Stabilization of p53 in Human Cytomegalovirus-initiated Cells Is Associated with Sequestration of HDM2 and Decreased p53 Ubiquitination*

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Human cytomegalovirus (HCMV) induces serum- or density-arrested human lung (LU) cells to traverse the cell cycle, providing it with a strategy to replicate in post-mitotic cells that are its cellular substrate in vivo. HCMV infection also induces high cellular levels of p53, seemingly in contradiction to the observed cell cycle progression. This study was undertaken to examine the mechanism(s) of the increased p53 abundance. HCMV infection caused a 4-fold increase in p53 that preceded a substantial increase in p53 transcripts by more than 24 h. p53 was stabilized in HCMV-infected cells (from a half-life of less than 30 min to about 8 h) and was less sensitive to proteasome-mediated degradation. Ubiquitination of p53 in mock-infected LU cells was sensitive to inhibition by trans-4-iodo, 4′-boranyl-chalcone, consistent with HDM2-catalyzing ubiquitination of p53. In HCMV-infected cells, ubiquitination of p53 was essentially undetectable. Although HDM2 had a nuclear distribution in mock-infected LU cells, in HCMV-infected cells HDM2 was translocated to the cytoplasm beginning at 12 h and demonstrated decreased cellular abundance thereafter. HDM2 was stabilized in the HCMV-infected cells by MG132, indicating a shift from p53 to HDM2 ubiquitination. p53 demonstrated a predominantly nuclear distribution in HCMV-infected cells through 48 h, resulting in p53 and HDM2 in distinct subcellular compartments. The principal mechanism responsible for increased p53 stabilization was nuclear export and degradation of HDM2. Thus, HCMV uses a shift from p53 to HDM2 ubiquitination and destabilization to obtain protracted high levels of p53, while promoting cell cycle traverse.

Human cytomegalovirus (HCMV), a large β-herpesvirus, infects the majority of humans, producing life long persistent infection (1, 2). Efficient replication of HCMV in stationary cells is dependent on the progression of infected cells through the cell cycle to a point at or near the G1/S boundary (3, 4). Consistent with this requirement, HCMV infection stimulates potent mitogenic signaling in infected cells, with some evidence suggesting that HCMV mitogenic signaling is more robust than that of serum growth factors, because HCMV, but not serum growth factors, induces the entry of density-arrested cells into the cell cycle. Among the immediate early and early signaling events induced by HCMV are hydrolysis of phosphatidylinositol 4,5-bisphosphate (5), activation of phospholipases (6), increased cellular levels of secondary messengers (3, 6, 7), production of robust and protracted signaling from some components of these pathways (8), stimulation of several protein kinases (6, 9), and activation of a number of cellular transcription factors (9, 10). Some of this signaling derives from HCMV binding to its cellular receptors (epidermal growth factor receptor (11) and integrin αvβ3 (12)), but HCMV infection also modifies the cell with induction of cellular (e.g. activation of m- and μ-calpains (13)) and viral (e.g. cellular transcriptional activation by IE2-86 (14)) activities that contribute to cell cycle entry and progression. These signaling events are related to substantially modified cellular transcription patterns (15), including up-regulation of cyclin E (14). In addition, HCMV infection stimulates the translocation of cyclin-dependent kinase 2 from the cytoplasm to the nucleus (16) and the degradation of p21Cip1 (p21 cyclin-dependent kinase-interacting protein 1) mediated by the ubiquitols calpains (13). Thus, HCMV uses cellular signaling pathways to drive infected cells into the cell cycle, providing this large DNA virus with the opportunity to replicate in the post-mitotic cells that are its cellular substrate in vivo (3).

In HCMV-infected cells stimulated to enter the cell cycle and progress through G1, the level of p53 has been reported to rise substantially (17–19). Expression of either of the prominent HCMV immediate early proteins (IE1-72 and IE2-86) results in increased p53 levels in transfectod fibroblasts (20) and endothelial cells (21). Several studies have suggested that HCMV-induced alterations in p53 are related to the pathogenesis of HCMV disease (22). Increased levels of p53 were observed in smooth muscle cells associated with restenosis (60), and a correla-

early gene product; LU, lung; M, mock-infected; MDM2, murine double minute 2; MG132, carbobenzoxy-l-leucyl-l-leucyl-l-leucinal; MOPS, 33-(N-morpholino)propanesulfonic acid; PFU, plaque-forming units; PI, post-infection; TIBC, trans-4-iodo, 4′-boranyl-chalcone; E3, ubiquitin-protein isopeptide ligase.
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EXPERIMENTAL PROCEDURES

Cell Culture and Growth Arrest—Human diploid lung (LU) fibroblasts (37), passage 6–20, were grown in Eagle’s minimum essential medium containing 10% fetal bovine serum (Intergen Co., Purchase, NY) and penicillin (100 units/ml)/streptomycin (100 µg/ml) (growth medium) at 37 °C in a 5% CO2 in air atmosphere. The cells were density-arrested as described previously (4).

Virus Stocks and Productive Infection—The AD169 strain of HCMV was propagated in LU cells as described previously (38). The infectivity of virus stocks was determined by plaque assay (37). Virus stocks typically had infectivities between 8.0 × 10⁶ and 4.0 × 10⁷ plaque-forming units (PFU)/ml. LU cells were infected with HCMV at a multiplicity of 5 PFU/cell to provide a uniform infection, as described in detail previously (38), and re-fed with reserved growth medium (4). Virus stocks and cell cultures were routinely examined for mycoplasma and were free from detectable contamination.

Northern Hybridization—Northern hybridization was performed as described in our previous report (13). RNA was extracted using TriReagent (Molecular Research Center, Inc., Cincinnati, OH). Total cellular RNA (10 µg/lane) was evaluated under denaturing conditions in formaldehyde-agarose gels, containing 1% agarose, 20 mM MOPS, 1 mM EDTA, 8 mM sodium acetate, and 2.2 M formaldehyde. After separation, the RNA was transferred to MSI nylon membranes (Micron Separations, Inc., Westborough, MA) for 18 h. The RNA was pre-hybridized in Rapid-Hyb buffer (Amersham Biosciences), containing 100 µg/ml denatured salmon sperm DNA at 65 °C for 1 h. A 32P-labeled DNA probe was prepared from the plasmid pc-w53ASN (generously provided by Dr. B. Vogelstein), which harbors a p53 insert. A probe derived from the plasmid p5B (39), which contains the cDNA for 18 S rRNA, was used to ensure uniform loading of gels. Plasmids were introduced into competent DH5α Escherichia coli cells, and amplified DNA fragments comprising p53 and 18 S CDNA were excised from isolated plasmid DNA using the restriction endonucleases BamHI and BamHI-EcoRI, respectively. The probes were labeled using the multiprimer labeling kit (Amersham Biosciences). 32P-labeled p53 or 18 S probe was added and hybridized for 3 h at 65 °C. Membranes were washed twice with 2× SSPE, 0.1% SDS for 15 min at 65 °C, once with 1× SSPE, 0.1% SDS at 42 °C, and twice with 0.1× SSPE, 0.1% SDS at 42 °C. The probe remaining on the filter was detected by autoradiography (Eastman Kodak Omnifilm for 1–16 h at −80 °C).

