Nuclear import of isoforms of the cytomegalovirus kinase pUL97 is mediated by differential activity of NLS1 and NLS2 both acting through classical importin-α binding

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The multifunctional protein kinase pUL97 of human cytomegalovirus (HCMV) strongly determines the efficiency of virus replication. Previously, the existence of two pUL97 isoforms that arise from alternative translational initiation and show a predominant nuclear localization was described. Two bipartite nuclear localization sequences, NLS1 and NLS2, were identified in the N terminus of the large isoform, whilst the small isoform exclusively contained NLS2. The current study found the following: (i) pUL97 nuclear localization in HCMV-infected primary fibroblasts showed accumulations in virus replication centres and other nuclear sections; (ii) in a quantitative evaluation system for NLS activity, the large isoform showed higher efficiency of nuclear translocation than the small isoform; (iii) NLS1 was mapped to aa 6–35 and NLS2 to aa 190–213; (iv) using surface plasmon resonance spectroscopy, the binding of both NLS1 and NLS2 to human importin-α was demonstrated, stressing the importance of individual arginine residues in the bipartite consensus motifs; (v) nuclear magnetic resonance spectroscopy of pUL97 peptides confirmed an earlier statement about the functional requirement of NLS1 embedding into an intact α-helical structure; and (vi) a bioinformatics investigation of the solvent-accessible surface suggested a high accessibility of NLS1 and an isoform-specific, variable accessibility of NLS2 for interaction with importin-α. Thus, the nucleocytoplasmic transport mechanism of the isoforms appeared to be differentially regulated, and this may have consequences for isoform-dependent functions of pUL97 during virus replication.

INTRODUCTION

Human cytomegalovirus (HCMV) is a member of the family Herpesviridae and shows a ubiquitous distribution worldwide. As a major human pathogen, primary HCMV infection of immunocompetent persons is generally weakly symptomatic (mononucleosis), whilst in immunocompromised persons, HCMV frequently causes systemic disease, sometimes with life-threatening consequences (Mocarski et al., 2007). Furthermore, HCMV is the most common cause of congenital viral infections eventually leading to a severe, generalized cytomegalic inclusion disease (Adler et al., 2007). HCMV replication is regulated through a balance of multiple interactions between viral and cellular protein, whereby various protein kinases display crucial functions and are interlinked in several aspects of their regulatory properties (Prichard, 2009; Lee & Chen, 2010; Marschall et al., 2011). Herpesviruses encode viral protein kinases of the serine/threonine type, each phosphorylating a number of viral and cellular substrate proteins. In the case of HCMV, the kinase pUL97 (the prototype of the
herpesvirus UL-type kinases; Marschall et al., 2011) exerts important functions in determining the efficiency of replication. For instance, pUL97 is able to phosphorylate pUL44, pUL69, pp65, nuclear lamins, the multifunctional adaptor protein p32, retinoblastoma protein, RNA polymerase II and the translational elongation factor EF-1α (reviewed by Marschall et al., 2011). These phosphorylations have specific regulatory consequences, such as increased RNA transport activity of phosphorylated pUL69 (Thomas et al., 2009) and a phosphorylation-dependent destabilization of the nuclear lamina (Milbradt et al., 2010). Deletion of the UL97-coding sequence from the viral genome or pharmacological inhibition of the pUL97 kinase activity results in a rigorous reduction in virus replication efficiency by a factor of 100–1000 (Prichard et al., 1999; Wolf et al., 2001; Biron et al., 2002; Marschall et al., 2002; Herget et al., 2004). Recently, we reported the formation of two distinct isoforms of pUL97 that may provide specific functions during the phosphorylation-dependent steps of HCMV replication (Webel et al., 2011).

The main regulatory steps underlying pUL97 activity are nuclear processes, such as viral DNA synthesis and transcription, and the nucleocytoplasmic egress of capsids (Prichard, 2009; Lee & Chen, 2010; Marschall et al., 2011). In this context, we postulated the positions of two nuclear localization signals (NLSs) in the N terminus of pUL97, with one located at aa 6–35 and the second downstream of this position (Webel et al., 2011). The identification of two typical bipartite-type NLSs strongly suggests that nuclear import of pUL97 may be regulated through the classical importin-α/β pathway (Fontes et al., 2003; Alvisi et al., 2008; Marfori et al., 2011). This pathway is characterized by direct binding of cargo proteins to the importin-α adaptor molecule, which is then associated with the importin-β molecule serving as a direct receptor for the nuclear pore complex. Interestingly, the loading of importin-α with cargo proteins is mediated through two distinct NLS-binding sites (Cingolani et al., 1999; Marfori et al., 2011). In contrast to monopartite NLSs, which preferentially bind to the major binding site of importin-α, bipartite NLSs simultaneously occupy both the major and minor binding sites.

