Association of p56\(^{lk}\) with the cytoplasmic domain of CD4 modulates HIV-1 expression

Michel Tremblay\(^1,2\), Sylvain Meloche\(^2,3\), Sophie Gratton\(^2,4\), Mark A.Wainberg\(^5\) and Rafick-P.Sékaly\(^2,6\)

\(^1\)Département de Microbiologie, Laboratoire d'Infectiologie, Centre de Recherche du CHUL, Université Laval, Ste-Foy, Québec G1V 4G2, \(^2\)Laboratoire d'Immunologie, Institut de Recherches Cliniques de Montréal, Montréal, Québec H2W 1R7, \(^3\)Division of Experimental Medicine, McGill University, Montréal, Québec H3G 1A4, \(^4\)Department of Medicine, Lady Davis Institute, Jewish General Hospital and McGill AIDS Centre, McGill University, Montréal, Québec H3T 1E2, and \(^5\)Département de Microbiologie et Immunologie, Université de Montréal, Canada

\(^6\)Present address: Centre de Recherche, Hôtel-Dieu de Montréal, Montréal, Québec H2W 1T8, Canada

Communicated by R.A.Weiss

To investigate the role played by the cytoplasmic domain of the CD4 glycoprotein in the process of HIV infection, we have transfected two CD4-negative human T cell lines with cDNAs encoding the full-length CD4 and a truncated form of the molecule, lacking most of the cytoplasmic domain. Levels of viral replication were significantly higher in cells carrying the truncated version of CD4, in comparison with cells expressing the full-length CD4, as measured by the percentage of cells expressing viral p24 protein and the number of infectious particles released into culture supernatants. The extent of viral entry and reverse transcription was similar in each case, as monitored by an enzymatic test and quantitative PCR. Quantitative differences at RNA and protein levels were responsible for changes in viral production. To further characterize the mechanisms responsible for decreased rates of HIV replication in CD4-expressing cells we have treated the different cell lines, very early after HIV infection, with azidothymidine and soluble CD4, two antiviral agents that inhibit replication of HIV at different stages in the virus replicative cycle. Results from these experiments indicate that a cellular signal is mediated by the CD4 molecule, which negatively regulates the expression of viral DNA already present in such cells. This signal would be initiated following oligomerization of the CD4 molecule by the virus itself. Results from experiments with a CD4 construct containing mutations of the cysteine residues which are responsible for association of CD4 with p56\(^{lk}\) demonstrate that p56\(^{lk}\) is implicated in the transduction of the signal negatively regulating HIV replication.

Key words: AIDS/CD4/HIV

Introduction

The CD4 molecule is a 55 kDa surface protein that contains four extracellular domains that have structural homology with an immunoglobulin V region (Ryu et al., 1990; Wang et al., 1990). The CD4 glycoprotein is highly expressed on T cells involved in recognition of antigen in the context of class II major histocompatibility complex (MHC) molecules (Swain, 1983). Interaction of CD4 with a non-polymorphic region of class II molecules was reported to enhance the avidity between the T cell receptor and its ligands (Gay et al., 1987; Sleckman et al., 1988; Lamarre et al., 1989). Data suggesting that the CD4 molecule is directly involved in signal transduction came from numerous reports. Negative signaling via CD4 was reported to occur based on the fact that some anti-CD4 monoclonal antibodies (mAbs) can inhibit T cell activation in the absence of accessory molecules (Wilde et al., 1983; Bank and Chess, 1985; Geppert and Lipsky, 1987).

The observation that CD4 is physically associated with the tyrosine protein kinase p56\(^{lk}\) further reinforced the notion that this integral membrane glycoprotein participates in signal transduction (Rudd et al., 1988; Veillette et al., 1988). p56\(^{lk}\) is a member of the src family of cytosolic protein tyrosine kinases and is expressed predominantly in T lymphocytes (Rudd et al., 1988; Veillette et al., 1988). Cross-linking of CD4 with CD4-specific mAbs induces a rapid increase in the autophosphorylation state and in the kinase activity of p56\(^{lk}\) (Veillette et al., 1989; Koretzky et al., 1990; Luo and Sefton 1990). Mutations in CD4 that abolish the interaction with p56\(^{lk}\) have led to decreased IL-2 secretion following antigenic stimulation, clearly indicating that this molecule plays an important role in T cell activation (Glaichenhaus et al., 1991). Moreover, the ability of a T cell hybridoma to secrete IL-2 in response to antigen stimulation was increased following the introduction of an active form of p56\(^{lk}\) (Abraham et al., 1991). The essential function of p56\(^{lk}\) in T cell ontogeny was also illustrated using \(\text{lk}^{-}\)-deficient mice in which no mature functional T lymphocytes could be detected (Molina et al., 1992). Altogether these studies indicate that the CD4 molecule plays an important role in T cell activation through its association with p56\(^{lk}\).

