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# Immunolocalization of PsNLEC-1, a Lectin-Like Glycoprotein Expressed in Developing Pea Nodules[1]

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**The pea (*Pisum sativum*) nodule lectin gene *PsNlec1* is a member of the legume lectin gene family that is strongly expressed in infected pea nodule tissue. A full-length cDNA sequence of *PsNlec1* was expressed in *Escherichia coli* and a specific antiserum was generated from the purified protein. Immunoblotting of material from isolated symbiosomes revealed that the glycoprotein was present in two antigenic isoforms, PsNLEC-1A and PsNLEC-1B. The N-terminal sequence of isoform A showed homology to an eight-amino acid propeptide sequence previously identified from the cDNA sequence of isoform B. In nodule homogenates the antiserum recognized an additional fast-migrating band, PsNLEC-1C. Fractionation studies indicated that PsNLEC-1C was associated with a 100,000*g* nodule membrane fraction, suggesting an association with cytoplasmic membrane or vesicles. Immunogold localization in pea nodule tissue sections demonstrated that the PsNLEC-1 antigen was present in the symbiosome compartment and also in the vacuole but revealed differences in distribution between infected host cells in different parts of the nodule. These data suggest that PsNLEC-1 is subject to posttranslational modification and that the various antigenic isoforms can be used to monitor membrane and vesicle targeting during symbiosome development.**

The legumes are unique among flowering plants because most of the 20,000 species establish an $N_2$-fixing symbiosis with *Rhizobium* spp. (Allen and Allen, 1981), which allows them to exploit the bacterial capacity for biological $N_2$ fixation to convert atmospheric $N_2$ into a form that can be utilized for plant growth. Establishing this intimate symbiosis requires a complex series of molecular interactions and signal exchanges between plant and bacterium (Brewin, 1991). Nodule tissue is formed from a new meristem initiated in the root cortex in response to *Rhizobium*-derived signal molecules known as lipochitin oligosaccharides.

Tissue and cell invasion by *Rhizobium* involves a series of poorly understood developmental interactions, which progressively allow bacteria to colonize the root hair surface, the intercellular space, and the infection thread (a transcellular cellulosic tube within which bacteria grow and divide). Finally, bacteria are released through the plasma membrane into the host cytoplasm, where they remain enclosed by a plant-derived membrane that modulates their metabolism. In this "symbiosome" compartment, the rhizobia (now termed bacteroids) develop the capacity for $N_2$ fixation under the low-concentration $O_2$ conditions that prevail in the central tissue of the nodule. The product of fixed $N_2$ is excreted from symbiosomes as $NH_3$, which is then assimilated in the host cytoplasm and ultimately translocated to other parts of the plant.

Throughout this process, from root colonization to the assimilation of the products of symbiotic $N_2$ fixation, there is evidence for the involvement of legume lectins and lectin-like proteins. At least three separate locations and putative functions have been recognized for these proteins during the development of the legume root nodule. At the surface of root hairs, they may promote the aggregation of rhizobia in the infectible zone of the root (Díaz et al., 1995); in the nodule primordium, they may lower the threshold of response to *Rhizobium*-derived nodulation factors, thereby stimulating mitotic activity in root cortical cells (Bauchrowitz et al., 1996; Díaz et al., 1996; Brewin and Kardailsky, 1997); and in the central tissue of mature, $N_2$-fixing nodules they may function as part of a transient N reserve (Law, 1996a, 1996b).

Legume lectins were first identified as nonenzymatic, carbohydrate-binding components that could be isolated from seed extracts (Peumans and Van Damme, 1995). In seeds lectins are present in large amounts and accumulate in specialized storage vacuoles termed protein bodies (Nakamura and Matsuoka, 1993; Hinz et al., 1995). Different legumes have lectins with different carbohydrate-binding specificities, although all of these proteins show strong sequence homology and all belong to the same gene family (Rini, 1995; Kardailsky et al., 1996). The subunits of legume lectins have a similar conformational structure, despite differences in posttranslational processing and multimerization. However, the functional role of legume lectins is still unclear and much remains to be learned about their molecular interactions and possible functions in plant cell morphogenesis (Brewin and Kardailsky, 1997).

Elucidating the roles of legume lectins in the process of nodule development has been complicated by the discovery that several different lectin genes may be present and functional within a single species. Moreover, the posttrans-

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Abbreviations: ConA, concanavalin A; dpi, days postinfection; ECL, enhanced chemiluminescence; PBF, peribacteroid (symbiosomal) fluid; PBM, peribacteroid (symbiosomal) membrane; PSL, pea seed lectin; PsNLEC, *Pisum sativum* nodule lectin.

lational processing of a particular gene product may differ in different parts of the same plant. In pea (*Pisum sativum* L.), for example, three classes of genes belonging to the legume lectin family have been identified as RNA transcripts or protein products. PSL is encoded by a small gene family, of which only one gene (*Psl*) appears to be functional (Gatehouse et al., 1987). This gene is strongly expressed in developing seeds (Van Driessche, 1988). There is also weak expression of *Psl* in roots, and some lectin is secreted into the rhizosphere. It is also present on the surface of root hair cells, where it appears to accumulate *Rhizobium* and other bacteria (Díaz et al., 1995).

In addition to *Psl*, two other members of the legume lectin gene family have recently been identified in pea. Bud lectin (*Blec*) mRNA is expressed in the epidermis of vegetative apices, where it accumulates specifically in meristematic epidermal cells of vegetative and floral organs but not in the protodermal cells (Maiti et al., 1993; Mandaci et al., 1994). Nodule lectin (encoded by *PsNlec1*) was identified as a major component of pea nodule symbiosomes (Kardailsky et al., 1996). When compared with sequences of other legume lectins, it was shown that the complete cDNA sequence of *PsNlec1* is more closely related to *Blec* than to any other legume lectin (Kardailsky et al., 1996). Transcript expression studies indicated that *PsNlec1* was much more strongly expressed in the central infected tissue than in any other part of the plant. Vegetative lectins present in peanut (*Arachis hypogaea*) nodule tissues (Law, 1996a, 1996b) and in the bark of yellow wood (*Cladastris lutea*; Van Damme et al., 1995) and false acacia (*Robinia pseudoacacia*; Yoshida et al., 1994) have been proposed to function as storage components in these tissues under conditions of N abundance.

In this paper we report the heterologous expression of *PsNlec1* as a full-length cDNA clone in *Escherichia coli* and describe the use of a specific antiserum developed against this polypeptide as a probe to localize the corresponding antigen (PsNLEC-1) in infected nodule cells. We also show that different antigen isoforms of PsNLEC-1 exist in different cellular compartments and that these glycoprotein isoforms differ with respect to processing of the polypeptide and carbohydrate side chains. The symbiosome compartment is a unique element in the endomembrane system of infected nodule cells (Brewin, 1991) and, because PsNLEC-1 has proved to be the major glycoprotein component of pea nodule symbiosomes, it is likely to be useful as a cytological marker to investigate the vesicle-targeting pathways leading to biogenesis of the peribacteroid membrane.

