Interactions between Human Cytomegalovirus IE1-72 and Cellular p107: Functional Domains and Mechanisms of Up-Regulation of Cyclin E/cdk2 Kinase Activity

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Previous work has demonstrated that the human cytomegalovirus IE1-72 protein is able to bind to the N terminus of p107, and IE1-72 alone is sufficient for alleviation of p107-mediated cell growth suppression. However, the mechanism of this alleviation is unclear. Here, we show that IE1-72 can alleviate p107 inhibition of cyclin E/cdk2 kinase activity. We cotransfected various IE1-72 and p107 constructs into C33A cells and demonstrated that IE1-72 could activate the kinase activity of cyclin E/cdk2. Conversely, IE2-86 did not activate this activity, suggesting that the interaction between p107 and IE1-72 and the subsequent kinase activation are specific. By the use of a series of deletion and point mutants of IE1-72 and p107, we observed that a mutation of the loop region of helix-loop-helix-turn-helix in exon 3 of IE1-72 as well as a mutation of the leucine zipper-2 region in exon 4 of IE1-72 abolished binding to p107. In addition, these two IE1-72 mutants did not alleviate p107 inhibition of cyclin E/cdk2 kinase activity and also failed to alleviate p107 inhibition of the E2F-responsive promoter. Meanwhile, deletion of the N-terminal aa 1 to 175 of p107 abolished both p107 binding with IE1-72 and p107 inhibition of cyclin E/cdk2 kinase activity. This result confirms that the N-terminus aa 1 to 175 region of p107 is a common region where both IE1-72 protein and cyclin E/cdk2 bind. We propose a mechanism in which binding of IE1-72 to p107 displaces cyclin E/cdk2 from p107. Once released from p107, cyclin E/cdk2 is able to function as an active kinase.

Human cytomegalovirus (HCMV), a member of the beta-herpesvirus family, rarely causes symptomatic disease in healthy, immunocompetent individuals (19). However, it may lead to severe illness in immunocompromised and immunosuppressed individuals. HCMV induces the synthesis of the cellular proteins associated with DNA replication (2–4, 15, 37). It has recently been demonstrated that infection of rat smooth muscle cells with HCMV increases smooth muscle cell proliferation and migration (45). These observations strongly support the notion that HCMV infection can alter cellular metabolism to generate an environment conducive to proliferation. We are interested in determining if HCMV disrupts cell cycle regulation through interactions of viral early-immediate gene products with cell cycle-regulating proteins.

The HCMV immediate-early (IE) proteins, IE1-72 and IE2-86, are nuclear proteins that play important roles in the regulation of both viral and cellular gene expression. IE2-86 can transactivate a number of viral and cellular promoters (dihydrofolate reductase and thymidine kinase genes, etc.) which are associated with cell proliferation and DNA replication (2, 11, 14, 16, 20, 23, 26, 28). Expression of IE2-86 induces the progression of quiescent cells into S phase and delays cell cycle exit. Analogous to IE2-86, expression of IE1-72 protein can induce several S-phase-associated genes, including dihydrofo-

late reductase and DNA polymerase α (15, 27, 37). In the absence of p53, IE1-72 expression can also induce S phase and delay cell cycle exit (7).

The cyclins are key cell cycle regulatory factors that are controlled by a number of mechanisms, including their gene expression, phosphorylation, subcellular localization, and degradation, as well as activation of cyclin-dependent kinases (cdk's) (1, 32, 34). Cyclin E/cdk2 kinase activity can be activated by the immediate-early genes, IE1 and IE2, in HCMV-infected cells (4, 21). Transactivation of the cyclin E promoter by IE2 has been reported (4), but the mechanism for IE1 activation of cyclin E/cdk2 kinase activity in HCMV-infected cells is still unclear.

Another class of proteins involved in regulating the cell cycle includes the retinoblastoma tumor suppressor protein (pRb) and its related proteins, p107 and 130. These pRb-related proteins serve as cell growth suppressors and share a homologous sequence called the pocket domain, which can bind to E2F, a protein known to promote proliferation (9, 36). pRb is thought to bind to and inhibit the activity of E2F, preventing cell entry into S phase (18, 39). p107 contains two independent domains implicated in cell growth suppression. The p107 pocket domain is located at the C terminus and, like the pocket domain of pRb, can bind to and inhibit the activity of E2F₄, causing cell growth arrest in the G₁ phase of the cell cycle. The other growth-suppression domain is located at the N terminus of p107 and interacts with cyclin/cdk kinases. p107 forms complexes with cyclin A/cdk2 or cyclin E/cdk2 to repress the activity of these kinases (46, 47). The interaction between p107 and cyclins and cdk's that results in inhibition of kinase activity has

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been demonstrated to be a way to regulate the cell cycle (6, 36, 40, 41, 46, 47). The formation of complexes between p107 and cyclin/cdk kinases appears in a temporally defined manner. The p107-cyclin E/cdk2 complex is seen in late G₁ phase, and the p107-cyclin A/cdk2 complex is seen later in S phase (24, 48).

Both IE1-72 and IE2-86 are able to bind to pRb family proteins. IE2-86 binds to pRb (8, 13), while IE1-72 binds to p107 (22, 30). The interaction between IE1-72 and p107 is dependent on the N terminus of p107 and exons 2, 3, and 4 of IE1-72 (22). Because the N terminus of p107 is necessary for binding to IE1-72 as well as to cyclin E/cdk2, we hypothesized that IE1-72 could disrupt the complex between p107 and cyclin E/cdk2. Thus, when HCMV infects cells and IE1-72 is expressed, the subsequent interaction of IE1-72 with p107 frees cyclin E/cdk2. This allows cyclin E/cdk2 to become an active kinase. To assess this hypothesis and to further define the interacting domains binding between p107 and IE1-72, we employed a series of deletion and point mutants of IE1-72 and p107. In this communication, we demonstrate that the interaction of IE1-72 with p107 does indeed activate cyclin E/cdk2 kinase activity. The helix-loop-helix-turn-helix (HLH) domain in exon 3 and leucine zipper-2 (LZ-2) in exon 4 of IE1-72 are necessary for its binding to p107. In addition, amino acids (aa) 1 to 175 of the N-terminal region of p107 are essential for its binding to IE1-72.

