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MANIPULATION OF THE CELL CYCLE BY HUMAN CYTOMEGALOVIRUS

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1. ABSTRACT

The human cytomegalovirus-induced changes to the transcriptome and proteome of infected cells in many ways resemble an abortive mitogenic response. The virus induces quiescent cells to re-enter the cell cycle, but they are prevented from entering the S phase, where the synthesis of the cellular genome would compete with that of the virus for the available precursors for DNA replication. The mechanisms of these cell cycle alterations include transcriptional induction and repression, post-translational modifications and changes in protein stability. Essentially every class of cell cycle regulators is affected, and some of the key proteins are targeted by multiple different mechanisms. While the effects on cell cycle progression of viral infection, and of individual viral genes outside the context of viral infection have been described, it is now important to synthesize these two experimental approaches to gain a more complete understanding of how and why human cytomegalovirus infection affects cell cycle progression.

2. INTRODUCTION

This review focuses on the ability of human cytomegalovirus (HCMV) to manipulate the cell cycle of the infected host cell, thus altering the intracellular environment to one in which the virus enjoys a growth advantage. After a brief introduction to the virus and a short general review of cell cycle progression, we will discuss the observed effects of viral infection on cell cycle progression. Next we will look at the effects of viral infection or of individual viral proteins on different classes of cell cycle regulatory proteins, including tumor suppressors, oncogenes, cyclins, the cyclin-dependent kinases and their inhibitors. Finally we discuss how individual viral proteins affect cell cycle progression, and address future avenues of research that may lead to a more complete vision of how the virus benefits from its ability to modulate the cell cycle.

3. HUMAN CYTOMEGALOVIRUS

HCMV (reviewed in 1) is a beta-herpesvirus that is maintained in a latent state for the life of the infected individual. Primary infection or reactivation of a latent infection causes severe disease in the absence of an effective immune system, such as in patients with AIDS and those treated with immunosuppressive drugs during organ transplantation. Congenital infection produces birth defects, most commonly hearing loss. The majority of the population is infected by HCMV.

The viral genome is a linear, double-stranded DNA molecule of approximately 235 kilobases that contains more than 225 open reading frames encoding proteins larger than 100 amino acids. In the virion, the DNA is enclosed in an icosahedral capsid, which is surrounded by a set of about 30 viral proteins (2) and 5 RNAs (3) organized into a structure termed the tegument, and this region in turn is surrounded by a host-cell derived lipid bilayer containing many virally encoded glycoproteins. Thus upon infection, viral envelope and tegument proteins can begin to modulate the infected cell prior to gene expression from the viral genome. Viral gene expression progresses in an ordered cascade of immediate early, early, and late transcription that is typical of all herpesviruses. Subsequent to viral DNA replication and the synthesis of late genes, virions are assembled and released completing the infectious cycle. It should be noted that for HCMV, completion of an infectious cycle takes a relatively long time, somewhere between 72 and 96 hours. That the virus is maintained in the host cell for such an extended period implies that it must interact efficiently with the host cell to ensure completion of the infectious process.

There are four known HCMV genes that affect cell cycle progression. Two are immediate early proteins (IE1 and IE2), which are the first class of proteins to by synthesized upon infection. The other two (UL69 and pp71) are found in the viral tegument and are delivered to the cell upon infection, thus they have the potential to act even before immediate early genes. It is possible that there are also additional, as yet unidentified viral regulators of the cell cycle. The effects of each of these proteins will be addressed below.

4. CELL CYCLE

Cell cycle transit (reviewed in 4,5) in mammalian cells is controlled by a family of cyclin-dependent kinases (cdk's), whose activity is dependent upon the correct subset of phosphorylations, and their physical association with a cyclin protein. The synthesis and degradation of cyclin proteins are tightly regulated, and this, as well as the action of the cyclin-dependent kinase inhibitors (cki's) contributes to the control of cell cycle progression.

In early G1 (or quiescent, G0 cells), cdk's are inactive, either because their obligate cyclin partner has not yet been synthesized, or because of the action of the cki's. Because of this, the product of the retinoblastoma tumor suppressor gene (Rb) is hypophosphorylated, and is found in a complex with the E2F family of transcription factors (6). This complex represses transcription from E2F responsive promoters, and since many genes required for S phase progression respond to E2F, cellular DNA replication is prevented. Mitogenic stimulation induces cellular transcription factors as well as kinases and phosphatases which participate in signal transduction cascades leading to cell cycle entry (7,8).

As cells progress through G1, cdk4/cyclin D and cdk5/cyclin D become activated, and phosphorylate Rb, liberating E2F, which can now activate transcription from promoters with E2F binding sites, such as the cyclin E promoter (9). Synthesis of this cyclin, and its subsequent pairing with cdk2 produces an active kinase which is thought to be important for late G1/early S phase events. As cells enter S phase, cyclin E-associated kinase activity decreases and cyclin A-associated kinase activity increases, leading to cellular DNA replication, and further cell cycle progression. Cyclin A- and cyclin B-associated kinase activity increases throughout S and G2 phase, and triggers entry into mitosis. Prior to completion of mitosis, they are degraded by the APC/cyclosome complex, allowing cytokinesis and the start of another cell cycle (reviewed in 10).

