Modulation of T-Cell Activation through Protein Kinase C- or A-Dependent Signalling Pathways Synergistically Increases Human Immunodeficiency Virus Long Terminal Repeat Induction by Cytomegalovirus Immediate-Early Proteins

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By using human CD4+ lymphoblastoid T cells transiently cotransfected with human immunodeficiency virus (HIV) and cytomegalovirus (CMV), we tested whether modulation of T-cell activation through the protein kinase C (PKC) or the protein kinase A (PKA) pathway synergized with CMV immediate-early (IE) proteins in HIV long terminal repeat (LTR) transactivation. Stimulation with phorbol myristate acetate, tumor necrosis factor, or cross-linked antibodies to CD3 and CD28 resulted in modest enhancement (two- to fourfold) of the activity of a luciferase expression vector under control of the HIV LTR. Cotransfection of a vector expressing the CMV IE1 and IE2 proteins under the control of their own promoter enhanced HIV LTR activity 16- to 49-fold. Combination of any one of the above stimuli and CMV IE expression amplified HIV LTR activity 99- to 624-fold. Stimulation of PKA-dependent pathways with forskolin, 8-bromo cyclic AMP, or prostaglandin E2 had a minimal effect on HIV LTR activity, whereas such stimuli resulted in synergistic amplification in cells cotransfected with CMV IE (three- to fivefold increases over the effects of CMV IE alone). This synergism was independent of the NF-κB binding motifs within the HIV LTR. CMV IE2, but not IE1, protein induced HIV transactivation and synergized with signals modulating T-cell activation. The intense synergism observed was superior to the increase in IE protein expression following PKC activation by phorbol myristate acetate. Treatment of cells with PKC inhibitor GF109203X blocked most of the observed synergism. These results show that stimulation of transduction pathways normally unable to induce HIV LTR transcription may become effective in cells doubly infected with HIV and CMV. Furthermore, our results imply that a cellular factor(s) or phosphorylation events induced during cell activation are important for full transactivating efficiency of CMV IE proteins on HIV LTR transactivation.

Study of the mechanisms involved in reactivation of human immunodeficiency virus (HIV) from its normal quiescent state in resting human T lymphocytes is key to understanding the pathogenesis of AIDS (49). Both cell activation and coinfection with other viruses have been shown to increase HIV replication or transactivate its promoter (17, 38, 49).

Among the many opportunistic viruses found in patients with AIDS, cytomegalovirus (CMV) is one of the possible cofactors involved in HIV reactivation. Both clinically and epidemiologically, CMV infection plays a major role in the pathogenesis and outcome of AIDS (14, 44, 63). HIV and CMV can infect T lymphocytes, and both viruses have been described to be present within the same cell (6, 8, 41, 48). Major immediate-early (IE) proteins of CMV, mainly IE2, can transactivate viral and cellular promoters (23, 58, 59). In vitro, CMV infection or IE protein expression can transactivate the HIV long terminal repeat (LTR) and increase HIV replication (2, 3, 10, 13, 24, 39, 46, 47). The mechanism through which this transactivation occurs is unclear. Recent reports have shown that CMV IE proteins may transactivate the HIV promoter through a specific region located near the initiation of RNA transcription (−6/−1) and that these viral proteins may have a role in HIV RNA elongation (2, 3).

Another way of inducing HIV genome transcription in vitro is cell activation. Treatment of T lymphocytes with mitogens (phytohemagglutinin [PHA] and phorbol myristate acetate [PMA]), cytokines (tumor necrosis factor [TNF]), cross-linked antibodies to CD3 and CD28, or specific antigen recognition results in enhanced activity of the HIV LTR or increases HIV replication (15, 20, 22, 28, 31, 32, 38, 40, 49). Such stimuli transactivate the HIV LTR by inducing nuclear translocation of the protein complex NF-κB, which binds to two consensus repeats in the LTR enhancer region (4, 15, 16, 18, 20, 22, 28, 31–33, 40, 61). Their effect as transactivators of the HIV LTR is thought to be dependent mainly, if not solely, on protein kinase C (PKC)-dependent but not PKA-dependent pathways (18, 32, 33, 61).

