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The Carboxy-Terminal Segment of the Human Cytomegalovirus DNA Polymerase Accessory Subunit UL44 Is Crucial for Viral Replication▽

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The amino-terminal 290 residues of UL44, the presumed processivity factor of human cytomegalovirus DNA polymerase, possess all of the established biochemical activities of the full-length protein, while the carboxy-terminal 143 residues contain a nuclear localization signal (NLS). We found that although the amino-terminal domain was sufficient for origin-dependent synthesis in a transient-transfection assay, the carboxy-terminal segment was crucial for virus replication and for the formation of DNA replication compartments in infected cells, even when this segment was replaced with a simian virus 40 NLS that ensured nuclear localization. Our results suggest a role for this segment in viral DNA synthesis.

Human cytomegalovirus (HCMV) encodes a DNA polymerase which is composed of two subunits, UL54, the catalytic subunit, and UL44, an accessory protein (8, 12, 21). UL44 can be divided into two regions, a 290-residue amino (N)-terminal domain and a 143-residue carboxy (C)-terminal segment. The overall fold of the N-terminal domain is markedly similar to that of processivity factors such as herpes simplex virus type 1 (HSV-1) UL42 and eukaryotic proliferating cell nuclear antigen (6, 22, 41), which function to tether catalytic subunits to DNA to ensure long-chain DNA synthesis. *In vitro*, the N-terminal domain of UL44 is sufficient for all of the established biochemical activities of full-length UL44, including dimerization, binding to double-stranded DNA, interaction with UL54, and stimulation of long-chain DNA synthesis, consistent with a role as a processivity factor (4, 5, 8, 11, 23, 24, 39). In contrast, little is known about the functions of the C-terminal segment of UL44 other than its having been reported from transfection experiments to be important for downregulation of transactivation of a non-HCMV promoter (7) and to contain a nuclear localization signal (NLS) (3). Neither the importance of this NLS nor the role of the entire C-terminal segment has been investigated in HCMV-infected cells.

We first examined whether the N-terminal domain is sufficient to support DNA synthesis from HCMV *ori*Lyt in cells using a previously described cotransfection-replication assay (27, 28). A DpnI-resistant fragment, indicative of *ori*Lyt-dependent DNA synthesis, was detected in the presence of wild-type (WT) UL44 (pSI-UL44) (34) and in the presence of the UL44 N-terminal domain (pSI-UL44ΔC290), but not in the presence of UL44-F121A (6, 34), a mutant form previously shown not to support *ori*Lyt-dependent DNA synthesis (34) (Fig. 1A). Thus, the N-terminal domain alone is sufficient to support *ori*Lyt-dependent DNA synthesis in a transient-transfection assay.

These results were somewhat unexpected, as the C-terminal segment contains a functional NLS identified in transfection assays (3). We therefore assayed the intracellular localization of WT and mutant UL44 following transient transfection using pcDNA3-derived expression plasmids. Since the anti-UL44 antibodies that we have tested do not recognize the N-terminal domain of UL44, we constructed *UL44* genes to encode N-terminally FLAG-tagged full-length UL44 (FLAG-UL44) or a FLAG-tagged N-terminal domain, the latter by inserting three in-frame tandem stop codons after codon 290 (FLAG-UL44-290stop, Fig. 1B). We also constructed a mutant form encoding a FLAG-tagged N-terminal domain, followed by the simian virus 40 (SV40) T-antigen NLS (15–17), followed by three tandem stop codons (FLAG-UL44-290NLSstop, Fig. 1B). Vero cells were transfected with each construct using Lipofectamine 2000, fixed with 4% formaldehyde at 48 h posttransfection, and assayed by indirect immunofluorescence (IF) using anti-UL44 (Virusys) or anti-FLAG antibody (Sigma). We observed mostly nuclear localization of WT UL44 or FLAG-UL44 with either diffuse or more localized intranuclear distribution (Fig. 1C, parts a to c and d to f, respectively) and some occasional perinuclear staining, which may be due to protein overexpression. In cells expressing FLAG-UL44-290NLSstop, we observed mostly diffuse nuclear localization with little to no perinuclear staining (Fig. 1C, parts j to l). In cells expressing FLAG-UL44-290stop, we observed mostly cytoplasmic staining, but with some cells exhibiting some nuclear staining (Fig. 1C, parts g to i), which may explain the ability of truncated UL44 to support *ori*Lyt-dependent DNA replication in a transient-transfection assay (Fig. 1A).

