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Permeabilized mammalian cells as an experimental system for nuclear import of geminiviral karyophilic proteins and of synthetic peptides derived from their nuclear localization signal regions

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The plant-infecting geminiviruses deliver their genome and viral proteins into the host cell nucleus. Members of the family *Geminiviridae* possess either a bipartite genome composed of two ~2·6 kb DNAs or a monopartite genome of ~3·0 kb DNA. The bipartite genome of *Bean dwarf mosaic virus* (BDMV) encodes several karyophilic proteins, among them the capsid protein (CP) and BV1 (nuclear shuttle protein). A CP is also encoded by the monopartite genome of *Tomato yellow leaf curl virus* (TYLCV). Here, an *in vitro* assay system was used for direct demonstration of nuclear import of BDMV BV1 and TYLCV CP, as well as synthetic peptides containing their putative nuclear localization signals (NLSs). Full-length recombinant BDMV BV1 and TYLCV CP mediated import of conjugated fluorescently labelled BSA molecules into nuclei of permeabilized mammalian cells. Fluorescently labelled and biotinylated BSA conjugates bearing the synthetic peptides containing aa 3–20 of TYLCV CP (CP-NLS) or aa 84–106 of BDMV BV1 (BV1-NLS) were also imported into the nuclei of permeabilized cells. This import was blocked by the addition of unlabelled BSA–NLS peptide conjugates or excess unlabelled free NLS peptides. The CP- and BV1-NLS peptides also mediated nuclear import of fluorescently labelled BSA molecules into the nuclei of microinjected mesophyll cells of *Nicotiana benthamiana* leaves, demonstrating their biological function in intact plant tissue. BV1-NLS and CP-NLS were shown to mediate specific binding to importin α, both *in vitro* and *in vivo*. These results are consistent with a common nuclear-import pathway for CP and BV1, probably via importin α.

INTRODUCTION

The plant-infecting geminiviruses, which possess ssDNA genomes, must deliver their genome and viral proteins into the cell nucleus for transcription and replication (Gafni & Epel, 2002; Lazarowitz & Beachy, 1999; Noueiry *et al.*, 1994; Rojas *et al.*, 2001; Sanderfoot *et al.*, 1996). Members of the family *Geminiviridae* possess either a bipartite genome composed of two ~2·6 kb DNA molecules, designated DNA A and DNA B, or a monopartite genome composed of a single ~3·0 kb DNA component (Brown, 2001; Hanley-Bowdoin *et al.*, 2000; Rojas *et al.*, 2005). Viruses that possess a bipartite genome, such as *Bean dwarf mosaic virus* (BDMV) and *African cassava mosaic virus*, encode several karyophilic proteins, among them the capsid protein (CP) encoded by DNA A and the BV1 protein encoded by DNA B (Gafni & Epel, 2002; Rojas *et al.*, 1998). A karyophilic CP is also encoded by the single DNA molecule of monopartite geminiviruses, e.g. *Tomato yellow leaf curl virus* (TYLCV) (Gafni & Epel, 2002; Rojas *et al.*, 2001). At present, little is known about the motility of the infectious form of geminiviruses into the nucleus of the host plant cells. For example, it is not known whether the geminate virions or some other nucleoprotein complex, formed following disassembly of the virion, enter the nucleus to initiate the infection process.

The geminivirus karyophilic proteins seem to have two different types of activity, one that mediates import of the viral genome into the nucleus and another that

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mediates nuclear export of the nascent viral DNA out of the nucleus (Gafni & Epel, 2002; Lazarowitz & Beachy, 1999; Palanichelvam *et al.*, 1998; Pascal *et al.*, 1994; Rojas *et al.*, 1998, 2001). Upon initiation of infection, the only viral protein present appears to be the CP, which has been implicated in mediating transport of viral ssDNA into the nucleus of host cells (Gafni & Epel, 2002; Palanichelvam *et al.*, 1998; Pascal *et al.*, 1994; Rojas *et al.*, 2001). In the case of TYLCV, the karyophilic properties of its CP have been demonstrated by import of fluorescently labelled CP or CP–green fluorescent protein (GFP) fusions into the nuclei of insect or plant cells (Kunik *et al.*, 1998; Rojas *et al.*, 2001). On the other hand, genetic studies have clearly established that the CP of bipartite begomoviruses is not required for infection and thus for essential steps involved in viral nucleocytoplasmic transport (Gafni & Epel, 2002). Indeed, various lines of evidence suggest that an additional protein, BV1, is required to transport the viral genome from the nuclei of the infected host cell to the cytoplasm and, through an association with another movement protein, BC1, to other cells (Haley *et al.*, 1995; Noueiry *et al.*, 1994; Rojas *et al.*, 1998; Zhang *et al.*, 2001). Thus, it appears that the BV1 protein of the bipartite geminiviruses functions as a nucleocytoplasmic shuttle protein and, as such, should contain a nuclear localization signal (NLS) as well as a nuclear export signal (NES) domain. Using molecular genetic approaches such as site-directed mutagenesis, NLS domains have been identified within CP and BV1 and have been shown to mediate nuclear import in transfected and microinjected cells (Kunik *et al.*, 1998; Rojas *et al.*, 2001). It should be noted, however, that both CP and BV1 are of relatively low molecular mass, around 30 kDa, and as such could enter host cell nuclei via a diffusion process (Hallan & Gafni, 2001; Sanderfoot *et al.*, 1996). However, as CP and BV1 have been implicated in promoting nuclear import or export of the viral ds/ssDNA (Noueiry *et al.*, 1994; Palanichelvam *et al.*, 1998; Rojas *et al.*, 1998), it is possible that a high molecular mass BV1–DNA nucleoprotein complex or even a complete virus particle enters the cell nucleus, thereby requiring an NLS (Kunik *et al.*, 1998). It has been suggested that BV1 enters cell nuclei unassisted, possibly via diffusion. Thus, its putative NLS may not necessarily be required to mediate nuclear import directly at this point. Thus, the possibility exists that the NLS domain of BV1, and perhaps also of CP, are required not for the mediation of direct binding to a nuclear-import receptor, but rather for binding to another cellular karyophilic protein.

To clarify these questions, we took an alternative approach to site-directed mutagenesis. This involved the synthesis and utilization of synthetic peptides bearing putative NLS sequences to mediate translocation of non-nuclear carrier proteins into nuclei of permeabilized cells. Permeabilized mammalian cells have been instrumental in dissecting the molecular components of the various nuclear import pathways that operate in eukaryotic cells, as well as the cellular factors required to promote NLS-mediated nuclear entry (Görlich, 1997; Nigg, 1997; Nigg *et al.*, 1991). Therefore, we tested whether permeabilized mammalian cells could also be used to study nuclear import of TYLCV CP and BDMV BV1, as well as of synthetic peptides derived from the NLS sequences of these geminiviral proteins. Our results may open the way for use of this *in vitro* nuclear import assay system to identify the nature of the viral nucleoprotein complex (i.e. virions or another nucleoprotein complex) that is imported into the cell nucleus.

