Human cytomegalovirus inhibits a DNA damage response by mislocalizing checkpoint proteins

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The DNA damage checkpoint pathway responds to DNA damage and induces a cell cycle arrest to allow time for DNA repair. Several viruses are known to activate or modulate this cellular response. Here we show that the ataxia-telangiectasia mutated checkpoint pathway, which responds to double-strand breaks in DNA, is activated in response to human cytomegalovirus DNA replication. However, this activation does not propagate through the pathway; it is blocked at the level of the effector kinase, checkpoint kinase 2 (Chk2). Late after infection, several checkpoint proteins, including ataxia-telangiectasia mutated and Chk2, are mislocalized to a cytoplasmic virus assembly zone, where they are colocalized with virion structural proteins. This colocalization was confirmed by immunoprecipitation of virion proteins with an antibody that recognizes Chk2. Virus replication was resistant to ionizing radiation, which causes double-strand breaks in DNA. We propose that human CMV DNA replication activates the checkpoint response to DNA double-strand breaks, and the virus responds by altering the localization of checkpoint proteins to the cytoplasm and thereby inhibiting the signaling pathway.

ionizing radiation | ataxia-telangiectasia mutated pathway

The DNA damage checkpoint detects DNA damage and responds by activating signaling pathways, which cause a cell cycle halt while the damage is repaired or induce apoptosis if it cannot be repaired (1). There are two branches of the checkpoint response pathway that respond to different types of DNA damage (2, 3). The first responds to ionizing radiation (IR) and other agents that cause double-strand (ds) breaks in DNA. The ds break is recognized by the Mre11-Rad50-Nbs1 complex, which recruits and activates ataxia-telangiectasia mutated (ATM) kinase. The second branch responds to UV radiation (UV) and other agents that induce the accumulation of stalled replication forks and subsequently single-stranded DNA stretches. The single-stranded DNA is coated by replication protein A, and it recruits a complex of ATM- and Rad3-related (ATR) kinase and ATR-interacting protein, which is then activated by the Rad9-Rad1-Hus1 complex and other factors. ATM and ATR are large phosphatidyl-inositol-3-OH kinase-like kinases that target proteins involved in the checkpoint response. These include the serine-threonine kinases, Chk1 and Chk2 (4), which in turn are responsible for transducing the damage signal to cell cycle regulators. Although ATM and ATR respond to different types of damage, there is crosstalk between the two pathways. For example, IR can activate both ATM and ATR (2, 5, 6). When DNA damage is sensed in the G_1 phase of the cell cycle, ATM and/or ATR, depending on the type of damage, phosphorylate and activate Chk2 at Thr-68 and Chk1 at Ser-317 and -345, respectively. Chk1 and Chk2 in turn phosphorylate Cdc25A at Ser-123, inducing its degradation (7). Cdc25A is a phosphatase that removes a phosphate group from Cdk2 at Tyr-15, allowing its interaction with cyclin E. Because this is an essential step for entry into S-phase, the degradation of Cdc25A blocks cell cycle progression (8). To maintain the resulting cell cycle block, p53 must be phosphorylated by ATM at Ser-15 and by Chk2 at Ser-20 (9, 10). These modifications disrupt the interaction of p53 with MDM-2, stabilizing p53, which then induces the expression of p21, an inhibitor of the Cdk2–cyclinE complex. This reinforces and maintains the cell cycle block.

The replication of viral DNA in the nucleus has the potential to activate the DNA damage response, if the ends of viral genomes are exposed and recognized as ds-DNA breaks. Different viruses have evolved mechanisms to block this response or use it to their advantage. Polyoma viruses activate this response and use it as part of a strategy to induce a cell cycle block in S-phase, where viral DNA replication can proceed (11). Adenoviruses induce the degradation of Mre11, an essential component for the activation of the damage response (12). Herpes simplex virus and Epstein–Barr virus, representatives of the α- and γ-herpesviruses, have been shown to activate the DNA damage response, although how this is achieved and the consequences of this activation are still being unraveled (13–15).

