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Cyclic β-(1,2)-Glucan Synthesis in *Rhizobiaceae*: Roles of the 319-Kilodalton Protein Intermediate

OLGA A. CASTRO, ANGELES ZORREGUIETA,* VERÓNICA IELMINI, GUSTAVO VEGA, AND LUIS IELPI

Instituto de Investigaciones Bioquímicas, Fundación Campomar, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina

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Cyclic β-(1,2)-glucans are synthesized by members of the *Rhizobiaceae* family through protein-linked oligosaccharides as intermediates. The protein moiety is a large inner membrane molecule of about 319 kDa. In *Agrobacterium tumefaciens* and in *Rhizobium meliloti* the protein is termed ChvB and NdvB, respectively. Inner membranes of *R. meliloti* 102F34 and *A. tumefaciens* A348 were first incubated with UDP-[^{14}C]Glc and then solubilized with Triton X-100 and analyzed by polyacrylamide gel electrophoresis under native conditions. A radioactive band corresponding to the 319-kDa protein was detected in both bacteria. Triton-solubilized inner membranes of *A. tumefaciens* were submitted to native electrophoresis and then assayed for oligosaccharide-protein intermediate formation in situ by incubating the gel with UDP-[^{14}C]Glc. A [^{14}C]glucose-labeled protein with an electrophoretic mobility identical to that corresponding to the 319-kDa [^{14}C]glucan protein intermediate was detected. In addition, protein-linked radioactivity was partially chased when the gel was incubated with unlabeled UDP-Glc. A heterogeneous family of cyclic β-(1,2)-glucans was formed upon incubation of the gel portion containing the 319-kDa protein intermediate with UDP-[^{14}C]Glc. A protein with an electrophoretic behavior similar to the 319-kDa protein intermediate was "in gel" labeled by using Triton-solubilized inner membranes of an *A. tumefaciens exoC* mutant, which contains a protein intermediate without nascent glucan. These results indicate that initiation (protein glucosylation), elongation, and cyclization were catalyzed in situ. Therefore, the three enzymatic activities detected in situ reside in a unique protein component (i.e., cyclic β-(1,2)-glucan synthase). It is suggested that the protein component is the 319-kDa protein intermediate, which might catalyze the overall cyclic β-(1,2)-glucan synthesis.

Members of the *Rhizobiaceae* family synthesize cyclic β-(1,2)-glucans through a mechanism which involves oligosaccharides covalently linked to a large inner membrane protein. Upon elongation to a polymer of about 15 to 25 glucose units, the oligosaccharides are cycled and thus liberated from the protein anchor. The glucose acceptor role of the inner membrane protein and the transient character of its glucosylation have been clearly demonstrated in *Agrobacterium tumefaciens* and *Rhizobium meliloti* (35), *Rhizobium fredii* (4, 5), *Rhizobium loti* (19), and all biovars of *Rhizobium leguminosarum* (9). After neutral cyclic β-(1,2)-glucans are formed, some of them are substituted by phosphoglycerol and/or succinyl residues, probably inside the periplasmic space (3, 6, 13, 20, 21, 32).

The *A. tumefaciens chv* and *R. meliloti ndv* chromosomal regions code for the protein intermediates ChvB and NdvB, respectively, of approximately 319 kDa (15, 33). In addition, these regions code for the ChvA/NdvA protein, which is probably involved in the transport of β-(1,2)-glucans to the periplasmic space (16, 23, 28).

It is likely that formation of cyclic β-(1,2)-glucan requires at least the following three enzymatic activities: (i) one that catalyzes the transfer of the first glucose to an unknown amino acid residue in the protein intermediate, (ii) a glucosyltransferase activity responsible for chain elongation, and (iii) an activity responsible for glucan cyclization and release from the protein. Due to the fact that only cyclic glucan forms have been detected after release from the protein intermediate (34), cyclization and release reactions may proceed in the same reaction step (31, 36). In this paper, we present evidence indicating that a unique protein component carries all three activities. We also suggest that this protein component is likely to be the protein intermediate.

