Determination of the Site of Phosphorylation of Nodulin 26 by the Calcium-Dependent Protein Kinase from Soybean Nodules†

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ABSTRACT: Nodulin 26 is a nodule-specific protein that is associated with the symbiosome membrane of soybean root nodules. Nodulin 26 is an endogenous substrate for a novel calcium-dependent protein kinase (CDPK) of soybean root nodules. By phosphopeptide mapping of endoproteinase Lys-C-digested nodulin 26 and automated and manual peptide sequence analyses, we have identified the site on nodulin 26 phosphorylated by CDPK. We have also established that the phosphorylation site of nodulin 26 is identical to the phosphorylation site of CK-15, a synthetic peptide with the carboxyl-terminal sequence of nodulin 26. The phosphorylation of nodulin 26 occurs at position Ser^{262}, and the phosphorylation of CK-15 occurs at the analogous position, $Ser,^{6}$ in vitro. Thus, the CK-15 sequence apparently contains sufficient structural features of the phosphorylation site of nodulin 26 to be recognized by CDPK. On the basis of peptide mapping analysis of nodulin 26 from nodules that are metabolically labeled with [^{32}P]phosphate, it appears that the site of nodulin 26 that is phosphorylated in vitro is also labeled in vivo. The data indicate that the carboxyl terminus of nodulin 26 is phosphorylated by CDPK and provide initial sequence data for the phosphorylation site of an endogenous substrate for a plant CDPK.

Recently the existence of a novel class of calcium-dependent protein kinases (CDPK)[1] has been reported in plants [reviewed in Roberts and Harmon (1992)]. This protein kinase activity has been detected in extracts from a number of plant tissues, and it has been purified from soybean tissue culture cells (Harmon et al., 1987; Putnam-Evans et al., 1990). CDPK is distinguished from other calcium-dependent protein kinases since it binds to and is stimulated by micromolar concentrations of Ca^{2+}, it is calmodulin-independent, and its activity is unaffected by added phospholipids, including lipids that modulate the activity of protein kinase C [for a review, see Roberts and Harmon (1992)]. The deduced amino acid sequence of a cDNA of soybean tissue culture CDPK reveals a calmodulin-like regulatory domain containing four EF-hand calcium-binding domains and proposed catalytic and autoinhibitory domains that share the highest sequence identity (39%) with the catalytic and autoinhibitory domains from calmodulin-dependent protein kinase II (Harper et al., 1991). Overall, on the basis of its biochemical properties and sequence comparisons with other protein kinases, CDPK represents a distinct calcium-regulated protein kinase that is widespread in the plant kingdom.

Although a number of substrates for CDPK have been reported in vitro, only a few potential endogenous substrates have been identified (Schaller & Sussman, 1988; Weaver et al., 1991). One proposed endogenous substrate is nodulin 26 from nitrogen-fixing soybean root nodules (Weaver et al., 1991). Nodulins are nodule-specific, host-encoded proteins that are proposed to play a role in the establishment and maintenance of the nitrogen-fixing symbiosis between legumes and rhizobia bacteria [reviewed by Nap and Bisseling (1990)]. Nodulin 26 is among a group of nodulins that is targeted to the plant-derived symbiosome membrane (SM) (Fortin et al., 1987) that surrounds the endosymbiotic rhizobia bacteroids (Mellor et al., 1989). This membrane is responsible for the transportation of key metabolites, such as dicarboxylic acids, from the plant cytosol to the bacteroid to support the nitrogen-fixation process (Udvardi et al., 1988; Ouyang et al., 1990). The deduced amino acid sequence of nodulin 26 possesses limited sequence identity (30–40%) to a number of proteins that have been proposed to constitute a family of transmembrane channel proteins [reviewed by Pao et al. (1991)]. Thus, it has been suggested that nodulin 26 may play a role in the transport functions of the SM.

In previous work, we found that a synthetic peptide, CK-15, that contains the last 14 carboxyl-terminal amino acids of nodulin 26 is phosphorylated by CDPK purified from soybean root nodules (Weaver et al., 1991). Furthermore, a M_r 27 000 protein in isolated SM, that is recognized by antibodies prepared against the CK-15 peptide, is phosphorylated on serine residues by the soybean nodule CDPK under in vitro conditions. The same polypeptide was phosphorylated in intact soybean root nodules metabolically labeled with [^{32}P]-orthophosphate. On the basis of these findings, we proposed that this SM phosphoprotein was nodulin 26 (Weaver et al., 1991). Recently it has been demonstrated that phosphorylation of nodulin 26 correlates with an increase in the dicarboxylic acid transport activity of purified symbiosomes (Ouyang et al., 1991). This supports the possibility that nodulin 26 has a role in metabolite transport in the SM. In the present paper, we extend our studies of nodulin 26 by defining the site of the molecule that is phosphorylated by soybean nodule CDPK.

