Transduction of Activation Signal That Follows HIV-1 Binding to CD4 and CD4 Dimerization Involves the Immunoglobulin CDR3-like Region in Domain 1 of CD4*

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The role of CD4 during the human immunodeficiency virus type 1 (HIV-1) life cycle in T cells is not restricted to binding functions. HIV-1 binding to CD4 also triggers signals that lead to nuclear translocation of NF-κB and are important to the productive infection process. In addition to its cytoplasmic tail, in the ectodomain, the immunoglobulin (Ig) CDR3-like region of CD4 domain 1 seemed to play a role in this cascade of signals. We demonstrate in this work that the structural integrity of the CDR3-like loop is required for signal transduction. Substitutions of negatively charged residues by positively charged residues within the CDR3-like loop either inhibited NF-κB translocation after HIV-1 and gp120-anti-gp120 immune complexes binding to E91K,E92K mutants or induced its constitutive activation for E87K,D88K mutants. Moreover, A2.01-3B cells expressing the E91K,E92K mutant exhibited a lower HIV-1Lai replication. These cells, however, expressed p56lck, demonstrated NF-κB translocation upon PMA stimulation, bound HIV-1Lai envelope glycoprotein with high affinity, and contained HIV-1 DNA 24 h after exposure to virus. E91K, E92K, and E87K,D88K mutant CD4 molecules were unable to bind a CD4 synthetic aromatically modified exocyclic, CDR3.AME-(82–89), that mimics the viral promoter (15) and activates NF-κB and AP-1 transcription factors (16).

Beside its physiological function, CD4 is known to be the primary high affinity cellular receptor for HIV-1. The virus primary envelope glycoprotein (HIV-1gp120) can bind to a protruding ridge located within the IgV-like D1 domain of CD4 at the CDR2-like region (8, 9). Increasing evidence indicates that the role played by CD4 during the HIV-1 life cycle in T cells is not limited to its ability to serve as a receptor for the virus but probably plays other roles that are important to the productive infection process. Indeed, it has been found that transfected cells expressing mutated forms of CD4 that lack the cytoplasmic domain are fusion competent and yet present a defect in a latter stage of HIV-1 replication cycle which results in delayed HIV-1 production (10–12). Further studies have demonstrated that truncation of CD4 cytoplasmic domain blocks NF-κB translocation induced by HIV-1-mediated oligomerization of native CD4, leading to the conclusion that under certain circumstances HIV-1 can take advantage of the signal transduction function of CD4 to prepare the cell for postfusion events (13, 14). More recently, we have shown that HIV-1 binding to CD4 at the surface of peripheral blood mononuclear cells activates tyrosine phosphorylation of a number of transducing proteins, including phosphotyrosinol 4-kinase and mitogen-activated protein kinase-2 which are possible intermediates in the activation pathway(s) that regulates the activity of the viral promoter (15) and activates NF-κB and AP-1 transcription factors (16).

Although the CDR3-like loop in D1 of CD4 plays no role (17) or a minor role (18) for HIV-1 entry, this region may play an important role during HIV-1 replication. This was suspected from antiviral properties of antibodies directed to this region of D1 (17) and synthetic peptides resembling the CDR3-like loop (19). More recently, we have demonstrated that anti-CD4 mAb specific for the CDR3-like region inhibited HIV-1 promoter activity and HIV transcription in cells containing an integrated

