HIV-induced Apoptosis Requires the CD4 Receptor Cytoplasmic Tail and Is Accelerated by Interaction of CD4 with p56^{lck}

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Summary

The roles of the CD4 receptor and the src kinase p56^{lck} were examined in the process of HIV-induced apoptosis of CD4$^+$ T lymphocytes. The presence of the CD4 cytoplasmic tail was found to be essential in delivering an apoptotic signal, and interaction of CD4 with p56^{lck} potentiated HIV-induced apoptosis. Apoptosis, but not HIV replication, was abrogated by deleting the NH_2-terminal intracytoplasmic tail of CD4, or by mutating the two critical cysteines in this tail that are responsible for CD4–p56^{lck} interaction. Introduction of p56^{lck} in C8166-45 or MT-2 cells, CD4$^+$ T cell lines deficient for this protein, greatly increased HIV-induced apoptosis and syncytium formation. The ability of p56^{lck} to deliver an apoptotic signal did not depend on its kinase function, since a kinase-deficient mutant was as effective as its normal counterpart in inducing apoptosis, suggesting that p56^{lck} may act as an adapter to anchor other proteins to transduce the death signal.

HIV-1 infection of CD4$^+$ T lymphocytes in cell culture induces apoptotic cell death in the absence of HIV-specific antibody or immune response (1, 2). Binding of glycoprotein (gp) 120 to CD4 on already infected cells appears to be a necessary step in the induction of this form of apoptosis (3). This study was undertaken to analyze additional steps in HIV-induced apoptosis after binding of gp120 to the CD4 receptor.

The membrane glycoprotein CD4 is the primary receptor for HIV-1 (4) but its normal function is to enhance antigen-mediated activation of T cells restricted by class II molecules of the major histocompatibility complex (5–7). This function is initiated by CD4 aggregation and rendered possible through the noncovalent association of the cytoplasmic tail of the CD4 protein with p56^{lck} (8, 9). T lymphocytes bearing this receptor are eliminated throughout the course of HIV disease. The rate of the CD4$^+$ T cell depletion, a hallmark of AIDS, remains the primary prognostic marker of disease progression.

Both direct and indirect mechanisms of cytopathology have been postulated to be responsible for CD4$^+$ T cell depletion of AIDS. These include syncytium formation (10, 11), superinfection (12), gp120 binding to CD4 on uninfected cells (13), antibody-dependent cell cytotoxicity, killing by cytotoxic CD8$^+$ T cell of gp120-coated cells, random Vβ T lymphocyte deletion (14), complement-mediated killing, and apoptosis (1, 2).

Apoptosis is a normal cellular process culminating in the activation of a cellular endonuclease that digests chromosomal DNA, initially in large fragments (50–300 kbp), and ultimately, in small oligomers of 180 bp corresponding to a segment of DNA wrapped around a nucleosome and therefore protected from enzymatic digestion. Other phenotypic changes are also manifested during apoptosis. The cell shrinks and blebbing occurs to facilitate ingestion by phagocytic cells. Apoptosis is aimed at efficient removal of altered and unwanted cells without generating inflammatory responses and is part of an essential mechanism of cell attrition in developmental and regulatory processes (15, 16).

CD4 signaling is a complex process, and how this function is altered during HIV infection remains unclear. A number of conflicting reports concerning CD4 signaling have been presented. gp120 binding to the CD4 receptor has been reported to inhibit CD4-dependent antigen responses (17), and uncouples TCR signaling rendering the cell anergic (18). Delivery of a signal after gp120-mediated CD4 multimerization has been reported both to diminish or increase viral replication (19, 20).

The roles of the CD4 receptor and p56^{lck} in triggering HIV-induced apoptosis were investigated. We have found that apoptosis occurred only in A2.01 T cells in which the wild-type CD4 receptor was introduced. In contrast, a truncated form of CD4 or a mutant CD4 unable to bind p56^{lck} were not susceptible to apoptosis despite being read-

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ily infectable by HIV-1. Furthermore, CD4–p56^{lck} interaction was determined to be a requisite in delivering an apoptotic signal, and introduction and overexpression of p56^{lck} in CD4$^+$ T cells greatly increased apoptosis and syncytium formation. The ability of p56^{lck} to deliver an apoptotic signal did not depend on kinase function, as a kinase-deficient form of p56^{lck} was as effective as its normal counterpart in inducing apoptosis, implying that other molecules are involved in mediating the apoptotic signal.

Materials and Methods

Cell Lines. The A2.01 and HSB-2 parental cell lines were obtained from Dr. Rafick-Pierre Sékaly (Institut de Recherches Cliniques de Montréal, Montréal, Canada). The CD4 wild-type, 402 stop, and dicysteine mutant at positions 420 and 422 (C to A) have been previously described (19). MT-2 and C8166-45 are HTLV-1–transformed CD4$^+$ T lymphoblastoid cell lines, which do not express p56^{lck} (21). They were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program. The CEM CD4$^+$ T lymphoblastoid cell line was obtained from Dr. Dennis Carson (University of California, San Diego, La Jolla). The wild-type CD4 gene and mutants were introduced in A2.01 and HSB-2 using the retroviral vector MNC stuffer and the amphotropic packaging cell line Damp (22). The p56^{lck} gene (cloneHK28: Genbank accession number M36881) and phosphorylation-deficient mutant were introduced into the MT-2 and C8166-45 cell lines using the same methodology under the selection of gentamicin (800 μg/ml). Two MT-2 clones expressing high levels of p56^{lck} were selected (Nos. 10 and 11); three were chosen from the C8166-45 cells (Nos. 7, 8, and 9), as well as one representative clone (No. p-mutant) with substitution of an alanine at position 273 (ATP-binding site) for a lysine which rendered the protein incapable of phosphorylating itself (23).

