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Human Cytomegalovirus DNA Polymerase Catalytic Subunit pUL54 Possesses Independently Acting Nuclear Localization and ppUL44 Binding Motifs

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The catalytic subunit of human cytomegalovirus (HCMV) DNA polymerase pUL54 is a 1242-amino-acid protein, whose function, stimulated by the processivity factor, phosphoprotein UL44 (ppUL44), is essential for viral replication. The C-terminal residues (amino acids 1220–1242) of pUL54 have been reported to be sufficient for ppUL44 binding *in vitro*. Although believed to be important for functioning in the nuclei of infected cells, no data are available on either the interaction of pUL54 with ppUL44 in living mammalian cells or the mechanism of pUL54 nuclear transport and its relationship with that of ppUL44. The present study examines for the first time the nuclear import pathway of pUL54 and its interaction with ppUL44 using dual color, quantitative confocal laser scanning microscopy on live transfected cells and quantitative gel mobility shift assays. We showed that of two nuclear localization signals (NLSs) located at amino acids 1153–1159 (NLSA) and 1222–1227 (NLSB), NLSA is sufficient to confer nuclear localization on green fluorescent protein (GFP) by mediating interaction with importin α/β. We also showed that pUL54 residues 1213–1242 are sufficient to confer ppUL44 binding abilities on GFP and that pUL54 and ppUL44 can be transported to the nucleus as a complex. Our work thus identified distinct sites within the HCMV DNA polymerase, which represent potential therapeutic targets and establishes the molecular basis of UL54 nuclear import.

Key words: antiviral therapy, cytomegalovirus, cytoplasm, DNA, herpesvirus, nuclear transport, nucleus, PAP, polymerase, ppUL44, processivity factor, protein–protein interaction, pUL54, UL54, regulation, replication

Human cytomegalovirus (HCMV), the principal beta herpesvirus of humans, represents a serious pathogenic threat to immunocompromised individuals (1) and is a leading cause of severe disease following congenital infection (2). The replication of its double-stranded DNA genome occurs in the nuclei of infected cells, requiring several viral-encoded proteins, including six that form the replication fork machinery (3–6), of which the 1242-amino-acid, 140-kDa, phosphoprotein product of open reading frame UL54 (pUL54), the catalytic subunit of the viral DNA polymerase, is one (7–11). As a crucial enzyme in viral replication, it is the main target for anti-HCMV therapeutics, which are in use clinically (12–15). As antiviral drug resistance has become an issue of increasing clinical importance (16), there is a pressing need for new agents targeting novel HCMV functions. Understanding the structure and function of HCMV DNA polymerase and its interaction with the other five essential replication proteins at the replication origin should prove invaluable in developing new therapeutic agents in the future.

pUL54 can be immunoprecipitated from the infected cells together with its processivity factor, the 52-kDa phosphoprotein ppUL44 (8). ppUL44 is essential in viral DNA replication (6,17), conferring processivity to the DNA polymerase holoenzyme (4,8,18,19). Intriguingly, a recent report showed that small molecules interfering with the pUL54–ppUL44 interaction can also impair viral replication (20). The C terminus of pUL54 has been implicated as the domain responsible for ppUL44 binding (21), as well as containing a nuclear localization signal (NLS) (22). Molecules >50 kDa cannot diffuse passively into the nucleus through the aqueous channel delimited by the nuclear pore complex (NPC), instead being actively translocated into the nucleus in NLS-dependent fashion through the action of members of the importin (IMP) superfamily of intracellular transporters (23). The IMPs mediate docking of the NLS-containing protein to the NPC and translocation through it into the nucleus. A well-characterized class of NLS consists of a single cluster of basic amino acids such as that of the simian virus 40 large tumor antigen (Tag) (24,25), which is recognized by the IMPα/β heterodimer, where IMPα recognizes the NLS and IMPβ facilitates the IMPα–NLS interaction by affecting a conformational change in IMPα

to release an autoinhibitory domain from the IMPα–NLS binding site, thereby increasing the affinity of the interaction (26). IMPβ also mediates interactions with NPC components to facilitate passage through the NPC and then with the guanine-nucleotide-binding protein Ran to achieve cargo release in the nucleus (23).

Both pUL54 and ppUL44, with apparent molecular weights of 140 and 52 kDa, respectively, have to be actively transported into the nuclei of infected cells to replicate HCMV DNA. We have recently shown that ppUL44 dimers are transported to the nucleus by IMPα/β, which recognizes a modular motif named 'C2N', constituted by a protein kinase CK2 (CK2) site upstream of a basic NLS ('NLS2'), located at the C terminus of the protein, whose activity is enhanced by phosphorylation of the CK2 site (27,28). No data are available on the nuclear transport of pUL54, but the recent demonstration that Kaposi's-sarcoma-associated human herpesvirus (KSHV) DNA polymerase catalytic subunit pol-8, despite lacking a functional NLS, can be transported to the nucleus on coexpression with its processivity factor, PF-8, implies that the presence of a functional NLS within pUL54 cannot be assumed.

The aims of this study were to identify potential NLSs within pUL54 and determine their mechanism of action and to map the pUL54 domain responsible for interaction of it with ppUL44 *in vivo*. Our results identified a basic NLS on pUL54, located immediately upstream of the ppUL44 binding domain, which is able to confer high-affinity recognition by IMPα/β and nuclear targeting on heterologous proteins. We showed for the first time that pUL54 residues 1213–1242 are sufficient to bind ppUL44 in the cytoplasm of living mammalian cells and that the two proteins can be translocated to the nucleus as a complex, as determined by dual color confocal laser scanning microscopy (CLSM) analysis of mammalian cells coexpressing several differently labelled pUL54 and ppUL44 fusion proteins. Because both pUL54–ppUL44 interaction and nuclear import represent potential therapeutic targets, our results have relevance to the development of new pharmaceuticals to combat HCMV.

Results

pUL54 carries a functional NLS distinct from the ppUL44 binding domain

pUL54 has to localize to the nucleus of infected cells to mediate viral DNA replication during HCMV infection. By analogy to the herpes simplex virus type 1 (HSV-1) homologue, pUL30, previous studies identified a weak NLS (NLSB: PRRLHL^{1227}) located within the ppUL44 binding domain of pUL54 (21,22). Analysis of the pUL54 sequence revealed the presence of a putative basic NLS (NLSA: PAKKRAR^{1159}) upstream of the ppUL44 binding domain and downstream of two consensus phosphorylation sites for CK2 (Figure 1A), which has previously been shown to regulate the NLS activity of proteins such as Tag (29) and, intriguingly, HCMV ppUL44 (27). To test the function of NLSA and NLSB, we generated a series of constructs for ectopic expression of different fragments of the pUL54 C-terminal region in fusion with green fluorescent protein (GFP; Figure 1B). The constructs were transfected into COS-7 cells and their subcellular localization analyzed in live cells using CLSM.

