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Biochemistry

A gene required for transfer of T-DNA to plants encodes an ATPase with autophosphorylating activity

(*Agrobacterium/vir* genes/ATP hydrolysis/phosphorylation)

PETER J. CHRISTIE*, JOHN E. WARD, JR.†, MILTON P. GORDON‡, AND EUGENE W. NESTER§

Department of Microbiology, SC-42, University of Washington, Seattle, WA 98195

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ABSTRACT **The *virB* operon of the *Agrobacterium tumefaciens* pTiA6NC plasmid likely plays a role in directing T-DNA transfer events at the bacterial membrane, as determined previously by mutagenesis and cellular fractionation studies and by DNA sequence analysis of the ≈12-kilobase-pair operon. The DNA sequence analysis also revealed consensus mononucleotide binding domains in the deduced *virB5* and *virB11* gene products, suggesting that one or both of these proteins couple energy, by means of nucleotide triphosphate (NTP) hydrolysis, to T-DNA transport. In this report, the product of *virB11*, an essential virulence gene, was overproduced in *Escherichia coli* and purified by using immunoaffinity chromatography. The immunoaffinity purified protein, as well as NaDodSO$_4$/polyacrylamide gel-eluted protein, bound and hydrolyzed ATP in the absence of DNA effectors. VirB11 protein also demonstrated *in vitro* autophosphorylation activity. VirB11 protein was localized primarily to the cytoplasmic membrane by immunoblot analysis of membrane fractions. The deduced VirB11 protein exhibits sequence similarity to *comG* ORF1, a protein required for uptake of DNA by competent *Bacillus subtilis* cells. These findings suggest that phosphorylation may serve to activate a component(s) of the *A. tumefaciens* T-DNA transport apparatus and may also represent a general activation mechanism of other bacterial DNA transport systems.**

Agrobacterium tumefaciens is unique among the bacteria in its ability to transfer a segment of its genome (T-DNA) to plant cells where the DNA integrates into the plant nuclear genome (see refs. 1 and 2). Mobilization of T-DNA from a large plasmid (Ti) within the bacterium is mediated by a separate virulence (*vir*) region of the Ti plasmid, which consists of at least six coordinately regulated operons designed *virA*, *-B*, *-C*, *-D*, *-E*, and *-G* (3, 4). The *vir* region directs at least three early events required for T-DNA transfer, including a sensory transduction system to activate the entire transfer process, a T-DNA processing reaction, and a mechanism to transfer the T-DNA intermediate across the bacterial envelope. In response to plant signal molecules, such as acetosyringone (5), a two-component regulatory system consisting of VirA protein (a transmembrane sensory component) and VirG protein (a transcriptional activator) induces expression of the *vir* genes (6–9). A VirD endonuclease initiates T-DNA processing by site-specific, strand-specific cleavage within the 25-base-pair T-DNA border sequences (10–13). The nicking event is facilitated by product(s) of the *virC* operon interacting with "overdrive," a cis-acting sequence located near the border sequences (14, 15). Following cleavage, linear, single-stranded molecules (T strands) corresponding to the bottom strand of the T-DNA are formed, most probably by a replication-strand displacement reaction initiated at the 5′ cleavage site (11–13, 16). Recent studies also suggest that certain *vir*-encoded proteins associate with T strands during these processing reactions. VirD2 protein, one component of the VirD endonuclease, binds, probably covalently, to the 5′ ends of T strands (17–19). In addition, we (20) and others (21–23) have shown that the product of the *virE2* gene is a single-stranded DNA-binding protein. We further demonstrated an association of the VirE2 protein with T-DNA *in vivo* (20) and speculated that the VirE2 single-stranded DNA-binding protein may stimulate DNA polymerase activity during the replication-strand displacement reaction. The VirE2 protein may also associate with T strands during transfer. Therefore, we have proposed that a T strand capped at the 5′ end by VirD2 protein and coated along its length by VirE2 protein is transferred to the plant cell (20). These *vir*-encoded proteins may protect the T strand from bacterial and plant nucleases and may also promote its integration into the plant genome (20).

