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Evidence that the Human Cytomegalovirus IE2-86 Protein Binds mdm2 and Facilitates mdm2 Degradation

Zhigang Zhang,[1] David L. Evers,[1] Joseph F. McCarville,[2] Jean-Christophe Dantonel,[3] Shu-Mei Huong,[1] and Eng-Shang Huang[1,4,5]*

Lineberger Comprehensive Cancer Center,[1] Department of Biochemistry and Biophysics,[2] Department of Medicine,[4] and Department of Microbiology and Immunology,[5] University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7295, and Institut de Genetique Moleculaire, CNRS-UMR 5535, Montpellier, France[3]

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Levels of the p53 tumor suppressor protein are increased in human cytomegalovirus (HCMV)-infected cells and may be important for HCMV pathogenesis. In normal cells p53 levels are kept low due to an autoregulatory feedback loop where p53 activates the transcription of mdm2 and mdm2 binds and ubiquitinates p53, targeting p53 for proteasomal degradation. Here we report that, in contrast to uninfected cells, mdm2 was undetectable upon treatment of infected fibroblasts with the proteasome inhibitor MG132. Cellular depletion of mdm2 was reproducible in p53-null cells transfected with the HCMV IE2-86 protein, but not with IE172, independently of the endogenous mdm2 promoter. IE2-86 also prevented the emergence of presumably ubiquitinated species of p53. The regions of IE2-86 important for mdm2 depletion were those containing the sequences corresponding to the putative zinc finger and C-terminal acidic motifs. mdm2 and IE2-86 coimmunoprecipitated in transfected and infected cell lysates and in a cell-free system. IE2-86 blocked mdm2's p53-independent transactivation of the cyclin A promoter in transient-transfection experiments. Pulse-chase experiments revealed that IE2-86 but not IE1-72 or several loss-of-function IE2-86 mutants increased the half-life of p53 and reduced the half-life of mdm2. Short interfering RNA-mediated depletion of IE2-86 restored the ability of HCMV-infected cells to accumulate mdm2 in response to proteasome inhibition. Taken together, the data suggest that specific interactions between IE2-86 and mdm2 cause proteasome-independent degradation of mdm2 and that this may be important for the accumulation of p53 in HCMV-infected cells.

Human cytomegalovirus (HCMV) is a betaherpesvirus that infects the majority of the population and is best known for causing birth defects and health problems in immunocompromised individuals such as transplant recipients and AIDS patients (8). Seropositivity for HCMV also correlates with poorer clinical outcomes for inflammatory bowel diseases, allograft rejection, atherosclerosis, and coronary restenosis following angioplasty. There are a number of reports of increased p53 and phosphorylated p53 in HCMV-infected cells and cells expressing the HCMV immediate-early (IE) protein IE2-86 (32, 51, 58, 60). However, the detailed mechanism of such an increase and its biological significance are not well understood.

The effects of HCMV on coronary smooth muscle cells were correlated with p53 accumulation and IE2-86-mediated inhibition of p53 functioning (60). This was evidenced by reports that IE2-86 expression in HCMV-infected cells mirrored p53 induction and IE2-86 coimmunoprecipitated p53 (59). IE2-86 also blocked reporter gene activity from p53-responsive promoters without interfering with p53 binding to its target DNA sequences (61). Steady-state levels of p21, a cyclin-dependent kinase inhibitor whose levels are indicative of p53 transcriptional activity, are decreased in HCMV-infected cells (12, 23, 52). However, either IE1-72 or IE2-86 alone increased the relative amounts of p21 in transfected cells (10, 58).

* Corresponding author. Mailing address: CB #7295, Lineberger Comprehensive Cancer Center, Rm. 32-026, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7295. Phone: (919) 966-4323. Fax: (919) 966-4303. E-mail: eshuang@med.unc.edu.

p53 is a cellular tumor suppressor protein that is induced in response to a variety of stresses, including cellular DNA damage, and is critical in arresting cell growth and the induction of apoptosis (41). Levels of p53 are normally maintained in a regulatory circuit with mdm2. p53 induces the transcription of mdm2, a ubiquitin ligase that targets p53 for proteasomal degradation (49). A variety of posttranslational modifications to p53, such as serine 15 phosphorylation by ataxia telangiectasia-mutated and Rad 3-related kinases, are capable of breaking the p53-mdm2 regulatory loop and increasing levels of p53 (3).

HCMV IE proteins are promiscuous transactivators with multiple functions (11). They block apoptosis (71) and are capable of stimulating quiescent cells into G_1/S phase (70) and arresting cell cycle progression at the same border (43). IE1-72 and IE2-86 bind and phosphorylate retinoblastoma (Rb) family members, resulting in the upregulation of cyclin E/cdk2 kinase activity and E2F/DP transcription factor release (47, 54, 64, 68), respectively. Cells transfected with IE2-86 show predominant activation of E2F-responsive genes (57), while IE1-72 causes the release of cyclin E/cdk2 from its inactive complex (68). IE1-72 is a serine/threonine protein kinase (53) that appears to be dispensable for HCMV replication under high multiplicities of infection (29), while IE2-86 is not (46). Either IE1-72 or IE2-86 can induce p53 (51), but IE2-86 is considered the dominant factor that induces and inactivates p53 during HCMV infection for the reasons described above.

Productive infection for a number of DNA viruses is accompanied by increased levels of inactive p53 (15). While it remains unclear how these viruses induce p53, it is likely that upregulation of p53, particularly as an effector of apoptosis, is

not conducive to viral replication. Numerous DNA viruses encode proteins that undermine p53 functioning. The best known of these include the adenovirus E1 proteins, human papillomavirus E6 and E7, and the large T antigen of polyomaviruses (41).

While a great deal of attention has been paid to the effects of virus infection upon p53, relatively little work has been done to characterize mdm2 in virus-infected cells. Knockout experiments suggest that mdm2 is at least as biologically important as p53. p53-negative mice display mild phenotypes and are susceptible to spontaneous tumors (19). Mdm2 knockouts are embryonic lethal and can be rescued by codeletion of p53 (36, 50). While mdm2 levels can indicate p53 transcriptional activity, mdm2 is its own ubiquitin ligase and a number of p53-independent functions have been described for mdm2, such as cell cycle regulation, differentiation, and transcriptional transactivation (25).

IE2-86, p53, and mdm2 are all known to bind and/or affect a variety of proteins associated with transcriptional activation and cell cycle regulation. For example, both IE2-86 and mdm2 bind to p53, Rb, TATA box-binding protein, Sp1, and p300/CBP (11, 25). Mdm2 links the Rb and p53 tumor suppressor pathways as a trimeric complex of Rb-mdm2-p53 (31). Many biologically relevant aspects of the interactions among these proteins and their corresponding pathways remain unresolved.

There is peripheral evidence to suggest that mdm2 may be either upregulated or downregulated in HCMV-infected cells. HCMV infection proceeds with the phosphorylation of Akt, Rb, and mitogen-activated protein kinases ERK1 and -2 (32–34, 55). Each of these events has induced mdm2 in other systems. Inhibition of mdm2 resulted in p21 induction independently of p53 (69), yet p21 is downregulated in HCMV-infected cells (12, 52). These observations suggest that mdm2 might be upregulated in response to HCMV infection. However, the increase in p53 levels and the lack of p53 transcriptional activity in HCMV-infected cells (60, 61) suggest that mdm2 might be downregulated in HCMV infection. Also, Sp1 transcription factors are induced in HCMV-infected cells (67) and mdm2 has been shown to inhibit Sp1-mediated transcription (35).

We are aware of only one report that has examined the status of mdm2 during HCMV infection. In situ hybridization and immunohistochemistry of human tissue sections correlated p53 induction with HCMV infection, yet infected cells demonstrated mutually exclusive patterns of either p21 or mdm2 induction (26). This study was initiated as an inquiry into the mechanism of p53 accumulation in HCMV-infected fibroblasts and to examine the fate of mdm2 in cells productively infected with HCMV and its implications for the effects of this virus upon the p53-mdm2 regulatory loop.

