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Interaction between the Human Cytomegalovirus UL82 Gene Product (pp71) and hDaxx Regulates Immediate-Early Gene Expression and Viral Replication

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The human cytomegalovirus UL82-encoded pp71 protein is required for efficient virus replication and immediate-early gene expression when cells are infected at a low multiplicity. Functions attributed to pp71 include the ability to enhance the infectivity of viral DNA, bind to and target hypophosphorylated Rb family member proteins for degradation, drive quiescent cells into the cell cycle, and bind to the cellular protein hDaxx. Using UL82 mutant viruses, we demonstrate that the LXCXD motif within pp71 is not necessary for efficient virus replication in fibroblasts, suggesting that pp71's ability to degrade hypophosphorylated Rb family members and induce quiescent cells into the cell cycle is not responsible for the growth defect associated with a UL82 deletion mutant. However, UL82 mutants that cannot bind to hDaxx are unable to induce immediate-early gene expression and are severely attenuated for viral replication. These results indicate that the interaction between the human cytomegalovirus UL82 gene product (pp71) and hDaxx regulates immediate-early gene expression and viral replication.

Human cytomegalovirus (HCMV) is a ubiquitous human pathogen. Although HCMV infection is usually asymptomatic in healthy individuals, HCMV infection can result in severe disease in newborns infected in utero and in immunocompromised individuals (40).

Like that of all herpesviruses, HCMV transcription is temporally regulated in a coordinated cascade which consists of immediate-early (IE), early (E), and late (L) gene expression (37, 49, 50). Immediate-early genes are transcribed first and encode critical regulatory proteins that function in part to control expression of viral early and late genes. By definition, IE genes do not require de novo protein synthesis for their transcription (37, 44). However, certain virion tegument proteins which are delivered to the host cell from the infectious virion have been shown to play an important role in controlling efficient IE gene expression (6, 10, 15, 18, 30, 52). Specifically, we and others have demonstrated that the tegument protein pp71 is involved in regulating the expression of a number of IE genes (6, 15, 18, 30).

The UL82 gene encodes the pp71 tegument protein, named for its molecular size upon electrophoresis and the fact that it is phosphorylated during HCMV infection (38, 43, 45). Sequence analysis of pp71 at the nucleotide or amino acid level has not revealed a homolog encoded by other herpesviruses. However, based on pp71's ability to activate immediate-early gene expression (6, 15, 18, 30) and enhance the infectivity of viral DNA (1), pp71 is thought to be the functional homolog of the herpes simplex virus VP16 protein. The function of pp71 has not been completely elucidated. Through the use of a UL82 (pp71) deletion mutant, we have demonstrated that pp71 is required for efficient viral replication when cells are infected at a low multiplicity (6). Using the same mutant, we have also demonstrated that pp71 delivered to the host cell from the virus particle plays an important role in regulating IE gene expression during a productive infection (6). Other functions and interactions attributed to pp71 have recently been described. Using in vitro overexpression assays, Kalejta et al. demonstrated that pp71 is able to interact with and degrade retinoblastoma (Rb) family member proteins, resulting in quiescent cells entering the cell cycle (23, 24, 26). These studies indicated that pp71 degrades hypophosphorylated Rb tumor suppressor family members through an LXCXD motif within pp71, targeting the tumor suppressor protein for proteasome-dependent, ubiquitin-independent degradation (24, 26). They also demonstrated that replacement of the cysteine with a glycine within the LXCXD motif at residue 219 abolished pp71's ability to degrade Rb family members and blocked its ability to induce quiescent cells into the cell cycle (24, 26). Interestingly, pp71's effect on Rb family member degradation and the host cell cycle was not linked to its in vitro transactivation capabilities (25). pp71 has also been shown to interact with the cellular protein hDaxx (15, 22). The significance of pp71's interaction with hDaxx is not fully understood, but it is thought to assist in upregulating viral gene transcription at subnuclear sites (15, 22). During HCMV infection, pp71 colocalizes with hDaxx at specific nuclear domains (ND10 domains) (15, 22, 31) which are sites of active viral gene transcription (11, 19, 21, 32–34). Two hDaxx binding domains were mapped to amino acids 206 to 213 and 324 to 331 of pp71 (15). Transfection studies revealed that removal of either of these binding domains blocked pp71's interaction with hDaxx, prevented pp71 ND10 localization, and abolished pp71's ability to

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transactivate the major immediate-early promoter in transient reporter assays (15).

Despite the identification of cellular pp71 binding partners, the significance of these interactions has not been determined in the context of a productive viral infection where these proteins are expressed at physiological levels and in the presence of the full complement of viral proteins. Therefore, this study utilized HCMV UL82 mutants to identify which pp71 interactions are required to mediate IE gene expression and viral replication necessary to overcome the growth defect associated with the UL82 deletion mutant. Using viral mutants, we demonstrated that the LXCXD motif within pp71, which is required to degrade Rb family members and induce quiescent cells into the cell cycle, is not required to enhance the infectivity of viral DNA, control viral replication, or regulate IE gene expression. However, pp71 mutants that are unable to bind hDaxx are severely attenuated for virus replication. We also demonstrate that the interaction of pp71 and hDaxx plays an important role in controlling IE gene expression and that this interaction is involved in regulating pp71's ability to enhance the infectivity of viral DNA.

MATERIALS AND METHODS

Oligonucleotides. The following oligonucleotides were obtained from Integrated DNA Technologies for the site-directed mutagenesis and cloning procedures described here: C219G-S, GGAGCAGCTGGCCGGTTCGGACCCTAACACG; C219G-AS, CGTGTTAGGGTCCGAACCGGCCAGCTGCTCC; SC48, GCGGAAGCTTTGGTCGCCTGC; SC49, GAATTCGATGGCGCCGGCGCGAAAGG; SC50, GAATTCGAGCAGCTGGCCTGTTCGG; SC51, GCATGCCGTAGTGCGGCGTGCTGCACG; SC52, GAATTCGATGTTTTCCGGGAAAAAGATGG; SC53, GAATTCCCGCTACCCGATCGTGTGCG; ADVCGN-5′, CTCTGGATCCGGTACCATGGCTTCTAGCTACCTTATG; ADVCGN-3′, GCGGCGCCAAACTCACCCTGAAGTTCTC; LF-5′, CAACTAGTCGGCGTGACGGAGCGCGAGTC; LF-3′, GATTAATTAACCTAGGGGGCGGGATGGGGGGAGGGTCAGG; RF-5′, CTCTTAATTAACGGTCCGTGCCCGCGCCACGACC; and RF-3′, GTTACGTATCTACCGCCGCTTTTACG.

Plasmid construction. Plasmids pGS284 (46), pCGNpp71 (1), and pGEMTKan/LacZ (54) have been described elsewhere.

(i) Expression vectors. pCGNpp71-C219G was generated using the Stratagene QuikChange site-directed mutagenesis kit according to the manufacturer's protocol. Briefly, pCGNpp71 was digested with XbaI and SalI to drop out a 1.6-kb fragment from the UL82 open reading frame. The XbaI/SalI fragment was subsequently cloned into XbaI/SalI-digested pGEM11 (Clontech) to yield pGEMpp71. pGEMpp71 in conjunction with oligonucleotides C219G-S and C219G-AS were used to generate the plasmid pGEMpp71-C219G, which contains a cysteine-to-glycine point mutation at position 219. The HindIII/SacII fragment from pGEMpp71-C219G containing the mutation was then ligated into HindIII/SacII-digested pCGNpp71 to yield pCGNpp71-C219G. pp71-hDaxx binding mutant constructs pCGNpp71Δ206–213 and pCGNpp71Δ324–331 were generated by using primer pairs SC48/SC49 and SC48/SC52 to amplify the N terminus and primer pairs SC50/SC51 and SC51/SC53 to amplify the C terminus, respectively, of UL82, using pCGNpp71 as a template. Fragments were TA cloned into pGEMT-Easy (Clontech), and the corresponding N- and C-terminal regions were ligated together via the EcoRI restriction sites within the primers to yield plasmids pGEMTEpp71Δ206–213 and pGEMTEpp71Δ324–331. These plasmids were digested with HindIII and KpnI, and the fragments containing the deletions were ligated into HindIII/KpnI-digested pCGNpp71.