Human CMV (HCMV) is a β-herpesvirus that causes disease and mortality in immunologically immature or compromised individuals (16). It has a large ds-DNA genome and a prolonged replication cycle (17). HCMV both induces and blocks cell cycle progression (18). It induces cells in the G_0 compartment to enter G_1 by directing the degradation of the retinoblastoma protein (19), and it blocks progression from late G_1 into S, by preventing the formation of prereplication complexes (20). By manipulating the cell cycle, the virus generates an environment where its own DNA replication proceeds without competition from the cell for DNA precursors. HCMV DNA replication begins at $\approx$24 h postinfection (hpi), and peak levels of DNA accumulation are observed at 48–72 hpi of fibroblasts. DNA replication occurs via a rolling circle mechanism, producing concatemeric molecules that can be branched and have multiple exposed ends (21). This structure, in theory, could trigger the ds-DNA damage checkpoint. The stress response would reduce Cdk2 activity generating an unfavorable environment for viral DNA replication, or it could induce apoptosis before completion of the infectious cycle.

Here we show that HCMV, like other herpesviruses, induces the DNA damage checkpoint branch that responds to DNA ds breaks. ATM and Chk2 are activated but do not mediate an expected downstream event, namely Cdc25A degradation. ATM, Chk2, and other components of the checkpoint response pathway were mislocalized from the nucleus to the cytoplasm, blocking the response pathway.

Results

HCMV Infection Induces a DNA Damage Response. We initially infected fibroblasts with the AD169 strain of HCMV and monitored the levels and activation of several DNA damage checkpoint proteins by immunoblot assay (Fig. 1*A*). HCMV induced an increase in the protein and phosphorylation levels of ATM and in the phosphorylation levels of its downstream targets, Chk2, p53, and H2A.X. The first unambiguous increase

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Abbreviations: HCMV, human CMV; hpi, hours postinfection; ds, double strand; UV, UV radiation; IR, ionizing radiation; ATM, ataxia-telangiectasia mutated; ATR, ATM- and Rad3-related; pfu, plaque-forming unit.

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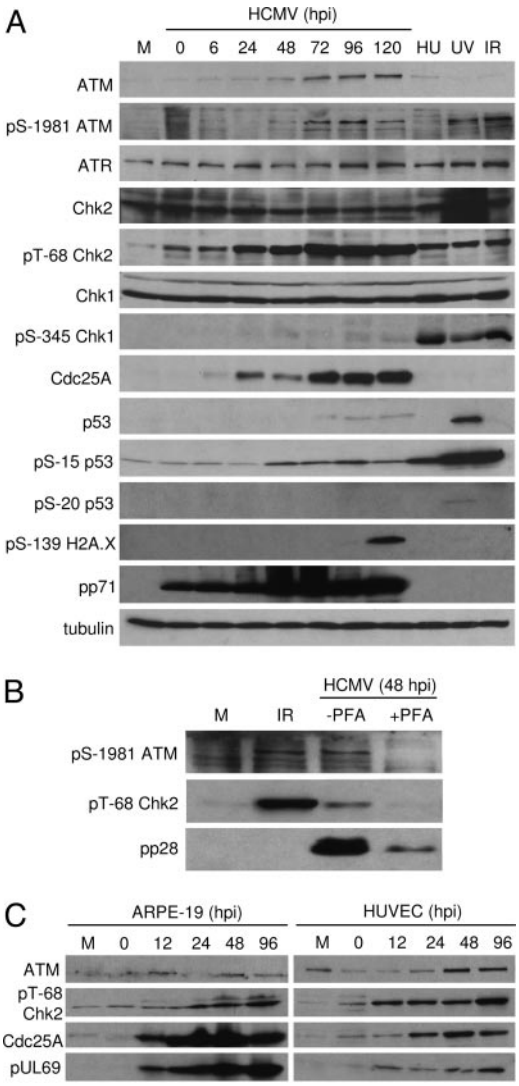

Fig. 1. HCMV activates a checkpoint response. Cell lysates were prepared at various times after infection (1 pfu per cell) and subjected to immunoblot assay by using antibodies to the indicated proteins. (*A*) Fibroblasts were infected with AD169. As controls, fibroblasts received 2 mM hydroxyurea (HU) for 12 h or were treated with UV or IR, and 4 h later, lysates were prepared. (*B*) Fibroblasts were infected with AD169 in the presence of 200 μg/ml phosphonoformic acid (PFA), and lysates were prepared at 48 hpi. (*C*) Human umbilical vein endothelial or ARPE-19 cells were infected with Toledo. M, mock-infected.