A third function of the *vir* genes, transfer of a T-DNA–protein complex across the bacterial envelope and into the plant cell, undoubtedly is a complex process. T-DNA transfer to plants is functionally analogous, by several criteria, to conjugal DNA transfer between bacteria (2, 20, 24–26). The conjugative transfer of F plasmid in *Escherichia coli*, a model plasmid-transfer system, requires that at least 13 *tra* genes be expressed to form a membrane apparatus for pilus assembly and disassembly and for conjugal DNA transfer (24). Although the *vir* genes do not appear to encode a pilus structure since initial attachment of the bacterium to plant cells is encoded by chromosomal genes (2, 27), a *vir*-determined membrane apparatus most probably directs transfer of a T-DNA–protein complex across the membrane. DNA sequence analysis of the ≈12-kilobase-pair *virB* operon of *A. tumefaciens* revealed that it likely codes for at least nine membrane proteins (25, 28). The membrane localization was confirmed for three VirB proteins by cell fractionation of *vir* mutants (29) and, more recently, for several additional VirB proteins by immunoblot analysis of cellular fractions (this paper; J.E.W. and P.J.C., unpublished results). Based upon their importance for virulence (4), abundance (29), and cellular localization (25, 28, 29), we predict that the VirB proteins direct T-DNA transfer events at the membrane.

We have initiated biochemical studies to identify specific functions of the VirB proteins encoded by the pTiA6NC plasmid in T-DNA transfer. Our molecular analyses of the *virB* sequence have revealed that the *virB5* and *virB11* gene

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Abbreviations: IPTG, isopropyl β-D-thiogalactoside; T-DNA, transferred DNA.

*Present address: Department of Biological Sciences, Stanford University, Stanford, CA 94305.
†Present address: Plant Science Institute, Department of Biology, Leidy Laboratories, University of Pennsylvania, Philadelphia, PA 19104.
‡Present address: Department of Biochemistry, University of Washington, Seattle, WA 98195.
§To whom reprint requests should be addressed.

products each contain consensus type A mononucleotide binding sequences (refs. 30 and 31; H. Bradshaw, personal communication). A similar analysis identified this sequence in the corresponding *virB4* and *virB11* genes of a second octopine-type Ti plasmid pTi15955 (28). Many mononucleotide binding proteins with diverse functions, including subunits of the periplasmic binding protein-dependent transport systems, protein kinases, and also DNA replication, recombination, and repair enzymes, retain the type A consensus sequence (30, 31). Many of these proteins share the property of autologous and/or heterologous regulation of activity through nucleotide hydrolysis and phosphorylation. Here, we have examined the enzymatic properties of the purified VirB11 protein and report that it binds ATP, possesses ATPase activity, and is autophosphorylated. This protein may play a role in activating the T-DNA transport process through ATP hydrolysis and phosphorylation.

MATERIALS AND METHODS

Protein Analysis, Immunological Techniques, and Localization Studies. Proteins were visualized and quantitated as described (20). An overproduced 70-kDa TrpE–VirB11 fusion protein, constructed by joining *trpE* to the fourth codon of *virB11* (25), was eluted from $NaDodSO_4$/polyacrylamide gels to raise VirB11 antiserum (20). Proteins from acetosyringone-induced *A. tumefaciens* A348, which carries the pTiA6NC plasmid, were separated into cytoplasmic, periplasmic, cytoplasmic membrane, and outer membrane fractions, and the quality of the fractionation was assessed as described (20). A *virB11*::*phoA* translational fusion under control of the *tac* promoter was constructed to determine whether VirB11 protein sequences could direct secretion of alkaline phosphatase, since this enzyme is active only when secreted into the periplasmic space. A 3.2-kilobase-pair *Bam*HI restriction fragment from pUCH2, which contains the *phoA* structural gene devoid of its promoter and signal sequence (32), was cloned in-frame into a *Bgl* II restriction site at codon 323 of *virB11*.

