The potential terminase subunit of human cytomegalovirus, pUL56, is translocated into the nucleus by its own nuclear localization signal and interacts with importin α

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Human cytomegalovirus (HCMV) DNA-binding protein pUL56 is thought to be involved in the cleavage/packaging process of viral DNA and therefore needs to be transported into the nucleus. By using indirect immunofluorescence analysis, HCMV pUL56 (p130) was found to be localized predominantly in the nucleus of infected cells. Solitary expression of wild-type as well as epitope-tagged pUL56 also resulted in nuclear distribution after transfection, suggesting the presence of an endogenous nuclear localization signal (NLS). Deletion of a carboxy-terminal stretch of basic amino acids (aa 816–827) prevented nuclear translocation, indicating that the sequence RVRATRKRPRR of HCMV pUL56 mediates nuclear targeting. The signal character of the NLS sequence was demonstrated by successful transfer of the NLS to a reporter protein chimera. Furthermore, sequential substitutions of pairs of amino acids by alanine in the context of the reporter protein as well as substitutions within the full-length pUL56 sequence indicated that residues at positions 7 and 8 of the NLS (R and K at positions 822 and 823 of pUL56) were essential for nuclear translocation. In order to identify the transport machinery involved, the potential of pUL56 to bind importin α (hSRP1α) was examined. Clear evidence of a direct interaction of a carboxy-terminal portion as well as the NLS of pUL56 with hSRP1α was provided by in vitro binding assays. In view of these findings, it is suggested that nuclear translocation of HCMV pUL56 is mediated by the importin-dependent pathway.

Introduction

Human cytomegalovirus (HCMV) is a member of the herpesvirus family and represents a major human pathogen, causing severe disease in newborns and immunocompromised patients, e.g. organ transplant recipients and patients with AIDS. HCMV is classified as a β-herpesvirus and is characterized by its narrow host range and prolonged replicative cycle in permissive cells (Britt & Alford, 1996; Mocarski, 1996). Maturational events of HCMV DNA replication and capsid assembly require efficient translocation of viral products into the nucleus of infected cells. Several gene products of HCMV have been identified that appear to be involved in the process of DNA replication, cleavage and packaging (Penfold & Mocarski, 1997; for a review see Gibson, 1996). There is little information, however, regarding their nuclear targeting. In a previous report, partial characterization of the gene product of HCMV ORF UL56 demonstrated that the encoded product, pUL56, is a 130 kDa, nucleocapsid-associated protein (Bogner et al., 1993). Recently, we showed that pUL56 binds specifically to viral DNA-packaging motifs and cleaves DNA bearing these motifs (Bogner, 1999; Bogner et al., 1998), suggesting an involvement of pUL56 in HCMV DNA packaging. These observations are in line with studies on ICP18.5, the homologous protein of herpes simplex virus type 1 (HSV-1) (Pellett et al., 1986) and pseudorabies virus (PrV) (Pederson & Enquist, 1989). By the use of virus mutants, it was demonstrated that the deletion of ICP18.5 leads to nuclear accumulation of naked nucleocapsids and uncleaved concatemeric DNA (Addison et al., 1990; Tengelsen et al., 1993; Mettenleiter et al., 1993). In view of these observations, nuclear translocation of HCMV pUL56 is expected to be essential for virus maturation.

Targetting of nuclear proteins has been shown to depend on intrinsic nuclear localization signals (NLS). The best-
characterized NLS motifs are the classical monopartite NLS of simian virus 40 (SV40) large T antigen (PKKKRKV; Kalderon et al., 1984), which consists of a single stretch of basic amino acids, and the bipartite nucleoplasm NLS of Xenopus laevis, which has two basic motifs separated by a mutation-tolerant 10 amino acid spacer (KRPAAKKAGQAKKK; Robbins et al., 1991). Although there is no clear consensus, it is known that a simple cluster of basic amino acids is generally not sufficient to serve as a nuclear transport signal (Chelsky et al., 1989; Dingwall & Laskey, 1991; Plafker & Gibson, 1998). Proteins such as the HSV-1 regulatory protein ICP27 (Mears et al., 1995), Epstein–Barr virus nuclear antigen 1 (EBNA-1; Fischer et al., 1997), little is known about one of the best-characterized human importin α homologues, hSRP1α (Weis et al., 1995).

**Methods**

**Cell culture and virus infection.** Human foreskin fibroblasts (HFF) or COS-7 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% foetal calf serum, 2 mM L-glutamine, penicillin (5 U/ml) and streptomycin (50 µg/ml). HFF at passages 10 to 15 were used for infection and experiments were carried out with subconfluent cell monolayers. HCMV AD169 stocks were prepared (Bogert et al., 1992) and infection of HFF at an m.o.i. of 1:0 was carried out as described previously (Radak et al., 1996).