5. VIRUS EFFECTS ON THE CELL CYCLE

There exists an extensive body of early literature about the effects of HCMV on the host cell metabolism (reviewed in 11). Though controversial, a consistent theme was that HCMV can induce cellular genomic DNA synthesis in non-permissive cells that undergo an abortive infection, but not in permissive cells where the viral replication cycle is completed, and progeny virions are produced. We will focus mainly on recent experiments performed almost entirely on fully permissive cells, most often human fibroblasts. It should be noted, however, that a lytic infection of fibroblasts in tissue culture may be somewhat different from what occurs during primary infection or reactivation from latency *in vivo*.

The effects of HCMV infection on cell cycle progression of permissive cells in tissue culture have been investigated mainly by two methods. In the first approach, the cell cycle position of infected cells is determined at different times post infection by quantitating the cellular DNA content by flow cytometry. The second approach has been to determine the level and activity of known cell cycle regulatory proteins after infection with HCMV.

A consensus view has arisen from these studies (12,13,14,15), in which HCMV can both stimulate as well as arrest cell cycle progression. The majority of the work has been performed on quiescent cells, where infection results in a synchronization of cells in late G1 or early S, and host genomic DNA synthesis is not observed (Figure 1). These cells maintain high levels of cyclin E and B protein and associated kinase activity, but cyclins D and A are absent (Figure 2).

HCMV-induced alteration of the cell cycle, while resembling a mitogenic response, has significant differences as well. For example, in sub-confluent quiescent cells, HCMV induced cyclin E (14) and cyclin B (W. Bresnahan, personal communication), but not cyclins D or A, and no genomic DNA synthesis was detected with a sensitive assay. This is quite different from serum stimulation of sub-confluent quiescent cells, where all of these cyclins are activated and cellular DNA replication is induced. Most importantly, the virus had identical cell cycle effects on contact inhibited cells (14), where serum stimulation does not induce cyclin or genomic DNA synthesis, indicating that the HCMV-induced alterations

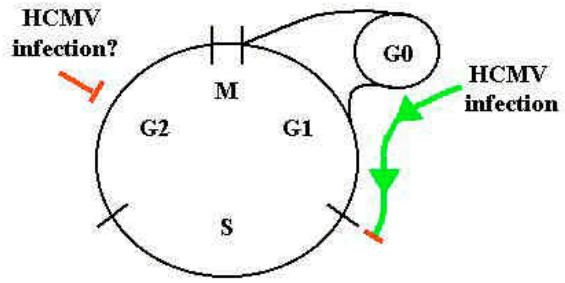

Figure 1. HCMV modulation of the cell cycle. Infection of quiescent (G0) cells with HCMV induces their re-entry into the cell cycle (green line with arrows). However, they arrest at the G1/S border before synthesis of the host cell's genomic DNA begins (red bar at end of green line). Infection of asynchronous, cycling cells results in a G1 arrest and, in some instances, an arrest in G2 (red bar).

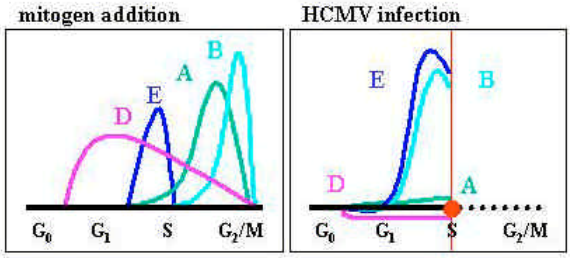

Figure 2. Cyclin synthesis is altered in HCMV-infected cells. Mitogen addition to quiescent (G0) cells induces a cascade of cyclin synthesis, with individual cyclins appearing at characteristic points during the ensuing cell cycle. Letters refer to individual cyclin proteins, and curves represent changes in the levels of cyclin mRNA and kinase activity. HCMV infection of quiescent cells results in elevated levels of cyclin E and B, but not D and A, and causes a cell cycle arrest at the G1/S border. The red dot and line mark the cell cycle arrest point, and the dotted black line indicates that this portion of the cell cycle is not reached.

are not only different from, but do not depend upon a mitogenic response. Other reports have also demonstrated a dysregulation of cyclin synthesis (12,16). However, interpretation of these studies is complicated by the experimental methods in which cells were simultaneously infected and induced with serum, making it impossible to determine which stimulus was responsible for the observed effects.

Thus, infection of quiescent cells stimulates their re-entry into the cell cycle and progression through G1 phase, with an eventual cell cycle arrest at the G1/S border (Figure 1). The ability to inhibit the G1/S transition was confirmed by demonstrating that prior infection can prevent entry into S phase after subsequent serum stimulation (13).

A few studies have also been performed on cycling cells. For example, cellular growth curves performed on HCMV infected fibroblasts demonstrated that infected cells were prevented from dividing (13). Moreover, infection of cycling cells did not have a dramatic effect on the percentages of cells in each stage of the cell cycle, implying that the cells arrested in both G1 and G2. However, a study of S phase synchronized and subsequently infected cells failed to demonstrate an arrest in G2 (16). Instead, the S phase infected cells appear to complete cellular DNA synthesis and mitosis prior to arresting in G1. Interestingly, the infectious cycle was slowed in the S phase infected cells, a finding which provides a rationale for why the virus prevents G1 cells from entering the S phase.

