Journal of Cellular Biochemistry 79:225–238 (2000)

Nuclear Import and DNA Binding of Human Papillomavirus Type 45 L1 Capsid Protein

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Abstract During the life cycle of human papillomaviruses (HPVs), the L1 capsid proteins seem to enter the nucleus twice: once after the virions infect the cells, and later during the productive phase when they assemble the replicated HPV genomic DNA into infectious virions. We established for the high-risk HPV45 that when digitonin-permeabilized HeLa cells were incubated with L1 homopentameric capsomers, the HPV45 L1 protein was imported into the nucleus in a receptor-mediated manner. In contrast, intact capsids were not able to enter the nucleus. Immunoisolation assays showed that HPV45 L1 capsomers interact with cytosolic karyopherin α2β1 heterodimers. HPV45 L1 bound strongly to karyopherin α2, and weakly to karyopherin β1, as did its nuclear localization signal (NLS). Nuclear import of HPV45 L1, or of a GST-$NLS_{HPV45L1}$ fusion protein was efficiently mediated by karyopherin α2β1 heterodimers, and only weakly by karyopherin β1. Nuclear import required RanGDP, but was independent of GTP hydrolysis by Ran. Together, these data suggest that the major nuclear import pathway for HPV45 L1 major capsid protein in infected host cells is mediated by karyopherin α2β1 heterodimers and that GTP hydrolysis by Ran is not required for import. Remarkably, HPV45 L1 capsomers can interact nonspecifically with different types of HPV-DNA, and the DNA binding region of HPV45 L1 overlaps with its NLS sequence. J. Cell. Biochem. 79:225–238, 2000.

Key words: DNA binding; HPV45; L1 capsid protein; nuclear import

Cervical cancer is one of the most frequent malignancies affecting women. Epidemiological studies indicate that human papillomavirus (HPV) is a major etiological agent [for reviews, see Galloway, 1994; Shah and Howley, 1996]. More than 70 types of HPVs have been isolated and classified either as "high risk," such as types 16, 18, 45, and 31, frequently found in cervical carcinomas, or "low risk," such as types 6 and 11, which are very rarely found in malignant tumors [Howley, 1996; Shah and Howley, 1996].

HPVs are small, nonenveloped, icosahedral DNA viruses that replicate in the nucleus of squamous epithelial cells. Replication of HPV is connected to the differentiation program of the host tissue, squamous epithelium. The virion particles (52–55 nm in diameter) consist of a single molecule of double-stranded circular DNA approximately 8,000 bp in size contained within a capsid. Two structural proteins form the capsid: L1, the major one, and L2, the minor one. L1 protein is capable of self-assembly in vivo and in vitro into capsidlike structures, referred to as viruslike particles. L1 is stable in both oligomeric configurations: homopentameric capsomers and capsids composed of 72 capsomers [Kirnbauer et al., 1992; Rose et al., 1993; Zhou et al., 1993; Hagensee et al., 1994; Belnap et al., 1996; Rose et al., 1998]. HPV L1 capsids can be disassembled into capsomers quantitatively by an agent that reduces disulfide bonds and reassembled by removing the reducing agent [Li et al., 1998; McCarthy et al., 1998].

The life cycle of PVs depends on the import of the viral genome and capsid proteins into the nucleus, where viral gene transcription, DNA replication, and viral maturation take place

Grant sponsor: American Cancer Society; Grant number: 99-210-01-MBC; Grant sponsor: Boston College.

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Received 4 April 2000; Accepted 4 May 2000

[for review, see Howley, 1996]. A study on the early phase of infection of cultured cells with bovine PV virions showed that although binding to the plasma membrane and uptake of virions into large endosomes could be monitored by electron microscopy, no complete virions could be observed in the nucleus of infected cells. In contrast, a strong nuclear fluorescent staining was observed for both L1 and L2 capsid proteins [Zhou et al., 1995].

Import of macromolecules into the nucleus is an active process and is mediated by the interactions of transport receptors (karyopherins/importins) with their respective cargoes as well as with the nuclear pore complexes (NPC). Nuclear import of classic nuclear localization signal (NLS)-proteins is mediated by karyopherin αβ1 heterodimers (known also as importin αβ, NLS receptor and p97, PTAC αβ, Kap60 and Kap95) [for reviews, see Corbett and Silver, 1997; Görlich and Kutay, 1999; Moroianu, 1999a]. Karyopherin α (Kap α) functions as an adapter: it binds directly to the classic basic NLS of the cargo and to karyopherin β1 (Kap β1). The trimeric complex is docked at the NPC via the interactions of Kap β1 with several peptide-repeat nucleoporins [Görlich et al., 1995; Moroianu et al., 1995b; Rexach and Blobel, 1995; Weis et al., 1995; Shah et al., 1998]. Nuclear import of the docked NLS-protein requires the GTPase Ran, and the RanGDP-interacting protein p10/NTF2 that mediates nuclear import of RanGDP [Melchior et al., 1993; Moore and Blobel, 1993, 1994; Paschal and Gerace, 1995; Schlenstedt et al., 1995]. There are several human Kap α that bind to the same Kap β1 and to distinct or overlapping types of NLS; the best characterized ones are Kap α2 (corresponding to Rch-1, hSRP1α, NPI-3) and Kap α1 (corresponding to NPI-1, hSRP) [O'Neill and Palese, 1995; Moroianu et al., 1995a, 1995b; Weis et al., 1995; Malik et al., 1997; Kohler et al., 1999]. Kap β1 can also bind directly to arginine-rich NLSs and function in nuclear import without Kap α adaptors [for reviews, see Mattaj and Englmeier, 1998; Görlich and Kutay, 1999; Moroianu, 1999b].

There are at least 14 members of the Kap β family: nine of these are import receptors, four are export receptors and one is uncharacterized [for reviews, see Pemberton et al., 1998; Wozniak et al., 1998; Görlich and Kutay, 1999; Moroianu, 1999b]. Except for Kap β1 (that can function both with and without adapters), the other Kap βs that mediate nuclear import bind the cargo directly through specific NLSs. For example, mammalian Kap β2/transportin, yeast Kap104p, and Kap111p/Mtr10p mediate import of mRNA-binding proteins. Mammalian Kap β3 and yeast Kap121p and Kap123p mediate import of a set of ribosomal proteins. The ribosomal proteins L23a, S7, and L5 can each be imported by several Kap βs: β1, β2/transportin, β3/Imp5, or Imp7. All Kap βs shuttle between the nucleus and the cytoplasm, and bind to nucleoporins at the NPC and to the GTPase Ran in its GTP bound form. In import, the interaction of RanGTP with Kap βs is involved in dissociation of import complexes with release of the cargo in the nucleus. The nucleotide state of Ran is regulated by RanGAP, which catalyses GTP hydrolysis to form RanGDP, and by RanGEF, which catalyzes nucleotide exchange to generate RanGTP. It is considered that the nuclear localization of RanGEF and the cytoplasmic localization of RanGAP result in nuclear RanGTP, which is responsible for the final step of nuclear import, the release of the cargo from Kap βs [for reviews, see Mattaj and Englmeier, 1998; Görlich and Kutay, 1999; Moroianu, 1999b].