CMV reactivation from a latent state in T cells may also be mediated by host cell activation. The CMV promoter contains DNA sequences homologous to NF-κB and cyclic AMP (cAMP) response element consensus binding regions (25, 51). Treatment of T cells with the inducer PHA, PMA, or PKA results in transactivation of the CMV promoter (25, 42, 54). It is therefore possible that activation of T cells doubly infected with HIV and CMV can induce reactivation of both viruses simultaneously or individually. Once this happens, the reported bidirectional interaction of the two viruses may occur, leading to increased replication of each virus.

We have tested the hypothesis that following activation of T cells, HIV transactivation by CMV IE proteins would be upregulated compared with the level of transactivation seen in nonactivated cells. For this, we employed T-cell stimuli which utilize two different types of protein kinase pathway: those which are PKC dependent for transactivation of the

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HIV LTR and those which induce the PKA pathway and are unable by themselves to increase HIV LTR transcription.

Our data demonstrate an NF-κB-independent synergistic effect of PKC-dependent stimuli (Ca²⁺/IE protein-induced HIV LTR transactivation). Furthermore, we show that PKA-dependent pathways may play an important role in transactivating the HIV LTR in cells also infected with CMV. These data suggest that IE proteins can cooperate with a cellular factor(s) induced by T-cell activation to exert full transactivating effects on HIV LTR activity.

MATERIALS AND METHODS

Reagents and antibodies. PMA was purchased from Sigma Chemical Co. (St. Louis, Mo.) and stored as a 500-mg/ml solution in phosphate-buffered saline (PBS) at −20°C. Human recombinant TNF-α was a gift of W. Fiers (Ghent, Belgium). Forskolin, Lariboisière, Paris, France) (62). Monoclonal antibody E13, which recognizes IE proteins (37), was kindly provided by M. C. Mazeron and A. Rosetto (Hôpital Lariboisière, Paris, France). Anti-CD3 was purchased from Ortho, Raritan, N.J. Anti-CD28 was purchased from Oncogene, Seattle, Wash.

Vectors. CMV plasmids pRL45, pMP17, and pMP18 were kind gifts from G. Hayward (Johns Hopkins University, Baltimore, Md.) and R. LaFemina (Merck Sharp & Dohme, West Point, Pa.) and have been described previously (43). pRL45 codes for IE1 (exons 1 through 5). pMP17 codes for IE1 (exons 1 through 4), and pMP18 codes for IE2 (exons 1 through 3 and exon 5). HIV LTR wild (w) covers the region from −644 (Xhol) to +78 (HindIII) of the HIV LTR (lymphadenopathy-associated virus type 1, Bru strain). HIV LTR dxB is deleted in the region from −105 to −77, which corresponds to the NF-κB-binding motifs. Both HIV LTR plasmids were a kind gift from A. Rabson, National Institutes of Health, Bethesda, Md. (12, 34). These HIV LTR plasmids were cloned upstream of the luciferase reporter gene, in plasmid pC-luc (11), as previously described (52). Plasmid pCMV-luc was constructed by inserting a HindIII-PvuII fragment containing CMV enhancer-promoter sequences (−524 to −95) (Towne strain) into pC-luc. All plasmids were purified by using Qiagen columns (Diagen, Düsseldorf, Germany), and purity, size, and restriction enzyme maps were verified by agarose gel electrophoresis.

Cells. J.Han, a CD4⁺ human lymphoblastoid cell line derived from the Jurkat cell line, was a kind gift of J. D. Fox (London, England). Cells were routinely tested for mycoplasma and maintained in RPMI 1640 with glutamine, antibiotics, and 5% fetal bovine serum.

Transfections. Five micrograms of the reporter gene and 5 µg of CMV IE plasmid or control (pC-luc) DNA were transfected by using the DEAE-dextran technique into 10⁵ cells, as previously described (27, 30, 43). Cells were then suspended in 10 ml of RPMI-10% serum, aliquoted into 24-well plates at 2 ml per well, and incubated at 37°C for 4 h. At that time, the different reagents were added. In experiments in which coated antibodies were used to stimulate transfected cells, 24-well plates were coated with antibodies in a phosphate solution (200 µl per well) at 4°C for 6 h and washed twice with PBS, and then cells were added. Cultures were then incubated for 18 h and harvested, and luciferase activity was read in a scintillation counter (LKB) by using a chemiluminescence measurement program as described previously (43). A 2.5-µl sample of the supernatant was used to dose total protein by using the Bio-Rad technique (7). Results were calculated as background counts per minute per microgram of protein. The experimental results shown in the figures are representative of three to five experiments performed under the same conditions.

