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Interaction of the Putative Human Cytomegalovirus Portal Protein pUL104 with the Large Terminase Subunit pUL56 and Its Inhibition by Benzimidazole-D-Ribonucleosides

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Herpesvirus DNA replication leads to unit length genomes that are translocated into preformed procapsids through a unique portal vertex. The translocation is performed by the terminase that cleaves the DNA and powers the insertion by its ATPase activity. Recently, we demonstrated that the putative human cytomegalovirus (HCMV) portal protein, pUL104, also forms high-molecular-weight complexes. Analyses now have been performed to determine the intracellular localization and identification of interaction partners of pUL104. In infected cells, HCMV pUL104 was found to be predominantly localized throughout the nucleus as well as in cytoplasmic clusters at late times of infection. The latter localization was abolished by phosphonoacetic acid, an inhibitor of viral DNA replication. Immunofluorescence revealed that pUL104 colocalized with pUL56, the large subunit of the HCMV terminase. Specific association of in vitro translated pUL104 with the carboxy-terminal half of GST-UL56C was detected. By using coimmunoprecipitations a direct interaction with pUL56 was confirmed. In addition, this interaction was no longer detected when the benzimidazole-D-nucleosides BDCRB or Cl_4RB were added, thus indicating that these HCMV inhibitors block the insertion of the DNA into the capsid by preventing a necessary interaction of pUL56 with the portal. Electron microscopy revealed that in the presence of Cl_4RB DNA is not packaged into capsids and these capsids failed to egress from the nucleus. Furthermore, pulsed-field gel electrophoresis showed that DNA concatemers synthesized in the presence of the compound failed to be processed.

The process of viral DNA packaging is multifunctional and determined by specific interactions of protein-DNA and protein-protein. An important role during this process is played by portal proteins. Portals are large macromolecules and are found throughout herpesviruses as well as those double-stranded DNA (dsDNA) bacteriophages examined to date (6). Portal proteins provide on one hand the channel for entry of the DNA during packaging and on the other the exit for releasing DNA during infection. They are incorporated at one unique vertex, a fivefold vertex, of icosahedral procapsids. All known portal proteins differ in size from 35.9 kDa (ϕ29) to 82.7 kDa (P22) for the monomer, but all are arranged in rings with a 12-fold symmetry (3, 17, 22, 24, 32, 33). Recently, Smith et al. (40) demonstrated that in the case of bacteriophage ϕ29 the internal force is approximately 50 pN at the end of packaging. Therefore, insertion of dsDNA into the procapsids requires the terminase ATPase activity (39). Portal proteins function, in addition, like a DNA pump as the docking site for the terminase-DNA complex and interact with proteins that seal the portal for preventing DNA loss and reopen it for ejection of the DNA into the nucleus of the host (21). These different functions lead to the suggestion that the portal protein must be comprised of multiple domains and has the ability to change its conformation.

Beside the portal the enzymes involved in the packaging process are responsible for site-specific duplex nicking and insertion of the DNA into the procapsids (5, 13), the so-called terminases. The human cytomegalovirus (HCMV) terminase is composed of two subunits, the large one encoding pUL56 and the small pUL89 (7, 8, 9); each protein has a different function. Whereas the large subunit mediates sequence specific DNA binding and ATP hydrolysis, pUL89 is only required for duplex nicking (23, 36, 37). The hydrolysis of ATP has multiple functions during the packaging process. It is also involved in the formation of the packaging complex. The anti-HCMV benzimidazole-D-nucleosides, BDCRB and TCRB, developed in Townsend's and Drach's laboratories (43) target the HCMV terminase. With Biron and coworkers, they showed for the first time that the HCMV maturation was inhibited by these compounds (27, 44). By marker transfer experiments, mutations in UL89 (44) as well as UL56 (26) were found.

Unlike ATP hydrolyzing terminase subunits of most other icosahedral virions, e.g., dsDNA bacteriophages and herpes simplex virus type 1 (HSV-1), the large subunit pUL56 is stably associated with the capsid and represents a structural component (4, 14, 38, 47). Only one terminase protein, P9 of bacteriophage PRD1, is also a structural component of the virion (31, 42). Recently, we showed by three-dimensional reconstruction that pUL56 forms a dimer with a C-2 symmetry (35).

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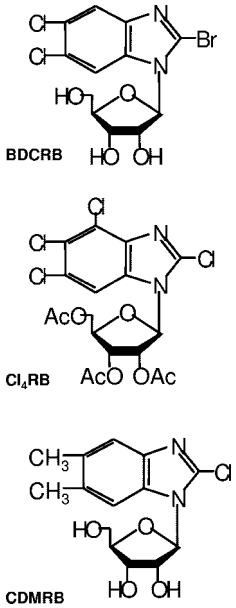

FIG. 1. Chemical structures of BDCRB, Cl_4RB, and CDMRB.

This structure is the prerequisite for the formation of a protein-DNA complex required for packaging into the procapsid.

The first herpesvirus portal protein encoded by open reading frame (ORF) UL6 of HSV-1 (11, 29, 34) was identified by Newcomb et al. (32). Recently, we could demonstrate that the HCMV ORF UL104 encodes a protein with an M_r of approximately 73,000 with a tendency to dimerize. Furthermore, pUL104 has the ability to bind sequence-independent to double-stranded DNA. Under native conditions pUL104 assembles into high-molecular-weight complexes (15). In this study, experiments are described concerning the colocalization of pUL104 with packaging proteins and a direct physical interaction regarding the HCMV large terminase subunit pUL56 and its inhibition by benzimidazole-D-ribonucleosides.