**Plasmid construction.** Restriction enzymes were purchased from New England Biolabs and used according to the instructions of the manufacturer. The construction of expression plasmids pRC/CMV-UL56 encoding wild-type HCMV pUL56 has been described previously (Bogert et al., 1993). To produce cDNA inserts for cloning, PCR was performed with the GeneAmp Kit (Perkin Elmer). All constructs in which PCR was used to generate coding sequences were analysed by DNA sequencing performed on a 377 DNA Sequencer (Applied Biosystems).

**Construction of an epitope-tagged pUL56 construct.** To generate the construct His–pUL56, which expressed 5’-Xpress epitope-tagged pUL56, plasmid pcDNA3.1/HisC (Invitrogen) was digested with KpnI and XhoI prior to insertion of a gene fragment encoding the ORF of HCMV pUL56. The respective fragment was obtained by using plasmid pRC/CMV-UL56 as the template for PCR and a pair of synthetic oligonucleotides (Table 1; restriction sites are underlined).

**Oligonucleotide-directed mutagenesis of pUL56 NLS.** Constructs His–pUL56NLS and His–pUL56nlsAA4 were generated by using the QuickChange site-directed mutagenesis kit (Stratagene) as recommended by the supplier and the synthetic oligonucleotides shown in Table 1 (construct His–pUL56 served as template).

**Plasmids encoding pUL56 NLS fused to the reporter protein β-galactosidase–green fluorescent protein (β-Gal–GFP).** Plasmids encoding β-Gal–pUL56NLS–GFP chimeras were constructed by using plasmid pHM829, which encodes a β-Gal–GFP fusion protein (Sorg & Stamminger, 1999). In order to generate the chimeras pHM829–pUL56NLS, plasmid pHM829–pUL56NLSAA1 to pHM829–pUL56nlsAA6 and pHM829–pUL56revNLS, plasmid pHM829 was digested with restriction endonucleases BamHI and Xhol. Gene fragments generated using pHM829 as template with a 3’ primer containing a BamHI site and 5’ oligonucleotides containing Xhol sites (Table 1; restriction sites underlined) were inserted into the digested plasmid.

**Generation of glutathione S-transferase (GST)–pUL56 fusion constructs.** To obtain the GST–pUL56NLS chimeras GST–pUL56NLS, GST–pUL56revNLS and GST–pUL56nlsAA4, plasmid pGEX-5X-1 (Amersham Pharmacia Biotech) was digested with restriction endonucleases EcoRI and PstI. Gene fragments generated using pGEX-5X-1 as template with the antisense oligonucleotide and sense oligonucleotides shown in Table 1 (restriction sites underlined) were inserted into the digested plasmid.

**Plasmid GST–pUL56C was generated by digestion of pRC/CMV-UL56 with EcoRI and pGEX-5X-1 (Amersham Pharmacia Biotech) with NolI. To create blunt ends, linearized plasmids were incubated with Klenow enzyme (Roche Diagnostics) as recommended by the supplier.**

**Transient transfection.** For transient expression, COS-7 cells were seeded on 60 mm Petri dishes with glass cover slips. COS-7 cells at 60% confluence were transfected with the appropriate DNA (10 µg per well) by the lipofectin method (Life Technologies). Antigen expression was analysed by indirect immunofluorescence at 40 h post-transfection.

**Immunofluorescence analysis and antibodies.** For immunofluorescence, mock-infected and infected HFF or transfected COS-7 cells grown on glass cover slips were fixed in 4% paraformaldehyde as described previously (Smuda et al., 1997). After fixation, incubation with the following primary antibodies was carried out for 60 min at room
temperature: HCMV pUL56-specific human polyclonal antibody pabUL56 (Bogner et al., 1993), which was purified from high-titre human serum by column affinity chromatography (Affigel 10/15–pUL56), mouse anti-NOR MAb (Novus Molecular), directed against the nucleolus-organizing region, diluted 1:100 in 3% BSA/PBS, MAb Anti-Xpress (Invitrogen), diluted 1:500 in 3% BSA/PBS, and β-Gal-specific MAb (Promega), diluted 1:1000 in 3% BSA/PBS. After extensive rinsing with PBS, further incubation was carried out for 45 min with FITC-labelled goat anti-human Fab fragment, FITC-labelled goat anti-mouse Fab fragment or, for double immunofluorescence analysis, Texas red-labelled goat anti-mouse Fab fragment (Dianova), diluted 1:100 in 3% BSA/PBS. In the case of double immunofluorescence staining of proteins, the primary antibodies were incubated together whereas secondary antibodies were added consecutively. A Zeiss microscope with digital photographic equipment (Spot camera system, version 2.1.2) Diagnostik Instruments was used for analysis of fluorescence signals and for taking phase-contrast images.

