Identification of Domains within the Human Cytomegalovirus Major Immediate-Early 86-Kilodalton Protein and the Retinoblastoma Protein Required for Physical and Functional Interaction with Each Other

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The human cytomegalovirus major immediate-early 86-kDa protein (IE2 86) plays an important role in the *trans* activation and regulation of HCMV gene expression. Previously, we demonstrated that IE2 86 contains three regions (amino acids [aa] 86 to 135, 136 to 290, and 291 to 364) that can independently bind to in vitro-translated Rb when IE2 86 is produced as a glutathione *S*-transferase fusion protein (M. H. Sommer, A. L. Scully, and D. H. Spector, *J. Virol.* 68:6223–6231, 1994). In this report, we have elucidated the regions of Rb involved in binding to IE2 86 and have further analyzed the functional nature of the interaction between these two proteins. We find that two domains on Rb, the A/B pocket and the carboxy terminus, can each independently form a complex with IE2 86. In functional assays, we demonstrate that IE2 86 and another IE protein, IE1 72, can counter the enlarged flat cell phenotype, but not the G₁/S block, which results from expression of wild-type Rb in the human osteosarcoma cell line Saos-2. Mutational analysis reveals that there are two domains on IE2 86 that can independently affect Rb function. One region (aa 241 to 369) includes the major Rb-binding domain, while the second maps to the amino-terminal region (aa 1 to 85) common to both IE2 86 and IE1 72. These data show that Rb and IE2 86 physically and functionally interact with each other via at least two separate domains and provide further support for the hypothesis that IE2 86 may exert its pleiotropic effects through the formation of multimeric protein complexes.

Human cytomegalovirus (HCMV) is an opportunistic pathogen that causes serious problems in newborns and immunocompromised individuals (for a review, see reference 6). It is the major viral cause of birth defects, and infection of the fetus can result in severe developmental abnormalities. Moreover, it is suspected that activation of latent virus may trigger cellular damage in infected organs of certain susceptible individuals, possibly contributing to coronary restenosis following angioplasty (57, 69). HCMV has long been known to affect the host cell in ways that mimic processes involved in cell activation (for reviews, see references 1 and 40). Infection is associated with the stimulation of cellular DNA, RNA, and protein synthesis, premature condensation of chromosomes, and metaphase abnormalities. Recent studies also demonstrate the mutagenic potential of the virus (2, 53). HCMV-infected cells have elevated levels of ornithine decarboxylase, thymidine kinase, DNA polymerase α , and dihydrofolate reductase. Additionally, we and others have found that HCMV infection affects key components of the cell cycle, resulting in cell cycle arrest (5, 14, 27, 36).

Temporal expression of the HCMV genome separates its gene products into three classes. The immediate-early (IE) gene products are the first to be expressed and go on to activate the next class of early genes. Viral DNA synthesis then follows, allowing expression of the late genes. The large body

of work that has focused on the IE genes has revealed much about their expression and potential functional interactions within the life cycle of the virus. The major IE (MIE) region of the HCMV genome encompasses two genetic units called IE1 and IE2 (for a review and references, see reference 40). The IE1 region yields a single mRNA consisting of four exons encoding a 72-kDa nuclear phosphoprotein (IE1 72). The IE2 region generates two transcripts via differential splicing which encode proteins of 86 kDa (IE2 86) and 55 kDa (IE2 55), respectively. Both IE2 transcripts contain the first three exons of IE1 fused to the IE2 region and encode proteins which share the amino-terminal 85 amino acids (aa) with IE1 72. Although the functions of the individual IE proteins in the context of the viral infection have yet to be defined, transient expression and in vitro transcription assays indicate that HCMV early promoters and heterologous viral promoters can be activated by the region of the genome specifying the IE1 and IE2 gene products. IE2 86 appears to play a major role in activating HCMV early promoters as well as repressing its own promoter, while IE1 72 can stimulate the MIE promoter and may augment the activating effect of IE2 86. It has also been reported that a late protein which consists of the carboxy-terminal 338 aa of IE2 (expressed from a construct designated 2203 in reference 28 and in this report) can repress the MIE promoter and activate some heterologous promoters in the presence of IE1 72.

Recent studies suggest that both IE1 72 and IE2 86 may have a role in mediating some of the activation events described above. IE1 72 alone can activate the DNA polymerase α promoter (22) and the dihydrofolate reductase promoter through its E2F binding site (39). There is also evidence indicating that IE1 72 interacts with the cellular proteins E2F-1 and p107 and can relieve p107-mediated transcriptional re-

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pression of an E2F-responsive promoter in transient expression assays (39, 44). The multifunctional IE2 86 protein binds to DNA in a site-specific manner (3, 10, 23, 34, 35, 38, 42, 50, 51) and counters histone-mediated repression of transcription (31). IE2 86 also forms complexes with multiple proteins, including components of the basal transcription complex, TBP (TATA-binding protein) and TFIIB; the transcriptional repressor Dr-1; the tumor suppressor proteins p53 and Rb (retinoblastoma protein); an HCMV early protein, UL84; and a number of cellular transcription factors, including Sp-1, Tef-1, c-Jun, JunB, Egr-1, and CBP/p300 (7, 8, 11, 16, 19, 20, 29, 33, 37, 48, 49, 51, 55–57, 61, 68). IE2 86 may be able to inactivate p53 as well as prevent Rb-mediated repression of a promoter with E2F-binding sites (11, 19, 57, 61). It has also been reported that HCMV IE1 72 and IE2 86 can block the induction of apoptosis by tumor necrosis factor alpha or by the adenovirus E1A proteins (70).

