Targeting of the gene product encoded by ORF UL56 of human cytomegalovirus into viral replication centers

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Abstract The highly conserved DNA-binding protein pUL56 of human cytomegalovirus (HCMV) was found to be predominantly localized throughout the nucleus as well as in viral replication centers of infected cells. The latter localization was abolished by phosphono acetic acid, an inhibitor of viral DNA replication. Immunofluorescence revealed that pUL56 co-localized in replication centers alongside pUL112–113 and pUL44 at late times of infection. By co-immunoprecipitations, a direct interaction with pUL44, a protein of the replication fork, was detected. These results showed for the first time that HCMV pUL56 is localized in viral replication centers, implicating that DNA replication is coupled with packaging.

Key words: Human cytomegalovirus; Nuclear distribution; Viral replication center; Co-localization

1. Introduction

Recently, we demonstrated that the human cytomegalovirus (HCMV) UL56 gene product [6], the homolog of the herpes simplex virus 1 (HSV-1) UL28 gene product, binds to HCMV DNA packaging motifs and cleaves DNA bearing these motifs [4,5]. These results are in line with studies on homologous proteins of HSV-1 ICP18.5 (pUL28) and pseudorabies virus showing by use of viral mutants that the deletion of UL28 leads to nuclear accumulation of naked nucleocapsids and uncleaved concatemeric DNA [1,15,19]. This observation led us to the assumption that pUL56 plays a key role in DNA packaging, and may functionally resemble a terminase subunit. This is consistent with previous genetic analysis which demonstrated that a new class of anti-HCMV agents inhibit HCMV DNA maturation by direct interaction with the viral UL89 [13] and UL56 [20] gene products.

It is known that herpes viral transcription, DNA synthesis and packaging occur in defined intranuclear globular structures called replication centers [8,9]. Previously, Ahn and Hayward [2] reported that formation of viral DNA replication compartments in HCMV-infected cells is initiated within granular structures that bud from the periphery of some of the promyelocytic leukemia protein oncogenic domains (PODs) and subsequently merge into larger structures that are flanked by PODs.

In this study, experiments are described regarding the localization of HCMV pUL56 in viral replication centers, the co-localization with two proteins, pUL112–113 and pUL44, known to be involved in DNA replication, and a direct physical interaction regarding pUL44. The work presented here is the first incidence for herpes viruses that such an association takes place.

2. Materials and methods

2.1. Cells and viruses

Human foreskin fibroblasts (HFF) were grown in Dulbecco's medium supplemented with 10% fetal calf serum, 2 mM glutamine and penicillin (5 U/ml) and streptomycin (50 μg/ml) as described previously. Preparation of HCMV AD169 and infection of HFF at a multiplicity of infection (moi) of 1.0 was carried out as described before [7].

2.2. Transient DNA expression

For DNA transfection experiments, COS-7 cells were seeded on 60 mm diameter petri dishes with glass coverslips. DNA (10 μg/well) was introduced for transient expression assays in 60% confluent cells using the lipofectin reagent (Life Technologies) as recommended by the manufacturer. Analysis was carried out at 40 h post transfection (p.t.) by indirect immunofluorescence.

2.3. Purification of the pUL56 antiserum

Affinity purification of the anti-UL56 antibody was done as follows. A β-galactosidase fusion protein, fusproII, containing a hydrophilic amino acid stretch from amino acid 324 to 524 of pUL56 was coupled to activated immunoaffinity supports Affi-Gel 10 and Affi-Gel 15 (1:2 Affi-Gel 10/Affi-Gel 15; Bio-Rad Laboratories) as described by Bio-Rad, yielding Affi-Gel 10/15-pUL56. Unspecific binding sites were blocked by incubation with buffer b (1 M ethanolamine–HCl pH 8.0, 0.02% NaN_3) for 1 h. The resin was equilibrated with start buffer (150 mM NaCl, 50 mM Tris–HCl pH 8.0, 0.02% NaN_3). High titer human convalescent serum was incubated overnight with the prepared affinity matrix. After the matrix was washed twice with 10 bed volumes of ice-cold start buffer, the bound antibody was eluted in 200 mM glycine–HCl (pH 2.5). The purified anti-pUL56 antibody, pabUL56, was neutralized with 100 mM Tris–HCl (pH 8.0). The specificity of the antibody was tested in Western blots (as shown in [6]).

