Human cytomegalovirus IE1 protein activates AP-1 through a cellular protein kinase(s)

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Introduction

Human cytomegalovirus (HCMV) is a member of the subfamily Betaherpesvirinae, and is endemic in the human population. HCMV genes are expressed in a temporally regulated manner upon infection of permissive cells, dividing the virus life-cycle into three stages: immediate-early (IE), early and late (Mocarski, 1996). The IE genes encode the first set of proteins expressed upon infection, some of which are involved in ‘fine-tuning’ of the virus life-cycle.

IE1 originates from the major IE region under the control of the major IE promoter. At least five exons are spliced alternatively from the major IE region, producing IE1 and IE2 (Stinski et al., 1983). A single mRNA species of 1.95 kb is transcribed for the 72 kDa IE1 protein, while there are multiple mRNAs for IE2, resulting in proteins of 86, 55 and 40 kDa. Transcripts for the IE1 and IE2 proteins share the first three exons, but differ in the structure of the last exons. The IE1 protein activates transcription from both homologous and heterologous viral and cellular promoters (Davis et al., 1987; Tevethia et al., 1987; Cherrington & Mocarski, 1989; Sambucetti et al., 1989; Hagemeier et al., 1992; Michelson et al., 1994; Hayhurst et al., 1995; Margolis et al., 1995; Yurochko et al., 1995).

AP-1 is a transcription factor composed of members of the Jun and Fos families (Angel & Karin, 1991; Angel & Herrlich, 1994). These proteins associate to form a variety of homo- and heterodimers that bind to a common site. The fine tuning of AP-1 activity is important for flexible regulation of cell growth, differentiation and development in response to different stimuli. It has been reported previously that induction of c-fos and c-jun, the major subunits of AP-1, occurs after HCMV infection (Boldogh et al., 1990, 1991, 1993; Hagemeier et al., 1992). AP-1 was found to be modulated in two phases during HCMV infection. At very early times after infection, there was a rapid increase in c-fos and c-jun that was not affected by either inactivation of the virus or inhibition of protein synthesis (Boldogh et al., 1990, 1991, 1993). These data indicated that activation of AP-1 was independent of de novo expression of HCMV IE proteins and possibly involved the interaction of viral particles with the cell. The second phase of the increase in c-fos RNA was detected 12 h post-infection (Hagemeier et al., 1992) and the HCMV IE products, IE1 and
IE2, were implicated (Hagemeier et al., 1992; Monick et al., 1992). IE2 was also shown to activate the HCMV early promoter via an AP-1-binding site (Scully et al., 1995). However, the precise mechanism of activation of AP-1 by IE proteins is not yet understood completely.

In this report, we present data showing that IE1 can up-regulate the activity of AP-1 efficiently. Mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1 (MEKK1), one of the protein kinases regulating AP-1 activity, acted synergistically with IE1 in transactivating AP-1. These results suggest that IE1 activates AP-1 through a protein kinase(s) involved in the cellular signal transduction pathway.

Methods

Cell culture. The human microglial cell line U373MG was grown in DMEM (Gibco BRL) supplemented with 10% fetal bovine serum (Gibco BRL), 100 U/ml penicillin and 100 µg/ml streptomycin under 5% CO₂ in a humidified incubator at 37 °C. The human promonocytic cell line U937 was grown in RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin.

Plasmids. The IE1 expression plasmid pRSV-IE1 was described previously (Kim et al., 1996). Other IE1 expression plasmids, pEQ273 and pEQ273 (Biegalke & Geballe, 1991), were provided by A. Geballe (Dept of Laboratory Medicine, University of Washington, Seattle, USA) and pEQ273 (Biegalke & Geballe, 1991), were provided by A. Geballe (Dept of Laboratory Medicine, University of Washington, Seattle, USA), pSR His-JNK1 into the pEQ273 (Biegalke & Geballe, 1991), were provided by A. Geballe (Dept of Laboratory Medicine, University of Washington, Seattle, USA). For high-level expression of kinase(s) involved in the cellular signal transduction pathway.