To define the domains of the IE2 86 protein involved in transactivation as well as the above-cited protein-protein and protein-DNA interactions, we previously constructed a panel of IE2 86 deletion mutants. Our studies showed that the binding patterns of IE2 86 to TBP, Rb, and c-Jun were indistinguishable and that three independent regions in the glutathione *S*-transferase (GST)–IE2 fusion protein (aa 86 to 135, 136 to 290, and 291 to 364) mediated the interactions (51, 55). IE1 72, however did not form a complex with any of these proteins. Using slightly different experimental conditions, Caswell et al. (8) and Hagemeyer et al. (19) also observed that the region of IE2 86 spanning aa 290 to 390 was involved in complex formation with Rb and TBP, as well as with TFIIB. We have chosen to further study the interactions of IE2 86 with Rb and to try to determine the functional significance of this interaction.

Rb functions as a tumor suppressor and plays a key role in regulating cell cycle progression and differentiation (for a review, see reference 64). It is believed that the growth-regulatory functions of Rb depend in part on its ability to form a complex with and inhibit the activity of the transcription factor E2F (24, 45, 46), whose target genes encode proteins involved in DNA synthesis and regulation of the G_1/S progression (13). Rb also appears to interact with and activate specific transcription factors such as NF-IL6 (9) and MyoD (17) that are important for differentiation.

Rb, like IE2 86, has at least two independent protein-binding domains (for reviews, see reference 59, 62, and 63), the first of which is the A/B pocket, consisting of regions A (aa 379 to 572) and B (aa 646 to 772) separated by an insert sequence (aa 572 to 646). This pocket region has been identified as the domain involved in complex formation with several viral oncoproteins, including adenovirus E1A, simian virus 40 (SV40) large T antigen, and human papillomavirus E7, as well as many cellular proteins containing an LXCXE binding motif (X is any amino acid). Expression of this A/B pocket domain, however, is not sufficient for Rb to exert growth suppression. The minimal domain for its growth-regulatory functions encompasses both the A/B pocket and the adjacent C-terminal residues up to aa 869. These additional amino acids are required for binding to D-type cyclins and the E2F family of transcription factors. The additional residues C terminal to the A/B pocket (aa 768 to 869), which are part of the minimal growth suppression domain, form another protein-binding domain (the C pocket) which can interact with c-Abl tyrosine kinase and mdm-2. Although it is still unclear how these various protein-binding domains interact in vivo, Wang (62) presents evidence that the minimal domain for growth suppression can potentially accom-

modate three separate Rb-binding proteins: an LXCXE protein, E2F, and a C-pocket-binding protein.

In this investigation, we have extended our studies to determine (i) which domains on Rb physically interact with IE2 86, (ii) the functional consequences of the interaction between Rb and IE2 86, and (iii) the domains on IE2 86 required for the observed biological effects. We demonstrate that the A/B pocket and C-terminal region on Rb can each independently form a complex with IE2 86. We also find that IE2 86 as well as IE1 72 can counter the enlarged flat cell phenotype resulting from expression of wild-type Rb in the human osteosarcoma cell line Saos-2. However, neither protein was able to abrogate the Rb-induced G_1/S block. By deletion analysis, we have mapped these biological functions to two independent domains on IE2 86. One region (aa 241 to 369) includes the major Rb-binding domain on IE2 86, while the other (aa 1 to 85) is common to both IE1 72 and IE2 86. The implications of these findings with respect to the multifunctional nature of the IE proteins are discussed.

MATERIALS AND METHODS

Cells. The Saos-2 human osteosarcoma cell line, which expresses a nonfunctional truncated Rb protein, was obtained from the American Type Culture Collection (HTB 85) and grown in Dulbecco's modified Eagle's media–high glucose (4.5 g/liter) supplemented with 15% heat-inactivated fetal bovine serum, L-glutamine (0.26 mg/ml), penicillin-streptomycin (180 U/ml), amphotericin B (Fungizone; 1.4 μ g/ml), and gentamicin sulfate (45 μ g/ml).

Expression vectors. The individual cDNAs corresponding to IE1 72 and IE2 86 were obtained from R. Stenberg (Eastern Virginia Medical School) and were cloned into the GST fusion vector pGEX-KG and the eukaryotic expression vector pSG5 (Stratagene) as described previously (31). Mutations in the IE2 86 coding region were generated as previously described (55), and the coding regions were recombined into pGEX-KG and pSG5. The IE2 86 plasmids 2203 (expressing aa 241 to 579), 2204 (2203 in the reverse orientation), and 2206 (full-length IE2 86) were obtained from E. Mocarski (Stanford University) and have been described previously (28). These cDNAs were directed by a combination of the SV40 early promoter and the R-U5 segment of the human T-cell leukemia virus type 1 long terminal repeat. The plasmid expressing the carboxy-terminal half of IE2 86, designated 370-end, was constructed by the following method. pSGIE86 was digested to completion with *NspI* and *BglII* to yield the region of IE2 86 encoding aa 370 to 579. A modified linker containing an *EcoRI* site, followed by a *HindIII* site (for diagnostic purposes) and an *NspI* 3' overhang, was then ligated to the *NspI* end of the fragment. The resulting DNA was then digested to completion and ligated into the pSG5 vector backbone that had been previously cut with *NspI* and *BglII* and treated with calf intestinal phosphatase. Colonies were then screened for inserts by restriction enzyme analysis with *HindIII* and sequenced through the linker to verify orientation and the presence of only a single insert.