2.4. Immunofluorescence and Immunoprecipitation

For immunofluorescence, cells were grown on glass coverslips. At various times post infection (p.i.)/p.t., coverslip cultures were fixed in 3% paraformaldehyde as described previously [18].

Detection of HCMV pUL56 was carried out with pabUL56 [5,6] for 45 min at room temperature prior to further incubation for 45 min with fluorescein-labeled goat anti-human $F(ab')_2$ fragments. For double staining, HCMV pUL56-specific human pabUL56 and monoclonal antibody (mAb) M23, against the gene products of HCMV pUL112–113 (provided by K. Hirai, Tokyo, Japan), or pUL44-specific antibody RG#1202 (Rumbaugh-Goodwin Institute for Cancer Research), or IE1/2-specific mAb (NEA9221 from NEN) was used in the first incubation. In the second incubation, fluorescein-labeled anti-human and Texas red-labeled anti-mouse $F(ab')_2$ fragments were used.

For immunoprecipitation, total cell extracts were prepared from [^{35}S]methionine-labeled cultures by solubilization in co-immunoprecipitation buffer (20 mM Tris–HCl pH 7.5, 100 mM NaCl, 1% NP-40,

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5 mM EDTA, 25 mM iodoacetamide, 0.4% Na-deoxycholate, 1 mM PMSF, 100 U/ml Trasylol) and ultrasonic treatment. Insoluble material was sedimented for 30 min at 100 000 × *g*. Comparable amounts of extracts and pUL56- (1:20), pUL44-specific antibody (1:200) or mAb 27-156 against glycoprotein B (1:10) were used for precipitation as described previously [7].

2.5. Western blot and autoradiography

The supernatants or precipitated proteins were electrophoresed on 8% polyacrylamide gels, transferred to nitrocellulose sheets and subjected to Western blot analysis as described previously [6]. The antibody RG#1202 (1:4000 in 3% bovine serum albumin (BSA)) specific for pUL44 (p52) was used as the primary antibody prior to incubation with horseradish peroxidase-conjugated anti-mouse $F(ab')_2$ fragments (1:300 in 3% BSA). After immunostaining, radioactive blots were subjected to autoradiography.

3. Results

3.1. Localization of HCMV pUL56 in viral replication centers

In order to precisely examine intracellular distribution of HCMV pUL56, immunofluorescence using pabUL56 [6], which was purified from high titer human serum by column affinity chromatography (Affi-Gel 10/15-pUL56), was performed. Coverslip HFF cultures were mock-infected or infected with HCMV (moi of 1) for 12, 24, 48 and 72 h prior to immunofluorescence. In HCMV-infected cells, pabUL56 detected antigen predominantly in the nucleus (Fig. 1A). Distribution and intensity of fluorescence changed during the course of infection: at 12 h p.i., weak and more homogeneous nuclear staining was observed, that increased with time until late time after infection when in addition to homogeneous nuclear staining bright intranuclear patches were detected. Fluorescence staining presumably in the nucleolar region was also detected only early after infection (Fig. 1A, a, b), but was no longer seen at later times p.i. In uninfected cells, no fluorescence was detected (Fig. 1A, e). To determine whether nuclear translocation of pUL56 was an early or late

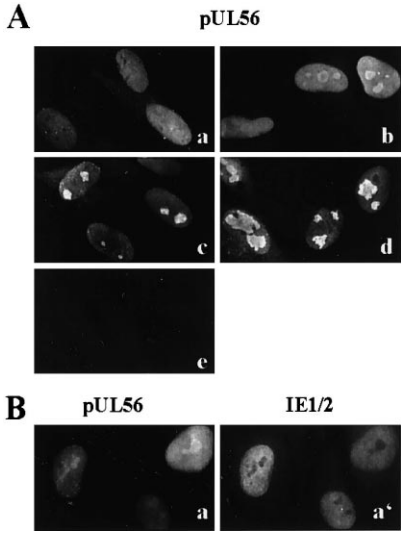

Fig. 1. Analysis of intranuclear distribution of HCMV pUL56. (A) HFF cells were mock-infected (e) or infected with HCMV for 12 (a), 24 (b), 48 (c) and 72 h (d) and subjected to immunofluorescence with antibody against pUL56, pabUL56. (B) HFF cells infected with HCMV in the presence of PAA were double-stained with antibodies against pUL56 (a) and against IE1/2 (a′) at 72 h p.i., respectively.