The GFP–UL54(1125–1242), GFP–UL54(1145–1242), GFP–UL54(1145–1231) and GFP–UL54(1145–1161) fusion proteins all localized in the nucleus of transfected cells, whereas the localization of GFP–UL54(1213–1242), GFP–UL54(1213–1231) and GFP–UL54(1145–1161m), in which residues PAKKRAR^{1159} are mutated to PAKKaAa^{1159}, was undistinguishable from that of GFP alone, indicating diffuse localization throughout the cell (Figure 2A). Digitized CLSM images were analyzed quantitatively (Figure 2B) to determine the nuclear to cytoplasmic ratio (Fn/c – *Materials and Methods*). No marked difference was observed between the Fn/c values (c. 1.5) of cells transfected to express GFP, GFP–UL54(1213–1242), GFP–UL54(1213–1231) and GFP–UL54(1145–1161m), whereas the cells expressing GFP–UL54(1125–1242), GFP–UL54(1145–1242), GFP–UL54(1145–1161) and GFP–UL54(1145–1213) all showed significantly higher ($p < 0.0001$) levels of nuclear accumulation (Fn/c of c. 10; Figure 2B). That GFP–UL54(1145–1161m) showed a significant reduction in nuclear accumulation (Fn/c of 1.5) compared with GFP–UL54(1145–1161) implied that the central arginine residues of NLSA were necessary for its nuclear targeting function. The finding that GFP–UL54(1125–1242), GFP–UL54(1145–1242), GFP–UL54(1145–1161) and GFP–UL54(1145–1231) all showed very similar levels of nuclear accumulation implied that the sequences flanking the pUL54-NLSA, including the putative CK2 phosphorylation sites and pUL54-NLSB, do not affect its activity. Similar results were obtained after transfection of Vero cells (data not shown).

pUL54-NLSA is a functional NLS

To test if pUL54-NLSA (PAKKRAR^{1159}) is sufficient to target an heterologous protein to the nucleus and to verify that the nuclear accumulation observed for GFP–UL54 fusion proteins is due to active nuclear transport rather than intranuclear binding, we performed live cell CLSM on COS-7 cells transfected to express GFP–UL54–NLSs–β-galactosidase (β-Gal) fusion proteins; the GFP–β-Gal and GFP–Tag–NLS–β-Gal encoding pHM830 and pHM830–Tag–NLS constructs were also examined as negative and positive controls, respectively (30). GFP–β-Gal fusion proteins have a predicted tetrameric molecular weight of c. 580 kDa and therefore can only translocate into the nucleus when fused to a functional NLS (30). CLSM images (Figure 3A) were analyzed to determine the Fn/c (Figure 3B). GFP–Tag–NLS–β-Gal and GFP–UL54(1153–1159)–β-Gal showed high levels of nuclear localization (Fn/c of c. 40 and 10, respectively), whereas GFP–UL54(1222–1227)–β-Gal showed cytosolic fluorescence

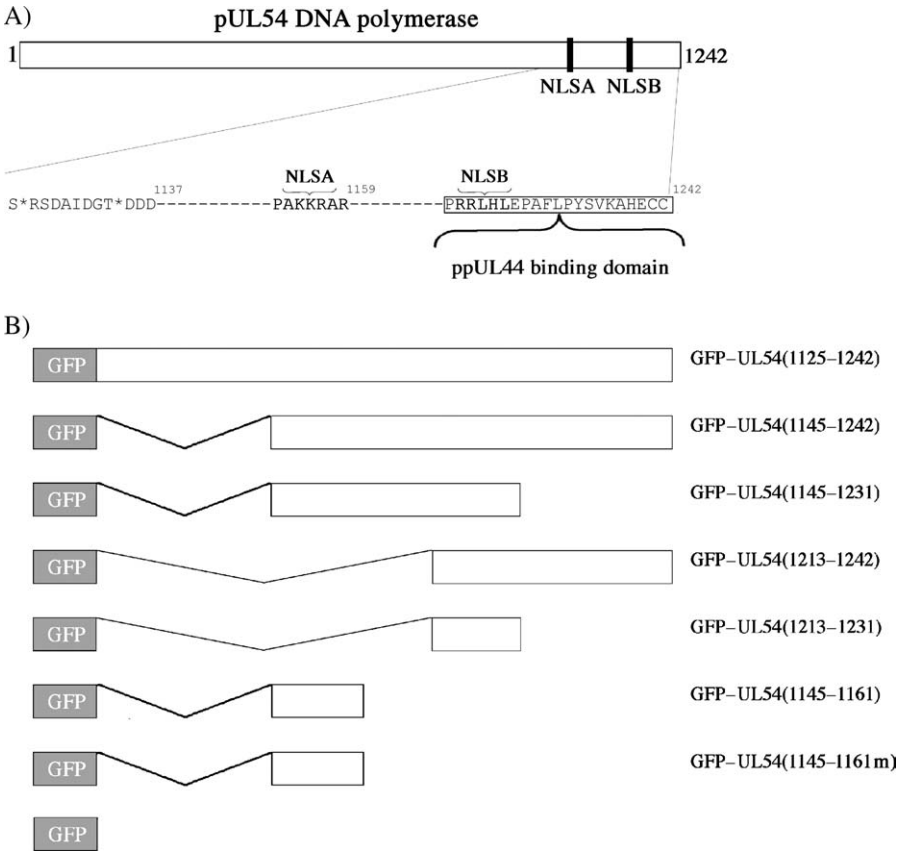


Figure 1: pUL54 contains a putative NLS (NLSA) immediately upstream of the ppUL44 binding domain. A) Schematic representation of the pUL54 coding sequence, highlighting the NLSs (black bars) and their position relative to the ppUL44 binding domain (boxed). The key sequences are shown in the single-letter amino acid code, with the putative CK2 phosphorylation site denoted by *. B) Schematic representation of the pUL54–GFP fusion proteins used in this study.

comparable with that observed for GFP–β-Gal (Fn/c of c. 0.2; Figure 3B). That GFP–UL54(1153–1159)–β-Gal showed nuclear localization implied that the nuclear accumulation observed for GFP–UL54 fusion proteins was due to active nuclear transport rather than binding to nuclear components, and that its levels of nuclear accumulation were lower than those for the control molecule GFP–Tag–NLS–β-Gal is consistent with that reported by previous thermodynamic studies performed on derivative mutants of the Tag NLS (31).