* Corresponding author. Mailing address: Instituto de Investigaciones Bioquímicas, Fundación Campomar, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Av. Patricias Argentinas 435, 1405-Buenos Aires, Argentina. Phone: (54-1) 863-4015. Fax: (54-1) 865-2246. Electronic mail address: azorre@iris.iib.uba.ar.

MATERIALS AND METHODS

Bacterial strains and culture media. *A. tumefaciens* and *R. meliloti* strains (Table 1) were grown in TY medium (0.5% tryptone and 0.3% yeast extract) and yeast extract-mannitol medium (1% mannitol, 0.1% yeast extract, 0.05% K_2HPO_4, 0.02% $MgSO_4$ and 0.02% NaCl), respectively. Bacteria were grown at 28°C in a rotary shaker.

Inner membrane preparation. Inner membranes were purified by fractional centrifugation as previously described (24) and resuspended in 30 mM Tris-HCl buffer, pH 8.2.

Native polyacrylamide gel electrophoresis. Native polyacrylamide gel electrophoresis (PAGE) was carried out in running gels of different acrylamide content (3, 5, or 7%) with an acrylamide/bisacrylamide ratio of 30:0.8, in 0.2 M Tris-HCl (pH 8.8)–0.1% Triton X-100. Gels were polymerized 20 h before electrophoresis in order to inactivate free radicals generated during polymerization. Agarose (0.7%) was added to the 3% polyacrylamide gel to improve manipulation. The stacking gels contained in all cases 3.5% acrylamide–0.1% Triton X-100–0.1 M Tris-HCl (pH 6.8) and were polymerized with 5 μg of riboflavine per ml, TEMED (*N,N,N′,N′*-tetramethylethylenediamine), and irradiation with UV light. Electrophoresis was carried out in a cool room employing 8 by 10 cm gels (0.75 mm of thickness) (Hoefer, San Francisco, Calif.) or 13 by 15 cm gels (1 mm of thickness). When 8 by 10 cm gels were used, electrophoresis was carried out at 30 V during the migration through the stacking gel and at 60 V during the electrophoretic separation. When 13 by 15 cm gels were used, electrophoresis was carried out at 120 V during migration through the stacking gel and at 250 to 300 V during the electrophoretic separation.

"In gel" protein intermediate activity. Inner membranes were solubilized on ice for 30 min with a loading buffer containing 50 mM Tris-HCl (pH 6.8), 1% Triton, 20% glycerol, and 0.1% bromophenol blue. The final protein concentration was approximately 5 mg/ml. Aliquots of the solubilized membranes (40 μg

TABLE 1. Strains used in this study

Strain	Relevant phenotype and genotype	Reference(s)
A. tumefaciens A348	Wild type	12
ME32	*chvB*^{t}, truncated protein intermediate	12, 33
ME42	*chvA*, deficiency in the secretion and modification of cyclic β1,2 glucan	12, 16
A5129	*exoC*, deficiency in the phosphoglucomutase enzyme	7, 30
R. meliloti 102F34	Wild type	11
LI1	*ndvA*, deficiency in the secretion and modification of cyclic β1,2 glucan	28

of protein) were loaded on 5% polyacrylamide (8 by 10 cm) gels and submitted to native PAGE. The gels were washed with 50 mM Tris-HCl (pH 8.2) and gently shaken at 4°C for the indicated times with 5 ml of a solution containing UDP-[^{14}C]Glc (2.9×10^6 cpm, 300 Ci/mol), 20 mM $MgCl_2$, and 50 mM Tris-HCl (pH 8.2). The labeled solutions were discarded, the gels were further washed, and the proteins were fixed with 20 volumes of 50% methanol–10% acetic acid for 1 h. Proteins were stained for 30 min with Coomassie brilliant blue and destained with 20% methanol–7% acetic acid. Radioactivity was detected by fluorography with 1 M salicylic acid (10). X-Omat films from Kodak were used. Relative intensities were determined with a Scan Jet 4C (Hewlett-Packard) and the Image Quant version 3.22 program (Molecular Dynamics).