METHODS

Cultured cells. A monolayer of HeLa cells or a suspension of human Colo-205 cells was grown in DMEM or RPMI 1640, respectively, as described previously (Armon-Omer *et al.*, 2004; Friedler *et al.*, 1998).

Design and synthesis of peptides. The NLS sequence of the TYLCV CP-derived synthetic peptide was designed based on mutagenesis experiments that localized its NLS domain within its N terminus (Kunik *et al.*, 1998). The sequence of the BV1-derived peptide was inferred based on an NLS suggested by site-directed mutagenesis and microinjection experiments (Noueiry *et al.*, 1994) and on sequence identity with the BV1 NLS of another bipartite begomovirus, *Squash leaf curl virus* (SLCV) (Sanderfoot *et al.*, 1996).

Peptides bearing the simian virus 40 (SV40) large T-antigen NLS (SV40-NLS, PKKKRKV; Kalderon *et al.*, 1984), the human immunodeficiency virus 1 (HIV-1) Vpr N-terminal NLS (VprN-NLS, NEWTLELLEELKNEAVRHF; Karni *et al.*, 1998), *Agrobacterium* VirE2-derived peptide (KLRPEDRYIQTEKYGRR; Citovsky *et al.*, 1988), TYLCV CP-NLS-derived peptide (^{3}KRPGDIIISTPVSKVRRR^{20}; Kunik *et al.*, 1998) and BDMV BV1-NLS-derived peptide (^{84}KIEPNR-SRSYIKLKRLRFKGTVK^{106}) were synthesized as described previously (Armon-Omer *et al.*, 2004; Friedler *et al.*, 1998).

Expression and purification of recombinant human importin α and β, BDMV BV1 and TYLCV CP. The expression vectors pET28-hIMP α 1 and pET28-hIMP β 1 were kindly provided by V. Citovsky (State University of New York, Stony Brook, NY, USA). Histidine (His)-tagged (Qiagen) human importins α and β were expressed and purified by standard protocols following growth at 37 °C and induction at 25 °C of the *Escherichia coli* strains. The TYLCV CP and BDMV BV1 genes were subcloned into the bacterial expression vectors pHISP1 and pRSETA (Invitrogen) (Rojas *et al.*, 1998, 2001), respectively. CP and BV1 were produced in *E. coli* using the T7 RNA polymerase expression system. Here, pHISP1-CP and pRSET-BV1 were introduced into *E. coli* strain BL21 (DE3) carrying the plasmid pLysS (Novagen). T7 RNA polymerase expression was then induced by the addition of 1 mM IPTG to liquid cultures of cells ($OD_{600}=0{\cdot}4$) harbouring the pRSET-BV1 and pHISP1-CP plasmids. Purification of the bacterially expressed recombinant TYLCV CP and recombinant BDMV BV1 and their subsequent analysis by SDS-PAGE were performed as described in the QIAexpressionist 2002 (Qiagen).

Transport substrates. The CP- and BV1-NLS peptides were covalently attached to either lissamine rhodamine B sulfonyl chloride (Pierce)-labelled BSA (Rho–BSA) or to caproylaminidobiotin BSA (Bb; Sigma) molecules using sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC; Pierce) as a cross-linker, as described previously (Armon-Omer *et al.*, 2004), to give a Rho–BSA–NLS or Bb–NLS conjugate, respectively. Purified CP or BV1 was covalently attached to either fluorescein isothiocyanate (FITC;

Sigma)-labelled BSA (FITC–BSA) or Bb molecules, using sulfo-SMCC as a cross-linker to give an FITC–BSA–protein or Bb–protein conjugate, respectively (Broder *et al.*, 1997).

***In vitro* nuclear import.** HeLa cells were cultured on 10 mm coverslips to subconfluent density and then permeabilized with digitonin as described previously (Armon-Omer *et al.*, 2004; Friedler *et al.*, 1998). Following 30 min of incubation under the conditions indicated for each experiment, import of Rho–BSA–NLS or FITC–BSA–protein conjugate into the nuclei of digitonin-permeabilized HeLa cells was observed by fluorescence microscopy as described previously (Armon-Omer *et al.*, 2004; Friedler *et al.*, 1998).

Nuclear import of Bb–NLS or Bb–protein conjugates was determined quantitatively using an ELISA-based system, essentially as described previously (Armon-Omer *et al.*, 2004; Friedler *et al.*, 1998). Briefly, a suspension of Colo-205 cells was permeabilized with digitonin, followed by the addition of the Bb–CP-NLS/protein conjugate (1 and 5 μg, respectively) or Bb–BV1-NLS/protein conjugate (3 and 2 μg, respectively). The cells were then incubated for 30 min at 37 °C to allow nuclear import. Cytosolic Bb conjugates were neutralized by the addition of an excess of avidin (Bayer & Wilchek, 1980) and unbound avidin was neutralized by the addition of an excess of biocytin (Armon-Omer *et al.*, 2004). After extensive washing, nuclei were disrupted with Triton X-100 and the attachment of the nuclear Bb–NLS/protein conjugates to microtitre plates (Nunclon) coated with anti-BSA was performed exactly as described previously (Armon-Omer *et al.*, 2004; Friedler *et al.*, 1998). The amount of bound Bb–NLS/protein conjugate was estimated following the use of a streptavidin–HRP conjugate as described above and previously (Armon-Omer *et al.*, 2004; Friedler *et al.*, 1998). Results given are means of three independent experiments and the SD was no greater than 20 % of the value of the mean.

After 30 min of incubation, nuclear import in permeabilized cells reached similar levels both in the presence and in the absence of rabbit reticulocyte extracts (Promega), as described in Results. Therefore, most of the import reactions were performed in the absence of these soluble factors and allowed to proceed to saturation.

Quantification of Bb–NLS conjugate binding to human importins. Binding of Bb–NLS conjugate to 96-well plates (Nunc) coated with importins (α and β) and the amount of bound Bb–NLS conjugate were estimated following the use of streptavidin–HRP conjugate as described above and previously (Armon-Omer *et al.*, 2004; Friedler *et al.*, 1998). The results given are means of three experiments and the SD never exceeded 20 %.

Detection of Bb–NLS conjugate binding to tomato importin α using a bimolecular fluorescence complementation (BiFC) assay. The coding sequences of BV1, CP or tomato importin α were fused downstream from either the yeast-enhanced GFP N terminus (N′yEGFP, aa 1–154), inserted in the yeast plasmid pAES423 (a generous gift from Professor D. Engelberg, Department of Biological Chemistry, The Hebrew University of Jerusalem) containing the histidine selection marker, or the yEGFP C terminus (C′yEGFP, aa 155–239) inserted in the yeast expression plasmid pAES426 (a generous gift from Professor D. Engelberg) containing the uracil selection marker. The following fusion proteins were constructed: N′yEGFP–tomato importin α, N′yEGFP–CP, C′yEGFP–BV1 protein and C′yEGFP–tomato importin α. Both plasmids were introduced into yeast strain EGY48 (Clontech) and then grown on agar medium lacking histidine and uracil for 2 days at 30 °C until colonies appeared. The plates were subsequently transferred to 23 °C for an additional 2 days. A sample of colonies was transferred to a solution of PBS and visualized by fluorescence microscopy.

