Human Cytomegalovirus Immediate-Early 2 Protein IE86 Blocks Virus-Induced Chemokine Expression

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The effect of human cytomegalovirus (HCMV) gene expression on cytokine (beta interferon) and chemokine (RANTES, MIG, MCP-2, MIP-1α, and interleukin-8) expression was examined. We demonstrate that HCMV gene expression is required to suppress the transcriptional induction of these cytokines and that the HCMV immediate-early 2 gene product IE86 can effectively block the expression of cytokines and proinflammatory chemokines during HCMV and Sendai virus infection. Additionally, we present data on viral mutants and ectopic protein expression which demonstrate that pp65, another identified HCMV cytokine antagonist, is not involved in regulating these proinflammatory cytokines. This is the first report to demonstrate that IE86 can act to suppress virus-induced proinflammatory cytokine transcript expression, extending the antiviral properties of this multifunctional viral protein.

Virus-infected cells respond to infection by inducing numerous signaling pathways that ultimately lead to the expression of cellular genes that limit viral replication and spread. This response is characterized by the induction of cytokines and proinflammatory chemokines (chemoattractant cytokines). Cytokine and chemokine production are critical for the host to be able to mount an effective antiviral response (20, 32, 33). For example, upon virus infection of the host cell, alpha/beta interferon (IFN-α/β) is expressed and secreted from the infected cell. This interferon then functions in an autocrine and paracrine fashion to induce a plethora of antiviral genes that can efficiently inhibit viral replication within the infected cell and the surrounding tissue. In addition, cells also produce chemokines (induced either directly upon infection or by interferon stimulation) which act to link the host innate immune response to the cell-mediated adaptive immune response. These small, secretory proteins aid in viral clearance by attracting leukocytes, including macrophages, natural killer (NK) cells, and T cells, to the site of infection by enhancing the cytotoxic activity of NK and T cells and by blocking entry of viruses that use chemokine receptors for entry into the host cell (5, 14, 41). However, as viruses have evolved, they have developed mechanisms to block these antiviral responses induced by interferon and chemokines, thereby allowing for viral persistence within the infected host. A number of viruses encode proteins that function to block the expression of interferon and chemokines (4, 31, 50), in addition to expressing chemokine analogues (3, 37, 53, 55), and virus-encoded chemokine receptors (2, 27, 40, 49).

Previous studies have demonstrated that HCMV infection regulates the expression of IFN-β (10, 11, 50, 58) and of a number of chemokines, including RANTES (regulated upon activation normal T cell expressed and secreted) (1, 10, 11, 21, 45, 58), MIG (monokine induced by interferon-γ) (1, 10, 11), monocyte chemotactic protein-1 and -2 (MCP-1 and -2) (10, 23), macrophage inflammatory protein-1 alpha (MIP-1α) (1, 11), and interleukin-8 (IL-8) (11, 15, 16, 34, 40). Interestingly, the expression of these cytokines was significantly enhanced when HCMV gene expression was inhibited, suggesting that one or more newly synthesized HCMV-encoded proteins may actively block the expression of these genes during infection (11, 23, 45, 59).

Recently, we demonstrated that the HCMV immediate-early 2 gene product IE86 can efficiently block the induction of IFN-β following viral infection (50). In addition, two studies using microarray analysis and viral deletion mutants reported that the HCMV UL83-encoded protein pp65 could partially inhibit the induction of IFN-β and of a number of chemokines following HCMV infection (1, 10). Since there is significant overlap in the signaling pathways and virus-activated transcription factors that regulate the expression of IFN-β and the various chemokines regulated by HCMV, we sought to determine the relative contributions made by IE86 and pp65 in the inhibition of IFN-β expression and to determine if IE86 can block the expression of chemokines following viral infection.

MATERIALS AND METHODS

Cell culture and virus infections. Telomerase 12 human foreskin fibroblast (HFF) cells (8) and 293 cells were cultured in Dulbecco’s modification of 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% CO2 at 37°C. HCMV stocks were purified by ultracentrifugation in an SW40 rotor for 1 hour at 20,000 rpm. Purified virus was resuspended in serum-free medium and used for infection. For HCMV infection, cells were infected at a multiplicity of 5 PFU/cell with either wild-type (WT) HCMV or UV-irradiated (360 mJ/cm2 in a Stratalinker instrument) HCMV (UV-HCMV) or with the mutant viruses IE2A5X, ΔUL83, or UL83Stop. Sendai virus (Charles River labs) infections were performed using 100 hemagglutinin (HA) units/ml as previously described (50).