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[1] Abbreviations: CK-15, synthetic peptide based on the last 14 carboxyl-terminal amino acid residues of nodulin 26; CDPK, calcium-dependent protein kinase; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HPLC, high-performance liquid chromatography; Lys-C, endoproteinase Lys-C; MIP, major intrinsic protein; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; SM, symbiosome membrane; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; TLE, thin-layer electrophoresis; Tris, tris(hydroxymethyl)aminomethane.

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EXPERIMENTAL PROCEDURES

Materials. Endoproteinase Lys-C and trypsin were purchased from Boehringer-Mannheim. Radionuclear chemicals were purchased from Du Pont–New England Nuclear. Triethylamine, phenyl isothiocyanate, Ultra-Pure Tris, and bicinchoninic acid protein assay reagents were purchased from Pierce. Burdick and Jackson acetonitrile, heptane, trifluoroacetic acid (TFA), and ethyl acetate were obtained from Baxter. High-purity formic acid was purchased from Gallard-Schlesinger. All other reagents were of reagent grade or higher. The synthetic peptide CK-15 was prepared on a Biosearch SAM II automated solid-phase peptide synthesizer by the Molecular Biology Resource at the University of Tennessee, Knoxville.

Symbiosome Membrane Purification. Root nodules were harvested from 28–35-day-old soybean plants (*Glycine max* cv Essex) infected with *Bradyrhizobium japonicum* USDA 110. SM were isolated essentially as described by Udvardi and Day (1989) with the following exceptions. Isolated symbiosomes were suspended in 20 mM MOPS–NaOH, pH 7.0, and 0.15 M KCl and were ruptured by extrusion through a 1.5-cm needle (26 gauge). The extrusion was repeated, and the ruptured symbiosomes were centrifuged in a Sorvall HB-4 swinging-bucket rotor at 6000*g* for 10 min at 4 °C. The supernatant fraction was decanted and saved. The pellet was resuspended in fresh 20 mM MOPS–NaOH, pH 7.0, and 0.15 M KCl, and the process was repeated. The resultant 6000*g* supernatant fractions were combined and centrifuged at 100000*g* for 1 h at 4 °C. The 100000*g* supernatant fraction was discarded, and the 100000*g* pellet, which contains the purified SM, was resuspended in 20 mM MOPS–NaOH, pH 7.0, and 0.5 mM EGTA.

Phosphorylation of SM Proteins and CK-15. For in vitro phosphorylation of nodulin 26, isolated SM were incubated in a reaction mixture containing 0.4 mM [γ-^{32}P]ATP (0.45 μCi/nmol), 0.5 mM $CaCl_2$, 25 mM MOPS–NaOH, pH 7.0, 10 mM magnesium acetate, and 1 mM DTT for 20 min at 30 °C. The labeled membranes were centrifuged at 100000*g* for 1 h at 4 °C. The pellet, which contains the labeled membranes, was rinsed twice with 50 mM Tris-HCl, pH 7.8.

CK-15 (200 μg) was phosphorylated in a reaction mixture containing symbiosome membranes (475 μg of protein), 0.4 mM [γ-^{32}P]ATP (0.45 μCi/nmol), 0.5 mM $CaCl_2$, 25 mM MOPS–NaOH, pH 7.0, 10 mM magnesium acetate, and 1 mM DTT for 20 min at 30 °C. CK-15 was purified from the reaction mixture by chromatography on a C_{18} SEP-PAK cartridge (Waters). After application of the reaction mixture, the cartridge was washed with 0.1% (v/v) TFA in water. The column was eluted in a stepwise fashion with 0.1% (v/v) TFA/10% (v/v) acetonitrile followed by 0.1% (v/v) TFA/32% (v/v) acetonitrile. All fractions were tested for CK-15 by a phosphocellulose filter assay (Weaver et al., 1991), and the peptide was found in the 0.1% (v/v) TFA/32% (v/v) acetonitrile fraction. This fraction was pooled, frozen, and lyophilized.

Metabolic [^{32}P]phosphate labeling of nodules was performed by incubating 7.0 g of soybean root nodules in 5 mL of 0.85 mg/L KH_2PO_4, 1.09 mg/L K_2HPO_4, 18.7 mg/L KCl, 123.3 mg/L $MgSO_4 \cdot 7H_2O$, 27.7 mg/L $CaCl_2$, 8.7 mg/L ferric monosodium EDTA, and 1 mCi of [^{32}P]orthophosphate for 2 h at room temperature with shaking. The nodules were extracted, and the SM were isolated as described above with the following exceptions: EGTA was omitted in the extraction buffer, and all buffers contained 10 mM EDTA, 10 mM NaF, and 10 mM sodium pyrophosphate.