The CD4 molecule is an integral membrane glycoprotein that contains four extracellular domains (D1–D4) showing structural homology with immunoglobulin Vx regions (1, 2). This molecule plays a key regulatory role in the immune system by stabilizing MHC class II-T cell receptor complex interactions (3–5) and also by acting as a signal-transducing molecule during T cell activation (6, 7), by its association with the protein-tyrosine kinase p56lck. Beside its physiological function, CD4 is known to be the primary high affinity cellular receptor for HIV-1. The virus primary envelope glycoprotein (HIV-1gp120) can bind to a protruding ridge located within the IgV-like D1 domain of CD4 at the CDR2-like region (8, 9). Increasing evidence indicates that the role played by CD4 during the HIV-1 life cycle in T cells is not limited to its ability to serve as a receptor for the virus but probably plays other roles that are important to the productive infection process. Indeed, it has been found that transfected cells expressing mutated forms of CD4 that lack the cytoplasmic domain are fusion competent and yet present a defect in a latter stage of HIV-1 replication cycle which results in delayed HIV-1 production (10–12). Further studies have demonstrated that truncation of CD4 cytoplasmic domain blocks NF-κB translocation induced by HIV-1-mediated oligomerization of native CD4, leading to the conclusion that under certain circumstances HIV-1 can take advantage of the signal transduction function of CD4 to prepare the cell for postfusion events (13, 14). More recently, we have shown that HIV-1 binding to CD4 at the surface of peripheral blood mononuclear cells activates tyrosine phosphorylation of a number of transducing proteins, including phosphotyrosinol 4-kinase and mitogen-activated protein kinase-2 which are possible intermediates in the activation pathway(s) that regulates the activity of the viral promoter (15) and activates NF-κB and AP-1 transcription factors (16).

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* The abbreviations used are: MHC, major histocompatibility complex; HIV-1, human immunodeficiency virus-1; mAb, monoclonal antibody; GAM, goat anti-mouse; PMA, phorbol myristate acetate; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; PCR, polymerase chain reaction; DPT, dithiotreitol; ECL, enhanced chemiluminescence; EMSA, electrophoretic mobility shift assays; AME, aromatically modified exocyclic; gp, glycoprotein; iHIV, heat-inactivated HIV-1.
providing data show that the CDR3.AME-(82–89) analog can bind to CD4. Moreover, molecular modeling data show that the activation signal transduction and in the binding of the CDR3-like loop play a critical role during the initiation of T cell activation. Recently, a constrained aromatically modified exocyclic (AME) analog that mimics the CDR3-like loop has been proposed that the AME prevents the formation of an essential homodimeric surface involving the CDR3-like region of CD4 (25).

Here we investigated further the structural requirements of the CDR3-like loop responsible for the cascade of signal transduction events that lead to NF-κB translocation after HIV-1 binding to CD4 and probed the role played by the negative charges in this region by using a series of A2.01 T cell clones expressing different mutants of CD4. We demonstrate that the negatively charged residues at positions 87, 88, 91, and 92 in the CDR3-like loop play a critical role during the initiation of the activation signal transduction and in the binding of the CDR3.AME-(82–89) analog to CD4. Moreover, molecular modeling data show that the CDR3.AME-(82–89) analog can bind to the CDR3-like loop and indicate that residues 87, 88, 91, and 92 are essential for CD4 dimerization.

**EXPERIMENTAL PROCEDURES**

**Monoclonal Antibody, Peptide, and Other Reagents**

Anti-CD4 mAbs 13B8–2/iotA4 (IgG1), specific for the CDR3-like loop, and BLA4/iotA4 (IgG2a) which binds to the D1–D2 of CD4 were provided by M. Hirn (Immunotech, Marseille, France), ST40/F142.63 (IgG1) which binds to the CDR3-like loop was provided by D. Carrière (Sanofi Corp., Montpellier, France); Leu 3a (IgG1) specific for the CDR2-like loop and FITC-conjugated OKT4 were purchased from Beckton Dickinson (Erembodegem-Aalst, Belgium) and Ortho Diagnostics Systems (Raritan, NJ), respectively. Anti-p24 