(ii) Retrovirus vectors. pRevTREpp71HA was generated by using primers ADVCGN-5′ and ADVCGN-3′ to PCR amplify the UL82 open reading frame and N-terminal hemagglutinin (HA) tag, using pCGNpp71 as a template. The BamHI-digested PCR product was then ligated into BamHI-digested pRevTRE vector (Clontech). pRevTREpp71Δ206–213 and pRevTREpp71Δ324–331 were generated by excising an RsrII/NotI fragment from pCGNpp71Δ206–213 and pCGNpp71Δ324–331, respectively, and ligating the fragments into pRevTREpp71HA that was digested with RsrII and partially digested with NotI.

(iii) Shuttle vectors for allelic exchange. To construct the pGS284ΔUL82-2A shuttle vector, UL82 flanking regions corresponding to nucleotides 121748 to 119164 and 117566 to 115926 of the HCMV AD169 strain genome were amplified using primer pairs RF-5′/RF-3′ and LF-5′/LF-3′, respectively, and cloned into the pGEMT-Easy vector (Clontech) to yield pUL82Flanks. pGEMTKan/LacZ (54) was digested with PacI to excise the kanamycin/LacZ cassette, which was then cloned into PacI-digested pUL82Flanks to yield pΔUL82Kan/LacZ. pΔUL82Kan/LacZ was digested with SphI and partially digested with NsiI (which drops out the UL82 flanks and Kan/LacZ cassette) and was cloned into NsiI- and SphI-digested pGS284 to yield pGS284ΔUL82-2A. pGS284ΔUL82-2A contains a Kan/LacZ cassette in place of the UL82 coding region from nucleotides 119171 to 117566. Shuttling vectors used for allelic exchange to generate the various recombinant viruses were generated by digesting pCGNpp71, pCGNpp71-C219G, pCGNpp71Δ206–213, and pCGNpp71Δ324–331 with XbaI and RsrII and ligating the corresponding fragments into pGS284ΔUL82-2A digested with AvrII and RsrII to generate pGS284UL82, pGS284C219G, pGS284Δ206–213, and pGS284Δ324–331, respectively. All constructs used in these studies were sequence verified.

Cell culture, BAC generation, and virus propagation. Human foreskin fibroblast (HFF) cells and cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal calf serum (Gemini), 100 units/ml penicillin, and 100 μg/ml streptomycin in an atmosphere of 5% CO_2 at 37°C. pADCREGFP bacterial artificial chromosome (BAC) was generated via allelic exchange using the HCMV AD169 BAC clone pAD/CRE (54) and the shuttling vector pGS284-UL21.5-GFP-Puro by methods that have previously been described (46, 54). This recombination results in the HCMV sequence from base pair 27105 to 27611 being replaced with a marker cassette containing the green fluorescent protein under control of a simian virus 40 promoter, followed by an internal ribosomal entry site and a puromycin resistance gene. This substitution removes the UL21.5 open reading frame of HCMV and replaces it with a GFP/puromycin cassette, allowing for the visualization of virus-infected cells by fluorescence microscopy. The UL21.5 open reading frame is nonessential for HCMV replication, and we have demonstrated that virus generated from this BAC, termed ADCREGFP, replicates to wild-type (WT) levels and has no observable growth defect (data not shown) (48). The pADΔUL82 BAC was generated by standard allelic exchange procedures described previously (46, 54), using the pADCREGFP BAC and pGS284ΔUL82-2A shuttle vector. pADUL82Rev, pADUL82-C219G, pADUL82Δ206–213, and pADUL82Δ324–331 BACs were generated by allelic exchange using the pADΔUL82 BAC and the pGS284UL82, pGS284UL82-C219G, pGS284UL82Δ206–213, and pGS284UL82Δ324–331 shuttle vectors, respectively. All HCMV BACs were screened by restriction digestion, Southern blot analysis, and direct sequencing to confirm proper recombination and incorporation of the mutations. Recombinant viruses were generated by transfecting BAC DNA (~1 μg) into 5×10^6 UL82-complementing cells (Tel UL82 #5) via electroporation (950 μF, 260 V). Cells were seeded into dishes and infectious virus harvested when 100% cytopathic effect was observed. Wild-type and UL82 recombinant viruses generated from BAC DNA were propagated as described previously (6). Infectious titers for all viruses were determined at the same time by plaque assay on UL82-complementing cells (Tel UL82 #5) as described previously (6). Mutations or deletions were also confirmed by directly sequencing DNA isolated from viral particles.

Retrovirus production and generation of stable cell lines. Retrovirus stocks were prepared as described previously (27). Briefly, 10 μg of the various pRevTRE plasmids was transfected into Phoenix A cells (provided by Garry Nolan) via the calcium phosphate method. At 48 h after transfection, supernatant containing the retrovirus was collected and cell debris was removed via centrifugation (3,000 × *g* for 10 min). Polybrene (4 μg/ml) was added to the retrovirus stock, and the solution was used to infect cells for 12 h. Telomerase 12 15-1neo cells were generated by transfecting Telomerase 12 cells (5) with ScaI-linearized pUHD15-1neo plasmid (13). At 48 h after transfection, cells were cultured in the presence of G418 (400 μg/ml). Individual clones were then picked, expanded, and screened for their ability to be regulated by tetracycline. One clone (Telomerase 12 15-1neo #3) was used for the generation of all subsequent cell lines. Cell lines expressing pp71, pp71Δ206–213, or pp71Δ324–331 were generated by transducing Telomerase 12 15-1neo #3 cells with the replication-defective retrovirus REVTREpp71, REVTREpp71Δ206–213, or REVTREpp71Δ324–331, which express the respective pp71 proteins. Cells were selected in hygromycin (150 μg/ml) and G418 (400 μg/ml). Individual clones were then picked, expanded, and screened for expression of pp71 by Western blot analysis.

Southern blot analysis. HCMV BAC DNA was digested with restriction enzymes, and fragmented DNA was separated on a 0.8% agarose gel. DNA was then transferred to an Optitran BA-S nitrocellulose membrane by using a Turboblotter according to the manufacturer's protocol for neutral transfer of DNA (Schleicher & Schuell). The membrane was then probed for UL82 sequence and a UL82 flanking region with [^{32}P]dCTP-labeled PCR products in ULTRAhyb

(Ambion) at 42°C. Membranes were then washed in ultra-high-stringency wash buffer (0.1× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% sodium dodecyl sulfate [SDS]) at 42°C and fragments detected by autoradiography.

Antibodies. The following antibodies were obtained from commercial sources: anti-pp65 (1205-S; Rumbaugh-Goodwin Institute); anti-hDAXX (M-112; Santa Cruz); anti-promyelocytic leukemia protein (PML) (PG-M3; Santa Cruz), antitubulin (TU-02; Santa Cruz); anti-HA (16B12; Babco); and anti-IE1/2 (MAB810; Chemicon). pp71 antibodies were a generous gift from T. Shenk and have been previously described (24)

Western blotting and immunoprecipitation analysis. Western blotting and immunoprecipitations were conducted as previously described (4). Briefly, cells were harvested by trypsinization, collected by centrifugation, and lysed in radioimmunoprecipitation assay buffer (50 mM Tris-HCl, 1% NP-40, 0.25% Na-deoxycholate). Cellular debris was removed by centrifugation and the supernatant fluids reserved. The protein concentration was determined by the method of Bradford (3). Equal amounts (40 μg) of protein were resolved by electrophoresis in the presence of SDS on 7.5 to 10% polyacrylamide gels (SDS-PAGE). Proteins were transferred to nitrocellulose membrane (Optitran; Schleicher & Schuell) and probed with primary and secondary antibodies. Immunoreactive proteins were detected by the ECL chemiluminescent system (Amersham).

