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VirB1, a Component of the T-Complex Transfer Machinery of *Agrobacterium tumefaciens*, Is Processed to a C-Terminal Secreted Product, VirB1*

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During genetic transformation of plant cells by *Agrobacterium tumefaciens*, 11 VirB proteins and VirD4 are proposed to form a transmembrane bridge to transfer a DNA-protein complex (T-complex) into the plant cytoplasm. In this study, the localization of the first product of the *virB* operon, VirB1, was studied in detail. While full-length VirB1 localized mostly to the inner membrane, an immunoreactive VirB1 product was found as soluble processed form, designated VirB1*. Equal amounts of VirB1* could be detected in concentrated culture supernatants versus associated with the cell. VirB1* was purified from the supernatant of *vir*-induced cells by ammonium sulfate precipitation and Q-Sepharose chromatography. Sequence analysis of the N terminus of VirB1* localized the processing site after amino acid 172 of VirB1. Cell-associated VirB1* was partly removed by vortexing, suggesting a loose association with the cell or active secretion. However, cross-linking and coimmunoprecipitation showed a close association of cell-bound VirB1* with the VirB9-VirB7 heterodimer, a membrane-associated component of the T-complex transfer machinery. Homologies of the N-terminal part of VirB1 to bacterial transglycosylases suggest that it may assist T-complex transfer by local lysis of the bacterial cell wall, whereas the exposed localization of the C-terminal processing product VirB1* predicts direct interaction with the plant. Thus, VirB1 may be a bifunctional protein where both parts have different functions in T-complex transfer from *Agrobacterium* to plant cells.

Secretion of virulence factors from pathogenic microorganisms plays a major role in the interaction with their hosts. In the case of gram-negative bacteria, these macromolecules (proteins or protein-DNA complexes cross two membranes of the pathogen and, depending on the system, up to two membranes of the host to reach their target in the cell. Current nomenclature defines four types of secretion or transfer systems (52). Type I systems contain three gene products which secrete proteins carrying a C-terminal recognition signal without a periplasmic intermediate (e.g., proteases from *Erwinia* species and hemolysins from *Escherichia coli*) (53). Type II systems (47) secrete proteins after Sec-dependent export into the periplasm and cleavage of the signal peptide by signal peptidase I or propilin peptidase. In the periplasm, at least partial folding of the proteins occurs, which in some cases depends on periplasmic chaperones. Also, periplasmic Dsb enzymes catalyze the formation of disulfide bonds, which is often necessary for further secretion. The type II machinery required for secretion through the outer membrane (e.g., of pectinases, cellulases, lipases, and pullulanase) consists of at least 13 proteinaceous components which localize in the inner and outer membranes. Homologous proteins are also required for assembly of type IV pili (27). Type III systems secrete proteins in a Sec-independent manner, and in the case of Yop proteins from *Yersinia pseudotuberculosis*, secretion was shown to depend on N-terminal signal sequences which were not cleaved (57). An interesting feature of type III systems is that contact with host cells triggers their action. Recently, evidence from animal and plant pathogen systems suggests that some of these systems are not secretion systems in the strict sense, but that their function is to directly transfer virulence factors into host cells (e.g., Avr proteins from phytopathogenic pseudomonads and xanthomonads, Yop proteins from yersiniae, Inv proteins from *Salmonella* and *Shigella* species, and virulence factors from *E. coli*) (3, 25, 51). Type IV systems transfer protein-DNA complexes from a donor to a recipient cell, and their action often depends on contact with host cells too (52). Examples are the conjugative transfer of plasmid-DNA in different incompatibility groups (IncW, IncP, and IncN) and the transfer of T-complex from *Agrobacterium tumefaciens* to plant cells. Also, the *ptl* (pertussis toxin liberation) operon from *Bordetella pertussis*, which is required for secretion of pertussis toxin, encodes nine proteins which show homology to those of plasmid conjugation systems (66), and *Helibacter pylori* uses a VirB4 homolog for secretion of an interleukin-8-inducing factor (64). Thus, type IV systems may use a common mechanism for secretion or transfer of widely different substrates, such as elongated large protein-DNA complexes versus much smaller toxins (33, 36, 67).

A common feature of all secretion and transfer systems is that they contain several membrane-associated and periplasmic components believed to assemble into complexes spanning one or both membranes of gram-negative bacteria, and in some cases they may even form a bridge for delivery of virulence factors to host cells. At least one of these proteins has a consensus nucleotide binding motif and may supply energy for secretion and/or complex assembly. Because of their role in host-pathogen interactions, these systems are currently the subject of intense studies with the goal of intervention, and the following questions are at the center of interest. First, how does the assembly of secretion/transfer complexes in the mem-

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branes proceed, and which protein-protein interactions play roles? Second, which of the multiple components play structural roles, which are catalysts, and which supply energy for the secretion/transfer process? Third, what is the basis of the strict substrate specificity observed in many systems? Fourth, direct contact between bacteria and their host cells triggers action of some type III systems, and type IV systems may function in a similar way. Is an interaction between the transfer machinery and host cells responsible for this phenomenon, and which are the components involved?