**Cells and Viruses**

The A3.01 (a CD4 positive cell line) (26) was provided by T. Folks (Center for Disease Control, Atlanta, GA). The previously described (12) A2.01/CD4 cells expressing the wild type CD4 and A2.01/CD4:403 cells expressing a truncated form of CD4 were provided by D. Littman (New York, NY). The A2.01 cell clones transfected with the wild type CD4 or CD4 mutants in the Ig CD50-like loop were previously described (18). Briefly, mutations were introduced in the CD4 cDNA by site-directed mutagenesis, and then the mutated sequence was substituted into the full-length CD4 cDNA present in the retroviral vector pMV7, and A2.01 cells were transfected with the mutant CD4 cDNA using a retrovirus shuttle. A2.01–2B was transfected with the wild type CD4 gene but fails to express CD4 although it is neomycin-resistant. A2.01–M1 clone expresses CD4 mutant E87G, A2.01–M2 mutant Q89L, A2.01–2B the double mutant E87K,D88K, and A2.01–3B clones (A2.01–3B-1 and A2.01–3B-2, respectively) the double mutant E91K,E92K. Cells were cultured at a density of 5 \times 10^6 per ml in RPMI 1640 medium containing penicillin/streptomycin antibiotic mixture, glutamax, and 10% fetal calf serum (Life Technologies, Inc., Egrany, France), in a 5% CO2 atmosphere. G418 (Life Technologies, Inc.) at 1 mg/ml was added to the culture medium of transfected A2.01 cells. The origin of CEM and MT2 cells was described previously (23).

Viral stocks (HIV-1lan) were prepared from the chronically infected CEM cell supernatants, as described previously (27), and kept frozen at −80 °C until use. After thawing, 100 μl of these stock viruses at 10^6 TCID₅₀ (50% tissue culture infective dose)/ml was used for infection assays. Heat-inactivated virus was prepared by incubation for 30 min at 56 °C.

**HIV Infection Assay**

Cells (5 \times 10^5) were incubated for 30 min at 4 °C in flat-bottom 96-microwell plates (cell culture top performance product (TPP)) with 100 μl of HIV-1 at 10^5 TCID₅₀ per ml. Thereafter, cells were washed five times and cultured at 37 °C in 24-microwell plates (TPP). The amount of virus produced by the cells was monitored twice a week by measuring reverse transcriptase activity in 1 ml of cell-free culture supernatant, as described previously (27).

**Flow Cytometry Analysis**

Cells (1 \times 10^6) were incubated for 1 h at 4 °C with PBS containing 0.2% BSA (PBS-BSA) or PBS-BSA supplemented with anti-CD4 mAb at concentrations necessary for saturation of cell surface CD4. After washing three times with PBS-BSA, bound mAb was revealed by addition of 50 μl of a 1:50 dilution of fluoresceinated GAM Ig (Immunotech). After 30 min staining, cells were washed with PBS-BSA, and fluorescence intensity was measured on an EPICS cytofluorometer (Coulter, Margency, France). In some experiments, cells (1 \times 10^6) were incubated for 2 h at 37 °C with 10 μl of gp120 solution (100 μg/ml), washed, and incubated for an additional 1 h at 4 °C with 50 μl of anti-gp120 mAb 110-4 (28) solution (10 μg/ml). Bound gp120-antibody complexes were revealed by FITC-labeled GAM Ig probe as above. In another set of experiments, A2.01, A2.01/CD4, A2.01–2B, and A2.01–3B T cell lines (2 \times 10^5) were incubated with FITC-conjugated CDR3.AME-(82–89) analog diluted at 1:50 in PBS-BSA or with a FITC-conjugated anti-CD4 mAb OKT4 for 1 h at 4 °C and then washed in cold PBS-BSA. Background staining was assessed with a FITC-conjugated mAb specific for chicken Ig (Sigma).