For immunoprecipitation experiments, cells were infected with the indicated viruses and harvested at the indicated time points. Cells were lysed in NP-40 buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.5% NP-40, 0.75% IPEGAL) containing protease inhibitor cocktail (Roche) for 20 min at 4°C. Lysates were cleared by centrifugation and protein concentrations determined by Bradford assay. One hundred μg protein was incubated with hDaxx antibody for 2 h at 4°C. Antibody complexes were recovered on protein A/G-agarose beads (Santa Cruz), washed three times in NP-40 buffer, and boiled in 2× SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2.5% SDS, 20% glycerol, 1% β-mercaptoethanol). Proteins were then separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed as described above.

Immunofluorescence analysis. HFF cells were seeded onto chamber slides (Falcon) and infected the following day with the indicated viruses. Cells were washed twice with phosphate-buffered saline (PBS) and subsequently fixed with 4% paraformaldehyde or 50:50 acetone:methanol solution for 20 min at the designated time postinfection. Cells were permeabilized with PBST (PBS plus 0.05% Tween 20 and 0.1% Triton X-100) for 25 min at room temperature and incubated with blocking solution (PBST plus 0.5% bovine serum albumin and 1% goat serum) for an additional 30 min. Cells were then incubated with primary antibody for 1 h at room temperature, washed three times in PBST, and incubated with Alexa-488- or Alexa-546-conjugated secondary antibody for 1 h. Slides were washed in double-distilled H_2O and nuclei stained with Hoechst 33258 for 5 min. Slides were sealed with coverslips, cells visualized using a Zeiss Axioplan 2 microscope, and images taken with a SPOT camera (Diagnostic Instruments).

Viral DNA purification and infectivity assay. HCMV ADCREGFP viral DNA was isolated as described previously (1). Briefly, confluent monolayers of HFF cells were infected with ADCREGFP virus at a multiplicity of 2 PFU/cell. Supernatants were collected ~6 days postinfection, and virions were pelleted through a 20% sorbitol cushion by ultracentrifugation in a SW28 rotor at 20,000 rpm for 1 h at room temperature. Infectious viral DNA was isolated, quantified, and used for infectivity assays. Viral DNA infectivity assays were done as previously described (1). Briefly, 5×10^6 HFF cells were transfected with 1 μg viral DNA and 5 μg plasmid DNA via electroporation. Transfected cells were plated into six-well dishes and overlaid (0.75% agarose-Dulbecco's modified Eagle's medium solution) 3 days posttransfection. Plaques were then fixed, stained, and counted 16 days posttransfection.

RESULTS

We previously reported on the generation and characterization of a UL82 deletion mutant termed AD*sub*UL82, which was created by homologous recombination within human fibroblasts (5, 6). The generation of large deletion mutants by this method is well established; however, it is not conducive to the generation of point mutants or small deletion mutants (2). Therefore, in order to rapidly create multiple UL82 mutants we utilized a BAC that contains the HCMV genome to generate our UL82 viral mutants. First, we generated a UL82 deletion mutant that could subsequently be used to reinsert a variety of mutated UL82 open reading frames (Fig. 1A). The UL82 deletion mutant was generated by replacing the UL82 open reading frame within the ADCREGFP BAC with a kanamycin resistance and LacZ cassette, using standard allelic exchange protocols (46, 54). This UL82 deletion mutant BAC has the same UL82 sequence deleted as AD*sub*UL82 and is termed pADΔUL82. Southern blot analysis of the pADΔUL82 DNA digested with BamHI confirmed that pADΔUL82 lacks the UL82 coding region and that the marker cassette had recombined properly within the viral genome (Fig. 1B). A revertant BAC, termed pADUL82Rev, was also generated using the pADΔUL82 BAC and the pGS284UL82 shuttling construct to demonstrate our ability to reincorporate either the wild-type sequence or mutated UL82 sequence. Southern blot analysis of pADUL82Rev DNA digested with BamHI revealed that the pADUL82Rev had undergone proper recombination and now contained the UL82 coding sequence. Stocks of wild-type ADCREGFP, ADΔUL82, and ADUL82Rev viruses were then generated as previously described (6). To confirm that ADΔUL82 was unable to express pp71, HFF cells were infected with wild-type, ADΔUL82, or ADUL82Rev virus at a multiplicity of 2 PFU/cell and harvested for Western blot analysis at 72 h postinfection (Fig. 1C). pp71 was expressed in cells infected with WT virus and the UL82 revertant virus but was not expressed in cells infected with the UL82 deletion mutant. UL83 (pp65) was expressed at similar levels in cells infected with any of the viruses.

Viral growth curves comparing replication of wild-type ADCREGFP, ADΔUL82, and ADUL82Rev viruses at a multiplicity of 0.01 or 4 PFU/cell were then conducted (Fig. 1D and E). Identically to AD*sub*UL82 (5, 6), ADΔUL82 failed to efficiently replicate on HFF cells when infected at a low multiplicity. However, the growth defect was overcome when cells were infected at a high multiplicity, thus confirming the multiplicity-dependent UL82 growth phenotype of ADΔUL82. The pADΔUL82 BAC was subsequently used to generate the other UL82 mutants in a fashion similar to that described for the UL82 revertant.

Mutation of the pp71 LXCXD domain does not affect virus replication. pp71 has been shown to bind to and induce degradation of hypophosphorylated Rb family member proteins (pRb, p107, and p130) in transfected cells (24, 26). The ability of pp71 to cause degradation of these proteins was mapped to an LXCXD motif within pp71 (26). When the cysteine residue at position 219 within this motif was replaced with a glycine residue, pp71 lost its ability to degrade Rb family member proteins and drive quiescent cells into the cell cycle (24, 26). To determine if pp71's ability to degrade Rb family members and induce quiescent cells is required for efficient virus replication, the C219G mutation was incorporated into the HCMV genome by using the pADΔUL82 BAC and pGS284C219G shuttle vector via allelic exchange. Recombinants were screened by restriction enzyme analysis, PCR, and direct sequencing of pADUL82-C219G BAC DNA (data not shown). pADUL82-C219G BAC DNA was then transfected into both UL82-complementing and noncomplementing HFF cells. ADUL82-C219G recombinant virus was isolated from both cell types. ADUL82-C219G viral stocks were generated on noncomplementing cells and assayed for viral growth. Growth curve anal-