Overproduction of VirB11 Protein. The *virB11* gene was cloned as a 1.4-kilobase-pair *Eco*RI–*Xho* I restriction fragment into *Eco*RI and *Sal* I sites in the polylinker of pUC118 (33) such that the cloned gene in the resulting plasmid, pJW322, was oriented in the same direction as the *lacZ* promoter. DNA (51 base pairs) residing between the translational start sites of *lacZ* and *virB11* was deleted by the site-directed mutagenesis method of Kunkel *et al.* (34). An oligonucleotide, CGGATCCACTTCCATAGCTGTTTCCTGTGT, was synthesized on a multiple-column DNA synthesizer (model 8600; Biosearch) such that the first half was complementary to 15 bases at the 5′ end of the *virB11* gene and the second half was complementary to 15 bases that immediately precede the *lacZ* translational start site. The synthetic 30-mer was hybridized to uracil-containing single-stranded pJW322 template to prime complementary strand synthesis *in vitro*, and the resulting DNA was introduced into *E. coli* SG935 [F^-, *lac, trp, pho, msl, htp-R, rpsL, supC*(ts), *tsx*::Tn*10, lon100*; from Steve Goff, Columbia University]. Transformed cells were screened for plasmids sustaining the 51-base-pair deletion and for overproduction of a 38-kDa protein, the expected size of VirB11 protein.

Purification of VirB11 Protein. Cells collected from a 1-liter culture of isopropyl β-D-thiogalactoside (IPTG)-induced *E. coli* SG935 carrying pPC39 were suspended in buffer A [25 mM Hepes (potassium salt), pH 7.0/12.5 mM $MgCl_2$/1 mM dithiothreitol/10% (vol/vol) glycerol] and disrupted by French pressure cell treatment. The suspension was centrifuged at 10,000 × *g* for 30 min, the resulting insoluble pellet was solubilized in buffer A containing 6 M guanidine hydrochloride, and material was renatured by step dialysis in buffer A and 40% glycerol to remove the guanidine hydrochloride. Soluble material was passed through an immunoaffinity column (bed volume, 5 ml) consisting of VirB11-specific antiserum coupled to CNBr-activated Sepharose 4B beads (Pharmacia), and bound protein was eluted in steps of 0.05, 0.5, 1.0, and 2.5 M NaCl. Fractions were concentrated, and salt was removed with Centricon 10 microconcentrator filters (Amicon). The resulting preparations were electrophoresed through $NaDodSO_4$/polyacrylamide gels and examined by Coomassie blue staining and immunostaining. VirB11 protein, detected primarily in the 1.0 and 2.5 M NaCl fractions, was eluted from gel slices and renatured according to the procedure of Hager and Burgess (35).

ATPase Assays. ATP hydrolysis was assayed by measuring the formation of free phosphate from [γ-^{32}P]ATP (3000 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear) as described by Weinstock *et al.* (36). Reaction mixtures (20 μl) consisting of VirB11 protein from the immunoaffinity chromatography fractionation or isolated from $NaDodSO_4$/polyacrylamide gels and ATP were incubated in buffer A at 30°C for the specified times. Aliquots (2 μl) were spotted onto polyethyleneimine-cellulose plates (Sigma) and developed in 1 M formic acid and 0.5 M LiCl. By using this solvent system, ATP migrates only slightly from the origin and P_i migrates near the solvent front (36). Following chromatography, [γ-^{32}P]ATP and P_i were identified by autoradiography, and the amount of radioactivity in the excised spots was determined by liquid scintillation spectroscopy.

Autophosphorylation Assays. Phosphorylation was assayed by incubating VirB11 protein with [γ-^{32}P]ATP (3000 Ci/mmol) in a 20-μl reaction mixture containing buffer A for 30 min at 30°C. Reactions were stopped by the addition of $NaDodSO_4$-containing sample buffer and heat treatment (80°C) for 5 min. Reaction mixtures were electrophoresed through $NaDodSO_4$/12.5% polyacrylamide gels, protein was transferred to nitrocellulose, and the nitrocellulose was exposed to x-ray film for autoradiography.

