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REVIEW

Human cytomegalovirus riding the cell cycle

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Abstract Human cytomegalovirus (HCMV) infection modulates the host cell cycle to create an environment that is optimal for viral gene expression, DNA replication, and production of infectious virus. The virus mostly infects quiescent cells and thus must push the cell into G_1 phase of the cell cycle to co-opt the cellular mechanisms that could be used for DNA synthesis. However, at the same time, cellular functions must be subverted such that synthesis of viral DNA is favored over that of the host. The molecular mechanisms by which this is accomplished include altered RNA transcription, changes in the levels and activity of cyclin-dependent kinases, and other proteins involved in cell cycle control, posttranslational modifications of proteins, modulation of protein stability through targeted effects on the ubiquitin–proteasome degradation pathway, and movement of proteins to different cellular locations. When the cell is in the optimal G_0/G_1 phase, multiple signaling pathways are altered to allow rapid induction of viral gene expression once negative factors have been eliminated. For the most part, the cell cycle will stop prior to initiation of host cell DNA synthesis (S phase), although many cell cycle proteins characteristic of the $S/G_2/M$ phase accumulate. The environment of a cell progressing through the cell cycle and dividing is not favorable for viral replication, and HCMV has evolved ways to sense whether cells are in S/G_2 phase, and if so, to prevent initiation of viral gene expression until the cells cycle back to G_1. A major target of HCMV is the anaphase-promoting complex E3 ubiquitin ligase, which is responsible for the ubiquitination and subsequent degradation of cyclins A and B and other cell cycle proteins at specific phases in the cell cycle. This review will discuss the effects of HCMV infection on cell cycle regulatory pathways, with the focus on selected viral proteins that are responsible for these effects.

Keywords Human cytomegalovirus · Cell cycle · Anaphase-promoting complex · Cyclins · Cyclin-dependent kinases

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Introduction

The large spectrum of clinical problems associated with human cytomegalovirus (HCMV), including birth defects, atherosclerosis, cardiovascular disease, organ transplant failure, and cancer, has led to an increasing interest in how the virus subverts host cell functions. During infection, there are multiple effects not only on the cell cycle, but also on the cell death pathways, signaling networks, metabolism, and innate immune defenses (for review, see [1]). HCMV disrupts and subverts the host cell cycle at many points, and thus I begin with a summary of this intricate process, focusing on the mechanisms and proteins specifically targeted by the virus.

The cell cycle consists of a highly controlled series of steps that promote duplication of the DNA, monitor the DNA for damage, and allow division into two identical daughter cells once all checkpoint surveillance requirements have been met (for review, see [2, 3]). The two classes of proteins that serve as the master regulators of each of the cell cycle steps are the cyclins and cyclin-dependent kinases (CDK), which form heterodimeric

complexes that phosphorylate numerous target proteins on serine and threonine residues to either activate or inhibit them. Phosphorylation and dephosphorylation of the CDKs themselves orchestrate the coordinated temporal passage of the cell through each phase. The CDKs are expressed constitutively, while the cyclins are regulated by transcriptional activation and protein degradation. Multisubunit E3 ubiquitin ligases that ubiquitinate proteins and target them for degradation by the proteasome also play a major role in cell cycle progression. The two most important cell cycle E3 ubiquitin ligases are the anaphase-promoting complex (APC) and the Skp1–cullin–F-box protein complex (SCF) (for review see [4]). The periodic activation and inactivation of the APC in particular are essential for accurate cell cycle progression. Specifically, it triggers exit from mitosis, prevents early onset of DNA replication in G_1, and blocks more than one round of DNA replication from a given origin. As will be discussed below, the APC is a main target of HCMV during the infection. In addition, there is regulated expression of specific proteins that inhibit the catalytic activity of each of the kinase complexes, adding another layer of control.

The cell cycle is divided into four major phases—G_1, S, G_2, and M. In addition, there is a phase of quiescence or resting state, referred as G_0, in which the cell has withdrawn from the cell cycle, usually during G_1 phase. The G_1 phase marks the period of time between mitosis (M) and DNA synthesis (S). Progression into the G_1 phase, from either G_0 phase or mitosis, is associated with expression of the D-type cyclins that combine with CDK4 or CDK6 to form active kinase complexes. In turn, D-type cyclin activity is blocked by binding of the INK4 family of inhibitors and p27 to CDK4 and CDK6. The G_1 phase is distinguished by the expression of multiple transcription factors and proteins involved in nucleotide metabolism and DNA replication. Prior to S phase, pre-replication complexes (pre-RC) assemble at the origins of cell DNA replication. The origin recognition complex (ORC) binds first to the DNA and provides a base for temporal addition of other factors. The binding of CDC6 and CDT1 to the complex follows, which allows recruitment of minichromosome maintenance 2-7(MCM 2-7) proteins [5]. Cyclin E1 synthesis (referred as cyclin E in the text) is induced at the transcriptional level and forms a complex with CDK2 prior to entry into S phase.