Oligonucleotides. Primers for shuttle vector construction were as follows: 83RF F, 5’-GGATCCGATATCATTTCGCGGACACGGC-3’; 83RF R, 5’-AGATCTACCTCTCGGTCACATCCC-3’; 83LF F, 5’-AGATCTCTCA CGCAGGGCCCTTTGATG-3’; 83LF R, 5’-GGATCCCCATGATCCGCT CGACGCC-3’; 83Stop F, 5’-GGATCTCTGACGACCATTTCCGGCC-3’; 83Stop R, 5’-GGATCTCTGACGACCATTTCCGGCC-3’. Lowercase letters in 83Stop F indicate mutated base pairs.

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time PCR primers were as follows: IFN-β F, 5'-CAGCAATTTCCTAAGTGCTCA AAAGCT-3'; IFN-β R, 5'-TATCTGGCTTCTTGAAGGACTAT-3'; GAPDH F, 5'-CTGGGCTCAGTCTGACCCAGAC-3'; GAPDH R, 5'-CCAGGCTCA AAGGTTGAAG-3'; RANTES F, 5'-TGCTCCTTGTGGCTACATTGC-3'; RANTES R, 5'-TTGGCCACTGTGTTAGAATTCTCTCTC-3'; MCP2 F, 5'-AGGAGAAGGTGGAAGAACAACCA-3'; MCP2 R, 5'-AGGGCTGCAAGAACCTTTCATCTGCT-3'; MIP1A F, 5'-TGTCCTGGCTCTCTTGCAAGAG-3'; MIP1A R, 5'-CATTTGTCTGCTGAGGACCA-3'; MIG F, 5'-TTACTGAGCTCAGGCTCAC-3'; MIG R, 5'-TGGACTGAGAGAAGAAGACCACT-3'; IL-8 F, 5'-AGAAGACCCCGGAGAAGACATCT-3'; and IL-8 R, 5'-AGAGGCTGAA CAAAGCAGAGAAGC-3'.

Shuttles vectors for allelic exchange. To construct the pGS284UL83 shuttle vector, UL83 flanking regions corresponding to nucleotides 118881 to 119388 and 121000 to 121500 of the HCMV AD169 strain genome were amplified by PCR using primers 83RF F and 83RF R for the right flanking region and 83LF F and 83LF R for the left flanking region of UL83. These PCR products were then cloned into the pGEMT-Easy vector (Clontech) to yield pUL83Flanks. pUCK4 (52) was digested with BamHI to excise the kanamycin resistance cassette, which was then cloned into BamHI-digested pUL83Flanks. pUCK4 was used for recombination with the pADCRE bacterial artificial chromosomes (BACs) were generated by standard allelic exchange procedures described previously (25). pGEMTE-UL83Stop was then digested with BbsI to remove the region containing the stop codons in all three open reading frames starting at nucleotide 119388. pGEMTE-UL83Stop was then cloned into the pGEMT-Easy cloning vector (Clontech) to create pGEMTE-UL83R.

The stop codon mutations were created by using PCR product generated with the pUL83Flanks primers, which contain the 5' and 3' UL83 sequence to create stop codons in all three open reading frames starting at nucleotide 119388. This PCR product was TA cloned into pGEMT-Easy to create pGEMTE-UL83StopR. pGEMTE-UL83Stop was then created by replacing the EcoRV fragment of pGEMTE-UL83R with the EcoRV fragment from pGEMTE-UL83StopR. Additionally, this mutation created a novel SpeI site to facilitate screening. pGEMTE-UL83Stop was then digested with BbsI to remove the region containing the stop codons and cloned into pGS284UL83, which was subsequently digested with BbsI to create pGS284UL83Stop. All constructs used in these studies were sequence verified.

BAC mutagenesis. All viral mutants were generated using previously reported allelic exchange protocols (48, 56). Briefly, the pADRECFFGP (12), pADRECFFPUL83, and pADRECFFGUL83 stop bacterial artificial chromosomes (BACs) were generated by standard allelic exchange procedures described previously. The shuttle vector pGS284UL83 was used for recombination with the pADRECFFGP BAC to create the ΔUL83 virus. The shuttle vector pGS284UL83Stop was used for recombination with the pADRECUL83 BAC to generate the UL83Stop virus. Following allelic exchange, all mutant BACs were screened by restriction enzyme digestion, Southern blot analysis, and direct sequencing to confirm proper recombination and incorporation of the desired mutations. Recombinant viruses were generated as described previously (48, 56).