In gel synthesis of β-(1,2)-glucan. Inner membranes were solubilized and submitted to native PAGE. The gels were washed as described above. Gel slices were cut in 1-mm slices, transferred to reaction tubes, and incubated in the presence of UDP-[^{14}C]Glc (2.5×10^5 cpm, 300 Ci/mol), 20 mM $MgCl_2$, and 50 mM Tris-HCl (pH 8.2) at 25°C for 60 min. Reactions were stopped by dilution and centrifugation. Supernatants were analyzed with DEAE-Sephadex (36). Radioactive material in the DEAE-Sephadex percolates was quantified.

HPLC. The compounds synthesized in vitro were further purified with Bio-Gel P4 (34) and submitted to high-performance liquid chromatography (HPLC). HPLC was performed on a reversed-phase ODS2 C18 column (5 μm, 4 by 250 mm; Pharmacia, Uppsala, Sweden), as previously described (9).

Acid hydrolysis and paper chromatography. Partial acid hydrolysis was carried out in 0.5 N HCl at 100°C for 20 min. HCl was removed by evaporation under an air stream, and hydrolysates were subjected to descending paper chromatography on Whatman no. 1 paper with a solution of isopropanol-acetic acid-water (27:4:9, vol/vol/vol).

Digestion with Glusulase. Compounds synthesized in situ were further purified by Bio-Gel P4 chromatography (34). Digestion with Glusulase and quantitation of the [^{14}C]glucose that was liberated was carried out as previously described (34).

RESULTS

Native PAGE of the protein intermediate. To test whether the 319-kDa protein intermediate carries out some or all of the activities required for cyclic β-(1,2)-glucan synthesis, we decided to employ native PAGE. We initially studied the behavior of the [^{14}C]glucan protein intermediate (15, 35). Inner membranes of *R. meliloti* 102F34 were incubated with UDP-[^{14}C]glucose as previously described (35). Membranes thus labeled were solubilized with 1% Triton X-100 and submitted to native PAGE in 3, 5, and 7% acrylamide gels. In all cases a radioactive band corresponding to the NdvB protein intermediate was detected by fluorography. As depicted in Fig. 1A, C, and D, a lower mobility corresponded to a higher acrylamide percentage in the gel. Addition of the ionic detergent deoxycholate to the solubilization buffer produced the sharpest bands (Fig. 1C). However, it could not be used because of a negative effect on [^{14}C]Glu incorporation in in vitro assays for the protein intermediate (data not shown). For optimal activity and resolution, native PAGE in the following experiments was performed in a 5% acrylamide gel in the presence of Triton X-100.

As mentioned above, it has been proposed that the ChvA/NdvA protein is involved in the transport of cyclic β-(1,2)-glucans to the periplasmic space (8, 16, 23, 28). In addition, a complex between the ChvB and ChvA proteins has been suggested (16). Triton-solubilized inner membranes of an *R. meliloti* LI1 *ndvA* mutant were analyzed simultaneously on the nondenaturing gel. The absence of the NdvA protein did not alter the electrophoretic behavior of the NdvB protein (Fig. 1).

A radioactive band of similar mobility to the one obtained with *R. meliloti* was detected when inner membranes of *A. tumefaciens* wild-type strain A348 incubated with UDP-[^{14}C]glucose were submitted to native PAGE (not shown). The latter bacterium was used in further experiments as the radioactivity associated with the protein intermediate was much higher.