The CD4 molecule is the primary cellular receptor for the human immunodeficiency virus (HIV) due to the high-affinity binding of the external viral envelope protein (gp120) to CD4 (reviewed in Sattentau and Weiss, 1988). The binding site of gp120 has been mapped to the N-terminal extracellular region of CD4 and more precisely within the first immunoglobulin-like domain termed D\(_{1}\) (Landau et al., 1988; Mizukami et al., 1988; Peterson and Seed, 1988; Arthos et al., 1989; Fleury et al., 1991). The cytoplasmic domain of CD4 is not required for HIV-1 entry into cells since infection was shown to occur despite the deletion of the whole domain (Bedinger et al., 1988; Maddon et al., 1988). The objective of these studies was to clarify the precise role played by the cytoplasmic domain of CD4 in the process of HIV infection. We demonstrate that the intracellular domain of CD4 negatively regulates the replicative rate of HIV-1 in T-lymphoid cells. Most
importantly, the association of CD4 with the protein tyrosine kinase p56^ck is shown to be required for this effect.

**Results**

**Expression of wild-type, truncated and mutated forms of CD4 in A2.01 and HSB-2 cells**

We have expressed in two CD4-negative T cell lines (A2.01 and HSB-2) wild-type (wt-CD4), truncated (t-CD4) and a mutated form of CD4 with substitution of cysteine residues 420 and 422 by alanine residues (C4202A). Independently derived clones of each construct were obtained and expression of CD4 was assessed by flow cytometry (Figure 1). It is of interest to note that higher levels of CD4, on a greater percentage of cells, were detected in clones expressing wt-CD4 when compared with cells transfected with t-CD4 or C4202A. The affinity of wild-type and truncated CD4 for radiolabeled gp120 was measured by a quantitative gp120 binding assay and was found to be similar in the various clones tested (data not shown), thereby indicating that deletion of most of the cytoplasmic domain of CD4 did not alter its affinity for gp120.

**Infection of A2.01 and HSB-2 cells expressing wt-CD4 and t-CD4 with HIV-1**

A2.01 cells expressing either wt-CD4 or t-CD4 were infected with the HIV-IIIB laboratory strain of HIV-1 at a multiplicity of infection (m.o.i.) (infectious virus/target cell) of 0.01. The percentage of p24-expressing cells was evaluated by indirect immunofluorescence. Three independently derived clones, obtained from the same transfection, expressing either wt-CD4 or t-CD4, were studied. These experiments consistently showed that a greater number of cells expressing specific viral p24 antigen was detected in cell lines carrying t-CD4 (Figure 2A) at 12 days post-infection. A 12- to 40-fold increase in the percentage of cells expressing p24 was noted in the three cell lines expressing t-CD4 as compared with cells transfected with wt-CD4. A 30- to 300-fold increase in the release of infectious viral particles was detected in culture supernatants originating from cells carrying t-CD4 as compared with cells transfected with wt-CD4 following HIV-1 infection (Figure 2B). These experiments were repeated several times using these different clones and consistently showed that cells expressing the truncated form of CD4 yielded faster kinetics of HIV-1 infection. The most prominent difference in the level of HIV-1 replication was observed between clones A2D8 and 3D4D8 transfected with wt-CD4 and t-CD4, respectively. Most of our subsequent experiments were performed using these two clones. Similar differences at the level of virus production were obtained in subsequent experiments performed using various m.o.i. ranging from 0.01 to 0.14 (Figure 2C). To validate our results further, other cellular clones were derived from an independent transfection. Again, a marked increase in the level of HIV-1 replication was observed in t-CD4 cells as compared with wt-CD4 cells (data not shown). To confirm that this observation was not strain specific, these same cells were infected with the SF-2 strain of HIV-1 (Cheng-Mayer and Levy, 1988). A 30-fold increase in the percentage of cells expressing viral p24 antigen was detected in t-CD4 cells as compared with wt-CD4 cells (Figure 2D). To confirm that the role of the cytoplasmic tail of CD4 in HIV-1 replication was not restricted to A2.01 cells, the same CD4 constructs (wt-CD4 and t-CD4) were also expressed in HSB-2, another CD4-negative cell line. Again, the replication of HIV-1 was reproducibly greater in cells expressing the truncated form of CD4 with a 4- to 11-fold increase in the percentage of p24-positive cells observed at 15 days post-infection (Figure 2E). Moreover, a 6-fold increase in the number of infectious viral particles was detected in culture supernatants originating from infected cells carrying t-CD4 in comparison with cells transfected with wt-CD4 (Figure 2F). These results indicate that the cytoplasmic domain of CD4 can modulate HIV-1 replication. More importantly, this observation is not restricted to a specific virus strain or T cell line.