Briefly, BAC DNA was transfected (−10 μg) into 5 × 10^6 human foreskin fibroblasts via electroporation (950 V; Electroporator) and lysed in radioimmunoprecipitation assay buffer (50 mM Tris-HCl, 1% NP-40, 0.25% Na-deoxycholate) containing 1 μg/ml Lipo-nectamycin (Invitrogen) to the virus inoculum. Expression of viral proteins following transduction was confirmed by Western blotting.

Western blot analysis. Western blotting was conducted as previously described (7). 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) containing a protease inhibitor cocktail (Roche) added at the time of lysis. Cellular debris was removed by centrifugation, and the supernatant fluids were reserved. The protein concentration was determined by using Bradford assay (6). Equal amounts of protein were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis on 8.5% to 10% gels. Proteins were transferred to nitrocellulose membrane (Optitran; Schleicher & Schuell) and probed with primary and secondary antibodies. Immunoreactive proteins were detected by use of an ECL system (Amersham).

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FIG. 1. HCMV gene expression is required to block chemokine expression. Telomerase 12 HFF cells were mock infected (M), infected with WT HCMV, or infected with UV-HCMV at a multiplicity of 5 PFU/cell. RNA was isolated 8 h postinfec-

RESULTS

HCMV gene expression blocks cytokine and chemokine induction. Previous reports have demonstrated that infection with transcriptionally inactive UV-irradiated HCMV or infection with wild-type HCMV in the presence of cycloheximide results in a dramatic induction of cytokine and chemokine expression compared with wild-type infection (11, 23, 45, 59). These results suggest that a newly synthesized HCMV gene product can inhibit the induction of these genes during infection. To confirm the previous results, we assayed for the expression of key cytokines and chemokines by Northern blotting. HFF cells were infected for 8 h at a multiplicity of 5 PFU per cell with either purified wild-type HCMV or purified UV-inactivated HCMV. As shown in Fig. 1, infection with UV-inactivated HCMV results in a robust induction of IFN-β, RANTES, MIG, and MCP-2 expression compared with wild-type infection. Blots were also probed for the immediate-early 1 (IE1) transcript to confirm that our UV irradiation protocol efficiently blocks viral gene expression.

Delayed IE86 expression correlates with increased cytokine induction. We have reported that the HCMV IE2 gene product IE86 can efficiently block the induction of IFN-β following
viral infection (50). In addition, two previous reports using a viral deletion mutant and gene array analysis demonstrated that HCMV UL83 gene product pp65 could also attenuate the expression of IFN-β and of a number of chemokines during HCMV infection (1, 10). Using mutant viruses, we attempted to elucidate the individual contributions of both IE86 and pp65 with regard to the inhibition of IFN-β and chemokine expression during HCMV infection. To accomplish this, we used two viral mutants. The first was an IE2 mutant virus termed IE2ΔSX, which has amino acids 136 to 290 deleted from exon 5 of IE2 and is fused at its carboxy terminus to green fluorescent protein (GFP). IE2ΔSX is viable but expresses IE86 at dramatically reduced levels and with delayed kinetics compared to IE86 expression during a wild-type or revertant virus infection (43). The second mutant was a UL83 deletion mutant termed ΔUL83. The ΔUL83 mutant has the UL83 ORF corresponding to nucleotides 119388 to 121000 of the AD169 genome replaced with a kanamycin cassette. The ΔUL83 virus was constructed to be identical to the previously described RVAd65 UL83 deletion mutant (44). To determine the relative roles that IE86 and pp65 play in the attenuation of cytokine and chemokine expression, cells were infected with either purified UV-irradiated HCMV, IE2ΔSX virus, IE2-Rev virus (a revertant virus of IE2ΔSX), or ΔUL83 virus. RNA was isolated at various times after infection and assayed for IFN-β and RANTES expression by Northern blot analysis. As shown in Fig. 2A, infection with both UV-irradiated HCMV and the IE2ΔSX virus resulted in a robust induction of IFN-β and RANTES expression. However, infection with the IE2 revertant virus did not induce the expression of either gene. Interestingly, the level of induction observed following infection with the IE2ΔSX virus closely paralleled that observed following infection with UV-inactivated virus. As previously described (1, 10), infection with the ΔUL83 virus also resulted in an increase in both IFN-β and RANTES expression (Fig. 2B). However, this increase in expression is delayed and significantly reduced compared to the response following infection with either the IE2ΔSX virus or UV-inactivated HCMV. This suggests that there is a greater cytokine response following infection with a virus that does not express IE86, at early times during infection, than after infection with a virus that does not express pp65.