Here, we defined the precise position of the two NLSs in pUL97, termed NLS1 and NLS2, at aa 6–35 and 190–213, respectively. We have provided evidence for differential activities of NLS1 and NLS2 and proved in both cases a direct binding to human importin-α. An additional bioinformatics investigation revealed differences in the solvent accessibility of NLS2 when present in the context of either the large or the small isoform of pUL97, which may affect the interaction with importin-α. These properties may have an impact on the fine regulation of nuclear transport and on the overall biological behaviour of pUL97. Putative regulatory consequences for the HCMV replication are discussed.

RESULTS

The predominant nuclear localization of pUL97 is based on the function of NLS1 (aa 6–35) and NLS2 (aa 190–213)

The kinase pUL97 phosphorylates a number of viral and cellular substrates. Most are nuclear proteins, but cytoplasmic proteins can additionally be phosphorylated efficiently (see above; Marschall et al., 2011). This reflects the multifunctional role of pUL97 in a variety of different regulatory processes within the nucleus and the cytoplasm (Hertel et al., 2007; Prichard, 2009; Goldberg et al., 2011). In view of this fact, it was expected that the main localization of pUL97 would be nuclear, and that some cytoplasmic localization would additionally be detected in HCMV-infected as well as in transiently UL97-transfected cells. Here, we demonstrated that, in primary human foreskin fibroblasts (HFFs) infected with HCMV strain AD169, a predominant nuclear localization was found by immunofluorescence staining. This confocal microscopy analysis showed that pUL97 was detected in virus replication compartments, confirming earlier data (Marschall et al., 2003), or was distributed evenly over the entire nucleus (Fig. 1a). Another interesting feature was that pUL97 to some extent could be detected in proximity to the nuclear envelope. Even when this type of localization involved only a small fraction of the pUL97 staining, the finding was strengthened by the fact that pUL97 partially co-localized with nuclear rim speckles containing viral pUL53 (Fig. 1a, insets; note the yellow speckles in panel 6), a typical marker of the nuclear egress complex (Milbradt et al., 2009). This co-localization was seen mostly at late time points of infection. Similar to the laboratory strain AD169, two clinical isolates showed a nuclear distribution of pUL97 with a marked accumulation at virus replication compartments (Fig. 1b). This proved to be the main type of pUL97 localization. In addition, co-staining with lamin A/C indicated some co-localization with the nuclear rim, although this was only detectable in a minority of cells (Fig. 1b).

Previously, two NLSs were identified within the N-terminal region of pUL97 (Webel et al., 2011). The first NLS is positioned at aa 6–35 (NLS1; Fig. 2a, panels 1–3) and consists of a bipartite structure in which two clusters of basic amino acids (R34R35 and RPR35R36R37R38R39) are separated by a spacer region of 16 aa. For the second NLS, two overlapping bipartite-type NLSs had previously been postulated, aa 164–198 and 190–213. In the present study, this NLS region was examined in more detail using additional GFP–β-galactosidase (β-gal) fusion constructs for confocal immunofluorescence analysis. A construct containing the entire region of interest, aa 164–213, was localized in the nucleus of transfected HeLa cells (Fig. 2a, panels 7–9). Two separate constructs containing either the first or the second postulated NLS sequence (aa 164–198 or 190–213) were used to narrow down the essential region. Whilst a cytoplasmic localization was noted for the
aa 164–198 construct (Fig. 2a, panels 10–12), a pronounced nuclear localization was detected for the aa 190–213 construct (Fig. 2a, panels 4–6). Thus, aa 190–213 was sufficient to confer nuclear import and represented NLS2. These observations could be rationalized from the primary amino acid sequence exhibiting two clusters of basic residues, namely \( ^{190} \text{RGGRKR} ^{195} \) and \( ^{211} \text{RRR} ^{213} \). Therefore, NLS2 appeared to consist of a bipartite sequence element in which a spacer region of 15 aa separated these two basic clusters.

The efficiency of experimental nuclear translocation is higher for the large isoform than for the small isoform of pUL97

To determine the significance of both NLS1 and NLS2 for nuclear translocation of the two isoforms of pUL97, further constructs were generated containing the entire wild-type pUL97 or the isoform-specific versions of pUL97 (N-terminally fused to \( \beta \)-gal in the absence of an additional GFP fusion to confine the sizes of the constructs). Whilst the construct for the large isoform was completely translocated to the nucleus (100 % of the cells; Table 1), the two constructs representing the small isoform lacked this efficiency (75 and 67 %, respectively). Thus, in this NLS mapping system, the nuclear translocation activity of pUL97 was higher for the large isoform than for the small isoform.