Northern (RNA) blot of transfected cells. Forty million cells were transfected with 20 µg of a control plasmid (pC-luc) or pRL45 as described above. After 16 h of incubation with the different stimuli, total RNA was harvested by using the hot-phenol technique. RNA was electrophoresed in gels containing 1% agarose, 20 mM MOPS (morpholinepropanesulfonic acid) (pH 7), 1 mM EDTA, 5 mM Na-actate, 0.7% formaldehyde, and 8 µg of ethidium bromide per ml at 70 V for 2 h. The same amount of RNA was loaded into each well, as quantified by ethidium bromide staining. The RNA was immediately transferred under vacuum to a nylon membrane (Hybond N⁺; Amersham) by using a 50 mM NaOH solution.

RESULTS

T-cell activation by PMA, TNF, or cross-linked antibodies to CD3 and CD28 synergizes with CMV IE in transactivation of the HIV LTR. We determined whether cellular stimuli known to transactivate the HIV LTR would synergize with the IE proteins of CMV. J.Han cells were transiently transfected with HIV LTR w-luc and subsequently treated with different stimuli. Treatment with TNF or PMA transactivated HIV LTR w-luc threefold. Cotransfection of a plasmid coding for CMV IE1 and IE2 (pRL45) induced greater HIV LTR w-luc transactivation (49-fold) (Table 1). However, when J.Han cells were cotransfected transiently with CMV IE and HIV LTR w-luc and subsequently treated with TNF or PMA, the HIV LTR was transactivated 225- and 624-fold, respectively. This level of transactivation, induced by the combination of CMV IE and PMA or TNF, was much higher than that induced by either stimulus alone.

We then determined whether other stimuli, such as those which mimic antigen presentation to T cells, would induce...
TABLE 1. Transactivation of the HIV LTR by CMV IE in synergy with T-cell activation

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>LTR w-luc</th>
<th>LTR δkB-luc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>CMV IE</td>
</tr>
<tr>
<td>None (control)</td>
<td>141</td>
<td>6,916 (49)</td>
</tr>
<tr>
<td>TNF</td>
<td>432 (3)</td>
<td>31,735 (225)</td>
</tr>
<tr>
<td>PMA</td>
<td>427 (3)</td>
<td>88,035 (624)</td>
</tr>
<tr>
<td>None (control)</td>
<td>126</td>
<td>2,083 (17)</td>
</tr>
<tr>
<td>Anti-CD3</td>
<td>177 (1)</td>
<td>4,269 (34)</td>
</tr>
<tr>
<td>Anti-CD28</td>
<td>117 (1)</td>
<td>1,695 (13)</td>
</tr>
<tr>
<td>Anti-CD3 + anti-CD28</td>
<td>340 (2)</td>
<td>12,477 (99)</td>
</tr>
<tr>
<td>None (control)</td>
<td>91</td>
<td>6,326 (69)</td>
</tr>
<tr>
<td>Forskolin</td>
<td>146 (2)</td>
<td>28,336 (311)</td>
</tr>
<tr>
<td>cAMP</td>
<td>101 (1)</td>
<td>18,745 (206)</td>
</tr>
<tr>
<td>PGE2</td>
<td>96 (1)</td>
<td>16,466 (181)</td>
</tr>
</tbody>
</table>

* J.Jhan cells (10⁷) were cotransfected with an HIV LTR (i) and a control or CMV IE plasmid (pRL45) and subsequently stimulated or not with (i) TNF-α (50 ng/ml) or PMA (20 ng/ml); (ii) cross-linked anti-CD3 (10 μg/ml), anti-CD28 (10 μg/ml), or both antibodies (total of 10 μg/ml); (iii) forskolin (10⁻⁴ M), 8-bromo cAMP (cAMP) (10⁻⁴ M), or PGE2 (10⁻⁶ M).