The L1 capsid proteins of HPVs are thought to enter the nucleus of host cells at two times during the virus life cycle: immediately after the virions infect the cells [Zhou et al., 1995], and during the late productive phase, when the newly synthesized L1 and L2 proteins assemble the replicated HPV genomic DNA into infectious virions. Previously, we established that the L1 major capsid protein of a low oncogenic risk HPV type (i. e., HPV-11) binds Kap α2 and it is imported into the nucleus via Kap α2β1 heterodimers [Merle et al., 1999]. In this study, we investigated the nuclear import pathways for the L1 capsid protein of high-risk HPV45. We found that HPV45 L1 capsomers (or/and monomers) can be imported into the nuclei of digitonin-permeabilized cells in a receptor-dependent manner. In contrast, intact L1 capsids cannot enter the nucleus. These data indicate that during infection, the L1 capsids must first be disassembled for the L1 proteins to enter the nucleus. Immunoisolation assays showed that HPV45 L1 capsomers interact with cytosolic Kap α2β1 heterodimers. Binding assays indicated that HPV45 L1 binds strongly to Kap α2, and only weakly to Kap β1, as does the HPV45 L1′NLS. In agreement with

the binding data, nuclear import of HPV45 L1 capsid protein, or of a GST-$NLS_{HPV45L1}$ fusion protein is efficiently mediated by Kap α2β1 heterodimers, and only weakly by Kap β1. We found that nuclear import of GST-$NLS_{HPV45L1}$ requires RanGDP, but is independent of GTP hydrolysis by Ran. These data suggest that the major nuclear import pathway for HPV45 L1 capsid protein in host cells is mediated by Kap α2β1 heterodimers. We also discovered that HPV45 L1 capsomers can interact with HPV-DNA in a nonspecific manner and that the DNA binding region of HPV45 L1 overlaps with its NLS sequence.

MATERIALS AND METHODS

Preparation of Recombinant Transport Factors

Recombinant full-length transport factors: His-tagged Kap α2 [Weis et al., 1995], His-tagged Kap β1, GST-Kap β1 [Chi et al., 1995], Kap β2 [Chook and Blobel, 1999], Ran [Coutavas et al., 1993], and His-tagged p10 [Moroianu et al., 1995b] were prepared as previously described. The purified proteins were dialyzed in buffer A (20 mM HEPES-KOH, pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 2 mM dithiothreitol) containing protease inhibitors, and stored in aliquots at −80°C until use.

Preparation of Recombinant HPV45 L1 Capsids and Capsomers

The recombinant HPV45 L1 capsids were generated in insect cells, purified as previously described [Rose et al., 1993, 1994a, 1994b], and stored at 4°C until use. Purity and lack of proteolytic degradation of the proteins were always checked by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), Coomassie staining, and immunoblotting before use. HPV45 L1 capsomers were obtained by incubating the L1 capsids with 5% mercaptoethanol overnight at 4°C [McCarthy et al., 1998]. HPV45 L1, which lacks 86 amino acids from the carboxy terminus (trpL1), was obtained by trypsin digestion of the full-length HPV45 L1 protein [Li et al., 1997].

Antibodies

Rabbit polyclonal antiserum R-122 was raised against HPV45 L1 capsids as previously described [Rose et al., 1998; White et al., 1998]. Murine antibodies raised against Kap α2/Rch-1 and Kap β2/transportin were from Transduction Laboratories; a mouse monoclonal antibody to karyopherin β1/p97 was from Affinity Bioreagents, Inc. and the goat anti-GST antibody was from Pharmacia Biotech.

Synthetic Peptides and Conjugates

The peptide corresponding to the NLS of SV40 T antigen was synthesized with an N-terminal Cys added for coupling purposes: CYTPPKKKKRKVED. The peptide was conjugated to human serum albumin (HSA) as previously described [Merle et al., 1999]. As assayed by SDS/PAGE, the NLS-HSA conjugates contained from 5 to 10 peptides per HSA.

HeLa cytosol from Cellex Biosciences Inc. was centrifuged, and stored in aliquots at −80°C.

Preparation of GST-$NLS_{HPV45L1}$ and GST-$NLS_{HPV11L1}$ Fusion Proteins

Two GST-NLS fusion constructs were made: one contains the potential NLS of HPV45 L1 ($_{479}$AGLRRRPTIGPRKRPAASTSTASRPAKRVRIRSKK$_{513}$) and the other contains the NLS of HPV11 L1 (APKRKRTKTKK). The GST-NLS fusion proteins and the GST protein were expressed in *Escherichia coli* BL21(DE3) and purified using standard procedures for GST-containing proteins.

In Vitro Nuclear Import Assays

Nuclear import assays in digitonin-permeabilized cells were carried out as previously described [Moroianu et al., 1995b; Merle et al., 1999]. Subconfluent HeLa cells grown on glass coverslips for 1 day were permeabilized with 70 μg/ml digitonin for 5 min on ice. Digitonin permeabilizes the plasma membrane but leaves the nuclear envelope intact and, as a consequence, digitonin-permeabilized HeLa cells retain intact import-competent nuclei, but are largely depleted of cytosolic transport factors. Unless otherwise specified, the import reactions contained the energy-regenerating system (0.5 mM ATP, 0.5 mM GTP, 5 mM phosphocreatine, and 0.4 U creatine phosphokinase), plus various transport factors (0.5–1 μM for karyopherins, 3 μM RanGDP, 0.2 μM p10) or HeLa cytosol, plus the L1 capsids/capsomers, or GST-NLS fusion proteins, as indicated in the figure legends. Final import reaction volume was adjusted to 20 μl

with buffer A. For visualization of nuclear import, the HPV45 L1 protein was detected by immunofluorescence with specific rabbit antibodies. The nuclei were identified by DAPI staining. Nuclear import was analyzed with a Nikon Eclipse TE 300 Microscope in fluorescence. Quantitation of import was done as described [Merle et al., 1999] by measuring the fluorescence of 25 nuclei with IPLAB software.

Immunofluorescence

For detection of nuclear import of HPV45 L1 capsomers/capsids, or GST-NLS fusion proteins by immunofluorescence, we used rabbit polyclonal antibodies against HPV45 L1 capsids and an anti-GST antibody, respectively. At the end of the import reactions, the cells were washed with transport buffer and fixed with 3.7% formaldehyde for 15 min on ice and then with methanol for 3 min at −20°C. After blocking nonspecific binding with 3% bovine serum albumin (BSA), 0.1% Tween in phosphate-buffered saline (PBS) for 1 h, the cells were incubated for 1 h with the first antibody (1:200 dilution) followed by detection with a secondary antibody labeled with fluorescein (1:100 dilution). Specificity controls consisted of the absence of the protein to be detected or of the first antibody.