MATERIALS AND METHODS

Cells and viruses. Human foreskin fibroblasts (HFF cells) were grown in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum, 2 mM glutamine, penicillin (5 U/ml), and streptomycin (50 μg/ml). Preparation of HCMV AD169 and infection of HFF with HCMV at a multiplicity of infection (MOI) of 1 was carried out as described before (10). Inhibition of the viral polymerase was achieved by addition of phosphonoacetic acid (PAA; 200 μg/ml) during the entire infection.

Benzimidazole nucleosides. 2-Bromo-5,6-dichloro-(1-β-D-ribofuranosyl)benzimidazole (BDCRB); 2,4,5,6-tetrachloro-1-(2,3,5-tri-O-acetyl-β-D-ribofuranosyl)benzimidazole (Cl_4RB) and 2-chloro-5,6-dimethyl-(1-β-D-ribofuranosyl)benzimidazole (CDMRB) were synthesized in the laboratory of L. B. Townsend (Fig. 1).

Pulsed-field gel electrophoresis. HFF cells were seeded in 6-cm wells, infected at an MOI of 3, and treated without or with 100 μM Cl_4RB for 3 days. Cells were resuspended in 200 μl L buffer (100 mM EDTA, 10 mM Tris-HCl [pH 7.5], 20 mM NaCl), mixed with 200 μl 2% SeaPlaque GTG agarose (Bio-Rad) and 10 mg/ml proteinase K, and embedded in blocks. Lysation was performed by incubation of the agarose block in 10 ml lysis buffer (100 mM EDTA, 10 mM Tris-HCl [pH 7.5], 20 mM NaCl, 1% sarcosyl sodium, 100 μg/ml proteinase K) at 50°C overnight. Proteinase K was inactivated in 10 volumes of TE buffer (10 mM Tris-HCl, 10 mM EDTA, pH 8.0) and 1 mM phenylmethylsulfonyl fluoride for 2 h at room temperature. Agarose blocks were treated with 50 U restriction enzyme NotI at 37°C over night and equilibrated in 0.5× Tris-borate-EDTA buffer for 30 min. The blocks and MidRange I pulsed-field gel marker (New England Biolabs) were loaded into a 1% agarose gel, and parameters were as follows: 20-h run at 14°C, a 120° pulse angle, a voltage gradient of 6 V/cm, and a pulse time of 2 s with a linear ramp to 20 s. DNA was stained with 1 μg/ml ethidium bromide for 30 min and photographed. The photograph was scanned, and the colors were inverted by using Adobe Photoshop CS. By using the MidRange I marker, the size of the monomers was determined to be 230 kb.

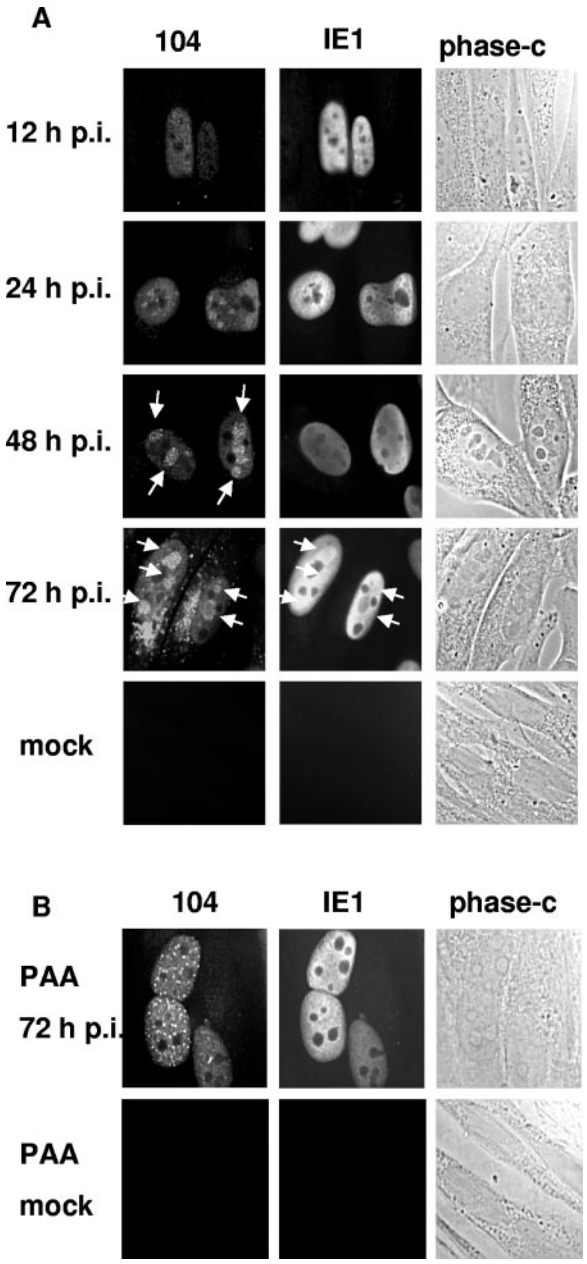

FIG. 2. Analysis of intracellular distribution of HCMV pUL104. (A) HFF cells were mock-infected or infected with HCMV strain AD169 for 12, 24, 48, and 72 h at an MOI of 1. After paraformaldehyde fixation, a consecutive immunofluorescence staining with antibody against pUL104, PAbUL104, and antibody against IE1, MAb63-27, was performed. (B) HFF cells infected with HCMV AD169 in the presence of 200 μg/ml PAA were 72 h p.i. fixed with 4% paraformaldehyde. Double-staining was performed with antibodies against pUL104 and IE1, respectively. As a control, phase contrast images were taken to visualize nuclei (phase-c).