In gel β-(1,2)-glucosyltransferase activity. To determine the possible presence of the above-mentioned enzymatic activities in the protein intermediate itself, solubilized inner membrane proteins of *A. tumefaciens* A348 and ME42 (*chvA* mutant) were submitted to native PAGE, and assayed for in gel protein intermediate activity as described in Materials and Methods

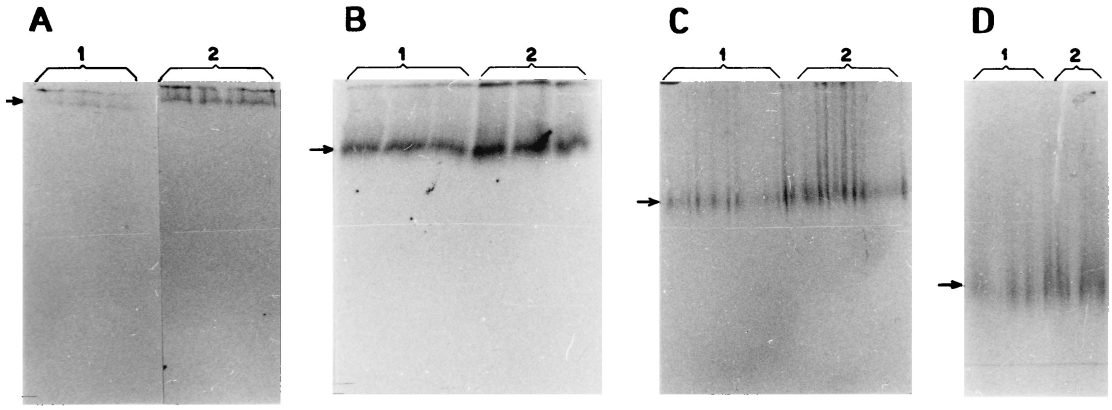

FIG. 1. Native PAGE of the solubilized [^{14}C]glucan protein intermediate. Inner membranes of *R. meliloti* 102F34 (lanes 1) and LI1 (lanes 2) were incubated with 2×10^5 cpm of UDP-[^{14}C]glucose (300 Ci/mol) and 10 mM $MgCl_2$ at 10°C for 40 min and solubilized on ice for 45 min with a solution containing 50 mM Tris-HCl (pH 6.8), 20% glycerol, and either 2% Triton X-100 (A, C, and D) or 2% Triton X-100 plus 0.5% deoxycolate (B). Solubilized inner membranes (150 μg of protein) were submitted to PAGE under native conditions on 7% (A), 5% (B and C) and 3% (D) acrylamide gels (see Materials and Methods). The running gel shown in panel B contained 0.025% deoxycolate in addition to 0.1% Triton X-100.

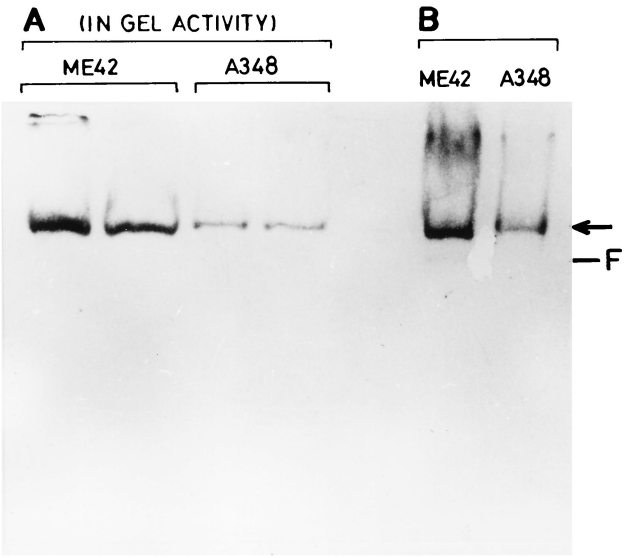

FIG. 2. In gel activity of the protein intermediate. (A) Duplicates of Triton X-100-solubilized inner membranes (50 μg of protein) of *A. tumefaciens* A348 and ME42 (*chvA*) were submitted to native PAGE and assayed for in gel protein intermediate activity as described in Materials and Methods. (B) Duplicates of inner membranes of *A. tumefaciens* A348 and ME42 incubated in vitro with UDP-[^{14}C]Glc under usual conditions (36) and solubilized with Triton X-100 were analyzed as controls. The sample lanes corresponding to the controls were fixed without any treatment and exposed as described in Materials and Methods. F, ferritine marker. The arrow shows the ChvB [^{14}C]glucan-protein intermediate.