The analysis of T-complex transfer from *Agrobacterium* to plant cells as a model system is well suited to address these questions because recent studies on the transfer machinery have laid the foundation for deeper understanding of its structure and function. The T-complex contains a single-stranded copy of the T-region on the Ti plasmid, which can be up to 22 kb long (29, 68, 72). According to a widely held view, T-complex transfer proceeds as follows. The T-DNA is covalently linked at the 5′ end to VirD2 and covered on its entire length by up to 600 molecules of a single-stranded DNA binding protein, VirE2. The T-complex passes two bacterial membranes and the plant cytoplasmic membrane and, after entry to the nucleus, integrates into plant chromosomal DNA (13, 14, 70). However, indirect evidence suggests that the VirB complex also transfers VirE2 to plant cells alone, and an alternative scenario implying separate transfer of a VirD2–T-DNA complex and VirE2 was proposed (10, 43, 60, 71). The transfer to plant cells requires 10 products of the *virB* operon and VirD4, and antibodies against all components were generated and used for analysis of their localization in the membranes (5a, 8, 9, 12, 19, 21, 23, 32, 41, 55, 56, 62). Further, analysis of PhoA fusions, investigation of accessibility to proteases, and immunoelectron microscopic studies gave clues toward understanding the membrane topology of the components of the transfer complex (7, 19, 63). Another important achievement was the generation and analysis of in-frame deletions in all 11 *virB* genes by Berger and Christie (9), which established that VirB2 through VirB11 are absolutely essential for T-complex transfer. In contrast, a 100-fold reduction of virulence was observed after loss of VirB1. Deletion of some *virB* genes, e.g., *virB7* and *virB9*, led to reduction of steady-state levels of several VirB components, which implicated protein-protein interactions involved in assembly or stabilization of the transfer complex (9). Complementation analysis of deletion mutants further supported the stabilizing effect of a subset of VirB proteins (20, 32).

We have addressed T-complex transfer from a biochemical approach aimed at characterizing topology and protein-protein interactions of the transfer machinery. To address VirB proteins possibly involved in contact formation between *Agrobacterium* and plant cells, we focused on VirB products with signal peptides. Such proteins may localize to the exterior of the bacterial cell and directly contact plant cells after attachment at the wound site. This contact could trigger formation of a channel or conjugation bridge through which the T-complex travels to the plant cytoplasm. Here we report analysis of VirB1. While full-length VirB1 was found predominantly in the inner membrane, a major fraction was processed to a C-terminal product VirB1*. VirB1* was found to be weakly associated with the T-complex transfer machinery and partly secreted, and it may therefore be involved in contact formation with plant cells. In addition, homology of the N terminus of VirB1 to bacterial transglycosylases and putative components of type II and III systems implies that this aspect of its function is conserved in a wide variety of macromolecular secretion/transfer systems (6, 15, 38, 59).

MATERIALS AND METHODS

Bacterial strains and growth conditions. *A. tumefaciens* nopaline strain GV3850 (69) was used for all analyses. In a typical induction experiment, cultures were grown for 48 h in YEB medium (0.5% beef extract, 0.1% yeast extract, 0.5% peptone, 0.5% sucrose, 2 mM $MgSO_4$) at 22°C in the presence of carbenicillin (100 μg/ml), diluted to an A_{600} of 0.1 in phosphate-buffered M9 medium (pH 5.5) containing 10 mM $MgSO_4$, 1 mM $CaCl_2$, and 0.2% glucose. After 3 to 5 h in M9 medium, the *vir* system was induced by addition of acetosyringone (AS; 200 μM in dimethyl sulfoxide), or an equal amount of dimethyl sulfoxide was added for control cultures, and samples were taken at different time points for up to 18 h.

Cell fractionation methods. Cells from 500-ml cultures were harvested and washed three times in 50 mM sodium phosphate buffer, pH 6 (P buffer). Cells were then suspended in 5 ml of P buffer containing 0.2 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 1 μg each of pepstatin and leupeptin per ml. Lysis was achieved by three passages through a French pressure cell (Aminco) at 20,000 lb/in^2, followed by addition of lysozyme at 0.2 mg/ml and incubation on ice for 30 min. Centrifugation at 4°C in a Beckman JA-20 rotor at 10,000 rpm for 10 min removed unbroken cells; after addition of potassium chloride at 0.2 M, the supernatant was separated into soluble (cytoplasmic and periplasmic) and total membrane fractions by centrifugation in a Beckman Ti 50.3 rotor for 1 h at 150,000 × *g*.

To separate inner and outer membranes, the total membrane pellet was suspended by sonication in 1 ml of 5 mM EDTA (pH 6)–0.2 mM DTT–20% sucrose and layered onto a discontinuous sucrose gradient (3.2 ml of 53% sucrose over 1.1 ml of 70% sucrose) in 5 mM EDTA (pH 6). After centrifugation at 4°C for 15 h at 100,000 × *g* in a Beckman SW50.1 rotor, fractions of 0.5 ml were collected and separation of inner and outer membrane was assessed by measurement of NADH oxidase activity (inner membrane marker [42]) and 2-keto-3-deoxyoctonate content (outer membrane marker [34]). In contrast to the procedure used by other groups, we performed fractionations at pH 6, because we found that VirB5 was unstable at the standard pH of 7.6 even in the presence of protease inhibitors (5a). Loss of VirB5 might lead to alterations of transfer complex structure, and we tried to avoid such effects by lowering the pH to 6, where VirB5 is stable.

Removal of loosely surface associated proteins was attempted by vortexing of cells from AS-induced and control cultures. Cell pellets from 100-ml cultures were resuspended in 5 ml of M9 induction medium followed by vortexing for up to 10 min. Cells from 500-μl samples were pelleted by centrifugation for 10 min at 15,000 rpm in a Beckman microcentrifuge, and proteins in the supernatant were precipitated with 3 volumes of acetone for 1 h on ice. Cell pellets and acetone precipitates were boiled in equal volumes of Laemmli sample buffer (35) prior to electrophoretic separation.

Cell pellets from 20-ml AS-induced and control cultures were resuspended in 500 μl of M9 induction medium or phosphate-buffered saline (PBS; (8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 per liter [pH 7.2]), followed by incubation for 15 min at 23°C with or without 200 μg of pronase (Calbiochem), proteinase K (Calbiochem), or thermolysin (Sigma) per ml. In the latter case, 1 mM $ZnSO_4$ was added to ensure enzymatic activity. Total cell lysates were prepared by lysis in a French pressure cell as described above for membrane preparations and were incubated with or without proteases as indicated. Phenylmethylsulfonyl fluoride (at 5 μM) and EDTA (at 10 mM) were then added to inhibit proteases, followed by pelleting of the cells and boiling in Laemmli sample buffer.