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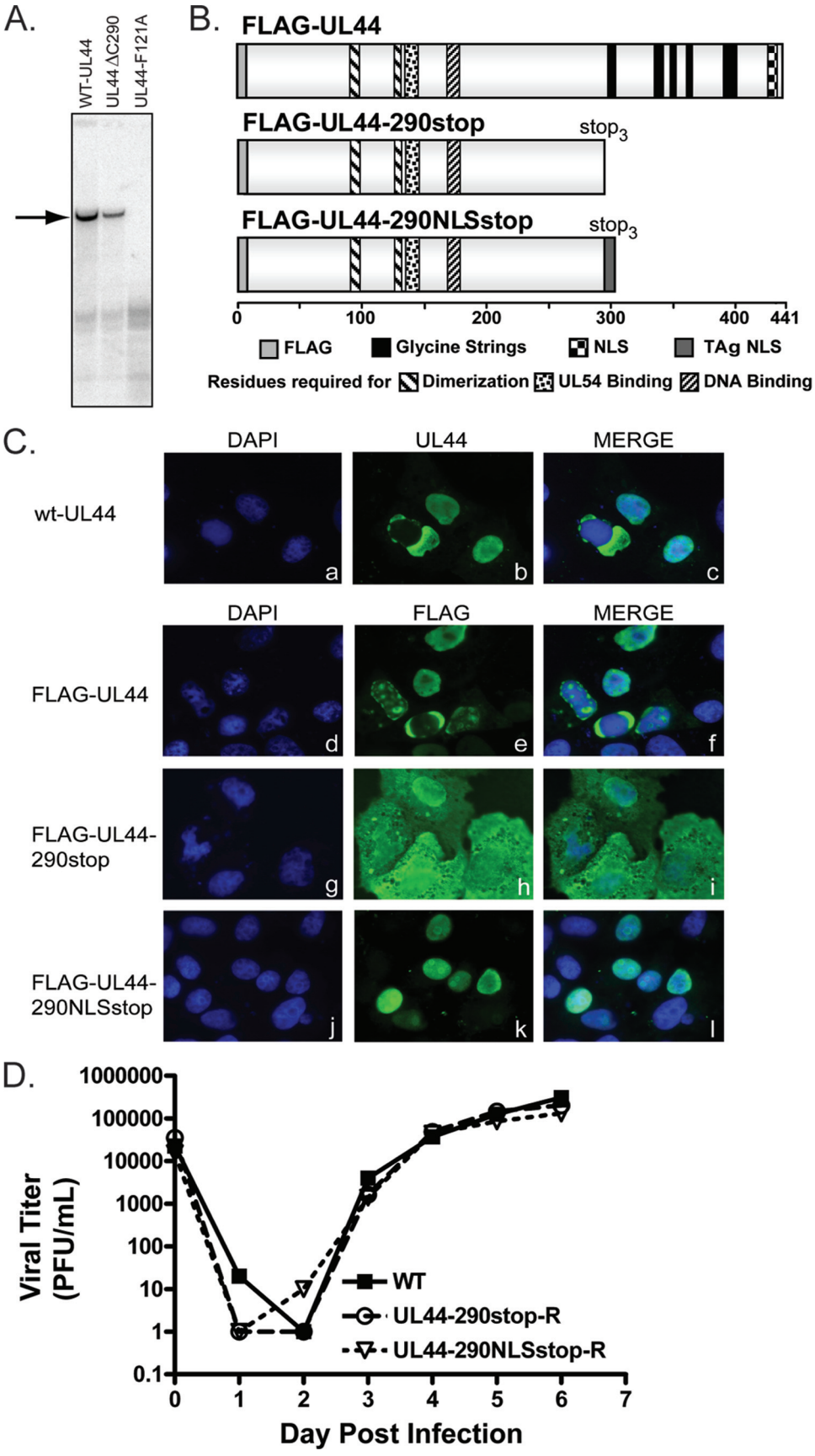