- **In vitro binding assays.** The TnT T7-coupled reticulocyte lysate system (Promega) was used to synthesize [35S]methionine-labelled (Amersham) importin α in a coupled transcription/translation reaction by using 1 µg plasmid pSET8–hSRP1α (Weis et al., 1995) as template according to the protocol of the supplier (Promega). For *in vitro* binding analysis, GST–pUL56 fusion proteins were expressed in *E. coli* BL21 and protein purification was carried out according to the manufacturer’s instructions (Pharmacia). Equal amounts of GST–pUL56 fusion proteins loaded on glutathione–Sepharose 4B (Amersham Pharmacia Biotech) were incubated overnight at 4 °C with *in vitro*-translated importin α in 500 µl binding buffer (0.05% NP-40, 50 mM HEPES–NaOH pH 7.3, 10% glycerol, 0.1% BSA, 300 mM NaCl). Samples were washed with binding buffer and subsequently subjected to SDS–PAGE, consecutive fixation and autoradiography (Bonner & Laskey, 1974). A Biolmager (Raytest) was used for quantification of radioactive signals.

- **Immunoprecipitation.** Immunoprecipitation of *in vitro*-translated importin α with MAb Anti-Xpress (Invitrogen) was carried out as described previously (Radsak et al., 1990). All incubation and washing cycles were performed with immunoprecipitation buffer (0.02 M Tris–HCl pH 9.0, 0.3 M NaCl, 10% (v/v) glycerol, 0.001 M CaCl₂, 0.5 mM MgCl₂, 0.002 M EDTA, 0.5% (v/v) NP-40) as described by Blanton & Tevethia (1981). Immunoprecipitates were analysed by SDS–PAGE and autoradiography.

**Results**

**Nuclear translocation of HCMV pUL56 in infected cells**

Our previous experiments showed that HCMV pUL56 is able to bind viral DNA packaging elements (pac1 and pac2) and has a specific nuclease activity (Bogner et al., 1998). In order to address the question of whether pUL56 is indeed transported into the nucleus to function in viral DNA maturation, the intracellular distribution of this protein in infected cells was examined by using double immunofluorescence analysis. Coverslip cultures of HFF were mock-infected or infected with HCMV strain AD169 (m.o.i. of 3) for 12, 24 and 48 h prior to immunofluorescence with the monospecific polyclonal antibody pabUL56 (Bogner et al., 1993) and MAb anti-NOR against the nucleolus-organizing region. In HCMV-infected cells, pabUL56 detected antigen predominantly in the nucleus (Fig. 1A; 12, 24 and 48 h p.i.).

**Table 1. Oligonucleotide primers used in PCR**

For the primers used in the generation of reporter protein fusions, dashes indicate residues identical to the first primer in the group.
Fig. 1. Analysis of intracellular distribution of HCMV pUL56. (A) HFF were mock-infected (mock) or infected with HCMV strain AD169 for 12, 24 and 48 h at an m.o.i. of 3. After paraformaldehyde fixation, consecutive immunofluorescence staining was performed with antibodies against pUL56 or nucleolus-organizing regions (NOR). Arrows with open heads indicate intranuclear inclusion bodies; filled arrow heads represent some of the nucleoli. (B) Immunofluorescence staining with antibody pabUL56 of COS-7 cells transfected with an expression construct encoding authentic pUL56 (left image). As a control, phase-contrast images were taken to visualize nuclei or nucleoli (right image). (C) Solitary expression of epitope-tagged full-length His–pUL56 in COS-7 cells, as revealed by immunofluorescence staining at 40 h after transfection with MAb Anti-Xpress. Again, nuclei are shown by a phase-contrast image. In (B) and (C), the pUL56 amino acid sequences are shown as grey boxes and the recombinant Anti-Xpress epitope is indicated. Numbers indicate the positions of residues of the pUL56 sequence. Some of the nucleoli are indicated by arrows.

The distribution and intensity of fluorescence changed during the course of infection: at 12 h post-infection (p.i.), weak and more homogeneous nuclear staining was observed, together with a weak accumulation in nucleolar regions (24 h p.i.). The fluorescence intensity increased with time until late times after infection (48 h p.i.); bright intranuclear patches were detected in addition. Fluorescence signals in nucleoli were no longer detectable. The analysis of these intranuclear inclusion bodies was the object of a previous investigation (Giesen et al., 2000). Uninfected cells exhibited no specific fluorescence signals (Fig. 1A; mock).