## MATERIALS AND METHODS

### Heterologous Expression of PsNLEC-1

PsNLEC-1 protein was expressed in *Escherichia coli* using the pRSET expression system (Invitrogen, San Diego, CA), which incorporates an N-terminal 6-His tag and a peptide epitope from the bacteriophage T7 gene 10 protein. Oligonucleotides ATGGCTTTTTATCGCACAA (upstream) and GGAATTCATATGTTGGAGAC (downstream) were used to amplify the complete open reading frame of *PsNLEC-1* using nodule cDNA as a template for reverse transcriptase-PCR. Amplification was for 35 cycles with *Taq* polymerase (Perkin-Elmer) at 94°C for 30 s, 50°C for 1 min, and 72°C for 2 min. The PCR product was blunt-ended with T4-DNA polymerase, digested with *Eco*RI to produce a sticky end on the 3′ end of the open reading frame, and ligated with the pRSET-C plasmid cut with *Pvu*II and *Eco*RI. This produced clone Nlex1 and several similar clones.

When junction sites of the recombinant clones were verified by sequencing, all were found to have 1 or 2 bp missing at the 5′ end of the open reading frame (the site of blunt-end ligation). This created an undesired frame shift in the translation fusion. To restore the open reading frame, the *Bam*HI-*Eco*RI fragment of the clone Nlex1 (which was missing 2 bp) was ligated with the *Bam*HI-*Eco*RI-digested pRSET-A plasmid. This produced recombinant clone Nlex1.1. The entire open reading frame of the inserted segment in pNlex1.1 was verified by sequencing. *E. coli* strain BL21 (DE3), which carries a gene encoding T7 RNA polymerase under the control of the *lac-UV5* promoter (Tabor and Richardson, 1985), was transformed with the recombinant plasmid pNlex1.1, and the resulting transformant was given the strain name B817.

To produce the recombinant His-tagged PsNLEC-1 protein, a single colony of B817 cells was grown in Luria-Bertani medium containing 200 $\mu$g/mL ampicillin and 50 $\mu$g/mL kanamycin. The cells were incubated at 30°C with shaking to an $A_{600}$ of 0.6 unit. Synthesis of PsNLEC-1 was induced in cells by culturing for 2 h in the presence of 1 mM isopropyl $\beta$-D-thiogalactopyranoside. A 1-mL aliquot of the cell suspension was taken before induction and another aliquot was taken 2 h after induction. The rest of the cells from the induced culture were centrifuged, resuspended in 2 mL of PBS, and stored at −20°C.

To purify expressed PsNLEC-1 protein from *E. coli*, $Ni^{2+}$-charged resin (ProBond, Invitrogen) was used. The cells expressing PsNLEC-1 were first lysed under denaturing conditions (6 M guanidinium, 0.02 M sodium phosphate, and 0.5 M NaCl, pH 7.8) and the supernatant was mixed with the $Ni^{2+}$-charged resin in the presence of the binding buffer (8 M urea, 0.02 M sodium phosphate, and 0.5 M NaCl, pH 7.8). Under these conditions, the His-tagged leader peptide of the recombinant fusion protein binds with high affinity to the resin. After extensive washing with denaturing wash buffer (8 M urea, 0.02 M sodium phosphate, and 0.5 M NaCl, pH 6.0), the His-tagged PsNLEC-1 was eluted using low-pH elution buffer (8 M urea, 0.02 M sodium phosphate, and 0.5 M NaCl, pH 4.0). A mouse monoclonal antibody (T7-Tag, Invitrogen), which recognizes the bacteriophage T7 gene 10 protein, was used to recognize the corresponding epitope in the new recombinant protein.

### Production and Purification of Specific Antisera

The recombinant PsNLEC-1 protein, purified by elution from $Ni^{2+}$-charged resin, was used as an immunogen to produce specific antiserum in two rabbits. After SDS-PAGE, the PsNLEC-1 protein band visualized by Coomassie blue staining was removed with a scalpel. (Because the recombinant protein was difficult to solubilize under non-

denaturing conditions, the N-terminal His tag and T7 epitopes were not removed.) Approximately 100 μg of the isolated protein was mixed with 1 mL of Freund's adjuvant for each injection. Complete adjuvant was used for the initial injection and incomplete adjuvant was used for all of the subsequent boosts, which were given at intervals of 4 to 5 weeks. Prior to the experiment, samples of preimmune serum were obtained.

Both immune and preimmune antisera contained antibodies that cross-reacted with *E. coli*. These were removed by preabsorption with *E. coli* cell material, as described previously (Gruber and Zingales, 1995). The resulting antiserum derived from one of the rabbits was termed R76 and was used for further experiments in the study. R76 is defined as the antiserum after preabsorption with *E. coli*. The other antiserum (which gave identical results in all tests) was retained as reserve material. For immunogold localization and silver-enhancement studies, antiserum R76 was made even more specific by immuno-adsorption to gel-purified recombinant PsNLEC-1, as described previously (Smith and Fisher, 1984). It was noted that R76 also recognized a 70-kD band in extracts from bacteroids, although this antigen was absent from extracts of free-living rhizobia (data not shown). The nature of this antigenic material has not been established. However, because immunopurified R76 antiserum gave very little labeling of bacteroids in fixed tissue sections, it was not found necessary to remove the cross-reacting antibodies by preadsorption of R76 with bacteroid lysates.

### Carbohydrate-Binding Activity of PsNLEC-1

Rabbit reticulocytes were used for the study of agglutination activity of PsNLEC-1. Nodule lectin was derived either as a component of symbiosomal fluid prepared by fractionation of nodule homogenates (Brewin et al., 1985; Perotto et al., 1991) or from the bacterial lysate of an induced culture of B817. Hemagglutination tests were as described by Lis et al. (1994). Fetuin agarose (Sigma) was used as a complex matrix to investigate the carbohydrate-binding activity of PsNLEC-1 protein. Fetuin agarose slurry (100 μL) was mixed with 100 μL of lectin in the presence of protease inhibitors and was left on ice for 5 min. The matrix was then washed three times with 1-mL aliquots of PBS to remove unbound material. Elution of putative lectins was investigated using serial dilutions (5–150 mM) of the following sugars: L-Fuc,D-Gal,D-Glc,D-Man, *N*-acetyl glucosamine, *N*-acetyl galactosamine, and inositol. A mixture of all of these sugars, each at a final concentration of 200 mM, was also used. Eluted material was screened on both western blots and dot blots using R76 or anti-T7 antibodies. In both of these assays an equivalent concentration of PSL was used as a positive control.

### Plant Material

For pea (*Pisum sativum*) nodulation, germinating seedlings of cv Wisconsin Perfection were inoculated with *Rhizobium leguminosarum* bv *viciae* wild-type strain 3841 (Wood et al., 1989). Peas were grown in gnotobiotic conditions (i.e. controlled microbiological conditions) in conical flasks on agar medium containing mineral salts but lacking an N source. Lupin nodules were a gift from Dr. J. Robertson (Palmerston North, New Zealand), and nodules of bean were obtained as previously described (Borthakur et al., 1986). Snap-frozen pea seeds were purchased from a local grocery store.

### Fractionation of Pea Nodule Homogenate

A simple Suc step gradient was used for the biochemical fractionation of the pea nodule homogenate in microcentrifuge tubes (Brewin et al., 1985). All procedures were carried out at 4°C in the presence of a cocktail of protease inhibitors (Boehringer Mannheim): *p*-amino benzamidine (100 μg/mL), 4-amidino phenylsulfonyl fluoride (10 μg/mL), phosphoramidon (5 μg/mL), E64 (10 μg/mL), and pepstatin (2 μg/mL).

