Human Cytomegalovirus (HCMV) DNA Polymerase Processivity Factor ppUL44 Dimerizes in the Cytosol before Translocation to the Nucleus†

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ABSTRACT: Replication of the human cytomegalovirus genome takes place in the nuclei of infected cells and is mediated by a viral-encoded DNA polymerase complex formed by the catalytic subunit pUL54 and the processivity factor ppUL44. Although it has recently been shown that the dimerization ability of recombinant pUL44 appears to be crucial for effective DNA binding *in vitro*, whether ppUL44 can dimerize or not in a cellular context is unknown. Here, we show, by using co-immunoprecipitation and dual-color live imaging approaches on cells expressing fluorescent and differently tagged ppUL44 fusion proteins, that ppUL44 dimerizes in the cytoplasm via its N-terminal domain, before translocating to the nucleus. Furthermore, we show that nuclear translocation of differently tagged ppUL44 heterodimers can occur even when one subunit carries a nonfunctional nuclear localization signal. Importantly, the latter cotransfection assay represents a system to test small-molecule inhibitors for their ability to impair ppUL44 dimerization.

The *β-Herpesviridae* subfamily member human cytomegalovirus (HCMV)[1] is a major human pathogen, causing severe diseases in immunocompromised individuals and in newborns following congenital infection (*1*). HCMV antiviral drug resistance has become an issue of increasing clinical importance (*2*), making necessary the development of new agents targeting novel functions of HCMV. The anti-HCMV therapeutics that are currently in use in the clinic (*3*−*6*) mainly target HCMV DNA polymerase catalytic activity and thereby viral genome replication, a process that occurs in the nuclei of infected cells and requires several viral-encoded proteins, six of which form the replication fork machinery (*7*−*10*).

A member of the latter group, the HCMV DNA polymerase holoenzyme, is formed by a catalytic subunit, the product of ORF UL54 (pUL54), and a processivity factor, the product of ORF UL44 (ppUL44) (*11*). ppUL44, also known as polymerase accessory protein (PAP) or infected cell protein 36 (ICP-36) is readily detectable by Western blotting as a family of proteins in lysates of infected cells, with its most abundant member being a 52 kDa phosphoprotein of 433 amino acids, with strong dsDNA-binding ability (*12*−*15*). ppUL44 is an essential factor for DNA replication both *in vitro* (*10*) and *in vivo* (*16*). During viral infection, a phosphorylation-regulated nuclear localization signal (NLS) mediates translocation of ppUL44 into the host cell nucleus (*17*, *18*), where it functionally interacts with pUL54 to confer processivity to the DNA polymerase holoenzyme (*11*, *13*, *19*, *20*).

Molecules > ca. 50−60 kDa cannot diffuse passively into the nucleus through the aqueous channel delimited by the nuclear pore complex (NPC); instead, they are actively translocated by members of the importin (IMP) superfamily of intracellular transporters, which recognize targeting signals on the cargo molecules. The IMPα/β heterodimer or IMPβ alone recognizes NLSs on cargo molecules mediating their nuclear translocation (*21*).

As alluded to above, the ppUL44 basic NLS (NLS2, PNTKKQK[431]), together with the sequences upstream thereof, comprising a protein kinase CK2 (CK2) site, define the C2N (CK2 and NLS) motif, responsible for CK2-enhanced nuclear transport of ppUL44 (*17*). While the C terminus of ppUL44 is essential for the phosphorylation-regulated nuclear import and for the transactivation activity of the protein (*17*, *22*), all of its known properties as a PAP, including the ability to bind to DNA and to dimerize *in vitro*, reside in its N-terminal 290 amino acids (*13*, *19*, *20*, *23*). Intriguingly, point

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[1] Abbreviations: HCMV, human cytomegalovirus; PAP, polymerase accessory protein; ICP-36, infected cell protein 36; NLS, nuclear localization signal; NPC, nuclear pore complex; IMP, importin; CK2, protein kinase CK2; GFP, green fluorescent protein; DMEM, Dulbecco's modified Eagle medium; CLSM, confocal laser-scanning microscopy; Fn/c, nuclear/cytoplasmic ratio; HSV-1, herpes simplex virus-1; KHSV, Kaposi-associated herpes virus; EBV, Epstein−Barr virus.