Isolation and Analysis of Nodulin 26 and CK-15 Phosphopeptides. Proteolytic digestion of phosphorylated nodulin 26 was performed by resuspending the purified SMs in 50 mM Tris-HCl, pH 7.8, and 0.1% (w/v) SDS to yield a protein concentration of 1 mg/mL. The sample was heated at 85 °C for 5 min and was centrifuged at 100000*g* for 1 h at 25 °C. The supernatant fraction was decanted and saved. Endoproteinase Lys-C (1:100 w/w protease to SM protein) or trypsin (1:50 w/w protease to SM protein) were added to the solubilized SM, and samples were incubated overnight at 37 °C. Proteolytic digestion of labeled CK-15 was performed by incubating purified, phosphorylated CK-15 with endoproteinase Lys-C (1:50 w/w protease to peptide) in 50 mM Tris-HCl, pH 7.8, overnight at 37 °C.

Peptides were isolated from other components in proteolytic digests by chromatography on C_{18} SEP-PAK cartridges. After application of the sample, cartridges were washed with 0.1% (v/v) TFA in water. The cartridges were eluted in stepwise fashion with 30% (v/v), 50% (v/v), and 90% (v/v) acetonitrile in 0.1% (v/v) TFA. The eluants were monitored by measuring Cerenkov radiation. All eluted phosphopeptides from digests of CK-15 and SM were recovered in the 30% (v/v) acetonitrile fractions. These fractions were pooled, frozen, and lyophilized.

Phosphopeptide mapping was accomplished by TLE and TLC analysis of C_{18} SEP-PAK-purified phosphopeptides. TLE was performed by spotting C_{18} SEP-PAK-purified phosphopeptides onto cellulose thin-layer plates (Whatman) along with [^{32}P]orthophosphate and [γ-^{32}P]ATP as standards. Electrophoresis was performed on a Pharmacia flatbed electrophoresis apparatus with a buffer containing formic acid/acetic acid/H_2O, 5:15:80 (v/v), at 1000 V for 40 min as described by Adam et al. (1989). TLC was performed by spotting phosphopeptide samples on cellulose thin-layer plates along with [^{32}P]orthophosphate and [γ-^{32}P]ATP standards as above. Plates were incubated in an atmosphere saturated with TLC buffer (butanol/pyridine/acetic acid/H_2O, 45:30:9:36 v/v) for 30 min. Ascending chromatography was performed in TLC buffer as described by King et al. (1983).

Two-dimensional analysis by TLC and TLE was performed by mixing Lys-C-digested phospho-CK-15 and labeled SM phosphopeptides. Mixed samples were spotted onto cellulose thin-layer plates, and TLE was performed as described above. The plate was allowed to dry completely, and TLC was performed as described above. Phosphopeptides were detected by autoradiography using Wicor X X-ray film.

For HPLC chromatography, C_{18} SEP-PAK phosphopeptides were resuspended in 200 μL of 0.06% (v/v) TFA in water (buffer A). Buffer B contained 0.052% (v/v) TFA/80% (v/v) acetonitrile. The samples were chromatographed on a Vydac C_{18} column (0.46 × 25 cm) by using a Waters HPLC. Phosphopeptides were eluted with a linear gradient of 12.5–43% buffer B over 50 min at a flow rate of 0.5 mL/min. One hundred microliter fractions were collected. Peptide elution was monitored at 210 nm, and phosphopeptide elution was monitored by measuring Cerenkov radiation.

Manual Edman degradation was performed essentially as described by Tarr (1986) and Girault et al. (1989). Purified phosphopeptides (2000 cpm) were placed into 6 × 15 mm borosilicate glass tubes and dried under vacuum. Each cycle of Edman degradation consisted of the following steps: addition of 5 μL of triethylamine, drying under vacuum, addition of 10 μL of 95% ethanol/triethylamine/phenyl isothiocyanate, 7:2:1 (v/v), incubation for 15 min at 50 °C, drying under vacuum, two washes with 200 μL of heptane/