Nodules 3 to 4 weeks postinfection were collected in the extraction buffer (50 mM Tris-HCl, 10 mM DTT, and 500 mM Suc, pH 7.4) and were ground using a mortar and pestle in the presence of polyvinylpolypyrrolidone (Sigma). The extract was filtered through two layers of Miracloth (Calbiochem). The symbiosomes and the nodule supernatant were separated by spinning the extract for 1 min at 10,000*g*. The nodule supernatant was spun further at 10,000*g* for 15 min to separate the 10,000*g* nodule membrane pellet from nodule supernatant. The nodule supernatant was spun further at 100,000*g* for 30 min at 4°C in a bench-top ultracentrifuge (TLA 100, Beckman) to separate small cytoplasmic vesicles and other nodule membrane components from the nodule supernatant.

The symbiosomal material was released from intact symbiosomes by using osmotic shock and mechanical force in the form of vortexing and pipetting (Brewin et al., 1985). Released bacteroids were removed by centrifugation at 10,000*g* for 2 min. The symbiosomal material was further fractionated by centrifugation at 100,000*g* for 30 min at 4°C to separate the PBF and PBM fractions.

### Gel Electrophoresis and Western Analysis

Samples were separated by SDS-PAGE (Laemmli, 1970) using 12% (w/v) polyacrylamide minigels. Coomassie brilliant blue was used for protein staining. For western blotting, protein and glycoprotein antigens were transferred electrophoretically to nitrocellulose membranes (Schleicher & Schuell), using a constant 40 V for 1 h at room temperature. The transfer buffer was 0.1 mM Caps (3-[cyclohexylamino]1-propane sulfonic acid, Sigma), pH 10.5, with 10% (v/v) methanol.

### Monoclonal Antibodies and Lectin Probes

Rat monoclonal antibodies MAC266 and MAC254 react with carbohydrate epitopes associated with glycoproteins (including PsNLEC-1) that are targeted to the PBF (Perotto et al., 1991). Similarly, rat antibody JIM84 (Horsley et al., 1993), obtained from Dr. C. Hawes (Oxford Brookes University, UK), identifies a carbohydrate epitope on Golgi-

derived glycoproteins. Rat monoclonal antibody YZ 1/2.23 (McManus et al., 1988), recognizing the heptasaccharide side chain of horseradish peroxidase, was kindly provided by Dr. D. Ashford (University of York, UK). Biotinylated ConA was obtained from Sigma.

### Immunostaining of Western Blots

Procedures for immunostaining of antigens on nitrocellulose sheets were described previously (Perotto et al., 1991). Sheets were incubated with antisera in the presence of blocking buffer with 3% (w/v) BSA in TBS (50 mM Tris-HCl and 200 mM NaCl). Binding of anti-rabbit, anti-rat, or anti-mouse secondary antibodies conjugated with alkaline phosphatase was detected with nitroblue tetrazolium (0.1 mg/mL) and 5-bromo-4-chloro-indolyl phosphate (0.05 mg/mL) in 90 mM Tris-HCl, pH 9.6, containing $MgCl_2$ (4 mM).

Alternatively, secondary antibodies conjugated to horseradish peroxidase were used, and antigen bands were revealed by ECL (Amersham) using the conditions recommended by the manufacturer. (To achieve simultaneous labeling of the same blot with two independent antibodies, the immunophosphatase and peroxidase-ECL systems were operated concurrently.) In the case of biotinylated probes, streptavidin-peroxidase conjugates (Amersham) were used for detection by ECL.

### Microscopy

Pea nodules were fixed and embedded in London Resin White, as described previously (VandenBosch et al., 1989). Gold sections (90–150 nm thick) were collected for immunogold labeling on gold grids coated with 4% (w/v) parlodion and carbon. The sections were incubated overnight at 4°C with primary antibody followed by secondary antibody conjugated to 20-nm colloidal gold (Amersham). The sections were counterstained in 2% (w/v) aqueous uranyl acetate for 5 min and were viewed and photographed in a transmission electron microscope (JEM-1200, Jeol) at 80 kV. Silver enhancement was carried out as described previously (VandenBosch, 1992; Gardner et al., 1996).

## RESULTS

### Heterologous Expression of *PsNlec1* in *E. coli* and Production of Specific Antiserum

The full-length open reading frame of *PsNlec1* was expressed in *E. coli* to produce protein in sufficient quantities to be purified for use as an antigen. After induction of *E. coli* cells and analysis by SDS-PAGE (Fig. 1A, lane 2), a new band at 32 kD was visible, which apparently had no counterpart in uninduced cells (Fig. 1A, lane 1). After western blotting and immunostaining with T7-Tag antibody, it was confirmed that the new band was the expressed recombinant protein (Fig. 1B, lane 2). Similarly, antiserum raised against the purified recombinant protein apparently also recognized the same band as the T7-Tag antibody (Fig. 1C), because it gave an identical pattern of antigen staining on

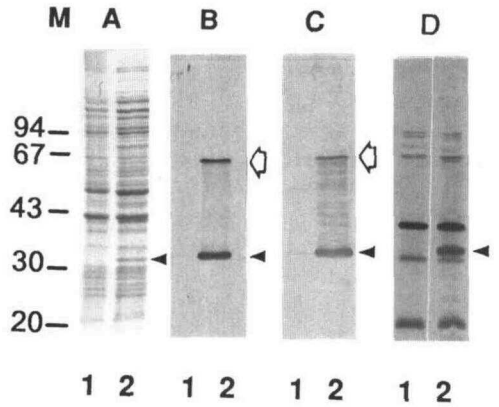


**Figure 1.** Heterologous expression of PsNLEC-1 protein by *E. coli* strain B817 and development of specific antiserum R76. Lanes 1, Uninduced culture; lanes 2, induced culture. A, Polyacrylamide gel stained with Coomassie blue. B, Western blot treated with T7-Tag monoclonal antibody followed by immunostaining with alkaline phosphatase. C, Similar blot treated with specific antiserum (R76) raised against purified PsNLEC-1. D, Blot treated with crude rabbit antiserum before removal of nonspecific antibodies by preabsorption with *E. coli* lysate. M, Molecular weight markers (×1000). Arrowheads mark the position of the 32-kD polypeptide expressed in B817 under inducing conditions. Open arrows identify an apparent dimer of the PsNLEC-1 derivative (62 kD) that reacts with both R76 and the T7-tag antibody.

western blots. This antiserum (R76) appeared to have been rendered monospecific after irrelevant *E. coli* antibodies had been removed from the initial antiserum (Fig. 1D) by preadsorption with *E. coli* lysate (Fig. 1C).

Preimmune serum also reacted with the same *E. coli* antigens labeled in Figure 1D, lane 1 (data not shown). However, the purified antiserum R76 was found not to react with immunoblots derived from extracts of uninduced *E. coli* (Fig. 1C, lane 1) or with extracts from pea root homogenates (data not shown). Similarly, after preadsorption, the preimmune serum did not react with homogenates from induced *E. coli* cultures or with pea root or nodule homogenates (data not shown). Tests with various irrelevant recombinant proteins generated using the pRSET vector system revealed that R76 contained antibodies reacting with all of these recombinant proteins, indicating that the antiserum probably contained antibodies directed against the His tag and T7 epitopes associated with all of these constructs. However, further tests conducted with T7-Tag antibody demonstrated that there were no cross-reacting antigens, either in tissue sections or in nodule extracts (data not shown).