**Nuclear translocation of HCMV pUL56 in the absence of other viral proteins**

In order to determine nuclear import of solitary HCMV pUL56, transient transfections were performed of expression plasmids encoding pUL56 and epitope-tagged His–pUL56 into COS-7 cells. The subcellular localization of the proteins was again examined by immunofluorescence by using pabUL56 for recombinant wild-type pUL56 (Fig. 1B) and MAb Anti-Xpress for epitope-tagged His–pUL56 (Fig. 1C). Phase-contrast images were taken in order to visualize nuclei and nucleoli. Immunofluorescence revealed nuclear staining for both recombinant proteins (Fig. 1B, C). Interestingly, the nucleolar regions remained unstained. Identical results were obtained with transient expression of recombinant pUL56 in HCMV-permissive human astrocytoma cells (U373, ATCC HTB17; data not shown). No specific fluorescence signal was obtained in COS-7 and U373 cells transfected with vector DNA (data not shown). These observations showed that
Nuclear localization of HCMV pUL56

A

| HCMV pUL56: | aa 816 R R V A T R K R P R R R 827 |
| polyoma virus large-T: | aa 189 R R V S R K R P P 196 |
| NLS consensus sequence: | K R X K |

(X position in most cases filled with A, K, L, P, R or V)

**Fig. 2.** A carboxy-terminal stretch of basic amino acids of full-length pUL56 mediates its nuclear translocation. (A) Alignment of NLS sequences of HCMV pUL56 (strain AD169; Chee et al., 1990) with that of polyomavirus large T (Richardson et al., 1986) and the NLS consensus sequence predicted by Chelsky et al. (1989), aligned by using the BESTFIT program of the Heidelberg UNIX Sequence Analysis Resources (HUSAR). Residues corresponding to the classical monopartite consensus sequence are enclosed in a grey box; adjacent identical and similar residues are framed in the open box. Numbers indicate the relative positions of residues within the authentic proteins. (B) Immunofluorescence staining at 40 h after transfection of deletion construct His–pUL56ΔNLS, which lacks the NLS sequence (aa 816–827), into COS-7 cells. Antibody MAb Anti-Xpress (His–pUL56) was used and nuclei are shown by a phase-contrast image (phase-c). (C) As a control, construct His–pUL56, encoding epitope-tagged pUL56, was expressed transiently in COS-7 cells. Immunofluorescence staining was performed as described above at 40 h after transfection. Again, nuclei and nucleoli are visualized by a phase-contrast image. In (B) and (C), black boxes represent the Anti-Xpress epitope and grey boxes indicate the HCMV pUL56 sequence. Numbers indicate positions of residues of HCMV pUL56; residues of the NLS sequence are shown and basic residues are underlined. The gap sign marks the position of a deletion. Arrows indicate positions of some of the nucleoli.

singly expressed pUL56 is translocated into the nucleus, suggesting the presence of an endogenous NLS.

**Nuclear translocation of full-length HCMV pUL56 is mediated by a carboxy-terminal stretch of basic amino acids**

In order to identify specific domains responsible for nuclear import, the amino acid sequence of pUL56 (850 aa) was scanned by computer analysis for the presence of putative NLS motifs. By using criteria such as predicted surface exposure (Roberts et al., 1987; Rost & Sander, 1993) and comparison of the pUL56 amino acid sequence with known monopartite and bipartite NLS motifs (Boulikas, 1993; Dingwall & Laskey, 1991; Robbins et al., 1991), a putative monopartite NLS motif was identified at the extreme carboxy terminus of pUL56. The sequence consisted of a stretch of 12 amino acids, eight of which had a basic character (aa 816–827; Fig. 2 A). The motif exhibited similarity to the polyomavirus large T NLS (Richardson et al., 1986) and contained the putative NLS consensus sequence K[K/R]X[K/R] (Chelsky et al., 1989).

In order to determine the functionality of the putative pUL56 NLS sequence, the deletion construct His–pUL56ΔNLS, which lacked the carboxy-terminal stretch of basic residues (aa 816–827), was constructed and transfected into COS-7 cells. Immunofluorescence at 40 h after transfection with MAb Anti-Xpress revealed predominantly cytoplasmic staining for the mutant protein (Fig. 2B). As a control, expression of full-length His–pUL56 in COS-7 cells again showed a nuclear distribution (Fig. 2C). Phase-contrast images of the cells served as a control to visualize nuclei and nucleoli (Fig. 2B, C; phase-c).
Amino acid residues 816–827 of HCMV pUL56 mediate nuclear targeting of a cytosolic reporter protein

