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The Protein Tyrosine Kinase p56^{lck} Is Required for Triggering NF-κB Activation upon Interaction of Human Immunodeficiency Virus Type 1 Envelope Glycoprotein gp120 with Cell Surface CD4

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We have previously shown that NF-κB nuclear translocation can be observed upon human immunodeficiency virus type 1 (HIV-1) binding to cells expressing the wild-type CD4 molecule, but not in cells expressing a truncated form of CD4 that lacks the cytoplasmic domain (M. Benkirane, K.-T. Jeang, and C. Devaux, EMBO J. 13:5559–5569, 1994). This result indicated that the signaling cascade which controls HIV-1-induced NF-κB activation requires the integrity of the CD4 cytoplasmic tail and suggested the involvement of a second protein that binds to this portion of the molecule. Here we investigate the putative role of p56^{lck} as a possible cellular intermediate in this signal transduction pathway. Using human cervical carcinoma HeLa cells stably expressing CD4, p56^{lck}, or both molecules, we provide direct evidence that expression of CD4 and p56^{lck} is required for HIV-1-induced NF-κB translocation. Moreover, the fact that HIV-1 stimulation did not induce nuclear translocation of NF-κB in cells expressing a mutant form of CD4 at position 420 (C420A) and the wild-type p56^{lck} indicates the requirement for a functional CD4-p56^{lck} complex.

The CD4 protein is an integral membrane glycoprotein of 58 kDa that contains four extracellular domains showing structural homology with immunoglobulin (Ig) Vκ regions and that is predominantly expressed at the surface of helper T lymphocytes (29, 36, 48). CD4 function as an adhesion or accessory molecule that facilitates cell-to-cell contact by interacting directly with the major histocompatibility complex (MHC) class II molecules at the surface of the antigen-presenting cells and stabilizing the T-cell receptor (TCR)–MHC-II interaction (8, 26). Furthermore, CD4 can actively participate in transmembrane signal transduction, since coaggregation of the TCR-CD3 complex and CD4 in multimeric clusters (40, 49) potentiates a variety of biochemical responses, including protein tyrosine phosphorylation, production of cytoplasmic inositol triphosphate, and release of intracellular Ca^{2+} (58), that ultimately regulate cell proliferation (2). During the past few years, some ligands of CD4 were shown to modulate T-cell activation in MHC-independent systems, suggesting that activation signals can be transduced directly through the CD4 molecule (3, 5, 10, 16).

Beside its crucial role in immune function, the CD4 molecule has been identified as the primary high-affinity cellular receptor for human immunodeficiency virus type 1 (HIV-1) (19, 32). The initial step in the infection of human T lymphocytes by HIV-1 involves binding of the viral envelope glycoprotein (gp120) to the cell surface CD4 molecule. Because it is a ligand capable of cross-linking CD4, the possibility that HIV-1 can activate T cells has been considered, and it is now generally accepted that HIV-1 and recombinant HIV-1 gp120 can modulate T-cell activation, although there is some controversy as to the nature of the signals delivered to the target cells (5, 10, 15, 16, 27, 28, 31, 33). Conceivably, the noted differences derive, at least in part, from differences in experimental design, the origin of the ligand for CD4 (heat-inactivated HIV-1, gp120–anti-gp120 immune complexes, virus-extracted gp120, recombinant gp120/gp160), and the nature of the $CD4^+$ cells used (peripheral blood mononuclear cells [PBMCs], purified $CD4^+$ lymphocytes, $CD4^+$ T-cell lines, CD4-transfected cell lines). Moreover, for viral ligands, differences in the interactions between molecules (of viral or cellular origin) expressed on the virus envelope and cell surface molecules other than the virus receptors may also influence signaling.

Using CD4-transfected T-lymphoblastoid cell lines as a model, we reported direct evidence indicating that heat-inactivated HIV-1 (iHIV-1)-mediated oligomerization of CD4 triggers the delivery of an activation signal to T cells which can be monitored by measuring the nuclear translocation of NF-κB (5). This result was confirmed by the work from Chirmule and coworkers (15). Next, we demonstrated similar effects of iHIV-1 on primary lymphocytes; the binding of iHIV-1 to infected resting PBMCs promotes progression in the cell cycle, induces cell surface expression of CD25, stimulates provirus integration, induces NF-κB translocation, and commits the cell to produce virus (10). Indeed, it is well established that virus production requires cell activation and that nuclear translocation of NF-κB enhances the κB-dependent early transcription of HIV-1. These results suggest that besides using CD4 as a receptor, HIV-1 takes advantage of the signal-tranduction function of CD4 to modulate the intracellular virus life cycle and/or to regulate the equilibrium between viral latency, viral replication, and virus-induced apoptosis. However, the mechanism(s) by which HIV-1 induces immune activation is still poorly understood.

To better understand the mechanism of cell signaling that

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results from HIV-1 interaction with CD4, signal transduction studies have been performed which demonstrate that CD4 ligation by HIV-1 or gp120 stimulates protein kinase C (PKC) (60), generates PKC-dependent phosphorylation of CD4 (25), induces a rise in intracellular calcium (33), and activates $p56^{lck}$ (27, 28), as well as phosphatidylinositol-3-kinase (PI-3K) (9), phosphatidylinositol-4-kinase (PI-4K) (50), Ras (34), Raf-1 (43), and extracellular-regulated protein kinase (ERK) (6). Besides the identification of a panel of molecules that are activated upon engagement of CD4 with HIV-1, the consequences of activation for the virus and the cell and the signaling pathway(s) used remain unclear.