in ATM protein and its phosphorylation at Ser-1981 occurred at 48 hpi. However, an increase in Chk2 phosphorylation at Thr-68 was evident at the start of infection, and a further increase was evident at 24–48 hpi. The phosphorylation of p53 Ser-15 was elevated beginning at 48 hpi, and an increase in the amount of p53 protein was detected beginning at 72 hpi. Finally, there was an increase in the amount of phosphorylated H2A.X Ser-139 at 96 and 120 hpi. In contrast to ATM and its targets, the amount of ATR protein did not detectably increase after infection, and phosphorylation of its downstream target, Chk1 Ser-345, was increased to only a modest extent beginning at 48 hpi. This modest ATR-dependent response might result from an ATM-dependent activation of ATR (6) in the subset of cells that are blocked in G_2 rather than in G_1 by virus infection (18).

Induction and phosphorylation of ATM with phosphorylation of Chk2 Thr-68, p53 Ser-15, and H2A.X Ser-139 indicate that the branch of the DNA damage checkpoint pathway responding to ds breaks is activated after HCMV infection. However, the full pathway is not activated, because the level of Cdc25A is not decreased, as would be expected (7); rather, the amount of this phosphatase increases substantially with time after infection (Fig. 1*A*). Further, there is no detectable increase in the phosphorylation of p53 Ser-20. In the absence of infection, both of these proteins are modified in response to activation of Chk2, suggesting that, even though it is phosphorylated by ATM, Chk2 does not function properly. The level of the HCMV-coded pp71 protein was monitored as a marker for infection, and α-tubulin was assayed as a loading control (Fig. 1*A*). As positive controls for activation of DNA damage pathways, cells were treated with hydroxyurea, UV, or IR.

Because the branch of the DNA damage checkpoint that is activated responds to ds-DNA breaks, and the peak activation occurs during the late phase of the HCMV replication cycle, it seemed possible that the checkpoint was activated in response to the accumulation of viral DNA. We tested this idea by blocking viral DNA replication with phosphonoformic acid (Fig. 1*B*). The drug inhibited activation of the DNA damage checkpoint, as evidenced by reduced levels of phosphorylated ATM and Chk2. The accumulation of the viral late protein pp28 was assayed as a control for viral infection and for the inhibition of viral DNA replication, because the production of this protein requires viral DNA replication.

The experiments described above used the AD169 laboratory strain of HCMV. This virus grows efficiently in fibroblasts, but not in other cell types that are infected by clinical isolates of HCMV (22, 23). To test whether clinical isolates also activated the DNA damage pathway and to determine whether the activation occurred in cell types other than fibroblasts, we analyzed the effect of infection with the Toledo clinical strain of HCMV in epithelial (ARPE-19) and endothelial (human umbilical vein endothelial) cells (Fig. 1*C*). Even though Toledo enters these cells inefficiently (23), it induced a similar DNA damage response, as was seen for AD169 in fibroblasts. Chk2 Thr-68 was highly phosphorylated, and Cdc25A was not degraded. Accumulation of virus-coded UL69 protein was assayed to monitor HCMV infection.

HCMV Induces the Mislocalization of DNA Damage Checkpoint Proteins. To further characterize the activation of the DNA damage checkpoint by HCMV DNA replication, we analyzed the localization of checkpoint proteins (Fig. 2*A*). As expected, the checkpoint proteins localized to the nucleus in uninfected cells and early after HCMV infection. However, between 24 and 48 hpi, ATM, ATR, Chk1, Chk2, and phosphorylated Chk2 were mislocalized from the nucleus to the cytoplasm. Importantly, however, Cdc25A maintained its normal nuclear localization. Two clinical strains of HCMV, Toledo and FIX, also induced the mislocalization of ATM and Chk2 at 48 hpi (Fig. 2*B*).

The observation that HCMV induces the mislocalization of checkpoint proteins at 48 h, but not at 24 hpi, suggested that a late viral function is required to reposition the cellular proteins. To test this possibility, we treated infected cells with phosphonoformic acid, and Chk2 remained in the nucleus (Fig. 2*C*). Because this drug inhibits viral DNA replication and the accumulation of numerous late virus-coded proteins to different extents, a late HCMV gene product is likely responsible for the relocation.

