Phosphorylation of Nodulin 26 on Serine 262 Affects Its Voltage-sensitive Channel Activity in Planar Lipid Bilayers*

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Jung Weon Leet, Yuxin Zhangt, C. David Weaver§, Nirah H. Shomer*, Charles F. Louis†, and Daniel M. Roberts§

From the §Department of Biochemistry and Center for Legume Research, University of Tennessee, Knoxville, Tennessee 37996 and the ¶Department of Veterinary Pathobiology and Biochemistry, University of Minnesota, St. Paul, Minnesota 55108

Nodulin 26 is a member of the MIP (major intrinsic protein) channel protein family, but its role in symbiosome membranes remains unknown. Recently, the in vitro activity of purified soybean nodulin 26 was studied by reconstitution into planar lipid bilayers for single channel conductance measurements (5). Nodulin 26 formed channels with a large single channel conductance and weak anion selectivity (5). Furthermore, nodulin 26 channels showed sensitivity to high applied voltages, including more active gating and the tendency to occupy discrete lower subconductance states (5).

Previous work also showed that nodulin 26 is phosphorylated by a calcium-dependent protein kinase on the symbiosome membrane (4). This kinase has characteristics of the calmodulin-like domain protein kinase family (4). Members of this family possess a protein kinase catalytic domain fused to a calmodulin-like regulatory domain with four EF-hand calcium-binding sites (6, 7). Based on protein sequence analysis, in vivo and in vitro phosphorylation of nodulin 26 occurs at only one residue, serine 262 within the hydrophilic, cytoplasmic COOH-terminal domain (4, 8).

The finding that nodulin 26 is phosphorylated by a symbiosome membrane CDPK suggests that calcium signaling may be involved in its regulation. A correlation between nodulin 26 phosphorylation and changes in metabolite transport have been observed with isolated symbiosomes (9), but a role for nodulin 26 and phosphorylation in symbiosome membrane transport is still not defined. To study the effect of phosphorylation on nodulin 26, we have investigated the channel activities of wild-type recombinant nodulin 26 before and after in situ phosphorylation by CDPK, as well as the activities of nodulin 26 mutant proteins with substitutions at position 262 that imitate the unphosphorylated or phosphorylated states.

Materials and Methods

Molecular Cloning Techniques—A full-length nodulin 26 cDNA was obtained and cloned into M13mp19 as described previously (10), and site-directed mutagenesis was done by using a Bio-Rad mutagenesis kit. Mutagenesis primers were 5'-AAGAGTGTGGTCTTTCTCAAG-3' for the Ser-262 → Ala substitution, and 5'-AAGAGTGTGGTCTTTCTCAAAG-3' for the Ser-262 → Asp substitution. Mutants were confirmed by dideoxynucleotide chain termination DNA sequencing with a Sequenase kit (U. S. Biochemical Corp.). Nodulin 26 cDNAs were excised from M13mp19 by BamHI digestion and were cloned into the BamHI site of the pSET A His-tag expression vector (Invitrogen). The resulting

The establishment of symbioses between legumes and rhizobia bacteria represents a specialized developmental pathway that leads to the formation of a root nodule on the plant host. The bacteria infect this structure and become enclosed in intracellular organelles known as symbiosomes (1). The symbiosome membrane encloses the bacterium and controls the exchange of metabolites and nutrients between the host and the bacterial symbiont (2). During nodule formation, nodule-specific genes are induced that encode proteins that aid in the establishment and maintenance of the symbiosis. Among these is nodulin 26, which is a major integral symbiosome membrane protein of soybean nodules (3, 4).


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§Present address: Dept. of Pharmacology, Vanderbilt University School of Medicine, Nashville, TN 37232-6600.

†To whom correspondence should be addressed. Tel.: 423-974-4070; Fax: 423-974-6306; E-mail: dmr@utkvx.utk.edu.