MATERIALS AND METHODS

Reagents. MG132 was purchased from Sigma (St. Louis, MO). Antibodies to p53 (DO-1 and FL-393) and mdm2 (SMP-14) were purchased from Santa Cruz (Santa Cruz, CA). Antibodies to HCMV IE1-72 and IE2-86 proteins were prepared as described previously (39). Antibody to β-actin (CP01) was from Oncogene (San Diego, CA). Protein G-Sepharose and glutathione-Sepharose 4B were from Amersham Biosciences (Piscataway, NJ). All other reagents were from Sigma and were of at least reagent grade.

Plasmids. pCMV-HDM2 and pcDNA3-myc3-HDM2 were gifts from Yue Xiong. (Elsewhere in this publication we employ the term mdm2 regardless of the organism of origin.) The reporter plasmid expressing firefly luciferase from the cyclin A promoter (WT-Luc) was prepared as described previously (14). Control plasmid expressing *Renilla* luciferase from a herpes simplex virus thymidine kinase promoter (pRL-TK) was purchased from Promega (Madison, WI).

pcDNA3IE2 containing IE2-86 cDNA was used as a template for PCR to produce full-length protein and the following deletions: IE2-26-579, encoding amino acids (aa) 26 to 579; IE2-D325-448, encoding aa 1 to 324 and 449 to 579; IE2-1-324, encoding aa 1 to 324; and IE2-1-538, encoding aa 1 to 538. A C-terminal Flag tag was incorporated into the primer design of each construct. The primers used to generate deletion mutants were IE2N (5′-CGACGGATCCATGGAGTCCTCTGCCAAGAG-′3), IE2C (5′-GTCGAATTCCTTGTCGTCGTCATCCTTGTAGTCCTGAGACTTGTTCCTC-3′), IE2-26-579N (5′-CGACGGATCCATGGAGACACCCGTGACCAAGG-3′), IE2-1-324C (5′-GTCGAATTCCTTGTCGTCGTCATCCTTGTAGTCCTTCTTGCGGGGTCC-3′), IE2-D325-448N (5′-GGACCCCGCAAGAAGGTGACACATCCACC-3′), IE2-D325-448C (5′-CGGGTGGATGTGTCACCTTCTTGCGGGGTCC-3′), and IE2-1-538C (5′-GTGGAATTCCTTGTCGTCGTCATCCTTGTAGTCCTCGTAGATAGGCAGC-3′).

Each PCR fragment contained a BamHI restriction site at the 5′ terminus and a 3′-terminal EcoRI restriction site. These restriction sites were used to insert PCR fragments into the multiple cloning sequence of the pcDNA3 expression vector to construct Flag-tagged pcDNA3IE2f, pcDNA3IE2-26-579f, pcDNA3IE2-D325-448f, pcDNA3IE2-1-324f, and pcDNA3IE2-1-538f. Ligation of the same fragments into plasmid pGEX-2T resulted in N-terminal glutathione *S*-transferase (GST) expression vectors pGEX-2TIE2f, pGEX-2TIE2-26-579f, pGEX-2TIE2-D325-448f, pGEX-2TIE2-1-324f, and pGEX-2TIE2-1-538f.

Cell culture. Human embryonic lung fibroblasts (HEL 299), BHK-21, and p53-null H1299 cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 5 to 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in an atmosphere of 6% CO_2. Cells were infected with HCMV Towne strain (passages 36 to 42) that had been purified through a sucrose cushion to eliminate cytokines and growth factor contamination at a multiplicity of infection (MOI) of 2 to 5 PFU/cell.

Western blot assays. Following treatments and incubation periods in 100-mm dishes, cell monolayers were harvested by washing in phosphate-buffered saline (PBS) and scraping in 2× sodium dodecyl sulfate (SDS) loading buffer. Samples were boiled and proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to Immobilon-P membranes (Millipore; Bedford, MA). Membranes were blocked for 30 min in 5% (wt/vol) nonfat dry milk in PBS with 0.1% Tween 20 (PBS-T). Blots were probed with primary antibodies overnight at 4°C. Membranes were washed with PBS-T, probed with secondary horseradish peroxidase-conjugated antibodies, and then washed and developed by enhanced chemiluminescence (ECL) according to the manufacturer's instructions (Amersham).

p53 ubiquitination assay. HEL cells incubated in DMEM without serum for 48 h were infected with HCMV at an MOI of 2 to 5 PFU/cell. Fourteen hours postinfection, MG132 was added to cells to a final concentration of 30 μM. Cells were harvested following 10 hours treatment with MG132, corresponding to 24 h postinfection.

Immunoprecipitation for p53 ubiquitination. The p53-null H1299 cells were transfected with vectors encoding mdm2, p53, IE1-72, IE2-86, and IE2-86 deletions in the combinations indicated in the figure legends using Effectene (QIAGEN, Valencia, CA) according to the manufacturer's instructions. Sixty-two hours posttransfection, the proteasome inhibitor MG132 was added to a final concentration of 30 μM and presumably ubiquitinated species were allowed to accumulate for approximately 10 h. Cells were harvested by scraping in ice-cold PBS and pelleted and then lysed in denaturing buffer containing 50 mM Tris-HCl, 0.5 mM EDTA, 1% SDS, and 1 mM dithiothreitol, followed directly by boiling for 10 min to avoid coimmunoprecipitation of another ubiquitinated protein(s). Lysates were diluted 10-fold in EBC (0.05 M Tris-HCl, pH 8.0, 0.12 M NaCl, 0.5% NP-40, 0.1 M NaF, 0.2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) buffer, followed by immunoprecipitation with anti-p53 (FL-393).

Protein-protein interaction detection. Cells were harvested by scraping in ice-cold PBS and pelleted and then lysed in EBC. Cell extracts of 500 μg of protein were first precleared with protein G-Sepharose beads for 1 h at 4°C and extracts were incubated with the appropriate antibody for 4 h. Protein complexes were precipitated by incubation with protein G-Sepharose beads overnight at 4°C. Precipitated complexes were washed four times with 1 ml of ELB^+ buffer (0.25 M NaCl, 0.1% NP-40, 0.05 M HEPES, pH 7.0, 1 mM phenylmethylsulfonyl fluoride, 5 mM EDTA, and 0.5 mM dithiothreitol) and then resuspended in 2×

sample buffer and boiled for 8 min. Proteins were separated by SDS-PAGE and analyzed by Western blotting.

Protein interaction. Transcription-translation of mdm2 was performed with a TnT-coupled reticulocyte lysate system (Promega) for ^{35}S labeling using pcDNA3-myc3-HDM2 as the template DNA. Equal amounts of the resulting protein were mixed individually with 25 μl of a complex of glutathione, Sepharose 4B, GST, and IE2-86 (described below) and its deletions at 4°C overnight in 0.3 ml of ELB$^+$ buffer. Beads were washed with ELB$^+$ buffer four times prior to boiling in 2× SDS-PAGE loading buffer and Western blotting and autoradiography.

GST–IE2-86 and its deletions were expressed in *Escherichia coli* BL21 bacteria and purified with glutathione-Sepharose 4B. Expression from pGEX-2T vectors containing IE2-86 or its deletion cDNAs was induced in transformed BL21 cells with 0.5 mM isopropylthiogalactopyranoside (IPTG) for 2 h. Bacteria were centrifuged at 2,500 × *g* for 10 min and pellets were resuspended in 1 ml cold PBS with 0.1 mM phenylmethylsulfonyl fluoride for each 10 ml of original culture. Suspensions were freeze-thawed three times, sonicated, and centrifuged at 13,000 × *g* for 15 min. Supernatants were mixed with 25 μl glutathione-Sepharose 4B beads for 4 h at 4°C on a rocking platform. Beads were washed with 1 ml cold PBS with 0.1 mM phenylmethylsulfonyl fluoride four times. These complexes of glutathione, Sepharose, GST, and IE2-86 or its deletions were used for pulldown experiments.

Pulse-chase. H1299 cells were incubated to 80% confluence in 60-mm dishes and were transfected with 2 μg of each expression plasmid indicated in Fig. 6 to 8 and adjusted to a total of 6 μg with pcDNA3 empty vector to standardize input plasmid DNA. Twenty-four hours posttransfection, cells were pulse-labeled with [^{35}S]methionine Translabel (MB Biosciences, Irvine, CA) in medium lacking methionine for 2 h, washed twice with PBS, and chased with fresh medium with 10% fetal bovine serum for the times indicated in Fig. 6 to 8.