In addition to the antigen band of a predicted molecular mass (32 kD), a second antigen band of lower mobility was observed after SDS-PAGE of samples from induced cultures of *E. coli* when immunostained with R76 (Fig. 1C, lane 2). This antigen band (migrating at approximately 62 kD) was presumed to be related to the recombinant PsNLEC-1 protein because it was found only in extracts from induced cultures and because it apparently reacted with both T7-Tag antibody and R76 on immunoblots (Fig. 1, B and C). We interpret these observations to suggest that the recom-

binant PsNLEC-1 protein from the *E. coli* lysate has a strong tendency to dimerize under standard denaturing conditions.

### Immunoblotting of PsNLEC-1 Protein Isolated from Pea Symbiosomes

The purified antiserum R76 was used on immunoblots to compare PsNLEC-1 protein expressed heterologously in *E. coli* (Fig. 2B, lane 1) with the same protein isolated from PBF (Fig. 2B, lane 2). Two antigenic bands were identified in PBF that corresponded to molecular masses of 32 and 27 kD (Fig. 2B, lane 2). To confirm that these antigens were related to the PsNLEC-1 polypeptide, antiserum R76 was further purified by adsorption to the recombinant PsNLEC-1 protein from *E. coli* (following SDS-PAGE and transfer to a sheet of nitrocellulose). It was demonstrated that after two cycles of immunoadsorption the released antibodies still reacted with the 32- and 27-kD bands from PBF, and the intensity of staining of these bands was unaltered relative to the *E. coli* protein (data not shown). It is therefore presumed that these two bands represent different antigenic isoforms of nodule lectin, provisionally termed PsNLEC-1A and PsNLEC-1B, respectively.

In addition, the immunoblots revealed two antigen bands of lower mobility (approximately 60 kD), which reacted with R76 (Fig. 2B, lane 2). As previously seen with the material derived from *E. coli* (Figs. 1C, lane 2, and 2B, lane 1), it is presumed that these bands from PBF extracts

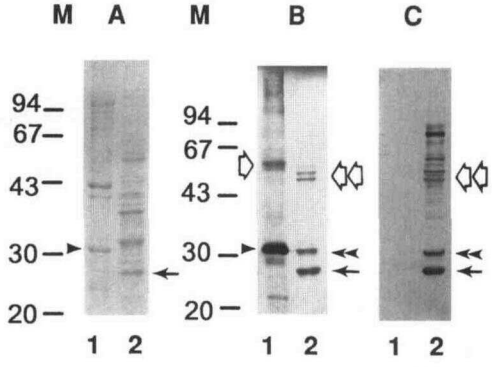


**Figure 2.** Comparison of PsNLEC-1 chimeric protein derived from *E. coli* with corresponding protein antigens isolated from pea nodule symbiosomes as revealed by immunostaining with R76 antiserum (which had been preadsorbed with *E. coli* antigens). Lanes 1, Induced culture of *E. coli* B871 after affinity purification on a $Ni^{2+}$ column; lanes 2, sample of PBF after fractionation of pea nodule homogenate. A, Polyacrylamide gel stained with Coomassie blue (loaded at 10 times the sample concentration used for western blotting). B, Western blot treated with R76 (followed by immunophosphatase staining) to reveal PsNLEC-1 polypeptide antigens. C, Blot immunostained with MAC266 to reveal associated carbohydrate epitopes. M, Molecular weight markers (×1000). Arrowheads and open arrows, respectively, mark the positions of the 32-kD PsNLEC1 polypeptide and dimer expressed in *E. coli*. Arrows mark the positions of the 27-kD antigen that was previously purified by MAC266 immunoaffinity chromatography (Kardailsky et al., 1996). Double arrowheads mark the positions of the 32-kD antigen band, which also reacts with R76 and MAC266. Open double arrows identify apparent dimers of PsNLEC-1 in material derived from PBF.

may correspond to dimerized forms of PsNLEC-1 isoforms PsNLEC-1A and PsNLEC-1B.

### N-Terminal Sequence of PsNLEC-1A and PsNLEC-1B

The *PsNlec1* cDNA was originally isolated by 3′ rapid amplification of cDNA ends using amino acid sequence information derived from the N terminus of a 27-kD protein purified from pea nodule symbiosomes (Kardailsky et al., 1996). Because the antiserum R76 had been raised against recombinant PsNLEC-1 expressed in *E. coli*, we expected it to react with the 27-kD peptide from PBF, but the fact that R76 also recognized a 32-kD band in the PBF was somewhat surprising. During the original purification of the 27-kD glycoprotein from pea nodule symbiosomes (Kardailsky, 1995), it was noted that a 32-kD protein was co-purified and, like the 27-kD protein, it also reacted with monoclonal antibody MAC266.

The N-terminal sequence of the 32-kD band had been determined in the same way as that of the 27-kD band, but the sequence information had not been good enough to design a primer for PCR. After having obtained the full-length sequence information of PsNLEC-1, and in the light of the new information regarding cross-reactivity of the 27- and 32-kD bands with R76, we carefully re-examined the N-terminal sequence data. Despite the uncertainties in reading this sequence, we found that the N-terminal sequence of the 32-kD band showed strong homology to the sequence of eight amino acids positioned upstream to the N terminus of the 27-kD band, as deduced previously from cDNA sequence information (Table I). Therefore, although the N-terminal sequences of the two PsNLEC-1 antigen isoforms (A and B) are different, both polypeptides are likely to be derived from the same gene product, or from closely related gene products, as a result of different degrees of posttranslational processing.

### Analysis of Glycoproteins Isolated from PBF

Both of the antigen isoforms, PsNLEC-1A and PsNLEC-1B, were also recognized by MAC266 (Fig. 2C, lane 2). This monoclonal antibody recognizes a periodate-sensitive (carbohydrate) epitope associated with glycosylated proteins of PBF and PBM (Perotto et al., 1991). The antibody was used initially to isolate the 27-kD PsNLEC-1B glycoprotein from symbiosomes (Kardailsky, 1995; Kardailsky et al., 1996). Because the recombinant form of PsNLEC-1 expressed in *E. coli* is not glycosylated, it was not recognized by MAC266 (Fig. 2C, lane 1). Further analysis of the carbohydrate epitopes associated with the isoforms A and B was made by using different antibodies and carbohydrate-specific staining of PBF-derived components, as shown in Figure 3.