1 The abbreviations used are: MIP, major intrinsic protein; CDPK, calmodulin-like domain protein kinase; FPLC, fast protein liquid chromatography; OG, 1-O-n-octyl β-D-glucopyranoside; MOPS, 3-(N-morpholino)propanesulfonic acid; CK-15, synthetic peptide based on the last 14 carboxyl-terminal amino acid residues of nodulin 26; S262 nodulin 26, recombinant nodulin 26 which has a serine at residue 262; S262A nodulin 26, recombinant nodulin 26 with an alanine substitution at residue 262; S262D nodulin 26, recombinant nodulin 26 with an aspartate substitution at residue 262; NS, nonsignificant.
Phosphorylation of Nodulin 26 Channel

Expression and Purification of Recombinant Nodulin 26—E. coli clones were cultured in 40 ml of LB medium, 50 μg/ml carbenicillin, and 34 μg/ml chloramphenicol at 37°C with shaking until the A_{600} reached 0.6. The culture was placed at 4°C overnight. The cells were collected by centrifugation at 5000 × g for 5 min at 4°C, washed with 50 ml of washing buffer (3.9 mM Tris-HCl, 0.9 mM NaCl, 1% (w/v) OG (column buffer)), and incubated on ice. The His-tag at the trans- and cis-end of nodulin 26 was solubilized by resuspending the pellet in 20 ml of 40 mM Tris-HCl, pH 7.9, 0.5 M NaCl, 1 μM pepstatin A, 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, MgCl_{2} (10 mM), and DNase I (20 μg/ml) and the suspension was incubated at room temperature until the viscosity was reduced. The suspension was centrifuged at 100,000 × g for 1 h at 4°C, the pellet was resuspended in 25 ml of 20 mM Tris-HCl, pH 7.9, 1 μM KI, 1 μM pepstatin A, 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin and was stored at 4°C with shaking. The mixture was centrifuged at 100,000 × g for 1 h at 4°C, and the supernatant fraction was applied to a Ni^{2+}-imino-di-acidic acid-Superose column (1.3 cm × 19 cm) attached to a Pharmacia FPLC system. The column was equilibrated with 20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, 1% (w/v) OG (column buffer). The column was washed with column buffer until the A_{600} reached 0.2, base line sensitivity, and nodulin 26 was eluted with a linear gradient of 0–300 mM imidazole (Δ12 mA/m/l) in column buffer. One-mL fractions were collected and screened for nodulin 26 by SDS-polyacrylamide gel electrophoresis and Western blot analysis (4, 10). Fractions containing purified recombinant nodulin 26 were combined and stored at −80°C.

Protein and Enzyme Analyses—CDPK protein kinase activity was determined as described previously (4). Reaction mixtures (50 μl) contained 20 mM MOPS-KOH, pH 7.4, 10 μM dithiobisretiol, 1 mM MgCl_{2}, 0.1 mM [γ-32P]ATP (190 cpn/pmol), 3 mM of bovine serum albumin, and varying amounts of the CK-15 peptide substrate (4). Protein kinase reactions were initiated by the addition of 0.867 μg of CDPK (K M23–6H2, Ref. 12) and were incubated for 30 min at 25°C. 32P incorporation was determined by the phosphocellulose filter assay method (4). The concentration of CK-15 was determined by amino acid composition analysis (13). Other protein determinations were done by the BCA (14) or Bradford (15) methods.

RESULTS

Expression and Purification of Recombinant Nodulin 26—For expression in E. coli, nodulin 26 CDNA was cloned into the pSET5 A vector as a His-tag fusion under the control of the T7 promoter. Based on Western blot analysis, nodulin 26 was successfully expressed in E. coli containing these vectors and is associated with the membrane fraction. The His-tag at the amino terminus of nodulin 26 allowed it to be separated from other E. coli membrane proteins by Ni^{2+}-chelate chromatography. Initial attempts to isolate recombinant nodulin 26 by stepwise Ni^{2+}-chelate chromatography were not successful because of the contamination by other metal binding proteins in E. coli membranes (data not shown). However, sequential Ni^{2+}-chelate chromatography with the application of a linear gradient of imidazole resulted in excellent separation of recombinant nodulin 26 from other E. coli membrane proteins (Fig. 1). The final product was pure based on SDS-polyacrylamide gel electrophoresis (Fig. 1A), and its identity as nodulin 26 was confirmed by Western blot analysis (Fig. 1B).

