Synergistic induction of intercellular adhesion molecule-1 by the human cytomegalovirus transactivators IE2p86 and pp71 is mediated via an Sp1-binding site

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Human cytomegalovirus (HCMV) infection of transplant recipients is frequently associated with allograft vasculopathy and rejection. One potential mechanism is vascular injury from HCMV-triggered, immunologically mediated processes. HCMV infection has been shown to increase the expression of intercellular adhesion molecule-1 (ICAM-1). The objective of this study was to determine the molecular basis of HCMV-enhanced ICAM-1 gene expression. Transient transfection experiments identified the IE2p86 protein as a potent activator of the ICAM-1 promoter. The tegument protein pp71 showed a strong synergistic effect on IE2p86-mediated ICAM-1 promoter activation. Mutagenesis experiments defined a DNA element from −110 to −42 relative to the transcription start site as responsive for IE2p86. Further point mutations within this DNA element identified an Sp1-binding site that was essential for strong synergistic activation by IE2p86 and pp71. To confirm the activation of ICAM-1 gene expression, human fibroblasts (HFF) as well as endothelial cells (HUVEC) were infected with recombinant IE2p86- and pp71-expressing baculoviruses, respectively. In FACS analysis, a synergistic induction of ICAM-1 was detectable when cells were co-infected with the two recombinant baculoviruses. These findings clearly demonstrate that IE2p86 and pp71 are crucial regulatory factors for HCMV-induced ICAM-1 upregulation.

INTRODUCTION

Human cytomegalovirus (HCMV), a β-herpesvirus, is a ubiquitous human pathogen with a prevalence of infection in the adult population of 50–90%. Although HCMV rarely causes severe disease on primary infection in immunocompetent people, it represents a highly pathogenic agent for immunosuppressed patients such as transplant recipients, as well as newborns, and establishes lifelong persistence in the infected host (Alford & Britt, 1990). So far, the exact site of latency is still uncertain; monocytes, haematopoietic precursor cells and endothelial cells have been discussed as potential reservoirs for latent HCMV (Fish et al., 1995; Sindre et al., 1996).

Recent evidence suggests that HCMV not only causes acute infection in transplanted patients but may also be associated with extensive transplant vasculopathy, ultimately leading to chronic allograft rejection (Grattan et al., 1989; Skowronski et al., 1993; Koskinen et al., 1993; Lautenschlager et al., 1997a; Schnitzler et al., 1997; Borchers et al., 1999). One of the molecular mechanisms underlying the acceleration of vascular disease processes by HCMV infection may be enhancement of transplant immunogenicity. Several studies indicate that this involves the HCMV-induced upregulation of cellular adhesion molecules, which augments the adherence and infiltration of inflammatory cells that are capable of promoting vascular disease (Eisele et al., 1994; Lemstrom et al., 1995; Steinhoff et al., 1995; Koskinen et al., 1996; Yilmaz et al., 1996; Martelius et al., 1998; Waldman et al., 1998; Lautenschlager et al., 1999; The et al., 2001). For instance, cell culture experiments have demonstrated that HCMV-infection of endothelial cells as well as fibroblasts leads to increased cell-surface expression of intercellular adhesion molecule-1 (ICAM-1, CD54) (Sedmak et al., 1994; Grundy & Downes, 1993; Ito et al., 1995; Knight et al., 1999). In vivo evidence for a pathogenetic role of CMV-induced upregulation of ICAM-1 is provided by animal models: rat cytomegalovirus infection in kidney allograft recipients has been linked to increased expression of ICAM-1 and its ligand leukocyte function antigen-1 (LFA-1) in the vascular endothelium and this correlated with enhanced histological changes of chronic allograft rejection (Kloover et al., 2000).