Protein analysis and purification. For analysis of VirB1 products in the supernatants, *vir*-induced cultures were centrifuged in a Beckman JA-14 rotor for 20 min at 10,000 rpm. The upper two-thirds of the supernatant was carefully decanted, and proteins were concentrated by addition of 3 volumes of acetone (−20°C), incubation on ice for at least 30 min, and centrifugation at 4°C. The resulting pellets were suspended in sodium dodecyl sulfate (SDS) sample buffer and boiled for 5 min. Cells from an equal amount of culture were lysed the same way. Cell lysates and solubilized culture supernatants were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) in denaturing 10% polyacrylamide gels, using a Tricine buffer system (54), and proteins were transferred in a semidry blotting apparatus to polyvinylidene difluoride (PVDF) membranes (Immobilon P; Millipore). Incubations with primary VirB1-specific antiserum or with secondary alkaline phosphatase- or horseradish peroxidase-coupled anti-rabbit antibody and detection were performed according to standard protocols (26).

Determination of the isoelectric point (pI) of VirB1* by two-dimensional gel electrophoresis was accomplished as follows. For the first dimension, nonequilibrium pH gradient electrophoresis was performed basically as described by O'Farrell et al. (40). Wide-pH-range gels contained 2% Ampholines (LKB), pH 3.5 to 10. Narrow-pH-range gels contained 1.6% Bio-Lyte Ampholytes (Bio-Rad), pH 4 to 6, and 0.4% Ampholines (LKB), pH 3.5 to 10. For wide-pH-range gels, the original protocol was followed, the electrophoresis toward the cathode with the acidic reservoir on the top. Narrow-pH-range gels were electrophoresed toward the anode so that more-acidic proteins would enter the gel first. Gels were electrophoresed for 2,000 V h unless otherwise indicated. After electrophoresis, gels were extruded, equilibrated twice for 1 h in SDS sample buffer, and stored at −80°C. Cytochrome *c* (pI = 9.6) and methyl red (pI = 3.8; Sigma) were used as colored pI markers. Soybean trypsin inhibitor (pI = 4.6; Sigma) and

ovalbumin (pI = 5.1; Pharmacia) were used as pI standards. Second-dimension SDS-PAGE was followed by Western blotting and detection with VirB1-specific antiserum.

Partial purification of VirB1* from 1 liter of supernatant of *vir*-induced GV3850 cells was achieved as follows. The pH of the supernatant was adjusted to 7, and after cooling on ice, ammonium sulfate powder was slowly added to 70% saturation. After 1 h of incubation on ice, the sample was centrifuged in a Beckman JA-14 rotor at 10,000 rpm at 4°C for 15 min, and the supernatant was filtered through 4 layers of cheesecloth. Ammonium sulfate was added to the supernatant to 90% saturation, and the pellet was collected by centrifugation as described above after incubation on ice for 10 to 15 h. The pellet was suspended in 10 ml of 50 mM HEPES (pH 7)–50 mM NaCl (HN buffer) and dialyzed in 2 liters of the same buffer for 15 h at 4°C to remove salt. The sample was applied to a 2-ml Q-Sepharose column (Pharmacia) with a flow rate of 1 ml/min. The column was washed with HN buffer followed by elution with a linear gradient of 50 mM to 1 M NaCl in 50 mM HEPES (pH 7). The fractionation of VirB1* in all steps of the purification procedure was monitored by Western blotting analysis.

The two peak fractions which contained the highest amounts of VirB1* were concentrated by addition of trichloroacetic acid (TCA) at 10%, incubation on ice for 1 h, and centrifugation. The pellet was solubilized by boiling in SDS sample buffer for 10 min, and proteins were subjected to SDS-PAGE and blotted onto a PVDF membrane. The VirB1*-containing band was identified by staining with Ponceau S and Western blotting analysis of an adjacent lane with VirB1-specific antibodies. The N-terminal sequence of VirB1* on the PVDF membrane was determined on an ABI 477A Sequencer by Edman degradation.

Cross-linking and immunoprecipitation. AS-induced cultures of 20 ml were harvested, and cells were washed three times in PBS (pH 7.2) and suspended in 500 μl of the same buffer followed by addition of the homobifunctional amino-cross-linking agent BS^3 or DSP (Pierce) at 1 mM. After 30 min of incubation at 23°C, 500 μl of 1 M Tris-HCl (pH 6) was added to react with the remaining cross-linking agents, and the cells were pelleted and washed with 1 ml of ice-cold PBS (pH 6).

For analysis of cross-linked samples, cell pellets were suspended, lysed by boiling for 5 min in Laemmli sample buffer either with (BS^3 cross-links) or without (DSP cross-links) β-mercaptoethanol, and subjected to SDS-PAGE followed by Western blotting and detection with specific antisera. To analyze the composition of DSP-cross-linked products, the lane containing the cross-linked sample was excised after electrophoresis in the first dimension and incubated in Laemmli sample buffer with 100 mM DTT for 30 min at 22°C to break the internal disulfide bridge of the cross-linking agent. The lane was then subjected to a second SDS-PAGE and Western blotting followed by detection with VirB1-specific antisera.