Viral Preparations. High titer stocks of HIV-1_{LAI} (5×10^7 tissue culture infectious dose $[TCID]_{50}$/ml) were prepared and titered using the end point dilution method of Kärber (24). High titer stocks were prepared by inoculating CEM cells at a multiplicity of infection (moi)[1] of 0.001 and growing the cells for 10 d. 10 ml of this culture was added to 400 ml of uninfected CEM (5×10^5 cells/ml) and grown for 5–7 d until abundant syncytia were present. The cells were pelleted (300 *g*/10 min) resuspended in 1/100 of the initial vol for 8 h. The supernatant was clarified by centrifugation (800 *g*/10 min).

HIV-1 Infection. The infection protocol was identical for each of the cell types used. Briefly, $5–10 \times 10^6$ cells were inoculated with HIV-1_{LAI} at moi of 0.5 or 1 in 1 ml culture medium in which 2 μg/ml of polybrene was added to facilitate infection. The cells were incubated for 3 h at 37°C in 5% CO_2 in air. The cells were then washed once with cold RPMI 1640 and resuspended at a density of 5×10^5 cells/ml in culture medium. Aliquots of $1–2 \times 10^6$ cells were taken daily to perform the analyses.

Detection of Apoptosis-associated Chromatin Degradation and Cell Viability by Flow Cytometry. Cells ($\approx 2 \times 10^6$) were washed in PBS and resuspended in 30% ethanol and kept at 4°C. The cells were stained with propidium iodide (PI) as previously described (25, 26) with slight modifications. The cells were centrifuged and resuspended in PBS containing 0.1 mM EDTA$(Na)_2$, RNase A at 50 μg/ml (50 U/mg), and PI (50 μg/ml). The cells were then washed twice with PBS before analysis by flow cytometry with a fluorescence-activated cytometer (Elite; Coulter Corp., Epics Div., Hialeah, FL) and the cell cycle was analyzed with doublet discrimination protocol. PI was excited using a 488-nm line of an argon laser and detected with a 620–700-nm long pass filter. This assay has an SE of ±3%. The percentage of apoptotic cells was obtained for each time point by subtracting the percentage of apoptotic cell death in uninfected control cultures from the HIV-infected culture. Cell viability was determined by incubating 10^6 cells with 2 μM of EthD1 (Molecular Probes, Inc., Eugene, OR), which stained DNA if membrane integrity was not preserved. The cells were then fixed in 1% paraformaldehyde in PBS and analyzed within 3 h.

Determination of the Level of p56^{lck}, p56^{lck} and CD4 Association by Immunoprecipitation and Generation of p56^{lck} Autophosphorylation-deficient Mutant. Determination of the level of p56^{lck} protein present in stably transduced C8166-45 and MT-2 cells was carried out as follows. 10^6 cells were solubilized in SDS-PAGE electrophoresis Laemmli buffer under reducing conditions. The samples were boiled 10 min and subjected to electrophoresis on a 10% SDS-acrylamide gel. The gel was then electroblotted onto a nylon membrane. Detection was carried out using a combination of a monoclonal anti-p56^{lck} mAb (clone 3A5 at a 1:3,000 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA) and an affinity-purified peroxidase-conjugated sheep anti–mouse IgG2b antibody (1:5,000) (The Binding Site, Birmingham, UK). Immunoreactive bands were visualized using the ECL Detection System (Amersham Corp., Arlington Heights, IL) according to the manufacturer's instructions. Similarly, the association of p56^{lck} to CD4 was determined by solubilizing 2×10^7 cells in 500 μl of a lysis buffer consisting of 1% NP-40, 20 mM Hepes, pH 7.9, 150 mM NaCl, 20 mM NaF, 1 mM Na_3VO_4, 1 mM $Na_4P_2O_7$, 1 mM EDTA, 1 mM EGTA, 10 μg/ml aprotinin, and 10 μg/ml leupeptin for 45 min at 4°C. The suspension was centrifuged at 14,000 rpm for 15 min. The supernatant was then incubated at 4°C for 30 min, with protein A–Sepharose (Pharmacia, Uppsala, Sweden) and then 2 h with protein A–Sepharose previously coupled with anti-CD4 antibody (OKT4; Johnson and Johnson, Raritan, NJ) (50 μl of protein A–Sepharose + 4 μg of OKT4 antibody overnight at 4°C). The mixture was centrifuged at 10,000 rpm for 10 min and resolubilized directly in SDS sample buffer. The samples were electrophoresed, blotted, and immunoreactive bands were visualized using the ECL Detection System.

An autophosphorylation-deficient p56^{lck} was generated by substituting the lysine residue at position 273 with alanine using PCR overlap extension procedure (27). Wild-type and mutated p56^{lck} constructs were then subcloned in the eukaryotic expression retroviral vector MNC stuffer. The amphotropic helper packaging cell line DAMP was transfected with the constructs by calcium phosphate coprecipitation. The resulting recombinant amphotropic retrovirus particles containing the human p56^{lck} cDNA driven by the MuLV LTR and the neo gene driven by the SV40 promoter were used to stably infect C8166-45 and MT-2 cells selected with 0.8–1 mg/ml of the antibiotic G418 (Gibco-BRL, Gaithersburg, MD).

