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Human Cytomegalovirus IE2 86-Kilodalton Protein Binds p53 but Does Not Abrogate G_1 Checkpoint Function

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Physical interactions between human cytomegalovirus (HCMV) immediate-early (IE) proteins and key cell cycle regulatory proteins have been suggested as a mechanism whereby this herpesvirus modifies cellular control of proliferation. Observed similarities to interactions of other DNA virus proteins (human papillomavirus type 16 E6 and E7, simian virus 40 large T antigen, and adenovirus type 5 E1A and E1B) with cell cycle modulatory proteins such as p53 and Rb have suggested that HCMV IE proteins may likewise alter the G_1-to-S phase transition. The IE2 region gene product IE86 has been shown to specifically bind p53, potentially modifying p53 G_1 checkpoint function. To examine this possibility, p53-mediated G_1 arrest in the presence of IE86 was assessed. Retroviral constructs were created to facilitate the stable expression of IE86 and IE72, another IE protein implicated in HCMV-mediated alteration of cell cycle progression. Western analysis and immunoprecipitation confirmed IE protein expression and binding of IE86 to p53, respectively. Chloramphenicol acetyltransferase assays examining the ability of IE86 to repress activity from the HCMV major IE promoter or activate the HCMV early promoter for the 2.2-kb class of RNAs demonstrated the functional integrity of the IE86 protein. Induction of DNA damage in normal, uninfected fibroblasts (FB) or FB expressing IE86 by actinomycin D (Act D) resulted in increased p53 levels, a predominance of the hypophosphorylated form of Rb, and increased expression of both $p21^{CIP1/WAF1}$ and mdm-2. Fluorescence-activated cell sorting revealed that both uninfected and IE86-expressing FB experienced dramatic G_1 arrest following exposure to Act D. The clear demonstration of these p53-dependent responses in the presence of IE86 indicates that binding to this viral protein does not compromise the ability of p53 to elicit growth arrest following DNA damage.

Human cytomegalovirus (HCMV) is a linear double-stranded DNA virus that is usually considered to be rather innocuous to the general population. Although primary exposure is commonly uneventful for the immunocompetent individual, the virus establishes a latent presence with periodic reactivation throughout the individual's lifetime, perhaps reflecting a weakened immune status (for reviews, see references 18, 21, 22, 31, and 34). The complex pathogenicity of this virus is evidenced by a wide variety of clinical manifestations ranging from mononucleosis-like symptoms in immunocompetent individuals to interstitial pneumonitis and disseminated HCMV infection in immunosuppressed or immunocompromised individuals. For this reason, HCMV presents a serious threat to transplant recipients and individuals infected with human immunodeficiency virus (HIV). Accumulating evidence suggests that the pathogenicity of HCMV may involve modulation of key proteins involved in cell cycle progression (7, 11, 23, 36, 41, 46, 52, 55, 60, 62, 65, 72). The potential concomitant mitogenic effects associated with such pathogenic mechanisms have created particular interest in the role of HCMV in proliferative pathologies such as atherosclerosis and postangioplasty restenosis (62). Detection of HCMV DNA in atherosclerotic plaque further supports a viral etiology of these vascular diseases (30, 47, 48, 61), although the high prevalence of viral nucleic acids in the adult population hinders the clear demonstration of a role for HCMV (6, 28, 29). Data from Lemstrom et al. (42), however, support the hypothesis that this virus may indeed promote smooth muscle cell replication, an early event in plaque formation.

A product of the immediate-early (IE) region 2 of the HCMV genome, IE86, has been shown to interact with a number of viral (13, 64) and cellular (24) promoters in a TATA-dependent manner. Expressed in the absence of de novo protein synthesis, IE gene products are important in the regulation of their promoter, the major IE enhancer-promoter (MIEP) (1, 10, 31), as well as HCMV early viral promoters (45, 59). Binding to the *cis* repression signal, IE86 represses activity at the MIEP, decreasing its expression (9, 43, 54). IE86 may act in conjunction with the IE region 1 product, IE72, to activate a number of HCMV early promoters (EP) (45, 59). IE86 has also been shown to transactivate heterologous viral promoters such as the HIV long terminal repeat (LTR) (3, 13) and may thereby promote progression from HIV infection to AIDS (34). In addition, HIV has been shown to transactivate HCMV promoters in vitro, indicating the potential synergistic effects of HCMV and HIV on viral gene expression (34). IE86-mediated transactivation of cellular promoters including IL-2, IL-2R, c-*fos*, c-*myc*, and hsp70 has also been reported (20, 24). More recently, IE86 has been shown to interact with several cellular proteins involved in transcription, thereby permitting this viral effector to modulate cellular synthetic processes. Interactions with the TATA-binding protein (TBP) and the transcription factor TFIID have been described by Jupp et al. (37) and Hagemeier et al. (24), respectively. As typifies proteins expressed by several other DNA viruses including simian virus (SV40), adenovirus, human T-cell leukemia virus type 1, Epstein-Barr virus, and human papillomavirus (HPV), IE86 binds several key proteins involved in cell cycle progression. The product of the retinoblastoma gene, Rb, has been shown to interact directly with IE86, relieving IE86-mediated repression of the MIEP (23). Choi et al. (11) found that Rb binding also blocks IE86-mediated transactivation of heterologous viral pro-

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moters (SV40, HIV, and mouse mammary tumor virus) as well as the c-*fos* promoter. Conversely, binding of IE86 to Rb removed Rb-mediated inhibition of E2F-dependent transcription in a manner similar to that of the adenovirus protein E1A (23). This interaction was further characterized by Sommer et al. (60), who found that IE86 possesses three independent phosphorylation-sensitive sites that can each bind TBP and Rb, suggesting that IE86 may participate in complex protein-protein relationships, binding multiple cellular factors simultaneously.

More recently, Speir et al. (62) reported that IE86 binds to the G_1 cell cycle checkpoint protein p53. Furthermore, p53-mediated transactivation of a promoter containing multiple p53-responsive elements was inhibited in the presence of IE86, suggesting the existence of both physical and functional interactions between IE86 and this tumor suppressor protein (62). Increased expression of p53 has often been associated with HCMV infection (36, 52, 62). Muganda et al. (52) found that coexpression of IE1 and IE2 genes also elevated p53 expression in fibroblasts (FB). Speir et al. (62) specifically attributed the increased p53 expression in HCMV-infected smooth muscle cells to the presence of IE86 since its expression kinetics paralleled the rise in p53 levels. These findings were interpreted to suggest that the binding of IE86 to p53 might stabilize the protein and abolish its checkpoint function, permitting unhindered cell replication and promoting cellular accumulation typical of proliferative diseases such as atherosclerosis and postangioplasty restenosis (62).

To more closely examine this hypothesis, we employed retroviral vectors to stably express IE86 in human foreskin FB. p53 function and several of its downstream effectors were examined in cells expressing this IE gene product in the context of DNA damage induced by actinomycin D (Act D), an agent known to effect single-strand DNA breaks (66) and, consequently, p53-dependent G_1 phase arrest (38). In addition, FB were infected with retroviral constructs containing the IE72 cDNA since this protein promotes the expression of several S phase genes and reportedly interacts with cell cycle regulatory proteins (27, 46, 55).

MATERIALS AND METHODS

Cell culture. FB were isolated from human neonatal foreskin as previously described (5) and grown in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL) supplemented with 10% fetal bovine serum (FBS; Hyclone), penicillin (Gibco BRL; 100 U/ml), and streptomycin sulfate (Gibco BRL; 100 μg/ml). Cells were passaged upon confluency at a split ratio of approximately 1:3. PE501 and PG13 were also grown in DMEM supplemented with 10% FBS containing penicillin and streptomycin sulfate.

Retroviral infections. Expression of HCMV IE72 and/or IE86 in FB was achieved with the retroviral vectors pLXSH and pLXSN (provided by A. D. Miller [50]). cDNAs (provided by J. Nelson [63]) were isolated from pIE72 and pIE86, respectively, by PCR, with polymerase fidelity confirmed by sequencing. IE72 and IE86 cDNAs were directionally cloned into pLXSH and pLXSN, respectively, downstream of the Moloney murine leukemia virus promoter (MMLV LTR). Retroviruses expressing these genes were prepared by modifications of published methods (25). Briefly, retroviral constructs (10 μg of plasmid DNA) were transfected into the ecotropic mouse FB packaging cell line PE501 by calcium chloride precipitation. The cells were incubated with precipitates overnight in DMEM supplemented with 10% FBS at 37°C under 3% CO_2. Viral supernatants (2 ml) generated by PE501 were filtered and placed on 30 to 40% confluent cultures (100-mm plate) of the mouse FB packaging cell line PG13 in 5 ml of medium containing 4 μg of Polybrene per ml. After overnight incubation at 37°C under 5% CO_2, the cells were washed with DMEM supplemented with 10% FBS and subcultured 24 h later to allow clone isolation from selected cells. Cells were selected in DMEM supplemented with 10% FBS containing 300 U of hygromycin per ml (IE72/pLXSH) or 1 mg of G418 per ml (IE86/pLXSN). Clones of PG13 expressing the gene of interest as determined by Western analysis (anti-HCMV IE72 R8575; polyclonal antibody [PAb] against exon 4 amino acids 383 to 420 [1:1,000], and anti-HCMV IE86 R638, PAb against the whole protein [1:2,000], generously provided by J. Nelson) were used to create viral supernatants for infection of FB. Fifteen-hour supernatants were collected from proliferating cultures, filtered, and added to 30 to 40% confluent cultures of human FB in 5 ml of DMEM supplemented with 10% FBS containing 4 μg of Polybrene per ml. After overnight incubation, the cells were washed with DMEM supplemented with 10% FBS and subcultured 24 h later to facilitate clone isolation. FB expressing IE72 were selected in DMEM supplemented with 10% FBS containing 300 U of hygromycin per ml; cells expressing IE86 were selected in DMEM supplemented with 10% FBS containing 1 mg of G418 per ml. Stable expression of IE72 and IE86 in clonal FB was confirmed by Western analysis as performed for PG13 clones. Sequencing of the IE86 gene in FB expressing the IE86 protein confirmed that there were no significant alterations.