Western Blotting—Western blot analysis was performed as described in our previous report (13). Cells were harvested by dislodging the cells with a cell lifter in phosphate-buffered saline, collected by sedimentation, and lysed in lysis buffer (50 mM Tris–HCl, pH 7.4, containing 150 mM NaCl, 0.5% Igepal CA-630, with 1 mM dithiothreitol, 1 mM NaVO₃, 50 mM NaF, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 25 µg/ml trypsin inhibitor, 25 µg/ml aprotinin, 1 mM benzamidine, and 25 µg/ml pepstatin A) added just before use). Cellular debris was removed by sedimentation, and the supernatant fluids were reserved. The protein concentration was determined by the BCA protein assay (Pierce). Whole cell extracts (40 µg/lane) were fractionated on 4–12% NuPAGE™ (1.0 mm x 10 wells, NP0321; Invitrogen) SDS-polyacrylamide gels, and the polypeptides were transferred to nitrocellulose membranes (Bio-Rad). Monoclonal antibody specific for p53 (DO-1, sc-126) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A polyclonal antibody preparation specific for p300 (C-20, sc-585X) was also obtained from Santa Cruz Biotechnology. Monoclonal antibody specific for HDM2 (2A9C1.18; target amino acids 153–222) was the gift of Dr. J. Chen (40). A second monoclonal antibody (Ab3; clone 4B11, target amino acids 383–491) specific for HDM2 was purchased from Oncogene Research Products (La Jolla, CA), and a polyclonal antibody preparation (C18, sc-812), which targets the C terminus of HDM2, was obtained from Santa Cruz Biotechnology. Antigen-antibody reactions were detected with the enhanced chemiluminescent assay (Amersham Biosciences) following the manu-
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A.

| Time PI (h) | 0 | 3 | 6 | 9 | 12 | 24 | 48 | 72 | 96 |
|-------------|---|---|---|---|----|----|----|----|----|
| Mock        | p53 |  |  |  |  |  |  |  |  |
| Calpain     |  |  |  |  |  |  |  |  |  |
| HCMV        |  |  |  |  |  |  |  |  |  |

B.

| Time PI (h) | 0 | 3 | 6 | 9 | 12 | 24 | 48 | 72 | 96 |
|-------------|---|---|---|---|----|----|----|----|----|
| Mock        | p53 |  |  |  |  |  |  |  |  |
| Calpain     |  |  |  |  |  |  |  |  |  |
| HCMV        |  |  |  |  |  |  |  |  |  |

C.

Relative abundance of p53 (DO-1) at 0 h PI with S.D. shown (A, mock-infected cells; HCMV-infected cells).

D.

Relative abundance of p53 (DO-1) at 0 h PI with S.D. shown (A, mock-infected cells; HCMV-infected cells).

FIGURE 1. A, abundance of p53 protein after HCMV infection or mock infection of LU cells. LU cells were density-arrested and infected with HCMV (5 PFU/cell) or mock-infected, as described under “Experimental Procedures.” Whole cell lysates were prepared at the times indicated in the figure, and extracts were prepared for analysis of protein. Aliquots of protein (40 g/lane) were resolved by PAGE in the presence of SDS and transferred to nitrocellulose membranes. The nitrocellulose filters were probed with antibody to p53 (DO-1), and polypeptides reactive with the anti-p53 antibody were detected by ECL. The blots were then stripped and re-probed with antibody to m-calpain (41), as described under “Experimental Procedures.” The results of a representative experiment are illustrated in A. The results of the experiment illustrated in A and two additional experiments were evaluated by densitometric analysis, normalized for m-calpain, and the means plotted as the abundance relative to the mock-infected control at 0 h with standard deviation shown (C, mock-infected cells; HCMV-infected cells). Datum points marked with an asterisk are significantly different from the values obtained for mock-infected cells at the indicated time. B, abundance of p53 transcripts as determined by Northern blot analysis after HCMV infection or mock infection. Parallel cultures of density-arrested LU cells were treated as described in the legend to A. RNA was isolated at the times indicated in the figure, resolved by electrophoresis (10 g/lane), and transferred to nitrocellulose membranes, as described under “Experimental Procedures.” Membranes were probed with 32P-labeled cDNA specific for p53 and detected by autoradiography. The membranes were then stripped and re-probed for 18 S RNA. Ribosomal 18 S RNA (18 S) was used as a loading standard. The results of the representative experiment illustrated in B and of two additional independent experiments were evaluated by densitometric analysis and normalized for 18 S RNA, and the means were plotted as the abundance relative to the mock-infected control at 0 h PI with S.D. shown (D, mock-infected cells; HCMV-infected cells). Datum points marked with an asterisk are significantly different from the values obtained for mock-infected cells at the indicated time.

Immunocytochemistry and Laser Scanning Confocal Microscopy—LU cells were grown on glass coverslips and density-arrested as described previously (16). Coverslip cultures were infected with HCMV at a multiplicity of 5 PFU/cell. At the time of harvest, the cells were washed three times in phosphate-buffered saline, air-dried, and fixed in acetone/methanol (1:1) at -20 °C for 10 min. The subcellular distribution of specific proteins was identified with antibody preparations specific for p53 (DO-1), HDM2 (2A9C1), p300 (C-20), or HCMV IE2-86 (ESH-IE2–84), and either fluorescein- or Alexa Fluor 546-labeled secondary antibody (rabbit anti-mouse IgG (F(ab')2 fragment), AQ160F; Chemicon International, Temecula, CA; goat anti-rabbit IgG (H + L) [F(ab')2 fragment]); Pierce; goat anti-mouse IgG (H + L) (F(ab')2 fragment); A-11018; Molecular Probes, Eugene, OR). Monoclonal antibody to HCMV IE2-86 was generously provided by Dr. E. S. Huang. Fluorescence was localized with a Zeiss LSM 510 UV META microscope equipped with 401/2.2 and 63/1.2 C-planapochromat water immersion objectives. Images were captured as MDB files and analyzed using the Zeiss LSM software (version 3.2).

Measurement of p53 Half-life—Density-arrested LU cells were HCMV- or mock-infected, and at selected times thereafter, protein synthesis was blocked with cycloheximide. At selected times after the cycloheximide block, HCMV- and mock-infected cell cultures were harvested, and protein extracts were prepared as described above. The polypeptides were separated by PAGE and evaluated by Western blot analysis for p53. The abundance of p53 was determined by densitometry (with normalization to the loading controls), and the p53 abundance at each harvest time was plotted relative to the time of harvest after initiating the cycloheximide block. The GraphPad Prism program (GraphPad Software, Inc., San Diego) was used to calculate the half-life of p53 under the various treatment conditions.

Image Processing and Statistical Analysis—All experiments were repeated at least twice, and either data from a representative experiment or means of data from two or more experiments were selected for presentation. Cell cultures (2 or more 100-mm dishes/datum point) were harvested at times selected to represent the major phases of HCMV replication as follows: 0 – 6 h, immediate early; 12 – 24 h, early; and 48 – 96 h, late. Chemiluminescent samples were exposed for intervals that ensured a linear response, as determined by standardization. All radiographic films were analyzed using an Alphalmager 2000 Documentation and Analysis System (Alpha Innotech Corp., San Leandro, CA) and the manufacturer’s software (Alphalmager 2000, version 4.0). The images were quantified and recorded as tagged image format files (TIFF). The TIFF files were used to prepare the graphic images illustrated in this report. The image densitometric quantification data were expressed as mean ± S.D. The Origin computer program (OriginLab Corp., Northampton, MA) was used for the statistical evaluation of the results and plotting of the data. Statistical significance was determined by independent t tests corrected for the number of comparisons. The two-sided probability level of 0.05 was used as the criterion for significance.
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A. 12-36 h PI

| Time PT (h) | 0 | 0.5 | 1 | 2 | 3 | 6 | 9 | 12 | 24 |
|-----------|---|-----|---|---|---|----|---|----|----|
| Time PI (h) | 12 | 12.5 | 14 | 15 | 18 | 21 | 24 | 36 |

Mock

Calpain

HCMV

**p53**

B. 48-72 h PI

| Time PT (h) | 0 | 1 | 2 | 3 | 6 | 9 | 12 | 24 |
|-----------|---|---|---|---|----|---|----|----|
| Time PI (h) | 48 | 49 | 50 | 51 | 54 | 57 | 60 | 72 |

Mock

Calpain

HCMV

**p53**

**Chemicals**—MG132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucinal) was purchased from Peptides International, Inc. (Louisville, KY). trans-4-Iodo, 4′-boranyl-chalcone (TIBC) was obtained from EMD Biosciences (San Diego). Penicillin, streptomycin, cycloheximide, diethyl pyrocarbonate, Tris, Igepal CA-630, NaCl, NaVO₃, NaF, phenylmethylsulfonyl fluoride, diithiothreitol, trypsin inhibitor, aprotinin, benzamide, and pepstatin A were purchased from Sigma.

