Nuclear import strategies of high-risk HPV18 L2 minor capsid protein

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Abstract

We have investigated the nuclear import strategies of high-risk HPV18 L2 minor capsid protein. HPV18 L2 interacts with Kap α2 adapter, and Kap β2 and Kap β3 nuclear import receptors. Moreover, binding of RanGTP to either Kap β2 or Kap β3 inhibits their interaction with L2, suggesting that these Kap β/L2 complexes are import competent. Mapping studies show that HPV18 L2 contains two NLSs: in the N-terminus (nNLS) and in the C-terminus (cNLS), both of which can independently mediate nuclear import. Both nNLS and cNLS form a complex with Kap α2β1 heterodimer and mediate nuclear import via a classical pathway. The nNLS is also essential for the interaction of HPV18 L2 with Kap β2 and Kap β3. Interestingly, both nNLS and cNLS interact with the viral DNA and this DNA binding occurs without nucleotide sequence specificity. Together, the data suggest that HPV18 L2 can interact via its NLSs with several Kaps and the viral DNA and may enter the nucleus via multiple import pathways mediated by Kap α2β1 heterodimers, Kap β2 and Kap β3.© 2006 Elsevier Inc. All rights reserved.

Keywords: HPV; L2 minor capsid protein; Nuclear import; Karyopherins

Introduction

About 120 distinct human papillomavirus (HPV) genotypes have been identified, infecting either the skin or oral/anogenital mucosal epithelial tissues. Mucosal HPVs have shown varying degrees of oncogenic potential: high-risk HPV types are frequently detected in invasive cervical carcinomas and other anogenital cancers, whereas the low-risk HPV types are associated with genital warts (benign exophytic condylomas). HPV16 is the most common type found in cervical cancers (50%), followed by HPV18 (15%), HPV45 (8%), and HPV31 (5%). The remaining cervical cancers contain other less common high-risk HPV types (33, 35, 39, 51, 52, 54, 56, 58, 59, 66, 68, and 69) (zur Hausen, 2000). Although HPV16 is the most prevalent type in cervical cancers, patients with HPV18-associated tumors have a relative risk of death several times greater than that of patients with HPV18-associated carcinomas (Lombard et al., 1998).

HPV virions (55–60 nm in diameter) consist of a single molecule of 8 kb double-stranded circular DNA contained within an icosahedral capsid comprising the L1 major and L2 minor capsid proteins (Doorbar and Gallimore, 1987). The abundance of the L2 minor capsid protein is about 1/30 that of L1 (Roden et al., 1996). The L2 protein, which interacts with the L1 homopentamers (Chen et al., 2000), is essential for the infectivity of virions, and its roles in the viral infection are starting to be elucidated. Analysis of the HPV life cycle in raft cultures has shown that L2 contributes to at least two steps in the production of infectious virus (Holmgren et al., 2005). HPV16 L2 minor capsid protein binds to cells (Kawana et al., 1998; Yang et al., 2003a) and also interacts with β-actin and these interactions are essential for infection (Yang et al., 2003b). Recently, it has been shown that L2 contains a membrane-destabilizing peptide that is required for egress of papillomavirus genome from endosomes during the initial phase of infection (Kamper et al., 2006). Interestingly, BPV1 pseudovirions containing wild-type L1 and derivatives of L2 lacking either the N-terminal or the C-terminal positively charged termini are noninfectious despite efficient binding to the cell surface and endocytosis (Roden et al., 2001). These BPV1 L2 termini can function as NLSs in nuclear import of L2 via a

Abbreviations: HPV, Human papillomavirus; NLS, Nuclear localization signal; GST, glutathione S-transferase; Kap, karyopherin.

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classical pathway and the cNLS is also the major DNA binding site of BPV1 L2 (Fay et al., 2004). Moreover, a BPV1 L2 mutant encoding a scrambled version of the cNLS, which supports production of virions, rescues the DNA binding but not the interaction with the karyopherins and nuclear import (Fay et al., 2004). These data together with the finding that the incoming L2 and genome colocalize in the nucleus in the initial stage of infection (Day et al., 2004) would suggest that L2 may facilitate the nuclear import of the genome after virion disassembly. L2 is also imported into the nucleus in the productive stage of infection when the newly synthesized L2 together with L1 encapsidate the newly replicated viral DNA into virions. Interestingly, expression and nuclear import of L2 during the productive stage of the viral cycle precede the expression and nuclear translocation of L1 in natural lesions (Florin et al., 2002).

