Human Cytomegalovirus UL36 Protein Is Dispensable for Viral Replication in Cultured Cells

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Consistent with earlier analyses of human cytomegalovirus UL36 mRNA, we find that the UL36 protein is present throughout infection. In fact, it is delivered to the infected cell as a constituent of the virion. Curiously, much less UL36 protein accumulated in cells infected with the AD169 strain of human cytomegalovirus than in cells infected with the Towne or Toledo strain, and localization of the protein in cells infected with AD169 is strikingly different from that in cell infected with the Towne or Toledo strain. The variation in steady-state level of the proteins results from different stabilities of the proteins. The UL36 proteins from the three viral strains differ by several amino acid substitutions. However, this variability is not responsible for the different half-lives because the AD169 and Towne proteins, which exhibit very different half-lives within infected cells, exhibit the same half-life when introduced into uninfected cells by transfection with expression plasmids. We demonstrate that the UL36 protein is nonessential for growth in cultured cells, and we propose that the ability of the virus to replicate in the absence of UL36 function likely explains the striking strain-specific variation in the half-life and intracellular localization of the protein.

MATERIALS AND METHODS

Biological reagents. Primary human foreskin fibroblasts (HFFs) between passages 6 and 12 were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum. Wild-type HCMV strains AD169 (14), Towne (12), and Toledo (13) were used in this study. To construct the AD169 UL36-deficient derivative, ADΔUL36, the HindIII fragments of cosmid pCM1015 (6) were subcloned into pGEM-7Zf(−) (Promega), and then the HindIII-J clone was further subcloned by ligation of the 5-kb fragment generated by BamHI cleavage into pGEM. The resulting clone was digested with Sall; the ends were blunted with the Klenow fragment of DNA polymerase I and then treated with phosphatase. This DNA was joined using ligase to a blunt-ended DNA fragment containing the coding regions for the green fluorescent protein (GFP) and a puromycin resistance marker that were separated by an internal ribosomal entry site. The resulting plasmid contains the HCMV DNA sequences flanking the UL36 coding region surrounding the marker cassette that is situated in a reverse orientation compared to the direction of transcription of the UL36-38 locus. The marker cassette surrounded by UL36 flanking sequences was removed from the plasmid by digestion with PstI plus XbaI and then transfected into HFFs together with AD169 virion DNA plus a pp71 expression vector (2). The transfected cells were plated, puromycin was added 24 h later, and after an additional 24 h during which puromycin-sensitive cells were killed, the drug was removed and fresh fibroblasts were added to regenerate confluent cultures. GFP-positive plaques were isolated, and virus was prepared and used to infect fresh HFF cultures. After three cycles of puromycin selection followed by isolation of GFP-positive plaques, pure clones of mutant virus were obtained, and the structure of the mutant DNA was confirmed by Southern blot assay. To produce virus stocks, HFFs were infected at a multiplicity of 0.01 PFU/cell with mutant or wild-type viruses. After 5 to 7

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days, when the cells showed full cytopathic effect, the medium was harvested and cellular debris was removed by centrifugation at 5,000 x g for 20 min at 4°C. The supernatants were stored at −80°C and served as virus stocks which were titered by plaque assay on HFF. Virus particles were purified by centrifugation through a sorbitol cushion as previously described (15).

Derivatives of pCGN, which fuses a nine-amino-acid epitope derived from the influenza virus hemagglutinin protein to the amino terminus of proteins expressed under control of the HCMV major immediate-early promoter (16), were constructed by inserting PCR-amplified UL36-specific cDNA sequences. The PCR primers incorporated KpnI or BamHI restriction endonuclease cleavage sites into the 5’ and 3’ ends, respectively, of the amplified UL36-specific DNA fragment, facilitating its incorporation into pCGN.

To produce a puUL36-specific monoclonal antibody, the carbonyl-terminal 196-amino-acid coding region of UL36 was cloned into the pGTV bacterial expression vector, and it produced a 45-kDa glutathione S-transferase fusion protein. After purification, the soluble fusion protein was used to immunize mice. Anti-body 5G11, which specifically recognizes the 52-kDa puUL36, was generated. The IE1-specific 1B12 monoclonal antibody was used as previously described (18), and anti-hemagglutinin tag mouse monoclonal 12CA5 (8) was used to visualize epitope-tagged proteins.