Fluorescence Labeling of Proteins

For in vitro nuclear import assays, the NLS-HSA conjugate was labeled with fluorescein 5′ maleimide dissolved in dimethylformamide at a 1:1 molar ratio. The reaction was allowed to proceed for 2 h on ice, quenched with 50 mM mercaptoethanol, and the labeled protein was dialyzed against transport buffer overnight at 4°C.

Immunoisolation Assays

HeLa cytosol (100 μl) was incubated first with HPV45 L1 capsomers (4 μM) for 30 min at room temperature (r.t.) to allow the formation of L1/karyopherin complexes and then with antibodies against HPV45 L1 for another 60 min. The complexes were immunoisolated by incubating with Protein A-Sepharose beads for 2 h at 4°C. The beads were washed four times with transport buffer and eluted with 1.5 M $MgCl_2$. The eluted proteins were subjected to SDS-electrophoresis and then transferred to nitrocellulose. The blots were probed with the following murine antibodies: anti-Kap β1/p97, anti-Kap β2/transportin, and anti-Kap α2/Rch-1, followed by an anti-mouse horseradish peroxidase (HRP) antibody. Positive immunoblotting controls consisted of blots of HeLa cytosol containing the Kaps. Immunoisolation controls consisted of omission of HPV45 L1 capsomers during the isolation.

Overlay Blot Assays

HPV45 L1, HPV11 L1 and Kap α2 (2 μg/lane) were subjected to SDS-PAGE and transferred onto a nitrocellulose membrane. The blots were stained with Ponceau and blocked overnight at 4°C with nonfat 5% milk in PBS. Then the blots were incubated for 30 min at r.t. with either Kap α2 (0.2 μM), or GST-Kap β1 (0.2 μM), or GST-Kap β1 in the presence of RanGTP (2x molar excess), or GST-Kap β2 (0.2 μM). Bound Kap α2 was detected by immunoblotting with a specific antibody. Bound GST-Kap β1 or GST-Kap β2 were detected by immunoblotting with an anti-GST antibody.

Immunoblotting

Blots were stained with Ponceau, blocked overnight at 4°C with nonfat 5% milk in PBS, and then incubated for 1h at r.t. in the first antibody (1:500–1:1,000 dilution). After washing, the blots were incubated for 1h at r.t. with the corresponding secondary antibody-HRP conjugate (1:1,000 dilution). Immunoblotting controls consisted of the absence of the first antibodies. The signal was detected with an ECL Detection Kit (Amersham Pharmacia) and exposure to Biomax film (Kodak, Rochester, NY).

In Solution Binding Assays

GST-$NLS_{HPV45L1}$ and GST-$NLS_{HPV11L1}$ fusion proteins immobilized on glutathione-Sepharose beads (2 μg protein/10 μl beads) were incubated with purified His-tagged Kaps in solution binding assays, as previously described [Rexach and Blobel, 1995]. Specificity control experiments consisted of incubating GST immobilized on glutathione-Sepharose beads with the purified Kaps. The bound fractions were analyzed by SDS-PAGE followed by Coomassie staining.

DNA Mobility Shift Assays

Capsomers of HPV45 L1 or HPV11 L1, or GST-$NLS_{HPV45L1}$, or GST-$NLS_{HPV11L1}$, or GST

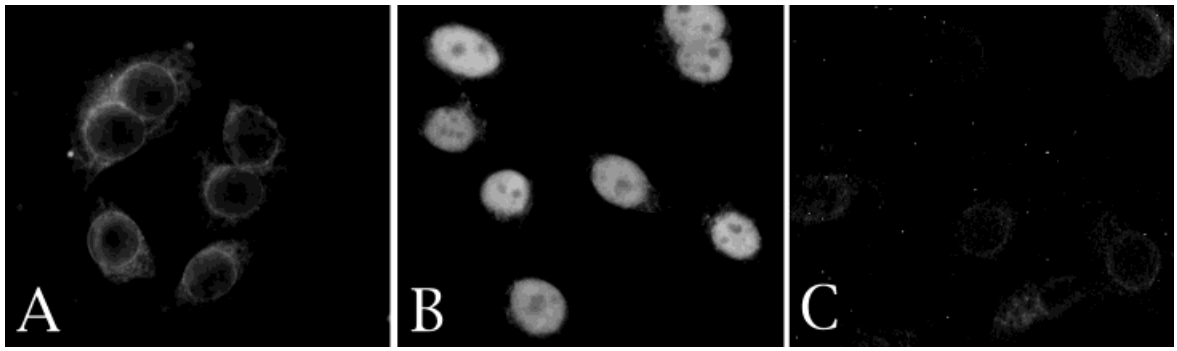

Fig. 1. Nuclear import of HPV45 L1 capsid protein. Digitonin-permeabilized HeLa cells were incubated for 30 min at room temperature with: HPV45 L1 capsomers (A and B) in the presence of only buffer A (**A**), or HeLa cytosol (**B**), or with HPV45 L1 capsids in the presence of HeLa cytosol (**C**). HPV45 L1 was detected by immunofluorescence with specific antibodies to HPV45 L1 viruslike particles. Note the nuclear import of HPV45 L1 in (B).

(as a control) were incubated with either genomic HPV-DNAs, or plasmid DNA for 30 min at 25°C in buffer A. The DNA or DNA–protein complexes were analyzed by electrophoresis in 0.7% agarose gels and staining with ethidium bromide.

RESULTS

Nuclear Import of HPV45 L1 Requires Disassembly of the Capsids

The nuclear import of HPV45 L1 was investigated in either the capsid or capsomer conformation. We used in vitro nuclear import assays in digitonin-permeabilized cells that have been used to investigate many nuclear import pathways mediated by Kap βs/importins [Melchior et al., 1993; Moore and Blobel, 1993, 1994; Adam and Adam, 1994; Chi et al., 1995; Görlich et al., 1995; Moroianu et al., 1995a, 1995b; Weis et al., 1995; Schwoebel et al., 1998; Merle et al., 1999; Ribbeck et al., 1999]. Digitonin-permeabilized HeLa cells were incubated with 1) L1 capsomers in only buffer A; 2) L1 capsomers plus cytosol; or 3) L1 capsids plus cytosol, and the HPV45 L1 protein was detected by immunofluorescence staining with specific antibodies. We found that HPV45 L1 capsomers, in contrast with L1 capsids (Fig. 1B), could be imported into the nuclei of digitonin-permeabilized cells in the presence of cytosol and an energy source. Nuclear import of HPV45 L1 did not occur in the absence of cytosol (Fig. 1A), indicating that it is mediated by transport factors present in the cytosol. Within the nucleus, the L1 protein was distributed throughout the nucleoplasm, although staining was less intense in regions that most likely are nucleoli (Fig. 1B). Indeed, double immunostaining with a monoclonal antibody to human nucleoli confirmed that these regions correspond to nucleoli (data not shown). A similar nuclear distribution with exclusion of the nucleoli was observed when the L1 protein of bovine PV is expressed alone in BPHE-1 fibroblast cells [Day et al., 1998].