As a next step, NLSs were deleted individually from these \( \beta \)-gal fusion constructs. Deletion of NLS1 from the wild-type construct resulted in a reduction in nuclear translocation activity, as manifested in a nucleocytoplasmic (58 %) or cytoplasmic distribution (42 %) (Table 1 and Fig. 2b, panels 1–8). Deletion of NLS2 also led to a reduction in nuclear import activity, similarly causing a shift to a more nucleocytoplasmic (49 %) or cytoplasmic distribution (11 %) (Table 1 and Fig. 2b, panels 9–16). As the effect of the NLS1 deletion was more drastic than that of the NLS2 deletion (residual nuclear signal of 0 versus 40 %), we concluded that NLS1 possesses a stronger overall NLS efficiency than NLS2. Deletion of both NLSs from wild-type pUL97 led to a massive reduction in nuclear localization with only marginal residual nuclear signals (2 %; Table 1 and Fig. 2b, panels 17–20). Deletion of NLS2 in a construct expressing only the small isoform resulted in the complete loss of nuclear signals (0 %) and a purely cytoplasmic localization (Table 1 and Fig. 2b, panels 21–24). This finding indicated the importance of NLS2 specifically for nuclear import of the small isoform. It should be noted that an antibody directed against the pUL97 part of the fusion protein...
protein generally produced a higher portion of residual nuclear signals after NLS deletion compared with the antibody specific for β-gal. This may have resulted from a small fraction of degraded pUL97 fragments diffusing passively into the nucleus, although the main detectable portion of the fusion protein was non-degraded and stable when monitored on Western blots (data not shown).

Independent of this, any residual nuclear localization of the NLS1 and NLS2 deletion mutants could be explained by a tight interaction of pUL97 with NLS-carrying cellular proteins, possibly providing an additional indirect nuclear import mechanism.

In vitro, both NLS1 and NLS2 are recognized and bound efficiently by human importin-α

The two identified NLSs were analysed in vitro with respect to their affinity for the responsible nucleocytoplasmic import pathway. Typically, proteins containing bipartite NLSs are transported via the classical importin-α/β pathway. Therefore, the interaction of pUL97 with importin-α was investigated by surface plasmon resonance (SPR) analyses using recombinantly expressed human importin-α and synthetic pUL97 peptides spanning NLS1 or NLS2 (Fig. 3). Importin-α was immobilized on CM5

Fig. 2. Identification of two bipartite NLSs, NLS1 (aa 6–35) and NLS2 (aa 190–213), using the GFP–β-gal NLS mapping system. HeLa cells were seeded on coverslips, transfected with the fusion constructs indicated, fixed at 2 days post-transfection and analysed by confocal laser-scanning microscopy. (a) For NLS1, a clear nuclear localization was detected for aa 6–35. For NLS2, the responsible sequence for nuclear localization was narrowed down to aa 190–213 (also detectable for the N-terminally extended construct aa 164–213). Other constructs, such as aa 164–198, showed a cytoplasmic localization. (b) The importance of NLS1 and NLS2 for pUL97 isoforms was illustrated using pUL97 deletion mutants N-terminally fused to β-gal (inserted scheme). For constructs pUL97(Δ6–35) and pUL97(Δ190–213), two representative microscopic fields are shown (panels 1–4 and 5–8, and panels 9–12 and 13–16, respectively). A quantitative evaluation is given in Table 1. Magnification, ×63.
sensor chips to 1000 or 10 000 response units (RU), and the interaction with pUL97 peptides was measured over a 2–10 μM range. For NLS1, the peptide comprising aa 6–35 [Pep97(6–35)] showed a strong interaction with importin-α. In comparison, a peptide with N-terminally truncated NLS1, Pep97(16–35), showed a >50 % reduced binding response. An even more reduced binding response was measured for the further truncated Pep97(27–35) (Fig. 4a). However, the differences in the binding responses between Pep97(27–35) and Pep97(16–35) could in part be explained by the lower molecular mass of Pep97(27–35), which is known to affect the SPR response. This theory was supported by the dissociation constants, which were in the same order of magnitude for these two peptides (Table 2). Nevertheless, our findings clearly demonstrated a direct NLS1–importin-α binding and suggested that the cluster of basic residues 2RRPSRARRR35 invariably contained in these peptides is an important binding determinant.