CAT assays. PG13 cultures that were approximately 50% confluent were transfected with 20 μg of p93CAT or p148CAT (EP constructs, generously provided by D. Spector) or 20 μg of pMIEP(−1145/+112)CAT (MIEP constructs, generously provided by J. Nelson) by the calcium chloride method described above. Cells were harvested 48 h after transfection, resuspended in 100 mM Tris (pH 7.8), and lysed by a series of rapid freeze-thaw steps. Equal amounts of protein were used for chloramphenicol acetyltransferase (CAT) assays according to a previously published method (53). Briefly, cell extracts were incubated in a reaction mixture containing 100 mM Tris (pH 7.8), 1.0 mM chloramphenicol, and 0.1 mM [^{14}C]acetyl coenzyme A (NEC-313L). The reaction mixture was overlaid with 5 ml of Econofluor 2 (Dupont-NEN) and incubated at room temperature [pMIEP(−1145/+112)CAT] or at 37°C (p93CAT or p148CAT). Samples were counted at increasing time intervals to determine the linear range, at which time the fold induction or repression was determined. Experiments were performed three times in duplicate for both the MIEP and the EP constructs.

Coimmunoprecipitation of p53 and HCMV IE86. The cells were rinsed with phosphate-buffered saline (PBS) and scraped from plates in cold PBS. Pelleted cells were resuspended in 2 volumes of lysis buffer (50 mM Tris HCl [pH 8.0], 150 mM NaCl, 0.5% Nonidet P-40 [NP-40], 25 μg of leupeptin per ml, 2.5 mM Pefabloc SC) for 30 min on ice. The cell lysates were precleared, for 30 min with rocking at 4°C, with 20 μl of protein A-Sepharose (10%, vol/vol) equilibrated in lysis buffer. Precleared lysates were incubated with 1 μg of anti-p53 (OP03, Ab-1, PAb 421; OP09, Ab-2, PAb 1801; OP29, Ab-3, PAb 240; OP33, Ab-5, PAb 1620; OP43A, Ab-6, DO-1 [Oncogene Science]) or anti-HPV-16L1 (as a negative control) overnight at 4°C with rocking. One volume of protein A-Sepharose equilibrated in lysis buffer was added to each lysate-Ab sample and incubated for 2 h at 4°C with rocking. Beads pelleted by centrifugation at 16,000 × *g* for 5 min were washed twice for 10 min each in 400 μl of lysis buffer at 4°C with rocking. Pelleted beads were boiled in 1× Laemmli sample buffer containing 1.25% 2-mercaptoethanol and run on a 7.5% acrylamide minigel. The proteins were transferred to nitrocellulose membranes and blocked with Blotto solution (5% nonfat dry milk, 1× TBST [10 mM Tris, pH 8.0, with 150 mM NaCl and 0.05% Tween 20]) for ≥6 h. The membranes were probed for IE86 (R638; 1:2,000; from J. Nelson), and the corresponding proteins were detected by generation of chemiluminescent signals (Renaissance; NEN). Supernatants remaining after immunoprecipitation (the entire volumes) were subjected to precipitation with an equal volume of 10% trichloroacetic acid followed by two washes with cold 90% acetone. The pellets were dissolved in Laemmli sample buffer and run on a 7.5% acrylamide minigel. The proteins were transferred to nitrocellulose membranes and blocked with Blotto solution. The membranes were probed for p53 (PC35, Ab-7, 1:2,500; biotinylated rabbit anti-sheep, 1:100,000 [Oncogene Science]), and the corresponding proteins were detected by generation of chemiluminescent signals (Renaissance).

Western analysis. The cells were rinsed and scraped up in cold PBS. Pelleted cells were resuspended and triturated in an equal volume of 25 mM Tris HCl (pH 7.5)–125 mM NaCl–2.5 mM EDTA–0.05% sodium dodecyl sulfate–0.05% NP-40–0.5% deoxycholate–10% glycerol–1 mM dithiothreitol–2.6 mM PFSC–25 μg of leupeptin per ml–10 μg of pepstatin per ml–10 μg of aprotinin per ml–80 mM β-glycerophosphate–0.5 mM Na_3VO_4–50 mM NaF. After incubation on ice for 3 to 5 min, the samples were sonicated in a cup horn sonicator (Branson Sonifier 450; output setting, 40) for 2 min in an ice slurry. The insoluble fraction was pelleted by centrifugation at 16,000 × *g* for 30 min at 4°C. Twenty micrograms of protein was boiled in 1× Laemmli sample buffer containing 1.25% 2-mercaptoethanol and subjected to SDS-polyacrylamide gel electrophoresis. The proteins were transferred to nitrocellulose membranes, which were subsequently blocked for ≥6 h. The following Abs were used for Western analysis: anti-p53 (0.1 μg/ml, OP43; Oncogene Science), anti-Rb (0.5 μg/ml, 14001A; Pharmingen), anti-IE72 (R8575, 1:1,000; from J. Nelson), anti-IE86 (R638, 1:2,000; from J. Nelson), anti-mdm2 (1 μg/ml, OP46; Oncogene Science) and anti-p21$^{CIP1/WAF1}$ (0.1 μg/ml, OP64; Oncogene Science). The corresponding proteins were detected by generation of chemiluminescent signals (Renaissance).

Act D-induced growth arrest and FACS analysis. FB cultures (60-mm plate; 50% confluent) were exposed to 0.5 nM Act D for 24 h and then to 10 μM bromodeoxyuridine (BrdU) for 4 h. Alternatively, cells were exposed to BrdU in the absence of Act D. Samples were stained for BrdU and with propidium iodide for cell cycle analysis using published procedures with slight modifications (38, 69). Briefly, cells were trypsinized, resuspended in PBS supplemented with 5% FBS, and pelleted. The cells were resuspended in 1.5 ml of cold PBS and fixed by the addition of 3 ml of cold 95% ethanol. All subsequent centrifugation steps were performed at room temperature at 2,000 × *g* for 5 min. Following centrifugation, the pellets were resuspended in 3 ml of 0.08% pepsin and incubated for 20 min at 37°C. The nuclei were pelleted, resuspended in 1.5 ml of 2 M HCl, and