Cyclin A2 (referred in the text as cyclin A) accumulates in S phase and forms an active kinase complex with CDK2. Regulation of cyclin A occurs at both the protein and mRNA levels [6–13]. The initiation of DNA replication requires the CDK2/cyclin A complex and the CDC7/DBF4 kinase (for review, see [14, 15]). There is another mechanism in place to prevent rereplication of DNA and polyploidy by blocking the activity of CDT1 after initiation of DNA synthesis at each origin. This is accomplished by targeted CDT1 degradation and by the binding of CDT1 to geminin, which as an APC substrate accumulates during S, G_2, and M phases when the APC is inactive.

G_2 phase occurs after DNA has been completely replicated and is characterized by the accumulation of proteins required for separation of the chromosomes and mitosis. The levels of cyclin B1 (referred in the text as cyclin B) increase, and it associates with CDK1. Activation of the kinase activity of the CDK1/cyclin B complex requires dephosphorylation of CDK1 by CDC25 phosphatase [16]. The CDK1/cyclin B and CDK1/cyclin A kinases have specific targets that promote the ordered segregation of the chromosomes to the daughter cells. The two daughter DNA molecules (sister chromatids) are attached to each other by protein crosslinks that concentrate at the centromere when the chromosomes are fully condensed. Additional proteins attach to the centromere forming the kinetochore. During mitosis, it is essential that all sister chromatids are correctly aligned and attached via the kinetochore to microtubules radiating from opposite poles of the mitotic spindle before segregation to daughter cells. This requires an intimate association of the spindle with the APC, which remains inactive until this checkpoint is silenced. The activation of the APC leads to the ubiquitination of securin and its degradation by the proteasome. This releases the protease separase, which cleaves the protein cohesin that keeps the sister chromatids bound to each other, thus allowing segregation of the chromatids and passage of the cell from metaphase to anaphase. Completion of mitosis also requires inactivation of the CDK1 complexes, which occurs via degradation of cyclins A and B by the proteasome following ubiquitination by the APC. At this time, geminin is also targeted by the APC for proteasome-mediated degradation, thus allowing loading of the pre-RCs onto the chromatin during G_1 phase and initiation of a new cycle of DNA synthesis and cell division [17]. This degradation of the cyclins and geminin continues until the onset of S phase when the APC is inactivated [18].

In addition to the above checkpoint for correct spindle formation, there are other checkpoints to regulate passage from one phase of the cell cycle to the next (for review, see [19]). The tumor suppressor p53 and the retinoblastoma (RB) family of pocket proteins (RB, p107, and p130) play a central role in these checkpoints. RB is the most important pocket protein in checkpoint surveillance, and at the beginning of G_1 phase, it is monophosphorylated by cyclin D/CDK4/6. In this form, RB binds to the E2F family of transcription factors and represses RNA synthesis from promoters that are activated by these factors. Hyperphosphorylation of RB by cyclin E/CDK2 in late G1 leads to dissociation of the RB–E2F complexes, which then allows the E2F factors to stimulate transcription of multiple genes,