To test for coimmunoprecipitation of VirB proteins after cross-linking, the DSP-treated samples from 50-ml cultures were lysed by addition of 500 μl of 10 mM Tris (pH 8)–150 mM NaCl–0.1% Tween 20 (TBST) containing 2% SDS and boiling for 10 min. Then 4.5 ml of TBST was added to the lysates, followed by incubation on ice for 10 min and centrifugation in a Beckman JA-20 rotor for 10 min at 10,000 rpm to remove cell debris. Next, antisera specific for different VirB proteins were added; after incubation at 4°C for 90 min, 100 μl of protein A-Sepharose beads (Sigma) was introduced, and the mixture was further incubated for 90 min to bind the immunoprecipitates. The beads were washed three times with TBST and boiled for 5 min in Laemmli sample buffer to break the DSP cross-links and the antigen-antibody interactions. Finally, the samples were subjected to SDS-PAGE, and after Western blotting, VirB1-specific antiserum was used to analyze the composition of the immunoprecipitates.

Image processing. The images presented in this report were generated after scanning of blots in a scanner (Microtek) at a resolution of 300 dots per inch and processed on a Power Macintosh 7100 computer with the software Photoshop 3.0. Prints were generated with a Phaser 440 printer (Tektronix).

RESULTS

Processing and partial secretion of VirB1. In our earlier work on the membrane association of VirB1 and other VirB proteins, cell extracts were routinely electrophoresed in SDS–10% polyacrylamide gels, using the Laemmli buffer system, prior to Western blotting and immunological detection (62). However, when cell extracts were electrophoresed in a Tricine buffer system as described by Schägger and von Jagow (54), which gives higher resolution of low-molecular-weight proteins, we detected multiple VirB1-cross-reacting species (Fig. 1A). Similar to our earlier report, in extracts from AS-induced cells, we detected two cross-reacting proteins migrating closely together with molecular masses of 27 and 28 kDa, which may correspond to full-length VirB1 before and after removal of the signal peptide. Alternatively, these two forms of VirB1 may result from posttranslational processing. In addition, using the Tricine gel system, we detected a protein with an apparent molecular mass of 12 kDa, which did not appear in control lanes containing extracts from uninduced cells. As shown below, the low-molecular-mass protein is a processing product of VirB1, designated VirB1*.

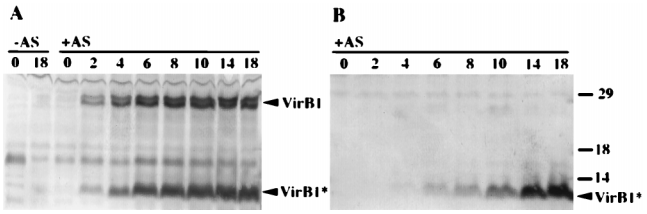

FIG. 1. Synthesis of VirB1 and VirB1* after induction of *vir* genes. Samples from *vir*-induced GV3850 cultures (+AS) or uninduced control cultures (−AS) were taken every 2 h up to 18 h after induction. Cell lysates (A) and equal volumes of concentrated culture supernatants (B) were subjected to SDS-PAGE and blotted on PVDF membranes followed by detection with VirB1-specific antibodies. Sizes are indicated in kilodaltons.

To assess a possible conversion of VirB1 to VirB1* after AS induction, we monitored the time course of induction of VirB1-cross-reacting proteins in cell lysates over a period of 18 h. Figure 1A shows that the ratio between VirB1 and VirB1* remains constant over the entire time of induction. However, when concentrated culture supernatants were analyzed for content of Vir proteins, we found that secretion of VirB1* occurred in increasing amounts from 6 to 14 h and that after 18 h of induction, equal amounts of VirB1* were associated with the cell and in the supernatant. To exclude that the occurrence of VirB1* in the supernatant was due to cell lysis, we analyzed culture supernatants with several Vir protein-specific antisera (anti-VirB4, -VirB7, -VirB9, -VirD2, and -VirE2) but failed to detect significant amounts of the membrane-associated or soluble components of the T-complex (data not shown).

The discovery that VirB1* was processed and partially secreted prompted an investigation of cell-bound VirB1 and VirB1*. AS-induced and uninduced GV3850 cells were lysed by passage through a French pressure cell, and total membranes were separated from the soluble fraction (periplasm and cytoplasm) by ultracentrifugation. Figure 2A shows that VirB1* was mostly soluble whereas VirB1 predominantly sedimented with the total membrane fraction. Further separation of inner and outer membranes by isopycnic sucrose gradient fractionation, monitored by markers for both membranes (data not shown), showed that VirB1 resided mostly in the inner membrane (Fig. 2A).

Thus, in contrast to all other components of the T-complex transfer apparatus, VirB1* is mostly soluble. To further characterize the cell-bound portion of VirB1*, *vir*-induced agrobacteria were subjected to vortexing for up to 10 min. Cells and supernatant were then analyzed separately for content of VirB1*. Figure 2B shows that vortexing partly stripped VirB1* from the cells, and the amount increased with the time of vortexing. Control experiments with VirB2-, VirB5-, and VirE2-specific antisera showed that this result cannot be explained by disintegration of the cell or the VirB complex (data not shown). To assess whether cell-bound VirB1* is exposed to the external medium, intact *vir*-induced agrobacteria and total cell lysates were incubated with different proteases (pronase, proteinase K, or thermolysin). Figure 2C shows that cell-bound VirB1* was not accessible to the enzymes, whereas rapid degradation occurred in total cell lysates. Therefore, cell-bound VirB1* probably resides predominantly in the periplasm and is

