A γ-2 Herpesvirus Nucleocytoplasmic Shuttle Protein Interacts with Importin α1 and α5*

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Herpesvirus saimiri (HVS) is the prototype γ-2 herpesvirus. This is an increasing important subfamily of herpesviruses due to the identification of the first human γ-2 herpesvirus, Kaposi’s sarcoma-associated herpesvirus. The HVS open reading frame (ORF) 57 protein is a multifunctional trans-regulatory protein homologous to genes identified in all classes of herpesviruses. Recent analysis has demonstrated that ORF 57 has the ability to bind viral RNA and to shuttle between the nucleus and cytoplasm, and is required for efficient nuclear export of viral transcripts. Here we have investigated the nucleocytoplasmic shuttling mechanism utilized by the ORF 57 protein. The yeast two-hybrid system was employed to identify interacting cellular proteins using ORF 57 as bait. We demonstrate that ORF 57 interacts with importin α isoforms 1 and 5. In addition, the binding of ORF 57 to importin α was mediated by the importin α hydrophobic internal armadillo repeats. An ORF 57 amino-terminal arginine-rich sequence, which functions as a nuclear localization sequence, was also required for this interaction. Furthermore, the ORF 57 protein is responsible for the redistribution of importin α into the nucleoli. These results identify novel cellular interactions essential for the functioning of this important herpesvirus regulatory protein.

A number of viruses including herpes, adenovirus, influenza, and retroviruses replicate in the host cell nucleus. In order to complete their virus replication cycle, they have devised a number of mechanisms to transport viral nucleic acids into and out of the nucleus. In particular, a variety of viruses encode nucleocytoplasmic shuttle proteins, which specifically mediate the nuclear export of viral RNA. Such virally encoded proteins include human immunodeficiency virus type 1 (HIV-1)1 Rev, herpes simplex virus type 1 (HSV-1) ICP27, influenza virus NEP, adenovirus E4orf6, and the herpesvirus saimiri (HVS) ORF 57 protein (1–6). Herpesvirus saimiri (HVS) is the prototype γ-2 herpesvirus, or rhadinovirus (7), an increasingly important family of viruses due to the recent identification of the first human γ-2 herpesvirus, Kaposi’s sarcoma-associated herpesvirus (8). Gene expression during HVS lytic replication is sequentially regulated and occurs in three main temporal phases: immediate-early, delayed-early, and late. The two major HVS transcriptional regulating proteins are encoded by the open reading frames (ORFs) 50 and 57 (9–12).

ORF 57 is a 52-kDa multifunctional trans-regulatory protein homologous to genes identified in all classes of herpesviruses. Transactivation of late viral genes by ORF 57 occurs independently of target gene promoter sequences and appears to be mediated at a post-transcriptional level (11). In addition to its transactivation properties, ORF 57 is responsible for repression of viral gene expression, which correlates with the presence of introns within the target gene (11–12). ORF 57 also redistributes both U2 and SC-35 splicing factors during an HVS infection into intense distinct nuclear aggregations (13). Recent analysis has demonstrated that the ORF 57 protein has the ability to bind viral RNA and shuttle between the nucleus and cytoplasm, and is required for efficient cytoplasmic accumulation of virus mRNA. This suggests that ORF 57 plays a pivotal role in mediating the nuclear export of viral transcripts (6).

An intriguing question regarding the functioning of virus-encoded nucleocytoplasmic shuttle proteins is the mechanism they utilize to be transported through the nuclear pore complex. Macromolecule trafficking into and out of the nucleus is mediated by soluble transport receptors (reviewed in Ref. 14). These receptors bind specific proteins or RNA cargoes and interact with nuclear pore proteins, which subsequently allow the translocation of the receptor cargo through the nuclear pore complex. Recently, CRM-1 (for chromosomal region maintenance 1) or exportin 1, a protein that shares homology with members of the importin-karyopherin nuclear transport pathway, has been identified as a nuclear export receptor for proteins, including HIV-1 Rev, carrying a leucine-rich NES in a process that also requires the GTP-bound form of Ran (15–16). Furthermore, CRM-1 has been shown to interact with nuclear pore complex proteins, namely the nucleoporins CAN/Nup214 and Nup88 (16), suggesting that CRM-1 is the bridging protein for the interactions of NES-containing proteins and the nuclear pore complex. However, it has recently been shown for the herpesviruses HSV-1 and Epstein-Barr virus that, although export of some viral RNAs require the CRM-1 pathway, a proportion of viral RNA export can be mediated by a CRM-1-independent pathway (17–18). This suggests that at least some herpesvirus nucleocytoplasmic shuttle proteins may function through a distinct, as yet unidentified, export mechanism.