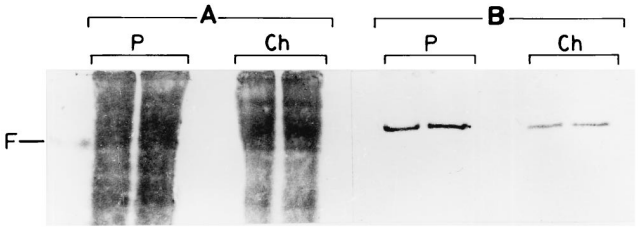

FIG. 3. In gel pulse and chase experiment. Four samples of solubilized inner membranes of *A. tumefaciens* A348 (50 μg of protein) were submitted to native PAGE and assayed for in gel protein intermediate activity. A gel portion corresponding to two lanes was further washed with fixed solution as described in Materials and Methods (lanes P, pulse). The gel portion corresponding to the remaining two lanes was further washed with 50 mM Tris-HCl, pH 8.2, and incubated at 4°C for 60 min and at room temperature for 30 min with 10 mM nonradioactive UDP-Glc–20 mM $MgCl_2$ in the same buffer. The gel was washed with fixed solution as described (lanes Ch, chase). F, ferritin marker. (A) Gels stained with Coomassie blue. (B) Gels with radioactivity detected by fluorography.

(Fig. 2A). Samples of inner membranes of both strains incubated with UDP-[^{14}C]Glc (35) prior to solubilization with Triton X-100 were used for comparison (Fig. 2B). As shown in Fig. 2A, an inner membrane protein of *A. tumefaciens* A348, depicting an electrophoretic mobility similar to that of the previously labeled protein intermediate (Fig. 2B), was in situ labeled. This result indicated that the activity responsible for [^{14}C]glucose labeling of the 319-kDa protein intermediate comigrated with this protein.

An identical but more strongly labeled protein was detected with Triton-solubilized inner membranes of the *A. tumefaciens* ME42 strain (a *chvA* mutant) (Fig. 2A). A higher [^{14}C]glucose incorporation on the protein intermediate, compared with that of the wild-type protein, was also observed by sodium dodecyl sulfate-PAGE upon incubating the inner membranes of ME42 and other *chvA* mutants with UDP-[^{14}C]Glc (16). Similar to the *R. meliloti* NdvB and NdvA proteins, the absence of a functional ChvA protein did not alter the [^{14}C]glucan protein intermediate mobility, labeled either previously (Fig. 2A) or in situ (Fig. 2B).

Pulse and chase in situ. To determine if the protein intermediate labeled in gel behaved in situ as a reaction intermediate, a pulse and chase in situ experiment was carried out. The result of adding nonradioactive UDP-Glc to half of the gel is shown in Fig. 3. It was observed that the radioactivity on the protein diminished, although it did not completely disappear. Relative quantitation showed a 55% reduction of the label. This result suggests that the activity responsible for glucan release from the protein comigrated with the 319-kDa protein intermediate. Nonionic detergents, such as Triton X-100 and Nonidet P-40, or ionic osmolytes strongly inhibited cyclization but not elongation (data not shown). An incomplete washing of ionic molecules or of Triton X-100 from the gel matrix may account, therefore, for the partial radioactivity displacement observed upon addition of unlabeled UDP-Glc.