Microinjection into plant tissues. Rho-labelled BSA–NLS conjugates, as well as recombinant purified BV1 and CP proteins labelled with Oregon green (OG; Molecular Probes) or Rho, and BDMV BC1 protein labelled with Rho were used in microinjection studies performed on detached mature leaves of *Nicotiana benthamiana* (Rojas *et al.*, 2001). Movement of fluorescent probes into nuclei or cell-to-cell movement out of injected cells was monitored and recorded by confocal laser scanning microscopy using a Leica upright confocal laser scanning microscope (model TCS-4D) equipped with a four-dimensional hydraulic micromanipulator system (Narishige). Microinjection was carried out as described previously (Rojas *et al.*, 2001).

Microinjection into mammalian cells. HeLa cells were microinjected with Rho–BSA–CP-NLS using the CompInject AIS2 automated microinjection system (Cell Biology Trading) as described previously (Krichevsky *et al.*, 2003), using a microinjection method developed by Graessmann & Graessmann (1983).

RESULTS

Import of BSA–BV1 and BSA–CP conjugates into the nuclei of permeabilized mammalian cells

To assess nuclear import of BDMV BV1 and TYLCV CP in the *in vitro* assay system, both proteins were chemically conjugated to fluorescent and biotin-labelled BSA molecules (see Methods). This intensified the sensitivity of the nuclear import assay system and increased the size of these relatively low molecular mass proteins (~30 kDa) (Hallan & Gafni, 2001) and the solubility of the relatively insoluble recombinant BV1 and CP (see also Armon-Omer *et al.*, 2004).

Fluorescent microscopic studies with FITC–BSA–BV1 (Fig. 1a–e) and with FITC–BSA–CP conjugates (Fig. 1g–k) revealed that both BV1 protein (Fig. 1a) and CP (Fig. 1g) mediated import of the conjugated FITC–BSA molecules into the nuclei of permeabilized HeLa cells. Note that in previous studies it has been established that FITC–BSA remains in the cytoplasm of these cells (Broder *et al.*, 1997). Nuclear import was not observed at 4 °C (Fig. 1b and h) or in the absence of ATP (Fig. 1d and j), which is consistent with the involvement of active transport across the nuclear pore complex (Görlich, 1997). Furthermore, addition of GTPγS almost completely blocked import (Fig. 1c and i), implying involvement of the Ran G protein in the nuclear import process of both proteins (Melchior *et al.*, 1993). Nuclear import of both conjugates was also inhibited in the presence of wheat germ agglutinin (WGA) (Yoneda *et al.*, 1987) (Fig. 1e and k). Essentially the same results were obtained using the quantitative nuclear import assay system (Fig. 1f and l). Thus, the major fundamental properties of nuclear import in mammalian cells were retained in the permeabilized cells.

Over relatively extended periods of incubation (i.e. $\geqslant$30 min), nuclear entry in permeabilized HeLa cell monolayers, as well as in the suspension of permeabilized Colo-205 cells, occurred both in the presence