The embedding of NLS1 into a specific structural environment, including a flanking α-helix, has been postulated previously and was confirmed here by nuclear magnetic resonance (NMR) (Supplementary Method, Fig. S1 and Tables S1–S3, available in JGV Online). It has been shown experimentally that α-hydrogen chemical shifts >0.1 p.p.m. relative to the random-coil values are qualitative indicators of

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**Table 1. Evaluation of the intracellular distribution of pUL97 isoforms as analysed in β-gal fusion constructs**

Construct pUL97(M38/74/111/157) represents the large isoform and constructs pUL97(M1L) and pUL97(74–707) the small isoform of pUL97.

<table>
<thead>
<tr>
<th>Expression construct</th>
<th>Nuclear localization (%)</th>
<th>Nucleocytoplasmic localization (%)</th>
<th>Cytoplasmic localization (%)</th>
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<tbody>
<tr>
<td>pUL97</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pUL97(M38/74/111/157)</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pUL97(M1L)</td>
<td>75</td>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>pUL97(74–707)</td>
<td>67</td>
<td>15</td>
<td>18</td>
</tr>
<tr>
<td>pUL97(Δ6–35)</td>
<td>0</td>
<td>58</td>
<td>42</td>
</tr>
<tr>
<td>pUL97(Δ190–213)</td>
<td>40</td>
<td>49</td>
<td>11</td>
</tr>
<tr>
<td>pUL97(Δ6–35/Δ190–213)</td>
<td>2</td>
<td>16</td>
<td>82</td>
</tr>
<tr>
<td>pUL97(M11/Δ190–213)</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
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</table>

**Fig. 3.** Schematic presentation of the two identified pUL97 isoforms. The positions of functional elements including NLS1 and NLS2 are indicated. The data obtained for the importin-α interaction with synthetic pUL97 peptides are summarized as: NS, not soluble; ND, not determined; –, no interaction; +, interaction; +/–, low interaction (reduced with respect to non-mutated peptide).
protein secondary structure. A minimum of four adjacent residues with an upfield shift is indicative of an $\alpha$-helix, whereas $\beta$-sheets require a minimum of three residues with downfield shifts (Wishart et al., 1992). The $\alpha$-hydrogen chemical shift index of Pep97(1–20) indicated the presence of a relatively weak $\alpha$-helical structure that may include...
residues Ser-3 to Ser-11 (Fig. S1a). Pep97(16–35) seemed to adopt mainly a largely disordered random-coil conformation (Fig. S1b). In contrast, an extensive α-helical structure including all residues of Pep97(31–50) was observed (Fig. S1c). This analysis, however, was based on the NMR determination of synthetic peptides of 20 aa in length and might not be absolutely significant for the entire native protein. Nevertheless, the finding is consistent with a computer-based empirical structure prediction indicating α-helical structural elements in the N terminus of pUL97 (Webel et al., 2011).

Further SPR analyses were performed to characterize peptides containing NLS2. A strong importin-α interaction was detected for Pep97(190–213), including the complete NLS2 sequence, which was drastically reduced for Pep97(195–213), lacking 5 aa on the N-terminal side (Fig. 5a). It should be noted that the peptides Pep97(190–213) and Pep97(195–213) both showed a delayed dissociation and did not produce clear-cut concentration-dependent responses. This may relate to the specific characteristics of these peptides (for details, see legend to Fig. 5). Furthermore, the additionally postulated NLS sequence at aa 164–198 (Webel et al., 2011) was examined. However, for Pep97(164–198), only marginal binding to importin-α was detected, which was similar to the response of Pep97(190–198). This was explained by the fact that both peptides covered an insufficient portion of NLS2 (Fig. 5; NLS-Npl was used as an importin-α-binding control). Thus, the experimental observations provided a mapping of NLS2 to region 190–213 and suggested that the two clusters with a high density of basic residues, namely 190RGGRKR195 and 211RR213, are required for the strong interaction between the bipartite-structured NLS2 and importin-α.

Using an accepted procedure for simple analyte–ligand systems (Rich & Myszka, 2010), quantification of interaction stoichiometry was performed according to a simple 1:1 Langmuir binding model (Table 2). In general, however, importin-α possesses two individual binding sites to generate favourable contacts with NLSs (major and minor sites; Alvisi et al., 2008; Marfori et al., 2011). Additionally, the two elements in the bipartite sequences of NLS1 and NLS2 may principally be able to mediate independent contacts with importin-α, suggesting a more complex mode of interaction. Therefore, alternative binding models for the SPR sensorgrams, such as heterogeneous ligand and bivalent analyte fittings, were additionally applied (Table S4), illustrating our concept of direct NLS1/NLS2–importin-α binding.