**Qualitative differences in viral proteins are not responsible for changes in virus replication**

To determine whether differences in the levels of viral replication were associated with qualitative and/or quantitative defects of specific viral proteins, Western blot analysis was performed on infected cell lines using antisera from infected patients (Figure 3A). Similar amounts of total cellular lysates obtained from A2D8 and 3D4D8 cells infected with HIV-1 for 7, 12 and 15 days were analyzed.
Controls consisted of uninfected A2D8 and 3D4D8 cells, and of U937 cells chronically infected with HIV-IIB. All major structural HIV-1 proteins (gp120, p66, p55, gp41 and p24) were detected in chronically infected U937 cells. These specific viral proteins were detected in 3D4D8 cells as early as 7 days following HIV-1 infection. In contrast, viral proteins were observed in A2D8 cells only 15 days following HIV-1 infection. A time-dependent increase in the amount of viral proteins was observed in both cell lines. These experiments clearly demonstrated the absence of qualitative changes in viral protein synthesis despite a marked increase in the synthesis of major structural viral proteins in cells carrying t-CD4 as compared with cells transfected with wt-CD4. These results are in agreement with those obtained with indirect immunofluorescence (percentage of p24-positive cells) and co-culture (number of infectious particles in culture supernatants) assays.

**Quantitative differences in HIV-1 RNA directly correlate with levels of HIV-1 proteins**

Since we observed marked differences in the expression of viral proteins, it was important to evaluate if such changes were associated with different RNA levels. For this purpose, Northern blot analysis of A2D8 and 3D4D8 RNA was carried out at different times after HIV-1 infection using a DNA probe which detects the three specific viral RNA species (Figure 3B, upper panel). Chronically infected U937 cells, used as control, express all three specific RNA bands. The full-length unspliced 9.2 kb mRNA encompasses the viral genomic mRNA and the mRNA for the gag-pol and gag proteins; the intermediate (~4–5 kb) corresponds to vpu, env, vif, vpr and tat mRNAs, while the small multiply spliced (~2 kb) mRNA produces the regulatory proteins tat, rev and nef. The full-length genomic RNA was detected in 3D4D8 cells as early as 7 days after viral infection. RNA levels increased in a time-dependent manner. However, when A2D8 cells were analyzed with the same probe, it was not possible to detect any specific RNA before 12 days after infection with HIV-1. To quantitate the levels of viral RNAs more precisely, slot-blot analysis was performed using total RNA extracted from infected A2D8 and 3D4D8 cells, and from chronically infected U937 cells (Figure 3B, lower panel). Ratios of HIV-1 mRNA and actin mRNA clearly indicate a 4-fold increase in the levels of HIV-1 mRNA in cells expressing t-CD4 as compared with cells transfected with wt-CD4. Results from this analysis further supported...
HIV-1 regulation by p56\(^{ck}\)–CD4

**Fig. 3.** (A) Western blot analysis of specific viral protein expression following HIV-1 infection of transfected A2.01 cells. Cellular lysates (100 \(\mu\)g) were resolved on a 10% polyacrylamide gel, transferred to nitrocellulose filter, incubated with polyclonal HIV-1-positive sera (1:500) and then with \([^{125}I]\)recombinant protein A/G. Samples were from wt-CD4 A2.01 cells (A2D8) either uninfected (1) or at 7 (3), 12 (5) and 15 days post-infection (7) and from t-CD4 A2.01 cells (3D4D8) either uninfected (2) or at 7 (4), 12 (6) and 15 (8) days post-infection. Chronically HIV-IIIB-infected U937 cells served as control (9). (B) Upper panel: Northern blot analysis of total RNA (10 \(\mu\)g) from cells infected with HIV-1 using pBH-10 (HIV-1) as probe. Lower panel: quantification of RNA content by slot-blot analysis. Total RNA (1.25 \(\mu\)g) was hybridized to pBH-10 and mouse actin probes, respectively. Samples were from A2.01 cells carrying wt-CD4 (A2D8) at 7 days (3) and 12 days post-infection (5) or A2.01 cells expressing t-CD4 (3D4D8), at 7 days (4) and 12 days post-infection (6), and U937 cells chronically infected with HIV-IIIB (7). Autoradiographs were analyzed using the RAS-1000 (Research Analysis System) image analysis system to evaluate HIV-1 and actin band intensity which are expressed in optical density (OD/pixels).

the observation that viral RNAs were more abundant in HIV-1 infected cells carrying t-CD4, as compared with cells expressing wt-CD4.