The cytoplasmic location of the checkpoint proteins appeared to coincide with the virion assembly zone (24), where virus-coded structural proteins accumulate. To test whether Chk2 becomes colocalized with virion proteins in the cytoplasm, we performed an immunofluorescence assay at 48 hpi by using an antibody specific for Chk2 together with antibodies recognizing several structural proteins (Fig. 3*A* and data not shown). The

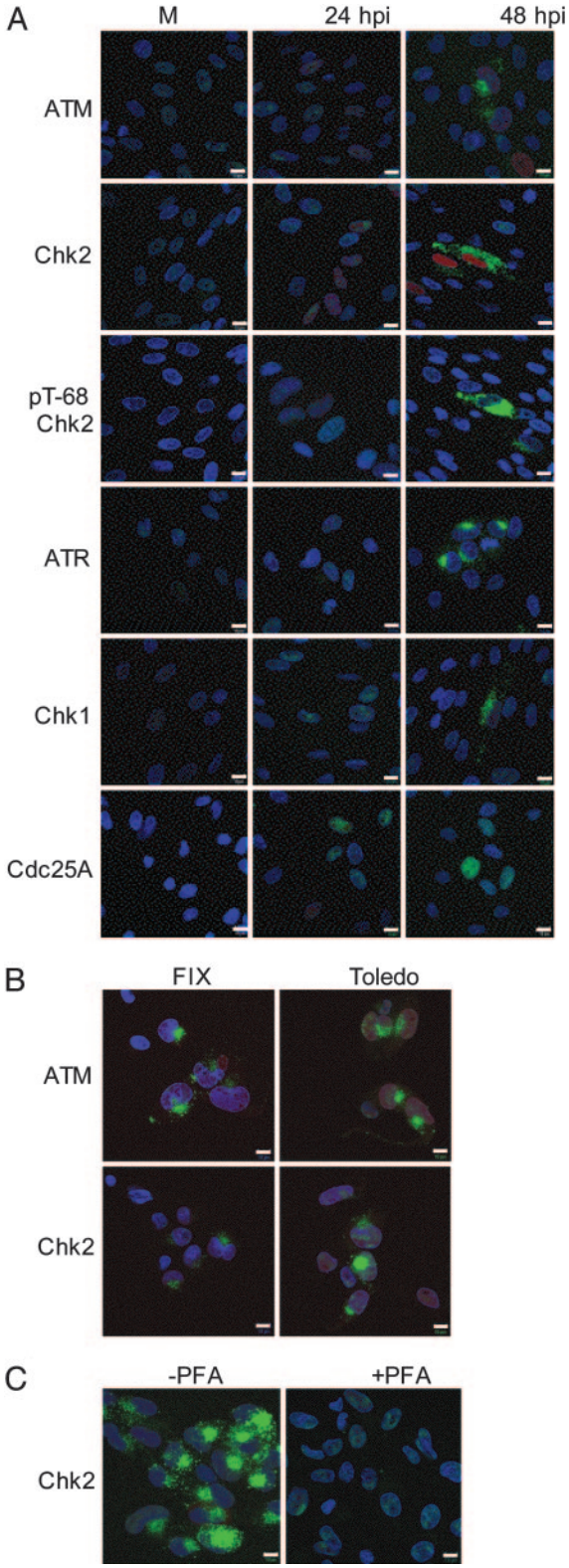

Fig. 2. Checkpoint proteins are mislocalized by HCMV. (*A*) Fibroblasts were infected with AD169 (0.1 pfu/cell) and processed 24 or 48 h later. Immunofluorescence used antibodies to the indicated cell proteins (green) and virus-coded IE2 protein (red). M, mock-infected. (*B*) Fibroblasts were infected with FIX or Toledo and assayed 48 h later by immunoflourescence with antibodies to ATM or Chk2 (green) and IE2 (red). (*C*) Fibroblasts were infected with AD169 (1 pfu/cell) in the absence or presence of 200 μg/ml phosphonoformic acid and assayed 48 h later by immunoflourescence with antibodies to Chk2 (green) and IE2 (red). DNA was stained with DAPI (blue). (Scale bars, 10 μm.)