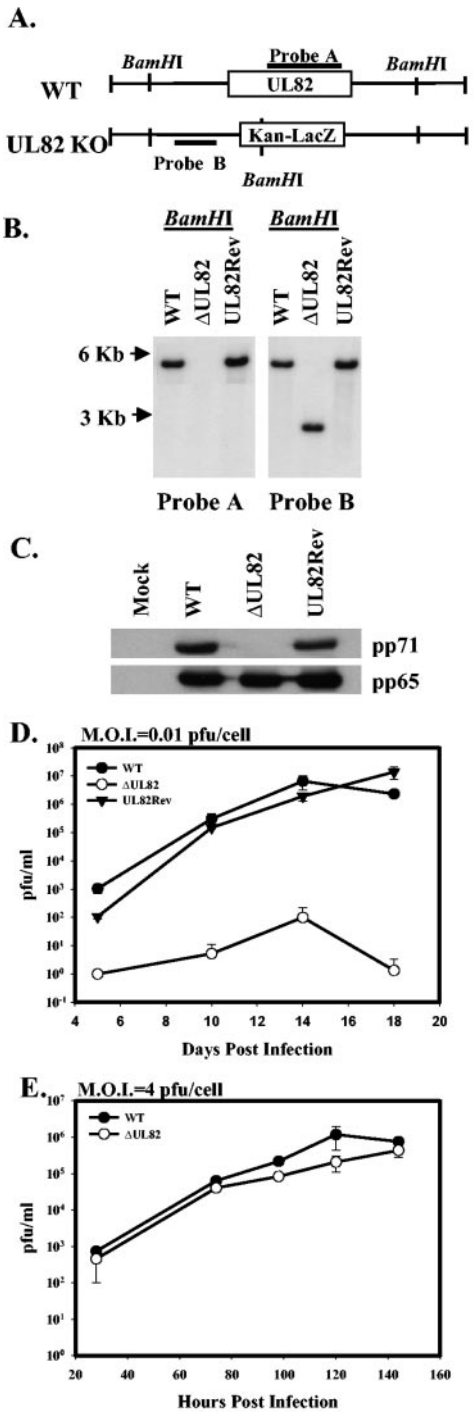

FIG. 1. Generation of the UL82 deletion mutant BAC and recombinant virus. (A) Schematic representation of WT and UL82 deletion mutant genomes. (B) Southern blot analysis of WT pADCREGFP, pADΔUL82, and pADUL82Rev BACs digested with BamHI restriction enzyme and probed for either UL82 or the UL82 right flanking region. (C) Western blot analysis of pp71 expression at 72 h postinfection in HFF cells mock infected (M) or infected with WT, ADΔUL82, or ADUL82Rev virus. As a control, the expression of pp65 was also measured. (D and E) HFF cells were infected at a multiplicity of infection (M.O.I.) of 0.01 PFU/cell (D) or 4 PFU/cell (E) with WT ADCREGFP (●), ADΔUL82 (○), or ADUL82Rev (▼) virus. Cultures were harvested at the indicated times postinfection, and infectious virus was quantified by plaque assay n UL82-complementing cells. Error bars indicate standard deviations.

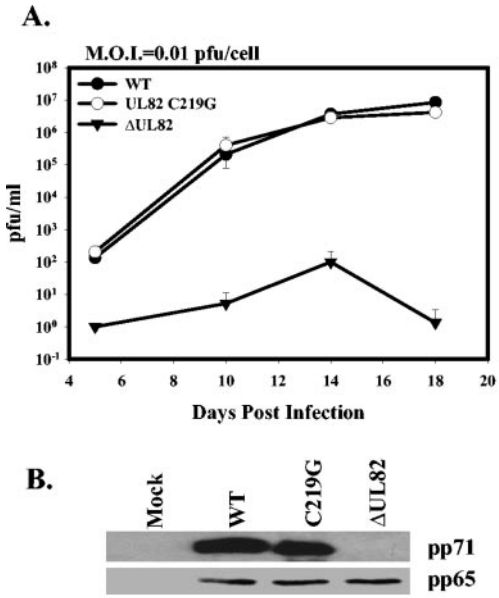

FIG. 2. Growth kinetics of ADUL82-C219G mutant virus. (A) HFF cells were infected (multiplicity of infection [M.O.I.] of 0.01 PFU/cell) with WT ADCREGFP (●), ADUL82-C219G (○), or ADΔUL82 (▼) virus. Cultures were harvested at the indicated times postinfection, and infectious virus was quantified by plaque assay on UL82-complementing cells. Error bars indicate standard deviations. (B) Western blot analysis of pp71 expression at 72 h postinfection in HFF cells mock infected or infected with WT, ADUL82-C219G, or ADΔUL82 virus at a multiplicity of 2 PFU/cell. As a control, the expression of pp65 was also measured.

ysis was conducted by infecting HFF cells with either wild-type ADCREGFP, ADUL82-C219G, or ADΔUL82 virus at a multiplicity of 0.01 PFU/cell. Virus was harvested at various times postinfection, and infectious virus was quantified by plaque assay on UL82-complementing cells. As shown in Fig. 2A, the ADUL82-C219G mutant virus replicated to wild-type levels, whereas replication of the ADΔUL82 deletion mutant was severely attenuated, showing a greater than 4-log reduction in virus production. To confirm the expression of pp71 from the ADUL82-C219G virus, HFF cells were infected at a multiplicity of 2 PFU/cell and harvested 72 h after infection for Western blot analysis. As shown in Fig. 2B, cells infected with the ADUL82-C219G virus expressed pp71 at wild-type levels. Expression levels of pp65 were also examined. ADUL82-C219G viral DNA was also isolated and sequenced to confirm that the C219G mutation was retained within the viral genome. These data demonstrate that pp71's LXCXD motif is not necessary for efficient virus replication, suggesting that pp71's ability to degrade Rb family member proteins and induce quiescent cells into the cell cycle is not required for efficient virus replication and that these functions of pp71 are not responsible for the growth defect associated with the UL82 deletion mutant.

Interaction between pp71 and hDaxx regulates efficient virus replication. Using yeast two-hybrid and transient-overexpression assays, it has been shown that pp71 can interact with the cellular protein hDaxx (15, 22). In those studies, the authors identified two separate eight-amino-acid regions within pp71 (amino acids 206 to 213 and 324 to 331) that are required for the interaction between pp71 and hDaxx (15). Deletion of

either region independently abolished pp71's interaction with hDaxx and inhibited pp71's ability to transactivate the HCMV major immediate-early promoter in transient-transfection assays (15). To determine the importance of pp71's interaction with hDaxx during a productive infection, we incorporated the two small deletions (Δ206–213 or Δ324–331) into the viral genome via allelic exchange using the pADΔUL82 BAC and shuttle vectors pGS284Δ206–213 and pGS284Δ324–331. DNAs from all mutant BACs were directly sequenced to confirm proper recombination and incorporation of the deletions (data not shown). In an attempt to recover mutant virus, BAC DNA was transfected into both UL82-complementing and noncomplementing HFF cells. We were unable to recover infectious virus from the noncomplementing cells. However, we were able to propagate the two hDaxx binding mutants on UL82-complementing cells. The failure to propagate the two hDaxx binding mutants on noncomplementing cells following transfection suggested that pp71's interaction with hDaxx is required for efficient virus replication on HFF cells. To confirm this, growth curves of wild-type virus, the hDaxx binding mutant viruses (ADUL82Δ206–213 and ADUL82Δ324–331), and the UL82 deletion virus (ADΔUL82) were analyzed at a low multiplicity of 0.01 PFU/cell on noncomplementing HFF cells. As shown in Fig. 3A, the ADΔUL82 deletion virus and the two hDaxx binding mutants failed to efficiently replicate on noncomplementing cells. In fact, the growth curves for the two hDaxx binding mutants paralleled that of the UL82 deletion mutant, demonstrating a greater than 4-log reduction in infectious virus compared with wild-type virus. However, when noncomplementing cells were infected at a high multiplicity of 4 PFU/cell, the growth defect was abolished and the UL82 mutants replicated to wild-type levels (Fig. 3B), demonstrating the multiplicity-dependent growth phenotype of the UL82 mutants. To confirm that the growth defect associated with these viruses was due to the mutations within UL82 and not elsewhere in the genome, UL82-complementing cells were infected at a multiplicity of 0.01 PFU/cell for growth curve analysis with wild-type, ADΔUL82, ADUL82Δ206–213, or ADUL82Δ324–331 virus. As shown in Fig. 3C, all viruses replicated to wild-type levels on complementing cells, demonstrating that the growth defect of the UL82 deletion mutant and the two hDaxx binding mutants is a direct result of the mutations within UL82 and not the result of a secondary mutation elsewhere in the genome.

pp71 expression and subcellular localization were also examined following infection with wild-type, ADΔUL82, ADUL82Δ206–213, and ADUL82Δ324–331 viruses. HFF cells were infected at a multiplicity of 2 PFU/cell, and lysates were harvested 72 h after infection for Western blot analysis. As shown in Fig. 4A, the two UL82 hDaxx binding mutants express pp71 but at somewhat reduced levels compared to wild-type virus. However, the subcellular localization of the mutant pp71 expressed from the two hDaxx binding mutants during infection of HFF cells displayed a staining pattern identical to that observed with wild-type pp71 (Fig. 4B).