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RNA preparation and quantitative RT–PCR. Total cellular RNA was isolated by using Trizol reagent (Gibco BRL) according to the manufacturer’s instructions. For a positive control, LNCX cells (U373MG and U937 cells) were treated with phosphor 12-myristate 13-acetate (PMA) (50 ng/ml) for 2 h. RNA (2 µg) was reverse-transcribed by using AMV reverse transcriptase (Promega) and 2 µg oligo(dt) primer (Pharmacia). The resulting cDNA was diluted to a final volume of 150 µl and 10 µl of the diluted cDNA was used in a PCR reaction. In addition to the cDNA, the reaction mixture included 2.5 units ExTaq polymerase (Takara), 200 nM each primer and 0.25 mM each dNTP in 50 µl. The reaction was incubated for 30 cycles of 94 °C for 1 min, 65 °C for 1 min and 72 °C for 90 s. The levels of c-fos and c-jun RNA were quantified by competitive PCR with their respective competitors. The competitive template for c-fos was generated by insertion of a 296 bp heterologous DNA fragment (isolated from the Neo gene) into the NcoI site of the human c-fos gene. The primers used to amplify the c-fos fragment were 5′ AGCCGGCTCTGTCGACAGA 3′ and 5′ GTGGGAGCTGCAGACAGTG 3′. A 210 bp fragment from c-fos and a 506 bp fragment from the competitive template were produced with these primers. The competitive template for c-jun was generated by insertion of a 246 bp heterologous DNA fragment (isolated from the IE1 gene) into the BamHI site of the human c-jun gene. The primers used to amplify the c-jun fragment were 5′ AGATGCCGGCCGAGCCGCC 3′ and 5′ AGCCGGCGCGGTCTCTCTCTTT 3′. A 314 bp fragment from c-jun and a 506 bp fragment from the competitive template were produced with these primers. The primers used for β-actin were 5′ GCCGAGCCGAGAATCTGCG 3′ and 5′ ACATGGAGGCGGCGGACTC 3′, which amplified a 500 bp fragment. The c-fos gene was kindly provided by M. Yoshida (Institute of Medical Science, University of Tokyo, Japan), and the c-jun gene by T. Deng (University of Florida, Gainesville, USA).

Results

IE1 activates transcription from a promoter containing AP-1-binding sites

To test whether IE1 activates AP-1, we transfected cells with an AP-1 reporter plasmid (AP-1 CAT) containing a CAT of a control plasmid that contained the β-galactosidase gene. CAT activity was determined according to a standard method (Gorman et al., 1982). The amount of acetylation was quantified by phosphorimager (Fuji).

Construction of cells stably expressing IE1. To construct a retroviral vector expressing IE1, a 2.9 kbp HindIII/ClaI fragment from pRSV-IE1 was subcloned into HindIII/ClaI-digested LNCX (Miller et al., 1993), resulting in LNC-IE1. LNC-IE1 was transfected into 293T cells, together with expression vectors for amphotrophic Moloney-murine leukaemia virus env (phHIT456) and gag-pol (phHIT60), by the calcium-phosphate method, as described elsewhere (Soneoka et al., 1995). The parental retroviral vector LNCX was used as a negative control. At 48 h post-transfection, virus supernatants were harvested and filtered through a 0.45 µm filter. U373MG and U937 cells were transduced by the retroviral vectors in the presence of 8 µg/ml polybrene, and G418 (1 mg/ml) was added 24 h after transduction. This medium was changed every 3 days and selection was continued for a further 4 weeks. G418-resistant U373MG and U937 cells were subcloned by using a cloning cylinder. For U937 cells, resistant cells were subcloned by limiting dilution. The presence of IE1 was confirmed in both cells by Western blot analysis, immunoprecipitation and immunofluorescence with a monoclonal antibody that can detect IE1 (MAb810; Chemicon).

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AP-1 activation by HCMV IE1

Fig. 1. Effects of IE1 on cellular transcription factors AP-1, Sp1 and Egr-1. (a) Reporter plasmids. The AP-1 reporter plasmid (AP-1 CAT) contains a bacterial CAT gene under the control of a truncated c-fos promoter and five consensus AP-1-binding sites. The truncated c-fos promoter (Δ56) contains 56 bp upstream of the RNA start site, and the TATA box is its only transcriptional element. The Sp1 reporter plasmid (tk CAT9) contains two copies of a synthetic oligonucleotide derived from GC boxes III and IV of the SV40 early promoter, approximately 50 bp upstream of the transcriptional start site of a truncated HSV tk promoter (−37tk CAT). The Egr-1 reporter plasmid (PDGF-A CAT) was constructed by fusing a PDGF-A promoter to a CAT gene. (b) IE1 activates AP-1-driven transcription. U373MG or U937 cells were transfected with 3 µg of the various reporter plasmids, together with 3 µg of the IE1 expression plasmid pRSV-IE1 or the control plasmid pRc/RSV, by using the DEAE-dextran method. For CAT assays, 100 µg protein was incubated with [14C]chloramphenicol for 1 h at 37 °C. The results shown here are the means of five independent transfection assays.

