Dissecting human cytomegalovirus gene function and capsid maturation by ribozyme targeting and electron cryomicroscopy

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Human CMV (HCMV) is the leading viral cause of birth defects and causes one of the most common opportunistic infections among transplant recipients and AIDS patients. Cleavage of internal scaffolding proteins by the viral protease (Pr) occurs during HCMV capsid assembly. To gain insight into the mechanism of HCMV capsid maturation and the roles of the Pr in viral replication, an RNase P ribozyme was engineered to target the Pr mRNA and down-regulate its expression by >99%, generating premature Pr-minus capsids. Furthermore, scaffolding protein processing and DNA encapsidation were inhibited by 99%, and viral growth was reduced by 10,000-fold. 3D structural comparison of the Pr-minus and wild-type B capsids by electron cryomicroscopy, at an unprecedented 12.5-Å resolution, unexpectedly revealed that the structures are identical in their overall shape and organization. However, the Pr-minus capsid contains tenuous connections between the scaffold and the capsid shell, whereas the wild-type B capsid has extra densities in its core that may represent the viral Pr. Our findings indicate that cleavage of the scaffolding protein is not associated with the morphological changes that occur during capsid maturation. Instead, the protease appears to be required for DNA encapsidation and the subsequent maturation steps leading to infectious progeny. These results therefore provide key insights into an essential step of HCMV infection using an RNase P ribozyme-based inhibition strategy.

RNase P | herpesvirus | antisense | catalytic RNA | structure

Human CMV (HCMV) is a member of the human herpesvirus family, which also includes herpes simplex virus 1 (HSV-1) and HSV-2, varicella-zoster virus, Epstein-Barr virus, and Kaposi's sarcoma-associated herpesvirus (1–4). The double-stranded DNA genome is encapsidated by an icosahedral protein capsid, which is surrounded by an amorphous layer of proteins called tegument. The entire particle is enclosed in a lipid bilayer envelope containing viral glycoproteins. The capsid is assembled initially with the formation of an internal protein scaffold, followed by proteolytic cleavage and removal of the scaffold and packaging of viral genome in the core (1–4).

HCMV is the leading viral cause of birth abnormalities (5) and causes one of the most common opportunistic infections encountered in AIDS patients and organ transplant recipients (6). Like other herpesviruses, HCMV virions contain multiple layers whose assembly requires several steps. In HCMV, UL80.5, which encodes preassembly/scaffold protein (preAP), overlaps with the 3' coding sequence of UL80 that codes for the protease (Pr). The UL80 product processes itself internally to release an N-terminal fragment, proteolytic protein (~30 kDa), NP1n, and a C-terminal fragment, NP1c protein, which overlaps with preAP (7). The Pr also cleaves preAP at its C-terminal region, which is shown to interact with the capsid shell (8), although this interaction has not been well defined in HCMV. For all herpesviruses, four intermediate capsid types are thought to form in cells during viral maturation (1–4, 8, 9). The earliest to form is the transient procapsid, a spherical and

porous structure (8, 9). The mature stable capsids (A, B, and C) have a more angular shape and sealed structure. The mechanism and pathway by which the herpesvirus procapsid transforms into the different mature capsids remain unclear or controversial (1–4).

Electron cryomicroscopy (cryoEM) has been used to study the structures of mature capsids of HCMV as well as other herpesviruses (10–14). Moreover, several previous studies have used *in vitro* assembly and genetic complementation to examine the effects of Pr knockout on capsid assembly in HSV-1 (12, 15–18). However, such studies have not been possible in HCMV because of either lack of an *in vitro* capsid assembly system or the difficulty to construct complementing cells that express capsid proteins and support the growth of HCMV mutants with the deletion of these essential genes (3). Consequently, little is known about the role of Pr and its cleavage of AP in HCMV capsid maturation. Novel approaches are needed to clarify the location of the Pr within the 3D structure of the capsid and to understand the functional and structural roles of Pr in the viral replication cycle.