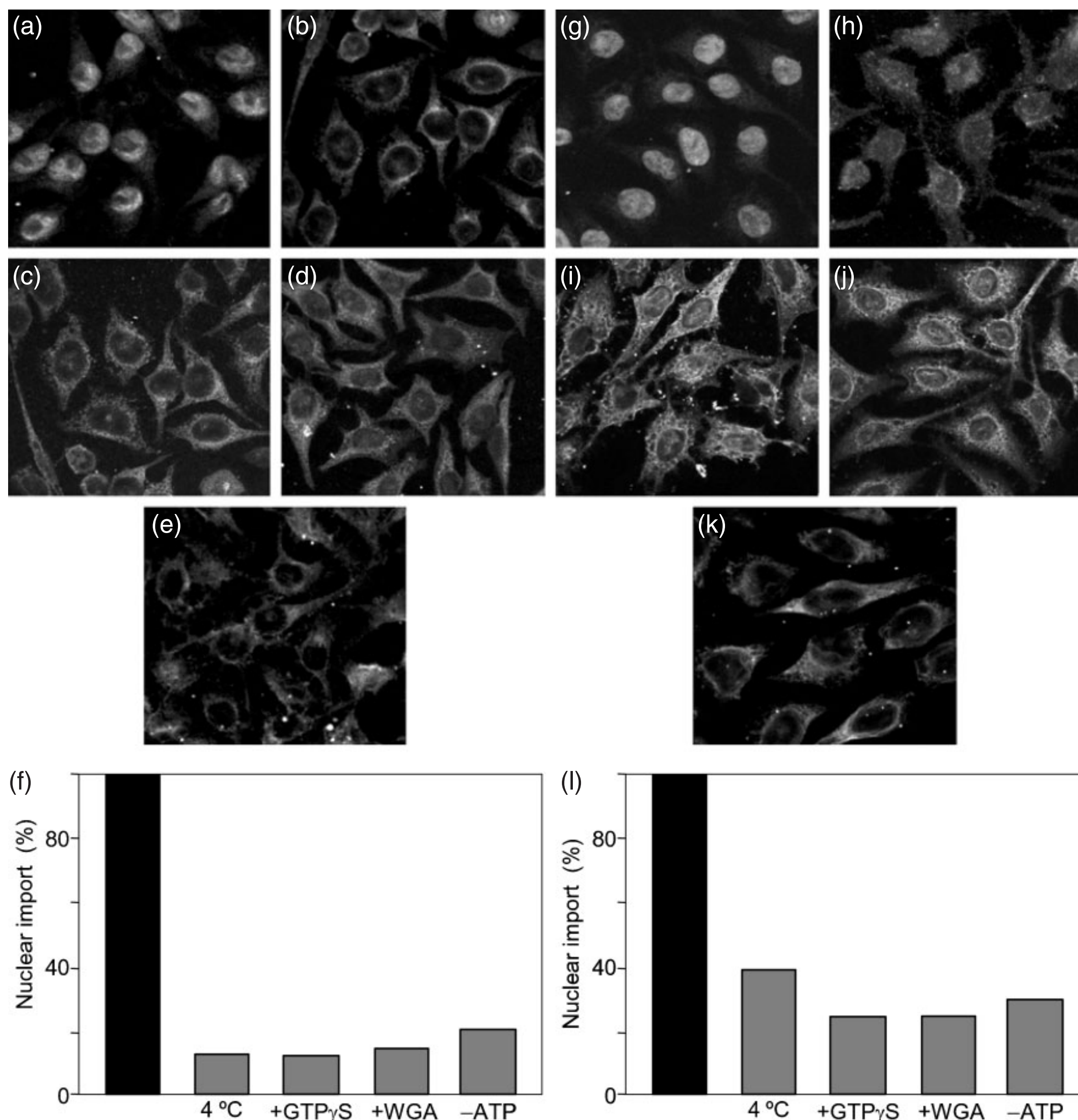

Fig. 1. Active nuclear import mediated by recombinant BV1 and CP. Digitonin-permeabilized HeLa cells were incubated with FITC–BSA–BV1 (a–e) or FITC–BSA–CP (g–k). These transport substrates were incubated at 37 °C (a, g) or at 4 °C (b, h), with GTPγS (1·6 mM) (c, i), in the absence of ATP (d, j) or pre-incubated for 20 min at 4 °C with 25 μg WGA before the addition of the transport substrate (e, k). ATP was depleted as described in Armon-Omer *et al*. (2004). Nuclear import was quantified by an ELISA-based system (Armon-Omer *et al*., 2004). Bb–BV1 (f) and Bb–CP (l) were added to permeabilized Colo-205 cells and incubated for 1 h at 37 °C, at 4 °C, with GTPγS (1·6 mM), with WGA (25 μg) or with ATP-depleted, permeabilized cells. The degree of nuclear import obtained for 1 h at 37 °C was taken as 100 %.

(Fig. 1a) and in the absence of externally added cytosolic factors (reticulocyte extract, data not shown). However, during a shorter time course (i.e. up to 15–20 min of incubation), nuclear import reached saturation only in the presence of the cytosolic factors, while, in their absence, the import process remained incomplete (data not shown). These results suggested that permeabilized mammalian cells retain small amounts of functional cytoplasmic factors necessary for nuclear import, but that these quantities are insufficient to promote the maximal rate of nuclear import during short incubation periods. As described in Methods, most of the nuclear import experiments described in this work were allowed to reach saturation and therefore were performed in the absence of the cytosolic factors.

Interestingly, permeabilized plant protoplasts have also been shown to retain cytoplasmic factors necessary for nuclear import (Hicks & Raikhel, 1995).

Nuclear import can be mediated by synthetic peptides derived from BV1 and CP

A region of the SLCV BV1 protein, which contains aa 81–96, is required for promoting its nuclear import (Sanderfoot *et al*., 1996) and has homology with the BDMV BV1 NLS-like region (Noueiry *et al*., 1994). The NLS of TYLCV CP has been mapped more precisely and N-terminal deletion mutants of this protein fail to exhibit nuclear import in transfected protoplasts and microinjected cells (Kunik *et al*., 1998; Rojas *et al*., 2001). Thus, peptides bearing aa 84–106 of BDMV BV1 and aa 3–20 of TYLCV CP were synthesized and chemically conjugated to labelled BSA molecules (see Methods). Both of these peptides mediated nuclear import in permeabilized mammalian cells (Figs 2 and 3) and were therefore designated BV1-NLS and CP-NLS, respectively.

Microscopy observations and quantification of nuclear import of both BSA–BV1-NLS (Fig. 2) and BSA–CP-NLS conjugates (Fig. 3) revealed that the import occurred at 37 °C (Figs 2a and g, and 3a and f), but was inhibited at 4 °C (Figs 2b and g and 3f), as well as in the presence of WGA or GTPγS (Figs 2g and 3f). These results are consistent with active nuclear import, translocation via the nuclear pore complex and a requirement for the Ran protein, respectively (Mattaj & Englmeier, 1998). The energy requirement for active nuclear import was also evident from only a low degree of import observed in ATP-depleted cells (Figs 2g and 3f).

Competition experiments showed that the addition of excess unlabelled BSA conjugates bearing either BV1- or CP-NLS blocked nuclear import mediated by the respective NLS peptides (see Figs 2c and 3b). Interestingly, the BSA–CP-NLS conjugate blocked nuclear import mediated by BV1-NLS (Fig. 2d) and the BSA–BV1-NLS conjugate inhibited, to some extent, nuclear import mediated by CP-NLS (Fig. 3c). In addition, an unlabelled BSA conjugate bearing the SV40-NLS peptide partially inhibited nuclear import mediated by BV1 (Fig. 2e) and completely inhibited import mediated by CP-NLS (Fig. 3d). Together, these results are consistent with a common nuclear import pathway for CP and BV1, probably via the importin α-dependent pathway (Fontes *et al*., 2000; Hübner *et al*., 1999). The specificity of the inhibition is strengthened by the finding that the addition of unlabelled BSA conjugate bearing a peptide derived from the *Agrobacterium* VirE2, which does not interact with importin α (Tzfira & Citovsky, 2001), did not have any effect on the NLS-mediated nuclear import (Figs 2f and 3e). Inhibitory effects were also obtained by the addition of free NLS peptides, although much higher peptide-to-conjugate ratios were required to obtain the extent of inhibition observed with the BSA-NLS conjugates (not shown). Thus, it appears that the nuclear import