Fig. 2. Effect of mutant IE1s on AP-1. U937 cells were transfected with 3 µg AP-1 reporter plasmid and 3 µg IE1 deletion expression plasmids. CAT assays were performed as described in Fig. 1. The results are the means of three independent transfection assays.

gene fused to a promoter with AP-1-binding sites upstream of a truncated c-fos promoter (Fig. 1a). Transient expression of the CAT enzyme was used to measure the inducibility of AP-1 by IE1. As controls, we also measured the inducibility of Sp1 and Egr-1 by using similar reporter plasmids containing binding sites for these transcription factors (Courey et al., 1989; Khachigian et al., 1995). These reporter plasmids were transfected into the human microglial cell line U373MG and the human promonocytic cell line U937, together with the IE1 expression plasmid pRSV-IE1. When various reporter plasmids were co-transfected with pRc/RSV (lacking IE1), the levels of CAT activity were very low. However, co-transfection with pRSV-IE1 resulted in a large increase in CAT activity from the AP-1 reporter plasmid, while it had no significant effect on CAT expression from a control promoter lacking the AP-1-binding sites (Fig. 1b). As a further control, we used pRc/RSV containing the IE1 sequence in the antisense orientation. This plasmid had no effect on CAT expression driven by any of the promoters described above (data not shown). These data indicated that IE1 could activate transcription from promoters containing AP-1-, but not Sp1- or Egr-1-binding sites.

We also tested the effect of previously characterized IE1 mutants (Lafemina et al., 1989) on activation of AP-1. Plasmids expressing mutant IE1 were transfected to U937 cells together with the AP-1 reporter plasmid. Results of AP-1 activation were compared between wild-type and mutant IE1s. No mutant IE1s induced AP-1-dependent transcription (Fig. 2). This result is consistent with previous reports suggesting that domains responsible for transcriptional activation are spread over the IE1 protein (Lafemina et al., 1989; Stenberg et al., 1990).

In order to perform more quantitative analyses, we constructed cell lines stably expressing IE1. U373MG cells and U937 cells were transduced by a retroviral vector, LNC-gIE1, containing the IE1 gene. The parental vector lacking the IE1 sequence, LNCX, was used as a negative control. Following selection in G418, drug-resistant cells were subcloned and more than 12 subclones were derived from each of the U937
Fig. 3. (a) Expression of IE1 proteins in U937 IE1 subclones. Whole-cell extracts were subjected to 8% SDS–PAGE and electroblotted onto PVDF membrane (Gelman). Immunoblotting was performed with an anti-IE1 monoclonal antibody against exon 2 and 3 (MAB810; Chemicon). The blot was visualized by chemiluminescence by using peroxidase-labelled anti-mouse antibody (Amersham). Lane 1, LNCX cells; 2, IE1-2 cells; 3, IE1-3 cells. (b) AP-1-driven transcription is increased in U373MG and U937 cells expressing IE1 constitutively. Two µg reporter plasmid was transfected into IE1 or LNCX cells and 20 µg protein was analysed. The means of five independent assays are presented.

Fig. 4. Quantitative analysis of mRNA expression of AP-1 subunits in cells expressing IE1 and control cells. Competitor DNAs have binding sites for the PCR primers but contain additional sequences, so that larger-sized products were produced. For U937 cells, PCR template was cDNA from: LNCX cells (—, lanes 1, 5, 9); IE1-2 cells (+, lanes 2, 6, 10); IE1-3 cells (+, lanes 3, 7, 11); and PMA-treated LNCX cells (P, lanes 4, 8, 12). For U373MG cells, PCR template was cDNA from: LNCX-2 cells (—, lanes 1, 5, 9); IE1-1 cells (+, lanes 2, 6, 10); IE1-2 cells (+, lanes 3, 7, 11); and PMA-treated LNCX-2 cells (P, lanes 4, 8, 12). The result shown here is representative of more than five independent assays.
and U373MG populations. The expression of IE1 in the subclones was confirmed by Western blot analysis with a monoclonal antibody specific for an epitope present in exons 2 and 3 of IE1. More than six subclones from each cell line were found to express IE1. Two representative subclones derived from U937, IE1-2 and IE1-3, expressed a protein of approximately 72 kDa, which was not present in the control cell line U937 LNCX (Fig. 3a), and were used in subsequent analysis.

Both IE1-expressing and control cells (U373MG and U937 cells) were transfected with the AP-1, Sp1 and Egr-1 reporter plasmids. Transfection of these reporter plasmids alone or their respective control plasmids resulted in very low levels of CAT activity in cells lacking the IE1 protein. However, transfection of the AP-1 reporter plasmid into cells expressing IE1 always resulted in a dramatic increase in CAT activity (Fig. 3a and b). Again, IE1 had no significant effect on CAT expression from the Sp1 or Egr-1 reporter plasmids. These results confirmed that IE1 could induce AP-1-driven transcription.