Synergistic transactivation of the HIV LTR in the presence of CMV IE proteins. To test this, J.Jhan cells transiently transfected with the HIV LTR were incubated in the presence of cross-linked anti-CD3 receptor antibodies alone or in combination with anti-CD28 antibodies. When HIV LTR w-luc was transiently cotransfected with CMV IE into J.Jhan cells which were subsequently activated with cross-linked anti-CD3 and anti-CD28 antibodies, synergistic transactivation of the HIV LTR was observed (150-fold) (Table 2). Only a two- to threefold increase in the level of HIV LTR transactivation was observed when anti-CD3 and anti-CD28 antibodies were combined. Anti-CD3 antibodies alone induced HIV LTR transactivation 1.5- to 2-fold, while anti-CD28 antibodies alone did not induce HIV LTR transactivation. Cotransfection with CMV IE in the absence of antibody stimulation transactivated HIV LTR w-luc 16-fold.

T-cell activation of cAMP-dependent pathways synergizes with the transactivating effect of CMV IE on the HIV LTR. The PKA-dependent pathway is functional in T cells, and its stimulation has been associated with transactivation of different cellular genes (1, 9). However, stimulation of this pathway in promonocytic cell lines does not increase HIV replication (32). We tested whether induction of cAMP, a mediator in the PKA pathway, could transactivate the HIV LTR in J.Jhan cells. Different agents which increase or are agonists of intracellular cAMP (forskolin, PGE2, and 8-bromo cAMP) did not transactivate the HIV LTR when added individually to T cells transiently transfected with HIV LTR w-luc. However, when these stimuli were added to J.Jhan cells cotransfected with HIV LTR w-luc and CMV IE, synergistic transactivation of the HIV LTR was observed (311-fold for forskolin, compared with 69-fold induction by CMV IE in the absence of forskolin) (Table 1).

Synergism between T-cell activation and CMV IE proteins is independent of the NF-κB binding motifs in the HIV LTR. Cellular stimulation with TNF, PMA, or anti-CD3 alone or combined with anti-CD28 antibodies results in HIV LTR transactivation or increased HIV replication, probably by inducing nuclear translocation of NF-κB (4, 40). This effect can be decreased by inhibitors of the PKC pathway, such as H7 (32, 61). To test whether the synergistic effect provided by these signals and CMV IE proteins in the transactivation of HIV LTR w-luc was NF-κB dependent, HIV LTR luc deleted of its NF-κB binding sites was used (HIV LTR δkB-luc). As shown in Table 1, HIV LTR transactivation mediated by CMV IE was synergistically increased when T cells were treated with TNF, PMA, or a combination of anti-CD3 and anti-CD28 antibodies, despite the absence of the NF-κB binding motif within the HIV LTR.

It has been reported that in vitro activation of the PKA pathway may lead to NF-κB nuclear translocation (53). To exclude the possibility of an NF-κB-dependent effect of PKA-mediated cell activation and CMV IE on the HIV LTR, cells were transfected with HIV LTR δkB-luc and treated with PKA-stimulating agents in the presence or absence of CMV IE. The synergistic effect continued to be observed despite the lack of the NF-κB binding site (Table 1).

CMV IE2 but not IE1 proteins synergistically transactivate the HIV LTR. The region encoding the major CMV IE

**TABLE 2. Transcriptional activity of the CMV promoter induced by T-cell activation and CMV IE proteins**

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>CMV promoter</th>
<th>Control plasmid</th>
<th>CMV IE plasmid</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>1,611</td>
<td>1,205</td>
<td>1,957 (1.6)</td>
</tr>
<tr>
<td>TNF</td>
<td>2,525 (1.5)</td>
<td>2,092 (1.7)</td>
<td>2,632 (1.3)</td>
</tr>
<tr>
<td>PMA</td>
<td>4,009 (2.7)</td>
<td>3,178 (2.6)</td>
<td>6,532 (3.3)</td>
</tr>
<tr>
<td>Forskolin</td>
<td>4,000 (2.4)</td>
<td>3,170 (2.6)</td>
<td>3,759 (2.0)</td>
</tr>
</tbody>
</table>