Autophosphorylation of p56^{lck} mediated by cross-linking of cell surface CD4 was carried out as follows: C8166-45 cells (10^6) were incubated with anti-CD4 Q428 antibody (5 μg/10^6 cells) before incubation with goat anti–mouse IgG (20 μg/10^6 cells). Cells were resuspended in lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 10% glycerol, 0.025 mM *p*-nitrophenyl guanidinobenzoate, 10 μg/ml aprotinin, 10

[1] *Abbreviations used in this paper:* moi, multiplicity of infection; PI, propidium iodide.

μg/ml leupeptin, 1 mM sodium orthovanadate, and 10 mM sodium fluoride) and electrophoresed on a 10% SDS-polyacrylamide gel. Immunoreactive bands were visualized using the ECL Detection System.

CD4 Staining and p24 ELISA. For the quantitation of surface HIV-infected cells (10^6) were washed in PBS and resuspended in 200 μl of PBS + 2% FBS. The anti-CD4 mAb OKT4 was added (20 μl) to the cells (except secondary antibody used as a control for nonspecific binding) and kept at 4°C for 30 min for staining. The cells were then washed twice in PBS and resuspended in 200 μl of PBS + 2% FBS to which 4 μl of goat anti–mouse IgG-FITC (Tago Inc., Burlingame, CA) was added and kept at 4°C for 30 min. The cells were then washed in PBS and resuspended in 400 μl of 0.5% paraformaldehyde in PBS and kept in the dark at 4°C until analyzed by FACS®. HIV p24 antigen was measured by an enzyme immunoassay as described by the manufacturer (Abbott Laboratories, North Chicago, IL).

Results

Binding of gp120 to CD4 mediates apoptosis in HIV-1–infected cells; however, the intracytoplasmic tail of CD4 has been shown to be dispensable for HIV replication. The ability of HIV-1 to induce apoptosis was investigated in two CD4 T cell lines. The wild-type full-length CD4 receptor was stably introduced into both the A2.01 and HSB-2 CD4-negative lymphoblastoid T cell lines. Stable transfectants carrying a truncated form of the CD4 receptor, with a premature stop codon at position 402, and a mutated form with two point mutations replacing the two cysteine residues at positions 420 and 422 by alanines, were also generated (Fig. 1). The expression of all three forms of the CD4 receptor rendered the cell fully infectable by HIV-1. The truncated form is predicted to escape CD4 downregulation by viral Nef protein (28) and presumably unable to deliver a signal through association with cytoplasmic signaling proteins including $p56^{lck}$. The dicysteine mutant is predicted to retain all the capabilities of the wild-type CD4 receptor except the ability to bind $p56^{lck}$ protein and transmit a signal via this pathway (29).

The CD4-negative A2.01 parental cell line was uninfectable, and did not undergo apoptosis despite being subjected to a high inoculum of $HIV\text{-}1_{Lai}$ (moi:1). This is consistent with the notion that the CD4 receptor is essential for high efficiency infection. A background amount of apoptosis (2.1%) was observed and represents the cell death of uninfected cells occurring with in vitro cell culture conditions. The HIV p24 antigen values obtained 3 d after inoculation were very low and corresponded to residual inoculum persisting after washes (9 ng/ml). In contrast, when the CD4 wild-type, CD4 stop, and CD4 dicysteine mutants were inoculated, high levels of virus replication were observed as determined by HIV p24 antigen production (341, 283, and 286 ng/ml, respectively). Only the A2.01 expressing wild-type CD4 underwent apoptosis (24.9% at day 3 in this representative experiment. The range was 22–34% in four experiments). The cells expressing CD4 stop and the CD4 dicysteine mutants in which HIV replicated to high levels similar to the cells expressing wild-type CD4, displayed little or no apoptosis during the course of the experiment (Fig. 2 *A*). A small amount of apoptosis was initially detected in CD4 dicysteine mutant, but later, at day 7, the percentage of cell undergoing apoptosis returned to background level (<5%). Cell viability as assessed by flow cytometry was >95% for uninfected cells, >90% for HIV-infected cells, except A2.01 with wild-type CD4, which was 55% at day 3.

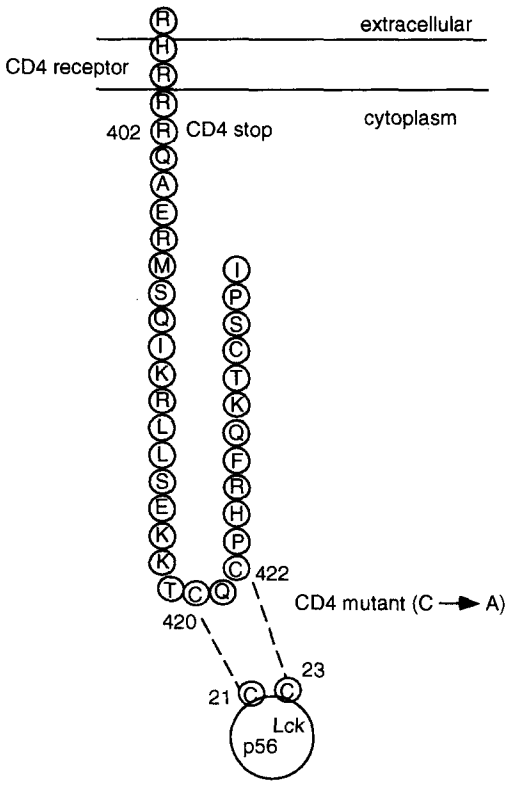


Figure 1. Schematic diagram of the intracytoplasmic tail of the CD4 receptor. The wild-type CD4 receptor was stably introduced in both A2.01 and HSB-2 lymphoblastoid T cell lines. A truncated form of the CD4 receptor with no cytoplasmic tail was generated by insertion of a stop codon at amino acid position 402. A dicysteine mutant was also generated by replacing the two cysteines at amino acid positions 420 and 422 by alanines rendering this molecule incapable of efficiently associating with the two cysteines (amino acid positions 21 and 23) of $p56^{lck}$.