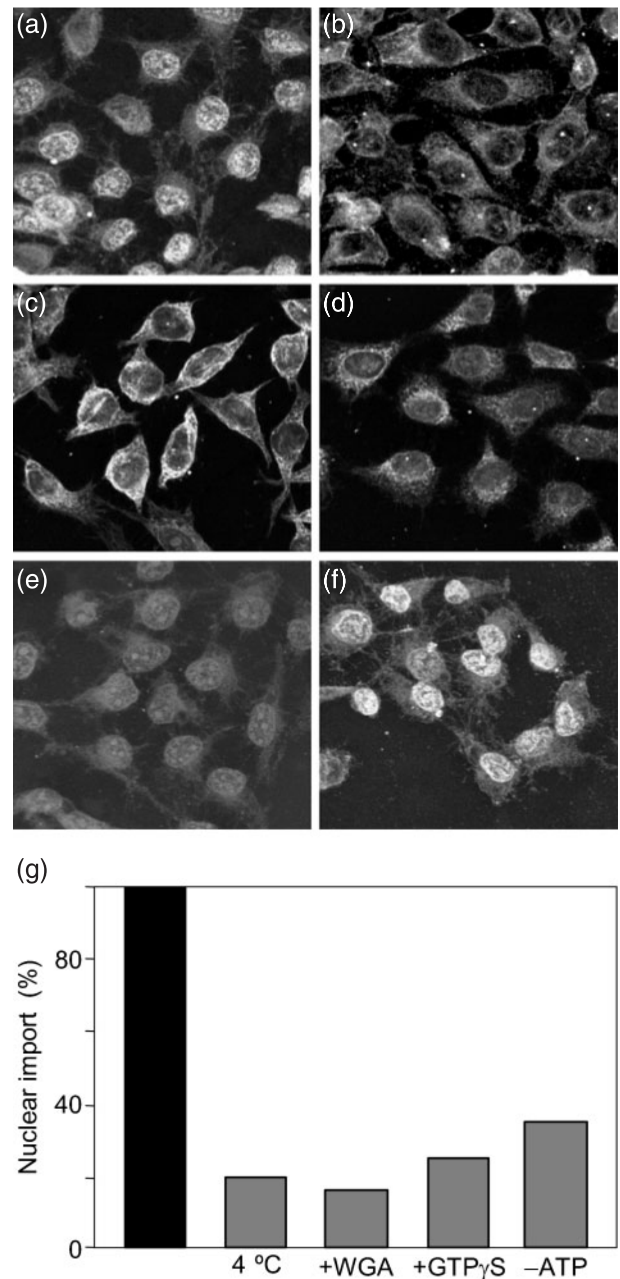

Fig. 2. Specific inhibition of active nuclear import mediated by the BV1-NLS peptide. (a)–(f) Nuclear import of Rho–BSA–BV1-NLS conjugate in permeabilized HeLa cells was performed at 37 °C (a) or at 4 °C (b) or with the addition of the following unlabelled BSA–peptide conjugates: BV1-NLS (c), CP-NLS (d), SV40-NLS (e) and VirE2-derived peptide (f). The molar ratio between Rho–BSA–BV1-NLS and the BSA peptides was 1 : 2. (g) Digitonin-permeabilized Colo-205 cells were incubated at 37 °C with Bb–BV1-NLS and nuclear import was quantified as described in Fig. 1(k). All other experimental conditions were as described in Fig. 1, Methods and Armon-Omer *et al*. (2004).

mediated by the BV1- and CP-NLS peptides is specific and consistent with all of the features that typify active nuclear import.

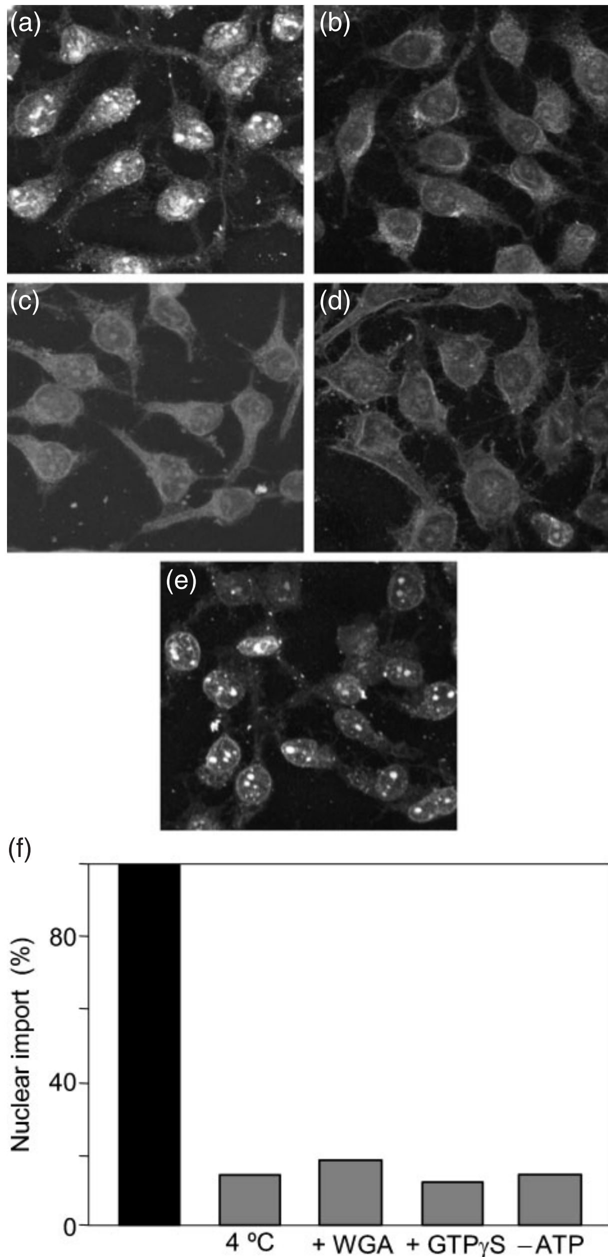

Fig. 3. Specific inhibition of active nuclear import mediated by the CP-NLS peptide. (a)–(e) Nuclear import of Rho–BSA–CP-NLS in permeabilized HeLa cells was performed at 37 °C (a) or with the addition of the following unlabelled BSA–peptide conjugates: CP-NLS (b), BV1-NLS (c), SV40-NLS (d) and VirE2-derived peptide (e). The molar ratio between the Rho–BSA–CP-NLS and BSA peptides was 1 : 2. (f) Digitonin-permeabilized Colo-205 cells were incubated at 37 °C with Bb–BV1-NLS and nuclear import was quantified as described in Fig. 1(k). All other experimental conditions were as described in Fig. 1, Methods and Armon-Omer *et al.* (2004).

Nuclear import mediated by the full-length BV1 and CP molecules is inhibited by the NLS peptides

The next question was whether translocation of the recombinant proteins and NLS peptides was mediated by the same import pathway. Nuclear import of the fluorescently labelled (Fig. 4a–d) and biotinylated (Fig. 4i) BSA–BV1 conjugates was inhibited by unlabelled conjugates bearing the BV1-NLS (Fig. 4b) and CP-NLS (Fig. 4c) peptides. Similarly, conjugates bearing the SV40-NLS completely blocked nuclear import mediated by the BV1 protein (Fig. 4d). Essentially the same results were obtained when the effect of these NLS peptides on the ability of the full-length CP to mediate nuclear import was studied (Fig. 4e–h). However, it should be noted that conjugates bearing the BV1-NLS (Fig. 4g), as well as the SV40-NLS (Fig. 4h), were somewhat less inhibitory compared with BSA–CP-NLS (Fig. 4f). This could indicate a difference in the binding affinities of the various conjugates. Collectively, these results strongly indicate that nuclear import of the recombinant proteins is mediated by the same pathway and probably by the NLS domains that were used as the basis for the synthesis of the BV1- and CP-NLS peptides. Essentially the same results were obtained using the quantitative nuclear import assay system with Bb–BV1 (Fig. 4i) and Bb–CP (not shown) conjugates as transport substrates.