The GST-Rb deletion clones (65) and plasmid pCD-Rb, which contains the full-length wild-type Rb gene directed by the SV40 early promoter, were a kind gift of J. Y. J. Wang (University of California, San Diego). RSV-SV40 T, which contains the cDNA for the SV40 large T antigen directed by the Rous sarcoma virus promoter, was provided by S. Subramani (University of California, San Diego).

Binding assays. Expression and purification of GST fusion proteins were carried out as previously described (55). In vitro transcription-translation reactions were carried out by using the TNT Coupled Reticulocyte Lysate system (Promega) according to the manufacturer's instructions. Binding studies were carried out as described previously (55). Briefly, GST fusion protein-bead complexes were resuspended in NETN buffer (20 mM Tris [pH 8.0], 1 mM EDTA, 100 mM NaCl, 0.5% Nonidet P-40) containing 1 mM benzamidine, 1 mM sodium metabisulfate, 0.2 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol, and in vitro-translated proteins were added. Following constant rotation for 1 h at room temperature, one-third of the complexes were removed and precipitated, representing the input fraction. The remaining two-thirds of the complexes were washed five times in NETN buffer, resuspended in Laemmli reducing sample buffer (2% sodium dodecyl sulfate [SDS], 10% glycerol, 100 mM dithiothreitol, 60 mM Tris [pH 6.8], bromophenol blue dye, aprotinin and leupeptin [each at 2 μ g/ml], phenylmethylsulfonyl fluoride [1 mM]), and boiled for 5 min. Proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and exposed to Kodak X-Omat film following fluorographic enhancement. The gels were quantitated with a Molecular Dynamics PhosphorImager.

Transfections. All transfections were performed by a modified calcium phosphate-DNA precipitation method in which the DNA precipitates are formed in *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) as described previously (4). Briefly, Saos-2 cells were seeded at a density of 2×10^6 /10-cm-diameter

plate the night before transfection. The next day, transfections were performed, using a total of 16 μ g of DNA/transfection. The DNA (in a total of 1 ml of BES solution containing 125 mM CaCl_2) was added dropwise to 10 ml of fresh medium added to the cells just prior to the transfection. The plates were then incubated at 37°C and 3% CO_2 for 16 to 20 h. Cells were washed twice in warm phosphate-buffered saline (PBS) and refed with normal medium. The following day, the cells were trypsinized, counted, and either reseeded at a density of 10^6 cells/plate for flat cell assays or processed for fluorescence-activated cell sorting (FACS) analysis.

Flat cell assays. Saos-2 cells were transfected as described above. All transfections were done by using a mass ratio of 1:5 of Rb construct to HCMV test construct (or 2.5 μ g of Rb and 12.5 μ g of test plasmid/transfection). It should be noted that several of the test constructs were also tested by using a mass ratio of 1:1, with similar results. In addition, 1 μ g of the pBABE selection plasmid (41; a gift from J. Y. J. Wang) was added to each transfection in order to provide puromycin resistance. After replating the cells at a density of 10^6 /plate as described above, the cells were placed under puromycin selection (1.2 μ g/ml) for 7 to 10 days. The only cells that remain after this selection period are large, flat nondividing single cells and small, very rapidly dividing puromycin-resistant colonies, which can easily be distinguished from each other. The plates were fixed and stained with a 1% crystal violet solution in 20% ethanol. Single flat cells were counted by using a grid with 1.5- by 1.5-mm squares which completely covered the viewing field. Twenty such fields covering several different areas of the plate for each transfection were counted. Each test transfection data point is an average of at least two trials unless otherwise noted.

Immunofluorescence. Saos-2 cells were transfected by using a 1:5 ratio of Rb to test plasmid as described above. Two days after transfection, the cells were washed twice in warm PBS and then simultaneously fixed and permeabilized in -20°C methanol for 10 min. The cells were rinsed three times in PBS. Coverslips were then double labeled for both Rb and the HCMV IE protein of interest. Depending on the HCMV construct being detected, either a rabbit or mouse antibody was used. For IE2 86, dMN, 1-85, and IE1 72, the mouse monoclonal antibody CH16.0 (a gift from L. Pereira, University of California, San Francisco) was used; for 2203 and 370-end, the polyclonal antibody 1218 (provided by J. Nelson, University of Oregon) was used. Depending on which antibody was used for the HCMV protein, Rb was detected with either G3-245 (mouse monoclonal antibody from Pharmingen) or N9 (rabbit polyclonal antibody from J. DeCaprio, Harvard Medical School). Coverslips were incubated in primary antibodies diluted in PBS-0.2% gelatin for 10 min. After extensive washing in PBS, the cells were incubated with the appropriately conjugated secondary antibodies (one coupled to fluorescein isothiocyanate; the other coupled to rhodamine) from Jackson Laboratories (diluted 1:400 in PBS-0.2% gelatin) for 10 min. Following several additional washes, the cells were finally incubated in Hoechst dye (Calbiochem) for 2 min to illuminate the DNA, washed, and mounted onto slides by using glycerol-paraphenylenediamine (an antiphotobleaching agent). It should be noted that each slide was stained sequentially first for one primary-secondary pair and then for the other. Slides were then analyzed on a Nikon EFD 3 fluorescence microscope equipped with Neofluor objectives and an MTT CCD2 camera.