The monoclonal antibodies JIM84, MAC254, and MAC266, all of which react with complex glycosylated antigens, reacted with both isoforms of PsNLEC-1 and their putative dimers. These PsNLEC-1 bands, which were also recognized by R76 (Fig. 3B), proved to be the most intense bands recognized by all of the monoclonal antibodies. In addition, biotinylated ConA also apparently cross-

**Table I.** *N-terminal amino acid sequence of PsNLEC-1 isoforms*

| Polypeptide[a] | Sequence | Isoform |
|---|---|---|
| 32-kD glycoprotein | **LSF**XTTPLTPX**N** | PsNLEC-1A |
| 27-kD glycoprotein (I) | T**AN**SGATY**QGDAQ** | PsNLEC-1B |
| | R V **F** | |
| (II) | T**AN**SGVVL**QGD**XSI | |
| PsNLEC-1 open reading frame ——-leader–V-31 | QALSFNFTKLTTANSGVTFQGDAQI | PsNLEC-1 |

[a] The 27- and 32-kD glycoproteins were purified from PBF: in the case of the 27-kD glycoprotein, results of two independent sequence determinations are presented, together with ambiguities. PsNLEC-1 open reading frame was obtained by translation of the full-length cDNA sequence of the *PsNlec1* gene: The position of the leader peptide is shown, ending with –V-31 QA; propeptide (beginning at position L-34) is underlined (Kardailsky et al., 1996); homologies between open reading frame and observed amino acid sequences are shown in bold.

reacted with the PsNLEC-1 isoforms as the major components among many other putative glycoprotein bands, including one band that apparently migrated only slightly slower than the 32-kD band of PsNLEC-1 (Fig. 3, lane F). Periodate silver staining of PBF on SDS-PAGE further supported the interpretation that the PsNLEC-1A and PsNLEC-1B isoforms were the most abundant glycoproteins present in the PBF.

### Distribution of PsNLEC-1 Isoforms in Fractions from Pea Nodule Homogenates

Pea nodule homogenate was fractionated using Suc density gradient centrifugation to study the distribution of different antigen isoforms of PsNLEC-1 among different cytoplasmic compartments. In the total nodule homogenate (Fig. 4B, lane 1) a new isoform (C) was identified in addition to isoforms A and B represented in PBF. PsNLEC-1C corresponds to a band of faster mobility (25 kD). MAC266 did not react with isoform C (Fig. 4C) but was recognized (data not shown) by biotinylated ConA and by a monoclonal antibody, YZ 1/2.23 (McManus et al., 1988), which is specific for the heptasaccharide of horseradish peroxidase (D. Ashford, personal communication).

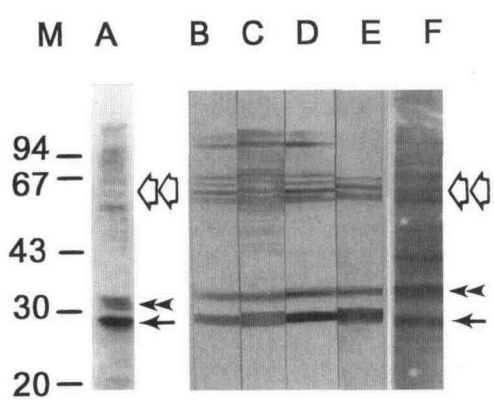


**Figure 3.** Analysis of carbohydrate epitopes associated with PBF fraction. A, Polyacrylamide gel after staining for glycoconjugates by treatment with periodate and silver ions. B to F, Strips from an immunoblot stained by immunophosphatase following treatment with R76 (B), JIM84 (C), MAC254 (D), and MAC266 (E). F, Strip treated with biotinylated ConA followed by streptavidin-peroxidase conjugate detected by ECL. Arrows mark positions of PsNLEC-1-derived polypeptides, as indicated in Figure 2.

The relative abundance of antigen isoforms A, B, and C was clearly different in the different fractions derived from nodule homogenates by centrifugation through Suc density cushions (Fig. 4). All symbiosomal fractions, i.e. PBF and PBM, had only isoforms A and B (Fig. 4B, lanes 3 and 4). Isoform B was more abundant than isoforms A and C in the nodule membrane fraction isolated by centrifugation for 15 min at 10,000*g* (Fig. 4B, lane 5), whereas isoform C was most strongly represented in the nodule membrane fraction after centrifugation at 100,000*g* (Fig. 4B, lane 2). The nodule supernatant fraction (derived from nodule homogenates after centrifugation at 100,000*g*) had relatively low concentrations of all isoforms (lane 6). These results show that isoforms A, B, and C were recovered in different relative amounts by centrifugation of pea nodule homogenates.

### Immunogold Staining and Silver Enhancement

R76 antisera and MAC266 antibody were used to label median longitudinal sections of pea nodule tissue to localize the PsNLEC-1 antigen. An adjacent section was counterstained with basic fuschin to reveal general anatomical

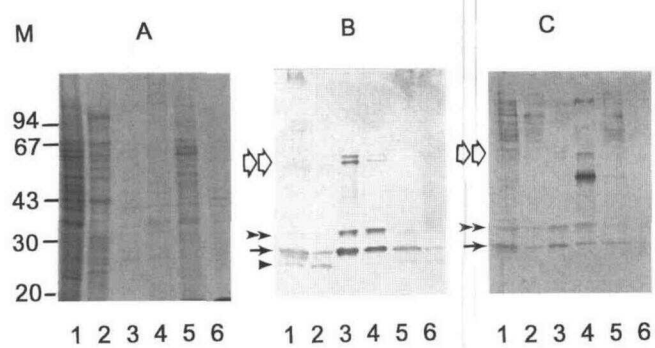


**Figure 4.** Fractionation of pea nodule homogenates and immunostaining of western blots using R76 and immunoperoxidase (B), together with MAC266 and immunophosphatase (C). A is a polyacrylamide gel of the same samples stained with Coomassie blue. Lanes 1, Nodule homogenate; lanes 2, nodule membrane 100,000*g* pellet; lanes 3, symbiosomal fluid 100,000*g* supernatant; lanes 4, symbiosomal membrane 100,000*g* pellet; lanes 5, nodule membrane 10,000*g*; and lanes 6, nodule supernatant 100,000*g*. Arrows and open and closed double arrowheads mark the positions of PsNLEC-1 antigens as indicated in Figure 3. The single arrowhead marks the position of a fast-migrating PsNLEC-1 isoform that does not react with MAC266.

features (Fig. 5). In nodules harvested 28 dpi, PsNLEC-1 antigen was uniformly distributed throughout the infected tissue containing symbiosomes (Fig. 6A). There was no evidence of antigen accumulation in uninfected cells in the central tissues of the nodule. The distribution of PsNLEC-1 (revealed by R76 antiserum) was broadly consistent with that observed for the group of glycoprotein antigens detected by monoclonal antibody MAC 266 (Fig. 6B), except that R76 identified antigen in vacuoles, whereas MAC266 showed very little labeling of these areas. There was no evidence of extracellular label with R76 and, similarly, this antiserum did not give any labeling of cortex or parenchyma cells. In younger nodules, harvested 17 dpi, the label was very weak, accumulating in cells containing $N_2$-fixing bacteroids (data not shown). Preimmune sera and irrelevant antibodies yielded no signal over infected cells, indicating that immunogold labeling was antigen specific.

### Immunogold Localization of the R76 Antisera in Pea Nodule Tissue

Immunoabsorbed R76 was used to localize the PsNLEC-1 antigen at the ultrastructural level in thin sections of pea nodule tissue (28 dpi). The gold label was most abundant in the mature $N_2$-fixing cells, confirming the results obtained with silver enhancement. By looking at the pattern of the R76 labeling, we could distinguish two types of infected cells. In the relatively young host cells that contained bacteroids just beginning to express nitrogenase, as judged by previous studies (Kannenberg et al., 1994), the PsNLEC-1 antigen was distributed diffusely in the symbiosomal space and appeared to be associated with the peribacteroid membrane. In addition, there was label in cytoplasmic membranes and the vacuolar compartment (Fig. 7, A and B).