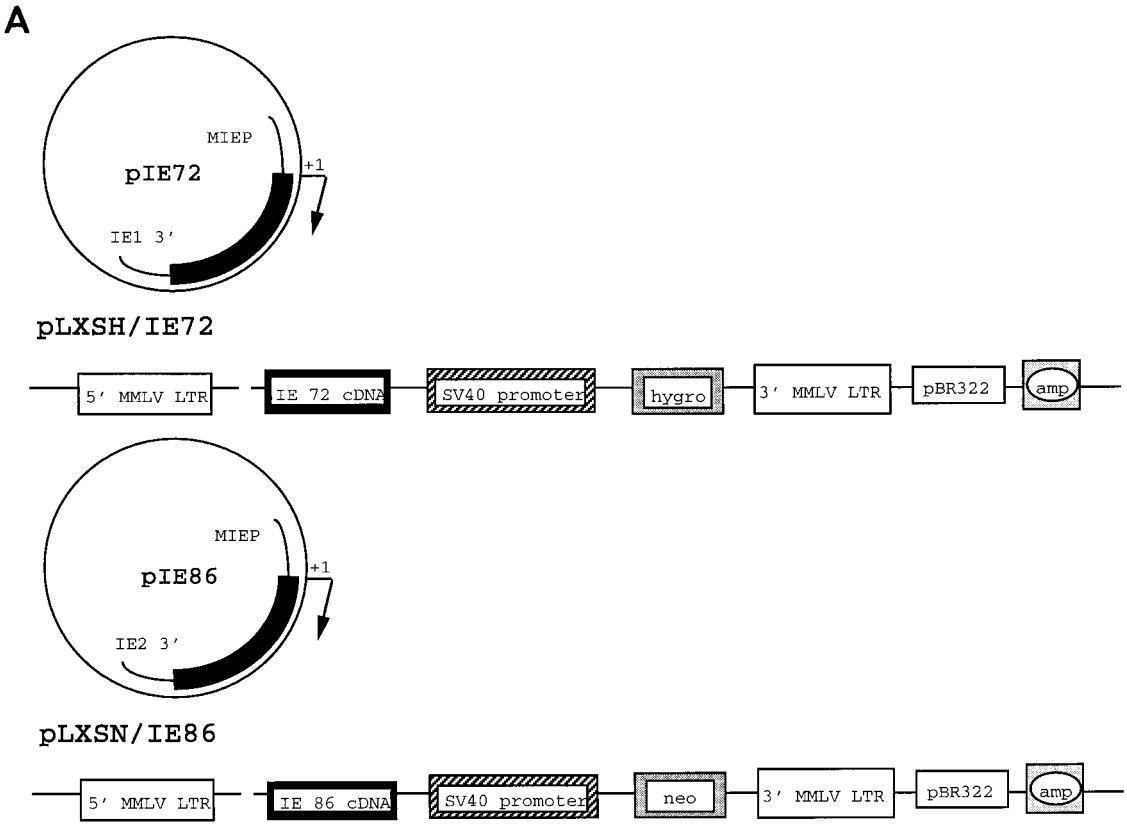

FIG. 1. (A) Retroviral constructs for the expression of IE72 and IE86. cDNAs for IE72 and IE86 were amplified from pIE72 and pIE86, respectively, and cloned into the retroviral vectors pLXSH and pLXSN, as shown (solid boxes). Expression of IE genes in these retroviral vectors is under the control of the MMLV LTR. pLXSH contains a hygromycin resistance gene, which was used to select clonal cells expressing IE72; pLXSN contains a neomycin (G418) resistance gene, which was used to select clonal cells expressing IE86. (B) Western analysis of FB expressing IE72 or IE86. Lanes: 1, uninfected; 2, LXSN only; 3, LXSN plus IE86; 4, LXSN plus IE86 expressing a truncated form of IE86; 5, LXSH only; 6, LXSH plus IE72.

incubated for 20 min at 37°C. A 3-ml volume of 0.1 M sodium borate was added, and the nuclei were again pelleted. The pellets were resuspended in 2 ml of IFA (10 mM HEPES [pH 7.4], 150 mM NaCl, 4% FBS, 0.1% sodium azide)–0.5% Tween 20. The nuclei were pelleted and resuspended in 100 μl of anti-BrdU-fluorescein isothiocyanate (1:5 in IFA; Becton Dickinson) and incubated for 30 min at 4°C. A 2-ml volume of IFA–0.5% Tween 20 was added, and the nuclei were subsequently pelleted and then resuspended in 250 μl of IFA. The samples were incubated with RNase A (5 μg/ml) for 15 min at 37°C. Propidium iodide was then added to a final concentration of 50 μg/ml, and the nuclei were incubated for ≥1 h at 4°C prior to fluorescence-activated cell sorting (FACS) analysis by FACScan (Becton Dickinson). For each sample, 10,000 events were analyzed; the data were displayed and quantitated with Cellquest software (Becton Dickinson).

RESULTS

Stable expression of IE72 or IE86 in FB. A retroviral infection system was employed to efficiently and stably express HCMV IE72 and IE86 in normal human foreskin FB. The retroviral constructs are shown in Fig. 1A. Following selection for hygromycin or neomycin resistance, clonal FB were analyzed for protein expression. As shown in Fig. 1B, expression of IE72 or IE86 was confirmed by Western analysis of clonal cell lysates. Uninfected FB and LXSN- or LXSH-expressing FB did not express IE72 or IE86. Stable expression was determined by Western analysis of both early- and late-passage clones. All subsequent experiments were performed with clonal FB. Of the FB infected with supernatant from PG13 expressing high levels of IE86, approximately 35% of the clones surviving selection consistently demonstrated the expression of a truncated form of IE86 of approximately 65 kDa; the mechanism and relevance of this form of IE86 are unknown at this time.

Retrovirally expressed IE86 activates the HCMV EP for the 2.2-kb class of RNAs and represses the HCMV MIEP. IE86 has been shown to activate the promoter for the 2.2-kb class of early RNAs (59). To assess this functional attribute of IE86 in the present system, CAT assays were performed on cells expressing LXSN or IE86 that were each transfected with the EP-CAT construct (wild type; p148CAT) or an EP-CAT construct containing a 5′ deletion to nucleotide −58 (p93CAT), which reportedly decreases activation by 10- to 20-fold (59). In these experiments, IE86-expressing cells transfected with p148CAT demonstrated CAT activity levels that were 140.33% of the levels expressed by control LXSN cells transfected with p148CAT (Fig. 2). In contrast, IE86-expressing cells transfected with p93CAT demonstrated CAT activity levels that were 105.81% of the levels expressed by LXSN cells transfected with p93CAT (data not shown). The percent increase in the CAT

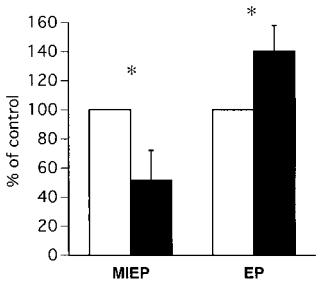

FIG. 2. Repression of the HCMV MIEP and activation of the HCMV EP for the 2.2-kb class of RNAs by retrovirus-expressed IE86. Cells expressing LXSN alone or LXSN plus IE86 were transfected with 20 μg of pMIEPCAT, p93CAT, or p148CAT. CAT activity detected in control transfected LXSN was set at 100% to normalize for interexperimental variability, and activity detected in LXSN-plus-IE86 cells is expressed as a percentage of that in the control transfected LXSN cells. Data shown are means ± standard errors SE for three experiments, each of which was performed in duplicate. Open bars, LXSN; solid bars, LXSN plus IE86; asterisks, different from control (LXSN) ($P < 0.05$ by the Mann-Whitney U test).

activity of p148CAT-transfected IE86 cells relative to that of p93CAT-transfected IE86 cells was approximately sevenfold. Another functional characteristic of IE86 is its ability to negatively autoregulate the MIEP (9, 37, 43, 54). To assess this aspect of IE86 function in cells infected with retrovirus to express this protein, CAT assays were performed on cells expressing LXSN or IE86 that were each transfected with the MIEP-CAT construct [pMIEP(−1145/+112)CAT]. As shown in Fig. 2, CAT activity was decreased in IE86-expressing cells to levels that were 51.67% of those observed in LXSN cells.

IE86 binds to p53. To determine if the retrovirus-expressed IE86 binds to p53 in FB, cell lysates were immunoprecipitated with anti-p53 antibodies. As shown in Fig. 3A, anti-p53 antibodies recognizing a variety of epitopes revealed that IE86 specifically binds to p53 in this system. Furthermore, the IE86-p53 interaction did not impair epitope recognition for any of the anti-p53 antibodies used in these experiments; these antibodies were employed to obtain information about the physical nature of the p53-IE86 interaction. IE86 was not detected in immunoprecipitates of uninfected FB or cells expressing IE72 or the LXSN vector alone. Likewise, IE86 was not detected when lysates of IE86-expressing cells were immunoprecipitated with an irrelevant antibody (anti-HPV-16L1 as a negative control for nonspecific binding [data not shown]). IE86 was, however, detected in immunoprecipitates of cell lysates containing the truncated form of IE86. Further characterization of the truncated IE86 protein may provide further information about the interaction between IE86 and p53. p53 Western analysis of supernatants remaining after immunoprecipitation with anti-p53 revealed that the majority of cellular p53 was pulled down (Fig. 3B).

IE86 binding to p53 does not abrogate Act D-induced G_1 arrest. Following the confirmation of a physical relationship between IE86 and p53, experiments were designed to investigate the functional consequences of this interaction. p53 provides a critical checkpoint in G_1 phase of the cell cycle, eliciting G_1 growth arrest in cells with genomic aberrations (4, 38, 44, 71). To examine the ability of p53 to effect arrest in the presence of IE86, proliferating FB were exposed to the DNA-damaging agent Act D (0.5 nM) (38, 66) for 24 h followed by the introduction of BrdU to assess DNA incorporation as an index of synthetic activity. Act D treatment consistently elicited G_1 arrest in uninfected FB (Fig. 4). Exposure to Act D also elicited a G_1 arrest in cells expressing either the full-length or truncated form of IE86. Likewise, FB expressing IE72, LXSN, or LXSH demonstrated accumulation of cells in G_1 following exposure to Act D. As a positive control for abrogation of Act D-mediated arrest, FB expressing the HPV-16E6 were included in this paradigm. The E6 viral oncogene is known to bind p53 (68), targeting it for degradation via a ubiquitin-dependent pathway (58). Consequently, HPV-16E6-expressing