ICAM-1 is an inducible cell-surface glycoprotein and a
member of the immunoglobulin supergene family. It is expressed in both haematopoietic and non-haematopoietic cells (such as endothelial cells and fibroblasts) and mediates cellular adhesive interactions by binding to its receptors on leukocytes, LFA-1 and Mac-1. Therefore, increased ICAM-1 cell-surface expression is accompanied by consequential leukocyte recruitment as well as activation and plays a central role in a wide range of inflammatory and immune responses (van de Stolpe & van der Saag, 1996; Roebuck & Finnegan, 1999). Induction of high levels of ICAM-1 occurs in response to various mediators of inflammation, including bacterial lipopolysaccharides, phorbol esters, oxidant stress and pro-inflammatory cytokines as well as viral infections [e.g. infections with rhinovirus (Papi & Johnston, 1999), respiratory syncytial virus (Stark et al., 1996), hepatitis B virus (Hu et al., 1992), human immunodeficiency virus type 1 (Dhawan et al., 1997), Epstein–Barr virus (Mehl et al., 2001) and HCMV (Sedmak et al., 1994; Ito et al., 1995)]. So far, the mechanism by which HCMV upregulates ICAM-1 cell-surface expression has been poorly understood.

Productive infection with HCMV results in a regulated cascade of immediate-early (IE), early (E) and late (L) viral proteins. In this context, the major IE proteins IE1p72 and IE2p86, which act as strong transactivators of viral early promoters and are therefore required for efficient productive infection (Stenberg et al., 1989; Pizzorno et al., 1996; Marchini et al., 2001). In transient transfection analysis, IE2p86 acts as a transcriptional regulator of cellular promoters such as the hsp70 and cyclin E promoters, as well as of heterologous viral control elements (e.g. human immunodeficiency virus long terminal repeat, LTR) (Biegalka & Geballe, 1991; Ghazal et al., 1991; Hagemeier et al., 1992b; Bresnahan et al., 1998). Therefore, IE2p86 seems to act as a transcription factor that exerts a broad activation pattern. Since IE2p86 interacts with the basal transcription factors TBP (Hagemeier et al., 1992a; Sommer et al., 1994) and TFIIIB (Caswell et al., 1993) and with distinct cellular transcription factors such as CREB, Ap-1, Egr-1, Spi-1/PU.1 or Sp1, protein interactions are believed to be essential for transactivation (Lukac et al., 1994; Lang et al., 1995; Scully et al., 1995; Yoo et al., 1996; Yurochko et al., 1997; Wara-Aswapati et al., 1999).

In this report we provide evidence for the mechanism by which HCMV upregulates ICAM-1 on the cell surface of endothelial cells as well as fibroblasts. We show that the regulatory proteins IE2p86 and pp71 act as strong synergistic inducers of the ICAM-1 promoter. The Sp1 site at −53 bp upstream of the ICAM-1 transcriptional start site was identified as a major response element for HCMV transactivation. Importantly, the synergistic action of IE2p86 and pp71 on endogenous ICAM-1 gene expression could be confirmed by baculovirus-mediated gene transfer followed by FACS analysis. Thus, we identified IE2p86 and pp71 as important mediators of HCMV-induced ICAM-1 upregulation.

**METHODS**

**Plasmid constructions and in vitro mutagenesis.** The ICAM-1 promoter–luciferase constructs, termed pIC677, pIC397, pIC277, pIC135, pIC34, pC277A56MA (−277NF-kBdel) and pC277A2P (−277STAP/Sp1del) were kindly provided by Paul T. van der Saag (van de Stolpe et al., 1994). The ICAM-1 promoter constructs with mutations within the STAT (at −75 bp) and Sp1 (at −53 bp) binding sites, respectively, were generated using the Quick-Change Site-Directed Mutagenesis Kit (Stratagene). Plasmid pM1568 (−277STATmut) was generated by PCR amplification using primers ICAM-1STAT (5′-GGGGAAGGCCAGGTAAACCTGTAAGCAGCACCGGACC-ACGCCccc-3′) and ICAM-1STAT (5′-GGGGCGGTGCTGCTTA-CACGGTTACCTGCGCCCTCCC-3′) and the pC277 plasmid as a template. For generation of plasmid pHM1569 (−277Sp1mut), the primers ICAM-1-5Sp1 (5′-CGGGAAAGGCCAGCGACCTGGCCCTG-GCCC-3′) and ICAM-1-3Sp1 (5′-GGGGCGGTGCTGCTTA-CACGGTTACCTGCGCCCTCCC-3′) were employed. Plasmid pHM1571 (−57Sp1) with a single Sp1-binding site upstream of the ICAM-1 minimal promoter was constructed by cleavage of plasmid pC677 with BamHI/HindIII followed by ligation with the double-stranded oligonucleotide Sp1bs (5′-GATCCAGCAGCACCGCCCTTGGCCCG-3′-CTAGCGGG-CCAGGCGTGCCTGCTG-5′). The promoterless luciferase vector pX2PM was generated by digestion of pC677 with BamHI and BglII and religation of the vector backbone. The eukaryotic expression plasmids for IE2p86 (pHM134 and pHM137) and for the pp71 protein (pCB6-pp71) were as described previously (Arlt et al., 1994; Lang et al., 1995; Hensel et al., 1996). In plasmid pHM134, IE2p86 expression is under the control of the HCMV MIEP, and in pHM137 under the control of the LTR of the Rous sarcoma virus (RSV). For construction of the pHM1587 shuttle vector (used for generation of recombinant pp71 baculoviruses), the open reading frame of pp71 was amplified by PCR using the primers p71-5ecoRl (5′-ATGGAATTCATGTCTCAGGCATCGTCCTG-3′) and pp71-3xhol (5′-ATGTCTAGACTATGATGGCTGGGTTGCGCTG-3′) with pCB6-pp71 as the template. After amplification, the PCR product was cleaved with EcoRI/Xhol and subcloned into pHM1479 to construct plasmid pHM1479. Plasmid pHM1479 was generated by digestion of pCDNA3 with BglII/Xhol and insertion of the resulting fragment into the pFASTBacPl vector. All oligonucleotides were purchased from Sigma-ARK.