RESULTS

Visualization of VirB11 Protein. As shown in Fig. 1, antibodies raised against the 70-kDa TrpE–VirB11 fusion protein reacted strongly with a 38-kDa protein present in extracts

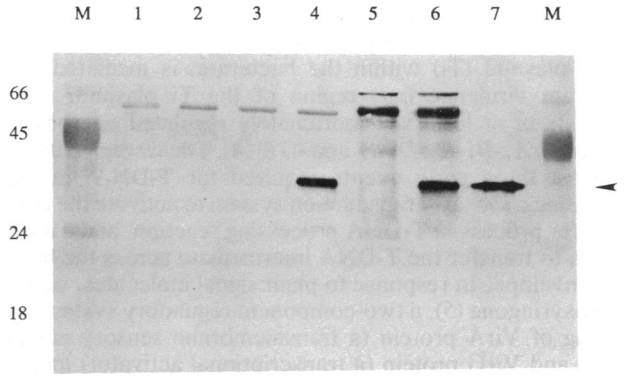

FIG. 1. Immunoblot showing expression of VirB11 protein in wild-type and mutant *A. tumefaciens* and in recombinant *E. coli* strains. Total proteins were electrophoresed through a $NaDodSO_4$/12.5% polyacrylamide gel and transferred to nitrocellulose; the blot was probed with anti-VirB11 antiserum. Uninduced and acetosyringone-induced *A. tumefaciens* A136(pTi368), a *virB11* mutant (4) (lanes 1 and 2); uninduced and induced *A. tumefaciens* A348 (lanes 3 and 4); IPTG-induced vector-only control *E. coli* SG935(pUC118) (lane 5) and induced SG935(pPC39) (lane 6); and material (0.5 μg) in pooled and concentrated 1.0 and 2.5 M NaCl fractions from immunoaffinity column chromatography of the solubilized SG935(pPC39) pellet (lane 7) are shown. Molecular mass markers (lane M), with sizes in kDa, are indicated at the left. The arrowhead indicates the position of VirB11 protein.

from acetosyringone-induced (lane 4) but not uninduced (lane 3) *A. tumefaciens* A348. No immunoreactive material of this size was present in extracts from induced or uninduced A136(pTi368) (Fig. 1, lanes 1 and 2), a *virB11* mutant (ref. 4; J.E.W., unpublished data). A 38-kDa immunoreactive protein also was visualized in extracts from IPTG-induced SG935(pPC39) (Fig. 1, lane 6) but not SG935(pUC118) (Fig. 1, lane 5). These analyses confirm that the antiserum specifically recognizes the 38-kDa *virB11* gene product. All *A. tumefaciens* and *E. coli* strains contained immunoreactive material of ≈60 kDa in total protein extracts, but this material was not present in the insoluble pellet used as starting material for the VirB11 protein purification or in preparations of the purified VirB11 protein (Fig. 1, lane 7).

VirB11 protein, as deduced from the DNA sequence, is highly hydrophilic and lacks consensus signal peptide and stop-transfer sequences (25). The relative amount of VirB11 protein present in subcellular fractions from induced *A. tumefaciens* A348 was determined by immunoblot analysis with anti-VirB11 antibodies. Fig. 2 shows that, although the cytoplasmic fraction contained some VirB11 protein (lane 2), most was localized to the cytoplasmic membrane fraction (lane 4). The protein was barely detected in outer membrane preparations (Fig. 2, lane 5) and not detected in periplasmic fractions (data not shown). These localization data were complemented by topological studies in which *phoA*, the structural gene for alkaline phosphatase, was translationally fused near the carboxyl end of *virB11*. *E. coli* (pJW351) produces an ≈85 kDa VirB11–PhoA fusion protein as determined by immunoblot analysis using anti-PhoA and anti-VirB11 antibodies, but this fusion protein has no alkaline phosphatase activity. Therefore, at least the carboxyl end of VirB11 protein is not exported to the periplasm. Taken together, our data suggest that VirB11 protein associates with the inner side of the cytoplasmic membrane either by interacting with integral membrane proteins, such as other VirB proteins, or by forming a membrane anchoring domain when properly folded.