Purification of GST fusion protein. A fresh overnight culture of *Escherichia coli* BL21 carrying the plasmid pGEX-6P-1-UL104 (15) was grown in 250 ml Luria-Bertani medium. After the cells reached an A_{600} of 0.6, glutathione *S*-transferase (GST) fusion protein expression was induced by the addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubation for 2 h at 32°C. Sedimented cells were lysed in phosphate-buffered saline containing lysozyme (10 mg/ml) by incubation on ice for 30 min. After cycled freezing/thawing, 0.5% Triton X-100 was added prior to incubation for 30 min at 4°C. After separation of undissolved material the proteins were loaded on a GSTrap column. The purification was performed at 4°C using an ÄKTA Prime (Amersham Biosciences) according to the instruction of the manufacturer. The isolated GST-104 was stored at −80°C prior to covalent binding to Affi-Gel.

In vitro translation. The plasmids encoding for pUL104, pcDNA-UL104 (0.5 μg), and luciferase (0.5 μg) were incubated with [^{35}S]methionine (10 mCi/ml) and 20 μl TNTT7 Quick Master Mix (Promega) in a final volume of 30 μl for 1 h at 30°C. Translation products were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE).

In vitro binding assay. The fusion protein pGEX-UL56C (amino acids [aa] 404 through 850) (20) and pGEX-UL89 (23) were expressed in *E. coli* strain BL21, and protein purification was carried out according to the instructions of the manufacturer (Amersham Bioscience). Equal amounts of solutions containing GST-UL56C, GST-UL89 fusion proteins, or GST alone were loaded on 50 μl of 50% glutathione Sepharose 4B beads (binding capacity, 250 μg of GST; Amersham Bioscience) prior to incubation with 20 μl of in vitro translated pUL104 or the luciferase for 2 h at 4°C in 500 μl binding buffer (0.05% Nonidet P-40, 50 mM HEPES [pH 7.3], 10% glycerol, 0.1% bovine serum albumin, 300 mM NaCl). Samples were washed with binding buffer and subsequently subjected to SDS–PAGE (10% polyacrylamide gel), fixation, and autoradiography.

Antibody against pUL104. HCMV pUL104-specific human polyclonal antibody UL104 (PAbUL104) was purified from high-titer human serum by column affinity chromatography (Affigel 10/15-pUL104) as described previously (15).

Immunofluorescence and immunoprecipitation. For immunofluorescence cells were grown on coverslips. At the appropriate time point cells were fixed with 3% paraformaldehyde as described previously (41).

AD169-infected cells were stained with a polyclonal human anti-pUL104 antibody, PAbUL104 (1:10), and Cy3-labeled anti-human F(ab)$_2$ fragments as secondary antibody. For coimmunofluorescence, staining of pUL104 was followed with pUL56-specific human PAbUL56 (1:10 [19]), monoclonal antibody M23 (MAbM23) against the gene products of HCMV pUL112-113 (provided by K. Radsak, Marburg, Germany [46]), MAb28-4 against the major capsid protein (MCP) (1:10, provided by W. Britt, Birmingham, AL [28]), or MAb63-27 against IE1 (1:10, provided by T. Stamminger [2]). After 45-min incubation with the primary antibodies, the coverslips were washed prior to treatment with Cy2-conjugated anti-mouse F(ab)$_2$ fragments and diluted 1:500 (Dianova). In case of PAbUL56, to avoid cross-linking, unconjugated goat anti-human F(ab)$_2$ fragments followed by fluorescein isothiocyanate (FITC)-conjugated anti-goat F(ab)$_2$ fragments were used. The samples were mounted in Fluoroprep (bioMerieux, France) with 2.5% (wt/vol) 1,4-diazabicyclo[2.2.2]octan and examined by laser scanning confocal microscopy using a Zeiss LSM 410 (Carl Zeiss, Oberkochen, Germany).

For coimmunoprecipitation, HFF cells were infected in the absence or presence of 100 μM BDCRB, CDMRB, or Cl_4RB. Total cell extracts were prepared 72 h postinfection (p.i.) by solubilization in immunoprecipitation buffer (20 mM Tris-HCl [pH 7.5], 100 mM NaCl, 1% NP-40, 5 mM EDTA, 25 mM iodacetamide, 0.4% Na-deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 100 U/ml Trasylol) containing the protease inhibitor mix M (Serva) and ultrasonic treatment. Insoluble material was sedimented for 30 min at 100,000 × *g*. Comparable amounts of extracts and pUL56-specific antibody (1:10) were used for precipitation as described previously (10).

Western blotting. Coimmunoprecipitates were separated on an 8% (wt/vol) polyacrylamide gel and transferred to nitrocellulose sheets and subjected to Western blot analysis as described previously (20). The PAbUL104 antibody (1:10) (15) specific for pUL104 was used as the primary antibody prior to incubation with horseradish peroxidase-conjugated anti-human F(ab′)$_2$ fragments (1:5,000 in 3% bovine serum albumin).