**Cell culture.** Spodoptera frugiperda (SF) 158 insect cells, primary human foreskin fibroblasts (HFF) and U373MG cells were cultivated as described previously (Kronschnabl et al., 2002). Primary human umbilical vein endothelial cells (HUVEC) were obtained by chymotrypsin treatment of single umbilical vein cords and were cultivated as described previously (Kronschnabl et al., 2002).

**Generation of recombinant baculoviruses.** Recombinant baculoviruses were generated by using the Bac-to-Bac system, as described by the manufacturer (Invitrogen). Construction of the IE2p86-expressing baculovirus baculo-IE2p86 and the baculo-control virus have been described previously (Kronschnabl et al., 2002). The pp71-expressing baculovirus baculo-pp71 was generated by transduction of the SF21 insect cells with the baculovirus baculo-pp71 as the donor virus.
baculovirus (baculo-pp71) was generated by transformation of the shuttle vector pHM1587 into the baculoviral genome that is maintained as a BACmid in E. coli cells (bMON14272). BACmid-DNA isolated from positive colonies was used for transfection of SF158 cells in order to reconstitute infectious baculoviruses. After further amplification by passing in SF158 cells, virus titres were determined by plaque assay.

**Western blotting.** Samples were subjected to SDS-PAGE, and the proteins were blotted on to nitrocellulose membranes (Schleicher and Schuell). Immunoblot analysis was carried out as described previously (Hofmann et al., 2000). For detection of the ICAM-1 protein, the monoclonal antibody anti-CD54 (mAb1379; Chemicon) was used; the polyclonal anti-serum anti-pHMI78 (Hofmann et al., 2000) was used for the detection of IE2p86, and the polyclonal anti-serum SA1718 (kindly provided by B. Plachter, Mainz, Germany) for detection of pp71. Anti-rabbit and anti-mouse horse-radish peroxidase-conjugated secondary antibodies were obtained from Dianova.

**RT-PCR.** Total RNA from HFF cells was isolated with the High Pure RNA Isolation Kit (Roche). The reverse transcription reaction was performed according to the manufacturer’s protocol using the Titan One Tube RT-PCR System (Roche). GAPDH mRNA was amplified as an internal control by using a specific primer pair creating a 698 bp fragment [5′ primer (698GAP-5) 5′-GTACGT-GTGGAGGTTCACTGG-3′; 3′ primer (698GAP-3) 5′-TCCACCACCCGTGTGCTGTA-3′]. The specific primer pair for the amplification of the ICAM-1 mRNA generated a 380 bp fragment [5′ primer (ICAMmRNA5) 5′-ACATGCAGCACCTCTGTG-3′; 3′ primer (ICAMmRNA3) 5′-CCCGTGTCGTGACCTCACTGAG-3′]. PCR cycling parameters were chosen as follows: 10 cycles of 94 °C for 10 s, 55 °C for 45 s and 68 °C for 45 s, followed by an additional 25 cycles with 5 s elongation of each polymerization step at 68 °C per cycle.