pUL54 is translocated to the nucleus by the IMPα/β heterodimer

pUL54-NLSA (PAKKRAR) matches quite well the consensus for IMPα/β binding through IMPα (31). To clarify the nuclear import mechanism of pUL54, gel mobility shift assays were performed with His_6GFP–UL54(1125–1242) fusion protein and bacterially expressed IMPs, using the His_6GFP–UL44(410–433) fusion protein as a positive control (27; Figures 4 and S1). Incubation of the GFP fusion proteins with increasing amounts of the IMPα/β heterodimer (Figure 4A,B) or IMPα alone (Figure 4C) revealed that pUL54 binds IMPα/β with high affinity, in similar fashion to ppUL44 [dissociation constant (K_d): c. 150 nM; Figure 4D]. Binding to IMPα was less efficient (K_d: c. 650 nM; Figure 4E), consistent with autoinhibition of NLS binding by IMPα in the absence of IMPβ1 (26). Therefore, pUL54 is likely to be translocated to the nucleus by the IMPα/β heterodimer. To verify if pUL54-NLSA was sufficient to mediate IMP binding, we also performed gel mobility shift assays with His_6GFP–UL54(1145–1161) and His_6GFP–UL54(1213–1222) fusion proteins and bacterially purified mouse IMPs. Results showed that only His_6GFP–UL54(1145–1161) was able to interact specifically with IMPα/β and to a lesser extent with IMPα alone (Figure 5). The clear implication was that pUL54-NLSA mediates pUL54 nuclear import by conferring interaction with the IMPα/β heterodimer.

pUL54 C-terminal amino acids bind to ppUL44 in live cells

To test if the UL54–GFP fusion proteins analyzed as mentioned above were functional in terms of ppUL44 binding in the live cell context, they were coexpressed with DsRed2 fusions of wild-type ppUL44 and a ppUL44 mutant derivative (ppUL44ΔNLS2), which, through mutation of the ppUL44-NLS2, localizes in the cytoplasm, often resulting in the formation of aggregates probably as a consequence of overexpression (27,28). The ability of the ppUL44 fusion proteins to modulate localization of GFP–UL54 fusion proteins was determined by dual color CLSM (Figure 6). As expected, all the GFP–UL54 fusion proteins containing pUL54 amino acid 1220–1242, the ppUL44 binding domain (UL44 BD); GFP–UL54(1125–1242),

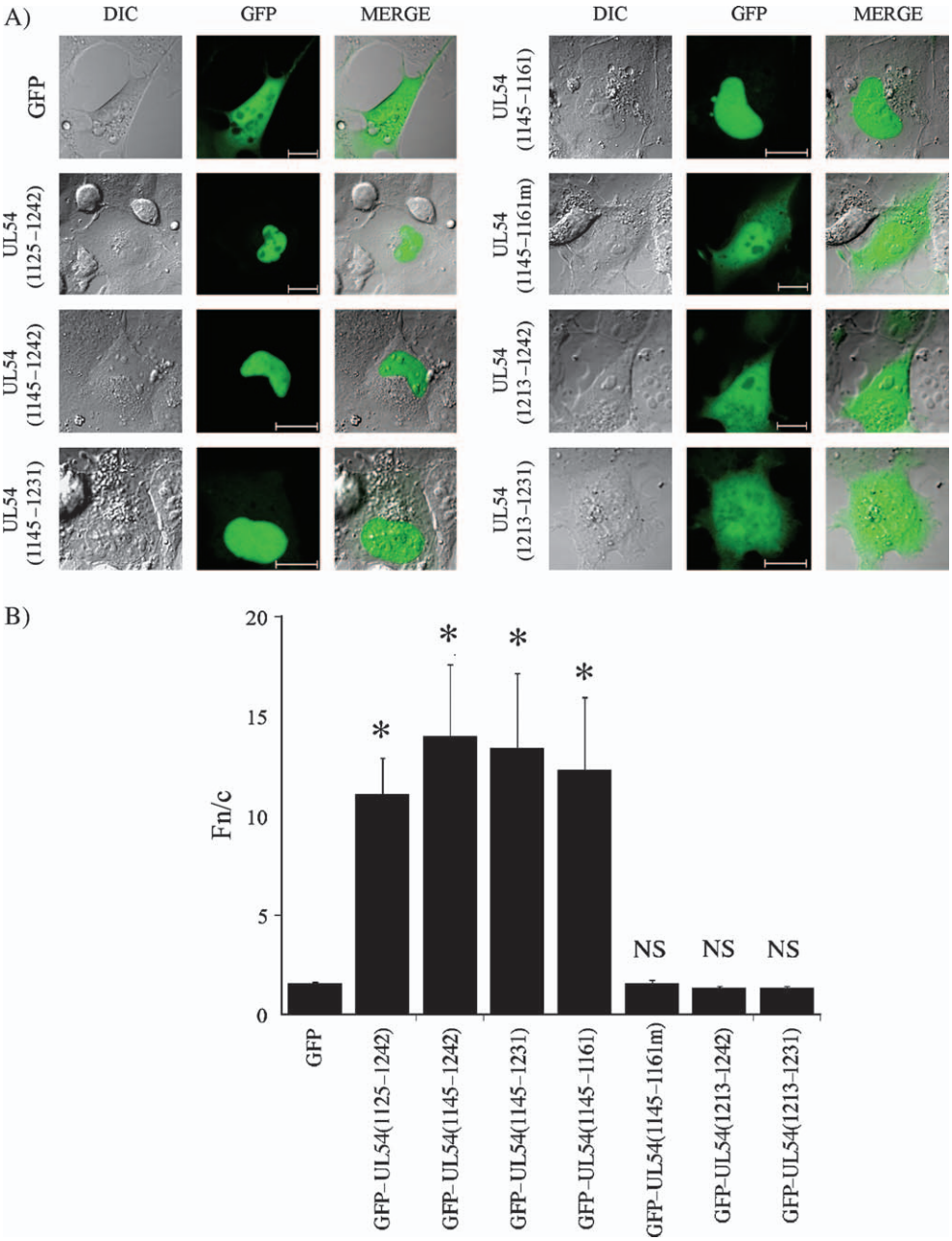


Figure 2: pUL54-NLSA can target GFP to the nucleus. A) CLSM images of COS-7 cells 16 h after transfection to express GFP–UL54 fusion proteins. DIC images are shown on the left, with merged images of the green (GFP) and the DIC channels on the right. B) Results for the determination of the Fn/c, where confocal images, such as those shown in (A), were analyzed using the ImageJ software as described in *Materials and Methods*. Data are given as mean ± SEM ($n > 15$), with values for the test of significance between the expressed protein and GFP shown. DIC, differential interference contrast; NS, not significant. *$p < 0.0001$. Scale bars represent 50 μM.