Signal-mediated nuclear import of proteins is performed by import receptors belonging to the karyopherin β/importin β (Kap β/Imp β) superfamily that interact with nucleoporins at the nuclear pore complex to transport the proteins into the nucleus (Fried and Kutay, 2003; Moroianu, 1999). There are more than 20 members of the karyopherin β superfamily in higher eukaryotes transporting a variety of proteins into the nucleus. Kap β1/importin β, the first one identified, together with a Kap α/importin α adapter can mediate nuclear import of proteins that contain basic monopartite or bipartite NLSs via the classical nuclear import pathway. Kap β1 can also function without adapters in the import of proteins containing longer basic NLSs such as ribosomal proteins, core histones, and several viral proteins, or in conjunction with Imp7, it mediates nuclear import of histone H1. Mammalian Kap β3/transportin mediates nuclear import of hnRNP A1 and A2, ribosomal proteins, and core histones. Interestingly, the ribosomal proteins and core histones can interact with several nuclear import receptors (Kap β3/transportin, Kap β3/Imp5, Kap β4, and Imp7) and enter the nucleus via multiple redundant pathways. Binding of nuclear Ran-GTP to the Kap β3/importins leads to the dissociation of the import complexes with the release of the transported proteins inside the nucleus (Fried and Kutay, 2003; Moroianu, 1999).

We have previously determined that the L2 capsid protein of high-risk HPV16 interacts with several karyopherins and can enter the nucleus via multiple import pathways (Darshan et al., 2004). In this study, we investigated the interactions of high-risk HPV18 L2 minor capsid protein with the karyopherins and with the viral DNA and mapped and characterized its NLSs and DNA binding sites.

**Results and discussion**

HPV18 L2 has two NLSs that can mediate nuclear import via a classical pathway

Our previous analysis of HPV16 L2 revealed that it contains two NLSs, localized near the N-terminus and the C-terminus, which can independently mediate nuclear import of HPV16 L2 (Darshan et al., 2004). These sequences, rich in lysine and arginine residues, are partially conserved in HPV18 L2. To determine if these sequences in HPV18 L2 function as NLSs, we made GST fusion proteins containing the corresponding N-terminal sequence (nNLS = MVSHRAARRKRA) and the C-terminal sequence (cNLS = PKKRKRV) of HPV18 L2 and analyzed them in nuclear import experiments, overlay blot, and binding assays.

We used nuclear import assays in digitonin-permeabilized cells to analyze the ability of each of these potential NLSs of HPV18 L2 to mediate nuclear import of a GST reporter protein. 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. Digitonin-permeabilized HeLa cells were incubated with either GST-nNLS, GST-cNLS, GST-NLS16L1, or GST itself in the presence of either only transport buffer (Fig. 1, panels A–D) or exogenous cytosol (Fig. 1, panels E–H). We found that both nNLS and cNLS were able to mediate nuclear import of the GST reporter protein in the presence of
cytosol (Fig. 1, panels E and F), as well as the positive control GST-NLS_{16L1} (Fig. 1, panel G). As expected, the GST-negative control was not imported into the nuclei in the presence of cytosol (Fig. 1, panel H). We also tested in nuclear import a GST fusion protein containing a middle sequence \((287 \text{LTR} \text{R}_{	ext{GTVRFSRLGQRATMFTR}}_{308})\) of HPV18 L2 rich in basic amino acids (called mL2_{18}). This mL2_{18} was unable to mediate nuclear import of the GST reporter in digitonin-permeabilized cells in the presence of exogenous cytosol (data not shown). Moreover, in transfection experiments with EGFP-L2 and EGFP-L2\Delta N\Delta C variant (lacking both nNLS and cNLS) in GP2-293 cells, the EGFP-L2\Delta N\Delta C localized in the cytoplasm (Fig. 2, panel E) in contrast with the wild-type EGFP-L2, which was mostly in the nucleus (Fig. 2, panel D). We obtained similar results in transfection experiments with EGFP-L2 and EGFP-L2\Delta N\Delta C variant in HeLa cells (data not shown). To note, that although the EGFP is small enough to passively diffuse through the nuclear pore complex, the EGFP-L2 (as well as L2 itself) is well above the limit of passive diffusion and its nuclear localization requires NLS(s). These data suggest that when both nNLS and cNLS are deleted, the HPV18 L2 protein is no longer able to enter the nucleus.