Thin sections. HFF cells were infected with HCMV at a multiplicity of infection of 1. Three days postinfection, cells were fixed with harvesting buffer (20 mM HEPES, pH 7.4) containing 4% paraformaldehyde, 2.5% glutaraldehyde, and 1% tannin according to the method of Gelderblom et al. (18). After dehydration and poststaining with 0.2% (wt/vol) uranyl acetate the specimen was embedded in glycid ether 100 (Carl Roth) with 1.5% (wt/vol) 1-methyl-5-norbornene-2,3-dicarboxylic acid anhydride, methylnadic anhydride (MNA; Carl Roth). Poly-

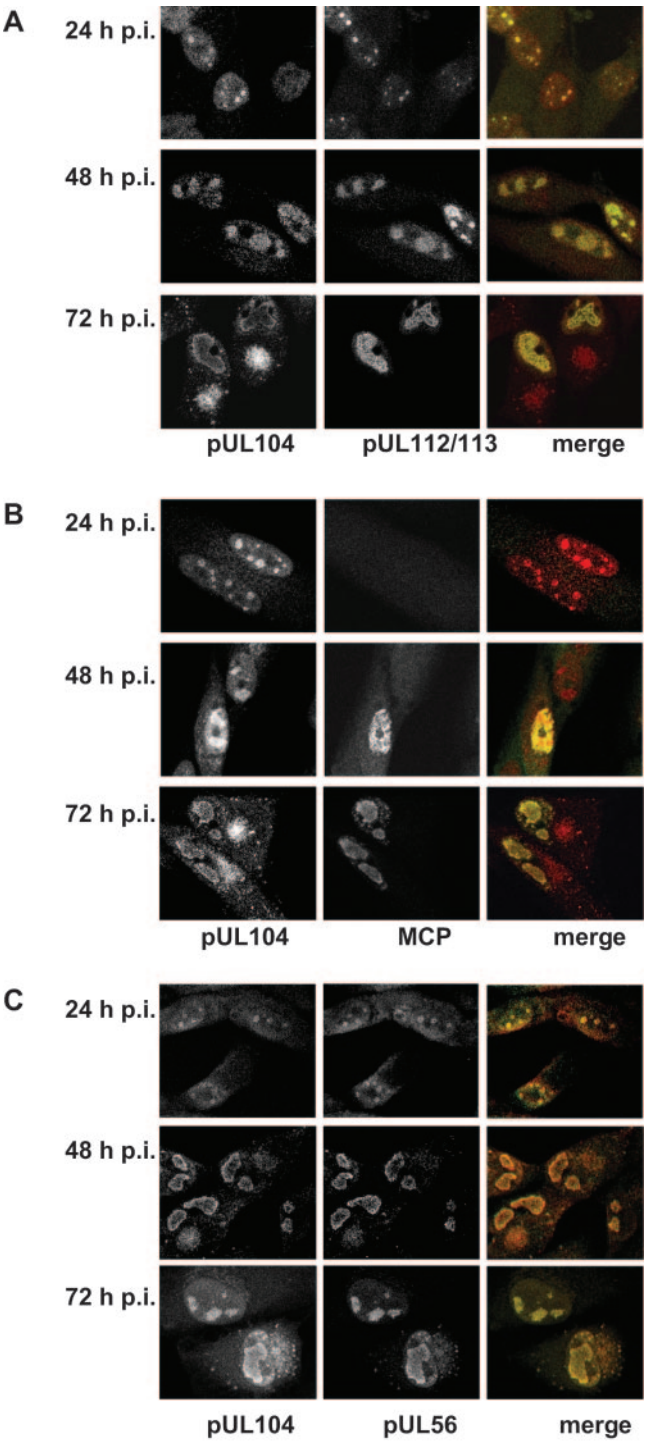

FIG. 3. Confocal microscopy analysis of HCMV pUL104 co-localization with packaging proteins. HFF cells mock infected or infected for 24, 48, and 72 h with HCMV AD169 at an MOI of 1 were double stained with PAbUL104 and MAbM23 against the pUL112-113 gene products (A), MAb28-4 against MCP (B) or PAbUL56 against the terminase subunit pUL56 (C). After paraformaldehyde fixation consecutive immunofluorescence staining was performed. The images represent the central section of the analyzed cells by laser scanning confocal microscopy. A merge of the Cy3- and Cy5-stained cells is shown in addition.

