The EMBO Journal vol.13 no.23 pp.5559–5569, 1994

The cytoplasmic domain of CD4 plays a critical role during the early stages of HIV infection in T-cells

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Communicated by M.Dorée

The role played by the cytoplasmic domain of the CD4 molecule in the process of HIV infection was investigated, using A2.01 cells which express different forms of the CD4 gene. A delay in HIV production was consistently observed in cells expressing a truncated CD4 which lacks the cytoplasmic domain (CD4.401) compared with cells expressing the wild type CD4. The delay was much less in cells expressing a hybrid CD4–CD8 molecule (amino acids 1–177 of CD4 fused to the hinge, transmembrane and cytoplasmic domains of CD8). Yet the extent of viral entry and reverse transcription, monitored by semi-quantitative PCR, was similar in each cell type studied. For further study of the mechanism responsible for delayed HIV replication in the A2.01/CD4.401 cell line, cells were treated with phytohaemagglutinin (PHA), 24 h after HIV infection. Under such experimental conditions HIV production was detected at the same time in the culture supernatants of A2.01/CD4 and A2.01/CD4.401 cells. Moreover, we found that CD4 oligomerization by HIV-1 induced NF-κB translocation in A2.01/CD4 and A2.01/CD4–CD8 but not in A2.01/CD4.401 cells. This was consistent with CAT assay experiments which provided evidence for Tat-independent NF-κB mediated activation of HIV-1 LTR promoter after HIV binding to CD4 in A2.01/CD4 and A2.01/CD4–CD8 but not in A2.01/CD4.401 cells. In contrast to results published recently by Tremblay *et al.* (1994, *EMBO J.*, 13, 774–783), we propose that a positive cellular signal initiated following oligomerization of the CD4 by the virus itself is involved in NF-κB-dependent early HIV transcription in A2.01/CD4 cells.

Key words: CD4/HIV infection/signalling

Introduction

The human immunodeficiency virus type 1 (HIV-1) is the primary aetiological agent of acquired immunodeficiency syndrome (AIDS) and associated diseases (Barré-Sinoussi *et al.*, 1983; Gallo *et al.*, 1984). In individuals infected with HIV, the $CD4^+$ T-lymphocyte is one of the major cell types producing the virus (Klatzmann *et al.*, 1984a), and progression towards AIDS is mainly characterized by the depletion of the $CD4^+$ T-lymphocyte population. Indeed, the primary high-affinity cellular receptor for HIV is the CD4 molecule (reviewed in Sattentau and Weiss, 1988), an integral membrane glycoprotein of ~55 kDa that contains four extracellular domains showing structural homology with immunoglobulin (Ig) V regions (Ryu *et al.*, 1990; Wang *et al.*, 1990). The binding site for the external HIV-1 envelope glycoprotein (HIV-1gp120^{env}) is located within the IgVκ-like D1 domain of CD4 (reviewed in Arthos *et al.*, 1990).

The function of CD4 during the HIV infection process has been mainly analysed using either anti-CD4 monoclonal antibodies (mAbs) or cells expressing mutant forms of CD4. Anti-CD4 mAbs have been shown to interfere with the virus life cycle either by inhibiting virion-anchored gp120 binding to the CD4 cell surface during the infection process, or by inhibiting syncytia formation, or by interfering with both events (Dalgleish *et al.*, 1984; Klatzmann *et al.*, 1984b; Dougal *et al.*, 1985). More recently, some anti-CD4 mAbs have been shown to be able to interfere with the HIV replicative life cycle at a post-binding step either before or after fusion has occurred (Burkly *et al.*, 1992; Hasunuma *et al.*, 1992; Moore *et al.*, 1992; Rieber *et al.*, 1992; Corbeau *et al.*, 1993). Among these mAbs, some act by modulating the CD4 pathway of T-cell activation (Benkirane *et al.*, 1993). These latter results suggest that the role of CD4 in the post-fusion step of the HIV replicative cycle is likely to be underestimated. In parallel, studies using cells expressing truncated CD4, CD4–CD8 hybrid molecules or mutant forms of CD4 have shown that the cytoplasmic domain of CD4 is not required for the binding of gp120^{env} and internalization of HIV (Bedinger *et al.*, 1988; Maddon *et al.*, 1988; Golding *et al.*, 1993; Tremblay *et al.*, 1994). Although cells expressing a truncated form of CD4 or a CD4–CD8 hybrid molecule are susceptible to HIV-1 infection, these cells have shown a variability to support HIV replication which is characterized by a delay in viral particle production (Poulin *et al.*, 1991). The above considerations emphasize the fact that the CD4–CD8 and mutated forms of CD4 are fusion competent but that cells carrying these molecules present a defect resulting in delayed HIV production.

Although HIV can enter resting T-cells (Zack *et al.*, 1990), mitogenic stimulation is required to ensure complete reverse transcription and/or integration, and to induce subsequent progeny virus production (Tong-Starksen *et al.*, 1989; Stevenson *et al.*, 1990a,b; Zack *et al.*, 1992). Provirus transcription is under the control of recognition sequences located in the 5′ long-terminal repeat (LTR) that bind host cell transcription factors (reviewed in Gaynor, 1992). The first transcripts, generated during so-called 'basal transcription', encode regulatory proteins

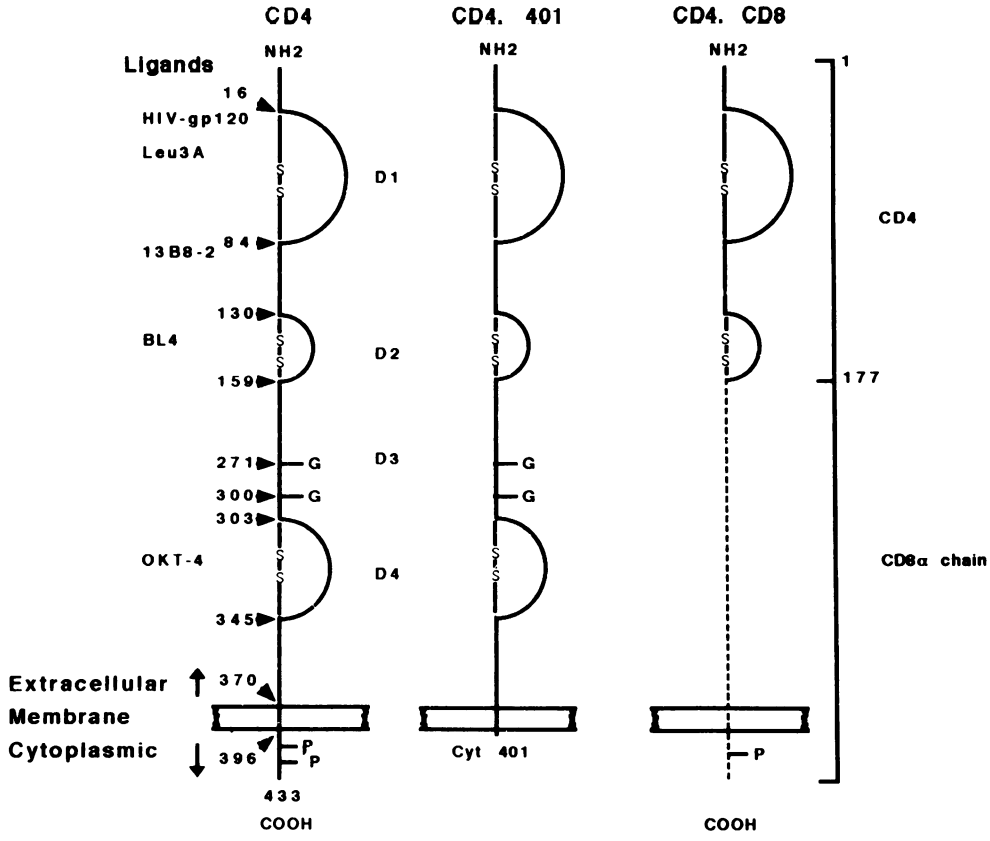

Fig. 1. Schematic diagram of the different forms of human CD4 glycoprotein expressed on the surface of the transfected A2.01 human T-cell lines used in this study: CD4, refers to the wild type CD4 molecule; CD4.401, refers to a truncated form of CD4 at position 401; and CD4–CD8 refers to a hybrid molecule in which the hinge, transmembrane and cytoplasmic domain of CD8 α chain is substituted into CD4 downstream of the amino acid at position 177. Numbering of the CD4 protein is based on the structure of the mature processed protein. Figure adapted from the work of Bedinger *et al.* (1988).

including Tat, which is necessary for mRNA elongation (Jeang and Berkhout, 1992).