pp71-hDaxx binding mutant proteins are unable to complement the UL82 deletion mutant growth defect. To eliminate the possibility that the lower levels of pp71 expressed from the ADUL82Δ206–213 and ADUL82Δ324–331 viruses were responsible for the growth phenotype, we generated stable cell lines that express the pp71 mutant proteins which are unable to bind hDaxx. Wild-type or pp71-hDaxx binding mutant cDNAs were inserted into replication-deficient retrovirus constructs and used to generate stable cell lines expressing wild-type pp71, pp71Δ206–213, or pp71Δ324–331. Stable clones were screened for pp71 expression by Western blot analysis and assayed for their ability to complement the growth defect of the ADΔUL82 deletion mutant. HFF cells, UL82-comple-

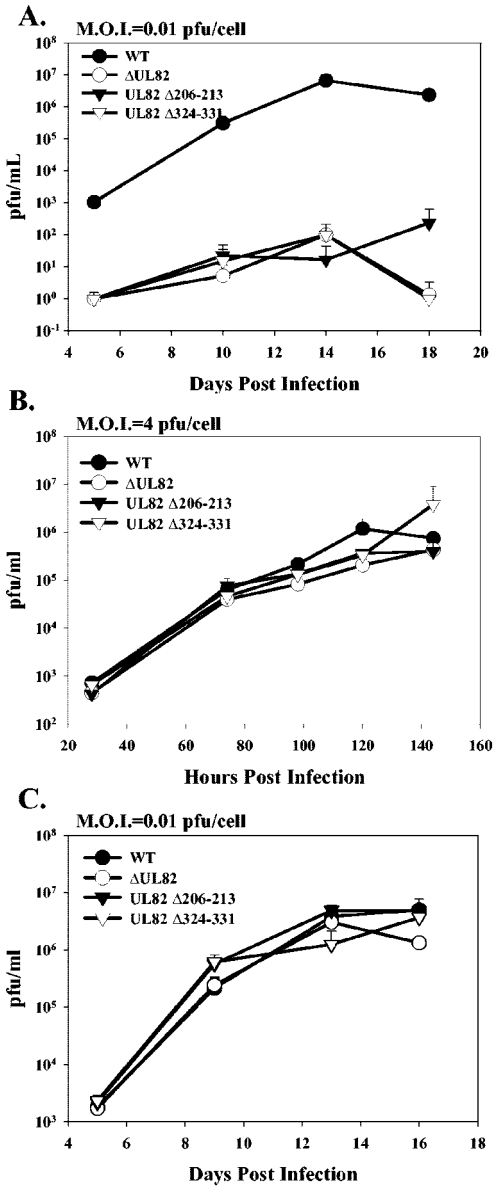

FIG. 3. Growth kinetics of wild-type and UL82-hDaxx binding mutants. (A and B) HFF cells were infected with (A) 0.01 PFU/cell or (B) 4 PFU/cell of WT (●), ADΔUL82 (○), ADUL82Δ206–213 (▼), or ADUL82Δ324–331 (▽) virus. Cultures were harvested at the indicated times postinfection, and infectious virus was quantified by plaque assay on UL82 complementing cells. (C) UL82-complementing cells were infected (0.01 PFU/cell) with WT (●), ADΔUL82 (○), ADUL82Δ206–213 (▼), or ADUL82Δ324–331 (▽) virus. Cultures were harvested at the indicated times postinfection, and infectious virus was quantified by plaque assay on UL82-complementing cells. M.O.I., multiplicity of infection. Error bars indicate standard deviations.

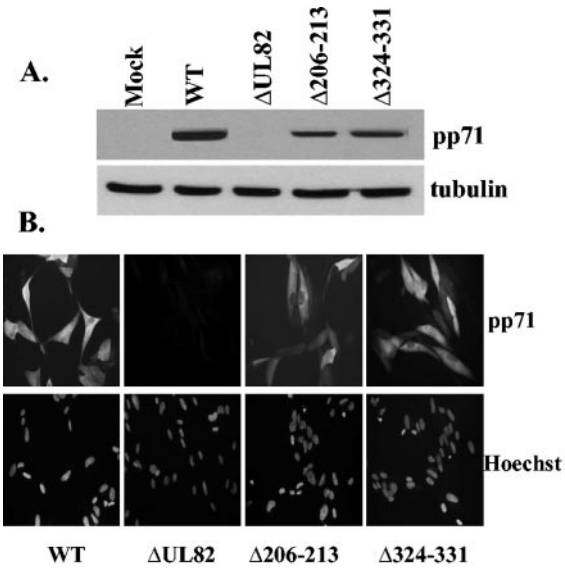

FIG. 4. Expression of pp71 from UL82 mutants. (A) HFF cells were infected with WT, ADΔUL82, ADUL82Δ206–213, or ADUL82Δ324–331 virus at a multiplicity of 2 PFU/cell. Lysates were prepared at 72 h postinfection and assayed for pp71 expression by Western blotting. Antitubulin was included as an internal loading control. (B) HFF cells were infected at a multiplicity of 1.0 PFU/cell with WT, ADΔUL82, ADUL82Δ206–213, or ADUL82Δ324–331 virus. Cells were fixed at 72 h postinfection and immunostained with monoclonal antibody against pp71. Cell nuclei were detected with Hoechst stain.

menting cells, pp71Δ206-213-expressing cells, or pp71Δ324-331-expressing cells were infected with wild-type or ADΔUL82 virus at a multiplicity of 0.01 PFU/cell. Virus was harvested at 15 days postinfection, and infectious virus was quantified by plaque assay on UL82-complementing cells. We tested eight clones each that expressed either pp71Δ206–213 or pp71Δ324–331, and none of the clones were capable of complementing the growth defect of the UL82 deletion mutant. However, all clones supported wild-type virus replication, and the UL82 deletion mutant replicated to wild-type levels on the cell lines expressing wild-type pp71. Representative expression levels and complementation results for two clones of each cell type are shown in Fig. 5A and B. All of the pp71Δ206–213 and pp71Δ324–331 cell clones expressed protein levels equal to or greater than that of the wild-type pp71-complementing cell line but were unable to complement the UL82 deletion mutant growth defect. These results suggest that the decreased pp71 expression levels observed with the hDaxx mutants are not responsible for the growth phenotype associated with the two hDaxx binding mutants.

It is also possible that the pp71 protein with the hDaxx binding domain deleted may be defective for pp71 tegument incorporation, leading to the growth phenotype observed. To rule out this possibility, stable cell lines expressing either wild-type pp71 or the pp71-hDaxx binding mutant proteins (pp71Δ206–213 or pp71Δ324–331) were infected with the ADΔUL82 virus at a multiplicity of 4 PFU/cell. At 96 h postinfection virus particles were harvested and purified by ultracentrifugation. Virions were then lysed, and virion proteins were separated by SDS-PAGE, transferred to membranes, and examined for pp71 incorporation by Western blot analysis. As shown in Fig. 5C, pp71 expressed from the pp71Δ206–213 or pp71Δ324–331 cell lines incorporated into virions at the same or greater efficiency compared to wild-type pp71 expressed from the UL82-complementing cell lines. Additionally, levels of pp65 were examined as an internal control for tegument incorporation. These results demonstrate that the growth phenotype associated with the pp71-hDaxx binding mutants is not due to a block in pp71's ability to incorporate into the tegument of the virus particle.

pp71 expressed from ADUL82Δ206–213 or ADUL82Δ324–331 is unable to interact with cellular hDaxx and does not colocalize to ND10 domains. We next examined the expression of hDaxx following infection and confirmed that pp71 expressed from the ADUL82Δ206–213 and ADUL82Δ324–331 mutants could no longer bind hDaxx. HFF cells were infected with wild-type virus at a multiplicity of 3 PFU/cell, and lysates were harvested at various times postinfection and examined for