**Reverse Transcriptase-Polymerase Chain Reaction (PCR)**

PCR detection of reverse-transcribed RNAs was performed according to the previously published procedure with slight modifications (17). Briefly, total RNA was extracted in guanidinium thiocyanate from 4 \times 10^6 cells and resuspended in 40 μl of H₂O, 0.1% diethyl pyrocarbonate. To reduce the amount of DNA originating from lysis, supernatants were treated with RNase-free DNase (Boehringer Mannheim, 10 units/ml) for 30 min at 20 °C and then for 5 min at 65 °C. To 2 μg of RNA sample (10 μl) was added 200 ng of oligo(dT) primer (1 μl) for 10 min at 65 °C. Each sample was made up with reaction buffer (50 m M Tris-HCl, pH 8.3, 10 m M KCl, 8 m M MgCl₂, 9 m M DTT, 320 m M dNTPs) to a final volume of 25 μl and supplemented with 20 units of RNase inhibitor (Boehringer Mannheim) and 25 units of avian myeloblastosis reverse transcriptase (Boehringer Mannheim), and then incubated for 90 min at 42 °C. PCR were carried out on 4 μl of sample supplemented with an amplification mixture containing the p56-I/p56-II or TK I/TK II oligonucleotide primer pair (23), and 2 units of Taq DNA polymerase. The amplification reaction was run in a PHC2 thermal cycler (Technie, Cambridge, UK). The amplified products were electrophoresed in a 2% agarose gel, blotted for 2 h onto Hybond N+ membrane (Amersham Corp., Buckinghamshire, UK), as described previously (17). The CD4 CDR3.AME-(82–89) analog was amplified using PCR kit (Boehringer Mannheim). Phorbol myristate acetate (PMA) was purchased from Sigma and was used at 10 ng/ml in cell cultures.

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Western Blot Assay

Cellular lysates were electrophoresed onto 12.5% SDS-polyacrylamide gel electrophoresis and blotted to polyvinylidene difluoride membrane (Millipore). The blot was then incubated for 1 h at room temperature with a blocking solution (PBS containing 10% milk and 0.05% Tween 20) prior to addition of mAb. After 1 h at 20 °C, the blot was washed three times with PBS, 0.05% Tween 20 and incubated for 30 min with 1:5000 dilution of GAM Ig peroxidase conjugate (Immuno-tech). After 3 washes, bound mAb was detected by incubating the membrane for 1 min with ECL reagent (Amersham Corp.). The membrane was then exposed for 0.5–5 min to hyperfilms-ECL (Amersham Corp.).

NF-κB Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were prepared as described previously (13). Briefly, cells (2 × 10^6) were centrifuged, transferred into 1.5-ml Eppendorf tubes and washed 3 times in PBS by centrifugation at 2,000 rpm for 10 min at 4 °C. The pellet was resuspended in 400 μl of buffer A (containing 10 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, 4 μg/ml leupeptin, and 10 mM Hepes, pH 7.8). After 15 min on ice, 50 μl of a solution of 10% Nonidet P-40 was added to the sample, and cells were homogenized by vortexing and microcentrifuged at 4 °C for 30 s. The pellets were resuspended in 100 μl of buffer B (containing 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, 4 μg/ml leupeptin, 10% glycerol, and 50 mM Hepes pH 7.8) and incubated for 20 min at 4 °C on a shaker. The nuclear extracts were microcentrifuged at 4 °C for 5 min, and the supernatants were stored at −80 °C until used. The NF-κB mobility shift assay was performed using 2 μg of protein of nuclear extract, 1 × 10^6 cpm of 32P-labeled probe (a double-stranded oligonucleotide NF-κB with a HIV-1 sequence: sense strand only, 5′-GGAGG CGTGG CCTGG GCGGG ACTGG GGAGT-3′) in buffer C (containing 100 mM KCl, 1 mM DTT, 1 μM ZnSO₄, 20% glycerol, 0.01% Nonidet P-40, and 50 mM Hepes pH 7.9), supplemented with BSA, TRNA, and poly(dI-dC) in a final volume of 20 μl. After 20 min at room temperature, the mixture was run at 120 V in a 10% polyacrylamide gel. Sp-1 mobility shift assay was performed using a double-stranded oligonucleotide probe (sense strand only, 5′-GGAGG CCTGG CCTGG GCGGG ACTGG GGCAG-3′).

Molecular Modeling

Construction of Mutants—Molecular modeling was carried out using QUANTA (Molecular Simulations, Inc., Cambridge, MA). The coordinates of CD4 were obtained from Brookhaven Protein data base (30), and the solvent molecules were removed. The mutant models were minimized while holding the remainder of the structure fixed except CDR3-like region, preserving the overall structure. The entire structure was subjected to conjugate gradient energy minimization for 2000 cycles to convergence, followed by an equilibration and production run of molecular dynamics at 300,000 for 60 ps. Molecular dynamics was performed to allow a change in the conformation in the loop, if more favorable. All energy calculations were performed at a dielectric constant of 1. Final energy values were calculated using CHARMM which is part of QUANTA. Electrostatic calculations were performed with GRASP (31). Charged amino acid groups were assigned full charges as provided in GRASP. The electrostatic calculations were performed with distance-dependent dielectric constants from 1, at the interior, to 80, at the outer surface.