**RESULTS**

Increased Abundance of p53 Protein in HCMV-initiated Cells—Western blot analysis of protein extracts of mock-infected, density-arrested, LU cells revealed a relatively constant amount of p53 through 96 h (Fig. 1A). In parallel cultures of HCMV-infected cells, p53 levels began to increase by 3 h post-infection (PI) and continued to increase through 24 h, when the abundance of p53 started to plateau. From 24 to 72 h PI, the abundance of p53 in HCMV-infected cells was about 4-fold greater than that observed in mock-infected cells (Fig. 1C). By 96 h, the cellular level of p53 had declined somewhat. The levels of p53 in HCMV-infected cells were significantly ($p < 0.01$) greater than those observed in mock-infected cells from 6 to 96 h PI.

*p53 Transcription during Productive HCMV Infection*—To evaluate if the elevation of p53 protein was temporally correlated with an increase in p53 transcription, LU cells were HCMV- or mock-infected, and total cellular RNA was evaluated by Northern blot hybridization. The results revealed that p53 transcripts increased in a biphasic manner in HCMV-infected cells (Fig. 1B). The first increase, observed at 3 h PI, although modest and not significantly different from the abundance of p53 transcripts in mock-infected cells, was consistently obtained. The p53 RNA levels dropped to near base line by 6 h and remained at a low level through 12 h. p53 RNA levels increased significantly between 24 and 48 h PI and remained at significantly elevated levels through 72 h PI ($p < 0.05$). The second increase in p53 RNA was much more robust than the first with nearly a 4-fold increase compared with a less than 1.6-fold increase at 3 h PI (Fig. 1D). The results illustrated in Fig. 1 failed to show a close temporal correlation between p53 RNA and protein levels, in that the increase in p53 protein preceded the major increase in p53 RNA by more than 24 h. These results suggest that mechanisms other than transcriptional regulation were involved in the p53 protein increase observed in HCMV-infected cells, particularly at early times.

*p53 Protein Is Stabilized in HCMV-infected LU Cells*—The effect of HCMV infection on p53 stability was monitored by blocking protein synthesis with cycloheximide and following the decay of p53 by Western blot analysis beginning at 12 and 48 h PI. These two intervals were chosen because of the contrasting findings for the relative abundance of p53 protein and RNA (Fig. 1) at these times (12 h PI; high protein, low RNA; 48 h PI; high protein, high RNA). Analysis of p53 protein decay (Fig. 2) demonstrated that p53 protein was stabilized in HCMV-infected cells, even when monitored from 48 to 72 h PI (Fig. 2B) when there were abundant p53 transcripts (Fig. 1B). Decay of p53 in mock-infected cells at 12–36 h (Fig. 2A) or 48–72 h (Fig. 2B) demonstrated a biphasic pattern, with substantially more rapid decay evident in the first component of the decay curves than the second. The half-life of p53 was less than 0.5 h in mock-infected cells evaluated at either time, which is consistent with previous results (28). In the HCMV-infected cells evaluated from 12 to 36 h or from 48 to 72 h PI (Fig. 2), p53 protein stability was increased significantly ($p < 0.05$). Only a single component was evident in the decay curves for p53 in HCMV-infected cells at either of the intervals monitored. The slope of the p53 protein decay curve in HCMV-infected cells was similar to the slope of the lower component of the p53 decay curves in mock-infected LU cells. The half-life of p53 protein in HCMV-infected cells was about 7.6 h, when monitored from 12 to 36 h PI, and about 8.4 h when measured from 48 to 72 h PI. These results suggest that the initial component in the p53 decay
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A. 12–36 h PI

B. 48–72 h PI

C. HCMV Inhibitor Decreases p53 Ubiquitination in LU Cells

D. HCMV Inhibitor Decreases p53 Ubiquitination in LU Cells

**FIGURE 3.** The effect of proteasome inhibition on the stability of p53 in HCMV-infected LU cells. The results of a representative experiment are shown in which density-arrested LU cells were HCMV- (5 PFU/cell) or mock-infected, as described in the legend to Fig. 1, and then exposed to cycloheximide (100 μg/ml), with or without MG132 (10 μM), beginning at either 12 (A) or 48 h (B) PI. Cell lysates were prepared for protein analysis at the times indicated in the figure. Polypeptides (40 μg/lane) were resolved by PAGE in the presence of SDS, as described under "Experimental Procedures." The gels were blotted onto nitrocellulose and probed with antibody against p53 (DO-1). The blots were stripped and re-probed with antibody against m-calpain (41). The change in abundance of p53 after cycloheximide treatment in the presence or absence of MG132 was determined by densitometry of the results of three experiments, normalized to m-calpain, and plotted relative to the p53 abundance ± S.D. in HCMV- or mock-infected cells in the absence of MG132 at the time of the cycloheximide block (C, 12–36 h PI), mock infection in the absence of MG132; C, mock infection in the presence of MG132; ○, HCMV infection in the absence of MG132; ●, HCMV infection in the presence of MG132. D, (48–72 h PI), mock infection in the absence of MG132; ●, mock infection in the presence of MG132; ○, HCMV infection in the absence of MG132; ●, HCMV infection in the presence of MG132. Treatment of mock-infected, but not HCMV-infected, LU cells with MG132 significantly (p < 0.05) increased the half-life of p53 beginning at 12 h PI. The half-life of p53 in mock-infected LU cells treated with MG132 was similar and not significantly different from the half-life of p53 in HCMV-infected LU cells in the absence of MG132, when measured beginning at 12 h PI. Treatment with MG132 beginning at 48 h PI significantly (p < 0.05) increased the half-life of p53 in both mock- and HCMV-infected LU cells.

curves in mock-infected cells, which depicts the rapid degradation of p53, was compromised by HCMV infection.

**Proteasome-mediated Degradation of p53 in LU Cells**—The ubiquitin/proteasome pathway is the principal degradative mechanism through which p53 levels are regulated in unstressed cells (29). Inhibition of degradation by MG132, an inhibitor of proteasome-mediated proteolysis (42), beginning at 12 (Fig. 3A) or 48 h PI (Fig. 3B), significantly (p < 0.05) increased the half-life of p53 in mock-infected cells from less than 0.5 h to about 8 h (Fig. 3C) or to more than 24 h (Fig. 3D), respectively. MG132 had no significant effect on the half-life of p53 in HCMV-infected cells evaluated from 12 to 36 h PI (Fig. 2A), suggesting that proteasome-mediated degradation of p53 was inhibited in HCMV-infected cells at this time. The p53 half-life in HCMV-infected cells evaluated from 12 to 36 h PI was about 7.6 h in the absence of MG132 and not significantly different from the half-life (9.6 h) in the presence of MG132. In the absence of MG132, the half-life of p53 was about the same during the late phase of HCMV infection (about 8.4 h, 48–72 h PI) as observed during the early phase. In contrast to the results in mock-infected cells, the presence of MG132 significantly (p < 0.05) increased the half-life of p53 in HCMV-infected cells monitored from 48 to 72 h PI to more than 24 h (Fig. 3D). The results of this series of experiments suggested that ubiquitin/ proteasome-mediated degradation is responsible for the short half-life of p53 in mock-infected LU cells and that this mechanism is substantially inhibited during the early phase of HCMV infection. These findings also suggest that MG132-sensitive proteolytic mechanisms, facilitating a relatively slow loss of p53, that are absent during the early phase of HCMV infection may be present during the late phase of HCMV replication.