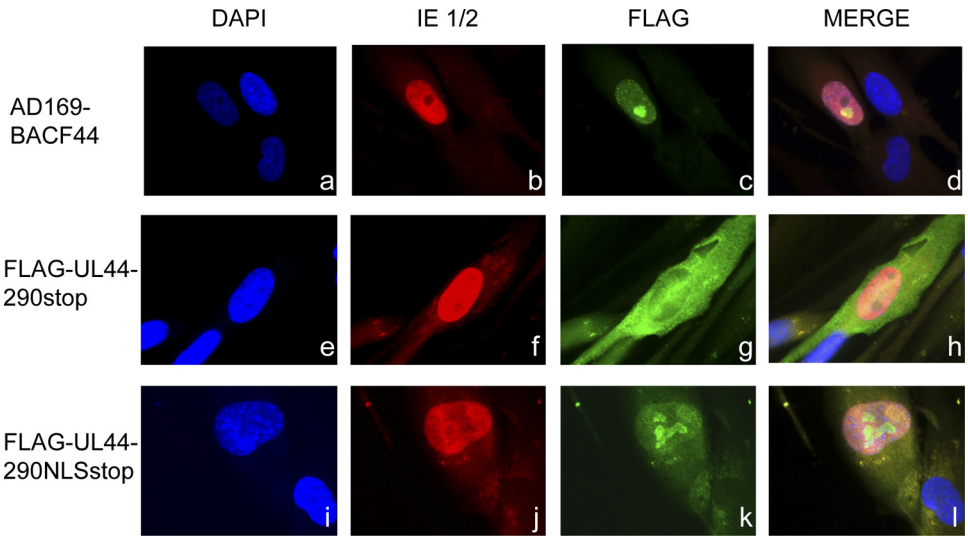

FIG. 2. Localization of IE1/IE2 and FLAG-UL44 proteins in electroporated cells. HFF cells were electroporated with AD169-BACF44 (panels a to d), BAC-UL44-290stop (panels e to h), or BAC-FLAG-UL44-290NLSstop (panels i to l). At 48 h posttransfection, cells were fixed and probed with anti-IE1/2 (Virusys) or anti-FLAG (Sigma). Secondary antibodies coupled to fluorophores were used for visualization of IE1/2 (anti-mouse Alexa 594; panels b, f, and j) and FLAG (anti-rabbit Alexa 488; panels c, g, and k) antibodies. DAPI was used to counterstain the nucleus (panels a, e, and i). Panels d, h, and l are merged images of the panels in the other columns. Magnification: ×1,000.

We next investigated whether the C-terminal segment of UL44 is necessary for viral replication. We reasoned that we could investigate whether any requirement for this segment could be due to a requirement for an NLS by testing whether the SV40 NLS could substitute for the loss of the UL44 C terminus. We therefore constructed HCMV UL44 mutant viruses by introducing the UL44-290stop and UL44-290NLSstop mutations into a WT AD169 bacterial artificial chromosome (BAC) using two-step red-mediated recombination as previously described (35, 38). We also constructed the same mutants with a FLAG epitope at the N terminus of UL44 (BAC-FLAG-UL44-290stop and BAC-FLAG-UL44-290NLSstop) to monitor UL44 expression, and we constructed rescued derivatives of the mutant BACs by replacing the mutated sequences with WT *UL44* sequences, as described previously (35). We introduced BACs into human foreskin fibroblast (HFF) cells using electroporation (35, 38). In several experiments using at least two independent clones for each mutant, cells electroporated with any of the mutant BACs did not exhibit any cytopathic effect (CPE) within 21 days. In contrast, within 7 to 10 days, cells electroporated with the WT AD169 BAC, a BAC expressing WT UL44 with an N-terminal FLAG tag [AD169-BACF44 (35)], or any of the rescued derivatives began displaying a CPE and yielded infectious virus. The rescued derivatives of the nontagged mutants displayed replication kinetics similar to those of the WT virus following infection at a multiplicity of infection (MOI) of 1 PFU/cell (Fig. 1D). The rescued derivatives of the FLAG-tagged mutants also replicated to WT levels (data not shown). Thus, the replication defects of the mutants were due to the introduced mutations that result in truncated UL44 either with or without the SV40 NLS. We therefore conclude that the C-terminal segment of UL44 is required for viral replication.

To investigate the stage of viral replication at which the UL44 C-terminal segment is important, we first assayed the subcellular localization of immediate-early proteins IE1 and IE2 and FLAG-UL44 in cells electroporated with BAC DNA expressing the FLAG-tagged WT or the two mutant UL44s using IF at 2 days postelectroporation. IE1/IE2 could be detected diffusely distributed in nuclei of cells electroporated with all three BACs (Fig. 2b, f, and j). In cells electroporated with AD169-BACF44 or BAC-FLAG-UL44-290NLSstop,