FACS analysis. Saos-2 cells were transfected as described above. All transfections were done by using a mass ratio of 1:4 of Rb to HCMV test construct (3 μ g of Rb and 12 μ g of test plasmid/transfection) unless otherwise noted. Each transfection also contained 1.5 μ g of CD4-neo (a gift from S. Hedrick, University of California, San Diego). This construct contains the mouse CD4 gene directed by the β -actin promoter and was used as a cell surface marker for cells that had been successfully transfected. Two days after transfection, cells were washed twice with warm PBS and then harvested by using 1 mM EDTA in PBS. The cells were counted, and 10^6 cells from each transfection were washed once more in PBS and then pelleted in Eppendorf tubes. The cell pellets were resuspended in 100 μ l of PBS, and 1 μ l of fluorescein isothiocyanate-conjugated anti-mouse CD4 (Pharmingen) was added. The cells were covered and placed on ice for 30 min. They were then washed twice with PBS, resuspended in 300 μ l of PBS, and fixed by adding 200 μ l of 95% ethanol dropwise while gently vortexing. The cells were again covered, incubated on ice for 30 min, and then washed with PBS to remove the fixative. After the cells were stained with propidium iodide, they were sorted for CD4-positive cells, and this population was analyzed for DNA content. Each data point represents the average of at least two transfections (unless otherwise noted).

Western blot analysis. Cells were seeded and transfected as described above. Two days posttransfection, cells were trypsinized, washed, and counted. Cells were then resuspended in Laemmli reducing sample buffer. Cells were then sonicated, boiled for 5 min, and spun at 15,000 rpm for 10 min at 4°C to pellet any debris. Equal amounts of lysate were then loaded onto SDS-polyacrylamide minigels. Proteins were then transferred to Immobilon P (Millipore). Primary antibodies used included mouse CH16.0 and rabbit 1218 (to detect the N and C termini, respectively, of IE2 86) and mouse PMG-345 (to detect Rb). Goat anti-rabbit and anti-mouse secondary antibodies conjugated with horseradish peroxidase (Amersham) were used to detect primary antibody binding. Proteins were visualized by using enhanced chemiluminescence reagents as instructed by the manufacturer (Pierce).

FIG. 1. Domains on Rb involved in complex formation with IE2 86. IE2 86 Δ 5X was translated in vitro in the presence of [35 S]methionine and added to the GST-Rb proteins immobilized on glutathione-agarose beads. Following incubation for 1 h at room temperature, an aliquot was removed (IN) and the remaining beads were washed in NETN buffer with 100 mM NaCl. Proteins in the input (IN) and bound (BD) fractions were resolved by SDS-PAGE. The A and B pockets are indicated with the numbers at the top indicating positions of the amino acid residues at the border of each pocket. The C $\rightarrow$ F designation in the RB-Ase/End (706) mutant indicates a cysteine-to-phenylalanine change at aa 706 in the Rb protein.

RESULTS

IE2 86 binds independently to two distinct domains on the Rb protein. Previously, we had determined which domains on IE2 86 could form a complex with Rb, but we had not defined which regions of Rb were involved in this interaction (55). To address this question, we performed binding experiments using in vitro-translated IE2 86 and the indicated GST-Rb mutants. We used a variant form of IE2 86 with an internal in-frame deletion of aa 136 to 290 (IE2 Δ 5X) because it bound Rb more efficiently than full-length IE2 86. However, the same pattern of binding was observed with a full-length clone (data not shown). In addition, this mutant, which leaves the major Rb binding domain of IE2 86 intact, is indistinguishable from full-length IE2 86 in terms of its ability to bind DNA and activate the HCMV early promoters (50, 51, 55).

As shown in Fig. 1, IE2 86 binds to both the A/B pocket region (aa 379 to 776) and the carboxy-terminal region of Rb (aa 768 to 928). Although IE2 86 binds weakly to a subfragment of the carboxy terminus (aa 768 to 834), significant levels of binding were detected only when the intact carboxy terminus was present (compare Rb-Ssp/End, Rb-Ssp/Mun, and Rb-Mun/End). The binding of IE2 86 to the carboxy terminus is probably not due to a simple conformational change of this region resulting from deletion of the A/B pocket since IE2 86 also binds to the Rb-Ase/End (706) mutant. This clone has a single amino acid change at position 706 that prevents A/B pocket binding (30). It should be noted that when full-length GST-IE2 86 was incubated with an in vitro-translated version of this mutant construct, equally efficient binding was observed (data not shown). The ability of IE2 86 to interact with both protein-binding domains on Rb individually is in contrast to previously published results showing that other proteins seem to bind to either the A/B pocket or the carboxy-terminal region or to require the presence of both (59, 62, 63).