Characterization of products synthesized in situ. The reaction products synthesized in situ were characterized in order to further define the reaction(s) catalyzed within the gel matrix. After native PAGE, sample lanes corresponding to proteins of both *A. tumefaciens* A348 and ME42 were cut in 1-mm slices and assayed for cyclic β-(1,2)-glucan synthesis as described in Materials and Methods. The soluble products formed by the gel slices were analyzed with DEAE-Sephadex. Radioactive compounds in the percolates (neutral compounds) were only obtained with gel slices where the protein intermediate migrated. Partial acid hydrolysis of the soluble and neutral compounds yielded [^{14}C]Glc, [^{14}C]sophorose (Glc-β1,2-Glc), and a series of higher homologous ^{14}C-oligosaccharides (data not shown). A similar pattern was obtained upon partial acid hydrolysis of cyclic β-(1,2)-glucans of *A. tumefaciens* (34). Therefore, gel slices corresponding to the positions of the protein intermediate of *A. tumefaciens* A348 and ME42 synthesized β-(1,2)-glucans in situ. HPLC analysis of the β-(1,2)-glucans showed that although their size range was similar to that of compounds synthesized by isolated inner membranes of *A. tumefaciens* A348, a higher proportion of compounds with a larger degree of polymerization were formed by the gel-trapped enzyme preparation (Fig. 4).

The cyclic nature of the β-(1,2)-glucans synthesized in situ was studied by treatment with snail intestinal juice as previously reported (34). After an overnight treatment, 75% of the Bio-Gel P4 purified β-(1,2)-glucan was resistant to degradation with Glusulase. A similar value was obtained by digestion of a sample of authentic cyclic β-(1,2)-glucan. Treatment carried out with a partial hydrolysate of cyclic β-(1,2)-glucans as a control released more than 80% of [^{14}C]Glc. These results agree with the conclusion that the β-(1,2)-glucans formed in situ have a cyclic structure (34). Thus, the activity responsible for glucan cyclization also comigrated with the 319-kDa protein intermediate.

In gel activity of the protein intermediates of *exoC* and *chvB*^{t} mutants. *A. tumefaciens exoC* mutants are phosphoglucomutase negative and thus do not synthesize in vivo UDP-glucose and glucan. On the other hand, inner membranes of the same mutants transferred [^{14}C]glucose to the protein intermediate and synthesized cyclic β-(1,2)-glucans when incubated with UDP-[^{14}C]Glc (30). Uttaro et al. proposed that the protein intermediate of *exoC* mutants is free of nascent glucan chains (30). In order to investigate whether the product of the first step of β-(1,2)-glucan synthesis, i.e., the transfer of the first