The expression of pp65 could be affected during infection with the IE2ΔSX virus, or deletion of pp65 could somehow alter the expression of IE86 during infection. To address these possibilities, we examined the protein levels of IE86 and pp65 by Western blot analysis. As previously reported, infection with the IE2ΔSX virus resulted in a dramatic reduction and delay in IE86 expression compared to infection with wild-type HCMV (43). The truncated form of IE86 was not detected until 24 h after infection, whereas IE86 was abundantly expressed by 4 h following wild-type infection. There was also a slight decrease in the expression of pp65 at 4 h postinfection with the IE2ΔSX virus compared to the wild-type virus (Fig. 3A). These results are consistent with previous reports demonstrating the IE2ΔSX virus expresses lower levels of pp65 (43). However, pp65 was clearly present within the infected cell by 4 h postinfection with the IE2ΔSX virus. The levels of two other tegument proteins, pp71 and pp28, were approximately the same following infection with the IE2ΔSX virus compared to infection with wild-type HCMV.

FIG. 2. Cytokine response following infection with HCMV IE2ΔSX and ΔUL83 mutant viruses. Telomerase 12 HFF cells were mock infected (lanes M), infected with IE2ΔSX or IE2ΔSX-Rev virus (A), or infected with ΔUL83 or WT virus (B) at a multiplicity of 5 PFU/cell. RNA was isolated 4, 8, and 24 h postinfection and was assayed for IFN-β, RANTES, and GAPDH expression by Northern blot analysis. IFN-β and RANTES expression was quantified by phosphorimaging analysis and standardized to GAPDH expression. Numbers below the blots represent the increase (x-fold) over mock-infected cells. Results for UV-HCMV are also shown.

FIG. 3. Expression of IE and tegument proteins following infection with IE2ΔSX or ΔUL83 mutant viruses. Telomerase 12 HFF cells were mock infected (lanes M), infected with IE2ΔSX or IE2ΔSX-Rev virus (A), or infected with ΔUL83 or WT virus (B) at a multiplicity of 5 PFU/cell. Cell lysates were harvested 4, 8, and 24 h postinfection and assayed for IE86, pp65, pp71, pp28, and tubulin expression by Western blot analysis.
the wild-type virus (Fig. 3B). IE86 was abundantly expressed by 4 h postinfection following wild-type infection, whereas IE86 was not expressed until 8 h postinfection with the ΔUL83 virus. We obtained similar results when a second UL83 deletion mutant virus termed RVAd65 (44) was used (data not shown). Interestingly, there was also a significant delay in the expression of pp71 following infection with the ΔUL83 virus (Fig. 3B). Taken together, these results identify a connection between delayed IE86 expression and increased accumulation of IFN-β and RANTES transcripts.

**IE86 blocks virus-induced IFN-β and RANTES transcript expression.** Since there was a decrease in the expression of pp65 following infection with the IE2ΔSX virus, we could not rule out the possibility that decreased expression of pp65 was also contributing to the cytokine induction observed during infection with the IE2ΔSX virus. Therefore, we wanted to determine whether expression of IE86 or pp65 alone was capable of blocking virus-induced cytokine expression. To accomplish this, we used replication-deficient adenoviruses that express IE86, pp65, or GFP. Cells were transduced with adenoviruses that express either IE86, pp65, or GFP at a multiplicity of infection of 3 PFU/cell. Twenty-four h after transduction, the cells were infected either with UV-HCMV (Fig. 4A and B) or with Sendai virus (Fig. 4C and D). RNA was isolated 8 h postinfection and reverse transcribed, and real-time PCR was performed to quantitate the abundance of IFN-β and RANTES transcripts. As shown in Fig. 4A to D, infection with either UV-irradiated HCMV or Sendai virus alone resulted in a dramatic accumulation of IFN-β and RANTES message. However, expression of IE86 prior to infection with UV-HCMV or Sendai virus greatly reduced or abolished the induction of IFN-β or RANTES (Fig. 4A-D). Prior expression of either pp65 or GFP had no effect on the induction of IFN-β or RANTES following either type of infection. A Western blot is also shown (Fig. 4E) to confirm the expression of IE86, pp65, and GFP at the time of infection. These results confirm the role of IE86 as an IFN-β antagonist and also demonstrate its ability to block the induction of RANTES following virus infection.