HCMV IE proteins can counter some of the growth-suppressing functions of Rb. To assess the biological consequences of the interaction between Rb and IE2 86 in vivo, we used the human osteosarcoma cell line Saos-2, which expresses

TABLE 1. Results of flat cell assays and FACS analyses^a

Construct(s) tested	No. of flat cells/20 fields	% CD4 ⁺ cells in S/G ₂ /M
pSG5 alone	1 ± 2	60 ± 2
Rb + pSG5	64 ± 16	30 ± 9
Rb + wt IE2 86	6 ± 4	29 ± 3
Rb + 2206	0 ± 0	ND
Rb + wt IE1 72	11 ± 4	24 ± 10
Rb + wt IE2 86 + wt IE1 72	1 ^b	26 ± 2
Rb + SV40 T antigen	0 ^b	76 ± 4
wt IE86 + pSG5	0 ± 0	71 ^b
wt IE2 86 + wt IE1 72 alone	ND	73 ^b

^a Flat cell assays and FACS analyses were performed as described in Materials and Methods. Data are expressed as mean ± standard deviation, where each represents at least two trials, unless otherwise noted. Flat cell assays were performed with a 1:5 mass ratio of Rb to test plasmid (except for the assay with IE2 86 plus pSG5, which was done at a ratio of 1:1). FACS analysis assays were performed with a 1:4 mass ratio of Rb to test plasmid (except for the assay with IE2 86 plus IE1 72 alone and for one of the two trials when they were cotransfected with Rb, which were performed at a ratio of 1:2). ND, not determined.

^b Only done once.

a nonfunctional cytoplasmic form of Rb. These cells are extremely sensitive to the growth-suppressing properties of wild-type Rb and undergo cell cycle arrest in G₁ when Rb function is restored. The size of the cells also increases, resulting in the appearance of enlarged flat cells which resemble cells in senescence. One common assay for determining the functional interaction between Rb and another protein involves cotransfection of an expression vector for Rb, a puromycin resistance plasmid, and a construct expressing the protein to be tested (25, 26, 60). Cells that express wild-type Rb (and the selectable puromycin resistance gene) do not divide but maintain protein synthesis. By doing so, they are able to remain resistant to puromycin and appear as large flat single cells on the selection plates. When a protein to be tested is cotransfected along with Rb, one of two phenotypes is observed. If the protein cannot counter the growth suppression block imposed by Rb, the cells remain flat and comparable in number to cells transfected with Rb alone. However, if the cotransfected protein can somehow interfere with Rb function, the cells will divide (or die), and the number of flat cells on the plate will be reduced.

In accord with previous observations, Table 1 shows that the large flat cell phenotype appeared when the Rb expression vector was cotransfected with the control plasmid pSG5 and was abrogated by cotransfection with an expression vector for SV40 T antigen, which is known to physically interact with Rb and block its growth suppression function (46). As expected, no enlarged flat cells appeared when the Saos-2 cells were transfected with the control pSG5 or the vector with IE2 86 (construct wt IE2 86) in the absence of Rb. However, when the wt IE2 86 expression vector was cotransfected with Rb into Saos-2 cells, there was a marked reduction (10-fold) in the number of flat cells. This effect was not due to the plasmid backbone or the promoter driving IE2 86, as another IE2 86 expression vector, designated 2206, in which the transcription of the IE2 86 RNA was directed by a chimeric promoter consisting of the SV40 early promoter-enhancer and sequences from the human T-lymphotropic virus type 1 instead of the SV40 promoter alone, had a similar blocking effect on Rb function. Interestingly, wt IE1 72, alone or in combination with wt IE2 86, also was able to reduce significantly the number of flat cells.

As a second assay for the ability of IE2 86 to interfere with Rb function, we examined whether cotransfection of the IE

proteins into the Saos-2 cells would release the Rb-induced G₁ block. For these experiments, Rb and the IE expression vectors were cotransfected with a plasmid encoding the mouse cell surface protein CD4. After coating the cells with an antibody to the murine CD4 and staining the DNA with propidium iodide, we subjected the cells to FACS analysis to determine the distribution of the cotransfected cells in the different phases of the cell cycle. As summarized in Table 1, when the cell surface marker was transfected with the control plasmid pSG5 alone, approximately 60% of the CD4-positive cells were in S/G₂/M. As expected, when Rb was also transfected with the control plasmid into the cells, the number of CD4-positive cells in S/G₂/M dropped to 30%. For a positive control, we documented that SV40 T antigen was able to prevent this Rb-dependent G₁ block. Table 1 shows that 76% of the CD4-positive cells were in S/G₂/M following cotransfection of Rb with SV40 T antigen. However, constructs wt IE2 86 and wt IE1 72, either alone or in combination, were not able to override the G₁ block imposed by Rb. This G₁ block resulting from cotransfection of Rb with the IE proteins was not due solely to the presence of the IE proteins, as transfection of these proteins in the absence of Rb gave values for the percentage of CD4-positive cells in S/G₂/M essentially equal to those with the pSG5 control alone.

One possible explanation for the ability of the IE proteins to prevent some of the Rb-imposed growth suppression was that the expression or subcellular localization of Rb was altered as a result of their cotransfection. To verify that Rb and the IE proteins were expressed in the same cells and that cotransfection did not alter the subcellular localization of Rb, the cells were fixed 2 days after transfection and the proteins were visualized by double-label immunofluorescence staining with the antibodies to Rb and the IE proteins as described in Materials and Methods. Figure 2 shows that both Rb and the IE proteins colocalized in the nucleus and that the coexpression of the IE proteins did not appear to affect the subcellular distribution of Rb or its relative intensity of staining. We did note that a number of cells scored as positive for the IE proteins but negative for Rb. This is likely due to the fact that the expression vectors for the IE proteins were in fivefold excess relative to the Rb plasmid during the cotransfection. Thus, some cells might have taken up the IE expression vector but not the Rb vector. We consider it unlikely that the expression of the IE proteins was suppressing the expression of Rb since the percentage of cells which stained positive for Rb did not decrease when Rb was cotransfected with the IE vectors instead of the control pSG5.