* J.Jhan cells (10⁷) were transfected with (i) pCMV-luc and stimulated or not with TNF (50 ng/ml), PMA (20 ng/ml), or forskolin (10⁻⁴ M) or (ii) pCMV-luc and a control plasmid or CMV IE1 and IE2 (pRL45) and stimulated or not with the stimuli listed.
proteins is divided into the IE1 and IE2 subregions, both of which are present in pRL45 (43, 55). To determine which of the two CMV IE regions mediates transactivation of the HIV LTR and synergizes with T-cell activation, J.Jhan cells were cotransfected with HIV LTR w-luc and vectors permitting expression of either CMV IE1 (pMP17), CMV IE2 (pMP18), or both (pRL45). CMV IE2 proteins induced HIV LTR transactivation, which was further (synergistically) amplified following T-cell activation with PMA or forskolin (Fig. 1). CMV IE1 proteins alone did not induce transactivation of HIV LTR w-luc or synergize with PMA or forskolin stimulation. Nonetheless, the transactivation mediated by CMV IE1 and IE2 together (pRL45) was greater than that mediated by CMV IE2 alone (pMP18).

Synergy between T-cell activation and CMV IE proteins correlates with an increase of CMV IE protein expression following stimulation with TNF and forskolin. The synergistic transactivating effect of PMA may be mediated by a mechanism other than just an increase of CMV IE protein expression. Different stimuli, such as PMA and forskolin, are known to transactivate the major IE promoter region of CMV (25, 42, 54). It is possible that the synergistic effects provided by cell activation in association with CMV IE proteins were due to an increase in the quantity of CMV IE proteins. To test this hypothesis, the following experiments were performed.

We first determined whether the promoter of CMV present within the pRL45 vector could be transactivated by different T-cell stimuli. For this, the CMV enhancer-promoter was linked to the luciferase reporter gene, yielding pCMV-luc. This plasmid was transiently transfected into J.Jhan cells which were subsequently stimulated with TNF, PMA, or forskolin. As shown in Table 2, a slight (two- to threefold) increase over the baseline activity of the CMV promoter was observed with these stimuli.

Since CMV promoter activity is enhanced by the CMV IE proteins (23, 35, 60) and because in the cotransfection experiments the CMV IE proteins encoded by pRL45 may have autoregulated their own promoter, we tested whether stimuli such as PMA and forskolin increased the quantity of CMV IE proteins, which would in turn further transactivate the CMV promoter. For this purpose, J.Jhan cells were transiently cotransfected with the CMV promoter (pCMV-luc) and plasmid pRL45 (CMV IE1 and IE2), followed or not by stimulation with PMA or forskolin. The increase in pCMV-luc activity induced by PMA or forskolin relative to that in untreated cells was not proportionally increased in the presence of CMV IE proteins coded by pRL45 (Table 2).

To determine whether the transactivation of pCMV-luc induced by cellular activation correlated with a similar increase in IE steady-state RNA, we performed Northern blot analysis of J.Jhan cells transiently transfected with pRL45 and stimulated or not with TNF, PMA, and forskolin. As shown in Fig. 2, a slight increase in CMV IE RNA was observed in stimulated cells compared with unstimulated cells. Densitometric scanning showed 1.3-, 3.1-, and 2.1-fold increases in steady-state RNA following TNF, PMA, and forskolin, respectively, compared with that obtained from unstimulated CMV IE-transfected cells.

We next assessed the amount of IE protein produced in J.Jhan cells transiently transfected with pRL45 and stimulated or not with TNF, PMA, or forskolin. With Western blot techniques, a moderate increase in the amount of the 76- and 82-kDa IE proteins was observed with monoclonal antibody E13 (Fig. 3). Densitometric analysis of the bands disclosed that CMV IE proteins were increased 2.3-, 4.7-,
and 3.6-fold following stimulation with TNF, PMA, and forskolin, respectively. The increase in both IE RNA and proteins following T-cell activation correlated with the increase in CMV promoter transactivation (Table 2).