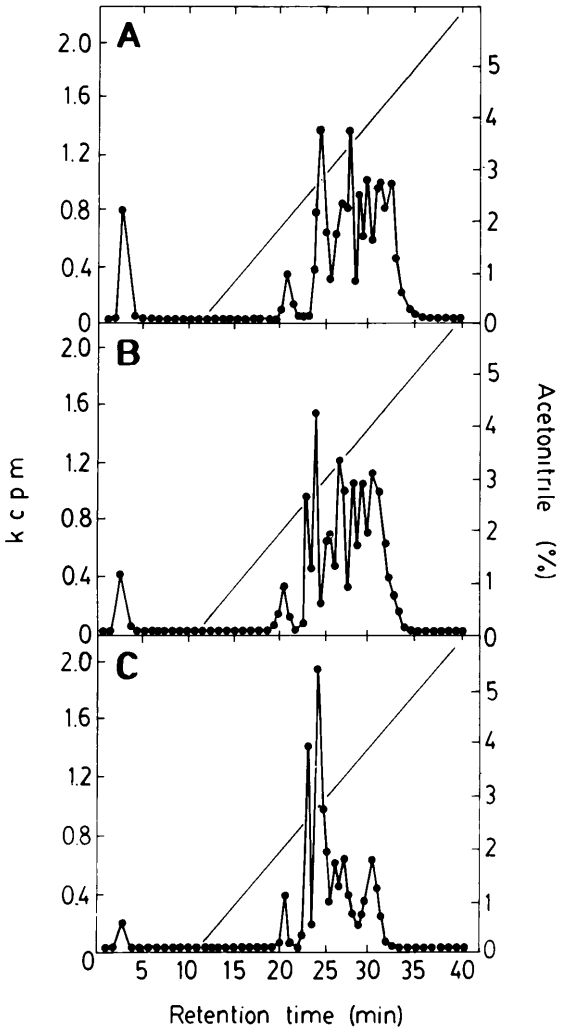

FIG. 4. HPLC patterns of products synthesized in situ. Triton X-100-solubilized inner membranes of *A. tumefaciens* A348 and ME42 were submitted to native PAGE. Half of the gel was assayed for in gel activity as a marker of the protein intermediate position. Samples lanes of the other half of the gel were cut into 1-mm slices and assayed for cyclic β-(1,2)-glucan synthesis in a total volume of 100 μl. The products formed by the gel slices corresponding to the protein intermediate position were further purified with Bio-Gel P4 and analyzed by HPLC. (A) *A. tumefaciens* ME42. (B) *A. tumefaciens* A348. (C) Cyclic β-(1,2)-glucan formed by inner membranes of *A. tumefaciens* A348 (36).

glucose to an amino acid residue, also comigrated with the 319-kDa protein intermediate under native electrophoresis conditions, the glucosyltransferase activity of an *exoC* mutant (*A. tumefaciens* A2159) was assayed in situ. As shown in Fig. 5A, the solubilized protein intermediate of the mutant was in gel labeled, thus indicating that indeed the activity responsible for the transfer of the first glucose unit comigrated with the 319-kDa protein intermediate. However, in contrast to the wild-type protein, the label on the ExoC protein intermediate was not chased after nonradioactive UDP-glucose was added (Fig. 5B). This is consistent with previous data that showed a low displacement of the radioactivity from the ExoC protein intermediate (i.e., cyclization and release reactions) by the nonradioactive sugar nucleotide in an in vitro assay (30).

The *A. tumefaciens* ME32 *chv*^{t} mutant synthesizes a truncated protein intermediate with an estimated molecular mass of 150 kDa which is active in β-(1,2)-glucan synthesis (33). We

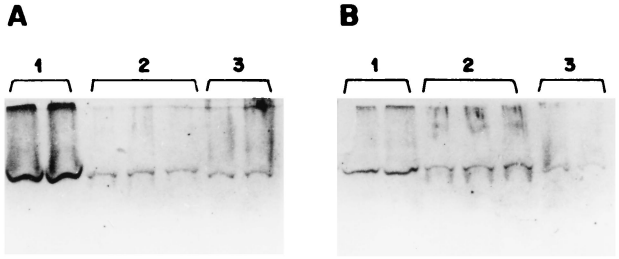

FIG. 5. In gel activity of the protein intermediates of *exoC* and *chvB*^{t} mutants. Duplicates of Triton-solubilized inner membranes (50 μg of protein) of *A. tumefaciens* A348 (lanes 1), A2159 (*exoC* mutant) (lanes 2) and ME32 (*chvB*^{t} mutant) (lanes 3) were submitted to native PAGE and assayed for in gel protein intermediate activity. Half of the gel was further washed with fixed solution (pulse) (A). The other half of the gel was further washed with 50 mM Tris-HCl (pH 8.2) and incubated at 4°C for 60 min and at room temperature for 30 min with 10 mM nonradioactive UDP-glucose–20 mM $MgCl_2$ in the same buffer. The gel was washed with fixed solution (chase) (B). Radioactive material was detected in both gels by fluorography.

tested if the glucosylation and glucan cyclization and release activities comigrated with this truncated protein. The solubilized truncated protein, which showed a slightly higher mobility on native electrophoresis compared with the wild-type species, was also labeled in situ (Fig. 5A). Furthermore, 40% of the label was chased after nonradioactive UDP-Glc was added (Fig. 5B). Thus, the carboxy-terminal half of the protein was dispensable for in situ [^{14}C]glucose incorporation. This supports a previous suggestion that only the 150-kDa amino-terminal portion of the protein intermediate is essential for cyclic β-(1,2)-glucan formation (33).