Ribozymes represent promising gene-targeting agents used in both basic research and clinical therapy. Altman and colleagues (19–22) have shown that RNase P of *Escherichia coli*, a tRNA processing enzyme, contains a catalytic RNA subunit (M1 RNA) that can be engineered to cleave tRNA substrates and other target RNAs, including specific mRNAs. A sequence-specific ribozyme, M1GS, can be constructed by attaching to M1 RNA a guide sequence complementary to a target mRNA (Fig. 1*a*) and is effective in blocking mRNA expression in cultured cells (23, 24). Unlike other nucleic acid-based interference approaches such as antisense oligonucleotides and RNA interference (25, 26), M1GS-based strategy is unique because of the use of M1 RNA, one of the most efficient catalytic RNAs found in nature (22). In this study, we constructed a M1GS ribozyme to inhibit the expression of HCMV Pr and showed that a reduction of Pr expression significantly blocks viral DNA encapsidation and growth, leading to accumulation of Pr-minus capsid-like intermediates. Using comparative cryoEM analyses, we determined the 3D structures of both Pr-minus and mature B capsids to 12.5-Å resolution, the highest ever for an HCMV particle. Our data also provide significant insight into HCMV capsid assembly and suggest a general pathway that explains the origin of different types of herpesvirus capsids. These results also show that engineered RNase P ribozyme is a highly effective gene-targeting agent for studies of gene function.

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Abbreviations: HCMV, human CMV; HSV-1, herpes simplex virus type 1; cryoEM, electron cryomicroscopy; AP, assembly/scaffold protein; Pr, protease; moi, multiplicity of infection; V6, variant 6.

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Fig. 1. Construction of M1GS ribozymes, their expression in U373MG cells, and their effects on HCMV gene expression. (a) Schematic diagrams of ptRNA (Left; substrate for RNase P), external guide sequence (EGS) hybridized to mRNA (Center), and M1GS RNA hybridized to target mRNA (Right). (b) Substrate pr39 used with the target sequence binding to the guide sequence of ribozymes highlighted and the site of cleavage indicated by the arrow. (c) Overall cleavage rate $[(k_{cat}/K_m)^S]$ and binding affinity (K_d) in reactions of pr39 with ribozymes (average values of triplicate experiments). (d) Cleavage of pr39 by M1GS ribozymes. ^{32}P -labeled Pr mRNA substrates (10 nM) were incubated alone (–) or in the presence of 1 nM AP1, 2 nM P1, or 10 nM AP2. Cleavage products were separated on 15% polyacrylamide gels containing 8 M urea. (e) Western analysis of HCMV proteins in infected cells. Protein samples were isolated from parental U373MG cells or M1GS-expressing cells that were either mock-infected (lane 8) or infected with HCMV (moi = 1; lanes 5–7), separated by SDS/PAGE, and transferred to membranes. Membranes were reacted with antibodies to HCMV AP. NP1c, which shares the carboxyl-terminal sequence with AP, was barely detected, because UL80, the precursor to this protein, is expressed at a much lower level than AP, and the anti-AP serum does not exhibit high affinity to the AP sequence (P.T., J. Zhu, and F.L., unpublished results).

Materials and Methods

Viruses, Cells, and Antibodies. HCMV strain AD169 was propagated in human foreskin fibroblasts and astrocytoma U373MG cells, as described (24). Antibodies against HCMV Pr and AP were kindly provided by Annette Meyer (Pfizer Diagnostics). Antibodies against UL44, IE1, and gH were purchased from either Goodwin Institute for Cancer Research (Plantation, FL) or Biondesign (Kennebunk, MN) (24).