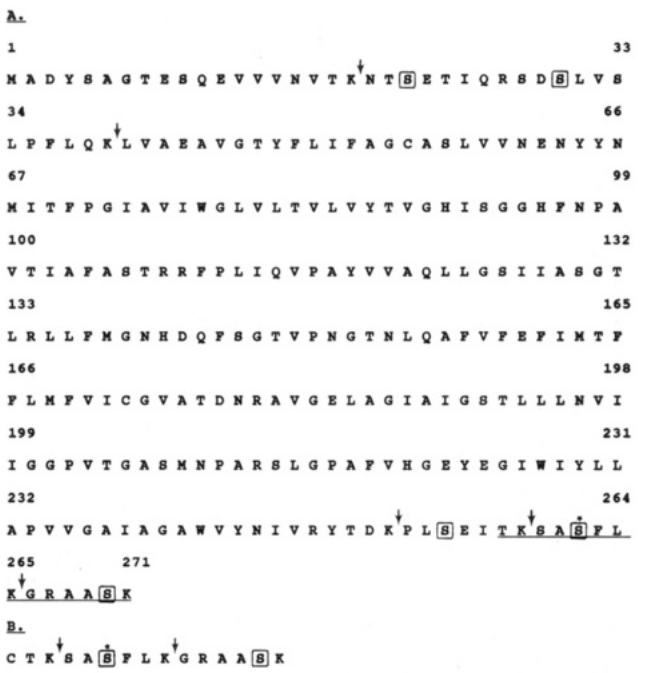

FIGURE 1: Amino acid sequence of nodulin 26 and CK-15. (A) Deduced amino acid sequence of nodulin 26 (Fortin et al., 1987; Sandal & Marker, 1988); (B) amino acid sequence of CK-15 (Weaver et al., 1991). The region of homology between the COOH terminus of nodulin 26 and CK-15 is underlined. Asterisks show the site of phosphorylation by CDPK on each molecule as determined by this study. Arrows indicate predicted sites of cleavage for endoproteinase Lys-C on each molecule. Boxes indicate serine residues that occur within basic-Xaa-Xaa-Ser sequences.

ethyl acetate, 17:1 (v/v), and drying under vacuum. Cleavage was accomplished by the addition of 20 μL of TFA and incubation for 10 min at 50 °C. Following incubation, the samples were dried under vacuum. At the completion of each cycle, one tube for each peptide was withdrawn from the process. Radioactive products were eluted from the sequencing tubes with 100 μL of TLE buffer (acetic acid/formic acid/H_2O, 8:2:90 v/v). The samples were dried under vacuum and resuspended in 8 μL of TLE buffer. The samples were spotted onto cellulose thin-layer plates, along with [^{32}P]orthophosphate as a standard, and were subjected to TLE in TLE buffer at 100 V for 2 h. After electrophoresis, the plates were allowed to dry, and radioactive products were detected by autoradiography using Wicor X X-ray film.

Miscellaneous Methods. SDS–PAGE was performed with the system of Laemmli (1970). Protein concentration was determined using the bicinchoninic acid method (Smith et al., 1985). Automated Edman degradation was performed on 55 pmol of HPLC-purified SM phosphopeptide by using an Applied Biosystems Model 473 automated peptide sequencer. The Pth-amino acid products were analyzed by on-line HPLC as described by Hewick et al. (1981). Amino acid composition analysis was performed on acid hydrolysates as described by Bidlingmeyer et al. (1984).

RESULTS

The deduced amino acid sequence of nodulin 26 contains hydrophilic amino- and carboxyl-terminal regions that have five serine residues (Ser^{22,30,255,262,270}, Figure 1) that reside in sites (basic-Xaa-Xaa-Ser) phosphorylated by CDPK [for a review, see Roberts and Harmon (1992)]. A synthetic peptide (CK-15) based on the last 14 carboxyl-terminal amino acid residues of nodulin 26 contains 2 of these sites (Figure 1) and is phosphorylated by soybean nodule CDPK. Thus,

Table I: Amino Acid Composition of CK-15 Lys-C Phosphopeptide[a]

residue	total pmol	mol of residues/mol of peptide
S	622	2.1
A	263	0.9
F	389	1.3
L	353	1.2
K	353	1.2

[a] Results are from the analysis of 300 pmol of isolated phosphopeptide. Hydrolysis was performed in 6 N HCl at 110 °C for 24 h. The peptide was purified by reverse-phase HPLC of a Lys-C digest of labeled CK-15 by using the conditions described in Figure 5.