The cells were scraped and washed twice with PBS and lysed in 300 μl EBC buffer at 4°C on ice for 1 h. Lysates were mixed with extra 300 μl EBL$^+$ buffer and 25 μl of protein G-Sepharose 4B for preclearance, and 0.5 μg anti-p53 DO-1 or 0.5 μg anti-mdm2 antibody was added. Samples were placed on a rocking platform at 4°C for 1 h, and 25 μl protein G-Sepharose beads was added, and samples were gently mixed at 4°C for 3 h. Immunoprecipitation complexes were run out by SDS-PAGE and gels were dried and autoradiographed. The resulting films were analyzed with New ImageQuant TL v2003.01.

Reporter gene assays. BHK-21 cells were transfected with various combinations of plasmids as indicated using Effectene. Cells were grown in six-well plates and transfected when a density of 90% confluence was reached. A total of 1.0 μg of DNA was used in each transfection. The mixture of vectors and Effectene reagent was added to the cells in 1.5 ml of DMEM without serum. Cells were incubated at 37°C and 6% CO_2 for an additional 18 h prior to dual luciferase assays that were performed according to the manufacturer's instructions (E1910; Promega). Firefly and *Renilla* luciferase activities were quantified on an LB 9501 luminometer (Berthold Technologies; Bad Wildbad, Germany) and raw data were normalized to the *Renilla* luciferase activities, which served as internal controls.

IE2-86 knockdown by siRNA. The short interfering RNA (siRNA) oligonucleotide 5′-AAACGCAUCUCCGAGUUGGACdTdT-3′ and the complement 5′-GUCCAACUCGGAGAUGCGUUUdTdT-3′ targeting HCMV IE2-86 were synthesized by Dharmacon (Lafayette, CO) as described previously (65). We employed siRNA-Cy3 (QIAGEN) (sense sequence, UUCUCCGAACGUGU CACGUdTdT; 3′-Cy3) as a nonspecific control. HEL cells were grown to confluence in 100-mm dishes at 37°C in an atmosphere of 6% CO_2 and serum starved for 24 h. Dishes of cells were transfected with 10 μl Lipofectamine 2000 and 5 μl 20 μM siRNA according to the manufacturer's instructions (Invitrogen). Four hours later cells were washed twice with DMEM and the medium was replaced with DMEM. Twenty-four hours posttransfection cells were infected with 2 to 5 PFU/cell HCMV. Six hours postinfection MG132 was added to the cell culture medium to a final concentration of 30 μM, and plates were incubated an additional 10 h prior to harvesting for Western blot assays.

RESULTS

Status of mdm2 and p53 in HCMV-infected cells. We were unable to measure endogenous mdm2 levels in HEL fibroblasts by Western blotting (Fig. 1, lane 1 of upper panel), presumably due to the low levels and/or high turnover rates described elsewhere for this protein (24, 37). Mdm2 remained undetectable upon infection with HCMV (Fig. 1, lane 2 of upper panel). In

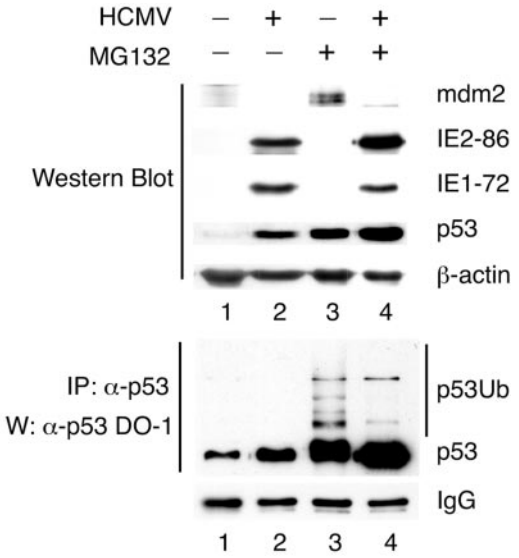

FIG. 1. Effects of HCMV infection upon mdm2 and ubiquitinated p53 as measured by immunoblotting. HEL fibroblasts were infected with HCMV and harvested at 24 h postinfection with or without a 10-h treatment with the proteasome inhibitor MG132 (30 μM). The upper panel represents direct loading and immunoblotting with the antibodies indicated, while the data in the bottom panel were obtained from immunoprecipitation and Western blotting. IP, immunoprecipitation; W, Western blotting; Ub, ubiquitin; IgG, immunoglobulin G.

HCMV-infected cells treated with the proteasome inhibitor MG132, p53 and IE2-86 levels were higher than in HCMV-infected cells without MG132 (Fig. 1, lanes 2 and 4 of upper panel). Mdm2 was detectable by Western blotting when uninfected cells were treated with MG132, yet mdm2 remained undetectable when HCMV-infected cells were treated with MG132 (Fig. 1, lanes 3 and 4 of upper panel).

Since mdm2 is known to function as a ubiquitin ligase and facilitate p53 ubiquitination and degradation (49), we examined the same system for ubiquitinated species of p53 by immunoprecipitation and Western blotting. Slowly migrating species of p53 consistent with ubiquitin conjugation were detected in uninfected cells treated with MG132 but little accumulated in the corresponding HCMV-infected cells (Fig. 1, lanes 3 and 4 of bottom panel).

HCMV IE2-86 reduced both mdm2 levels and p53 ubiquitination. Previous reports have established (51) and we have reproduced the observation (data not shown) that p53 accumulation is detectable from 4 h post-HCMV infection of fibroblasts. This prompted examination of the effects of IE1-72 and IE2-86 upon p53 ubiquitination. Mammalian expression vectors encoding these immediate-early gene products, mdm2, and p53 from CMV promoters were transfected into H1299 (p53-null) cells. Slower-migrating species of p53 suggesting ubiquitination were reduced in cells transfected with IE2-86, mdm2, and p53 (Fig. 2, lane 2 of bottom panel), but not in cells transfected with IE1-72, mdm2, and p53 (Fig. 2, lane 3 of bottom panel). mdm2 levels were reduced in cells transfected with IE2-86, mdm2, and p53 compared to those in cells transfected with mdm2 and p53 in the presence or absence of IE1-72 (Fig. 2, compare lane 2 to lanes 1 and 3 in upper panel). This suggested that IE2-86 expression, but not IE1-72, results

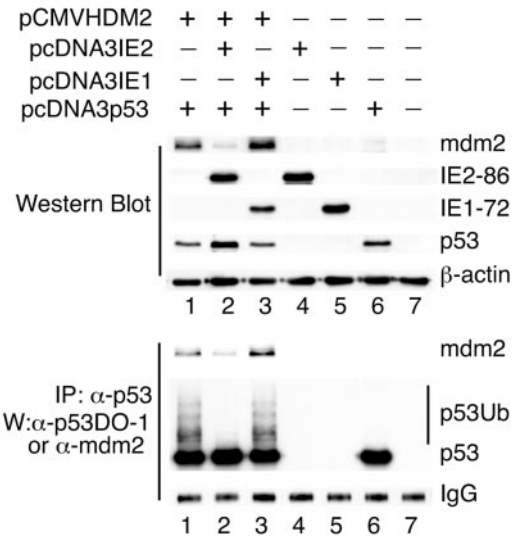

FIG. 2. Effects of IE2-86 upon mdm2 and ubiquitinated p53. H1299 (p53-null) cells were transfected with expression vectors encoding mdm2, IE1-72, IE2-86, and p53. Lysates of H1299 cells transfected with the indicated expression victors were analyzed by immunoblotting (upper panel) or by immunoprecipitation followed by Western blotting (bottom panel). IP, immunoprecipitation; W, Western blotting; Ub, ubiquitin; IgG, immunoglobulin G.

in the inhibition of p53 ubiquitination and the depletion of mdm2, independently of their endogenous promoters.