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mutations that interfere with ppUL44 dimerization in vitro also result in decreased DNA binding (*23*), leading to the hypothesis that ppUL44 dimerization could be an attractive therapeutic target (*24*). However, it has not been demonstrated that ppUL44 dimerizes *in vivo*, and the cellular compartment in which the process takes place has not been identified either.

The aim of our study was to ascertain if ppUL44 can dimerize *in vivo* and to identify the cellular compartment in which the process takes place. Performing immunoprecipitation experiments and live-cell imaging, we show here for the first time that ppUL44 dimerizes in the cytoplasm and that the process requires the N-terminal domain (amino acids 1−155), which thus represents a new potential therapeutic target on HCMV ppUL44. We also test the ability of ppUL44 derivative mutants impaired in normal intracellular localization to interfere with the localization of the wild-type protein, demonstrating that a functional NLS on one subunit is sufficient to mediate the nuclear import of the dimer. Importantly, the cotransfection assay that we describe will be of potential application in screening for new molecules able to interfere with HCMV replication by inhibiting ppUL44 dimerization.

EXPERIMENTAL PROCEDURES

ppUL44 Expression Plasmid Construction Using the Gateway Technology. All mammalian constructs expressing ppUL44 fusion proteins were generated using the Gateway system (Invitrogen). Primers including the attB1 and attB2 recombination sites were used to amplify the ppUL44 sequences of interest using plasmids pDNR-UL44 and pDNR-UL44ΔNLS2 (*17*) as templates. PCR fragments were introduced into plasmid vector pDONOR207 (Invitrogen) via the BP recombination reaction, according to the recommendations of the manufacturer, to generate the entry clones pDNR-UL44(156−433) and pDNR-UL44(156−433)ΔNLS2.

pDNR−UL44 constructs were then used to perform LR recombination reactions with the Gateway system compatible expression ("DEST") vectors pDEST26 (Invitrogen), pEPI-DEST-GFP (*25*), and pBkCMVDsRed2-DEST (*26*), according to the recommendations of the manufacturer to express N-terminally tagged V5, GFP, or DsRed2 fusion proteins, respectively.

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

Expression construct ppUL44-FLAG, encoding residues 1−433 of ppUL44 fused to the FLAG epitope (*14*), was a generous gift from M. Marschall (Erlangen), whereas expression construct pHM830, encoding a fusion protein between GFP and β-galactosidase (*27*) was obtained from T. Stamminger (Erlangen).

Cell Culture and Transfection. African green monkey Vero and COS-7 cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% (v/v) fetal bovine serum, 50 units/mL penicillin, 50 units/mL streptomycin, and 2 mM L-glutamine. For confocal laser-scanning microscopy (CLSM), cells were trypsinized and 2×10^5 cells were seeded onto coverslips in 6-well plates 1 day before transfection, which was performed using Lipofectamine 2000 (Invitrogen), according to the recommendations of the manufacturer. For immunoprecipitation experiments, cells were trypsinized and 2×10^5 cells were seeded onto a 6-well multiwell plate 1 day before transfection.

CLSM and Image Analysis. Subcellular localization of UL44 fusion proteins in living cells was visualized 24 h after transfection by CLSM using a Perkin−Elmer Fluoview, equipped with a differential interference contrast (DIC) apparatus. A Nikon 60× water immersion lens was used in combination with a heated stage. The nuclear/cytoplasmic ratio (Fn/c) was determined as previously described (*28*, *29*) using the Image J 1.62 public domain software (NIH), from single-cell measurements for each of the nuclear (Fn) and cytoplasmic (Fc) fluorescences, after the subtraction of fluorescence because of autofluorescence/background.