Binding of BSA–BV1/CP-NLS conjugates and the BV1 and CP recombinant proteins to importin α

Inhibition of nuclear import by the SV40-NLS, as described above, is suggestive of nuclear import via the importin α pathway (Fontes *et al.*, 2000). The ability of the BV1/CP-NLSs to interact directly with human importin α was studied using Bb–NLS conjugates and an ELISA-based system. The human importin α was employed in the ELISA binding experiments due to difficulties in obtaining purified, recombinant tomato importin α (Kunik *et al.*, 1999). However, a high degree of similarity between plant and mammalian importin α has been demonstrated (Kunik *et al.*, 1999). Binding mediated by BV1-NLS to importin α showed a typical saturation curve and the specificity of the assay system was evident from the results demonstrating no binding to the BSA molecules alone (Fig. 5a). Furthermore, the interaction between BV1-NLS and importin α was competitively inhibited by the addition of unlabelled BSA conjugates bearing the NLS sequences of BV1 (Fig. 5b), CP (Fig. 5c) or SV40 (Fig. 5d). No such inhibition was observed under the same conditions by a conjugate bearing the HIV-1 VprN-NLS (Fig. 5b–d), which, according to some previous reports and in our hands, does not interact with importin α (A. Krichevsky and A. Loyter, unpublished; Jans *et al.*, 2000; Jenkins *et al.*, 1998). Similarly, CP-NLS also bound to importin α (Fig. 5e). Evidence of the specificity of the NLS–importin α interaction came from results showing competitive inhibition by BSA–CP-NLS (Fig. 5f) and BSA–SV40-NLS (Fig. 5h). Furthermore, CP-NLS did not bind to

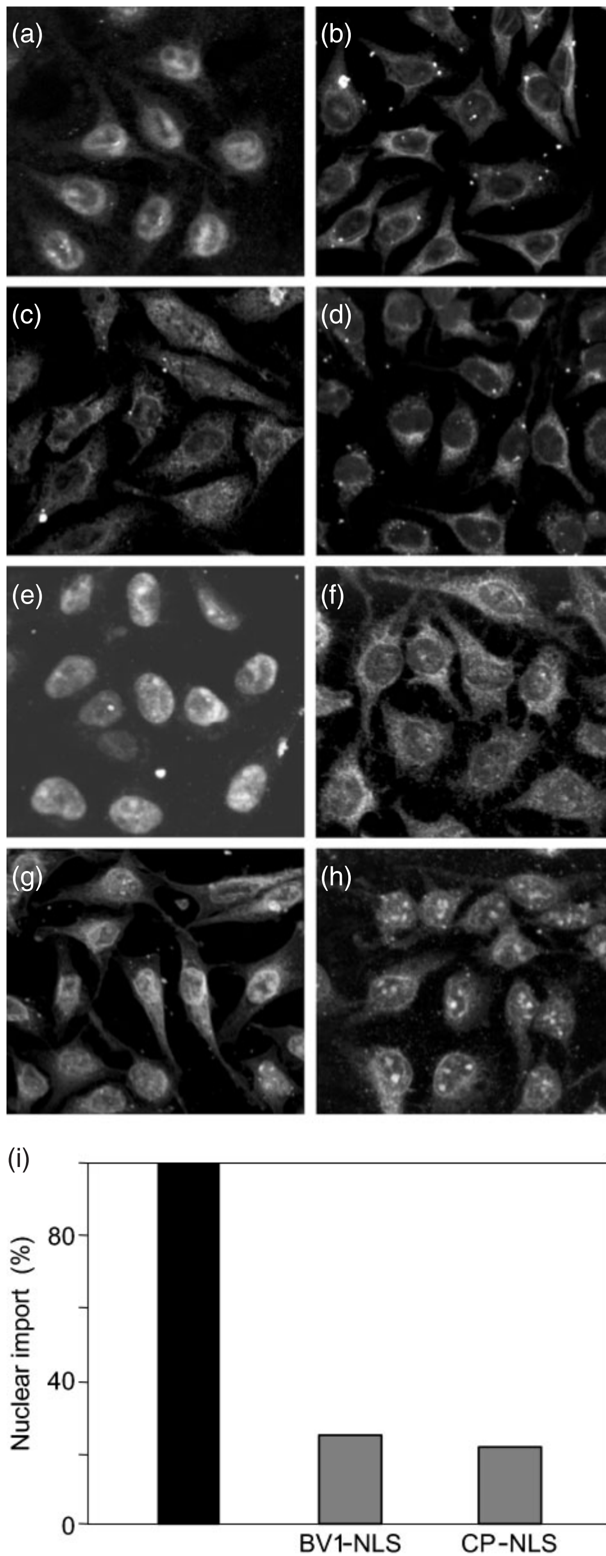

Fig. 4. Nuclear import mediated by the full-length BV1 and CP proteins is specifically inhibited by BSA–NLS conjugates. (a)–(h) Nuclear import mediated by BV1 protein (a–d) and by CP (e–h) was performed as described in Fig. 1(a). Import was inhibited by the addition of the following unlabelled BSA–NLS conjugates (BSA–NLS : FITC–BSA, 30 : 1 molar ratio): BV1-NLS (b, g), CP-NLS (c, f) and SV40-NLS (d, h). (i) Nuclear import of the Bb–BV1 protein was inhibited by the addition of BSA molecules conjugated to BV1-NLS and CP-NLS in an ELISA-based assay as described in Fig. 1(f and l) and in Methods (BSA–NLS : Bb–BV1, 30 : 1 molar ratio).

importin β under similar conditions (Fig. 5e). It should be noted that a fourfold molar excess of BSA–CP-NLS was sufficient to provide complete inhibition of BV1-NLS-mediated binding to importin α (Fig. 5c). This was not the case for BV1-NLS conjugates, which, even at a 30-fold molar excess, provided only about 50 % inhibition of CP-NLS-mediated binding to importin α (Fig. 5g).

The specific interaction of BV1 and CP with importin α was also demonstrated directly *in vivo* by the BiFC assay (Atmakuri *et al.*, 2003; Hu & Kerppola, 2003; Hu *et al.*, 2002; Tsuchisaka & Theologis, 2004). In this approach, a molecule of yEGFP (Cormack *et al.*, 1997) is divided into two parts, the N-terminal (N′yEGFP) and C-terminal (C′yEGFP) parts, neither of which fluoresces when expressed alone. However, fluorescence is restored when N′yEGFP and C′yEGFP are brought together as fusions via interacting proteins (Atmakuri *et al.*, 2003; Hu & Kerppola, 2003; Hu *et al.*, 2002; Tsuchisaka & Theologis, 2004). Using this approach, we showed that BV1 (Fig. 6a) and CP (Fig. 6b) interacted with tomato importin α in yeast cells and that the interacting proteins localized within the cell nucleus (Fig. 6a and b). In negative controls, no BiFC signal was observed when C′yEGFP-tagged importin α was co-expressed with free N′yEGFP (Fig. 6c and d).