As both nNLS and cNLS resemble classical monopartite type of NLSs, we first tested the interaction of HPV18 L2 and its NLSs with the Kap\(\alpha_2\) adapter using overlay blot assays, as we described for HPV16 L2 (Darshan et al., 2004). Incubation of HPV18 L2 blots with increasing concentrations of Kap\(\alpha_2\) resulted in increased binding of Kap\(\alpha_2\) to L2 (Fig. 3, lanes 3–5). Overlay blot analysis of the interactions of HPV18 L2 NLSs with Kap\(\alpha_2\) showed that the cNLS interacted with Kap\(\alpha_2\) with high affinity (Fig. 4, lanes 7–10) and the nNLS with much lower affinity than the cNLS (Fig. 4, compare lanes 2–5 with lanes 7–10). As expected, the NLS of HPV16 L1, used as a positive control, interacted with Kap\(\alpha_2\) (Fig. 4, lanes 12–15), whereas the GST itself did not (Fig. 4, lane 17).

In agreement with these data, the binding assays with the GST-NLSs immobilized on glutathione–Sepharose beads and incubated with the Kaps in solution revealed that both nNLS and cNLS formed a complex with Kap\(\alpha_2\beta_1\) heterodimers (Fig. 5, lanes 1 and 2) via interaction with Kap\(\alpha_2\) (Fig. 5, lanes 3 and 6), although the nNLS had a lower affinity (Fig. 5, compare lanes 1 and 3 with lanes 4 and 6). Neither the nNLS nor the cNLS interacted directly with Kap\(\beta_1\) (Fig. 5, lanes 2 and 5). As expected, the NLS_{16L1}, used as a positive control, formed a complex with Kap\(\alpha_3\beta_1\) heterodimers via interaction with Kap\(\alpha_3\) (Fig. 5, lanes 7 and 9), whereas the GST did not (Fig. 5, lanes 10 and 12).

Analysis of nuclear import of the GST-NLSs of HPV18 L2 in digitonin-permeabilized cells revealed that both nNLS and the cNLS could mediate nuclear import of a GST reporter protein in the presence of recombinant Kap\(\alpha_3\beta_1\) heterodimers plus RanGDP, with the cNLS being more efficient than the nNLS.
As expected, the GST-NLS16L1, used as a positive control for the classical pathways, was also imported in the presence of Kap α2β1 heterodimers plus RanGDP (Fig. 6, panel G), whereas the GST-negative control was not (Fig. 6, panel H). Together, the data indicate that HPV18 L2 has two NLSs, one at the N-terminus and another at the C-terminus, which can form complexes with Kap α2β1 heterodimers and independently mediate nuclear import via a classical pathway. Although HPV16 L2 also has two NLSs interacting with Kap α2β1 heterodimers, there are differences in the binding affinities of the two NLSs of HPV16 L2 versus HPV18 L2: the cNLS of HPV18 L2 has a higher affinity than the nNLS, whereas the opposite is true for HPV16 L2 (Darshan et al., 2004). The biological significance of this difference between the NLSs of HPV16 L2 and HPV18 L2, if any, remains to be established.

We next analyzed if HPV18 L2 can interact directly with Kap β nuclear import receptors. The L2 blots were incubated with increasing concentrations of either GST-Kap β2 (Fig. 7A, lanes 3–6) or GST-Kap β3 (Fig. 8A, lanes 3–5), and the bound GST-Kaps were detected with an anti-GST antibody. We determined that GST-Kap β2 bound to L2 at concentrations of 5 and 10 μg/ml (Fig. 7A, lanes 5 and 6) but not at concentrations of 1 and 2.5 μg/ml (Fig. 7A, lanes 3 and 4). The GST alone did not interact with HPV18 L2 (Fig. 7A, lane 2). Significantly, Ran-GTP inhibited the interactions between GST-Kap β2 and L2 (Fig. 7B, compare lanes 1 and 2), suggesting that the complex between L2 and Kap β2 can be

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**Fig. 4.** The nNLS and cNLS of HPV18 L2 interact with Kap α2 adapter with different affinities. Blots containing GST-nNLS18L2 (lanes 2–5), GST-cNLS18L2 (lanes 7–10), or GST-NLS16L1, as a positive control (lanes 12–15), or GST, as a negative control (lane 17), were incubated with increasing amounts of Kap α2 and the bound Kap α2 was detected with an anti-Kap α2 antibody. The different GST-NLS proteins and the GST were detected with an anti-GST antibody (lanes 1, 6, 11, and 16 respectively).