MATERIALS AND METHODS

Cell culture and infection. Human tumor cell line C33A and 293 cells were obtained from the American Type Culture Collection (Rockville, Md.) and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (GIBCO BRL, Rockville, Md.) and penicillin-streptomycin (100 U and 100 µg/ml, respectively) at 37°C in a 6% CO₂ incubator (30). The transfections or cotransfections for immunoprecipitation (IP) and Western analysis were performed as described in the Effectene transfection reagent handbook (Qiagen, Valencia, Calif.).

Preparation of cell lysates for Western analysis, IP, and kinase assays. Procedures for preparation of whole-cell lysates for Western blotting and IP were performed as described previously (30). Cells were harvested at the indicated times posttransfection. Cells were pelleted by low-speed centrifugation and washed twice with phosphate-buffered saline (PBS). Cells were then incubated in an EBC buffer (0.05 M Tris-HCl [pH 8.0], 0.12 M NaCl, 0.5% Nonidet P-40, 0.1 M NaF, 0.2 mM Na-orthovanadate, 1 mM phenylmethylsulfonyl fluoride [PMSF], 10 µg of leupeptin/ml, and 1 µg of aprotinin/ml) at 4°C for 1 h, or in 0.2% Tween 20 lysis buffer (50 mM HEPES [pH 7.3], 150 mM NaCl, 2.5 mM EGTA, 1 mM EDTA, 0.2% Tween 20, 1 mM dithiothreitol, 1 mM PMSF, 1 µg of aprotinin/ml, 1 mM sodium orthovanadate, 10 mM β-glycerophosphate, and 1 mM NaF) at 4°C for 4 h. Microcentrifugation at 13,600 × g for 15 min at 4°C was used for clarification. Supernatant was removed, and protein concentrations were determined with the Bio-Rad (Richmond, Calif.) protein assay kit.

To prepare whole-cell extracts for the kinase assays, cells were rinsed twice with PBS prior to harvest. Cell pellets were washed with PBS once and resuspended in 0.2% Tween 20 lysis buffer and lysed for 4 h on ice. Cellular debris was removed by centrifugation for 15 min at 13,600 × g at 4°C. The supernatant was recovered and stored at -70°C until use (10).

Transfection and CAT assays. C33A cells were transfected with various combinations of plasmids as indicated using the Effectene kit, following the manufacturer's protocol (Qiagen). Cells were grown in six-well plates and transfected when a density of 50% confluence was reached. A total of 0.8 µg of DNA was used in each transfection. The mixture of DNA and Effectene reagent was suspended in 600 µl of DMEM and added to the cells in 1 ml of DMEM-free serum. The cells were incubated at 37°C and 6% CO₂ for 24 h, and then 1 ml of DMEM with 12% fetal bovine serum was added (30). After 24 h, cells were harvested and chloramphenicol acetyltransferase (CAT) assays were performed, as described previously (12).

Immunocomplex kinase assays. Immunocomplex kinase assays were performed as described previously (10). Cyclin E kinase complexes were immunoprecipitated from 200 µg of total cell protein with anti-cyclin E mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, Calif.) for 4 h at 4°C and collected on 15 µl of protein A-Sepharose CL-4B beads (Amersham, Piscataway, N.J.) overnight at 4°C. Immunoprecipitates were washed twice with lysis buffer (0.2% Tween 20 lysis buffer) and twice with kinase buffer (50 mM HEPES [pH 7.3], 10 mM MgCl₂). They were then resuspended in 15 µl of kinase buffer (containing 1 mM dithiothreitol, 1 mM sodium orthovanadate, 2.5 mM EGTA, 20 µM ATP, 10 mM β-glycerophosphate, and 1 mM NaF) with 1.5 µg of histone H1 substrate (Upstate, Lake Placid, N.Y.) and 5 µCi of [γ-³²P]ATP (ICN, Irvine, Calif.). The kinase reaction was allowed to proceed for 30 min at 30°C and was stopped by the addition of 15 µl of 2× sodium dodecyl sulfate (SDS) sample buffer. Samples were boiled for 5 min and then separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were vacuum dried, and phosphorylated substrate was visualized by autoradiography.

IP. Cell extracts of 500 µg of protein were first precleared with protein A- or protein G-Sepharose CL-4B beads (Amersham) for 1 h at 4°C. Beads were spun out, and then the precleared cell extract was incubated with appropriate antibody for 4 h. The protein complexes were precipitated by incubation with protein A- or G-Sepharose CL-4B beads overnight at 4°C. Precipitated complexes were washed three times with 0.2% Tween 20 lysis buffer and then resuspended in 2× sample buffer and boiled for 3 min. Proteins were separated by SDS-PAGE and analyzed by Western blotting.

In vitro protein interaction. All transcription-translation of proteins was done with the Promega TnT-coupled reticulocyte lysate system (Promega, Madison, Wis.) for [³⁵S]methionine labeling. The manufacturer's protocol was followed. Proteins were translated individually and then mixed in the indicated combinations for 1 h at 30°C. IPs were performed with 20 µl of translated protein, 0.5 µg of antibody, and 25 µl of protein G beads, in 0.3 ml of ELB⁺ buffer. The reactions were allowed to proceed overnight, and the beads were washed five times with ELB⁺ before boiling and separation by SDS-PAGE. The gels were dried and exposed to Kodak X-ray film.

Western blotting (immunoblotting) analysis. Equal protein amounts of whole-cell extracts (35 to 100 µg, depending on the specific antigen to be tested) were separated by SDS-PAGE gels and then transferred for 3 to 4 h to Immobilon-P membranes (Millipore, Billerica, Mass.). Blots were blocked in 5% (wt/vol) nonfat dry milk dissolved in PBS-0.1% Tween 20. Blots were probed with primary antibody in PBS-0.1% Tween 20 (1:1,000 dilution for anti-p107 [Santa Cruz Biotechnology], 1:20,000 for anti-IE1-72 [our lab], 1:20,000 for anti-IE2-86 [our lab], and 1:1,000 for anti-cyclin E and anti-cdk2 antibody [Santa Cruz Biotechnology]). After extensive washing, blots were probed with an appropriate peroxidase-labeled secondary antibody. Blots were again washed extensively and developed by enhanced chemiluminescence (ECL; Amersham).