In addition, it is tempting to speculate that the virally encoded nucleocytoplasmic shuttle proteins must interact with cellular nuclear import pathways. The most widely characterized transport pathway mediates the nuclear import of proteins that contain a classical nuclear localization signal (NLS) (re-
viewed in Ref. 14). These basic, generally lysine-rich NLS serve as recognition sites for an NLS receptor termed importin α or karyopherin α (19), which forms a heterodimeric complex with importin β or karyopherin β. Importin β functions as a transport adapter molecule binding to the nuclear pore complex via a direct interaction with specific nucleoporins (20, 21). Once in the nucleus, binding of Ran-GTP to importin β causes dissociation of the import complex (22, 23). Once released from the cargo, importin subunits are then recycled to the cytoplasm. Importin β is recycled rapidly, whereas the export of importin α is mediated by the nuclear export factor, CAS, which binds importin α preferentially in the presence of Ran-GTP (24–26).

In the cytoplasm the importin molecules are released by the action of RanBP1 and RanGAP1 (27–29), allowing participation in additional rounds of nuclear import.

In this report we have investigated the nucleocytoplasmic shuttling mechanism utilized by the HVS ORF 57 protein. The yeast two-hybrid system was employed to identify interacting cellular proteins using ORF 57 as bait. Here we show that ORF 57 interacts with importin α isoforms 1 and 5. Confirmation of this interaction was provided by co-immunoprecipitation experiments from transfected and infected cells. In addition, the binding of ORF 57 to importin α is mediated via the hydrophobic, internal, importin α armadillo (arm) repeat. Moreover, an ORF 57 amino-terminal arginine-rich sequence, which functions as an NLS, is required for this interaction. Furthermore, the ORF 57 protein is responsible for the redistribution of importin α into the nucleoli.

**EXPERIMENTAL PROCEDURES**

**Yeast Two-hybrid Screen for ORF 57-Interacting Proteins—**The GAL4-based yeast two-hybrid system screening technique (30) was employed to identify ORF 57-interacting proteins. The “bait” plasmid was constructed by PCR amplification of the ORF 57 coding region using forward and reverse primers. These oligonucleotides incorporated BamHI and PstI restriction sites for the convenient cloning of the PCR fragment into pGBT9 (CLONTECH), to derive the GAL4 DNA-binding domain fusion, pDBD57. A human kidney cDNA-GAL4 activation domain fusion, pADGBD, to derive the GAL4 DNA-binding domain fusion, pAD57. Each of these library plasmids demonstrated a requirement for the pDBD57 plasmid for induced expression of HIS3 or lacZ reporter genes were considered further and selected for DNA sequencing.

**Plasmid Constructs—**The yeast two-hybrid importin α and α5 deletion series were produced by a PCR-based method using a series of forward and reverse primers. The oligonucleotides incorporated BamHI and XhoI restriction sites for the convenient cloning of the PCR products. Each fragment was inserted into the yeast two-hybrid expression vector, pACT2, in frame with the GAL4-AD, to derive the deletion series pADGα1–6 and pADGα5–1. The bacterial expression importin α and α5 deletion series were produced by cloning the α1 and α5 deletion PCR fragments into pGEX3T, to derive the GST fusion constructs, pGSTα1–4 and pGSTα5–1.