It remains possible that HIV particle binding to CD4 may modulate basal HIV transcription by modifying the activation status of T-cells through the CD4 signal. Indeed, CD4, besides being the HIV receptor, is primarily an adhesion molecule that stabilizes the MHC class II–T-cell receptor complex interactions (Lamarre *et al.*, 1989), and a signal-transducing molecule by its association with the protein tyrosine kinase (PTK) $p56^{lck}$ during T-cell activation (Rudd *et al.*, 1988). Cross-linking of surface CD4 by anti-CD4 mAb or HIV-1gp120^{env} induces autophosphorylation of $p56^{lck}$ and/or phosphorylation of $p56^{lck}$ substrates (Veillette *et al.*, 1989; Hivroz *et al.*, 1993), and mutations in CD4 that abolish interaction with $p56^{lck}$ result in decreased IL-2 secretion following antigenic stimulation (Glaichenhaus *et al.*, 1991). Accordingly, HIV may use CD4 not only as a receptor but may also take advantage of the signal transduction function of this molecule to prepare the cell ready for post-fusion events.

The objective of this study was to gain a better understanding of the role played by the cytoplasmic domain of CD4 in the process of HIV infection and, in particular, to determine precisely whether it could modulate HIV expression. We found that HIV-1-mediated oligomerization of CD4 induces NF-κB translocation in CEM cells thereby allowing κB-dependent early transcription of HIV to occur.

Results

Delayed HIV-1 production in cells expressing truncated CD4 or hybrid CD4–CD8 molecules

We first compared the capacity of HIV-1LAI to infect and replicate in $CD4^+$ CEM cells and A2.01 (CD4 negative T-cell line) transfected either with the wild type human CD4 gene (A2.01/CD4), a truncated form of CD4 lacking the whole cytoplasmic domain (A2.01/CD4.401), or a hybrid molecule containing the first 177 amino acids of CD4 fused to the CD8 hinge, transmembrane and cytoplasmic domain of CD8 α chain (A2.01/CD4–CD8) (Figure 1). At high virus concentration ($10^5\times$ $TCID_{50}$) the RT reached a plateau within 7 days of infection in CEM and A2.01/CD4, and was also detected in A2.01/CD4–CD8 and A2.01/CD4.401 cells (Figure 2a). However, RT activity was much lower in A2.01/CD4.401 cell culture supernatants. At lower virus input ($10^4\times$ and $10^3\times$ $TCID_{50}$) a delay in viral production was consistently observed in A2.01/CD4.401 cells compared with other cell lines including A2.01/CD4–CD8. It is noteworthy that at $10^4\times$ $TCID_{50}$ viral production in A2.01/CD4–CD8 was slightly delayed compared with cells expressing the

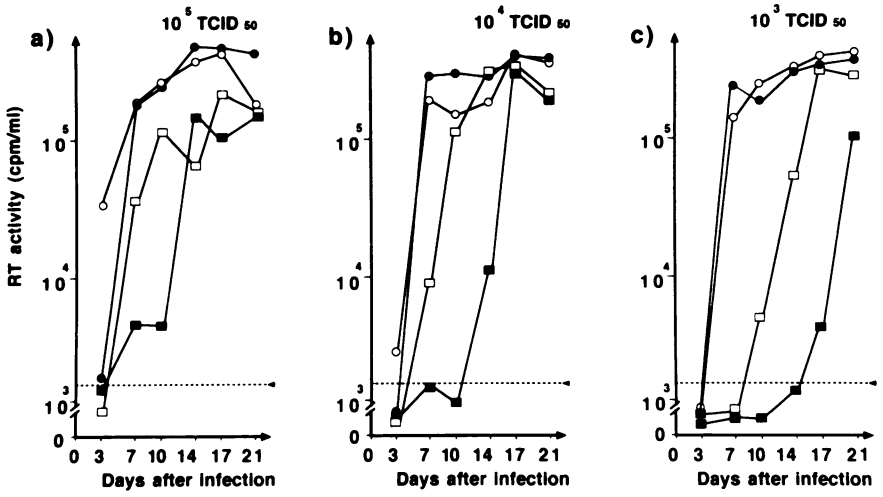

Fig. 2. Infection of different cell lines with HIV-1. CEM (○), A2.01/CD4 (●), A2.01/CD4−CD8 (□), and A2.01/CD4.401 cells (■) were exposed to $10^5\times$ $TCID_{50}$ (**a**), $10^4\times$ $TCID_{50}$ (**b**) or $10^3\times$ $TCID_{50}$ (**c**) of HIV-1LAI and extensively washed before culture. Viral particle production was followed by measuring RT activity in the cell-free culture supernatant. RT activity less than 1.5×10^3 c.p.m./ml was considered as negative (dashed line). The data has been calculated from duplicate experiments. Representative experiment out of three.

full-length CD4 and that this delay was higher at $10^3\times$ $TCID_{50}$ (Figure 2b and c). Syncytia were observed in each culture positive for viral production (data not shown).

To investigate the reason for delayed viral particle production in A2.01/CD4−CD8 and A2.01/CD4.401 cells, we first analysed the expression of CD4 and mutant forms of CD4 on the surface of transfected A2.01 cells by cytofluorometry using Leu 3A (an anti-CD4 mAb specific for the Ig CDR2-like region of the D1 domain), 13B8-2 (an anti-CD4 mAb specific for Ig CDR3-like region of the D1 domain), BL4 (an anti-CD4 mAb specific for the D2 domain) and OKT4 (an anti-CD4 mAb specific for the D4 domain) (Figure 3). Except for A2.01/CD4−CD8, which lacks expression of the OKT4 epitope as a consequence of the D3 and D4 domains of CD4 being changed for CD8 sequence, all other cell lines were stained with the four anti-CD4 mAbs. Moreover, this analysis revealed that A2.01/CD4−CD8 and A2.01/CD4.401 expressed ~2-fold more CD4 molecules than the cells expressing the wild type CD4.

These results indicate that the major epitopes in the D1 and D2 domains of CD4 are conserved on the A2.01/CD4, A2.01/CD4.401 and A2.01/CD4−CD8 cells and that the number of CD4 molecules expressed on the cell surface cannot account for the lag period in the production of progeny virus observed in A2.01/CD4−CD8 and A2.01/CD4.401 cells compared with A2.01/CD4 cells.

Internalization and retrotranscription of HIV-1 do not require the cytoplasmic domain of CD4

We examined whether the delay observed for the appearance of RT activity in A2.01/CD4.401 and, to a lesser extent, A2.01/CD4−CD8 cultures could be attributed to a difference in virus binding to and/or entry into cells, or to the ability of HIV-1 to be fully retrotranscribed in those cell lines. The presence and structure of HIV-1 DNA in infected cells was studied by means of M667/M668 and M667/GAG3 oligonucleotide primer pairs, which detect respectively all intermediate molecules formed during reverse transcription or only full-length viral DNA (Benkirane *et al.*, 1993). PCR amplification using the primer pair β globin I/β globin II was performed as an internal control for DNA extraction. PCR quantification was performed using the 8.E5 cell line. This cell line contains a single integrated copy of HIV-1, therefore allowing assessment of the number of HIV copies present in the samples tested. Using the M667/M668 oligonucleotide primer pair, HIV-1 DNA was found in all cell types 6 h after viral exposure (Figure 4a). No HIV DNA was found in the controls that consisted of uninfected CEM cells or CEM cells exposed to heat-inactivated HIV-1LAI. Next, the extent of reverse transcription in those cells was tested using the M667/GAG3 oligonucleotide primer pair, which detects completely reverse transcribed HIV DNA molecules. As shown in Figure 4b, we found full-length viral DNA in the sample prepared from each of the cell lines exposed to HIV-1, 24 h after viral exposure, regardless of the expression of the native or mutant forms of CD4.

We then analysed HIV-1LAI RNA transcription in infected cells on days 3, 7, 10 and 14, following exposure to HIV-1. We used the M667/VPR2 oligonucleotide primer

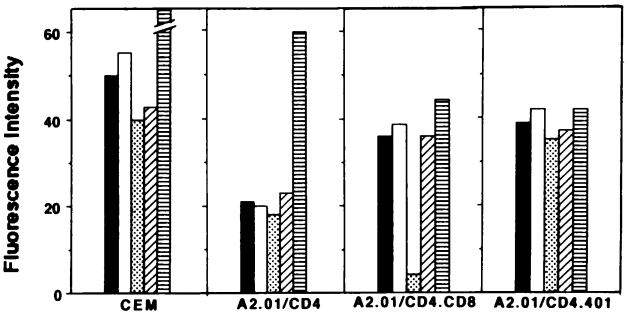

Fig. 3. Cell-surface expression of CD4, truncated CD4 and CD4−CD8 in transfected A2.01 cells. CEM, A2.01/CD4, A2.01/CD4−CD8 and A2.01/CD4.401 cells were incubated with medium alone (to determine the background), anti-CD4 mAb Leu 3A (■), 13B8-2 (□), OKT4 (▨), or BL4 (▨), or anti-β2m mAb B1-1G6 (▤). mAb binding was detected by a FITC-labelled RAM Ig. The mean fluorescence intensity was recorded using the linear channels 0–1024. The figure shows the mean fluorescence intensity after the background value has been subtracted (this value varies from 4 to 8 depending on the cell line analysed).