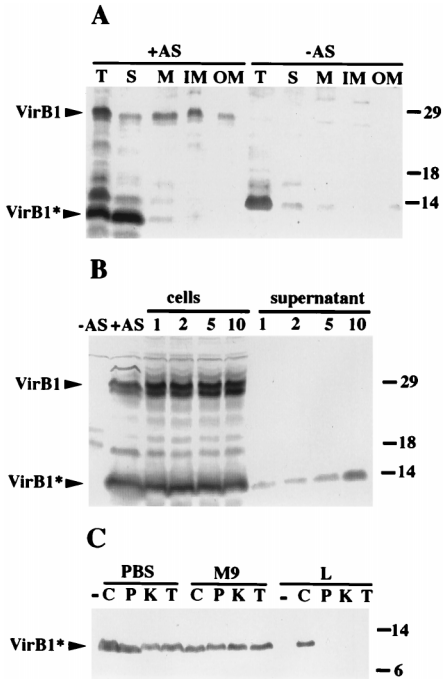

FIG. 2. Cellular localization of VirB1 and VirB1*, determined by Western blotting analysis with VirB1-specific antibodies after SDS-PAGE. (A) Detection of VirB1 and VirB1* after lysis of *vir*-induced (+AS) and control cells (−AS) and fractionation by differential centrifugation. Lanes: T, total cell lysates; S, soluble fraction; M, total membrane fraction; IM, inner membrane; OM, outer membrane. (B) *vir*-induced cells were vortexed for the indicated times (minutes) and pelleted, followed by analysis of cell-associated (cells) and stripped (supernatant) VirB1 and VirB1*. (C) *vir*-induced cells were suspended in PBS or M9 medium; total cell lysates were prepared by lysis through a French pressure cell (L). After incubation with proteases (pronase [P], proteinase K [K], or thermolysin [T]) or without proteases (C) for 30 min at 23°C, VirB1* content was analyzed. -, control lanes with lysates from noninduced cells. Sizes are indicated in kilodaltons.

not accessible to proteases in intact cells. However, its partial release by vortexing suggests either loose association with the cell or secretion from the periplasmic pool.

VirB1* corresponds to a C-terminal part of VirB1. To obtain direct evidence that VirB1* is a processing product of VirB1, VirB1* was purified from supernatant of AS-induced GV3850-cells. Figure 3A shows that differential precipitation of the culture supernatant with ammonium sulfate resulted in approximately equal amounts of VirB1* in the 70 and 90% pellets. However, the 70% pellet contained massive amounts of viscous material, presumably exopolysaccharides, which might interfere with further purification, and was therefore discarded. The 90% pellet was dissolved in HN buffer, dialyzed in the same buffer to remove salt, and then subjected to chromatography over a Q-Sepharose column. Gradient elution concentrated VirB1* into two fractions containing partially purified VirB1* protein (Fig. 3B). The VirB1*-containing fractions were concentrated by precipitation with TCA, and the sample was subjected to SDS-PAGE and electrotransferred onto a PVDF membrane. VirB1* was localized by staining with Ponceau S, and the N-terminal protein sequence was determined by Edman degradation. The N-terminal sequence of VirB1* was determined to be AQQLVPPL; thus, VirB1* begins at amino acid 173 of VirB1 (Fig. 3C). Therefore, VirB1* contains the C-terminal 73 amino acids of VirB1, and its predicted molecular mass of 7.9 kDa differs from its mobility at 12 kDa. The anomalous mobility in the electrophoretic separation may

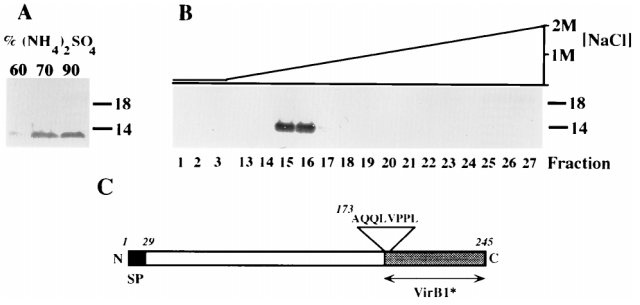

FIG. 3. Purification of VirB1* and location of the processing site. (A) Detection of VirB1* in pellets after precipitation with increasing amounts of ammonium sulfate following electrophoretic separation of the samples and Western blotting analysis. Sizes are indicated in kilodaltons. (B) Purification of VirB1* by Q-Sepharose chromatography. Fractions from column flowthrough (fractions 1 to 3) and gradient elution (50 mM to 2 M NaCl; fractions 13 to 27) were concentrated with TCA, and the VirB1* content was determined after electrophoretic separation and Western blotting. (C) Localization of the processing site of VirB1* in the sequence of VirB1 determined by Edman degradation. N, N terminus; C, C terminus; SP, signal peptide.

be attributed to its high proline content of 15% (39). Alternatively, the slow migration might be caused by an unidentified modification of VirB1*. A protein corresponding to the N-terminal 144 amino acids (VirB1 after removal of the signal peptide and VirB1*) was never detected, either due to its low antigenicity or because of rapid degradation after processing.

VirB1* in the supernatant and VirB1* in the periplasm of AS-induced cultures migrate identically in SDS-PAGE and may therefore be identical. To obtain direct evidence for this assumption, we compared the pIs of VirB1* from both locations by two-dimensional gel electrophoresis: isoelectric focusing (IEF) in the first dimension and SDS-PAGE in the second dimension followed by Western blotting (40). Figure 4 shows that indeed, the pIs of VirB1* from both sources were identical. However, the measured pI was 4.6, identical to that of the trypsin inhibitor marker, and differs from the predicted value of 4.25. Together with the above-mentioned aberrant mobility of VirB1* in SDS-PAGE and the occurrence of two forms of