The yeast two-hybrid ORF 57 deletion series was produced by a PCR-based method using a series of forward and reverse primers. The oligonucleotides incorporated BamHI and PstI restriction sites for the convenient cloning of the PCR products. Each fragment was inserted into the yeast two-hybrid expression vector, pGBT9, in frame with the GAL4-DBD, to derive the deletion series pDBD57α1–5. The ORF57-GFP and ORF 57 amino-terminal deletion series were again generated by PCR amplification, again using a series of forward and reverse primers. These oligonucleotides incorporated BamHI and PstI restriction sites to facilitate cloning of the PCR product into the eukaryotic expression vector, pcDNA4GFp (Invitrogen), to yield p57GFp and p57N–5–10. Expression of p57NLS–GFp, oligonucleotides encoding the putative ORF 57 NLS within nucleotides 877–917 were synthesized. These oligonucleotides incorporated BamHI and XhoI restriction sites, for convenient cloning. The oligonucleotides were annealed and ligated with pEGFP-C1 (CLONTECH) to create an in-frame carboxy-terminal fusion of the NLS sequence and GFP. To produce p57NLS–GFp, oligonucleotides encoding the SV40 NLS and GFP, respectively, were synthesized using fluo- rescence microscopy. To produce pSV40NLS–GFp, oligonucleotides encoding the SV40 NLS, PKKKRKV, were synthesized. These oligonucleotides incorporated BglII and FmiI restriction sites, for convenient cloning into pBKΔRSV57 (11), previously digested with BglII and FmiI, thereby replacing the wild type coding region with the site-directed mutated sequence.

The importin α1–GFp and α5–GFp constructs were generated by PCR amplification of the complete α1 and α5 cDNA sequences using forward and reverse primers. These oligonucleotides incorporated BamHI and XhoI restriction sites to facilitate cloning of the PCR products. Each fragment was inserted into the eukaryotic expression vector, pcDNA4GFp (Invitrogen) to yield pα1–GFp and pα5–GFp, respectively. The carboxy-terminal 30 amino-terminal armadillo repeats were synthesized encompassing nucleotide sequences bp 78677–78774 of the published sequence. These oligonucleotides incorporated BglII and FmiI restriction sites, for convenient cloning into pBKΔRSV57 (11), previously digested with BglII and FmiI, thereby replacing the wild type coding region with the site-directed mutated sequence.

**Viruses, Cell Culture, and Transfections—**HVS (strain A11) was propagated in oval monkey kidney cells, which were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum. COS-7 cells were also maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Plasmids used in the transfections were prepared using Qiagen plasmid kits according to the manufacturer’s directions. Transfections were performed using LipofectAMINE (Life Technologies, Inc.) as described by the manufacturer, using 2 μg of the appropriate DNAs.

**Co-immunoprecipitation Assays—**COS-7 cells either remained untransfected, were transfected with 2 μg of the appropriate DNAs, or were transfected with a multiplicity of infection of 4. A total of 20–30 h cells were harvested and lysed with lysis buffer (0.5 M NaCl, 1% Triton X-100, 50 mM HEPES buffer, pH 8.0) containing protease inhibitors (leupeptin and phenylmethylsulfonyl fluoride). For each immunoprecipitation, 10 μl of the ORF 57 polyclonal (31) or GFP monoclonal antiserum (CLONTECH) were incubated with protein A-Sepharose beads (Amersham Pharmacia Biotech) for 16 h at 4°C. The beads were washed three times with lysis buffer and incubated with each respective cell lysate for 3 h at 4°C. The beads were then pelleted and washed, and precipitated polypeptides were resolved on a 12% SDS-polyacrylamide gel and analyzed by immunoblot analysis.

**Immunoblot Analysis—**Poly peptides were resolved on a 12% SDS-polyacrylamide gel, then soaked for 10 min in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol (v/v)). The proteins were transferred to nitrocellulose membranes by electroblotting for 3 h at 250 mA. After transfer, the membranes were soaked in PBS and blocked by preincubation with 2% (w/v) nonfat milk powder for 2 h at 37°C. Membranes were incubated with a 1:4000 dilution of the anti-importin α5 polyclonal antiserum or a 1:1000 dilution of anti-GFP (CLONTECH), washed with PBS, and then incubated for 1 h at 37°C with a 1:1000 dilution of secondary immunoglobulin conjugated with horseradish peroxidase (Dako) in blocking buffer. After five washes with PBS, the nitrocellulose membranes were developed using ECL (Pierce).