When the same constructs were introduced into the CD4-negative T cell line HSB-2, even the wild-type CD4-transfected cell line failed to undergo apoptosis despite high levels of viral replication equivalent to that obtained in the A2.01 cells (Fig. 2 *B*). The level of surface CD4 expressed on both of these cell lines were similar (results not shown) and could not account for this discrepancy. However, HSB-2 cells have a mutated form of the src kinase $p56^{lck}$ (substitution V28L, insertion of QKP at amino acid position 230, substitution A353V, and substitution P447L) and despite a mutation at amino acid position 28 (V to L), in proximity to two critical cysteines at amino acid residues 21 and 23, still bound the CD4 receptor (Fig. 3). Furthermore HSB-2 $p56^{lck}$ has been reported to be catalytically activated and transforming in NIH 3T3 assays (30).

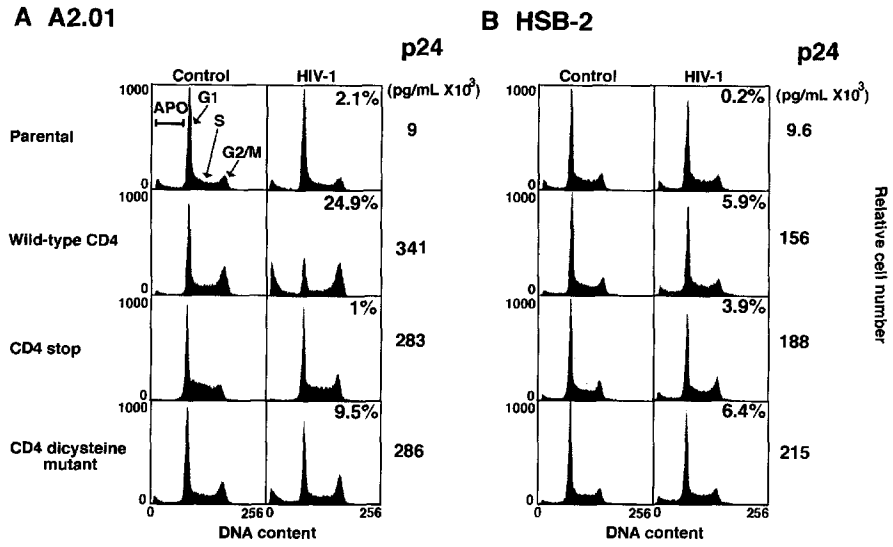


Figure 2. Flow cytometric profiles of cell cycle analyses of A2.01 cells (*A*) and HSB-2 cells (*B*). Cell cycle analyses of parental cell line, wild-type CD4, CD4 stop mutant cells, and CD4 dicysteine mutant cells obtained at day 3 with and without inoculation of HIV-1_{LAI} (moi: 0.5). Cell cycle phases are indicated in upper right panel. The proportion of cells in the region marked APO represent cells with sub-G1 DNA content and were considered apoptotic and quantified. The difference in the percentage of apoptotic of cells present in HIV-infected cells vs control is indicated in the top right part of the HIV-1–inoculated group. Relative cell number is plotted on the y-axis. 10,000 cells were analyzed for each condition. A representative experiment is shown ($n = 4$). Note the extensive apoptosis occurring in A2.01 wild-type CD4.

The constitutive activation of the $p56^{lck}$ of HSB-2 cells may therefore preclude the induction of apoptosis.

Cell cycle analysis and generation of DNA profiles of infected A2.01 parental cells and those expressing each of the three forms of CD4 confirmed that only the cells expressing wild-type CD4 were susceptible to HIV-induced apoptosis and that the extent of apoptosis was time dependent and correlated with infection. The presence of apoptotic cells increased with time and anomalies in DNA profiles were readily detectable, a constant feature being a diminution of the proportion of cells in the G1 phase of the cell cycle, a concomitant increase in the fraction of cells present in G2/M, and the appearance of cells with a sub-G1 DNA content representing apoptotic cells (Fig. 4, *A* and *B*). These cell cycle anomalies were previously described to occur in primary $CD4^+$ T lymphocytes and in the lymphoblastoid $CD4^+$ T cell lines SupT1 (3). This effect was not due to inappropriate downregulation of the CD4 receptor because cells expressing wild-type CD4 were efficiently downregulated by HIV-1 (87% by day 3) compared to 68% for the CD4 stop and 95% for the CD4 dicysteine mutant at day 3. The CD4 stop mutant was downregulated slightly slower possibly due to the fact that Nef, the principal protein responsible for the downregulation of the CD4 receptor, requires the cytoplasmic tail of the receptor to exert its effect (28). Therefore, the downregulation obtained for the CD4 stop mutant may represent the contribution of receptor endocytosis due to virus binding or alternatively to more efficient trapping of the full-length CD4 receptor by gp160 in the endoplasmic reticulum.