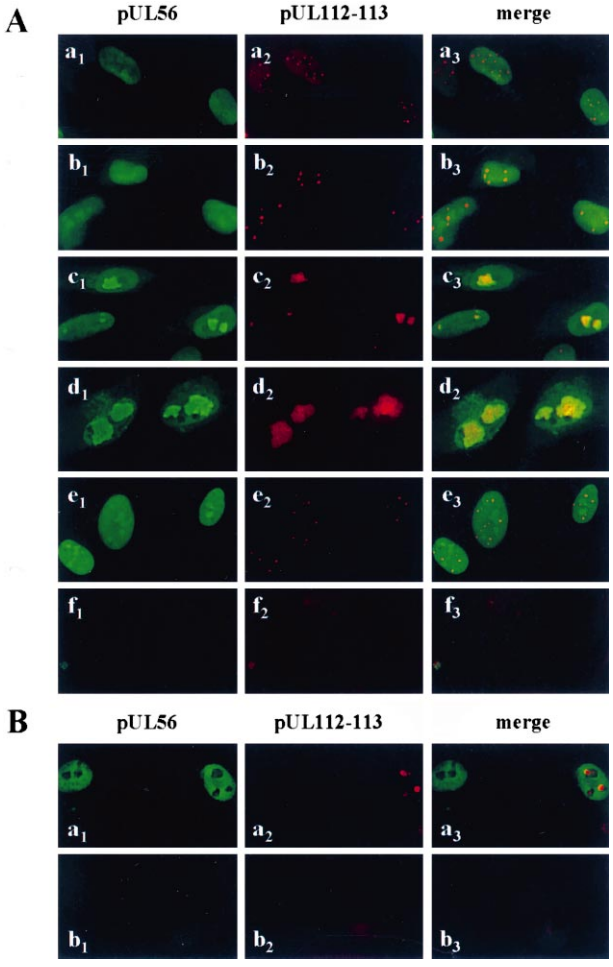

Fig. 2. Co-localization of pUL56 with HCMV gene products of ORF UL112–113. (A) HFF mock-infected (f) or infected with HCMV were double-stained with pabUL56 and mAb M23 against pUL112–113 at 12 (a), 24 (b), 48 (c) and 72 h (d) p.i. HFF infected with HCMV in the presence of PAA were double-stained with pabUL56 and mAb M23 against ORF UL112–113 at 72 h p.i. (e). A merge of the FITC and Texas red-stained cells is shown in addition (a_3–f_3). (B) COS-7 cells were transfected with vector (b) or co-transfected with UL56 and the 43K form of ORF UL112–113. Double staining with pabUL56 (a_1, b_1) and mAb M23 (a_2, b_2) was performed at 40 h after transfection. A merge of the FITC and Texas red-stained cells is shown in addition (a_3–f_3).

event, cells were infected in the presence of 200 μg/ml phosphono acetic acid (PAA) and analyzed at 72 h p.i. by double staining with monospecific pabUL56 and mAb against the HCMV IE1/2 protein. IE1/2 antigen was restricted to the nucleus, as expected. The pUL56 fluorescence pattern of PAA-treated cells equaled that of untreated cells at 24 h p.i. (Fig. 1B, a). This observation suggested that viral DNA replication is required for translocation of pUL56 into intranuclear patches but not for its expression.

3.2. Co-localization of HCMV pUL56 with the gene products of open reading frame (ORF) UL112–113

Double staining of infected cells was performed to determine whether compartmentalization of pUL56 to nuclear replication centers late p.i. was comparable to that of gene products of the ORF UL112–113 [12], which encode four proteins that are required for DNA replication. In infected cells,