CD4 lacks intrinsic tyrosine kinase activity but associates with $p56^{lck}$, a 56-kDa cytoplasmic membrane-associated member of the *Src* family of nonreceptor protein tyrosine kinases expressed primarily in T lymphocytes (46, 57) through interaction of its cytoplasmic domain with two cysteine residues located at the N-terminal domain of the kinase (51, 56). Although association with Lck was demonstrated to be necessary for CD4-mediated antigen responsiveness, it has not been clearly established that the kinase activity of Lck plays a role in CD4-dependent T-cell activation. Although $p56^{lck}$ is the usual partner of CD4 in CD4-dependent signal transduction, a number of results suggest that $p56^{lck}$ also plays a major role in the transduction of signals following HIV-1 binding to CD4. Cross-linking of CD4 with gp120, gp120-derived peptides, or anti-CD4 monoclonal antibodies (MAbs) known to be specific for the HIV-1 gp120 binding site results in a rapid phosphorylation of $p56^{lck}$ on both tyrosine and serine residues and an increase in $p56^{lck}$ activity (9, 27, 28, 31, 42, 54). Moreover, we found that the integrity of the CD4 cytoplasmic domain is required for HIV-1-induced nuclear translocation of NF-κB (5) and HIV-1-induced activation of ERK (6), which represents a possible downstream substrate for $p56^{lck}$ (23). Finally, $p56^{lck}$–Raf-1 coimmunoprecipitation after HIV-1 binding to CD4 has been reported (43).

The objective of this study was to assess the involvement of the $p56^{lck}$ in the transduction of activation signal(s) induced by iHIV-1 oligomerization of CD4. To this end, we used a series of nonlymphoid HeLa cell lines stably transfected with wild-type or mutant forms of the human CD4 molecule with or without murine wild-type $p56^{lck}$. We demonstrate that the $p56^{lck}$-CD4 interaction is required for triggering the NF-κB nuclear translocation that follows HIV-1 interaction with CD4.

Main characteristics of transfected HeLa cell lines used in this study. All cell lines included in the present study derive from the HeLa parental cell line. The HeLa CD4.2G3 cell line (37), referred to below as HeLa $CD4^+$, expresses the human wild-type CD4. The HeLa $CD4^+/p56^{lck}$ line was obtained by supertransfection of the HeLa CD4.2G3 cell line with both the pSM expression vector encoding $p56^{lck}$ and the pBabe/Hygro vector encoding the gene conferring hygromycin resistance (41). HeLa $CD4^+$ Cyt^- expresses a tailless CD4 molecule truncated at amino acid 402 (38). This truncation deletes all but seven amino acids of the cytoplasmic domain. HeLa CD4 (S408A)/$p56^{lck}$ and CD4 (C420S)/$p56^{lck}$ express mutant forms of CD4 with an alanine and a serine substitution for S408 and C420, respectively (41). Both cell lines were supertransfected with the pSM vector and the pBabe/Hygro vector and express the murine $p56^{lck}$. The S408A mutation removes a critical residue in the cytoplasmic domain of CD4 which has been shown to be phosphorylated in response to phorbol esters (52), and the Ser-408 CD4 mutant molecule does not dissociate from $p56^{lck}$ under phorbol myristate acetate (PMA) stimulation (7, 53). The cysteine residue at position 420 of CD4 is required for binding to $p56^{lck}$, and mutation at this site completely disrupts CD4-$p56^{lck}$ association (56). Finally, HeLa CH4 cells express a chimeric molecule consisting of the first two domains of CD4 linked to Thy1, a cell surface antigen with an external domain attached to the membrane by a glycosylphosphatidylinositol (GPI) anchor (30). An additional cell line, HeLa $p56^{lck}$, was included, consisting of HeLa cells expressing $p56^{lck}$ but not CD4. All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM), 10% fetal calf serum, 100 U of penicillin per ml, and 0.1 mg of streptomycin (Gibco BRL Life Technologies, Paisley, Scotland) per ml. One milligram of G418 (Gibco) per ml was added to culture medium of cells expressing the human CD4. Two hundred micrograms of hygromycin (Gibco) per ml was added to the culture medium of cells expressing $p56^{lck}$. The pSM-Lck construct and HeLa CH4 cells were kindly provided by Dan Littman (Skirball Institute of Biomolecular Medicine, New York University Medical Center, New York).

CD4 and $p56^{lck}$ expression in HeLa-transfected cell lines. CD4 expression was assessed by indirect immunofluorescence staining and flow cytometry. Cells (5×10^5) were incubated for 30 min at 4°C with 50 μg of BL4 anti-CD4 antibody (Immunotech-Coulter Comp., Marseille, France) per ml directed against the D1-D2 region of CD4. After washing in phosphate-buffered saline–bovine serum albumin (PBS-BSA), bound MAb was revealed by addition of 50 μl of a 1/50 dilution of fluoresceinated goat anti-mouse (GAM) Ig (Immunotech-Coulter). Fluorescence intensity was recorded in the log mode on an EPICS PROFILE XL4C cytometer (Coulter, Coultronics, Margency, France). Representative cytofluorometric profiles are shown in Fig. 1. As expected, no CD4 expression was detected on HeLa $p56^{lck}$, which was only transfected with the pBabe/Hygro vector and the pSM vector encoding the $p56^{lck}$ gene. Although variations in the expression level of CD4 were observed among the different cell lines, this antigen was expressed at the surface of HeLa cells transformed with wild-type and mutant forms of CD4 or the chimeric CH4 molecules.

Expression of the $p56^{lck}$ human gene was tested by Western blotting. Cell lysates were electrophoresed onto sodium dodecyl sulfate-polyacrylamide (10%) gel electrophoresis (SDS-PAGE) gels and blotted to polyvinylidene difluoride (PVDF) membrane (Millipore, St Quentin Yvelines, France). The blot was saturated for 1 h in PBS–10% milk–0.05% Tween 20 prior to the addition of anti-$p56^{lck}$ (Santa-Cruz Biotechnologies, Santa Cruz, Calif.) or antiactin (Immunotech-Coulter) antibody. Antibody staining was revealed by addition of a 1:3,000 dilution of peroxidase-GAM Ig. After three washes, bound antibody was detected by incubating the membrane with the ECL (enhanced chemiluminescence) reagent (Amersham, Les Ullis, France). Western blot detection of $p56^{lck}$ is shown in Fig. 2. Expression of $p56^{lck}$ was detected in the HeLa $CD4^+/p56^{lck}$, HeLa $p56^{lck}$, HeLa CD4 (S408A)/$p56^{lck}$, and HeLa CD4 (C420S)/$p56^{lck}$ cell lines, with the highest expression level found in the HeLa $p56^{lck}$ cell line. As expected, no $p56^{lck}$ protein was detected in total protein lysates from HeLa $CD4^+$, HeLA $CD4^+$ Cyt^-, and HeLa CH4 cell lines that were not transfected with the $p56^{lck}$ expression vector.