In Vitro Assays of Ribozymes. Plasmids V6, pFL117, and pC102 contain the DNA sequences coding for variant 6 (V6) RNA, M1 RNA, and mutant C102, respectively (24, 27). Mutant ribozyme C102 contains several point mutations (e.g., $A_{347}C_{348} \rightarrow C_{347}U_{348}$ and $C_{353}C_{354}C_{355}G_{356} \rightarrow G_{353}G_{354}A_{355}U_{356}$) at the catalytic domain (P4 helix). The DNA sequences encoding ribozymes AP1, P1, and P2 were constructed by PCR with V6, pFL117, and pC102 as the templates, respectively. The 5' and 3' PCR primers were AF25 (5'-GGAATTCTAATACGACTCACTATAG-3') and M1AP1 (5'-CCCGCTCGAGAAAAATGGTGTCTCTCCAGCCG-

GCGCTGTGTGGAATTGTG-3'), respectively. The DNA sequence encoding substrate pr39 was constructed by annealing oligonucleotides AF25 and sAP1 (5'-CGGGATCCGAGCGC-CGGCTGGAGA GCGAGAGGCCCGCCTATAGTGAGTCG-TATTA-3'). The procedures for *in vitro* cleavage and binding analyses and construction of ribozyme-expressing cells were carried out as described (24) (see *Supporting Text*, which is published as supporting information on the PNAS web site).

Assays for Inhibition of Viral Growth, Gene Expression, and Genome Replication. Cells were infected with HCMV at a multiplicity of infection (moi) of 1–3. To determine the level of viral growth, the cells and medium were harvested at 1-day intervals throughout 7 days after infection (24). Viral stocks were prepared and their titers determined by infecting 1×10^5 human foreskin fibroblasts and counting the number of plaques 10–14 days after infection (24). The values obtained were averages from three independent experiments each with duplicated wells.

Northern and Western analysis were used to determine the expression levels of viral mRNAs and proteins, respectively (details are provided in *Supporting Text*) (24). To quantify the results of the expression levels of viral mRNAs and proteins, Northern and Western assays were carried out by using a 2-fold serial dilution of RNA and protein samples isolated from parental U373MG cells infected with HCMV. The measurement values using the IMAGE-QUANT software (Molecular Dynamics) were all within the linear range of the RNA and protein detection (i.e., 2-fold changes in RNA and protein samples result in a 2-fold change in signal bracketing the range of experimental values). The values obtained were averages from three independent experiments each with duplicated wells. A PCR-based assay was used to quantify the level of viral genome replication and DNA encapsidation (*Supporting Text*).

CryoEM Studies of Capsid. Pr-minus and wild-type B capsids were isolated by using a protocol modified from the density-gradient purification procedure described (*Supporting Text*) (8). To isolate Pr-minus capsids, ≈ 40 2-liter roller bottles of AP1 ribozyme-expressing cells were infected with HCMV at a moi of 1–3. Standard thin-section EM was performed to monitor HCMV capsid assembly and to verify the presence of accumulated large-cored Pr-minus capsids in the cell nuclei. Western blot analysis confirmed that preAP was present and not cleaved, and that Pr was barely detected, indicating that the capsids present in the infected cells were Pr-minus capsids. Established cryoEM procedures (14, 28) were used to image the Pr-minus and wild-type B capsids with a JEOL 2010F electron microscope equipped with a charge-coupled device camera (see *Supporting Text*).

Results

Construction of M1GS Ribozymes and Ribozyme-Expressing Cells. We used previously described strategies to map solvent-accessible regions of HCMV Pr mRNA in infected cells (24) and to select M1GS variants that were highly efficient [e.g., V6 RNA (a $C_{235} \rightarrow U_{235}$ substitution and insertion of an adenine immediately 5' to C_{228})] (27) and would be expected to result in the most effective inhibition of HCMV Pr expression (see below and Table 1). Ribozyme AP1 was constructed by covalently linking the 3' terminus of V6 RNA with a guide sequence of 18 nucleotides complementary to the targeted Pr mRNA sequence, which does not overlap with UL80.5 (Fig. 1). Two other M1GS ribozymes, P1 and P2, were also constructed in a similar way and included in the study. P1 was derived from the wild-type M1 RNA sequence. P2 was derived from C102 RNA, an M1 mutant containing several point mutations at the catalytic P4 domain ($A_{347}C_{348} \rightarrow C_{347}U_{348}$ and $C_{353}C_{354}C_{355}G_{356} \rightarrow G_{353}G_{354}A_{355}U_{356}$) (24). P2 was at least 10,000-fold less active than M1 RNA in cleaving a preRNA (24). A control ribozyme, AP2, was derived from AP1