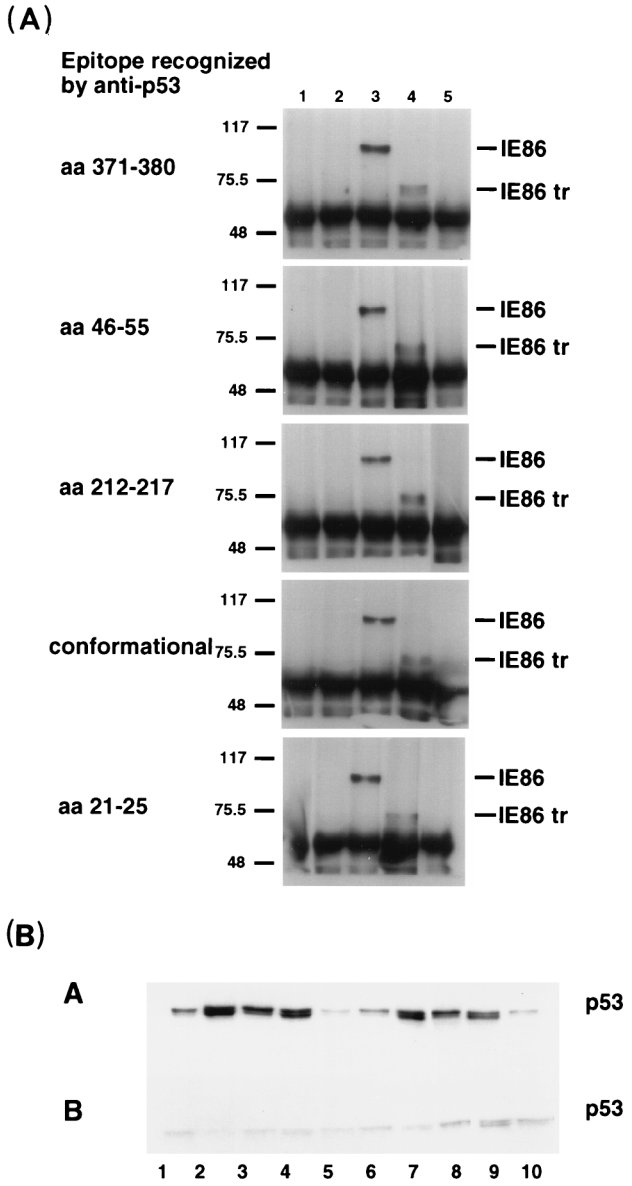

FIG. 3. (A) Immunoprecipitation of IE86 with p53. FB lysates were incubated with antibodies recognizing different p53 epitopes as shown here and described in Materials and Methods; immunoprecipitates were subjected to Western analysis for IE86. Lanes: 1, uninfected; 2, LXSN only; 3, LXSN plus IE86; 4, LXSN plus IE86 expressing a truncated form of IE86; 5, LXSH/E72. (B) Analysis of p53 remaining after immunoprecipitation of p53. FB lysates were incubated with sequence-specific MAbs recognizing two different p53 epitopes (amino acids [aa] 371 to 380 [lanes 1 to 5] or aa 46 to 55 [lanes 6 to 10]). Immunoprecipitates were subjected to Western analysis for p53 (row A; PAb Ab-7). The entire volumes of post-immunoprecipitation supernatants were subjected to Western analysis for p53 (row B; PAb Ab-7). Lanes: 1 and 6, uninfected; 2 and 7, LXSN only; 3 and 8, LXSN plus IE86; 4 and 9, LXSN plus IE86 expressing a truncated form of IE86; 5 and 10, LXSH plus IE72.

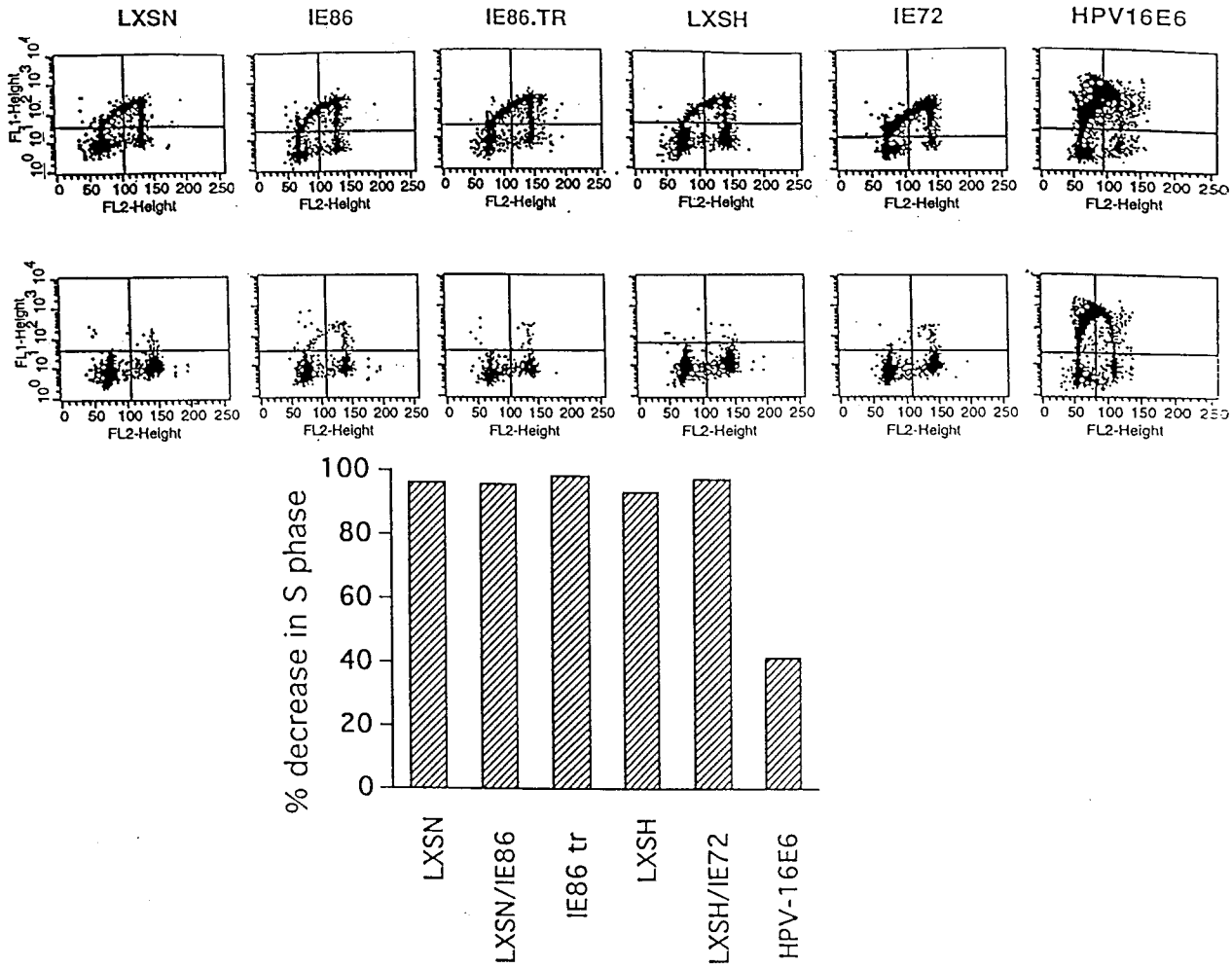

FIG. 4. FACS analysis of untreated FB and FB treated with Act D to induce p53-dependent G_1 arrest. Proliferating FB, untreated or treated with 0.5 nM Act D for 24 h, were pulsed for 4 h with 10 μM BrdU and then subjected to FACS analysis to determine the percentage of cells in the S phase. In the upper panel, the top row shows results for untreated FB and the bottom row shows results for Act D-treated FB (representative experiment). In the lower panel, the percent decrease in the number of FB in the S phase ($n = 3$) is shown.

FB were able to escape Act D-induced G_1 arrest due to the absence of p53 (39).

p53 expression levels are increased following Act D treatment. The G_1 growth arrest elicited by Act D-induced DNA damage is believed to be mediated by p53 since cells with mutated p53 or no p53 escape arrest in G_1 and continue through S phase (38). G_1 arrest in cells without detectable p53 mutations was associated with increased p53 protein levels (38). Therefore, elevated p53 levels following DNA damage would support the hypothesis that this p53-dependent response pathway is functional in the presence of IE86. FB expressing IE86 were exposed to Act D as in growth arrest experiments and then examined by Western analysis for p53 protein levels (Fig. 5). Act D-treated uninfected FB demonstrated increased p53 levels compared to untreated cells. Increased p53 levels were also observed in Act D-treated FB expressing IE86 or IE72 or the respective vector controls LXSN or LXSH. Basal p53 levels in FB expressing IE86 did not appear to be higher than those observed in untreated uninfected, IE72-expressing, or vector-expressing cells.

Levels of $p21^{CIP1/WAF1}$ are elevated in Act D-treated FB. Another index of p53 function is the induction of *CIP1/WAF1* following exposure to Act D. Increased expression of p53 following exposure to DNA-damaging agents has been shown to temporally correlate with a transient G_1 arrest (38). El-Deiry et al. (17) subsequently found that *CIP1/WAF1* gene expression is directly transactivated by p53, and its product, $p21^{CIP1/WAF1}$, was shown to mediate the growth-inhibitory effects of

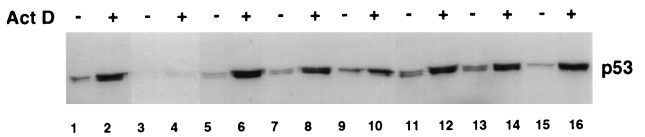

FIG. 5. Western analysis of untreated FB and FB treated with 0.5 nM Act D (24-h exposure) for determination of p53 protein levels. Lanes: 1 and 2, uninfected; 3 and 4, HPV-16 E6; 5 and 6, HPV-16 E7; 7 and 8, LXSN only; 9 and 10, LXSN plus IE86; 11 and 12, LXSN plus IE86 expressing a truncated form of IE86; 13 and 14, LXSH only; 15 and 16, LXSH plus IE72.