DISCUSSION

Synthesis of bacterial polysaccharides proceeds through oligosaccharide-lipid or oligosaccharide-protein intermediate formation. Oligosaccharide-polyprenyl phosphate precursors were clearly demonstrated as intermediates in heteropolysaccharide synthesis of several bacteria (14, 29). On the other hand, cyclic β-(1,2)-glucans are synthesized through oligosaccharides covalently linked to an inner membrane protein of about 319 kDa (ChvB or NdvB in *A. tumefaciens* or *R. meliloti*, respectively) as intermediates.

In vitro, the 319-kDa protein intermediate is labeled on incubation of inner membranes with UDP-[^{14}C]Glc (35). As reported here, we have solubilized the [^{14}C]glucan-protein intermediate with Triton X-100 from inner membranes of *R. meliloti* and *A. tumefaciens*. The protein was resolved on electrophoresis under native conditions by using a large porous acrylamide gel. A labeled band corresponding to the native [^{14}C]glucan-protein intermediate was detected.

It may be envisaged that cyclic β-(1,2)-glucan synthesis involves at least three enzymatic activities. The first one may catalyze the transfer of the first glucose from UDP-Glc to an unknown amino acid of the protein intermediate. The second enzymatic activity [UDP-Glc:β-(1,2) oligosaccharide glucosyltransferase] may be responsible for chain elongation. Finally, the third activity may catalyze glucan cyclization and release from the protein. The data presented here demonstrate that the three steps are catalyzed by the same protein band after Triton-solubilized inner membrane proteins are resolved in native electrophoresis.

The results reported in this paper support the notion that the ChvB/NdvB protein promotes an autoglucosylation reaction. Nevertheless, the possibility that certain reaction steps

could be catalyzed in situ by another inner membrane polypeptide in strong association with the ChvB/NdvB protein cannot be ruled out. In eukaryotic systems, for homopolysaccharides such as glycogen, nascent α-1,4 chains are built up joined to a carrier protein (18, 25–27). It has been suggested that the glucose transfer from UDP-Glc from the second glucose up to a few units is catalyzed by the same carrier protein (26). On the other hand, there is biochemical evidence suggesting that the UDP-Glc:protein transglucosylase is the first-step enzyme and the acceptor protein in starch biosynthesis (2, 22).

As expected, the ChvA protein was not required for the reactions detected in situ. As mentioned above, it has been previously proposed that this protein might be involved in glucan extrusion to the periplasmic space.

Kinoshita et al. (17) purified to apparent homogeneity a β-(1,2)-glucan synthase (synthase I) with an estimated molecular mass of 350 kDa from a crude extract of *Agrobacterium radiobacter*. Nevertheless, the only activity assayed by these authors was that of elongation. It seems probable that synthase I represents the 319-kDa protein intermediate (ChvB/NdvB) described in several species of *Rhizobiaceae*. If this was the case, the results obtained on the biochemical characterization of synthase I would support the evidence described in this paper.

On the other hand, it has been proposed that size distribution of the cyclic β-(1,2)-glucan products, which varies with species, depends on competition between elongation and cyclization reactions (31). The relative levels of both activities in the ChvB/NdvB protein intermediates would then determine the size range of cyclic β-(1,2)-glucans.

It has been suggested, by indirect genetic evidence, that only the *chvA*/*ndvA* and *chvB*/*ndvB* genetic loci are required for the synthesis of neutral cyclic β-(1,2)-glucans. Cyclic β-(1,2)-glucan synthesis ability was transferred to *Azospirillum brasilense* (which does not produce the glucan) by introduction of a cosmid containing the entire *chv* region (1). Nevertheless, it must be remarked that the cosmid insert contained additional places for other putative genes. In addition, it was demonstrated that *chvA*/*ndvA* mutants synthesize neutral cyclic β-(1,2)-glucans in vitro, as was confirmed in the present paper. Therefore, from these data taken together, it may be concluded that only the *chvB*/*ndvB* loci are essential for neutral glucan synthesis. Since the *ndvB* locus only encodes the 319-kDa protein intermediate (15), it may be concluded that this protein is solely required for glucan synthesis.

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