In the very mature cells at the base of the nodule, the label was restricted to the vacuoles and the peribacteroid space; there was no evidence of cytoplasmic labeling. In these cells the antigen in the symbiosomal space appeared to be associated with a protein aggregate resembling a small inclusion body in the symbiosomal space (Fig. 7C). There was no evidence of intercellular label either between the infected cells or in the uninfected cortical parenchyma. However, in older nodules, there was some labeling of the tonoplast of parenchymal cells associated with the uninfected inner cortex (data not shown). Preimmune antisera was used as a control (Fig. 7D) and this did not show any label in any of the samples examined.

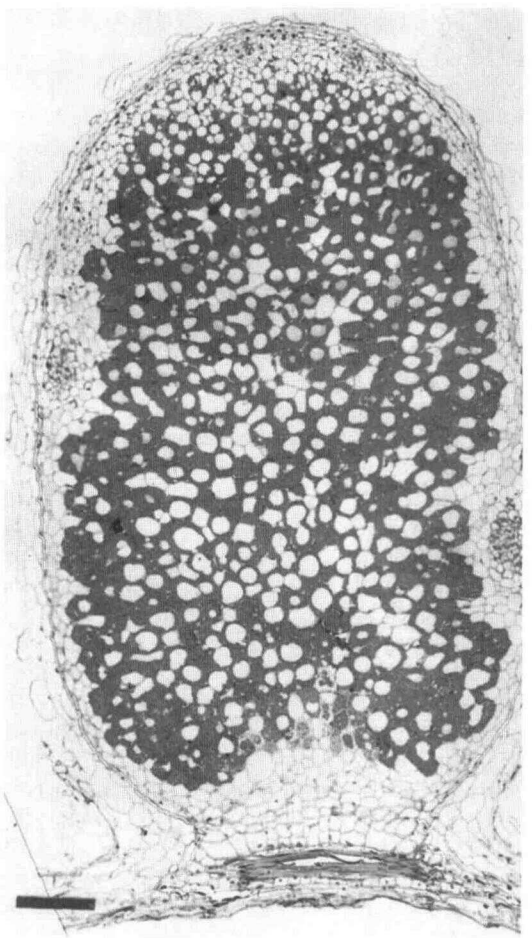

**Figure 5.** Median longitudinal section of pea nodule tissue harvested 28 dpi and counterstained with basic fuschin. This section was derived from the same nodule used for immunolocalization of PsNLEC-1, as illustrated in Figure 6. Bar = 80 $\mu$m.

### Carbohydrate-Binding Activity of PsNLEC1

R76 antiserum was used as a molecular probe to investigate the possible lectin-like activity of PsNLEC-1 in relation to agglutination of rabbit reticulocytes or the ability to bind to a complex carbohydrate matrix (fetuin agarose). All such experiments gave negative results using PsNLEC-1 derived from PBF or from heterologous expression in *E. coli*. In parallel experiments, PSL was used as a control and this gave positive results for hemagglutination and binding to fetuin agarose; moreover, its lectin activity was inhibited in the presence of 200 mM D-Glc/Man (data not shown).

## DISCUSSION

PsNLEC-1 is an example of a vegetative lectin (or lectin-like protein) being expressed in pea nodules but not in seeds (Kardailsky et al., 1996). The development of a specific antiserum recognizing this polypeptide has now made it possible to study some of the biochemical properties of PsNLEC-1 and to investigate its localization within tissues and cells. The results of this study have highlighted the differences between PSL and PsNLEC-1, which were predicted from a comparison of DNA sequence for their respective cDNA clones.

Although both genes belong to the legume lectin gene family, they are no more closely related to each other than to any other member of the gene family from any other legume (Kardailsky et al., 1996). Other examples of legume species containing several lectin genes that are not closely related include peanut (*Arachis hypogaea*; Law, 1996a, 1996b), false acacia (*Robinia pseudoacacia*; Yoshida et al., 1994), and yellow wood (*Cladastris lutea*; Van Damme et al., 1995). Many of these lectins are found in vegetative loca-

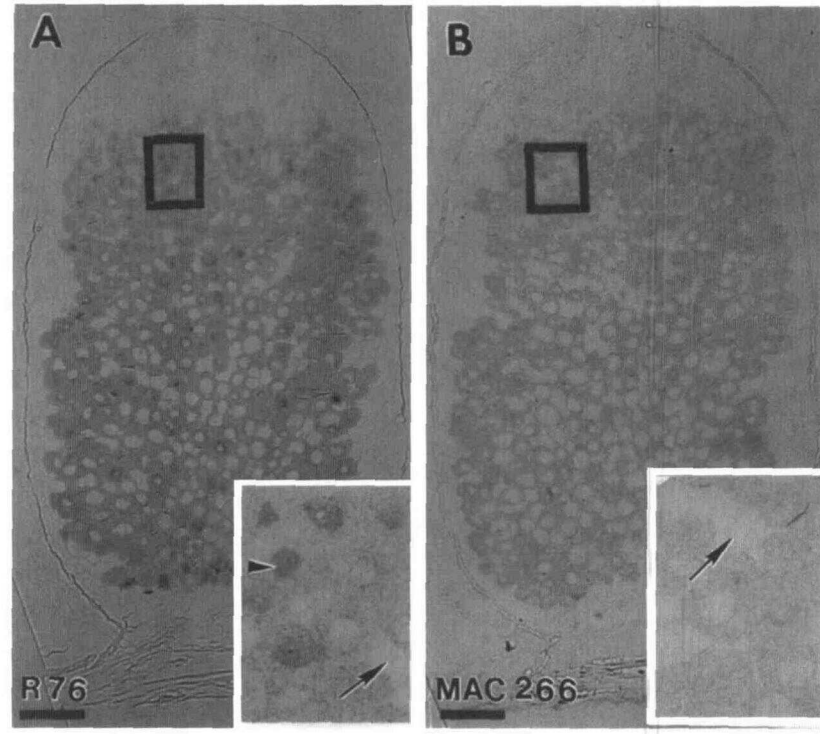


**Figure 6.** Localization of PsNLEC-1 antigen by immunogold staining and silver enhancement in tissue sections derived from the same nodule illustrated in Figure 5. A, Treated with R76; B, treated with MAC266. Most of the antigens recognized by R76 and MAC266 are associated with the central infected tissue and particularly with the symbiosomes (Fig. 7). Insets show 4-fold magnification of boxed areas from main micrographs. The arrowhead indicates the presence of PsNLEC-1 antigen in vacuoles of young infected cells from 28-dpi nodules (detected with R76 antiserum but not with MAC266 antibody). Arrows indicate uninfected cells from the same area, showing the absence of antigen. Bar = 80 $\mu$m.

tions, e.g. shoot, root, bark, or nodule. The discordant homology of vegetative lectins with respect to seed lectins of the same species suggests that the genes diverged before radiation of the family and presumably evolved under different functional constraints (Brewin and Kardailsky, 1997).