**Table 2. Evaluation of the importin-α 1 : 1 binding of pUL97 peptides by SPR spectroscopy**

<table>
<thead>
<tr>
<th>pUL97 peptides</th>
<th>Amino acid sequence</th>
<th>Kinetic constants</th>
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<tr>
<td></td>
<td></td>
<td>$K_D$ (µM)</td>
</tr>
<tr>
<td><strong>NLS1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pep97(6–35)</td>
<td>RSRARASLGGTQTGWDPPPLRRPSARRR</td>
<td>0.68 ± 0.02</td>
</tr>
<tr>
<td>Pep97(16–35)</td>
<td>TTTQGWDPPLRRPSARRR</td>
<td>2.33 ± 0.46</td>
</tr>
<tr>
<td>Pep97(27–35)</td>
<td>RRRPSARRR</td>
<td>5.83 ± 0.60</td>
</tr>
<tr>
<td>Pep97(16–35_A32L)</td>
<td>TTTQGWDPPLRRPSARRR</td>
<td>6.26 ± 1.49</td>
</tr>
<tr>
<td><strong>NLS2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pep97(190–213)*</td>
<td>RGRGRKPLRPLPPPLVSLARTPLCRR</td>
<td>ND</td>
</tr>
<tr>
<td>Pep97(195–213)*</td>
<td>RPLRPLVSLARTPLCRR</td>
<td>ND</td>
</tr>
<tr>
<td>Pep97(164–198)</td>
<td>RDGDVTSSVRALLFTGSDPSDSVSVGVRGGRKPLR</td>
<td>5.60 ± 2.87</td>
</tr>
<tr>
<td>Pep97(190–198)</td>
<td>RGRGRKPLR</td>
<td>10.84 ± 2.82</td>
</tr>
</tbody>
</table>

*The kinetic constants of peptides Pep97(190–213) and Pep97(195–213) were not determined as that their binding characteristics are more complex than standard binding models can account for.*

To identify the residues within the bipartite consensus motif of NLS1 essential for importin-α binding, peptides carrying replacement mutations were analysed by SPR (Fig. 3). Pep97(16–35_A32L) carrying an exchange at position 32 (alanine to leucine) showed no detectable differences from Pep97(16–35) in terms of importin-α-binding efficiency and kinetics (Fig. 4a; Table 2). In contrast, peptides carrying replacement mutations of basic residues with hydrophobic residues (arginine to alanine) exhibited a significantly reduced interaction with importin-α, as shown for Pep97(16–35_R34A), Pep97(16–35_R35A) and Pep97(16–35_R34/35A). The double-mutation R34A and R35A led to a nearly complete inhibition of binding (Fig. 4b). Further analysis of peptides with arginine to alanine exchanges, such as Pep97(16–35_R31A), Pep97(16–35_R33A) and Pep97(16–35_R31/33A), confirmed the importance of arginine residues for NLS1–importin-α binding activity (data not shown; summarized in Fig. 3).
Computational analysis suggests that NLS2 of the small isoform might be locked by numerous salt bridges in a conformation inaccessible to nuclear import factors.

The strong importin-α binding detected in vitro for NLS1 and NLS2 indicated that these two NLSs do not require flanking parts of pUL97 for functionality. This property is common to NLSs that are independent elements usually located outside globular protein domains in order to allow their undisturbed interaction with nuclear import factors (Kosugi et al., 2009). Thus, the differences in nuclear import detected for the small and large isoforms of pUL97 may result from different degrees of accessibility of the NLSs. To investigate this point in more detail, molecular dynamics (MD) simulations were performed for aa 1–270 in the large isoform or aa 74–270 in the small isoform, respectively. These stretches are predicted to exhibit a large conformational flexibility that may facilitate intramolecular interaction with the NLS2 sequence, thereby shielding it.