To get insight into the nuclear import pathways that HPV45 L1 might use, we first investigated whether the classic NLS peptide of SV40 T antigen can compete for nuclear import with HPV45 L1 protein. Digitonin-permeabilized HeLa cells were incubated for 30 min with HPV45 L1 capsomers in the presence of either 1) HeLa cytosol, or 2) HeLa cytosol plus an excess of the NLS peptide of SV40 T antigen. We found that the classic NLS peptide inhibited nuclear import of HPV45 L1 (Fig. 2). We also found that vice versa, the nuclear import of NLS-HSA substrate could be inhibited by HPV45 L1 capsomers in excess (data not shown). Together, these data suggest that HPV45 L1 capsid protein might use the classic nuclear import pathway.

Interaction of HPV45 L1 Capsid Protein With Karyopherins

The complexes that HPV45 L1 capsomers form with the Kaps present in HeLa cytosol were immunoisolated and then probed by immunoblotting with anti-Kap α2, anti-Kap β1/p97, or anti-Kap β2/transportin. Blots of HeLa cytosol (containing the karyopherins) were used as positive immunoblotting controls. We

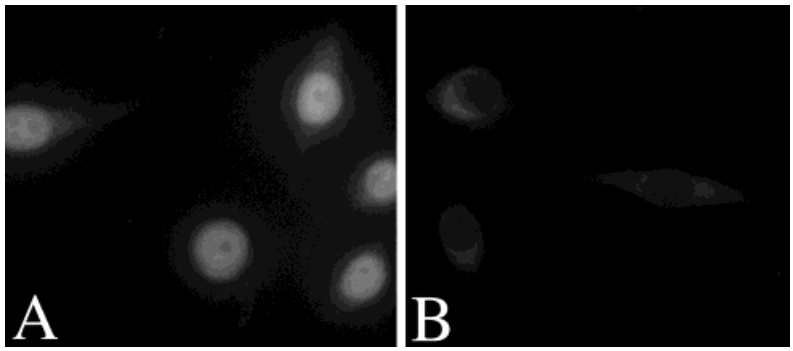

Fig. 2. Nuclear import of HPV45 L1 is inhibited by the classic NLS peptide of SV40 T antigen. Digitonin-permeabilized HeLa cells were incubated with HPV45 L1 capsomers in the presence of either HeLa cytosol (**A**), or HeLa cytosol plus the NLS peptide of SV40 T antigen (100x molar excess)(**B**).

found that both Kap α2 and Kap β1, but not Kap β2, were specifically immunoisolated from HeLa cytosol together with HPV45 L1 (Fig. 3A). As a specificity control, in the absence of HPV45 L1 capsomers during the incubations, no Kaps were immunoisolated (data not shown). These data suggest that in the cytoplasm of host cells, HPV45 L1 can form complexes with Kap α2β1 heterodimers.

Overlay blot assays were used to investigate the direct interactions between the HPV45 L1 capsid protein and the Kaps. We found that Kap α2 bound strongly to HPV45 L1 (Fig. 3B, lane 1), as well as to HPV11 L1 (Fig. 3B, lane 2), that we have previously shown to interact with Kap α2 [Merle et al., 1999]. The potential NLS of HPV45 is basic, but it is more extended than HPV11 L1' NLS and it is rich in arginine residues (Table 1), suggesting that HPV45 L1 might also interact directly with Kap β1. Indeed, we found that Kap β1 could bind directly to HPV45 L1, but with less efficiency than Kap α2 (Fig. 3C, lane 1). As a control, Kap β1 bound to Kap α2 (Fig. 3C, lane 3). Kap β1, when complexed with RanGTP, did not bind to either HPV45 L1 (Fig. 3C, lane 2), or to the control Kap α2 (Fig. 3C, lanes 3 and 4). In agreement with the immunoisolation data, Kap β2 did not bind to HPV45 L1 in this assay (data not shown), suggesting that it does not have any affinity for HPV45 L1′NLS.

HPV45 L1′NLS Mediates Nuclear Import Via Karyopherin α2β1 Heterodimers

It was previously established that the L1 capsid protein of HPV16 contains two classic NLSs [Zhou et al., 1991]. One NLS consists of six basic amino acids at the carboxy-terminal of HPV16 L1; the other NLS is of the bipartite type and overlaps with the first monopartite NLS [Zhou et al., 1991]. Sequence analysis revealed that the carboxy-terminal NLS of HPV16 L1 is well conserved in HPV11 L1, but less in HPV45 L1 (Table I). The C-terminus of HPV45 L1 contains a potential basic NLS rich in arginine residues and more extended than a classic monopartite NLS (Table I). Indeed, an HPV45 trpL1 protein, which lacks the C-terminal region (86 amino acids), is no longer imported into the nucleus (data not shown). We tested a GST-fusion protein containing the potential NLS of HPV45 L1 (Table I) in nuclear import assays. As a control, we used a GST-fusion protein containing the classic monopartite NLS of HPV11 L1. Both the GST-$NLS_{HPV45L1}$ and the GST-$NLS_{HPV11L1}$ were imported into the nucleus in the presence of cytosol, but not in the presence of only buffer A (Fig. 4A). As expected, a GST control was not imported into the nucleus in the presence of cytosol (data not shown).

We further investigated the nuclear import of GST-$NLS_{HPV45L1}$ in the presence of recombinant transport factors. Digitonin-permeabilized HeLa cells were incubated with GST-$NLS_{HPV45L1}$ in the presence of either 1) Kap β1 and RanGDP, or 2) Kap β1, RanGDP, and p10, or 3) Kap β1, Kap α2, RanGDP, or 4) Kap β1, Kap α2, RanGDP, and p10 (Fig. 4B). The GST-$NLS_{HPV45L1}$ was efficiently imported into the nucleus in the presence of Kap α2β1 heterodimers plus either RanGDP, or RanGDP and p10 (Fig. 4B, panels C and D). In the presence of only Kap β1, plus either RanGDP, or