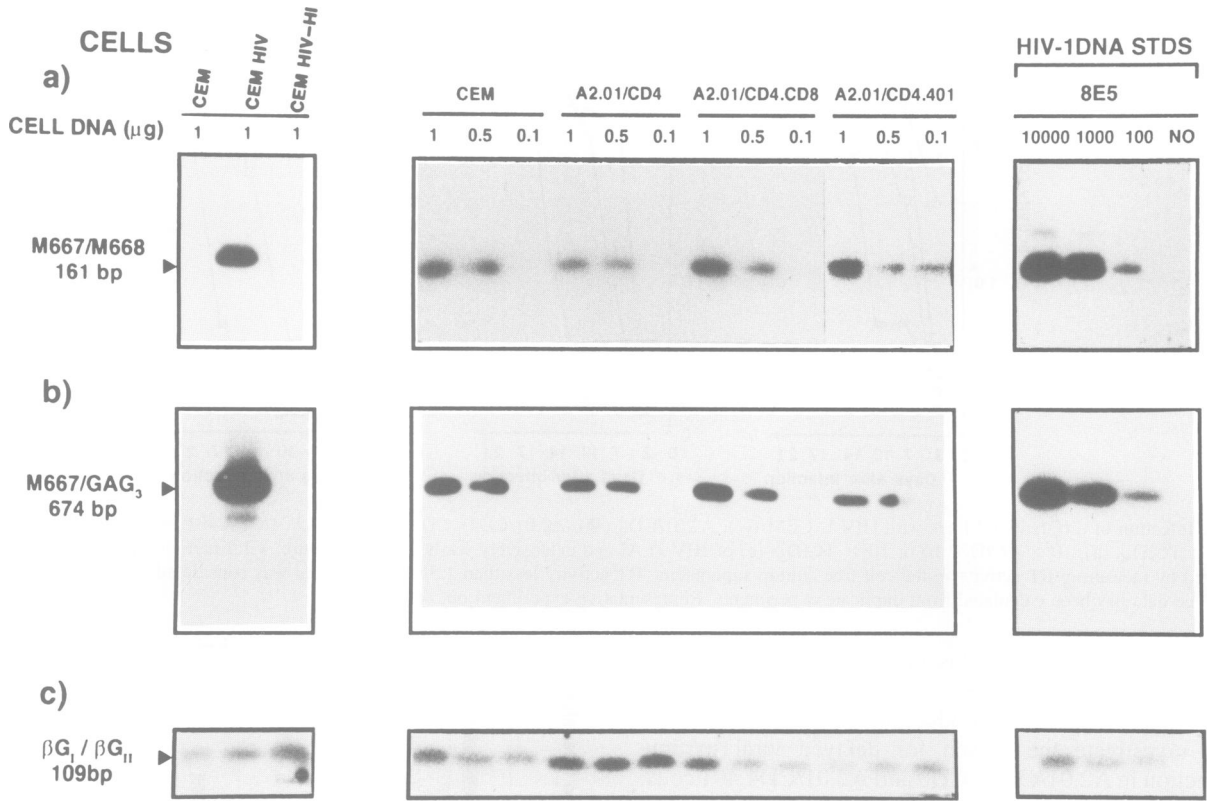

Fig. 4. PCR analysis of viral DNA in HIV-1 exposed CEM, A2.01/CD4, A2.01/CD4.401 and A2.01/CD4−CD8 cells. Cells were exposed to HIV-1LAI ($10^3\times$ $TCID_{50}$) and extensively washed before culture. HIV-1 DNA was monitored by PCR analysis using the M667/M668 (**a**) or the M667/GAG_3 (**b**) oligonucleotide primer pairs PCR were performed on total DNA extracts (1 μg, 0.5 μg and 0.1 μg) prepared from cells at 6 h (a) or 24 h (b) following viral exposure. The controls for PCR were virus-free CEM cells (CEM), HIV-1LAI chronically infected CEM cells (CEM HIV) and CEM cells exposed to heat-inactivated HIV-1LAI (CEM HIV-HI). A control for estimation of viral DNA copies number consisted of total DNA extracts prepared from CEM cells mixed with a variable known number of 8.E5 cells. The amplified products were electrophoresed, blotted and hybridized with a radiolabelled HIV-1 probe. Labelled viral DNA products were visualized by autoradiography. Product of PCR amplification using β globin I/β globin II oligonucleotide primer pair, hybridized with ^{32}P-labelled β globin probe, is shown as control (**c**).

pair, designed to flank the common splice donor and acceptor sites of the Env, Tat and Rev genes (Benkirane *et al.*, 1993). As shown in Figure 5a, spliced mRNAs were found in A2.01/CD4 cells on day 3 post-infection, whereas they were evidenced in A2.01/CD4−CD8 cells on day 7 post-infection and in A2.01/CD4.401 only on day 14 post-infection.

These results demonstrate that the presence of the cytoplasmic domain of CD4 is not required for complete reverse transcription and that the delays in viral production correlate with an event taking place after reverse transcription and before transcription.

Lack of delay of HIV-1 production in phytohaemagglutinin treated A2.01/CD4.401 and A2.01/CD4−CD8 cells

Viral production is known to be under the control of cellular DNA and RNA binding proteins which are themselves influenced by the activation status of the cell. To determine whether the delay observed for the appearance of RT activity in A2.01/CD4.401 and, to a lesser extent, in A2.01/CD4−CD8 culture supernatants could be attributable to the activation status of the cell, we compared viral production in infected A2.01/CD4, A2.01/CD4.401 and A2.01 CD4−CD8 cells treated or not with phytohaemagglutinin (PHA). Cell activation by PHA is known to stimulate HIV-1 production *in vitro* (Tong-Starksen *et al.*, 1989). As shown in Figure 6b, when cells were exposed to $10^3\times$ $TCID_{50}$ of HIV-1LAI and treated with PHA we no longer observed the delay in viral production that we found in cells not treated with PHA (Figure 6a). Similar results were obtained after phorbol myristate acetate (PMA) treatment (data not shown).

These results suggest that there is a defect either during integration or during the early HIV-1 provirus transcription in A2.01/CD4.401; this is also evidenced to a lesser extent in the A2.01/CD4−CD8 cells. This defect is likely to be responsible for the delayed production of progeny virions in those cells.

A positive signal is transduced via the CD4 molecule after HIV-1 binding

We and others have previously reported that infection of CEM cells by HIV-1 induces IL2-R (Benkirane *et al.*, 1993) and HLA class II (Benkirane *et al.*, 1994) expression on the surface of those cells. These results indicate that an activation signal is likely to be delivered to T-cells by HIV. To study whether such an activation signal may follow binding of HIV to CD4, we compared the capacity of heat-inactivated HIV-1 and PMA to induce translocation of NF-κB in cells expressing CD4 or the mutant forms of CD4. As shown in Figure 7a, using an electrophoretic

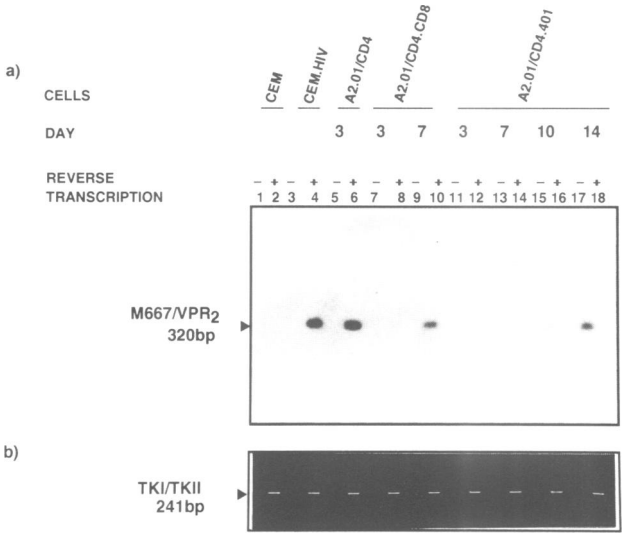

Fig. 5. Analysis of HIV-1 gene transcription. (**a**) PCR analysis of HIV-1 spliced mRNA in HIV-1LAI exposed cells. Total RNAs were extracted from cells and retrotranscribed (+) or not (−) into DNA using (or not using) AMV RT as described in Materials and methods. PCR amplification was then performed using the M667/VPR2 oligonucleotide primer pair. Amplified fragments were hybridized to a α-^{32}P-labelled HIV-1LAI probe and visualized by autoradiography (a). Products of PCR amplification of retrotranscribed thymidine kinase (TK) RNA, stained with ethidium bromide are shown as control (**b**). The different samples analysed by PCR were prepared from virus-free CEM cells (lanes 1 and 2), HIV-1LAI chronically infected CEM cells (lanes 3 and 4), A2.01/CD4 cells 3 days after infection (lanes 5 and 6), A2.01/CD4−CD8 cells 3 days (lanes 7 and 8) and 7 days after infection (lanes 9 and 10) and A2.01/CD4.401 cells, 3 days (lanes 11 and 12), 7 days (lanes 13 and 14), 10 days (lanes 15 and 16) and 14 days after infection (lanes 17 and 18).