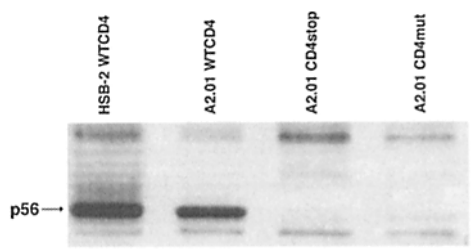
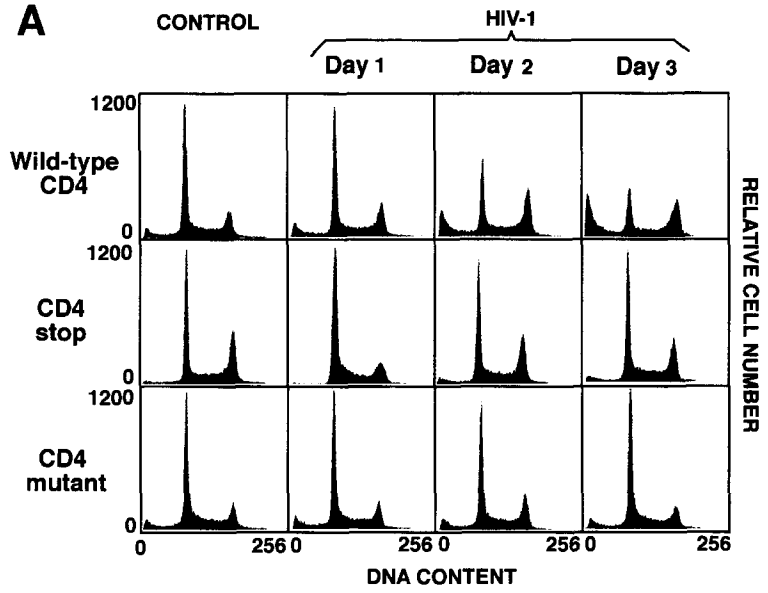


Figure 3. $p56^{lck}$ and CD4 association and immunoprecipitation. 2×10^7 cells were lysed and the supernatant reacted with anti-CD4 antibody (OKT4) coupled to protein A–Sepharose. 10 μl was loaded on a 12% SDS-PAGE gel. $p56^{lck}$ was detected as described in Materials and Methods.

The A2.01 cells expressing the CD4 dicysteine mutant did not undergo apoptosis. This mutant differs from the wild-type CD4 only in its ability to bind $p56^{lck}$. Moreover, HSB-2 cells did not undergo apoptosis despite the reintroduction of the wild-type form of the CD4 receptor (Fig. 2 *B*) suggesting that the constitutively active form of $p56^{lck}$ present in these cells may be responsible for the resistance of these cells to apoptosis. These observations hinted that $p56^{lck}$ may contribute in modulating HIV-induced apoptosis.

To evaluate the effect of $p56^{lck}$ in HIV-induced apoptosis, we stably introduced the wild-type gene and a kinase-inactive $p56^{lck}$ mutant by transduction in two HTLV-1–transformed, IL-2–independent, $CD4^+$ T cell lines, C8166-45 and MT-2. These two $CD4^+$ T cell lines do not express $p56^{lck}$, but are highly susceptible to HIV-1 infection. Stable clones were generated and a number of high expressing clones were selected. Reintroduction of this gene had no detectable effect on the growth of these cells (results not shown). A Western blot demonstrating the levels of expression of $p56^{lck}$ protein obtained in parental C8166-45, C8166 cells transduced with the vector alone, in one of the high expressor clones (lck#7) and in a clone expressing a kinase-deficient form of $p56^{lck}$ is presented in Fig. 5 *A*. Both parental and vector-only C8166-45 (lanes *1* and *2*, respectively) did not express any detectable $p56^{lck}$ in contrast to clone lck#7 (lane *3*) and a kinase-negative mutant (lane *4*). To verify the phenotype of the kinase-inactive $p56^{lck}$, the CD4 receptor of both C8166-45 cells transfected with wild-type $p56^{lck}$ (clone lck#7) and the clone expressing the kinase-negative form of $p56^{lck}$ were cross-linked with anti-CD4 antibodies. This type of cross-linking will mediate, within 5 min, a potent autophosphorylation of



B

	APOPTOSIS (%) ON DAY			CD4 (%) downregulation on day 3	p24 (ng/mL)
	1	2	3		
A2.01 parental	< 1	< 1	< 1	nd	3.5
Wild-type CD4	1.4	15.8	24	87	775
CD4 stop	0.6	< 1	< 1	68	460
CD4 dicysteine mutant	1.9	3.7	< 1	95	505

Figure 4. Flow cytometric profiles of cell cycle analyses of A2.01 cells (*A*). Cell cycle analyses of parental cell line, wild-type CD4, CD4 stop mutant cells, and CD4 dicysteine mutant cells obtained daily for 3 d after infection with HIV-1_{LAI} (moi: 0.5). The profiles of control cells at day 3 are also presented. Relative cell number is plotted on the y-axis. 10,000 cells were analyzed for each condition and a representative experiment is shown. *B* provides the percentage of apoptosis present for each day as well as the percentage of CD4 downregulation and amount of p24 antigen at day 3.