**Transfection and reporter assays.** Plasmid transfection in U373MGs was performed by the DEAE-dextran method as described previously (Arlt et al., 1994). Routinely, 1 μg luciferase target and 2-3 μg transactivator plasmid were used. In co-transfection experiments with two transactivator plasmids, 1:15 μg of each were transfected. The total amount of transfected DNA was kept constant by using the cloning vector pcDNA3 (Invitrogen). In superinfection experiments with HCMV or baculovirus, U373MG cells were transfected with 1 μg of the luciferase target vector alone and superinfected at 24 h after transfection. At 48 h after transfection, cells were harvested and luciferase assays were performed as described previously (Kronschnabl et al., 2002). Each transfection was determined in triplicate and was repeated at least three times.

**Flow cytometry (FACS).** HFFs or HUVECs were infected with baculo-IE2p86, baculo-pp71 or baculo-control at an m.o.i. of 50. In co-infection experiments, the total amount of virus was kept constant (m.o.i. of 50) by co-infection with baculo-control. Cells were fixed with 3 % formaldehyde for 15 min at room temperature. For antibody staining, cells were first Fc-blocked with 0-5 mg Cohn-II fraction for 15 min on ice. Cells were then stained with a mouse monoclonal anti-CD54 R-Phycoerythrin (PE) conjugate at a 1:50 dilution. ICAM-1 cell-surface expression was measured by FACS analysis using a Becton Dickinson FACScan.

**RESULTS**

**HCMV induces ICAM-1 promoter activity as well as mRNA and protein expression**

It has previously been reported that infection of primary human fibroblasts or endothelial cells with HCMV results in an enhancement in the cell-surface expression of ICAM-1 (Grundy & Downes, 1993; Sedmak et al., 1994; Ito et al., 1995). In order to confirm these results, we performed a FACS analysis of primary human fibroblasts that were infected with HCMV, strain AD169, at an m.o.i. of 1. As shown in Fig. 1(A), this resulted in a marked increase of ICAM-1 on the surface of infected cells. As a control, the induction following treatment with TNF-α resulted in an even stronger signal. Western blot analysis was performed in order to distinguish between enhanced surface presentation and upregulation of ICAM-1 protein levels. Using whole-cell lysates of HCMV-infected HFFs, we observed that a considerable induction of ICAM-1 protein expression was already present at 24 h post-infection (p.i.) and further increased up to 72 h p.i. (Fig. 1B, lanes 2, 4 and 6). Therefore, the ICAM-1 induction on the cell surface correlated with increased ICAM-1 protein levels in HCMV-infected HFFs. Since a previous publication indicated that ICAM-1 mRNA levels are upregulated after infection with HCMV, we performed RT-PCR to confirm this regulatory step after infection of HFFs under our conditions (Burns et al., 1999). In parallel with the ICAM-1 protein expression, an increase in ICAM-1 mRNA levels was already detectable by 24 h p.i. and further increased up to 72 h p.i. (Fig. 1C, lanes 2, 4 and 6).

Furthermore, we wanted to determine whether the HCMV-induced increase in expression was regulated at the level of ICAM-1 promoter activation. The nuclear transcription factors known from previous publications to be involved in the regulation of the ICAM-1 promoter include the factors AP1, NF-κB, C/EBP, Sp1 and members of the Ets and STAT families (Fig. 2A; van de Stolpe & van der Saag, 1996; Roebuck & Finnegnan, 1999). To analyse the influence of HCMV on ICAM-1 promoter activation, U373MG cells were transfected with a luciferase reporter construct containing the ICAM-1 promoter up to 140 bp relative to the transcription start site (−277NF-κBdel; Fig. 2B, bars 7) had no significant influence on promoter activation, although this NF-κB element has been shown to be responsible for the induction of ICAM-1 by several mediators of inflammation (such as TNF-α and IL-1β; van de Stolpe & van der Saag, 1996; Roebuck & Finnegnan, 1999). In contrast, the internal deletion of sequences between nt −110 and −42 (−277 STAT/Sp1del) containing binding sites for STAT and Sp1...
factors resulted in a strong decrease in promoter activation. Activity was reduced to a minimal level close to that observed with a construct containing only the ICAM-1 TATA box (Fig. 2B, bars 8 and 6, respectively). For further characterization of the responsible element, site-directed mutagenesis of the Sp1 and the STAT binding sites was performed. This revealed that a mutation of the STAT binding site did not diminish HCMV-mediated transactivation. In contrast, after mutagenesis of the Sp1 site, we observed a strong reduction in promoter stimulation (Fig. 2C, bar 4). Thus, we identified an Sp1-binding site within the ICAM-1 promoter as a strong response element for HCMV-mediated activation.