Immunoprecipitation. ppUL44 fusion proteins were immunoprecipitated using the magnetic μMACS Protein G Microbeads in combination with μMACS column technology (Miltenyi Biotech) and the anti-FLAG (SIGMA) and anti-V5 (Invitrogen) monoclonal antibodies, according to the recommendations of the manufacturer. Briefly, 6×10^5 COS-7 cells were transfected to express ppUL44-FLAG and V5-UL44 fusion proteins and 24 h later were washed twice with cold PBS in ice. Cell lysates were harvested in 300 μL of lysis buffer [150 mM NaCl, 1% Triton X-100, 50 mM Tris-HCl (pH 8.0), and complete protease inhibitors (Roche)] and incubated for 30 min on ice. Cell debris were removed by centrifugation at 14 000 rpm for 10 min at 4 °C, and the supernatant containing the cleared cell lysates was incubated for 30 min at 4 °C with 20 μL of Protein G Microbeads (Miltenyi Biotech) and 1 μg of either the anti-FLAG or anti-V5 mouse monoclonal antibodies. The mixtures were then loaded on μMACS columns (Miltenyi Biotech), which were pre-equilibrated with 200 μL of lysis buffer, washed 4× with 200 μL of lysis buffer, and then eluted with 50 μL of preheated elution buffer [50 mM Tris-HCl (pH 6.8), 50 mM dithiothreitol (DTT), 1% sodium dodecyl sulfate (SDS), 0.005% bromophenol blue, and 10% glycerol].

Western Blot Analysis. A total of 15 μL of the mixture containing the eluted proteins was loaded onto 4−20% bis-trisacrylamide NuPage precast gels (Invitrogen) and separated by polyacrylamide gel electrophoresis (PAGE). Electrophoretically separated proteins were then transferred to a Whatman nitrocellulose paper as previously described (*16*). The membrane was blocked in buffer A [5% bovine serum albumin (BSA) (w/v) and TBS 1×] for 1 h at room temperature and washed 3 times with buffer B (0.05% Tween and TBS 1×). Detection of ppUL44 fusion proteins was performed by incubating the membranes with either the anti-FLAG (SIGMA; 1:1000) or anti-V5 (Invitrogen; 1:500) mouse primary monoclonal antibodies and peroxidase-coupled secondary antibodies (SIGMA; 1:10 000). The immunoblots were developed with ECL plus (Amersham Biosciences), according to the recommendations of the manufacturer.

RESULTS

Differently Labeled ppUL44 Fusion Proteins Can Be Co-immunoprecipitated from Transfected Cells. To verify if ppUL44 dimerizes in a cellular context, we first performed a co-immunoprecipitation assay using differently tagged ppUL44 derivatives (Figure 1). Extracts of COS-7 cells transfected to express the V5-UL44 fusion protein in the

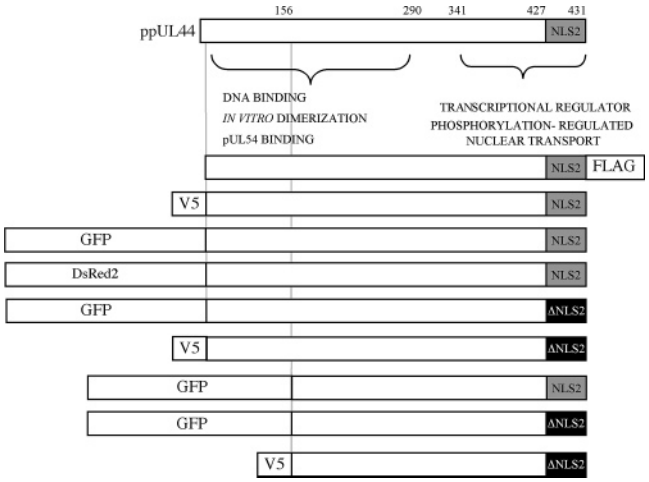

FIGURE 1: Proteins expressed in this study. Schematic representation of HCMV ppUL44 functional domains as well as the ppUL44 fusion proteins expressed in this study. The wild-type ppUL44-NLS2 (PNTKKQK^{431}) is shown as a gray box, whereas the mutant derivative ΔNLS2 (PNTVAQL^{431}) carrying a nonfunctional mutation is shown as a black box.

