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Characterization of Membrane and Protein Interaction Determinants of the *Agrobacterium tumefaciens* VirB11 ATPase

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The VirB11 ATPase is a putative component of the transport machinery responsible for directing the export of nucleoprotein particles (T complexes) across the *Agrobacterium tumefaciens* envelope to susceptible plant cells. Fractionation and membrane treatment studies showed that ~30% of VirB11 partitioned as soluble protein, whereas the remaining protein was only partially solubilized with urea from cytoplasmic membranes of wild-type strain A348 as well as a Ti-plasmidless strain expressing *virB11* from an IncP replicon. Mutations in *virB11* affecting protein function were mapped near the amino terminus (Q6L, P13L, and E25G), just upstream of a region encoding a Walker A nucleotide-binding site (F154H;L155M), and within the Walker A motif (P170L, K175Q, and ΔGKT174-176). The K175Q and ΔGKT174-176 mutant proteins partitioned almost exclusively with the cytoplasmic membrane, suggesting that an activity associated with nucleotide binding could modulate the affinity of VirB11 for the cytoplasmic membrane. The *virB11F154H;L155M* allele was transdominant over wild-type *virB11* in a merodiploid assay, providing strong evidence that at least one form of VirB11 functions as a homo- or heteromultimer. An allele with a deletion of the first half of the gene, *virB11Δ1-156*, was transdominant in a merodiploid assay, indicating that the C-terminal half of VirB11 contains a protein interaction domain. Products of both *virB11Δ1-156* and *virB11Δ158-343*, which synthesizes the N-terminal half of VirB11, associated tightly with the *A. tumefaciens* membrane, suggesting that both halves of VirB11 contain membrane interaction determinants.

Translocation of oncogenic nucleoprotein particles across the *Agrobacterium tumefaciens* envelope during infection of plant cells requires the functions of at least 10 of the 11 *virB* gene products (4, 5, 20, 22, 48, 52, 54). Recent studies have shown that the VirB proteins are highly related in sequence to Tra components of conjugal transfer machineries dedicated to transmission of the IncP (25), IncN (32), and IncW (21) broad-host-range plasmids to bacterial recipient cells. The similarities between these systems extend to the level of function in that the VirB and Tra proteins promote conjugal transfer of a common substrate, the non-self-transmissible IncQ plasmid RSF1010 (3, 6, 7, 26). These findings have led to the view that the VirB and Tra proteins assemble as mechanistically conserved conjugal mating pores or channels for intercellular transmission of nucleoprotein particles. Interestingly, the VirB and Tra proteins also bear sequence similarities to the *Bordetella pertussis* Ptl proteins, which function in secretion of pertussis toxin (50, 52). There is also an increasing body of evidence that the VirB proteins direct the export not only of DNA in the form of nucleoprotein particles but also of protein only (6, 10, 30, 36, 43).

Two VirB proteins, VirB4 and VirB11, possess conserved Walker A nucleotide-binding motifs that are required for function (references 4, 18, 40, and 42 and this study). Purified forms of both proteins exhibit weak ATP hydrolysis activities (9, 40), and purified VirB11 has been shown to autophosphorylate in vitro (9). Recent studies have shown that VirB11 is a member of a large family of evolutionarily related ATPases whose ATP-binding and/or hydrolysis activities are indispensable for assembly or function of a diverse array of DNA or protein transport systems in eubacteria. While VirB11 is most highly similar to TraG, TrbB, and TrwD of the IncN, IncP, and IncW broad-host-range plasmids, respectively, it also displays sequence similarities to ATPases whose functions are required for secretion of exoenzymes or toxins (PtlH [*B. pertussis* pertussis toxin], PulE [*Klebsiella oxytoca* pullulanase], XcpR [*Pseudomonas aeruginosa* exotoxin A], and EpsE [*Vibrio cholerae* cholera toxin]), assembly of type IV fimbriae (PilT and PilB [*P. aeruginosa*]), and establishment of competence (ComG1 [*Bacillus subtilis*]) (26, 33).

Members of this family of ATPases, like the ATPase subunits of the ATP-binding cassette (ABC) transporter superfamily (13), are generally hydrophilic proteins that lack obvious transmembrane domains and amino-terminal export signals. Despite these features, three members of this family, PulE (33), EpsE (38), and VirB11 (9), have been shown to associate predominantly with the cytoplasmic membrane. In this study, we examine the nature of the membrane association of VirB11. We also identify several functional domains of VirB11 by analysis of a collection of *virB11* alleles generated by random and site-directed mutagenesis. Finally, we report the isolation of two transdominant alleles of *virB11*. One allele is comprised of the 3′ half of the gene, providing strong genetic evidence that the C-terminal half of VirB11 possesses a protein interaction domain.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Table 1 lists bacterial strains and plasmids used in this study. Conditions for growth of *Escherichia coli* and *A. tumefaciens* and for induction of the *A. tumefaciens vir* genes with induction medium (IM) containing acetosyringone have been described previously (14). Plasmids were introduced into *A. tumefaciens* by electroporation as described previously (4). Plasmids were maintained in *E. coli* or *A. tumefaciens* by addition of carbenicillin (50 μg/ml), kanamycin (50 μg/ml), tetracycline (5 μg/ml), or chloramphenicol (35 μg/ml).

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TABLE 1. Bacterial strains and plasmids used