**Ubiquitination of p53 Is Decreased during HCMV Infection**—Proteins are targeted for proteasome-mediated degradation by ubiquitination (43). To examine the possibility that the stabilization of p53 in HCMV-infected cells was a result of compromised p53 ubiquitination, density-arrested LU cells were HCMV- or mock-infected and treated with MG132 (10 μM) at 24, 48, and 72 h PI. The cells were harvested 6 h after MG132 treatment and subjected to Western blot analysis. Slower migrating p53 bands consistent with ladders of polyubiquitinated p53 were detected in mock-infected cells in the presence, but not in the absence, of MG132 at each of the times evaluated (Fig. 4). Slower migrating p53 species (ladders of polyubiquitinated p53) were not detected in protein extracts from the HCMV-infected cells treated or not treated with MG132 at 24, 48, or 72 h PI in the experiment illustrated in Fig. 4, nor in any of the several repetitions of the experimental protocol. Thus, ubiquitination of p53 appeared to be inhibited both at early and late phases of the p53 protein increase in HCMV-infected LU cells.

**HDM2 Inhibitor Decreases p53 Ubiquitination in LU Cells**—It has been reported that MDM2/HDM2 is the primary E3 ligase for p53 (44). Other E3 ligases for p53, such as Pirh2 (45), COP1 (46), and Topors (47) have been reported recently. To determine whether HDM2 was a major E3 ligase for p53 in LU cells, the effects of a cell-permeable MDM2/HDM2 inhibitor, TIBC, on p53 ubiquitination were measured in mock- and HCMV-infected LU cells. This MDM2/HDM2 inhibitor binds strongly to MDM2/HDM2 and irreversibly disrupts MDM2/ HDM2-p53 protein complexes (48). Consistent with Fig. 4, slower migrating species of p53 were detected in mock-infected
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FIGURE 4. Decreased ubiquitination of p53 in HCMV-infected cells. Density-arrested cells were either HCMV- (5 PFU/cell) or mock-infected as described in the legend to Fig. 1. At 24, 48, or 72 h PI, HCMV- and mock-infected cell cultures were treated with MG132 (10 μM) or mock-treated. Whole cell lysates for protein analysis were prepared 6 h after MG132 treatment, as described under "Experimental Procedures." Polypeptides (40 μg/lane) were resolved by PAGE in the presence of SDS. The gels were blotted onto nitrocellulose and probed with antibody against p53 (DO-1). Films were exposed for a longer time (about 1 h) than that illustrated in Figs. 1–3 to detect the presumably ubiquitinated-p53 species.

FIGURE 5. The effect of the HDM2 inhibitor, TIBC, on p53 ubiquitination in mock- or HCMV-infected LU cells. Density-arrested LU cells were either mock (M)- or HCMV (5 PFU/cell)-infected, and at 48 h PI, the cells were either treated with the HDM2 inhibitor TIBC (0, 25, 50, or 100 μM) or its vehicle (--) in the absence or presence of MG132 (5 μM). At 6 h post-treatment (PT), cell cultures were harvested, and whole cell lysates were prepared for protein analysis as described under "Experimental Procedures." Polypeptides (40 μg/lane) were resolved by PAGE in the presence of SDS as described in the legend to Fig. 1A. The gels were blotted onto nitrocellulose and probed with antibody against HDM2 (2A9C1.18 supernatant, 1:6). The blots were stripped and re-probed with antibody to m-calpain (41). The results of a representative experiment are illustrated. B, results of the experiment illustrated and two additional experiments were evaluated by densitometry and normalized to m-calpain, and the means were plotted as the abundance relative to the mock-infected control at 0 h PI with S.D. shown (C, mock-infected cells; ⦿, HCMV-infected cells). Datum points marked with an asterisk are significantly different from the values obtained for mock-infected cells at the indicated time.

these results and those illustrated in Fig. 4 indicate were suppressed in HCMV-infected LU cells.

The Effect of HCMV Infection on the Abundance of HDM2—The decrease in p53 ubiquitination in HCMV-infected cells could result from inhibition of one or more components of the ubiquitin pathway. Because HDM2 is the major E3 ligase in LU cells, we examined if the cellular abundance of HDM2 changed during HCMV infection. The same cellular lysates of HCMV- and mock-infected LU cells that were examined for p53 abundance (Fig. 1A) were reevaluated by Western blot analysis for HDM2 (Fig. 6). The level of HDM2 in mock-infected cells increased modestly (by about 30%) and insignificantly through 6 h, and then fell to about 90% of the initial level by 9 h and remained at about that level through 96 h (Fig. 6B). In HCMV-infected cells, the abundance of HDM2 also increased modestly and insignificantly through 6 h PI and then decreased over the next 90 h. There was significantly less HDM2 in HCMV-infected cells than mock-infected cells from 24 to 96 h PI (Fig. 6B) (24 h PI, p < 0.05; 48–96 PI, p < 0.01). Similar results were obtained when the blots were analyzed with other HDM2-specific antibodies (Ab3; C18) (data not shown).
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MG132 Protects HDM2 Protein from Degradation during HCMV Infection—Because HDM2 is a target of its own E3 ubiquitin ligase activity (49), we investigated if the decrease in HDM2 abundance in HCMV-infected cells was because of a proteasome-mediated mechanism. HCMV- and mock-infected, density-arrested, LU cells were treated with MG132 and HDM2 at 24, 48, or 72 h PI and 6 h later were harvested and the proteins subjected to Western blot analysis with anti-HDM2 monoclonal antibody. HDM2 was significantly (24 h PI, p < 0.05; 48–96 h PI, p < 0.01) less abundant in the HCMV-infected cells than in the mock-infected cells at each of the times examined (Fig. 7, A and B). MG132 significantly stabilized HDM2 in both mock-infected (p < 0.01) and HCMV-infected cells (p < 0.05) but to a lesser extent in the HCMV-infected cells. Increases of about 5-fold (5.0 ± 0.12–1.2, 5.1 ± 0.21–1.1, and 5.0 ± 0.30–fold at 24, 48, and 72 h respectively) were observed in the MG132-treated mock-infected cells. Smaller increases (2.3 ± 0.22–1.2, 2.1 ± 0.12–1.1, and 2.5 ± 0.14–fold, respectively) were observed in the MG132-treated HCMV-infected cells. These results suggest that HDM2 auto-ubiquitination is active in HCMV-infected, as well as mock-infected LU cells.