Analysis of CD4-$p56^{lck}$ interaction by coimmunoprecipitation. The nature of the CD4-$p56^{lck}$ interactions in each cell line was characterized by a coimmunoprecipitation assay. Adherent cells were washed once in Ca^{2+}-, Mg^{2+}-free PBS, harvested by scraping into PBS, and centrifuged at 1,500 rpm for 5 min at 4°C in a GR412 Jouan (Jouan, St. Herblain, France). Cell pellets were resuspended in lysis buffer (20 mM Tris-HCl [pH 8.0], 3% Nonidet P-40, 150 mM NaCl, 2 mM EDTA) and protease inhibitors (1 mM phenylmethylsulfonyl fluoride [PMSF] and 10 μg [each] of leupeptin, antipain, and pepstatin per ml). Detergent-insoluble material was removed by centrifugation at 4°C for 20 min at full speed in an Eppendorf microcentrifuge.

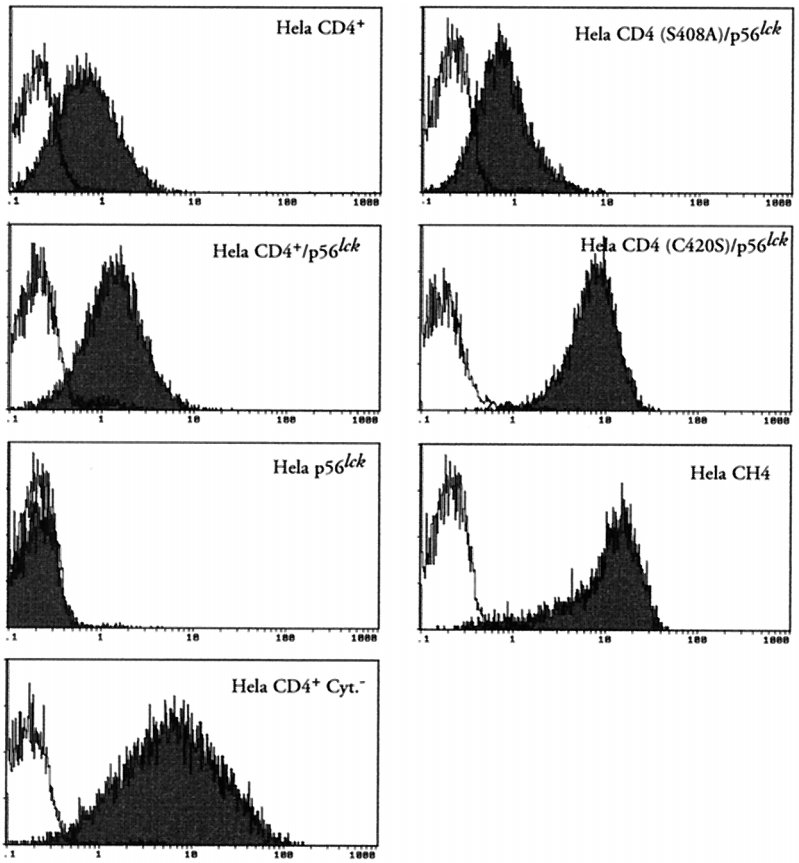

FIG. 1. Cell surface expression of CD4 molecules in HeLa cell lines. Cells were incubated with medium alone (white histograms) or 50 μg of anti-CD4 MAb BL4 per ml (black histograms). MAb binding was detected by a fluorescein isothiocyanate-labeled GAM Ig. The fluorescence intensity was recorded in the log mode.

The supernatants were collected, and the protein concentration in each sample was determined. Supernatants were precleared by incubation for 30 min with 50 μl of packed prewashed protein A-Sepharose (Sigma Chemical Company, Ltd.). CD4 was immunoprecipitated at 4°C by adding 4.5 μg of 13B8-2 anti-CD4 MAb (Immunotech-Coulter) for 1 h and protein A-Sepharose for an additional 1.5 h. The beads were collected by centrifugation, washed three times in lysis buffer, resuspended in an equal volume of SDS-PAGE sample buffer containing 50 mM dithiothreitol (DTT), and analyzed on SDS-PAGE (10% polyacrylamide) gels. Proteins were transferred onto PVDF membrane (Millipore), and the blot was saturated for 1 h in PBS–10% milk–0.05% Tween 20. CD4-p56^{lck} immune complexes were revealed by addition of anti-p56^{lck} antibody (Santa-Cruz Biotechnologies) for 1 h. After three washes, MAb staining was revealed by addition of a 1:3,000 dilution of peroxidase-GAM Ig (Immunotech-Coulter). After three washes, bound MAb was detected by incubation of the membrane with ECL reagent (Amersham).

As shown in Fig. 3, CD4-associated-p56^{lck} was detected in the HeLa CD4$^+$/p56^{lck} and HeLa CD4 (S408A)/p56^{lck} cell lines. In contrast, proteins migrating at the expected size for p56^{lck} were not observed in HeLa CD4$^+$, HeLa CH4, or HeLa CD4$^+$ Cyt$^-$ cells, which only express CD4 molecules, nor in HeLa p56^{lck} cells, which express p56^{lck} but lack expression of the CD4 protein. Finally, CD4/p56^{lck} coimmunoprecipitates were not detected by immunoprecipitation with anti-CD4 MAb in a HeLa CD4 (C420S)/p56^{lck} cell line that expresses a CD4 mutant molecule and p56^{lck}, indicating, as previously shown (56), that the p56^{lck} kinase does not interact with the mutated form of CD4 expressed in these cells.