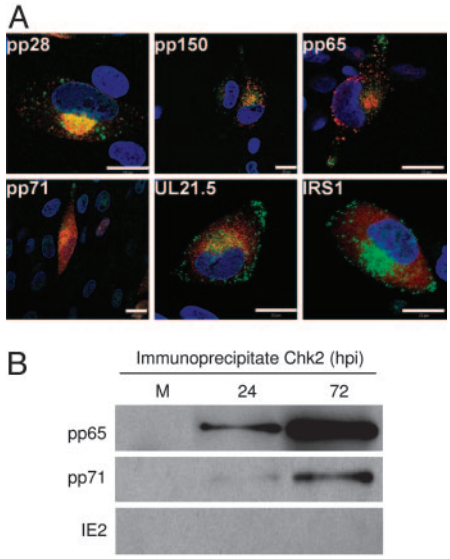

Fig. 3. Chk2 localizes and interacts with HCMV virion proteins. (*A*) Fibroblasts were infected with AD169 (0.1 pfu/cell), and fixed 48 h later. Immunofluorescence used antibodies to Chk2 (green) and the indicated virion proteins (red). DNA is stained with DAPI (blue). (Scale bars, 20 μm.) (*B*) Fibroblasts were infected with AD169 (1 pfu/cell), and lysates were processed for immunoprecipitation with Chk2-specific antibody 24 and 72 h later. M, mock-infected.

results showed a high level of colocalization of Chk2 with UL32-coded pp150, UL99-coded pp28 and pUL49, a lower level of colocalization with UL83-coded pp65 and UL82-coded pp71, and little colocalization for the pIRS1 and UL21.5 proteins.

The colocalization of cytoplasmic Chk2 with virion proteins prompted us to assay for a physical interaction between the cell and virus proteins. Chk2 was immunoprecipitated from cell lysates, and the presence of virus-coded proteins in the precipitates was assayed by immunoblot (Fig. 3*B*). Antibody to Chk2 coprecipitated the two abundant virion proteins that were tested, pp65 and pp71. These interactions were observed at 24 and 72 hpi. UL122-coded IE2, a nuclear nonstructural virus-coded protein, was not coprecipitated. This result argues that mislocalized Chk2 is associated with complexes containing virion proteins. Late after infection, newly synthesized pp65 protein initially accumulates in the nucleus and subsequently moves to the cytoplasm (25). Thus, there might be a connection between the exit of viral proteins from the nucleus and the mislocalization of checkpoint proteins.

UV, but Not IR, Treatment Inhibits the Production of Progeny Virus. Because HCMV inactivates the DNA damage checkpoint response, virus replication could be substantially resistant to the induction of DNA damage. Accordingly, we tested the effect of UV (which induces the accumulation of stalled replication forks and subsequently stretches of single-stranded DNA) and IR (which induces ds-DNA breaks) on the yield of HCMV (Fig. 4*A*). IR treatment reduced virus yields to only a modest extent at 6 days postinfection. The IR dose used, 10 Gy, activated the checkpoint response, because ATM and Chk2 were phosphorylated in other experiments (Figs 1*A* and 5*A*). In contrast, UV substantially reduced virus yields when it was administered after infection. When administered at 1 hpi, it reduced the yield by a factor of ≈42. We measured cell viability at 24 h after irradiation (Fig. 4*B*). Not surprisingly, both radiation treatments led to some cell death, but there was little difference between UV and IR, ruling out differential toxicity as the basis for their different effects on HCMV replication. Caffeine inhibits the checkpoint response (26), and it has previously been shown to enhance

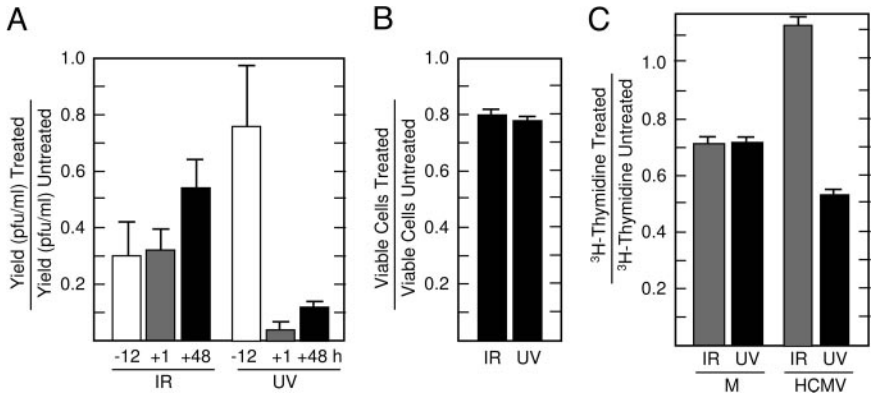

Fig. 4. HCMV replication is more resistant to IR than to UV. (*A*) Fibroblasts were treated with UV or IR before (−12 h) or after (+1 or +48 h) infection with AD169 (1 pfu/cell), and virus yields were determined 6 days later. (*B*) Fibroblasts were irradiated with UV or IR, and cell viability was measured 24 h later. Samples were analyzed in triplicate. (*C*) Fibroblasts were infected with AD169 (1 pfu/cell), 48 h later cells were treated with UV or IR, and ^{3}H-thymidine incorporation from 49–57 hpi was quantified for three experiments. M, mock-infected. Mean and standard error are presented for three experiments.