**Fig. 5.** Both nNLS and cNLS of HPV18 L2 form a complex with Kap α2β1 heterodimers. GST-nNLS18L2 (lanes 1–3), GST-cNLS18L2 (lanes 4–6), GST-NLS16L1 (lanes 7–9), and GST (lanes 10–12) immobilized on glutathione–Sepharose (2 μg/10 μl beads) were incubated with either Kap α2 (lanes 1, 4, 7, and 10), Kap β1 (lanes 2, 5, 8, and 11), or Kap β1 + Kap α2 (lanes 3, 6, 9, and 12). Bound proteins were eluted with sample buffer and analyzed by SDS–PAGE and Coomassie blue staining. The input of Kap α2 and Kap β1 is shown in lanes 13 and 14.

**HPV18 L2 interacts directly with Kap β2 and Kap β3 nuclear import receptors and deletion of its nNLS inhibits these interactions**
dissociated by nuclear Ran-GTP to release L2 into the nucleus. Interestingly, we also found that L2 binds with high affinity to the Kap $\beta_3$ receptor (Fig. 8A, lanes 4 and 5) and Ran-GTP inhibited the interaction between GST-Kap $\beta_3$ and L2 (Fig. 8B, compare lanes 1 and 2). The GST-negative control did not bind to the L2 protein (Fig. 8A, lane 3). The high affinity binding of HPV18 L2 to Kap $\beta_3$ would suggest that the Kap $\beta_3$-mediated nuclear import pathway may be preferentially used by L2 in conditions of competition with host proteins for the classical pathway.

To determine the binding domain(s) of HPV18 L2 for Kap $\beta_2$ and Kap $\beta_3$ import receptors, we performed binding assays with the wild-type L2 and the corresponding L2 variants (L2ΔN lacking the nNLS, L2ΔC lacking the cNLS, and L2ΔNΔC lacking both NLSs). GST-Kap $\beta_2$ and GST-Kap $\beta_3$ immobilized on glutathione–Sepharose beads were incubated with either wild-type L2 or the L2 mutants (Fig. 9). The wild-type L2 and the L2ΔC mutant bound to both Kap $\beta_2$ and Kap $\beta_3$ (Fig. 9, lanes 2, 4, 7, and 9), whereas the L2ΔN and L2ΔNΔC mutants did not (Fig. 9, lanes 3, 5, 8, and 10). The wild-type L2 did not interact with the GST itself (Fig. 9, lane 12). These data indicate that the nNLS of HPV18 L2 is essential for the interaction with both Kap $\beta_2$ and Kap $\beta_3$ receptors. However, in binding assays the GST-nNLS immobilized on glutathione–Sepharose beads did not interact with either Kap $\beta_2$ or Kap $\beta_3$ (data not shown). This suggests that although the nNLS is required for the interaction with Kap $\beta_{2/3}$ it is not sufficient, and the binding sites may contain additional amino acids.

Previously, we have shown that the high-risk HPV16 L2 interacts with Kap $\alpha_2\beta_1$ heterodimers and Kap $\beta_2$ and Kap $\beta_3$ import receptors (Darshan et al., 2004). Thus, HPV18 L2 has a similar pattern of interactions as HPV16 L2. Although the BPV1 L2 protein interacts with Kap $\alpha_2\beta_1$ heterodimers, it does not interact with either Kap $\beta_2$ or Kap $\beta_3$ receptors (Fay et al., 2004). These data show that the HPV L2 capsid proteins evolved multiple interactions with the host nuclear import receptors in comparison with the bovine papillomavirus L2 protein, and consequently they are able to enter the nucleus of host cells via different import pathways.

Fig. 6. Both nNLS and cNLS can mediate nuclear import via a classical pathway. Digitonin-permeabilized HeLa cells were incubated with either GST-nNLS (panels A and E), GST-cNLS (panels B and F), GST-NLS$_{16L1}$ (panels C and G), or GST as a negative control (panels D and H) in the presence of either only transport buffer (panels A–D) or Kap $\alpha_2\beta_1$ heterodimers plus RanGDP (panels E–H). Note the nuclear import in panels E–G.

Fig. 7. HPV18 L2 interacts with Kap $\beta_2$ nuclear import receptor and RanGTP inhibits this interaction. (A) HPV18 L2 blots were incubated with either anti-L2 Ab (lane 1), or with 5 μg/ml GST (lane 2), or with increasing amounts of GST-Kap $\beta_2$ (lanes 3–6). Bound GST-Kap $\beta_2$ and GST were detected with an anti-GST Ab. (B) HPV18 L2 blots were incubated with GST-Kap $\beta_2$ in the absence (lane 1) or presence of RanGTP (lane 2).