Thus, by both an induction of quiescent cells and an inhibition of entry into S phase, HCMV appears to synchronize infected cells in late G1/early S, where they express cyclin E but not cyclin A. This unique cell cycle compartment is presumably favorable for viral replication since the precursors for DNA replication are available but not being consumed in the synthesis of the host cell's genomic DNA. The delay in the viral life cycle observed in cells infected during the S phase (16) supports this hypothesis. Since a G2 arrest has been observed under some (12,13) but not other (16) experimental conditions, the significance of preventing cells from entering or completing mitosis is not clear.

6. VIRUS EFFECTS ON CELL CYCLE REGULATORY PROTEINS

6.1. Rb tumor suppressor family

As described above, the Rb proteins regulate progression from G0 through G1 and into S phase by repressing transcription from E2F responsive promoters. The Rb family consists of three members, Rb, p107 and p130 (17), and the E2F family has six members (18). The multiple family members presumably allow for a tighter, yet more versatile regulation. The mRNA level for Rb in infected cells is ten-fold higher than in mock-infected cells (19,20). Thus, it is not surprising that the protein accumulates in infected cells. Interestingly, only hyperphosphorylated forms are observed in infected cells. This is likely to result from both the specific degradation and phosphorylation of the hypophosphorylated forms of Rb (see below).

HCMV has at least three proteins that attack the Rb family and E2F-mediated transcriptional mechanisms, each of which may be more prominent at different times of the infection process. IE1 appears to target E2F-mediated promoters in at least three ways. First, IE1 binds to p107 in infected cell lysates (21), and *in vitro* was shown to bind to the N-terminus of p107 (22), a region without homology to Rb. Thus it is not surprising that IE1 can alleviate p107-mediated, but not Rb-mediated repression of an E2F responsive promoter (21). IE1 also could overcome p107-mediated cell cycle repression in Soas-2 cells (22). Second, IE1 binds to E2F-1 itself, and transactivates the DHFR promoter only if the E2F binding sites are intact (23). Third, IE1 has protein kinase activity (24), and its substrates include p107 and p130 (but not Rb), as well as E2F-1, E2F-2, and E2F-3, (but not E2F-4 or E2F-5). IE1 also autophosphorylates. A conserved ATP binding site in

the protein is required for kinase activity as well as activation of E2F-dependent transcription. IE1 can also disrupt E2F-4/p107 complexes (24), but with an efficiency less than that of E1A (22). Because these proteins disrupt the complex by different mechanisms [E1A by displacement (25) and IE1 by phosphorylation (24)], perhaps this is not surprising. It is interesting that IE1 appears to target p107 since this protein is found at its highest levels only during the S phase. Thus, during infection of quiescent cells, perhaps other mechanisms (see below) are more important to initiate cell cycle progression, and once cells approach late G1/early S, IE1 functions to maintain that response.

IE2 has been reported to bind to Rb (26,27,28,29). Binding to p107 or p130 has not been reported. IE2 binding to Rb was shown to inactivate the ability of Rb to repress a synthetic promoter through its E2F binding sites (26). Overexpressed Rb also inhibited the ability of IE2 to activate (27) and repress (26) transcription. However, IE2 was unable to counteract a G1 arrest imposed by ectopic expression of Rb in Saos-2 cells (28). While IE2 prevented morphological changes induced in these cells by Rb, the significance of this is unclear not only because the relationship between cellular morphology and cell cycle progression in this system is not understood, but also because the regions required for Rb binding (see below) were dispensable in this assay (28).

The interaction of IE2 and Rb has been demonstrated most often by monitoring the binding of *in vitro* translated, radiolabeled proteins to GST-fusion proteins produced in bacteria, although other assays such as far-western blots and a dual immunoprecipitation from HCMV infected cells labeled with ^{32}P-orthophosphate have also been employed (26). In one of the reports, a region between IE2 amino acids 290-390 was shown to be required for this *in vitro* interaction (26). This region is adjacent to, but does not overlap the domain required for TBP binding to IE2. In addition, an Rb mutant with a deletion in the pocket domain was unable to bind IE2. This is interesting since the interaction of other viral and cellular proteins that bind in the RB pocket is mediated by an LxCxE motif, and since IE2 does not have such a sequence, it may interact with the RB pocket in a novel way.

A subsequent report (28) identified three regions of IE2 that could bind to Rb. However, some of these regions also inhibited this interaction when the identities of the GST-fusion and labeled proteins employed in the assay were switched. Also, the only mutations that reduced binding more than 50% required the removal of almost half of the coding sequence, from amino acids 85-370. In these experiments, removal of amino acids 289-369 which had been previously implicated in binding to Rb (26) reduced the interaction by only 7%. Furthermore, in these assays the binding of IE2 to Rb was indistinguishable from that of IE2 to TBP (28). Since Rb can associate with TBP (30) and since TBP is undoubtedly in the *in vitro* transcription/translation extracts employed, it is possible that TBP or its associated proteins mediate the observed interaction of IE2 and Rb in these assays.