Regions of IE2-86 governing mdm2 depletion and p53 ubiquitination. To examine the domains of IE2-86 that downregulated mdm2, full-length and four deletion mutants of IE2-86 (pcDNA3IE2-26-579f, pcDNA3IE2-D325-448f, pcDNA3IE2-1-324f, and pcDNA3IE2-1-538f) were transfected into H1299 cells with mdm2 and p53 as indicated in Fig. 3. The expression of Flag-tagged full-length and deletion IE2-86 proteins was confirmed by Western blotting (Fig. 3a). Full-length IE2-86 and the mutant lacking amino acids 1 to 25 (IE2-26-579f) reduced the levels of mdm2 and stabilized those of p53 (Fig. 3a, lanes 3 and 4), yet the other three (IE2-D325-448f, IE2-1-538f, and IE2-1-324f) mutants did not (Fig. 3a, lanes 5, 6, and 7). When we immunoprecipitated p53, cells transfected with full-length IE2-86 and IE2-26-579f did not display slowly migrating species of p53 that would be consistent with ubiquitination (Fig. 3b, lanes 3 and 4). Similarly, little mdm2 was detected in complex with p53 upon transfection with IE2-86 or IE2-26-579f (Fig. 3b, lanes 3 and 4).

Immunoprecipitation of IE2-86 and its deletion mutants by their Flag epitopes allowed for the identification of interacting proteins. The mutant IE2-86 lacking amino acids 325 to 448 (IE2-D325-448f) was the most efficient at pulling down p53 despite slightly lower levels of p53 in this sample (Fig. 3c and a, lanes 5). Full-length IE2-86 and those mutants lacking amino acids 1 to 25 (IE2-26-579f), 325 to 448 (IE2-D325-448f), or 539 to 579 (IE2-1-538f) coimmunoprecipitated p53 (Fig. 3c, lanes 3, 4, 5, and 6), yet the 325 to 579 deletion mutant (IE2-1-324f) did not (Fig. 3c, lane 7). Full-length IE2-86, IE2-26-579f, and IE2-1-538f coimmunoprecipitated mdm2 (Fig. 3d, lanes 3, 4, and 6), yet mutants IE2-D325-448f and IE2-1-324f did not (Fig. 3d, lanes 5 and 7). This was observed despite the

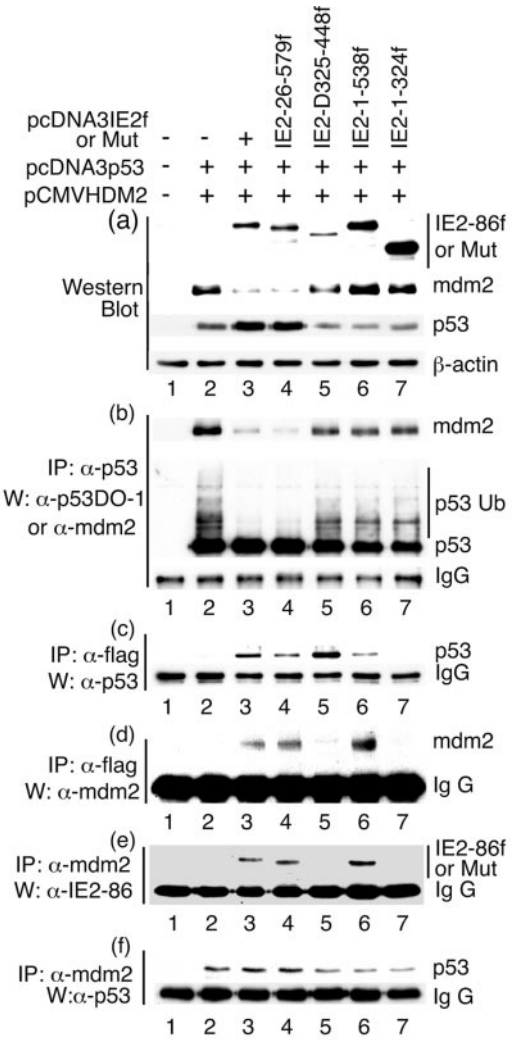

FIG. 3. Mapping the regions of IE2-86 responsible for mdm2 depletion, inhibition of p53 ubiquitination, and protein interactions. H1299 (p53-null) cells were transfected with expression vectors encoding mdm2, p53, IE2-86, and IE2-86 deletion mutants. (a) Whole-cell lysates were directly analyzed by Western blotting. (b) p53 was immunoprecipitated from lysates by anti-p53 (FL-393) antibody to determine mdm2 binding or p53 species. (c and d) IE2-86 and mutants were immunoprecipitated by their Flag epitopes and samples were examined for p53 and mdm2 complexes. (e and f) mdm2 was immunoprecipitated and complexes were examined for IE2-86 and its mutants as well as p53. IP, immunoprecipitation; W, Western blotting; Ub, ubiquitin; IgG, immunoglobulin G.

lower levels of mdm2 in cells transfected with full-length IE2-86 and IE2-26-579f (Fig. 3a, lanes 3 and 4). We observed a similar pattern when we immunoprecipitated mdm2 with anti-p53 (FL393) antibody (Fig. 3b, lanes 3 and 4). mdm2 efficiently pulled down full-length IE2-86, IE2-26-579f, and IE2-1-538f (lanes 3, 4, and 6 in Fig. 3e) but not IE2-D325-448f or IE2-1-324f (Fig. 3e, lanes 5 and 7). However, mdm2 coimmunoprecipitated p53 regardless of the presence or absence of IE2-86 and its mutants (Fig. 3f).

Taken together, the data suggested that the putative zinc finger (amino acids 428 to 452) and/or acidic (amino acids 551 to 579) domain of IE2-86 (45) may be necessary for mdm2

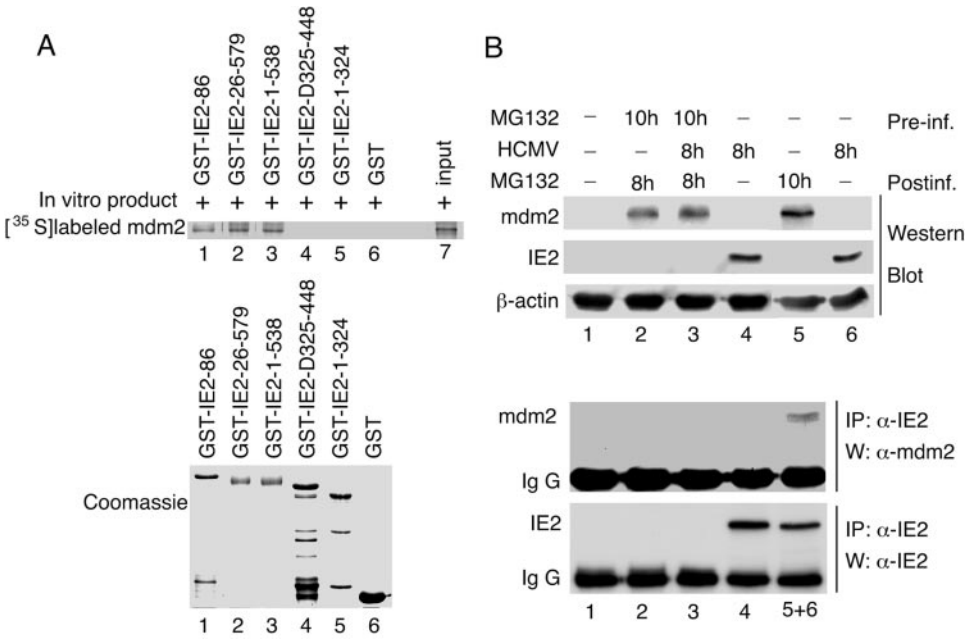

FIG. 4. Direct interactions between IE2-86 and mdm2. (A) In vitro translation reveals direct interaction between IE2-86 and mdm2. GST-tagged IE2-86 and its deletion mutants were expressed in bacteria (as verified by Western blotting; data not shown) and collected on glutathione-Sepharose beads that were incubated with [^{35}S]mdm2 produced by in vitro transcription-translation. Specifically bound mdm2 was determined by autoradiography. Complexes formed by [^{35}S]mdm2 and GST-IE2-86 or its deletion mutants were analyzed by SDS-PAGE and autoradiography (upper panel) and expression of GST-IE2 and its deletion mutants in BL21 cells was determined by SDS-PAGE and Coomassie blue staining (bottom panel). (B) Coimmunoprecipitation of IE2-86 and mdm2 from lysates of MG132-treated or HCMV-infected HEL cells. The upper panel shows Western blots for mdm2 and IE2-86 in cell lysates. HEL cells were treated with 20 μM MG132 for 10 h prior to and 8 h postinfection (lanes 2 and 3) or 30 μM MG132 for 10 h (lane 5) after the time at which the cells in lanes 3, 4, and 6 were infected. The bottom panel shows immunoprecipitation with anti-IE2 antibody and Western blots for mdm2 and IE2-86. Lanes 1 to 4 received the same treatments in the upper and lower panels. In lane 5+6, the lysates of lanes 5 and 6 from the upper panel were mixed at 4°C for 1 h and the mixture was subjected to immunoprecipitation with subsequent Western blotting. IP, immunoprecipitation; W, Western blotting; Ub, ubiquitin; IgG, immunoglobulin G.

reduction and the inhibition of p53 ubiquitination. Since IE2-D325-448f efficiently pulled down p53 (Fig. 3c, lane 5) but poorly bound mdm2 (Fig. 3d, lane 5), and IE2-1-538 efficiently interacted with mdm2 (Fig. 3d and e, lanes 6) but did not reduce mdm2 and inhibit p53 ubiquitination (Fig. 3a and b, lanes 6), it appeared possible that the physical and functional interactions between IE2-86 and mdm2 might be independent of the described physical and functional associations between IE2-86 and p53 (60, 61) and between p53 and mdm2 (41).

