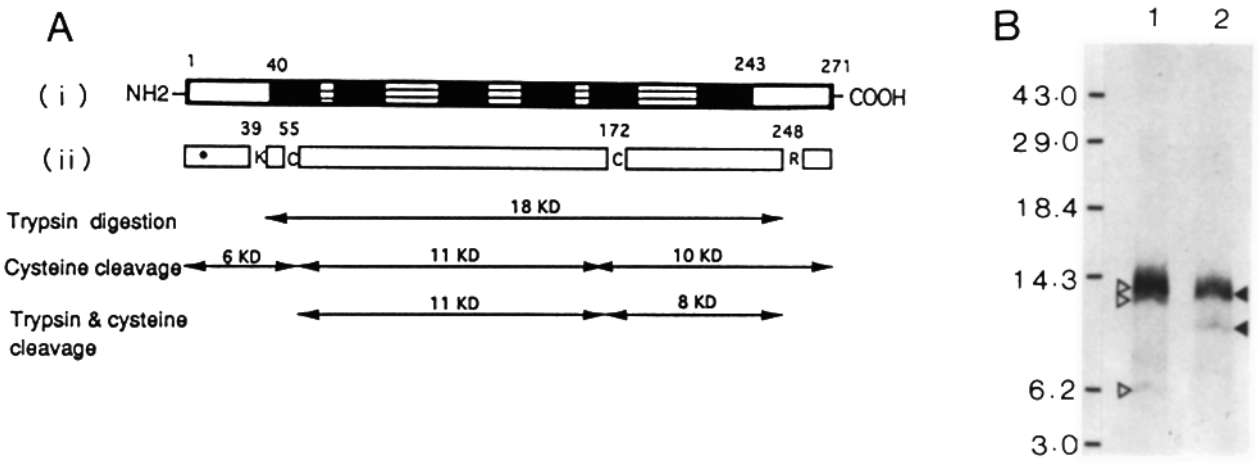

Figure 6. Chemical cleavage at cysteine residues and topological mapping of nodulin-26 in microsomal membranes. (*A*) Diagrammatic representation of the predicted products of trypsin digestion and cysteine cleavage. (*B*) In vitro translation product of nodulin-26 mRNA in the presence of microsomes, followed by cleavage at cysteine residues alone (lane *1*, *open arrowheads* correspond to three fragments of sizes of 6, 10, and 11 kD), and cysteine cleavage after trypsin digestion (lane *2*, *solid arrowheads* correspond to the fragments of ~8 and 11 kD). (See Materials and Methods for details).

The Protein Kinase Phosphorylating Nodulin-26 Is Ca^{2+} Dependent but Calmodulin Independent

To determine whether the activity of protein kinase phosphorylating nodulin-26 depended on Ca^{2+} or calmodulin, either EGTA (1 mM) or trifluroparazine (TFP), a calmodulin antagonist, was included in the reaction buffer. EGTA almost completely inhibited the phosphorylation of nodulin-26 (Fig. 7 *A*, lane *6* and Fig. 7 *C*, lane *2*), while TFP had no effect (Fig. 7 *C*, lane *3*). Although a phosphorylated 27-kD band is absent in Fig. 7 *A* (lane *6*), the presence of nodulin-26 protein was verified by reaction with antibody (*arrow*). This suggests that the activity of the protein kinase that phosphorylates nodulin-26 is Ca^{2+}-dependent but calmodulin-independent. When the PBM was dissolved in 2% Triton X-100 before reaction, the efficiency of nodulin-26 phosphorylation significantly decreased (Fig. 7 *C*, lane *4*). After removal of the detergent with Amberlite XAD-2 (Aldrich) or incorporating purified PBM proteins into liposomes, the efficiency of nodulin-26 phosphorylation was restored almost completely (data not shown), suggesting that an intact membrane structure and lipid bilayer might be necessary for the kinase to function properly.

We were unable to detect any endogenous phosphatase activity that dephosphorylates nodulin-26. It was interesting to note, however, that while alkaline phosphatase from calf intestine had no effect on phosphorylation, acid phosphatase from potato effectively dephosphorylated nodulin-26 (Fig. 7 *C*, lanes *5* and *6*, respectively). Acid phosphatase had no effect on other phosphorylated proteins in the PBM (data not shown).

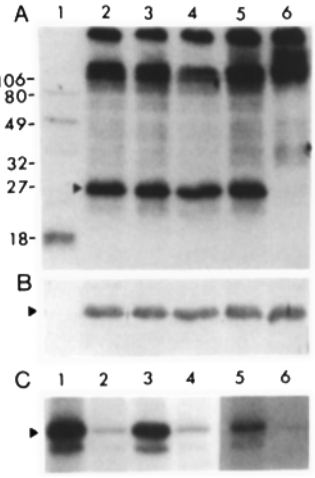

Figure 7. Phosphorylation of nodulin-26 by a protein kinase located in the PBM. (*A*) Autoradiogram of phosphorylated PBM proteins. (Lane *1*) Nodule soluble extract; (lane *2*) PBM; (lane *3*) PBM plus leaf soluble extract; (lane *4*) PBM plus root soluble extract; (lane *5*) PBM plus nodule soluble extract; (lane *6*) PBM plus EGTA (1 mM). (*B*) Western blot of the samples as in *A* reacted with antibody against nodulin-26. (*C*) Characterization of nodulin-26 phosphorylation. (Lane *1*) PBM; (lane *2*) PBM plus 1 mM EGTA; (lane *3*) PBM plus 1 mM TFP (trifluoperazine); (lane *4*) PBM in 2% Triton X-100; (lane *5*) phosphorylated PBM plus alkaline phosphatase; (lane *6*) phosphorylated PBM plus acid phosphatase. Arrowheads indicate the position of nodulin-26.

