THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 28, Issue of July 9, pp. 29528–29533, 2004
Printed in U.S.A.

Protein Interactions Involved in Nuclear Import of the *Agrobacterium* VirE2 Protein *in Vivo* and *in Vitro**

Received for publication, March 22, 2004, and in revised form, April 12, 2004
Published, JBC Papers in Press, April 28, 2004, DOI 10.1074/jbc.M403159200

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***Agrobacterium*, the only known organism capable of trans-kingdom DNA transfer, genetically transforms plants by transferring a segment of its DNA, T-DNA, into the nucleus of the host cell where it integrates into the plant genome. One of the central events in this genetic transformation process is nuclear import of the T-DNA molecule, which to a large degree is mediated by the bacterial virulence protein VirE2. VirE2 is distinguished by its nuclear targeting, which occurs only in plant but not in animal cells and is facilitated by the cellular VIP1 protein. The molecular mechanism of the VIP1 function is still unclear. Here, we used *in vitro* assays for nuclear import and quantification of protein-protein interactions to directly demonstrate formation of ternary complexes between VirE2, VIP1, and a component of the cellular nuclear import machinery, karyopherin α. Our results indicate that VIP1 functions as a molecular bridge between VirE2 and karyopherin α, allowing VirE2 to utilize the host cell nuclear import machinery even without being directly recognized by its components.**

Agrobacterium, the only known organism capable of trans-kingdom DNA transfer (1), elicits neoplastic growths on many plant species. Moreover, although plants represent the natural hosts for *Agrobacterium*, this microorganism can also transform a wide range of other eukaryotic species, ranging from fungi (2, 3) to human cells (4). This genetic transformation is achieved by transporting a single-stranded copy (T-strand)[1] of the bacterially transferred DNA (T-DNA) from the tumor-inducing plasmid into the plant cell nucleus and then by integration into the host genome (5, 6). These processes are likely mediated by two *Agrobacterium* proteins VirD2 and VirE2, which are believed to directly associate with the T-strand, forming a transport (T-) complex (7). In the T-complex, one molecule of VirD2 is covalently attached to the 5′ end of the T-strand, whereas VirE2, a single-stranded (ss)DNA binding protein, is presumed to cooperatively coat the remainder of the ssDNA molecule (5, 7, 8). Both VirD2 and VirE2 proteins are targeted to the host cell nucleus (9–15), but VirE2 alone is sufficient to transport ssDNA into the nucleus of the plant cell (16).

Although VirE2 accumulates in the cell nucleus even in very diverse plant species (9), it fails to enter the nucleus of yeast or animal cells (15, 17–19). VirE2 nuclear import in non-plant systems is promoted by expression of an *Arabidopsis* protein, VIP1, that interacts with VirE2 (19). Because VIP1, a basic leucine zipper motif protein, shows no significant homology to known animal or yeast proteins, it was suggested to be a cellular factor responsible, at least in part, for plant-specific VirE2 nuclear import (19). The role of VIP1 in the nuclear import of transfer complexes is also consistent with observations that VIP1, which by itself is unable to associate with ssDNA, is able to interact with VirE2, whereas the latter is bound to the ssDNA, forming ternary VIP1-VirE2-ssDNA complexes *in vitro* (19). Exactly how VIP1 facilitates nuclear import of VirE2, however, remains unknown.

Here, we used purified VirE2 in an *in vitro* nuclear import assay to study the dependence of its nuclear import on the presence of purified VIP1. *In vitro* nuclear import in plant-permeabilized cells occurs without the addition of cytosolic factor, making these cells unsuitable for testing the roles of individual import factors (20, 21). Therefore, we used permeabilized HeLa cells for *in vitro* studies of nuclear import. To shed light on the molecular mechanism by which VIP1 assists nuclear uptake of VirE2, a quantitative *in vitro* binding assay was used to examine interactions between VirE2, VIP1, and karyopherin α. Our results demonstrate the critical role of VIP1 for nuclear accumulation of VirE2 and suggest that VIP1 functions as a molecular bridge between VirE2 and karyopherin α.