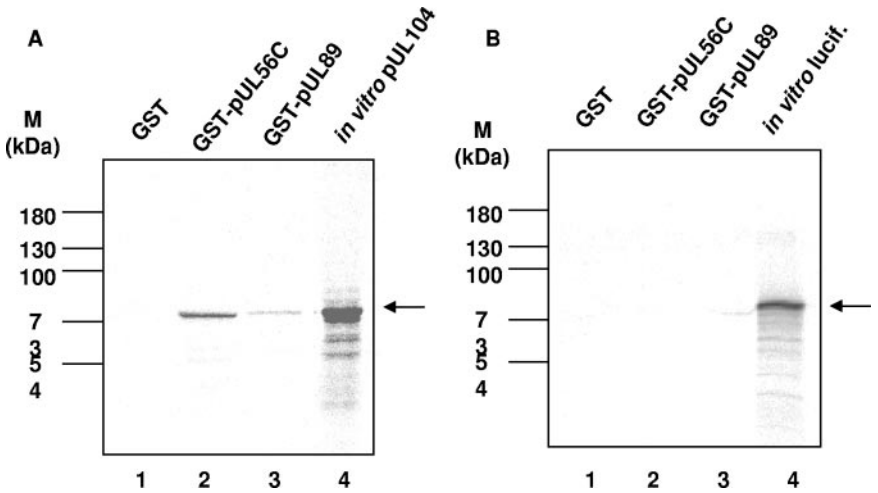

FIG. 4. Binding of pUL104 to an immobilized carboxy-terminal portion of pUL56 (aa 404 through 850). Glutathione-Sepharose 4B beads loaded with GST, GST-UL56C or GST-UL89 were incubated with in vitro translated UL104 (in vitro UL104) or luciferase (in vitro luciferase). The bound material was subjected to SDS–10% PAGE prior to autoradiography. The arrows indicate the positions of pUL104 and luciferase, the molecular weight markers (M) are shown on the left.

merization was performed at 60°C for several days prior to sectioning with ultracut S (Reichert-Jung). The sections were transferred to slot grids coated with pioloform (Plano, Wetzlar, Germany), stained for 10 min with 1% (wt/vol) uranyl acetate in 40% EtOH followed by lead citrate staining for an additional 10 min prior to analysis by electron microscopy.

RESULTS

Identification of pUL104 in infected cells. In order to examine intracellular distribution of pUL104 during viral infection coverslip HFF cultures were mock infected or infected with HCMV AD169 for 12, 24, 48, and 72 h, immunofluorescence was detected with the PAbUL104 antibody and with an antibody against the IE1 protein, MAb63-27. In HCMV-infected cells PabUL104 detected antigen predominantly in the nucleus. At 24 h p.i., a diffuse nuclear staining was observed but the protein was also beginning to aggregate in ring- or circle-shaped structures (Fig. 2). At 48 h p.i., in addition to numerous ring structures, pUL104 could be found in enlarged clusters as well (Fig. 2). Late in infection (72 h p.i.), pUL104 also either appeared in the cell cytoplasm as perinuclear clusters or was distributed over the cytoplasm in small dots (Fig. 2). IE1 antigen was restricted to the nucleus, as expected (Fig. 2, IE1). Mock-infected HFF cells revealed no specific immunofluorescence signals (Fig. 2). Phase contrast images were taken in order to visualize nuclei, respectively. These observations showed that pUL104 is translocated into the nucleus as well as late times of infection to the cytoplasm.

Co-localization of pUL104 with packaging proteins. In order to identify an interaction with proteins known to be involved in DNA replication, double staining using MAb M23 against the replication center proteins pUL112-113, MAb28-4 against the MCP, and PAbUL56 against the terminase subunit pUL56 was undertaken. HFF cells were mock infected or infected with HCMV AD169 and immunofluorescence was performed at 24, 48, and 72 h postinfection. At early times after infection pUL112-113 was already found in prereplication centers while pUL104 still showed a diffuse distribution and an outer rim staining of the prereplication centers as can be seen 24 h p.i. The distribution of pUL112-113 changed after 48 h p.i. to a distinct staining of replication centers, where it colocalized with pUL104 (Fig. 3A). Only at late times after infection (72 h p.i.) were MCP and pUL104 observed together in large intranuclear patches, representing replications centers (Fig. 3B). In infected cells, pUL104-specific immunofluorescence colocalized with that of pUL56 at all observed time points, which indicates a close association of both proteins (Fig. 3C). These results demonstrated that the putative HCMV portal protein colocalizes with the terminase subunit pUL56 and the major capsid protein and also localizes to the replication centers.

Interaction of pUL104 with the large terminase subunit pUL56. To examine a possible interaction between pUL104 and pUL56 GST pull down assays were carried out using GST fusion proteins. To this end, the GST-UL56C containing the carboxy-terminal portion of UL56 (aa 404 through 850), GST-UL89 or GST itself was immobilized on glutathione-Sepharose beads and incubated either with in vitro-translated [^{35}S]methionine-labeled pUL104 (in vitro UL104) (Fig. 4A, lane 4) or [^{35}S]methionine-labeled luciferase (in vitro luciferase) (Fig. 4B, lane 4). The amount of bound material was analyzed by SDS–10% PAGE prior to autoradiography. As shown in Fig. 4, pUL104 interacted specifically with GST-UL56C (Fig. 4A, lane 2) but not with GST alone (Fig. 4A, lane 1). In addition, almost no interaction was observed with GST-UL89 (Fig. 4A, lane 3). In vitro translated luciferase, used as a negative control, did neither interact with GST-UL56C nor GST (Fig. 4B, lanes 1 and 2). These experiments demonstrated for the first time an interaction of pUL104 with pUL56.