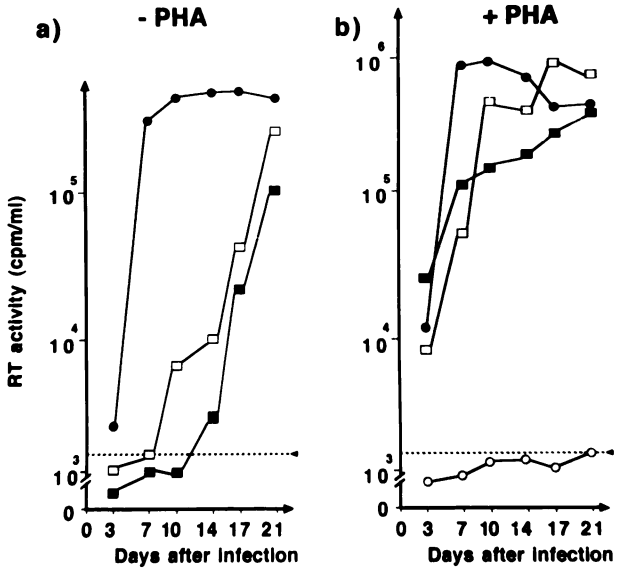

Fig. 6. Modulation of viral particle production after PHA treatment. A2.01/CD4 (●), A2.01/CD4−CD8 (□), and A2.01/CD4.401 cells (■) were exposed to $10^3\times$ $TCID_{50}$ of HIV-1LAI at 4°C for 30 min and extensively washed before culture at 37°C for 24 h. Then cells were trypsinized to eliminate extracellular virus, washed and replated in fresh medium, in the absence of PHA (**a**) or in medium supplemented with PHA (**b**). Viral particle production was followed by measuring RT activity in the cell-free culture supernatant. RT activity less than 1.5×10^3 c.p.m./ml was considered as negative (dashed line). A control that consists of cells exposed to HIV-1 at 4°C for 30 min and treated with trypsin before they were cultivated at 37°C is also shown (o). Representative experiment out of three.

mobility shift assay (EMSA), we observed a shift of labelled NF-κB oligonucleotide when mixed with nuclear extracts from PMA-treated CEM (lane 3), A2.01/CD4 (lane 6), A2.01/CD4−CD8 (lane 9) and A2.01/CD4.401 (lane 12) cells. Similarly, a shift in the oligonucleotide migration was observed when the oligonucleotide was mixed with nuclear extracts from CEM (lane 4), A2.01/CD4 (lane 7) or A2.01/CD4−CD8 (lane 10) exposed to heat-inactivated HIV-1. However, no protein−oligonucleotide complex was found when the oligonucleotide was mixed with nuclear extracts from A2.01/CD4.401 cells exposed to heat-inactivated HIV-1 (lane 13). To demonstrate that the constitutive DNA binding activities of the extracts were unchanged, labelled Sp-1 oligonucleotide was mixed with the different nuclear extracts and assayed by EMSA. As shown in Figure 7b, a shift of labelled Sp-1 oligonucleotide was observed with the different extracts. We next provided evidence that the shift observed in Figure 7a could be ascribed to the NF-kB protein by competition experiment and South-western blotting analysis. When EMSA was performed in the presence of a 10^3-fold excess of unlabelled NF-κB oligonucleotide (Figure 7C; lanes 3, 5, 7, 9, 11, 13 and 15) or even in the presence of a 100- or 10-fold excess of unlabelled NF-κB oligonucleotide (Figure 7d), complex formation between the nuclear protein and the labelled NF-κB oligonucleotide was inhibited. Using South-western blotting analysis we found that the retarded complex contained a protein with an apparent mol. wt equivalent to that of NF-κB (data not shown).

To confirm that NF-κB translocation resulted from heat-inactivated HIV-1LAI binding to CD4 and not from infection of cells by residual infectious particles, and that neither could it be attributable to interaction between target cell and an unidentified compound present in the virus preparation, two set of experiments were performed. First, A2.01/CD4 cells were exposed to HIV-1LAI and heat-inactivated HIV-1LAI, and the RT activity measured in the cell-free culture supernatants of those cells during 21 days. As shown in Figure 7e, when A2.01/CD4 cells were exposed to HIV-1LAI, virus particle production was evidenced on day 7 post-infection, whereas there was no evidence of residual infectious particles within the heat-inactivated HIV-1LAI preparation (the RT activity remained negative for the duration of the experiment). Second, to provide evidence that induction of NF-κB translocation following cell exposure to heat-inactivated HIV-1LAI required interaction between HIV-1LAI and cell surface CD4, we analysed the consequence of treatment of cells with heat-inactivated HIV-1LAI (HIV-HI) or HIV-HI previously incubated with 10 μg/ml sCD4 for 2 h at 37°C (HIV-HI/sCD4) on NF-κB translocation. As shown in Figure 8, using EMSA, we observed a shift of labelled oligonucleotide migration when mixed with nuclear extracts from A2.01/CD4 cells exposed to PMA (lane 2) or HIV-HI (lane 3) but not with extracts from A2.01/CD4 cells exposed to HIV-HI/sCD4 (lane 4). Similar results were obtained with nuclear extracts from CEM cells treated with PMA, HIV-HI or HIV-HI/sCD4 (data not shown). When extracts from A2.01/CD4.401 cells were