GFP–UL54(1145–1242) and GFP–UL54(1213–1242); were found to co-localize with the DsRed2–UL44 fusion proteins, whereas GFP, GFP–UL54(1145–1231) and GFP–UL54(1213–1231), which lack UL44 BD, did not. As mentioned above, GFP–UL54(1213–1242) localizes in both the nucleus and the cytoplasm when expressed alone (Figure 2A), but when coexpressed with the nuclear localizing wild-type or cytoplasmically localized ppUL44 mutant derivative, it was retained predominantly in the nuclear (Figure 6A) or the cytoplasmic (Figure 6B) compartment, respectively. That GFP–UL54(1145–1242) and GFP–UL54(1125–1242) but not GFP–UL54(1145–1231) were able to interact with wild-type ppUL44 is highlighted by their co-localization with DsRed2–UL44 in distinctive nuclear speckles (Figure 6A). On the other hand, the GFP fusion proteins carrying the pUL54-NLSA but lacking the

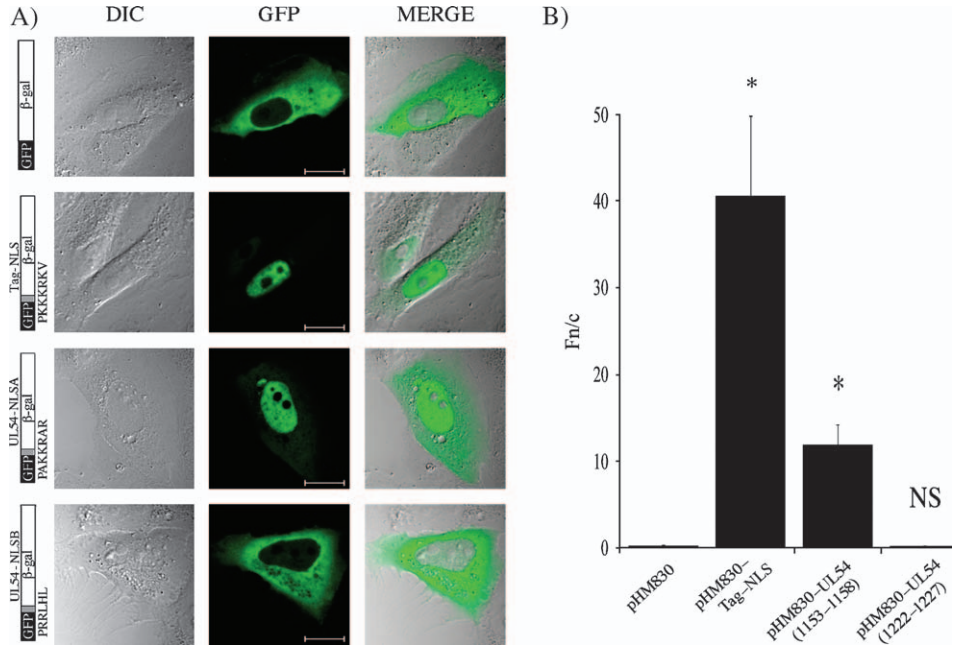


Figure 3: pUL54-NLSA but not pUL54-NLSB is able to confer nuclear localization on a large heterologous protein. A) Images of COS-7 cells imaged live 16 h after transfection to express GFP–β-Gal fusion proteins using CLSM and a 40× water immersion objective ($n > 40$). B) Confocal images, such as those shown in (A), were analyzed for the Fn/c as per legend to Figure 2. Data are given as mean ± SEM ($n > 15$), with values for the test of significance between the expressed protein and GFP–β-Gal shown. NS, not significant. *$p < 0.0001$. Scale bars represent 50 μM.

UL44 BD localized to the nucleus and did not co-localize with DsRed2–UL44 in the nuclear speckles. The clear implication is that pUL54 NLS (residues 1153–1159) and ppUL44 binding (residues 1220–1242) activities are modular and able to function independently.

When GFP–UL54 fusion proteins containing both NLSA and UL44 BD were coexpressed with DsRed2–UL44ΔNLS2, they were partially retained in the cytoplasm, as shown by their co-localization in cytoplasmic aggregates (Figure 6B). Quantitative analysis of the levels of nuclear accumulation of DsRed2–UL44ΔNLS2 revealed that coexpression with the latter GFP–UL54 fusion proteins increased its nuclear accumulation by threefold (Figure 7A), whereas no effect was detected on coexpression with the other GFP–UL54 fusion proteins, in fashion similar to the negative control GFP. To verify if higher expression levels of pGFP–UL54 fusion proteins correlated with stronger DsRed2–UL44ΔNLS2 nuclear localization, we classified coexpressing cells on the basis of their GFP fusion proteins expression levels (*Materials and Methods*); our results showed that higher expression of GFP–UL54(1125–1242) resulted in stronger nuclear accumulation of DsRed2–UL44ΔNLS2 (Fn/c levels increased by fivefold), whereas cells expressing lower levels of GFP–UL54(1125–1242) showed reduced nuclear accumulation, by comparison. In contrast, higher expression levels of GFP–UL54(1145–1161) and of the negative control GFP did not (Figure 7B). These results clearly indicate that the HCMV DNA polymerase can be transported to the nucleus as a complex.

Discussion

This is the first study to examine the nuclear import of the two HCMV DNA polymerase subunits – pUL54 and ppUL44 – simultaneously in a live cell system. We previously showed that ppUL44 dimerizes in the cytoplasm before being translocated to the nucleus through a transport process that depends on a phosphorylation-regulated, IMPα/β-recognized NLS (27,28). In this study, we showed that pUL54-NLSA (PAKKRAR^{1159}) is similarly able to mediate IMPα/β-dependent nuclear import of pUL54, although there appears to be no role for phosphorylation in regulating NLS activity. Analysis of pGFP–UL54 fusion protein derivatives revealed UL54 residues 1145–1161, encompassing NLSA, to be sufficient to confer nuclear accumulation and IMP binding abilities to GFP, with two arginines located at positions +3 and +5 with respect to the core of NLSA essential for its functionality, consistent with findings for other IMPα/β recognized NLSs (31). GFP–UL54(1145–1161) nuclear accumulation is an active process rather than the result of intranuclear binding, as indicated by the ability of UL54 residues 1153–1159