Influence of benzimidazole ribonucleosides on the interaction of pUL104 with pUL56. To further investigate a direct interaction between pUL104 and pUL56 coimmunoprecipitation prior to Western blot analysis using PAbUL104 was performed. In precipitations with PAbUL56, a 145-kDa dimer of pUL104 was observed (Fig. 5A, lane 2). pUL104 also was detected using immunocomplexes in HCMV-infected cell ex-

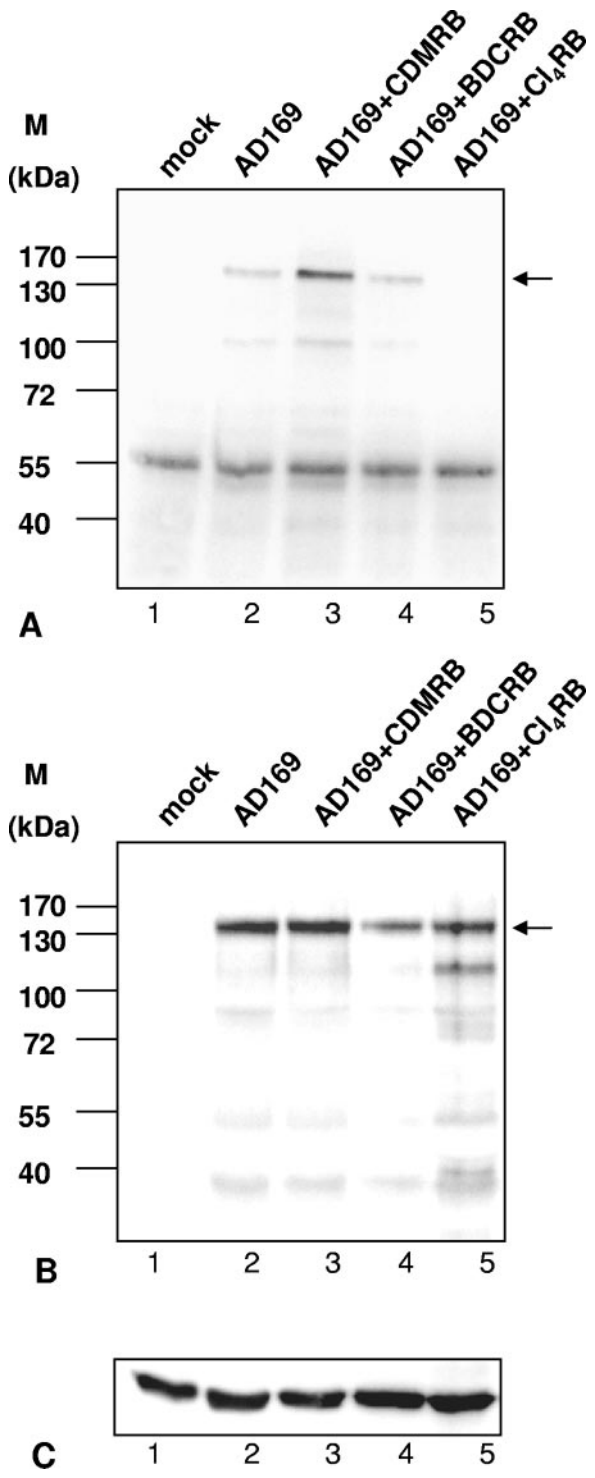

FIG. 5. Direct interaction of pUL104 with the terminase subunit pUL56 and inhibition by benzimidazole ribonucleosides. (A) Mock-infected (lane 1) or HCMV AD169 infected at an MOI of 1 in the absence (lane 2) or in the presence of 100 μM CDMRB (lane 3), 100 μM BDCRB (lane 4), or 100 μM Cl_4RB (lane 5). HFF cells were coimmunoprecipitated with PAbUL56 at 72 h p.i. and subjected to SDS–8% PAGE. The separated probes were electrotransferred onto nitrocellulose prior to immunoblot analysis using the antibody PAbUL104. (B) Cell extracts from the identical probes of A were separated on an SDS–8% PAGE gel. After transfer onto nitrocellulose immunoblot analysis were performed by using the antibody PAbUL104. (C) As a loading control the immunoblot was consecutively stained with an antibody against actin. The arrows indicate the position of pUL104, the molecular weight markers (M) are shown on the left.

tracts (Fig. 5B, lane 2). In mock-infected cell extracts as well as in precipitates, no specific proteins were detected (Fig. 5A and B, lanes 1). These experiments showed a physical interaction of the portal protein with the large terminase subunit pUL56.

In addition, experiments were carried out in the presence of 100 μM BDCRB or Cl_4RB to analyze whether these drugs had an influence on the essential binding of portal with the large terminase subunit. The amount of proteins in all samples was adjusted by using actin (Fig. 5C). A control benzimidazole-D-ribonucleoside which is inactive against HCMV replication—the 5,6-dimethyl-analog of TCRB (Fig. 5A and 5B, lanes 3, CDMRB)—had no inhibitory effect on the interaction. In contrast, Cl_4RB blocked the binding (Fig. 5A and 5B, lanes 5) as did BDCRB (lanes 4). The effect of BDCRB was less apparent when measured by antibody to pUL56 (Fig. 5A) compared to pUL104 antibody (Fig. 5B). It also appeared to be less effective than Cl_4RB in its effect measured by pUL56 antibody. Interestingly, this effect is consistent with the higher potency of Cl_4RB against HCMV replication compared to BDCRB. These observations indicate that one effect of benzimidazole-D-ribonucleosides as a specific HCMV inhibitor is mediated by preventing the binding between pUL104 and the ATPase pUL56.

Effect of Cl_4RB on cells. To determine the effect of the compound Cl_4RB on viral maturation electron microscopy of thin sections was performed and nuclear particles were measured. HFF cells were infected at an MOI of 1, incubated for 3 days in the absence or presence of 100 μM Cl_4RB, fixed, and embedded. In infected cells treated with the compound, DNA packaging was blocked and only B capsids were formed (Fig. 6B), whereas in untreated cells all forms of capsids, were observed (Fig. 6A). In the latter 5.9% A, 51% B, and 43% C capsids were found, whereas in the treated cells, over 90% were B capsids (Table 1). These result demonstrated that Cl_4RB like BDCRB prevents the formation of replicative structures.