The interaction of IE2 and Rb has not been explored by IP-western experiments in transfected or infected cells even though all the required materials (expression vectors, antibodies, etc.) are readily available. In light of the recent data on the ability of IE2 to alter the cell cycle in a unique manner (see below), the way in which IE2 may interact with the Rb family members and the consequences of any such interaction should be thoroughly re-examined.

Finally, pp71, through an LxCxD motif, targets the hypophosphorylated members of the Rb family for degradation by the proteasome, resulting in the stimulation of quiescent cells to enter the cell cycle and proceed to the S phase (Kalejta and Shenk, unpublished observations). As pp71 is located in the virion, it has the potential to be the first viral protein to act directly on the Rb family members. Thus HCMV has multiple proteins that attack the Rb family of tumor suppressors, each with its own unique mechanisms.

6.2. p53 tumor suppressor family

The p53 tumor suppressor (reviewed in 31) senses cellular stress, such as DNA damage, activated oncogenes, or abnormal proliferation, and is responsible for ensuring that cells that have lost the ability to regulate their own growth are terminated. It accomplishes this either by inducing a cell cycle arrest in G1 or G2 until the cellular damage can be fixed, or by inducing apoptosis if the cell is beyond repair. p53 is extremely labile but becomes stabilized under conditions of cellular stress, and acts as a sequence-specific DNA binding transcription factor. It activates as well as represses transcription, and also has some transcription-independent activities. The p53 pathway is impaired in the majority of human cancers most often by mutation of p53 itself, although it is also thought to occur by cytoplasmic sequestration. Thus, it is perhaps the most important tumor surveillance mechanism in human cells.

Viruses also target p53, presumably to inhibit both its cell cycle and apoptotic functions to ensure efficient viral replication. Viral proteins such as adenovirus E1B 55K and SV40 T Antigen bind to and inhibit the transcriptional abilities of p53, and papillomavirus E6 induces its degradation.

HCMV disruption of the p53 pathway was first established in smooth muscle cells (SMC) of restenosis lesions (32). Restenosis is a proliferation and migration of smooth muscle cells into arteries after angioplasty. A large percentage of SMC from restenotic lesions had high levels of p53, which correlated with the presence of HCMV. Cells cultured from such lesions expressed IE2 and high levels of p53. Moreover, HCMV infection of SMC *in vitro* also induced p53 accumulation in a time frame consistent with IE2 expression (32). Subsequent studies confirmed the ability of HCMV to induce high levels of p53 (12,33,34), and determined that the induction occurred by a stabilization of the protein, not by an increase in transcription (35). p53 accumulation was also detected in cells transfected with an expression construct that made both IE1 and IE2 (33). Recently, either IE1 or IE2

individually have been demonstrated to increase cellular p53 levels (36).

Binding studies detected an interaction between IE2 and p53 that inhibited the ability of p53 to activate transcription (32) and to induce apoptosis (37). Further work confirmed this result and concluded that IE2 bound to p53 acts as a transcriptional repressor, with the N terminus of IE2 binding to p53 and its C-terminus functioning as a repressor domain (38). An IE2/p53 complex is also thought to repress transcription from the HCMV UL94 late promoter (39).

In one report (40), an interaction between IE2 and p53 was observed but the ability of p53 to arrest cells in G1 was not compromised. However, that study was performed with cells lines that constitutively express IE2 from an integrated retrovirus, and those cells were only able to stimulate the HCMV UL 112-113 early promoter by 1.4 fold. Transient transfection assays with this promoter detected a 62-fold increase of promoter activity by IE2 (41,42). Thus it is likely that the IE2 protein constitutively produced by those cells is at least partially non-functional. It was later found that the IE2 allele in these cells contains mutations which change amino acids in critical regions of the protein (43). In fact, no cell line exists that makes a completely functional IE2 (i.e. one that can complement a viral deletion mutant of IE2), and it is difficult, although not impossible to generate recombinant retroviruses, baculoviruses or adenoviruses that synthesize a fully functional IE2. This is most likely due to the induction of mutations in essential cellular genes by IE2 (see below).

In addition to IE2, the mtrII oncoprotein of HCMV has been shown to bind p53 and inhibit its ability to activate transcription (44). Recently the open reading frame that encodes the 79 amino acid mtrII protein has been shown to be part of a newly discovered viral homologue of interleukin-10 (45). The significance of this gene fragment to viral transformation and modulation of p53 is unclear.

The subcellular localization of p53 is also disrupted by HCMV. As mentioned above, p53 is known to be sequestered in the cytoplasm. This was observed in infected endothelial cells, and correlated with their resistance to apoptosis induced by serum withdrawal (34). Another report has demonstrated the accumulation of p53 in electron dense nuclear structures that resemble viral replication centers (35). More work will be required to further define how binding by viral proteins and cellular redistribution work to antagonize the p53 pathway, presumably leading to more efficient viral replication. Likewise, the role, if any, of the other p53 family members (reviewed in 46) will need to be addressed before we have a complete picture of the role of p53 during HCMV infection.