Reconstitution of Recombinant Nodulin 26 into Planar Lipid Bilayers and Measurement of Ion Channel Activity—Purified recombinant nodulin 26 was reconstituted into proteoliposomes, and its single channel properties were investigated in planar lipid bilayers as described for soybean nodulin 26 (5). Addition of proteoliposomes to the bilayer chamber resulted in the appearance of ion channel activity, which is inhibited by the addition of the anti-nodulin 26 IgG but not preimmune IgG (Fig. 2). This observation suggests that the interaction of the antibody with nodulin 26 protein blocks the channel, and thus channel activity is due to the insertion of nodulin 26 into the bilayer.

A current-voltage relationship for recombinant S262 nodulin 26 is shown in Fig. 3. The current-voltage relationship is linear with conductance values of 3.1 nS (plot 1) or 1.6 nS (plot 2) calculated depending upon the ionic strength of the recording solutions (Fig. 3). Under asymmetric recording conditions (cis 0.2 M KCl, 20 mM MOPS-KOH, pH 7.4, trans 1.0 M KCl, 20 mM MOPS-KOH, pH 7.4) a +4 mV reversal potential (E_{r}) was obtained. Based on the Goldman-Hodgkin equation, this represents...
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Figure 1. Purification of recombinant nodulin 26 from E. coli membranes. A, OG-solubilized E. coli membrane proteins were separated by FPLC on Ni²⁺-imidoacetic acid-Superose. Proteins were eluted with a linear imidazole gradient (0–300 mM). Shown are column fractions separated by SDS-polyacrylamide gel electrophoresis on a 15% (w/v) polyacrylamide gel stained with Coomassie Blue. Lane 1, unadsorbed fraction; lanes 2 and 3, fractions eluted between 48 and 84 mM imidazole, showing metal-binding E. coli contaminants; lanes 4–12, fractions eluted between 96 and 216 mM imidazole showing purified recombinant nodulin 26 protein. Apparent molecular weight values from standards are indicated. B, Western blot of purified nodulin 26 (0.25 μg of total protein) probed with anti-nodulin 26 IgG (4 μg/ml). Lane 1, S262 nodulin 26; lane 2, S262A nodulin 26; lane 3, S262D nodulin 26.

Figure 2. Sensitivity of ion channel activity to nodulin 26 antibodies. The recording solution was as follows: cis 0.2 M KCl, 20 mM MOPS-KOH, pH 7.4, trans 1.0 M KCl, 20 mM MOPS-KOH, pH 7.4. Voltage potentials were applied by alternating 500-ms pulses from a holding potential of 0 to 80 and −80 mV. A, recombinant S262 nodulin 26 channel; B, channel after addition of 56 μg of preimmune IgG to both chambers (incubation time, 25 min); C, channel after addition of 40 μg of anti-nodulin 26 IgG to both chambers (incubation time 15 min).

Figure 3. Voltage-current relationship of single recombinant S262 nodulin 26 ion channel in planar lipid bilayers. The traces were obtained by using a ramp protocol (−90 to 90 mV, 0.09 mV/ms) as described under “Materials and Methods.” Plot 1, the recording solution was cis 0.2 M KCl, 20 mM MOPS-KOH, pH 7.4, trans 1.0 M KCl, 20 mM MOPS-KOH, pH 7.4. Plot 2, the same channel in symmetrical 0.2 M KCl, 20 mM MOPS-KOH, pH 7.4.

Properties of Mutant Recombinant Nodulin 26 Channels—To address the effects of phosphorylation on nodulin 26, the channel activities of recombinant S262 nodulin 26 as well as S262A and S262D nodulin 26 mutant channels were analyzed. As discussed previously, CDPK phosphorylates only one site on nodulin 26, Ser-262 (8). S262A nodulin 26 possesses an Ala substitution at 262 and thus is not susceptible to phosphorylation by CDPK. Conversely, the S262D mutant (Ser to Asp) was designed to have a permanent negative charge at residue 262 to mimic the phosphorylated form of nodulin 26.