The Redistribution of HDM2 and p53 in HCMV-infected LU Cells—In unstressed cells, the abundance of p53 is regulated by protein complexes, consisting of MDM2/HDM2-p53-p300. Immunocytochemistry and confocal microscopy were used to evaluate the effect of HCMV infection on the subcellular distribution of these proteins. In mock-infected LU cells stained with anti-p53 and fluorescein-conjugated secondary antibody at 0 h, a dim punctate fluorescence was observed within the cytoplasm, whereas within the nucleus a few fine punctate bodies with faint fluorescence were evident (Fig. 8 A, 0 h). A similar pattern of fluorescence was observed in mock-infected cells from 24 to 96 h PI. The pattern of fluorescence observed in HCMV-infected cells in parallel coverslip cultures exposed to anti-p53 antibody was distinct (Fig. 8 A) from that observed for mock-infected cells (Fig. 8 B), in that p53 fluorescence was enhanced in HCMV-infected cells and associated with the HCMV nuclear inclusion. From 3 to 12 h PI, the number and intensity of fluorescent bodies in the nucleus of HCMV-infected cells increased substantially, whereas much less fluorescence was observed in the cytoplasm (data not shown). The pattern of nuclear p53 fluorescence at 24 h consisted of numerous brightly fluorescent punctate bodies (Fig. 8 A). By this time very little fluorescence was associated with the cytoplasm. At 48 h PI, large bodies with bright fluorescence were present in most nuclei. Other nuclei contained fine punctate fluorescence throughout the nucleus, except in the nucleoli. Fluorescence was very infrequently observed in the cytoplasm, and when it was, it was dim. By 72 h PI, bright fluorescence was evident throughout the nucleoplasm with the exception of the nucleoli, in which fluorescence was absent. The cytoplasm of some cells at 72 h PI contained both diffuse and punctate fluorescence, but it was not as bright as the nuclei of most cells. The pattern of fluorescence in HCMV-infected cells was very similar at 96 h PI (data not shown).

Because HDM2 activity is regulated by its subcellular location (50), we investigated the effect of HCMV infection on the subcellular distribution of HDM2 within LU cells. In mock- or HCMV-infected cells stained at 0 h PI with anti-HDM2 antibody, bright fluorescence was primarily restricted to areas of the nucleus that resembled the nucleoli (Fig. 8 B, 0 h). Smaller punctate bodies with dimmer fluorescence were identified in the nuclei of some cells (Fig. 8 B, 0 h). A similar pattern of nuclear fluorescence was observed throughout the time course of these studies in mock-infected LU cells (data not shown). HDM2 fluorescence was not detected in the cytoplasm of mock-infected cells at any of the times examined (Fig. 8 B, 0 h, and data not shown).

HCMV infection induced a substantial redistribution of HDM2 over the 96-h course of these studies (Fig. 8 B, 0–72 h). From 3 to 12 h PI after HCMV infection, fluorescence markedly increased in the cytoplasm, in which both dim diffuse fluorescence and bright punctate fluorescence were evident. Within the nucleus the larger punctate bodies (nucleoli) with bright fluorescence became dimmer and each appeared as an aggregate of multiple small punctate bodies with diminished fluorescence by 12 h (data not shown). This trend of diminished nuclear fluorescence continued through 72 h PI. By 24 h PI, most of the HDM2-associated fluorescence was located in the cytoplasm in structures that appeared to be associated with the early HCMV-induced cytoplasmic inclusion. The fluorescence associated with the nucleus was much dimmer than at earlier times, although the larger nuclear fluorescent structures con-
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The Cellular Distribution of the HCMV Immediate Early Protein IE2-86—Recently, results were reported suggesting that HCMV IE2-86 binds HDM2 in cells overexpressing these proteins, as well as p53 (51). Accordingly, we examined the distribution of IE2-86 in parallel coverslip cultures of HCMV-infected LU cells. IE2-86 was detectable by 1 h PI in the nuclei of HCMV-infected cells as we (52) and others (53) have previously observed; and by 6 h numerous bright punctate fluorescent bodies were present in most nuclei, whereas only faint fluorescence was visible in the cytoplasm. By 24 h PI, numerous bright punctate fluorescence bodies were detected in nearly all nuclei in the absence of detectable cytoplasmic fluorescence (Fig. 8C). Large areas of bright fluorescence were evident at 48 h PI in all nuclei, except the nucleolus. These fluorescent areas consisted of aggregates of brightly fluorescent punctate bodies. Cytoplasmic fluorescence was not detected at this time. The large areas of nuclear fluorescence increased in size and in fluorescent intensity by 72 h PI. No evidence of cytoplasmic fluorescence was observed at this time (Fig. 8C). Accordingly, we did not observe evidence supporting the co-localization of HCMV IE2-86 and HDM2 by laser scanning confocal microscopy during the course of this study.

Detection of p300, a Ubiquitin Conjugation Factor E4 Ligase Involved in p53 Polyubiquitination,

A. p53

0 h

24 h

48 h

72 h

B. HDM2

0 h

24 h

48 h

72 h

C. IE2-86

0 h

24 h

48 h

72 h

FIGURE 8. The effect of HCMV infection on the subcellular distribution of p53 (A), HDM2 (B), and HCMV IE2-86 (C). Coverslip cultures of density-arrested LU cells were infected with HCMV (5 PFU/cell) or mock-infected, as described in the legend to Fig. 1. At 0, 24, 48, and 72 h, coverslip cultures were harvested and fixed with acetone/methanol (1:1) at −20 °C ("Experimental Procedures"). The cells were exposed to monoclonal antibody specific for p53 (DO-1, 1:75), MDM2/HDM2 (2A9C1.18 supernatant, 1:100), or to HCMV IE2-86 (ES-HIE2–84, 1:2000) and counterstained with fluorescein-labeled rabbit anti-mouse (Fab’), AQ160F (Chemicon International), 1:75) or with Alexa Fluor 546-labeled goat anti-mouse IgG (H + L) (Fab’), A-11018 (Molecular Probes), 1:75. The nuclei were stained with DAPI ("Experimental Procedures"). Fluorescence was detected with a Zeiss LSM 510 UV META confocal microscope equipped with a C-planapochromat 63/1.2 water-immersion objective. Images were acquired as MDB files and analyzed using the Zeiss LSM software (version 3.2). The results from a representative experiment are shown. Blue fluorescence, DAPI staining of nuclei; green fluorescence, fluorescein staining of p53 (A) or HDM2 (B); red fluorescence, Alexa Fluor 546 staining of HCMV IE2-86 (C). The final magnification of all images is the same, with the scale bar indicating 10 μm.
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FIGURE 9. The p300 abundance and subcellular distribution in HCMV-infected LU cells. A, density-arrested LU cells were HCMV- (5 PFU/cell) or mock-infected as described in the legend to Fig. 1. Whole cell lysates were prepared for protein analysis at the times indicated in the figure. Polyacrylamide gels were resolved by PAGE in the presence of SDS. The gels were blotted onto nitrocellulose and probed with antibody against p300 (C-20). The results of a representative experiment are illustrated. B, means of the densitometric analysis, normalized for m-calpain, of the results shown in A, and two additional experiments with S.D. shown were plotted relative to the value for mock-infected cells at 0 h PI ( ). mock-infected cells; HCMV-infected cells. Datum points marked with an asterisk are significantly different from the values obtained for mock- or HCMV-infected cells at 0 h PI. C, subcellular distribution of p300 in HCMV-infected LU cells. Coverslip cultures of density-arrested LU cells were prepared and HCMV- (5 PFU/cell) or mock-infected in parallel with the cultures described above (A and B). At 72 h PI, the coverslip cultures were harvested, and the cells were fixed with acetone/methanol (1:1) at −20 °C. The cells were exposed to antibody against p300 (C-20) and fluorescein-labeled rabbit anti-mouse (AQ160F) antibody. The cells were counter-stained with DAPI (“Experimental Procedures”). Fluorescence was detected with a Zeiss LSM 510 UV META confocal microscope (63/1.2 C-planapochromat, scale bar = 20 μm). Images were acquired as MDF files and analyzed using the Zeiss LSM software (version 3.2). 0 h, mock-infected LU cells. 72 h, HCMV-infected cells at 72 h PI. Blue fluorescence, DAPI staining of nuclei; green fluorescence, fluorescein staining of p300. The final magnification of all images is the same, with the scale bar indicating 20 μm.

infection, there was a substantial increase in the intensity and distribution of p300-associated fluorescence, due in part to an increase within the cytoplasm (data not shown). By 48 h PI, p300-associated fluorescence was increased substantially in the nucleus, where it was evident as aggregates of punctate fluorescence. Similar results were obtained at 72 h (Fig. 9C). Accordingly, neither a lack of abundance of p300 nor its sequestration from p53 appeared to contribute to the failure to ubiquitinate p53 in HCMV-infected cells.