In PSL each functional unit is a 2$\alpha$-2$\beta$ tetramer with carbohydrate-binding sites at opposite ends of the two polypeptide domes, which are composed predominantly of antiparallel $\beta$-strands (Van Eijsden et al., 1994). The *Psl* gene encodes a 49-kD tetrameric protein, which is the result of posttranslational processing and cleavage of two identical polypeptide chains, each generating $\alpha$- and $\beta$-subunits. During this maturation, signal peptide is removed, both ends of the polypeptide are trimmed, and the remainder is proteolytically cleaved into two subunits. In many legume lectins, however, the $\alpha$- and $\beta$-subunits are not separated by proteolytic cleavage (Rini, 1995); consequently, these lectins are isolated as dimers comprising two identical subunits of approximately 30 kD, each corresponding to the combined $\alpha$- and $\beta$-chains of PSL. In the case of PsNLEC-1 protein (Fig. 2), even the smallest antigenic form, PsNLEC-1C, has an estimated molecular mass of 25 kD, which is greater than would be expected if separate $\alpha$- and $\beta$-chains had been generated by proteolytic cleavage.

The specific antiserum R76 also revealed antigen bands of lower mobility (approximately 62 kD on SDS gels), both in nodule extracts (Fig. 2) and following heterologous expression in *E. coli* (Fig. 1). Because the band present in lysates of *E. coli* corresponded to approximately twice the monomer molecular mass of PsNLEC-1, it is presumed to correspond to a protein dimer that was stable even under SDS-denaturing conditions. This possibility is supported by the observation that the 62-kD antigen band also cross-reacted with the T7-tag antibody. Similarly, in nodule extracts (Fig. 2) there was an antigen band migrating in the region of a putative dimer, and when such a component was purified and subjected to N-terminal sequence analysis (Kardailsky, 1995), it was found to show strong homology (if not identity) to that of PsNLEC-1, as previously determined (Kardailsky et al., 1996). The reason for the apparent stability of these putative dimers may be that this protein, like all legume lectins, has a very high content of $\beta$-pleated sheet, which could account for its very stable conformation.

Using antiserum R76, we identified two different antigen isoforms of PsNLEC-1 polypeptide in isolated symbiosomes (Fig. 2) and these have been termed PsNLEC-1A and PsNLEC-1B. N-terminal sequence analysis of isoform A (Table I) revealed significant homology with an eight-amino acid propeptide deduced from the complete cDNA sequence previously obtained by PCR-based cloning from isoform B (Kardailsky et al., 1996). However, the N-terminal sequence information for isoform 1A was not reliable enough to determine whether antigen isoforms A and B correspond to the products of two distinct but homologous genes or whether they represented different posttranslational products of a single gene. Clearly, the difference in mobility between isoforms A and B is more than could be accounted for by the presence or absence of an eight-amino acid peptide, but this discrepancy could be explained by other modifications to the two components, either at the C terminus or involving the glycan substituent.

Similar arguments apply to a third, smaller isoform, PsNLEC-1C, which has also been identified in nodule ho-

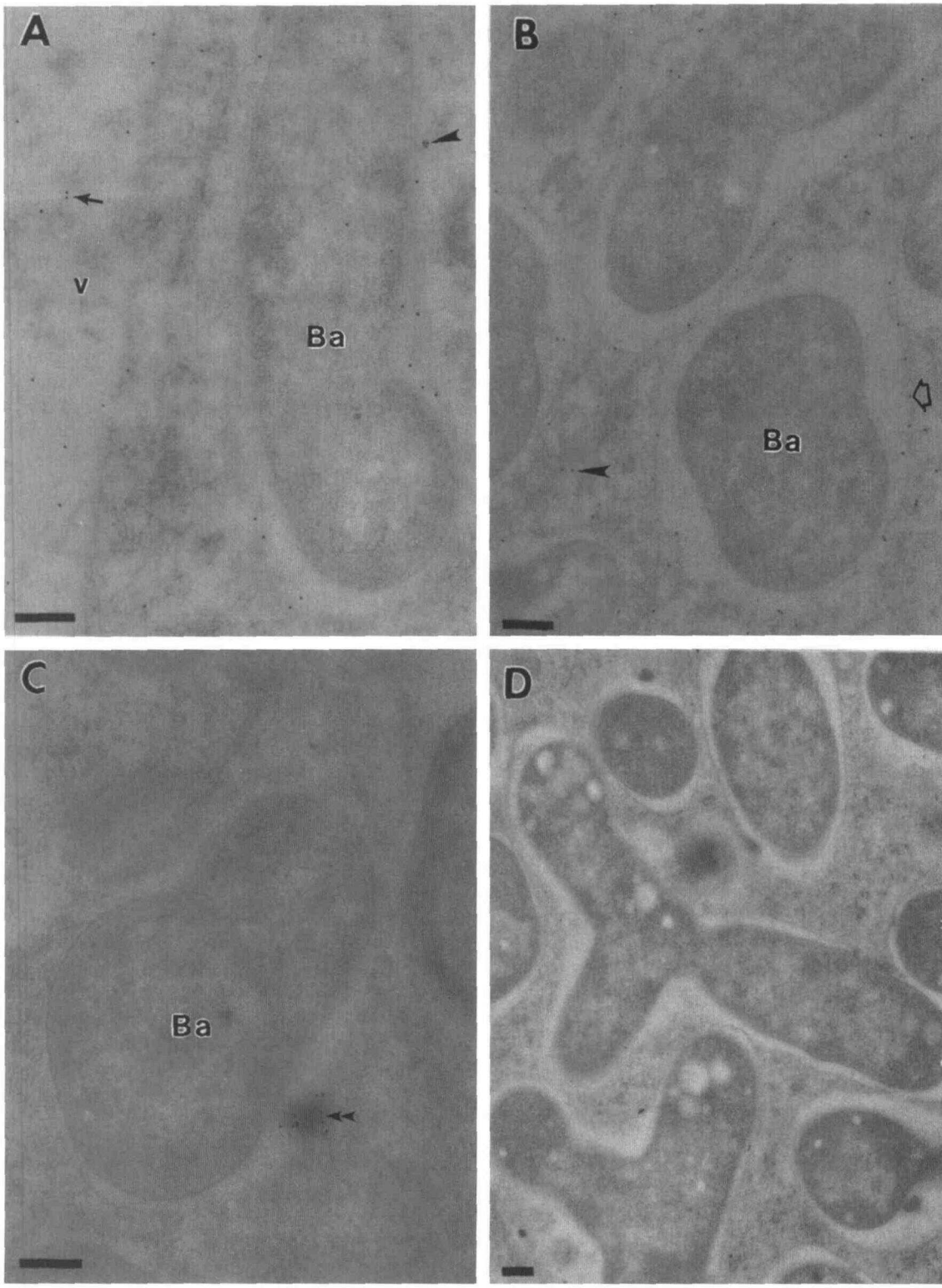


**Figure 7.** Electron micrographs of pea nodule (28 dpi) showing bacteroids (Ba) and vacuole (v) following immunogold staining of sections with R76 antiserum (A–C) or preimmune serum (D). A and B, Early symbiotic zone showing antigen associated with cytoplasmic membranes (arrowhead), PBM (open arrow), and vacuolar compartment (arrow). C and D, Late symbiotic zone showing accumulation of label as inclusion bodies in PBF (double arrowhead) and the relatively low level of label in the host cytoplasm. Bars = 0.25 $\mu$m.

mogenates (Fig. 3), but the nature of its N-terminal sequence has not been determined. Because this isoform was not found in isolated symbiosomes, it is apparently associated with another cellular location. The close antigenic relationship between isoforms A, B, and C was further demonstrated by the fact that R76 antiserum, immunopurified by adsorption to recombinant PsNLEC1 from *E. coli*, still reacted to all three isoforms with equal intensity (data not shown).