Transfections. HFFs were grown to approximately 90% confluence, released from culture plates by trypsinization, and washed twice with DMEM containing 10% fetal calf serum. The washed cells were resuspended at a concentration of 10⁵ cells/ml in DMEM containing 10% fetal calf serum, and 0.4 ml was added to a electroporation cuvette (4.0-mm electrode gap). Viral (2 μg) and/or plasmid (1 to 15 μg) DNAs were added to the cell suspension and mixed thoroughly. DNA was electroporated into cells at (200 V and 960 μF) in a Gene Pulser (Bio-Rad).

Analysis of gene structure and expression. For Western blot, cell extracts were prepared by adding buffer containing 100 mM Tris (pH 6.8), 10 mM EDTA, 4% sodium dodecyl sulfate (SDS), 40% glycerol, 5% β-mercaptoethanol, and 0.015% bromophenol blue directly to cells in tissue culture dishes. After electrophoresis in an SDS-containing 8% polyacrylamide gel, proteins were transferred to an Immobilon-P membrane (Millipore) and blocked with 10% dry milk in PBS (phosphate-buffered saline [PBS] containing 0.1% Triton X-100 and 0.05% Tween 20). The immobilized proteins were reacted sequentially with primary mouse monoclonal antibody and secondary horseradish peroxidase-conjugated goat anti-mouse antibody (Amersham). The Amersham ECL (enhanced chemiluminescence) detection system was then used for visualization of protein bands.

For Northern analysis, total cellular RNA was isolated by using the TRIzol Reagent (Gibco-BRL); then 5-μg aliquots of RNA were resolved by electrophoresis in a 1% formaldehyde-agarose gel, transferred to a Nytran membrane, and cross-linked to the membrane with UV light. RNA phoresis in a 1% formaldehyde-agarose gel, transferred to a Nytran membrane aliquots of RNA were resolved by electrophoresis in a 1% formaldehyde-agarose gel, transferred to a Nytran membrane and cross-linked to the membrane with UV light.

RESULTS

Marked differences in UL36 expression and localization among HCMV strains. A puUL36-specific monoclonal antibody was produced by using the C-terminal 196-amino-acid segment of the protein as an immunogen, and it was used to characterize the expression of puUL36 in cells infected with three strains of HCMV. AD169 (14) and Towne (12) are laboratory-adapted strains, and Toledo (13) is a clinical isolate that has been passaged in cultured cells to a minimal extent. These viruses were used to infect HFFs at a multiplicity of 3 PFU/cell, and extracts were prepared for Western analysis at various times after infection. Although the three strains of virus were used to infect cells at the same input multiplicity, puUL36 accumulated to markedly lower levels in AD169-infected cells than in cells receiving the Towne or Toledo strain (Fig. 1A). This was not the case for all virus-encoded proteins; AD169 directed the accumulation of the same quantity of IE1 protein as did the other two HCMV strains (Fig. 1B). The puUL36-specific antibody recognized a doublet of proteins migrating at the predicted molecular mass for the viral protein, 52 kDa. The doublet was evident in cells infected with the Towne and Toledo strains (Fig. 1A), and it was also seen in the AD169-infected cell sample when longer exposures of the autoradiogram were examined (data not shown). Given their size and the fact that both bands are lost when a mutant virus lacking the UL36 gene is tested (see below), we conclude that both bands contain puUL36. The doublet could result from post-translational modification of the protein. For all three HCMV strains, puUL36 was evident at 4 h after infection, the earliest time assayed (Fig. 1A), consistent with the earlier demonstration that its mRNA can be detected during the immediate-early phase of AD169 infection (9, 17).

We also examined the intracellular localization of puUL36 by using the monoclonal antibody (Fig. 2). HFFs were assayed at 24 h after infection or mock infection. Little fluorescent signal was evident in mock-infected cells, and AD169-infected cells displayed uniform nuclear as well as cytoplasmic staining with occasional intense fluorescent spots in the cytoplasm. In marked contrast, Towne-infected cells exhibited significantly less nuclear fluorescence. Rather, the cytoplasm of Towne-infected cells contained intensely fluorescent spots and worm-shaped structures. The fluorescent analysis was repeated at
various times after infection (data not shown). Towne does not exhibit a localization similar to that observed for AD169 during an earlier phase of infection, and AD169-infected cells do not eventually accumulate the worm-like structures observed for Towne. The Toledo strain was also tested, and it exhibited a fluorescent pattern similar to that seen for Towne (data not shown). We have attempted to colocalize this structure with several organelle markers. The pUL36 Towne structure does not colocalize with the Golgi apparatus, lysosomes, mitochondria, recycling and early endosomes, endoplasmic reticulum, or death effector filaments (data not shown). Consistent with these findings, treatment of Towne-infected cells with brefeldin A does not change the pUL36 structure (data not shown).