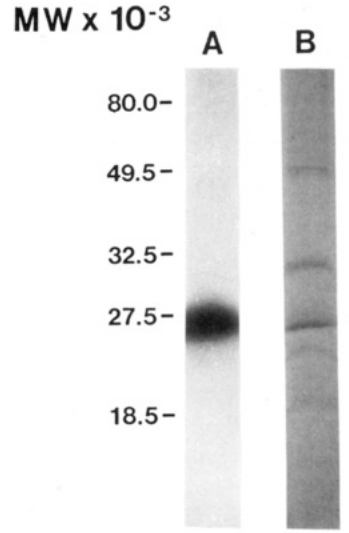

FIGURE 2: Analysis of in vitro phosphorylation of symbiosome membrane preparations. Purified SMs were incubated with [γ-^{32}P]-ATP in the presence of 0.5 mM $CaCl_2$, were resolved by SDS–PAGE on 15% (w/v) polyacrylamide gels (5.0 μg of total protein), and were subjected to autoradiography. (A) Autoradiogram of labeled SM proteins. (B) Coomassie Blue staining pattern of labeled SM proteins.

it has been proposed that the site of phosphorylation resides at the carboxyl terminus of nodulin 26 (Weaver et al., 1991). In order to determine which potential site of CK-15 is phosphorylated, we subjected the labeled CK-15 to digestion with the endoproteinase Lys-C, isolated the labeled peptide, and determined its amino acid composition. As shown in Table I, the composition is consistent with the peptide Ser-Ala-Ser-Phe-Leu-Lys, spanning residues 4–9 of the peptide sequence (Figure 1).

In order to elucidate the actual site of phosphorylation in native nodulin 26, and its relationship to the CK-15 sequence, phosphopeptide maps were generated from nodulin 26 phosphorylated in vitro in SM. Incubation of purified SM preparations with [γ-^{32}P]ATP and calcium results in the labeling of one major band (Figure 2) with an apparent molecular weight of 27 000 on SDS–PAGE. This band corresponds to a major protein in SM that reacts with anti-CK-15 antibodies, and thus we have proposed that it represents nodulin 26 (Weaver et al., 1991). Since this band is the only phosphoprotein detected in our preparations, we used isolated, labeled SM as our source for peptide mapping experiments with endoproteinase Lys-C. On the basis of the location of lysine residues in the sequence of nodulin 26, Lys-C digestion should cleave between all putative phosphorylation sites with the exception of the two sites between residues 20 and 39 (Figure 1). Thus, this protease is especially suited to our analyses since a limited number of peptides should be generated, and we should be able to distinguish most of the

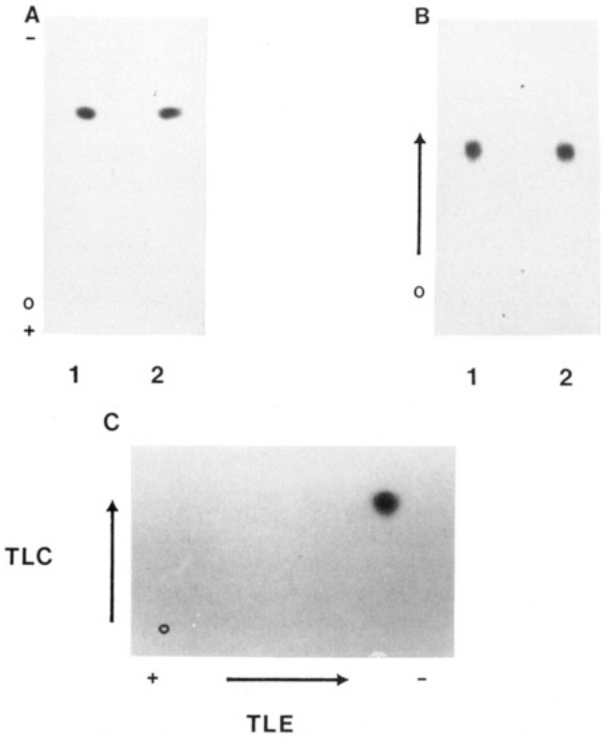

FIGURE 3: Phosphopeptide mapping of in vitro-labeled nodulin 26 and CK-15. Nodulin 26 and CK-15 were labeled in vitro, digested with endoproteinase Lys-C, and analyzed by TLE and TLC as described under Experimental Procedures. (A) 3000 cpm of (1) nodulin 26 and (2) CK-15 phosphopeptides were subjected to TLE and autoradiography. (B) 3000 cpm of (1) nodulin 26 and (2) CK-15 phosphopeptides were subjected to TLC and autoradiography. (C) 3000 cpm of nodulin 26 and CK-15 phosphopeptides were mixed and subjected to two-dimensional analysis (TLE followed by TLC) and autoradiography. o, origin.