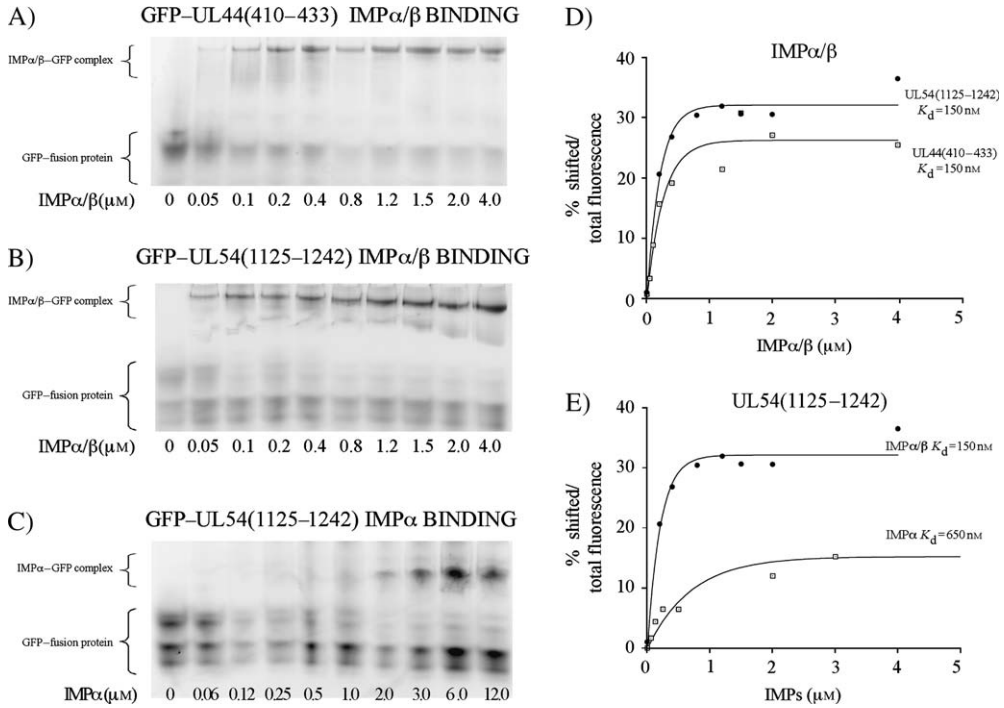


Figure 4: The IMPα/β heterodimer recognizes pUL54(1125–1242) with high affinity as shown by native PAGE fluorimaging. A–C) Fluorescent images of native gels after electrophoresis for 8 h at 80 mA. The position of the control molecule His_6GFP–UL44C2N (A;2 μM) or His_6GFP–UL54(1125–1242) (B and C;2 μM) is shown in the absence (left lane) or presence of incubation with increasing amounts of precomplexed GST-mouse IMPα/β (A and B; 50–4000 nM) or IMPα (C; 60–12 000 nM) prior to native PAGE. D) Results of quantification of specific fluorescence from (A) and (B) for the relative amount of His_6GFP–UL44C2N and His_6GFP–UL54(1125–1242) shifted fluorescence due to IMPα/β binding to the total fluorescence. The values for the apparent K_d representing the IMP concentration yielding half-maximal binding for shifted relative to total fluorescence ratio are indicated. E) Results of quantification of specific fluorescence from (B) and (C) for the relative amount of His_6GFP–UL54(1125–1242) shifted fluorescence due to IMPα/β and IMPα binding to the total fluorescence. The values for the apparent K_d representing the IMP concentration yielding half-maximal binding for shifted relative to total fluorescence ratio are indicated.

(PAKKRAR) to target the c. 580-kDa tetrameric fusion protein GFP–β-Gal to the nucleus (Figure 3). Our quantitative gel mobility shift assay results showed that bacterially expressed His_6GFP–UL54(1125–1242) is recognized by IMPα/β with a c. fivefold higher affinity than by IMPα alone (K_d: c. 150 and 650 nM, respectively), clearly implying that pUL54 is likely to be translocated to the nucleus via IMPα/β, although it is not formally possible to exclude the possibility that other members of the IMP superfamily may also be able to mediate pUL54 nuclear transport.

Nuclear import of HCMV pUL54 appears to be very different from that of the KSHV DNA polymerase catalytic subunit pol-8, which lacks a functional NLS and whose