Analysis of DNA processing. To analyze whether Cl_4RB has the ability to inhibit the cleavage of concatemeric, viral DNA into unit length genomes intracellular DNA was separated by pulsed-field electrophoresis and stained with ethidium bromide. DNA of AD169-infected cells was cleaved into unit length genomes in the absence or presence of restriction endonuclease NotI treatment that cleaves monomers from the concatemer (Fig. 7, w/o). In contrast, cells infected in the presence of Cl_4RB were unable to cleave viral DNA in the absence of the endonuclease (Fig. 7, Cl_4RB), thus indicating that this benzimidazole ribonucleoside also targets DNA processing.

DISCUSSION

The mechanism which results in translocation of viral DNA into empty procapsids is still not understood. However, it is

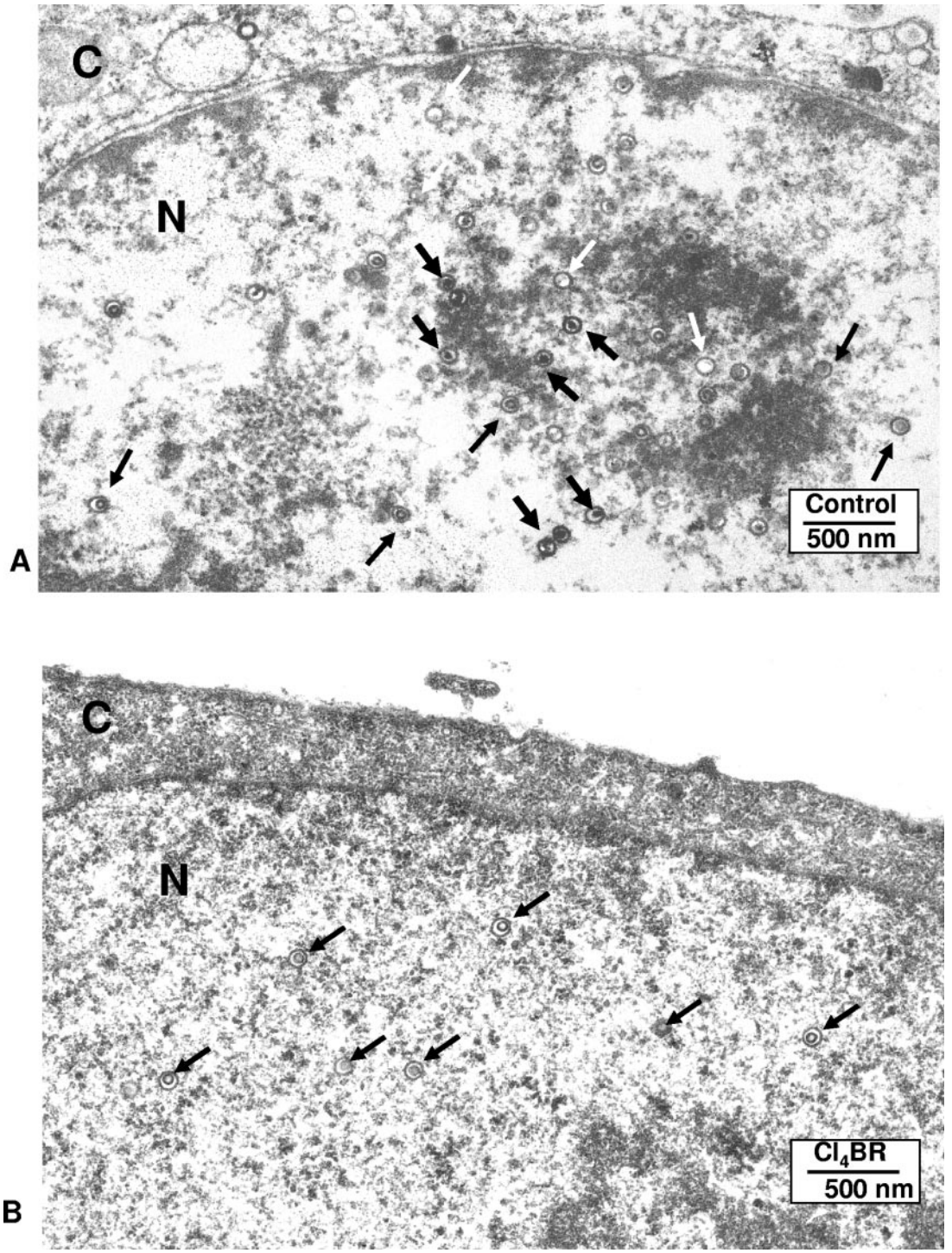

FIG. 6. Electron microscopy analysis of ultrathin section of HCMV infected cells in the absence (A) or presence of 100 μM Cl_4RB (B). After 3 days, cells were fixed in 4% paraformaldehyde and 2.5% glutaraldehyde with 1% tannin and prepared for electron microscopy. B capsids are indicated by black arrows, A capsids by white arrows, C capsids by bold arrows. N indicates the nucleus, C the cytoplasm.

known that this complex process requires a concerted interaction of the terminase-DNA complex with the portal protein located on one unique vertex of the procapsids. In addition to the already known components of this process, the capsid proteins and the viral terminase, the HCMV portal protein has not been characterized thus far. In this study, experiments were undertaken aimed to identify the intracellular localization, the expression in the infectious cycle and the interaction with packaging proteins of the putative portal protein encoded by ORF UL104.