Purification of VirB11 Protein. Induced *E. coli* strains carrying pJW322 produced VirB11 protein in amounts detectable only by immunostaining with VirB11-specific antiserum. The protein yield was increased by deleting sequences between the *lacZ* ATG and the first codon of *virB11* by using a synthetic oligonucleotide. As shown in Fig. 3, IPTG-induced SG935(pPC39) overproduced VirB11 protein (lane 2), which partitioned primarily to the pellet fraction after centrifugation of extracts from French press-disrupted cells (lane 4). Solubilization of the pellet followed by chromatography through an anti-VirB11 immunoaffinity column resulted in purification of VirB11 protein to apparent homogeneity as determined by $NaDodSO_4$/PAGE and staining (Fig. 3, lane 5). The sequence of the first 16 amino acids—Met-Glu-Val-Asp-Pro-Gln-Leu-Arg-Phe-Leu-Leu-Lys-Pro-Ile-Leu-Glu—corresponds precisely to the amino terminus of the *virB11* gene product deduced from the DNA sequence (25).

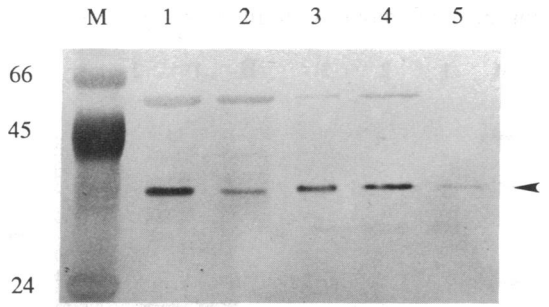

FIG. 2. Immunoblot analysis of *A. tumefaciens* A348 cellular components. Proteins from the various fractions were electrophoresed through a $NaDodSO_4$/10% polyacrylamide gel and transferred to nitrocellulose, and the blot was probed with anti-VirB11 antiserum. Lanes: 1, total protein; 2, cytoplasmic protein; 3, total membrane protein; 4, cytoplasmic membrane protein; 5, outer membrane protein. Anti-VirB11 antiserum did not react with any material in the periplasmic fraction (data not shown). The arrowhead indicates the position of VirB11 protein.

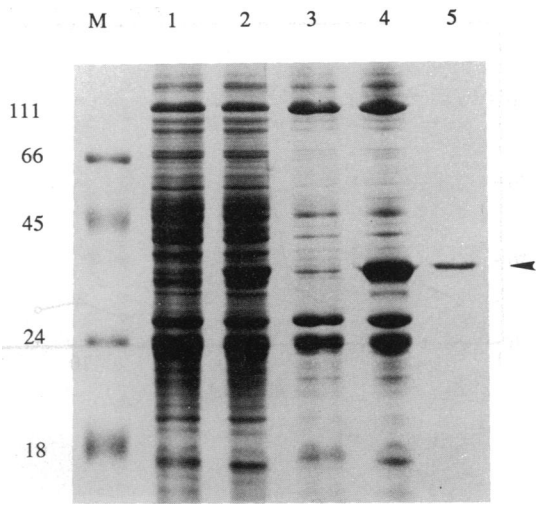

FIG. 3. Overproduction and purification of VirB11 protein from IPTG-induced *E. coli* SG935(pPC39). A $NaDodSO_4$/12.5% polyacrylamide gel stained with Coomassie blue shows stages of the purification. Total protein from the IPTG-induced vector-only control *E. coli* SG935(pUC118) (lane 1) and induced SG935(pPC39) (lane 2), pellet from centrifugation of crude extract obtained by French press treatment of SG935(pUC118) (lane 3) and SG935(pPC39) (lane 4); and material (1.0 μg) in pooled and concentrated 1.0 and 2.5 M NaCl fractions from immunoaffinity column chromatography of the solubilized SG935(pPC39) pellet (lane 5) are shown. The arrowhead indicates the position of VirB11 protein.