HCMV replication (24). As anticipated, it enhanced the production of progeny virus to a limited extent (data not shown).

We next compared the effect of UV and IR on DNA synthesis in infected cells (Fig. 4*C*). At 48 hpi or mock infection, cells were irradiated and then labeled with ^{3}H-thymidine for 8 h. This experiment detected DNA repair synthesis and viral DNA replication but not cell DNA replication, because HCMV blocks cell cycle progression. Both radiation treatments inhibited ^{3}H-thymidine incorporation to the same extent in mock-infected cells, but UV had a much greater inhibitory effect than IR in infected cells, consistent with its ability to markedly reduce the yield of virus.

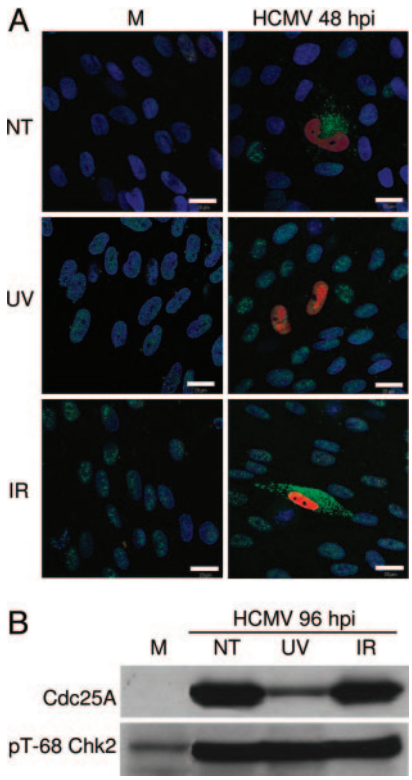

Fig. 5. UV but not IR restores Chk2 activity in HCMV-infected cells. (*A*) Fibroblasts were subjected to UV or IR at 48 hpi with AD169 (0.1 pfu/cell), and after an additional 4 h, they were processed for immunofluorescence with antibodies to pThr-68 Chk2 (green) and virus-coded IE2 (red). DNA was stained with DAPI (blue). (Scale bars, 20 μm.) (*B*) Fibroblasts were subjected to UV or IR at 96 hpi with AD169 (1 pfu/cell), and after an additional 4 h, lysates were prepared and analyzed by immunoblot by using antibodies to Cdc25A and pThr-68 Chk2. NT, not treated; M, mock-infected.

UV but Not IR Treatment of Infected Cells Blocks the Mislocalization of Checkpoint Proteins and Induces the Degradation of Cdc25A. To better understand the different effects of UV and IR on infected cells, we irradiated cells at 48 hpi and assayed the location of checkpoint proteins 4 h later (Fig. 5*A*). UV reversed the mislocalization observed at 48 hpi, whereas IR did not. We interpret this result to indicate that UV irradiation inhibits viral DNA replication, preventing synthesis of the late viral gene product responsible for the mislocalization. The restoration of Chk2 to the nucleus observed upon UV irradiation raised the possibility that Cdc25A is degraded in these circumstances. Accordingly, cells were UV- or IR-treated at 96 hpi, and the level of Cdc25A was analyzed by immunoblot 4 h later (Fig. 5*B*). As expected, UV induced the degradation of Cdc25A, but IR did not. The amount of phosphorylated Chk2 at 96 hpi did not change after treatment with UV or IR. Consequently, the degradation of Cdc25A was not simply the result of a change in the amount of activated kinase.