**GST Pull-down Assays—**The importin α-deletion series were expressed as GST fusion proteins in E. coli DH5α. A fresh overnight culture of transformed E. coli was diluted 1 in 20 with LB medium. After growth at 37°C for 2 h, the culture was induced with 1 mM IPTG at 37°C for an additional 4 h. The bacteria were pelleted and resuspended in 0.1 volume of lysis buffer (100 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1% Triton X-100). Cells were sonicated and stored on ice for 30 min, and cellular debris pelleted. The recombinant protein was purified from crude lysates by incubation with glutathione-Sepharose 4B affinity beads. The protein-bound beads were then incubated with untransfected or appropriately transfected COS-7 cell lysates previously...
treated with lysis buffer for 16 h at 4 °C. The beads were then pelleted and washed, and precipitated polypeptides resolved on a 12% SDS-polyacrylamide gel. The proteins were then transferred to nitrocellulose membranes by electroblotting and probed as described previously.

**Immunofluorescence Analysis**—Cells were fixed with 100% ice-cold methanol for 10 min. The cells were rinsed in PBS and blocked by preincubation with 1% (w/v) nonfat milk powder for 1 h at 37 °C. A 1:100 dilution of anti-ORF 57 polyclonal antisemur (31) or 1:50 dilution of B23 monoclonal antibody was layered over the cells and incubated for 1 h at 37 °C. Texas Red-conjugated immunoglobulin (Dako; 1:200 dilution) was added for 1 h at 37 °C. After each incubation step, cells were washed extensively with PBS. The immune fluorescence slides were examined using a Zeiss Axiovert 135TV inverted microscope with a Neofluar 40× oil immersion lens.

**Northern Blot Analysis**—Northern blot analysis was performed as previously described (6). Total, nuclear, and cytoplasmic RNA was isolated from transfected cells and separated by electrophoresis on 1% denaturing formaldehyde-agarose gel. The RNA was transferred to Hybond-N membranes and hybridized with 32P-radiolabeled random-primed probes specific for gB and actin coding sequences.

**RESULTS**

**Importin α1 and α5 Interact with HVS ORF 57**—To identify ORF 57-interacting cellular proteins, 1.5 × 10^6 independent cDNA clones of a human kidney cDNA library fused to the GAL4 activation domain were screened. 24 clones were identified that activated histidine and β-galactosidase reporter gene expression in the presence of the ORF 57-DBD fusion protein. The specificity of the interaction was confirmed by transforming the putative ORF 57-interacting cellular clones into yeast strains harboring no plasmid, yeast containing pGBT9 vector only, yeast containing pLAM5 (a GAL4 human lamin C fusion), or pDBDS7. Only those library plasmids demonstrating a requirement of pDBDS7 for induced expression of histidine and β-galactosidase reporter genes were considered further.

Clones that fulfilled all these criteria were sequenced and BLAST searched against the EMBL/GenBank® data base. Analysis revealed that seven ORF 57-interacting clones corresponded to importin α1/karyopherin α2/Rch1 and that four clones corresponded to importin α5/karyopherin α1/hSRP1.

**In Vitro Co-immunoprecipitation of ORF 57 and Importin α1 and α5**—To confirm whether the observed interaction of ORF 57 with importin α1 or α5 could also be observed in vitro, co-immunoprecipitation studies were performed. Control untransfected COS-7 cells were compared with cells transfected with pRSVORF57, a eukaryotic expression vector encoding the complete coding region of ORF 57 (11), or HVS-infected cells (multiplicity of infection of 1). After 24 h, the cells were harvested and cell lysates utilized in co-immunoprecipitation analysis using an anti-ORF 57 polyclonal antisemur. Polypeptides precipitated from untransfected, transfected, and infected cellular extracts were then resolved on SDS-PAGE and transferred to a nitrocellulose membrane. Immunoblot detection was performed using antisemur specific for importin α1 or α5. The results demonstrate that, in both ORF 57-transfected and HVS-infected cells, ORF 57 specifically interacts with both importin α1 and α5 (Fig. 1).

**Importin α1 and α5 Interact with ORF 57 via Their Armadillo Repeats**—To map the domains within importin α1 and α5 required for their specific interaction with ORF 57, a series of importin α1 and α5 truncations were expressed as fusions with GAL4-DBD (Fig. 2a). Competent yeast strain HF7c was co-transformed with pDBDS7, and each importin truncation AD plasmid, and assessed for their ability to grow on selective medium. The results indicated that the ORF 57 binding region maps to amino acids 291–450 of importin α1 and amino acids 253–459 of importin α5, encompassing the central 5–8 arm repeats in both cases. Smaller deletions within these regions of either importin α abolished the interaction, suggesting that all the 5–8 armadillo repeats are required for ORF 57 binding.