6.3. Oncogenes

Although other viruses have been implicated as cancer-causing agents, the role, if any, of HCMV in the induction of human cancers is a controversial issue (reviewed in 47), one complicated by the ubiquitous nature of this infectious agent. Creation of a malignant cell generally involves cellular transformation as well as an inhibition of apoptosis, and HCMV encodes proteins that can transform cells (reviewed in 47) and inhibit apoptosis (48,49). HCMV infection also induces changes in cellular oncogenes that play roles in cell cycle progression, and a more thorough examination of the roles that cellular oncogenes may play in HCMV infection is warranted.

For example, when quiescent cells are stimulated with mitogens to enter the cell cycle, signal transduction pathways become mobilized (7,8) and result in the activation of cellular oncogenes, which in turn stimulate cellular growth and division. As mentioned above, infection with HCMV leads to a cellular response similar to, yet distinct from mitogenic stimulation (reviewed in 11,50,51). HCMV infection induces the transcription of the oncogenes fos, jun and myc, but not others such as ras, myb or mos (52,53). The increased transcription of these genes did not require viral protein synthesis and thus likely results from either the interaction of the virus with receptors on the cell membrane or from the action of transcription factors released from the viral tegument upon infection.

Other experiments have demonstrated that, while HVMC does not activate the MAP kinase pathway in infected quiescent cells, if this pathway is first activated by serum stimulation prior to infection, HCMV maintains the activity of the pathway for a much longer time than observed in non-infected cells (54). The maintenance of the pathway appears to be a result of a decreased rate of de-phosphorylation of the Erk family of protein kinases. Moreover, the viral IE2 protein was shown to be phosphorylated *in vivo* on map kinase sites and *in vitro* by Erk2. Substitution of these sites with alanine residues created a protein with increased ability to transactivate certain promoters (55), leading to the speculation that Erk phosphorylation, though it stimulates cellular transcription factors, was a way to down regulate the activity of IE2 for the benefit of the infectious process. However, recent experiments indicate that a virus encoding only the non-phosphorylatable IE2 has identical growth kinetics and transcription of viral genes as the wild type virus (56), indicating that these phosphorylations may not play a significant role during lytic infection *in vitro*.

6.4. Cyclins and cyclin regulators

When quiescent cells are stimulated with mitogens, synthesis of the D type cyclins is induced (57,58). Cyclin D performs two functions to help induce progression into the S phase. It phosphorylates Rb disrupting Rb-E2F complexes, thus liberating E2F to transactivate the expression of many genes, among them cyclin E, and it binds to and sequesters the cyclin dependent kinase inhibitors p21 and p27, both of which inhibit cyclin E-dependent kinase activity. Both of these activities lead to induction of cyclin E dependent kinase activity, which further stimulates cell cycle progression. Interestingly, HCMV infection does not induce cyclin D

expression, yet still induces high levels of cyclin E dependent kinase activity. It appears to accomplish this by the same general strategy as cyclin D, decreasing the levels of the cki's p21 and p27, and transcriptionally activating the cyclin E promoter, but achieves this by entirely different means.

6.4.1. Cyclin-dependent kinase inhibitors

HCMV attacks p21 by three mechanisms: modulation of mRNA levels, direct binding and inactivation, and degradation. Microarray analysis demonstrated that in contact inhibited fibroblasts infected with HCMV, the p21 mRNA was decreased four-fold (20). Furthermore, IE2 interacts with p21 *in vitro* and in a yeast two-hybrid screen, although an *in vivo* interaction in transfected or infected cells has not been demonstrated (59). This interaction impairs the ability of p21 to act as a cki *in vitro*. HCMV infection also induces the degradation of p21, not by the proteasome, but by the calcium-dependent protease calpain (60). Thus, a combination of these three activities likely results in impaired p21 function, allowing the high levels of cyclin E-dependent kinase activity observed in infected cells to be achieved, which, presumably could phosphorylate another cki, p27. Phosphorylation of p27 induces its degradation, and decreased levels of p27 have been observed in HCMV infected cells (14). However, the decrease in p27 protein is also likely to be a result of decreased protein synthesis, since the mRNA is downregulated seven-fold upon infection (20). Interestingly, the decrease in mRNA level was observed with both active and UV-inactivated virus, implying that it is an event triggered either during or shortly after viral entry by a component of the virion.

6.4.2. Cyclins

HCMV infection results in elevated levels of cyclin E mRNA, protein, and associated kinase activity (12,14,20). Since cyclin E induction is one of the universally agreed upon results of HCMV infection, many groups have been investigating how the cyclin E promoter is activated by viral infection or by individual viral proteins. In one study (61), IE2 was shown to transactivate a cyclin E promoter luciferase construct in transient assays. IE2 can activate transcription both by directly binding to DNA and by interacting with members of the basal transcription machinery (reviewed in 1). IE2 was found to bind to the cyclin E promoter *in vitro*, and binding required the DNA-binding zinc-finger of IE2 as well as a consensus IE2 binding site identified in the cyclin E promoter (61). Interestingly, the E2F sites that regulate the cell cycle dependent expression of cyclin E were not required for the IE2-mediated induction. Although that study (61) was preformed on artificial promoter constructs, the result is supported by recent evidence from microarray analysis of mRNA from cells infected with a recombinant adenovirus expressing IE2, in which cyclin E, as well as many E2F-responsive genes were found to be induced (62). The ability of IE2 to induce cyclin E transcription is also consistent with its ability to stimulate cell cycle progression (see below).