Positioning of AME Cyclic Peptide—The CDR3-AME cyclic peptide adopts very similar conformation of the native CDR3 loop of CD4 (25). A dimeric model of CD4 was built with D1 as dimeric interface using an antibody VH-VL domain as template. The CDR3-AME peptide was positioned by superimposing the CDR3-AME to the CDR3 of one of the dimeric molecule. Since this region is solvated, no attempt was made to dock this peptide to the CD4 molecule. The positioning was done only for reference purpose.

RESULTS

Expression of CD4 and p56lck in A2.01 Cells Transfected with Mutant CD4—To analyze whether the CDR3-like region in the first extracellular domain of the CD4 molecule may be involved in HIV-1-induced NF-κB translocation, we used a series of CD4

![Fig. 1. Schematic diagram of the different forms of the human CD4 glycoprotein expressed on the surface of the transfected A2.01 human T cell lines used in this study. CD4 refers to the wild type (WT) CD4 molecule and CD4.403 refers to a truncated form of CD4 at position 403. Numbering of the CD4 protein is based on the structure of the mature processed protein. TM, transmembrane; Cyt, cytoplasmic.](image)

![Fig. 2. Cell-surface expression of CD4 and mutant forms of the CD4 molecule in transfected A2.01 cells. Cells were incubated with medium alone (to determine the background), anti-CD4 mAb Leu 3a, 13B8-2, ST40, or BL4, or anti-HLA class I mAb B9–12-1. mAb binding was detected by a FITC-labeled GAM Ig. The fluorescence intensity was recorded using the log mode.](image)
Human CD4-CDR3-like Loop

We previously demonstrated that heat-inactivated HIV-1 (iHIV) binding to CD4 induced NF-κB activation in cells expressing wild type CD4 but failed to induce NF-κB activation in cells expressing a truncated CD4 molecule. To study whether such an activation signal may follow binding of HIV-1 to cells expressing CD4 mutants, electrophoretic mobility shift assays (EMSA) were performed. As shown in Fig. 4A, a shift of labeled NF-κB oligonucleotide was observed when mixed with nuclear extracts from A3.01 (lane 2), A2.01-M2 (lane 10), and A2.01-M1 (lane 12), exposed to iHIV. In contrast, no protein-NF-κB oligonucleotide complex was found when the oligonucleotide was mixed with nuclear extracts from A2.01/CD4.403 cells (lane 4) or A2.01–3B exposed to iHIV (lane 6). Similar results were obtained using two different clones (A2.01–3B-1 and A2.01–3B-2) of A2.01–3B cells (Fig. 5 lanes 3–6), indicating that the lack of activation by iHIV was likely related to the mutation in the CDR3-like loop. Unexpectedly, the A2.01–2B cells constitutively expressed a large amount of nuclear NF-κB (Fig. 4A, lane 7), and no detectable increase of nuclear NF-κB was observed after iHIV binding to this CD4 mutant (Fig. 4A, lane 8).

Such observation was reproducible using two different clones (A2.01–2B-1 and A2.01–2B-2) of A2.01–2B cells (Fig. 5, lanes 7–10).