The viral transactivators IE2p86 and pp71 synergistically induce ICAM-1 promoter activation

Our next objective was to clarify which viral protein was responsible for ICAM-1 promoter stimulation by HCMV. For this purpose, U373MG cells were co-transfected with the ICAM-1 promoter–luciferase reporter construct (pIC677) and expression plasmids for several viral regulatory proteins (including IE1p72, IE2p86, pp71, ppUL69, ppUL26 and pUL84). As shown in Fig. 3(A), the potent viral transactivator IE2p86 under the control of the HCMV MIEP (CMV-IE2) (Fig. 3A, bar 1) strongly stimulated the ICAM-1 promoter approximately 50-fold while IE1p72 and the tegument protein pp71 alone had little influence on promoter activation (Fig. 3A, bars 1, 2 and 4). Only minor effects could be detected after co-transfection of expression constructs for ppUL69, ppUL26 or pUL84 (data not shown). Surprisingly, after co-transfection of CMV-IE2 together with pp71, the ICAM-1 promoter activation was dramatically increased up to 750-fold (Fig. 3A, bar 5). When IE2p86 expression was driven from the RSV LTR (RSV-IE2), the ICAM-1 promoter was activated about 18-fold and promoter activation was synergistically enhanced to 170-fold when pp71 was co-transfected (Fig. 3A, bars 6 and 7). To clarify whether increased protein levels of IE2p86 were responsible for this synergy, a Western blot analysis was performed. This showed that CMV-IE2 protein expression was increased in the presence of pp71, while pp71 did not alter RSV-IE2 expression (Fig. 3B, bars 2 and 4).

To analyse whether promoter induction by IE2p86 and pp71 in co-transfection experiments is mediated via the same response element as determined for transactivation under the conditions of HCMV infection, U373MG cells were transfected with various ICAM-1 promoter constructs together with expression plasmids for IE2p86 and pp71. As shown in Fig. 4 (bars 3–5), deletion of the NF-kB site or mutation of the STAT element had no significant influence, while the loss of the Sp1-binding site resulted in a considerable decrease in promoter activation by IE2p86 and pp71. Furthermore, a reporter construct with a single...
Sp1-binding site upstream of the minimal ICAM-1 promoter (TATA box) was stimulated as strongly as the wild-type promoter (Fig. 4, bars 7 and 2, respectively). This clearly demonstrates that the viral transactivator proteins IE2p86 and pp71 activate the ICAM-1 promoter via the same Sp1-binding site identified in HCMV infection experiments.

Recombinant baculoviruses for IE2p86 and pp71 are efficient tools for gene transfer into primary human cells and confirm the ICAM-1 promoter upregulation

We addressed the question of whether IE2p86 and pp71 are capable of activating endogenous ICAM-1 gene expression...
in HFF and HUVEC cells. Due to the low transfection rates obtained with primary human cells, we generated recombinant baculoviruses for efficient gene transfer of IE2p86 and pp71 into HFF and HUVEC cells. As shown in previous studies, baculoviruses efficiently infect mammalian cells and express the recombinant proteins when the respective coding region is under the control of the HCMV MIEP (Shoji et al., 1997; Dwarakanath et al., 2001; Kronschnabl et al., 2002). With indirect immunofluorescence analysis transduction rates of 70–90% were detectable after infection of HFF and HUVEC cells with the recombinant baculoviruses (data not shown). To monitor the protein expression of the recombinant baculoviruses, Western blot analysis was performed using whole-cell lysates of HFFs infected with baculo-IE2p86 or baculo-pp71. As shown in Fig. 5, both IE2p86 (lane 3) and pp71 (lane 6) were abundantly expressed 24 h p.i. The functional competence of IE2p86 and pp71 derived from recombinant baculoviruses was analysed by their ability to activate the ICAM-1 promoter in luciferase reporter assays. U373MG cells were transfected with the reporter plasmid pIC277 containing the wild-type ICAM-1 promoter followed by superinfection with baculo-IE2p86, baculo-pp71, or a combination of both viruses. As a control, a baculovirus carrying only the HCMV MIEP was used. After infection with baculo-IE2p86, a clear ICAM-1 promoter stimulation was measurable (Fig. 5B, bar 2), which synergistically increased in the presence of baculo-pp71 (Fig. 5B, bar 4). Infection with baculo-pp71 or the baculo-control virus alone had little influence on ICAM-1 promoter activation (Fig. 5B, bars 1 and 3). This clearly demonstrates the functionality of IE2p86 as well as pp71 expressed by recombinant baculoviruses and confirms the IE2p86/pp71-specific activation of the ICAM-1 promoter.