In order to determine further the signal character of the pUL56 12 amino acid motif, it was fused to an adequate reporter by using vector pHM829 (Sorg & Stamminger, 1999), which enables expression of protein fragments that are fused to β-Gal and GFP. After subcloning of the coding sequence for aa 816–827 of pUL56 into the multiple cloning site of vector pHM829, the fusion construct was expressed transiently in COS-7 cells (Fig. 3). In order to demonstrate that the inserted amino acid sequence was expressed correctly, the subcellular localization of the chimeric proteins was examined by GFP signals and immunofluorescence staining with an anti-β-Gal MAb and a Texas red-conjugated secondary antibody (β-Gal). A phase-contrast image was taken to visualize nuclei and nucleoli (phase-c). In addition to homogeneous distribution of fluorescence signals, bright intranuclear patches were detected (Fig. 3 A; β-Gal, GFP) representing nucleoli, which were seen as darker regions in phase-contrast images (Fig. 3 A; phase-c). These patches appeared not to be identical to the inclusion bodies

Fig. 3. The NLS of HCMV pUL56 mediates nuclear import of cytosolic reporter proteins. β-Gal- and GFP-specific protein segments are indicated. The residues of the amino acid sequence of pUL56 NLS and the reversed NLS are shown. Relative positions are indicated and basic residues are underlined. Some of the nucleoli are marked by arrows. (A) Subcellular distribution of HCMV pUL56 NLS sequence fused to reporter protein β-Gal–GFP was examined at 40 h after transfection by detection of GFP fluorescence (GFP) and staining with an anti-β-Gal MAb and a Texas red-conjugated secondary antibody (β-Gal). A phase-contrast image was taken to visualize nuclei and nucleoli (phase-c). (B) Subcellular distribution after transient expression of the reversed pUL56 NLS sequence fused to reporter protein β-Gal–GFP in COS-7 cells. Detection of chimeric reporter protein was performed as described above. (C) As a control, construct pHM829 encoding the β-Gal–GFP reporter protein was expressed transiently in COS-7 cells. Detection of proteins was carried out at 40 h after transfection as described above.
observed in cells at late times after infection (Fig. 1A; 48 h p.i.). The order of basic and neutral amino acids within the external parts of the pUL56 NLS sequence (RRVR or RPRR) is similar, implying that the signal could also be functional in the reverse orientation. In order to determine the subcellular distribution of the reversed pUL56 NLS, the respective residues were fused to the reporter protein β-Gal–GF. Chimeric protein constructs were transfected transiently into COS-7 cells prior to immunofluorescence analysis at 40 h after transfection, as described above. This approach revealed retention of fusion proteins in the cytoplasm (Fig. 3B; β-Gal, GFP). As a control, expression of vector pHM829 alone revealed clear retention of the reporter proteins in the cytoplasm (Fig. 3C). These results demonstrated that the newly identified monopartite NLS (aa 816–827) of HCMV pUL56, but not the reversed NLS sequence, can serve as an efficient nuclear-targeting signal in mammalian cells.

**Amino acid residues 7 and 8 of the HCMV pUL56 NLS sequence are essential for nuclear translocation of reporter proteins**

The 12 amino acid NLS sequence of pUL56 contains two clusters of basic residues that represent possible consensus motifs for classical monopartite NLS sequences (RRVR and KPRR; Chelsky et al., 1989). Mutational analysis was performed in order to evaluate the role of these motifs. For this purpose, pairs of residues of the pUL56 NLS sequence were substituted sequentially by alanine by using fusion protein constructs pHM829–pUL56NLS as the template. COS-7 cells were transfected with the constructs containing NLS mutations pUL56nsAA1 to pUL56nsAA6 and the subcellular localization of the altered proteins was again determined by GFP signals and immunofluorescence staining with anti-β-Gal MAb and a Texas red-conjugated secondary antibody. In order to visualize nuclei and nucleoli, phase-contrast micrographs were taken of the cells (Fig. 4). After substitution of residues 5 and 6, nuclear distribution was obtained comparable to that of the wild-type NLS sequence fused to β-Gal and to GFP (Fig. 4C). In the case of substitutions of residues 1 and 2, 3 and 4 or 11 and 12 of the pUL56 NLS, exclusively intranuclear localization was observed, but no fluorescence signals were obtained in nucleolar regions of the nuclei (Fig. 4A, B, F). Substitution of residues 9 and 10 by alanine did not interfere with nuclear translocation, although some fluorescence was also observed in the cytoplasm (Fig. 4E). On the other hand, substitution of residues 7 and 8 resulted in clear exclusion of the fusion protein from the nucleus and its distribution throughout the cytoplasm (Fig. 4D). As a control, expression of the wild-type pUL56 NLS fused to reporter proteins again showed nuclear distribution (Fig. 4G). These results demonstrated that residues at positions 7 and 8 (R and K) of the HCMV pUL56 NLS are required for nuclear localization of the reporter product.