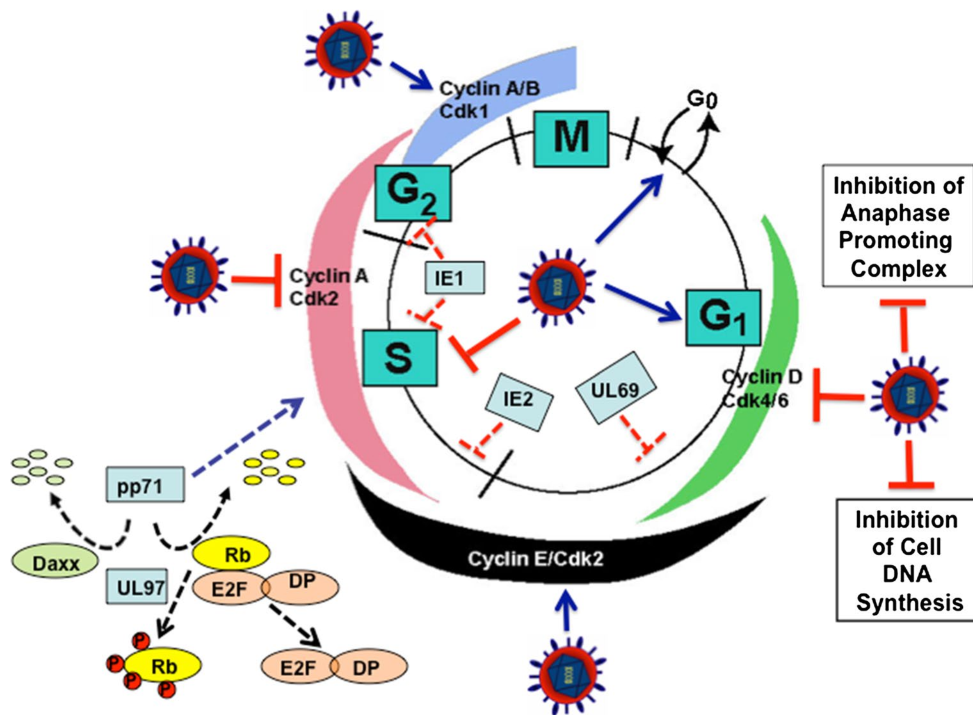

Fig. 1 Cell cycle arrest in HCMV-infected cells is multifactorial. The cell cycle consists of four phases—G_1, S, G_2, and M. Cells in the G_0 resting state are stimulated through growth signals to express cyclin D and enter G_1 phase. In uninfected cells, the anaphase-promoting complex (APC), an E3 ubiquitin ligase, remains active and targets proteins for degradation by the proteasome. A pre-replication complex is established at cellular origins of DNA replication, and cyclin E is induced. During S phase, cyclin A accumulates and the cellular DNA is replicated. G_2 phase marks the transition prior to cell division in M phase. Both cyclin A and cyclin B are required during the G_2/M period. HCMV infection of cells during G_0/G_1 phase induces progression through G_1. However, the cell cycle is blocked before the replication of cellular DNA, and the expression of the cyclins and cyclin-dependent kinases is disrupted. HCMV inhibits the expression of cyclin D and cyclin A, but promotes accumulation of high levels of cyclin E and cyclin B. Host cell DNA replication is inhibited, and activity of the APC is blocked. The effects of several viral proteins on the cell cycle are also shown. The virion protein UL69 can independently prevent the cells from progressing through G_1 phase, while the virion protein pp71 can accelerate progression of cells through the G_1 phase into S phase. pp71 interacts with the hypophosphorylated forms of the RB family of proteins and targets them for ubiquitin-independent degradation by the proteasome, allowing release of E2F/DP as an active transcription factor. Hyperphosphorylation of RB by the viral kinase UL97 also inactivates it. pp71 additionally interacts with the transcription repressor DAXX and promotes its ubiquitin-independent degradation, facilitating immediate early transcription. The viral immediate early protein IE1-72 has the capacity to block the cell cycle in the S and G_2/M phases, while IE2-86 can block the cycle at the G_1/S boundary

many of which encode proteins required for DNA replication and cell proliferation [20, 21]. p53 has pleiotropic functions and can serve as a transcriptional activator and repressor [22, 23]. When p53 is phosphorylated, it can associate with the murine double minute (MDM2) protein, which acts as an E3 ubiquitin ligase targeting p53 for proteasome degradation (for review, see [24]). p53 is stabilized in response to multiple signals of stress, including DNA damage and nutrient deprivation. In turn, p53 can activate the transcription of several pro-apoptotic genes as well as p21, which is an inhibitor of most CDKs.

Cell cycle arrest in infected cells is multifactorial

The initial observation that cells infected with HCMV in the G_0/G_1 phase of the cycle do not proceed through mitosis was made almost 20 years ago [25–28]. What was most surprising, however, was that the cells arrested in a pseudo-G_1 state, where there was expression of selected G_1-phase, S-phase, and M-phase gene products and a block in cellular DNA synthesis (see Fig. 1) [25–33]. Subsequent studies showed that this viral-mediated disruption of the cell cycle occurred at multiple levels of gene expression—transcription, posttranscriptional processing, translation, posttranslational modification, protein stability, and cellular localization of proteins. For example, the levels of the G_1-/S-phase cyclin E and the G_2-/M-phase cyclin B accumulate in infected cells, and the cyclin E/CDK2 and cyclin B/CDK1 complexes are active kinases [27, 31, 34]. In contrast, one of the G_1-phase cyclins (D1) and the S-phase cyclin A remain at very low levels [25, 27, 29, 31]. The accumulation of cyclin B is due to increased protein stability, while the infection appears to affect the RNA levels of cyclins E and D1 [29, 34]. The localization of cyclin B