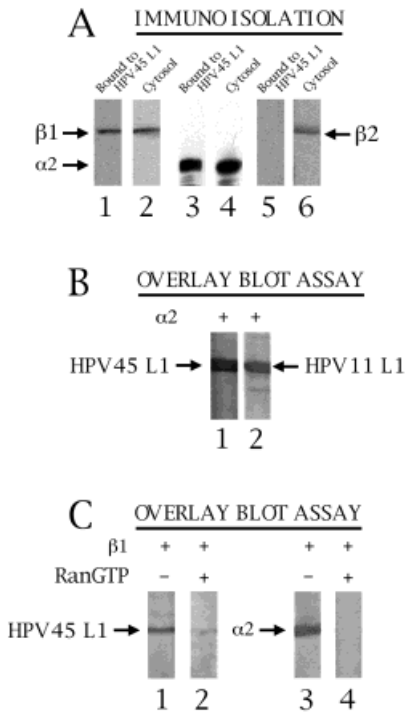

Fig. 3. Interactions of HPV45 L1 with karyopherins. **A:** HPV45 L1 forms complexes with cytoplasmic Kap α2β1. The complexes that HPV45 L1 forms with the Kaps present in HeLa cytosol were immunoisolated as described (see Materials and Methods) and then subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane. The blots were probed with anti-Kap β1/p97 (**lane 1**), or anti-Kap α2 (**lane 3**), or anti-Kap β2/transportin (**lane 5**). As positive immunoblotting controls, we used HeLa cytosol probed with anti-Kap β1 (**lane 2**), or anti-Kap α2 (**lane 4**), or anti-Kap β2 (**lane 6**). **B:** HPV45 L1 and HPV11 L1 were subjected to SDS-PAGE and transferred onto a nitrocellulose membrane. The blots were probed with Kap α2 and the bound Kap α2 was detected with a specific antibody. **C:** HPV45 L1 and Kap α2 were subjected to SDS-PAGE and transferred onto a nitrocellulose membrane. The blots were incubated with GST-Kap β1 (**lanes 1** and **3**), or GST-Kap β1 in the presence of RanGTP (2x molar excess) (**lanes 2** and **4**). The bound GST-Kap β1 was detected with an anti-GST antibody.

RanGDP plus p10, import of GST-$NLS_{HPV45L1}$ was not efficient (Fig. 4B, panels A and B). We obtained similar data when digitonin-permeabilized HeLa cells were incubated with HPV45 L1 capsomers in the presence of recombinant transport factors (Fig. 5). The data suggest that the sequence between amino acids 479 and 513 of HPV45 L1 capsid protein functions as an NLS and can mediate efficient nuclear import via Kap α2β1 heterodimers. Most likely, because the interaction between HPV45 L1 and Kap β1 is weak, it does not result in efficient nuclear import.

Analysis of the Interactions Between HPV45 L1′ NLS and Karyopherins

We analyzed the interactions between GST-$NLS_{HPV45L1}$ immobilized on glutathione-Sepharose and His-tagged Kaps by solution binding assays. As a control, we used GST immobilized on glutathione-Sepharose. The GST-$NLS_{HPV45L1}$ interacted strongly with Kap α2, whereas the GST control did not (Fig. 6, lanes 1 and 4). In addition, the GST-$NLS_{HPV45L1}$ interacted weakly with Kap β1, whereas the GST control did not (Fig. 6, lanes 2 and 5). These data indicate that the NLS of HPV45 L1 can interact strongly with Kap α2, and weakly with Kap β1, in agreement with the overlay blot data (Fig. 3B,C). As expected, in the presence of both karyopherins, binding of Kap β1 was increased via its interaction with Kap α2 bound to the HPV45 L1′NLS (Fig. 6, lane 3).

Nuclear Import Mediated by HPV45 L1′NLS Does Not Require GTP Hydrolysis by Ran

We investigated whether GTP hydrolysis by Ran is required for nuclear import of GST-$NLS_{HPV45L1}$ or GST-$NLS_{HPV11L1}$ by comparing the nuclear import in the presence of recombinant transport factors and either GTP, or the nonhydrolyzable GTP analogues, GTPγS and GMP-PNP. Both the GST-$NLS_{HPV45L1}$ (Fig. 7) and the GST-$NLS_{HPV11L1}$ (data not shown) were efficiently imported in the presence of either GTP, or GTPγS, or GMP-PNP. These data strongly suggest that GTP hydrolysis by Ran is not required for the nuclear import mediated by HPV45 L1′NLS or HPV11 L1′NLS.

HPV45 L1 Can Interact With DNA Via Its NLS

We used DNA mobility shift assays to investigate the DNA-binding properties of HPV45 and HPV11 L1 capsid proteins and their corresponding NLS sequences. HPV11-DNA was incubated for 30 min with GST-$NLS_{HPV11L1}$, or GST-$NLS_{HPV45L1}$, or GST (as a control), and the DNA–protein complexes were analyzed by agarose gel electrophoresis. We found that HPV11 L1′NLS interacted with HPV11-DNA, indicating that the DNA-binding activity and the NLS sequence of HPV11 L1 overlap (Fig. 8A, lane 4). Interestingly, we discovered that HPV45 L1′NLS also interacted with HPV11-

TABLE I. Nuclear Localization Signals (NLSs) of Human Papillomavirus (HPV) L1 Capsid Proteins

HPV type	NLS sequences
HPV16 L1	KRKatpttssts**t**taKRKKRK
HPV11 L1	KRpavskpstapKRKRtKtKK
HPV45 L1	RRRptigpRKRpaaststastasRpaKRvRiRsKK

DNA causing a similar mobility shift (Fig. 8A, lane 3). We further analyzed the interactions of HPV45 L1′NLS, or HPV11 L1′NLS with the genomic DNA of high risk HPV16. We found that both HPV45 L1′NLS and HPV11 L1′NLS interacted with HPV16-DNA (Fig. 8B, lanes 3 and 5). Also, the capsomers of HPV45 L1 and HPV11 L1 interacted with HPV16-DNA (Fig. 8B, lanes 4 and 6). We obtained similar interactions between HPV45 L1′NLS, or HPV11 L1′NLS and either the genomic DNA of HPV18 or an unrelated plasmid DNA (data not shown). These data suggest that the interactions between either HPV45 L1′NLS or HPV11 L1′NLS and DNA are nonspecific. Both the number of GST-NLS molecules and L1 capsomers bound to one molecule of DNA varied with experiments. The range of bound GST-NLS molecules per DNA molecule was between 20 and 80. The range of bound L1 capsomers per DNA molecule was between 1 and 8.

DISCUSSION

Nuclear Import of HPV45 L1 Capsomers

The study of nuclear import of L1 capsid protein of high-risk HPV45 revealed that the intact capsids cannot be transported through the NPC into the nuclei of digitonin-permeabilized HeLa cells (Fig. 1). This suggests that capsid disassembly is required for nuclear entry of incoming HPV45 L1 protein during viral infection. Disassembly of the capsids (at least to capsomers) before nuclear import would be expected because the capsid diameter (55 nm) is bigger than the functional diameter of the NPC (25 nm). We have obtained similar data for the nuclear import of L1 capsid proteins of low-risk HPV11 [Merle et al., 1999] and high-risk HPV16/18 (unpublished observations). During the import reactions in digitonin-permeabilized cells in the presence of cytosol, it is possible that the L1 capsomers might be further disassembled into monomers. Hence, we do not know whether L1 proteins were transported into the nucleus as monomers, or as capsomers, or both. Disulfide bonds are involved in capsomer–capsomer interactions and stability of the capsids, and reducing agents can break these bonds [Li et al., 1998; McCarthy et al., 1998]. This would suggest that during the early stages of the viral infection, exposure of HPV capsids to the reducing environment of the cytoplasm of host cells would break some of the stabilizing disulfide bonds leading to capsid disassembly [Li et al., 1998; McCarthy et al., 1998]. The same reducing environment could perhaps prevent capsid assembly in the cytoplasm of host cells during the late productive phase of viral life cycle when the L1 proteins are synthesized and transported into the nucleus. The interactions between HPV capsomers/monomers and the corresponding host karyopherins could play a role in preventing capsid assembly in the cytoplasm. The molecular mechanisms of HPV capsid disassembly and prevention of capsid assembly in the cytoplasm of host cells remain to be established.