Discussion

Membrane Translocation and Insertion of Nodulin-26

Understanding the formation of intracellular compartments in eukaryotic cells is of great importance in cell biology. How specific proteins are targeted to a membrane compartment has helped to elucidate the biogenesis of various subcellular compartments. Different mechanisms govern the targeting and insertion of proteins into various organelle membranes (Lingappa, 1991). Vacuole or lysosome membrane proteins are generally synthesized in membrane-bound polysomes and inserted cotranslationally (Klausner, 1989). Some plasma membrane proteins are also posttranslationally inserted into membranes (Mueckler and Lodish, 1986). In the root nodules, the bacteroid compartment enclosed in PBM represents a new organelle which differs from other eukaryotic organelles both morphologically and functionally. Although posttranslational and cotranslational protein targeting routes have been hypothesized for PBM nodulins (Mellor and Werner, 1987), very little is known about how specific proteins are actually targeted to this subcellular compartment. Our data showed that nodulin-26 is cotransla-

tionally inserted into the membrane without signal cleavage. In contrast, targeting of another PBM nodulin, nodulin-24, involves signal sequence cleavage (Katinakis and Verma, 1985). The mechanism of insertion of nodulin-26 into the PBM is apparently similar to that of most ER, plasma membrane, and vacuole membrane proteins. This is consistent with our previous suggestion (Fortin and Verma, 1985; Verma and Miao, 1991) that PBM is initiated from engulfed plasma membrane through continuous fusion of endosome membranes during maturation of PBM.

Targeting of proteins to membranes requires specific signal sequences that are recognized by specific receptors (Wickner and Lodish, 1985). While secreted proteins and group I-type integral membrane proteins contain a cleavable signal sequence at the amino-terminal end, group II-type membrane proteins usually contain an internal uncleavable signal sequence (Singer, 1990). Insertion of proteins which span the membrane several times may involve multiple signal sequences (Anderson et al., 1983; Friedlander and Blobel, 1985). The internal signal sequence can act as a membrane insertion signal as well as a membrane anchor, while a cleavable sequence serves only as signal for membrane translocation. Lack of a cleavable signal in nodulin-26 suggests that internal signal sequence(s) are involved in directing the membrane insertion of this protein. The first two membrane-spanning domains (consisting of 103 amino acid residues) are able to insert into the membrane, suggesting that this region contains the signal for membrane insertion. Since both terminals of nodulin-26 are located on the cytoplasmic surface (Figs. 6 and 8), the first membrane-spanning domain of nodulin-26 appears to serve as a membrane insertion signal, while the second membrane-spanning domain may act as a stop transfer sequence (Lipp et al., 1989). The first transmembrane domain alone is unable to insert into membrane (Fig. 4, lane *8*), suggesting that stable membrane integration may require a second membrane-spanning domain which may help to anchor this peptide in the membrane.

Co- and Posttranslational Modifications of Nodulin-26: Glycosylation, Phosphorylation, and Topology in PBM

Residues at positions 16, 20, and 150 of nodulin-26 are potential N-linked glycosylation sites. Topological mapping of nodulin-26 showed that the residue at positions 16 and 20 are on the cytoplasmic side, while the residue 150 is translocated and faces the lumen of the microsomes as shown in Fig. 8. The first two sites are unlikely to be glycosylated because N-linked glycosylation is catalyzed by the enzyme oligosaccharyl transferase which is located in the ER membrane (Kornfeld and Kornfeld, 1985). Con-A binding of the 18-kD fragment which resists trypsin digestion and contains residue 150 but not 16 and 20 demonstrated that the asparagine at residue 150 is glycosylated. Nodulin-26 inserted into the PBM remains glycosylated, indicating that glycosylation may play a role in the function or subcellular location of this protein. TIP, a homologue of nodulin-26 from the tonoplast membrane of bean, is not glycosylated (Maeder and Chrispeels, 1984); whether MIP-26 is glycosylated or not is unclear due to contradictory reports (Brockhuyse and Kuhlmamn, 1978; Wong et al., 1978).