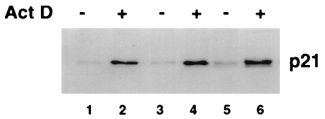

FIG. 6. Western analysis for determination of $p21^{CIP1/WAF1}$ protein levels in untreated FB and FB treated with 0.5 nM Act D (24-h exposure). Lanes: 1 and 2, uninfected; 3 and 4, LXSN; 5 and 6, LXSN plus IE86.

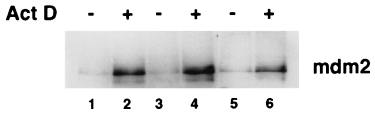

FIG. 8. Western analysis of untreated FB and FB treated with 0.5 nM Act D (24-h exposure) for determination of mdm-2 protein levels. Lanes: 1 and 2, uninfected; 3 and 4, LXSN; 5 and 6, LXSN plus IE86.

p53. Therefore, increased levels of p53 resulting from exposure to DNA-damaging agents such as Act D would be expected to correlate with elevated $p21^{CIP1/WAF1}$ expression, reflecting p53-mediated transactivation of the *CIP1/WAF1* promoter. Western analysis of lysates derived from cells cultured in the presence or absence of Act D revealed that $p21^{CIP1/WAF1}$ levels are increased in Act D-treated uninfected, IE86-expressing, IE72-expressing, and vector-expressing FB compared to untreated cells (Fig. 6).

Phosphorylation of Rb is inhibited in Act D-treated FB. The reported effects of irradiation and DNA-damaging agents on p53 levels (38) and the ability of irradiation-induced p53 to inhibit the G_1-to-S phase transition via the induction of *CIP1/WAF1* (16) are consistent with the present findings. The increased $p21^{CIP1/WAF1}$ levels associated with Act D-induced arrest in G_1 would also be predicted to correlate with an inhibition of G_1 CDK activities (16, 26). The cyclin-dependent kinase inhibitor (CDKI) $p21^{CIP1/WAF1}$ suppresses the activation of several cyclin-CDK complexes including the G_1-associated cyclin E-CDK2 and the D-type cyclins for which CDK4 is the catalytic subunit (26). Although the mechanism is not clearly understood, Rb appears to be a substrate for cyclin D-CDK4 and also possibly for cyclin E-CDK2 (56, 67). Phosphorylation of this tumor suppressor protein correlates with and appears to be necessary for the G_1-to-S phase transition as it relieves the inhibitory effect of Rb upon E2F-dependent transcription of S-phase genes (32, 33, 67). Therefore, predominance of the faster-migrating or hypophosphorylated form of Rb typifies cells arrested in G_1 as a consequence of DNA damage. In the present studies, Western analysis indicated that the hypophosphorylated form of Rb predominates in Act D-treated uninfected FB as well as in IE86-, IE72-, or vector-expressing FB exposed to Act D (Fig. 7). It is interesting to note that FB expressing IE72 demonstrate a high level of the slower-migrating or hyperphosphorylated form of Rb compared to uninfected FB or FB expressing LXSH.

Act D increases mdm-2 protein levels in FB expressing HCMV IE86. mdm-2 represents another downstream effector of p53 activation in a pathway that is apparently distinct from the $p21^{CIP1/WAF1}$ pathway. Elevated p53 levels have been associated with increased expression of mdm-2 (2), an event directly attributed to the binding of p53 to a specific intronic site within the mdm-2 gene and subsequent transactivation by p53 (70). The mdm-2 gene product has also been shown to bind to p53, preventing continued transactivating activity and providing a negative feedback loop for the regulation of mdm-2 expression (70). Chen et al. (8) have shown that irradiation increases steady-state mdm-2 mRNA levels in cells expressing wild-type p53 but not in cells with dysfunctional p53 or $p53^{-/-}$ cells, indicating that modulation of mdm-2 expression is dependent upon the functional competence of p53. Consistent with these reports, Western analysis of uninfected FB exposed to Act D (0.5 nM) for 24 h revealed increased mdm-2 protein levels (Fig. 8). Likewise, FB expressing IE86 demonstrated increased mdm-2 protein levels following Act D-induced DNA damage, as did FB expressing IE72 or the vector controls.

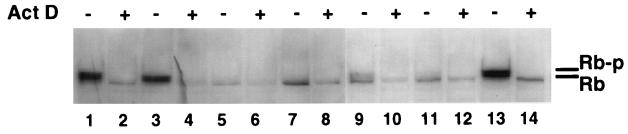

FIG. 7. Western analysis for determination of Rb protein levels and phosphorylation status of untreated FB and FB treated with 0.5 nM Act D (24-h exposure). Lanes: 1 and 2, uninfected; 3 and 4, HPV-16 E7; 5 and 6, LXSN; 7 and 8, LXSN plus IE86; 9 and 10, LXSN plus truncated IE86; 11 and 12, LXSH; 13 and 14, LXSH plus IE72.

DISCUSSION

A common strategy among DNA viruses for ensuring viral propagation is modulation of cellular transcription. This seems to be especially true of the small DNA viruses such as HPV, SV40, and adenovirus, perhaps because synthetic enzymes necessary for viral replication are not encoded by their genomes (11). The abilities of HPV-16 E6 and E7, SV40 large T antigen, and adenovirus E1B and E1A to bind and inactivate the tumor suppressor proteins p53 and Rb are well-characterized mechanisms for promoting the transcription of cellular genes needed for both DNA and viral replication. The genomes of larger DNA viruses such as the human herpesviruses do contain sequences for enzymes involved in DNA replication (57), and, presumably, these viruses would not require the induction of cellular genes for viral transcription. In contrast to the alphaherpesviruses, the betaherpesvirus HCMV demonstrates a rather protracted replication period (57), perhaps suggesting that the availability of virally derived enzymes may be insufficient for genomic duplication in the host cell. It is conceivable that an additional factor(s) or events are needed for replication of the HCMV genome. In this light, the reported binding of an IE gene product to the cell cycle regulatory protein and transcriptional factor p53 (62) is of particular interest with regard to an understanding of the basic transcriptional mechanisms of HCMV as well as the significant implications that such an interaction might have for viral regulation of cell proliferation.

The present studies were performed to investigate the consequences of IE86 binding to p53 in the context of growth control and in the presence of DNA damage. This physical interaction has been interpreted by Speir et al. (62) to suggest that IE86 expression in HCMV infection may alleviate p53-mediated G_1 checkpoint function, thereby promoting cell proliferation. To examine this hypothesis, p53 function was assayed in FB stably expressing HCMV IE86. Specific binding of p53 to IE86 in a cellular context was confirmed by immunoprecipitation of cell lysates from uninfected, IE86-expressing, IE72-expressing, or vector-expressing FB with antibodies to p53 followed by Western analysis of immunoprecipitates for IE86. CAT assays validated the functional competence of the IE86 protein based on its ability to activate an HCMV EP and repress the HCMV MIEP. Sequencing confirmed that were no significant alterations in the integrated LXSN/IE86 sequences. FACS analysis of Act D-treated FB revealed that IE86-ex-

pressing cells consistently arrested in G_1, as did uninfected and vector control cells but not FB expressing HPV-16E6. p53 effector pathways involving p21 and Rb as well as mdm-2 were also shown to be functionally competent. These data suggest that the presence of IE86 does not impair the ability of p53 to detect genomic damage and initiate pathways leading to growth arrest.

The ability of Act D to increase p53 protein levels in cells expressing IE86 further demonstrates the functional competence of this pathway. An increase in the half-life of p53 has been observed following DNA damage induced by several different agents including UV or gamma irradiation, 1-β-D-arabinofuranosylcytosine (ara-C), and Act D, and this effect was attributed to posttranscriptional mechanisms rather than increased transcription (38). It is thought that phosphorylation, protein binding, or oligomerization may be important events in stabilizing p53 (4, 19, 40). Whatever the mechanism, increased expression of p53 seems to be dependent on some degree of p53 functionality since UV irradiation of FB expressing large T antigen did not elicit increases in p53 levels (38); these cells were also able to escape G_1 arrest induced by DNA damage. Similarly, cells expressing HPV-16E6 are also able to bypass Act D-induced growth arrest (15; see above). The present results suggest, however, that binding of p53 to IE86 has different consequences for cell cycle progression than p53 binding to SV40 large T antigen or HPV-16 E6 since DNA damage-induced growth arrest is not impaired when p53 is complexed with IE86.

Increased basal expression levels of p53 have been reported in FB and smooth muscle cells following infection with HCMV (36, 52, 62). Speir et al. (62) attributed this effect to the presence of IE86 based on the expression kinetics of IE86 and p53 and further suggested that the binding of IE86 to p53 was responsible for stabilization of this tumor suppressor protein. Results from the present studies, however, suggest that IE72 and/or IE86 is not responsible for this effect. Considering the possibility that both IE72 and IE86 are required to increase p53 levels, Western analysis was performed on lysates from FB stably expressing both IE gene products. Coexpression of IE72 and IE86 also failed to elicit an increase in basal p53 levels relative to uninfected or vector control FB (data not shown). These data would suggest that another HCMV protein may contribute to the increase in p53 basal expression observed in infected cells. This hypothesis is not inconsistent with a report by Muganda et al. (52) that p53 levels are increased in FB transiently transfected with a plasmid containing both the IE1 and the IE2 genomic regions under control of the MIEP (pHD101SV1). In those studies, it is conceivable that another IE gene product such as IE55 might have been transcribed and could have been responsible either independently or in conjunction with IE72 and/or IE86 to effect the observed increase in p53 levels. Since alternative splicing of our IE86 cDNA-retrovirus construct could also permit the expression of IE55, we are further characterizing the present system to consider the potential role of this IE gene product in the regulation of p53. Elevated p53 levels in FB or smooth muscle cells infected with HCMV have also been described (36, 62), and it is conceivable that a protein necessary for this effect is available in viral infection models that is not available in the present retroviral expression system which utilizes the IE72 or IE86 cDNAs.