**Generation of a UL83 stop codon mutant.** The previous results demonstrate that ectopic expression of pp65 does not block the induction of IFN-β or RANTES during virus infection. They further suggest the increased cytokine response observed in Fig. 2B following infection with the ΔUL83 virus is not the result of abolishing pp65 expression but is likely due to the delayed expression of IE86. The delay in pp71 protein expression following infection with the ΔUL83 virus (Fig. 3D) also suggests that deletion of the UL83 ORF from the viral genome in some way alters expression of the downstream UL82 ORF, which codes for pp71. Interestingly, pp71 is a critical regulator of immediate-early gene expression (9). In order to determine whether loss of pp65 expression or deletion of the UL83 gene sequence from the genome is responsible for the cytokine induction observed following infection with the ΔUL83 virus, we constructed a second UL83 viral mutant. This mutant, termed UL83Stop, contains point mutations that introduce stop codons in all three reading frames 35 bp downstream from the UL83 start codon. Therefore, like the ΔUL83 virus, the UL83Stop virus will not be capable of expressing pp65. However, unlike the ΔUL83 virus, the UL83Stop virus does not delete any genomic sequences and still contains the entire UL83 ORF (Fig. 5A) and therefore should not interfere with the expression of pp71. The UL83Stop virus was constructed by standard allelic exchange protocols described previously (48, 56). To confirm proper recombination and insertion of the stop codons within the UL83 ORF, restriction enzyme analysis of wild-type, ΔUL83, and UL83Stop BAC DNA was performed. As indicated in Fig. 5B, wild-type and UL83Stop DNA digested with XhoI generates the predicted 9.5-kb fragment that is absent from ΔUL83-digested DNA due to a novel XhoI site in the kanamycin cassette. The stop codon mutations within UL83Stop also create a novel SpeI restriction enzyme site in the UL83 ORF (Fig. 5A). To confirm deletion of UL83 from ΔUL83 and distinguish UL83Stop DNA from wild-type DNA, PCR was used to amplify across the recombination junction to produce a specific fragment using purified wild-type, ΔUL83, or UL83Stop BAC DNA as the template. As shown in Fig. 5C, there is a 1.5-kb PCR product generated by using wild-type and UL83Stop DNA as the template. No amplification product was observed using ΔUL83 BAC DNA. Digestion of the 1.5-kb UL83Stop PCR fragment with SpeI produced a 1.0-kb fragment and a 0.5-kb fragment, indicating

![FIG. 4. Expression of IE86 blocks the induction of IFN-β and RANTES during virus infection. Telomerase 12 HFF cells were transduced with replication-defective adenoviruses expressing IE86, pp65, or GFP. Twenty-four h later, the transduced cells were infected with UV-HCMV (A and B) or with 100 HA units/ml of Sendai virus (C and D). RNA was isolated 8 h postinfection and analyzed for IFN-β and RANTES transcripts. As shown in Fig. 4A to D, infection with either UV-irradiated HCMV or Sendai virus alone resulted in a dramatic accumulation of IFN-β and RANTES message. However, expression of IE86 prior to infection with UV-HCMV or Sendai virus greatly reduced or abolished the induction of IFN-β or RANTES (Fig. 4A-D). Prior expression of either pp65 or GFP had no effect on the induction of IFN-β or RANTES following either type of infection. A Western blot is also shown (Fig. 4E) to confirm the expression of IE86, pp65, and GFP at the time of infection. These results confirm the role of IE86 as an IFN-β antagonist and also demonstrate its ability to block the induction of RANTES following virus infection.](http://jvi.asm.org/)
the stop codon mutations were properly located within the viral genome. The fragment amplified from WT DNA was not digested with SpeI. Finally, to confirm that the UL83Stop is viral genome. The fragment amplified from WT DNA was not the stop codon mutations were properly located within the UL83Stop virus. The asterisk represents the stop codon mutation within the UL83Stop virus. (B) Wild-type, UL83, or UL83Stop mutant viruses. The asterisk indicates the diagnostic fragment which is absent in the UL83 digest and present in the WT and UL83Stop digests. (C) WT, ΔUL83, or UL83Stop BAC DNA was used as the template to amplify a specific genomic region spanning the 5' end of the UL83 ORF and the adjoining flanking sequence. The PCR product was then digested with SpeI to confirm that the stop codon was properly inserted within the UL83 ORF. (D) HFF cells were either mock infected or infected with WT, ΔUL83, or UL83Stop virus. Cell lysates were prepared 72 h postinfection and assayed for pp65 expression by Western blot analysis. Tubulin expression was included as an internal loading control.