is also affected, with a significant fraction remaining in the cytoplasm and concentrated at the centrosome [29, 34]. Interestingly, cyclin A expression is affected at the level of both transcription and protein stability [25, 27, 29, 31, 35–37]. Geminin and p53 also accumulate to high levels due to increased protein stability, and p53 is sequestered in viral replication centers [25, 27, 38–42]. In addition, hypophosphorylated RB can be targeted for degradation by the input virion protein pp71 or hyperphosphorylated by the viral kinase UL97, thus releasing E2F/DP as an active transcription factor [27, 43]. A role for HCMV-encoded microRNAs in cell cycle control is also suggested by the finding that one of the HCMV viral miRNAs, miR-US25-1, binds to the 5′ UTR of several cell mRNAs involved in the cell cycle [44]. One gene identified was cyclin E2, which is different from the cyclin E1 that is the main cyclin studied in association with the cell cycle. At later times in the infection, miR-US25-1 appears to downregulate levels of cyclin E2 that were induced earlier.

Input virion proteins initiate modulation of the cell cycle

The proteins UL69 and pp71 (UL82) have been shown to affect cell cycle progression (see Fig. 1). These proteins are part of the virion tegument and thus can function without any de novo viral gene expression. It should be noted that the cell cycle effects have mostly been studied with the isolated proteins in the absence of viral infection, as the many other viral proteins affecting the host cell cycle can obscure the potential function of a single protein.

Overexpression of UL69 results in accumulation of cells in G_1 phase of the cell cycle [45], and cells infected with a mutant virus lacking functional pUL69 do not undergo cell cycle arrest as efficiently [46]. The mutant-infected cells also produce significantly less virus than WT, but this may be due to other functions of pUL69 relating to nuclear RNA export and translation [47–49].

The deletion of pp71 also creates a growth-impaired virus, which can be complemented by the expression of the protein in trans [50, 51]. As noted above, pp71 interacts with the hypophosphorylated forms of the RB family of proteins (RB, p107, and p130) [52] and targets them for ubiquitin-independent degradation by the proteasome, allowing release of E2F/DP as an active transcription factor [52]. Expression of this protein alone accelerates progression of cells through the G_1 phase into S phase. In addition, pp71 interacts with ND10-associated transcription repressor DAXX and promotes its ubiquitin-independent and proteasome-mediated degradation, as well as dissociation of its binding partner ATRX, thus facilitating the activation of major immediate early (IE) RNA synthesis [53–60].

Role of the major IE proteins IE1 and IE2

The major region of immediate early transcription includes two genes, IE1 and IE2. IE1 RNA has four exons; a single ORF (UL123) initiates in exon 2 and specifies a 72-kDa protein (IE1-72). The IE2 gene product, IE2-86 (ORF UL122), is encoded by an alternatively spliced RNA with the first three exons of IE1 and a different terminal exon. IE2 also encodes abundant late unspliced RNAs that specify 60- and 40-kDa proteins corresponding to the C-terminus. Studies with mutant viruses have demonstrated that IE1-72 is required at low but not high MOI, while IE2-86 is essential regardless of the MOI.

Although there is only limited evidence that IE1-72 and IE2-86 play a major role in altering the cell cycle in the context of the infection, both have been shown to activate, as well as block, cell cycle progression in heterologous systems in the absence of other viral proteins (see Fig. 1). For example, transient expression of IE1-72 in asynchronously cycling cells results in the accumulation of the cells in the S and G_2/M phases [61]. IE1-72 alleviates p107-mediated repression of E2F-responsive promoters in transient transfection assays and thus may stimulate S-phase entry. Alternatively, the ability of IE1-72 to induce nuclear accumulation of p53 in transient assays and expression of p21 may be responsible for stimulating quiescent cells to enter S phase [62]. IE2-86 is the major transactivator of HCMV early genes and in in vitro assays binds to multiple proteins, including RB and p53 [63–67]. IE2-86 also has been implicated in inducing many genes required for host cell DNA synthesis, but again most studies have not been done in the context of the infection [68]. Several studies have also shown that transient expression of IE2-86 alters cell cycle progression, with a block at the G_1/S boundary in a $p53^{+/+}$ cell or after entry into S phase in a p53 mutant cell [31, 69–72]. It has been suggested that IE2-86 may drive cells into S phase and then inhibit cellular DNA synthesis, possibly by interacting with minichromosome maintenance 3 (MCM3)-associated protein (MCM3AP) [73].