Table 1. Inhibition of viral gene expression in cells expressing ribozymes AP1, AP2, and P1, compared with cells not expressing a ribozyme (U373MG)

Target	Viral gene class	Percent inhibition			
		U373MG	AP2	P1	AP1
IE1 mRNA	α	0	1	1	1
US2 mRNA	β	0	1	2	3
Pr mRNA	γ	0	7	80 $\pm$ 6	99 $\pm$ 9
IE1 protein	α	0	1	1	1
UL44 protein	β, γ	0	1	1	2
Pr protein	γ	0	4	78 $\pm$ 6	99 $\pm$ 9
Glycoprotein H	γ	0	1	2	1

Values shown are means derived from three independent experiments, each with duplicated wells. Standard deviation values <5% are not shown.

by introducing into M1 RNA the same mutations found in C102; this control is therefore expected to be catalytically inactive.

AP1 and P1 efficiently cleaved substrate pr39, which contains the targeted Pr mRNA sequence of 39 nucleotides (Fig. 1*d*, lanes 1 and 2). In contrast, cleavage by AP2 was barely detected (Fig. 1*d*, lane 3). Detailed kinetic analyses of overall ribozyme cleavage efficiency (measured as $[k_{\text{cat}}/K_m]^*$) indicated that AP1 is at least 20-fold more active than P1 in cleaving pr39, whereas AP2 and P2 are barely active because of the mutations in their catalytic domains (Fig. 1*c*). Gel-shift assays using different concentrations of the ribozymes and pr39 revealed that the binding affinities of AP2 and P2 to substrate pr39, measured as the dissociation constant (K_d), are similar to those of AP1 and P1, respectively (Fig. 1*c*). Because AP2 and P2 contain the same antisense guide sequence and bind to pr39 as well as AP1 and P1, respectively, but are catalytically inactive, these ribozymes were used as a control for the antisense effect in our experiments in cultured cells.

To construct cell lines expressing M1GS ribozymes, LXS-N-M1GS DNAs in which M1GS is driven under the control of the small nuclear U6 RNA promoter were transfected into amphotropic packaging cells (PA317) to produce M1GS-expressing retroviruses (24, 29). Human U373MG cells were then infected with these retroviruses, and the ribozyme-expressing cells were cloned. The constructed cell lines and a control (vector-only) cell line were indistinguishable in terms of cell growth and viability for up to 2 months, suggesting that the expressed ribozymes do not exhibit significant cytotoxicity (data not shown). The level of M1GS RNA in each cell clone was determined by Northern analysis with a DNA probe that is complementary to M1 RNA, using the expression level of H1 RNA, the RNA subunit of human RNase P (22), as the internal control (Fig. 6*a*, which is published as supporting information on the PNAS web site). Only the cell lines that expressed similar levels of these ribozymes were used for further studies.

Inhibition of Viral Pr Expression by M1GS Ribozyme. Pr mRNA expression in HCMV-infected cells was analyzed by Northern blotting, using the level of viral immediate-early 5-kb mRNA as an internal control (Fig. 6*b*). A reduction of 99 $\pm$ 9%, 80 $\pm$ 6%, and 7 $\pm$ 3% (average of three experiments) in Pr mRNA expression was found in cells expressing AP1, P1, and AP2, respectively (Table 1) (see *Supporting Text* for experimental details), suggesting that the significant reduction of Pr mRNA expression in cells expressing AP1 and P1 was due to targeted cleavage by the ribozyme. The low level of inhibition observed in AP2-expressing cells was probably due to an antisense effect, because AP2 bound to the target mRNA sequence as well as AP1 but is catalytically inactive (Fig. 1*c*). Western analyses with anti-Pr or anti-IE1 (control) antibodies indicated that Pr protein expression was reduced by 99 $\pm$ 9%, 78 $\pm$ 6%, and 4 $\pm$ 3% (average of three experiments) (see *Supporting Text* for experimental details) in cells expressing AP1, P1, and AP2,

respectively (Fig. 6*c* and Table 1). In HCMV-infected cells, the anti-Pr serum predominantly detected a smaller Pr species ($\approx$ 30 kDa), which results from efficient self-cleavage of the UL80 product (Fig. 6*c*). Thus, AP1 is highly effective in blocking Pr expression.