We then examined the expression of IE86 and pp71 following infection with either wild-type or UL83Stop virus. Fibroblasts were infected with either virus, and cell lysates were prepared at various times postinfection. As shown in Fig. 6, the expression of IE86 and pp71 following UL83Stop virus infection occurred with kinetics the same as those observed following wild-type infection; the expression levels were also similar to those observed following wild-type infection. These results suggest that the delay in IE86 and pp71 expression observed with the ΔUL83 virus (Fig. 3B) is the result of deleting the UL83 ORF from the genome and was not due to abolishing pp65 expression.

pp65 is not responsible for blocking IFN-β and RANTES expression. We next compared the cytokine responses following infection with the UL83Stop and ΔUL83 mutants. Fibroblasts were infected with wild-type, UV-HCMV, ΔUL83, or UL83Stop virus. Cells were harvested and RNA was extracted 8 h postinfection and assayed for IFN-β expression by Northern blot analysis. As shown in Fig. 7A, infection with UV-HCMV resulted in a strong induction of IFN-β transcript. Infection with ΔUL83 also resulted in IFN-β transcript accumulation, although it was not nearly as robust as that observed for UV-HCMV. Interestingly, infection with either wild-type or UL83Stop virus completely blocked the induction of IFN-β (Fig. 7A). Similar results were obtained for RANTES expression (data not shown). Northern blotting was used to determine if prior infection with the UL83Stop virus could block Sendai virus-induced cytokine expression. Cells were infected with either wild-type, ΔUL83, or UL83Stop virus for 8 h and then infected with Sendai virus. RNA was isolated 8 h after Sendai virus infection and assayed for IFN-β and RANTES expression. As shown in Fig. 7B, infection with Sendai virus
alone results in a dramatic induction of both IFN-β and RANTES expression. However, when cells were infected with either the wild-type virus or the UL83Stop virus prior to Sendai virus infection, the induction of both IFN-β and RANTES was almost completely blocked, demonstrating that an HCMV gene other than pp65, which is expressed within the first 8 h of infection, was responsible for this inhibition. Prior infection with the ΔUL83 virus also resulted in an attenuated cytokine response. However, this inhibition was not as dramatic as that observed for the wild-type virus or the UL83Stop virus. Since the UL83Stop and ΔUL83 viruses do not express pp65, the difference in their abilities to inhibit the cytokine response following viral infection is likely due to the different mutation strategies adopted to prevent pp65 expression.

**HCMV IE86 blocks induction of multiple proinflammatory chemokines.** We also examined the ability of IE86 to block the induction of a number of other chemokines that were previously reported to be blocked by pp65 following infection with the ΔUL83 virus. Cells were transduced with adenoviruses that express either IE86, pp65, or GFP and then infected with UV-HCMV for 8 h. RNA was isolated and assayed by real-time PCR for expression of IFN-β, RANTES, MCP-2, MIG, MIP-1α, and IL-8. As shown in Table 1, infection with UV-HCMV resulted in a robust induction of all cytokines and chemokines tested compared to wild-type infection. All chemokines were induced to levels similar to that observed for UV-HCMV in cells expressing either pp65 or GFP, demonstrating that these gene products are not capable of blocking chemokine induction during HCMV infection. However, cells expressing IE86 effectively blocked chemokine induction following UV-HCMV infection. ISG56, which is induced by both WT HCMV and UV-HCMV (11, 47), was included to show that prior expression of IE86 efficiently blocked IFN-β expression, confirming previous microarray results. However, compared to the IFN-β and RANTES transcript when compared to those seen with wild-type infection. The levels of induction observed following infection with either the IE2ΔSX virus or UV-irradiated HCMV were quite similar (Fig. 2A). Infection with the ΔUL83 virus also resulted in an induction of IFN-β and RANTES expression, confirming previous microarray results. However, compared to the IFN-β and RANTES levels observed following infection with the IE2ΔSX virus or with UV-inactivated HCMV, the induction observed following infection with the ΔUL83 virus was severely attenuated, suggesting pp65 could be only partially responsible for blocking the response. Western blot analysis of viral proteins expressed at early times after infection revealed that IE86 expression not only is delayed with the ΔUL83 virus, the induction observed following infection with the ΔUL83 virus or with UV-inactivated HCMV, in which the immediate-early 2 protein IE86 blocks expression of proinflammatory cytokines at the level of mRNA abundance.