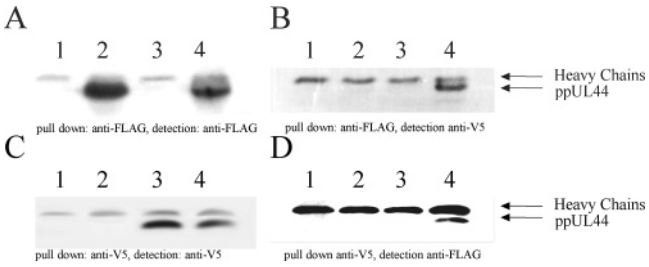

FIGURE 2: ppUL44 can dimerize in transfected cells. COS-7 cells were mock-transfected (lane 1) or transfected to express the following ppUL44 fusion proteins: ppUL44-FLAG, lane 2; V5-UL44, lane 3; and ppUL44-FLAG + V5-UL44, lane 4. Cells were harvested 24 h after transfection and immunoprecipitated using mouse anti-FLAG (A and B) or anti-V5 (C and D) monoclonal antibodies, as described under the Experimental Procedures. After SDS−PAGE separation and transfer to nitrocellulose, purified proteins were detected using anti-FLAG (A and D) or anti-V5 (B and C). The positions of UL44 fusion proteins and IgG heavy chains are indicated by arrows on the right.

presence or absence of the ppUL44-FLAG fusion protein were immunoprecipitated using monoclonal antibodies directed against either the FLAG or V5 epitope. Precipitated proteins were separated on denaturing polyacrylamide gels and transferred to nitrocellulose. The anti-FLAG and anti-V5 monoclonal antibodies were then used to detect the purified ppUL44 fusion proteins (Figure 2). After immunoprecipitation with the anti-FLAG antibody and detection using the anti-V5 antibody, a protein with the expected molecular weight of ca. 60 kDa was specifically detected only from cells transfected to co-express the UL44-FLAG and V5-UL44 fusion proteins (parts A and B of Figure 2); a similar result was obtained after immumoprecipitation using the anti-V5 antibody and detecting with the anti-FLAG antibody (parts C and D of Figure 2). These results suggest that ppUL44 can dimerize in a cellular context.

ppUL44 Is Translocated to the Nucleus as a Dimer. The nuclear import of ppUL44 is dependent upon a C-terminally located NLS (NLS2, PNTKKQK^{431}), on the basis of the fact that the NLS derivative mutant ppUL44ΔNLS2 (PNTVAQL^{431}) localized in the cytoplasm of several cell lines (*17*). Because our immunoprecipitation results suggest that ppUL44 is likely to be a dimer or a higher oligomer *in vivo*, we hypothesized that the co-expression of the wild type and the cytoplasmically localizing mutant fused to different tags could result in the formation of ppUL44/ppUL44ΔNLS2 heterodimers, where either the wild type or NLS mutant could colocalize with the dimerized partner. Vero cells were transfected to co-express DsRed2-UL44, together with GFP-UL44ΔNLS2 or GFP-β-GAL, as a negative control. Live cells were imaged microscopically 24 h after transfection. When expressed alone, both GFP-UL44ΔNLS2 and GFP-β-GAL localized in the cytoplasm (Figure 3A), whereas GFP-UL44ΔNLS2 but not GFP-β-GAL was able to colocalize with DsRed2-UL44 within the nucleus when the proteins were co-expressed in the same cell (Figure 3B). Similar results were obtained in COS-7 cells. ppUL44 would thus appear to dimerize in the cytoplasm before being translocated into the nucleus with a single NLS sufficient to mediate translocation of the dimer.

ppUL44 Amino Acids 1−155 Are Required for UL44 Dimerization in Live Cells. Bacterially expressed pUL44 has previously been shown to dimerize through its N-terminal amino acids (*23*). To verify if the N-terminal domain of ppUL44 was also required for *in vivo* dimerization, we expressed the GFP-UL44(156−433) and GFP-UL44(156−433)ΔNLS2 fusion proteins in Vero cells and analyzed their localization using CLSM as compared to that of full-length GFP-UL44 fusion proteins (Figure 4A). Quantification of the levels of specific nuclear and cytoplasmic fluorescence