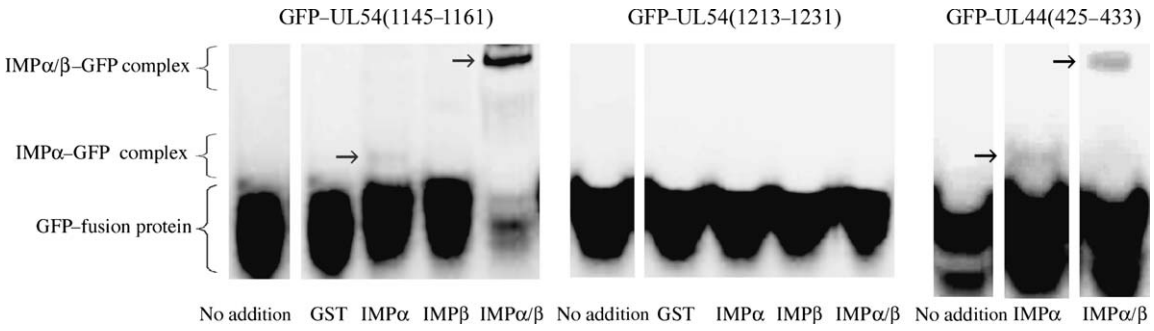
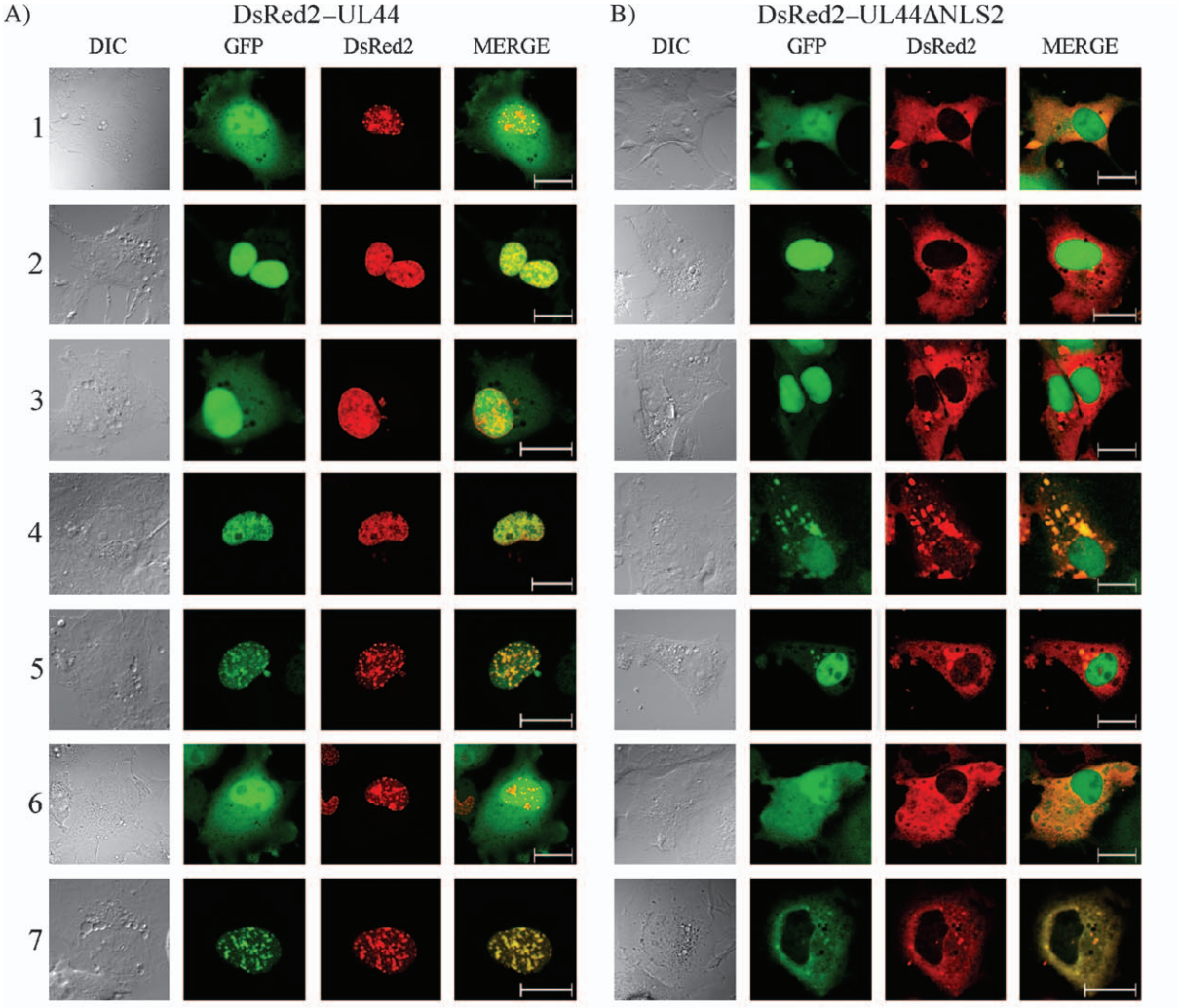


Figure 5: pUL54-NLSA but not pUL54-NLSB is recognized specifically by IMPα/β. The indicated GFP fusion proteins were incubated as described in *Materials and Methods* in the absence or presence of 10 μM GST or GST–IMPs prior to native gel electrophoresis and fluorimaging. The position of GFP fusion proteins and fusion protein–IMP complexes are indicated.



C)

Panel row	GFP–UL54 fusion protein	Presence of functional domains		Subcellular localization of GFP–UL54 fusion proteins			DsRed2–UL44 co-localization
		NLSA	UL44 BD	No addition	+ DsRed2–UL44	+ DsRed2–UL44ΔNLS2	
1	GFP	–	–	N/C	N/C	N/C	No
2	GFP–UL54(1145–1231)	+	–	N	N	N	No
3	GFP–UL54(1165–1231)	+	–	N	N	N	No
4	GFP–UL54(1125–1242)	+	+	N	N; Nsp	N; Ca	Yes
5	GFP–UL54(1145–1242)	+	+	N	N; Nsp	N; Ca	Yes
6	GFP–UL54(1213–1231)	–	–	N/C	N/C	N/C	No
7	GFP–UL54(1213–1242)	–	+	N/C	N; Nsp	C; Ca	Yes

Figure 6: pUL54-NLSA and UL44 BD are modular and independent. COS-7 cells were transfected to express the indicated GFP–UL54 fusion proteins or GFP as a control (see C for key) in the presence of A) DsRed2–UL44 or B) DsRed2–UL44ΔNLS2. Cells were imaged 24 h after transfection by CLSM. Differential interference contrast images are shown on the left and merge images of the green (GFP) and the red (DsRed2) channels shown on the right, with yellow coloration indicative of co-localization. C) Summary of the effect of expression of DsRed2–UL44 fusion proteins on the subcellular localization of GFP–UL54 fusions. C, mainly cytoplasmic (Fn/c of <1); Ca, co-localizing with DsRed2–UL44ΔNLS2 in cytoplasmic aggregates; N, mainly nuclear (Fn/c of >2); N/C, present in both nucleus and cytoplasm ($1 <$ Fn/c < 2); Nsp, co-localizing with DsRed2–UL44 in nuclear speckles. Scale bars represent 50 μM.