Previous reports have demonstrated that HCMV gene expression can attenuate the host proinflammatory cytokine response and that this attenuation is dependent on a newly synthesized viral protein expressed early during infection (11, 18, 23, 45, 58, 59). We have shown that the HCMV immediate-early 2 gene product IE86 can efficiently block the induction of IFN-β following viral infection (50). Additionally, two labs have independently reported microarray studies that demonstrate an increase in the cellular antiviral cytokine response during infection with a UL83 deletion mutant, suggesting the tegument protein pp65 is involved in attenuating cytokine expression (1, 10). Therefore, we wanted to investigate the respective roles that IE86 and pp65 play during the immediate-early events following virus infection and determine if either protein is responsible for suppressing the host cytokine response.

To determine the relative roles of IE86 and pp65, we used two HCMV viral mutants. The first mutant, termed IE2ΔSX (43), is an IE2 mutant that expresses a truncated IE86 protein; this protein is expressed with severely delayed kinetics. The second mutant, termed ΔUL83, is a UL83 deletion mutant which does not express pp65. After infection with the IE2ΔSX virus, we observed dramatically increased levels of both IFN-β and RANTES transcript when compared to those seen with wild-type infection. The levels of induction observed following infection with either the IE2ΔSX virus or UV-irradiated HCMV were quite similar (Fig. 2A). Infection with the ΔUL83 virus also resulted in an induction of IFN-β and RANTES expression, confirming previous microarray results. However, compared to the IFN-β and RANTES levels observed following infection with the IE2ΔSX virus or with UV-inactivated HCMV, the induction observed following infection with the ΔUL83 virus was severely attenuated, suggesting pp65 could be only partially responsible for blocking the response. Western blot analysis of viral proteins expressed at early times after infection revealed that IE86 expression not only is delayed with the IE2ΔSX virus, as reported previously (43), but also is impaired following infection with the ΔUL83 virus. Together, these results support the conclusion that impaired IE86 expression correlates with increased cytokine expression.

Experiments with the IE2ΔSX and ΔUL83 mutants suggest that IE86 can attenuate cytokine expression more efficiently than pp65. To test this, we used replication-defective adenoviruses that express IE86 or pp65 to determine if prior expression of either protein could block the expression of cytokines induced following infection with UV-HCMV or Sendai virus. Prior expression of IE86 efficiently blocked IFN-β and RANTES expression.
TES expression following infection with either UV-HCMV or Sendai virus. The results demonstrate that IE86 not only functions as an IFN-β antagonist but also can function as a RANTES antagonist. Interestingly, prior expression of pp65 had no effect on the induction of IFN-β and RANTES expression observed following UV-HCMV or Sendai virus infection. These results suggest that the delay in IE86 expression observed following infection with the ΔUL83 virus, and not a lack of pp65 expression, is likely responsible for the increased cytokine response.