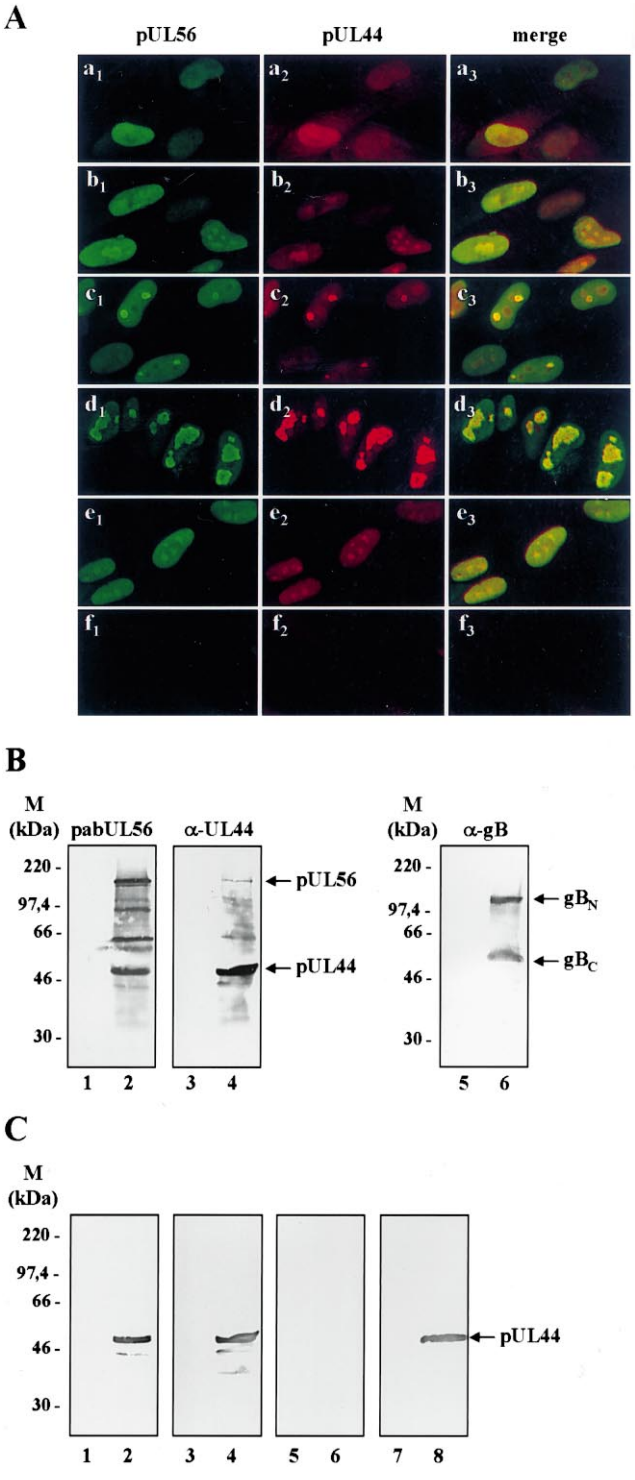

Fig. 3. Co-localization of pUL56 with HCMV pUL44. (A) HFF mock-infected (f) or infected with HCMV were double-stained with pabUL56 and mAb RG#1202 against pUL44 at 12 (a), 24 (b), 48 (c) and 72 h (d) p.i. HFF infected with HCMV in the presence of PAA were double-stained with pabUL56 and mAb RG#1202 against pUL44 at 72 h p.i. (e). (B) Mock-infected (1, 3, 5) or HCMV-infected (2, 4, 6) HFF at 72 h p.i. were co-immunoprecipitated with pabUL56 (1, 2), mAb RG#1202 (3, 4) or anti-gB mAb 27-156 (5, 6) and subjected to SDS–PAGE prior to transfer onto nitrocellulose and autoradiographic analysis. (C) The identical immunoprecipitates of mock-infected (1, 3, 5) or HCMV-infected (2, 4, 6) HFF together with extracts of mock- (7) and HCMV-infected cells (8) were subjected to immunoblot analysis using antibody RG#1202. The arrows indicate the position of pUL56, pUL44 and gB, the molecular weight markers (MW) are indicated on the left. ←

pUL56-specific immunofluorescence of intranuclear replication centers at 48 and 72 h p.i. indeed partially co-localized with that of pUL112–113 (Fig. 2A, c, d). Infection in the presence of PAA on the other hand revealed a punctate staining for pUL112–113 while pUL56 again was homogeneously distributed with some accumulation presumably in nucleoli (Fig. 2A, e).

To determine whether the gene product of ORF UL112–113 is able to translocate pUL56 into replication centers, immunofluorescence after transient transfection of pUL56 and the 43K form of UL112–113, which is the predominant product in infected cells [12], was performed. While pUL112–113 was detected in prereplication centers [8], pUL56 showed a homogeneous nuclear staining (Fig. 2B). This result suggested that translocation of pUL56 in replication centers in infected cells is independent of interaction with pUL112–113.