Fig. 8. HPV18 L2 interacts with Kap $\beta_3$ nuclear import receptor and RanGTP inhibits this interaction. (A) HPV18 L2 blots were incubated with either anti-L2 Ab (lane 1) or with 5 μg/ml GST (lane 2) or with increasing amounts of GST-Kap $\beta_3$ (lanes 3–6). Bound GST-Kap $\beta_3$ and GST were detected with an anti-GST Ab. (B) HPV18 L2 blots were incubated with GST-Kap $\beta_3$ in the absence (lane 1) or presence of RanGTP (lane 2).
Fig. 9. Deletion of the nNLS of HPV18 L2 inhibits its interaction with Kap \( \beta_2 \) and Kap \( \beta_3 \) receptors. GST-Kap \( \beta_2 \) immobilized on glutathione–Sepharose beads (lane 1) was incubated with either wild-type L2 (lane 2), L2\( \Delta N \) (lane 3), L2\( \Delta C \) (lane 4), or L2\( \Delta N \Delta C \) (lane 5). GST-Kap \( \beta_3 \) immobilized on glutathione–Sepharose beads (lane 6) was incubated with either wild-type L2 (lane 7), L2\( \Delta N \) (lane 8), L2\( \Delta C \) (lane 9), or L2\( \Delta N \Delta C \) (lane 10). GST immobilized on glutathione–Sepharose beads (lane 11) was incubated with wild-type L2 (lane 12). The input of wild-type L2 (lane 13), L2\( \Delta N \) (lane 14), L2\( \Delta C \) (lane 15), and L2\( \Delta N \Delta C \) (lane 16).

Both nNLS and cNLS of HPV18 L2 protein can interact with the viral DNA

Previous results with BPV1 L2 revealed that it interacts with the DNA in a sequence-independent manner and that its cNLS is the major DNA binding site (Fay et al., 2004). Here we analyzed the interactions of the nNLS and cNLS of HPV18 L2 and HPV16 L2 with the HPV16 DNA plasmid using DNA mobility shift assays as described (Fay et al., 2004). We found for both HPV18 L2 and HPV16 L2 that both nNLS and cNLS interacted efficiently with the DNA, with the NLSs of HPV18 L2 having a higher affinity than the NLSs of HPV16 L2 (Fig. 10A). To investigate if the interactions are DNA sequence specific, we also incubated an unrelated DNA ladder with the GST-NLSs of HPV18 L2 and HPV16 L2. Both nNLS and cNLS of HPV18 L2 and HPV16 L2 interacted with the unrelated DNA demonstrating that the interactions are independent of the DNA sequence (data not shown). We also tested for DNA interactions the Arg/Lys-rich sequences located in the middle of HPV18 L2 (mL2\( \Delta 18 \): \( \Delta 285LTSSRRTGTVRSRLGQRAGMFTR\Delta 308 \)) and HPV16 L2 (mL2\( \Delta 18 \): \( \Delta 296SSRTGIRYSRIGIN\Delta 308 \)). The basic residues of these sequences are mostly conserved in HPV L2 proteins, but they are not in the BPV1 L2 protein. DNA mobility shift assays showed that both mL2\( \Delta 16 \) and mL2\( \Delta 16 \) interact with the DNA (Fig. 10B, lanes 2–7). However, comparison of the interactions of wild-type HPV L2 and the L2\( \Delta N \Delta C \) double mutant with the DNA revealed that deletion of both NLSs of HPV18 L2 strongly inhibits its interaction with the DNA (Fig. 10C, compare lanes 2 and 3 with lanes 4, 5, and 6). Similar results were obtained for HPV16 L2 (data not shown). These data suggest that the major DNA binding sites for the L2 minor capsid proteins of HPV18/16 are the nNLS and cNLS. However, we cannot exclude the possibility that the Arg/Lys mL2 sequence may contribute to the interaction of L2 with the DNA during the viral cycle. For BPV1 L2, we have previously found that only the cNLS interacts efficiently with the DNA (Fay et al., 2004). Moreover, a BPV1 L2 mutant encoding a scrambled version of the cNLS, which rescues the production of infectious virions (Rodên et al., 2001), rescued the DNA binding but not the interaction with the karyopherins and nuclear import, suggesting that the role of the BPV1 L2 cNLS in infectivity may be its interaction with the viral DNA (Fay et al., 2004). For HPV18 and HPV16 L2 capsid proteins, both nNLS and cNLS can interact with the DNA, suggesting that HPV L2 proteins have the potential to facilitate nuclear import of the viral DNA in the initial phase of infection after virion disassembly. Future studies will determine if HPV L2 proteins can simultaneously interact with both the viral DNA and the karyopherins and mediate nuclear import of the DNA.