CD4-p56^{lck} interaction is required for iHIV-1-induced nuclear translocation of NF-κB in HeLa cell lines. We have previously demonstrated that iHIV-1 binding to CD4 induced NF-κB activation in T-lymphoblastoid cell lines expressing a wild-type CD4 molecule (5) and in primary lymphocytes (10). To assess whether such an activation signal requires p56^{lck} expression, electrophoretic mobility shift assays (EMSAs) were performed to analyze the ability of iHIV-1–CD4 interaction to stimulate NF-κB nuclear translocation in HeLa cell lines expressing either wild-type or mutated CD4 molecules and/or p56^{lck}. To this end, 2×10^6 cells were exposed for 4 h either to 100 μl of an iHIV-1 solution stock corresponding to 1,000 50% tissue culture infective doses ($TCID_{50}$) of infectious virus per ml or to 20 ng of PMA per ml (Sigma). Briefly, cells were washed three times in PBS and lysed in buffer containing 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 4 μg of leupeptin per ml, and 10 mM HEPES (pH 7.8). After 15 min on ice, a 50-μl solution of 10% Nonidet P-40 was added to the sample, and cells were microcentrifuged at 4°C for 30 s. The pellets were resuspended in 100 μl of buffer containing 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 4 μg of leupeptin per ml, 10% glycerol, and 50 mM HEPES (pH 7.8) and incubated for 20 min at 4°C. The supernatants were collected after centrifugation for 5 min at 4°C. The EMSAs were performed with 2 μg of protein of nuclear extract, 10^5 cpm of a ^{32}P-labeled oligonucleotide corresponding to the NF-κB sequence binding site from the HIV-

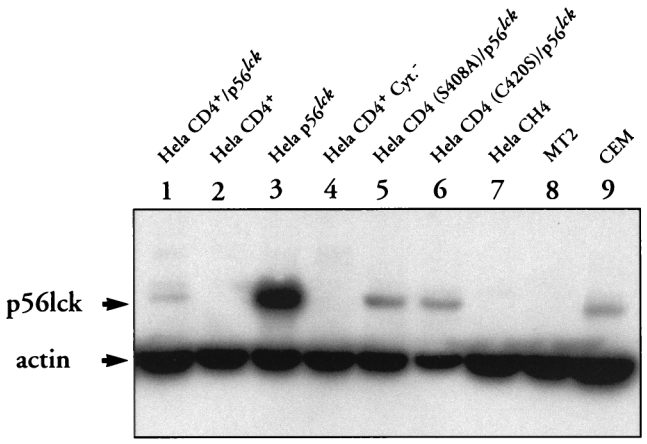

FIG. 2. Detection of $p56^{lck}$ expression by Western blot analysis. HeLa $CD4^+/p56^{lck}$, HeLa $CD4^+$, HeLa $p56^{lck}$, HeLa $CD4^+$ Cyt^-, HeLa CD4 (C420S)/$p56^{lck}$, HeLa CD4 (S408A)/$p56^{lck}$, and HeLa CH4 extracts containing 50 μg of total cellular proteins were electrophoresed in an SDS–10% polyacrylamide gel and blotted to a PVDF membrane. The membrane was incubated with a mixture of anti-$p56^{lck}$ and antiactin MAbs and then reacted with GAM Ig-peroxidase conjugate. Bound MAbs were revealed by incubation of the membrane with ECL reagent and exposure to Hyperfilm-ECL. Controls consist of lysates from MT2 cells (a human T-cell leukemia virus type 1-transformed $CD4^+$ T-cell line which lacks $p56^{lck}$ expression) and CEM cells (a $CD4^+$ T-cell line which expresses $p56^{lck}$).

1 long terminal repeat (LTR) (5), and 100 mM KCl, 1 mM DTT, 1 mM $ZnSO_4$, 20% glycerol, 0.01% Nonidet P-40, and 50 mM HEPES (pH 7.9), supplemented with BSA, tRNA, and poly(dI-dC). After 20 min at room temperature, the mixture was run at 120 V in a 10% polyacrylamide gel.

As shown in Fig. 4, a shift of labelled NF-κB oligonucleotide was observed when mixed with nuclear extracts from HeLa $CD4^+/p56^{lck}$ cells exposed to iHIV-1 compared with the basal activation level identified from unstimulated HeLa $CD4^+/p56^{lck}$ cells (lanes 2 and 1, respectively). In contrast, no shift was observed when HeLa $CD4^+$ (lane 5) or HeLa $p56^{lck}$ cells (lane 8), lacking either CD4 expression or $p56^{lck}$ expression, respectively, were exposed to iHIV-1. As a control, a strong activation of NF-κB was observed when these two cell lines were incubated in presence of 20 ng of PMA per ml (lanes 6 and 9). These observations show that in nonlymphoid cell lines expressing wild-type CD4 and $p56^{lck}$ molecules, iHIV-1 binding to CD4 induces an activation signal that leads to NF-κB nuclear translocation and is transduced via the CD4 and $p56^{lck}$ molecules.

Our experiments provide direct evidence that the coexpression of CD4 and $p56^{lck}$ is required for full signal transduction after oligomerization of CD4 by the envelope glycoprotein of HIV. It is worth noting that the absence of the CD4 receptor abolishes the nuclear translocation of NF-κB that is usually observed upon iHIV-1 stimulation, providing direct evidence that the activation events that we describe are highly specific for the interaction of the virus envelope glycoprotein gp120 with CD4. The analysis of the HeLa $CD4^+$ cell line demonstrates the crucial role of $p56^{lck}$ in signal transduction induced by HIV-CD4 interaction. These data corroborate recent data from other groups; Di Somma et al. (21) reported that activation of NF-κB and AP-1 induced by CD4 triggering with anti-CD4 MAb is strongly inhibited by a dominant-negative mutant of Lck. Furthermore, Merzouki and coworkers (39) demonstrated that HIV-1 gp120/160-expressing cells upregulate HIV-1 LTR-directed gene expression in $CD4^+$ CEM cells transfected with an HIV-1 LTR-reporter gene construct, whereas expression of the reporter gene was not induced in $CD4^+$ U937 cells, which lack $p56^{lck}$.