The potential role of IE2-86 in the accumulation of cyclin E during the infection has been the focus of several studies [31, 43, 69, 74]. The majority of the experiments have used transient expression assays to examine the regulation of the cyclin E promoter driving a reporter gene [74]. The work demonstrating that cyclin E transcription was induced when IE2-86 was expressed from an adenovirus vector reinforced the hypothesis that IE2-86 expression may contribute to the upregulation of cyclin E in infected cells [68]. In one of the few studies involving infected cells, it was shown that a recombinant virus with a deletion of amino acids (aa) 30–77 in the N-terminal region shared by both IE1-72 and IE2-86 is deficient in upregulating cyclin

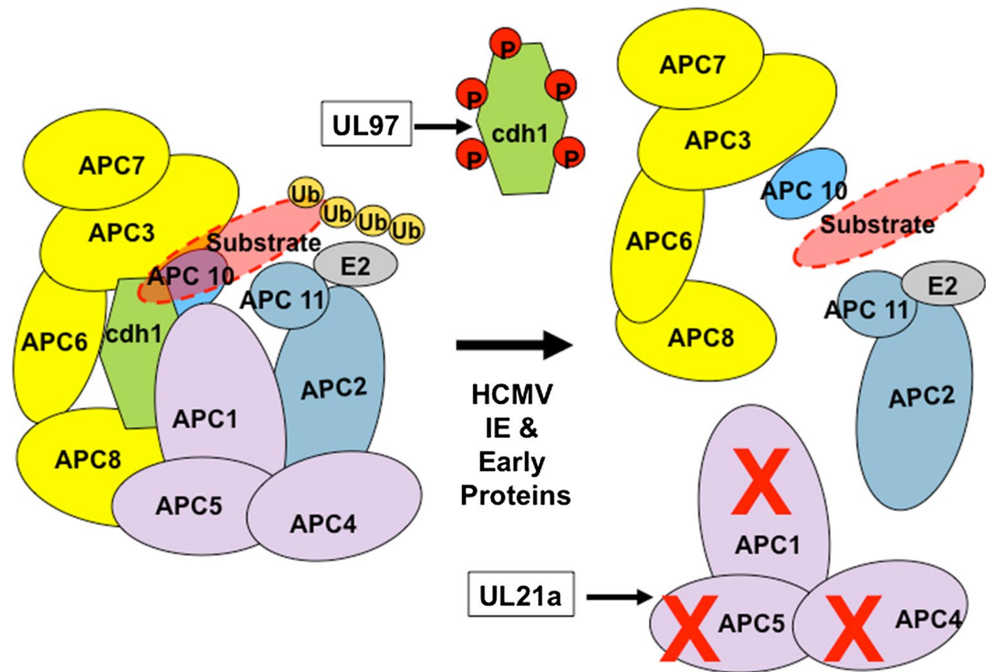

Fig. 2 Anaphase-promoting complex (APC) is disabled during the early phase of the HCMV infection. This inhibition is associated with multiple changes in the composition of the APC, including: degradation of the APC1, APC4, and APC5 subunits that is mediated by the viral protein UL21a, hyper-phosphorylation of the CDH1 regulatory subunit by the viral kinase UL97, and disassembly of the complex. Not all subunits of the APC are shown. The inhibition of the APC results in the accumulation of substrate proteins (e.g., cyclin B, geminin, and CDC6) that would normally be ubiquitinated and targeted for degradation by the proteasome

E [75]. A role of IE2-86 was suggested by the finding that the recombinant virus lacking aa 30–77 is still unable to increase the levels of cyclin E in complementing cells that express IE1-72 [43, 75]. Whether IE2-86 plays a direct role or the increase in cyclin E is due to the effect of IE2-86 on early gene expression remains to be determined [43]. Moreover, it is likely that cyclin E expression is regulated at the level of both RNA transcription and protein stability during the infection.

Targeting the anaphase-promoting complex (APC)

The ubiquitin proteasome degradation pathway is fundamental for the regulation of numerous cellular functions, including transcription, immune defense, metabolism, neural differentiation, DNA replication and progression of the cell cycle [76–78]. The prior attachment of ubiquitin to a protein is required for most, but not all (e.g., RB and DAXX discussed above), proteins to be targeted for degradation by the 26S proteasome. Attachment of ubiquitin to the protein involves three major steps. First, the enzyme E1 forms a thioester bond between itself and ubiquitin. The ubiquitin is then transferred to an E2 ubiquitin-conjugating enzyme. Finally the E2 enzyme transfers the ubiquitin to a ubiquitin protein ligase E3, which has high specificity and attaches ubiquitin to a lysine on selected target proteins. The transfer of additional ubiquitin proteins generates a polyubiquitin chain. Once ubiquitinated, the protein moves to the proteasome, where it is degraded.