Among the proteins that share homology to nodulin-26, MIP-26 (Lampe and Johnson, 1990) and TIP (Johnson et al., 1990) have been demonstrated to be phosphorylated, but GlpF is not (Sweet et al., 1990). Using synthetic oligopeptide, Weaver et al., (1991) have also shown that soybean nodulin-26 is phosphorylated and they suggested that this phosphorylation is catalyzed by a Ca^{2+}-dependent protein kinase that is present in all parts of the plant. Our studies demonstrate that this kinase is Ca^{2+}-dependent and lipid-stimulated, and that its activity is confined to the PBM. Because we used native protein as substrate (as opposed to a synthetic peptide employed in studies by Weaver et al., 1991), our data more likely represent in vivo events. Localization of both nodulin-26 and protein kinase in the PBM may allow for efficient regulation of phosphorylation of this channel. TIP has also been suggested to be phosphorylated by a membrane-bound protein kinase (Johnson and Chrispeels, 1991). Phosphorylation of some proteins may facilitate membrane transcytosis (Casanova et al., 1990). Mutations created in nodulin-26 to replace serine residues at positions 262 and 270 did not have any effect on membrane insertion, however.

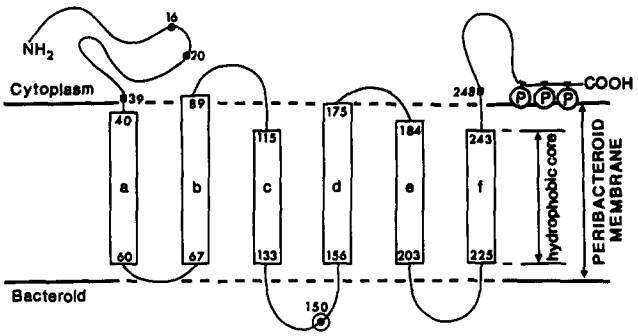

Figure 8. Proposed topology of soybean nodulin-26 in the peribacteroid membrane: (*a–f*) Six membrane-spanning domains, determined by computer analysis. (▼) Phosphorylation sites; (●) potential glycosylation sites that are not glycosylated; (⊙), glycosylation site facing the bacteroid is in fact glycosylated as shown by Con-A binding; (■) trypsin target sites closest to the first and last transmembrane domains; (*p*) phosphate residues interacting with the membrane.

Based on our topological mapping of in vitro translated nodulin-26 in microsomes and glycosylation and phosphorylation studies, we propose that the orientation of the native nodulin-26 in PBM is as shown in Fig. 8. This structure is consistent with the function of this protein as a channel. The expression of nodulin-26 occurs prior to and independent of nitrogen fixation (Morrison and Verma, 1987), suggesting that it participates in the transport of specific metabolites to support bacteroid function. Such transport of metabolites through PBM would create a concentration gradient between the peribacteroid fluid and the host cell to be controlled by nodulin-26 type channel(s), and active uptake by bacteroids from peribacteroid fluid could follow. This transport may be regulated by the phosphorylation of nodulin-26 as is the case in other known channels directly regulated by phosphorylation (Tabcharani et al., 1991).

Structure of Nodulin-26 and Evolutionary Conservation

Although proteins of the nodulin-26 family show similar hydropathic properties, the peptide folding and turning pat-

tern of nodulin-26 is similar to GlpF, while those of MIP-26 and JM7a are very similar (Verma et al., 1990). Moreover, the isoelectric point of nodulin-26 is similar to that of GlpF (pI 6.93 and 6.97, respectively), while those of MIP-26 and JM7a are similar (pI 9.94 and 9.61). Furthermore, the first half of the protein (membrane-spanning domains 1–3) shares sequence homology with the last half of the protein (membrane-spanning domains 4–6) among the members of this family (Pao et al., 1991), suggesting that intragenic gene duplication of an ancestral gene encoding three transmembrane domains has given rise to the six transmembrane domains of proteins in this family. The most highly conserved regions in this family are located in the loops between membrane-spanning domains 2 and 3 and domains 5 and 6, rather than within the membrane-spanning domains themselves. Structural analysis of the nodulin-26 gene revealed that membrane-spanning domains correspond to specific exons (Miao, G.-H., and D. P. S. Verma, manuscript in preparation). Amino acid residues on the surface of the membrane constitute domains interacting with the substrate(s) for most of the channel proteins. The degree to which amino acids are conserved in these regions suggests that these proteins are likely to perform similar roles in different organisms.

The subcellular locations of several members of the nodulin-26 family e.g., JM7a, Bib, and TobRB7, are not known. While MIP-26 is located in the plasma membrane of the eye lens cell, TIP is a vacuolar-type membrane protein induced during seed development. The homology between TIP and nodulin-26 (Johnson et al., 1990) suggests that the PBM compartment shares some features common to vacuoles. α-Mannosidase, a vacuolar marker enzyme, is found in the peribacteroid compartment (Mellor et al., 1984). Recently, a vacuole-type proteinase inhibitor has been identified as a nodulin of the peribacteroid space (Manen et al., 1991). Both vacuole and plasma membrane type ATPases (Bassarab et al., 1986; Blumwald et al., 1986) exist in this membrane, which has structural properties similar to the plasma membrane (Verma et al., 1978). Thus, the PBM appears to be a mosaic membrane possessing features of both the plasma membrane and the vacuolar membrane.

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