Plasmid constructs. The IE1-72 and IE2-86 expression vectors (pcDNA3IE1-72 and pcDNA3IE2-86) have been previously described (43). The adenovirus E2CAT and the E2CAT mutant were gifts from Steve Bachenheimer and were described previously (25). The p107 expression vector (pCMV-neop107) and the p107 vector used for transcription-translation (pBSKII p107) were obtained from Liang Zhu (46-48). pGEX2Tp107 used to produce glutathione S-transferase (GST)-p107 was generated from pBSKp107 by inserting a BamHI fragment of p107 into pGEX2T at the BamHI site. The direction of the insertion was identified by DNA sequencing.

Construction of IE1-72 mutants. The cDNA fragment encoding IE1-72 in pGEX2TIE1-72 was mutated by using the GeneEditor in vitro site-directed mutagenesis system (Promega) and subcloned into pcDNA3 plasmid at the BamHI site. The site-directed mutants of IE1-72 were created using primers with the following sequences: M10 (179-184), 5'pGCATGATGTGAGCGAGGCCG CCGTACCGAGTTGGGGGTGC; M11 (192-197), 5'pGCACTGCAGGCT ACGGCCCGTGCTACAACGGATGAACCTTAGG; M12 (10-16), 5'pCCAAG AGAAGAGTGGCCCTGCTAATCTGCCGCGGGCCCTTCTCC; M13-1 (45-52), 5'pGTTGAGGAAGGAGGTTACCACTCCGCTGAGTCTGGCAGC CCCGCTGTTTCAGAG; M13-2 (71-77), 5'pCTTTTGAAACAAGTACGCCG GCTTCCACCGCGAAACCCGAGAAAGATGTGC; M13-3 (57-64), 5'pGAC CCGCTGTTTCCAGCGTTGGCCGAGCATCCCTCACAACCTTTGAACA AGTG; M14 (106-114), 5'pCAAGGACACATGCTGACAAACATCTACCCA GACGGCAGCGCAATTCAGTGGCGCTTAAATATG; and M15 (312-318), 5'pGAGTCTGCTCGGGTGCAGTCTCTCTGTCTTTGCGGAGACTAGT GTGATGC. The cDNAs and the orientation of IE1-72 or its mutants in vectors were verified by DNA sequencing. DNA and amino acid sequences and the schematic location of the point mutants of IE1-72 are shown in Table 1 and below in Fig. 4A, respectively.

TABLE 1. Mutated sequences of the IE1-72 protein

Mutant	DNA sequence change	Amino acid sequence change
M10 (179-184) (Mut ATP binding)	From AAGGGCGCCGCTAACAAAG To GAGGCCGCGCTACCGAG	KGAANK EAAATA
M11 (192-197) (Mut NL-2)	From AAGGCCCGTGCTAAAAAG To ACGGCCCGTGCTACAACG	KARAKK TARATT
M12 (10-16) (Mut exon 2)	From GACCCTGATAATCCTGACGAG To GCCCCTGCTAATCCTGCCGCG	DPDNPDE APANPAA
M13-1 (45-52) (Mut exon 3 loop)	From AACAGTCAGCTGAGTCTGGGAGAC To ACCAGTCCGCTGAGTCTGGCAGCC	NSQLSLGD TSPLSLAA
M13-2 (71-77) (Mut exon 3 turn)	From GAGGATTGCAACGAGAACCC To GCGGCTTCCACCGCAAAACC	EDCNENP AATAKAP
M13-3 (57-64) (Mut exon 3 helix)	From GAGTTGGCCGAAGAATCCCTCAA To GCGTTGGCCGAGCATCCCTCACA	ELAEESLK ALAAASLT
M14 (106-114) (Mut NL-1)	From AAAAAATATACCCAGACGGAAGAGAAA To ACAACATCTACCCAGACGCGCAAA	KKYTQTEEK TTSTQTAAQ
M15 (312-318) (Mut LZ-2)	From CTGTGCTGCTATGTCTTAGAG To CAGTCTCCTCTGTCTTTGCG	LCCYVLE QSSSVFA

Construction of p107 deletions and mutants. The GeneEditor site-directed mutagenesis kit (Promega) was used to create mutants of p107. The pCMVneop107 plasmid was used as a template DNA. The DNA sequences of primers used for generating the p107 point mutants were as follows: p107M151 (151-156), 5'pGAT ATATTTCAAATCCATCTAAAAAACCACAAACGTTACCACGAAGCCG; p107M201 (201-208), 5'pCTCTTATCATTTTTTTCTAAGCAGCTTTGCTCTGA TTTTGCC; p107M291 (291-297), 5'pGAGAATGCCTCCTGGCCCATCCAA-GATATACTGCTAATAGCAAAGCAG; and p107M323 (323-329), 5'pGAGGA TCITTTTGAAGGAGCCGAGCAGGGGCAATTGGAACACCTCG. These constructed mutants were then used to create the constructs pcDNA3p107M151-flag, pcDNA3p107M201-flag, pcDNA3p107M291-flag, and pcDNA3p107M323-flag by ligating PCR products of C-terminal flag-tagged p107 mutant fragments flanked with *Bam*HI and *Xba*I sites into *Bam*HI and *Xba*I cloning sites of the pcDNA3 plasmid. The following common primers were used: 5' primer, CATCGGATCCA TGTTGAGGACAAGCCCCAC; 3' primer, GCTCTAGATTACTTGTCTGTCG TCATCCTTGTAGTCATGATTTGCTCTTTCACTG. pcDNA3p107-flag was created by a similar strategy.

Deletions of p107N30-flag and p107N175-flag, in which the first 30 or 175 aa of p107 were deleted and the flag sequence was tagged at the C terminus of the deletion, were produced by a similar strategy to that mentioned above, but the template DNA was pCMVneop107 and the 5' primers were p107N30-flag (5'C ACTGGATCCATGGACGAGGGGAGCGCG) and p107N175-flag (5'CGGC GGATCCATGTTCTGTTGGACACTTTTG). The common 3' primer mentioned above was used. DNA and amino acid sequences and the schematic of the p107 deletion and point mutants are shown in Table 2 and also below in Fig. 8A.