The VirB11 Protein Has ATPase Activity. Purified VirB11 protein obtained from the anti-VirB11 immunoaffinity column hydrolyzed [γ-^{32}P]ATP to ADP and P_i as shown by thin-layer chromatography. Three control experiments confirmed that the VirB11 protein and not a contaminant was responsible for ATPase activity. Proteins from the vector-only strain SG935(pUC118) were treated by the identical procedure used to purify VirB11 protein. The concentrated immunoaffinity chromatography fractions contained no detectable material, as determined by $NaDodSO_4$/PAGE and staining. These fractions possessed no ATPase activity. Second, immunoaffinity chromatography purified VirB11 protein was electrophoresed through $NaDodSO_4$/polyacrylamide gels, and material in 0.5-cm gel slices was eluted, renatured, and tested for ATPase activity. As shown in Fig. 4, material in the gel slice containing VirB11 protein possessed ATPase activity, but material obtained from other gel slices did not hydrolyze ATP. Finally, as shown in Table 1, the addition of anti-VirB11 immunoglobulin inhibited ATPase activity by 80%, whereas immunoglobulins from preimmune serum inhibited at most 5–10% of the activity.

Characterization of the VirB11 ATPase Activity. VirB11 ATPase activity was characterized under standard reaction conditions (see *Materials and Methods*) where hydrolysis was proportional to protein concentration and linear with time. The VirB11 ATPase is optimally active at a pH of 6.5–7.5. ATPase activity requires Mg^{2+}, Zn^{2+}, or Mn^{2+} at optimal concentrations of 10–25 mM, whereas similar concentrations of K^+ and Ca^{2+} reduce activity by 50%. The reaction is strongly inhibited 80–90% by 25 mM NaCl. VirB11 ATPase activity was assayed in the presence of ATP (10 μM)

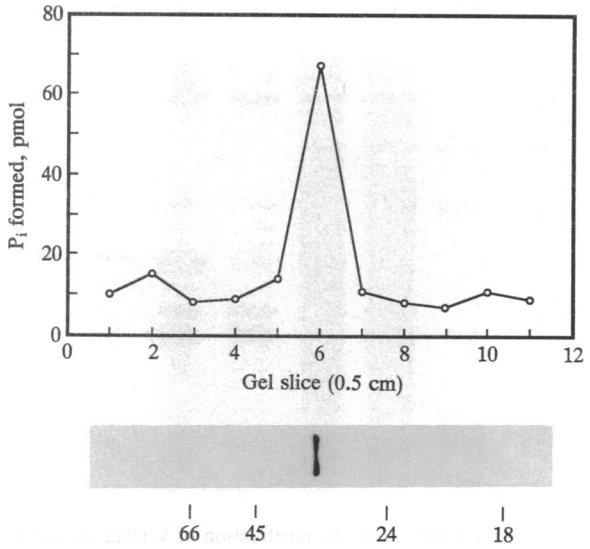

FIG. 4. ATPase activity of gel-eluted and renaturated VirB11 protein. Gel lane depicts immunoaffinity purified VirB11 protein (1.0 μg) electrophoresed through a NaDodSO$_4$/12.5% polyacrylamide gel and stained with Coomassie blue. The apparent molecular mass (kDa) of material in the gel slices is indicated. The graph shows ATPase profiles of material obtained from 0.5-cm gel slices cut along an adjacent lane loaded with 2.5 μg of total protein.

and competitor nucleotides (50 μM). ADP reduces ATP hydrolysis by 80%. GTP, CTP, UTP, and dATP also decrease activity by 50–80%, whereas dGTP, dCTP, and dTTP inhibit activity only by 10–25%. A double-reciprocal plot of P_i formation versus ATP concentration yields an apparent K_m for ATP of 550 μM (data not shown). However, optimal ATPase activity may depend on the presence of additional cofactors or a purification scheme that does not denature the protein.

ATPase activity was stimulated at most 5–10% with additions of poly(dA), dA_{30}, and single-stranded circular pUC118 but was not stimulated with additions of single-stranded calf thymus DNA or dT_{30} or with double-stranded dA_{30}·dT_{30} or linear (by *Eco*RI digestion) or circular pUC118 or pBR322. Single- or double-stranded 30-mers homologous to border sequences or overdrive also did not stimulate ATPase activity. Altering DNA concentrations did not affect the level of stimulation. Although activity was slightly stimulated by some single-stranded polynucleotides, our results strongly suggest that the VirB11 protein ATPase activity does not depend on DNA effectors, including T-DNA or flanking sequences.