**Fig. 5.** SPR analysis showing the interaction of human importin-α with synthetic pUL97 peptides covering NLS2 (a) or with the heterologous peptide NLS-Npl used as a binding control (KRPAATKKAGQAKKKK) (b). The peptides were injected as in Fig. 4 (sensor chip immobilized to 1000 RU). Kinetic analyses (heterogeneous ligand fitting) were performed for Pep97(164–198), Pep97(190–198) and NLS-Npl. Note that Pep97(190–213) showed a delayed dissociation that hampered the detailed SPR analysis (even after prolonging the dissociation time from 120 to 1200 s). The peptides Pep97(190–213) and Pep97(195–213) showed an unexpected course of concentration-dependent responses, which could not be resolved by repeated rounds of analysis. The reason for this variation may be derived from a peptide-specific succession of amino acid clusters, promoting a very strong, irreversible mode of binding, thereby lacking efficient dissociation.
Fig. 6. SASA and structural features of the pUL97 NLSs. (a–d) SASA was determined for NLS1 in the large isoform (a), NLS2 in the large isoform (b), NLS2 in the small isoform (c) and NLS2 in the GFP–β-gal reporter construct (d). Evaluations of the entire NLSs and NLS-inherent basic residues are shown as red and black curves, respectively. Analysis of the mean SASA for the final 60 ns of the simulation resulted in the following accessibilities for the entire NLSs and NLS-inherent basic residues, respectively: (a) 1970 and 600 Å²; (b) 1762 and 635 Å²; (c) 1372 and 415 Å²; (d) 1914 and 1044 Å². (e) Structures of the N-terminal region of pUL97 in the large (left) and small (right) isoforms obtained by computer simulation. The NLSs are shown as stick presentations, with NLS1 in blue (aa 6–35, missing in the small isoform) and NLS2 in green (aa 190–213). The non-NLS parts of the N termini are shown as a solid surface with an electrostatic potential colouring scheme (basic residues in blue, acidic residues in red). The basic arginine and lysine residues of NLS2 are highlighted by arrows: violet arrows indicate that the respective arginine/lysine residues are fixed by salt bridges, whilst green arrows indicate the absence of such an electrostatic interaction.
from an interaction with importin-α. To analyse the degree of shielding, the solvent-accessible surface area (SASA) of both NLSs was investigated as a function of the simulation time (Fig. 6a–c). As far as the large isoform was concerned, a high accessibility for NLS1 and NLS2 specifically for the NLS-inherent basic arginines was detected (Fig. 6a, b), suggesting that both NLSs feature nuclear import. In the case of NLS2, the mean SASA showed a slight reduction in relation to NLS1, whereas the mean accessibilities of arginine residues were similar. Moreover, the accessibility of the isolated minimal sequence of NLS2 contained within the GFP–β-gal fusion construct was even higher than in its natural protein context, in particular the accessibility of its basic arginine residues (Fig. 6d). This finding is compatible with the high nuclear import activity measured for this minimal construct (Fig. 2a, panels 4–6). Importantly, however, the SASA of the entire NLS2 and the SASA of the NLS2-specific arginine residues in the small isoform (Fig. 6c) were both diminished with respect to the large isoform (Fig. 6b). This indicated a reduced interaction potential of NLS2 in the small isoform with nuclear import factors. These results were in accordance with data from the quantitative NLS evaluation system showing a less efficient nuclear translocation of the small isoform (Table 1). In Fig. 6(e), representative structures of the N-terminal region of both pUL97 isoforms are depicted, highlighting the two NLSs and the important arginine/lysine residues within NLS2. Only three basic residues of NLS2 are fixed within the N terminus of the large isoform by salt bridges, whilst the NLS2 of the small isoform exhibits eight fixed arginine/lysine residues (Fig. 6e, violet arrows). In conclusion, the number of intramolecular electrostatic interactions and the shielding from the solvent detected in the computer simulations was generally much higher for NLS2 in the small isoform than in the large isoform, thus suggesting structural reasons for the reduced nuclear import of the small isoform.

**DISCUSSION**

Previous investigations of pUL97 have demonstrated that this viral kinase is expressed as two isoforms. The current analysis of viral protein expression in HFFs infected with various strains of HCMV confirmed the occurrence of pUL97 isoforms. No basic difference in isoform expression patterns was observed between laboratory strains and clinical isolates of HCMV (data not shown). A closer examination of parameters, such as m.o.i. and kinetics using clinically relevant HCMVs and pUL97 mutants, may provide further insight into the functional nature of pUL97 isoforms (work in progress). The large and small isoforms of pUL97 both exhibit a pronounced nuclear localization, which is mediated through two distinct bipartite NLSs (Webel et al., 2011). Here, we provided evidence for the positioning of NLS1 and NLS2 at aa 6–35 and 190–213, respectively. The data indicated that both NLSs mediated direct binding of pUL97 to importin-α, emphasizing the importance of NLS arginine residues. Furthermore, bioinformatics data suggested a high accessibility of NLS1 and an isoform-dependent accessibility of NLS2 for interaction with importin-α, supporting the experimental finding that the large isoform showed higher efficiency of nuclear translocation than the small isoform. This may reflect an individual fine regulation of the nuclear import of the isoforms.