**Cellular parameters and viral entry are similar in cells expressing wt-CD4 and t-CD4**

Next, we started to examine which step in the virus replicative cycle was responsible for such marked changes in the levels of viral production between the two cell lines. Growth curves were determined for A2D8 and 3D4D8 cells, either infected or not with HIV-IIIB (m.o.i., 0.14). Doubling times were similar for each cell line and could not be responsible for the enhanced viral replication detected in cells expressing t-CD4 (data not shown). Cell viability, as assessed by Trypan blue exclusion, was also determined and the percentage of live cells up to 21 days post-infection was close to 100% for both cell lines (data not shown). Modulation of the CD4 molecule from the cell surface may also influence the rate of viral entry, thereby affecting levels of viral replication. For this purpose, A2D8 and 3D4D8 cells were incubated at 37°C with HIV-IIIB (m.o.i., 0.14) and CD4 surface expression was monitored at specific time points following viral infection. Results from these experiments demonstrated that the levels of CD4 expressed on the surface of cells transfected with wt-CD4 (A2D8) or t-CD4 (3D4D8) remained unchanged for 7 days post-infection. However, a

<table>
<thead>
<tr>
<th>Cell line</th>
<th>p24 antigen level (pg/ml)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2D8 (control)</td>
<td>386 ± 93</td>
</tr>
<tr>
<td>A2D8 + HIV-1</td>
<td>6800 ± 141</td>
</tr>
<tr>
<td>3D4D8 (control)</td>
<td>254 ± 78</td>
</tr>
<tr>
<td>3D4D8 + HIV-1</td>
<td>3975 ± 106</td>
</tr>
</tbody>
</table>

More rapid down-modulation of CD4 expression was detected over longer periods of time on cells expressing t-CD4 which correlates with the higher rate of HIV-1 replication in such cells (data not shown).

Experiments aimed at quantitating the virus load entering cells very early after infection were also carried out. The
above-described cell lines were incubated with HIV-1 at 4°C to permit viral binding and were subsequently transferred at 37°C to allow viral entry. The cells were then incubated at pH 3.0 to remove viruses which had not entered cells. Levels of viral p24 protein as monitored using a commercial enzymatic assay (Table 1), revealed that viral entry was not increased in cells carrying t-CD4. To quantitate precisely the extent of viral entry, semi-quantitative PCR was performed at 6 h after infection. To decrease PCR amplification of premature HIV-1 reverse transcripts, we have used a set of primers (M667/M661) that recognize only full-length or nearly completely synthesized viral DNA (Zack et al., 1990). To rule out the possibility that PCR amplification could be due to the presence of partial reverse transcripts in infectious mature HIV-1 particles (Lori et al., 1992; Trono, 1992), PCR amplifications were carried out on cells previously incubated with heat inactivated virus (Figure 4B, lanes 3 and 4). Linearity of the PCR assay was assessed by using different concentrations of ACH-2 cells previously reported to contain one copy of viral DNA (Figure 4A). Results of the experiments clearly indicated that increased viral replication in cells expressing the truncated form of CD4 could not be attributed to facilitated entry of the virus into cells. They suggested that other mechanisms could be responsible for this phenomenon.

**The full-length CD4 molecule negatively regulates HIV-1 replication**

To investigate the possibility that viral dissemination by cell-free virus and/or cell-to-cell contact might be more efficient in cells expressing t-CD4, the antiviral drug azidothymidine was added at a non-toxic inhibitory concentration (1 μM) to both cell lines 24 h after their initial contact with HIV particles. Addition of azidothymidine inhibits any further cycle of viral integration mediated either by cell-free virus or via cell-to-cell contact. However, it does not affect replication of viral DNA which is already integrated. Consequently, infected cells are derived only from the initial pool of infected cells. As expected, in the absence of drug, higher viral expression was observed in cells carrying t-CD4 (100%) in comparison with cells transfected with wt-CD4 (47%) (Figure 5A). In azidothymidine-treated t-CD4 cells, the number of HIV-1-positive cells remains almost negative.
An increase in autophosphorylation activity of the CD4-associated lck is induced by cross-linking of CD4. Cells (10^7) were incubated on ice with 1F3 anti-CD4 antibody at 5 µg/ml (lanes 2, 4 and 6) or with medium (lanes 1, 3 and 5) for 30 min, prior to cross-linking with a goat anti-mouse serum for 2 min at 37°C. CD4 was then immunoprecipitated and an in vitro kinase assay was performed. Proteins were resolved on a 7.5% SDS–polyacrylamide gel. Lanes 1 and 2, HSB-2 CD4 (A4); lanes 3 and 4, A2.01 A2D8; lanes 5 and 6, Jurkat cell line.

until ~12 days post-infection; however, the number of HIV-1 particles released into the medium (data not shown) was so important that despite the presence of azidothymidine, effective HIV-1 replication was observed over time with ~50% of cells expressing p24 22 days post-infection. Interestingly, viral replication was totally abrogated in cells expressing wt-CD4 even at the later time points (22 days post-infection). This experiment was repeated twice and yielded similar results. Results from these experiments suggest that viral replication is down-regulated in cells expressing wt-CD4 into which viral binding and internalization has occurred. This down-regulation is not observed in cells expressing t-CD4.