Discussion

Herpesviruses activate the branch of the DNA damage checkpoint that responds to ds breaks in DNA. Herpes simplex virus and Epstein–Barr virus induce the phosphorylation of ATM and Chk2 (13–15). HCMV also activates this checkpoint, as evidenced by phosphorylation of ATM, Chk2, p53,and H2A.X (Fig. 1 *A* and *C*). HCMV-induced phosphorylation of Chk2 and p53 has been demonstrated (27). Phosphonoformic acid inhibited the phosphorylation of ATM and Chk2 (Fig. 1*B*), demonstrating that viral DNA replication is required to activate the checkpoint response. HCMV infection induces elevated levels of Cdk2 activity, and viral replication is inhibited by roscovitine, a Cdk2 inhibitor (28). Because activation of the DNA damage checkpoint normally results in inhibition of Cdk2 activity via the degradation of Cdc25A and the induction of p21, HCMV clearly blocks the downstream consequences resulting from activation of the DNA damage checkpoint. Indeed, we observe elevated levels of Cdc25A during HCMV infection (Fig. 1 *A* and *C*), which is inconsistent with the high levels of phosphorylated Chk2 observed.

Recently, it was reported that the exogenous expression of the HCMV IE1 protein induces the phosphorylation of ATM Ser-1981 and p53 Ser-15 and -20 (29). We did not detect elevated

phosphorylation of these substrates at 6 hpi (Fig. 1*A*), a time at which immediate-early proteins such as IE1 are highly expressed. Further, phosphorylation of ATM Ser-1981 was not detected in the presence of phosphonoformic acid (Fig. 1*B*), which blocks viral DNA replication but allows efficient expression of immediate-early proteins. Ectopic expression of IE1 has been shown to damage DNA (30), so it is not surprising that expression of IE1 outside the context of an HCMV infection can lead to phosphorylation of ATM and p53. Another immediate-early HCMV protein might prevent a DNA damage response at 6 hpi.

DNA damage checkpoint proteins are normally localized to the nucleus and organize into repair centers upon induction of DNA damage. These repair centers are formed at or near the site of damage (31). The localization of multiple checkpoint proteins is dramatically altered by HCMV, blocking their normal function. They accumulate in the cytoplasm during the late phase of infection (Fig. 2*A*). As was the case for phosphorylation of the checkpoint proteins (Fig. 1*B*), phosphonoformic acid blocked their mislocalization (Fig. 2*C*). This argues that a late viral gene function mediates the abnormal localization. As yet, we have not identified the putative mislocalizing protein. The UL84-coded HCMV protein is an intriguing candidate for this role. It is expressed with early kinetics and associates with the IE2 protein as the infection enters the late phase (32). The UL84 protein binds several members of the α-karyopherin family (KPNA1, -3, -4, and -5), a family of proteins that function in nuclear transport (33). Indeed, Chk2 and Nbs1 have been shown to depend on KPNA2 for their nuclear localization (34, 35). Thus, it is possible that UL84 or a UL84/IE2 protein complex binds to KPNA2 and inhibits its nuclear transport function. This could cause Chk2 and possibly other checkpoint proteins to accumulate in the cytoplasm. Alternatively, virion structural proteins might ferry checkpoint proteins from the nucleus to the cytoplasm during the late phase of infection. This notion is consistent with the observation that the pp65 and pp71 virion proteins are coimmunoprecipitated with Chk2 (Fig. 3*B*).

Although we did not detect significant phosphorylation of ATR (Fig. 1*A*), it is nevertheless mislocalized to the cytoplasm late after infection (Fig. 2*A*). This indicates that HCMV also blocks ATR function, but UV treatment, which induces signaling through ATR, inhibits HCMV replication (36). However, we observed that UV most effectively inhibited HCMV replication when administered during the early phase of infection (Fig. 4*A*), and the treatment caused phosphorylated Chk2 to accumulate in the nucleus (Fig. 5*A*) with degradation of Cdc25A (Fig. 5*B*). UV presumably blocks production of the late viral product that directs the cytoplasmic accumulation of checkpoint proteins. IR does not induce the return of phosphorylated Chk2 to the nucleus and consequently does not cause degradation of Cdc25A.

In sum, HCMV escapes the consequences of activation of the DNA damage checkpoint by inducing the mislocalization of checkpoint proteins.

Materials and Methods

Biological Reagents. Primary human fibroblasts were cultured in DMEM containing 10% newborn calf serum, ARPE19 endothelial cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM/Ham's F-12 (1:1) medium containing 10% of FBS, and primary human umbilical vein endothelial cells were cultured in EGM-2 medium (Cambrex, East Rutherford, NJ). Three HCMV strains were used in these studies: the AD169 laboratory strain (37), the Toledo clinical isolate (38), and the FIX clinical isolate (39). Viruses were propagated in fibroblasts, and titers were determined by tissue culture infectious dose ($TCID_{50}$) assay on fibroblasts.