Ribozyme Targeting Reveals Essential Roles of Pr in HCMV Lytic Replication. To determine the role of Pr in viral replication, viral yield was analyzed by measuring viral titers of stocks from HCMV-infected cells that expressed ribozymes. At 5 days postinfection, viral yields were reduced by at least 10,000- and 100-fold in cells expressing AP1 and P1, respectively, whereas no significant reduction was observed in cells expressing AP2 (Fig. 2). Thus, an inhibition of Pr expression results in significant reduction of viral growth. Three sets of experiments were further performed to study the effects of AP1-mediated blocking of Pr expression on HCMV lytic replication and to elucidate the function of Pr. We first analyzed the expression of viral IE1 mRNA (an immediate-early transcript), US2 mRNA (an early transcript), UL44 (an early-late protein), and gH (a late protein) (Fig. 6*b*, lanes 11–18, Fig. 6*c*, lanes 11–14, and Table 1). No significant difference in the expression of these genes was found among cells expressing AP1 or AP2, suggesting that Pr does not regulate the expression of other viral genes. More importantly, these results imply that the engineered RNase P ribozyme is highly specific in blocking the expression of its target.

The second set of experiments investigated whether AP processing is blocked as a result of the inhibition of Pr expression. As expected, only the uncleaved precursor form of AP, preAP, was found in cells expressing AP1, whereas the cleaved form was found in cells that do not express a ribozyme or express inactive ribozyme AP2 (Fig. 1*e*, lanes 5–8). NP1c, which shares the C-terminal sequence with AP, was barely detected, because its precursor, UL80, is expressed at a much lower level than AP, and the anti-AP serum does not exhibit high affinity to the AP sequence (P.T., J. Zhu, and F.L., unpublished results). The third set of experiments examined the effect of the ribozyme on viral genomic DNA replication and packaging. Total DNA was isolated from lysates of the HCMV-infected cells that were either treated with DNase I or not. The encapsidated viral DNAs would be resistant to DNase I digestion, whereas those not packaged in the capsid would be susceptible. When assaying the DNA samples from cell lysates that were not treated with DNase I, no significant difference in the level of intracellular HCMV DNA (determined by PCR detection of HCMV IE1 using human β -actin DNA as the internal control) was observed in all cell lines (Fig. 3*a*, lanes 4–6), suggesting that the total levels (both encapsidated and unencapsidated DNAs) of intracellular viral DNAs are similar among these cells, and that replication of the viral genome is not affected by the reduction of Pr expression. However, when the DNase I-treated samples were assayed, the “encapsidated” DNA was hardly detected in cells that expressed AP1 (Fig. 3*a*, lanes 1–3). These observations imply that inhibition of Pr expression blocks the step at which the viral genome is packaged into the capsids. Accumulation of unique HCMV capsid intermediates that contain a uniformly scaffold-like core without the electron-dense viral DNA was observed in AP1-expressing cells (Fig. 3*b*), whereas mature DNA-containing capsids were observed in cells that did not express the ribozyme (Fig. 3*c*) or express control ribozyme AP2 (data not shown). Thus, HCMV DNA encapsidation and capsid maturation appeared to arrest prematurely in the absence of viral Pr. Surprisingly, the Pr-minus capsids inside the nucleus exhibited the characteristic angular shape of the wild-type capsids, in contrast to the circular HSV-1 Pr-minus capsids observed inside host nuclei (17).

3D Structural Comparisons of Pr-Minus and Wild-Type B Capsids. To further study the function of Pr in viral capsid maturation, Pr-minus capsids and wild-type B capsids were isolated from AP1-expressing cells and the parental cells, respectively. The angular shape of

Fig. 2. Growth of HCMV in U373MG cells and cell lines expressing M1GS RNA. Cells (5×10^5) were infected with HCMV at moi = 1–3. Values are means derived from three independent experiments, each with duplicated wells. Standard deviation is indicated by error bars.