The negative signal is transduced via the CD4 molecule

To determine if the negative signal on viral replication was transduced by the CD4 molecule, different concentrations of soluble CD4 (sCD4) were added 24 h following HIV-1 infection of wt-CD4 and t-CD4 A2.01 cells. In these experiments, viruses which are already integrated are allowed to replicate. However, sCD4 will bind to viral particles released from infected cells, thereby preventing their interaction with wt-CD4 or t-CD4 on the surface of transfected cells. Results from these experiments are illustrated in Figure 5B. Addition of sCD4 at different concentrations (2 and 20 µg/ml) led to a significant decrease in the percentage of p24^+ cells in both wt-CD4- and t-CD4-expressing cells. The number of p24^+ cells ranged between 4.3 and 7% in cells incubated in the presence of 20 µg/ml sCD4 at 24 days post-infection, while all of the cells were infected in the absence of sCD4. Interestingly, in the presence of sCD4, we observed for the first time a similar percentage of p24^+ cells in both cell lines. In triplicate experiments with 20 µg/ml of sCD4, the percentage was ~4.3% in wt-CD4 cells and 7% in t-CD4 cells. Similar results were also observed when 2 µg/ml of sCD4 were added to these cultures (Figure 5B). These results strongly suggest that the CD4 molecule is responsible for transducing the signal which down-regulates HIV-1 replication.

The protein tyrosine kinase p56^{lb} plays a dominant role in the transduction of the negative signal

The CD4 molecule is associated in T cells with the src family tyrosine kinase p56^{lb} (Rudd et al., 1988). Cross-linking of CD4 using anti-CD4 antibodies or gp120 leads to an increase in autophosphorylation activity of the CD4-associated lck (Veillette et al., 1989; Koretzky et al., 1990; Luo and Sefton, 1990; Hivroz et al., 1993). It is thus possible that the above-described regulatory signal generated through CD4 involves p56^{lb}. We first verified if the CD4-associated lck could be activated in the HSB-2 CD4 A4 and the A2.10 A2D8 cell lines, following cross-linking with an anti-CD4 mAb that prevents gp120 binding to CD4. We observed a 3.2-fold (HSB-2 CD4 A4) and a 3.7-fold (A2.01 A2D8) enhancement in the autophosphorylation activity of the CD4-associated lck following a 2 min CD4 cross-linking at 37°C (Figure 6). This increase in lck autophosphorylation activity was comparable with the 3.6-fold enhancement observed upon CD4 cross-linking on Jurkat, which is a mature T cell line. Furthermore, a similar pattern of tyrosine phosphorylation of substrates was observed after cross-linking of CD4 at the surface of both Jurkat and A2.01 A2D8 cells (data not shown), suggesting that the CD4-lck association in HSB-2 CD4 and A2.01 A2D8 cell lines is functional.

To examine the involvement of the CD4-associated tyrosine kinase p56^{lb} in the above-described down-regulation of HIV-1 replication, a CD4 mutant lacking the residues responsible for p56^{lb} association was transfected into A2.01 and HSB-2 cells. Infection of A2.01 and HSB-2 cells expressing C4202A mutant was carried out as described previously and compared with infection of wt-CD4

HIV-1 regulation by p56^{lb} - CD4
cells and t-CD4 cells. Results are illustrated in Figure 7A (A2.01) and B (HSB-2). A 6- to 20-fold increase in the percentage of p24+ cells was detected in A2.01 (25%) and HSB-2 (21.6%) cells expressing the mutated form of CD4 when compared with A2.01 (1.25%) or HSB-2 (3.9%) cells transfected with the wt-CD4. This difference was observed throughout the different time points of the experiment. Furthermore, the percentage of p24+ cells was almost identical in cells expressing the mutated form of CD4 and t-CD4 A2.01 (31.5%) and t-CD4 HSB-2 cells (16.6%). Results of these experiments indicate that the association of p56cck to CD4 contributes to the decreased viral replication observed in cells expressing the full-length form of CD4.

**Discussion**

We present evidence suggesting that the full-length CD4 molecule transduces a signal that will negatively regulate HIV-1 replication. Cells expressing a truncated form of CD4 or a mutant CD4 molecule which can no longer associate with p56cck permitted increased levels of viral production. These results were obtained using independently derived cell clones for each construct and two different strains of HIV-1 (HIV-1IIIB and SF-2), thus eliminating the possibility of a strain-specific phenomenon. A very low m.o.i. was intentionally used in each experiment to parallel physiological conditions. Viral p24 protein levels and quantitative PCR were used to determine that the efficiency of viral entry and reverse transcription was similar following HIV-1 infection of cells expressing either wt-CD4 or t-CD4. Our results strongly suggest that the event responsible for the different levels of viral expression occurs following reverse transcription of viral RNA and prior to initiation of transcription.