MATERIALS AND METHODS

Protein Expression—All proteins were expressed in the *Escherichia coli* strain BL21(DE3) using the T7 RNA polymerase system (22) and purified to near homogeneity (95–98% pure as determined by silver-stained SDS-polyacrylamide gels). VirE2 (23) was produced from the pET3b vector (22) and purified as described previously for purification of the gene I protein of cauliflower mosaic virus (24). Histidine-tagged VIP1 (19) and human karyopherin α (hKAPα) (25) were produced from the pET28a(+) vector (Novagen) and purified on nickel beads (Qiagen)

* This work was supported by a grant from the U. S.-Israel Binational Science Foundation (BSF) (to A. L. and V. C.). This work is also supported by grants from National Institutes of Health, National Science Foundation, U. S. Department of Agriculture, and U. S.-Israel Binational Research and Development Fund (BARD) (to V. C), from BARD and Human Frontiers Science Program (to T. T.), and from BARD (to A. L. and R. L. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: T-, transfer or transport; ssDNA, single-stranded DNA; hKAPα, human karyopherin α; NLS, nuclear localization signal; VPR, human immunodeficiency virus protein R; BSA, bovine serum albumin; R-BSA, lissamine rhodamine-labeled BSA; bBSA, biotinylated BSA; sulfo-SMCC, sulfosuccinimidyl 4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate; PBS, phosphate-buffered saline; R-VirE2, rhodamine-labeled VirE2; F-VirE2, fluorescein-labeled VirE2.

according to the manufacturer's instructions and standard protocols (26).

Labeling of Peptides and Proteins—Linear peptides corresponding to the nuclear localization signal (NLS) sequences of the SV40 large T antigen (PKKKRKV) (27), human immunodeficiency virus protein R (VPR) (NEWTLELLEELKNEAVRHF) (28), and VIP1 (KRILANRQSAARSKERKIR) (19) were synthesized as described before (29). These peptides and recombinant full-length VirE2 and hKAPα proteins were covalently attached to unlabeled bovine serum albumin (BSA) (Sigma), lissamine rhodamine-labeled BSA (R-BSA), or biotinylated BSA (bBSA) (Sigma) tags using sulfo-SMCC as a cross-linker as described previously (29). In addition, VirE2 and BSA were chemically labeled with lissamine rhodamine or fluorescein isothiocyanate (Molecular Probes, Inc.) as described (21). Labeled peptides and proteins were purified from uncoupled labels or sulfo-SMCC by gel filtration on a Sephadex G-25 column as described (21), and then aliquoted and stored at −70 °C until use.

Microinjection into Plant Tissues—Purified VirE2 labeled with lissamine rhodamine or fluorescein isothiocyanate was used in microinjection studies (30) that were performed on detached mature leaves of *Nicotiana benthamiana*. Movement of fluorescent probes out of target mesophyll cells was monitored and recorded by confocal laser scanning microscopy, using a Leica confocal laser scanning microscope (model TCS-4D, Heidelberg, Germany) equipped with a Narishige four-dimensional hydraulic micromanipulator system (31).

Nuclear Import in Permeabilized HeLa Cells—HeLa cells were grown for 2 days on 10-mm coverslips to a subconfluent density and permeabilized with digitonin as described (29). The permeabilized cells, supplemented with rabbit reticulocyte cytosolic extracts, were incubated with fluorescently labeled import substrates as described (29), and nuclear import was observed by confocal laser scanning microscopy using a Zeiss LSM 5 Pascal confocal laser scanning microscope system. Each experiment was repeated at least three times.

Enzyme-linked Immunosorbent Assay for Protein-Protein Interactions—Binding of bBSA-VIP1 NLS, bBSA-hKAPα, or bBSA-VirE2 conjugates to hKAPα or VIP1 was assayed and quantified essentially as described previously (26, 32). Briefly, MaxiSorb plates (Nunc) were incubated overnight at 4 °C with a solution containing either 5 μg of hKAPα or VIP1 in 200 μl of 0.05 M sodium carbonate buffer, pH 9.6. The protein solutions were removed, the plates were washed with PBS, and 200 μl of a blocking solution (5% w/v BSA in PBS) was added. Following incubation at 37 °C for 2 h and a wash with PBS, the appropriate bBSA conjugates in PBS were added, and blocking solution was added to a total volume of 200 μl. After incubation at 37 °C for 3 h, the plates were washed with PBS, and 200 μl of avidin-peroxidase (Roche Diagnostics) dissolved in the blocking solution was added. Following an additional incubation for 1 h at 37 °C, the plates were washed with PBS, and horseradish peroxidase substrate was added according to the manufacturer's instructions. The horseradish peroxidase enzymatic activity was quantified by monitoring the absorbance of its reaction product at 490 nm (A_{490}). In competition experiments, competitors were added to the coated plates and incubated at 37 °C for 1 h before addition of bBSA conjugates.