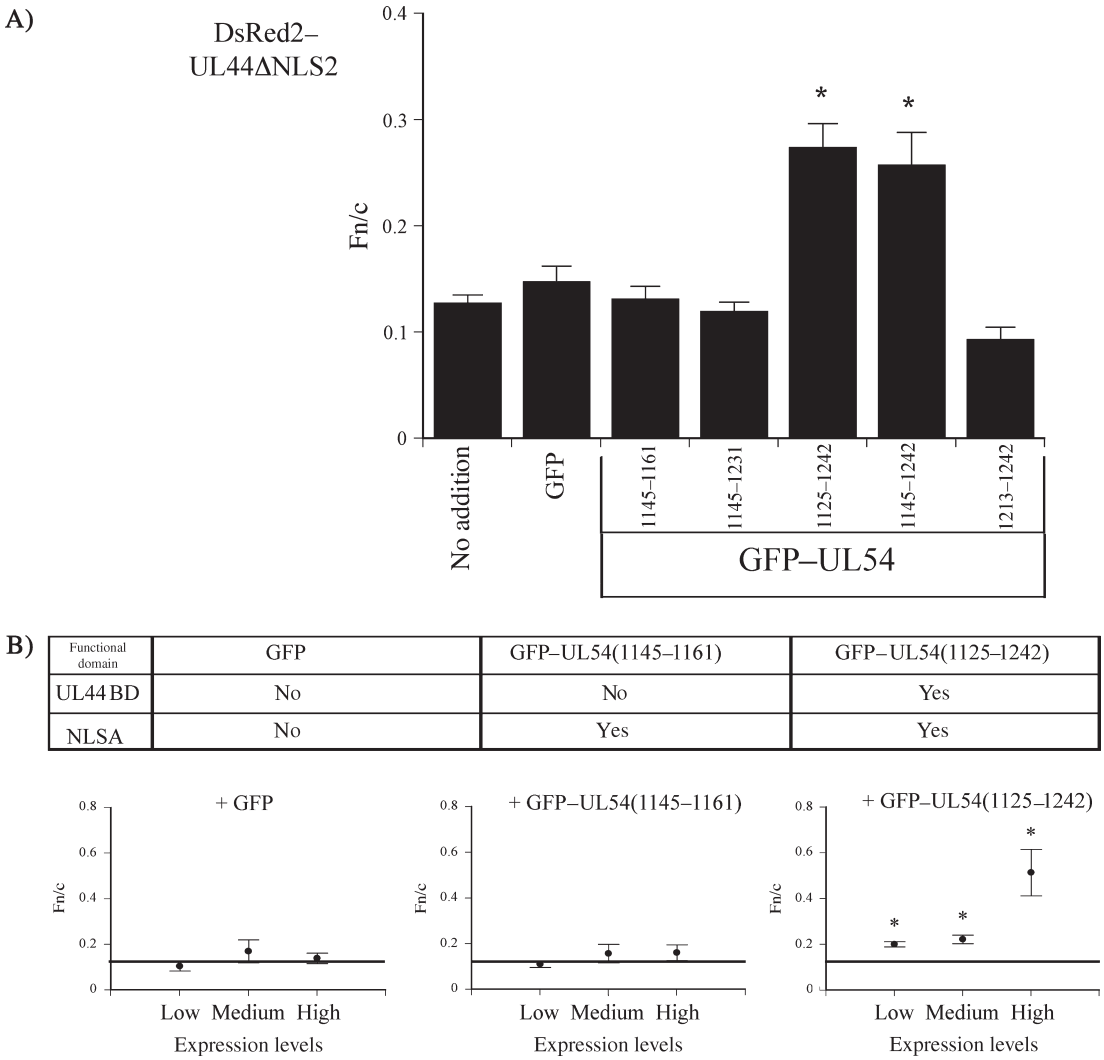


Figure 7: DsRed2–UL44ΔNLS2 is partially relocalized to the nucleus of cells expressing high levels of GFP–UL54(1125–1242). A) Results for the Fn/c of DsRed2–UL44ΔNLS2 in the absence or presence of the indicated GFP or GFP–UL54 fusion proteins, where confocal images, such as those shown in (6B), were analyzed as described in the legend to Figure 2. Data are given as mean ± SEM ($n > 15$), with significant differences ($p < 0.0001$) between DsRed2–UL44ΔNLS2 expressed alone and in the presence of the indicated GFP–UL54 fusion proteins denoted by *. B) COS-7 cells imaged in (A) were scored according to the expression levels of the indicated GFP–UL54 fusion proteins (*Materials and Methods*), and the Fn/c relative to coexpressed DsRed2–UL44ΔNLS2 is shown. The Fn/c of DsRed2–UL44ΔNLS2 in the absence of GFP–UL54 fusion proteins is indicated with a gray horizontal line. Data are given as mean ± SEM ($n > 5$), with significant differences ($p < 0.0001$) between DsRed2–UL44ΔNLS2 expressed alone and in the presence of the indicated GFP–UL54 fusion proteins denoted by *. Scale bars represent 50 μM.

nuclear transport is dependent on piggy back transport through its processivity factor PF-8 (32). Intriguingly, a putative NLS (PAKRPRE), highly homologous to HCMV pUL54-NLSA, is located on the HSV-1 pUL30, upstream of the accessory protein binding domain (22), implying that it may also be capable of independent transport to the nucleus, in a fashion similar to that by pUL54. Currently, we are testing the functionality of this sequence in terms of nuclear import. The fact that both HCMV DNA polymerase subunits possess functional NLSs and are therefore able to localize to the nucleus independently, as well as to be transported to the nucleus as a complex, presumably represents a mechanism to maximize their nuclear import possibilities. Because pUL54 is only able to partially relocate ppUL44ΔNLS2 to the nucleus, the implication is that the NLSs of both pUL54 and ppUL44 may be required for optimal accumulation of the DNA polymerase holoenzyme. ppUL44 is expressed in excess with respect to pUL54 during viral infection, so that a proportion of ppUL44 is likely to be transported to the nucleus independently of pUL54. Intriguingly, ppUL44 has recently been reported to be able to relocalize to the nucleus the otherwise cytoplasmic viral-encoded uracil-DNA glycosylase pUL114, proposed to be part of the HCMV DNA polymerase

complex (33). It is important to note in this context that ppUL44 structure is highly similar to that of other processivity factors, including non-viral factors such as the trimeric proliferating cell nuclear antigen (PCNA), and that PCNA can simultaneously bind to several partners through a domain that strongly resembles the domain of ppUL44 responsible for the recognition of pUL54 (34,35). As ppUL44 exists as a dimer containing two functional pUL54 binding sites (34), it can be speculated that ppUL44 dimers can simultaneously bind pUL54 and ppUL114, consistent with the hypothesis that ppUL44 could function in part as a scaffold, promoting the preassembly in the cytoplasm of various factors involved in HCMV replication prior to translocation to the nucleus (28). As ppUL44 nuclear transport is regulated by phosphorylation of its C2N motif (27), regulation of ppUL44 nuclear transport could in turn modulate nuclear import of other complexed HCMV DNA replication complex proteins.

Regardless of whether ppUL44 can interact with pUL54 and ppUL114 simultaneously, pUL54 catalytic activity clearly requires both interaction with ppUL44 and nuclear localization (36). We have thus identified two potential targets for the development of antivirals with respect to HCMV replication. It is important to mention in this context that a peptide corresponding to the last 22 amino acids of pUL54 selectively inhibited the activity of the DNA polymerase holoenzyme *in vitro*, presumably by preventing pUL54/ppUL44 interaction (37), and that small molecules able to interfere with such interactions have been shown to inhibit HCMV replication in cultured human foreskin fibroblasts (20). Because we showed in this study that GFP–UL54(1213–1242) is able to co-localize both with wild-type UL44 in nuclear speckles and in the cytoplasm with the NLS mutated cytoplasmically localizing ppUL44 point mutant, an intriguing possibility may be to use coexpression of differently tagged pUL54 and ppUL44 fusion proteins followed by live cell imaging to screen for molecules able to interfere specifically with pUL54/ppUL44 interaction or with pUL54 nuclear translocation, thus inhibiting viral replication. Future work in our laboratories is focused on validating and developing this approach.