Peptides bearing the NLS domains of CP and BV1 can mediate nuclear import in plant and mammalian cells

The ability of the CP- and BV1-NLS peptides to mediate nuclear import of BSA molecules was assessed in intact plant tissues. Rho–BSA–CP-NLS and Rho–BSA–BV1-NLS conjugates were microinjected directly into the cytoplasm of mesophyll cells of *N. benthamiana* leaves and nucleocytoplasmic and cell-to-cell transport were evaluated. Control injections established that fluorescence associated with BSA–Rho and fluorescently labelled 10 kDa dextran (F-dextran) remained in the cytoplasm of injected cells, whereas Lucifer yellow and BC1–Rho moved from cell to cell; none of these substrates accumulated in the nucleus (Table 1). In contrast, both the CP-NLS and BV1-NLS conjugates accumulated efficiently in the plant cell nucleus (Table 1 and Fig. 7). Microscopic observations revealed strong nuclear staining and some residual cytoplasmic signal (Fig. 7a and b). Essentially identical nuclear localization patterns were observed in microinjection experiments with full-length recombinant Rho–BV1 (Fig. 7c) and CP–OG (Rojas *et al.*, 2001), although it took longer for the NLS peptide-linked conjugates to accumulate in the nucleus

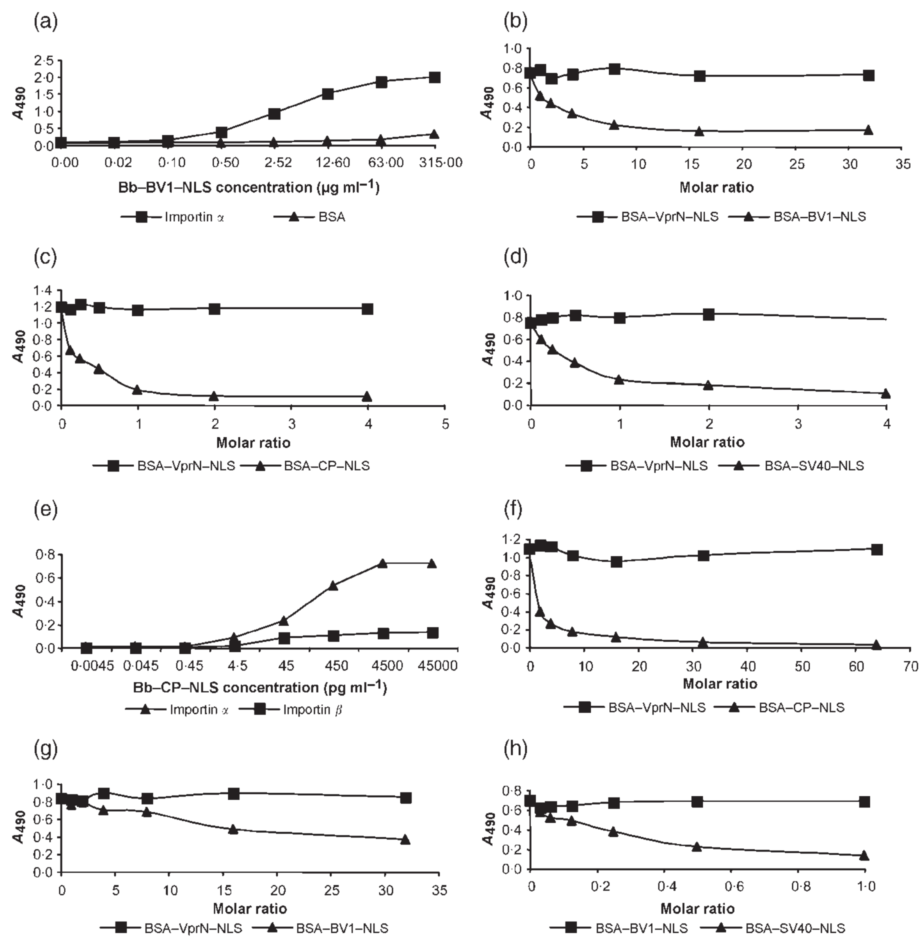

Fig. 5. BV1- and CP-NLS peptides mediate specific binding to importin α *in vitro*. Increasing amounts of Bb–BV1-NLS (a–d) and of Bb–CP-NLS (e–h) were incubated with importin α- and/or importin β-coated plates and the degree of binding was estimated by an ELISA-based system as described in Methods. For competition experiments, Bb–BV1-NLS (3·15 μg ml^{-1}) (b–d) or Bb–CP-NLS (0·45 μg ml^{-1}) (f–h) were incubated with importin α-coated plates in the presence of increasing amounts of the indicated unlabelled competitors at the competitor : Bb–BV1-NLS or competitor : Bb–CP-NLS molar ratio specified in each panel.

compared with microinjected fluorescently labelled BV1 and CP (Rojas *et al.*, 2001). In negative-control experiments, microinjected fluorescently labelled BSA molecules remained cytoplasmic (Fig. 7d). Neither the microinjected BSA–NLS conjugates nor the BV1–OG or CP–OG protein moved from cell to cell following microinjection into leaf mesophyll cells (Table 1; Rojas *et al.*, 2001). Together, these results indicate that these different fluorescent markers did not alter the subcellular targeting of these proteins (Table 1) and that the NLS peptides were biologically active and mediated import into the nuclei of living plant cells.

Similarly, microinjection of the Rho–BSA–CP-NLS conjugate into the cytoplasm of intact cultured HeLa cells resulted in the accumulation of fluorescent dots in the nucleus, suggesting nuclear import (Fig. 7e). Co-microinjection of Rho–BSA–CP-NLS with unlabelled BSA–CP-NLS resulted in inhibition of nuclear import (Fig. 7f), again indicating a receptor-mediated process.

DISCUSSION

Permeabilized mammalian cells as an experimental system for studying the karyophilic properties of geminivirus proteins

We demonstrated that both BDMV BV1 and TYLCV CP are capable of interacting with the nuclear import machinery of animal cells in a manner similar to that of animal virus proteins (Görlich, 1997; Mattaj & Englmeier, 1998). This is consistent with previous reports of nuclear import of plant karyophilic proteins in digitonin-permeabilized mammalian cells (Citovsky *et al.*, 2004; Jiang *et al.*, 1998; Ziemienowicz *et al.*, 2003) and import of TYLCV CP into

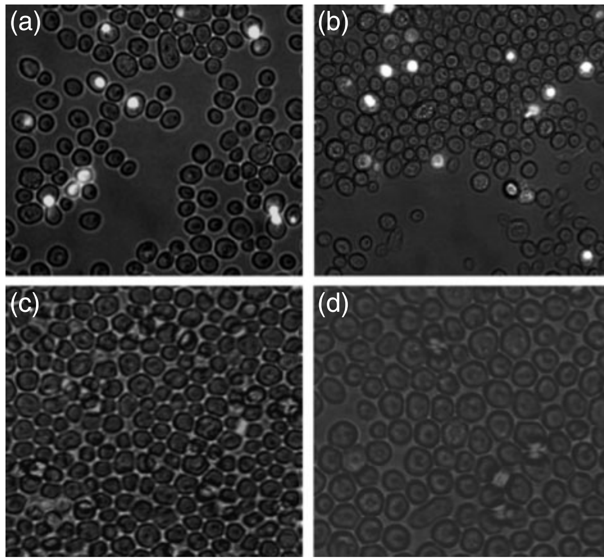

Fig. 6. BV1- and CP-NLS peptides mediate specific binding to importin α *in vivo*. Interactions of BV1 and CP with tomato importin α and subcellular localization of the interacting proteins in yeast cells were detected by the BiFC assay as described in Methods: (a) C′yEGFP–BV1 + N′yEGFP–importin α; (b) C′yEGFP-CP1 + N′yEGFP–importin α; (c, d) free C′yEGFP + N′yEGFP–importin α.

nuclei of cultured insect cells and *Drosophila* embryos (Kunik *et al.*, 1998). Our results on import into the nuclei of mammalian cells of CP and BV1 protein further strengthen the view that NLS-containing proteins, as well as the nuclear import pathways, show a high degree of conservation among diverse eukaryotic organisms.