It is possible that the lack of effects on p53 levels and p53-dependent G_1 growth control observed in the present IE86 expression system reflects a mutation in the retrovirus-expressed IE86 protein that renders it nonfunctional. However, examination of the IE86 gene in FB expressing the IE86 protein indicates that there are no significant sequence differences. In addition, CAT assays demonstrated the clear activation of the HCMV promoter for the 2.2-kb class of early RNAs and repression of the HCMV MIEP in IE86-expressing cells compared to cells expressing the LXSN vector alone, thereby excluding this hypothesis. Promoter activation and repression results from these experiments are similar to other reports in the literature with regard to the ability of IE86 to modify HCMV promoter activity (37, 59) and suggest that the retrovirus-expressed IE86 described in the present studies is functionally competent.

The apparent integrity of the p53 DNA damage response pathway in the presence of IE86 might be due to the effects of p53 that remains unbound by IE86. Although accurate quantitative assessment of protein-protein interactions is limited by the efficiency of the antibody, p53 Western analysis of supernatants remaining after anti-p53 immunoprecipitation suggests that the majority of cellular p53 was removed by the immunoprecipitation. These experiments do not assess the actual proportion of precipitated p53 that is bound by IE86, but the specificity of this interaction as well as the high levels of IE86 in cell lysates and in p53 immunoprecipitates suggest that most of the precipitated p53 is bound to IE86. Additional investigations to consider this point are in progress. The possibility also exists that lower levels of IE86 expression in the present system relative to viral infection will permit free cellular p53 to effect the observed responses. This appears rather unlikely since retroviral expression of IE86 is driven by the MMLV LTR rather than the HCMV MIEP, which is known to be negatively regulated by IE86.

The association of a truncated form of IE86 with p53 observed in these experiments is of particular interest and may eventually provide information about the physical aspects of the protein-protein interaction between p53 and the full-length IE86. Identification of specific binding sites for IE86 on p53 may reveal any functional consequences of this interaction. Studies are in progress to further characterize this form of IE86 and its association with p53.

One manifestation of DNA damage-induced increases in p53 levels and the ensuing growth arrest (38) is p53-dependent transactivation of the *CIP1/WAF1* promoter (17). Increased expression of this CDKI subsequently prevents Rb phosphorylation by G1 cyclin-CDK complexes, an event required for the G_1-to-S transition (16, 26). Predictably, cells subjected to DNA-damaging agents undergo growth arrest and accumulate in G_1, as observed here for cells treated with Act D. In the present studies, increased levels of $p21^{CIP1/WAF1}$ were also observed in Act D-treated FB in both the presence and absence of IE86 expression. Consistent with this model, a shift in the phosphorylation state of Rb toward the hypophosphorylated form was observed following Act D exposure of either uninfected or IE86-, IE72-, or vector-expressing FB. Although *CIP1/WAF1* expression can be induced by p53-independent pathways (49), the coordinate increase in p53 and growth arrest observed in these cells suggests that the $p21^{CIP1/WAF1}$ response is likely mediated by p53 (17). Furthermore, *CIP1/WAF1* induction following Act D-induced DNA damage provides evidence for the functional competence of p53 in this system. The overexpression of IE86 and p53 has previously been reported to repress the reporter activity of a CAT construct containing multiple p53 responsive elements (62). Because extrapolation of these data to a physiological system is rather difficult, the present studies assessed cellular p53 transactivating activity as reflected by transactivation of the endogenous *CIP1/WAF1* promoter following Act D-induced DNA damage, an event dependent upon p53 (17). Data from these experiments clearly indicate that complex formation with IE86

does not compromise p53 function, as reflected by the induction of *CIP1/WAF1* and the predominance of the hypophosphorylated form of Rb. Bresnahan et al. (7) have reported that $p21^{CIP1/WAF1}$ levels are decreased during HCMV infection. Although it is possible that IE86 binding to p53 alters the transcriptional regulation of $p21^{CIP1/WAF1}$, this seems rather unlikely since the present data demonstrate that basal $p21^{CIP1/WAF1}$ levels are unchanged by the presence of IE86. Furthermore, the $p21^{CIP1/WAF1}$ levels in Act D-treated cells expressing IE86 still increase compared to those in untreated IE86-expressing cells. The decrease in $p21^{CIP1/WAF1}$ expression during viral infection (7) would appear to be mediated by other viral products, although it is quite interesting with regard to numerous reported cell cycle modulatory effects of HCMV.

Further evidence for p53 functionality in the presence of IE86 is provided by the increased levels of mdm-2 in response to Act D-induced DNA damage in FB expressing IE86. These data indicate that the binding of IE86 to p53 does not prevent the physical interaction between p53 and the mdm-2 gene that is responsible for mdm-2 induction (70). mdm-2 has also been shown to bind to p53, and Wu et al. (70) have suggested that the oncogenicity of mdm-2 may be manifested through its ability to prevent p53-dependent induction of genes associated with growth arrest. In this regard, overexpression of mdm-2 was shown to inhibit irradiation-induced GADD45 expression (70). Although the present studies do not reveal whether IE86 binding to p53 alters the ability of mdm-2 to complex with p53 (70), the effect of IE86 binding on mdm-2 regulation of the transactivating activity of p53 would certainly be of interest. It is clear, however, that p53-dependent induction of mdm-2 expression is functional in the presence of IE86.

The current data indicate that the association of IE86 and p53 in FB is specific but does not seem to compromise the ability of p53 to mediate events leading to G_1 arrest. In this regard, the Epstein-Barr virus nuclear protein EBNA-LP or EBNA-5 has also been shown to bind p53 without impairing its growth arrest function (35). In these studies, a $p53^{-/-}$ cell line was transiently transfected to express wild-type p53 in a manner that would maximally stimulate (approximately 40-fold increase) CAT activity from a reporter containing multiple upstream p53-responsive elements. Expression of EBNA-5 (or EBNA-LP) in these cells did not abrogate p53-mediated transactivation, indicating that EBNA-LP binding did not hinder the ability of p53 to stimulate promoter activity. Furthermore, treatment of Epstein-Barr virus-immortalized human B cells with the DNA-damaging agent cisplatin elicited rapid and dramatic increases in p53 and $p21^{CIP1/WAF1}$ levels and resulted in an apparent p53-dependent apoptosis. Other functions of p53, however, may be impaired as a consequence of this interaction with IE86. Crook et al. (12), for example, have separated the transactivational activities of p53 from its ability to repress *ras*-dependent viral transformation.

Although p53 has not been identified as a specific target for IE86-mediated modulation of G_1 checkpoint function, this viral effector may still intervene in cell cycle progression by associating directly with Rb. The transactivational consequences of this interaction, as mentioned previously, have been more clearly characterized (11, 23, 60) and suggest that Rb inactivation by IE86 may play a role in HCMV-induced modulation of cell cycle progression.

Of particular interest in the present studies is the observation that high levels of hyperphosphorylated Rb were detected in lysates from untreated FB expressing IE72 compared to those in lysates from uninfected or vector control FB. In addition, these cells expressed lower levels of p53. These results may indicate an action of IE72 to modify cyclin-CDK-CDKI activity in G_1 to effect the phosphorylation of Rb and promote the G_1-to-S transition. In this regard, IE72 has been shown to transactivate the dihydrofolate reductase promoter in an E2F-dependent manner (46). A physical interaction between IE72 and E2F1 was also noted in these studies (46). It is possible that IE72 effects the phosphorylation of Rb, releasing E2F and promoting the transcription of genes involved in DNA replication, several of which are known to contain E2F sites (14). Suppression of p53 expression may represent a coordinate event in this pathway. These data may suggest an important mechanism whereby the expression of IE72, an early event in HCMV infection, may promote cellular as well as viral replication. It is interesting to note that while IE1 gene products are requisite for low multiplicities of infection, viral replication with moderate to high levels of input does not require the contribution of this IE region (51). Tegument-derived viral products are thought to compensate for IE1 expression at high multiplicities of infection, suggesting that IE1 gene products may be somewhat dispensable under these conditions (51). Considering these observations, the IE1 72-kDa protein may be important in events promoting or permitting viral replication during reactivation from latency or during low levels of replication in persistent, subclinical infection.

In summary, data are presented from experiments designed to assess the functional consequences of the physical interaction between IE86 and the tumor suppressor protein p53. Although these results clearly demonstrate a specific interaction between p53 and IE86, known cellular activities dependent upon p53 are seemingly unaffected. However, modulation of p53 function by IE86 binding may require the association of another factor with this complex. Jault et al. (36) recently described increased steady-state levels of p53 following HCMV infection of FB in the absence of *CIP1/WAF1* induction. The increase in p53 expression was not associated with growth arrest, and the authors attributed these results to IE86-mediated inhibition of p53 transactivation of the *CIP1/WAF1* promoter. Considering the results presented here, these data (36) may be interpreted to suggest that HCMV-mediated stabilization of p53 in the absence of growth arrest may require coordinate binding of another viral and/or cellular protein(s) to IE86 in addition to p53. In this regard, Sommer et al. (60) have described the existence of multiple binding sites on IE86 that might facilitate simultaneous association of both TBP and Rb. Data presented here alternatively suggest that the IE gene product IE72 may modulate events leading to the G_1/S transition, possibly through an interaction with Rb. A combined effect of multiple IE gene products should not be ruled out, however, since the coordinate interactions of viral gene products with multiple cellular regulatory proteins have been shown to significantly affect cell cycle progression.