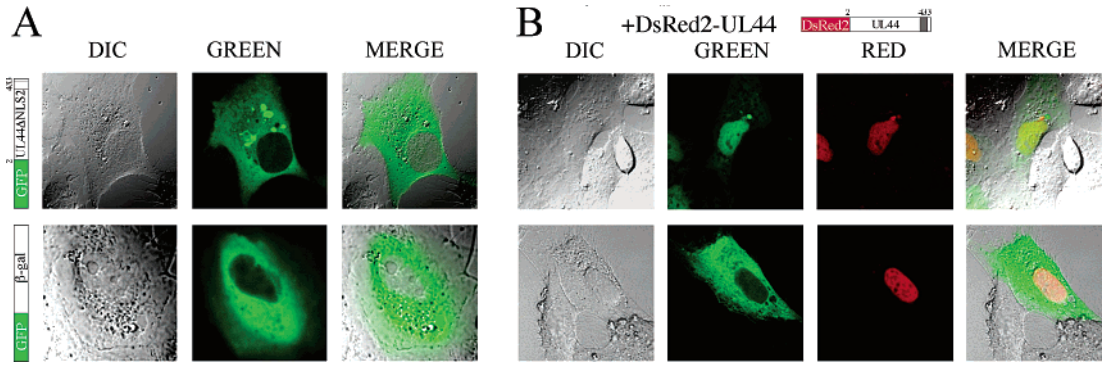

FIGURE 3: GFP-UL44ΔNLS2 localizes in the nucleus in the presence of DsRed2-UL44. Vero cells were transfected to express the indicated GFP fusion proteins in the absence (A) or presence (B) of DsRed2-UL44. Cells were imaged 24 h after transfection by CLSM. DIC images are shown on the left, and merge images of the green (GFP) and red (DsRed2) channels are shown on the right, with yellow coloration indicative of co-localization.