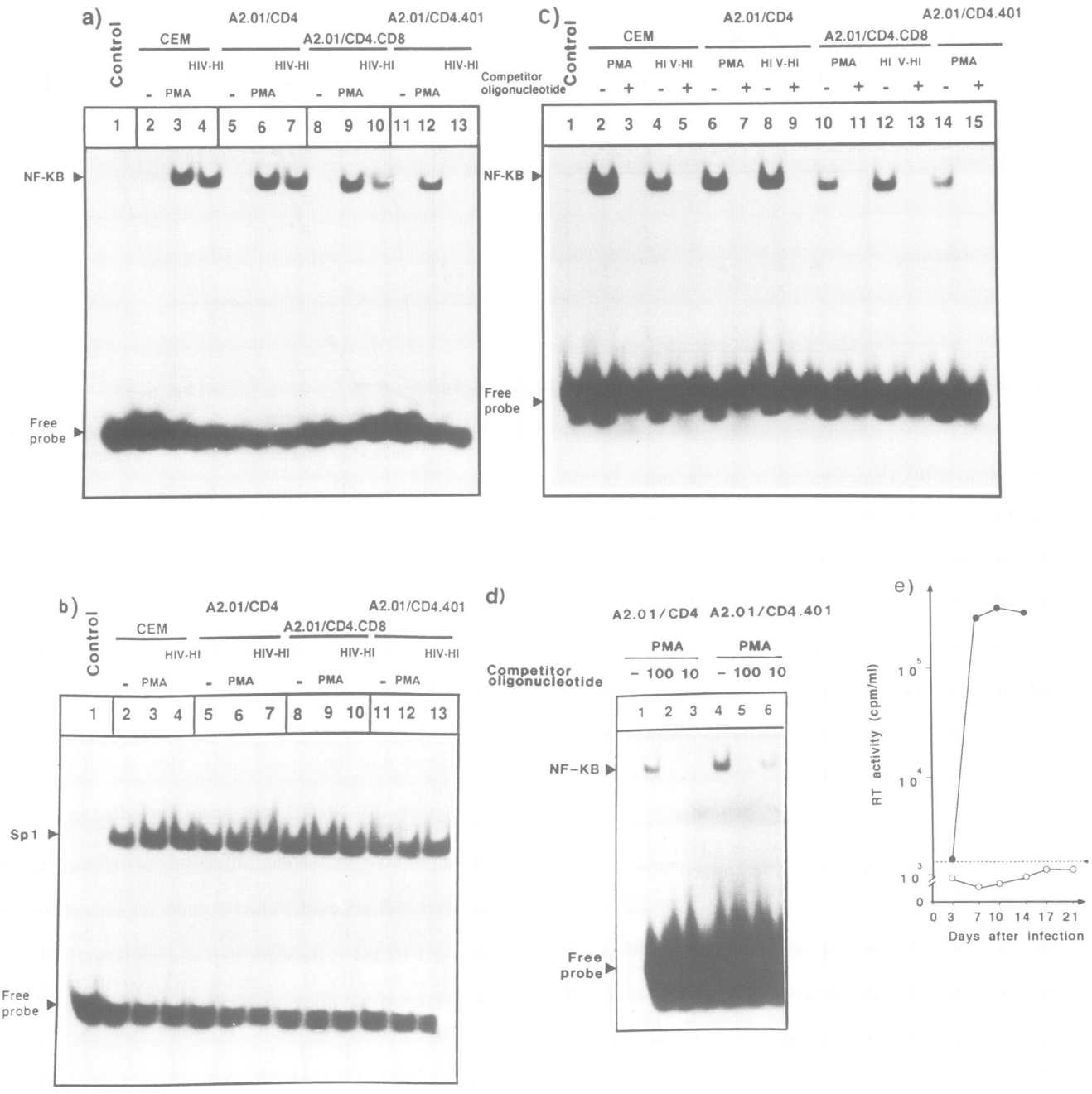

Fig. 7. Translocation of NF-κB after exposure of cell lines to HIV-1. (**a**) Analysis of NF-κB translocation in CEM (lanes 2–4), A2.01/CD4 (lanes 5–7), A2.01/CD4−CD8 (lanes 8–10) and A2.01/CD4.401 cells (lanes 11–13), using electrophoretic mobility shift assay. Nuclear extracts were prepared from untreated cells (−) (lanes 2, 5, 8 and 11), cells treated with PMA (PMA) (lanes 3, 6, 9 and 12) and cells exposed to heat-inactivated HIV-1LAI (HIV-HI) (lanes 4, 7, 10 and 13) were reacted with radiolabelled double-stranded NF-κB oligonucleotide probe. Control consisted of a sample without nuclear extract (lane 1). (**b**) Analysis of Sp-1 in nuclear extracts of CEM (lanes 2–4), A2.01/CD4 (lanes 5–7), A2.01/CD4−CD8 (lanes 8–10) and A2.01/CD4.401 cells (lanes 11–13), using EMSA [see (a) for details]. (**c**) Specificity of protein double-stranded NF-κB oligonucleotide interaction. Each time a retarded complex was evidenced [see (a)], the specificity of the binding was studied by competitive experiment using a 10^3-fold excess of unlabelled NF-κB oligonucleotide. A reaction performed in the absence of competitor is referred to as (−) and a reaction performed in the presence of unlabelled competitor is referred to as (+). (**d**) Competitive experiment in which a 100- or 10-fold excess of unlabelled NF-κB oligonucleotide was used. (**e**) Control of HIV-HI preparation. An infection experiment is shown. In this experiment cells were exposed to $10^3\times$ $TCID_{50}$ of HIV-1LAI (●) or HIV-HI at a concentration corresponding to $10^4\times$ $TCID_{50}$ of infectious HIV-1 (o). Viral particle production was followed by measuring RT activity in cell-free culture supernatants.

used (lanes 5–8), a shift in the oligonucleotide migration was observed with nuclear extracts from cells treated with PMA only (lane 6). Moreover, we found that the A2.01/CD4, A2.01/CD4−CD8 and A2.01/CD4.401 cells expressed similar amounts of cytoplasmic NF-κB (Figure 9) as evidenced by EMSA of labelled NF-κB oligonucleotide incubated with extracts containing 1% deoxycholate (DOC).

These results indicate that NF-κB is translocated into the nucleus and binds to the κB unit after oligomerization of CD4 by HIV-1, and that the cytoplasmic domain of CD4 is required for this event to occur. It is worth noting

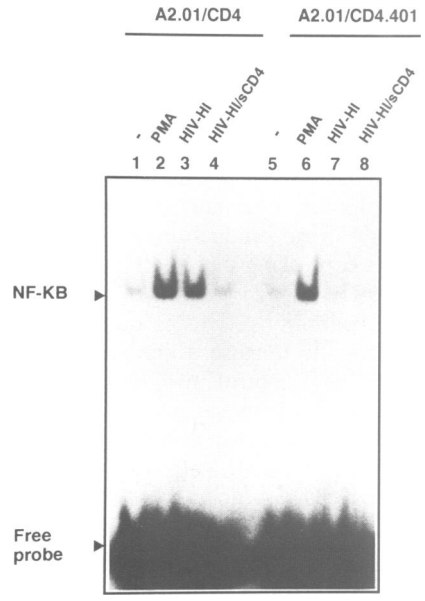

Fig. 8. Effect of sCD4 treatment on HIV-HI induced NF-κB translocation using electrophoretic mobility shift assay. Nuclear extracts from A2.01/CD4 (lanes 1–4), and A2.01/CD4.401 cells (lanes 5–8) were prepared from untreated cells (−) (lanes 1 and 5), cells treated with PMA (PMA) (lanes 2 and 6), cells exposed to heat-inactivated HIV-1LAI (HIV-HI) (lanes 3 and 7) and cells exposed to heat-inactivated HIV-1LAI (HIV-HI) previously incubated with 10 μg/ml sCD4 for 2 h at 37°C (lanes 4 and 8) and were reacted with radiolabelled double-stranded NF-κB oligonucleotide as in Figure 7a.

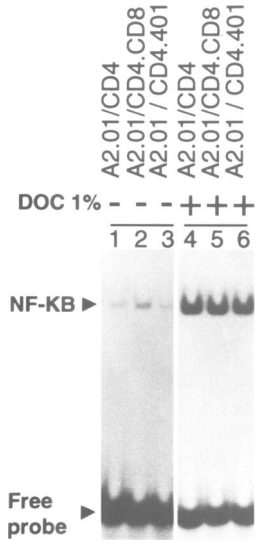

Fig. 9. Deoxycholate (DOC) released cytoplasmic NF-κB binding protein. Cytoplasmic extracts were isolated from A2.01/CD4 (lanes 1 and 4), A2.01/CD4−CD8 (lanes 2 and 5) and A2.01/CD4.401 cells (lanes 3 and 6). EMSA was conducted as described in the legend of Figure 7a with 2 μg protein/cell line and radiolabelled double-stranded NF-κB oligonucleotide probe either in the presence (+) or absence (−) of 1% DOC which release NF-κB from its cytoplasmic association with IκB.

that the cytoplasmic tail of CD4 may be replaced by the cytoplasmic domain of the CD8 α chain, a domain able to bind p56^{lck}.

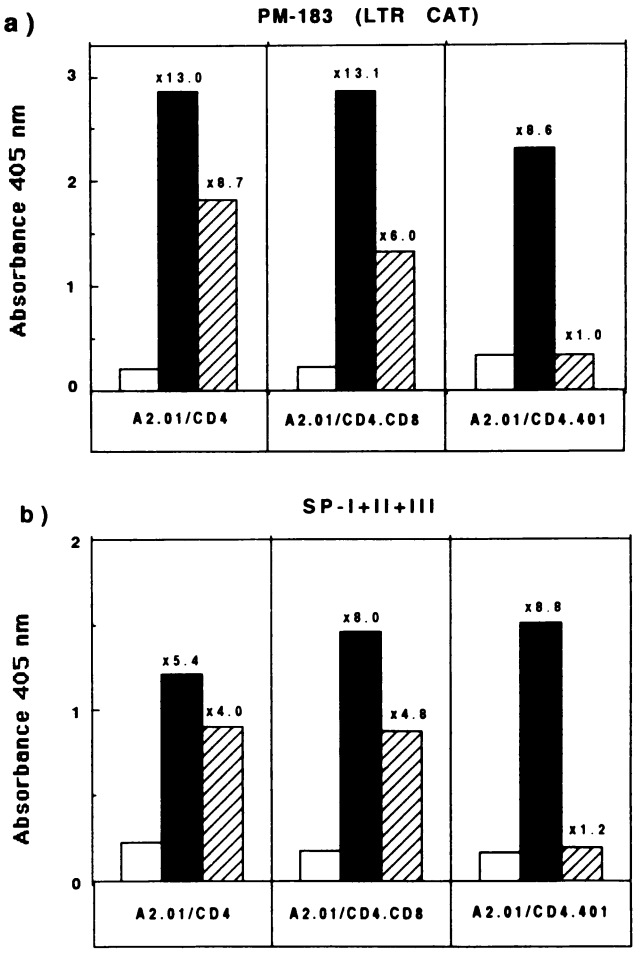

Fig. 10. CAT assay. A2.01/CD4, A2.01/CD4−CD8 and A2.01/CD4.401 cells were transfected with HIV-1 LTR CAT (**a**) or SPI+II+III (b) at 20 μg DNA/5×10^6 cells by electroporation. HIV-1 LTR CAT is a CAT gene expression vector in which CAT is driven by HIV-1 LTR. SPI+II+III is a CAT gene expression vector driven by a promoter containing two wild type HIV-1 NF-κB sites and three mutated Sp-1 sites inserted 5′ to a minimal promoter (Berkhout and Jeang, 1992); the SPI+II+III plasmid allows the direct study of NF-κB-driven gene transcription. The different CAT activity assays consisted of untreated transfected cells (□), cells cultivated in medium containing PMA (■) and cells exposed to heat-inactivated HIV-1LAI (▨). CAT activity was measured as described in Materials and methods. The fold increase in CAT activity following activation is indicated. Representative experiment out of two.