The above-described experiments had indicated that viral entry was identical in cells expressing the different forms of CD4, suggesting that the quantitative differences observed in HIV-1 expression were due to later events in the viral replication cycle. Addition of azidothymidine to cells which had integrated comparable amounts of HIV-1 resulted in an enhancement of the differences observed in the percentage of p24+ cells between t-CD4 cells and wt-CD4 cells. A 50-fold difference could be observed 23 days post-infection. These results can be explained by a model whereby viral particles released from cells infected prior to addition of azidothymidine bind to CD4 on such HIV-1-infected cells and induce multimerization of CD4. This leads to the transduction of a signal that negatively regulates the expression of already integrated viral DNA (Figure 7A). Alternatively, the negative signal could affect integration of viral DNA. This negative signal is not transmitted in cells carrying t-CD4 since deletion of the cytoplasmic domain of CD4 abrogates the interaction of CD4 with p56cck (Shaw et al., 1990; Turner et al., 1990), thus leading to enhanced viral replication. To confirm that CD4 was in fact playing a primordial role in the transduction of a putative negative signal, sCD4 was added to the cells soon after infection with HIV-1. This antiviral agent was used to abrogate multimerization of CD4 induced by HIV-1 particles released from cells into which viral integration had already occurred (Figure 8B). sCD4 also prevents de novo infection of CD4+ cells mediated by cell–cell contact or cell-free virus. Results from this set of experiments demonstrated that the rate of virus replication was similar in cells carrying the wt-CD4 or t-CD4, again confirming that the initial infection was comparable in cells expressing both forms of CD4. These experiments clearly demonstrated that the negative regulatory effect results from the binding of HIV-1 to CD4. Most importantly, experiments using a mutated form of CD4 which is not associated with p56cck clearly demonstrated that this tyrosine kinase is implicated in the transduction of the negative signal (Figure 7).

Our results contrast with those of Poulin et al. (1991) who observed a delay of HIV-1 replication in A2.01 cells expressing a truncated version of CD4, lacking most of the cytoplasmic domain. These differences may be explained by the fact that they have used at least 100- to 1000-fold more virus particles for infection. Our results are consistent with a previous report which demonstrated that a glycoprotein-anchor CD4 molecule, lacking membrane spanning and cytoplasmic domains, was associated with enhanced HIV-1 replication in the same human T-lymphoid cell line (HSB-2).
that has been used in our experiments (Diamond et al., 1990). Two recent studies have also proposed that the CD4 glycoprotein could influence replication of HIV-1. Binding of recombinant HIV-like particles (containing only the envelope glycoprotein of HIV) to CD4 was shown to inhibit induction of latent HIV-1 (Haffar et al., 1992). Interestingly, viral particles which contained another viral protein (gag) but not env did not mediate this effect on HIV-1 replication. Moreover, expression of increasing levels of CD4 in HSB-2 cells was shown to correlate with a marked decrease in HIV-1 replication, which is consistent with our results (Marshall et al., 1992). In this report, similar levels of virus entry and reverse transcription were observed in cells expressing low and high levels of CD4, which is also similar to our data.

Results from our experiments suggest that binding of HIV-1 to cell surface CD4 stimulates signal transduction pathways in T cells. Recent evidence consistent with this scenario came from the observation that treatment of CD4-positive cells with gp120, followed by anti-gp120 antibodies, results in increased tyrosine kinase activity of p56Lck (Juszczak et al., 1991). Our results also indicate that the signal leads to decreased viral replication. Cross-linking of CD4 molecules with CD4-specific mAbs or with gp120 prior to T cell receptor mediated stimulation has been shown to inhibit T cell activation and to induce T cell death (Newell et al., 1990; Banda et al., 1992). Several mechanisms including negative signaling by p56Lck (Juszczak et al., 1991) or sequestration of p56Lck by CD4 (Haughn et al., 1992) have been suggested to play a role in this down-regulation of T cell activation by CD4. T cell activation leads to enhanced HIV-1 replication indicating the presence of common pathways between these two responses. It is thus possible that signals which will negatively regulate T cell activation will also result in down-regulation of HIV-1 replication.

Numerous studies have demonstrated that various factors can affect negatively HIV expression at transcriptional or post-transcriptional levels. Transcriptional activity of HIV is regulated by virally encoded factors, such as tat, and also by inducible cellular transcription factors. Some of these factors (L-P-1 and USF/MLTF) have been shown to regulate negatively HIV-1 transcription (Kato et al., 1991; Giacca et al., 1992). Another report has indicated that integrated HIV-1 genome is maintained in an inactive state by cellular factors binding to a region of the promoter located in the long terminal repeat between -120 and +80 relative to the transcription initiation site (Drysdale and Pavlakis, 1991). One particularly well-characterized nuclear transcription factor, NF-xB, has been reported to greatly stimulate the HIV-1 enhancer (Böhnlein et al., 1988). An 1xB-like molecule has been demonstrated to induce negative regulation of HIV-1 transcription by blocking the formation of the 65 kDa plus 50 kDa NF-xB heterodimer (Raziuddin et al., 1991). Dissociation of NF-xB from its inhibitor 1xB is achieved following phosphorylation of 1xB by protein kinase C. It is thus possible that one of these factors is involved in the negative regulation of HIV-1 that we have observed in our experiments.