Nuclear Import in Mammalian Cells—For transient expression in mammalian cells, PCR-amplified *virE2* gene was cloned into the XhoI-BamHI sites of pcDNA3.1(−)Myc-HisA (Invitrogen), resulting in pcDNA-E2-Myc. The VIP1-expressing pCB6-VIP1 construct was previously described (19). pcDNA-E2-Myc, pCB6-VIP1, or a mixture of both constructs (1:1 molar ratio) were transformed into COS1 cells as described (19), and subcellular localization of each of the proteins was determined by indirect immunofluorescence 24 h after transformation using a Zeiss LSM 5 Pascal laser scanning confocal microscope. VirE2 was detected using mouse anti-Myc antibodies (Invitrogen) and visualized with Cy5-labeled anti-mouse secondary antibodies, and VIP1 was detected using anti-VIP1 antibodies raised in the rabbit and visualized with Alexa Fluor-labeled anti-rabbit secondary antibodies.

RESULTS

Nuclear Import of Purified VirE2 in Plant Tissues—To assess the biological functionality of the purified intact VirE2, we examined its nuclear import in plant tissues. To this end, two preparations of fluorescently tagged VirE2, rhodamine-labeled (R-VirE2) and fluorescein-labeled (F-VirE2), were produced and microinjected directly into the cytoplasm of the mesophyll cells of *N. benthamiana* leaves. R-VirE2 efficiently accumulated in the plant cell nucleus (Fig. 1*A*, *arrow*), resulting in strong nuclear staining and only very faint, residual cytoplasmic signal. A virtually identical nuclear localization pattern was observed with microinjected F-VirE2 (Fig. 1*B*, *arrow*), indicating that the chemical composition of the fluorescent tag had no effect on the subcellular targeting of VirE2. In control experiments, R-BSA (Fig. 1*C*) and fluorescent-labeled BSA (data not shown) remained cytoplasmic following microinjection into leaf mesophyll cells. These results demonstrate that the recombinant purified VirE2 is biologically active and is imported into the cell nucleus in living plant cells.

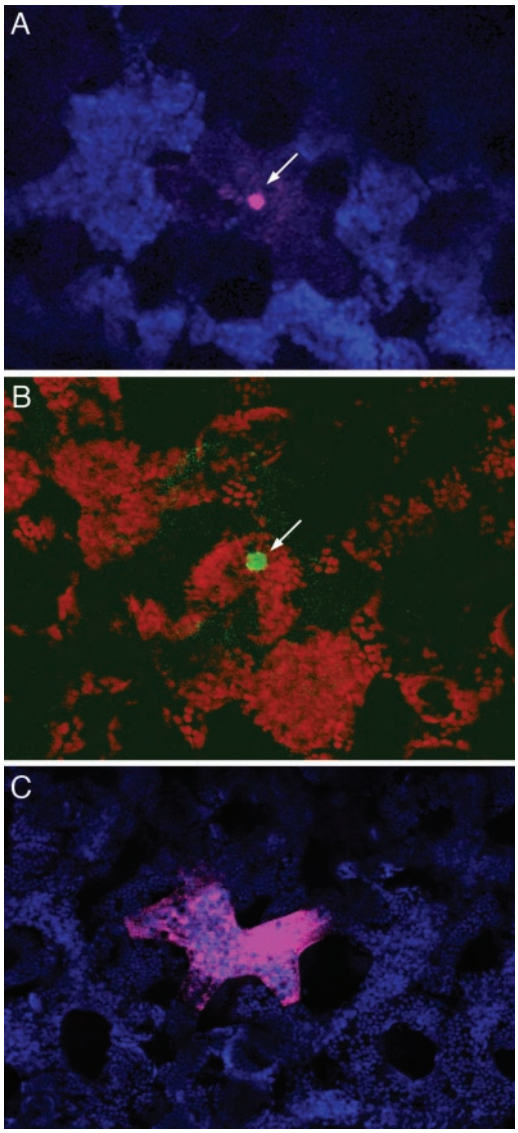

FIG. 1. **Nuclear import of VirE2 in living plant cells.** *A*, R-VirE2; *B*, F-VirE2. *A*, R-BSA is shown. Rhodamine signal is indicated in *pink*, fluorescein signal is indicated in *green*, and plastid autofluorescence is in *red* (*A* and *C*) or in *blue* (*B*). All images are projections of several confocal sections. *Arrows* indicate cell nuclei that accumulated fluorescently labeled VirE2.