Table 1. Inhibition of ATPase activity with anti-VirB11 antibodies

Protein	Antibody*	% ATPase activity†
None	None	0
None	Preimmune	9
None	Anti-VirB11	7
VirB11	None	100
	Preimmune	92
	Anti-VirB11	20

The reaction mixtures consisting of 0.26 μm VirB11 protein purified by immunoaffinity chromatography (where indicated), 10 μM ATP, and 10 μg of antibodies as specified were incubated for 60 min at 30°C.

*Antibodies were purified by protein A-Sepharose chromatography.

†One hundred percent ATPase activity corresponds to the hydrolysis of 7.8% of input ATP.

VirB11 Protein Is Phosphorylated *in Vitro*. VirB11 protein incubated with [γ^{32}-P]ATP was phosphorylated, as determined by analysis of reaction products by NaDodSO$_4$/PAGE, transfer to nitrocellulose, and autoradiography. As shown in Fig. 5*B*, VirB11 protein (0.5 μM) was specifically labeled by incubation with [γ^{32}-P]ATP (lane 1) but not by [α^{32}-P]ATP (lane 2), [γ^{32}-P]GTP (lane 3), [γ^{32}-P]dATP (data not shown), or [α^{32}-P]dATP (data not shown) of comparable specific activities. In control experiments, rabbit serum albumin (0.5 μM) was not labeled when incubated with any of the labeled mononucleotides. These results demonstrate that VirB11 protein is labeled specifically by incubation with [γ^{32}-P]ATP. Whereas most of the phosphorylated VirB11 remained intact when exposed to 1.0 M KOH for 2 hr at 55°C, almost all of the label was released by exposure to 16% trichloroacetic acid for 60 min at 90°C. Therefore, phosphate most likely is covalently attached to a hydroxyl group (37).

DISCUSSION

The consensus mononucleotide binding domains in the deduced *virB5* and *virB11* gene products of the pTiA6NC plasmid suggests that NTP binding or hydrolysis is involved in the *A. tumefaciens* T-DNA transformation system. We examined this possibility in detail by purifying the VirB11 protein from a VirB11-overproducing *E. coli* strain by using immunoaffinity chromatography. In preliminary studies, we demonstrated that the native form of VirB11 protein, but not a heat-denatured form or lysozyme, is photolabeled by the photoaffinity ATP analog 8-azido-[α^{32}-P]ATP (P.J.C., unpublished data). Here, we have shown that VirB11-containing fractions from immunoaffinity chromatography possessed ATPase activity. The corresponding immunoaffinity fractions obtained by chromatography of solubilized proteins from the vector-only control strain possessed no detectable proteins or ATPase activity. In addition, purified VirB11 protein eluted from NaDodSO$_4$/polyacrylamide gels exhibited ATPase activity, whereas material obtained from higher and lower molecular size positions in the gel had no activity (Fig. 4). Finally, anti-VirB11 antibodies strongly inhibited the ATPase activity (Table 1). We conclude from these results that the VirB11 protein is an ATPase.

The VirB11 ATPase activity was stimulated slightly by some single-stranded DNA polynucleotides but not at all by double-stranded DNA. Although some VirB11 protein was retained on single-stranded DNA affinity columns when protein extracts from induced *A. tumefaciens* A348 were chromatographed and fractions were analyzed by immunoblotting (P.J.C., unpublished data), the purified protein did not bind DNA, in the absence or presence of ATP and Mg^{2+}, by using gel shift mobility or filter binding assays. These