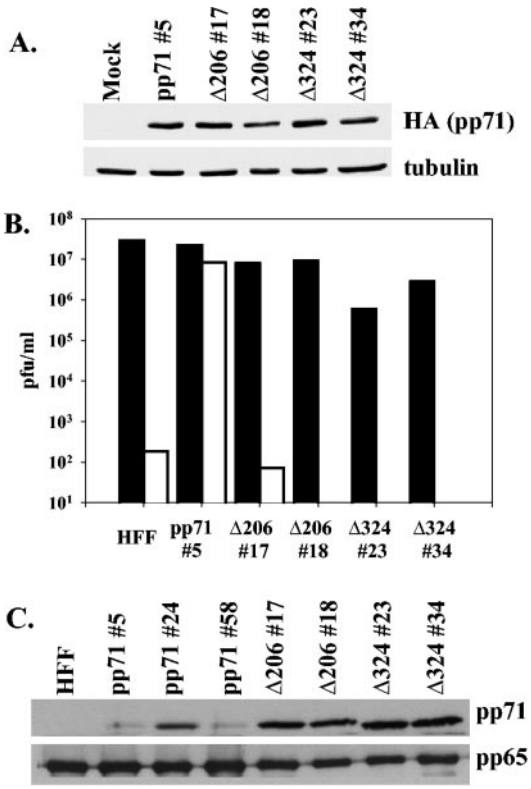

FIG. 5. Cell lines expressing pp71-hDaxx mutant proteins fail to complement the UL82 deletion mutant growth defect. (A) Cell lysates from stable HFF cell lines expressing WT pp71 or pp71 which is unable to interact with hDaxx were examined for pp71 expression by Western blot analysis. Two clones of each pp71-hDaxx binding mutant and the pp71-complementing cells (pp71 no. 5) were examined. Antitubulin was included as an internal loading control. (B) HFF cells or cells expressing the various pp71 mutant proteins were infected (0.01 PFU/cell) with WT (black bars) or ADΔUL82 (open bars) virus. Cultures were harvested and infectious virus was quantified by plaque assay on UL82-complementing cells. (C) HFF cells or cells expressing the WT or various pp71 mutant proteins were infected (4 PFU/cell) with ADΔUL82 virus. At 96 h postinfection, virus was collected and purified via ultracentrifugation. Virus particles were lysed and examined for tegument incorporation of pp71 by Western blot analysis. As a control, the tegument protein pp65 was also examined.

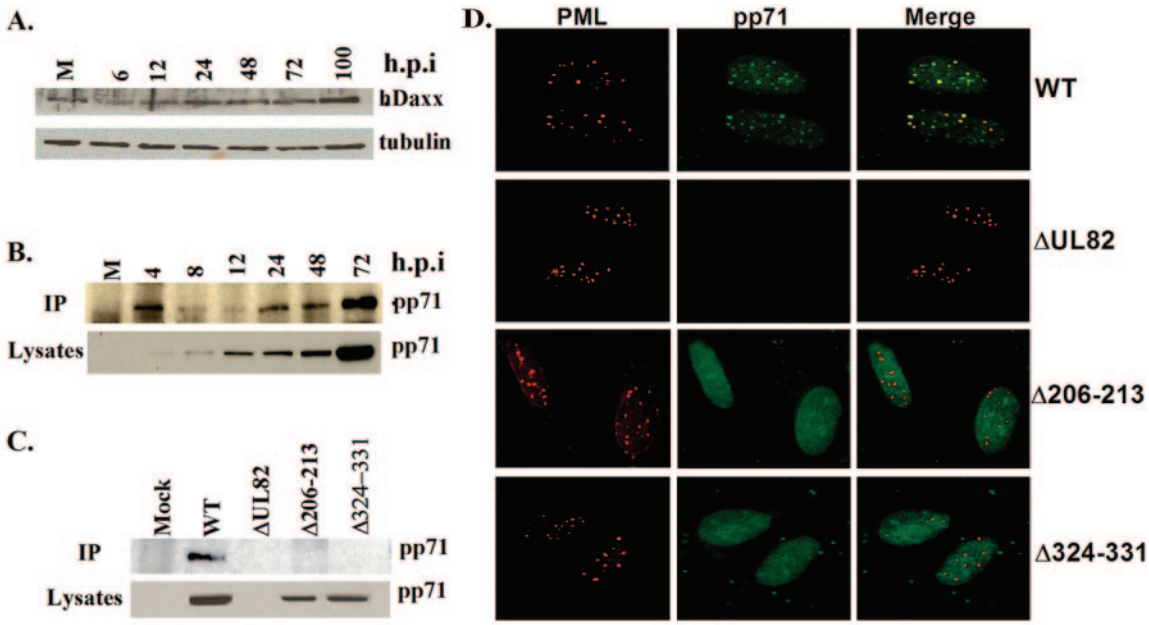

FIG. 6. pp71 interaction with hDaxx during virus infections. (A) HFF cells were infected with WT virus at a multiplicity of 3 PFU/cell. Lysates were prepared at the indicated times postinfection (p.i.) and assayed for hDaxx expression by Western blotting. (B) HFF cells were infected with WT virus at a multiplicity of 2 PFU/cell. Lysates were prepared at the indicated times postinfection and incubated with antibody against hDaxx. Immune complexes were collected, separated by SDS-PAGE, transferred to membranes, and probed for pp71 via Western blotting. Lysates were also examined for pp71 expression by Western blot analysis. IP, immunoprecipitation. (C) HFF cells were infected with WT, ADΔUL82, ADUL82Δ206–213, or ADUL82Δ324–331 virus (1 PFU/cell). Cell lysates were prepared at 72 h postinfection and incubated with hDaxx antibody. Immune complexes were collected, separated by SDS-PAGE, transferred to membranes, and probed for pp71 via Western blotting. Cell lysates were also examined for pp71 expression by Western blot analysis. (D) HFF cells were infected at a multiplicity of 10 PFU/cell with WT, ADΔUL82, ADUL82Δ206–213, or ADUL82Δ324–331 virus in the presence of 100 μg/ml cycloheximide. Cells were fixed at 2 h postinfection and immunostained with monoclonal antibody against pp71 (green) and polyclonal antibody against PML (red) to detect ND10 domains.

hDaxx expression by Western blot analysis. As shown in Fig. 6A, hDaxx is expressed throughout HCMV infection, with its expression increasing between 12 and 24 h postinfection. The kinetics of pp71's interaction with Daxx was then examined by immunoprecipitation assays. Cell lysates from HFF cells infected with wild-type virus were harvested at various times postinfection and incubated with hDaxx antibody. Immune complexes were collected, washed, separated by SDS-PAGE, and transferred to membranes. Membranes were then probed for pp71 expression by Western blotting. As shown in Fig. 6B, pp71 bound to hDaxx could be detected as early as 4 h postinfection and became more abundant at late time points when pp71 is highly expressed. The interaction between pp71 and hDaxx was present at all times assayed during infection. However, at 8 and 12 h postinfection we consistently observed a slight decrease in pp71 bound to hDaxx. Additionally, cell lysates were examined by Western blot analysis to demonstrate pp71 expression levels throughout the course of infection. To confirm that pp71 expressed from the ADUL82Δ206–213 and ADUL82Δ324–331 viruses was unable to interact with hDaxx, HFF cells were infected with either wild-type, ADΔUL82, ADUL82Δ206–213, or ADUL82Δ324–331 virus. Cell lysates were prepared at 72 h postinfection and assayed for the ability of pp71 to interact with hDaxx. As shown in Fig. 6C, pp71 expressed from wild-type HCMV was able to interact with hDaxx. However, we were unable to detect an interaction of pp71 with hDaxx from lysates infected with ADΔUL82, ADUL82Δ206–213, or ADUL82Δ324–331 virus. Identical results were obtained when earlier times postinfection were examined (data not shown), thus confirming that these mutations abolish pp71's ability to interact with hDaxx.