Nuclear Import of VirE2 in Permeabilized HeLa Cells—Next, we tested conditions for active nuclear import of VirE2 in an *in vitro* system, which allows better control over its molecular components than in living cells. In this experimental system composed of permeabilized HeLa cells and exogenously added reticulocyte cytosol, R-VirE2 showed no significant nuclear import, with very little if any intranuclear fluorescent signal (Fig. 2*A*), consistent with the previously reported inability of non-plant systems to support nuclear transport of VirE2 (15, 17–19). Recently, an *Arabidopsis* protein, VIP1, has shown to be

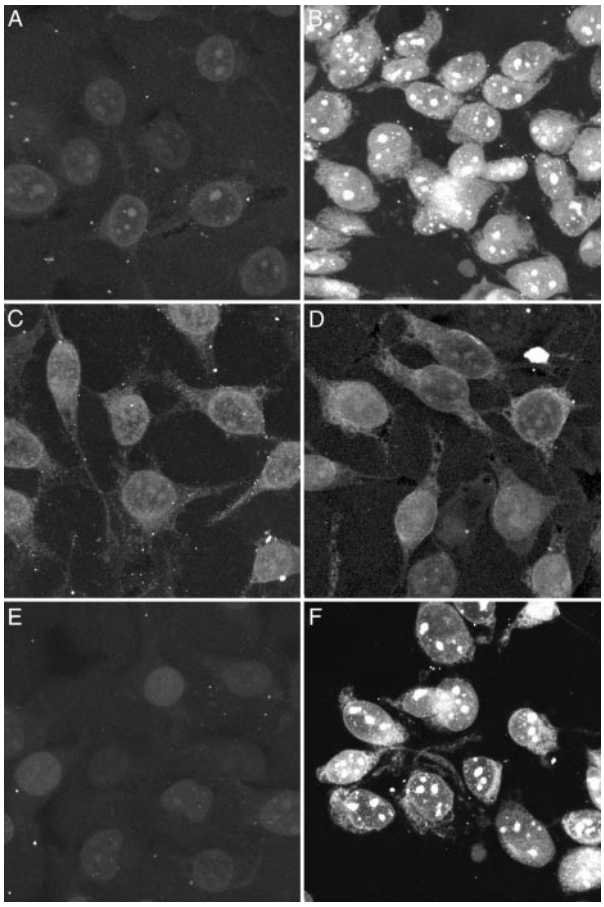

FIG. 2. **Nuclear import of VirE2 in permeabilized HeLa cells.** *A*, R-VirE2; *B*, R-VirE2 and VIP1. *C*, R-VirE2 and VIP1 in the presence of 0.5 mg/ml wheat germ agglutinin. *D*, R-VirE2 and VIP1 in the presence of 1 mM GTPγS. *E*, R-VirE2 and VIP1 in the presence of 2000 molar excess of BSA-SV40 NLS. *F*, R-VirE2 and VIP1 in the presence of 2000 molar excess of BSA. All images are single confocal sections.

required for *in vivo* nuclear accumulation of VirE2 fused to various reporters, such as β-glucuronidase and green fluorescent protein, in plant and mammalian cells (19). Here, we tested the ability of VIP1 to facilitate nuclear transport of unfused VirE2 *in vitro*. Fig. 2*B* shows that the addition of purified recombinant VIP1 to R-VirE2 promoted efficient uptake of the fluorescent signal into nuclei of permeabilized HeLa cells, consistent with nuclear import. This VIP1-dependent nuclear accumulation of R-VirE2 did not occur in the absence of the cytosolic factors (data not shown) and was greatly inhibited in the presence of wheat germ agglutinin (Fig. 2*C*), GTPγS (Fig. 2*D*) or at low temperature (4 °C) (data not shown). The wheat germ agglutinin lectin blocks nuclear import by interacting with the protein components of the nuclear pore. GTPγS, a non-hydrolyzable analog of GTP, blocks the activity of Ran, an essential regulator of nuclear import, and inhibition by low temperature is indicative of involvement of metabolic energy, which also is known to be required for nuclear import (33–35). That the VIP1-induced accumulation of R-VirE2 within the nuclei of permeabilized HeLa cells was blocked by these specific inhibitors indicates that it occurred by a true process of active nuclear import.

Next, we examined whether karyopherin α, the nuclear import receptor that directly recognizes the transported proteins (36–38), plays a role in the VIP1-induced nuclear uptake of VirE2. The amounts of karyopherin α in the *in vitro* import system can be effectively depleted by the addition of competing concentrations of SV40 NLS peptide, which is known to specifically bind karyopherin α (37, 38), preventing it from promoting nuclear import of the labeled substrate (39, 40). Indeed, addition of the SV40 NLS peptide conjugated to a BSA carrier virtually blocked accumulation of R-VirE2 in the nuclei of permeabilized HeLa cells even in the presence of VIP1 (Fig. 2*E*), whereas the addition of free BSA as expected had no effect on nuclear import of R-VirE2 (Fig. 2*F*). These results demonstrate involvement of karyopherin α in the VirE2 nuclear import. The molecular mechanism by which this involvement may occur is explored in the subsequent sections of this article.