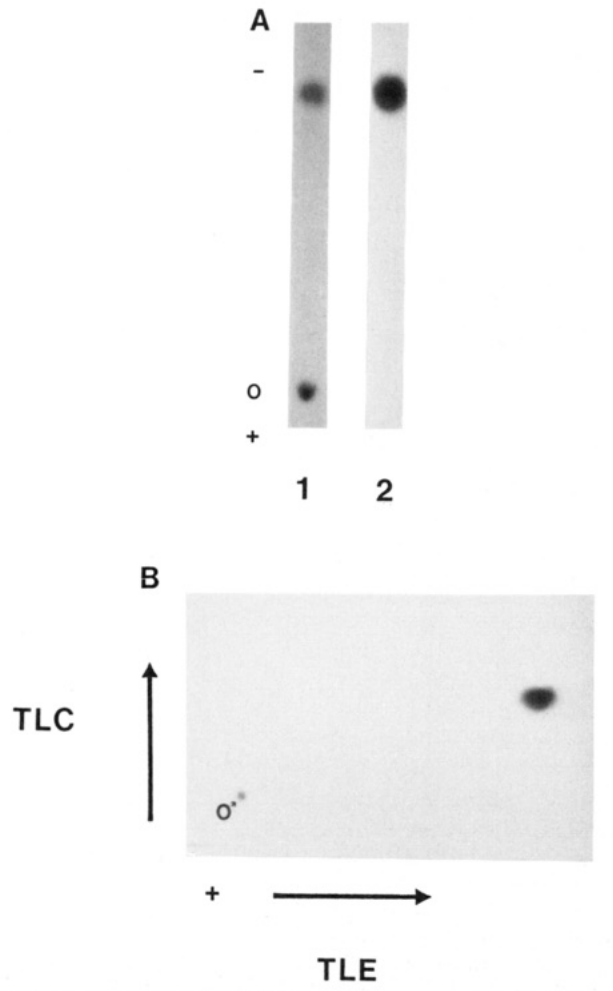

FIGURE 4: Phosphopeptide mapping of nodulin 26 phosphorylated in intact nodules. SM proteins from soybean root nodules labeled with [^{32}P]orthophosphate were digested with endoproteinase Lys-C and were analyzed by TLE and two-dimensional TLE/TLC analysis as described under Experimental Procedures. (A) Autoradiogram of TLE analysis of 500 cpm of (1) nodulin 26 phosphopeptides and (2) in vitro-labeled Lys-C-digested CK-15. (B) 500 cpm each of nodulin 26 phosphopeptides and of Lys-C-digested, phosphorylated CK-15 were mixed and subjected to two-dimensional analysis (TLE followed by TLC) and autoradiography. O, origin.

putative phosphorylation sites. As a control throughout our analyses, we used Lys-C-digested, phosphorylated CK-15 peptide.

TLE and TLC analyses of phosphopeptides generated by Lys-C digestion of CK-15 and nodulin 26 show a single radioactive spot (Figure 3), suggesting that there may be only one phosphopeptide in each preparation. Further, the Lys-C phosphopeptides from CK-15 and in situ-labeled nodulin 26 have the same mobility on single-dimension TLE and TLC (Figure 3A,B) and migrate as a single spot when mixed and subjected to two-dimensional TLE/TLC analysis (Figure 3C). Overall, the data show that the phosphopeptides derived from Lys-C digestion of CK-15 and nodulin 26 have indistinguishable chromatographic and electrophoretic properties and suggest that they may have the same sequence.

Tryptic peptide maps of CK-15 and in vitro-labeled SM were identical to those generated with Lys-C (data not shown). These data rule out three potential phosphorylation sites ($Ser^{22,30,270}$) in nodulin 26, since they occur in Lys-C peptides (20–30 and 265–271, see Figure 1) that have internal arginines. Thus, cleavage of these sequences with trypsin should result in an altered peptide map compared to that generated by Lys-C digestion.

In order to test whether the in vivo phosphorylation site of nodulin 26 is the same as in the in vitro phosphorylation site, SMs purified from nodules metabolically labeled with [^{32}P]-phosphate were analyzed by using the approach described above for in vitro-labeled SM. As in the case of in vitro-labeled SM, nodulin 26 is the only detectable labeled product in in vivo-labeled SM (Weaver et al., 1991). The major Lys-C phosphopeptide from metabolically labeled SMs behaves identically to that from SMs labeled in vitro in one-dimensional TLE and two-dimenstional TLE/TLC analyses (Figure 4). This finding suggests that nodulin 26 is the target of calcium-dependent phosphorylation on the same site in vivo as it is in vitro. A spot remaining at the origin for both TLC and TLE analyses was present in samples derived from metabolically labeled nodules (Figure 4). This spot was resistant to digestion by endoproteinase Lys-C, trypsin, and proteinase K. Therefore, we suggest that it may not be proteinaceous in nature.