3.3. Co-localization of HCMV pUL56 with HCMV pUL44

In order to identify an interaction with an additional protein known to be involved in DNA replication, immunofluorescence and immunoprecipitation using mAb against pUL44 was undertaken. Early after infection for both proteins homogeneous nuclear staining was observed (Fig. 3A, a, b), and both products were also found in presumably nucleolar regions. This distribution changed after 48 h p.i. to a distinct staining of replication centers (Fig. 3A, c). However, in contrast to the pUL56 fluorescence pattern, the nucleolar accumulation of pUL44 seemed to be preserved. The fluorescence pattern in the presence of PAA was for both proteins similar to that seen at early times after infection (Fig. 3A, e). Again, mock-infected HFF revealed no specific immunofluorescence signals (Fig. 3A, f).

To examine a putative interaction between both proteins, co-immunoprecipitation prior to Western blot analysis and autoradiography was performed. Autoradiographic analysis of the blotted precipitation with pabUL56 detected pUL56 and in addition a 50 kDa protein (Fig. 3B, lane 2). On the other hand, using antibody against pUL44 for immunoprecipitation, a protein of about 130 kDa was detected in the precipitates, in addition (Fig. 3B, lane 4). Additional co-precipitating proteins could represent the approximately 100 kDa precursor (expected molecular weight) of pUL56, which is not seen in urea gels as well as in virions, while the 65 kDa band might be pp65, which adheres to protein A Sepharose. Co-immunoprecipitation with an antibody against HCMV gB, mAb 27-156, recognized only gB-specific gene products (Fig. 3B, lane 6). Identical co-immunoprecipitates as well as cell extracts of mock- and HCMV-infected HFF were subjected to Western blot analysis using pUL44-specific mAb RG#1202. pUL44 was detected in all immunocomplexes (Fig. 3C, 2, 4) as well as HCMV-infected cell extracts (Fig. 3C, 8). No detection was observed in control co-immunoprecipitates with mAb against HCMV gB (Fig. 3C, 6). These experiments showed that the co-precipitated 50 kDa protein indeed was pUL44 and implicates a direct interaction between HCMV pUL56 and pUL44.

4. Discussion

The results presented are to our knowledge the first evidence that the highly conserved structural protein pUL56 of HCMV is associated with viral replication centers. Currently, no reports are available about similar associations of pUL56 homologs of other herpes viruses. In infected cells, pUL56 accumulates in discrete regions of the nucleus. Studies in the presence of PAA demonstrated that this intranuclear localization was dependent of viral DNA replication.

Interestingly, pUL56 was partially co-localized with the gene product of ORF UL112–113. Pari and Anders [16] reported that this ORF encodes four proteins, which are translated from differentially spliced transcripts and are required for HCMV DNA replication in transient complementation assays. In addition, Yamamoto et al. [22] showed by use of antisense strategy that UL112–113 gene products as well as viral DNA replication were concomitantly affected. It was previously reported that UL112–113 products form prereplication structures, and redistribute during viral DNA replication to replication compartments [3]. This report is in line with our observation that after solitary expression the 43K form of UL112–113 is localized in prereplication structures. Regarding pUL56, our results showed that its translocation into viral replication centers is independent of pUL112–113. Remarkably, we observed co-localization as well as co-precipitation of pUL56 with pUL44, a protein known to be predominantly localized in intranuclear viral replication centers and to increase DNA-binding specificity of the viral DNA polymerase [10,17,21]. At present, it is not possible to clearly distinguish between HCMV DNA replication, cleavage and packaging (for review, [2]). Our observation of the direct interaction between the structural protein pUL56 and the non-structural protein pUL44 is therefore intriguing and needs further elucidation.

Regarding our previous findings of pUL56 DNA-binding and cleavage [4,5], localization in replication centers is in line with this function. One may speculate that DNA-binding of pUL56 occurs concomitant and/or consecutive to viral DNA synthesis, DNA–protein complexes are then translocated to empty capsids where the protein remains attached after packaging. Interestingly, DNA of ssDNA phage ∅X174 is packaged into a procapsid by a unique mechanism in that this process is coupled with DNA replication [11]. Further, Lamberti and Weller ([14]) reported that at 6 and 8 h p.i., HSV capsids co-localize with the major DNA-binding protein in replication compartments suggesting that cleavage and packaging occur at this site. This finding implicates that DNA replication could be coupled with packaging also in the case of herpes viruses. This mechanism could explain our observations and future experiments will show whether this notion indeed reflects the processes in infected cells.

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