Protein Interactions Involved in Nuclear Import of VIP1—To better understand the molecular pathway by which VIP1 assists nuclear import of VirE2, we examined interactions between VIP1, VirE2, and hKAPα. To this end, we used an enzyme-linked immunosorbent assay in which one of the interacting proteins, usually VIP1, was immobilized on a plastic surface, incubated with a ligand such as hKAPα or VirE2, and conjugated to bBSA in the presence of a large excess of free BSA to quench nonspecific interactions. The degree of binding was then quantified by detection with peroxidase-conjugated avidin (see "Materials and Methods" and Ref. 26). The use of bBSA as a soluble tag was developed to enhance the solubility of VirE2 as well as to increase the sensitivity of the binding assay, thus allowing the use of relatively low, nanomolar-level concentrations of ligand proteins to avoid nonspecific interactions.

Fig. 3*A* shows that immobilized VIP1 efficiently interacted with bBSA-tagged hKAPα (bBSA-hKAPα). This dose-dependent binding occurred in a saturable fashion, characteristic of specific interactions. The specificity of VIP1-hKAPα binding was confirmed by competition experiments in which increasing concentrations of an unlabeled specific competitor, VIP1, efficiently inhibited binding (Fig. 3*B*).

We then tested whether hKAPα recognizes the bipartite NLS sequence of VIP1, located between amino acid residues 118 and 136 (19). The results shown in Fig. 3*C* demonstrate dose-dependent and saturable interaction of hKAPα with a peptide corresponding to the VIP1 NLS and conjugated to bBSA (bBSA-VIP1 NLS). A significant, 60–70%, inhibition of this interaction was achieved in the presence of a specific competitor, BSA-conjugated SV40 NLS peptide, which is well known to interact with karyopherin α (37, 38). Importantly, a BSA conjugate of another type of NLS, VPR, which is not recognized by karyopherin α (41, 42), did not compete with the VIP1 NLS (Fig. 3*C*), indicating specificity of the VIP1 NLS-hKAPα interaction.

The biological activity of the VIP1 NLS was directly demonstrated using permeabilized HeLa cells. In this system, a synthetic peptide corresponding to the VIP1 NLS sequence efficiently promoted nuclear import of rhodamine-labeled BSA conjugated to this peptide (R-BSA-VIP1 NLS) (Fig. 4*A*). Nuclear accumulation of R-BSA-VIP1 NLS was almost completely inhibited by an unlabeled specific competitor, BSA-VIP1 NLS (Fig. 4*B*), GTPγS, and low temperature (4 °C) (data not shown). Taken together, these results indicate that the VIP1 NLS is biologically functional, mediating active protein nuclear import via the karyopherin α-dependent pathway.

Protein Interactions Involved in Nuclear Import of VirE2 and Formation of Ternary VirE2-VIP1-hKAPα Complexes—To better understand the role of VIP1 in nuclear import of VirE2, we examined interactions between VIP1, hKAPα, and VirE2 and tested whether VIP1 can simultaneously bind both hKAPα, and VirE2. Fig. 5*A* shows that VIP1 bound bBSA-labeled VirE2 in a dose-dependent and saturable fashion and that the VIP1-VirE2 binding was inhibited by a specific competitor, BSA-VirE2, but not by a nonspecific competitor, BSA-VPR NLS,