Direct interactions between IE2-86 and mdm2. To assess the possibility that IE2-86 might interact directly with mdm2 in the absence of p53 or other cellular factors, we employed cell-free pulldown experiments. GST-tagged full-length and deletion mutants of IE2-86 were expressed in *E. coli* BL21 and collected on glutathione-Sepharose beads. These beads were incubated with [^{35}S]methionine-labeled mdm2 produced by in vitro transcription-translation. Complexes retained on beads were examined by autoradiography for mdm2 (Fig. 4A, upper panel) and by Coomassie blue staining to confirm the presence of IE2 species (Fig. 4A, bottom panel). These results suggested that full-length IE2-86 and its mutants encoding amino acids 26 to 579 and 1 to 538 specifically interacted with mdm2 (Fig. 4A, lanes 1, 2, and 3 of upper panel) and were consistent with the HCMV-infected cell experiments shown in Fig. 3.

To investigate whether or not the specific interaction between IE2-86 and mdm2 could be reproduced in the context of an actual virus infection, cells were pretreated with 20 to 30 μM MG132 for 10 h and then infected with HCMV for the analysis of mdm2-IE2-86 complexes by immunoprecipitation followed by Western blotting. We found that cells pretreated with MG132 poorly expressed IE2-86 upon infection (Fig. 4B, lanes 3 and 4 of upper panel). This was consistent with reports that pretreatment of cells with MG132 blocked HCMV activation of NF-κB and virus infectivity (16, 17). In order to provide a system where MG132-treated cells and virus-infected cells expressing IE2-86 could be examined together, we mixed the lysates of HCMV-infected cells with MG132-treated cells and subjected the mixture to immunoprecipitation followed by Western blotting (Fig. 4B, lanes 5 and 6 of bottom panel). These conditions reproduced the mdm2-IE2-86 complexes observed by in vitro transcription-translation (Fig. 4A).

IE2-86 blocked mdm2's transactivation of the cyclin A promoter. In order to substantiate the possibility that IE2-86's downregulation of mdm2 was independent of p53, we employed a reporter gene assay using firefly luciferase expression from a cyclin A promoter. mdm2 was shown to increase cyclin A promoter activity independently of p53 (40). Figure 5 shows that mdm2 increased cyclin A promoter activity approximately sixfold in BHK-21 cells, similar to what was reported by others (40). Cotransfection with IE2-86 blocked mdm2-dependent transactivation in a dose-dependent manner but did not affect basal levels of cyclin A promoter activity (Fig. 5, lanes 2, 3, 4,

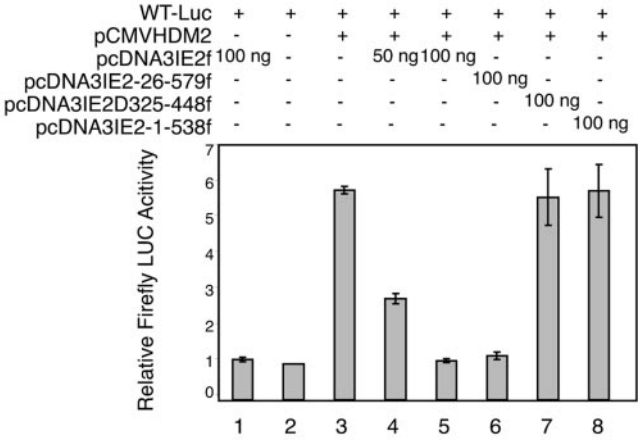

FIG. 5. Effects of HCMV IE2-86 upon mdm2-dependent transactivation of the cyclin A promoter. BHK-21 cells were transfected with a plasmid encoding firefly luciferase from a cyclin A promoter (WT-Luc) and combinations of expression vectors encoding mdm2 and/or IE2-86 and its mutants. Firefly luciferase activities were normalized to the *Renilla* luciferase activities of an internal control plasmid (pRL-TK) and relative firefly luciferase activities were determined by setting WT-Luc transfections to 1.0 arbitrary unit. Values are presented as the means ± standard deviation of four independent experiments.

and 5). IE2-26-579f reversed mdm2 activation of the cyclin A promoter (Fig. 5, lane 6), while mutants IE2-D325-448f and IE2-1-538f did not reproducibly affect this system (Fig. 5, lanes 7 and 8). These results suggested that IE2-86 specifically blocked mdm2-dependent, p53-independent transactivation of the cyclin A promoter.

IE2-86 increased the half-life of p53 and reduced the half-life of mdm2. Since IE2-86 decreased and increased the steady-state protein levels of mdm2 and p53, respectively, independently of their endogenous promoters, we examined the effects of IE2-86 on the half-lives of mdm2 and p53. Pulse-chase experiments were performed with p53-transfected [^{35}S]methionine-labeled H1299 cells cotransfected with combinations of mdm2, IE1-72, and IE2-86 expression vectors as described in the legend to Fig. 6. As expected, the half-life of p53 was reduced when it was cotransfected with mdm2 (Fig. 6A, compare a and b). When IE2-86 was cotransfected with p53 and mdm2, the effects of mdm2 on p53 were fully reversed (Fig. 6A, compare b and c) and the levels of mdm2 protein were reduced (Fig. 6B, compare b and c). In contrast, IE1-72 did not change the effects of mdm2 on p53 (Fig. 6A and B, compare b and d). These results suggested that IE2-86 stabilized p53 via increasing its half-life independently of its effects upon the endogenous p53 promoter and possibly by assisting the degradation of mdm2.

We also performed pulse-chase experiments with mdm2-transfected [^{35}S]methionine-labeled H1299 cells cotransfected with combinations of p53 and IE2-86 expression vectors. The half-life of mdm2 was increased upon cotransfection with p53 (Fig. 7A and B, compare a and c) and decreased upon cotransfection with IE2-86 (Fig. 7A and B, compare a and d). Cotransfection with p53 and IE2-86 reduced the half-life of mdm2 (Fig. 7A and B, compare a and b) in a manner indistinguishable from cotransfection with IE2-86 alone (Fig. 7A and B, compare a and d). Western blotting showed an increase in the