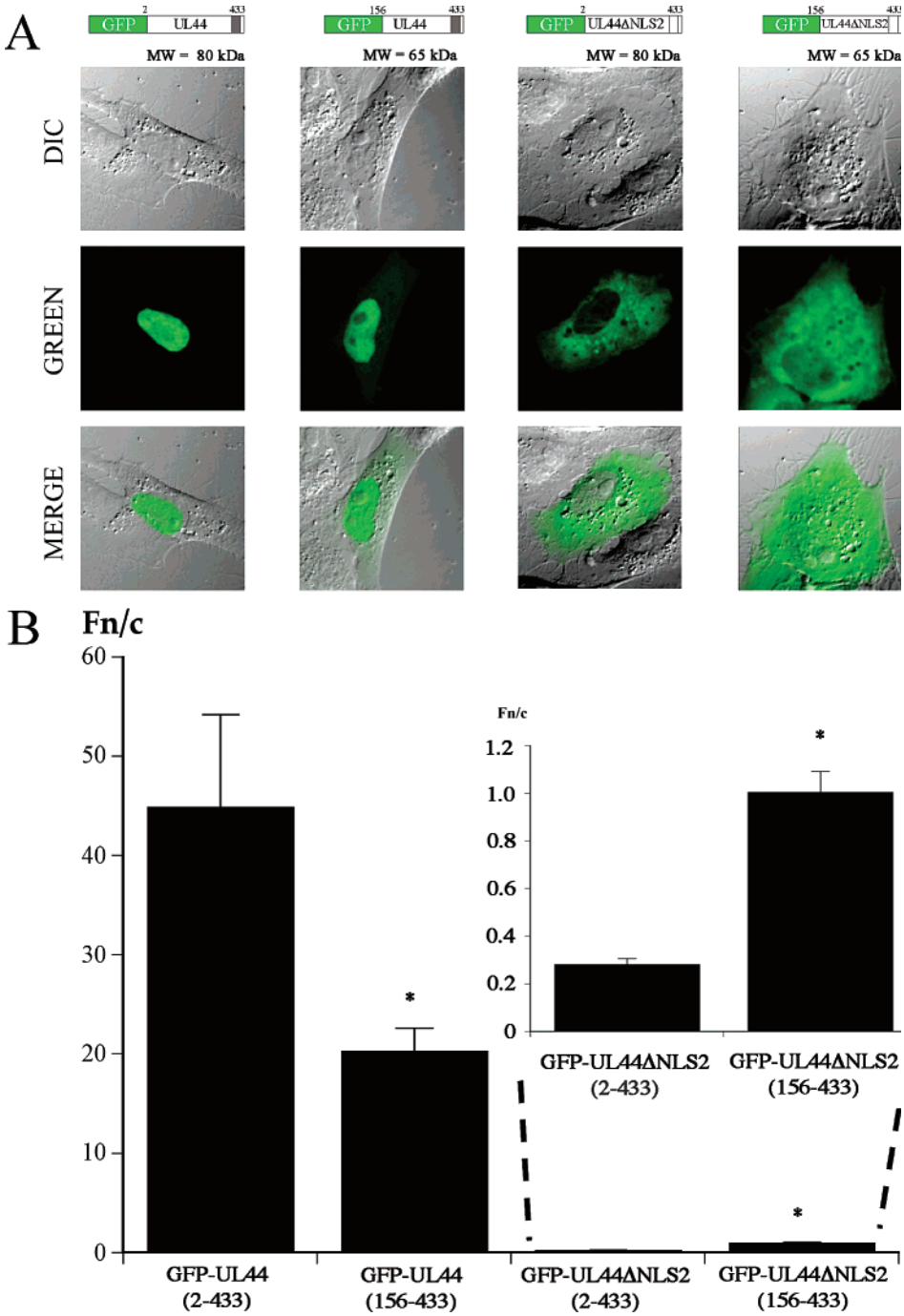

FIGURE 4: ppUL44 amino acids 1−155 modulate ppUL44 nuclear localization. (A) Vero cells were transfected to express the indicated GFP fusion proteins, and cells were imaged using CLSM 24 h after transfection. (B) Results for the quantification of the Fn/c, where confocal images, such as those shown in A, were analyzed using the ImageJ software as described under the Experimental Procedures. Data shown are for the mean ± standard error of the mean (SEM) ($n > 15$), with values for the test of significance between the full-length and the truncated proteins shown. (∗) $p < 0.0001$.

at the single-cell level revealed that nuclear accumulation of GFP-UL44(156−433) was lower than that of GFP-UL44 (Fn/c values of ca. 20 and 130, respectively) (Figure 4B), consistent with the reduced intranuclear binding ability of the truncation derivative mutant (*12*, *13*). Intriguingly, GFP-UL44(156−433)ΔNLS2 entered the nucleus more efficiently than GFP-UL44ΔNLS2, although both proteins were concentrated more in the cytoplasm than in the nucleus (Fn/c values < 1) (Figure 4B). The fact that the ca. 60 kDa GFP-UL44(156−433)ΔNLS2 fusion protein (Figure 5A) was able to enter the nucleus more efficiently than the ca. 80 kDa GFP-UL44ΔNLS2 fusion protein was most likely attributable to its inability to dimerize, because molecules larger than 60 kDa cannot diffuse passively through the NPC (*27*); conversely, diffusion of GFP-UL44ΔNLS2 across the NPC was prevented by its ability to dimerize in the cytoplasm (see Figures 2 and 3). To confirm this hypothesis, we co-expressed GFP-UL44(156−433)ΔNLS2 with DsRed2-UL44 in Vero cells, using GFP-UL44ΔNLS2 as a positive control. Cell were imaged by CLSM, with the results showing that GFP-UL44(156−433)ΔNLS2 was not able to colocalize with DsRed2-UL44 inside the nucleus (Figure 5B). When these