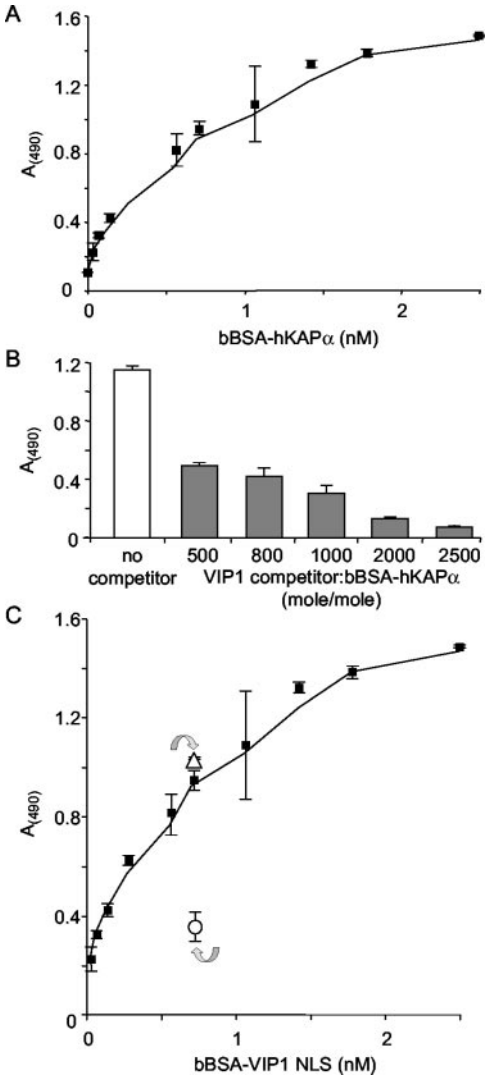

FIG. 3. **Binding of VIP1 and VIP1 NLS to hKAPα.** *A*, immobilized VIP1 coincubated with the indicated amounts of bBSA-hKAPα. *B*, immobilized VIP1 coincubated with 2 nM bBSA-hKAPα and free VIP1 competitor at the indicated molar excess ratios. *C*, immobilized hKAPα coincubated with bBSA-VIP1 NLS. *Open circle* and *triangle* (*arrows*) indicate binding in the presence of 2000 molar excess of unlabeled BSA-SV40 NLS or BSA-VPR NLS competitors, respectively. Standard deviations based on at least three independent experiments are indicated for each experimental condition.

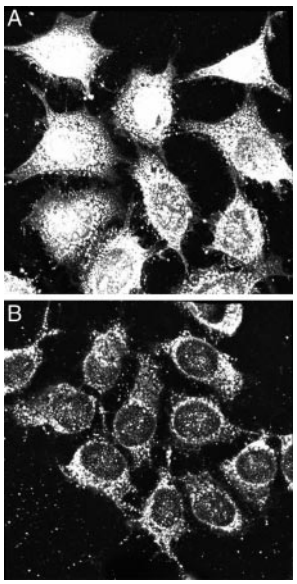

FIG. 4. **Nuclear import of BSA-conjugated VIP1 NLS in permeabilized HeLa cells.** *A*, R-BSA-VIP1 NLS; *B*, R-BSA-VIP1 NLS in the presence of unlabeled BSA-VIP1 NLS competitor (1:1000 mol/mol). All images are single confocal sections.

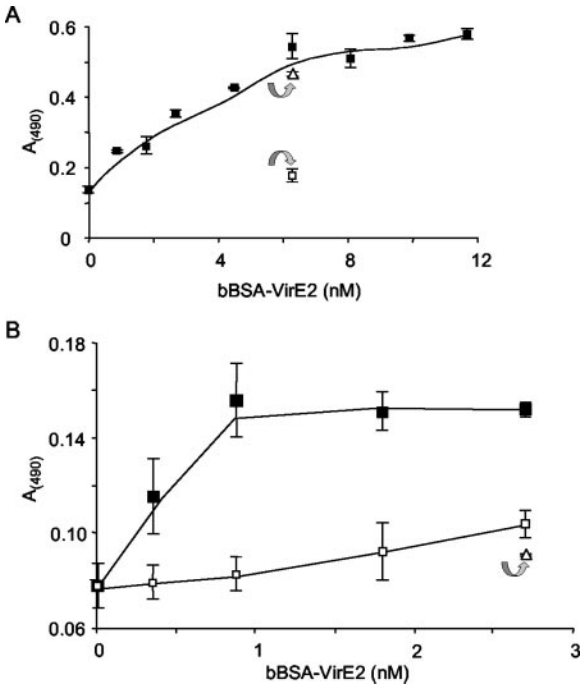

FIG. 5. **Binding of VIP1 to VirE2 and hKAPα.** *A*, immobilized VIP1 coincubated with the indicated amounts of bBSA-hKAPα. *Open square* and *triangle* (*arrows*) indicate binding in the presence of 2000 molar excess of unlabeled BSA-VirE2 or BSA-VPR NLS competitors, respectively. *B*, immobilized hKAPα coincubated with bBSA-VirE2 in the absence (*open squares*) or in the presence of 10 nM free, unlabeled VIP1 (*filled squares*). *Open triangle* (*arrow*) indicates binding in the presence of VIP1 and 2000-molar excess of unlabeled BSA-SV40 NLS competitor. Standard deviations based on at least three independent experiments are indicated for each experimental condition.

indicating a specific interaction. Unlike VIP1, VirE2 did not exhibit statistically significant interaction with hKAPα (Fig. 5*B*), and this result was consistent with previous observations that VirE2 did not interact with a plant karyopherin α, AtKAPα, in a yeast two-hybrid system (43). The addition of unlabeled VIP1, however, promoted dose-dependent and saturable association of VirE2 with hKAPα (Fig. 5*B*). This association could occur only if VIP1 bound both VirE2 and hKAPα together, in a single complex. This idea was further supported by the observation that BSA-SV40 NLS, which prevented VIP1-dependent nuclear import of VirE2 in permeabilized cells (Fig. 2*E*) and inhibited VIP1 NLS binding to hKAPα (Fig. 3*C*), also abolished the VIP1-dependent association of VirE2 and hKAPα (Fig. 5*B*).