To determine whether increasing quantities of CMV IE would result in increased synergy with T-cell activation, a fixed quantity of HIV LTR w-luc or dxB-luc (5 μg/10^7 cells) was cotransfected with increasing amounts of plasmid pRL45 in the presence or absence of PMA. J.Jhan cells were cotransfected with HIV LTR w-luc (A) or HIV LTR dxB-luc (B) and increasing doses of pRL45 and subsequently stimulated or not with PMA (20 ng/ml).

![Figure 4](http://jvi.asm.org/)

**FIG. 4.** Transcriptional activity of the HIV LTR induced by increasing quantities of a transfected CMV IE plasmid (pRL45) in the presence or absence of PMA. J.Jhan cells were cotransfected with HIV LTR w-luc (A) or HIV LTR dxB-luc (B) and increasing doses of pRL45 and subsequently stimulated or not with PMA (20 ng/ml).

TABLE 3. Effects of PKC inhibitor GF109203X treatment on transactivation of the HIV-1 promoter by a CMV IE expression vector and modulation of T-cell activation^d^

<table>
<thead>
<tr>
<th>LTR and stimulus</th>
<th>Mock transfection</th>
<th>CMV IE transfection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Luciferase U/μg of protein (% reduction vs control)</td>
<td></td>
</tr>
<tr>
<td>LTR and stimulus</td>
<td>LTRw</td>
<td>LTRw</td>
</tr>
<tr>
<td>PMA</td>
<td>2</td>
<td>150</td>
</tr>
<tr>
<td>Forskolin</td>
<td>2</td>
<td>1.178</td>
</tr>
<tr>
<td>PMA</td>
<td>4</td>
<td>2,991</td>
</tr>
<tr>
<td>Forskolin</td>
<td>3</td>
<td>1.1,78</td>
</tr>
</tbody>
</table>

^d^ J.Jhan cells (10^7) were cotransfected with an HIV LTR and a control or a CMV IE plasmid and then treated or not with GF109203X (500 nM) for 30 min before being stimulated or not with PMA (20 ng/ml) or forskolin (10^-4 M).

Discussion

Our data demonstrate that different T-cell activation pathways can synergize with the effects of CMV IE proteins in transactivation of the HIV LTR. The synergy between T-cell activation and CMV IE proteins can be mediated by IE2. Greater induction of HIV LTR activity by a plasmid that encodes both IE1 and IE2 proteins (pRL45) compared with one that encodes IE2 alone (pMP18), either as single effectors or in combination with T-cell activation, reflects the dominant stimulatory effect of IE1 proteins on the CMV promoter. This effect probably increases the availability of IE2 proteins, known as being the main transactivator proteins of homologous and heterologous promoters (2, 24, 43, 55, 58, 59).

T-cell stimuli which are known to transactivate the HIV promoter (LTR) or increase HIV replication (15, 16, 20, 22, 28, 31, 32, 40) can synergize with the effects of CMV IE proteins to increase transactivation of the HIV LTR, PMA, TNF, or stimulation of the CD3 receptor apparently activates the HIV LTR through a common mechanism: nuclear translocation of transcription factor NF-κB, which binds to the enhancer region of the LTR (4, 15, 18, 20, 22, 28, 31-33, 40, 61). Inhibition of the PKC pathway abolishes HIV LTR transactivation or viral replication mediated by PMA, TNF, or activation of the CD3 and CD28 surface receptors (32, 61). We have shown with our human T-cell model that these different stimuli synergize with CMV IE proteins in transactivation of the HIV LTR, even when the NF-κB motifs are deleted from the LTR. This indicates that the observed synergy is mediated through HIV LTR sequences different from the 10-bp repeat consensus motifs of the HIV enhancer which bind and respond to the NF-κB protein complex. Furthermore, by using HIV LTR vectors deleted of the TAR or SP1 region, we have excluded the possibility that the described synergy requires the presence of these regions (data not shown).