Bacterial strain or plasmid	Relevant characteristics	Source or reference
Strains		
E. coli		
DH5α	λ$^-$ ϕ80d/*lacZ*Δ*M15* Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 gyrA relA1*	GIBCO BRL
CJ236	*dut ung thi relA*; pCJ105 (Cam^r)	Bio-Rad
XL1-Red	*endA1 gyr96 thi-1 hsdR17 supE44 relA1 lac mutD5 mutS mutT* Tn*10* (Tet^r)	Stratagene
A. tumefaciens		
A136	Strain C58 cured of pTiC58	49
A348	A136 containing octopine-type Ti plasmid pTiA6NC	19
PC1011	Formerly A348Δ*virB11*; A348 derivative sustaining a *virB11* gene deletion from pTiA6NC	5
Plasmid vectors		
pUC118	Crb^r, cloning vector	37
pUC119	Crb^r, cloning vector	37
pMY1133	Cam^r, cloning vector with P_{tac} promoter	53
pBSIISK$^+$	Crb^r, cloning vector	Stratagene
pBSIISK$^+$*Nde*I	Crb^r, pBSIISK$^+$ containing an *Nde*I site at the translational start site of *lacZ*	5
pBSIISK$^+$*Nco*I	Crb^r, pBSIISK$^+$ containing an *Nco*I site at the translational start site of *lacZ*	This study
pUI1188::Kan	Source of ΩKan^r cassette	J. Eraso and S. Kaplan
pSW172	Tet^r, broad-host-range IncP plasmid containing P_{lac} and downstream polylinker sequence	8
pET15b	Crb^r, T7 *lac* expression vector for constructing translational fusions between His_6 and the N terminus of a protein of interest	Novagen
Plasmids		
pJW325	Crb^r, pUC119 carrying *virB9–virB11* on a 3.6-kb *Eco*RI-*Xho*I fragment	48
pJW353	Cam^r Tet^r, pMY1133 with the *virB11* gene expressed from the P_{tac} promoter, ligated at *Hin*dIII site to similarly digested pSW172	J. Ward
pJW237	Crb^r, pUC119 with bp 1–969 of *virB11* carried on a *Sal*I-*Bgl*II fragment from pJW325 (48) inserted into *Sal*I and *Bam*HI sites, resulting in *virB11*Δ*323-343*	J. Ward
pJWB237	Crb^r, Tet^r, pJW237 ligated at *Kpn*I site to similarly digested pSW172	This study
pGSB3	Cam^r Tet^r, pJW353 containing a Q6-L codon substitution by random mutagenesis	This study
pGSB4	Cam^r Tet^r, pJW353 containing a P13-L codon substitution by random mutagenesis	This study
pGSB5	Cam^r Tet^r, pJW353 containing an E25-G codon substitution by random mutagenesis	This study
pGSB6	Cam^r Tet^r, pJW353 containing a P170-L codon substitution by random mutagenesis	This study
pPC39	Crb^r pUC118 containing *virB11* expressed from the P_{lac} promoter	9
pPCB7111	Crb^r Tet^r, pPC39 ligated at *Kpn*I site to pSW172	This study
pPCB7112	Crb^r Tet^r, pPC39 with a deletion of G174, K175, and T176 codons of *virB11* ligated at the *Kpn*I site to pSW172	This study
pPCB7113	Crb^r Tet^r, pPC39 with a K175-Q codon substitution ligated at the *Kpn*I site to pSW172	This study
pPC914KS$^+$	Crb^r, pBSIIKS$^+$ with a 1.37-kb *Hin*dIII fragment containing P_{virB} and *virB1* with an *Nde*I site at its translational start site	14
pPC9118	Cam^r, pBCSK$^+$*Nde*I with *virB11* carried on a 1.4-kb *Nde*I-*Hin*dIII fragment	5
pPC9119	Crb^r, pET15b with *virB11* carried on a 1.4-kb *Nde*I-*Xho*I fragment from pPC9118 such that the 5′ end of *virB11* is fused in frame to sequence encoding a His_6 tag	This study
pSR1	Crb^r, pPC914KS$^+$ with a 1.4-kb *Nde*I-*Kpn*I fragment from pPC9118 introduced into similar sites downstream of P_{virB}, substituting *virB11* for *virB1*	This study
pSR2	Crb^r, pBSIISK$^+$*Nco*I with *His$_6$-virB11* introduced as a 1.60-kb *Nco*I-*Xho*I fragment from pPC9119	This study
pSRB2	Crb^r Tet^r, pSR1 ligated at *Kpn*I site to similarly digested pSW172	This study
pSR3	Kan^r Cam^r, pPC9118 with F154-H and L155-M codon substitutions and with the ΩKan^r cassette from pUI1188::Kan inserted at *Hin*dIII site	This study
pSRB3	Kan^r Cam^r Tet^r, pSR3 ligated at *Xba*I site to similarly digested pSW172	This study
pSR4	Crb^r, pSR1 deleted of a 1-kb *Sph*I fragment, resulting in *virB11*Δ*158-343*	This study
pSRB4	Crb^r Tet^r, pSR4 ligated at *Kpn*I site to similarly digested pSW172	This study
pSR5	Kan^r Cam^r, pSR3 deleted of a 0.46-kb *Nde*I fragment, resulting in *virB11*Δ*1-156*	This study
pSRB5	Kan^r Cam^r Tet^r, pSR5 ligated at *Xba*I site to similarly digested pSW172	This study

Random mutagenesis. In preliminary experiments, we demonstrated that plasmid pJW353, which constitutively expresses *virB11* from the P_{lac} promoter, fully restores virulence of the Δ*virB11* mutant PC1011. Mutations were introduced into *virB11* in vivo (17) by use of the *E. coli* mutator strain XL1-Red (Stratagene). Several hundred XL1-Red colonies transformed with plasmid pJW353 were pooled and used as the inoculum for growth of a 1-liter culture to stationary phase with vigorous shaking at 37°C. One milliliter of this culture was used as the inoculum for growth of a second 1-liter culture to stationary phase. Mutagenized plasmid DNA (pJW353*) was isolated and purified by CsCl density gradient centrifugation for introduction into electrocompetent PC1011 cells. Eight hundred fifty *A. tumefaciens* transformants were assayed at least three times for virulence by inoculation onto *Kalanchöe diagremontiana* leaves. Fifteen strains exhibiting attenuated virulence or avirulence were assayed at least five more times for virulence on *K. diagremontiana*. Plasmid DNA was isolated from these strains and reintroduced into strain PC1011, and resulting transformants were assayed two more times for virulence. Plasmid DNA from nine strains that consistently exhibited avirulence or severely attenuated virulence was isolated, and the presumptively mutated *virB11* alleles were sequenced with modified T7 DNA polymerase (Sequenase 2.0; United States Biochemical) according to the manufacturer's instructions. Sequence analysis was performed by using four primers spaced at 250-bp intervals along the length of the 1-kb *virB11* gene. *A. tumefaciens* strains carrying these alleles were assayed for VirB11 protein abun-

dance by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and immunostaining with anti-VirB11 antiserum (see below).

Site-directed mutagenesis. Two mutations predicted to diminish or abolish ATP binding or hydrolysis were introduced into the Walker A nucleotide-binding site (Gly-Pro-Thr-Gly-Ser-**Gly-Lys-Thr**) positioned between amino acid residues 169 and 176 of VirB11 (46, 47). The K175 codon (shown underlined) was changed to a glutamine codon with the oligonucleotide 5′-GCTCATTGTTGTCT GGCCGCTTCCGG-3′, and codons specifying G174, K175, and T176 (shown in bold type) were deleted with the oligonucleotide 5′-GGTCTTGCTCATTGTG CTTCCGGTAGGGCC-3′. A third mutated allele encoded a VirB11 derivative with F154-to-histidine and L155-to-methionine substitutions. This allele was generated as a result of the introduction of an *Nde*I site at bp 461 with the oligonucleotide 5′-GCATGCGTGCATATGCGCTTCCAA-3′ for subcloning and expression of the C-terminal half of the protein. *E. coli* CJ236(pPC39) served as the source of single-stranded template DNA for introduction of mutations by the method of Kunkel et al. (24). Sequence analysis confirmed that plasmids pPCB7113 and pPCB7112 harbored the K175-Q and ΔGKT174-176 mutations, respectively, and that pSR3 harbored the *Nde*I restriction site at bp 461.