Nuclear Import of VirE2 in Living COS1 Cells—The role of VIP1 in the VirE2 nuclear import demonstrated *in vitro* (Fig. 2) was confirmed *in vivo* in living mammalian cells. Previously, this VIP1 function has been shown only with green fluorescent protein-VirE2 fusions (19), whereas here we used VirE2 tagged with a Myc epitope as the import substrate. VirE2 and VIP1 were expressed in COS1 cells and detected using mouse anti-Myc antibodies with Cy5-labeled anti-mouse secondary antibodies and rabbit anti-VIP1 antibodies with Alexa Fluor-labeled anti-rabbit secondary antibodies.

Fig. 6 shows that VirE2 expressed alone remained completely cytoplasmic (Fig. 6*A*, dispersed *blue* fluorescent signal

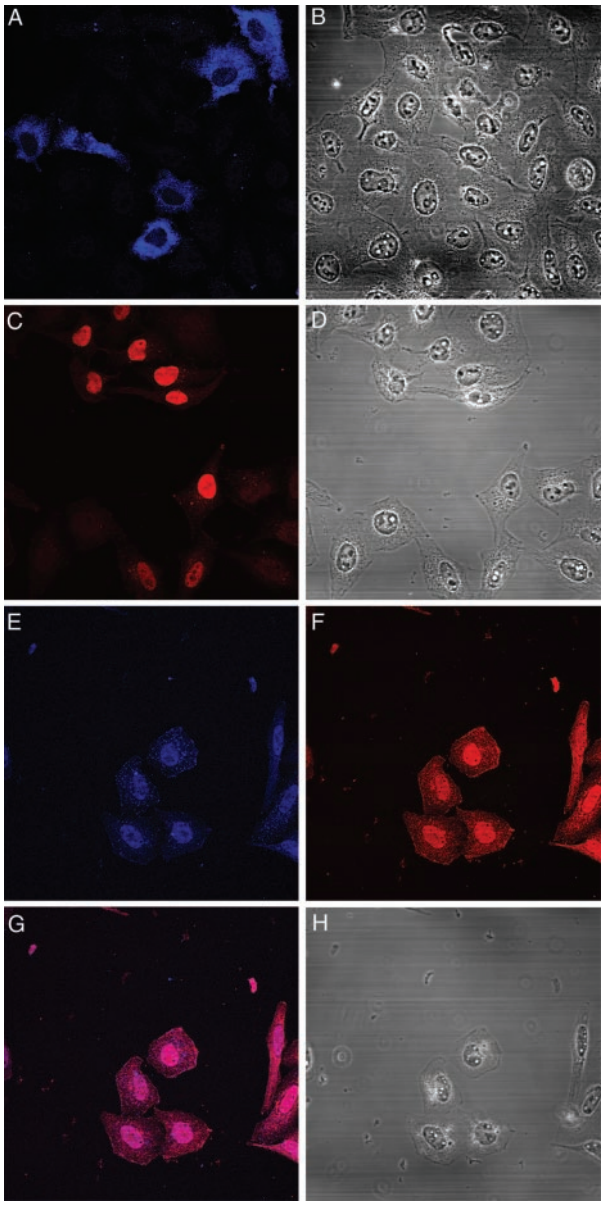

FIG. 6. **Nuclear import of intact VirE2 in living COS1 cells.** VirE2 and VIP1 were detected using secondary antibodies labeled with Cy5 or Alexa Fluor, respectively. *A* and *B*, VirE2; *C* and *D*, VIP1; *E–H*, VirE2 and VIP1. *A*, *C*, and *E–G*, fluorescence images; *B*, *D*, and *H*, phase-contrast images of the corresponding cells. *G*, merged Cy5-Alexa Fluor image. Cy5 signal is indicated in *blue*, Alexa Fluor signal is indicated in *red*, and merged Cy5-Alexa Fluor signals are in *pink*. All fluorescence images are single confocal sections.

surrounding fluorescence-free *black* nuclei), whereas VIP1 efficiently localized to the cell nucleus (Fig. 6*C*, *red* fluorescent signal almost exclusively concentrated within cell nuclei). Coexpression of VirE2 with VIP1 resulted in a substantial accumulation of VirE2 in the cell nucleus; simultaneous detection of VirE2 (Fig. 6*E*) and VIP1 (Fig. 6*F*) localized a significant proportion of these proteins within the cell nucleus. Fig. 6 also demonstrates that, in these coexpressing cells, merged images of VirE2 (Fig. 6*E*, *blue* signal) and VIP1 (Fig. 6*F*, *red* signal) indicated colocalization of these proteins within the cell nucleus (Fig. 6*G*, *pink* signal). It is important to note that in Fig. 6 the confocal optical sections with the plane of focus through the cell nucleus detect intranuclear accumulation of the fluorescent label rather than its perinuclear binding. Thus, VIP1 facilitates nuclear import of intact VirE2 in an animal system *in vivo*.