As a complement to the immunostaining experiment, we also used Western blot analysis to verify that IE2 86 did not affect the synthesis of Rb, or vice versa. Cell extracts were prepared 2 days after transfection, and the proteins were resolved on SDS-polyacrylamide gels and analyzed by Western blotting with antibodies to Rb and the IE2 86 protein. Figure 3 demonstrates that the levels of Rb and IE2 86 were not affected by their coexpression. Moreover, the absence of a significant change in the migration of Rb in the gels indicated that IE2 86 did not alter the phosphorylation state of Rb. As expected, the control lane (IE2 86 plus pSG5) did not show the presence of any Rb protein migrating at the position of 110 to 116 kDa, as these cells do not possess a full-length Rb protein (54).

IE2 86 contains two separate domains that can functionally interact with Rb. To delineate the regions of IE2 86 involved in the above-described functional interactions with Rb, we tested a panel of vectors that expressed various deletion mutants of IE2 86 for the ability to prevent the Rb-induced en-

FIG. 2. Cotransfection of Rb and the IE proteins does not affect localization of Rb. Cells were seeded onto coverslips and then cotransfected with either Rb plus IE2 86 (a to c), Rb plus IE1 72 (d to f), or Rb plus control plasmid pSG5 (g to i) at a mass ratio of 1:5 (Rb to test plasmid). Coverslips were fixed and stained 2 days posttransfection, using monoclonal antibody CH16.0 (to detect IE proteins) and polyclonal antibody N9 (to detect Rb). Magnification, $\times 460$.

larged flat cell phenotype. A representative sample, which most clearly illustrates the regions required to interfere with Rb function, is shown in Fig. 4. A mutant IE2 86 protein that lacked 240 aa from the N terminus but still retained the major Rb-binding domain (vector 2203) was as effective as the wild-type protein in countering the flat cell phenotype. We verified that the reduction in the number of Rb-induced flat cells also required protein expression by showing that a vector with the above-cited truncated IE2 86 coding sequences in the reverse orientation, designated 2204, was not able to override the flat cell phenotype. However, in the sense configuration, a further

deletion of 130 aa from the IE2 86 N terminus that removed the Rb-binding domain yielded a protein (370-end) that was completely nonfunctional in the flat cell assay. Thus, in the construct 2203, the Rb-binding domain appeared to be required for IE2 86 to interfere with Rb function.

To determine if the Rb-binding region was the only functional domain, we tested an internal in-frame deletion mutant of IE2 86 that lacked all three Rb-binding domains (designated dMN) but retained 85 aa from the N terminus as well as the C-terminal amino acids present in the vector 370-end. This mutant, was also able to counter the flat cell phenotype almost

FIG. 3. Cotransfection with IE2 86 does not affect Rb protein levels or phosphorylation. Equal amounts of all constructs were transfected as described in Materials and Methods. Two days posttransfection, the cells were harvested and lysed in Laemmli sample buffer, and lysates were analyzed for protein expression via Western blotting. For Rb + pSG5 and Rb + IE2 86 lanes, 2×10^5 cell equivalents were loaded. For the IE2 86 + pSG5 lanes, 10^5 cell equivalents were loaded. In panel A, the IE2 86 protein was detected with antibody CH16.0. Panel B represents the same blot after stripping and reprobing to detect Rb by using antibody PMG-345. Sizes are indicated in kilodaltons.

as efficiently as the wild-type IE2 86 protein. In fact, assays of additional mutants showed that expression of the N-terminal 85 aa of IE2 86 (protein 1-85), which is the region shared with wt IE1 72, was sufficient to block the appearance of the Rb-induced large flat cells. As noted above, IE1 72 was also capable of interfering with Rb function in this assay.

As a control for the foregoing experiments, we used both immunostaining and Western blot analysis to ascertain that all of the IE2 86 mutants were expressed in the nucleus and that the coexpression of the IE proteins did not affect the synthesis or subcellular localization of Rb. Figure 5 shows that following transfection into Saos-2 cells, the IE2 86 mutant proteins were expressed at comparable levels, with the exception of the short N-terminal protein 1-85, whose steady-state concentration appeared to be lower. However, because the level of expression of protein 1-85 seems to be comparable to those of other mutant proteins when detected via immunofluorescence (Fig.

6), we believe that the lower level observed on the Western blot is likely due to inefficient detection of the epitope on this small protein by antibody CH16.0 when it is fully denatured. Moreover by immunostaining (Fig. 6), all of the mutant proteins could be detected in the nucleus, although the two proteins which represented the N-terminal and C-terminal deletion mutants, 370-end and 1-85, respectively, were also present in the cytoplasm. In all cases, Rb was found in the nuclei of cells expressing the IE proteins, and its intensity of staining was not significantly affected by the coexpression of the IE2 86 mutant proteins.