Pr-minus capsids was confirmed by cryoEM. The cryoEM images of Pr-minus (Fig. 3*d*) and wild-type B capsids (Fig. 3*e*) were very similar, both showing polyhedral capsids with electron-dense internal cores that represent the protein scaffold. It is worth noting that improved imaging technique using a charge-coupled device camera played a key role in our structural studies using limited samples of wild-type B capsids and Pr-minus capsids. By merging 959 particle images, we reconstructed the Pr-minus capsid to a resolution of 12.5 Å (Fig. 4*a*). The wild-type B capsid was reconstructed from >2,000 particles and was filtered to the same resolution for comparison. Even though the Pr-minus capsid structure was compared with that of the wild-type B capsid at 12.5 Å, the shaded surface representation of the Pr-minus capsid structure was indistinguishable from that of the wild-type B capsid when viewed from the outside of the capsid (Fig. 4 and data not shown). Both are polyhedral in shape, with angular vertices, and exhibit the arrangement of pentons, hexons, and triplexes typical of sealed mature capsids.

Despite their outward similarity, there are some important differences between the insides of the Pr-minus and wild-type B capsids. We compared 3.6-Å-thick slices through the center of each 3D reconstruction, perpendicular to a three-fold axis (Fig. 4 *b* and *c*). Both capsids had a spherical gap just inside the capsid shell, indicating that the scaffold makes no or very little contact with the capsid. However, closer examination of the Pr-minus slice revealed several string-like connections (boxed regions in Fig. 4*d*) between the scaffold density and the inner capsid floor. These connections are absent from wild-type B capsids (Fig. 4*e*). We attribute these tenuous densities to the attachment of the C termini of preAP to the floor domain of the major capsid protein. That these attachments were still present in the Pr-minus capsid, despite its angular shape, suggests that cleavage of the scaffolding protein by the Pr, which removes the C-terminal region of preAP, is not required for the procapsid to achieve the stable angular morphology. Furthermore, our results indicate that neither the Pr nor any of its self-cleaved products (e.g., NP1c) plays any roles in this process. Conversely, the weaker signal of these tenuous densities indicates that not all of the sites were equally occupied at this stage of procapsid maturation, suggesting the possibility that the scaffolding protein dissociates from the major capsid protein as the capsid angularizes.

Additional differences between the Pr-minus and wild-type B capsids are revealed in their radial density profiles (Fig. 7, which is published as supporting information on the PNAS web site), which plot the spherically averaged density distribution of the 3D reconstructions as a function of radius. Two of the main features, the shell

Fig. 3. Effects of ribozyme inhibition of HCMV Pr expression on viral DNA packaging, capsid assembly, and maturation. (a) Levels of total intracellular (Right) and encapsidated (Left) viral genomic DNA. Total DNA (lanes 4–6) and DNase I-treated DNA samples (lanes 1–3) were isolated from HCMV-infected cells that either did not express a ribozyme (lanes 3 and 6) or expressed ribozymes AP1 (lanes 1 and 4) or AP2 (lanes 2 and 5). Levels of viral IE1 DNA sequence were determined by PCR by using primers that amplified the IE1 sequence. Human β -actin DNA sequence was used as internal control. (b and c) Negative-stain electron micrographs of thin sections of HCMV-infected cells that expressed AP1 (b) or did not express any ribozymes (c). In c, a representative A, B, and C capsid is denoted by A, B, and C, respectively. (d and e) cryoEM images of purified Pr-minus capsids (d) and wild-type B capsids (e).