Construction of *His$_6$-virB11* and *virB11*Δ alleles. Standard procedures were used to construct recombinant plasmids (37). A wild-type *virB11* allele with additional coding information for an N-terminal His_6 tag was constructed by introducing a ~1.0-kb *Nde*I-*Xho*I restriction fragment carrying *virB11* from pPC9118 into similarly digested pET15b, resulting in pPC9119. To express *His$_6$-virB11* from the P_{tac} *promoter for expression in A. tumefaciens*, a ~1.60-kb *Nco*I-*Xho*I fragment carrying *His$_6$-virB11* was isolated from plasmid pPC9119 and introduced into similarly digested pBSIISK$^+$*Nco*I to make pSR2. Three plasmids expressing *virB11*Δ alleles from the P_{lac} *or* P_{virB} promoter were constructed as follows. Plasmid pJW237, which expresses *virB11*Δ*323-343*, was constructed by ligating a 1.3-kb *Sal*I-*Bgl*II fragment from plasmid pJW325 into *Sal*I and *Bam*HI sites of vector plasmid pUC119. Plasmid pJW237 was a gift from J. Ward. Plasmid pSR5, which expresses *virB11*Δ*1-156* from P_{lac}, was constructed by introducing an *Nde*I restriction site into pPC9118 at bp 462 by site-directed mutagenesis as described by Kunkel et al. (24) and then removing a ~460-bp *Nde*I fragment by digestion and religation. This placed *virB11*Δ*1-156* in-frame with the translational initiation codon of *lacZ* carried on pBSIIKSK$^+$*Nde*I. Plasmid pSR4, which expresses *virB11*Δ*158-343*, was constructed by introducing *virB11* from pPC9118 as an *Nde*I-*Kpn*I fragment into pPC914KS to make pSR1. Plasmid pSR1 was then digested with *Sph*I and religated to remove the 3′ half of *virB11*.

Protein analysis and immunoblotting. Proteins were resolved by SDS-PAGE using 12.5 or 15.0% polyacrylamide gels, transferred to nitrocellulose membranes, and visualized by immunoblot development with goat anti-rabbit antibodies conjugated to alkaline phosphatase (14). Polyclonal antiserum against VirB11 was previously described (9). Anti-VirA antiserum (51) was kindly provided by S. Winans. For comparisons of steady-state levels of wild-type and mutant forms of VirB11, immunoblots were scanned with a PDI scanning densitometry system. Protein loading on SDS-polyacrylamide gels routinely was normalized with respect to total protein content and, when possible, VirE2 protein abundance to ensure that the *vir* genes were induced equivalently in all strains (5).

Cell fractionation. For fractionation of *A. tumefaciens*, acetosyringone-induced cells from a 500-ml culture were suspended in 3.5 ml of a 50 mM Tris-HCl buffer (pH 8.0) containing 20% sucrose, 0.2 mM dithiothreitol (DTT), 0.2 mg of DNase per ml, and 0.2 mg of RNase per ml. Iodoacetamide was added at a final concentration of 1 mM to cells immediately prior to lysis to exclude the possibility of spontaneous oxidation of cysteine sulfhydryls in vitro. Cells were disrupted by three passages through a French pressure cell at 14,000 lb/in^2. Lysozyme was added to a final concentration 0.4 mg/ml, and the crude extract was incubated for 30 min on ice. The volume was brought to 5 ml with Tris-HCl buffer, and the lysate was centrifuged two times for 15 min each at 14,000 × *g* to remove unbroken cells and cell debris. The resulting cleared lysate represented the total protein fraction. KCl was added to the total protein fraction to a final concentration 0.2 M, and soluble and insoluble material was separated by centrifugation with an SW55 Ti rotor at 150,000 × *g* for 4 h at 4°C. The supernatant represented the total cytoplasmic fraction. Pelleted material was resuspended in 1 ml of ice-cold 50 mM Tris-HCl (pH 7.4) buffer containing 0.2 M KCl by sonication. The volume was brought up to 5 ml with the same buffer, and membranes were repelleted at 150,000 × *g* for 2 h. The washed membrane preparation represented the total membrane fraction. Total membranes were suspended in 1 ml of 20% sucrose (wt/vol)–5 mM EDTA–0.2 mM freshly prepared DTT and centrifuged through isopycnic sucrose gradients prepared with 1 ml of 70% and 3 ml of 53% sucrose at 200,000 × *g* for 22 h at 4°C. Fractions of equal volume were taken from the bottom of the gradient. The quality of the fractionation was assessed by measuring absorption of fractions at 280 nm and enzymatic activity of the cytoplasmic membrane marker NADH oxidase (27, 28). Protein peaks corresponding to cytoplasmic and outer membrane proteins were found in fractions with buoyant densities of 1.143 and 1.247 g/cm^3, respectively, consistent with previous findings (10, 14, 27, 28). Fractionated material was applied to SDS-polyacrylamide gels on a per-cell equivalent basis, proteins were electrophoresed and transferred to nitrocellulose membranes, and the relative amount of VirB11 in each of the samples was quantitated by immunostaining and scanning densitometry.

Membrane extraction. For membrane extraction studies, total membranes were suspended in 50 mM Tris-HCl (pH 8.0) and sonicated immediately prior to treatment to eliminate possible sequestration of VirB11 in membrane vesicles formed during the fractionation procedure. Membranes were treated with the following reagents at a final concentration of 1 mg of protein/ml: 2 M NaCl, 2 M $NaClO_4$, 10 mM EDTA, and 5 M urea. Alternatively, membranes were suspended in glycine-NaOH buffer (pH 11.3) or in glycine-HCl buffer (pH 3.2). For treatment with different detergents, total membrane material was incubated in Tris-HCl buffer (pH 8) containing 2% Triton X-100 (TX-100); 2% TX-100 and 5 mM EDTA; 2% TX-114; 2% octylglucoside (OG), 10% glycerol, 10 mM DTT, and 5 mM $MgCl_2$; 1% SDS; 1% deoxycholate (DOC); or 1% 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS), with slow shaking for 1 h at 4°C or, for the SDS treatment, at 25°C. Treated samples were centrifuged in a TLA-100.2 rotor at 100,000 × *g* for 45 min. Pellets were resuspended in the same buffers at volumes equal to those of the corresponding supernatants. Soluble and particulate material was analyzed for the presence of VirB11 and VirA proteins by immunostaining with antisera specific for these proteins.

Virulence assays. *A. tumefaciens* strains were tested for virulence on uniformly wounded *K. diagremontiana* leaves as previously described (4, 5). Controls for the tumorigenesis assays included coinoculation of the same leaf with the wild-type strain A348 and the Δ*virB11* mutant PC1011. Assays were repeated at least four times for each strain on separate leaves. Tumors were photographed after 4 to 5 weeks of inoculation.