**Endogenous ICAM-1 gene expression is synergistically activated by IE2p86 and pp71**

To elucidate the influence of IE2p86 on endogenous ICAM-1 gene expression, HFFs and HUVECs were infected with either the recombinant baculo-IE2p86 or a baculo-control virus. ICAM-1 cell-surface expression was determined by FACS analysis at various time points p.i. By 48 h, and with higher intensity at 72 h.p.i., we observed an increased ICAM-1 expression on HUVEC cells that had been infected with the
baculo-IE2p86 virus (Fig. 6). In contrast, infection with the baculo-control virus did not significantly affect ICAM-1 cell-surface expression. Similar results were obtained in an experiment performed in parallel with HFFs (data not shown). From these data, we concluded that IE2p86 is the minimal factor of HCMV-encoded regulators that is capable of stimulating endogenous ICAM-1 gene expression.

To investigate the synergistic effect of the viral pp71 protein on IE2p86-mediated induction of endogenous ICAM-1 gene expression, HUVEC cells were co-infected with baculo-IE2p86 in combination with baculo-pp71 or the baculo-control virus. Cells were harvested at 48 h p.i. and the ICAM-1 cell-surface expression determined by FACS analysis. The double infection with baculo-IE2p86 and baculo-pp71 viruses (Fig. 7C) resulted in a significantly increased ICAM-1 cell-surface expression when compared with infection with baculo-IE2p86 virus alone (Fig. 7A). Surprisingly, under these conditions infection with baculo-pp71 was also able to increase the ICAM-1 cell-surface expression on both HUVEC and HFF cells (Fig. 7B; data not shown). However, a quantitative evaluation illustrated that induction by baculo-pp71 was weaker than that with baculo-IE2p86 or the combination of both (Fig. 7D). In summary, the synergistic action of IE2p86 and pp71 has a clearly measurable effect on endogenous ICAM-1 expression, suggesting that these two proteins are mainly responsible for ICAM-1 induction during HCMV infection.

**DISCUSSION**

HCMV infection has been associated with several forms of vascular pathology including transplantation-associated atherosclerosis (Grattan et al., 1989; Normann et al., 1991; Koskinen et al., 1993; Skowronska et al., 1993; Lautenschlager et al., 1997a), restenosis after balloon angioplasty (Speir et al., 1994; Zhou et al., 1996) and even the development of native atherosclerotic lesions (Hendrix et al., 1990; Melnick et al., 1994; Horvath et al., 2000; Streblow et al., 2001; Levi, 2001). However, the pathogenic mechanisms by which HCMV infection contributes to these processes remain to be elucidated. Several studies indicate that vascular injury could result from HCMV-triggered, immunologically mediated processes, in particular in transplant patients (Koskinen et al., 1996; Yilmaz et al., 1996; Lautenschlager et al., 1997b; Martelius et al., 1998; Waldman et al., 1998). Investigations examining the influence of HCMV on immunomodulating molecules on infected cells identified a marked increase in ICAM-1 induction as well as mRNA levels in endothelial cells and fibroblasts (Grundy & Downes, 1993; Sedmak et al., 1994; Ito et al., 1995; Burns et al., 1999; Knight et al., 1999, 2000). The in vivo relevance of cytomegalovirus-mediated ICAM-1 induction in the development of vascular lesions is strongly supported by results obtained with the rat model, where it has been shown that prolonged expression of ICAM-1 after infection of kidney allograft recipients with rat
cytomegalovirus correlated with accelerated chronic allograft nephropathy (Yilmaz et al., 1996; Kloover et al., 2000). Furthermore, upregulation of ICAM-1 directly contributes to the dissemination of HCMV via the peripheral blood, and thus this adhesion molecule also plays an important role in the pathogenesis of acute infections (Fish et al., 1995; Waldman et al., 1995; Grundy et al., 1998). Recent reports have demonstrated that ICAM-1-mediated cell interactions are essential for microfusion events taking place between polymorphonuclear leukocytes (PMNLs) and infected endothelial cells (Gerna et al., 2000, 2002). Virus is delivered via these fusion events from the endothelium to transiently adherent PMNLs, which are implicated in the subsequent haematogenous spreading of HCMV. Transmission could be prevented by inhibition of the contact between the two cell populations and by the use of monoclonal antibodies to CD54 and ICAM-1. Therefore, ICAM-1 upregulation by HCMV is a crucial determinant of viral dissemination and is even proposed as a surrogate marker of pathogenicity as well as attenuation of HCMV strains (Gerna et al., 2000; Knight et al., 2000).