Discussion

The results of this study confirm that HCMV infection results in increased cellular levels of p53 (17, 18, 21). The kinetics of the p53 increase observed in our study was similar to that observed by Muganda et al. (17), with a substantial increase in cellular levels of p53 through 24 h PI and maintenance of relatively high levels thereafter (Fig. 1A). The substantial decrease in p53 levels observed by Jault et al. (18) after 12 h PI may be a result of differing experimental conditions. Both Western blot analysis (Fig. 1A) and laser scanning fluorescence microscopy (Fig. 8B) indicate that substantial levels of p53 are maintained well into the late phase of HCMV infection.

It is difficult to account for the early increases in p53 protein based on transcriptional activation of p53 as proposed by Muganda et al. (19), because there is a considerable lag (more than 24 h) in the major p53 RNA increase relative to the increase in p53 protein (compare Fig. 1, C and D). Aside from an insignificant and short-lived increase in p53 transcripts at 3 h, p53 RNA synthesis was near baseline through at least 24 h PI (Fig. 1D), during which p53 protein rose rapidly beginning by 6 h PI and reached near maximal levels by 24 h PI (Fig. 1C). Transcripts for p53 were elevated from 48 to 72 h PI, suggesting that transcriptional activation may have contributed to the p53 protein levels observed during the late phase of HCMV replication. Earlier work (55), however, showed that the synthesis of p53 was decreased relative to mock-infected cells at this time, so that the increased p53 RNA may not be reflected in increased synthesis of p53 protein. Additional experiments demonstrate that even during the late phase of HCMV infection p53 protein stabilization contributes substantially to the increased levels of p53.

The steady-state level of p53 is regulated by the balance between its synthesis and degradation. Indeed, in mock-infected, density-arrested LU cells, a moderate steady-state level of p53 was observed throughout the 96-h course of these experiments (Fig. 1A). Much earlier work has shown that HDM2-mediated ubiquitination is an important component in regulating the level of p53. Our results demonstrate that the ubiquitin-proteasome pathway is intact in LU cells, because p53 extracted from these cells demonstrated prominent, presumably ubiquitin, ladders (Fig. 4) and a substantial increase in p53 stability when proteasome-mediated degradation was inhibited by MG132 (Fig. 3). Furthermore, the findings that inhibition of HDM2 activity by TIBC caused an concentration-dependent decrease in the abundance of these p53 ladders (Fig. 5) are consistent with these ladders representing ubiquitinated p53 and indicate that HDM2 is the major E3 ligase for p53 in density-arrested LU cells, as in many other cells (29, 56). In the presence of MG132, the half-life of p53 was extended from less than 30 min to about 8 h at 12–36 h PI and to more than 24 h at 48–72 h PI (Fig. 3). These findings indicate that the half-life of p53 in mock-infected, density-arrested LU cells is highly sensitive to inhibition of the ubiquitin/proteasome pathway.

The lack of correlation between the changes in abundance of p53 RNA and protein identified in this study during the early progression of HCMV infection suggests that the balance between p53 synthesis and degradation is altered during HCMV infection. Evidence from several experimental approaches is consistent with the view that HCMV infection stabilizes p53. Protein decay studies in cycloheximide-treated cells indicate that p53 protein is significantly stabilized during HCMV infection with the half-life extended from less than 30 min to about 8 h (Fig. 2), similar to the effect of MG132. The rapid component in the decay curves for p53 observed in mock-infected cells is not evident during the early or late phase of
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HCMV replication (Fig. 2), suggesting that mechanisms facilitating the rapid decay of p53 in mock-infected cells are compromised by HCMV infection. The decreased effect of MG132 in extending p53 half-life in HCMV-infected cells (Fig. 3) is consistent with the absence of the rapid decay component (Fig. 2) and the increased stability of p53. In fact, much of the increased abundance of p53 in HCMV-infected cells appears to be due to its stabilization, even at times (48 h PI) when a high level of p53 transcripts was available (Fig. 1D). Also consistent with these results were the observations that ladders of presumably ubiquitinated p53 were not detected in HCMV-infected cells in this study (Figs. 4 and 5), even in the presence of MG132, nor in the study by Zhang et al. (51) using cells transfected with HCMV IE2-86. Although ladders of polyubiquitinated p53 were not detected in the HCMV-infected cells, paradoxically, treatment with MG132 resulted in additional stabilization of p53 in the LU cells at 48–72 h PI, but not at early times (Fig. 3B). It is possible that during the late phase of HCMV infection MG132 indirectly stabilized p53 by stabilizing one or more proteins, for example, which either inhibited other proteases capable of degrading p53 or were involved in p53 post-translational modifications that influenced p53 degradation.

The results of this investigation indicate that much of the stabilization of p53 in HCMV-infected cells is a consequence of modifications of HDM2 homeostasis, so that ubiquitination of p53 and its proteasome-mediated degradation is inhibited. A ternary complex consisting of MDM2/HDM2, p300, and p53 has been reported to regulate p53 stability (57). Indeed, in the LU cells that were the cellular substrate for these studies, ubiquitination of p53 was dependent on the activity of HDM2 (Fig. 5), consistent with the findings in a number of earlier studies that MDM2/HDM2 is the primary E3 ligase monoubiquitinating p53. The E3 ligase activity of MDM2/HDM2 is controlled by its subcellular location. The shuttling of MDM2/HDM2 between the nucleoplasm, where it is active, and the nucleolus and the cytoplasm, where it is inactive, regulates ubiquitination of p53 and consequent p53 degradation in uninfected cells (58). MDM2/HDM2-mediated ubiquitination of p53 is dependent on the binding of MDM2/HDM2 to p53 and, accordingly, on the two molecules residing within the same subcellular compartment within the nucleoplasm (59). From the first few hours after HCMV infection, most p53 is located in the nucleus but exclusive of the nucleoli (Fig. 8B). Thus, the translocation of HDM2 to the cytoplasm, with a relatively small amount remaining within the nucleoli (Fig. 8A), and the decreasing cellular abundance of HDM2 (Fig. 7) indicate that HCMV infection causes the redistribution of HDM2 and p53 to distinct subcellular compartments and stimulates degradation of HDM2. These results suggest that there should be a substantial reduction of p53 ubiquination in HCMV-infected cells. Our immunoblot analysis confirms this, both at early (24 h PI) and at late (48, 72 h PI) phases of HCMV infection (Fig. 4). This is the first time that HCMV, or any other virus that we are aware of, has been demonstrated to block the HDM2-p53 interaction by redistributing HDM2 (essentially quantitatively removing HDM2 from the nucleoplasm), and thus stabilizing p53.