RESULTS

Analysis of VirB11 association with the membrane. In wild-type A348 cells, VirB11 associates predominantly but not exclusively with the cytoplasmic membrane (9) (see below). Treatment of membranes with 1 M NaCl fails to release the membrane-associated form of VirB11 into the soluble fraction (16, 42), which could indicate that this form of VirB11 is tightly associated with the membrane. However, an alternative explanation is that VirB11 is solubilized from the membranes but then forms aggregates that sediment along with membranes during centrifugation. To distinguish between these possibilities and to further characterize the nature of the association of VirB11 with the *A. tumefaciens* envelope, we first tested for solubilization by treatment of membranes with a variety of reagents or conditions. As a control, we assessed the effects of applied treatments on the integrity of the cytoplasmic membrane by monitoring extractability of the integral cytoplasmic membrane protein VirA (51).

Figure 1A shows the distribution of VirB11 into soluble and particulate fractions after various treatments of A348 membranes. Buffers with high ionic strength (2 M NaCl), a high concentration of chaotropic ions (ClO_4^-), and low or high pH did not release any VirB11 into the soluble fraction. In preliminary studies, we determined that treatment of membranes with 6 M urea, a condition previously shown to extract the majority of VirB11 (16), also extracted as much as 20% of the integral membrane protein VirA, indicating that this concentration of urea partially disrupts the integrity of *A. tumefaciens* membranes. By contrast, treatment with 5 M urea did not extract detectable amounts of VirA, and this concentration of urea reproducibly extracted only about one-half of the membrane-associated VirB11 (Fig. 1A). Treatments of membranes of *A. tumefaciens* A136(pJW353), a Ti-plasmidless strain that constitutively expresses *virB11* from P_{lac}, with the reagents indicated in Fig. 1A gave similar results (data not shown).

Next, to distinguish whether particulate VirB11 was aggregated or membrane associated, we fractionated the particulate material recovered from the membrane treatments through sucrose gradients. Figure 2 shows representative distribution patterns following centrifugation of particulate material recovered from high-pH and high-salt treatments of A348 and A136(pJW353) membranes. In both cases, VirB11 was found exclusively in low-density fractions near the tops of the sucrose gradients. The same fractions also contained the cytoplasmic membrane markers VirA (Fig. 2) and NADH oxidase activity

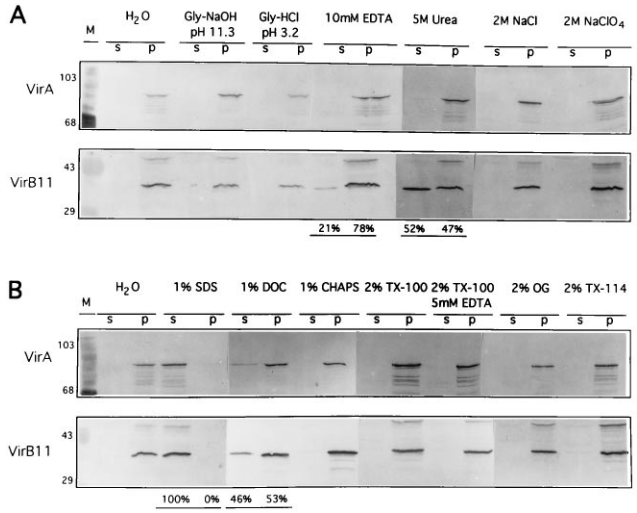

FIG. 1. Extractability of membrane-bound VirB11 protein. Total membranes from wild-type A348 cells were concentrated by centrifugation and treated as indicated at the top (A) and with detergents (B). Soluble (s) and particulate (p) material was separated by centrifugation and analyzed by SDS-PAGE and immunoblotting for the presence of VirA and VirB11 proteins. Lane M, molecular mass markers with sizes indicated at the left in kilodaltons. Percentages at the bottoms of the panels refer to the relative amounts of VirB11 in the corresponding fractions, as determined by scanning densitometry of the immunoblots.

(data not shown). Sucrose gradient analyses of particulate material recovered from treatments of A348 and A136(pJW353) membranes with the other reagents indicated in Fig. 1A yielded similar results (data not shown). Together, these findings establish that all reagents except urea fail to extract the cytoplasmic membrane-associated form of VirB11. Furthermore, even urea only partially extracts this form of VirB11 when used at a concentration that does not disrupt cytoplasmic membrane integrity.

Parallel membrane extraction studies were carried out with the nonionic detergents 2% TX-100, 2% OG, and 2% TX-114 and an amphoteric detergent, 1% CHAPS. In all cases, VirB11 was found exclusively in the particulate fractions following centrifugation of treated membranes (Fig. 1B and data not shown). These findings are consistent with the earlier demonstration by Finberg et al. (16) that VirB11 partitions with the particulate fraction following treatment of membranes with TX-100. Treatment of membranes with 1% DOC released approximately one-half of the total amount of membrane-associated VirB11 into the soluble fraction, and treatment with 1% SDS released 100% of this form of protein into the soluble fraction. Interestingly, VirA displayed fractionation patterns very similar to those observed for VirB11 (Fig. 1B).

Sucrose gradient analyses of 2% OG-treated A348 and A136(pJW353) membranes showed that both VirB11 and the integral membrane protein VirA partitioned exclusively in high-density fractions near the bottoms of the gradients (Fig. 2). Neither protein was present at detectable levels in fractions from the upper regions of the gradients where untreated cytoplasmic membranes of *A. tumefaciens* normally partition (14), strongly suggesting that 2% OG effectively solubilized the *A. tumefaciens* cytoplasmic membrane. Therefore, the particulate forms of both VirA and VirB11 recovered from the detergent treatments most likely consist of dense protein-lipid or protein-detergent complexes. It is unlikely that the patterns of distribution of VirB11 in these gradients can be attributed to an in vivo association with outer membrane, which, in contrast to cytoplasmic membrane, may not be completely solubilized with nonionic detergents. We (14) and others (9, 42, 44) have shown that the membrane-associated form of VirB11 partitions almost exclusively with the cytoplasmic membrane during cell fractionation. In addition, we have shown that VirB11 is not degraded by addition of proteases to *A. tumefaciens* spheroplasts, suggesting that VirB11 resides at the cytoplasmic face of the cytoplasmic membrane (11, 14).