Representative channel records and conductance amplitude histograms are shown in Fig. 4. The data shown are from a typical, representative channel incorporation, but several channel incorporations (17 separate S262 channels, eight S262A channels, and nine S262D channels) have been analyzed and show similar single-channel conductances and voltage-dependent behavior. Similar to native soybean nodulin 26, all recombinant nodulin 26 channel proteins show a maximal single channel conductance of 3.1 nS under standard recording conditions (Fig. 4) and at low applied voltages (e.g. 30 mV) showed a principal single channel conductance of 3.1 nS with only infrequent occupancy of lower subconductance levels (Fig. 4A). We showed previously that native soybean nodulin 26 from symbiosome membranes showed increased channel gating and a tendency to preferentially occupy lower conductance substates at high applied voltages (e.g. 70 mV, Ref. 5). In contrast, at 70-mV potentials, the recombinant S262 and S262A nodulin 26 channels still remained completely open with a principal single channel conductance of 3.1 nS and only infrequent transitions to lower conductance states (Fig. 4B). Based on the amplitude histogram (Fig. 4), these channels exist in the 3.1-nS state greater than 97% of the time. However, the recombinant S262D nodulin 26 channel shows more frequent gating to lower subconductance states at 70 mV (Fig. 4B). Based on the amplitude histogram, the S262D nodulin 26 channel shows three major conductance states at 70 mV: 3.1, 1.8, and 0.6 nS (Fig. 4B), with a preference for the lower substates. The percent occupancy times for the 3.1-, 1.8-, and 0.6-nS states were 13.1, 35.1, and 51.8%, respectively. The data suggest that a negative charge at residue 262 confers voltage-sensitive behavior on the channel and that the phosphorylation of Ser-262 of nodulin 26 by CDPK may regulate voltage-sensitive channel activity. This was tested by direct phosphorylation of S262 nodulin 26 by
Phosphorylation of Nodulin 26 in Situ in Planar Lipid Bilayers by CDPK—

The effects of phosphorylation of the recombinant nodulin 26 channel were investigated using recombinant KJM23–6H2 CDPK (12). KJM23–6H2 is derived from the expression of an Arabidopsis cDNA clone in E. coli and has a substitution of six amino acids in its autoinhibitor site resulting in a highly active, constitutive enzyme activity (12). Because the KJM23–6H2 CDPK is easily prepared at high concentrations in a constitutively active form, we selected this enzyme for phosphorylation studies. Purified KJM23–6H2 CDPK readily phosphorylates CK-15 (a synthetic peptide substrate containing the CDPK recognition sequence and the unique phosphorylation site, Ser-262 of nodulin 26 (8)) showing hyperbolic kinetics, and an apparent $K_m$ of 142 $\mu$M (Fig. 5), and thus has kinetic properties similar to the soybean nodule CDPK activity (4).

The effects of phosphorylation of Ser-262 nodulin 26 were studied by in situ phosphorylation with CDPK after incorporation into planar lipid bilayers (Fig. 6). S262A nodulin 26, which possesses an Ala-262, was used as a negative control. Experiments were performed in symmetric 0.2 M KCl. Under these conditions, both channels show a maximum single channel conductance of 1.6 nS (Figs. 3 and 6). Addition of MgCl$_2$ and ATP did not affect channel properties (data not shown). However, subsequent addition of CDPK resulted in changes in the gating behavior of S262 nodulin 26 at 70 mV (Fig. 6B). At 70 mV, CDPK-treated S262 nodulin 26 showed several conductance substates including 1.6 nS (28.3%), 1.0 nS (24.4%), and 0.6 nS (42.6%), as well as a completely closed state (4.7%) (Fig. 7A). Conductance of S262 nodulin 26 at low voltage potentials (e.g. 30 mV) was not significantly affected by CDPK treatment. Furthermore, CDPK appears to mediate this effect on nodulin 26 by phosphorylation of Ser-262. This is supported by the control experiments that show that S262A nodulin 26 only occupies the fully open 1.6 nS conductance state even after prolonged treatment with CDPK (Fig. 7B).