$p56^{lck}$, which can be detected using an antiphosphotyrosine antibody in a Western blot. Constitutive expression of the phosphorylated form of $p56^{lck}$ was strong in cells stably transduced with wild-type $p56^{lck}$ but was minimal in cells carrying kinase-inactive form of $p56^{lck}$ (Fig. 5 *B*, lanes *1* and *3*, respectively). Upon cross-linking with anti-CD4 antibodies only the wild-type $p56^{lck}$-expressing cell line showed an increase in phosphorylated $p56^{lck}$ (compare lanes *1* and *2*). The autophosphorylation-deficient mutant of $p56^{lck}$ did not generate phosphorylated $p56^{lck}$ (lane *4*). Thus, both wild-type $p56^{lck}$ and an autophosphorylation-deficient form of $p56^{lck}$ can be stably introduced and expressed in C8166-45. Similar results were obtained with the MT-2 cell line (results not shown).

Upon HIV-1 infection, the presence of either the wild-type or the autophosphorylation-deficient form of $p56^{lck}$ greatly exacerbated the cytopathic effects of the virus. Both HIV-induced apoptosis and syncytium formation appeared with an earlier onset and greater intensity than controls. The cytopathic effects also seemed to correlate with the amount of $p56^{lck}$ expressed in the cell lines. DNA content profiles were obtained 3 d after infection for both controls and HIV-infected cultures of C8166-45, C8166-45 transduced with the vector only, with wild-type $p56^{lck}$ (lck#7), or with an autophosphorylation-deficient mutant. Despite high levels of initial viral replication in all infected cells as measured by p24 antigen production, only the wild-type $p56^{lck}$ and the autophosphorylation-deficient $p56^{lck}$ mutant showed extensive apoptosis early in the infection process, as determined by the percentage of cells with sub-G1 DNA content (Fig. 6, *A* and *B*). Levels of p24 antigen were actually lower in both Lck#7 and the autophosphorylation-deficient $p56^{lck}$ mutant at day 3 due to extensive cytopathic effects and loss of HIV-1–producing cells. This effect correlated with the persistence of cell surface CD4 during the

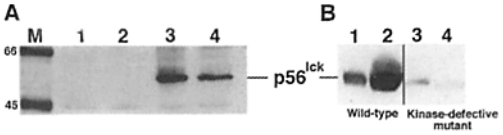
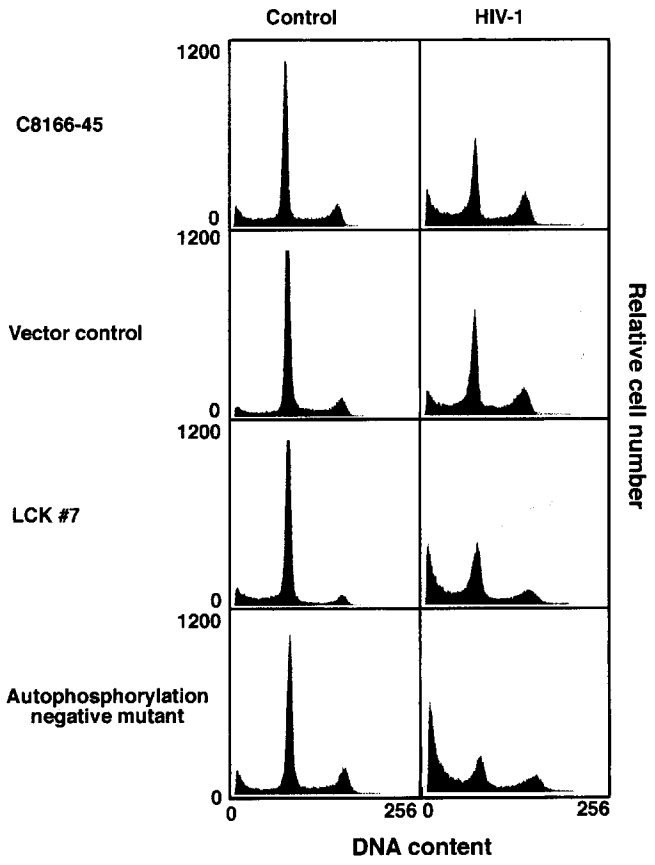


Figure 5. Presence of $p56^{lck}$ and verification of kinase activity. (*A*) Western blot of C8166-45 (lane *1*), vector only (lane *2*), lck#7 (lane *3*), and kinase defective mutant (lane *4*) (10^6 cells/condition) were solubilized directly in SDS-PAGE buffer and run on a 12% acrylamide gel. $p56^{lck}$ was revealed with an anti-$p56^{lck}$ antibody and enhanced chemiluminescence detection kit. (*M*) Molecular weight marker in kilodaltons indicated on the left side of the panel. (*B*) Western blot of phosphorylated $p56^{lck}$ as detected by a phosphotyrosine antibody. Clone lck#7 unstimulated (lane *1*) and stimulated by cross-linking with anti-CD4 antibody (lane *2*). Kinase-defective mutant unstimulated (lane *3*) and cross-linked (lane *4*).