Isolation of VirB11 mutants with altered function. To identify residues or domains important for protein function, the *virB11* coding sequence was mutagenized by cycling plasmid pJW353 through the *E. coli* mutator strain XL1-Red. Four of the nine alleles that conferred an attenuated or avirulent phenotype when expressed in *A. tumefaciens* PC1011 (see Materials and Methods) possessed single-point mutations within the *virB11* coding sequence. Three of these mutations resulted in amino acid substitutions at the N terminus of VirB11 (Fig. 3A). PC1011(pGSB3) and PC1011(pGSB4) cells, encoding Q6-L and P13-L mutant proteins, respectively, exhibited severely attenuated virulence, and PC1011(pGSB5) cells encoding an E25-G mutant protein exhibited avirulence (Fig. 3B). The fourth mutation resulted in a substitution of leucine for P170, one of the nonconserved residues within the Walker A nucleotide-binding site. PC1011(pGSB6) cells expressing P170-L reproducibly exhibited severely attenuated virulence (Fig. 3B). These PC1011 strains accumulated each of the four mutant proteins at levels comparable to the wild-type protein synthesized in A348 and in A136(pJW353) cells, as judged by immunoblot analysis. By contrast, PC1011 strains that exhibited attenuated virulence but possessed wild-type *virB11* alleles according to sequence analysis accumulated VirB11 at reduced steady-state levels, possibly as a result of mutations elsewhere on the plasmid that affected copy number, plasmid mainte-

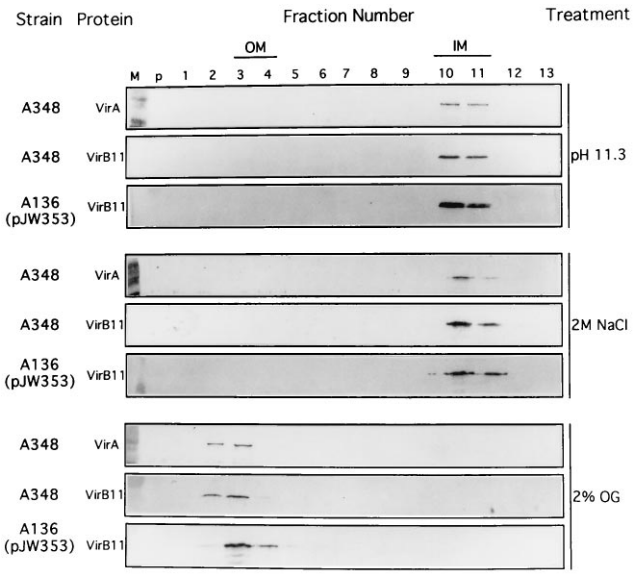

FIG. 2. Distribution of VirB11 in sucrose density gradients after treatments of membranes from A348 and A136(pJW353) cells. Membranes treated with glycine-NaOH buffer (pH 11.3), 2 M NaCl, or 2% OG were centrifuged through isopycnic sucrose gradients. Pelleted material (p) and fractions 1 to 13 (bottom to top of gradients) were analyzed by SDS-PAGE and immunoblotting for the presence of VirA and VirB11. OM and IM correspond to fractions where outer and cytoplasmic membranes from untreated membranes were expected to distribute, based on sucrose densities of these fractions (see text and references 10, 14, 27, and 28). Lane M, molecular mass markers.

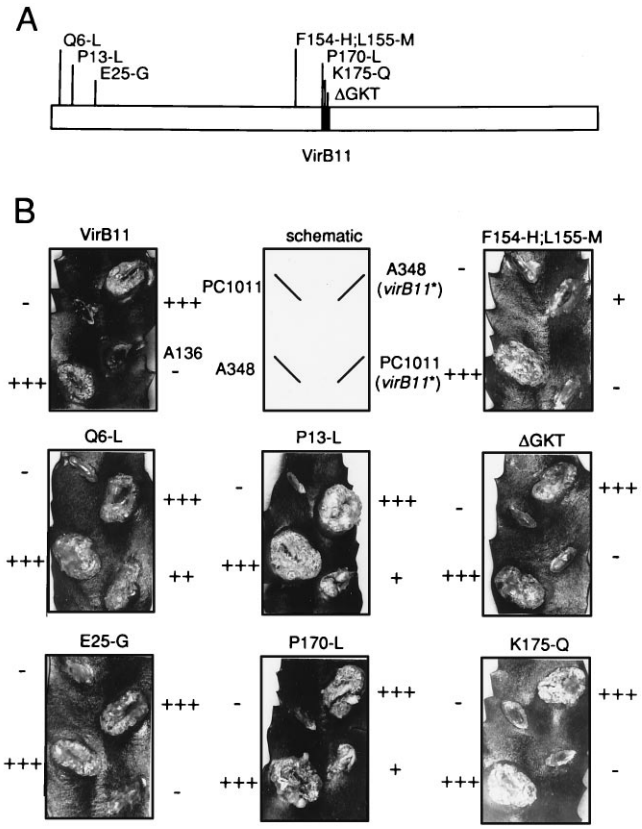

FIG. 3. VirB11 derivatives with point mutations conferring altered function phenotypes. (A) Positions and types of residue substitutions that affected VirB11 function. The black box denotes the position of the Walker A NTP-binding motif. (B) Representative virulence assays of PC1011 and A348 cells expressing wild-type (*virB11*) and mutated (*virB11**) alleles. Strains were inoculated on each leaf according to the schematic shown at the top center. Panel VirB11 (control leaf) shows results of virulence assays with wild-type *virB11* allele and with Ti-plasmidless A136 as a negative control. Relative virulence was scored as avirulent (−), severely attenuated (+), slightly attenuated (++), and wild-type virulence (+++).

nance, or *virB11* expression efficiencies from the P_{lac} promoter (data not shown).

We also changed conserved residues within the Walker A nucleotide-binding motif by site-directed mutagenesis. PC1011(pPCB7112) and PC1011(pPCB7113) cells, synthesizing ΔGKT174-176 and K175-Q, respectively, were avirulent (Fig. 3B), confirming a previous report that an intact nucleoside triphosphate (NTP)-binding domain is essential for VirB11 function (42). A third mutation, generated as a result of the introduction of an *Nde*I restriction site at bp 461 (see Materials and Methods), resulted in F154-H and L155-M substitutions. PC1011(pSRB3) cells, which synthesize the VirB11F154-H; L155-M mutant protein, also exhibited avirulence (Fig. 3B). These and other VirB11 derivatives examined in this study accumulated in their respective hosts at levels comparable to the abundance of native VirB11 in A348, as judged by immunoblot analysis (data not shown). Together, these investigations led to the identification of three regions—near the N terminus, just upstream of the Walker A motif, and the Walker A motif—which are critical for VirB11 function.

Identification of a dominant *virB11* allele. A previous study showed that A348 merodiploids coexpressing wild-type *virB11* and either the *virB11K175A* or *virB11K175R* allele exhibit wild-type virulence (42), indicating that VirB11 derivatives with mutations in the Walker A box do not interfere with any of the processes related to T-DNA transfer to plants. Virulence assays showed that all but one of the merodiploids expressing the mutated *virB11* alleles generated in this study also exhibited wild-type virulence (Fig. 3B). The one exception, A348(pSRB3), which expresses the *virB11F154H;L155M* allele, reproducibly induced the formation of tumors that were significantly smaller than tumors induced by A348 cells (Fig. 3B). Given the presumed role of VirB11 in T-complex transport, a likely explanation for this transdominant effect is that the mutant protein competes with wild-type VirB11 for interactions with the transport machinery. This dominant effect of *virB11F154H;L155M* on virulence represents the first genetic evidence that VirB11 functions as a homo- or heteromultimer.