To confirm whether ORF 57 interacts with importin α, co-immunoprecipitations were performed. Control total lysate (lane 1), untransfected (lane 2), p57-transfected (lane 3), and HVS-infected (lane 4) cell extracts were immunoprecipitated using ORF 57 antisemur. Bound proteins were resolved on a 12% SDS-PAGE gel and the presence of importin α1 (a) and α5 (b) were detected by Western blot analysis.
The results demonstrate that the amino-terminal arginine-rich sequence of ORF 57 is required for its interaction with importin α1 and α5 (Fig. 4).

To determine if the arginine-rich sequence functions as a NLS, the subcellular localization of the ORF 57 amino-terminal deletion series was analyzed. These constructs contained a carboxyl-terminal GFP fusion tag, allowing direct visualization. Transient p57N_D1–3 transfections were performed, and the resulting fluorescence pattern was subsequently evaluated. pcDNAGFP was used as a control and displayed, as expected, a fluorescence signal throughout the cell nucleus and cytoplasm. In contrast, p57GFP and p57N_D1 resulted in a distinct nuclear localization reminiscent of that observed previously with HVS-infected and ORF57-transfected cells (11). However, p57N_D2 and p57N_D3 resulted in fluorescence restricted to the cytoplasm. This indicated that the arginine-rich sequence contained within the amino terminus is required to direct the ORF57 protein to the nucleus (Fig. 5a).

To confirm that the arginine-rich sequence functions as a NLS, and enables nuclear import of a heterologous protein, the NLS was fused with GFP. COS-7 cell monolayers were transfected with either pEGFP-C1 or p57NLS-GFP, and the subcellular localization of GFP was observed. The results show that cells transfected with pEGFP-C1 displayed a fluorescence pattern throughout the cell, in both the nucleus and cytoplasm. However, the fluorescence pattern observed in p57NLS-GFP-transfected cells was confined to the nucleus and in particular the nucleolus (Fig. 5b).

Furthermore, to determine the importance of the arginine and lysine residues for ORF 57 nuclear localization, a range of site-directed mutations were constructed, p57NLSM1–4, incorporated the alteration of varying residues within the putative NLS to alanine (Fig. 5c). COS-7 cell monolayers were transfected with either p57GFP or p57NLSM1–4, and the subcellular localization of GFP was observed. The results show that cells transfected with p57GFP displayed a nuclear fluorescence pattern as observed previously. However, the fluorescence pattern observed in p57NLSM1–4-transfected cells was confined to the cytoplasm (Fig. 5d). This suggested that the ORF 57...
amino-terminal arginine-rich sequence functions both as a NLS and possibly as a nucleolar localization signal, and the arginine and lysine residues are essential for this function.

**ORF 57 Redistributes Importin α1 and α5 into the Nucleolus**—To determine the effect of ORF 57 on the subcellular localization of importin α1 and α5, indirect immunofluorescence was performed. Initially, the cDNAs of importin α1 and importin α5 were inserted into the eukaryotic expression vector, pcDNAGFP, allowing direct visualization of the importin α1 and α5 proteins. To confirm the molecular weight of importin α1-GFP and importin α5-GFP fusion proteins, Western blot analysis of transiently transfected cells was performed. Western blot analysis demonstrated that importin α1-GFP and importin α5-GFP encode the predicted 82- and 86-kDa proteins, respectively (data not shown).

To ascertain the subcellular localization of each importin α1 and α5, dual immunofluorescence was performed using importin α1-GFP and ORF57 co-transfected (Fig. 6a) or HVS-infected cells (data not shown). Cells only expressing importin α1 resulted in the distinct perinuclear staining as previously described. In contrast, cells expressing ORF 57 showed a drastic redistribution of the importin α1-GFP (Fig. 6a). This analysis showed that ORF 57 expression resulted in a strong nuclear fluorescence of importin α1-GFP. Moreover, the fluorescence was concentrated in nuclear compartments, varying in number between 2 and 5 per cell, that resembled nucleoli (Fig. 6a). Essentially identical results were observed with importin α5-GFP in the presence of the ORF 57 protein (data not shown).