IE1 does not have a significant effect on the levels of c-fos and c-jun mRNA

To test whether the increase in AP-1 activity induced by IE1 was due to overexpression of AP-1 components, we compared the amounts of mRNA for c-fos and c-jun in various IE1-expressing subclones. Because the levels of c-fos and c-jun mRNA were too low to be detected by conventional Northern blot hybridization analysis, a quantitative RT–PCR method was used. Total RNAs were prepared from two IE1-expressing subclones and one control subclone from both U373MG and U937 cells, followed by RT–PCR. For quantitative analysis, serially diluted competitors of c-fos and c-jun were added to the fixed amount of cDNA in the PCR reaction. As a control, β-actin RNA was also amplified. When the control cells were treated with PMA, levels of c-fos and c-jun mRNA were significantly increased (Fig. 4a, b; compare lane 5 with lane 8). However, the ratio of target to competitor products was reproducibly identical for a given amount of competitor between IE1-expressing and control cells (Fig. 4a, b; compare, for example, compare lane 5 with lanes 6 and 7). The same experiments were performed with several other subclones and similar results were obtained (data not shown). These results indicated that the levels of c-fos and c-jun mRNA were not changed by expression of IE1 and that IE1 might regulate AP-1 at the post-transcriptional level.

Protein kinase inhibitors suppress IE1 activation of AP-1 in a cell type-dependent manner

Some mitotic stimuli, such as phorbol esters and growth factors, activate AP-1 through the protein kinase C (PKC) pathway, while other reagents, such as transforming growth factor (TGF) and cyclic AMP, seem to control AP-1 independently of PKC (Muller et al., 1989).

To investigate the involvement of protein kinases in the IE1 activation of AP-1, we tested the effects of various protein kinase inhibitors. Several IE1-expressing subclones were transfected with the AP-1 reporter plasmid and were treated with the PKC-specific inhibitor GF109203X (bisindolylmaleimide I) (Toullec et al., 1991), the general protein kinase inhibitor staurosporine (Tamaoki et al., 1986), the protein kinase A (PKA) inhibitor HA1004 [N-(2-guanidinoethyl)-5-isoquinolinesulfonylamide] (Asano & Hidaka, 1984) and the PKC and PKA inhibitor H7 [1-(5-isoquinolinesulfonyl)-2-methylpiperazin] (Hidaka et al., 1984). The results from one representative subclone from U373MG and U937 cells expressing IE1 are shown in Fig. 5.

In U373MG cells expressing IE1, AP-1 activity was blocked efficiently by 10 nM staurosporine but not by GF109203X, HA1004 or H7 (Fig. 5). In contrast, in U937 cells expressing IE1, staurosporine did not have any effect, even at a high concentration, while 5 μM H7 blocked AP-1 activation almost completely. These results suggested that a protein kinase(s) other than PKC and PKA might be involved in AP-1 activation by IE1, and that the mechanism by which IE1 activates AP-1 might differ between the two cell types.
MEKK1 has a highly synergistic effect on AP-1 activation by IE1

AP-1 activity can be modulated through phosphorylation. It is well established that some of the mammalian mitogen-activated protein kinase (MAPK) pathways regulate AP-1 activity (Cano & Mahadevan, 1995; Karin, 1995). The [Raf-1 → MAPK/ extracellular signal-regulated kinase1 (MEK1) → extracellular signal-regulated kinase 1, 2 (ERK 1, 2)] pathway induces c-Fos by activating the c-fos promoter through a serum response element (SRE), while the [MEKK1 → MAPK kinase 4 (MKK4) → c-Jun N-terminal kinase (JNK)] pathway activates c-Jun by phosphorylating c-Jun at its N terminus.

Based on the above results from quantitative RT–PCR and protein kinase inhibitor assays, we tested the possible involvement of the [MEKK1 → MKK4 → JNK] pathway in the IE1 activation of AP-1. U937 IE1 cells and LNCX cells were transfected with plasmids expressing Ras, MEKK1, MKK4 or JNK1, together with the AP-1 reporter plasmid. Co-transfection of an expression vector for MKK4 or JNK1 appeared to have little effect on AP-1 activation by IE1, while Ras increased the level of CAT activity by an average of 2-fold. Co-transfection of an MEKK1 expression vector had a reproducible dramatic effect, resulting in an average 10-fold increase in the level of CAT activity (Fig. 6a). This increase by MEKK1 occurred in a dose-dependent manner (data not shown). To be certain that this observation was specific for IE1, we performed a similar experiment with the Egr-1 reporter plasmid (PDGF-A CAT). IE1 did not increase transcription from this promoter, and none of the above expression vectors had any positive effect on the level of CAT activity (Fig. 6b).