Different half-lives for pUL36 in AD169-infected compared to Towne-infected cells. To ascertain whether the HCMV strain-specific differences in the level of pUL36 might result from differences in mRNA accumulation, we performed a Northern blot analysis. The three virus strains produced similar amounts of UL36 mRNA at 24 h after infection (Fig. 3A), a time at which the protein encoded by the mRNA has accumulated to much lower levels in AD169-infected cells than in Towne- or Toledo-infected cells (Fig. 1A). Duplicate RNA samples were also probed for a cellular mRNA (phospholipase A2 [19]) as a loading control and for IE1 mRNA to monitor the virus infection (Fig. 3A).

Finding no difference in mRNA accumulation, we next examined the stability of pUL36 in infected cells with the AD169 and Towne strains of HCMV. A pulse-chase experiment was performed at 24 h after infection of HFFs (Fig. 3B). 35S-labeled protein was immunoprecipitated and subjected to electrophoresis, and pUL36-specific radioactivity was quantified. The two pUL36-specific bands comprising the 52-kDa doublet exhibited the same stability. The half life for pUL36 in AD169-infected cells was 1.5 h. In contrast, the pUL36 half life was 13.5 h in Towne-infected cells. In a second independent experiment (data not shown), the half-lives of pUL36 were determined to be 1.5 and 10.5 h in cells infected with AD169 and Towne, respectively. Thus, the different steady-state levels of pUL36 result from differences in its stability.

Different half-lives are not intrinsic properties of pUL36 encoded by different strains of HCMV. UL36-specific cDNAs were prepared by reverse transcription and PCR amplification, and two independently produced clones for each strain were sequenced. The protein encoded by Towne has eight single amino acid substitutions compared to the AD169 protein, and the protein encoded by Toledo has five substitutions (Fig. 4). The Towne and Toledo proteins share three of the amino acid differences in comparison to AD169 pUL36, and it seemed possible that one or more of these substitutions is responsible for the altered stability and localization of pUL36 observed for Towne and Toledo versus AD169. To test this supposition, HFFs were transfected with plasmids expressing the pUL36 variants. Pulse-chase analysis revealed that pUL36 from
AD169 and Towne decayed at similar rates, exhibiting half-lives of 2.6 and 3.2 h, respectively (Fig. 5A). We also examined the localization of pUL36 variants in transfected cells by immunofluorescence, and both displayed uniform nuclear and cytoplasmic staining (Fig. 5B).

We conclude that the differences in half-life and localization for UL36 observed in cells infected with different strains of HCMV are not intrinsic properties of the proteins. The differences are observed only in infected cells; variations in stability and intracellular location require the activity of additional viral gene products.

UL36 is not essential for HCMV growth in cultured cells. One explanation for the marked variability in the half-life and localization of pUL36 in cells infected by different HCMV strains could be that the protein is not required and does not function during replication of the virus in cultured cells. To test this possibility, we constructed a mutant derivative of AD169. We replaced the UL36 coding region with a DNA segment expressing GFP and a puromycin resistance marker under control of the simian virus 40 early promoter to produce a mutant termed ADul36 (Fig. 6A).

Southern analysis of virion DNA cut with BamHI and KpnI confirmed that the UL36 coding region was replaced with the GFP-puromycin resistance cassette in the mutant virus. Wild-type and ADul36 DNAs were distinguished by two different probes. One probe, derived from the region adjacent to and containing the UL36 locus, identified the altered restriction enzyme-generated fragments in the mutant predicted for the substitution (Fig. 6B, left); the other probe, corresponding to the UL36 cDNA, detected a UL36-specific band in the digest for wild-type but not mutant DNA (Fig. 6B, right). The mutant virus was also tested for expression of pUL36 to be certain that a functional copy of the gene had not been inserted by an aberrant recombination event at a new location within the mutant genome (Fig. 6C). Whereas an extract prepared at 24 h after infection at a multiplicity of 3 PFU/cell with wild-type virus contained the protein, no pUL36 was detected in extracts of cells infected with two independent isolates of the mutant. The extracts from mutant-infected cells contained normal amounts of IE1 proteins, confirming that the cells were infected.