In order to confirm the data suggesting that the phosphorylated residues of nodulin 26 are identical to that of CK-15, the Lys-C phosphopeptide from labeled SM was purified by reverse-phase HPLC and analyzed by automated and manual Edman sequencing. Upon HPLC separation of SM phosphopeptides generated by Lys-C digestion, only a single peak of radioactivity was detected (Figure 5). The retention time of the radioactive peak corresponded with that of a major peptide absorbance peak that was well separated from other peptide peaks (Figure 5). The data support the proposal that only one phosphopeptide is generated by Lys-C digestion of nodulin 26 (Figure 5). HPLC analysis of the Lys-C phosphopeptide from digests of CK-15 showed that it has an identical retention time (data not shown). The HPLC-

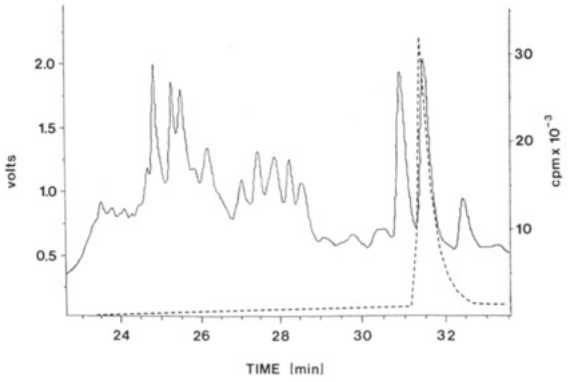

FIGURE 5: Purification of the major phosphopeptide of nodulin 26. Purified SMs were incubated with [γ-^{32}P]ATP in the presence of 0.5 mM $CaCl_2$, were digested with endoproteinase Lys-C, and were chromatographed using a C_{18} SEP-PAK cartridge as described under Experimental Procedures. C_{18} SEP-PAK fractions containing radioactivity were pooled, and peptides were separated by reverse-phase HPLC using a C_{18} column (Vydac). Peptides were detected in the eluate by monitoring the absorbance at 210 nm (solid line). Phosphopeptides were detected in the eluate by collecting 0.1-mL fractions and measuring Cerenkov radiation (dashed line).

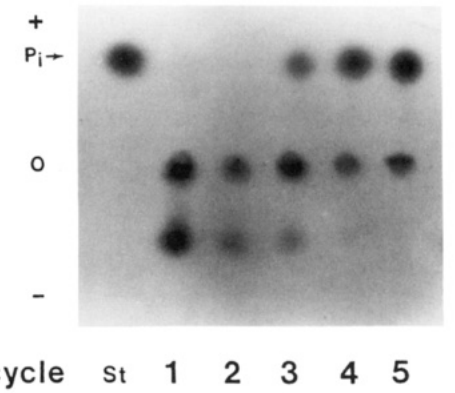

FIGURE 6: Identification of the phosphorylated residue in nodulin 26 by manual Edman degradation. The endoproteinase Lys-C phosphopeptide from in vitro-labeled nodulin 26 was isolated by HPLC and was subjected to manual Edman degradation. St indicates inorganic phosphate standard (P_i). O indicates origin. Numbers indicate how many cycles of manual Edman degradation were performed prior to elution and analysis.

fractionated SM–phosphopeptide was sequenced by automated Edman degradation, and yielded results consistent with the peptide sequence Ser-Ala-Ser-Phe-Leu-Lys. This sequence is consistent with amino acid composition analysis of the phosphopeptide generated by Lys-C digestion of CK-15 (Table I), and corresponds to a unique sequence (residues 260–265) in the carboxyl-terminal portion of nodulin 26 that is shared with CK-15 (Figure 1). Overall, the peptide mapping, HPLC, and protein chemistry data substantiate the claim that nodulin 26 is in fact the substrate of the SM-associated CDPK of soybean root nodules and that the phosphorylation site of nodulin 26 is in the hydrophilic carboxyl-terminal portion of the molecule.

There are two serine residues (Ser^{260} and Ser^{262}) within the nodulin 26 phosphopeptide sequence. In order to address which serine residue of CK-15 and nodulin 26 is phosphorylated, Lys-C phosphopeptides from CK-15, as well as from in vitro- and in vivo-labeled nodulin 26, were subjected to manual Edman degradation. This approach is based on the observation that the phosphodiester bond of amino-terminal phosphoserine residues is hydrolyzed during the cleavage reaction of Edman degradation. Thus, [^{32}P]phosphate should only be released during the cycle that the phosphorylated residue is cleaved from the peptide chain. While the released phosphate molecule remains bound to the glass fiber filter in the automated gas-phase sequencer (Wang et al., 1988), it can be eluted and analyzed by using the manual Edman technique (Girault et al., 1989). As shown in Figure 6, [^{32}P]-phosphate first appears at cycle 3 of the manual Edman degradation of the in vitro-labeled nodulin 26 Lys-C phosphopeptide. Identical results were obtained with Lys-C phosphopeptides from CK-15 and in vivo-labeled nodulin 26 (data not shown). No release is observed in the first cycle, which would be consistent with the phosphorylation of Ser^{260}. Therefore, phosphorylation of nodulin 26 occurs at Ser^{262}, and phosphorylation of CK-15 occurs at the analogous residue, Ser^{6} (Figure 1).