The cytoplasmic domain of CD4 is required for activation of LTR-driven CAT gene transcription following heat-inactivated HIV-1LAI binding to CD4

To study the influence of signal transduction and NF-κB translocation on viral transcription, A2.01/CD4, A2.01/CD4.401 and A2.01/CD4−CD8 cells were transfected by means of electroporation either with the PM-183 plasmid containing the CAT gene under the control of the wild type HIV-1LAI LTR promoter, or with the SPI+II+III plasmid containing the CAT gene under the control of HIV-1 LTR NF-κB sites only. Basal CAT gene transcription was evaluated by cultivating the cells in medium alone whereas PMA-induced transcription was used as a positive control. Assays were performed on cells treated with HIV-HI. As shown in Figure 10, the addition of PMA to cells transfected with PM-183 (Figure 10a) or SPI+II+III (Figure 10b) resulted in an ~5- to 13-fold increase in CAT

synthesis compared with untreated cells. Similarly, CAT expression was also induced in A2.01/CD4 and A2.01/CD4−CD8 following contact with viral antigens of heat-inactivated particles. Finally, CAT expression was not induced by HIV-HI in A2.01/CD4.401 cells.

We conclude that cross-linking of CD4 by HIV activates transcription of genes under the control of HIV-1 LTR, that this activation involves NF-κB and that the cytoplasmic domain of CD4 is required for such an activation.

Discussion

The ability of cells expressing different forms of CD4 to be infected by HIV-1LAI and to produce viral particles was examined. The variable lag periods in virus production observed in cells expressing the mutant forms of CD4 were no longer seen when cells were treated with PHA. Moreover, rapid viral production was found to correlate with the capacity of the CD4 molecule to transduce an activation signal, resulting in the translocation of NF-κB and up-regulation of HIV-1 LTR driven gene transcription. This signal was demonstrated to be transduced following CD4 cross-linked by HIV-1 itself, and required the integrity of the cytoplasmic domain of CD4 or CD8 α chain for its induction. We propose that the binding of HIV-1 to cell surface CD4 stimulates a signalling pathway that results in translocation of NF-κB into the nucleus, and that this factor in turn regulates the rate of the early transcription of the provirus genes before Tat is synthesized.

We have observed a delay in HIV-1 replication in A2.01 cells expressing hybrid and truncated forms of CD4 when compared with cells expressing the wild type CD4 molecule. This delay was not attributable to the density and accessibility of CD4 molecules on the surface of the different cell lines. The delay in the appearance of RT activity in these cells was more pronounced when cells were exposed to low viral concentrations. This corroborates with observations previously reported by Poulin *et al.* (1991). Yet, at slight variance with Poulin and co-workers (Poulin *et al.*,1991), we reproducibly found that the delay of HIV-1 production was longer in A2.01/CD4.401 (truncated CD4) cells than in A2.01/CD4−CD8 (hybrid CD4−CD8 molecule) cells. We made similar observations when HIV-2ROD isolate grown in CEM cells was used for infection of these cells (data not shown). Although viral production was delayed in cells expressing mutant forms of CD4, we showed that the rate of viral entry was identical in cells expressing the different forms of CD4, and this also corroborates with results from others (Bedinger *et al.*, 1988; Maddon *et al.*, 1988; Golding *et al.*, 1993; Tremblay *et al.*, 1994). In addition, we report here the first evidence that the retrotranscription was complete in cells expressing the different forms of CD4 24 h after infection.

We demonstrate here that native CD4 molecules, once oligomerized by HIV-1, transduce a signal that leads to NF-κB translocation which in turn positively regulates HIV-1 replication. This is relevant to previous observations by Suzan and co-workers (Suzan *et al.*, 1991) indicating that *in vitro* infection with HIV of mature cells from the monocytic cell lineage induces NF-κB translocation. We also found that this signal transduction is not induced in the A2.01/CD4.401 cells which express a truncated form of CD4 unable to associate with $p56^{lck}$. This observation suggests strongly that a protein kinase, probably $p56^{lck}$, is implicated in the transduction of the signal. However, our results contrast with those of Tremblay and co-workers (Tremblay *et al.*, 1994), who recently reported that cells expressing a truncated form of CD4, or a mutant molecule which can no longer associate with $p56^{lck}$, permitted increased levels of viral production. It is noteworthy that their A2.01 cells expressing the wild type CD4 apparently do not produce HIV-1 $p24^{gag}$ antigen when exposed to high HIV-1 concentrations. This is quite surprising and unfortunately they did not use a known infectable CD4-positive cell line as a control in their study. The reason for such discrepancies between our results and theirs is not clear at present since we apparently used a similar model that consist of A2.01 cells expressing wild type CD4 or truncated CD4. A difference may consist of the cells in which HIVs were replicated; our HIV-1LAI (present work) and HIV-2ROD (data not shown) viruses were produced in CEM cells whereas the HIV-1IIIB and HIV-1SF2 used by Tremblay *et al.* were harvested from culture fluids of chronically infected H-9 and HUT-78 cell lines. It is known that the process of T-cell activation is highly complex (reviewed in Janeway and Bottomly, 1994; Weiss and Littman, 1994). It remains possible that the nature of the signal transduced via CD4 may be different if a second cell-surface molecule is engaged at the same time, and it is known that several cell surface molecules which could serve as counter-receptor to cell surface molecules are incorporated into budding virions (reviewed in Benkirane *et al.*, 1994). If cell surface molecules incorporated into budding virions differed, co-stimulatory signals may have triggered T-cell activation in different ways. We have attempted to investigate this possibility by exposing A2.01/CD4, A2.01/CD4−CD8 and A2.01/CD4.401 cells to HIV-1LAI grown in MT2 cells (an HTLV-I immortalized T-cell line) and found similar results to those we obtained with HIV-1LAI and HIV-2ROD (data not shown). Additional research is needed to address this problem.

It is generally admitted that HIV-1 and HIV-1$gp120^{env}$ modulate T-cell activation although there is still some controversy about the nature of the signals which are delivered to the target cells (Mann *et al.*, 1987; Shalaby *et al.*, 1987; Diamond *et al.*, 1988; Fields *et al.*, 1988; Kornfeld *et al.*, 1988; Mills *et al.*, 1988; Hofman *et al.*, 1990; Horak *et al.*, 1990; Neudorf *et al.*, 1990; Juszczak *et al.*, 1991; Haffar *et al.*, 1992; Soula *et al.*, 1992; Hivroz *et al.*, 1993; Hofman *et al.*, 1993). Among other evidence that T-cell activation signals are delivered to target cells by HIV-1 antigens, Kornfeld and co-workers (Kornfeld *et al.*, 1988) first reported that HIV-1 $gp120^{env}$ binding to CD4 induces IL2-R expression. Similarly, we have found that HIV-1 infection of CEM cells induces IL2-R on the surface of those cells (Benkirane *et al.*, 1993). We have also observed that HIV-1 induces cell surface expression of HLA class II antigens (Benkirane *et al.*, 1994) another marker of T-cell activation. Here we found direct evidence for signal transduction after HIV binding to CD4 and we demonstrate that the cytoplasmic domain of CD4 (or CD8 α chain) is required for the transduction of this signal. Accordingly, a protein kinase, probably $p56^{lck}$ which can