A



B

	APOPTOSIS (%) ON DAY			CD4 (%) downregulation			p24 (ng/mL) X10^3
	1	2	3	1	2	3	
C8166-45	1.6	<1	13.3	nd	nd	nd	120
Vector control	0.3	3.3	17.8	24	48	73	48
Lck #7	1.7	26.8	23.6	18	35	28	1
Phos. Mutant	0.2	22.1	36.4	10	24	34	28

Figure 6. Flow cytometric profiles of cell cycle analyses of C8166-45 cells. (*A*) Cell cycle analyses obtained at day 3 of parental, vector control, lck#7, and autophosphorylation-negative mutant without (*control*) and with infection by HIV-1_{LAI} (moi: 0.5). 10,000 cells were used for each analysis and a representative experiment is shown. *B* provides the percentage of apoptosis and CD4 down-regulation present for each day as well as the amount of p24 antigen at day 3.

course of the infection (Fig. 6 *B*). 3 d after infection, C8166-45 cells transduced with the vector only had down-regulated its CD4 receptors by 73% as opposed to 28% for wild-type $p56^{lck}$ and 34% for the autophosphorylation-deficient $p56^{lck}$ mutant. Cells stably expressing $p56^{lck}$ showed marked cytopathic effects due to HIV-1 infection and the rapid appearance of syncytia. Only 6% of the parental C8166-45 and cells transduced with the vector formed syncytia as assessed by flow cytometric analysis where cells with a DNA content greater than 4n (aneuploid) would be considered to be a syncytium. It should be noted that syncytia >60 μM in diameter would be excluded from the analysis due to the presence of exclusion filters in the flow cytometer. Syncytia were seen in 15% of wild-type $p56^{lck}$ and 17% of autophosphorylation-deficient $p56^{lck}$ cells (representative of three independent experiments). Nevertheless, the majority of the cells still died at the single cell level through apoptosis. The extent of cytopathic effects could readily be detected (Fig. 7). Balloon cells could be seen as early as 16 h after infection at an moi of 0.5 for both lck#7 and the autophosphorylation mutant cell lines.

Discussion

HIV-1 infection downregulates the surface expression of the CD4 receptor by at least two mechanisms. The *nef* gene product efficiently downregulates the expression of surface CD4 early in infection (28, 31). The *vpu* and the *env* gene products act in concert to trap the CD4 molecule in the endoplasmic reticulum and facilitate its digestion to prevent its expression on the cell surface (32, 33). Downregulation of CD4 might benefit the virus because the persistence of high levels of CD4 expression on the surface of an infected cell would diminish continued virus replication. If the cell

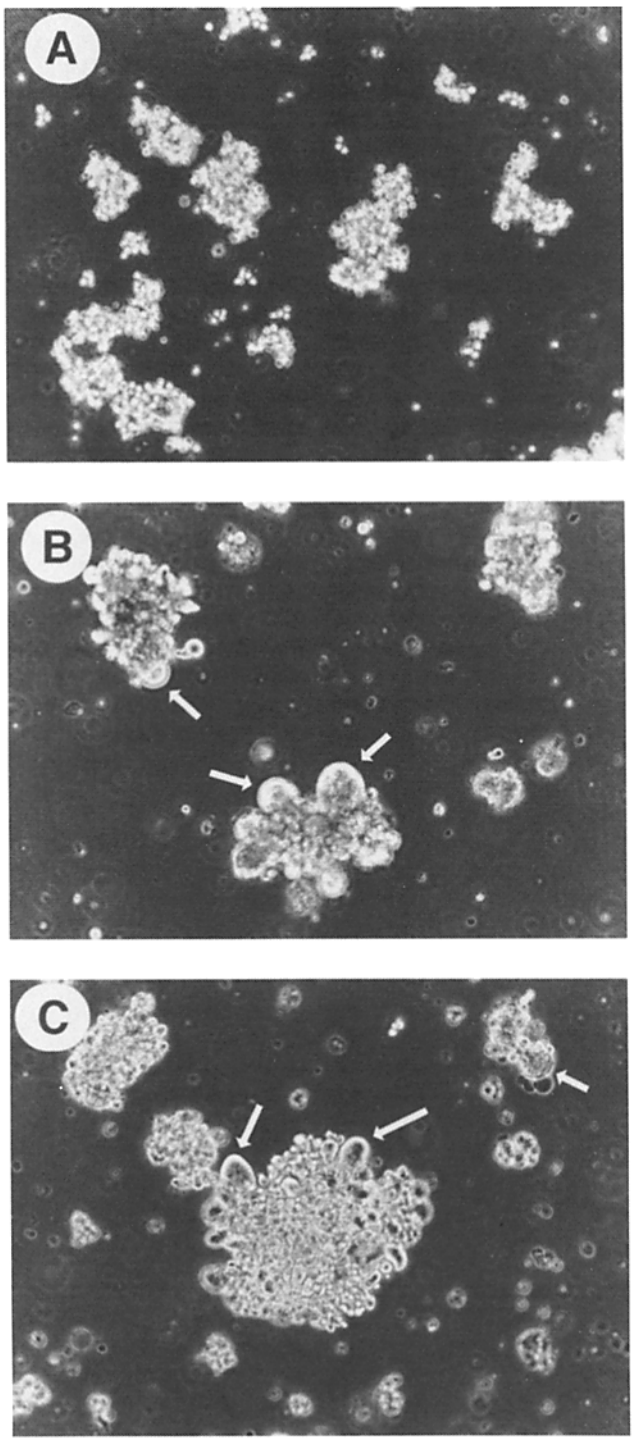


Figure 7. Effects of the introduction of $p56^{lck}$ in C8166-45 lymphoblastoid $CD4^+$ T cells. Photomicrographs taken at day 2 of uninfected cells (*A*), HIV-inoculated clone lck#7 cells (*B*) and HIV-inoculated lck autophosphorylation-negative mutant cells (*C*). The presence of syncytia can readily be detected (*arrows*). ×50.

were to retain full CD4 expression, it might be more susceptible to the deleterious consequences of superinfection, the induction of anergy by gp120 binding to the receptor, syncytium formation, and, as indicated in the current studies, apoptosis. In these studies, clear evidence was presented indicating that the cytoplasmic tail of CD4 is required to mediate and modulate HIV-induced apoptosis. Apoptosis provides a mechanism to abort continuing virus replication in a cell. It is interesting to note that $CD4^+$ T cell lines that constitutively express low CD4 levels can be made to be chronic producer lines of HIV-1 (Hut 78, for example) but not $CD4^+$ T lymphoblastoid cell lines that express high levels of CD4, such as SupT1 and MT-4 (Corbeil, J., unpublished observation; and 34). This evidence points to an important role for downmodulation of CD4 expression during HIV infection to enhance continuing virus replication.