As shown above (see Fig. 3), A2.01–3B cells express p56\(^{lck}\) and therefore should be able to transduce CD4-dependent signals stimulating NF-κB translocation. To confirm that the lack of NF-κB translocation in A2.01–3B can be ascribed neither to a defect in the ability of the A2.01–3B clones to translocate NF-κB nor to a lack of gp120 binding to the E91K,E92K mutant CD4 molecule, two sets of experiments were performed. First, the ability of A2.01–3B cells to translocate NF-κB after stimulation with PMA was tested using EMSA. As shown in Fig. 6, PMA induced a shift of labeled oligonucleotide migration when mixed with nuclear extracts from A3.01 and A2.01–3B cells (lanes 4 and 8, respectively). In addition, NF-κB translocation was found with extracts from A3.01 cells exposed to iHIV (lane 2) but was not found with extracts from A3.01 cells exposed to iHIV previously incubated with 10 μg/ml sCD4 for 2 h at 37 °C (lane 3), indicating that induction of NF-κB translocation following A3.01 cell exposure to iHIV required interaction between HIV-1 and cell surface CD4.

Next, we compared the ability of the A3.01, A2.01, and A2.01–3B clones to bind recombinant gp120 envelope glycoprotein (gp120) from HIV-1.Lai. As

![Image](69x387 to 287x729)

**Fig. 3.** Comparative analysis of lck mRNA and p56\(^{lck}\) protein in A3.01 and CD4-transfected A2.01 cells. A, semi-quantitative PCR analysis of avian myeloblastosis virus-reverse transcribed lck mRNA in A3.01 (lanes 1–4 corresponding to dilutions 1:1, 1:10, 1:50, and 1:100 of the sample, respectively), A2.01-3B (lanes 5–8), A2.01-2B (lanes 9–12), A2.01-M1 (lanes 13–16), A2.01/CD4.403 (lanes 17–20), A2.01-M2 (lanes 21–24), and A2.01-c26 (lanes 25–28) was performed using the p56-lck/p56-II oligonucleotide primer pair. The amplified products were electrophoresed, blotted, hybridized with an α-\(^{32}P\)-labeled probe, and visualized by autoradiography. An autoradiogram of PCR amplification visualized by autoradiography. An autoradiogram of PCR amplification

**Fig. 4.** A, Western blot analysis of lck protein in A3.01, A2.01, and CD4-transfected cells. Lysates from A3.01 (lane 1), A2.01-M1 (lane 2), A2.01-M2 (lane 3), A2.01-2B (lane 4), A2.01–3B (lane 5), A2.01/CD4.403 (lane 6), A2.01-c26 (lane 7), and A2.01 (lane 8) containing 30 μg of total cellular protein extract were electrophoresed onto SDS-polyacrylamide gel electrophoresis and blotted to polyvinylidene difluoride membrane. The membrane was treated as described under "Experimental Procedures," incubated with a mixture of anti-p56\(^{lck}\) and anti-actin mAb, and then reacted with goat-anti-mouse Ig (GAM) peroxidase conjugate. Bound mAb was detected by incubating the membrane with ECL reagent and mAb were detected by incubating the membrane with ECL reagent

**Fig. 5.** A, Western blot analysis of lck protein in A3.01, A2.01, and CD4-transfected cells. Lysates from A3.01 (lanes 1–4), A2.01-M1 (lanes 5–8), A2.01-3B (lanes 9–12), A2.01-M2 (lanes 13–16), A2.01/CD4.403 (lanes 17–20), A2.01-M1 (lanes 21–24), and A2.01-c26 (lanes 25–28) was performed using the p56-lck/p56-II oligonucleotide primer pair. The amplified products were electrophoresed, blotted, hybridized with an α-\(^{32}P\)-labeled probe, and visualized by autoradiography. An autoradiogram of PCR amplification visualized by autoradiography. An autoradiogram of PCR amplification

**Fig. 6.** A, Western blot analysis of lck protein in A3.01, A2.01, and CD4-transfected cells. Lysates from A3.01 (lanes 1–4), A2.01-M1 (lanes 5–8), A2.01-3B (lanes 9–12), A2.01-M2 (lanes 13–16), A2.01/CD4.403 (lanes 17–20), A2.01-c26 (lanes 25–28) was performed using the p56-lck/p56-II oligonucleotide primer pair. The amplified products were electrophoresed, blotted, hybridized with an α-\(^{32}P\)-labeled probe, and visualized by autoradiography. An autoradiogram of PCR amplification visualized by autoradiography. An autoradiogram of PCR amplification