Overall our data in this study suggest that the L2 minor capsid protein of high-risk HPV18 can interact via its two NLSs with several karyopherins and the viral DNA and may enter the nucleus via multiple import pathways mediated by Kap \( \alpha_2 \beta_1 \), heterodimers, Kap \( \beta_2 \), and Kap \( \beta_3 \).

Fig. 10. The nNLS and cNLS of high-risk HPV18 L2 and HPV16 L2 are the major DNA binding sites. (A) HPV16 DNA plasmid (lane 1) was incubated with increasing amounts (1, 2, or 5 \( \mu \)g of protein) of either GST-nNLS\( \Delta 16L2 \) (lanes 2–4), GST-nNLS\( \Delta 16L2 \) (lanes 5–7), GST-cNLS\( \Delta 16L2 \) (lanes 8–10), GST-cNLS\( \Delta 16L2 \) (lanes 11–13), or 10 \( \mu \)g of GST (lane 14) and analyzed via agarose gel electrophoresis. (B) HPV16 DNA plasmid was incubated with 1, 2, and 5 \( \mu \)g of either GST-mL2\( \Delta 16 \) (lanes 2–4), GST-mL2\( \Delta 16 \) (lanes 5–7), 1 and 2 \( \mu \)g of GST-NLS\( \Delta 16L1 \), as a positive control (lanes 8 and 9), or 5 \( \mu \)g of GST (lane 10). (C) HPV16 DNA plasmid (lane 1) was incubated with 0.5 and 1 \( \mu \)g of wild-type HPV18 L2 (lanes 2 and 3), 0.5, 1, and 2 \( \mu \)g of L2\( \Delta N \Delta C \) double mutant (lanes 4–6), or 5 \( \mu \)g of GST (lane 7).
Materials and methods

Preparation of recombinant human nuclear import factors

His-tagged Kap α3/hSRP1α (Weis et al., 1995) and His-tagged Kap β1/p97 (Chi et al., 1996) were expressed in *Escherichia coli* BL21 Star and *E. coli* BL21(DE3), respectively (3 h induction with 2 mM IPTG at 30 °C), and the soluble His-tagged proteins were purified in their native state on Talon beads using a standard procedure. GST-Kap β1 (Chi et al., 1996), GST-Kap β2 (Chook and Blobel, 1999) were expressed in *E. coli* BL21(DE3), and GST-Kap β3 (Yaseen and Blobel, 1997) was expressed in *E. coli* BL21-CodonPlus (3 h induction with 1 mM IPTG at 30 °C), and the soluble GST-Kap β fusion proteins were purified in their native state on glutathione–Sepharose beads using a standard procedure. Human Ran (Coultavas et al., 1993) was prepared as described (Fler and Blobel, 1996). Ran was loaded with GTP by incubation with 100 mM GTP, 0.5 M EDTA, 1 M DTT, and 1 M HEPES (pH 7.4) for 30 min at RT and the reaction was stopped with 1 M magnesium chloride (pH 7.4). All proteins were checked for purity and lack of proteolytic degradation by SDS–PAGE and Coomassie blue staining. The purified proteins were dialedyzed in transport buffer (20 mM HEPES-KOH, pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 1 mM EGTA, 2 mM DTT, plus protease inhibitors) and stored in aliquots at −80 °C until use. HeLa cytosol from the National Cell Culture Center (Minneapolis, MN) was centrifuged and stored in small aliquots at −80 °C.

Preparation of HPV18 L2 protein and L2 mutants

The pProEX HTb plasmid vector containing the HPV18 L2 wild type (Rodén et al., 1994) was a kind gift from Dr. Richard Roden. To prepare the L2 deletion mutants (L2ΔN lacking aa 1–12, L2ΔC lacking aa 445–451, and the L2ΔNΔC double mutant), we used the QuikChange Site-Directed Mutagenesis Kit from Stratagene. The constructs were transformed into XL1 blue bacteria and confirmed by automated sequence analysis (MGH DNA Sequencing Department). The His-tagged HPV18 L2 wild-type (Rodén et al., 1994) and the corresponding L2 mutants were expressed in *E. coli* BL21-CodonPlus (2 h induction with 2 mM IPTG at 25 °C) and purified from inclusion bodies using the Novagen Protein Refolding kit as previously described (Roden et al., 2000). A mouse antibody to Kap α3/Rch1 was from Transduction Laboratories; a mouse antibody to 6× His tag and a goat anti-GST antibody were from Santa Cruz Biotechnology.