Given that the APC is one of the two most important cell cycle E3 ubiquitin ligases, it is not surprising that HCMV as well as other viruses subvert its function (for review, see [79]). The recent cryo-electron microscopy reconstruction of a human APC/C-co-activator-substrate complex at 7.4 Å resolution showed that it consists of three major subcomplexes: the tetratricopeptide repeat (TPR) subcomplex (subunits 3, 6, 7, and 8), the catalytic E3 subcomplex (subunits 2 and 11), and the base or platform subcomplex (subunits APC1, APC4, and APC5), which attaches the TPR subunits to the catalytic core (see Fig. 2) [80]. Other TPR accessory subunits (APC12, APC13, and APC16, and APC10) assist the regulatory subunits in substrate recognition, and the subunit APC15 bridges APC5 and APC8. The TPR subcomplex interacts with APC/C co-activators CDH1 or CDC20, which serve as specificity factors at distinct times in the cell cycle by binding both the APC and the target protein, thus activating ubiquitination of the target protein. CDC20 is the major regulatory protein for the APC at the beginning of mitosis through anaphase, while APC/CDH1-mediated ubiquitination is required for completion of mitosis and passage through G_1 phase. The APC is inactive during the S and G_2 phases, during which the SCF E3 ubiquitin ligase is active.

The early observations from our laboratory and others that several substrates of the APC (e.g., cyclin B, CDC6, and geminin) abnormally accumulate early in the HCMV infection led to the hypothesis that APC activity is down-regulated during the infection [42, 81–83] Surprisingly, cyclin A, which is also a target of the APC, did not accumulate. Subsequently, we and others showed that the APC

is dysregulated at multiple levels (see Fig. 2) [81, 84–86]. The regulatory subunit CDH1 is hyperphosphorylated in a CDK-independent manner by the viral UL97 kinase, and the APC becomes destabilized, as evidenced by the dissociation of CDH1 and the complex composed of the subunits that have the tetratricopeptide repeat (TPR) motif (APC3, APC6, APC7, and APC8). This disassembly is also associated with specific proteasome-dependent degradation of the APC4, APC5 and APC1 subunits, although it is not clear whether the disassembly occurs before or after degradation of the proteins [84, 86]. In addition to the disassembly and degradation of specific subunits, there is also altered localization of the TPR subunits to the cytoplasm [85].

Initially, it was hypothesized that hyperphosphorylation of CDH1 by UL97 was associated with disruption of the APC, since in uninfected cells, hyperphosphorylation of CDH1 by CDKs occurs during S phase and is associated with inactivation of the APC. CDH1 becomes phosphorylated during HCMV infection beginning 8–12 h p.i., and this phosphorylation still occurs in the presence of the CDK inhibitor roscovitine. The use of a UL97 deletion virus confirmed that this kinase was responsible for the phosphorylation of CDH1. However, in mutant-infected cells, CDH1 still dissociated from the APC, the subunits APC1, APC4 and APC5 were degraded, and the APC substrates accumulated, albeit with delayed kinetics [84] (Clark and Spector unpublished results). Moreover, expression of UL97 alone in uninfected cells does not result in accumulation of APC target proteins (Clark and Spector, unpublished results).

Given the above observations, it appears that the major mechanism of APC inactivation is likely due to the decreased levels of specific APC subunits. The kinetics of the degradation of the APC4 and APC5 subunits during the infection indicated that the potential viral protein(s) involved were likely expressed at IE or early times of the infection or brought in with the viral tegument. The results of several experiments, however, demonstrated that input virion proteins and IE gene expression are not sufficient and that a viral early gene product or cellular gene induced at early times targets APC4 and APC5 [84]. Subsequently, it was discovered by Fehr et al. [86] that a small HCMV protein encoded by UL21a was responsible for this degradation. My lab has confirmed this result and has further found that the APC1 subunit is also degraded. Expression of UL21a alone in uninfected cells leads to degradation of the subunits and inhibition of the APC. It remains to be determined, however, whether UL21a first disrupts the APC subcomplex of APC1/APC4/APC5 and this leads to degradation of the proteins, or UL21a itself directs the degradation of these proteins. Surprisingly, in cells infected with a mutant virus containing a deletion of UL21a or UL21a and UL97, although there was a delay in the accumulation of APC target proteins, there was clearly a specific increase in the levels of these proteins at late times in the infection, suggesting that other viral proteins or cellular proteins induced during the infection can contribute to blocking APC/C function in the absence of UL21a and UL97. The fact that the virus devotes several HCMV gene products to inactivate the APC highlights the importance of this for the viral infection, which in vivo primarily occurs in cells in either G_0/G_1 phase where the APC is active.