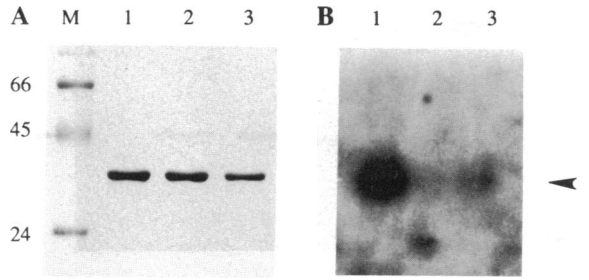

FIG. 5. Nucleotide specificity of the VirB11 phosphorylation. Reaction mixtures were electrophoresed through a NaDodSO$_4$/12.5% polyacrylamide gel and stained with Coomassie blue (*A*) or proteins were transferred to nitrocellulose and the blot was autoradiographed (*B*). VirB11 protein (0.5 μM) was incubated in the presence of [γ-^{32}P]ATP (lanes 1), [α-^{32}P]ATP (lanes 2), or [γ-^{32}P]GTP (lanes 3). The arrowhead indicates the position of VirB11 protein. M, molecular size markers in kDa.

observations suggest that VirB11 protein does not interact directly with DNA but may still associate with other DNA-binding proteins (i.e., VirD2 or VirE2). VirB11 protein may regulate gene expression through its interactions with other transcriptional factors, but there is compelling evidence that only VirA and VirG proteins (4, 6–8), through phosphorylation of VirG protein by VirA protein (S. Jin, T. Roitsch, R. G. Ankenbauer, M.P.G., and E.W.N., unpublished data), are required for *vir* gene induction. Furthermore, an involvement in T-DNA processing is unlikely, since the mutagenesis studies of Stachel *et al.* (12) demonstrated an absolute requirement only for the 5′ end of the *virD* locus for T-strand formation. Taken together, these findings suggest that VirB11 protein does not participate in *vir* gene induction or in the T-DNA processing reaction but instead plays a role in the T-DNA transport process.

The intriguing finding that VirB11 protein autophosphorylates *in vitro* suggests that VirB11 protein modulates its own activity and, possibly, the activities of other *vir* proteins, by phosphorylation. Phosphorylation may induce a conformational change in the VirB11 protein, allowing it to bind other components of the membrane apparatus. Alternatively, VirB11-phosphate may function as a kinase that activates one or more components of the apparatus. In other multicomponent membrane systems involved in signal or energy transduction or substrate transport (see refs. 30, 31, and 38–40), a proposed function of ATP binding, autophosphorylation, and/or kinase activity is to alter the allosteric interactions among components of the membrane apparatus. For example, the well-characterized periplasmic binding protein-dependent transport systems are activated to transport substrates across the cytoplasmic membrane in response to substrate binding and ATP binding or hydrolysis (see ref. 40). One model proposes that a conformational change in the membrane apparatus results in the formation of a pore, which allows passage of the substrates (40). VirB11 autophosphorylation or kinase activity may similarly induce a conformational change in a *virB*-encoded membrane apparatus, resulting in pore formation to allow passage of T-DNA or a T strand–protein complex across the bacterial envelope. Indeed, activation of the transport apparatus may also require binding of a component of the T strand–protein complex, such as VirD2 protein associated with the 5′ end of the T strand.

A DNA sequence analysis of the *Bacillus subtilis comG* operon recently revealed that the deduced *comG* ORF1 protein exhibits sequence similarity to the VirB11 protein (41). The hydrophilic *comG* ORF1 protein, like VirB11 protein, possesses a consensus nucleotide binding domain and may regulate the activities of membrane proteins involved in the development of genetic competence by *B. subtilis*. The *comG* operon, like the *virB* operon, codes for several membrane-associated proteins, some or all of which may form a membrane apparatus involved in directing transfer of DNA across the membrane (41). In addition, whereas *A. tumefaciens* mediates the transfer of mobilizable plasmids to plants by a conjugation-like process (25, 26), some of the *comG*-encoded proteins share sequence similarities with known bacterial pilin proteins required for conjugation (41). These observations suggest the intriguing possibility that the T-DNA transfer system, as well as bacterial conjugation and transformation systems, each utilize a multicomponent membrane apparatus for transport of DNA across the membrane, which is conserved both in structure and function and activated, at least in part, through phosphorylation.

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