Our immunoisolation experiments indicated that HPV45 L1 forms complexes with cytoplasmic Kap $\alpha 2\beta 1$ heterodimers when incubated with cytosol (Fig. 3A). In binding assays, we found that HPV45 L1 and its NLS interacted strongly with Kap $\alpha 2$, and weakly with Kap $\beta 1$ (Fig. 3B,C and Fig. 6). In agreement with these data, HPV45 L1 capsomers and a GST-$NLS_{HPV45L1}$ fusion protein were efficiently imported into the nuclei of digitonin-permeabilized cells in the presence of Kap $\alpha 2\beta 1$ heterodimers, and only very weakly in the presence of Kap $\beta 1$ (Fig. 4B and Fig. 5). Together these data suggest that HPV45 L1 interacts with cytoplasmic Kap $\alpha 2\beta 1$ heterodimers via an NLS located at the C-terminus of the protein and can exploit this interaction to enter the nuclei of host cells.

Six Kap α family members have been identified in higher eukaryotes. These sequences are

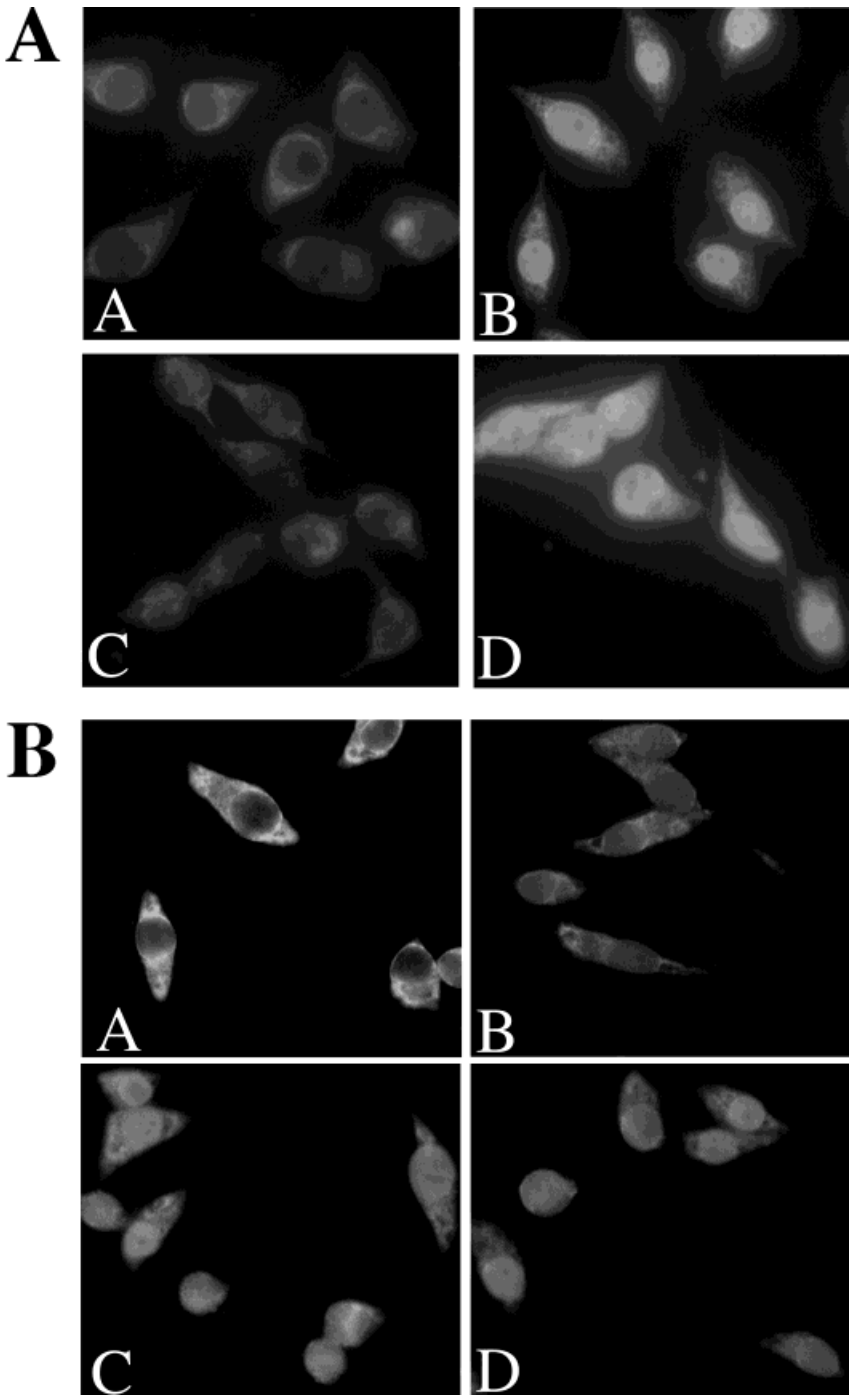

Fig. 4. Nuclear import of GST-$NLS_{HPV45L1}$ can be efficiently mediated by Kap α2β1 heterodimers. **A:** Digitonin-permeabilized HeLa cells were incubated for 30 min at room temperature with either GST-$NLS_{HPV45L1}$ (A and B), or GST-$NLS_{HPV11L1}$ (C and D) in the presence of either buffer A (A and C) or HeLa cytosol (B and D). Note the nuclear import in panels B and D. **B:** Digitonin-permeabilized HeLa cells were incubated for 30 min at room temperature with GST-$NLS_{HPV45L1}$ in the presence of either Kap β1 + RanGDP (A), or Kap β1 + RanGDP + p10 (B), or Kap β1 + Kap α2 + RanGDP (C), or Kap β1 + Kap α2 + RanGDP + p10 (D). Note the nuclear import of GST-$NLS_{HPV45L1}$ in the presence of Kap β1 + Kap α2 + RanGDP (C and D). The addition of p10 (D) did not increase the nuclear import.

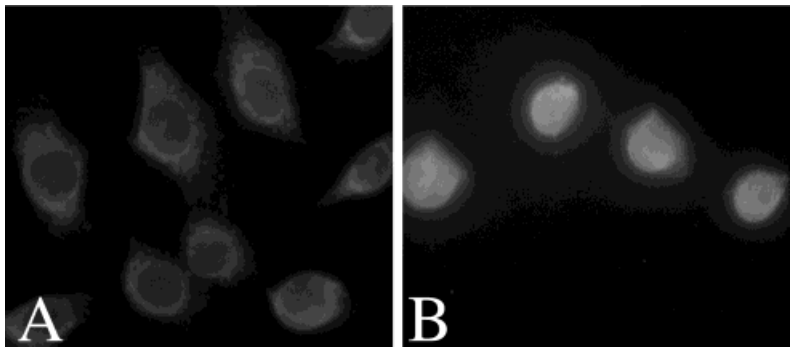

Fig. 5. Nuclear import of HPV45 L1 protein can be efficiently mediated by Kap α2β1 heterodimers. Digitonin-permeabilized HeLa cells were incubated for 30 min at room temperature with HPV45 L1 capsomers in the presence of either Kap β1 + RanGDP (**A**), or Kap β1 + Kap α2 + RanGDP (**B**). Note the nuclear import in (B).