We have previously generated evidence that the initial entry of HIV-1 resulted in infection and viral replication in permissive cells but only subsequent signaling by gp120 to CD4 at the cell surface of these infected cells triggered apoptosis. We have demonstrated that inoculation of SupT1 cells by HIV-1 (moi = 1) did not induce apoptosis when AZT (10 μM) was added 2 h earlier, suggesting that at least reverse transcription and possibly the production of viral proteins had to occur to render the cells susceptible to apoptosis. In the same setting, the addition of the protease inhibitor saquinavir could not block apoptosis even when added 2 h before inoculation. In this case viral production is mostly unhampered but yields noninfectious virions. Interestingly, adding dextran sulfate (10 μg/ml) at a concentration to exclude cell surface signaling and new infection, 7 h after inoculation to allow the first part of the virus cycle to proceed, inhibited apoptosis completely despite the production of large amounts of virus. These studies thus suggest that a target cell had to be infected and then resignaled at the cell surface to undergo apoptosis (3).

The present studies demonstrate that the apoptotic signal is delivered through the cytoplasmic tail of CD4 and that further interaction with the src-related protein tyrosine kinase $p56^{lck}$ augments the extent of apoptosis observed. CD4 is physically associated with $p56^{lck}$ (29, 35). Cell surface engagement of CD4 leads to enzymatically activation of the associated $p56^{lck}$ and the phosphorylation of T cell proteins on tyrosine residues (36). In the context of HIV infection, $p56^{lck}$ is required to prolong the presence of CD4 on the cell surface (37) permitting the delivery of the apoptotic signal and to anchor other proteins to transduce the signal. Although Lck is not an absolute requirement for inducing apoptosis, it appears to have some kinase-independent modulatory role in regulating apoptosis mediated through the cytoplasmic tail of CD4 because an autophosphorylation-negative mutant stably introduced in C8166-45 induced levels of apoptosis similar to wild-type $p56^{lck}$. This mutant cannot autophosphorylate because of a mutation at the ATP-binding site (aa273: L to A). Additional evidence using the wild-type CD4 HSB-2 T cell line, which has a constitutively activated mutant form of $p56^{lck}$, confirmed that disruption of this pathway abrogates HIV-induced apoptosis, implying that constitutive activation prevents the apoptotic signal triggered by HIV-1. Alternatively, the HSB-2 cells may have mutated downstream effectors, enabling it to survive constitutively active Lck, which may impact on the HIV-induced apoptosis. Candidate mole-

cules to transmit the apoptotic signal through the CD4 signaling pathway would be ZAP-70 which has been reported to associate directly through the SH2 domain interaction of $p56^{lck}$ (38). Alternatively, a 32-kD GTP protein has also been reported to associate directly with $p56^{lck}$ when $p56^{lck}$ is bound to the CD4 receptor (39); this association may convey the message to undergo apoptosis. Another candidate molecule to associate with $p56^{lck}$ would be Raf-1–related p110 which serves as a bridge between the CD4–$p56^{lck}$ complex and the serine/threonine kinase pathways of T cell activation (40, 41).

A mutational analysis of the SH2 domain and myristoylation of $p56^{lck}$ may reveal if the putative interactions mentioned above require CD4 and $p56^{lck}$ colocalization at the cytoplasmic membrane. It should be noted that the subsequent interactions of $p56^{lck}$ with ZAP-70, p110 raf-1, or p32-kD proteins have not been demonstrated in HIV-infected cells. Defective protein tyrosine phosphorylation and altered levels of $p56^{lck}$ in $CD4^+$ T cells obtained from HIV-1–infected patients have been reported however (42). Furthermore, HIV-1 may actually interfere with appropriate signal transduction through the CD4 signaling cascade and induce apoptosis. Interaction between the viral protein Nef with the CD4 cytoplasmic tail may disrupt $p56^{lck}$ binding (43) or attachment of other cellular factors, resulting in inappropriate signaling and ultimately apoptosis.

HIV-1 replication proceeds at extremely high rates in infected individuals (44, 45) and the quantity of virus present and the proportion of cells infected could account for the rate of depletion observed (46–49). $CD4^+$ T cell depletion is certainly not due only to HIV-induced apoptosis; a substantial contribution would be provided by the action of cytotoxic T cells in the background of cell death due to the general state of activation of the immune system in HIV-infected individuals, which has been shown to contribute to apoptosis detected in lymph nodes (50). It is interesting to note that the prevention of apoptosis in HIV-infected cells would result in the production of more virus and generate a state of chronic infection (51). Therefore, therapeutic modalities aimed at preventing apoptosis, which is a normal physiological process, as suggested recently (52) should be viewed with caution as such interventions may result in the production of more virus and possibly immortalization of cells (lymphomas) which would not be beneficial to the host.

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