DISCUSSION

CDPK represents a novel type of protein kinase that is widely distributed in plants and algae. CDPK is characterized by its ability to directly bind to and become activated by calcium without the requirement for other effector molecules [reviewed by Roberts and Harmon (1992)]. On the basis of the amino acid sequence of CDPK deduced from its cDNA sequence (Harper et al., 1991; Choi & Suen, 1991), this enzyme is homologous to other protein kinases, with the highest identity (39% identity) shared with the catalytic domain of calmodulin-dependent protein kinase II (Harper et al., 1991). CDPK and calmodulin-dependent protein kinase II also possess similar catalytic properties and phosphorylate some of the same proteins and peptides in vitro (Polya et al., 1989; Roberts, 1989; Putnam-Evans et al., 1990; Weaver et al., 1991). On the basis of a comparison of these substrates, it has been suggested that CDPK, similar to calmodulin-dependent protein kinase II, recognizes basic-Xaa-Xaa-Ser/Thr sequences as phosphorylation sites (Roberts & Harmon, 1992).

The deduced amino acid sequence of nodulin 26 shows five serines that are found within basic-Xaa-Xaa-Ser sequences (Figure 1). However, we find that only one of these sites, Ser^{262}, is phosphorylated by the SM-associated CDPK. Therefore, it seems that the substrate recognition requirements for this CDPK include other elements besides those in the four amino acid phosphorylation motif. However, on the basis of studies with CK-15, it appears as if the 14 amino acids at the carboxyl terminus of nodulin 26 contain enough structural features surrounding Ser^{262} to allow phosphorylation. The specific structural features that allow recognition by CDPK, and whether other sequences outside this region are involved in the interaction with the enzyme, are not yet clear. However, by defining Ser^{262} as the single site of phosphorylation, it is now possible to investigate the properties of this site that allow phosphorylation by the SM-associated CDPK.

Although the phosphorylation site of nodulin 26 has been identified, the effect of phosphorylation of Ser^{262} on nodulin 26 function has not been unequivocally established. Nodulin 26 has been proposed to be a member of a homologous family of membrane proteins, some with known channel activities (Pao et al., 1991), and it has been proposed that nodulin 26 may have a transport role in the SM (Sandal & Marcker, 1988). There is evidence for phosphorylation of other members of the nodulin 26 protein family, and that phosphorylation may affect transport activity. For example, the major intrinsic protein from bovine lens, MIP, is phosphorylated by cyclic AMP dependent protein kinase and protein kinase C (Johnson et al., 1986; Lampe et al., 1986; Lampe & Johnson, 1989). Similar to nodulin 26, MIP is phosphorylated on a serine residue within the hydrophilic carboxyl-terminal region

(Lampe & Johnson, 1990). It has been reported that phosphorylation of MIP results in an alteration of its in vitro channel activity (Ehring et al., 1990).

Recent evidence also suggests that the phosphorylation of nodulin 26 in situ may result in altered metabolite transport. The major transport activity found in the SM is a specific dicarboxylic acid transporter (Udvardi et al., 1988; Ouyang et al., 1990), which is proposed to play a critical role in providing dicarboxylic acids (e.g., malate) to the rhizobia bacteroids to serve as fuel to support nitrogen fixation [reviewed by Day et al. (1990)]. It has been shown that treatment of intact, purified symbiosomes with phosphatase results in a decrease in malate transport that correlates with a decrease in the phosphorylation of nodulin 26 (Ouyang et al., 1991). Subsequent addition of calcium and ATP results in the rephosphorylation of nodulin 26 and the complete restoration of malate transport (Ouyang et al., 1991). This result is consistent with the proposal that nodulin 26 has a transport function, and raises the possibility that nodulin 26 is the dicarboxylate transporter. However, it is also possible that nodulin 26 may play another, possibly indirect, role that is related to dicarboxylate transport. A more thorough investigation of biochemical transport activities associated with nodulin 26, and how phosphorylation of Ser^{262} affects these activities, may provide further insight into the biological function of nodulin 26, and the role of calcium and CDPK in the regulation of nitrogen fixation.

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