As demonstrated by several experiments in this study, the nuclear import of pUL97 was conferred through the direct binding of two bipartite NLSs to importin-α. The two clusters of basic amino acids of NLS1, comprising residues 6RSRAR10 and 27RRPSRARRR35, resided to some extent in regions with a propensity for forming α-helical structures (Fig. S1a, c). The NMR experiments revealed that the spacer region connecting these two clusters adopted a flexible random-coil structure (Fig. S1b), allowing orientational freedom of the structured elements that may be vital for the strong binding to importin-α. Comparing NLS1 with NLS2, the spacer regions comprised an almost identical length (a stretch of 16 or 15 residues, respectively) that may refer to a similar mode of interaction. In general, classical NLSs are characterized by such regions with a high portion of basic residues. For example, the arginine-rich motif of the Tat protein encoded by human immunodeficiency virus type 1, serving as a NLS and additionally as an RNA-binding domain (Friedler et al., 2000), is consistent with this model. Nuclear import of classical NLS-carrying proteins is commonly initiated by binding to importin-α, functioning as the primary import receptor. Thereafter, the complex is attached to importin-β, which confers contact to the nuclear pore complex, finally resulting in nuclear import driven by an energy-dependent process (Görlich, 1998). We provided new evidence that both isoforms of pUL97 are transported via this classical pathway through the combined activity of NLS1 and NLS2 (large isoform) or NLS2 only (small isoform). However, the measurable differences in their nuclear import efficiencies in experimental systems indicated the possibility that the transport mechanism may be differentially regulated in an isoform-specific manner.

The solvent accessibility of NLS1 and NLS2 in the two isoforms was investigated by computational MD simulations to determine whether structural features influenced the NLS regulation of pUL97. The major goal was to predict the structural features that might lead to reduced nuclear transport of the small isoform. As studies of large-scale structural rearrangements are computationally extensive, we limited our investigation to the pUL97 N terminus (aa 1–270 or 74–270), which has been predicted to adopt a non-globular structure (Webel et al., 2011). Due to its flexibility, this region is most likely to be a candidate for structural changes that may result in shielding of the NLSs. To allow a more comprehensive sampling of the motions in the N terminus, we used an implicit solvent model. Although this method is an established procedure to study large-scale motions and the folding of small
proteins (Simmerling et al., 2002), simulations on a nanosecond timescale give only limited information on the structural properties of larger proteins. Consequently, the conformational sampling of the pUL97 N termini was not sufficiently exhaustive to allow functional conclusions based on the simulation data alone. However, the structural features detected in the simulations can give initial information about conformational preferences of the small and large pUL97 isoforms that need to be considered carefully in the context of the experimental data. Consistent with the experimental observation of reduced nuclear import of the small isoform, NLS2 of the small isoform exhibited the lowest accessible surface area among all constructs computationally investigated, thus offering a initial explanation for the reduced interaction with importin-α. However, the overall differences in solvent accessibility were only in the range of 20–35%, suggesting that additional structural factors also affect importin-α recognition. Structural modelling, as shown in Fig. 6(e), suggested that not only the degree of accessibility itself but also the number of intramolecular electrostatic interactions formed by the arginines of the NLSs play a crucial role in nuclear import. The structural simulation suggested that the basic residues of NLS2 may be fixed by only three salt bridges in the large isoform but by eight in the small isoform. These salt bridges would need to be broken to make the arginines available for importin-α recognition, thereby representing a structural feature that may contribute to the reduced nuclear import of the small isoform. This bioinformatics-based concept needs to be substantiated further in future experiments to verify the functional consequences of differentially regulated nuclear import of pUL97 for HCMV replication. Possibly, differences in nuclear import of the two isoforms may be translated into so far unrecognized functional specificities of pUL97. To this end, the generation of recombinant HCMVs expressing either of the two isoforms separately is presently under way for phenotypic characterization. It will be pertinent to our understanding if a differential impact of one of the isoforms is displayed during early or late times of infection or, alternatively, if a preferential phosphorylation of the various substrates of pUL97 is detectable. As isoform formation has not been reported for the UL-type protein kinases of other herpesviruses or animal cytomegaloviruses, such functional specification may be a unique property of HCMV.

METHODS

Plasmid constructs. To generate UL97 deletion mutants (Δ6–35, Δ190–213, Δ6–35/Δ190–213 and Δ111/Δ190–213) in the NLS mapping vector pHM830 (Sorg & Stamminger, 1999), fragments were amplified by an overlap-extension PCR method from the template pcDNA-Ul97-F (Marschall et al., 2001) or pcDNA-Ul97(M1L)-F (Schregel et al., 2007). PCR was performed as described previously (Schregel et al., 2007) using primers containing HindIII and XbaI restriction sites (synthetic primers purchased from Biomers). The GFP expression cassette was removed from vector pHM830 by HindIII and XbaI digestion, producing UL97–β-gal NLS constructs lacking GFP. The generation of UL97–β-gal NLS constructs M38/74/111/157–707 (carrying replacement mutations M38A, M74L, M111L and M157A), M117L, 74–707 and 1–707 has been described previously (Webel et al., 2011). To generate GFP–UL97–β-gal NLS constructs for testing the putative NLSs at aa 6–35, 190–213, 164–198 and 164–213, fragments were amplified with primers carrying AffI or Xhol restriction sites (Table S5) and inserted into pHM830.