RESULTS

Cyclin E/cdk2 kinase activity is inhibited by p107 overexpression but is activated by IE1-72 overexpression. To determine whether IE1-72 activates cyclin E/cdk2 kinase activity,

lysates of the C33A cells transfected with p107 or IE1-72 or IE2-86 expression vector were immunoprecipitated with anti-cyclin E antibody, anti-p107 antibody, anti-IE1 antibody, or anti-IE2 antibody. The various IP complexes were tested in kinase assays for the ability to phosphorylate histone H1. Kinase activity in complexes immunoprecipitated with anti-cyclin E antibody was inhibited by p107 overexpression (Fig. 1B, lane 2), but it was increased by IE1-72 overexpression (Fig. 1B, lane 4). Complexes immunoprecipitated from cell lysate by anti-p107, anti-IE1-72, or anti-IE2-86 antibodies (Fig. 1B, lanes 3, 5, and 6) had little or no kinase activity, suggesting that either cyclinE/cdk2 did not complex with these proteins or that cyclin E/cdk2 in complexes with these proteins lacked kinase activity.

Coexpression of p107 and IE1-72 resulted in cyclin E/cdk2 kinase activity, and kinase activity increased with increased expression of IE1-72. Cotransfection of p107 and IE1-72 expression constructs into the C33A cell line was performed, and lysates of these cells were prepared. Cyclin E/cdk2 complexes were immunoprecipitated by anti-cyclin E antibody from these lysates, and the kinase activities of the IP complexes were examined (Fig. 2). p107 overexpression alone inhibited histone H1 phosphorylation catalyzed by cyclin E/cdk2 (Fig. 2, lane 2). However, IE1-72 coexpression resulted in restoration of cyclin E/cdk2 kinase activity (Fig. 2, lanes 3 to 5), and this kinase activity increased with increased IE1-72 protein expression (Fig. 2, lanes 3 to 5). It was reported previously that the cyclin

TABLE 2. Sequences of p107 mutants

Mutant name	DNA sequence change	Amino acid sequence change
p107N30 (del.1-30)	Bases 1–90 deleted	aa residues 1–30 deleted
p107N175 (del. 1-175)	Bases 1–525 deleted	aa residues 1–175 deleted
p107M151 (151-156)	From TATGAAGAACCACCAAG To TCTAAAAAACCACAAACG	YEEPPK SKKPQT
p107M201 (201-208)	From TTACTTCTATGCTGCTTGGAT To TTTTCTCTAAGCAGCTTTGCT	LLLCCLDL FFLSSFAL
p107M291 (291-297)	From GACCTTTCAAGTTTACTGAT To GCCCATCCAAGATATACTGCT	DLSSFTD AHPNYTA
p107M323 (323-329)	From GGAGCAGACGAGAGAGGAA To GAAGGAGCCGAGCAGGGGCA	GADAEED EKAAAKA

FIG. 1. Overexpressed p107 inhibited cyclin E/cdk2 kinase activity, while overexpressed IE1-72 activated cyclin E/cdk2 kinase. (A) Western blot analyses demonstrating expression levels of p107, IE1-72, and IE2-86 in C33A cells transfected with the indicated constructs. (B) Kinase assays of complexes immunoprecipitated with anti-cyclin E antibody from mock-transfected C33A cells (lane 1), cells overexpressing p107 (lane 2), and cells overexpressing IE1-72 (lane 4). Lanes 3, 5, and 6 show kinase assays of complexes immunoprecipitated with anti-p107, anti-IE1-72, or anti-IE2-86 antibodies, respectively. His.H1, histone H1.

E promoter was activated by HCMV IE2-86 expression (4). Therefore, the kinase activity from pcDNA3IE2-86- and pCMVp107-transfected C33A cell lysate was used as a positive control in this experiment. In lane 7 of Fig. 2, an increase in cyclin E/cdk2 kinase activity induced by IE2-86 was observed. This kinase activity was mainly due to IE2-86 up-regulation of the cyclin E promoter and not from the release of cyclin E/cdk2 from the p107-cyclin E/cdk2 inactive complex. This result suggests that either the interaction of p107 with IE1-72 or an

up-regulation of cyclin E/cdk2 by IE1-72 alone may contribute to the increase of cyclin E/cdk2 kinase activity. However, we found IE1-72 and p107 expression alone or together in C33A cells did not significantly increase endogenous cyclin E and cdk2 expression, as shown below in lanes 2, 3, and 12 of Fig. 5A.

IP complexes containing p107 showed kinase activity when mixed with IP complexes containing IE1-72, and kinase activity increased with increasing levels of IE1-72. We hypothesized that IE1-72 binding to p107 displaces cyclin E/cdk2 from the p107-cyclin E/cdk2 complex, thereby activating the cyclin E/cdk2 kinase activity. To test this hypothesis, p107, IE1-72, and IE2-86 expression constructs were transfected into C33A cells. Protein complexes were immunoprecipitated from cell lysates using either anti-p107 antibody or anti-cyclin E antibody. These IP complexes were then mixed with IP complexes immunoprecipitated with either anti-IE1-72 antibody or anti-IE2-86 antibody. Finally, the kinase activity of the mixed IP complexes was analyzed for histone H1 phosphorylation. Anti-p107 antibody IP complexes immunoprecipitated from cells transfected with pcDNA3 p107 mixed with anti-IE1-72 IP complexes immunoprecipitated from cells transfected with pcDNA3 (expression vector alone) had minimal kinase activity (Fig. 3C, lane 1). However, anti-p107 antibody IP complexes from the cells transfected with 2 μg of pcDNA3p107 mixed with anti-IE1-72 antibody IP complexes from cells transfected with 2 μg of pcDNA3IE1-72 showed kinase activity (Fig. 3C, lane 4). The level of kinase activity in the mixed IP complexes increased with the level of IE1-72 expression (Fig. 3C, lanes 2 to 4). Anti-p107 antibody IP complexes from the cells transfected with 2 μg of pcDNA3 p107 mixed with anti-IE2-86 antibody IP complexes from cells transfected with pcDNA3IE2-86 showed no kinase activity (Fig. 3C, lane 5).