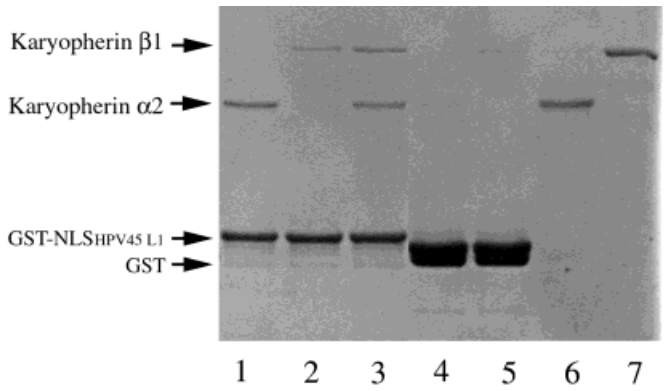

Fig. 6. Analysis of the interactions between HPV45 L1′NLS and karyopherins. GST-$NLS_{HPV45L1}$ immobilized on glutathione-sepharose beads (2 μg protein/10 μl beads) was incubated with either 1 μg of Kap α2 (**lane 1**), or 1 μg of Kap β1 (**lane 2**), or both Kap α2 and Kap β1 together (**lane 3**). The bound Kaps were eluted with sample buffer and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. As a specificity control, GST immobilized on glutathione-sepharose (4 μg protein/10 μl beads) was incubated with either Kap α2 (**lane 4**) or Kap β1 (**lane 5**); 1 μg of Kap α2 (**lane 6**) and 1 μg of Kap β1 (**lane 7**).

between 45% and 85% identical to one another, and all Kap α isoforms bind to Kap β1 [Görlich et al., 1995; O'Neill and Palese, 1995; Moroianu et al., 1995a, 1995b; Weis et al., 1995; Malik et al., 1997; Kohler et al., 1999; for review see Mattaj and Englmeier, 1998]. The import substrate specificity of all six Kap α isoforms has been analyzed for four cargoes (a classic NLS-BSA, nucleoplasmin, P/CAF and hnRNP K). The data indicate that when the protein cargoes were tested singly, they were imported by all Kap α isoforms with similar efficiencies. Differences in cargo preferences of various Kap α proteins appeared when two cargoes were presented simultaneously [Kohler et al., 1999]. It is likely that HPV45 L1 interacts also with other members of the Kap α family and could exploit these interactions for import into the nucleus of host cells.

HPVs infect the proliferating cells of the basal layers of the epithelium, whereas expression of L1 and L2 capsid proteins occurs only in the terminally differentiated keratinocytes located in the more superficial layers of the epithelium. As a consequence, in the initial stage of infection, HPV L1 proteins are transported into the nuclei of proliferating epithelial cells, whereas in the late productive phase they are transported into the nuclei of terminally differentiated keratinocytes. The nuclear import assays in this study have been carried out in digitonin-permeabilized HeLa cells, that are commonly used in investigating nuclear import pathways mediated by different Kaps [Melchior et al., 1993; Adam and Adam, 1994; Chi et al., 1995; Görlich et al., 1995; Paschal and Gerace, 1995; Weis et al., 1995; Schwoebel et al., 1998; Merle et al., 1999; Ribbeck et al.,

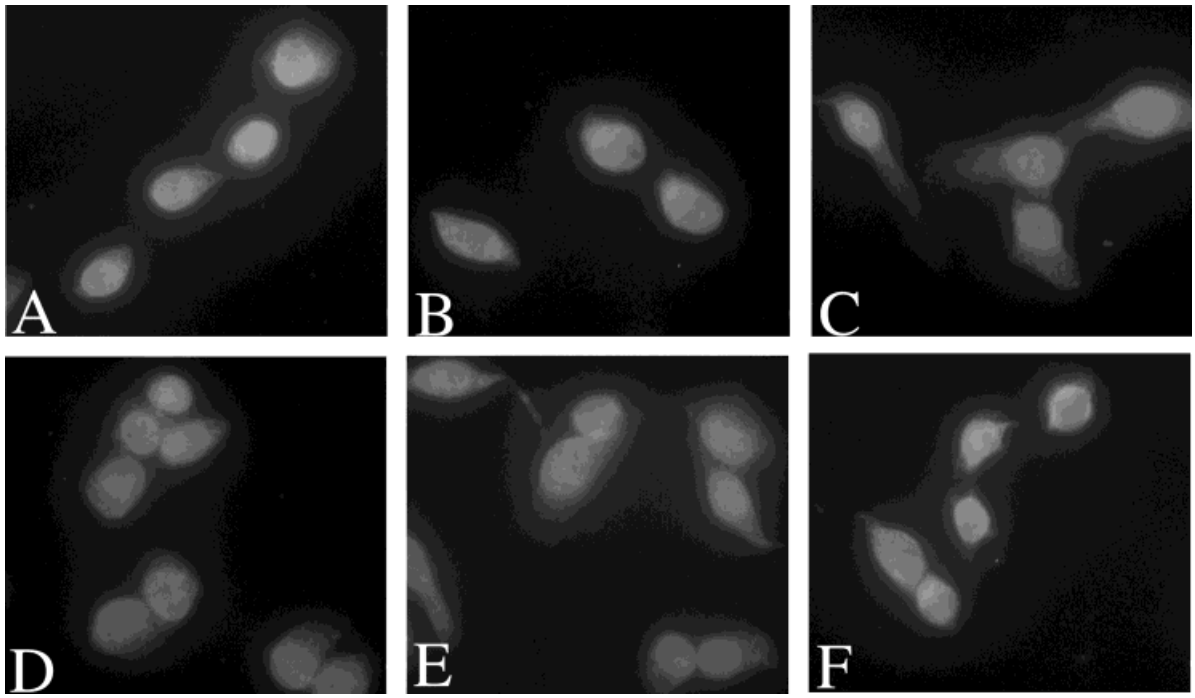

Fig. 7. GTP hydrolysis is not required for karyopherin α2β1-mediated nuclear import of GST-$NLS_{HPV45L1}$. Digitonin-permeabilized HeLa cells were incubated for 30 min at room temperature with GST-$NLS_{HPV45L1}$ in the presence of either Kap α2, β1, RanGDP (**A–C**) or Kap α2, β1, RanGDP and p10 (**D–F**). The added NTPs were as follows: GTP (A and D); GTPγS (B and E), and GMP-PNP (C and F). Note the nuclear import of GST-$NLS_{HPV45L1}$ in the presence of either GTP, or GTPγS, or GMP-PNP.

