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

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Nodulin 26 is a symbiosome membrane protein of soybean nodules that shows ion channel activity in planar lipid bilayers. Serine 262 of nodulin 26 is phosphorylated by calmodulin-like domain protein kinase. To study the effects of phosphorylation, nodulin 26 with Ser, Ala, or Asp at position 262 were expressed in Escherichia coli. The expressed protein possessed a histidine-rich leader sequence for purification by Ni²⁺-chelate fast protein liquid chromatography. Upon reconstitution into planar lipid bilayers, the recombinant proteins showed a large single channel conductance (3.1 nanosiemens (nS) in cis_{2,o}+trans_{1,o}+KCl) and 1.6 nS in cis_{2,o}+trans_{1,o}+KCl) and weak anion selectivity, similar to native soybean nodulin 26. Nodulin 26 with Ser- or Ala-262 occupied the maximal open conductance state greater than 97% of the time (3.1 nS in cis_{2,o}+trans_{1,o}+KCl) regardless of applied voltage. However, nodulin 26 with Asp-262 showed increased gating and preferential occupancy of lower subconductance states (1.8 and 0.6 nS in cis_{2,o}+trans_{1,o}+KCl) at high applied voltages (e.g. 70 mV). In situ phosphorylation of Ser-262 of nodulin 26 by calmodulin-like domain protein kinase also resulted in increased voltage-dependent gating and preferential occupancy of lower subconductance states. These results suggest that phosphorylation of serine 262 of nodulin 26 modulates channel activity by conferring voltage sensitivity.

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).

Nodulin 26 is a member of the MIP³ 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 discreet 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 DNA 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'-AAGAGTGCCTGCTTTCCTCAA-3' for the Ser-262 → Ala substitution, and 5'-AAGAGTGCATTTCTCCTGATTTCCCT-CAA-3' for the Ser-262 → Asp substitution. Mutants were confirmed by dideoxy nucleotide chain termination DNA sequencing with a Sequence-it (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

³ 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, nanosiemens.
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 A₀₂₈₀ reached 0.6. The culture was placed at 4 °C overnight. The cells were collected by centrifugation at 10,000 g for 10 min at 4 °C and resuspended in 40 mL of buffer containing 20 mM Tris-HCl, pH 7.9, 100 mM KI, 1 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, MgCl₂ (10 mM), and DNase I (20 μg/ml) and were added to 1 liter of LB medium, 50 μg/ml carbenicillin, and 34 μg/ml chloramphenicol and were grown at 37 °C with shaking until the A₀₂₈₀ reached 0.6. Isopropyl-1-thio-β-D-galactopyranoside (1 mM) was added, and the culture was incubated at 37 °C with shaking for 2.5–3.0 h. The cells were harvested by centrifugation at 5000 × g for 5 min at 4 °C and washed twice at –80 °C.

The cell pellet was thawed at 22–25 °C in 70 mL of extraction buffer (20 mM Tris-HCl, pH 7.9, 0.5 mM NaCl, 1 mM pepstatin A, 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin). MgCl₂ (10 mM), and DNase I (20 μg/ml) were added, 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, and the pellet was resuspended in 25 mL of 20 mM Tris-HCl, pH 7.9, 1 mM KCl, 1 mM pepstatin A, 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin and was incubated for 30 min at 37 °C. The sample was centrifuged at 100,000 × g for 1 h at 4 °C, and the pellet was washed with 25 mL of extraction buffer.

Nodulin 26 was solubilized by resuspending the pellet in 10 mL of 20 mM Tris-HCl, pH 7.9, 0.5 mM NaCl, 1 mM pepstatin A, 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 0.1 mM leupeptin and was incubated for 1 h at 4 °C, and the supernatant fraction was dialyzed at 4°C against diacetic 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 mM NaCl, 1% (w/v) Glycerol (OG) (column buffer). The column was washed with column buffer until the A₀₂₈₀ reached base line, and nodulin 26 was eluted with a linear gradient of 0–300 mM imidazole (Δ12 mM/ml) 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.

Nodulin 26 in Two-Dimensional Electrophoresis and Electrophysiological Methods—Nodulin 26 proteoliposomes were prepared by reconstitution into phosphatidylcholine bilayers by a dialysis method (5). Thirty-five μg of purified protein was used for each preparation with a mole ratio of nodulin 26 to phosphatidylcholine of 1:2000. This low nodulin 26 to lipid ratio helps ensure the likelihood of incorporation of a single channel into the bilayer (5). After dialysis, proteoliposomes were sedimented at 100,000 × g for 1 h at 4 °C, and the supernatant fraction was dialyzed at 4°C against diacetic 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 mM NaCl, 1% (w/v) Glycerol (OG) (column buffer). The column was washed with column buffer until the A₀₂₈₀ reached base line, and nodulin 26 was eluted with a linear gradient of 0–300 mM imidazole (Δ12 mM/ml) 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.

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a permeability ratio of $P_{Cl}/P_{K}$ of 1.26. Both the single channel conductance and ion selectivity are consistent with values previously determined for soybean nodule nodulin 26 (5).

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 states (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 states at high applied potentials (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 Channel

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 μM (Fig. 5), and thus has kinetic properties similar to the soybean nodule CDPK activity (4).

The effects of phosphorylation of S262 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 results in the restoration of voltage-insensitive behavior (Fig. 8). Furthermore, this appears to be the result of removal of phosphate from S262 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
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

**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

Voltage (data not shown).

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 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 voltagge-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 the regulation of channel gating (24). In the case of nodulin 26 and MIP, one possibility is that phosphorylation of the COOH-terminal domain results in the interaction of this part of the protein with the channel pore, resulting in a change in the kinetic properties of the channel. Evidence is accumulating that cytosolic “gating domains” at the termini of channel proteins interact with the channel pore, resulting in a change in gating kinetics (34). For example, site-directed mutagenesis studies show that removal of the cytosolic amino-terminal domain of the Shaker K⁺ channel results in an open channel that cannot be inactivated (25). Inactivation can be restored by the addition of a synthetic peptide corresponding to the missing residues (25). Similar cytosolic gate domains have been demonstrated in other channels (for review, see Ref. 26), and this may be a common mechanism for controlling channel activity. Evidence that this may be the case in MIP comes from the observation that proteolytic removal of the COOH-terminal region results in a channel with the same single channel conductance that is no longer voltage-sensitive (23). However, the interaction of the phosphorylated COOH-terminal region with MIP and nodulin 26 is likely to be complex since phosphorylation results in the appearance of at least two to three well defined subconductance states and a closed state, rather than just simple channel closure.

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 (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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