Mapping of a protein interaction domain within the C-terminal half of VirB11. To localize a protein interaction domain, corresponding studies were carried out with alleles that code for the N- and C-terminal halves of VirB11 and an allele that codes for a C-terminal truncation derivative deleted of the last 20 residues (Fig. 4). We also assessed the effects of an N-terminal fusion of a ~2-kDa His_6 tag on protein function. PC1011 cells expressing *His_6-virB11* exhibited wild-type virulence (Fig. 4), which is consistent with a previous report that VirB11 function in vivo is not perturbed by fusions at the N terminus (42). By contrast, PC1011 cells expressing the deletion alleles were avirulent, demonstrating that the corresponding mutant proteins were nonfunctional (Fig. 4). To test whether any of these alleles displayed transdominance, A348 merodiploids coexpressing the mutated and wild-type *virB11* alleles were assayed for virulence. Merodiploids expressing wild-

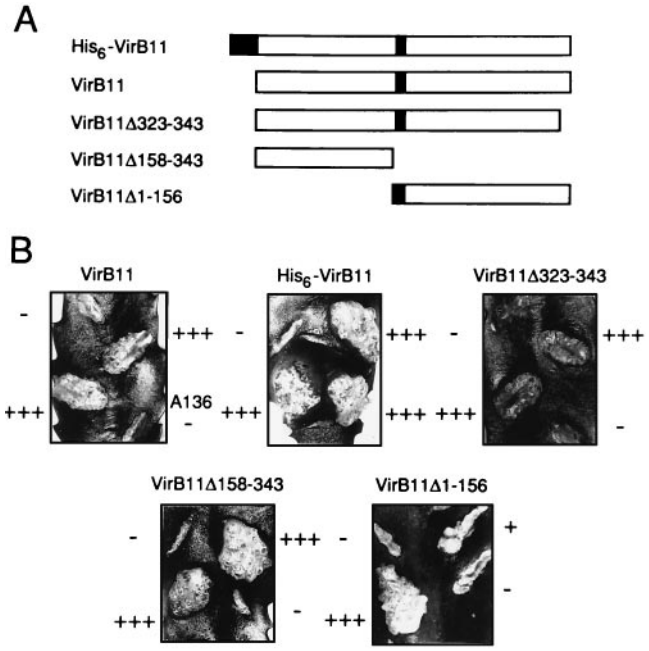

FIG. 4. VirB11 truncation derivatives with altered function phenotypes. (A) Designations at the left identify deleted residues. His_6 was fused to full-length VirB11. The schematic shows the position and extent of each truncation relative to full-length VirB11. The smaller black boxes denote the position of the Walker A NTP-binding motif. (B) Representative virulence assays of PC1011 and A348 cells expressing wild-type and mutated alleles. Strains were inoculated on each leaf according to the schematic shown in Fig. 3. Panel VirB11 (control leaf) shows results of virulence assays with the wild-type *virB11* allele and with Ti-plasmidless A136 as a negative control. Relative virulence was scored as avirulent (−), severely attenuated (+), and wild-type virulence (+++).

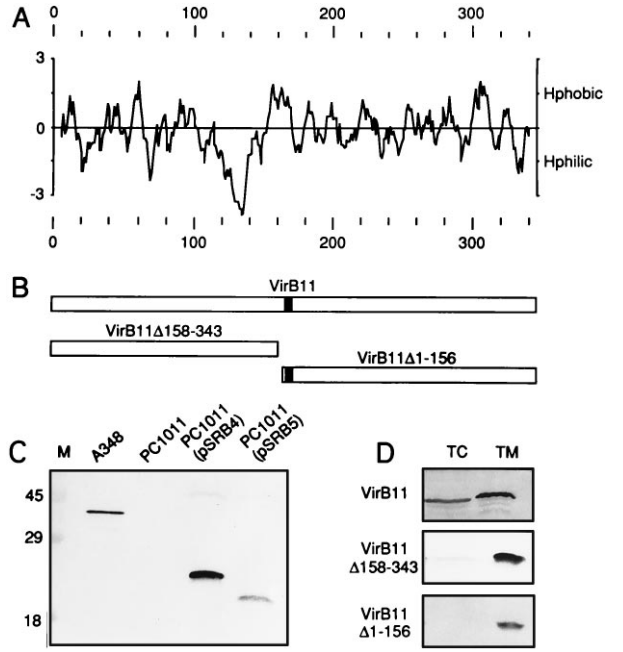

FIG. 5. Subcellular localization of N- and C-terminal truncation derivatives. (A) Hydropathy profile of VirB11 from pTiA6NC (46, 47), using the Kyte-Doolittle algorithm and a window of 19 residues. Hphobic, hydrophobic; Hphilic, hydrophilic. (B) Full-length VirB11 and the two truncation derivatives, VirB11Δ158-343 and VirB11Δ1-156, generated by subcloning as described in the text. (C) Immunodetection of truncation products by SDS-PAGE and development of blots with polyclonal anti-VirB11 antiserum. (D) Distribution of native VirB11, VirB11Δ158-343, and VirB11Δ1-156 synthesized in strains shown in panel C into total cytoplasmic (TC) and total membrane (TM) fractions. Sucrose gradient analyses confirmed that each of these proteins associated almost exclusively with the cytoplasmic membranes (data not shown). Positions of mass markers (M) are indicated in kilodaltons.

type *virB11*, *His$_6$-virB11*, *virB11Δ158-343*, and *virB11Δ323-343* in *trans* exhibited wild-type virulence (Fig. 4). This lack of transdominance cannot be attributed to low protein abundance, since strains expressing these alleles accumulated the corresponding gene products at wild-type levels (Fig. 5 and data not shown). By contrast, the A348(pSRB5) merodiploid, which expresses *virB11Δ1-156*, reproducibly induced the formation of tumors that were significantly smaller than tumors induced by A348 cells (Fig. 5). Interestingly, strains expressing *virB11Δ1-156* appeared to accumulate lower amounts of the truncation product than of the wild-type protein, although it is alternatively possible that the faintness of the stained band in the immunoblots is due to low antigenicity of this region of VirB11 (Fig. 5). The transdominant effect of the *virB11Δ1-156* allele strongly suggests that a protein interaction domain is located in the C-terminal half of VirB11. In addition, the corresponding lack of transdominance of *virB11Δ323-343* could indicate that the extreme C terminus is important for VirB11 homo- or heteromultimerization. Thus far, efforts to further localize a protein interaction domain(s) by expression of fragments from within the 3′ half of *virB11* have been unsuccessful due to instabilities of the encoded products in *A. tumefaciens*.