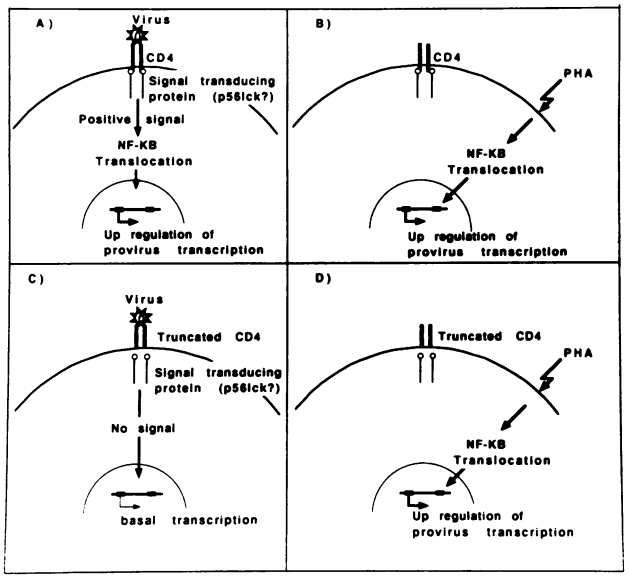

Fig. 11. Hypothetical model of cellular mechanisms that take place following binding of HIV-1 or activation with PHA or PMA. Addition of PHA to A2.01/CD4 (**B**) or A2.01/CD4.401 (**D**) will result in transduction of a signal probably via PKC that will induce translocation of NF-κB and consequently will up-regulate provirus transcription. Addition of HIV-1 to A2.01/CD4 cells (**A**) will result in oligomerization of wild type CD4 and transduction of a signal probably via $p56^{lck}$ that will induce translocation of NF-κB and consequently will up-regulate provirus transcription. Although addition of HIV-1 to A2.01/CD4.401 cells (**C**) will also result in oligomerization of truncated CD4, it will fail to transduce a signal probably because it requires $p56^{lck}$ association with the cytoplasmic domain of CD4 that is missed.

associate with the cytoplasmic domain of CD4 and CD8 α chain, plays a major role in HIV-1 induced T-cell activation. Protein kinase activity (frequently shown to be a $p56^{lck}$ activity) induced by HIV-1 or HIV-1gp120^{env} was reported (Fields *et al.*, 1988; Juszczak *et al.*, 1991; Soula *et al.*, 1992; Hivroz *et al.*, 1993). $p56^{lck}$ was shown to activate second messengers (Veillette *et al.*, 1989; Thompson *et al.*, 1991; Ettehadieh *et al.*, 1992; Kanner *et al.*, 1992; Weber *et al.*, 1992; Duplay *et al.*, 1994). Binding of HIV-HI to cell surface CD4 could modulate the induction of HIV-1 by modulating the activation by $p56^{lck}$ of one of, or a combination of, the second messengers.

Our data indicate that heat-inactivated HIV-1LAI stimulates the NF-κB pathway of activation when it binds CD4 and that HIVs might take advantage of NF-κB translocation to regulate their rate of provirus gene transcription before Tat is synthesized. This conclusion is supported by the CAT assay experiments showing that heat-inactivated HIV-1LAI induced HIV-1 wild type LTR- and HIV-1 NF-κB sites-driven CAT gene transcription in A2.01/CD4 and A2.01/CD4−CD8 cells, and by the observation that NF-κB was found in the nuclear extracts of A2.01/CD4 and A2.01/CD4−CD8 cells exposed to heat-inactivated HIV-1LAI. The fact that virus production in A2.01/CD4−CD8 is delayed (although the CD8 α chain cytoplasmic domain is likely to be able to associate with protein kinases) and induced NF-κB translocation after binding to heat-inactivated HIV-1LAI suggests that the hybrid CD4−CD8 molecule is less efficient than the wild type CD4 molecule in signal transduction. This is consistent with the observation that induction of LTR-driven CAT gene expression is slightly lower in A2.01/CD4−CD8 cells than in A2.01/CD4 cells.

We hypothesize that the delay in virus production seen in A2.01/CD4.401 cells will consist mainly of a defect in the 'early' Tat-independent transcription of HIV-1 genome. Previous studies have shown NF-κB to be an important factor in HIV-1 gene expression in systems in which Tat was not present (Leonard *et al.*, 1989). It has been suggested that 'early' transcription, or basal transcription, is regulated by NF-κB protein whereas 'late' transcription is regulated by Tat and Sp-1 (Berkout and Jeang, 1992). As illustrated by the model presented in Figure 11, we propose that in the A2.01/CD4.401 cells, because there is no association between CD4 and protein kinase, the oligomerization of CD4 does not result in NF-κB translocation. HIV early transcription in those cells will truly refer to basal transcription (Figure 11C). In contrast in the A2.01/CD4 (Figure 11A) and A2.01/CD4−CD8 cells, the oligomerization of wild type CD4 by HIV-1 will induce NF-κB translocation which will in turn activate HIV-1LTR driven CAT gene expression in the absence of Tat and Sp-1. HIV-1 early gene transcription in those cells will be up-regulated by NF-κB. Accordingly, Tat and Sp-1 dependent 'late' transcription will begin more rapidly in those cells, and RT will be detected earlier, than in the A2.01/CD4.401 cells. Finally, when PHA (Figure 11B and D) or PMA is used to induce NF-κB translocation, HIV transcription and viral production in the A2.01/CD4, A2.01/CD4−CD8 and A2.01/CD4.401 cells will be induced concomitantly. This model corroborates previous results indicating that molecular clones of HIV-1 that differed in their NF-κB elements showed a different replication rate in T-cell lines (Englund *et al.*, 1991; Ross *et al.*, 1991).

Although the relevance of these observations to a more physiological situation, such as the infection of T-cells during virus propagation in infected patients, will need to be established, the above results suggest a remarkable adaptation of HIV which can induce into its target T-cell line cellular factors required for its own transcription, prior to fusion and via cell surface molecules.

Materials and methods

Monoclonal antibodies and reagents

Purified anti-CD4 mAbs Leu 3A and OKT4 were purchased from Becton Dickinson (Mountain View, CA) and Ortho Diagnostic Systems, Inc. (Raritan, NJ). Purified anti-CD4 mAbs 13B8-2 and BL4 were provided by M.Hirn (Immunotech SA, Marseille, France). FITC-labelled rabbit anti-mouse (RAM) was purchased from Immunotech. Phorbol myristate acetate (PMA) was purchased from Sigma Chemical Co. (St Louis, MO) and was used at 10 ng/ml in cell cultures. PHA (Boehringer Mannheim, Germany) was used at 2 μg/ml in cell cultures. Because PMA at 10 ng/ml and PHA at 2 μg/ml was slightly toxic for A2.01/CD4, a concentration 10-fold lower was used for treatment of those cells. Recombinant sCD4 was provided by D.Klatzmann (Hôpital de la Pitié-Salpétrière, Paris, France).

Oligonucleotides

HIV-1 oligonucleotide primers: M667 (5′-GGCTAACTAGGGAACCC-ACTG-3′ nucleotides 496–516 sense), M668 (5′-TTTCAGGTCCC-TGTTCGGGCGCC-3′ nucleotides 637–656 antisense), VPR2 (5′-CTAGGATCTACTGGCTCCATTTC-3′ nucleotides 5836–5859 antisense), GAG3 (5′-GACCTGACTGCTGTGTCCTGTG-3′ nucleotides 1149–1171 antisense); cellular oligonucleotide primers: β globin I (5′-ACACAACTGTGTTCACTAGC-3′ nucleotides 14–33 sense), β globin

II (5′-CAACTTCATCCACGTTCACC-3′ nucleotides 104–123 antisense), TK I (5′-GAGTACTCGGGTTCGTGAAC-3′ nucleotides 24–43 sense mRNA), TK II (5′-GGTCATGTGTGCAGAAGCTG-3′ nucleotides 246–265 antisense mRNA). Double-stranded oligonucleotides NF-κB used for electrophoretic mobility shift assay(s): HIVLTR5′-1 (sense strand only: 5′-GCTGGGGACTTTCCAGGGAGGCGT-3′). These oligonucleotides were synthesized by J.-P.Capony (CRBM, Montpellier, France). Double-stranded oligonucleotides Sp-1 used for EMSA: HIVSp-1 (sense strand only: 5′-GGAGGCGTGGCCTGGGCGGGACTGGGGAGTGGCGA-3′) were purchased from Eurogentec (Seraing, Belgique).

Cells and viruses

The $CD4^+$ lymphoblastoid CEM cell line was obtained from the American Type Culture Collection (Bethesda, MD, USA). The 8.E5 cell line (Folks *et al.*, 1986) was provided by F.Barré-Sinoussi (Institut Pasteur, Paris, France). The A2.01/CD4, A2.01/CD4.401 and A2.01/CD4−CD8 cell clones provided by D.Littman (Department of Microbiology and Immunology, UCSF, CA, USA), have been previously described (Bedinger *et al.*, 1988). All cell lines were cultured to a density of 5×10^5 cells/ml in RPMI 1640 medium supplemented with a penicillin-streptomycin antibiotic mixture, glutamine (Axcell-Novotec, Lentilly, France) and 10% fetal calf serum (ATGC-Biotechnologie, Noisy-Le-Grand, France), in a 5% CO_2 atmosphere. G418 (Life technologies, Eragny, France), at 1 mg/ml was added to the culture medium of transfected A2.01 cells.