Materials and Methods

Analysis of sequence motifs within pUL54

Putative NLSs and phosphorylation sites within the pUL54 coding sequence were identified using the PSORTII (38) and NetPhos (39) softwares, respectively.

Construction of expression plasmids

All prokaryotic and mammalian constructs expressing pUL54–GFP fusion proteins were generated using the Gateway™ system (Invitrogen, Carlsbad, CA, USA). Primers including the attB1 and attB2 recombination sites were used to amplify the UL54 sequences of interest, with plasmid PolPrset as a template (8). Polymerase chain reaction fragments were introduced into plasmid vector pDONOR207 (Invitrogen) via the BP recombination reaction, according to the manufacturer's recommendations, to generate the entry clones pDNR–UL54(1125–1242), pDNR–UL54(1145–1242), pDNR–UL54(1145–1231), pDNR–UL54(1213–1242), pDNR–UL54(1213–1231), pDNR–UL54(1145–1161) and pDNR–UL54(1145–1161m), in which residues PAKKRAR^{1159} were mutated to PAKKaAa^{1159}.

Entry clones were used to perform LR recombination reactions with the Gateway system compatible expression (DEST) plasmids pGFPRfB (40) and pEPI-DEST-GFP (41), according to the manufacturer's recommendations.

LR recombination reactions with the pDNR–UL44 and pDNR–UL44ΔNLS constructs (27), encoding wild-type UL44 and a mutant derivative thereof in which UL44–NLS2 (PNTKKQK^{431}) is substituted by PNTvaQI^{431}, respectively, and the Gateway system compatible expression plasmid pBkCMV-DsRed2 (42) were used to generate mammalian cell expression constructs expressing DsRed2–UL44 fusion proteins.

To generate mammalian cell expression constructs pHM830–UL54(1153–1159) and pHM830–UL54(1222–1227) encoding fusion proteins containing the HCMV pUL54-NLSA (PAKKRAR^{1159}) and pUL54-NLSB (PRRLHL^{1227}), respectively, inserted between the coding sequences of GFP and β-Gal, appropriate oligonucleotide pairs were annealed and cloned into expression vector pHM830 (30). The mammalian cell expression construct pHM830–Tag encoding a fusion protein with the Tag NLS (PKKKRKV^{132}) between the coding sequences of GFP and β-Gal has been described (27).

The integrity of all constructs was confirmed by DNA sequencing (MWG-BIOTECH, Ebersberg, Germany).

Cell culture and transfection

COS-7 and Vero cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% (v/v) fetal bovine serum, 50 U/mL penicillin, 50 U/mL streptomycin and 2 mM L-glutamine. For live cell imaging by CLSM, cells were trypsinized and 8×10^4 cells were seeded onto coverslips in six-well multiwell plates 1 day before transfection, which was performed using Lipofectamine 2000 (Invitrogen), according to the manufacturer's specifications.

CLSM/image analysis

Subcellular localization of GFP–UL54 fusion proteins in living cells was visualized 16 h after transfection by CLSM using an Olympus Fluoview 1000 (Olympus, Tokyo, Japan), equipped with a differential interference contrast apparatus. An heated Planapo ×60 water immersion lens (Nikon, Tokyo, Japan) was used in combination with a heated stage. The Fn/c values were determined as previously mentioned (43,44) using the ImageJ 1.62 public domain software (National Institutes of Health), from single cell measurements for each of the nuclear (Fn) and cytoplasmic (Fc) fluorescence, subsequent to the subtraction of fluorescence due to autofluorescence/background. Subcellular localization of GFP–UL54 and DsRed2–UL44 fusion proteins in living cells was visualized as mentioned above 24 h after transfection. Single cells were scored for the expression levels of GFP–UL54 fusion proteins on the basis of the Photomultiplier high voltage (PMT HV) used to obtain maximum *F* values of c. 250. Cells requiring a PMT HV of <600 were regarded as expressing high levels of fusion protein, cells requiring a PMT HV of >700 were classified as expressing low levels of fusion protein and cells requiring $600 <$ PMT HV < 700 were esteemed to express medium levels of fusion proteins. The settings of gain (1%), offset (0) and the intensity of the 488-nm argon laser (4%) were kept constant during each experiment.

Expression and purification of His_6 and glutathione-S-transferase-tagged fusion protein

His_6GFP–UL54(1145–1161), His_6GFP–UL54(1125–1242), His_6GFP–UL54(1213–1231), His_6GFP–UL44(425–433) and His_6GFP–UL44(410–433) were expressed in *Escherichia coli* strain M15 carrying plasmid pREP4,

essentially as previously described (27). Protein expression was induced for 6 h with isopropyl β-D-thiogalactoside (1 mM) at 28°C, and proteins were purified by nickel affinity chromatography as previously described (27). Expression and purification of glutathione S-transferase (GST)-tagged mouse IMPs α1 and β1 were performed as previously described (43).

Gel mobility shift assay

To test the ability of IMPs to bind pUL54, native polyacrylamide gel electrophoresis (PAGE)/fluorimaging was used as previously described (27,45). GFP fusion proteins (2 μM) were incubated for 15 min in PBS at room temperature with increasing amounts of mouse IMPα–GST, IMPβ–GST, precomplexed IMPα/IMPβ–GST [performed as previously described by Hubner et al. (43)] or GST as a control (10 μM). Sucrose was added to reactions to a final concentration of 15% (w/v) and the mixture electrophoresed at 80 V for 8 h on a native PAGE 4–20% gradient gel (Gradipore, Frenchs Forest, New South Wales, Australia) run in TBE buffer. The gels were visualized using a Wallac Arthur 1422 Multi-wavelength Fluorimager (Perkin Elmer, Boston, MA, USA) using side illumination and exposure times of 0.1–5 seconds.

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Supplemental Material

Figure S1: Gel mobility shift assays allow IMPs to be directly identified by Coomassie staining after fluorimaging. The indicated GFP fusion proteins (2 μM) are shown in the absence (left lane) or presence of preincubation with increasing amounts of precomplexed GST-mouse IMPα/β (15–3000 nM) prior to native PAGE, followed by both fluorimaging A) and Coomassie staining B).

Supplemental materials are available as part of the online article at http://www.blackwell-synergy.com

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