Altered localization of VirB11K175Q and VirB11ΔGKT. Fractionation studies of A348 cells have shown that although VirB11 copartitions predominantly with the cytoplasmic membrane, a significant amount of protein also is detected in the soluble fraction (9, 14). We have estimated by densitometry scanning that soluble VirB11 comprises approximately 30% of the total amount of VirB11 present in A348 cells (Table 2).

TABLE 2. Distribution of VirB11 during cellular fractionation

Strain	VirB11 derivative	Amount of VirB11[a] in:	
		Cytoplasmic fraction	Membrane fraction
A348	Wild type	29.6	70.4
A136(pJW353)	Wild type	28.6	71.4
PC1011(pPCB7111)	Wild type	28.5	71.4
PC1011(pPCB7112)	ΔGKT	13.6	86.4
PC1011(pPCB7113)	K175Q	12.9	87.1

[a] Expressed as a percentage of combined amounts of VirB11 in cytoplasmic and membrane fractions. The value for each strain represents the mean value from five independent fractionation experiments. Standard deviations from the mean values were less than 3%.

Furthermore, we have detected a comparable ratio of cytoplasmic to membrane-bound forms of VirB11 upon fractionation of cells that express *virB11* in *trans* from an IncP replicon, including A136(pJW353) and PC1011(pPCB7111) (Table 2). IncP replicons have been estimated to reside in *A. tumefaciens* at fivefold higher levels than Ti plasmids (6, 8). Thus, apparent overproduction does not shift the ratio of soluble to membrane-associated forms of VirB11.

We have further determined that the soluble form of VirB11 withstands sedimentation even after prolonged centrifugation of cell extracts at 200,000 × *g* for over 12 h, which is consistent with the notion that this form of VirB11 may exist in vivo. Experimental support for this possibility was gained by analyzing the subcellular localizations of the VirB11 mutant proteins. Among the collection of substitution mutants, most displayed a wild-type pattern of distribution into soluble and membrane-associated forms (Table 2). By contrast, the two derivatives with mutations in the conserved residues of the Walker A box, VirB11K175Q and VirB11ΔGKT174-176, reproducibly partitioned almost exclusively with the total membrane fraction (Table 2). As with wild-type VirB11, these mutant proteins also copartitioned in sucrose gradients exclusively with the cytoplasmic membrane (data not shown). By analogy to other proteins with similar mutations in the Walker A motif (4, 45), these mutant proteins are predicted to be defective in nucleotide binding and hydrolysis. Thus, these findings suggest that an activity associated with nucleotide binding, i.e., binding, hydrolysis, and/or autophosphorylation, could modulate the affinity of VirB11 for the cytoplasmic membrane.

Membrane association of the N- and C-terminal halves of VirB11. The hydropathy plot for VirB11 shows a lack of contiguous stretches of hydrophobic residues long enough to span the cytoplasmic membrane (Fig. 5A). To localize regions that could be responsible for a membrane association, we analyzed the subcellular localizations of the VirB11 truncation derivatives. Interestingly, all three of these mutant proteins, VirB11Δ1-156 and VirB11Δ158-343 (Fig. 5C) and VirB11Δ323-343 (data not shown), copartitioned almost exclusively with the total membrane. Sucrose gradient analyses further showed that both halves of VirB11 copartitioned with cytoplasmic membranes, confirming that the particulate forms of these mutant proteins were indeed membrane associated (data not shown). Thus, both the N- and C-terminal halves of VirB11 possess domains that contribute to an interaction with the cytoplasmic membrane. However, in light of a possible modulating effect of ATP binding and/or hydrolysis on the membrane affinity of native VirB11, further studies are needed to establish the physiological importance these domains.

DISCUSSION

In this study, we have undertaken a structure-function analysis of VirB11, first by investigating the nature of the membrane association of the wild-type protein and second by identifying functional domains through the characterization of partial or nonfunctional derivatives sustaining point mutations or truncations. VirB11 is devoid of stretches of contiguous hydrophobic residues long enough to form a membrane-spanning α helix (Fig. 5). Despite this hydropathy pattern, our fractionation studies showed that only ~30% of VirB11 partitions as soluble protein. Approximately one-half of the remaining membrane-associated protein is released from the membrane with urea, whereas the remaining half is completely resistant to extraction with all reagents tested, including urea. These patterns of distribution are atypical of peripheral or integral membrane proteins and could be suggestive of a topology that is intermediate between that of a peripheral protein and that of an integral protein. We have shown elsewhere that VirB11 is inaccessible to proteases when added to *A. tumefaciens* spheroplasts, suggesting that VirB11 does not span the cytoplasmic membrane (11, 14). Thus, VirB11 may attach to the membrane via lipophilic posttranslational modification or formation of an amphipathic helix for partial insertion into the membrane. It is possible that the nature of the membrane attachment ultimately is responsible for the recovery of apparently different forms of VirB11 during cell fractionation. Alternatively, as discussed in more detail below, the different patterns of distribution could reflect a dynamic association of VirB11 with the cytoplasmic membrane, possibly analogous to that observed for the SecA protein translocase of *E. coli* (12, 35).

Our fractionation and membrane treatment studies confirm and extend the work of Finberg et al. (16) and Stephens et al. (42). The present investigations demonstrated conclusively that VirB11 remains tightly associated with the cytoplasmic membrane upon treatment with various chemical reagents and does not simply cosediment with membranes as protein aggregates (Fig. 1 and 2). Thus far, we have been unable to detect any differences in the membrane association of VirB11 when synthesized in the presence or absence of other VirB proteins. This is somewhat surprising in view of recent experimental evidence that VirB11 is stabilized by synthesis of other membrane-associated VirB proteins. Our laboratory (14, 15, 41) and the laboratory of Das (1) have shown that VirB7, a predominantly outer membrane lipoprotein, forms a disulfide bridge with VirB9. Dimer formation is essential not only for stabilization of both VirB7 and VirB9 but also for stabilization of VirB11 and other VirB proteins (15, 41). Mutants defective in dimer formation, either as a result of a deletion of the *virB7* or *virB9* gene or because of the expression of a transdominant *virB7*::*phoA* allele in A348 merodiploids, accumulate aberrantly low levels of these VirB proteins (5, 15, 41). Conversely, strains carrying constructs that allow for the regulated synthesis of the heterodimer accumulate VirB11 and other VirB proteins at levels roughly proportional to heterodimer abundance (15). Together, our studies suggest that VirB11 has a strong, intrinsic affinity for the cytoplasmic membrane, but it nevertheless adopts a more stabilized configuration at the membrane when assembled as a component of the transport machinery. Conceivably, the VirB proteins have a stabilizing effect on one form of VirB11, such as that which loosely associates with the cytoplasmic membrane.