In a recent report, which examined the effect of overexpression of exogenous HCMV IE2-86, p53, and HDM2 on p53 protein levels, it was noted that IE2-86 bound HDM2, leading to the proteasome-independent degradation of HDM2 and consequently the stabilization of p53. Our results are inconsistent with important aspects of this report, even though both studies indicate that HCMV infection results in decreased ubiquitination of p53 and substantially reduced abundance of HDM2. We did not observe results supporting the co-localization of endogenously expressed IE2-86 and HDM2 in HCMV-infected cells. In fact, endogenously expressed HDM2 and IE2-86 were localized in distinct subcellular compartments as a result of HCMV infection (Fig. 8, compare A and C). It may be noteworthy that IE2-86-HDM2 complexes were not detected in whole cell lysates of HCMV-infected cells in the study by Zhang et al. (51), which was attributed to the rapid degradation of HDM2. Only when lysates of HCMV-infected cells were mixed with lysates of MG132-treated uninfected cells were IE2-86-HDM2 complexes observed. As an immediate early HCMV protein, IE2-86 is synthesized rapidly after infection and reaches abundant levels by 1 h PI (5). If IE2-86 binding were directly responsible for the degradation of HDM2 in HCMV-infected cells, then a measurable decrease in HDM2 would be anticipated within the first few hours after HCMV infection. Our results indicate that HDM2 levels increased at 3 h PI (Fig. 6), when IE2-86 is abundant. Furthermore, in our study, HDM2 was stabilized by MG132 in HCMV-infected cells, indicating that HDM2 degradation was dependent on the activity of the proteasome (Fig. 7). Thus, the results of this study do not support a mechanism in which p53 stabilization is based on the loss of HDM2 through direct binding of IE2-86 to HDM2, leading to proteasome-independent degradation of HDM2. Neither the results of this study nor those of Zhang et al. (51), however, rule out the possibility that the binding of IE2-86 to p53, which has been reported for some time, disrupts HDM2-p53-p300 complexes leading to the destabilization of HDM2.

A number of factors, in addition to the abundance and subcellular distribution of HDM2, have been identified in the ubiquitin-proteasome pathway for degradation of p53. p300 has been identified as a ubiquitin conjugation factor E4 ligase for p53. p300 cellular levels are enhanced by HCMV infection (Fig. 9), and p300 remains predominantly in the nucleus (Fig. 9C). Considered together, these results suggest that if small amounts of p53 were monoubiquitinated by any HDM2 that was within our level of detection in the nucleus, then p53 would be polyubiquitinated. Polyubiquitinated p53 was not detected in HCMV-infected cells in this study (Figs. 4 and 5), indicating that very little p53 is monoubiquitinated in the HCMV-infected cells. Additionally, monoubiquitination of p53 exposes its nuclear export signal, facilitating its export from the nucleus (61). Very little p53 was associated with the cytoplasm by 24 h PI (Fig. 8A), when HDM2 had been translocated to the nucleoli and cytoplasm (Fig. 8B).

Increasing evidence suggests that the nucleolus may be a pivotal subcellular site, integrating stress signals that are converged through regulation of the balance in the MDM2/HDM2-p53 pathway (62). Rubbi and Milner (62) have also suggested that nucleolar disruption may be necessary for p53 stabilization. Unlike infection by some viruses, such as alphaviruses (63), HCMV infection leaves the nucleolus morphologi-
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...cally intact (64, 65). Thus, our results indicate that nuclear disruption is not always necessary for p53 stabilization. It has been reported that a substantial number of nucleolar proteins, such as p14ARF (66–68), L5 (69, 70), L11, L23, PML, nucleophosmin (NPM, B23), and nucleolin, are capable of interacting with MDM2/HDM2, thereby inhibiting its activity and leading to the stabilization and activation of p53. It is possible that one or more of these cellular proteins or an HCMV-specified protein is involved in the nucleolar sequestration of HDM2 observed in this study (Fig. 8B). The amount of HDM2 observed in the nucleolus of HCMV-infected cells, however, is only a small fraction of the cellular HDM2 (Fig. 8B). The overwhelming HDM2 modification in response to HCMV infection in permissive LU cells is export to the cytoplasm and degradation. It is not known at this time if the small amount of HDM2 observed in the nucleolus of HCMV-infected cells is a part of the pathway directing HDM2 toward nuclear export and degradation (Figs. 6 and 7). Studies of these changes and the proteins governing the nucleolar sequestration of HDM2 or a switch to HDM2 autoubiquitination in HCMV-infected cells are beyond the scope of this study. It may be noteworthy, however, that recently several groups have obtained results suggesting that HCMV activates the ataxia-telangiectasia mutated gene (71–73), which is known to phosphorylate HDM2 in x-irradiated cells, leading to HDM2 degradation (74). It is not presently known if phosphorylation of HDM2 leads to autoubiquitination and degradation of HDM2 in HCMV-infected cells, although we have observed some preliminary evidence of HDM2 phosphorylation.

The strategies DNA viruses used to induce cells to enter and traverse the cell cycle are quite diverse. As might be anticipated for viruses that replicate in post-mitotic cells, a general cellular target of these viruses appears to be inactivation of tumor suppressor gene products that arrest cell cycle entry and progression (22). HCMV appears to use a different strategy, which resembles the stress-activated pathway, converting HDM2- mediated p53 ubiquitination to ubiquitination and degradation of HDM2. Extraordinarily, in these mitogenically activated cells (3, 4), p53 is stabilized principally through this mechanism, resulting in a substantial increase in its cellular abundance. The HCMV strategy for promoting cell cycle entry and progression, while preserving high cellular levels of p53, involves activating the ubiquitous cellular calpains and degrading the p53-responsive, cyclin kinase inhibitor, p21Clp1 (13). By removing HDM2 from the nucleoplasm, HCMV infection inhibits ubiquitination and, possibly, neddylation of p53, because neddylation requires HDM2 (75). It has been proposed that HDM2/MDM2 forms a complex with E2F transcription factor 1 and DP-1 that is required for transcription necessary for the G1/S phase transition. If this proposal is correct, then the strategy used by HCMV of excluding HDM2 from the nucleoplasm would be anticipated to contribute to inhibition of G1/S transition. Indeed, we (4) and others have demonstrated previously that stationary cells productively infected and initiated by HCMV infection are arrested at or near the G1/S boundary. Thus, utilization of the cellular stress response of shifting HDM2 E3 ligase activity from p53 to HDM2 ubiquitination facilitates accumulation of p53 and possibly contributes to the inhibition of the G1/S transition (4) in HCMV-infected cells. This strategy, together with the activation of other cellular pathways (7, 9), provides HCMV with the means to efficiently replicate in post-mitotic cells with uncompeted access to abundant DNA precursors (3, 4).