DISCUSSION

Permeabilized animal cells have been instrumental in dissection of nuclear import pathways, allowing for identification of essential soluble components of the import machinery (44, 45). This approach, however, is not feasible in plants because nuclear import in permeabilized plant protoplasts is independent of the addition of soluble cellular factors (20, 21). Instead, permeabilized animal cells also have been used to study plant nuclear import, and such studies have demonstrated that plant cytosolic factors support nuclear uptake in permeabilized HeLa cells (21) and that *Agrobacterium* karyophilic proteins are imported into animal cell nuclei (46). Here, we used this *in vitro* nuclear import system to study the molecular requirements for nuclear import of VirE2 of *Agrobacterium*, a protein known to play a central role in the *Agrobacterium*-mediated genetic transformation of plants (5, 47). We also used a quantitative, enzyme-linked immunosorbent-based *in vitro* binding assay to elucidate protein-protein interactions involved in the VirE2 nuclear import.

Our results indicate that animal cytosolic factors were insufficient for *in vitro* nuclear import of VirE2. On the other hand, our microinjection studies in plant tissues demonstrated that VirE2 is a karyophilic protein in plant cells. An explanation for this difference is that VirE2 is not directly recognized by the animal cell nuclear import machinery. Thus, plant cells may contain a protein factor that mediates the targeting of VirE2 into the cell nucleus, whereas this factor is absent in animal cells. Indeed, recent studies indicated that the *Arabidopsis* protein, VIP1, that binds VirE2 is important for VirE2 nuclear import and *Agrobacterium* tumorigenicity *in vivo* (19, 43). The data presented here establish that VIP1 is responsible for the nuclear uptake of VirE2 because its addition to the *in vitro* import reaction mediated efficient accumulation of VirE2 in the nuclei of permeabilized HeLa cells. Nuclear accumulation of VirE2 in living mammalian cells also depended on the presence of VIP1, demonstrating similar requirements for VIP1 during nuclear import of VirE2 in animal cells both *in vitro* and *in vivo*. Presently, we cannot explain why, in previous studies by others, VirE2 was reported to enter animal cell nuclei *in vitro* in the absence of exogenously added VIP1 (46, 48).

Specific protein-protein interactions underlying the VIP1 role in the VirE2 nuclear import were examined and dissected using a quantitative *in vitro* binding assay (26). VIP1 specifically interacted with human karyopherin α, and this binding was most likely mediated by the VIP1 NLS signal, which was itself capable of specific binding to karyopherin α. These interactions indicate that VIP1 is imported into the cell nucleus by a karyopherin α-mediated pathway via its NLS; indeed, the VIP1 NLS sequence also promoted *in vitro* nuclear import of an unrelated cargo molecule. Unlike VIP1, VirE2 was not recognized by human karyopherin α, explaining the inability of VirE2 to enter the nuclei of animal cells *in vitro* and *in vivo*. Furthermore, VirE2 exhibited a specific interaction with VIP1, consistent with the notion that VIP1 may serve as a physical link between VirE2 and karyopherin α. Although this idea has been proposed previously (19, 43, 47, 49), it has never been proven directly. Our *in vitro* binding experiments provided this evidence by demonstrating specific and saturable association of VirE2 with karyopherin α in the presence of VIP1, indicating formation of ternary VirE2-VIP1-hKAPα complexes in which VIP1 serves as a bridge between VirE2 and hKAPα. In these complexes, VIP1 likely functions as a molecular adapter between VirE2 and karyopherin α, allowing VirE2 to utilize the host cell nuclear import machinery even without being directly recognized by its components. That VIP1 does act as such an adaptor rather than the import factor itself was supported by

our observations that nuclear accumulation of VirE2 in the presence of VIP1 was inhibited by the SV40 NLS peptide known to specifically block the karyopherin α-dependent nuclear import pathway (39, 40). Because VIP1 appears to be found only in plants but not in yeast or animal cells, VirE2 nuclear import also occurs only in plant cells. Potentially, this reliance on the cellular VIP1 protein for efficient infection may represent a result of a long evolutionary relationship between *Agrobacterium* and its plant hosts.

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