Peptide synthesis. Peptides were synthesized as C-terminal amides by Fmoc/t-Bu-based solid-phase synthesis, as described elsewhere (Franke et al., 2007). The N-terminal amino groups were acetylated. Cleaved peptides were purified by preparative HPLC, and their identities were confirmed by electrospray ionization/mass spectrometry.

Cell culture, plasmid transfections and HCMV infections. Transient transfection of HeLa cells using Lipofectamine 2000 (Invitrogen) and infection of primary HFFs with HCMV laboratory strain AD169 or clinical isolates R3 (Iso 3) and R4 (Iso 4) was performed as described elsewhere (Marschall et al., 2000; Efferth et al., 2002; Herget et al., 2004; Schregel et al. 2007).

Immunofluorescence assays and confocal microscopy. HeLa cells were seeded on coverslips, transfected and used for indirect immunofluorescence analysis (UL97–β-gal NLS constructs) as described previously (Mübradt et al., 2010) or for direct GFP fluorescence analysis (GFP–UL97–β-gal NLS constructs). For HCMV infection, HFFs were seeded on coverslips, infected at an m.o.i. of 0.5 and cultured for 2–4 days. Immunostaining was performed with a primary mAb against β-gal and a polyclonal antibody against UL97 (kindly provided by Donald Coen, Harvard Medical School, Boston, MA, USA) and a polyclonal antibody against UL53 (kindly provided by Paola Dal Monte, University of Bologna, Italy), and with secondary antibodies conjugated to Alexa Fluor 488 (green) and Alexa Fluor 555 (red). A mouse mAb (clone 636) was used to detect anti-lamin A/C; Santa Cruz Biotechnology). Confocal laser-scanning microscopy was carried out using a Leica TCS SP5 microscope.

Semi-automated interactive cell segmentation for the determination of nucleocytoplasmic intensity ratios. For objective quantification of the degree of nuclear versus cytoplasmic localization of UL97–β-gal fusion constructs, an automated image segmentation method was applied (Held et al., 2011) to outline the boundaries of individual nuclei from the DAPI channel and the boundaries of the corresponding cytoplasmic areas from the corresponding GFP channel. Errorneously segmented cells were corrected manually or removed after segmentation. Based on the segmentation results for each entity, the mean nuclear intensity (I_nucleus) and the mean cytoplasmic intensity (I_cytoplasm) were quantified automatically. To determine the varying patterns of intensity relationships, the intensity ratio R=I_cytoplasm/I_nucleus was analysed and computed by the support vector machine classifier: R<0.24 indicated cells with mainly nuclear localization of UL97–β-gal constructs; 0.24< R<1.4 indicated nucleocytoplasmic localization and R≥1.4 indicated mainly cytoplasmic localization (Schölkopf et al., 1999).

SPR analysis. SPR measurements were performed at 25 °C on a T100 or T200 (Biacore AB) and X100 instrument using CM5 sensor chips (GE Healthcare). A commercially available human importin-α expressed recombinantly in Escherichia coli (GenBank accession no. NP_002257; Jena Bioscience) was immobilized to 1000 or 10 000 RU via standard covalent amine coupling. The reference flow cell was treated identically without importin-α immobilization. Synthetic pUL97 peptides were dissolved in the running buffer HBS-EP+ (GE Healthcare) and used for binding analyses at concentrations of 2–10 μM. The samples were injected over the flow cells at a flow rate of 30 μl min⁻¹ with 60 s association and 120 s dissociation phases. Association and dissociation rate constants were obtained from the
resulting sensormgrams using the Biacore T100 evaluation software version 2.0.1 in accordance with the global curve fit model. The sensormgrams were fitted to the 1:1 (Langmuir), bivalent analyte and heterogeneous ligand-binding models, respectively, and the kinetic constants were calculated.

**MD simulations.** The constructs investigated by MD simulations included aa 1–270 for the large isoform and aa 74–270 for the small isoform. In addition, a control simulation was performed for NLS2 of pUL97 in the context of the GFP–β-gal fusion construct. For each of the three systems, separate MD simulations were carried out with the AMBER11 package (Case et al., 2010) and the ff99SB force field (Hornak et al., 2006). The simulations were performed using a protocol by Simmerling et al. (2002) that was developed to investigate protein folding in implicit solvent. All systems were first minimized, heated gradually from 0 to 310 K and subsequently simulated for 6 ns in accordance with the global curve fit model. The solvent-accessible surface was calculated using the DSSP program (Kabsch & Sander, 1983).

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**REFERENCES**