FIG. 1. Effects of UL44 C-terminal truncations in various assays. (A) HFF cells were cotransfected with the pSP50 plasmid (containing the *ori*Lyt DNA replication origin), a plasmid expressing WT or mutant UL44 (as indicated at the top of the panel), and plasmids expressing all of the other essential HCMV DNA replication proteins. At 5 days posttransfection, total DNA was extracted and cleaved with DpnI to digest unreplicated DNA and a Southern blot assay was performed to detect replicated pSP50. An arrow indicates DpnI-resistant, newly synthesized pSP50 fragments. (B) FLAG-tagged constructs analyzed in panel C are cartooned as horizontal bars. The names of the constructs are above the bars. The lengths of the constructs in amino acids are indicated by the scale at the bottom of the panel. The positions of residues required but not necessarily sufficient for features of the constructs are designated by shading, as indicated at the bottom of the panel. (C) Vero cells were transfected with plasmids expressing WT UL44 (parts a to c), FLAG-UL44 (parts d to f), FLAG-UL44-290stop (parts g to i), or FLAG-UL44-290NLSstop (parts j to l). At 48 h posttransfection, cells were fixed and stained with 4′,6-diamidino-2-phenylindole (DAPI) to visualize the nucleus (blue) (parts a, d, g, and j) and by IF with anti-UL44 (part b) or anti-FLAG (parts e, h, and k) and a secondary antibody conjugated with Alexa 488 (green). Parts c, f, i, and l are merged from images in the left and middle columns. Magnification: ×1,000. (D) Replication kinetics of rescued viruses. Rescued derivatives of UL44 mutant viruses (UL44-290stop-R and UL44-290NLSstop-R) or WT AD169 viruses were used to infect HFF cells at an MOI of 1 PFU/cell. The supernatants from infected cells were collected every 24 h, and viral titers were determined by plaque assays on HFF cells.

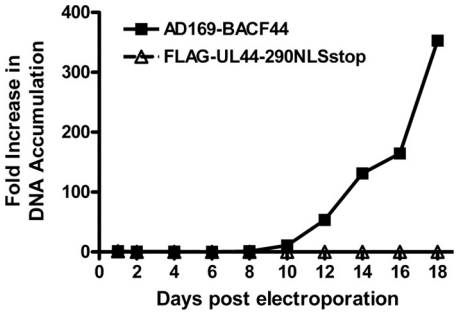

FIG. 3. Quantification of viral DNA accumulation in electroporated cells. HFF cells were electroporated with AD169-BACF44 or BAC-FLAG-UL44-290NLSstop, and total DNA was harvested on the days postelectroporation indicated. Viral DNA accumulation was assessed by real-time PCR by assessing levels of the *UL83* gene and normalizing to levels of the cellular β-*actin* gene (32). The data are presented as the fold increase in normalized viral DNA levels over the amount of input DNA (day 1).

FLAG-UL44 was localized largely within the nucleus (Fig. 2c and k, respectively). In contrast, in cells electroporated with BAC-FLAG-UL44-290stop, the FLAG epitope was mainly localized diffusely in the cytoplasm, with only a small amount diffusely distributed in the nucleus (Fig. 2g). These data indicate that IE proteins expressed from mutant BACs are properly localized and suggest that without its C-terminal segment, which includes the NLS identified in transfection assays (3), UL44 cannot efficiently localize to the nucleus in HCMV-infected cells. However, addition of the SV40 NLS was sufficient to efficiently localize the N-terminal domain of UL44 to the nucleus. Thus, the requirement for the C-terminal segment of UL44 for viral replication is not due solely to its NLS.

We next investigated if the block in viral replication due to the loss of the C-terminal segment could be attributed to a defect in viral DNA synthesis. Cells were electroporated with AD169-BACF44 or BAC-FLAG-UL44-290NLSstop, and viral DNA accumulation was assayed by quantitative real-time PCR at various times postelectroporation (Fig. 3) as previously described (32, 35). In HFFs electroporated with AD169-BACF44, viral DNA began to accumulate above the input levels by 8 days postelectroporation and increased over time, with as much as a 350-fold increase over the input DNA level by 18 days postelectroporation. In contrast, levels of viral DNA in cells electroporated with BAC-UL44-290NLSstop did not increase above input levels, even by 18 days postelectroporation. These data are consistent with the notion that the UL44 C-terminal segment is required for viral DNA synthesis, although we caution that the assay did not detect DNA synthesis from AD169-BACF44 until day 8, when viral spread had likely occurred (see below).