The HLHTH and LZ-2 regions of IE1-72 are required for interaction with p107 in vitro. In order to define the domains of the IE1-72 protein that interact with p107, eight point mutants of the IE1-72 gene were constructed (Table 1 and Fig. 4A) and expressed in an in vitro transcription-translation system containing [³⁵S]methionine. The [³⁵S]methionine-labeled gene products were analyzed for construct expression by SDS-PAGE and autoradiography (Fig. 4B). [³⁵S]methionine-labeled full-length IE1-72 or one of the eight mutant products was mixed with [³⁵S]methionine-labeled p107, and IE1-72 protein complexes were immunoprecipitated from these mixtures with anti-IE1-72 antibody. The IP complexes were analyzed for the presence of p107 with SDS-PAGE and autoradiography. [³⁵S]methionine-labeled p107 coimmunoprecipitated with full-length IE1-72 and IE1 mutants M10, M11, M12, M13-2, M13-3, and M14 (Fig. 4C, lanes 4–6, 7, and 9–11), but not with M13-1 or M15 (Fig. 4C, lanes 8 and 12). We noticed that in the coimmunoprecipitation of the in vitro-translated M13-1 mutant protein and of p107 using anti-IE1-72 antibody, the M13-1 protein level was less than that of the other mutants (Fig. 4C, lane 8). We do not understand the reason for this at this moment, but it is reproducible and the results are quite consistent. Although the M13-1 protein level is relatively low, no trace amount of coprecipitated p107 was detected. The IE1-72 antibody used for IP was a monoclonal antibody. It may have less affinity to the M13-1 mutant protein than to the wild type and other mutant proteins, but it is still very immunoreactive

FIG. 2. p107 overexpression inhibited histone H1 phosphorylation catalyzed by cyclin E/cdk2, and IE1-72 expression reversed this inhibitory effect. (A) Western blot analyses demonstrating expression of p107, IE1-72, and IE2-86 in C33A cells transfected with the indicated constructs. (B) Kinase assays of complexes immunoprecipitated with anti-cyclin E antibody from lysates of mock-transfected cells (lane 1), cells transfected with p107 plasmid alone (lane 2), cells cotransfected with p107 plasmid and increasing amounts of IE1-72 plasmid (lanes 3 to 5), or cells cotransfected with p107 plasmid and IE2-86 plasmid (lane 7).

FIG. 3. IP complexes containing p107 showed kinase activity when mixed with IP complexes containing IE1-72, and kinase activity increased with increasing levels of IE1-72. (A and B) Western blot analyses demonstrating p107, IE1-72, and IE2-86 expression in C33A cells transfected with the indicated expression vectors. (C) Kinase assays of mixtures of various IP complexes. Lanes 1 to 4, complexes immunoprecipitated with anti-p107 antibody from cells overexpressing p107 (see panel A, lanes 1 to 4) mixed with complexes immunoprecipitated with anti-IE1-72 antibody from mock-transfected cells (see panel B, lane 1) or cells transfected with increasing amounts of IE1-72 plasmids (see panel B, lanes 2 to 4). Lane 5, complexes immunoprecipitated with anti-p107 antibody from cells overexpressing p107 (see panel A, lane 5) mixed with complexes immunoprecipitated with anti-IE1-86 antibody from cells transfected with IE2-86 plasmid (see panel B, lane 5). Lane 6, complexes immunoprecipitated with anti-cyclin E antibody from cells overexpressing IE1-72 (see panel A, lane 6) mixed with complexes immunoprecipitated with anti-IE1-72 antibody from mock-transfected cells (see panel B, lane 6).

against the M13-1 protein (see Fig. 5A and B, 6B, and 7B) as detected by immunoblotting. To confirm that the M13-1 mutant protein does not interact with p107, we performed a GST-p107 pull-down experiment using *in vitro*-translated and [³⁵S]methionine-labeled IE1-72 proteins. The results demonstrated that neither the M13-1 nor the M-15 mutant bound to GST-p107 (Fig. 4D, lanes 6 and 10, respectively). This result agrees with the result shown in Fig. 4C.

Mutation 13-1 is located in the helix-loop-helix region of exon 3 (aa 45 to 52, with a sequence of NSQLSLGD), and mutation 15 is located in the LZ-2 region of exon 4 (aa 312 to 318; LCCYVLE). To examine whether the binding was specific, [³⁵S]methionine-labeled IE2-86 was used as a control. IE2-86 did not bind to p107 (Fig. 4C, lane 13). The anti-IE1-72 antibody reacted to IE1-72 but not to p107 (Fig. 4C, lanes 3 and 2).

HLTH and LZ-2 regions of IE1-72 are required for binding to p107 and induction of cyclin E/cdk2 kinase activity *in vivo*, and mutations in these regions inhibit IE1-72 activation

FIG. 4. The aa 45 to 52 (loop structure of HLTH) and aa 312 to 318 (LZ-2) sequences of IE1-72 are required for its interaction with p107. (A) Schematic diagram of the mutation sites of constructed IE1-72 mutants. (B) Autoradiographs of direct loads of *in vitro*-translated [³⁵S]methionine-labeled p107, IE1-72, IE1-72 mutant proteins, and IE2-86, as indicated. (C) Autoradiographs of [³⁵S]methionine-labeled proteins within complexes immunoprecipitated with various antibodies. Lane 1, [³⁵S]methionine-labeled p107 protein immunoprecipitated with anti-p107 antibody; lane 2, [³⁵S]methionine-labeled p107 protein immunoprecipitated with anti-IE1-72 antibody; lane 3, [³⁵S]methionine-labeled IE1-72 immunoprecipitated with anti-IE1-72 antibody; lanes 4 to 12, [³⁵S]methionine-labeled IE1-72 (wild type and mutants) and p107 within complexes immunoprecipitated with anti-IE1-72 antibody; lanes 13 to 15, [³⁵S]methionine-labeled proteins in complexes immunoprecipitated with anti-IE2-86 antibody (controls). (D) GST-p107 pull-down experiment using *in vitro*-translated and [³⁵S]methionine-labeled IE1-72 wild-type or mutant proteins. Results demonstrated that M13-1 and the M-15 mutant did not bind to GST-p107.