However, in a different series of experiments (63), IE2 was found not to transactivate the cyclin E promoter when expressed from a recombinant baculovirus either by itself, in combination with IE1, or with viral tegument proteins delivered by infection with UV inactivated HCMV virions. The baculovirus-expressed IE2 was demonstrated to be a competent transactivator by inducing expression from the viral UL112-UL113 early promoter (64). However, that promoter has two sites that respond to IE2, an ATF/CREB binding site which is primarily responsible for IE2 mediated transactivation at early times after infection, and a consensus IE2 binding site which mediates activation much later in infection (65). These sites are presumably targeted by IE2 through different mechanisms, with the ATF/CREB site activated by the ability of IE2 to interact with other transcription factors, and the IE2 binding site requiring IE2 binding to the DNA. Perhaps the baculovirus expressed IE2 retains its ability to bind to other transcription factors, but has lost its ability to bind DNA. As mentioned above, recombinant viruses and cell lines that express fully functional IE2 have been difficult to generate. This would explain why the baculovirus-expressed IE2 could activate the UL112-113 promoter but not cyclin E. In the same study, it was observed that during HCMV infection, viral early gene expression was required for cyclin E induction (63). Moreover, a novel DNA binding complex was detected on the endogenous cyclin E promoter. Although this complex was in the same region as the previously mapped IE2 binding site (61), it appears to require an intact E2F binding site and, because of its migration properties, was proposed to contain either uniquely phosphorylated E2F-4, Dp-1 or p130, or an additional, unidentified protein.

Finally, a recent microarray analysis observed that cyclin E mRNA levels increased five-fold in contact inhibited cells infected with UV-inactivated virus (20). As irradiated viruses are unable to synthesize viral proteins, this implies that IE or early genes are not required to activate transcription of the cyclin E gene, and that a virion protein can accomplish this. The viral envelope protein gB has been shown to stimulate the accumulation of many cellular mRNAs (66,67) and may be responsible for the increase in cyclin E. However, the tegument protein pp71 has been demonstrated to induce G0 cells to enter the cell cycle and proceed into the S phase (Kalejta and Shenk, unpublished observations), and thus may be responsible for the increase in cyclin E, either by direct transactivation or as a result of the induced cell cycle progression.

The induction of cyclin A by HCMV is somewhat controversial, as it has been detected in some experiments (12,59) but not in others (14), and has been seen to decrease in still others (16). As cyclin A is a central regulator for entry into the S phase, it is important to resolve the conflicting data. As of yet, no detailed analysis of how the virus or viral proteins may act at the cyclin A promoter has been undertaken.

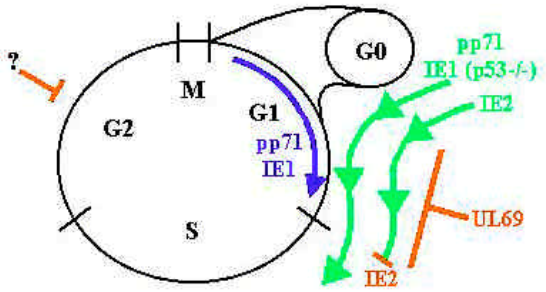

Figure 3. HCMV proteins that alter cell cycle progression. Both pp71 and IE1 can accelerate cycling cells through the G1 phase (blue arrow). pp71 and IE1 (in the absence of p53), can induce quiescent (G0) cells to enter the cell cycle and proceed into the S phase (green line with arrows). IE2 stimulates quiescent cells to re-enter the cell cycle but then arrests them at the G1/S border (green line with arrows and red bar). UL69 arrests cells with a G1 DNA content. The exact location of the arrested cells within the G1 phase has not been determined (red bar). A viral protein that may induce a G2 arrest has not been identified (question mark).

The ability of HCMV to dramatically increase the level and associated kinase activity of cyclin B has not been extensively examined, but a few interesting findings have surfaced. By simultaneously monitoring DNA content and cyclin B levels by flow cytometry, it was observed that cells that maintain a G1 DNA content contained high levels of cyclin B protein (15). This further strengthens the concept of an HCMV disruption of cell cycle proteins since cyclin B is normally absent from G1 cells. How do cells stopped in G1 accumulate cyclin B, and why? HCMV does not appear to dramatically affect the transcription of the cyclin B gene (16), thus regulation of the levels of this protein during HCMV infection likely occur post-translationally. Interestingly, B-type cyclin dependent kinase activity has been shown to prevent re-replication from origins in several eukaryotic species (reviewed in 68), and thus the high levels of cyclin B may play some role in the ability of the virus to prevent cellular DNA replication. Furthermore, in some instances HCMV has been shown to arrest cells in G2 (12,13), and high levels of cyclin B inhibits the completion of mitosis. The causal relationships between these phenomenon remain to be investigated.