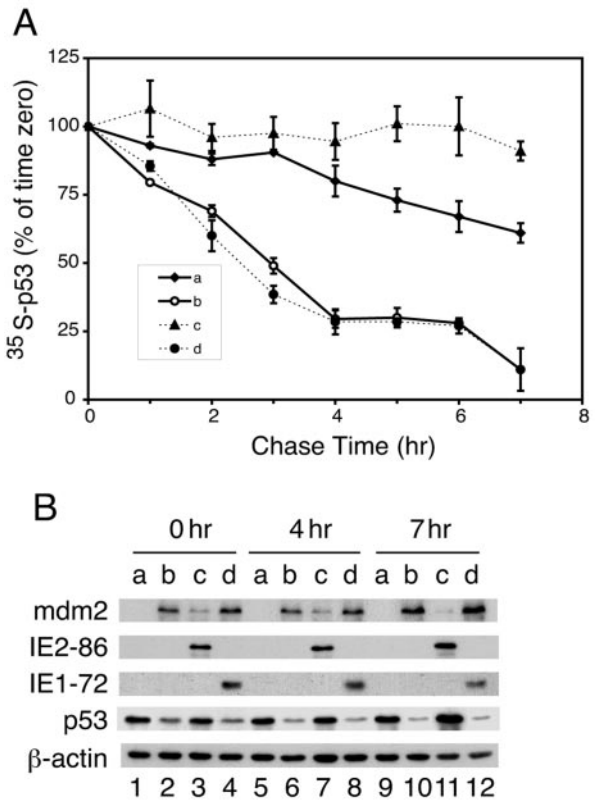

FIG. 6. Half-life of p53 is increased by IE2-86: pulse-chase. H1299 cells were transfected with expression vectors encoding p53 and the indicated combinations of mdm2, IE1-72, and IE2-86 and pulsed with [^{35}S]Met and then chased with unlabeled amino acids for the indicated times prior to harvesting. (A) Labeled p53 was detected by immunoprecipitation followed by SDS-PAGE separation and autoradiography, and then the resulting film was analyzed with New ImageQuant TL v2003.01 for detecting relative levels of labeled p53 at the indicated chase time points. (B) The amounts of p53, mdm2, IE2-86, and IE1-72 were determined by immunoblotting at the indicated chase time points. "a" refers to transfection with p53, "b" refers to transfection with p53 and mdm2, "c" refers to transfection with p53 and mdm2–IE2-86, and "d" refers to transfection with p53 and mdm2 and IE1-72.

steady-state levels of p53 when it was cotransfected with mdm2 and IE2-86 compared to the level with mdm2 alone, which correlated with decreased steady-state levels of mdm2 in the presence of IE2-86 (Fig. 7A or C, compare a with b and d).

These experiments were repeated using IE2-86 deletion mutants in order to correlate these experiments with the above results. We found that the half-life of mdm2 was decreased in cells cotransfected with full-length IE2-86 and IE2-86 lacking amino acids 1 to 25 (IE2-26-579f) (Fig. 8A and B, b and c), but not with any of the other corresponding deletion mutants employed (Fig. 8A and B, d, e, and f). These results suggested that IE2-86 directly decreased the half-life of mdm2 independently of p53 and that the putative zinc finger and/or acidic domain of IE2-86 was required for this effect.

IE2-86 depletion in HCMV-infected cells restored mdm2 induction. In order to determine the contribution of IE2-86 to mdm2 depletion in HCMV-infected cells, we intended to reproduce the above phenomena using recombinant viruses with IE2-86 deleted and/or mutated from infectious bacterial arti-

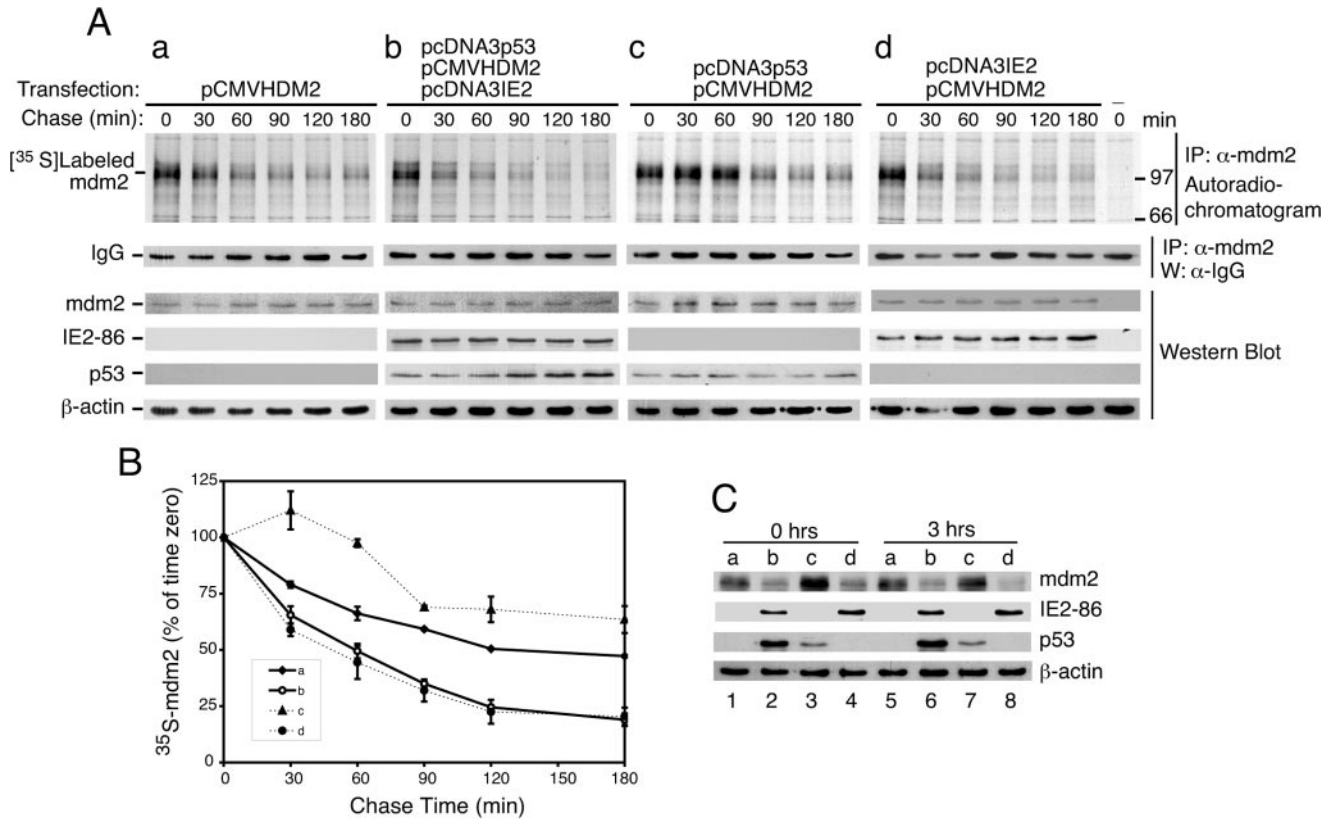

FIG. 7. Half-life of mdm2 is decreased by IE2-86: pulse-chase. H1299 cells were transfected with expression vectors encoding mdm2 and the indicated combinations of p53 and IE2-86 and pulsed with [^{35}S]Met and then chased with unlabeled amino acids for the indicated times prior to harvesting. (A) Labeled mdm2 was detected by immunoprecipitation followed by autoradiography and the levels of other proteins were determined by immunoblotting. (B) The autoradiographed film was analyzed with New ImageQuant TL v2003.01 to detect relative levels of labeled mdm2 at the indicated chase time points. (C) The amounts of p53, mdm2, and IE2-86 were determined by immunoblotting at the indicated chase time points. "a" refers to transfection with mdm2, "b" refers to transfection with mdm2 and p53 and IE2-86, "c" refers to transfection with mdm2 and p53, and "d" refers to transfection with mdm2 and IE2-86. IP, immunoprecipitation; W, Western blotting; Ub, ubiquitin; IgG, immunoglobulin G.

ficial chromosome clones. However, IE2-86 knockouts have been reported and they are replication incompetent (46). Therefore we employed an siRNA knockdown strategy reported previously (65) to significantly reduce IE2-86 expression in HCMV-infected cells. Treatment of HCMV-infected cells with IE2-siRNA disrupted IE2-86 expression, but not that of IE1-72 (Fig. 9, lanes 5 and 8). Specificity was also confirmed using a negative control siRNA (Fig. 9, lanes 9 to 11). In the context of HCMV infection, mdm2 levels accumulated upon IE2-siRNA and MG132 treatment (Fig. 9, lane 8), but not without MG132 or IE2-siRNA treatment (Fig. 9, lanes 5 and 6). The results suggested that only when the expression of IE2-86 was blocked could HCMV-infected cells accumulate measurable levels of mdm2 in response to MG132.