DISCUSSION

The functions of the HCMV IE1 and IE2 gene products have been the subject of many studies, and most agree that both play a key role in the regulation of viral gene expression at the level of transcription. Results of recent studies also suggest that both IE1 72 and IE2 86 may mediate some of the effects of the virus on the host cell cycle. There is an expanding list of cellular proteins that can form a complex with IE1 72 or IE2 86, and this list includes tumor suppressor proteins and both general and specific transcription factors. Although the biological relevance of most of these interactions remains to be elucidated, there is increasing evidence that these interactions may significantly affect cellular regulatory processes (19, 44, 55, 70). In this report, we have focused on the effects of the IE1 72 and IE2 86 proteins on Rb function and have defined the domains in IE2 86 required for both physical and biological interactions.

Both Rb and IE2 86 have the ability to form protein-protein complexes via at least two domains, and the results shown in Fig. 1 indicate that IE2 86 can interact with the two major binding domains of Rb independently. In contrast, most other Rb-binding proteins interact with either the A/B pocket or C-terminal region or require both domains to be present (59, 62, 63). Our results differ somewhat from those of Hagemeier et al. (19), who concluded that IE2 86 bound only to the A/B pocket based on their observation that a partial deletion of this region of Rb (aa 712 and 767) substantially reduced the inter-

FIG. 4. IE2 86 possesses two domains that can functionally interact with Rb. Flat cell assays were performed as described in Materials and Methods. On the left is a schematic depiction of full-length IE2 86, with defined domains represented by different patterns. The common region between IE2 86 and IE1 72 is also delineated. Underneath are the regions still encoded in each deletion mutant. Construct 2204 contains the same cDNA as 2203, but it is inserted in the opposite orientation to the promoter and thus should not produce any functional protein. On the right are the results of the flat cell assays performed with each construct in conjunction with Rb. Error bars represent 1 standard deviation from the mean, which is represented numerically to the right of each bar. Each bar represents an average of at least two trials.

FIG. 5. All mutant proteins are expressed in Saos-2 cells. All transfections were performed using 16 μ g of one construct alone. Cells were harvested 2 days posttransfection and lysed, and either 4×10^5 (A) or 6×10^5 (B) cell equivalents were loaded in each lane. Panel A represents protein lysates from two separate experiments (constructs IE2 86, dMN, and 2203 are from one experiment, and constructs 370-end and IE2 86 are from the other) run on the same gel, transferred, and probed for IE2 86 expression by using 1218 (polyclonal antibody directed against the C terminus of the protein). Panel B represents a third experiment (constructs 1-85 and dMN) on a separate blot which was probed using CH16.0 (monoclonal antibody directed against the very N terminus of IE2 86). Sizes are indicated in kilodaltons.

action of Rb with IE2 86. In our experiments, we found that IE2 86 bound efficiently to the Rb mutant in which the cysteine at aa 706 was changed to a phenylalanine. This mutation has been found to eliminate the binding of Rb to other proteins which require an intact A/B pocket for their interaction but does not affect the binding of Rb to c-Abl, which forms a complex with the C-terminal region (30, 65). It is possible that the deletion of aa 712 to 767 from Rb not only eliminates the binding ability of the A/B pocket but also changes the structure of the protein sufficiently to prevent the C-terminal region from engaging in protein-protein interactions. In this regard, it would be interesting to determine whether this mutant still can bind to c-Abl. However, it does appear that the A/B pocket is not essential for binding to the C-pocket domain since both c-Abl and IE2 86 can bind efficiently to the Rb deletion mutant which has retained only aa 768 to 928.

A key question raised by the results presented here is, what functional role is played by these multiple protein-binding domains on both Rb and IE2 86 with respect to the viral life cycle? The adenovirus E1A protein, SV40 T antigen, and the papillomavirus E7 protein bind to the A/B pocket of the unphosphorylated form of Rb, leading to the disruption of the E2F-Rb complex (for a review, see reference 12). IE2 86 also binds more efficiently to unphosphorylated Rb (19). Thus, analogous to these other DNA virus proteins, the interaction of HCMV IE2 86 with Rb may be necessary for the accumulation of free E2F and the subsequent activation of E2F-regulated promoters, including those directing expression of cell cycle-related factors required for the viral infection. As mentioned above, this hypothesis is supported by the experiments showing that IE2 86 can relieve Rb-mediated repression of a promoter with E2F-binding sites in transient expression assays (19). The observation that many transcription factors that bind to Rb also bind TBP has led to the proposal that Rb may block transcription by inhibiting communication between transcription factors and TBP (21). The IE2 86-Rb interactions possibly play a role in the release of factors that are required for promoter activation. Thus, the ability of IE2 86 to bind independently to both the A/B pocket and the C-terminal domain of Rb may allow a greater number of cellular regulatory factors to dissociate from Rb and become functionally active. Alternatively, the IE2 86-Rb interaction may interfere with the

ability of Rb to actively mediate the formation of specific complexes that regulate transcription either negatively or positively. These interactions may also have the effect of compromising some of the functions of IE2 86. Relevant to this observation, in transient expression assays, cotransfection of the expression vectors for IE2 86 and Rb relieves the IE2 86-induced repression of the MIE promoter and suppresses the activation of some heterologous promoters induced by IE2 86 (11, 19). The ability of IE2 86 to associate with either region of Rb may also facilitate potential interactions of the HCMV protein with other Rb-binding proteins; i.e. when the A/B pocket is occupied, IE2 86 can bind to the C-terminal domain, or vice versa. Likewise, by providing three independent sites for the binding of Rb and other cellular factors, IE2 86 may serve as the bridge which brings these proteins into close contact.