Mutation of CD4 at C420 and S408 influences iHIV-1 stimulation of NF-κB translocation. The HeLa CD4 (C420S)/$p56^{lck}$ cell line expresses a mutant CD4 molecule, CD4 (C420S), in which cysteine residue 420 was replaced by a serine. This mutation disrupts a site in the cytoplasmic tail of CD4 that is required for interaction with $p56^{lck}$ (51, 56). The coimmunoprecipitations shown in Fig. 3 and an in vitro kinase assay performed after immunoprecipitation with an anti-CD4 antiserum (41) confirmed the lack of association between CD4 (C420S) and $p56^{lck}$. When this cell line was incubated with iHIV-1, no NF-κB shift was observed by EMSA (Fig. 4, lane 17), although a strong activation was induced with PMA (lane 18). Thus, a physical interaction of the cytoplasmic tail of CD4 with $p56^{lck}$ is required to induce NF-κB activation upon CD4 cross-linking.

Additional information was provided by the analysis of the HeLa CD4 (S408A)/$p56^{lck}$ cell line coexpressing the S408A-mutated form of CD4 and $p56^{lck}$. The S408A mutation replaces a critical residue in the cytoplasmic domain of CD4 that is phosphorylated in response to phorbol esters (52) and is involved in both CD4 endocytosis and the dissociation of $p56^{lck}$ from CD4 (7, 53). When NF-κB activation was studied with this cell line, a very strong shift was observed upon stimulation by both iHIV-1 and PMA (Fig. 4, lanes 14 and 15, respectively). This shift was significantly higher than that observed in HeLa $CD4^+/p56^{lck}$ cells expressing both CD4 and $p56^{lck}$ wild-type molecules. The high activation observed in HeLa CD4 (S408A)/$p56^{lck}$ may be a consequence of high expression levels of $p56^{lck}$ or may be due to the lack of S408 phosphorylation, thus preventing $p56^{lck}$ dissociation. Altogether, these data suggest that the physical interaction of CD4 and $p56^{lck}$ is a prerequisite for transduction of the activation signal(s) induced by the CD4–iHIV-1 interaction.

Finally, we have studied an additional cell line, HeLa CH4, expressing a CD4–Thy-1 fusion protein on the cell surface which contains the HIV-1 binding site (D1 domain of CD4) and is anchored to the membrane by a GPI tail in place of a conventional membrane-spanning domain. The CD4–Thy-1 fusion protein can serve as an HIV-1 receptor, and HeLa CH4 cells can therefore be infected by HIV-1 (30). In addition, this molecule, like CD4, is downmodulated in its cell surface expression by exogenous gangliosides. Incubation of the HeLa CH4 cell line with iHIV-1 showed a slight shift of the NF-κB transcription factor. The signal transduction observed through

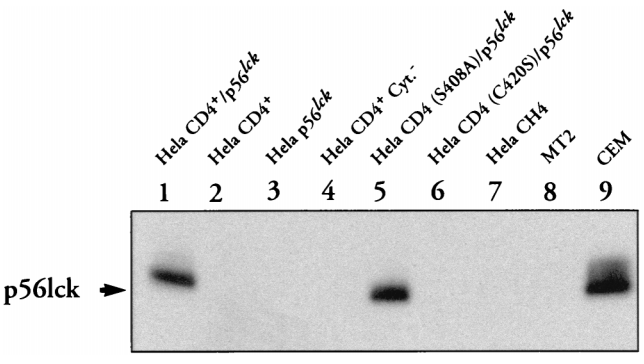

FIG. 3. Analysis of CD4-$p56^{lck}$ interaction by coimmunoprecipitation. One milligram of total cellular protein was immunoprecipitated with 13B8-2 anti-CD4 MAb. After washing, the immunoprecipitates were electrophoresed in SDS-PAGE (10% polyacrylamide) gels, transferred to PVDF membrane, and hybridized with anti-$p56^{lck}$ MAbs. MAb staining was revealed by incubation of the membrane with a 1:3,000 dilution of GAM Ig. Immunoprecipitates from MT2 and CEM cellular extracts are shown as controls.

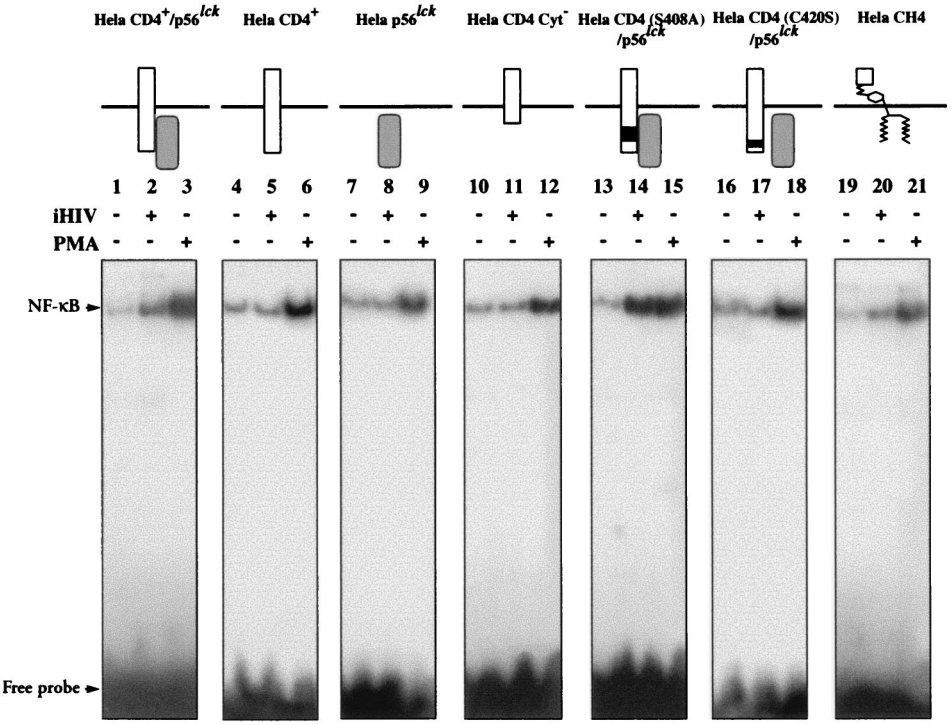

FIG. 4. Effect of iHIV treatment on NF-κB nuclear translocation in different HeLa cell lines analyzed by EMSA. Nuclear extracts prepared from HeLa $CD4^+/p56^{lck}$, HeLa $CD4^+$, HeLa $p56^{lck}$, HeLa $CD4^+$ Cyt^-, HeLa CD4(S408A)/$p56^{lck}$, HeLa CD4(C420S)/$p56^{lck}$, and HeLa CH4 cell lines cultured for 4 h in medium alone, medium containing iHIV-1 (iHIV +) or medium supplemented with 20 ng of PMA per ml (PMA +) were reacted with radiolabeled double-stranded NF-κB oligonucleotide (HIV-1Lai LTR sequence). The samples were electrophoresed and analyzed by autoradiography.