FIG. 5. aa 45 to 52 and 312 to 318 of IE1-72 are required for binding to p107 and for alleviating p107 repression of cyclin E/cdk2 kinase activity. (A) Western blotting showing expression of cyclin E, cdk2, p107, IE1-72, and IE1-72 mutant proteins expressed in C33A cells cotransfected with the indicated plasmid constructs. Note that cyclin E and cdk2 are endogenous proteins (lane 1). (B) Western blots demonstrating anti-IE1-72 immunoreactivity in complexes immunoprecipitated with anti-p107 antibody from cell lysates of the same transfected cells as in panel A. (C) Histone H1 kinase assays using immune complexes immunoprecipitated with anti-cyclin E antibody using cellular lysates as described in the legend for panel A.

of p107-cyclin E/cdk2 complex kinase activity. To determine if mutations in the HLTH and LZ-2 regions of IE1-72 would affect binding to p107 in vivo, the eight mutant IE1-72 constructs were cotransfected with pCMVp107 into C33A cells. Protein expression profiles were confirmed by Western blotting (Fig. 5A). Complexes were immunoprecipitated from cell lysates using anti-p107 antibody and analyzed by Western blotting using anti-IE1-72 antibody to detect coimmunoprecipitated IE1-72 or IE1-72 mutants (Fig. 5B). IE1-72 mutant proteins M13-1 and M15 did not coimmunoprecipitate with p107 (Fig. 5B, lanes 7 and 11, respectively). The kinase activity of the IP complexes immunoprecipitated with anti-cyclin E antibody from these lysates was determined (Fig. 5C). IP complexes from cells expressing IE1-72 mutants M13-1 and M15 showed no cyclin E/cdk2 kinase activity (Fig. 5C, lanes 7 and 11). Kinase activity was seen in IP complexes from cells expressing full-length IE1-72 as well as those cells expressing IE1-72 mutants M10, M11, M12, M13-2, M13-3, and M14 (Fig. 5C, lanes 3, 4, 5, 6, 8, 9, and 10, respectively).

To confirm which domains of IE1-72 are essential for activating the cyclin E/cdk2 kinase activity of the p107-cyclin E/cdk2 complex, p107, full-length IE1-72, and IE1-72 mutant constructs were transfected into C33A cells. In a protocol similar to that described for Fig. 3, anti-p107 antibody IP complexes were immunoprecipitated from cells transfected with pCMVp107 and mixed with anti-IE1-72 antibody IP complexes immunoprecipitated from cells expressing IE1-72 constructs. The IP complex mixtures were screened for kinase activity (Fig. 6C). As expected, IP complexes from cells expressing full-length IE1-72 induced kinase activity (Fig. 6C, lane 2). In addition, IP complexes from cells expressing IE1-72 mutants M10, M11, M12, M13-2, M13-3, and M14 also in-

FIG. 6. aa 45 to 52 and 312 to 318 of IE1-72 are necessary for activating cyclin E/cdk2 kinase activity. (A) Western blotting demonstrating expression of p107 or IE1-72 in lysates of C33A cells transfected with pCMV p107 (lanes 1 to 10) or pcDNA3 IE1-72 (lane 11). These lysates were subsequently immunoprecipitated with anti-p107 antibody or anti-cyclin E antibody (as indicated at the bottom of the panel), and the IP complexes were mixed with IP complexes represented in the corresponding lanes of panel B and assayed for kinase activity, as shown in panel C. (B) Western blot demonstrating IE1-72 or mutants of IE1-72 in lysates of C33A cells transfected by the indicated constructs. These lysates were subsequently immunoprecipitated with anti-IE1-72 antibody, and the IP complexes were mixed with IP complexes represented in the corresponding lanes of panel A and assayed for kinase activity, as shown in panel C. (C) Histone H1 kinase assays of mixed IP complexes (see panels A and B).

duced kinase activity. Conversely, IP complexes from cells expressing mutants M13-1 or M15 did not induce kinase activity (Fig. 6C, lanes 6 and 10). As seen in Fig. 6C, lane 11, IE1-72 expression alone in C33A cells led to very high kinase activity, suggesting a very high level of active cyclin E/cdk2 kinase.

IE1-72 expression alleviates p107-mediated repression of the E2FCAT promoter, and mutations of the HLTH and LZ-2 regions of IE1-72 abolish this alleviation. We have previously reported that IE1-72 alleviates p107-mediated repression of the E2F-responsive promoter (22, 30). To determine the domains of IE1-72 necessary for this function, C33A cells were transfected with E2FCAT, a reporter gene containing an E2F-responsive promoter, as well as with p107 and various IE1-72 constructs. Lysates from these cells were assayed for CAT activity. p107 overexpression inhibited E2FCAT expression (Fig. 7A, lane 6), but E2FCAT expression increased with increased IE1-72 expression (Fig. 7A, lanes 7 and 8).

CAT activity was also seen in cells cotransfected with IE1-72 mutants M10, M11, M12, M13-2, M13-3, and M14 (Fig. 7B, lanes 5, 6, 7, 9, 10, and 11, respectively). However, cells cotransfected with IE1-72 mutants 13-1 and 15 did not alleviate p107-mediated repression of the E2FCAT gene (Fig. 7B, lanes 8 and 12). The fold differences for the CAT assays in Fig. 7 are small (three to sixfold), but they are significant and consistent.

FIG. 7. IE1-72 coexpression with p107 alleviates p107-mediated repression of the E2FCAT promoter, and aa 45 to 52 and 312 to 318 of the IE1-72 protein are essential for alleviating p107-mediated suppression of the E2F-responsive CAT reporter construct. (A) Western blot showing expression of p107 and IE1-72 in C33A cells cotransfected with the indicated expression plasmids (upper panel). E2FCAT that contains an E2F-responsive promoter was subsequently used as a reporter gene to study the effect of IE1-72 on p107-mediated repression of the E2F-responsive gene. The results of CAT expression assays of lysates from cells transfected with various IE1-72 mutants are shown in the lower panel. (B) Western blots showing expression of p107 and IE1-72 in C33A cells cotransfected with the indicated expression plasmids (upper panel). The lower panel shows CAT expression assays of lysates from cells represented in the upper panel.

These results suggest that the HLHTL and LZ-2 regions of the IE1-72 protein are essential for the alleviation of p107-mediated suppression of the E2F-responsive CAT reporter.