To ascertain if the importin α nuclear aggregations in the presence of ORF 57 are concentrated in the nucleolus, indirect dual immunofluorescence was performed. Importin α1-GFP and ORF 57 co-transfected cells were labeled with a monoclonal antibody, specific for a major nucleolar protein, B23. Results demonstrated that in the presence of ORF 57, importin α1 was localized into nuclear aggregations, as described previously. Moreover, these distinct nuclear aggregations co-localized with B23 (Fig. 6b). This suggests that ORF 57 redistributes importin α1 and α5 into the nucleolus.
ORF 57 Reduces Nuclear Import of Other NLS-containing Proteins—As demonstrated above, ORF 57 redistributes importin α1 and α5 into distinct nucleolar aggregations. To determine whether this redistribution affected the nuclear import of secondary NLS-containing proteins via the importin nuclear import pathway, transient transfections were performed. COS-7 cells were transfected with pSV40NLS-RFP, a transfer vector encoding a carboxyl-terminal fusion of the SV40 NLS and RFP, in the absence or presence of ORF57. Results demonstrate that ORF 57/importin α nuclear aggregations are concentrated in the nucleolus, untransfected (i) or pα1-GFP- and p57-transfected cells were labeled with a specific B23 monoclonal antibody and detected using an anti-rabbit Texas Red conjugate (v). Importin α was directly visualized using fluorescence microscopy (vi).

DISCUSSION

HVS ORF 57 interacts with importin α1 and α5.

ORF 57 Reduces Nuclear Import of Other NLS-containing Proteins—As demonstrated above, ORF 57 redistributes importin α1 and α5 into distinct nucleolar aggregations. To determine whether this redistribution affected the nuclear import of secondary NLS-containing proteins via the importin nuclear import pathway, transient transfections were performed. COS-7 cells were transfected with pSV40NLS-RFP, a transfer vector encoding a carboxyl-terminal fusion of the SV40 NLS and RFP, in the absence or presence of ORF57. Results demonstrate that cells transfected with pSV40NLS-RFP displayed a fluorescence pattern confined to the nucleus. However, in contrast, cells expressing ORF 57 showed a reduced presence of SV40NLS-RFP in the nucleus and an increased amount in the cytoplasm (Fig. 7). This analysis suggests that ORF 57 may sequester importin α in the nucleolus, thereby reducing nuclear import of secondary NLS-containing proteins via the importin pathway.

ORF 57-Importin α Interaction Is Required for Viral RNA Nuclear Export—We have previously demonstrated that ORF 57 is a nucleocytoplasmic shuttle protein which mediates the nuclear export of viral mRNAs (6). To determine whether the interaction of ORF 57 and importin α is required for viral RNA nuclear export, Northern blot analysis was performed. Total, nuclear, and cytoplasmic RNA were isolated separately from COS-7 cells transfected with pUCgB, a transfer vector containing the full-length coding region and promoter of the HVS late glycoprotein B gene, in the absence and presence of pRSVORF57 or p57NLSΔ3. The RNA was then separated by electrophoresis, transferred to Hybond-N membranes, and hybridized with 32P-radiolabeled random-primed probe specific for the HVS gB and actin coding regions (Fig. 8). The results demonstrate, as described previously, ORF 57 is required for the efficient cytoplasmic accumulation of late viral transcripts (6). However, the deletion of the ORF 57 NLS results in the retention of late viral transcripts in the nucleus. This suggests that the ORF 57-importin α interaction is required for ORF 57’s nucleocytoplasmic shuttling ability and therefore the efficient nuclear export of late viral transcripts.
importin α1 and importin α5 share distinct binding affinities for various NLSs. However, recent analysis has demonstrated that all importin α isoforms could import most substrates with a similar efficiency (33). At present, we have not determined whether all the importin α isoforms have distinct ORF 57 specificities.

Interestingly, a number of viral proteins have been shown to interact with importin α and in turn exploit the importin-mediated pathway to enter the host cell nucleus. The influenza virus nucleoprotein (NP) has been shown to bind both importin α1 and α5 isoforms (36–38). Furthermore, the Epstein-Barr virus EBNA-1 protein, which is required for the replication and stable maintenance of the viral genome (reviewed in Ref. 39), has also been shown to interact with importin α1 and α5 isoforms (40, 41). Moreover, nuclear import of the human papillomavirus (strain 11) L1 major capsid has been shown to be mediated by the importin pathway via importin α5 binding (42). More recently, HIV-1 Rev has been shown to utilize the importin-mediated pathway but independently of importin α (43, 44). The HIV-1 Rev arginine-rich NLS has been demonstrated to interact directly with importin β which mediates the import of Rev, using in vitro import assays (43, 44).