Overlay blot assays

For analysis of the interactions of HPV18 L2 with the karyopherins, we used overlay blot assays as previously described for HPV16 L2 (Darshan et al., 2004). Briefly, the wild-type L2 and the L2 mutants, different GST-NLS fusion proteins, or GST (all the proteins at 2 μg/lane), were subjected to SDS–PAGE and transferred to nitrocellulose membrane. After blocking, the individual blots were incubated with different Kaps and the bound Kaps detected with specific Abs as indicated in the figure legends.

In solution binding assays

Binding assays were performed as previously described (Nelson et al., 2002). Briefly, the different GST-NLSs, or GST constructs, the corresponding forward and reverse oligonucleotides containing an EcoRI and XhoI restriction-cutting sites were annealed and inserted directly into the pGEX4T-1 vector, which had been double cut with the same enzymes. The GST-NLS constructs were transformed in XL1 blue bacteria and confirmed by automated sequence analysis (MGH DNA sequencing department). For protein expression, the GST-nNLS, GST-ML218, and GST-cNLS constructs were used to transform *E. coli* BL21 CodonPlus. After induction of the bacteria with 1 mM IPTG for 2 h at 28 °C, the GST fusion proteins were purified in their native state on glutathione–Sepharose using a standard procedure. The GST-NLSHPV16L1 containing the monopartite NLS (AKRKKRKL) of HPV16 L1 major capsid protein was prepared as previously described (Nelson et al., 2002).

Preparation of GFP fusion proteins for transfection assays

The EGFP expression plasmid pEGFP-C1 (Clontech, Inc.) was used to construct the EGFP fusion protein expression plasmids (EGFP-L2, and EGFP-L2ΔNΔC lacking both nNLS and cNLS). The pEGFP-C1 plasmid was double digested with EcoRI and BamHI, run on a 0.7% agarose gel, and extracted by the protocol from the QIAquick Gel Extraction Kit (Qiagen). DNA fragments spanning the HPV18 L2 or the L2 mutant plasmids were amplified using PCR oligonucleotides that added an EcoRI and BamHI restriction endonuclease sites and the DNA fragments were ligated into the EcoRI and BamHI cloning sites on pEGFP-C1. The resulting plasmids were transformed into XL1-Blue cells, and the purified plasmids were confirmed by sequence analysis.

Antibodies

An Ab to HPV16 L2 that recognizes HPV18 L2 was obtained as described (Rodén et al., 2000). A mouse antibody to Kap α3/Rch1 was from Transduction Laboratories; a mouse antibody to 6× His tag and a goat anti-GST antibody were from Amersham Biosciences. HRP-conjugated secondary antibodies (anti-rabbit, anti-mouse, and anti-goat) were from Santa Cruz Biotechnology.

Preparation of GST-NLS fusion proteins

The N-terminal sequence (MVSHRAARRKRA12), the middle sequence (287LTSRRGTVFSLGQRATMFR303), and the C-terminal sequence (445PKKRKRV451) of HPV18 L2 were separately fused to a GST reporter to obtain the GST-nNLS, GST-ML218, and GST-cNLS. To make the GST-NLS proteins and L2 mutants, the corresponding forward and reverse oligonucleotides containing an EcoRI and XhoI restriction-cutting sites were annealed and inserted directly into the pGEX4T-1 vector, which had been double cut with the same enzymes. The GST-NLS constructs were transformed in XL1 blue bacteria and confirmed by automated sequence analysis (MGH DNA sequencing department). For protein expression, the GST-nNLS, GST-ML218, and GST-cNLS constructs were used to transform *E. coli* BL21 CodonPlus. After induction of the bacteria with 1 mM IPTG for 2 h at 28 °C, the GST fusion proteins were purified in their native state on glutathione–Sepharose using a standard procedure. The GST-NLSHPV16L1 containing the monopartite NLS (AKRKKRKL) of HPV16 L1 major capsid protein was prepared as previously described (Nelson et al., 2002).