The in vitro studies presented here provide evidence that the cytoplasmic domain of CD4 can regulate the rate of HIV-1 expression in T cells. Our hypothesis is that, following binding of HIV-1 to CD4, a signal is transduced via p56Lck leading to phosphorylation of cellular and/or viral substrates and resulting in degradation of viral genome prior to integration or down-modulation of viral replication following efficient viral integration. The relevance of our observations to the in vivo situation is not known but it is tempting to speculate that it might play a role in the pathogenesis of this retroviral infection, particularly at the level of latency. The mechanism by which HIV remains latent in infected individuals is still unclear. It seems that most infected cells are transcriptionally silent since the frequency of cells expressing HIV-1 RNA has been shown to be one to two orders of magnitude lower than that of provirus-containing cells (Harper et al., 1986; Psallidopoulos et al., 1989; Schnittman et al., 1989). Binding of cell-free virus present in the circulation (Coombs et al., 1990; Ho et al., 1990) to infected cells could lead to transcriptional down-regulation of HIV-1 integrants. The hypothesis that the great majority of proviruses in infected individuals are functionally defective is of interest in this regard (Goodenow et al., 1989). Such defective particles, even though they could not initiate a full virus replicative cycle, might be able to multimerize the CD4 molecule, thus leading to the transduction of a signal that will negatively affect replication of integrated viral DNA and induce viral latency. This would prevent a quick destruction of the host and help maintain a sustained level of viral infection.

Materials and methods

Cells and media

A2.01 and HSB-2 are T-cell tumor cell lines which are CD4-negative and have been previously described (Folks et al., 1986; Hara et al., 1988). These cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 2 mM l-glutamine, 50 μM 2-mercaptoethanol and 1 mg/ml of gentamicin (G-418) when required. Cell lines producing recombinant retroviruses were maintained in DMEM medium supplemented with 10% FBS, 2 mM l-glutamine, 50 μM 2-mercaptoethanol and 1 mg/ml of G-418.

Expression of the full-length, truncated and mutated CD4 in A2.01 and HSB-2 cells

Wild-type and truncated CD4 coding regions were subcloned into the MNC retroviral vector containing a neomycin resistance gene (Peterson and Seed, 1988). The cytoplasmic domain of CD4 was truncated by insertion of a stop codon in the coding sequence. Only the first six membrane-proximal residues were conserved. The generation of the double cysteine mutant was performed by overlap extension procedure. Briefly, DNA fragments containing the mutation were amplified using 250 ng of MNC plasmid containing the wild-type CD4 cDNA as template, 100 ng of either of complementary primers containing the desired substitutions (5'-CCGGTGAGGGGCCTGGGCGGT-3' or 5'-AAGAAGACGCCAGCCAGGCTTCTT-3'), 100 ng of 5' or 3' primers hybridizing to MNC vector sequences and a reaction mix containing 2.5 U of Taq polymerase, 0.2 mM of each of the dNTPs, 100 mM KCl, 10 mM Tris—HCl pH 9.0, 1.5 mM MgCl₂, 0.01% gelatin and 0.1% Triton X-100. The DNA was amplified for 20 cycles consisting of denaturation for 30 s at 94°C, annealing for 2 min at 50°C and elongation at 72°C for 2 min, followed by an elongation cycle of 15 min at 72°C. The mutated cDNA was generated in a subsequent overlap-elongation amplification using 250 ng of each of the two overlapping fragments, 100 ng of each of the vector encoded 5' and 3' primers and the same amplification conditions as above. The full-length mutated cDNA was then cloned in the HindIII-BamH1 sites of the eukaryotic expression vector MNC stuffer. The whole cDNA was sequenced using Sanger's method to confirm the presence of the desired substitutions and the integrity of the rest of the DNA. The amphotropic helper packaging cell line DAME (Sleeman et al., 1988) was transfected by calcium phosphate co-precipitation with the different pMNC constructs and G-418 resistant DAME cells were selected and used as producer cell lines of the recombinant retrovirus. DAME cells transfected with the different pMNC-CD4 constructs were enriched for cells expressing high levels of membrane CD4 by cell sorting. For infection, DAME cells, at 70% confluence, were treated with 10 μg/ml of...
of mitomycin C at 3 h at 37°C and washed extensively with phosphate buffered saline (PBS) prior to co-culture with A2.01 and HSB-2 cells (1 × 10⁶) in complete RPMI medium in the presence of 8 µg/ml of polybrene for 24 h at 37°C. Cells were washed three times with PBS and resuspended in complete RPMI medium for 24 h at 37°C. The cells were finally seeded at a density of 50,000 cells per well (2 ml) in complete RPMI medium containing 1 mg/ml of G-418. Medium was changed twice a week and G-418 resistant A2.01 and HSB-2 cells were analyzed for the expression of CD4 molecules by flow cytometry.