DISCUSSION

The aim of this study was to examine the status of mdm2 in cells productively infected with HCMV and its implications for the mechanism of p53 accumulation in virus-infected cells. The steady-state levels of mdm2 and presumably ubiquitinated species of p53 were decreased in response to HCMV infection in a proteasome-independent manner (Fig. 1). These reductions in mdm2 and ubiquitinated p53 were reproducible upon transfection of cells with IE2-86 but not with IE1-72 (Fig. 2). IE1-72 and IE2-86 share the same 85 N-terminal amino acids and are splice variants of the UL123 and 122 genes (11). Our examination of the regions of IE2-86 responsible for perturbation of the mdm2-p53 autoregulatory loop suggested that the putative zinc finger and C-terminal acidic domains were important for mdm2 downregulation. IE2-86 amino acids 326 to 449 were required for mdm2 binding and reducing the half-life of mdm2, while amino acids 540 to 579 were required to reduce the half-life of mdm2 but not for mdm2 binding.

The unique regions of IE1-72 and IE2-86 (exons 4 and 5, respectively) have similar sequences that suggest zinc finger motifs (amino acids 428 to 452), yet their orientations (H_2C_2 and C_2H_2, respectively) are opposite (27). The primary amino acid sequence of IE2-86 also suggests the possibility of a double zinc finger or Lin-11, Isl-1, and Mec-3 (LIM) domain from residues 428 to 480 ($CX_5CX_{11}HX_5HX_9DXCX_{13}HXH$) (Fig. 10A). LIM domains are multiple binding and adapter modules containing specialized double zinc finger motifs that are important for protein-protein interactions (5). These are responsible for key interactions with coactivators, corepressors, competitors, and other transcription factors and are therefore of con-

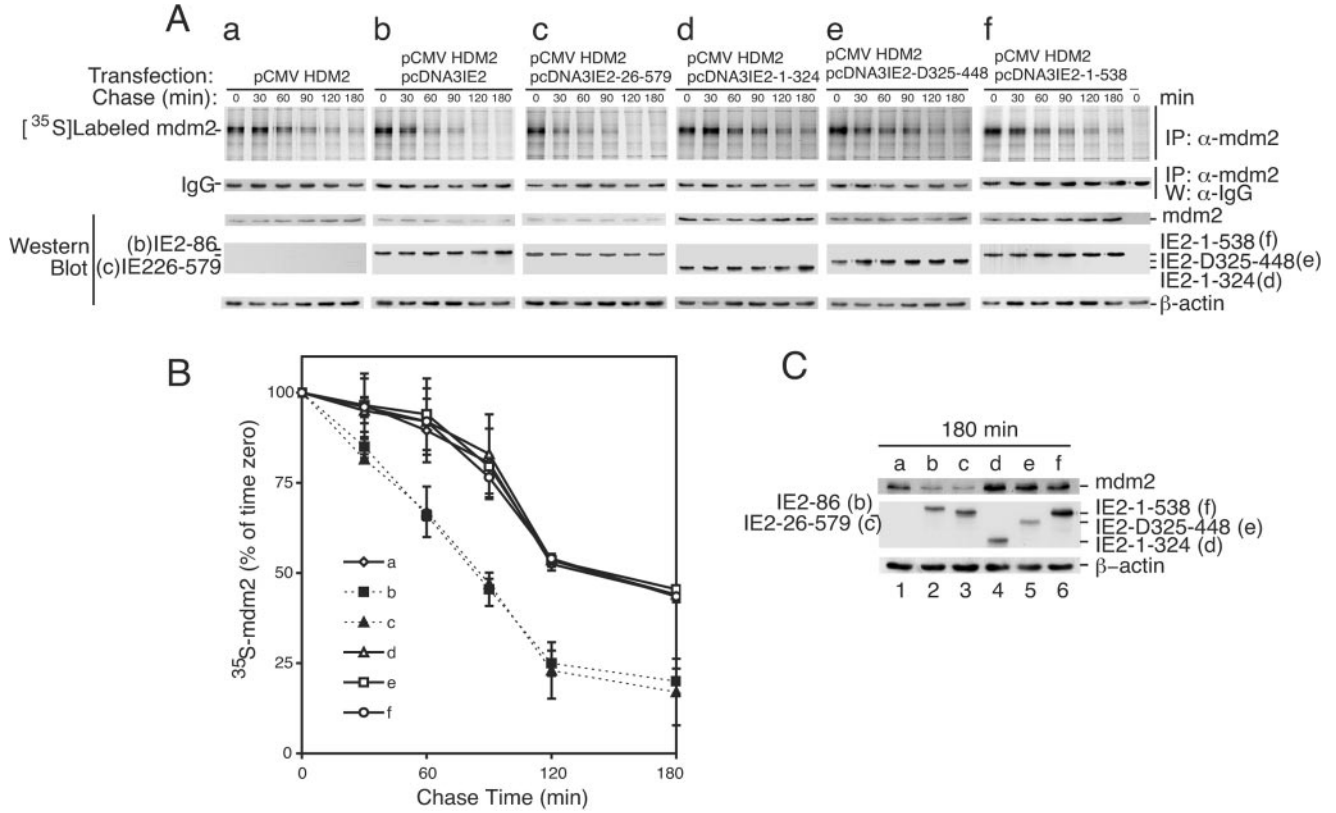

FIG. 8. The half-life of mdm2 is decreased only by full-length and IE2-26-579: pulse-chase. H1299 cells were transfected with expression vectors encoding mdm2 and IE2-86 and its deletion mutants. Cells were pulsed with [^{35}S]Met and chased with unlabeled amino acids for the indicated times prior to harvesting. (A) Labeled mdm2 was detected by immunoprecipitation followed by autoradiography and the levels of other proteins were determined by immunoblotting. (B) The autoradiographic film was analyzed with New ImageQuant TL v2003.01 to detect relative amounts of labeled mdm2 at the indicated chase time points. (C) The amounts of p53, mdm2, and IE2-86 and its mutants were determined by immunoblotting at the indicated chase time points. "a" refers to transfection with mdm2, "b" refers to transfection with mdm2 and IE2-86, "c" refers to transfection with mdm2 and IE2-26-579, "d" refers to transfection with mdm2 and IE2-1-324, "e" refers to transfection with mdm2 and IE2-D325-448, and "f" refers to transfection with mdm2 and IE2-1-538. IP, immunoprecipitation; W, Western blotting; Ub, ubiquitin; IgG, immunoglobulin G.

siderable importance for the regulation of associated transcriptional activity.

It is possible that the zinc finger/LIM domain of IE2-86 may mediate not only binding to mdm2, but also other protein-protein interactions potentially facilitating mdm2 degradation, such as interacting with the RING-H2 zinc finger protein RLIM (5). It was reported that the putative IE2-86 zinc finger (from C428 to H452) had no effect upon DNA binding, but H446L and H452L IE2-86 mutants were unable to bind DNA (4). It is possible that these mutations disrupted the putative LIM domain.

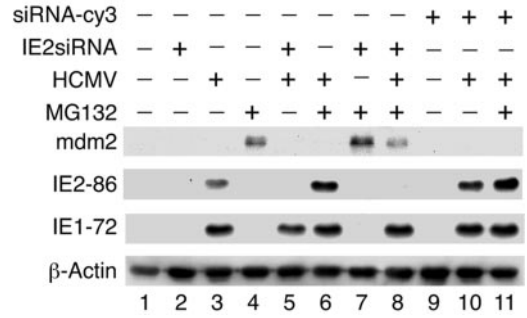

FIG. 9. Effect of siRNA directed to IE2-86 upon HCMV induction of mdm2 as measured by immunoblotting. HEL fibroblasts were transfected with siRNA to IE2-86 or a nonspecific control siRNA and then infected with HCMV and treated with the proteasome inhibitor MG132 (30 μM) for 10 h prior to harvesting. Control β-actin levels indicate that similar levels of protein were loaded in each lane.