(region D, ≈ 480 to ≈ 660 Å) and the scaffold ring (region B, ≈ 130 to ≈ 410 Å), are well conserved, with the shell densities being almost identical. A drop in density between ≈ 410 and ≈ 480 Å (region C) corresponds to the space between the scaffold ring and the capsid floor (Fig. 7). The Pr-minus capsids have a slightly higher density in that region, which might be attributed to the tenuous connections

Fig. 4. Comparison of Pr-minus and wild-type HCMV capsid structures. (a) Surface representation of the 12.5-Å 3D reconstruction of Pr-minus HCMV capsid, viewed from a threefold axis. The map is color-coded according to radial density (see color bar). P, C, and E indicate three types of quasiequivalent hexons, 5 indicates a penton, and Ta-Tf indicate six types of quasiequivalent triplexes. (b and c) A 3.6-Å-thick central slice through capsid reconstructions of Pr-minus (b) and wild-type B (c) capsids, perpendicular to a threefold axis. (d and e) Close-ups of boxed regions in b and c. Boxes in d highlight tenuous densities connecting scaffold to major capsid protein in Pr-minus capsid.

between the scaffold and shell. The greater B capsid density between ≈ 30 and ≈ 130 Å (region A) suggests that the bulk of the viral Pr proteins may be located within the core of the scaffold ring (Fig. 7).

Discussion

Engineered RNase P Ribozyme as a Tool for Studies of HCMV Assembly. Engineered RNase P ribozymes represent a nucleic acid-based gene interference approach to knock down gene expression (22). Unlike other strategies, such as antisense oligonucleotides and RNA interference (25, 26), the M1GS-based approach is unique, because it hydrolyzes RNA using M1 RNA, one of the most efficient catalytic RNAs found in nature (22). The AP1 ribozyme used in this study represents a variant generated through *in vitro* selection procedure (27). The level of efficacy of this ribozyme, to our knowledge, is among the most effective in blocking viral gene expression using a nucleic acid-based gene interference approach, leading to a reduction of 99% in the expression of viral Pr and a reduction of 10,000-fold in viral growth in HCMV-infected cells.

Further examination of HCMV lytic replication in AP1-expressing cells reveals that the activity of M1GS ribozymes is highly specific and can thus be used to study the functional roles of Pr in HCMV infection. First, the antiviral effect of the ribozyme appears to be the result of a reduction in the expression of Pr. This is because DNA encapsidation, as well as the amount of processed AP products, was significantly reduced in cells expressing AP1 but not in those expressing AP2 (Figs. 1, 3, and 6). Second, expression of M1GS in cells inhibits the expression of Pr mRNA but not the expression of other viral genes examined (IE1, US2, UL44, and gH) (Fig. 6 and Table 1). Moreover, the expression of M1GS and the reduction of Pr expression do not appear to affect the replication of viral genomic DNA (Fig. 3a). These results indicate that the Pr is required for viral DNA encapsidation but does not play a role in regulating viral gene expression and genome replication.

Insights into the Role of Pr in HCMV Capsid Maturation. All human herpesviruses can engage in lytic replication or establish latent infection, and all encode a Pr (1–4). Efforts to either establish an *in vitro* cell-free capsid assembly system or to engineer comple-

mentary cell lines that express Pr and support the growth of HCMV Pr deletion mutants have been fruitless to date.

Our constructed cell line expresses AP1 that is more efficient at cleaving the Pr mRNA than the wild-type ribozyme P1. In addition to demonstrating the effects of suppressing Pr expression on HCMV gene expression, DNA replication, and packaging, we have also used the cell line to generate Pr-minus HCMV capsids. Several lines of evidence strongly suggest that the capsid-like particles isolated from AP1-expressing cells represent Pr-minus capsids rather than wild-type B capsids. First, Western analyses showed that neither Pr nor processed AP was found in AP1-expressing cells or in the capsid-like particles isolated from these cells (Fig. 1e and data not shown). Second, the core of the AP1 capsid is less dense than that of the wild-type B capsid, because the Pr protein is thought to be localized in the core (Fig. 4). Third, although a 10,000-fold reduction in viral titers was observed in AP1-expressing cells, significantly larger amounts of capsid-like particles accumulated in these cells, compared with cells expressing no ribozymes or the control ribozyme AP2 (Fig. 3b and c). These observations suggest that wild-type B capsids, if there are any present in the nuclei of

Fig. 5. Model of HCMV capsid assembly and maturation. HCMV capsid proteins assemble into an intermediate circular procapsid, which spontaneously angularizes into A, B, or C capsids, depending on the timing of Pr cleavage of preAP and simultaneous recruitment of unit-length viral DNA to the portal complex.