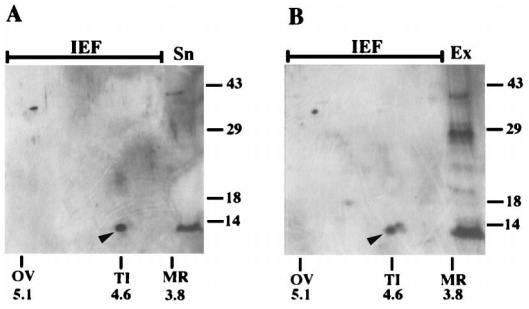

FIG. 4. Two-dimensional electrophoresis of cell-associated and secreted VirB1*. (A) VirB1* was partially purified from supernatant of *vir*-induced cells by ammonium sulfate precipitation. The VirB1*-containing pellet was solubilized and dialyzed in HN buffer, concentrated with acetone, and loaded on the first- and second-dimension gels. Sample from the supernatant was also resolved in the second-dimension gel only (Sn). (B) Total cell extract of *vir*-induced cells was also concentrated by acetone precipitation and electrophoresed in both dimensions. Cell extract was also resolved in the second-dimension gel only (Ex). Samples also contained the pI markers ovalbumin (OV), soybean trypsin inhibitor (TI), and methyl red (MR). After the first-dimension IEF, gels were extruded and resolved in the second dimension by SDS-PAGE followed by Western blotting and detection with VirB1-specific antibodies. Positions of VirB1* after second-dimension IEF are indicated by arrowheads; positions of IEF markers and their pI values are indicated at the bottom; sizes are indicated in kilodaltons on the right.

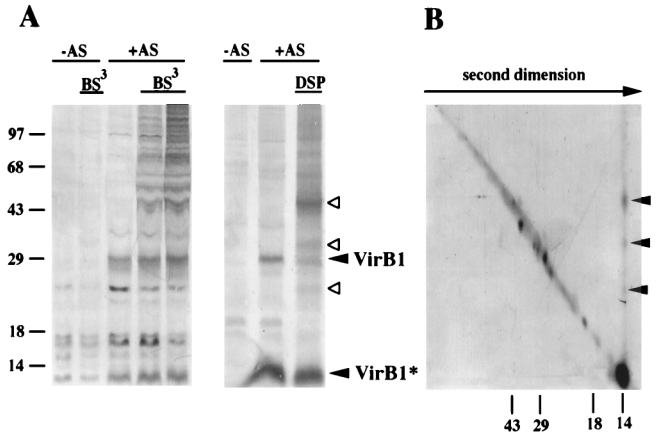

FIG. 5. Cross-linking of VirB1*. (A) Cells from *vir*-induced cultures (+AS) or controls (−AS) were incubated in the presence of BS^3 (0.25 or 1 mM) or DSP (1 mM), and lysates were resolved by SDS-PAGE (reducing in the case of BS^3; nonreducing in the case of DSP) followed by Western blotting and detection with VirB1-specific antibodies. Major bands visible only after DSP cross-linking are indicated by triangles. (B) A lane containing a DSP-cross-linked sample was incubated in sample buffer with DTT and subjected to SDS-PAGE in the second dimension followed by Western blotting and detection with VirB1-specific antibodies. VirB1* released from complexes of higher molecular weight, presumably from those indicated by triangles after first-dimension SDS-PAGE, is indicated by arrowheads. Sizes are indicated in kilodaltons.

VirB1 at 27 and 28 kDa (Fig. 1A), this finding suggests the possibility that an additional C-terminal processing or modification reaction can occur. For example, if VirB1* had 14 residues removed from the carboxyl end, its predicted pI would be 4.59.

VirB1 was detected in the total cell extract samples electrophoresed directly in SDS-polyacrylamide gels; however, it was not detected after IEF and SDS-PAGE. To check the possibility of degradation during pH gradient formation, we followed the time course of separation of VirB1 and VirB1* by second-dimension SDS-PAGE following IEF for 1, 2, 4, and 6 h. Between 2 and 4 h after loading of the sample, the VirB1-specific signal disappeared, suggesting VirB1 is unstable following IEF (data not shown).

VirB1* shows association with the VirB membrane complex. VirB1* was partly recovered from the culture supernatant, and one explanation of this result could be that it forms a brittle structure, such as a pilus, that may be stripped from the cell surface by shaking of the culture. The periplasmic pool of VirB1* may associate with the VirB complex to ensure reassembly of the pilus. Thus, we determined whether VirB1 and VirB1* associate with other proteins. AS-induced cells were incubated with the homobifunctional cross-linking agent BS^3, which links closely juxtaposed proteins primarily via accessible ε-amino groups of lysine residues. As shown in Fig. 5A, Western blotting analysis after cross-linking detected multiple VirB1-cross-reacting complexes of higher molecular weight, indicating that VirB1 and/or VirB1* are highly accessible to the agent. To determine which form of the protein was cross-linked, we performed the analysis with the cleavable BS^3-analogous agent DSP, and after nonreducing gel electrophoresis in the first dimension, cross-links were cleaved by incubation with DTT. The cleaved products were resolved in a second-dimension gel, and Western blotting analysis showed that mostly VirB1* was released from complexes of higher molecular weight (Fig. 5B). These experiments established that VirB1* associates closely with other proteins. Compared to cross-linking patterns observed after analysis with other VirB-specific antisera, VirB1* showed the highest degree of cross-linking (5a), underscoring its exposed location. However, the complexity of cross-linking patterns prevented a direct assignment of cross-linked complexes to other specific VirB proteins.

To gain more direct evidence for an association of VirB1* with other components of the T-complex transfer apparatus, we analyzed cross-linked complexes by immunoprecipitation. Exported VirB proteins, or those which are at least partially exposed to the periplasm, could be in close proximity to VirB1* in the transfer apparatus, so that cross-linking and coimmunoprecipitation might be feasible. AS-induced agrobacteria were incubated with or without the thiol-sensitive cross-linking agent DSP. After cell lysis, different VirB-specific antisera (anti-VirB1, -VirB5, -VirB8, -VirB9, and -VirB10) were added to bind their antigens, followed by protein A-Sepharose beads for immunoprecipitation. The beads were washed, sedimented, and boiled in Laemmli sample buffer to break the DSP cross-links and antigen-antibody interactions. Analysis of the immunoprecipitates by SDS-PAGE and Western blotting with VirB1-specific antiserum revealed that after cross-linking, coimmunoprecipitation of VirB1* and VirB9 occurred (Fig. 6). VirB1* did not bind to VirB9 in the control reaction without DSP, and no cross-reacting protein was detected in control reactions from uninduced cells (not shown). Since accompanying experiments have shown that VirB9 is found cysteine linked to VirB7 (2, 4, 58), we conclude that in the native T-complex transfer apparatus, VirB1* closely associates with the complex of VirB9 and VirB7. These latter proteins associate, presumably via the lipoprotein modification of VirB7 and protein-protein interactions, with both membranes of *Agrobacterium* (4, 19, 20). Thus, there is a link between the membrane-associated VirB complex and loosely cell associated VirB1*, which may mediate formation of the mating bridge to plant cells.