CH4 must involve interactions between the PI-linked molecule and a second messenger expressed in HeLa cells.

Our results indicate that signals transduced in the cell through CD4 upon iHIV-1 stimulation involve $p56^{lck}$ and require cysteine 420 in the cytoplasmic tail of CD4.

Involvement of $p56^{lck}$ in HIV-1 LTR activation induced after HIV-1 gp120-CD4 interaction. NF-κB nuclear translocation is a major start signal for HIV-1 early gene transcription. Having determined that $p56^{lck}$ is required for NF-κB activation induced by iHIV-1 binding to CD4, we next assessed the role of $p56^{lck}$ in HIV-1 LTR activation generated upon HIV-1 envelope binding to CD4. To this end, we used two HeLa $CD4^+$ HIV-1 LTR–β-galactosidase indicator cell lines (CD4-LTR/β-gal) expressing either the CD4 molecule alone and referred to as HeLa P4 (22), or coexpressing the CD4 and $p56^{lck}$ molecules and referred to as HeLa P4p56 (50a). These indicator cell lines either were exposed to medium alone, heat-inactivated virus (iHIV-1), or infectious HIV-1 or were cocultured with the $CD4^-$ human T-cell line A2.01 or A2.01 cells expressing HIV-1 gp120. A2.01 cells expressing HIV-1 gp120 (referred below to as A2.01/gp120), were constructed by transient transfections of A2.01 cells with the pV3 plasmid. (This plasmid derives from the pBRU3 vector that contains the complete HIV-1Lai genome [provided by L. Montagnier at the Pasteur Institute, Paris, France], in which the *Pst*I-*Apa*I *gag* sequence was deleted to construct a defective provirus. pV3 [45a] was used as an *env* expression vector.)

As shown in Fig. 5A, A2.01/gp120 cells were found by flow cytometry to express gp120 at the cell membrane following transfection compared to the parental A2.01 cells. Figure 5B shows that both HeLa P4 and HeLa P4p56 cells expressed cell surface CD4 and can therefore bind HIV-1. Moreover, Fig. 5C and D indicate that HeLa P4p56 cells express $p56^{lck}$ and that $p56^{lck}$ associates with CD4. The functional assays were performed as follows. The CD4-LTR/β-gal indicator cell lines were plated in 12-well plates at 8×10^5 cells/ml in DMEM with 10% FCS. On the next day, the cells were stimulated with 500 μl of iHIV-1 (iHIV-1 at a concentration equivalent to 1,000 × $TCID_{50}$ of infectious virus/ml) or exposed to 500 μl of infectious HIV-1 (at 1,000 × $TCID_{50}$ of infectious virus/ml). The plates were rocked every 30 to 45 min. After 2 h, the cells were washed, and 1 ml of DMEM supplemented with 10% FCS per well was added. In some experiments, stimulation were performed by coculturing the HeLa $CD4^+$-LTR/β-gal cell lines with 4×10^4 A2.01 or A2.01/gp120 cells. After 3 days in culture, nonadherent (A2.01) cells were removed, and adherent cells were washed three times in PBS and lysed in 300 μl of buffer containing 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 10 mM $MgSO_4$, 2.5 mM EDTA, 50 mM 2-β-mercaptoethanol, and 0.125% Nonidet P-40 for 15 min at room temperature. Cell lysates were clarified by centrifugation for 15 min at 13,000 rpm at 4°C. For β-galactosidase activity determination, 150 μl of total cellular extract was reacted for 1 h at 37°C in 500 μl of buffer containing 80 mM Na_2HPO_4, 10 mM $MgCl_2$, 1 mM 2-β-mercaptoethanol, and 6 mM *O*-nitrophenyl-β-D-galactopyranoside (ONPG). β-Galactosidase activity was evaluated by measuring A_{410}.

β-Galactosidase activities in HeLa P4 and HeLa P4p56 cells infected with HIV-1 were determined. The values obtained under such experimental conditions were used to fix the 100% β-galactosidase activity for each cell line. (Note that HIV-1 infection provides Tat transactivator to the target cell.) Subsequent values obtained in each assay were expressed as percentages of maximal β-galactosidase activity. As shown in Fig. 6A, a very weak β-galactosidase activity was detected in the HeLa P4 cell line after iHIV-1 stimulation or after coculture with the