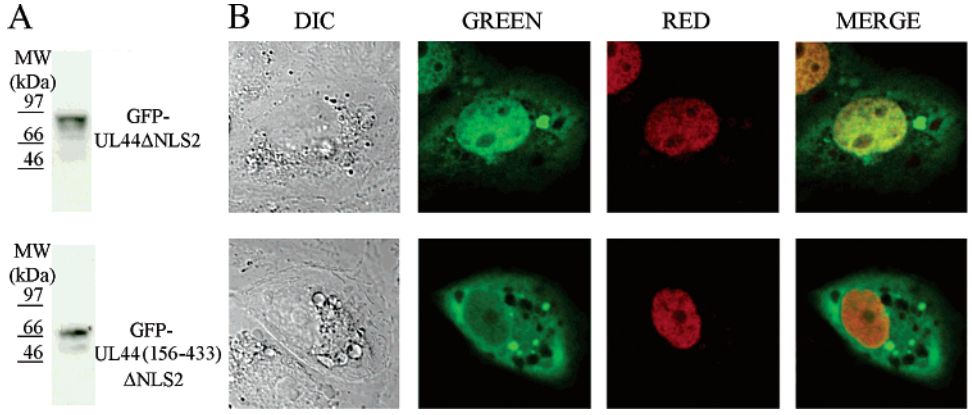

FIGURE 5: ppUL44 amino acids 1−155 are required for in vivo dimerization. (A) Lysates of COS-7 cells transfected to express the indicated GFP fusion proteins were harvested 24 h post-transfection, separated by PAGE, and transferred to nitrocellulose as described in the Experimental Procedures. GFP-UL44 fusion proteins were detected with the monoclonal antibody CH13 to ppUL44. (B) Vero cells transfected to express the indicated GFP fusion proteins in the presence of DsRed2-UL44 were imaged by CLSM. Merge images of the green (GFP) and red (DsRed2) channels are shown on the right, with yellow coloration indicative of co-localization.

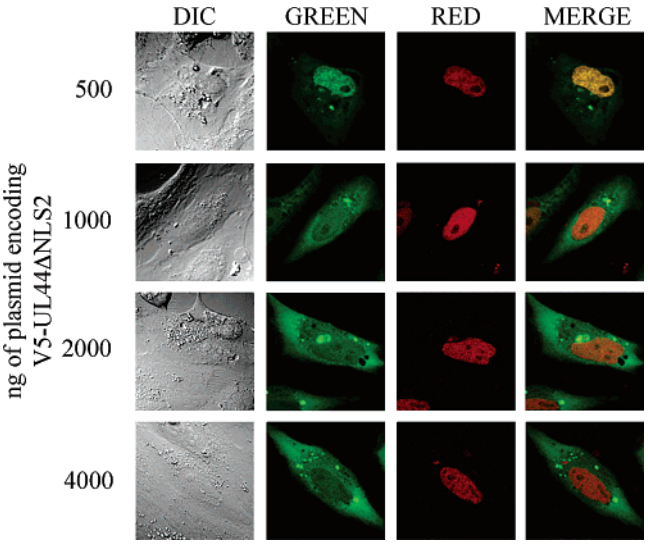

FIGURE 6: Live-cell-based system to monitor the inhibition of ppUL44 dimerization. Vero cells were transfected to express GFP-UL44ΔNLS2 and DsRed2-UL44 in the presence of increasing amounts of plasmids encoding V5-UL44ΔNLS2. Cells were imaged live 24 h after transfection by CLSM. DIC images are shown on the left, and merge images of the green (GFP) and red (DsRed2) channels are shown on the right, with yellow coloration indicative of co-localization.

results are taken together, they imply that residues 1−155 are required for ppUL44 dimerization in a cellular context, consistent with *in vitro* data (*23*).

ppUL44 Dimerization Can Be Inhibited. The DsRed2-UL44 fusion protein appears to be able to relocate GFP-UL44ΔNLS2 into the nucleus, apparently through generating DsRed2-UL44/GFP-UL44ΔNLS2 heterodimers. On the basis of this, we reasoned that cells co-expressing the latter proteins could be of potential utility in the identification of molecules able to interfere with ppUL44 dimerization. As a proof of this principle, we co-expressed the DsRed2-UL44 and GFP-UL44ΔNLS2 fusion proteins, together with increasing amounts of plasmid encoding V5-UL44ΔNLS2 fusion proteins, containing either the dimerization domain or lacking it [V5-UL44(156−433)ΔNLS2]. The overexpression of protein V5-UL44ΔNLS2 (Figure 6) but not the V5-UL44-(156−433)ΔNLS2 fusion protein (not shown) effected a significant reduction in GFP-UL44ΔNLS2 relocalization to the nucleus, presumably by preventing its interaction with DsRed2-UL44. This demonstrated that the efficiency of ppUL44 dimerization can be monitored in a live-cell system, by analyzing the extent of relocalization of GFP-UL44ΔNLS2 to the nucleus by co-expression with DsRed2-UL44.