The mutant virus was successfully propagated without supplying UL36 function to generate virus stocks with infectious
titers similar to wild-type virus. To more carefully evaluate the growth characteristics of AD169 subUL36, HFFs were infected at an input multiplicity of 1, and the production of progeny virus was monitored at intervals for the next 9 days. The growth kinetics observed for mutant and wild-type viruses were indistinguishable (Fig. 7A), demonstrating that pUL36 is dispensable for growth of HCMV in HFFs. The accumulation of viral DNA was also monitored in cells infected with wild-type versus AD169 subUL36 (Fig. 7B), and no differences greater than twofold were observed. These results also were obtained for cells infected at a multiplicity of 0.1 or 0.01 (data not shown).

**DISCUSSION**

As predicted by earlier studies demonstrating that UL36 encodes an immediate-early mRNA (9, 17), pUL36 can be detected at 4 h after HCMV infection and then continues to be present in infected cells through the early and late phases of infection (Fig. 1A). The protein consistently migrates as a doublet in SDS-containing polyacrylamide gels (Fig. 1A and 6C). Since there is no evidence for alternatively spliced versions of UL36 mRNA and a cloned cDNA directs the synthesis of both species, it seems likely that the protein is posttranslationally modified, but we have not identified the nature of the presumptive modification. HCMV strain AD169-infected cells contain much less pUL36 than cells infected with the Towne or Toledo strain (Fig. 1A). The steady-state levels of the protein are different because the half-life of the protein is shorter in cells infected with AD169 compared to Towne (Fig. 3B). Even

![Diagram](image)

**FIG. 7.** Similar growth and viral DNA replication kinetics of AD169 (□) and ADsubUL36 (○). (A) Growth kinetics of wild-type and mutant virus on HFFs infected at a multiplicity of 1 PFU/ml. Cultures were harvested at various times after infection, and infectious virus was quantitated by plaque assay on HFFs. (B) Accumulation of wild-type and mutant DNA in HFFs infected at a multiplicity of 1 PFU/ml. Cells were harvested at various times after infection, and viral DNA was quantified by slot blot analysis using an HCMV-specific DNA probe.
though the primary sequence of pUL36 differs among HCMV strains (Fig. 4), the difference in stability is not an intrinsic property of pUL36. When pUL36 was introduced into cells by transfection of expression plasmids, the half-lives for the AD169 and Towne proteins were very similar (Fig. 5). The difference in half-life is evident only in infected cells. Possibly, the differences in the pUL36 sequence from various HCMV strains cause the protein to interact differently with another viral protein or with a cell protein that is induced or modified by viral infection. The differences in such an interaction could, in turn, modulate the stability of the protein. Alternatively, there might be a mutation at a second locus in the viral genome that causes another viral gene product to interact with pUL36 differently, irrespectively of the alterations that we have observed.

In addition to changes in half-life, there are significant differences in the localization of pUL36 within AD169 compared to Towne- or Toledo-infected cells (Fig. 2). We have not been able to colocate the striking worm-like structures evident in Towne-infected cells with known markers of the endoplasmic reticulum, Golgi apparatus, lysosomes, endosomes, mitochondria, or death effector filaments. We would guess that the localization is influenced by the same event that leads to altered half-lives, but we cannot be certain until the modulatory factor has been identified.

An HCMV mutant (Fig. 6) unable to express pUL36 proved to be viable, growing with the same kinetics as its wild-type parent (Fig. 7). This observation is consistent with an earlier report that the murine cytomegalovirus ie2 gene is dispensable for viral replication in cultured mouse cells (4), since the first exon of the HCMV UL36 coding region exhibits homology to the ie2 gene encoded by the murine virus. UL36 is a member of the UL22 gene family, a group of 13 HCMV coding regions predicted to encode proteins with several shared motifs (4). Conceivably, another member of the UL22 family performs the same or a similar function and substitutes for pUL36. Alternatively, pUL36 might execute a function that is needed for efficient viral replication within its infected host, the human, but not for replication in cultured cells. Whatever its role, pUL36 might begin to act immediately after infection before the viral genome becomes transcriptionally active since the protein is packaged in virions (Fig. 8).

The lack of an essential role for pUL36 in cultured cells might explain why the AD169-coded protein has a reduced half-life and altered localization compared to the protein in cells infected with the Towne or Toledo strain. Although we cannot be certain, we suspect that the longer half-life and predominantly cytoplasmic localization in worm-like structures seen for the Towne and Toledo strains represent the wild-type phenotype for UL36 since the Toledo strain has not been extensively passaged in the laboratory. We propose that one or more mutations have arisen in the AD169 strain as a consequence of genetic drift and are responsible for the altered pUL36 accumulation and localization.

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