Deletion of aa 1 to 175 of p107 abolishes p107 binding to IE1-72 in vivo and abolishes inhibition of cyclin E/cdk2 kinase activity in vitro. To further map the domains at the N terminus of p107 that interact with the IE1-72 protein, we constructed a series of deletion and point mutants of p107 located at the N terminus of p107 (Table 2). The deletion and point mutants of p107 and full-length IE1-72 plasmids were expressed in an in vitro transcription-translation system with [³⁵S]methionine. The [³⁵S]methionine-labeled mutants of p107 were mixed with [³⁵S]methionine-labeled IE1-72. The mixture was immunoprecipitated with anti-IE1-72 antibody. The IP complexes were

FIG. 8. The aa 1 to 175 sequence of the p107 protein is required for interaction with the IE1-72 full-length protein. (A) Schematic diagram showing the mutation sites of various p107 mutants. (B) Autoradiograph of direct loads of in vitro-translated [³⁵S]methionine-labeled IE1-72 and mutants of p107, as indicated. (C) Autoradiographs showing the presence of [³⁵S]methionine-labeled IE1-72 and/or p107 or p107 mutant proteins in IP complexes from mixtures of the proteins, as indicated, immunoprecipitated with anti-IE1-72 monoclonal antibody (lanes 2 to 10) or with anti-p107 antibody (lane 1).

separated by SDS-PAGE for autoradiography. Of the various p107 mutants, only p107N175 did not coimmunoprecipitate with IE1-72 (Fig. 8C, lane 6). This mutant protein lacked aa 1 to 175.

To further confirm this result, either pcDNA3p107-f or pcDNA3p107N175-f (f denotes the presence of a flag tag) was cotransfected with pcDNA3IE1-72 into C33A or 293 cells. Cell lysates were immunoprecipitated with anti-flag antibody, and the IP products were screened by Western blotting using anti-IE1-72 antibody. IE1-72 did not coimmunoprecipitate with the p107N175 deletion mutant (Fig. 9A, lane 3). Functionally, the p107N175 mutant did not inhibit cyclin/cdk kinase activity in C33A cells transfected with the mutant construct (Fig. 9B, lane 3).

IE1-72 displaces cyclin E/cdk2 from the p107-cyclin E/cdk2 complex. It has been demonstrated that p107 forms a complex with cyclin E/cdk2 (35, 36), and the N terminus of p107 is required for this complex formation (35, 36). The physical interaction of this complex formation is thought to be the mechanism behind cyclin E/cdk2 kinase inactivation. We have

FIG. 9. aa 1 to 175 of p107 is required for interaction with IE1-72 in plasmid-transfected 293 cells, and the aa 1 to 175 fragment of p107 is essential for inhibiting cyclin E/cdk2 kinase activity. (A) Western blots showing expression of IE1-72, p107-f, and p107N175-f in 293 cells transfected with the indicated plasmids (upper panel). The bottom panel shows the presence or absence of IE1-72 by Western blot analysis of IP complexes immunoprecipitated with anti-flag antibody from lysates of the cells represented in the upper panel. Note that IE1-72 did not coimmunoprecipitate with the p107N175-f mutant (lane 3). (B) Western blot confirming expression of p107-f or p107N175-f in C33A cells transfected with the indicated plasmids (upper panel). Kinase activity of IP complexes immunoprecipitated from cell lysates with anti-cyclin E antibody is shown in the lower panel.

now demonstrated that this region is also required for binding to IE1-72. Since IE1-72 appears to activate kinase activity, we hypothesized that IE1-72 binding to p107 physically displaces cyclin E/cdk2. To assess this hypothesis, we used wild-type p107 and p107 mutants, each flag tagged at the C-terminal end to determine the displacement domain of p107 that interacts with IE1-72. Unfortunately, for unclear reasons, we had relatively poor expression of p107-f in plasmid-transfected C33A cells. As higher levels of expression were needed, we then utilized the transformed primary human embryonic kidney cell line, 293, to overexpress the various flag-tagged p107 constructs. The expressed p107-f and flag-tagged mutants were then used to perform protein displacement studies.

293 cells were cotransfected with various combinations of plasmids encoding p107-f, p107N175-f, IE1-72, and IE1-86 (Fig. 10A). IP complexes using anti-flag antibody were produced from cell lysates, and these complexes were probed for the presence of cyclin E, cdk2, and IE1-72. Cyclin E and cdk2 coimmunoprecipitated with p107-f in cells expressing p107-f alone (Fig. 10A and B, lane 1) as well as in cells coexpressing p107-f and IE2-86 (Fig. 10A and B, lane 3). Neither cyclin E nor cdk2 was present in complexes from cells coexpressing p107-f and IE1-72 (Fig. 10A and B, lane 2), but IE1-72 was seen in these complexes (Fig. 10C, lane 2). Neither cyclin E, cdk2, nor IE1-72 coimmunoprecipitated with the deletion mutant protein p107N175-f (Fig. 10, lanes 4 and 5).

DISCUSSION

In eukaryotes, cell growth and differentiation are governed through cell cycle progression, which is regulated by cell cycle-related proteins that act either to promote or restrain cell growth. The retinoblastoma family of proteins, which consists of pRb, p107, and p130, are well-studied growth suppression proteins that restrain cell growth by negatively regulating the E2F transcription factor and cyclin/cdk kinase activity (40, 46).

On the other hand, cell cycle progression is positively regulated by cyclin/cdk kinases that drive cell cycle progression by phosphorylating substrates such as the pRb family of proteins (40). Distinct phases of the cell cycle are controlled by specific cdk's that complex with their respective catalytic subunit cyclins. cdk4 and cdk6 complexed with cyclin D1 are responsible for cell cycle progression through G₁ (49), while cyclin E/cdk2 functions in the progression of the cell cycle from late G₁ to early S phase (33).

In general, pRb plays a critical role in G₁-S checkpoint control, whereby unphosphorylated pRb prevents cellular proliferation by binding with E2Fs and inhibiting cell cycle progression (29, 38). Phosphorylation of pRb by cyclin/cdk com-

FIG. 10. p107-f coimmunoprecipitates with cyclin E and cdk2, and IE1-72 disrupts the interaction. (A) Western blot showing cyclin E immunoreactivity in IP complexes immunoprecipitated with anti-flag antibody from lysates of cells transfected with the indicated plasmid. (B) cdk2 immunoreactivity in the IP complexes described in panel A. (C) IE1-72 immunoreactivity in the IP complexes shown in panel A.

plexes results in the release of active E2F species to stimulate the transcription of genes involved in DNA synthesis and S-phase progression (17, 31). Data from our laboratory and from others has demonstrated that infection by HCMV is capable of activating cellular DNA replication machinery and driving quiescent cells to enter an S-phase-like environment (2, 11, 14, 37). HCMV activation of the cellular proliferation machinery is attributed to two major mechanisms: (i) viral ligand and cellular receptor interactions trigger viral signaling to activate important cellular transcription factors, such as Sp1 and NF- κ B (42–44), and (ii) the expression of promiscuous, trans-activating IE1-72 and IE2-86 proteins that are able to disrupt cell cycle regulation through their interaction with pRb family proteins (13, 22, 30).