Viral stocks were prepared from the chronically infected CEM cell supernatants as previously described (Corbeau *et al.*, 1991) and kept frozen at −80°C until use. After thawing, 100 µl of these stock viruses corresponding to 100 $TCID_{50}$ (50% Tissue Culture Infective Dose) were used for infection assays. Heat-inactivated virus controls were prepared by incubation for 30 min at 56°C. All viral preparations were treated with 100 U/ml RNase-free DNase for 30 min at room temperature before use.

HIV infection assay

Cells (5×10^5) were incubated for 30 min at 4°C in flat-bottomed 96-microwell plates (Costar) with 100 µl of HIV at different concentrations ($10^5\times$, $10^4\times$, or $10^3\times$ $TCID_{50}$/ml). Thereafter, cells were washed five times and cultured in 24-microwell plates (Costar). The amount of virus produced by CEM cells was monitored twice a week by measuring RT activity in 1 ml of cell-free culture supernatant using a synthetic template primer which permitted the RT to neosynthesize radioactive DNA, as previously described (Corbeau *et al.*, 1991).

HIV-HI, PHA and PMA activation assay

HIV-HI treatment: cells (5×10^5) were incubated for 30 min at 4°C with 100 µl of HIV-HI at a concentration equivalent to $10^4\times$ $TCID_{50}$/ml. Thereafter, cells were washed five times and cultured as described for HIV infection assay.

PHA/PMA treatment: 24 h after infection (see HIV infection assay) cells were treated with trypsin (Life Technologies) for 30 min at 37°C, extensively washed and cultivated in medium containing PHA or PMA.

Flow cytometry

Cells (1×10^5) were incubated for 30 min at 4°C with 100 µl of a 20 µg/ml solution of anti-CD4 mAb diluted in PBS containing 0.2% BSA and 0.1% NaN_3 (PBS-BSA). After washing three times with PBS-BSA, bound mAb was then revealed by addition of 50 µl of a 1/50 dilution of fluoresceinated RAM (Immunotech SA). After 30 min staining, cells were washed with PBS-BSA, fixed in PBS containing 2% formaldehyde and fluorescence intensity measured on an EPICS PROFILE cytofluorometer (Coulter, Coultronics, Margency, France).

PCR of DNA and RNA

HIV-1 DNA was monitored by PCR according to the previously published procedure (Benkirane *et al.*, 1993). Briefly, total DNA was extracted from cells (1×10^6) by alkaline lysis and resuspended in 200 µl H_2O. To the amplification mixture [20 mM Tris−HCl pH 8.3 containing 120 µM dNTPs, 1.5 mM $MgCl_2$, 50 mM KCl, 0.005% Tween 20, 0.005% NP-40, 0.001% gelatin, 20 pmol of each oligonucleotide primer and 2 U of *Taq* DNA polymerase (Epicentre Technologies, Madison, WI, USA)] a solution containing various amounts of DNA (0.1, 0.5 and 1 µg) was added. The amplification reaction was run in a PHC2 thermal cycler (Techne, Cambridge, UK). The amplified products were analysed by electrophoresis in a 1% agarose gel, blotted for 2 h onto Hybond N^+ membrane (Amersham) and hybridized with α-^{32}P-labelled HIV-1 probe. Labelled viral DNA products were visualized by autoradiography (Kodak X-Omat AR Films). Standards used to quantitate HIV-1 DNA were prepared from the 8.E5 cell line.

PCR detection of retrotranscribed HIV-1-RNA: PCR detection of retrotranscribed HIV-RNA was performed according to the previously published procedure (Corbeau *et al.*, 1993). Briefly, total RNA was extracted from 1×10^6 cells and was treated with RNase-free DNase (Boehringer, 100 U/ml) for 30 min at room temperature and then phenol-extracted before retrotranscription. To the RNA sample was added 1 U of RNase inhibitor (RNase block II Stratagene, Ozyme, France), 0.25 U of AMV reverse transcriptase (BRL, Gaithersburg, MD), 40 pmol of oligo (dT) primer (New England Biolabs, Beverley, MA) and reaction buffer (50 mM Tris−HCl pH 8.3, 100 mM KCl, 10 mM $MgCl_2$, 10 mM DTT, 500 µM dNTPs). The primer hybridization was performed at 65°C and slowly cooled to 37°C, then retrotranscription was done at 37°C for 1 h. RNA was then destroyed by adding 0.1 M NaOH solution to this mixture followed by neutralization using 0.1 M HCl solution. Neosynthesized HIV-DNA was precipitated in 0.3 M sodium acetate pH 5.2, 10 mM $MgCl_2$ and absolute ethanol for 18 h at −80°C, washed with 70% ethanol and desiccated. Samples were resuspended in 80 µl H_2O and PCR carried out on 20 µl sample as described above.

Electrophoretic mobility shift assay

Nuclear and cytoplasmic extracts were prepared according to the published method (Costello *et al.*, 1993). Briefly, cells (1×10^6) were incubated for 30 min at 4°C with 200 µl of HIV-HI (at $10^4\times$ $TCID_{50}$/ml), PMA (1 ng/ml or 10 ng/ml; see reagents section for details) or medium alone, extensively washed and harvested after 16 h incubation at 37°C in culture medium (or medium supplemented with PMA in the case of PMA inductions). Cells were washed with Tris buffered saline (TBS) pH 7.8, transfered into 1.5 ml Eppendorf tubes and microfuged at 4°C for 15 s. The pellet was resuspended in 800 µl A buffer (containing 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 4 µg/ml leupeptin and 10 mM HEPES pH 7.8). After 15 min on ice, 50 µl solution of 10% NP-40 was added to the sample and cells homogenized by vortexing and microfuging at 4°C for 30 s. The supernatants (cytoplasmic extracts) were supplemented with KCl, NaCl, glycerol and HEPES (final concentration of B buffer) and stored at −80°C. The pellets (nuclear extracts) were resuspended in 100 µl B buffer (containing 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 4 µg/ml leupeptin, 10% glycerol and 50 mM HEPES pH 7.8). The nuclear extracts were microfuged at 4°C for 5 min, and the supernatants were stored at −80°C until used. The NF-κB mobility shift assay was performed using 2 µg protein of nuclear extract, 1×10^5 c.p.m. of labelled probe in C buffer (containing 100 mM KCl, 1 mM DTT, 1 µM $ZnSO_4$, 20% glycerol, 0.01% NP-40 and 50 mM HEPES pH 7.9), supplemented with BSA, tRNA and poly(dIdC) in a final volume of 20 µl. After 20 min at room temperature, the mixture was run at 120 V in a 4% polyacrylamide gel. The specificity of binding was determined by mixing the nuclear extracts with excess of cold NF-κB probe before addition of ^{32}P-labelled probes.

Transfections and CAT assay

Plasmid PM-183 (LTR-CAT) previously described (Benkirane *et al.*, 1993) was provided by M.Alizon (ICGM, Paris, France). Plasmid SPI+II+III has been previously described (Berkhout and Jeang, 1992). Transient transfections of cells (5×10^6 cells resuspended into 300 µl of PBS) with 20 µg of the LTR-CAT or SPI+II+III were carried out by electroporation (250 V, 1500 µF using a Celljet apparatus). Transfected cells were resuspended at 5×10^5 cells/ml in RPMI containing 20% FCS and antibiotics, and incubated for 18 h at 37°C in 5% CO_2 atmosphere before being exposed to heat-inactivated HIV-1. Cells were harvested after 24 h. The cell pellet was resuspended in PBS and microfuged at 4°C for 1 min then washed in TEN buffer (TEN: 40 mM Tris−HCl pH 7.4 containing 1 mM EDTA and 150 mM NaCl) and resuspended in 250 mM Tris−HCl pH 7.8. Cells were disintegrated by repeated (4×) freezing and thawing. After centrifugation, aliquots of the clear supernatant were used for CAT assay. A CAT antigen capture assay (Boehringer Mannheim, Germany) was used to estimate the amount of enzyme contained in the cytoplasmic extracts.

Acknowledgements

We are indebted to Marc Alizon, Françoise Barré-Sinoussi, Michel Hirn, David Klatzmann, Dan Littman and Luc Montagnier for kind gifts of plasmid, cells, mAbs, sCD4 and viruses. We thank Pierre Corbeau, Jean Imbert and Dan Littman for helpful discussions and Bernard Rossi

for critically reviewing the manuscript. This work was supported by institutional funds from the Centre National de la Recherche Scientifique (CNRS), the Institut National de la Santé et de la Recherche Médicale (INSERM), a grant No.93213 from the Agence Nationale de Recherches sur le SIDA (ANRS) to C.D., and funds from the Association des Artistes Contre le SIDA (AACS); M.B. is fellow of the ANRS.

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Received on July 8, 1994; revised on September 15,1994