DISCUSSION

Here we show that VirB1, the first protein of the *virB* operon involved in T-complex transfer from *Agrobacterium* to plant cells, undergoes processing, and the processed C-terminal part, VirB1*, is partially secreted into liquid media. This constitutes the first report on a soluble extracellular component of the transfer process, since all the other proteins of the transfer apparatus and the T-complex are either tightly associated with the bacterial membranes or cytoplasmic. The exposed localization of VirB1* makes it an excellent candidate for an *Agrobacterium* factor that forms the initial contact with plant cells. In addition, homologies of the N terminus of VirB1 with components of other type II, III, and IV secretion and transfer systems strongly suggest a function of this protein in complex assembly. Thus, both parts of VirB1 may have different functions in T-complex transfer to plant cells. Figure 7 summarizes

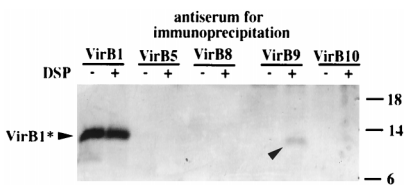

FIG. 6. Coimmunoprecipitation of VirB1* and VirB9 after cross-linking. Cells from *vir*-induced cultures were incubated in the presence (+) or absence (−) of DSP followed by cell lysis and immunoprecipitation with different VirB-specific antisera and protein A-Sepharose beads. Samples were resolved by reducing SDS-PAGE followed by Western blotting and detection with VirB1-specific antiserum. Sizes are indicated in kilodaltons.

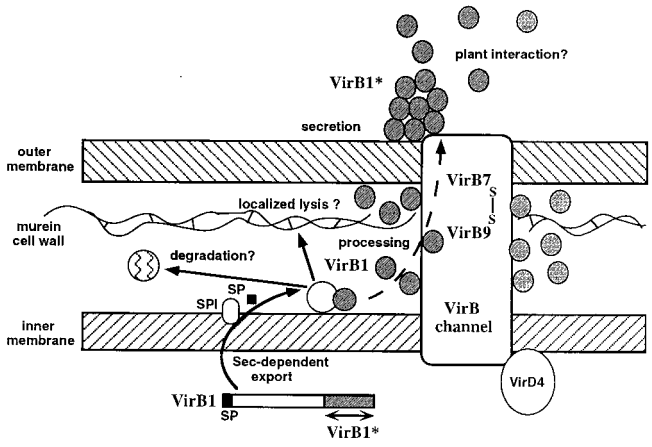

FIG. 7. A model for processing of VirB1 and VirB1* and their putative function(s) in T-complex transfer. After Sec-dependent export into the periplasm and cleavage of the signal peptide (SP) by signal peptidase I (SPI), VirB1 may facilitate assembly of the T-complex transfer apparatus by local lysis of the murein cell wall. Processing of VirB1 may terminate this activity, followed by quick degradation of the N-terminal part of the protein. The C-terminal VirB1* translocates to the outer surface of the bacterial cell and may assemble into a structure that contacts both the transfer apparatus via VirB9-VirB7 and plant cells at the wound site and may trigger formation of the conjugation bridge. Alternatively, soluble VirB1* might be a soluble virulence factor that interacts with plant cells to facilitate the infection process. Genetic transformation of plant cells requires the putative transmembrane VirB channel and VirD4.

our current understanding of its processing and possible role(s).

Homologies to components of other secretion systems suggest an enzymatic function for VirB1. Our independent database searches, as well as those performed by Bayer et al. (6) and Mushegian et al. (38), found that the N-terminal domain of VirB1 shows homologies to lysozymes and components of other type III and IV secretion/transfer systems, e.g., P19 of plasmid R1, TraL of IncN plasmid pKM101, TrbN of IncP plasmid RP4, IpgF of *Shigella flexneri*, and IagB of *Salmonella typhimurium*. Recently, Stone et al. (59) reported the sequence of an operon coding for a putative type II secretion system required for assembly of bundle-forming pili from enteropathogenic *E. coli*. One of the putative products (BfpH) shows homology to VirB1. Since the catalytic site and the substrate binding pocket, identified in the crystal structure of the Slt70 transglycosylase (16), show a high degree of conservation, the contribution of VirB1 and its homologs to the assembly of secretion or transfer complexes may be local lysis of the peptidoglycan cell wall. Interestingly, deletion of the respective genes led to a 10- to 100-fold reduction of DNA transfer for P19 (6), VirB1 (9), and TraL (44), whereas it had no detectable effect on viability of *E. coli* in the case of Slt70 (61) and invasion by *S. flexneri* in the case of IpgF (1). This finding may imply that transglycosylase-like activities either are dispensable or can be performed by redundant enzymes. In fact, disruption of the *sltY* and *mltB* genes, coding for two of the three known transglycosylases, had no detectable effect on viability of *E. coli*, implying they may functionally replace each other (18, 28).