Importance of cell cycle arrest for the viral infection

The multiple mechanisms used by HCMV to stop the cell cycle indicated that this was important for viral replication. In accord with this, it was shown that initiation of HCMV gene expression requires that the cells be in G_0 or G_1 at the time of infection [29, 87]. It was found that infection of cells that were in S phase blocked IE gene expression and allowed the cells to undergo mitosis. The block was not specific for the major IE promoter driving expression of IE1-72 and IE2-86, and other regions of IE gene expression, including the US3 and the UL36-38 loci, were also inhibited [88, 89]. This inhibition of gene expression was also not due to lack of translocation of the viral genome and matrix proteins to the nucleus, and was independent of the intrinsic early inhibition of viral transcription at ND10 domains involving DAXX, PML, and HDAC. Once the cells were back in G_1, the IE genes were expressed and replication ensued.

The mechanism by which this block to IE expression occurred was suggested by the following observations. Exposure of the cells to replicative stress or DNA damaging agents prior to and during the infection in S-phase cells relieved the inhibition of IE gene expression, and viral replication progressed to late gene expression in cells residing in the S/G_2 phase [88]. The finding that this lifting of the blockade did not occur in cells that had a stable knockdown of the checkpoint protein p53 suggested that the DNA damage-induced accumulation of this protein was likely important. Moreover, this effect of p53 appeared to be mediated by p21, which is transcriptionally regulated by p53. A prior study by Fortunato et al. [87] showing that inhibition of the proteasome, which would stabilize p53 and p21, allowed IE gene expression in S phase was also consistent with this hypothesis. The protein p21 is an inhibitor of CDK1 and CDK2 activity, and Zydek et al. [88] showed that there was a dose dependent increase in IE gene expression when a specific inhibitor of CDKs (CDK1, CDK2, CDK5, CDK7, and CDK9) was added for a short period. This effect, however, was abrogated by higher concentrations of the inhibitor and longer periods of treatment [87, 88], most likely due to its inhibition of CDK7 and CDK9, which are required

for HCMV viral gene expression at early times [90–92]. Interestingly, MCMV infection does not depend on the phase of the cell cycle for replication, and can block the cell cycle in either G_1 or G_2 phase [93, 94]. There is also no decrease in cyclin A levels in MCMV infected cells [95].

Regulation of cyclin A

The above studies focused attention on cyclin A/CDK2 as the mediator of inhibition of IE gene expression in S phase. Cyclin A/CDK2 normally increases in abundance during the transition from G_1 phase to the S phase as a result of increased transcription and protein stability [6–13]. However, in cells that are synchronized in G_0 and released into G_1 at the beginning of the infection, cyclin A expression is inhibited [27, 29]. This was somewhat surprising as cyclin A is a substrate for the APC, and almost all substrates of the APC were found to accumulate in infected cells. However, it was found that under these conditions, the inhibition of expression occurs at the transcriptional level [29]. At least some of the transcriptional inhibition involves the high-mobility group AT-hook 2 (HMGA2) protein [35], which in uninfected cells can activate the expression of cyclin A by relieving repression of the promoter [11]. The expression of this protein is significantly decreased in infected cells [35]. To determine if repression of HMGA2 is directly related to cyclin A inhibition and impacts on the infection, we constructed an HCMV recombinant virus that expressed HMGA2. In cells infected with the recombinant virus, cyclin A mRNA and protein were induced, and there was a significant delay in viral early gene expression and DNA replication, indicating that the repression of HMGA2 and cyclin A is important for viral replication. As will be discussed below, there are other mechanisms that can regulate the levels of the cyclin A protein, and progression of the infection depends on keeping cyclin A levels low.

The hypothesis that cyclin A/CDK2 was specifically responsible for the cell cycle dependency of the HCMV infection was further supported by studies using cells in which a cyclin A mutant resistant to APC degradation was stably expressed independent of the cell cycle [95]. Cells expressing this mutant cell cycle-independent cyclin A with an additional mutation that inhibited interaction with CDK2 were also used. It should be noted that interpretation of the results was complicated by the fact that the cells were infected while proliferating asynchronously and transformed cells had to be used, as primary cells are sensitive to continuous expression of cyclin A. Nevertheless, the data suggested that regardless of the phase of the cell cycle, there was almost complete inhibition of IE gene expression in cells expressing the mutant cell cycle-independent cyclin A that still bound to CDK2. In contrast, in control cells or cells expressing the form of cyclin A with an additional mutation that inhibited interaction with CDK2, IE expression occurred in cells that were in the G_1 phase, but not in S/G_2 phase. Thus, active cyclin A/CDK2 kinase activity was required for inhibition. Moreover, it was specific to cyclin A, as there was no restriction to IE expression in G_1 phase cells overexpressing cyclin B. Interestingly, the inhibitory effect of cyclin A overexpression did not extend to murine cytomegalovirus (MCMV). MCMV differs from HCMV in that initiation of infection is cell cycle independent, infection can block the cell cycle in either G_1 or G_2 phase, and cyclin A levels are not affected [93–95].