We also observed a delay and a decrease in the expression of pp71, a key regulator of IE86 expression (9), following infection with the ΔUL83 virus. This raised the possibility that the deletion of the UL83 ORF from the viral genome may have affected expression of the downstream UL82 ORF, which codes for pp71. In this scenario, the reduced expression of pp71 observed following ΔUL83 infection would be responsible for the observed delay in IE86 expression, which in turn would lead to the modest IFN-β and RANTES induction observed in Fig. 2B. To test this possibility, and to determine if pp65 expression was involved in blocking cytokine induction, we constructed an additional UL83 null mutant. This mutant, termed UL83Stop, contains stop codon mutations in all three reading frames 35 bp downstream of the UL83 AUG codon. The UL83 sequence containing these stop codon mutations was used to repair the deleted sequence within the UL83 genome. Importantly, the UL83Stop mutant does not have any UL83 or other genomic sequences deleted but is still unable to express pp65. When cells were assayed for IE86 and pp71 expression following infection with the UL83Stop virus, we observed no difference in the levels at which these proteins were expressed when compared to those for wild-type infection. Most importantly, infection with the UL83Stop virus now blocked the expression of IFN-β and RANTES to levels the same as those observed following wild-type infection (Fig. 6A). In addition, prior infection with the UL83Stop mutant also blocked the induction of IFN-β and RANTES observed during Sendai virus infection (Fig. 6B). These results demonstrate that the induction of IFN-β and RANTES observed following infection with the ΔUL83 virus is not the result of blocking pp65 expression, but more likely is the result of inhibiting the expression of pp71, which in turn regulates IE86 expression. In support of this hypothesis, we have demonstrated that infection of pp71-expressing fibroblasts with the ΔUL83 virus results in wild-type kinetics and expression of pp71 and IE86 (data not shown). In addition, infection with a UL82 deletion virus (12) resulted in an IFN-β and chemokine response similar to that observed following infection with the ΔUL83 virus. In fact, since the phenotype of the UL82 deletion mutant (i.e., reduced expression of IE genes) is multiplicity dependent, we observed a cytokine response following infection with the ΔUL82 virus at a low multiplicity that was even greater than the levels observed following low-multiplicity infection with the ΔUL83 virus (data not shown). Thus, using a UL82 stop codon mutant and a pp65-expressing adenovirus, we have demonstrated that pp65 is not involved in blocking the expression of IFN-β, RANTES, MIG, MCP-1, IL-8, or MIP1-α during HCMV infection. However, this does not mean that other genes identified by microarray analysis following infection with the ΔUL83 virus are not controlled by pp65. It also suggests that, in addition to regulating the chemokines identified here, IE86 may also regulate a number of other cellular genes which were previously identified as being regulated following ΔUL83 infection by gene array analysis. We are currently examining this possibility.

Our results clearly demonstrate that HCMV gene expression is required to efficiently block chemokine induction following HCMV infection and that the immediate-early 2 gene product IE86, in the absence of other HCMV gene products, is capable of blocking this induction during both HCMV and Sendai virus infection. Importantly, this is the first report to demonstrate the ability of IE86 to function as a proinflammatory cytokine antagonist. As reported by others, however, some chemokines are modestly induced during a wild-type virus infection, albeit at a much lower level than during an infection with inactivated virus (1, 10, 11, 13, 16, 18, 29, 34, 45, 58). Therefore, it is important to note that the low level of chemokine expression observed following wild-type infection may play an important role in the viral life cycle.

The mechanism by which IE86 inhibits chemokine expression is currently unknown. Similar to the induction of IFN-β transcription (54), inflammatory cytokines transcribed in response to virus infection require the activation of numerous transcription factors, which include interferon regulatory factor-3 (IRF-3), nuclear factor kappa B (NF-κB), and c-Jun (28, 32). An IE86-mediated block to cytokine induction may be facilitated though inhibition of transcription factor activation or binding to the various cytokine promoters. It is unlikely that IE86 targets the IRF-3 pathway, since previous reports have demonstrated the activation of IRF-3 (10, 17, 21), translocation (10, 17), and DNA binding following HCMV infection (10, 21, 36, 39). In addition, unpublished results from our laboratory have shown that IE86 does not block virus-induced activation of IRF-3. In addition, an IE86-mediated block to IRF-3 activation would not fully account for the array of cytokines inhibited in this report, as they do not all contain IRF-3 binding elements within their promoters. However, the inhibition of IRF-3 by IE86 cannot be ruled out, since IRF-3 binding to its cognate promoter element has not been assayed in the presence of IE86.

The NF-κB binding sequence is a common element within inflammatory cytokine promoters, and, interestingly, all of the cytokines shown to be regulated in this report contain NF-κB binding sites within their promoters (19, 41, 51). HCMV infection has been shown to result in NF-κB activation, and infection with UV-inactivated HCMV enhances NF-κB binding activity (15, 57). This suggests that HCMV gene expression may attenuate NF-κB activity. We are currently investigating the hypothesis that IE86 blocks NF-κB function through a variety of potential mechanisms, including protein degradation, inhibition of nuclear translocation, and inhibition of DNA binding. This latter mechanism is supported by a study from Browne and Shenk, which found NF-κB DNA binding activity is increased following infection with the ΔUL83 mutant virus (10). Their work supports the hypothesis that the impaired expression of IE86 observed following infection with the ΔUL83 virus leads to an increase in NF-κB DNA binding activity. Studies are currently under way to investigate this hypothesis and determine the mechanism by which IE86 blocks proinflammatory cytokine expression.
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