We also analyzed the localization patterns of UL44 and UL57, the viral single-stranded DNA binding protein, which is a marker for viral DNA replication compartments (1, 2, 18, 26, 29). At 8 days postelectroporation with AD169-BACF44, UL57 and FLAG-UL44 largely colocalized within a single large intranuclear structure that likely represents a fully formed replication compartment, with some cells containing multiple smaller globular structures within the nucleus that likely represent earlier stages of replication compartments (1, 2, 29) (Fig. 4a to d). Neighboring cells also stained for UL57 and FLAG-UL44, indicative of viral spread. In contrast, in cells electroporated with BAC-FLAG-UL44-290NLSstop, UL57 (Fig. 4f) was found in either punctate or small globular structures. This pattern of UL57 staining resembled that observed at very early stages of viral DNA synthesis in HCMV-infected cells, but the structures were larger and less numerous than those observed in HCMV-infected cells in the presence of a viral DNA polymerase inhibitor (2, 29). Staining for FLAG-UL44 was nuclear and largely diffuse, with some areas of more concentrated staining (Fig. 4g), which could also be observed in some cells at day 2 postelectroporation (Fig. 3k). This pattern of UL44 localization was generally similar to that observed in HCMV-infected cells at very early stages of infection or when HCMV DNA synthesis is blocked and also similar to the pattern in cells transfected with a *UL84* null mutant BAC (2, 29, 33, 40). Importantly, little colocalization of UL57 and UL44 was observed, with areas of concentration of UL57 or UL44 occupying separate regions in the nuclei of these cells (Fig. 4h). We are unaware of any other examples of this pattern of localization of these proteins in HCMV-infected cells

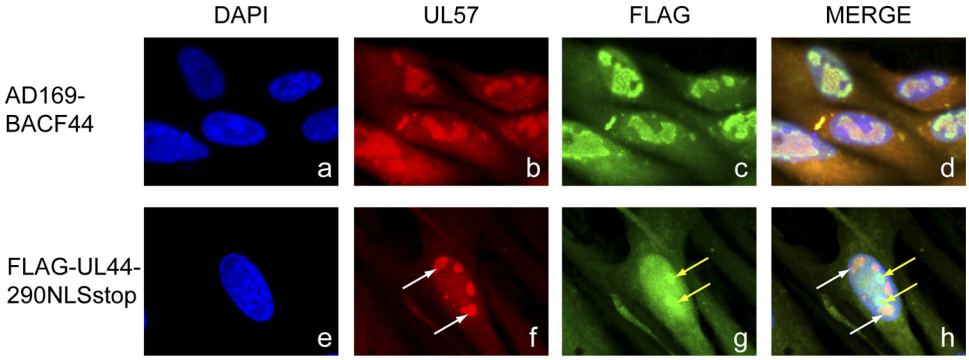

FIG. 4. Localization of UL57 and FLAG-UL44 proteins in electroporated cells. HFF cells were electroporated with AD169-BACF44 (panels a to d) or BAC-FLAG-UL44-290NLSstop (panels e to h). At 8 days posttransfection, cells were fixed and then stained with antibodies specific for UL57 (Virusys) or FLAG (Sigma), followed by a secondary antibody coupled to fluorophores to detect UL57 (anti-mouse Alexa 594; panels b and f) and FLAG (anti-rabbit Alexa 488; panels c and g) antibodies. DAPI stain was used to counterstain the nucleus (panels a and e). Panels d and h are merged images of the panels in the other columns. White arrows identify punctate UL57 staining. Yellow arrows identify areas of concentration of FLAG-UL44 staining. Magnification: ×1,000.

and suggest that it may be a result of the loss of the UL44 C-terminal segment. These results indicate that this segment is important for efficient formation of viral DNA replication compartments, again consistent with a requirement for this portion of UL44 for viral DNA synthesis.

Our results, taken together, argue for a role for the C-terminal segment of UL44 in HCMV-infected cells in efficient nuclear localization of UL44 and a role in viral DNA synthesis beyond its role in nuclear localization. It is possible that this segment interacts with host or viral proteins involved in DNA replication. Of the various proteins reported to interact with UL44 (10, 19, 30, 31, 35–37), interesting candidates include the host protein nucleolin, which has been shown to associate with UL44 and be important for viral DNA synthesis (35), and the viral UL112-113 proteins, which in transfection assays were shown to recruit UL44 to early sites of DNA replication (2, 29, 33). After this paper was submitted, Kim and Ahn reported that the C-terminal segment of UL44 is necessary for interaction with a UL112-113 protein and, similar to our findings, crucial for viral replication (19). However, contrary to our findings, they reported that this segment was not necessary for efficient nuclear localization of UL44 (19). It may well be that the C-terminal segment of UL44 also has some other role later in viral replication, perhaps in gene expression, as has been suggested (7, 13, 14).

A virus with a deletion of the C-terminal 150 amino acids of the HSV-1 polymerase accessory subunit UL42 displays no obvious defect in replication (9). Thus, it appears that HSV-1 and HCMV exhibit different requirements for the C-terminal segments of their respective accessory proteins. This and many other differences between these functionally and structurally orthologous proteins (5, 6, 20, 24, 25) suggest considerable selection for different features during evolution.

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