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REFERENCES

1. Rawlinson, W., and Scott, G. (2003) Aust. Fam. Physician 32, 789–793
2. Gandhi, M. K., and Khanna, R. (2004) Lancet Infect. Dis. 4, 725–738
3. Albrecht, T., Boldogh, I., Fons, M., Lee, C. H., Abubakar, S., Russell, J. M., and Au, W. W. (1989) Subcell. Biochem. 15, 157–202
4. Bresnahan, W. A., Boldogh, I., Thompson, E. A., and Albrecht, T. (1996) Virology 224, 150–160
5. Valyi-Nagy, T., Bandi, Z., Boldogh, I., and Albrecht, T. (1988) Arch. Virol. 101, 199–207
6. Abubakar, S., Boldogh, I., and Albrecht, T. (1990) Biochem. Biophys. Res. Commun. 166, 953–959
7. Albrecht, T., Boldogh, I., and Fons, M. P. (1992) J. Investig. Dermatol. 98, S29–S35
8. Nokta, M., Eaton, D., Steinsland, O. S., and Albrecht, T. (1987) Virology 157, 259–267
9. Evers, D. L., Wang, X., and Huang, E. S. (2004) Microbes Infect. 6, 1084–1093
10. Boldogh, I., Abubakar, S., and Albrecht, T. (1990) Science 247, 561–564
11. Wang, X., Huong, S. M., Chiu, M. L., Raab-Traub, N., and Huang, E. S. (2003) Nature 424, 456–461
12. Wang, X., Huang, D. Y., Huong, S. M., and Huang, E. S. (2005) Nat. Med. 11, 515–521
13. Chen, Z., Knutson, E., Kurosky, A., and Albrecht, T. (2001) J. Virol. 75, 3613–3625
14. Bresnahan, W. A., Albrecht, T., and Thompson, E. A. (1998) J. Biol. Chem. 273, 22075–22082
15. Zhu, H., Cong, J. P., Mamtorra, G., Gingers, T., and Shenk, T. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 14470–14475
16. Bresnahan, W. A., Thompson, E. A., and Albrecht, T. (1997) J. Gen. Virol. 78, 1993–1997
17. Muganda, P., Mendoza, O., Hernandez, J., and Qian, Q. (1994) J. Virol. 68, 8028–8034
18. Jault, F. M., Jault, J. M., Ruchti, F., Fortunato, E. A., Clark, C., Corbeil, J., Richman, D. D., and Spector, D. H. (1995) J. Virol. 69, 6697–6704
19. Muganda, P., Carrasco, R., and Qian, Q. (1998) Cell. Mol. Biol. (Noisy-le-grand) 44, 321–331
20. Castillo, J. P., and Kowalik, T. F. (2002) Gene (Amst.) 290, 19–34
21. Wang, J., Marker, P. H., Belcher, J. D., Wilcken, D. E., Burns, L. J., Vercellotti, G. M., and Wang, X. L. (2000) FEBS Lett. 474, 213–216
22. Collot-Teixeira, S., Bass, J., Denis, F., and Ranger-Roget, S. (2004) Rev. Med. Virol. 14, 301–319
23. Speir, E., Huang, E. S., Modali, R., Leon, M. B., Shawl, F., Finkel, T., and Epstein, S. E. (1995) Scand. J. Infect. Dis. Suppl. 99, suppl. 78–81
24. Rimsza, L. M., Vela, E. E., Frutiger, Y. M., Richter, L. C., Grogan, T. M., and Bellamy, W. T. (1996) Mol. Diagn. 1, 291–296
25. Vogelstein, B., Lane, D., and Levine, A. J. (2000) Nature 408, 307–310
26. Royds, J. A., and Jacopetta, B. (2006) Cell Death Differ. 13, 1017–1026
27. Guimearaes, D. P., and Hainaut, P. (2002) Biochimie (Paris) 84, 83–93
28. Levine, A. J. (1989) Princess Takamatsu Symp. 20, 221–230
29. Yang, Y., Li, C. C., and Weissman, A. M. (2004) Oncogene 23, 2096–2106
30. Chen, J., Marechal, V., and Levine, A. J. (1993) Mol. Cell. Biol. 13, 4107–4114
31. Oliner, J. D., Pietenpol, J. A., Thiagalingam, S., Gyuris, J., Kinzler, K. W., and Vogelstein, B. (1993) Nature 362, 857–860
32. Kussie, P. H., Gorina, S., Marechal, V., Eilenbaas, B., Moreau, J., Levine, A. J., and Pavletich, N. P. (1996) Science 274, 948–953
33. Chi, S. W., Lee, S. H., Kim, D. H., Ahn, M. J., Kim, J. S., Woo, J. Y., Tor-
Sequestration and Degradation of HDM2 during HCMV Infection

izawa, T., Kainosho, M., and Han, K. H. (2005) J. Biol. Chem. 280, 38795–38802
34. Freedman, D. A., and Levine, A. J. (1998) Mol. Cell. Biol. 18, 7288–7293
35. Grossman, S. R., Deato, M. E., Brignone, C., Chan, H. M., Kung, A. L., Tagami, H., Nakatani, Y., and Livingston, D. M. (2003) Science 300, 342–344
36. Deb, S. P. (2003) Mol. Cancer Res. 1, 1009–1016
37. Albrecht, T., and Weller, T. H. (1980) Am. J. Clin. Pathol. 73, 648–654
38. Albrecht, T., Li, M. L., Cole, N., Downing, E., and Funk, F. D. (1980) J. Gen. Virol. 51, 83–97
39. Bowman, L. H., Rabin, B., and Schlessinger, D. (1981) Nucleic Acids Res. 9, 4951–4966
40. Poehlmann, R., Fodera, B., Chen, L., Shao, W., Levine, E. A., and Chen, J. (1998) Oncogene 17, 2629–2636
41. Zhang, W., Lane, R. D., and Mellgren, R. L. (1996) J. Biol. Chem. 271, 18825–18830
42. Saito, Y., Tsukuba, S., Ito, H., and Kawashima, S. (1999) Neurosci. Lett. 259, 1–4
43. Ciechanover, A. (2005) Nat. Rev. Mol. Cell Biol. 6, 79–87
44. Brooks, C. L., and Gu, W. (2006) Mol. Cell 21, 307–315
45. Leng, R. P., Lin, Y., Ma, W., Wu, H., Lemmers, B., Chung, S., Parant, J. M., Chung, S., Parant, J. M., Lin, Y., Wu-Baer, F., Chen, D., Baer, R., and Gu, W. (2003) Science 302, 1972–1975
46. Rubbi, C. P., and Milner, J. (2003) EMBO J. 22, 6068–6077
47. Stanton, G. J., Osborne, L. C., and Albrecht, T. B. (1977) Proc. Soc. Exp. Biol. Med. 156, 109–112
48. Albrecht, T., Cavallo, T., Cole, N. L., and Graves, K. (1980) Lab. Invest. 42, 1–7
49. Albrecht, T., Graves, K., Cole, N. L., and Albrecht, T. (1981) J. Virol. 56, 97–104
50. Zhang, Y., Xiong, Y., and Yarbrough, W. G. (1998) Cell 92, 725–734
51. Shott, F. J., Bates, S., James, M. C., McConnell, B. B., Starborg, M., Brookes, S., Palmero, I., Ryan, K., Hara, E., Vousden, K. H., and Peters, G. (1998) EMBO J. 17, 5001–5014
52. Tao, W., and Levine, A. J. (1999) Proc. Natl. Acad. Sci. U.S.A. 96, 6937–6941
53. Marechal, V., Elenbaas, B., Piette, J., Nicolas, J. C., and Levine, A. J. (1994) Mol. Cell. Biol. 14, 7414–7420
54. Dai, M. S., and Lu, H. (2004) J. Biol. Chem. 279, 44475–44482
55. Song, Y. J., and Stinski, M. F. (2005) J. Virol. 79, 2597–2603
56. Shen, Y. H., Utama, B., Wang, J., Raveendran, M., Senthil, D., Waldman, W. J., Belcher, J. D., Vezzetti, E., Martin, D., Mitchell, B. M., and Wang, X. L. (2004) Circ. Res. 94, 1310–1317
57. Gaspar, M., and Shenk, T. (2006) Proc. Natl. Acad. Sci. U.S.A. 103, 2821–2826
58. de Toledo, S. M., Azzam, E. I., Dahlberg, W. K., Gooding, T. B., and Little, J. B. (2000) Oncogene 19, 6185–6193
59. Xirodimas, D. P., Saville, M. K., Bourdon, J. C., Hay, R. T., and Lane, D. P. (2004) Cell 118, 83–97