**Substitution of amino acid residues 822 and 823 in the carboxy-terminal NLS sequence of full-length HCMV pUL56 prevents nuclear translocation**

In order to determine whether the results obtained with the chimeric fusion proteins may be extended to full-length pUL56, double point mutagenesis was performed with the recombinant His–pUL56 construct. For this purpose, residues 822 and 823 (corresponding to residues 7 and 8 of the pUL56 NLS reporter protein chimeras) were substituted by alanine by site-directed mutagenesis, as described in Methods. The construct His–pUL56nsAA4 was expressed transiently in COS-7 cells prior to immunofluorescence analysis with MAb Anti-Xpress (Fig. 5A). At 40 h after transfection, immunofluorescence revealed predominantly cytoplasmic staining for the mutant protein (Fig. 5A). As expected, wild-type His–pUL56 was again detected in the nuclei of transfected cells (Fig. 5B). Phase-contrast images served as controls to visualize nuclei and nucleoli (Fig. 5 A, B; phase-c). These results suggested that residues 822 and 823 are essential for efficient nuclear translocation of full-length pUL56.

**The NLS of HCMV pUL56 specifically binds importin α (hSRP1α)**

The human recombinant protein hSRP1α was recognized recently as a member of the importin/karyopherin α family and shown to function as an NLS receptor (Weis et al., 1995). In vitro binding assays were performed in order to determine whether HCMV pUL56 and importin α (hSRP1α) interact with each other (Fig. 6 A). GST fusion proteins containing the carboxy-terminal portion (aa 404–850; GST–pUL56C), the NLS sequence (GST–pUL56NLS), a reversed NLS sequence (GST–pUL56revNLS) or the NLS sequence with substitution of residues 7 and 8 by alanine (GST–pUL56nsAA4) of pUL56 were used for this purpose. Fusion proteins or GST itself were immobilized on glutathione–Sepharose beads prior to incubation with equal amounts of in vitro-translated, [35S]-methionine-labelled importin α (Fig. 6 A; lanes 1, 2, 5, 6, 7). The amount of bound material was analysed by SDS–PAGE and autoradiography. Importin α interacted specifically with the carboxy terminus of pUL56 but not with GST alone (Fig. 6 A; lanes 1 and 2). Immunoprecipitation of Anti-Xpress epitope-tagged importin α with MAb Anti-Xpress as well as electrophoresis of in vitro translation products (10% of material used) served as controls (Fig. 6 A; lanes 3 and 4). Incubation of the fusion protein containing only the pUL56 NLS with in vitro-translated, [35S]-methionine-labelled importin α again revealed a specific interaction. However, no or reduced interaction was observed with GST alone and GST fused to the reversed NLS sequence or to the mutant NLS sequence of pUL56 (Fig. 6 A; lanes 5–7). Quantification was performed by Bioimager analysis of the autoradiographs in order to characterize further the intensity of interaction between pUL56 and importin α.
Fig. 4. For legend see facing page.
Comparison of binding of importin α to GST–pUL56C with that to GST alone resulted in a ratio of 100:2.6. On the other hand, the respective values for GST–pUL56NLS, GST–pUL56revNLS and GST–pUL56nlsAA4 were 100:4, 100:14.8 and 100:11 (Fig. 6B). As a control, equal amounts of the same GST fusion proteins used in the in vitro binding assays were immobilized on glutathione–Sepharose prior to separation by SDS–PAGE and detection by Coomassie brilliant blue staining (Fig. 6C). Taken together, these results demonstrated that the carboxy terminus as well as the NLS sequence of HCMV pUL56 can mediate specific binding to importin α (hSRP1α), suggesting that nuclear import of this viral protein occurs via the classical importin-dependent pathway (Görlich, 1997).