Localized action of a putative transglycosylase like VirB1 could aid in the assembly of the T-complex transfer machinery because of its cosynthesis and association with other VirB components (15). Support for transglycosylase activity of VirB1 comes from mutagenesis of putative active-site residues, which partially abolished complementation of tumor formation by a mutated gene in a *virB1* deletion strain (38). That deletion of the gene does not lead to complete impairment of complex assembly (9) may be explained by other transglycosylases and autolytic enzymes that constantly rebuild the murein of growing cells and may allow transmembrane assembly of the complex.

It is interesting that we never detected any protein corresponding to the remaining VirB1-specific N-terminal 144 amino acids. There are several possible explanations, such as low antigenicity of the N terminus or aberrant migration coincident with the two forms of VirB1 at 27 and 28 kDa. Whereas we cannot exclude the latter possibility, we consider the first unlikely, because we used a polyclonal antiserum. We favor the possibility that the N-terminal part is rapidly degraded as part of a regulatory mechanism, because after localized lysis of murein, the transglycosylase activity may no longer be needed. In the future, we will directly test the proposed enzymatic activity of VirB1 to assess its role in macromolecular secretion or DNA transfer. Whereas the enzymatic activity of VirB1 may reside in its N terminus, the secreted VirB1* may serve another function in T-complex transfer.

Does VirB1* form a pilus? That VirB1* was found partly in the supernatant of AS-induced cultures does not necessarily imply a true secretion event but may be due to its assembly into a loosely cell associated structure, such as a pilus, removed from the cell surface by the shearing imposed in liquid culture (5). In the natural infection process, it may contact plant cells at the wound site and mediate formation of a mating bridge by other VirB products. Since we analyzed cells after induction in liquid culture, we did not expect to find such a pilus structure attached to their outer surface. In accord with this assumption, cell-associated VirB1* was not accessible to proteases. However, vortexing of cells partly removed VirB1*, implying that it may constitute a periplasmic pool of subunits for assembly of the pilus structure. The extensive formation of higher-molecular-weight complexes that we observed after exposure of AS-induced cells to different cross-linking agents agrees with the idea that VirB1* assembles into a macromolecular structure. In fact, Fullner et al. (24) found that agrobacteria form a pilus after AS induction on solid media, and synthesis depended on the *virB* operon. However, the authors did not determine which of the 11 VirB proteins constitutes the pilus. Based on homologies of VirB2 to pilus subunits and their processing, VirB2 was proposed to constitute the *Agrobacterium* pilus (31, 33, 55). However, direct evidence is still lacking, and we did not detect processed VirB2 in the supernatant of AS-induced agrobacteria. In contrast, processed VirB2 associated tightly with the membranes and may be part of the transmembrane pore (5a, 55). Type II secretion systems contain up to four pseudopilins, and like propilin, they undergo N-terminal processing and methylation. However, in spite of intense studies, such as of the PulG pseudopilin component of *Klebsiella oxytoca* pullulanase secretion (48), there is currently no evidence for a pilus in any of these systems. The pseudopilins may have been adapted in evolution to form a transmembrane instead of an extracellular structure (27, 47, 49). Alternatively, a pilus-like structure composed of VirB2 may assemble after contact with the plant cell, as proposed for the TraA subunits of the F pilus from *E. coli* (22). That the putative VirB1-derived pilus is not essential for plant transformation may be explained by comparison to bacterial conjugation. Conjugative pili are believed to mediate attachment to recipients, and retraction of the pilus leads to close cell contact followed by formation of conjugative bridges (17). However, chromosomal attachment functions of *A. tumefaciens* mediate close contact to plant cells, which is further strengthened by cellulose fibrils (11). Thus, the putative VirB1* pilus also may facilitate contact formation but may not be absolutely required for T-complex transfer.

The partial extracellular localization of VirB1* after AS induction of agrobacteria in liquid culture suggests that it may be part of a pilus structure, but so far, there is no direct evidence for this hypothesis. Alternatively, VirB1* may be secreted in the supernatant and exert another function. For example, it may act as a soluble virulence factor that interacts with plant cells at the wound site to enhance the transformation process. In this model, the processing of VirB1 and secretion of VirB1* bears resemblance to that of other secreted virulence factors, which play a role in the interaction of animal pathogens with their hosts.

Comparison of VirB1 processing and secretion to other bacterial virulence factors. Filamentous hemagglutinin is the major extracellular virulence factor of *Bordetella pertussis*, and after export into the periplasm, it undergoes N- and C-terminal proteolytic processing prior to its secretion through the outer membrane, mediated by FhaC (30, 50). No functions in virulence have been assigned to products cleaved from N and C termini, but they act as intramolecular chaperones to assist the folding of filamentous hemagglutinin. Similarly, the propeptides of several secreted proteases, e.g., elastase from *Pseudomonas aeruginosa* and subtilisin from *Bacillus subtilis*, act as intramolecular chaperones aiding the correct folding of the protease, and following their autocatalytic removal, secretion of the protease occurs (37, 65). The immunoglobulin A1 protease from *Neisseria gonorrhoeae* also undergoes autocatalytic C-terminal processing after export into the periplasm (45). However, in addition to the function of the N-terminal protease domain, one of the C-terminal processing products, the α-peptide, was recently shown to enter the nuclei of tissue culture cells (46). Thus, it remains possible that processing products of secreted factors have a function in virulence.

We propose that VirB1 may be a bifunctional virulence factor of *Agrobacterium*. Future studies will address the proposed functions of the two parts of the protein, transglycosylase activity of the N terminus and formation of a pilus or action as soluble virulence factor by the C terminus. Also, the nature of the processing event and the need for chaperones or proteases will be investigated. Such studies may have broad significance, considering that VirB1 is member of a family of putative transglycosylases, which may play a role in the assembly of transmembrane complexes (6, 38). Since the homologies of VirB1 to some members of the transglycosylase family, e.g., TraL and TrbN, extend into the C-terminal VirB1* region, it will be interesting to study whether similar processing phenomena occur and to investigate the functions of their C termini.

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