If phosphorylation is responsible for the change in the voltage sensitivity of nodulin 26, then the effect should be reversed by dephosphorylation of Ser-262. In previous work (9) it was shown that nodulin 26 can be dephosphorylated in vitro by alkaline phosphatase. Alkaline phosphatase treatment of phosphorylated S262 nodulin 26 resulted in the restoration of voltage-insensitive behavior (Fig. 8). Furthermore, this appears to be the result of removal of phosphate from Ser-262 of nodulin 26 since phosphatase treatment of S262D nodulin 26 (Asp-262) has no effect on its voltage sensitivity (data not shown). These data show that phosphorylation at Ser-262 of nodulin 26 by

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**FIG. 5. Phosphorylation of CK-15 synthetic peptide by recombinant CDPK.** A purified, recombinant CDPK (KJM23–6H2, 12) expressed from an Arabidopsis cDNA clone was tested for its ability to phosphorylate the nodulin 26 peptide CK-15. CDPK activity was detected by the incorporation of $^32$P into CK-15 as described under "Materials and Methods." Each data point represents an average of duplicate determinations.

**FIG. 4. Effect of mutations at residue 262 on channel activity of recombinant nodulin 26 channels.** The traces represent currents at 30 mV (panel A) or 70 mV (panel B) obtained by using the pulsing protocol described under "Materials and Methods." The recordings were made in cis 0.2 M KCl, 20 mM MOPS-KOH, pH 7.4, trans 1.0 M KCl, 20 mM MOPS-KOH, pH 7.4. Channel openings are shown as downward deflections from the base line marked as C (for the closed state). Representative 1-s sweeps are shown with conductance levels determined from current-voltage relationships indicated. Conductance amplitude histograms show the event number versus the conductance level(s).
CDPK modulates its channel activity by affecting its voltage sensitivity.

**DISCUSSION**

We have purified recombinant nodulin 26 derivatives expressed in E. coli by Ni²⁺-chelate chromatography, and have shown that they form channels in planar lipid bilayers with large single channel conductances and weak anion selectivity, similar to nodulin 26 from soybean symbiosome membranes (5). However, nodulin 26 proteins with serine or alanine at residue 262 showed no voltage sensitivity, whereas nodulin 26 with aspartate 262 showed voltage-sensitive behavior that included more active gating and a tendency to preferentially occupy lower subconductance states. Nodulin 26 with serine 262 was converted to a similar voltage-sensitive state by CDPK phosphorylation and this effect was reversed by dephosphorylation with alkaline phosphatase. Overall, the data suggest that the presence of a negatively charged residue at position 262 confers voltage-sensitive behavior and that the phosphorylation of Ser-262 of nodulin 26 by CDPK modulates nodulin 26 channel activity.

The data show that the recombinant His-tag nodulin 26 derivatives have the same maximal single channel conductance and ion selectivity values as soybean nodulin 26. These results imply that recombinant nodulin 26 expressed in E. coli is structurally and functionally homologous to the native nodulin 26 molecule and that the presence of the His-tag sequence does not affect its conductance properties. Furthermore, the His-tag allows the purification of nodulin 26 in one step by FPLC on nickel chelate resins by using gradient elution conditions. The use of this system should allow the production of other site-directed mutations to further probe the nodulin 26 structure and function. Another advantage of expression in E. coli, which lacks CDPK, is the generation of nodulin 26 that is not phosphorylated on Ser-262. This is an important consideration for planar lipid bilayer studies since nodulin 26 purified from soybean probably exists as a mixture of phosphorylated and unphosphorylated forms, and it is unclear whether single-channel data represent the insertion of an unphosphorylated or phosphorylated nodulin 26 molecule. From the present study, it can be concluded that voltage-sensitive gating is observed only upon phosphorylation of Ser-262. Interestingly, all soybean nodulin 26 channels examined previously showed voltage-sensitive behavior similar to S262D and phosphorylated S262 recombinant nodulin 26 (5). Thus, a major population of nodulin 26 isolated from symbiosome membranes appears to be phosphorylated before or during purification. This is supported by the observation that alkaline phosphatase treatment of soybean nodulin 26 results in a channel that is less sensitive to

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**FIG. 6.** Single channel recordings of unphosphorylated and phosphorylated S262 nodulin 26. Recordings were made in symmetrical 0.2 M KCl, 20 mM MOPS-KOH, pH 7.4, 0.1 mM ATP, and 1 mM MgCl₂ by using the pulse protocol described under "Material and Methods." The traces (1-s sweeps) show the currents obtained with an applied voltage of 70 mV. Apparent single channel conductances calculated from the various current states are given. A, S262 nodulin 26 channel before CDPK addition; B, the same channel 35 min after the symmetrical addition of CDPK (4.1 μg/ml).