Antibodies to cell proteins were as follows: ATM Ser-1981, Chk1 Ser-345, p53, p53 Ser-15, and p53 Ser-20 (Cell Signaling Technology); Chk2 Thr-68, Chk2, Chk1, Cdc25A, and α-tubulin (Santa Cruz Biotechnology); H2A.X-Ser-139 (Upstate Biotechnology); ATM (Novus Biologicals, Littleton, CO); and ATR (Affinity Bioreagents, Neshanic Station, NJ). Antibodies to HCMV proteins were as follows: UL82-coded pp71 (19); UL32-coded pp150 and UL83-coded pp65 (40); UL99-coded pp28 and UL122-coded IE2 (41); pIRS1 (42); and pUL21.5 (43). Conjugated antibodies were as follow: horseradish peroxidase (HRP)-anti-mouse, HRP-anti-rabbit (Jackson Immunochemicals, West Grove, PA); and Alexa 546-anti-mouse and Alexa 488-anti-rabbit (Invitrogen).

UV and IR Treatment. For UV treatment, culture medium was removed, and cells were washed once with warm PBS and then uncovered and irradiated (50 J/m^2) in a Stratalinker (Stratagene). The same medium was added back to the cells and the cultures incubated for the indicated periods of time. Cells were exposed to IR (10 Gy) in culture medium, using a ^{137}Cs source at a dose rate of 0.97 Gy/min. Cells were assayed for viability by using the Cell Titer 96 AQ_{ueous} One Solution assay (Promega).

Thymidine Incorporation Assay. Cells were infected with HCMV AD169 at a multiplicity of one plaque-forming unit (pfu) per cell, irradiated at 48 hpi, and labeled from 49 to 57 hpi with 2 μCi/ml (1 Ci = 37 GBq) [^{3}H]-thymidine (NEN-Perkin-Elmer). Then cells were washed twice in ice-cold PBS, treated with 5% trichoracetic acid for 30 min at 4°C, washed twice with PBS, lysed in 0.5 M NaOH and 0.5% SDS for 5 min, resuspended, and added directly to scintillation fluid. Radioactivity was measured in a scintillation counter.

Protein Analysis. For immunoblot assay, cells were washed twice in ice-cold PBS and dissolved in lysis buffer (50 mM Tris, pH 7.4/1% Nonidet P-40/0.25% Na-deoxycholate/150 mM NaCl/1 mM EDTA/1 mM PMSF/1 μg/ml each of aprotinin, leupeptin, pepstatin/1 mM Na_3VO_4/1 mM NaF). Protein (100 μg) was mixed with an equal volume of 2× sample buffer (125 mM Tris, pH 6.8/4% SDS/20% glycerol/5% 2-mercaptoethanol), boiled for 5 min, and subjected to electrophoresis in an SDS-containing polyacrylamide gel. Proteins were transferred to nitrocellulose and probed with antibodies. For immunoprecipitation experiments, cell lysates (500 μg of protein) were precleared with protein A/G agarose beads (Santa Cruz Biotechnology) at 4°C for 2 h, incubated with Chk2-specific antibody overnight at 4°C, then incubated with protein A/G agarose beads at 4°C for 2 h followed by three washes in lysis buffer. Beads were boiled in sample buffer for 10 min, and solubilized protein was analyzed by electrophoresis in an SDS-containing polyacrylamide gel. Proteins were transferred to nitrocellulose and probed with antibodies. For analysis by immunofluorescence, cells were washed three times with PBS at 37°C, fixed for 15 min at room temperature with 2% paraformaldehyde in PBS, again washed three times with PBS, permeabilized for 15 min at room temperature with 0.1% Triton X-100 in PBS, washed three times with PBS with 0.05% Tween 20 (PBST), and blocked for 60 min with 2% BSA in PBST. Next, cells were incubated for 1 h with primary antibody diluted in PBST, washed three times with PBST, incubated for 30 min with fluorochrome-conjugated anti-mouse or anti-rabbit secondary antibody, and washed again three times with PBST. Samples were mounted in SlowFade solution (Invitrogen), and images were captured by using a Zeiss LSM510 confocal microscope.

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