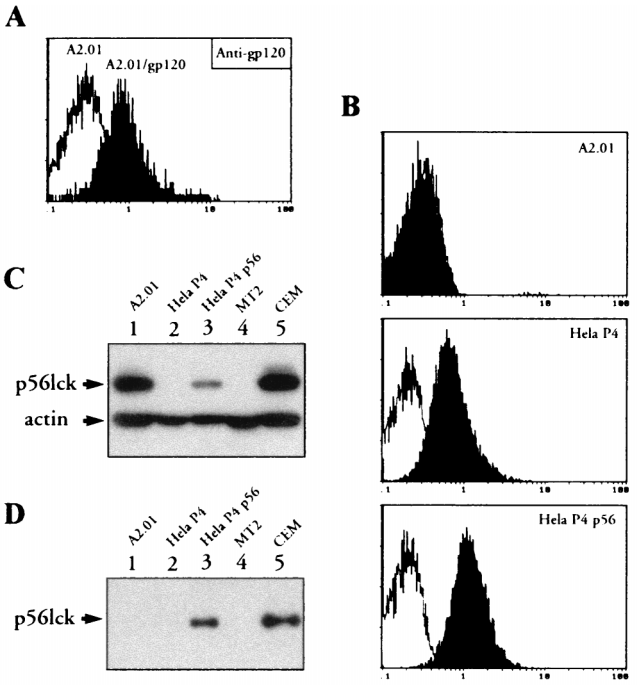

FIG. 5. Induction of HIV-1 LTR transactivation by gp120-CD4 interaction. (A) Expression of gp120 on A2.01/gp120 cells (black histogram) was monitored by indirect flow cytometry. Background reactivity of anti-gp120 antibodies with gp120-negative A2.01 parental cells is shown as a control (white histogram). (B) Expression of CD4 on HeLa P4 and HeLa P4p56 (black histogram) was monitored by flow cytometry, as described in the legend to Fig. 1. The background of the probe is shown (white histogram). The $CD4^-$ A2.01 cell line was used as a control. (C) Detection of $p56^{lck}$ expression in HeLa P4p56 by Western blot analysis. The experiment was performed as described in the legend to Fig. 2. (D) Analysis of CD4-$p56^{lck}$ interaction in HeLa P4p56 cells by coimmunoprecipitation. The experiment was performed as described in the legend of Fig. 3. The $CD4^-$ $p56^{lck}$-positive A2.01 cell line was used as a control.

A2.01/gp120 cells. In contrast (Fig. 6B), significant β-galactosidase activity was observed when the HeLa P4p56 cell line was incubated in the presence of either iHIV-1 (45.6% of maximal activation) or A2.01/gp120 cells (41.5% of maximal activation). When HeLa P4p56 cells were cocultured with gp120-negative parental A2.01 cells, no significant β-galactosidase gene expression driven by the HIV-1 LTR was detected in cell lysates. This result demonstrates that the reporter gene activity was specifically induced by gp120 expressed at the cell surface of the A2.01/gp120-stimulating cells. It is worth noting that the extent of stimulation of β-galactosidase synthesis by HIV-1 gp120-CD4 interaction was lower than that by infectious HIV-1. The higher β-galactosidase activity generated by infectious HIV-1 can probably be ascribed to Tat transactivation of the HIV-1 LTR and to virus propagation in cell cultures (the concentration of virus after 3 days of cell culture being much higher than the virus input), thereby increasing the percentage of β-galactosidase-positive cells.

PTX-sensitive G protein signalling is not required for iHIV-1-induced HIV-1 LTR activation. The G protein-coupled seven transmembrane chemokine receptor CXCR4 (24), also called fusin, has been identified as a cell surface coreceptor for T-cell-tropic viruses such as HIV-1Lai, which was used in the present study. Recently, CXCR4 was shown to transduce signals to T cells following interaction with HIV-1 (20). This molecule is expressed at the surface of HeLa cells (24), and we previously reported that HeLa P4 cells express about two- to fourfold excess of surface CXCR4 compared to HeLa P4p56 cells (18). This observation may explain why higher β-galactosidase activities were measured in HeLa P4 cells infected by HIV-1 compared to HIV-1-infected HeLa P4p56 cells (mean A_{410} of 0.944 for HIV-1-infected HeLa P4 cells and 0.472 for HIV-1-infected HeLa P4p56 cells). Although we have found (see above) that iHIV-1 binding to CD4 does not induce NF-κB translocation in HeLa $CD4^+$, HeLa $CD4^+$ Cyt^-, HeLa $p56^{lck}$, and HeLa CD4 (C420S)/$p56^{lck}$ cells, indicating that the interaction of HIV-1 gp120 with CXCR4 cannot account, by itself, for signal transduction triggering NF-κB translocation, we wanted to exclude the possibility that cosignaling through CXCR4 is required to activate HIV-1 LTR. We studied the antagonist activity of pertussis toxin (PTX), an inhibitor of protein Gi-mediated signals, on HIV-1 LTR activation induced after HIV-1 gp120 binding to the CD4-CXCR4 receptor complex by using a previously described protocol (18). Briefly, HeLa P4p56 cells were treated for 16 h with 250 ng of PTX per ml (a concentration of PTX that inhibits the CXCR4 natural ligand stromal cell-derived factor 1α [SDF-1α] induction of calcium fluxes in HeLa P4 cells without affecting the cells' viability [18]) or control medium. Next, the cells were stimulated with 500 μl of iHIV-1 or exposed to 500 μl of infectious HIV-1, and β-galactosidase activity was evaluated, as described above, after 3 days of cell culture. Under these experimental conditions (Fig. 6C), PTX did not significantly reduce the activation of HIV-1 LTR triggered by iHIV-1 gp120, indicating that CXCR4 signal transduction through Gi proteins is not required for HIV-1 LTR activation induced in HeLa P4p56 cells after iHIV-1 gp120 binding to the CD4-CXCR4 receptor complex. It is worth noting that we previously found that PTX did not modify the transactivation of HIV-1 LTR in HeLa P4 and HeLa P4p56 cells infected by HIV-1 (18).

Altogether, these data strongly support the hypothesis that NF-κB activation induced by HIV-1 gp120-CD4 receptor in-