**Fig. 7.** A, Western blot analysis of lck protein in A3.01, A2.01, and CD4-transfected cells. Lysates from A3.01 (lanes 1–4), A2.01-M1 (lanes 5–8), A2.01-3B (lanes 9–12), A2.01-M2 (lanes 13–16), A2.01/CD4.403 (lanes 17–20), A2.01-c26 (lanes 25–28) was performed using the p56-lck/p56-II oligonucleotide primer pair. The amplified products were electrophoresed, blotted, hybridized with an α-\(^{32}P\)-labeled probe, and visualized by autoradiography. An autoradiogram of PCR amplification visualized by autoradiography. An autoradiogram of PCR amplification

**Fig. 8.** A, Western blot analysis of lck protein in A3.01, A2.01, and CD4-transfected cells. Lysates from A3.01 (lanes 1–4), A2.01-M1 (lanes 5–8), A2.01-3B (lanes 9–12), A2.01-M2 (lanes 13–16), A2.01/CD4.403 (lanes 17–20), A2.01-c26 (lanes 25–28) was performed using the p56-lck/p56-II oligonucleotide primer pair. The amplified products were electrophoresed, blotted, hybridized with an α-\(^{32}P\)-labeled probe, and visualized by autoradiography. An autoradiogram of PCR amplification visualized by autoradiography. An autoradiogram of PCR amplification
shown in Fig. 7, gp120 binds to A2.01–3B clone to the same extent as to the A3.01 clone. The weak binding of gp120 to A2.01 cells that lack CD4 expression suggests that a fraction of recombinant gp120 binds to target cells independently of CD4 recognition and should be therefore considered as background.

We have previously reported that gp120-anti-gp120 immune complexes binding to wild type CD4 expressed at the surface of peripheral blood mononuclear cells induced NF-κB translocation similar to that induced by iHIV-1 (16). Since gp120 was found to bind A2.01–3B (see Fig. 7), we assessed whether gp120-anti-gp120 immune complexes were able to stimulate NF-κB translocation in these cells. As shown in Fig. 8, we observed a shift of labeled oligonucleotide migration when mixed with nuclear extracts from A3.01 cells exposed to iHIV (lane 2) and gp120-anti-gp120 immune complexes (lanes 5 and 8). NF-κB translocation was also observed with extracts from A3.01 cells exposed to iHIV (lane 2) and gp120-anti-gp120 immune complexes (lanes 5 and 8).
CD4 was investigated. As shown in Fig. 10, the FITC-conjugated CDR3.AME-(82–89) analog to bind the wild type CD4 molecule and mutant forms of CD4 (25). The ability of FITC-labeled CDR3.AME-(82–89) to bind to CD4 was previously shown to inhibit CD4-MHC class II binding and antagonize CD4 function through its binding to structure D1 (11, 12). In the crystal structure of CD4 (11, 12), the CDR3-like loop is stabilized by a disulfide bond, salt links, and several salt links. Fig. 11 shows the surface charge distribution of the CDR3-like loop (A2.01–3B, A2.01-2B, and A2.01/M2). The possibility that the CDR3.AME-(82–89) analog binds to the CDR3-like loop of CD4 was further examined by computer-assisted molecular modeling studies of the interaction between this analog and the first 2 domains of CD4. As shown in Fig. 11, A and B, the CDR3.AME-(82–89) analog (red) was predicted to bind to the CDR3-like loop (yellow) of CD4 (white), suggesting that the CDR3 loop represents a main dimerization site for CD4 molecules. In the crystal structure of CD4 (11, 12), the CDR3 loop is stabilized by a disulfide bond, salt links, and solvent molecules. Whereas the Glu-87/Asp-88 residues from CDR3-like loop occur at the periphery of the putative interface between the analog and CD4, residues Glu-91/Glu-92 occur at the core of the interface. Although the interactions cannot be docked accurately since these regions in the crystal structure contain water molecules, hydrophobic residues at the bottom of the peptide loop such as isoleucine at position 4 (Ile-4) and N-terminal residues would presumably interact with Ile-83 of CD4, if we assume that the role of the peptide is to displace water. Similarly, Tyr-12 at the top of the analog would interact with Asp-88 of CD4.