1999]. HeLa cells are undifferentiated, proliferating cells derived from a human cervical carcinoma cell line (HPV18 positive), and they express well Kap α2, Kap β1, and Ran. This would suggest that in the initial stage of infection, HPV45 L1 protein is imported into the nucleus via Kap α2β1 heterodimers. It is not known whether there are differences in the expression of Kap α2 and Kap β1 between the proliferating versus terminally differentiated epithelial cells that would translate into differences in nuclear import pathways. It has been reported that Kap β1 and Ran are ubiquitously expressed in various human adult tissues. Low levels of Kap β1 were found only in spleen [Kohler et al., 1999]. Most Kap α isoforms (including Kap α2) are well expressed in various human tissues, with lower levels in brain and liver [Kohler et al., 1999]. The expression of Kap α proteins and Kap β1 in terminally differentiated epithelial cells remains to be established in the future.

We previously established that the L1 capsid protein of low-risk HPV11 binds Kap α2 and can enter the nucleus of host cells via Kap α2β1-mediated pathway [Merle et al., 1999]. The NLSs of HPV11 L1 (this study) and HPV16 L1 [Zhou et al., 1991] are conserved (Table I). This suggests that HPV16 L1 most likely can use the Kap α2β1-mediated pathway to enter the nuclei of host cells. Other viral proteins, such as the influenza virus nucleoprotein and the matrix protein of HIV-1, can enter the nuclei of host cells via Kap α2β1 heterodimers [O'Neill et al., 1995; Gallay et al., 1996].

Previous studies, which used recombinant transport factors and nonhydrolyzable GTP analogues, suggested that GTP (or NTP) hydrolysis by Ran is not required for Kap α2β1, or Kap β2-mediated nuclear import of a classic NLS-HSA substrate, or M9-cargo, respectively [Schwoebel et al., 1998; Ribbeck et al., 1999]. We used nonhydrolyzable GTP analogues and determined that nuclear import of GST-$NLS_{HPV45L1}$, as well as GST-$NLS_{HPV11L1}$, requires RanGDP, but is independent of GTP hydrolysis by Ran (Fig. 4B and Fig. 7, and data not shown), in agreement with these previous data [Schwoebel et al., 1998; Ribbeck et al., 1999].

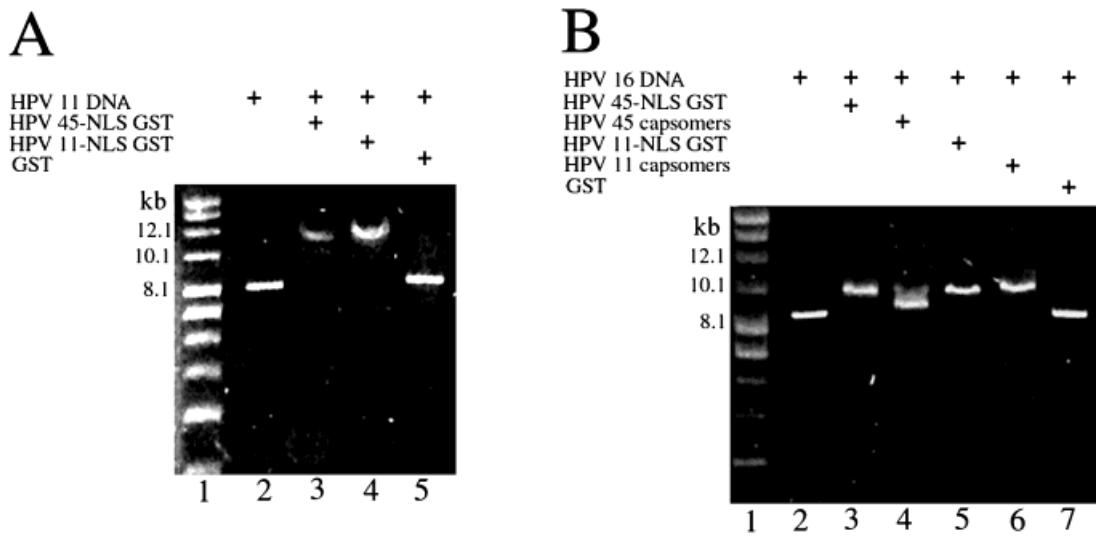

Fig. 8. HPV45 and HPV11 L1 proteins interact with different HPV-DNAs via their NLS sequences. **A:** Interaction of HPV11-DNA with HPV45 L1′NLS and HPV11 L1′NLS. HPV11-DNA (**lane 2**) was incubated for 30 min at room temperature with either GST-$NLS_{HPV45L1}$ (**lane 3**), or GST-$NLS_{HPV11L1}$ (lane 4), or GST (lane 5). **B:** Interaction of HPV16-DNA with HPV45 L1′NLS and HPV11 L1′NLS. HPV16-DNA (**lane 2**) was incubated for 30 min at room temperature with either GST-$NLS_{HPV45L1}$ (**lane 3**), or HPV45 L1 capsomers (**lane 4**), or GST-$NLS_{HPV11L1}$ (**lane 5**), or HPV11 L1 capsomers (**lane 6**), or GST (**lane 7**). The DNA and DNA–protein complexes were analyzed by agarose electrophoresis. A supercoiled DNA ladder was used for molecular weight markers (**lane 1**).

DNA Binding Activity of HPV45 L1 Capsomers

DNA mobility shift assays suggested that both HPV45 L1 and HPV11 L1 capsomers can interact nonspecifically with different HPV-DNAs (Fig. 8). Furthermore, we found that the DNA-binding region and the NLS sequence of either HPV45 L1, or HPV11 L1 overlap (Fig. 8). The overlap of the two binding activities could suggest that after L1 enters the nucleus, the interaction of L1 with the DNA would dissociate the Kap α2/L1 complex with release of Kap α2 for subsequent nuclear export and recycling for other rounds of import. Our data on HPV11 L1 are in agreement with previous southwestern assays that have shown that HPV11 L1 binds nonspecifically to plasmid DNA [Li et al., 1997].

The physiological significance of the interaction between HPV L1 capsomers and the viral DNA both in the early stages of infection, when the genomic DNA and capsid proteins are imported into the nucleus, and in the later stages of infection, when the newly replicated viral DNA is assembled into virions inside the host nucleus, requires further investigation.

ACKNOWLEDGMENT

We thank Karsten Weis for Kap α2/hSRP1α expression plasmid, Stephen Adam for Kap β1 expression plasmids, Yuh Min Chook and Günter Blobel for Kap β2 expression plasmid, and Peter Howley for HPV16/18-genomic DNA plasmids. We also thank Eric Merle for preliminary experiments and help with preparation of figures, and Meredith Garey for technical assistance. This work was supported by a Research Project Grant 99-210-01-MBC from the American Cancer Society to J.M. and an internal grant from Boston College to J.M.

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