Discussion

The data presented here extend previous observations on the highly conserved gene product of HCMV ORF UL56 (pUL56) (Bogner et al., 1993), which suggested involvement of HCMV pUL56 in viral DNA packaging and its function as a putative terminase subunit (Bogner, 1999; Bogner et al., 1998). In this context, it was of particular interest to examine the

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Fig. 4. Amino acid residues at positions 7 and 8 of the HCMV pUL56 NLS are essential for nuclear import of reporter proteins. β-Gal- and GFP-specific protein segments are indicated in all schemes. The residues of the sequence of the pUL56 NLS are shown and positions of residues are indicated. Basic residues are underlined. Open boxes indicate substitutions of residues within the pUL56 NLS by alanine. Some of the nucleoli are marked by arrows. (A)–(F) Plasmids were constructed that encoded the pUL56 NLS amino acid sequence with sequential double substitutions of residues by alanine (pUL56nlsAA1–pUL56nlsAA6) fused to reporter protein β-Gal–GFP. Subcellular localization of chimeras was examined by detection of GFP (GFP) and fluorescence staining with an anti-β-Gal MAb and a Texas red-conjugated secondary antibody (β-Gal). Phase-contrast images were taken to visualize nuclei and nucleoli (phase-c). (G) As a control, examination of the intracellular localization of the construct encoding the β-Gal–pUL56NLS–GFP fusion protein was carried out as described above.

Fig. 5. Nuclear translocation of full-length HCMV pUL56 is prevented by substitution of amino acids 822 and 823 by alanine. (A) Subcellular distribution of singly expressed His–pUL56nlsAA4 with substitutions of amino acids 822 and 823 introduced by site-directed mutagenesis. Transient transfection of the construct into COS-7 cells was performed prior to examination of intracellular distribution at 40 h after transfection by immunofluorescence with MAb Anti-Xpress (His–pUL56nlsAA4). Nuclei and nucleoli are visualized in the phase-contrast image (phase-c). (B) As a control, epitope-tagged His–pUL56 containing the wild-type NLS sequence was expressed transiently in COS-7 cells. Subcellular distribution was examined as described above. In the schemes of both panels, the recombinant Anti-Xpress epitope and the pUL56 sequence are indicated. Numbers indicate positions of residues of the HCMV pUL56 sequence. The sequence of the NLS at the carboxy terminus is shown, basic residues are underlined and substitutions are marked by an open box. Arrows indicate some of the nucleoli.
Fig. 6. HCMV pUL56 binds specifically to importin α (hSRP1α) via its NLS sequence. (A) Equal amounts of GST alone (lanes 1 and 5) and GST fusion proteins containing of the carboxy-terminal portion (aa 404–850, lane 2) of pUL56, the NLS (lane 6), the reversed NLS (lane 7) and the NLS sequence containing substitutions of residues 7 and 8 (lane 8) were immobilized on glutathione–Sepharose 4B beads and incubated with in vitro-translated [35S]methionine-labelled importin α (hSRP1α). Bound material was separated by SDS–PAGE and the amount of importin α bound was detected by autoradiography. Analysis of immunoprecipitates of in vitro-translated importin α with MAb Anti-Xpress and direct electrophoretic separation of in vitro translation products served as controls (lanes 3 and 4). Molecular mass markers (M) were myosin, phosphorylase b, BSA, ovalbumin and carbonic anhydrase. (B) Specifically immobilized radioactivity was quantified by BioImager analysis. The relative amounts of bound radioactivity are indicated. (C) As a control, equal amounts of the same GST fusion proteins described in (A) were immobilized on glutathione–Sepharose prior to washing and separation by SDS–PAGE (lanes 1–5). Fusion proteins were visualized by Coomassie blue staining. Molecular mass markers (M) are shown on the left.
intrinsic potential of pUL56 for nuclear targeting. By using immunofluorescence analysis of infected HFF, homogenous nuclear staining, with increased antigen accumulation in nucleolar regions, was obtained with monospecific antibody pabUL56 at early times after infection (12 h p.i.). At later times p.i. (48 h), pUL56-specific signals were also concentrated in discrete subnuclear structures, whereas no fluorescence signals were observed in the nucleoli (Fig. 1A). It has been shown that these bright nuclear inclusions obtained at late times after infection represent compartments of viral DNA replication in which viral proteins relevant for replication accumulate (Giesen et al., 2000; Penfold & Mocarski, 1997; Sarisky & Hayward, 1996). Our observations are consistent with findings on the PrV pUL56 homologue ICP18.5 (Pederson & Enquist, 1989), which is also expressed early after infection (2 h p.i.) and translocated into the nucleus (Pederson & Enquist, 1991). However, no reports are available regarding the localization of ICP18.5 in virus replication centres.

Furthermore, we demonstrated nuclear translocation of pUL56 in the absence of other viral proteins (Fig. 1B, C). After transfection of authentic pUL56 or epitope-tagged His–pUL56, homogenous intranuclear immunofluorescence signals were obtained, whereas nucleolar regions remained unstained. As expected, bright intranuclear inclusion bodies were not detectable under these conditions, suggesting that additional virus products, e.g. viral DNA and/or other viral proteins, may be needed for this particular localization.