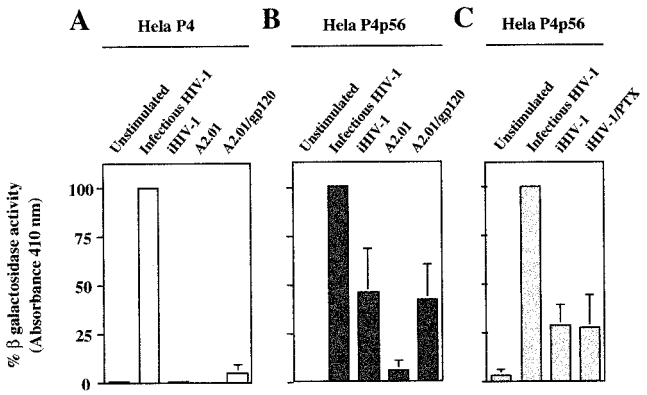

FIG. 6. β-Galactosidase activities in HeLa P4 and P4p56 cells. HeLa P4 (A) and HeLa P4p56 (B) cell lines expressing the β-galactosidase reporter gene cloned downstream of the HIV-1 LTR promoter were cultured in the presence of medium alone (column 1) or medium supplemented with infectious HIV-1 (column 2) or iHIV-1 (column 3). In columns 4 and 5, the LTR–β-galactosidase indicator cell lines were cocultured with A2.01 cells or the A2.01/gp120 cells, respectively. After 3 days in culture, adherent cells were harvested and lysed, and β-galactosidase activities were determined by incubation of 150 μl of total cellular extracts with ONPG in an appropriated buffer. (C) HeLa P4p56 cells were treated for 16 h with 250 ng of PTX per ml (column 4) or with control medium (columns 1 to 3) and next cultured for 3 days in the presence of medium alone (column 1), medium supplemented with infectious HIV-1 (column 2), or iHIV-1 (columns 3 and 4). β-Galactosidase activities were determined as described above. In order to compare the results obtained from different experiments, all values were normalized according to the β-galactosidase activities obtained following infection of cells by HIV-1 (100% β-galactosidase activity). Mean absorbances measured with HIV-1-infected samples were 0.944, 0.472, and 0.452 in panels A, B, and C, respectively.

teraction requires the formation of a functional CD4-p56 complex. Activation signals generated upon CD4 ligation are able to transactivate the HIV-1 LTR and to induce early viral gene transcription. These observations are in agreement with our previous results demonstrating that uninfectious HIV-1 and gp120–anti-gp120 immune complexes binding to CD4 on latently infected quiescent $CD4^+$ PBMCs upregulate latent HIV-1 and commit cells to produce virus (10).

$p56^{lck}$ plays a key role in transduction of signal(s) initiated upon HIV-1 interaction with CD4. During the past few years, many research groups have demonstrated the association of the $p56^{lck}$ tyrosine kinase with the cytoplasmic tail of CD4, providing a mechanism whereby CD4 could transduce signals through this kinase. However, a requirement for the association of CD4 with $p56^{lck}$ in transduction of signal(s) originating from CD4 is disputed. Indeed, $p56^{lck}$-independent CD4-mediated enhancement and inhibition of the T-cell activation pathway have been described (4, 17, 18, 35, 59). In contrast, it has been also demonstrated that $p56^{lck}$ linked to CD4 is critical for CD3- and antigen-dependent T-cell activation, since cells expressing a mutant form of $p56^{lck}$ lacking kinase activity demonstrated a profound inhibition of tyrosine phosphorylation in response to stimulation by anti-CD3 MAb (1, 14). Similar observations were performed with interleukin-16 (IL-16), a natural ligand of CD4, that was described to induce T-cell migration in a $p56^{lck}$-dependent fashion, whereas the motile response generated by IL-16–CD4 interaction was independent of CD4-$p56^{lck}$ coupling (47). Despite the increasing number of reports indicating the ability of gp120/160 to activate CD4-associated $p56^{lck}$ (9, 28, 31, 42, 54), there was no clear demonstration concerning the precise role played by $p56^{lck}$ in CD4-mediated T-cell activation, and the possibility remained that HIV-1-mediated signaling could be transduced following CD4 cross-linking independently of $p56^{lck}$ activation.

Beside activation of $p56^{lck}$, the ligation of multimeric gp120 to the CD4-CXCR4 receptor complex induces a variety of effects, including mobilization of intracellular Ca^{2+} (33), induction of protein phosphorylation, and activation of a number of cellular proteins, including PKC (60), Shc (3), PI-3K (9, 44) and PI-4K (44, 50), $p59^{fyn}$ (34), Zap70 (34), $p95^{vav}$ (34), Ras (34), Raf-1 (43), the Raf-1-related 110-kDa polypeptide (45), MEK-1 (12), ERK-1 (12), and ERK-2 (6), which have been identified as molecules activated in response to such stimuli. Some of these molecules are likely required (or have been shown to act) as signal messengers in activation of NF-κB (5, 10, 12, 13, 15) and AP-1 (5, 10, 12, 16) transcription factors that are specifically induced by virus-host interactions in lymphoblastoid cell lines and in primary T lymphocytes. We have previously suggested a pivotal role for $p56^{lck}$ in transduction of a CD4-dependent T-cell activation signal upon HIV-1 binding to CD4. (i) CD4 oligomerization by iHIV-1 induces $p42^{erk}$ activation in lymphoblastoid cell lines expressing the wild-type CD4 and a functional $p56^{lck}$ molecule but not in cells that express CD4 lacking the cytoplasmic domain (6). (ii) NF-κB translocation was induced by iHIV-1 in lymphoblastoid cell lines expressing the wild-type CD4 and a functional $p56^{lck}$ molecule but not in cells expressing the truncated CD4 (5). (iii) iHIV induced expression of a reporter gene driven by HIV-1 LTR in cells expressing the wild-type CD4 and a functional $p56^{lck}$ molecule but not in cells expressing a truncated form of CD4. Similar results have been obtained by Merzouki and coworkers (39), who reported that the activation of reporter gene driven by HIV-1 LTR was enhanced following envelope binding to CD4 in $p56^{lck}$-expressing cell lines but not in U937 cells (from the promonocytic lineage) that lack expression of this kinase. These data argued for the critical involvement of $p56^{lck}$ in CD4-mediated T-cell activation. We provide here the first direct demonstration that both CD4 and $p56^{lck}$ are required to transduce an activation signal leading to NF-κB nuclear translocation after iHIV-1 gp120 binding to CD4. The fact that cells expressing a mutant form of CD4 at position 420 (C420A) and the wild-type $p56^{lck}$ did not respond to iHIV-1 stimulation further indicates the requirement for a functional CD4-$p56^{lck}$ complex.

Understanding how HIV takes advantage of the cellular signaling pathways and modifies the physiology of a host cell is an important goal to improve our knowledge of the pathogenesis of AIDS (11, 55). Our results provide a molecular basis by which gp120 misappropriates the transduction function of the CD4-$p56^{lck}$ complex to act on CD4 T-cell signaling.

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