The aim of this study was to characterize in more detail the mechanism by which HCMV activates ICAM-1 gene expression. Since we and others have shown that ICAM-1 protein and mRNA levels are increased after HCMV infection, this suggested that regulation occurs at the level of promoter activation (Burns et al., 1999; Knight et al., 2000). Consistent with this, we observed strong ICAM-1 promoter activation after superinfection of transfected cells with HCMV. Deletion analysis revealed that 135 bp upstream of the transcriptional start site of the ICAM-1 promoter were sufficient for strong activation by HCMV. Interestingly, although the NF-κB element at −140 bp upstream of the transcriptional start site has been shown to be responsible for the induction of ICAM-1 by several mediators of inflammation such as IFN-γ, TNF-α or IL-1β (van de Stolpe & van der Saag, 1996; Roebuck & Finnegan, 1999), this transcription factor binding site was not influential on the ICAM-1 stimulation by HCMV, since an internal deletion of the NF-κB binding site did not change ICAM-1 promoter activation. Instead, the Sp1-binding site...
at -53 bp upstream of the transcriptional start site was identified, both by deletion analysis and by site-directed mutagenesis, as a response element for HCMV-mediated ICAM-1 promoter stimulation. This result is surprising in view of several previous publications indicating that HCMV infection induces NF-κB and that NF-κB binding sites constitute important control elements for virus-regulated gene expression (Sambucetti et al., 1989; Kowalik et al., 1993; Yurochko et al., 1995; Sun et al., 2001).

In transient transfection experiments we identified the IE2p86 protein as a potent transcriptional activator of the ICAM-1 promoter. In addition, the tegument protein pp71 was found to have a strong synergistic effect on promoter activation by IE2p86, while pp71 alone had no effect. One potential explanation for the observed synergistic stimulation could be enhanced IE2p86 expression due to pp71-mediated transactivation of the MIEP, since IE2p86 expression in plasmid pHM134 is driven by this promoter (Liu & Stinski, 1992). This possibility, however, was excluded by transfection experiments using an IE2p86-expressing plasmid under the control of the RSV LTR. With this construct, a comparable synergistic induction of ICAM-1 promoter activity by IE2p86 and pp71 was measurable while pp71 was not able to enhance IE2p86 protein expression (see Fig. 3). Furthermore, the synergy between IE2p86 and pp71 seems not to be confined to the ICAM-1 promoter, since this has previously been reported for the US11 promoter of HCMV (Chau et al., 1999). Thus, the results obtained by transient transfection analysis suggested that the viral tegument protein pp71, in conjunction with the IE protein IE2p86, is able to stimulate ICAM-1 gene expression.

In order to confirm that these two viral regulatory proteins are capable of stimulating the endogenous ICAM-1 gene, we generated recombinant baculoviruses expressing pp71 or IE2p86 under the control of the HCMV MIEP. Previously, it has been shown that recombinant baculoviruses can efficiently transduce a variety of mammalian cells, and we observed in Western blot experiments that IE2p86 and pp71 were easily detectable after infection of HFF cells. The functionality of the baculovirus-expressed proteins was assessed by transfection of an ICAM-1 promoter–luciferase