Thus although cyclin E appears to be a prime target during HCMV infection and we are beginning to understand the mechanism of its induction, the few experiments that have addressed the other prominent cyclins have produced some very interesting questions which merit further study. An obvious question is why doesn't HCMV just activate cyclin D in a similar fashion as mitogenic stimulation to effect cell cycle progression? The answer is unclear, but perhaps the ability of cyclin D to activate the transcription of cyclin A, which the virus downregulates, prevents the virus form using cyclin D and necessitates other means to activate cyclin E. In fact, cyclin D mRNA levels have been shown to drop almost ten-fold upon infection (19). Another interesting question is why of the three G1 cyclins, HCMV appears only to activate cyclin E. Are cyclin D and A responsible for a different subset of regulation that lead more directly to initiation at genomic origins of DNA replication, with cyclin E more responsible for making sure all of the enzymes that are required for DNA synthesis will be there? Further work will be needed to answer these intriguing questions.

6.4.3. Cyclin-dependent kinases

In addition to the cyclins, the role of the cyclin-dependent kinases themselves during HCMV infection has been examined. Because the virus induces high levels of cyclin E dependent kinase activity, it was not surprising to observe that HCMV infection requires cdk activity (69). The cdk inhibitor roscovitine was able to dramatically reduce virus yield. Late gene synthesis and DNA replication were also inhibited by roscovitine. In a complementary approach, a transiently transfected dominant negative cdk allele was shown to inhibit late gene synthesis in cells subsequently infected with HCMV (69). Much like the situation in uninfected cells, it is clear that cdk activity is an important cellular signal, but the critical substrates remain elusive. It will be interesting to examine if the substrate specificity of the cdk's is altered by viral infection.

7. VIRAL PROTEINS THAT ALTER CELL CYCLE PROGRESSION

To date, four viral proteins have been demonstrated to alter cell cycle progression outside the context of viral infection (Table 1; Figure 3). UL69 is a transcription factor found in the viral tegument, and arrests cells with a G1 DNA content by an unknown mechanism (70). The point in G1 at which the cells are arrested has not been examined. UL69 is required for efficient viral replication, and viruses lacking UL69 are still able to arrest cells in G1, but do so less efficiently than wild type virus (71). This implies that although UL69 plays a role in the viral induced G1-arrest, other proteins also participate (see below).

The pp71 protein is also a transcription factor located in the viral tegument (72). It can increase the infectivity of transfected viral genomic DNA (73), and is required for viral replication at low multiplicities of infection (74). A viral mutant lacking pp71 has decreased immediate early gene synthesis upon infection, which most likely contributes to the inability of the virus to replicate. pp71 also regulates cell cycle progression. It accelerates cells through G1 by an unknown mechanism, and can also induce quiescent cells to re-enter the cell cycle and enter S phase (Kalejta and Shenk, unpublished observations). Stimulation of quiescent cells occurs through the proteasome-mediated degradation of the hypophosphorylated forms of the Rb family members. Since transcript levels of several proteasome subunits increase more than 10-fold during infection (19), perhaps proteasomal degradation is a more pervasive event during HCMV infection than is currently realized.

Table 1. HCMV cell cycle regulators

Protein	Cell cycle affect	Mechanism	References
UL69	G1 arrest	Unknown	70, 71
pp71	Accelerate G1	Unknown	Kalejta & Shenk, unpublished
(UL82)	G0 to S	Degrade Rb family	Kalejta & Shenk, unpublished
IE1	Accelerate G1	Unknown	J.A. Clifford, personal comm.
(UL123)	G0 to S in p53-null cells	Unknown	36
IE2	G0 to early S block	Unknown	36, 43, 78, 79
(UL122)			

IE1 is a transcription factor that regulates many viral and cellular promoters. Like pp71, it is required for replication at low multiplicities of infection (75), and has been shown to accelerate cells through the G1 phase (J.A. Clifford, personal communication). IE1 has also been shown to induce DNA synthesis in quiescent cells, but only in the absence of wild type p53 (36) or p21 (T. Kowalik, personal communication). The mechanism for this stimulation is not known, however, IE1 has been implicated in altering E2F-regulated transcription through binding to the p107 protein, and has protein kinase activity. Experiments with IE1 mutants unable to bind p107 or act as a kinase should determine if either or both of these activities are required for its ability to stimulate cell cycle progression, or allow viral replication at low multiplicities of infection.

The other major immediate-early protein, IE2 is a promiscuous transcriptional activator and repressor. It associates not only with the basal transcription machinery, but also binds sequence-specifically to DNA. IE2 can also inhibit apoptosis, and along with E1A, can transform cells (48,76). A mutant viral genome lacking IE2 and propagated in *E. coli* as a bacterial artificial chromosome (BAC) does not produce progeny virus upon transfection into permissive cells (77), demonstrating that IE2 is strictly essential for viral replication. Indeed, many attempts to generate IE2 null viruses have failed, and no cell line that can complement an IE2-mutant virus has been described.