Previous reports have demonstrated that when expressed from plasmids, pp71 that contains the Δ206–213 or Δ324–331 deletion is unable to colocalize to ND10 domains following transient transfection (15). To confirm that pp71 expressed from the viral hDaxx binding mutants ADUL82Δ206–213 and ADUL82Δ324–331 was unable to localize to ND10 domains, HFF cells were infected with wild-type ADCREGFP, ADΔUL82, ADUL82Δ206–213, or ADUL82Δ324–331 in the presence of cycloheximide and fixed for immunofluorescence analysis 2 h postinfection. ND10 domains were detected by staining for PML. As shown in Fig. 6D, pp71 expressed from wild-type virus colocalized to ND10 domains. However, pp71 expressed from ADUL82Δ206–213 or ADUL82Δ324–331 did not colocalize to ND10 domains and displayed a diffuse nuclear staining pattern. These results demonstrate that the pp71 delivered from the virion of the hDaxx mutant viruses is unable to colocalize to ND10 domains.

pp71 interaction with hDaxx regulates efficient IE gene expression. We have previously reported that UL82 is required for efficient IE gene expression when cells are infected at a low multiplicity (6). Therefore, we examined whether pp71's interaction with hDaxx was involved in regulating efficient IE gene expression. HFF cells were infected at a multiplicity of 0.1 PFU/cell with wild-type, ADΔUL82, ADUL82Δ206–213,

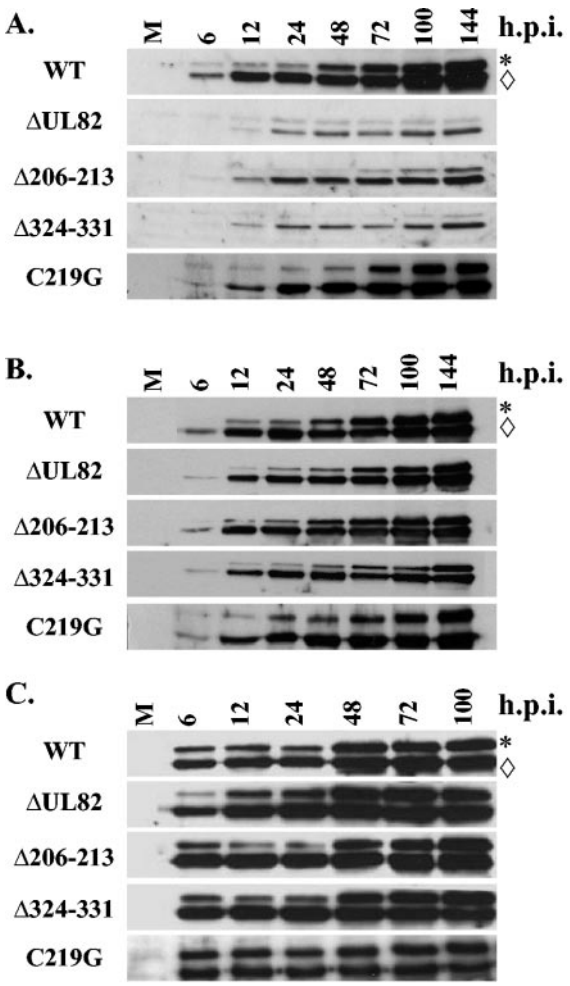

FIG. 7. IE gene expression following infection with UL82 mutant viruses. HFF cells were infected at a multiplicity of 0.1 PFU/cell (A) or 2.0 PFU/cell (C) with WT, ADΔUL82, ADUL82Δ206–213, ADUL82Δ324–331, or ADUL82-C219G virus. Cell lysates were prepared at the indicated times postinfection (p.i.) and assayed for IE1 (◇) and IE2 (*) expression by Western blotting. (B) UL82-complementing cells were infected (0.1 PFU/cell) with WT, ADΔUL82, ADUL82Δ206–213, ADUL82Δ324–331, or ADUL82-C219G virus. Cell lysates were prepared at the indicated times postinfection and examined for IE1 (◇) and IE2 (*) expression by Western blotting.

ADUL82Δ324–331, or ADUL82-C219G virus. Lysates were harvested at various times postinfection and assayed for IE1 and IE2 protein expression by Western blot analysis. As shown in Fig. 7A, IE1 expression was detectable by 6 h postinfection and IE2 was detectable between 12 and 24 h following infection with wild-type virus. However, IE1 expression was dramatically reduced and was delayed by 12 to 24 h in cells infected with the hDaxx binding mutants or the UL82 deletion mutant. IE2 expression was also dramatically reduced and only slightly above the limits of detection after infection with the UL82 mutant viruses (Fig. 7A). However, IE1 and IE2 levels were unaltered when cells were infected with the ADUL82-C219G virus, demonstrating that this mutation has no effect on IE gene expression. When the experiment was repeated on UL82-complementing cells, the UL82 mutants displayed wild-type IE gene expression levels and kinetics (Fig. 7B). Since the UL82 growth defect is multiplicity dependent, we wanted to determine if the effect on IE gene expression was also multiplicity dependent. To test this, noncomplementing HFF cells were infected with wild-type, ADΔUL82, ADUL82Δ206–213, ADUL82Δ324–331, or ADUL82-C219G virus at multiplicity of 2 PFU/cell. Cell lysates were harvested at various times postinfection and assayed for IE1 and IE2 expression by Western blotting. As shown in Fig. 7C, IE1 and IE2 expression levels and kinetics were the same regardless of the virus used to infect the cells. Taken together, these results indicate that like the growth phenotype of the UL82 deletion mutant, the ability of pp71 to regulate IE gene expression is multiplicity dependent and involves an interaction with hDaxx.

pp71-hDaxx binding mutants are unable to enhance the infectivity of viral DNA. pp71 enhances the infectivity of viral DNA when cotransfected into HFF cells (1). To determine if pp71's interaction with hDaxx is also involved in enhancing the infectivity of viral DNA, plasmids which express the various pp71 mutants were cotransfected into HFF cells with purified wild-type viral DNA and assayed for infectious virus production by plaque assay. As shown in Fig. 8A, cells transfected with empty vector, pp71Δ206–213, or pp71Δ324–331 plasmid produced ~30 plaques per microgram of transfected viral DNA. However, cotransfection of either wild-type pp71 or pp71C219G plasmid enhanced the infectivity of viral DNA and resulted in a five- to sevenfold increase in plaque production. Western blot analysis was conducted on an equal aliquot of the transfected cells to confirm equivalent transfection efficiencies

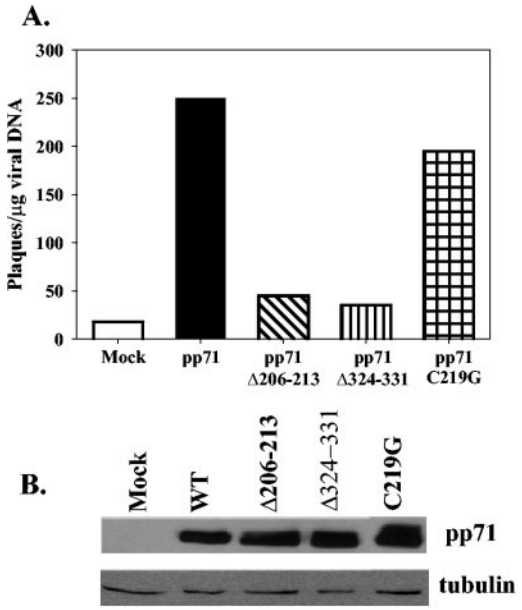

FIG. 8. Effect of pp71 mutant proteins on viral DNA infectivity. (A) HFF cells were transfected with WT ADCREGFP viral DNA (1 μg) and 5 μg pCGN vector (open bar), pCGNpp71 (black bar), pCGNpp71Δ206–213 (diagonally striped bar), pCGNpp71Δ324–331 (vertically striped bar), or pCGNpp71-C219G (crosshatched bar). Cells were plated in six-well dishes and overlaid with agarose. Plaques were fixed, stained, and counted at 15 days posttransfection. (B) Cell lysates from one well of each transfection described in A was harvested at 36 h posttransfection and assayed for pp71 expression by Western blot analysis. Antitubulin was included as an internal loading control.

and expression levels of pp71 (Fig. 8B). These data demonstrate that pp71's interaction with hDaxx is also involved in enhancing the infectivity of viral DNA.