In our experiments, we also found that IE1 72 was able to interfere with some of the growth suppression functions of Rb. This result was somewhat surprising in view of studies from our lab and others that showed that IE1 72 does not physically form a complex with Rb (18, 19, 55). It is possible that IE1 72 can inhibit the flat cell phenotype because of its ability to interact with E2F-1 and p107 (39, 44). However, this interaction is unlikely to be the means by which the small protein consisting of only the N-terminal 85 aa common to IE1 72 and IE2 86 interferes with Rb function, since IE2 86 does not form a complex with either E2F-1 or p107 (39, 44).

Any hypothesis regarding the underlying mechanisms by which the HCMV IE proteins interfere with Rb growth suppression must take into account the results of several other studies which have examined the relationship between this suppression and the formation of Rb-E2F complexes. Of particular interest are the experiments by Qin et al. (47) which demonstrated that coexpression of E2F-1 with Rb in Saos-2 cells was able to override the flat cell phenotype and prevent the G₁ arrest induced by Rb and, more importantly, that these two functions could be separated. The ability of E2F-1 to counter the flat cell phenotype required a functional DNA-binding domain but did not require that E2F-1 bind to Rb or activate an E2F-responsive promoter. In contrast, for E2F-1 to release the Rb-induced G₁/S block, it had to retain its functional DNA-binding and transactivation domains, although the latter could be replaced by a heterologous activation domain consisting of the acidic carboxy-terminal region from the herpes simplex virus VP16 protein. However, it is important to recognize that the simple inhibition of the E2F-responsive promoter is not sufficient to induce a G₁/S block, as evidenced by the findings that in Saos-2 cells, an E2F fusion protein consisting of the DNA-binding domain of E2F fused to the Rb A/B pocket (E2F1[1-368]/Rb[379-792]) both represses transcription and blocks G₁/S, while the fusion of E2F to the Kox1 transcriptional repression domain inhibits RNA synthesis directed by the E2F-responsive promoter but fails to induce a G₁/S block (52).

The results of the foregoing experiments as well as a number of studies by Wang and coworkers (63, 65-67) provide strong support for the hypothesis that Rb is a "matchmaker." In the model proposed by Wang et al. (63), the major premise is that Rb functions by recruiting factors that may not come in contact with each other under normal circumstances. If we consider that Rb actively coordinates the formation of multiple complexes with different combinations of proteins, then the HCMV IE proteins could affect Rb function by interacting with Rb or with one of the proteins involved either directly or indirectly with the activity of a specific Rb complex. Thus, we would propose that the HCMV IE proteins could interfere

FIG. 6. All mutant proteins are expressed in the nucleus and do not affect Rb expression or localization. Transfections were performed as described for Fig. 2. Cotransfection of Rb with 2203 (a to c), 370-end (d to f), dMN (g to i), and 1-85 (j to l) are pictured. For constructs 2203 and 370-end, IE2 86 was detected with 1218 (anti-C terminus) and Rb was detected with PMG-345; for constructs dMN and 1-85, IE2 86 was detected with CH16.0 (anti-N terminus) and Rb was detected with N9. Magnification, $\times 460$.

with complexes involved in controlling the development of the flat cell phenotype but not those governing the G₁/S block.

In accord with the above-described model, we hypothesize that the 85-aa N-terminal domain common to IE1 72 and IE2 86 does interact with a key protein, yet to be discovered, that is important for the activity of specific Rb complexes in controlling cell growth. One intriguing possibility is that this N-terminal region of the IE proteins interferes with the interaction between Rb or its family members with hBRG1 (human brahma-related gene 1 protein) or hBRM. The proteins hBRG1 and hBRM are found in a complex (designated SWI/SNF) which can remodel the chromatin by disrupting nucleosomes and facilitating the binding of specific factors to the DNA (32). These proteins also appear to be able to induce growth arrest and a flat cell phenotype through their interaction with members of the Rb family in certain cells (15, 58). Thus, it is possible that through this interaction, Rb and its family members are able to exercise additional controls over transcription. Interestingly, the N-terminal region common to IE1 72 and IE2 86 appears to contain a powerful transcriptional activation domain, as evidenced by the fact that a GAL4 fusion protein containing aa 25 to 85 from this region functions as a very strong activator of a promoter with five tandemly repeated GAL4-binding sites (43). Moreover, deletion of this region from IE2 86 completely eliminates its ability to activate transcription (51, 55).

In this report, we have focused on the protein-protein interactions involving IE2 86 and their potential role in defining the multifunctional nature of this viral regulatory protein. However, it should be pointed out that protein-DNA interactions are also likely to be important for some of the functions of IE2 86, and the two mechanisms are not necessarily mutually exclusive. The ability to bind to DNA and to engage in protein-protein interactions may allow IE2 86 to serve as the active recruiter for the formation of multiprotein-DNA complexes on HCMV early promoters to ensure that the proteins needed for high-level HCMV transcription are present. Such complexes on cellular promoters might also functionally interfere with either the activation or the repression of genes involved in growth regulation. The clinical consequences of such effects may include the induction of developmental abnormalities in the fetus and coronary artery occlusion in the susceptible adult.

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