**FIG. 7.** Amplitude histograms of S262 and S262A nodulin 26 before and after CDPK treatment. Recordings were made in symmetrical 0.2 M KCl, 20 mM MOPS-KOH, pH 7.4, 0.1 mM ATP, and 1 mM MgCl₂ by using the pulsing protocol as described under "Material and Methods." Recordings were made before CDPK addition (−CDPK), and after the symmetrical addition of KJM23–6H2 CDPK (4.1 μg/ml final concentration) (+CDPK). The solutions were stirred for 1 min after each addition and were incubated for 35 min (for S262) or 70 min (for S262A) before recording. Recordings were done at 30 and 70 mV. Absolute voltage potential values are used because of the symmetrical nature of the recording conditions and the channel behavior (Fig. 3, plot 2). A, S262 nodulin 26; B, S262A nodulin 26 (negative control).
Phosphorylation of Nodulin 26 Channel

Nodulin 26 is a member of a structurally homologous family of membrane channel proteins (16). In addition to nodulin 26, some other family members are phosphorylated by various protein kinases (17–21). Of particular interest is the similarity between the lens MIP and nodulin 26 with respect to the functional effects of phosphorylation. Similar to nodulin 26, MIP forms channels in planar lipid bilayers with a large unitary conductance and similar ion selectivity (22). Both proteins have an unique phosphorylation site (Ser-262 for nodulin 26 and Ser-243 for MIP) at homologous positions within their COOH-terminal domains (8, 20). However, whereas nodulin 26 is phosphorylated by CDPK, MIP is phosphorylated at Ser-243 by the CAMP-dependent protein kinase (20). Similar to our findings with nodulin 26, unphosphorylated MIP forms a voltage-insensitive channel, and phosphorylation with CAMP-dependent protein kinase results in voltage-sensitive gating behavior and partial channel closure (23). This suggests that phosphorylation within the COOH-terminal region of these proteins results in a similar change in their structure and function as manifested by their channel behavior in planar lipid bilayers.

The mechanism through which phosphorylation affects these proteins is not yet clear. Phosphorylation is a common mechanism for controlling ion channel activities, including through voltage (data not shown).

Nodulin 26 is an in vivo target of calcium-dependent phosphorylation by a calmodulin-like domain protein kinase on the symbiosome membrane of soybean nodules (4, 8). In light of previous results and the present findings, it is attractive to propose a role for calcium-dependent phosphorylation in the regulation of nodulin 26 channel activity in response to membrane potentials. This is further supported by the finding of an electrogenic H+-pumping ATPase on the symbiosome membrane, which is capable of producing large transmembrane potentials (27) that could affect the activity of phosphorylated nodulin 26. However, several potential factors will need to be taken into consideration before assessing the role of nodulin 26 and phosphorylation in symbiosome membrane function. First, the single-channel conductance of nodulin 26 in planar lipid membranes is very large and complete closure is infrequent, even with the phosphorylated form, a condition that may not be likely in vivo (2). However, other endogenous symbiosome membrane lipids or proteins that are absent from the reconstituted planar lipid bilayer system also may contribute to the modulation of nodulin 26 activity along with symbiosome membrane potentials. For example, it has been found that certain membrane lipids (e.g. cholesterol) can attenuate the conductance levels of MIP channels (28). Furthermore, many members of the MIP family are reported to form water channels (29) or channels for uncharged solutes such as glycerol (30). Although it has been reported that MIP is not a water channel (31), other recent evidence suggests that MIP can form a low activity water channel upon heterologous expression in Xenopus oocytes (32, 35). Regardless of these considerations, the planar lipid bilayer experiments have revealed that a fundamental change in the structure and function of nodulin 26 and MIP occurs upon phosphorylation. Further work, possibly in situ with symbiosome membranes, may provide further insight into the biological role of nodulin 26 phosphorylation.

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