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REFERENCES

1. **Baracchini, E., E. Glezer, K. Fish, R. M. Stenberg, J. A. Nelson, and P. Ghazal.** 1992. An isoform variant of the cytomegalovirus immediate-early auto repressor functions as a transcriptional activator. Virology **188:**518–529.
2. **Barak, Y., T. Juven, R. Haffner, and M. Oren.** 1993. mdm2 expression is induced by wild type p53 activity. EMBO J. **12:**461–468.
3. **Biegalke, B. J., and A. P. Geballe.** 1991. Sequence requirements for activation of the HIV-1 LTR by human cytomegalovirus. Virology **183:**381–385.

4. **Bischoff, F. Z., S. O. Yim, S. Pathak, G. Grant, M. J. Siciliano, B. C. Giovanella, L. C. Strong, and M. A. Tainsky.** 1990. Spontaneous abnormalities in normal fibroblasts from patients with Li-Fraumeni cancer syndrome: aneuploidy and immortalization. Cancer Res. **50:**7979–7984.
5. **Blanton, R. A., N. Perez-Reyes, D. T. Merrick, and J. K. McDougall.** 1991. Epithelial cells immortalized by human papillomaviruses have premalignant characteristics in organotypic culture. Am. J. Pathol. **138:**673–685.
6. **Bonin, L. R., and J. K. McDougall.** Unpublished data.
7. **Bresnahan, W. A., I. Boldogh, E. A. Thompson, and T. Albrecht.** 1996. Human cytomegalovirus inhibits cellular DNA synthesis and arrests productively infected cells in late G1. Virology **224:**150–160.
8. **Chen, C.-Y., J. D. Oliner, Q. Zhan, A. J. Fornace, B. Vogelstein, and M. B. Kastan.** 1994. Interactions between p53 and MDM2 in a mammalian cell cycle checkpoint pathway. Proc. Natl. Acad. Sci. USA **91:**2684–2688.
9. **Cherrington, J. M., E. L. Khoury, and E. S. Mocarski.** 1991. Human cytomegalovirus ie2 negatively regulates a gene expression via a short target sequence near the transcription start site. J. Virol. **65:**887–896.
10. **Cherrington, J. M., and E. S. Mocarski.** 1989. Human cytomegalovirus IE1 transactivates the a promoter-enhancer via an 18-base-pair repeat element. J. Virol. **63:**1435–1440.
11. **Choi, K. S., S.-J. Kim, and S. Kim.** 1995. The retinoblastoma gene product negatively regulates transcriptional activation mediated by the human cytomegalovirus IE2 protein. Virology **208:**450–456.
12. **Crook, T., N. J. Marston, E. A. Sara, and K. H. Vousden.** 1994. Transcriptional activation by p53 correlates with suppression of growth but not transformation. Cell **79:**817–827.
13. **Davis, M. G., S. C. Kenney, J. Kamine, and J. S. Pagano.** 1987. Immediate-early gene region of human cytomegalovirus trans-activates the promoter of human immunodeficiency virus. Proc. Natl. Acad. Sci. USA **84:**8642–8646.
14. **DeGregori, J., T. Kowalik, and J. R. Nevins.** 1995. Cellular targets for activation by the E2F1 transcription factor include DNA synthesis- and G_1/S-regulatory genes. Mol. Cell. Biol. **15:**4215–4224.
15. **Demers, G. W., S. A. Foster, C. L. Halbert, and D. A. Galloway.** 1994. Growth arrest by induction of p53 in DNA damaged keratinocytes is bypassed by human papillomavirus. Proc. Natl. Acad. Sci. USA **91:**4382–4386.
16. **Dulic, V., W. K. Kaufmann, S. J. Wilson, T. D. Tlsty, E. Lees, J. W. Harper, S. J. Elledge, and S. I. Reed.** 1994. p53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G1 arrest. Cell **76:**1013–1023.
17. **El-Deiry, W. S., T. Tokino, V. E. Velculescu, D. B. Levy, R. Parson, J. M. Trent, D. Lin, W. E. Mercer, K. W. Kinzler, and B. Vogelstein.** 1993. WAF1, a potential mediator of p53 tumor suppression. Cell **75:**817–825.
18. **Emery, V. C., and P. D. Griffiths.** 1990. Current status review. Molecular biology of cytomegalovirus. Int. J. Exp. Pathol. **71:**905–918.
19. **Finlay, C. A., P. W. Hinds, T.-H. Tan, D. Eliyahu, M. Oren, and A. J. Levine.** 1988. Activating mutations for transformation by p53 produce a gene product that forms an hsc70-p53 complex with an altered half-life. Mol. Cell. Biol. **8:**531–539.
20. **Geist, L. J., M. M. Monick, M. F. Stinski, and G. W. Hunninghake.** 1991. The immediate early genes of human cytomegalovirus upregulate expression of the interleukin-2 and interleukin-2 receptor genes. Am. J. Respir. Cell. Mol. Biol. **5:**292–296.
21. **Gibson, W.** 1993. Molecular biology of human cytomegalovirus, p. 303–329. *In* Y. Becker, G. Darai, and E.-S. Huang (ed.), Molecular aspects of human cytomegalovirus diseases. Springer-Verlag, New York, N.Y.
22. **Griffiths, P. D., and J. E. Grundy.** 1987. Molecular biology and immunology of cytomegalovirus. Biochem. J. **241:**313–324.
23. **Hagemeier, C., R. Caswell, G. Hayhurst, J. Sinclair, and T. Kouzarides.** 1994. Functional interaction between the HCMV IE2 transactivator and the retinoblastoma protein. EMBO J. **13:**2897–2903.
24. **Hagemeier, C., S. Walker, R. Caswell, T. Kourzarides, and J. Sinclair.** 1992. The human cytomegalovirus 80-kilodalton but not the 72-kilodalton immediate-early gene transactivates heterologous promoters in a TATA box-independent mechanism and interacts directly with TFIID. J. Virol. **66:**4452–4456.
25. **Halbert, C. L., G. W. Demers, and D. A. Galloway.** 1992. The E6 and E7 genes of human papillomavirus type 6 have weak immortalizing activity in human epithelial cells. J. Virol. **66:**2125–2134.
26. **Harper, J. W., G. R. Adami, N. Wei, K. Keyomarsi, and S. J. Elledge.** 1993. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell **75:**805–816.
27. **Hayhurst, G. P., L. A. Bryant, R. C. Caswell, S. M. Walker, and J. H. Sinclair.** 1995. CCAAT box-dependent activation of the TATA-less human DNA polymerase a promoter by the human cytomegalovirus 72-kilodalton major immediate-early protein. J. Virol. **69:**182–188.
28. **Hendrix, M. G. R., M. Daemen, and C. A. Bruggeman.** 1991. Cytomegalovirus nucleic acid distribution within the human vascular tree. Am. J. Pathol. **138:**563–567.
29. **Hendrix, M. G. R., P. H. J. Dormans, P. Kitslaar, F. Bosman, and C. A. Bruggeman.** 1989. The presence of cytomegalovirus nucleic acids in arterial walls of atherosclerotic and nonatherosclerotic patients. Am. J. Pathol. **134:** 1151–1157.
30. **Hendrix, M. G. R., M. M. M. Salimans, C. P. A. van Boven, and C. A. Bruggeman.** 1990. High prevalence of latently present cytomegalovirus in arterial walls of patients suffering from grade III atherosclerosis. Am. J. Pathol. **136:**23–28.
31. **Hermiston, G. P., C. L. Malone, and M. F. Stinski.** 1990. Human cytomegalovirus immediate-early two-protein region involved in negative regulation of the major immediate-early promoter. J. Virol. **64:**3532–3536.
32. **Hiebert, S. W.** 1993. Regions of the retinoblastoma gene product required for its interaction with the E2F transcription factor are necessary for E2 promoter repression and pRb-mediated growth suppression. Mol. Cell. Biol. **13:**3384–3391.
33. **Hiebert, S. W., S. P. Chellappan, J. M. Horowitz, and J. R. Nevins.** 1992. The interaction of Rb with E2F coincides with an inhibition of the transcriptional activity of E2F. Genes Dev. **6:**177–185.
34. **Huang, E.-S., and T. F. Kowalik.** 1993. The pathogenicity of human cytomegalovirus: an overview, p. 3–45. *In* Y. Becker, G. Darai, and E.-S. Huang (ed.), Molecular aspects of human cytomegalovirus diseases. Springer-Verlag, New York, N.Y.
35. **Inman, G. J., and P. J. Farrell.** 1995. Epstein-Barr virus EBNA-LP and transcription regulation properties of pRB, p107 and p53 in transfection assays. J. Gen. Virol. **76:**2141–2149.
36. **Jault, F. M., J.-M. Jault, F. Ruchti, E. A. Fortunato, C. Clark, J. Corbeil, D. D. Richman, and D. H. Spector.** 1995. Cytomegalovirus infection induces high levels of cyclins, phosphorylated Rb, and p53, leading to cell cycle arrest. J. Virol. **69:**6697–6704.
37. **Jupp, R., S. Hoffmann, A. Depto, R. M. Stenberg, P. Ghazal, and J. A. Nelson.** 1993. Direct interaction of the human cytomegalovirus IE86 protein with the *cis* repression signal does not preclude TBP from binding to the TATA box. J. Virol. **67:**5595–5604.
38. **Kastan, M. B., O. Onyekwere, D. Sidransky, B. Vogelstein, R. W. Craig.** 1991. Participation of p53 protein in the cellular response to DNA damage. Cancer Res. **51:**6304–6311.
39. **Kessis, T. D., R. J. Slebos, W. G. Nelson, M. B. Kastan, B. S. Plunkett, S. M. Han, A. T. Lorincz, L. Hedrick, and K. R. Cho.** 1993. Human papillomavirus 16 E6 expression disrupts the p53-mediated cellular response to DNA damage. Proc. Natl. Acad. Sci. USA **90:**3988–3992.
40. **Kraiss, S., A. Quaiser, M. Oren, and M. Montenarh.** 1988. Oligomerization of oncoprotein p53. J. Virol. **62:**4737–4744.
41. **Lang, D., S. Gebert, H. Arlt, and T. Stamminger.** 1995. Functional interaction between the human cytomegalovirus 86-kilodalton IE2 protein and the cellular transcription factor CREB. J. Virol. **69:**6030–6037.
42. **Lemstrom, K. B., J. H. Bruning, C. A. Bruggeman, I. T. Lautenschlager, and P. J. Hayry.** 1993. Cytomegalovirus infection enhances smooth muscle cell proliferation and intimal thickening of rat aortic allografts. J. Clin. Invest. **92:**549–558.
43. **Liu, B., T. W. Hermiston, and M. F. Stinski.** 1991. A *cis*-acting element in the major immediate-early (IE) promoter of human cytomegalovirus is required for negative regulation by IE2. J. Virol. **65:**897–903.
44. **Livingstone, L. R., A. White, J. Sprouse, E. Livanos, T. Jacks, and T. D. Tlsty.** 1992. Altered cell cycle arrest and gene amplification potential accompany loss of wild-type p53. Cell **70:**923–936.
45. **Malone, C. L., D. H. Vesole, and M. F. Stinski.** 1990. Transactivation of a human cytomegalovirus early promoter by gene products from the immediate-early gene IE2 and augmentation by IE1: mutational analysis of the viral proteins. J. Virol. **64:**1498–1506.
46. **Margolis, M. J., S. Pajovic, E. L. Wong, M. Wade, R. Jupp, J. A. Nelson, and J. C. Azizkhan.** 1995. Interaction of the 72-kilodalton human cytomegalovirus IE1 gene product with E2F coincides with E2F-dependent activation of dihydrofolate reductase transcription. J. Virol. **69:**7759–7767.
47. **Melnick, J. L., E. Adam, and M. E. DeBakey.** 1993. Human cytomegalovirus and atherogenesis, p. 80–91. *In* Y. Becker, G. Darai, and E.-S. Huang (ed.), Molecular aspects of human cytomegalovirus diseases. Springer-Verlag, New York, N.Y.
48. **Melnick, J. L., C. Hu, J. Burek, E. Adam, and M. E. DeBakey.** 1994. Cytomegalovirus DNA in arterial walls of patients with atherosclerosis. J. Med. Virol. **42:**170–174.
49. **Michieli, P., M. Chedid, D. Lin, J. H. Pierce, W. E. Mercer, and D. Givol.** 1994. Induction of WAF1/CIP1 by a p53-independent pathway. Cancer Res. **54:**3391–3395.
50. **Miller, A. D., M. A. Bender, E. A. Harris, M. Kaleko, and R. E. Gelinas.** 1988. Design of retrovirus vectors for transfer and expression of the human beta-globin gene. J. Virol. **62:**4337–4345.
51. **Mocarski, E. S., G. W. Kemble, J. M. Lyle, and R. F. Greaves.** 1996. A deletion mutant in the human cytomegalovirus gene encoding IE1 491aa is replication defective due to a failure in autoregulation. Proc. Natl. Acad. Sci. USA **93:**11321–11326.
52. **Muganda, P., O. Mendoza, J. Hernandez, and Q. Qian.** 1994. Human cytomegalovirus elevates levels of the cellular protein p53 in infected fibroblasts. J. Virol. **68:**8028–8034.
53. **Neumann, J. R., C. A. Morency, and K. O. Russian.** 1987. A novel rapid assay for chloramphenicol acetyltransferase gene expression. BioTechniques **5:** 444–447.