To gain further insights into the nature of VirB11's membrane association and its interactions with other proteins, we screened for mutations that resulted in partially functional or nonfunctional proteins. Such mutations mapped to three regions: near the N terminus of VirB11 (Q6-L, P13-L, and E25-G), near but not within the predicted nucleotide-binding fold (F154-H;L155-M), and within the nucleotide-binding fold (P170-L, K175-Q, and ΔGKT174-176). While the specific contribution of any of these regions to VirB11 function remains to be elucidated, it is interesting that the residues that abolished VirB11 function when mutated (E25, F154 and L155, K175, and ΔGKT174-176) also are present at corresponding positions in the VirB11 homologs TraG, PtlH, and TrbB (with the exception that TrbB lacks phenylalanine at the position corresponding to F154 of VirB11). Furthermore, the residues that conferred attenuated virulence when mutated to Leu (Q6, P13, and P170) fall within conserved regions but are not themselves conserved in the VirB11 homologs. It may also be significant that P170, which resides in the Walker A motif and conferred attenuated virulence when mutated to leucine, is not present in TrbB, TraG, or PtlH, but a proline is found at this position in almost all members of the so-called PulE family of ATPases, with which VirB11 shares sequence relatedness in the region surrounding the nucleotide-binding site (33). Together, these observations lead to a prediction that the residues identified in this study specify regions of functional importance not only for VirB11 but also for related transporter ATPases.

Biochemical and genetic studies of the VirB11 mutants thus far have resulted in two discoveries. First, subcellular localization studies showed that the two derivatives with mutations in conserved residues of Walker A NTP-binding motif fractionate differently than wild-type VirB11. Both VirB11K175Q and VirB11ΔGKT174-176 partitioned almost exclusively with the cytoplasmic membrane, and only small amounts of the mutant proteins were detectable in soluble fractions. The effects of these mutations on subcellular localization could indicate that ATP binding and/or hydrolysis modulates the association of VirB11 with the cytoplasmic membrane. While this remains to be tested directly, it is notable that studies of SecA have established a precedence for an ATP-dependent dynamic association of a transporter ATPase with the membrane (12, 35). Reminiscent of our findings for VirB11, Rajapandi and Oliver (35) recently showed that defects in the high-affinity ATP-binding domain cause a shift in the distribution of SecA to the membrane-associated form, leading to the hypothesis that this domain regulates SecA's insertion into and deinsertion from the membrane. VirB11 also autophosphorylates in vitro (9), a property that has not been demonstrated for SecA. Thus, it is possible that phosphorylation, not ATP binding or hydrolysis per se, modulates VirB11's interaction with the membrane or with other components of the T-complex transport apparatus. A precedence for such an activity is found with the PutA protein of *Salmonella typhimurium*, which exhibits a stronger affinity for the membrane in its phosphorylated state (29).

Second, we found that the *virB11F154-H;L155-M* allele exerts a transdominant effect over wild-type *virB11*. As noted above, the F154-H and L155-M mutations reside upstream of the Walker A motif and are not predicted to be part of the nucleotide-binding fold as deduced from modeling studies of other transporter ATPases (13). In view of the evidence that VirB11 assembles as a component of the transport apparatus, the simplest explanation for the transdominant effect of *virB11F154H;L155M* is that this allele encodes a mutant protein that competes with wild-type VirB11 for interactions with another component(s) of the transporter. However, consideration of the genetic data alone allows for the alternative possibility that the mutant proteins interfere with assembly of VirB11 homomultimers. In this regard, it is notable that a close homolog of VirB11, TrbB of the IncP RP4 plasmid, has been

reported to assemble as a homohexamer (31) and that several ATPase subunits associated with the ABC transporter superfamily have been shown to function as homodimers (13). Of course, VirB11 may assemble as both homo- and heteromultimers, the implication being that there are at least two distinct protein interaction domains, one involved in homomultimerization and a second involved in oligomerization with at least one other component of the T-complex transporter. By analogy, there is evidence to suggest that PulE, a putative ATPase required for pullulanase secretion from *K. oxytoca*, possesses distinct domains for homo- and heteroligomerization (33). Finally, while we favor a model that the observed transdominant effect is attributable to competitive or titrating interactions of the mutant protein, our data do not exclude the alternative possibility that the mutant protein catalytically counteracts a function supplied by wild-type VirB11, e.g., kinase or phosphatase activity.

Merodiploid studies of the *virB11* deletion alleles showed that *virB11Δ1-156* exerted a strong transdominant effect, constituting strong genetic evidence that a protein interaction domain maps within the C-terminal half of VirB11. The C-terminal half of VirB11 possesses the Walker A motif, and we have confirmed the original observation of Stephens et al. (42) that alleles with mutations that cause alterations in this motif are recessive in merodiploid assays. On the basis of this merodiploid phenotype, Stephens et al. (42) proposed that activities associated with ATP binding, e.g., ATP binding, ATP hydrolysis, and autophosphorylation, are required for a productive interaction between VirB11 and the transport apparatus. The transdominant phenotype of *virB11Δ1-156* and the lack of transdominance of the *virB11Δ323-343* allele prompt us to further suggest that VirB11 possesses at least two domains, the ATP-binding pocket and a distinct protein interaction domain located near the C terminus, whose combined activities are required for assembly of VirB11 as a component of the transporter. A reasonable hypothesis for further investigation is that ATP-induced conformational changes in this C-terminal domain modulate the oligomeric state of VirB11.

Several features identified in this and previous investigations distinguish VirB11 from other ATPases associated with macromolecular transport processes. First, fractionation studies have shown that other traffic ATPases, including HisP (2, 23), MalK (39), PulE (33), and EpsE (38), exhibit stronger membrane affinities and probably have different membrane topologies when synthesized in the presence of cognate transporter components. This observation implies that VirB11 associates with the membrane in a way which is quite distinct from that of other traffic ATPases. Second, protein interaction determinants have been mapped to the N termini of the traffic ATPases PulE (33) and EpsE (38). The N-terminal domain of EpsE mediates an interaction with the integral membrane protein EpsL, a second putative component of the toxin secretory apparatus (38). In contrast, our studies raise the possibility that a C-terminal domain is responsible for interactions between VirB11 and other T-complex transporter subunits. Finally, in contrast to the situation for VirB11, mutations that cause alterations in the Walker A motifs of PulE, EpsE, XcpR, and PilB exert transdominant effects (33, 38, 45). These genetic data support the notion that the physiological roles of ATP binding, hydrolysis, and/or phosphorylation could be quite distinct for VirB11 and these other ATPases. It is notable that among the ABC and PulE families of transporter ATPases, only VirB11 (9) and EpsE (38) have been shown to autophosphorylate, which highlights the importance of further studies aimed at defining the physiological role(s) of this biochemical activity.

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