To identify the regions of MEKK1 responsible for IE1 activation of AP-1, we used two previously characterized mutants, KMMEKK, containing a point mutation (K432M) in the catalytic domain and MEKKΔN, lacking the entire regulatory domain (Minden et al., 1994). As shown in Fig. 7, KMMEKK had no significant effect on AP-1 activation by IE1, while MEKKΔN could act synergistically with IE1 to a slightly greater degree than parental MEKK1. This result indicated that the kinase domain was sufficient for the synergistic effect of MEKK1 on AP-1 activation by IE1.

Discussion

We have demonstrated that the IE1 protein of HCMV activates transcription efficiently from a promoter containing AP-1-binding sites, and that this activation does not occur at
the transcriptional level. Although transcription was increased from the AP-1 reporter plasmid in transfection assays, we found by electrophoretic mobility shift assay that AP-1 DNA-binding activity was not increased by IE1 expression (data not shown). Based on the results from experiments with protein kinase inhibitors, we suggest that IE1 activates AP-1 probably through modulation of a protein kinase(s), but different protein kinases may be involved in different cell types. Our data suggest that MEKK1 may be a key factor in this process.

One possible mechanism for IE1 activation of AP-1 is that IE1 interacts directly with c-Fos or c-Jun and thereby modulates AP-1 activity. By various techniques, we tested whether IE1 binds to c-Fos or c-Jun, but failed to find direct association between the two proteins (data not shown). We propose two possible mechanisms involving protein kinase(s) in AP-1 activation by IE1. Firstly, IE1 itself may require phosphorylation by a protein kinase(s) for its function. Although IE1 has been shown to be a nuclear phosphoprotein, it is not yet clear whether phosphorylation is essential for its function. Because MEKK1 has recently also been shown to be present in the nucleus (Fanger et al., 1997), it may phosphorylate IE1 in the nucleus. Alternatively, a protein kinase(s) regulated by MEKK1 could phosphorylate IE1. Secondly, IE1 may control a protein kinase(s) that regulates c-Jun or c-Fos, resulting in activation of AP-1. Because the IE1 protein is present in the nucleus as well as in the cytosol (Otto et al., 1988), it is possible that IE1 may activate a cytosolic protein kinase(s) or a nuclear protein kinase(s). IE1 is known to have a kinase activity and this activity is required for transcription (Pajovic et al., 1997). Therefore, IE1 might regulate a cellular kinase(s) by phosphorylating it. We are currently testing these possibilities.

It is well established that AP-1 activity can be regulated at both the transcriptional and the post-transcriptional levels (Angel & Karin, 1991; Angel & Herrlich, 1994; Karin, 1995). Several viral proteins, such as hepatitis B virus X protein, Epstein–Barr virus latent membrane protein-1 and polyomavirus middle-sized tumour antigen, have been shown to activate AP-1 (Schöntal et al., 1992; Benn et al., 1996; Kieser et al., 1997). However, the underlying mechanism appears to be different depending on the activation protein. Polyomavirus middle-sized tumour antigen activates AP-1 by increasing c-Jun expression, Epstein–Barr virus latent membrane protein-1 up-regulates AP-1 via the JNK cascade and hepatitis B virus X protein induces AP-1 activity by increasing the level of c-fos mRNA and by stimulating protein kinases. Our data show that IE1 may employ an additional method of controlling AP-1. Therefore, all these DNA viruses have evolved to regulate AP-1, probably for their own benefit. Indeed, these DNA viruses can grow much more efficiently when the host cell is in an ‘activated’ state, in which AP-1 is critically involved.

AP-1 is a pleiotropic transcription factor that regulates many important genes involved in the immune response, cell differentiation and tumorigenesis. Induction of this factor by the IE1 protein may lead to the activation of cellular genes important for HCMV itself, and also cause the activation of other viruses residing in the same cells, such as human immunodeficiency virus (HIV). Indeed, a direct effect of HCMV superinfection on HIV-1 replication has been demonstrated (Ho et al., 1990). Furthermore, increased activity of AP-1 may disrupt normal cellular functions and lead eventually to uncontrolled cell growth, inflammation and tumorigenesis. Because HCMV is prevalent in the human population and we have shown IE1 to be a potent activator of the important cellular transcription factor AP-1, it is important to understand more about how IE1 expression activates AP-1 at the molecular level, and to identify the putative protein kinase(s) that is involved.

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