**Flow cytometry analysis**

Cells were incubated with 1F3 anti-CD4 monoclonal antibody at 5 µg/ml (a gift from A.Turne, SmithKline Beecham Pharmaceuticals, King of Prussia, PA, USA) for 30 min at 4°C, washed with PBS and incubated under similar conditions with FITC-coupled goat anti-mouse IgG at 2 µg/ml (Southern Biotechnology Associates, Inc., Birmingham, AL, USA). Cells were washed once and resuspended in PBS prior to analysis using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA).

**Western blot analysis**

Cellular lysates were prepared and subjected to electrophoresis through 10% polyacrylamide gels. The resolved proteins were then transferred onto nitrocellulose filters. Filters were saturated with a 5% milk solution and incubated overnight at 4°C with 1:5000 v/v dilution of polyclonal antiserum from HIV-1-infected subjects. Filters were washed several times and incubated for 60 min with 125I-labeled recombinant protein A/G (Calbiochem, La Jolla, CA, USA) (0.5 × 10⁶ c.p.m./ml), washed, dried and exposed to X-ray films.

**Northern blot analysis of HIV-1 RNA**

Total cellular RNA was isolated at specific time points following viral infection by a modified guанинов therapy iodophenyl procedure (Chomczynski and Sacchi, 1987). Similar amounts of total cellular RNA (10 µg) were resolved on formaldehyde ÷ 1.0% agarose gels and transferred to GeneScreenPlus membrane. Filters were hybridized with random-primed 32P-labeled products of PHB-10 (Shaw et al., 1984) and of mouse actin (Minty et al., 1981) to detect HIV-1 and actin, respectively. Filters were washed in 2 × SSC–0.1% SDS followed by 0.1 × SSC–0.1% SDS at 64°C. Blots were exposed to X-OMAT film (Kodak) at −70°C with intensifying screens for 24 h.

**[125I]rgp120 binding assay**

Soluble immunopurified recombinant gp120 (rgp120), kindly provided by Genentech (Dr T.Gregory), was radiolabeled by the lactoperoxidase method (Marchalonis, 1969). Cells were incubated with increasing concentrations of [125I]rgp120 for 4 h at 25°C with gentle mixing in a final volume of 200 µl RPMI-1640, 25 mM HEPES and 0.1% BSA (pH 7.4). Non-specific binding was evaluated by using a 100-fold excess of unlabeled rgp120. Cells were then layered onto a 200 µl oil cushion made of 8% silicone oil and 16% paraffin oil and were centrifuged at 17,000 g for 2 min. Cell pellets were obtained by cutting off the tip of the cell and cell-bound [125I]rgp120 radioactivity was measured in a gamma counter. Average of duplicate determinations of bound [125I]rgp120 were used for data analysis. Saturation binding curves were analyzed by non-linear least-squares curve fitting according to mass action law (DeLéan et al., 1982).

**Autophosphorylation assay**

An increase in autophosphorylation activity of the CD4-associated ick was observed following the cross-linking of CD4. Briefly, 10⁶ cells were incubated with anti-CD4 antibody 1F3 at 5 µg/ml with simultaneous medium (DMEM, BSA 0.5%, HEPES 10 mM) for 30 min on ice. Cells were then washed, treated with a goat-anti-mouse serum (Sigma) at 50 µg/ml for 2 min at 37°C, and then lysed in NP-40 lysis buffer (NP-40 2%, DOC 0.5%, Trit 50 mM, NaCl 150 mM, NaF 50 mM, NaPO₄ 10 mM, EGTA 2 mM, EDTA 10 mM, Na orthovanadate 500 µM, leupeptin 3 × 10⁻⁶ M, pepstatin A 2 × 10⁻⁶ M and PNBG 50 µM). CD4 was then immunoprecipitated with protein A–Sepharose precoated with a rabbit anti-CD4 serum. Autophosphorylation was performed as follows. After extensive washes, the immunoprecipitates were resuspended in 50 µl of kinase buffer (10 µCi of [γ-³²P]ATP, 50 µM of cold ATP, Na orthovanadate 100 µM, PNBG 50 µM, MgCl₂ 20 mM, MnCl₂ 10 mM, Triton X-100 0.1%, Na orthovanadate 100 µM, PNBG 50 µM) and washed three times before addition of sample buffer. Proteins were then resolved on a 7.5% SDS–polyacrylamide gel. The gel was then dried and exposed to an X-ray film for 2 h. The gel was also exposed to Kodak storage phosphor screens and quantitation was done on PhosphorImager devised by the ImageQuant Software Package (Molecular Dynamics, Sunnyvale, CA, USA).

**Acknowledgements**

We wish to acknowledge the kind editorial assistance of Mrs Nicole Guay and the expert flow cytometry assistance of Mr Claude Cantin. The Flow