AP1-expressing cells, probably represent 1 of 10,000 particles. Because our 3D reconstructions were obtained by averaging $\approx 1,000$ capsids, the features observed in the reconstruction of particles from AP1-expressing cells should mainly represent those of the Pr-minus capsids, not the wild-type B capsids.

Surprisingly, our structure of the Pr-minus procapsid reveals features of the capsid shell that are indistinguishable from those of the mature B capsid at a relatively high resolution of 12.5 Å, which permits functional domains to be resolved in HCMV capsids. These results suggest that the angularization/maturation process of herpesvirus procapsids is probably not triggered by the cleavage of scaffolding proteins by viral Pr as previously thought (18) but is rather a spontaneous process. Furthermore, these data provide direct evidence that Pr and/or processing of scaffold proteins is required for HCMV DNA encapsidation but has no role either in circular procapsid formation or triggering global conformational change during capsid maturation.

Based on these results, we have proposed a working model for HCMV capsid maturation (Fig. 5). A circular porous procapsid is initially formed from the major capsid protein, the minor capsid protein, and its binding protein mC-BP, preAP, pUL80, and portal protein pUL104, in a manner similar to that of HSV-1 (30). Our results indicate that angularization of the capsid is a spontaneous process independent of Pr activity. The type of capsid formed during this process may depend on at least two factors: binding of DNA and timing of scaffold protein cleavage by Pr. We believe that the Pr concentration determines the overall speed of cleavage of itself and its substrate scaffold proteins present in each capsid. This speed, or timing, of cleavage events in turn determines the fate of the capsid (Fig. 5). Correct timing of scaffold protein processing allows the cleaved proteins to be released from the capsid and DNA to simultaneously enter before angularization and sealing of the capsid pores are complete, leading to C capsid formation. If scaffold protein processing happens too quickly or prematurely, before DNA has been recruited to the procapsid, the procapsid will release the scaffold and be angularized, resulting in an empty A capsid. Conversely, if scaffold protein processing is somehow delayed, such that the capsid pores are sealed before the release of cleaved

proteins, the angularized capsid will trap a collapsed scaffold core inside, producing a B capsid. B capsids are believed to serve as the core for noninfectious enveloped particles that play an important role as a decoy to modulate immune response *in vivo* (1, 3). Our model suggests that reducing Pr expression or its availability in procapsid represents a mean for HCMV to generate B capsid and its related enveloped particles. This hypothesis is supported by our results that M1GS-mediated reduction of Pr expression led to the accumulation of angularized B capsid-like intermediates. Further studies in HCMV as well as other herpesviruses will determine whether regulated expression and/or procapsid packaging of Pr represent a general mechanism for generation of viral B capsid and its associated particles.

Our results also provide the direct evidence to suggest that engineered RNase P ribozymes, such as those variants generated through *in vitro* selection procedure (e.g., AP1) (27), can be much more active *in vitro* in cleaving mRNA substrates and more effective in inhibiting gene expression in human cells than the ribozyme derived from the wild-type M1 RNA sequence (e.g., P1). M1 RNA may further increase its activity in cultured cells by interacting with cellular proteins (22, 31). These properties, as well as the simple design of the guide sequence, make M1GS an attractive and unique gene-targeting agent that can be used generally for basic research and clinical applications. It has been technically difficult to construct complementing cell lines that support the replication of mutants with deletions of essential genes of HCMV and other herpesviruses, such as Epstein-Barr virus and Kaposi's sarcoma-associated herpesvirus (2–4). M1GS-mediated inhibition of the expression of viral essential genes offers an attractive approach to studying the function of these genes in viral infections. These studies will further facilitate the development of M1GS ribozymes for studies and treatment of human diseases.

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