Furthermore, the results showed that no plant-specific components were required for nuclear entry of these two viral proteins. What remains unclear is the form in which these proteins enter the nucleus. In the case of TYLCV, CP-mediated nuclear import of the viral genome may occur as whole virus particles or as a CP–DNA complex. The finding that the TYLCV CP can mediate import and export of DNA in microinjection experiments (where virion formation would not be expected to occur) are consistent with nucleocytoplasmic transport via a non-virion nucleoprotein complex. In the case of bipartite begomoviruses, which do not require CP for systemic infection, BV1 probably fulfils the role of CP (Gafni & Epel, 2002; Rojas *et al.*, 1998, 2001). Moreover, for infectious CP mutants of bipartite begomoviruses, it is clear that nucleocytoplasmic, cell-to-cell and long-distance movement events involve a non-virion nucleoprotein complex. An *in vitro* nuclear import assay system may provide further insight into the molecular mechanism by which the geminiviral genome finds its way into host cell nuclei.

Synthetic peptides as a tool to study functional NLSs

Blocking nuclear import of BV1 and CP may offer a new approach to inhibiting virus replication and/or spread and thus viral infection (Gafni & Epel, 2002). Therefore, efforts have been made to identify the NLS domains of BV1 and CP, as well as those of other geminiviral karyophilic proteins (Kunik *et al.*, 1998; Pascal *et al.*, 1993; Sanderfoot & Lazarowitz, 1995; Sanderfoot *et al.*, 1996).

In the present work, a direct approach was used to confirm that the sequences containing the putative NLSs of BV1 and CP were functional and could mediate nuclear import by themselves. This was achieved by showing that synthetic peptides bearing these sequences mediated active import of covalently attached BSA molecules into the nuclei of permeabilized mammalian cells. Our microinjection experiments proved unequivocally that the NLS synthetic peptides

Table 1. BDMV BV1-NLS peptide and TYLCV CP-NLS peptides mediate nuclear import of Rho-labelled BSA molecules in microinjected *N. benthamiana* mesophyll cells

Microinjection into plant cells was performed as described in Methods.

Injected agent	Nuclear accumulation*	Cell-to-cell movement†
10 kDa F-dextran	0/12	0/12
Lucifer yellow	0/10	10/10
TRITC–BC1	0/11	11/11
BV1–OG	15/15	0/15
Rho–BSA–BV1-NLS conjugate	14/14	0/14
Rho–BSA–CP-NLS conjugate	12/12	0/12

*Results are presented as the number of injections in which nuclear accumulation of fluorescent probe was observed/total number of injections performed. Fluorescence was detected in the nuclei of injected cells 1–5 min after injection; nuclei were identified by DAPI staining.

†Results are presented as the number of injections in which movement to neighbouring cells was observed/total number of injections performed.

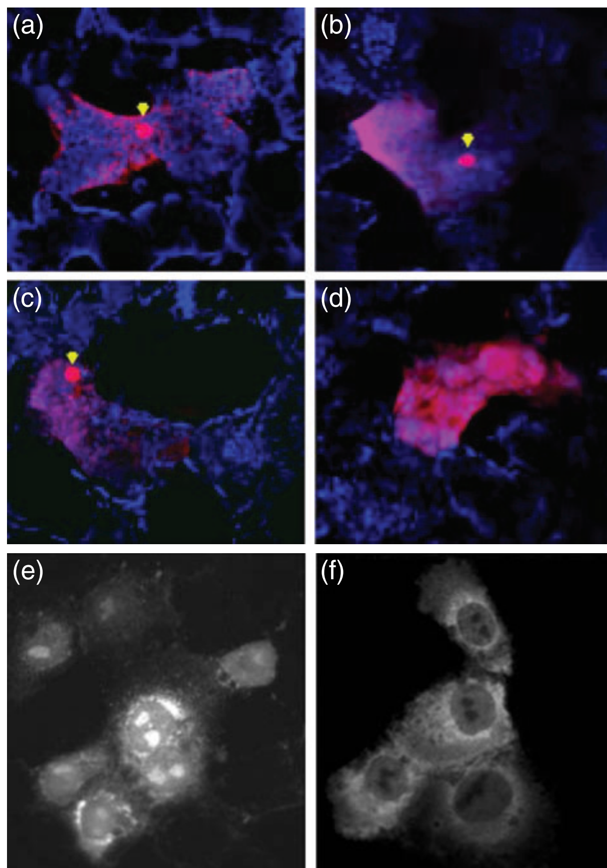

Fig. 7. Nuclear import mediated by the BV1-NLS and CP-NLS peptides in microinjected plant and mammalian cells. Mesophyll cells of *N. benthamiana* leaves were microinjected with the following transport substrates as described in Methods: (a) Rho–BSA–CP-NLS, (b) Rho–BSA–BV1-NLS, (c) Rho–BV1 and (d) Rho–BSA only. The rhodamine signal is pink and plastid autofluorescence is blue. Images were collected 20 min after injection and arrows indicate cell nuclei (confirmed with DAPI staining) that accumulated fluorescently labelled substrates. Cultured HeLa cells were microinjected with Rho–BSA–CP-NLS (e) or a mixture of Rho–BSA–CP-NLS and BSA–CP-NLS (BSA–CP-NLS : Rho–BSA–CP-NLS, 4 : 1 molar ratio) (f) as described in Methods. Images were recorded after 2 h of incubation at 37 °C.

used were biologically active and could function within the intracellular environment, further confirming the results obtained using the *in vitro* assay system. Furthermore, the same features that characterized import of BV1 and CP, including inhibition by SV40-NLS, characterized translocation of the BSA–NLS peptides into the nuclei of permeabilized cells (Armon-Omer *et al.*, 2004; Friedler *et al.*, 1998).

Thus, the BV1- and CP-NLS peptides can be used to select anti-NLS sequences (Krichevsky *et al.*, 2003) that could block nuclear import of these virus proteins and, when expressed in transgenic plants, may interfere with virus infection. The availability of functional NLS peptides as well as *in vitro* nuclear import and ELISA systems should enable the selection of inhibitory anti-NLS peptides. Experiments to achieve these goals are currently under way in our laboratory.

Nuclear import pathway used by BV1 and CP proteins

Our results clearly indicated that nuclear import of the BV1 and the CP proteins is mediated by the same receptor, most likely importin α. This assumption is based on our observations that conjugates bearing the BV1/CP-NLS peptides competitively blocked nuclear import of the full-length BV1 and CP. Furthermore, nuclear import of the NLS conjugates, as well as that of the BV1/CP proteins, was blocked by SV40-NLS, which is known to interact with the importin α pathway to mediate nuclear import (Görlich, 1997). Import by this pathway was also supported by our *in vitro* ELISA binding experiments and the *in vivo* BiFC assay.

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