Induction of the PKA pathway in T cells through an increase of intracellular cAMP results in transactivation of the promoter of human T-cell lymphotropic virus type I (45) but not of the HIV LTR, as reported by others for monocytic cell lines (32) and confirmed with T cells in the present study.
However, we demonstrate that this T-cell activation pathway can participate in HIV LTR transactivation in the presence of CMV IE proteins, resulting in a synergistic effect. Of particular clinical relevance is the fact that PGE₂, a widespread mediator of inflammation, can be effective in transactivating the HIV LTR in this model. PGE₂ is known to increase cAMP (30).

It is of interest that two very different and, in certain circumstances, antagonistic T-lymphocyte activation pathways such as those dependent on PKC and PKA (5, 27, 29, 30, 36, 57) can each transactivate the HIV LTR more efficiently in the presence of CMV IE proteins. Our findings suggest that in doubly infected cells, the repertoire of membrane signals which can induce HIV reactivation is wider than that in cells infected with HIV alone and includes signals leading to cAMP increase and PKA activation. Simultaneous activation with two different stimuli which interact in an antagonistic way, the PKC and PKA pathways (i.e., treatment with TNF and forskolin) not only did not induce a further increase in the level of transactivation of the HIV LTR but resulted in a slight antagonistic effect (data not shown), even though cross talk between these pathways has been described (64, 65).

The mechanism(s) involved in this intriguing synergism may be manifold. One possible mechanism is an increase in IE protein expression driven by the CMV promoter as a result of T-cell activation. This promoter contains NF-κB motifs (18-bp repeats) and cAMP response element-binding sites (19-bp repeats) which respond to cAMP increases (25, 42, 51, 54). The results of the different experimental approaches in this study suggest that T-cell activation leads to a moderate increase in the level of CMV promoter activity and thus of the quantity of CMV IE RNA and proteins within the cell. Such an increase correlates with the increased level of HIV LTR transactivation following treatment with stimuli such as TNF and forskolin. However, the quantitative increase of CMV IE proteins, as detected by Western blot following PMA treatment, does not fully correlate with the functional transactivation experiments of HIV LTR activity done in parallel. Interestingly, the synergy between CMV IE proteins and T-cell activation was observed in the transactivation of a heterologous promoter (the HIV LTR) but not of the homologous promoter, suggesting that CMV IE proteins function through different mechanisms for each promoter.

Therefore, a second or additional possibility to explain this phenomenon is that modulation of T-cell activation induces or modifies host cell proteins which interact with CMV IE proteins, rendering them more efficient, as is the case for the E1A protein of adenovirus (26), VP16 of herpes simplex virus (56), or the tax protein of human T-cell lymphotropic virus type I (50). In our model, CMV IE proteins were able to transactivate the HIV LTR in the absence of T-cell activation, suggesting that the mechanism involved, which could be interaction of a putative protein(s) with CMV IE proteins, is constitutively expressed, although further inducible, in the lymphoblastoid cell line used. Like CMV IE proteins, the tai protein of HIV transactivates the HIV LTR without other stimuli but T-cell activation further enhances its effects (21). The fact that very diverse activation pathways, such as PKC and PKA, converge to increase the transactivating function of CMV IE proteins is of interest. The synergism mediated by PMA was almost completely abolished by a newly developed PKC inhibitor shown to be more specific than staurosporin, since it did not inhibit the synergism mediated by forskolin (62). Phosphorylation of intermediate host cell proteins by PKC or PKA may render them fully functional in this interaction with CMV IE proteins. Such a situation would not be unprecedented, since the cellular transcription factor that binds the cAMP response element, CREB, needs phosphorylation to exert its full transactivating properties (19). Finally, phosphorylation of CMV-IE proteins themselves cannot be excluded.

In this study, we provide evidence that T-cell activation is especially efficient on HIV genome reactivation in cells coinfected with HIV and CMV. We have expanded the list of possible T-cell stimuli which can induce transactivation of the HIV LTR in the presence of CMV IE proteins by showing the functionality of stimuli such as those that induce PKA-dependent pathways. Cells coinfected with both viruses could be a source for dissemination of either virus following T-cell activation. Our observations make it possible to raise the intriguing concept that a cellular factor(s) associated with T-cell activation and dependent on phosphorylation modulates the transactivating effect of CMV IE proteins on HIV genome transcription.

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