A clue to the mechanism by which cyclin A/CDK2 inhibits HCMV IE expression was provided by observation that the HCMV virion tegument protein pp150 (UL32) contains not only multiple canonical CDK-phosphoacceptor sites, but also a putative cyclin A binding motif (RRLFG) [96]. It was shown that pp150 binds to cyclin A and that pp150-cyclin A binding and phosphorylation serve as a monitor for whether the cell environment is suitable for initiating the HCMV infection. When there are high levels of cyclin A/CDK2, there is inhibition of viral IE gene expression. Consistent with pp150 being a nucleocapsid-binding protein, the inhibition is cis-acting, affecting only the input parental virion genome. A mutation in the pp150 cyclin A binding motif relieved the S/G_2 block to HCMV IE expression. All known mammalian CMV pp150 homologs, even the closely related chimpanzee CMV pp150, lack the cyclin A binding motif, and chimpanzee CMV, like MCMV, is not subject to the S/G_2 block to IE expression. An important question is why only HCMV incoming viral particles are sensitive to cyclin A, with IE gene expression restricted to times in the cell cycle when cyclin A/CDK2 activity is low or absent (G_0/G_1 phase).

In addition to HCMV replication being sensitive to cyclin A levels, the expression of cyclin A was also repressed once the infection progressed to early phase. As noted above, initial studies in my lab showed that cyclin A expression early in the infection was inhibited, at least in part, at the transcriptional level when cells were infected in G_0/G_1 phase. However, when cells were infected in S phase and the cyclin A levels were already very high, the levels decreased more rapidly in the HCMV-infected cells than uninfected cells as the cells passed through the cell cycle, suggesting an effect on protein stability. Two groups independently discovered that the same HCMV protein that inhibited the APC, UL21a, also mediated the proteasomal degradation of cyclin A, and this required the cyclin A binding RXL/Cy motif on UL21a [36, 37]. This region of UL21a was distinct from the domain responsible for APC inhibition, and thus levels of cyclin A were much higher in UL21a RXL mutant-infected cells than in cells infected with the UL21a deletion mutant (due to some

APC activity). In cells infected in G_0/G_1 phase with either a UL21a deletion mutant or UL21a RXL mutant, some host cell DNA synthesis could be observed at 36–48 h p.i. in approximately 40 % of the cells, indicating that increased levels of cyclin A expression could result in host cell DNA replication. Premature chromosomal condensation and damage was also observed in some UL21a RXL mutant-infected cells, and this coincided with inhibition of the viral infection.

Inhibition of cellular DNA replication

In addition to the downregulation of cyclin A discussed above, the replication of cellular DNA appears to be inhibited by several other mechanisms in the infected cells. One mechanism involves interference with the formation of pre-RCs at the origins of DNA replication [42, 69]. In part, this may be due to decreased expression of several of the MCM proteins and inhibition of the loading of these proteins onto chromatin [42, 69]. As discussed above, geminin normally accumulates in S phase to block cdt1 activity and ensure that there is no refiring of origins as the cells proceed through S and G_2/M. Thus, its premature accumulation during G_1 phase due to viral inhibition of the APC would affect the loading of the MCM proteins. A second mechanism may involve a direct effect on the MCM proteins. Supporting this, latter hypothesis is the finding that a recombinant virus deficient in the expression of the viral protein UL117 is unable to inhibit host cell DNA synthesis as efficiently as WT virus [97]. This effect is associated with increased levels and loading of the MCM proteins onto the chromatin in mutant-infected cells. Thus, as we have seen with other effects on the cell cycle, HCMV has found several ways to block host cell DNA synthesis.

Conclusions

This review describes how the cell cycle is manipulated by HCMV and some of the mechanisms used by the virus to subvert cell cycle regulatory pathways. Key questions are which changes are actually important for viral replication and do they play a role in in vivo pathogenesis? It is intriguing that some of the effects are specific to HCMVs, and other animal cytomegaloviruses do not have a strict requirement that cells be non-cycling for infection to proceed. Is this due to differences in pathogenesis or is it cell-type specific? In addition, most studies to date have only looked at the effects in fibroblasts, and yet, the important in vivo targets include endothelial cells, smooth muscle cells, monocytes, and cells of the neural lineage. There is also a need to identify what other viral proteins are directly involved. The answers to these questions will greatly facilitate our understanding of HCMV disease and provide a basis for the development of new antiviral therapies.

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