TABLE 1. Nuclear capsids of infected cell in the absence or presence of Cl_4RB

Virus	No. of nuclei counted	No. of capsids formed per nucleus (%)		
		A	B	C
AD169	10	4 (5.9)	34.5 (51)	29 (43)
AD169+Cl_4RB	10	0.3 (2.4)	12.1 (97.6)	0

Using immunofluorescence analysis of infected cells, homogeneous nuclear staining with increased antigen accumulation in ring-like structures was obtained with antibody PAbUL104 at early times of infection (24 h p.i), suggesting that pUL104 is recruited to the outer rim of the prereplication centers before its involvement in DNA packaging. At later times (72 h p.i.), pUL104-specific signals also were concentrated in discrete cytoplasmic structures. It is reasonable to suggest that the latter localization may represent intracellular compartments (TGN or early endosomes) for final envelopment during viral maturation (30). Studies in the presence of PAA demonstrated that this cytoplasmic localization was dependent on viral DNA replication.

Yamamoto et al. (46) showed by use of antisense strategy against UL112-113 that the gene products as well as viral DNA replication were concomitantly affected. Furthermore, Anders and McCue (1) reported that UL112-113 products form prereplication structures, which change upon viral DNA replication to replication compartments. Our observations by immunofluorescence that the pattern changed starting with small dots to clusters are in line with this report. The partial colocalization with pUL104 demonstrated that (i) pUL104 is translocated in viral replication centers and that (ii) this localization is independent of pUL112-113.

Furthermore, we observed a physical interaction of pUL104 with the large terminase subunit pUL56 by coimmunoprecipitations. This result implied that a stable interaction of the terminase subunit with the putative portal is required for the packaging process in vivo. Beside the fact that translocation of DNA into an empty capsid is a poorly understood mechanism, it is a common agreement that interaction of the terminase with the portal protein is a prerequisite for this process. Interestingly, a direct interaction of the HSV-1 homologues pUL6 and pUL28 as well as pUL15 has been described recently (45). The insertion of a DNA molecule through the portal depends on ATP, but the portal protein itself can not hydrolyze ATP, thus leading to the presumption that the ATPase activity of pUL56 is important for the insertion of DNA into the capsid. Recently, a report from Komazin et al. (25) mapped a BDCRB-resistant mutant in pUL104, however this phenotype could not be found after insertion of the mutation into the HCMV genome. The authors hypothesized that the pUL104 mutation could compensate a conformational change in pUL56. Interestingly, genetic mapping of drug resistance with the compound BAY 38-4766 also identified a mutation in MCMV ORF 104 (12). Their findings are similar to the one from Komazin et al., because this mutant by itself has no effect on the development of resistance. In the current report we demonstrated that the interaction of pUL104 with pUL56 could be specifically inhibited by the benzimidazole-D-ribonucleosides BDCRB and Cl_4RB, but not by the virologically inactive, control dimethyl benzimidazole analog (16, 48). Since we saw an additional band in Fig. 5B, lane 5, we could not exclude the possibility that some amount of pUL104 was degraded in the presence of Cl_4RB. However, we hypothesize that the remaining pUL104 should be enough for an interaction in comparison with the one in the presence of BDCRB. Therefore it is reasonable to suggest that the effect is due to the inhibition of the pUL104-pUL56 interaction. By electron microscopy we demonstrated that Cl_4RB had a similar effect on the block of capsid maturation as other benzimidazole-D-ribonucleosides, because only B capsids were found in the nucleus of infected cells in the presence of the inhibitor. Furthermore, Cl_4RB inhibited viral DNA cleavage like BDCRB, TCRB (44) or 5′-deoxy-TCRB (27). Thus our results indicate that the phenotypic features caused by the inhibitors are due to direct interaction of pUL56 with the compound.

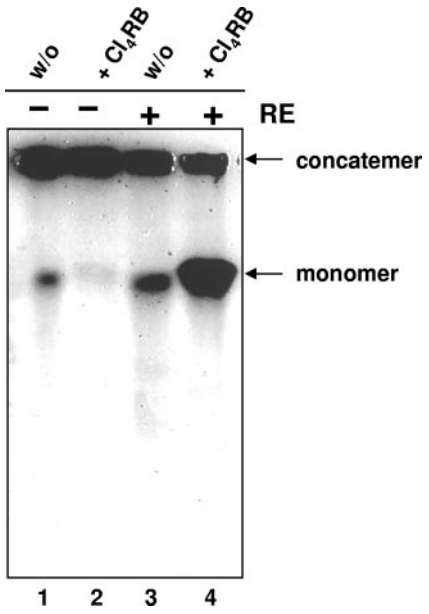

FIG. 7. Pulsed-field electrophoresis analysis of infected cells in the presence of Cl_4RB. HFF cells were infected with an MOI of 3 in the absence (w/o) or presence of 100 μM Cl_4RB. 72 h p.i. cells were embedded in agarose and digested with proteinase K and incubated with or without 50 U restriction endonuclease NotI. DNA was resolved by pulsed-field gel electrophoreses, stained with ethidium bromide, and photographed. Concatemers and unit length monomers are indicated.

In conclusion, the data from the present study demonstrated that (i) pUL104 directly interacts with the terminase subunit pUL56 and (ii) this interaction can be blocked by the new benzimidazole-D-nucleosides.

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