Whereas PSL is not glycosylated, PsNLEC-1 was first recognized and isolated by virtue of a carbohydrate epitope recognized by monoclonal antibody MAC266 and the probable site of glycosylation is at Asn-94 (Kardailsky et al., 1996). Different isoforms of PsNLEC-1 were found to react differently with a panel of monoclonal antibodies and ConA (Fig. 3), suggesting that they carry carbohydrate side chains of different complexity. Preliminary evidence indicates that the fastest-migrating isoform, PsNLEC-1C (which reacts strongly with ConA and monoclonal antibody YZ 1/2.23), probably carries the same heptasaccharide side chain as horseradish peroxidase (L.L. Guldemann and D. Ashford, personal communication), whereas isoforms A and B apparently carried a more complex side chain that was recognized by MAC266, MAC254, and JIM84 (Fig. 3).

PSL is a Man-/Glc-binding lectin. However, no such activity could be detected with PsNLEC-1 derived either from nodule homogenates or from extracts of *E. coli* following heterologous expression. It is difficult to interpret a negative result for the protein derived from *E. coli* because this polypeptide contains signal sequence and other N-terminal epitopes that could interfere with folding and activity. Furthermore, the use of urea for the isolation of the recombinant protein could result in carbamylation of the protein, a modification that might also affect carbohydrate-binding activity. Of greater significance is the fact that the antiserum R76 also failed to detect any binding of the native (ex-nodule) PsNLEC-1, either to carbohydrate matrices or to reticulocyte or bacterial cell surfaces. This is consistent with the tentative predictions of comparative sequence analysis, because PsNLEC-1 lacks any counterpart to the Gly-216 residue of PSL, which is thought to be an important component of the sugar-binding site (Kardailsky et al., 1996). Similarly, there is no counterpart to Gly-99, which has also been implicated in the sugar-binding site of PSL. Gly-99 is also absent from the metal-binding domain of the arcelin and protease-inhibitor groups, which also lack carbohydrate-binding activity (Mirkov et al., 1994). A lectin-like protein without detectable carbohydrate-binding activity has also been described in the bark of yellow wood (Van Damme et al., 1995).

The antiserum R76 raised against PsNLEC-1 polypeptide provided a specific probe for use in immunolocalization studies in nodule tissue. As expected, the major component of antigen in infected cells was localized in the lumen of symbiosomes, the original source of the glycoprotein used for purification and N-terminal amino acid sequencing (Kardailsky et al., 1996). In contrast to the situation in peanut nodules, none of the PsNLEC-1 antigen was localized in the extracellular matrix of infected cells or uninfected cortical cells (VandenBosch et al., 1994).

The histological distribution of PsNLEC-1 antigen was broadly consistent with the pattern of mRNA expression observed by in situ hybridization (Kardailsky et al., 1996). This distribution was also similar to that of the carbohydrate epitope recognized by MAC266 antibody and known to be present in isoforms PsNLEC-1A and PsNLEC-1B, as well as in other Golgi-derived glycoproteins. However, several interesting features emerged from this study, which suggested that the localization of PsNLEC-1 might reflect the physiological state of symbiosomes and their host cells. At the cellular level, two different patterns of PsNLEC-1 distribution were observed in infected host cells. In the first cell type, the antigen was evenly distributed through the PBF and was associated with the PBM; it was also found in the cytoplasmic space (presumably associated with small cytoplasmic vesicles) and in the tonoplast. In the second cell type, the antigen appeared to be aggregated as small inclusion bodies within the PBF; it was not found in any other locations except the tonoplast. Although the distribution of both cell types was sporadic throughout the infected cells of the nodule, the second type appeared to predominate toward the base of the nodule and was therefore presumed to be the more advanced condition in a developmental sense.

The results of subcellular fractions derived from nodule homogenates (Fig. 4) indicated that it was possible to identify cytoplasmic components in which different antigen isoforms of PsNLEC-1 were predominant. For example, the symbiosome fraction apparently lacked the isoform PsNLEC-1C, suggesting that this isoform was confined to the tonoplast and small cytoplasmic vesicles. It is possible that PsNLEC-1C should be considered as a degradation product, although we have no direct evidence for this.

Isoforms PsNLEC-1A and PsNLEC-1B were equally represented in the symbiosomal fractions isolated from pea nodule homogenates; indeed, they were the most abundant glycoproteins in the PBF (Fig. 3). But it is interesting to note that isoform PsNLEC-1B was more abundant than PsNLEC-1A in the 10,000*g* PBM. This might correlate with the observation that two types of host cell were distinguishable on the basis of immunogold localization with R76 antiserum. Conceivably, the first cell type (with the more diffuse distribution of PsNLEC-1) might harbor the PsNLEC-1B isoform, whereas the second cell type (with the more clumped distribution of PsNLEC-1) might harbor the PsNLEC-1A isoform. Because these two isoforms can be distinguished by the presence or absence of a propeptide sequence, an antibody recognizing this octapeptide could be used in the future to test the hypothesis that isoform PsNLEC-1B is confined to a subset of the symbiosomes present in nodule tissue.

In relation to the immunolocalization of PsNLEC-1, there seem to be several interesting paradoxes that we are unable to resolve at this time. First, *PsNLEC1* transcript labels extremely strongly by in situ hybridization (Kardailsky et al., 1996), giving a stronger signal than leghemoglobin. Paradoxically, however, the protein and antigen are relatively difficult to detect (as measured by direct protein

staining of SDS-polyacrylamide gels and by immunoblotting and immunogold staining of nodule tissues), suggesting a high rate of turnover for this protein. Second, as observed previously (Kardailsky et al., 1996), *PsNlec1* is weakly expressed in roots of nodulated plants and also in uninfected roots grown on $NO_3^-$, and therefore, its expression may reflect the N regime of the root system. Again, paradoxically, the transcript is still expressed strongly in symbiotically defective mutant nodules and therefore transcription is not exclusively under N control (Kardailsky et al., 1996). Third, as described in this paper, the protein antigen tends to accumulate in symbiosomes toward the base of the nodule (Fig. 6A), although the transcript is expressed equally strongly throughout the symbiosomal tissue. The resolution to this paradox could be that a high rate of protein turnover in the symbiosome compartment might indicate that the bacteroid uses protein hydrolysate, e.g. from the degradation of PsNLEC-1, as a nutritional source.

It is possible to propose a very speculative model in which protein antigen only tends to accumulate in infected tissue when the rate of synthesis of PsNLEC-1 (under N-abundant conditions) exceeds the rate of breakdown to satisfy the metabolic needs of bacteroids. Similarly, in peanut nodules it has been suggested that the nodule lectin acts as part of a transient N storage system and only accumulates in nodule tissue under conditions of N excess (Law, 1996a, 1996b).

In conclusion, this study of a vegetative lectin expressed in pea nodule tissue has revealed patterns of immunolocalization associated with the development and functioning of the symbiosome compartment. This phenomenon can be further investigated by the development of appropriate molecular probes capable of identifying specific isoforms of PsNLEC-1 in association with specific cytoplasmic compartments.

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