Also important for IE2-86 downregulation of mdm2 was the C-terminal acidic domain. The last 37 amino acids of IE2-86 have been shown to contain a transferable transactivation domain that is required for transactivation, autoregulation, and DNA binding (4, 13, 30). The acidic domains of other proteins were important for ubiquitination reactions (6, 42), suggesting the possibility of a role for this region of IE2-86 in catalysis. An IE2-86-mdm2 complex might recruit another coactivator(s), corepressor(s), kinase(s), or component(s) responsible for protein degradation by synergy of its zinc finger/LIM and acidic domains. While the specific function(s) of the identified IE2-86 domains remains speculative, the data indicated that both the zinc finger/LIM and acidic domains of IE2-86 were required to bring about mdm2 degradation. IE2-86 mutants IE2-D325-448 (zinc finger/LIM domain damage), IE2-1-324 (both zinc fin-

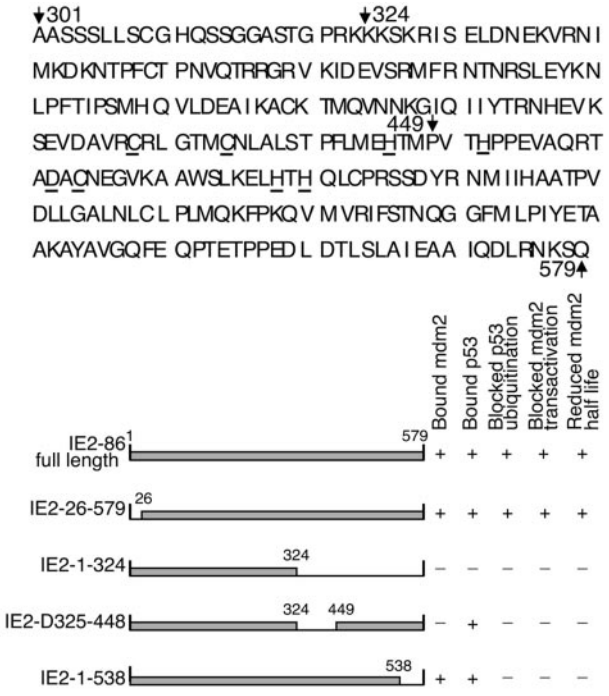

FIG. 10. Upper panel: putative LIM domain in IE2-86. Partial IE2-86 amino acid sequence from the Towne strain of HCMV. Residues suggesting a double zinc finger (LIM) domain are underlined. Lower panel: summary of the functions of IE2-86 mutants in this study. The altered regions of the IE2-86 mutants employed in this study are presented without epitope tags. Their corresponding effects upon the functions of mdm2 and p53 are indicated as positive (+) or negative (−).

ger/LIM and acidic region removal), and IE2-1-538 (acidic domain removal) were unable to cause mdm2 degradation (Fig. 3, 5, and 8).

Given that IE2-86 and mdm2 were known to bind at least five proteins in common (see introduction), it seemed reasonable to expect that these two proteins would be capable of coimmunoprecipitation. As shown in Fig. 3 and 4B, this prediction was validated. Pulldown experiments with in vitro-translated protein were employed to examine the binding properties of the isolated proteins. It was much more surprising that IE2-86 and mdm2 were capable of interactions in the absence of other cellular proteins (Fig. 4A). The inabilities of mutants GST–IE2-D325-448 and GST–IE2-1-324 to pull down mdm2 suggested that the binding was specific (Fig. 4A).

We examined five events that occurred in the p53-mdm2 regulatory loop in response to full-length and deletion mutants of IE2-86 (Fig. 10B). The IE2-D325-448 mutant bound p53 but not mdm2, suggesting distinct binding interactions for IE2-86–p53 and IE2-86–mdm2. Binding of IE2-86 to mdm2 was insufficient to block functional transactivation of the cyclin A promoter by mdm2 or to prevent p53 ubiquitination. The mutants of IE2-86 that were capable of binding mdm2 and downregulating its functions were the same as those that reduced the half-life of mdm2. This suggested that IE2-86-mediated functional downregulation of mdm2 resulted from mdm2 degradation.

The ability of IE2-86 to cause mdm2 depletion independent of mdm2's endogenous promoter suggested that regulation was not at the transcriptional level. It is not clear how IE2-86 facilitated mdm2 degradation. The inability to recover mdm2 in the presence of MG132 suggested that this phenomenon was proteasome independent. Since the binding of IE2-86 to mdm2 was not sufficient to degrade mdm2, it is possible that other functions of IE2-86 are required for mdm2 degradation. Possible functions include those that might mediate subcellular localization, catalysis, conformational changes, or the recruitment of another protein(s) that might degrade mdm2.

Since p53 transcriptional activity controls mdm2 expression and IE2-86 likely blocks p53 transcriptional activity, it seems unnecessary for HCMV to employ IE2-86 to facilitate mdm2 degradation. An apparent duplication of functions is also employed by HCMV to block cellular apoptosis. At least five cytomegalovirus proteins (vMIA, vICA, m41, IE1-72, and IE2-86) are independently capable of blocking apoptosis (9, 28, 56, 66, 71). vICA is dispensable for viral growth in cell culture (56) and IE1-72 is not required for efficient HCMV replication at high multiplicities of infection (29). This is consistent with the proposal that degenerate or multiple viral solutions to host countermeasures may be important virulence factors in vivo (2) and are not unique to mdm2. An alternative explanation is that p53 transcriptional activity may not be absent in HCMV-infected cells. Transfection of fibroblasts with IE2-86 did not prevent the induction of p53, mdm2, and p21 by actinomycin D, nor did it block cell cycle arrest (7, 44).

Another interesting question is how mdm2 depletion might facilitate HCMV replication. If the p53 induced by HCMV infection is not functional, then at issue are the p53-independent effects of mdm2. Several speculative explanations follow. HCMV IE1-72 and IE2-86 bring about the disruption of promyelocytic leukemia (PML) associated nuclear bodies (ND10 or PML oncogenic domains) causing diffuse nuclear redistribution of PML; however, IE2-86 alone does not disrupt ND10 (1). Coexpression of mdm2 with PML causes nuclear exclusion of PML (62) and thus could block a potentially important event in HCMV replication.

There is evidence to suggest that HCMV may alter the cell cycle of infected cells to a state resembling late G_1 to facilitate viral replication (18, 63). In limited systems mdm2 stimulated S-phase progression (20, 48) and thus functional mdm2 could alter the balance toward cellular rather than viral proliferation. The Sp1 transcription factor is stimulated in productive HCMV infection (67). mdm2 binds Sp1 and blocks Sp1 transcriptional activity (35) but can be reversed by Rb (35). A virion protein, pp71, causes degradation of Rb and related proteins (38). However, the accumulation of Rb during HCMV infection (32) suggests that downregulation of mdm2 to facilitate Sp1 induction may not be a sustained viral replication strategy. We also note that the fact that an event occurs during productive viral replication is not prima facie evidence that the event is conducive to viral replication. Infrequently they are not. For example, systematic gene deletions of the HCMV genome revealed that removal of 4 out of 162 genes resulted in enhanced viral fitness (21).

We have shown that mdm2 levels were decreased in HCMV-infected cells due to accelerated degradation and that the viral IE2-86 protein was likely responsible. The interactions between IE2-86 and mdm2 appeared to be specific and occurred in the absence of other candidate adaptor molecules. HCMV

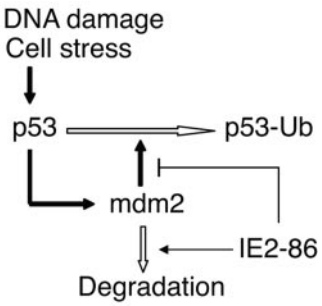

FIG. 11. Model for how HCMV infection results in increased levels of p53. In normal cells, p53 is kept at low levels by the p53-mdm2 autoregulatory feedback loop. In HCMV-infected cells, the feedback loop is blocked and p53 accumulates, at least partially via IE2-86 facilitating mdm2 degradation. Ub, ubiquitin.

infection results in cellular DNA damage (22) and the activation of signal transduction pathways that could be responsible for p53 accumulation. For example, HCMV infection or IE1-72 transfection induced ataxia telangiectasia-mutated kinase-dependent p53 phosphorylation on residue Ser 15 (10). However, others reported that in HCMV-infected cells or IE2-86-expressing cells there was a direct correlation between the relative amount of p53 and the relative amount of p53 phosphorylation (58). The data presented here suggest an alternative mechanism: p53 accumulation via IE2-86-dependent mdm2 degradation (Fig. 11). This mechanism is likely to play an important role in the context of HCMV infection because siRNA-mediated knockdown of IE2-86 restored the accumulation of mdm2 (Fig. 9).

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Zhigang Zhang and David L. Evers contributed equally to this publication.

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