Preparation of recombinant human nuclear import factors

His-tagged Kap α3/hSRP1α (Weis et al., 1995) and His-tagged Kap β1/p97 (Chi et al., 1996) were expressed in *Escherichia coli* BL21 Star and *E. coli* BL21(DE3), respectively (3 h induction with 2 mM IPTG at 30 °C), and the soluble His-tagged proteins were purified in their native state on Talon beads using a standard procedure. GST-Kap β1 (Chi et al., 1996), GST-Kap β2 (Chook and Blobel, 1999) were expressed in *E. coli* BL21(DE3), and GST-Kap β3 (Yaseen and Blobel, 1997) was expressed in *E. coli* BL21-CodonPlus (3 h induction with 1 mM IPTG at 30 °C), and the soluble GST-Kap β fusion proteins were purified in their native state on glutathione–Sepharose beads using a standard procedure. Human Ran (Coultavas et al., 1993) was prepared as described (Fler and Blobel, 1996). Ran was loaded with GTP by incubation with 100 mM GTP, 0.5 M EDTA, 1 M DTT, and 1 M HEPES (pH 7.4) for 30 min at RT and the reaction was stopped with 1 M magnesium chloride (pH 7.4). All proteins were checked for purity and lack of proteolytic degradation by SDS–PAGE and Coomassie blue staining. The purified proteins were dialyzed in transport buffer (20 mM HEPES-KOH, pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 1 mM EGTA, 2 mM DTT, plus protease inhibitors) and stored in aliquots at −80 °C until use. HeLa cytosol from the National Cell Culture Center (Minneapolis, MN) was centrifuged and stored in small aliquots at −80 °C.
itself, immobilized on glutathione–Sepharose beads (2 μg/10 μl beads) were incubated under rotation for 1 h at RT with the purified Kaps in binding buffer (transport buffer containing 0.01% Tween 20) as indicated in the figure legends. We also carried out binding assays with GST-Kap β2 and GST-Kap β3 immobilized on glutathione–Sepharose beads (2 μg/10 μl beads) incubated with wild-type L2 and the different L2 mutants (L2ΔN, L2ΔC, and L2ΔNΔC). The bound proteins were eluted with SDS–PAGE sample buffer and analyzed by SDS–PAGE and Coomassie Blue staining.

In vitro nuclear import assays

The nuclear import assays were carried out as previously described (Nelson et al., 2002). Briefly, subconfluent HeLa cells, grown on poly-L-lysine-coated glass coverslips for 24 h, were permeabilized with 70 μg/ml digitonin for 5 min on ice and washed with transport buffer. All import reactions contained an energy regenerating system (1 mM GTP, 1 mM ATP, 5 mM phosphocreatine, and 0.4 U creatine phosphokinase), plus either HeLa cytosol or various transport factors (1 μg Kap α2; 0.5 μg Kap β1; 3 μg RanGDP), plus the different GST-NLS fusion proteins (0.5 μg). Final import reaction volume was adjusted to 20 μl with transport buffer. For visualization of nuclear import, the GST-NLS fusion proteins were detected by immunofluorescence with an anti-GST antibody, as previously described (Nelson et al., 2002). The nuclei were identified by DAPI staining. Nuclear import was analyzed with a Nikon Eclipse TE 300 Microscope that has a fluorescence attachment and a Sony DKC-5000 CCD camera.

Transfection experiments

GP2-293 cells (BD Biosciences Clontech, CA) were plated on 12 mm poly-L-lysine-coated glass coverslips to 50–70% confluency 24 h prior to transfection. Cells in each well were transfected with 0.8 μg plasmid and 3 μl FUGENE 6 (Roche Applied Science, IN) in 500 μl DMEM prepared following product protocol. Media were changed to DMEM with 10% FBS and pen-strep after 6 h and the cells were fixed 24 h after the initial transfection with 10% formaldehyde in PBS (10 min). Coverslips were mounted using Vectashield–Dapi (Vector Labs, CA) mounting medium and examined by fluorescence microscopy using a Nikon Eclipse TE 300 Microscope.

DNA mobility shift assays

DNA mobility shift assays were performed as previously described (Fay et al., 2004). Briefly, the proteins to be tested for DNA binding were incubated with either HPV16 DNA plasmid or an unrelated DNA for 30 min at RT in transport buffer. GST-NLSHPV16L1 was used as a positive control and GST as a negative control. The DNA or DNA–protein complexes were analyzed by electrophoresis in 0.7% agarose gels.

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References