Intranuclear transport of pUL56 in the absence of other viral proteins implied the existence of an endogenous NLS. Two structurally different types of NLS have been described: the monopartite SV40 large T antigen NLS (Kalderon et al., 1984) and the bipartite NLS of Xenopus laevis nucleoplasmin (Robbins et al., 1991). A universal NLS consensus sequence for all nuclear proteins has not yet been established, other than an accumulation of basic residues within a short stretch of amino acids (Dingwall & Laskey, 1991), often containing a glycine or proline residue as a ‘helix-breaker’ (Dang & Lee, 1989). HCMV pUL56 contains an obvious stretch of basic amino acids at the extreme carboxy terminus (RRVRATRKRPRR; aa 816–827), an additional signal that mediates nucleolar transport including the function of the NLS is probably exposed at the surface and therefore more accessible to transport factors that interact with NLS sequences, such as importin α (Conti et al., 1998; Görlich et al., 1995). Both amino acids, arginine at position 822 and lysine at position 823, also appear to play a pivotal role in nuclear targeting of full-length His–pUL56 (Fig. 5).

When the pUL56 NLS sequence was fused to reporter proteins, not only diffuse nuclear staining but also nucleolar localization of the chimeras was detected (Figs 3 A and 4 C, G). Transport into the nucleolus is probably dependent on the cooperation of several domains and/or on interactions with other macromolecules within the nucleolus (Nosaka et al., 1989; Schmidt-Zachmann & Nigg, 1993). In some cases, the signals for nuclear transport have been shown to overlap with NLS sequences (Siomi et al., 1988; Warner & Sloboda, 1999). Therefore, one possible explanation for this particular distribution may be that the function of the pUL56 NLS is extended by adjacent residues of the reporter protein, creating an additional signal that mediates nucleolar transport including the interaction with nucleolar macromolecules. Interestingly, substitution of residues 1, 2, 3, 4, 11 and 12 (R, R, V, R, R and R) within the NLS motif by alanine did not influence nuclear import of the reporter proteins, but eliminated nucleolar localization (Fig. 4 A, B, F). Regarding the nuclear targeting function of the NLS of pUL56, the phenomenon of subnuclear compartmentation is intriguing, but of less interest.

Regarding the question of whether import of pUL56 into the nucleus is carrier mediated, the recombinant human NLS receptor subunit hSRP14 (Weis et al., 1995) of the importin complex was used for in vitro binding experiments. We were able to demonstrate that the importin α homologue hSRP14 interacts specifically with the carboxy terminus of HCMV pUL56 in an NLS-dependent manner (Fig. 6A). In order to support the significance of this interaction, GST constructs containing the reversed NLS of pUL56 (GST–pUL56revNLS)
or the pUL56 NLS containing substitutions of residues 7 and 8 by alanine (GST–pUL56nlsAA4) were used for in vitro binding assays. Fusion of the reversed pUL56 NLS sequence to the cytoplasmic reporter protein β-Gal–GFP resulted in a predominantly cytoplasmic localization of the chimera (Fig. 3B). The specific interaction between the reversed NLS fused to GST and hSRP1α was about 85% weaker than the binding of the GST–wild-type NLS chimera in vitro binding assays (Fig. 3B; lanes 6 and 7). These results are in line with experiments described for the SV40 large T antigen NLS. By using chemical cross-linking analysis, a synthetic peptide containing the reversed NLS sequence did not compete for the binding of importin α to wild-type SV40 NLS (Adam et al., 1989). These authors concluded that it is not only the charge density of NLS peptides that is responsible for binding and that there is a correspondence between the sequences directing nuclear transport in vivo and their ability to compete for NLS peptide binding of importin α in vitro. It has also been shown that the reversed peptide was incapable of directing nuclear transport in vivo (Lobl et al., 1990). Therefore, the weaker interaction between the reversed NLS sequence of HCMV pUL56 and hSRP1α may be explained by the requirement for a specific conformation in the context of adjacent residues that depends on a specific order of amino acids in this region.

Regarding the interaction between importin α and GST–pUL56nlsAA4, containing substitutions of residues 7 and 8 by alanine, a decrease of about 90% was observed when compared with the binding of wild-type pUL56 NLS fused to GST (Fig. 3B). This result was in line with the finding that pUL56nlsAA4 fused to the reporter protein β-Gal–GFP also resulted in the predominant retention of the reporter protein chimera in the cytoplasm (Fig. 4D).

Taken together, our results imply that HCMV pUL56 can be recognized via its NLS by importin α, the adaptor protein of the heterodimeric importin complex, in transfected or infected cells and is subsequently transported into the nucleus by the importin-dependent pathway. Our observations thus indicate that a pUL56-mediated step in virus maturation is probably dependent on the functional nuclear import machinery of the host cell.

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