![Fig. 7. Synergistic induction of endogenous ICAM-1 gene expression after co-infection with baculo-IE2p86 and baculo-pp71. HUVEC cells were either infected with baculo-IE2p86 (A) or baculo-pp71 (B) or both (C). At 72 h p.i., cells were harvested and ICAM-1 cell-surface expression was determined by staining with PE-conjugated antibody to CD54 followed by FACS analysis. Background staining was assessed by incubating cells with PE-conjugated IgG control antibody (not shown). (D) The diagram shows the mean fluorescence intensity values of the ICAM-1 antibody of each histogram.](http://vir.sgmjournals.org)
construct followed by superinfection with baculoviruses. This revealed synergistic promoter activation by co-infection with baculoviruses expressing pp71 and IE2p86. Most importantly, we were also able to detect an increase in endogenous ICAM-1 on the surface of baculovirus-infected HFF or HUVEC cells, as measured by FACS analysis. Again, pp71 was able to enhance the IE2p86-mediated ICAM-1 induction. Interestingly, however, there was a difference between the effects of pp71 on ICAM-1 promoter activation and cell-surface expression: while no significant promoter activation could be detected, we observed an increase in ICAM-1 surface expression after infection with baculo-pp71 alone. This could be due to the pleiotropic effects of this viral regulatory protein, which may not be confined to transcriptional regulation, as it has recently been proposed that pp71 may exert a function comparable with the ubiquitin E3 ligase ICP0 of herpes simplex virus (Marshall et al., 2002). The overall induction levels in the presence of both viral regulators correlated well with those observed after HCMV infection (see Fig. 1A). This strongly suggests that IE2p86 and pp71 are mainly responsible for HCMV-induced ICAM-1 upregulation, also supported by recent results demonstrating an inhibition of ICAM-1 stimulation by an antisense oligonucleotide complementary to the IE2 RNA (Cinatl et al., 2000).

In accordance with our results, Burns et al. (1999) also identified the IE2p86 protein of HCMV as a transactivator of the ICAM-1 promoter in transient transfection experiments. In their assays, a minimum of 370 bp of 5\' of the ICAM-1 promoter in transient transfection experiments. Moreover, we identified the Sp1-binding site at −53 bp as a target site for strong IE2p86 and pp71 activation. Even a promoter construct with a single Sp1-binding site upstream of the ICAM-1 minimal promoter was as strongly activated as the wild-type promoter. However, the synergistic action of pp71 was not strictly dependent on the Sp1-binding site, since pp71 also enhanced IE2p86 transactivation of the −277Sp1mut construct of the ICAM-1 promoter, although to a lesser degree (data not shown).

Previous studies have demonstrated that Sp1-binding sites are also crucial for maximal IE2p86-mediated activation of the HCMV DNA polymerase promoter and the cellular promoters of the NF-kB subunits p65 and p105/p50, respectively (Yurochko et al., 1995, 1997; Luu & Flores, 1997; Wu et al., 1998). In these reports, IE2p86 was shown to increase the Sp1 DNA-binding activity, which could be responsible for promoter activation. Results from other studies have suggested a direct interaction of the IE2p86 protein with the Sp1 transcription factor (Lukac et al., 1994; Yurochko et al., 1997). Furthermore, several recent publications have suggested that Sp1-mediated transcriptional activation could also be modulated by phosphorylation events (Fojas et al., 2001; Milanini-Mongiat et al., 2002). Thus, there are various possibilities as to how IE2p86, in conjunction with pp71, transactivates the ICAM-1 promoter via the Sp1-binding site. So far, we have not detected elevated protein levels of Sp1 after infection of HFF cells with baculo-IE2p86 in Western blot analysis (data not shown). In conclusion, we hypothesize that IE2p86 may either use Sp1 as a bridging factor to facilitate increased binding of the basal transcription apparatus or may modulate the phosphorylation status of Sp1. A further detailed analysis of the exact mechanism of transactivation will be important in order to develop strategies that can interfere with HCMV-mediated upregulation of ICAM-1 and might thus be able to inhibit viral dissemination as well as the development of vascular pathology.

ACKNOWLEDGEMENTS

We thank Dr Paul T. van der Saag and Dr B. Plachter for providing reagents, Dr TimMaisch for advice on the cultivation of HUVEC cells, Dr Peter Lischka and Dr Manfred Marschall for many helpful discussions and Dr B. Fleckenstein for continuous support. This work was supported by the DFG (SFB473) and the BMBF (IZKF Erlangen).

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