54. **Pizzorno, M. C., and G. S. Hayward.** 1990. The IE2 gene products of human cytomegalovirus specifically down-regulate expression from the major immediate-early promoter through a target sequence located near the cap site. J. Virol. **64:**6154–6165.
55. **Poma, E. E., T. F. Kowalik, L. Zhu, J. H. Sinclair, and E.-S. Huang.** 1996. The human cytomegalovirus IE1-72 protein interacts with the cellular p107 protein and relieves p107-mediated transcriptional repression of an E2F-responsive promoter. J. Virol. **70:**7867–7877.
56. **Resnitzky, D., and S. I. Reed.** 1995. Different roles for cyclins D1 and E in regulation of the G_1-to-S transition. Mol. Cell. Biol. **15:**3463–3469.
57. **Roizman, B.** 1991. Herpesviridae: a brief introduction, p. 841–847. *In* B. N. Fields (ed.), Fundamental virology. Raven Press, New York, N.Y.
58. **Scheffner, M., B. A. Werness, J. M. Huibregtse, A. J. Levine, and P. M. Howley.** 1990. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell **63:**1129–1136.
59. **Schwartz, R., B. Helmich, and D. H. Spector.** 1996. CREB and CREB-binding proteins play an important role in the IE2 86-kilodalton protein-mediated transactivation of the human cytomegalovirus 2.2-kilobase RNA promoter. J. Virol. **70:**6955–6966.
60. **Sommer, M. H., A. Scully, and D. H. Spector.** 1994. Transactivation by the human cytomegalovirus IE2 86-kilodalton protein requires a domain that binds to both the TATA box-binding protein and the retinoblastoma protein. J. Virol. **68:**6223–6231.
61. **Sorlie, P. D., E. Adam, S. L. Melnick, A. Folsom, T. Skelton, L. E. Chambless, R. Barnes, and J. L. Melnick.** 1994. Cytomegalovirus/herpesvirus and carotid atherosclerosis: the ARIC study. J. Med. Virol. **42:**33–37.
62. **Speir, E., R. Modali, E.-S. Huang, M. B. Leon, F. Shawl, T. Finkel, and S. E. Epstein.** 1994. Potential role of human cytomegalovirus and p53 interaction in coronary restenosis. Science **265:**391–394.
63. **Stenberg, R. M., J. Fortney, S. W. Barlow, B. P. Magrane, J. A. Nelson, and P. Ghazal.** 1990. Promoter-specific trans activation and repression by human cytomegalovirus immediate-early proteins involves common and unique protein domains. J. Virol. **64:**1556–1565.
64. **Tevethia, M. J., D. J. Spector, K. M. Leisure, and M. F. Stinski.** 1987. Participation of two human cytomegalovirus immediate early gene regions in transcriptional activation of adenovirus promoters. Virology **161:**276–285.
65. **Wade, M., T. F. Kowalik, M. Mudryj, E.-S. Huang, and J. C. Azizkhan.** 1992. E2F mediates dihydrofolate reductase promoter activation and multiprotein complex formation in human cytomegalovirus infection. Mol. Cell. Biol. **12:**4364–4374.
66. **Wassermann, K., J. Markovits, C. Jaxel, G. Capranico, K. W. Kohn, and Y. Pommier.** 1990. Effects of morpholinyl doxorubicins, doxorubicin, and actinomycin D on mammalian DNA topoisomerases I and II. Mol. Pharmacol. **38:**38–45.
67. **Weinberg, R. A.** 1995. The retinoblastoma protein and cell cycle control. Cell **81:**323–330.
68. **Werness, B. A., A. J. Levine, and P. M. Howley.** 1990. Association of human papillomavirus types 16 and 18 E6 proteins with p53. Science **248:**76–79.
69. **White, R., N. H. A. Terry, M. L. Meistrich, and D. P. Calkins.** 1990. Improved method for computing potential doubling time from flow cytometric data. Cytometry **11:**314–317.
70. **Wu, X., H. Bayle, D. Olson, and A. J. Levine.** 1993. The p53-mdm-2 autoregulatory feedback loop. Genes Dev. **7:**1126–1132.
71. **Yin, Y., M. A. Tainsky, F. Z. Bischoff, L. C. Stron, and G. M. Wahl.** 1992. Wild-type p53 restores cell cycle control and inhibits gene amplification in cells with mutant p53 alleles. Cell **70:**937–948.
72. **Zhu, H., Y. Shen, and T. Shenk.** 1995. Human cytomegalovirus IE1 and IE2 proteins block apoptosis. J. Virol. **69:**7960–7970.