DISCUSSION

This is the first study to demonstrate that ppUL44 dimerizes in a cellular context and that ppUL44 is translocated to the nucleus as a dimer. As a member of the *Herpesviridae* family, HCMV encodes a two-subunit DNA polymerase holoenzyme, comprising catalytic (pUL54) and accessory (ppUL44) subunits, both of which are essential for viral replication. Despite the fact that the HCMV PAP shares little sequence homology with functional homologues from other members of the *Herpesviridae* family, its crystal structure is extremely similar to that of herpes simplex virus 1 (HSV-1) pUL42, with the main difference being that pUL42 is a monomer, while HCMV ppUL44 appears to be a dimer (*23*, *30*, *31*). Intriguingly, the PAP encoded by Kaposi-associated herpes virus (KHSV PF-8) has been shown to dimerize during viral infection (*31*), whereas the PAP encoded by Epstein−Barr virus (EBV BMRF-1) forms multimers in solution and stimulates the catalytic subunit at a ratio of 2:1 (*32*, *33*). Here, we show for the first time that ppUL44 dimerizes *in vivo*, as shown by co-immunoprecipitation of differently tagged ppUL44 fusion proteins from COS-7 cells. Because ppUL44 is expressed at high levels during viral infection as determined by both Western blot and immunofluorescence analysis (*16*, *34*), our results imply that ppUL44 is also likely to be a dimer during viral infection, in a fashion similar to KHSV PF-8 (*31*).

The fact that ppUL44 can dimerize in a cellular context raises the question of whether ppUL44 is translocated to the nucleus as a dimer or a monomer and if ppUL44 mutant derivatives deficient in nuclear translocation can function as dominant negatives, retaining wild-type ppUL44 in the cytoplasm when co-expressed in the same cell. Nucleocytoplasmic shuttling of multimeric proteins can occur in several ways. Some of them, such as members of the NF-κB family, are believed to be translocated into the nucleus as dimers (*35*), whereas p53 is believed to be translocated through the NPC as a monomer and to multimerize only once

within the nucleus, in part because of the rapidity of the import process (*36*, *37*). Rapid nuclear import of ppUL44 is unlikely, because its NLS is rather weak and requires CK2-mediated phosphorylation of the upstream residue Ser^{413} for optimal nuclear accumulation when fused to heterologous proteins (*17*). Our dual-color live-cell imaging analysis here shows that ppUL44 is translocated to the nucleus as a dimer and that a single NLS on one subunit is sufficient to mediate the nuclear transport of the whole dimer (see Figures 3−5). It is therefore possible that ppUL44 dimerization enables fine modulation of its nuclear transport by regulation of the phosphorylation state of the C2N motif on each of the two subunits.

The dimerizing ability of ppUL44 implies that one subunit could function as a scaffold for the recruitment of other factors on the replication fork (*23*). Our findings that the NLS on one subunit is sufficient for nuclear transport of homodimers of ppUL44, together with the recent discovery that it can recruit the otherwise cytoplasmic viral-encoded uracil-DNA glycosylase pUL114 to the nucleus (*38*), supports the idea that certain components of the HCMV replication machinery could pre-assemble in the cytoplasm and be translocated to the nucleus as a complex. Whether ppUL44 dimerization is required for binding of ppUL114 is not clear at this stage, but ppUL44 dimerization has been shown to be essential for DNA binding *in vitro* (*23*). The fact that the DNA-binding activity of the structurally related HSV-1 UL42 (*30*) is essential for stimulation of its cognate DNA polymerase subunit (*39*) and that KSHV-1 ICP-8 dimerization is critical for DNA binding and stimulation of the KSHV-1 catalytic subunit Pol-8 (*31*) strongly suggests that ppUL44 DNA binding and hence its dimerization are important for viral replication. Protein−protein surface interactions generally involve large areas, thus rendering the identification of small molecules able to interfere with the interaction difficult (*40*). The fact that this may not be the case for ppUL44 homodimerization is implied by the fact that single amino acid substitutions appear to inhibit the process markedly (*23*), highlighting the potential of ppUL44 dimerization as a therapeutic target (*24*).

Automated microscopy applied on cell-based systems has been of great utility in screening for small molecules able to interfere with protein−protein interactions (*41*, *42*). Our results here show that it is possible to monitor the inhibition of ppUL44 dimerization in live cells simply by visualizing the ability of wild-type ppUL44 to relocalize the NLS mutant derivative ppUL44ΔNLS2 into the nucleus (see Figure 6); clearly, this represents the basis of an assay to identify small-molecule inhibitors able to interfere with ppUL44 dimerization. Validation and implementation of this is the focus of future work in our laboratories.

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