A common paradigm for the creation of a cellular environment favorable to virus DNA replication, established by the study of the small DNA tumor virus, is that only one of each virally encoded protein (E1A of adenovirus, E7 of human papillomavirus, and large T of simian virus 40) binds to all members of the pocket domain proteins—pRb, p107, and p130—and alleviates transcriptional repression by these proteins. However, data from our laboratory and from others have shown that two HCMV IE gene products, IE1-72 and IE2-86, interact specifically with different pocket proteins. HCMV IE1-72, but not IE2-86, complexes with cellular p107 during HCMV infection, while IE2-86 binds to pRb (13, 22, 30). The binding of IE1-72 to p107 was specific and did not extend to an interaction with the closely related pRb protein. These results suggest that HCMV might have a different mechanism for temporally regulating the pocket domain proteins to create a cellular environment favorable for viral DNA replication.

IE2-86 binding to pRb resulted in the release of E2F from the pRb-E2F complex, but the IE1-72 interaction with p107, although sufficient to functionally activate E2F-mediated transcription, did not release E2F from the p107-E2F complex (22). In addition, the N385 mutant of p107, which lacks the N-terminal aa 1 to 385 fragment that binds to the cyclin/cdk2 complex to inhibit its kinase activity, has previously been shown to suppress growth of SAOS-2 cells in an E2F-dependent manner (22). IE1-72 could not alleviate growth suppression of SAOS-2 cells by overexpression of the N385 mutant of p107. However, overexpression of the L19 mutant of p107, which has a deletion of the region (aa 781 to 1068) that binds to E2F₄ and represses E2F₄ transcription activity, led to IE1-72 alleviation of growth suppression (22). These results suggest that alleviation of p107-mediated growth suppression by IE1-72 may involve an E2F-dependent pathway as well as the cyclin E/cdk2 pathway. These data further support the notion that p107 regulates cell cycle progression by at least two distinct mechanisms: (i) inhibition of E2F transcription factor, and (ii) interaction with cyclin/cdk through a second domain to inactivate cyclin E/cdk kinase activity.

A critical question to be asked is, if the interaction between IE1-72 and the p107-E2F complex did not result in the dissociation of E2F from p107 (22), why did the expression of IE1-72 alleviate the repression of the p107-mediated E2F-responsive promoter? There are two possible mechanisms: (i) IE1-72 interacts with p107-E2F to form a positive preinitiation complex to up-regulate the E2F-responsive promoter, or (ii) IE1-72 interacts with p107-E2F and results in the activation of

FIG. 11. Model of the activation of cyclinE/cdk2 kinase activity by the overexpression of HCMV IE1-72.

cyclin/cdk2 kinase activity which, in turn, phosphorylates the pocket proteins and subsequently activates E2F transcriptional activity. A recent study by Calbo et al. (5) demonstrated that the phosphorylation of pocket proteins by G₁ cyclin/cdk regulated their interaction with E2Fs and the expression of genes controlled by these transcription factors. They found that the activation of cyclin E/cdk2 activity in the absence of D-type cyclin/cdk activity was sufficient to induce E2F₄ activation and promote the expression of E2F-dependent genes. Their findings strongly support the second notion described above.

Data presented in this communication suggest that the IE2-71 interaction with p107 modulates cyclin E/cdk2 activity (Fig. 1 to 3). The critical experimental result shown in Fig. 10 suggests that the binding of IE1-72 to p107 displaces cyclin E/cdk2 from p107. These results support a model in which IE1-72 binds to p107 and releases cyclin E/cdk2 (Fig. 11). This release results in activation of the cyclin E/cdk2 kinase, which is critical in cell cycle progression. IE1-72 appears to compete with cyclin E/cdk2 for p107 binding, as the alleviation of p107 inhibition of cyclin E/cdk2 kinase activity increased with IE1-72 protein expression (Fig. 3). We, therefore, speculate that IE1-72 binds to p107 with higher affinity than cyclin E/cdk2 does. However, further binding studies are required to confirm this suspicion.

In mapping the domains of IE1-72 that interact with p107, we previously demonstrated that exons 2 and 3 of IE1-72 are required for binding to p107. Because exons 2 and 3 are shared between IE1-72 and IE2-86, we hypothesized that the domain of interaction on the IE1-72 and p107 proteins may extend into exon 4, a region unique to IE1-72, to gain a correct protein conformation for this interaction. In this report, we show that a point mutation of the loop structure of the HLH region of exon 3 prevents binding of the mutated IE1-72 to p107. In addition, a point mutation of the LZ-2 region of exon 4 also abolished binding to p107. These results suggest that the loop structure of HLH and LZ-2 of IE1-72 work together to form a specific conformation necessary for the interaction of

IE1-72 with p107. Results of the interaction study, as demonstrated by co-IP and immunoblotting, were further confirmed by biochemical analysis of cyclin/cdk kinase activity using histone H1 as substrate (Fig. 5 and 6). Interestingly, IE2-86, which contains exon 5 instead of exon 4, did not interact with p107.

In this study, we further demonstrated that deletion of aa 1 to 175 of the p107 sequence abolished the p107 interaction with IE1-72 (Fig. 8). In addition, this mutated protein was not able to inhibit cyclin E/cdk2 kinase activity (Fig. 9B, lane 3). Thus, the aa 1 to 175 fragment at the N terminus of p107 is required for binding to both IE1-71 and cyclin E/cdk2. This experimental result is in agreement with the results obtained by Woo et al. that showed aa 1 to 110 of p107 play an important role in regulating cell cycle progression by inhibiting cyclin E/cdk2 kinase activity (40).

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