DISCUSSION

During HCMV infection, pp71 is delivered from the virion to the host cell nucleus and is involved in regulating HCMV IE gene expression (6, 10, 14, 15, 18, 21, 30, 52). Using a UL82 deletion virus, we have previously demonstrated that pp71 is required for efficient IE gene expression and virus replication when cells are infected at a low input multiplicity (6). Although several functions and interactions have been attributed to pp71, the function(s) and/or interaction(s) involved in efficient IE gene expression and virus replication has not been elucidated. More importantly, this has not been studied in the context of an HCMV infection. In this study, we utilized UL82 mutant viruses to identify an interaction that is critical for pp71's ability to enhance the infectivity of viral DNA, regulate IE gene expression, and promote viral replication.

The pp71 LXCXD motif is not required for viral replication. Rb family member proteins, which include pRb, p107, and p130, function to regulate cell cycle progression out of G_0 and through the G_1 phase of the cell cycle (51). pp71 has previously been shown to target hypophosphorylated Rb family member proteins for degradation in a proteasome-dependent, ubiquitin-independent fashion, which allows quiescent G_0 cells to enter the cell cycle and progress through G_1 (23–26). These studies also demonstrated that replacement of the cysteine residue within the pp71 LXCXD motif with a glycine residue blocked the degradation of hypophosphorylated Rb family members and the induction of quiescent cells into the cell cycle (24, 26). To determine if these functions of pp71 were required for efficient IE gene expression and virus replication, we generated a UL82 mutant that incorporated the cysteine-to-glycine substitution within the pp71 LXCXD motif. Growth curve analysis demonstrated that the ADUL82-C219G mutant virus replicated with wild-type kinetics and produced infectious virus at wild-type levels regardless of the multiplicity used to infect cells (Fig. 2). Additionally, the UL82-C219G mutation did not attenuate expression of IE1 or IE2 (Fig. 7), and expression of pp71-C219G was able to enhance the infectivity of viral DNA to levels similar to those observed with wild-type pp71 (Fig. 8A) (24). These results strongly suggest that pp71's ability to degrade hypophosphorylated Rb family members and induce quiescent cells into the cell cycle is not required for efficient virus replication in human fibroblasts and is not responsible for the growth phenotype associated with the UL82 deletion mutant. Even though pp71 degradation of Rb family members and induction of quiescent cells into the cell cycle is not required for efficient viral replication, IE gene expression, or viral DNA infectivity, it does not mean that HCMV's ability to affect these processes is not important for efficient virus replication. Other viral proteins involved in these processes may compensate for the loss of functions associated with the C219G mutant. For example, IE2 has also been shown to drive quiescent G_0 cells into the cell cycle (7, 8), while IE1 can bind to (41) and phosphorylate (39) Rb family members, indicating redundant mechanisms for HCMV regulation of the cell cycle. Consistent with this hypothesis, data from our laboratory have demonstrated that the UL82 deletion virus retains the ability to inhibit the expression of the G_0 marker GAS-1 (growth arrest-specific gene-1) and to induce expression of the late G_1 markers cyclin E and cyclin E kinase activity (data not shown). This suggests that other viral proteins in the absence of pp71 are capable of modulating G_0-G_1 cell cycle progression and that this function of pp71 is not responsible for the growth phenotype associated with the UL82 deletion mutant.

pp71 interaction with hDaxx regulates efficient HCMV replication. In addition to pp71's involvement with the cell cycle, pp71 has also been reported to interact with the cellular protein hDaxx during HCMV infection (15, 22). hDaxx was first identified as a proapoptotic protein (53) and later was shown to act as a transcriptional regulator of gene expression (9, 28, 29, 32, 35, 36). Although hDaxx has been associated with transcriptional activation, it is thought to function primarily as a transcriptional repressor. hDaxx represses Pax3 (17) and Ets-1 associated transcription (29) through its interaction with histone deacetylases (16). hDaxx has also been shown to regulate Fas-mediated apoptosis through a transcriptional repression mechanism (28, 47). hDaxx colocalizes with PML within the nucleus at ND10 domains, a site of active gene transcription and viral genome deposition (11, 15, 19–22, 32, 33) During HCMV infection, pp71 interacts with hDaxx and localizes to ND10 domains (15, 22, 31). Transient assays have demonstrated that disruption of the pp71-hDaxx interaction inhibits pp71 localization to ND10 domains and attenuates activation of the major immediate-early promoter. Based on these results, the authors suggested that pp71's interaction with hDaxx is important for viral IE gene expression and viral replication (15, 22). To test this hypothesis, we generated UL82 viral mutants that have the hDaxx binding domains deleted and assayed them for their effect on IE gene expression and viral replication. Growth curve analysis and kinetics of IE1 and IE2 expression with the hDaxx binding mutants were indistinguishable from those obtained with the UL82 deletion virus (Fig. 3 and 7). Replication of the hDaxx binding mutants and UL82 deletion mutant was severely attenuated when cells were infected at a multiplicity of 0.01 PFU/cell. However, this growth defect was overcome if cells were infected at a multiplicity of 4 PFU/cell or if UL82-complementing cells were infected (Fig. 3). Similar results were obtained when we examined the kinetics and expression levels of IE1 and IE2 following infection with the mutants. The UL82 deletion mutant and hDaxx binding mutants showed a dramatic decrease in the abundance of IE1 and IE2 and also demonstrated a significant delay in their expression compared to wild-type virus (Fig. 7). Although these results do not eliminate the possibility that the mutant viruses interfere with an undetermined function of pp71, the results suggest that pp71's ability to interact with hDaxx is required for efficient IE gene expression and virus replication.

pp71's interaction with hDaxx is involved in enhancing the infectivity of viral DNA. pp71 has been shown to enhance the infectivity of viral DNA (1). Since loss of the pp71-hDaxx interaction results in repressed and delayed IE gene expression and a multiplicity-dependent growth defect, we examined whether the pp71-hDaxx interaction was involved in pp71's ability to enhance viral DNA infectivity. Cotransfection of plasmids expressing the pp71-hDaxx binding mutants and viral DNA failed to enhance plaque production (Fig. 8A), demon-

strating that the interaction between pp71 and hDaxx is involved in controlling this function of pp71. It has been suggested that pp71 increases the infectivity of viral DNA by allowing for the efficient expression of IE genes and thus serves to "kick-start" the viral replication cycle (1, 15, 21). Previous reports have shown that cotransfection of IE1 and IE2 expression plasmids with viral DNA in the absence of pp71 was unable to increase the number of infectious plaques, suggesting that pp71's regulation of IE1 and IE2 alone is insufficient to enhance the infectivity of viral DNA (1). Therefore, it is likely that the regulation of all or some combination of IE genes by pp71 is required to enhance the infectivity of viral DNA and initiate the viral cascade of gene regulation. In support of this, we have determined that the pp71-hDaxx binding mutants were also unable to efficiently express other IE genes, including UL37x1, UL38, and UL115 (data not shown).

The mechanism by which the interaction between pp71 and hDaxx enables efficient virus replication, IE gene expression, and DNA infectivity has yet to be determined. pp71 and hDaxx have been shown to synergistically transactivate viral gene expression in transient assays, suggesting a model in which pp71 interacts with hDaxx at ND10 domains to transactivate IE genes (15). This model is consistent with studies indicating that ND10-sequestered hDaxx is inhibited in its ability to mediate transcriptional repression (28). However, other studies suggest that hDaxx retains its ability to mediate transcriptional repression from ND10 domains (12, 17). Therefore, it has also been suggested the pp71-hDaxx interaction may serve to downregulate cellular gene transcription and provide an advantageous site for viral gene expression (22). Although our results clearly demonstrate that the pp71-hDaxx interaction is involved in efficient IE gene expression and viral replication, they do not favor one model over the other. However, preliminary results from our laboratory and others indicate that overexpression of hDaxx inhibits wild-type infections (data not shown)(42), supporting a model in which pp71 relieves hDaxx-mediated repression and provides an environment beneficial for IE gene expression. Additional studies are under way to define the molecular mechanism by which the pp71-hDaxx interaction enhances viral gene expression, viral DNA infectivity, and viral replication.

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