IE2 has very interesting effects on the cell cycle. It arrests cycling cells with an apparent G1 DNA content (78). However, these cells are actually in early S phase (79), because they can incorporate a small amount of exogenously added BrDU into their DNA, indicative of a limited amount of DNA synthesis. They also arrest with high levels of cyclin E-dependent kinase activity, demonstrating that the arrest point is further through the cell cycle than the G1 restriction point. IE2 also prevents quiescent cells stimulated with serum from traversing through the S phase (43). The amount of DNA synthesis observed during the S-phase block can vary, perhaps depending upon experimental conditions (43,79). Interestingly, the ability of IE2 to activate transcription is not required for its ability to arrest cell cycle progression (78,79).

The ability of IE2 to arrest the cell cycle may explain some earlier experiments whose results were difficult to interpret at the time. For instance, IE2 can counteract Rb-mediated transcriptional repression in Saos-2 cells, but not the G1 arrest imposed by Rb (28). Also, IE2 can rescue the transcriptional defects in the TAF-250 mutant cell line ts-13, but can't restore cell cycle progression (80). Furthermore, the difficulty in generating cell lines that constitutively express a fully functional IE2 can now be explained. Along with its ability to prevent cells from progressing through the S phase, IE2 also has the ability to induce quiescent cells to re-enter the cell cycle and proceed at least until early S phase, where BrDU incorporation can be detected (36). In these experiments, BrDU incorporation was determined by counting positive cells after infection of quiescent cells with recombinant adenoviruses expressing IE2 and control proteins. It would be interesting to revisit these experiments using flow cytometry to determine how far these cells actually get into the S phase. Very recent work suggests that the ability to activate transcription is required for the cell cycle stimulation mediated by IE2 (C. Hagemeier, personal communication). As IE2 turns on cyclin E and other E2F-regulated genes, this is not surprising.

The mechanism of cell cycle arrest by IE2 is unknown. However, IE2 has been demonstrated to be mutagenic when expressed transiently in cells (76). Cells have safety mechanisms called checkpoints (reviewed in 81) which detect DNA damage and, by stopping entry into G1 (G1 checkpoint) or progression through the S phase (S phase checkpoint), prevent the damage from being fixed into the genome by DNA replication. When the damage is repaired, cell cycle progression can again proceed. G1 checkpoint-induced cell cycle arrest is mediated by a reduction of cdk activity. The means through which S-phase checkpoints stop DNA replication is not known. Since IE2-arrested cells retain high levels of cdk activity, perhaps DNA damage caused by IE2 activates S-phase, but not G1-phase checkpoints, thus arresting cells in S phase. The mechanism through which IE2 introduces mutations is unknown. However, determining if specific mutations in IE2 can separate these two affects (mutagenesis and cell cycle arrest) could begin to reveal if these two activities are linked.

Finally, it should be noted that, with its ability to stimulate quiescent cells yet subsequently arrest them in early S, IE2 appears to have very similar effects on the cell cycle as HCMV itself. A combination of the activities of pp71 and UL69 may have a similar effect. Thus, it appears once again that a redundancy exists in how HCMV can effect cell cycle progression, perhaps with these proteins all cooperating to achieve the desired goals.

8. PERSPECTIVES

Over the last few years, many observations have been made about how HCMV manipulates the cell cycle. Experiments were designed to determine what changes occur during infection, and what individual viral proteins can affect the cell cycle. Armed with this information, the challenges that lie ahead are to switch from experimental approaches that ask what happens, to ones that ask how does it happen. This will require determining the molecular mechanisms behind the cell cycle changes induced by HCMV and its individual proteins.

First and foremost, the interaction of viral proteins with cell cycle regulators such as Rb and p53 should be confirmed, preferably in infected cells, by co-immunoprecipitation experiments and western blotting with specific antibodies. In vitro binding of radioactive proteins to GST-fusions does not provide the specificity required to unequivocally demonstrate an interaction. Furthermore, small deletions and preferably, point mutations that disrupt binding should be generated and then tested for cell cycle function. Whenever possible, these cell cycle functions should be tested in fully permissive cells. Most importantly, point mutants defective in cell cycle regulation should be incorporated into viruses to test their effects on viral infectivity. Since BAC systems are now commonly employed to generate mutant viral genomes (reviewed in 82), this is no longer the daunting task it was a few short years ago. Once a defined system for latency is developed, the ability of these mutants to achieve and reactivate from latency should be examined.

Finally, it has become obvious that there is redundancy of function in how HCMV manipulates cell cycle progression. For example, the Rb family is attacked by no less than three viral proteins (IE1, IE2, and pp71). Not only are cell cycle regulators modulated by different proteins, but through different mechanisms as well, including phosphorylation and possibly other post-translational modifications, increases or decreases in transcript levels, alteration of protein stability, and direct binding to alter protein function. This redundancy should be kept in mind when designing and interpreting experiments.

We now have the basic information and technology needed to ask important, fundamental questions in HCMV biology as it relates to the cell cycle. Our results should not only provide information about HCMV replication in the hope of designing anti-viral agents, but also about cell cycle progression, and how dysregulation of the cell cycle can lead to cancer.

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