Further analysis of the ORF 57 (RPRSPFRKR), EBNA-1, and NP NLSs show very limited, if any, homology. The EBNA-1 NLS, KRPRSPSS, has been shown to be required for both importin α1 and α5 binding. Moreover, additional upstream and downstream sequences are required for importin α1 binding (45). In contrast, the influenza A NP contains two overlapping non-conventional NLSs (38). Mutational analysis by alanine scanning identified differing motifs required for importin α1 and α5 binding: SXGTKRSYXXM for importin α5 and TKRSXXXX for importin α1 binding (38). Although data presented in this report suggest that ORF 57 utilizes the same NLS for both importin α1 and α5 binding, it cannot be excluded that the NLS contains overlapping motifs that specify importin α isoform binding.

Insights into the binding of NLS-containing proteins to importin α have been revealed by examination of the crystal structure of yeast and mammalian importin α molecules (46, 47). Sequence analysis has revealed that importin α is composed of three distinct domains (20, 48): a basic amino-terminal domain in β-binding domain; a large central domain composed of 8–10 arm repeats; and an acidic carboxyl terminus, which mediates an interaction with the nuclear export factor, CAS (24–26). Co-crystallization studies of importin α and a monopartite NLS have identified two possible binding sites (46, 47). The major site lies at the amino-terminal end of the receptor between the first and fourth arm repeats, and the minor site is located at the carboxyl terminus between arm repeats 4 and 8. At both sites, the NLS binds in an extended antiparallel conformation, via tryptophan and asparagine residues (46, 47). Moreover, the bipartite nucleoplasmin NLS simultaneously binds to both major and minor sites (46, 47). From these studies we infer that ORF 57 contains a monopartite NLS, which specifically binds to the minor repeats at the carboxyl terminus between the fourth and eighth arm repeats. Similar observations have demonstrated that this is the minimal region required for the interaction of importin α with EBNA1-NLS (40) and importin α2 with LEF1-NLS (49).

Interestingly, upon nuclear import of the ORF 57-importin α complex, we have demonstrated that this complex is directed to the nucleolus. Results herein suggest that the ORF 57 amino-terminal arginine-rich domain mediates both nuclear and nucleolar localization. Similar nucleolar targeting has been observed with HIV-1 (50–52). However, no functional role has yet been attributed to this localization and it is the matter of some debate. It has recently been reported that Rev induces the redistribution of the nucleoporins Nup98 and Nup214, in addition to the nuclear export factor CRM-1, into the nucleolus (53). These findings suggest that assembly of the Rev-nuclear export complex occurs in the nucleolus. However, data generated utilizing an HIV-1 nucleolar-localized ribozyme suggest that HIV-1 transcripts undergo nucleolar trafficking (54). This has led to speculation that a ribonucleoprotein particle containing HIV-1 RNA, regulatory proteins, and cellular factors, involved in the post-transcriptional modification or export and translation of HIV-1 transcripts, occurs in the nucleolus. Whether the nucleolus plays similar roles in ORF 57 functioning is unknown and warrants further investigation.

In conclusion, our data demonstrate that the multifunctional ORF 57 nucleocytoplasmic shuttle protein interacts with importin α isoforms 1 and 5. The binding of ORF 57 to importin α is mediated by the hydrophobic, internal, importin α armadillo repeats. An ORF 57 amino-terminal arginine-rich sequence, which functions as an NLS, was also required for this interaction. Furthermore, the ORF 57 protein is responsible for the redistribution of importin α into the nucleoli. Moreover, preliminary experiments suggest that this redistribution reduces nuclear import of secondary NLS-containing protein via the importin-mediated nuclear import pathway. These results suggest that ORF 57 is a nucleocytoplasmic shuttle protein, which re-enters the nucleus via the importin β-mediated pathway and this interaction is necessary for the efficient nuclear export of viral RNA transcripts. Future studies will be directed to determine which cellular pathway ORF 57 utilizes to exit the nucleus and the functional significance of the nucleolus in the functioning of this important herpesvirus regulatory protein.

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