To highlight the role of Glu-87/Asp-88 and Glu-91/Glu-92 residues in CDR3.AME-(82–89) analog binding to CD4 and CD4 dimerization, mutant models of CD4 were constructed. Structural analysis reveals that in the wild type CD4 molecule (Fig. 11C, c), the conformation of the CDR3 loop is rigid and reinforced by a disulfide bond at the bottom of the loop and by several salt links. Fig. 11C shows the surface charge distribution in D1–D2 domains of CD4. Negatively charged residues (red) Glu-87/Asp-88 are located at the periphery of the putative dimeric interface (shown by circle) and are neutralized by neighboring positively charged lysine residues (Lys-90 at the interface, Lys-29, and Lys-35, below the CDR3 loop). Residues
Glu-91/Glu-92 are located at the core of the putative dimeric interface and are neutralized by lysine residues from CD4 N terminus and a water molecule in the crystal structure. Mutation E87K/D88K changes the charge spectrum at the dimeric interface (Fig. 11C, b). However, the core of the interface, where Glu-91 and Glu-92 provide some stability by interacting with N-terminal lysine residues, does not show significant changes. Conversely, mutation E91K/E92K changes the charge spectrum of the interface completely (Fig. 11C, c). The interface totally becomes positively charged (blue). The mutation not only alters the electrostatic property of the dimeric interface, it potentially alters the conformation of the CDR3 loop. This change occurs through repulsion between Lys-91, Lys-92, and a water molecule in the crystal structure. Mutations changing both charge distribution at the interface and CDR3-like loop conformation would prevent CD4 dimerization.

**DISCUSSION**

We demonstrate in this study that the CDR3-like loop of CD4 domain 1 plays an essential role in the signal transduction pathway that triggers activation of NF-κB after HIV-1 binding to CD4. We show that E91K,E92K substitutions within the CDR3-like loop result in the inability of this mutant molecule to transduce signals triggering NF-κB activation and also lead to impaired replication of HIV-1. Moreover these mutations suppress the binding of the CD4 CDR3.AME-(82–89) analog, a peptide derivative that mimics the CDR3-like loop structure and binds to native CD4, suggesting possible role played by this region in dimer formation.

In the past few years, several studies have reported evidence indicating that T cell activation signals are delivered to target cells by HIV-1 antigens (32–36). Most recently, we reported that binding of HIV-1 to the CDR2-like loop of CD4 domain 1 stimulates NF-κB translocation (13, 16) and that this event requires the integrity of the cytoplasmic domain of CD4 (13). It is worth noting that the interaction of Leu 3a mAb with the CDR2-like loop also triggers AP-1 translocation (36). Several reports have suggested that the “early” HIV-1 transcription events are regulated by NF-κB protein, and the “late” transcription events are regulated by Tat and Sp-1 (37–40). Based on these observations, we have proposed that HIV-1 binding to cell surface CD4 stimulates a signaling pathway(s) that results in nuclear translocation of NF-κB and thereby regulates the early transcription of HIV-1 (13). In agreement with this hypothesis, we have found that viral production was delayed in cells expressing mutant forms of CD4 that lack the cytoplasmic tail, although the rate of viral entry and retrotranscription was apparently not different from that found in cells expressing the wild type CD4 (10–13).

We have recently observed that binding of 13B8-2 mAb to the CDR3-like loop of CD4 domain 1 inhibits mitogen-activated protein kinase-2 activation (22) and NF-κB translocation induced by HIV-1 binding to CD4 (23), indicating that the CDR3-like loop may play an important role in T cell activation. To investigate this possibility, we studied the effects of mutations affecting four negatively charged residues within the CDR3-like region on signal transduction pathway that triggers activation of NF-κB after HIV-1 binding to CD4 and on viral production. The structural integrity of the CDR3-like loop mutant molecules was established by binding experiments using a panel of CD4 mAbs (see Fig. 2). Consistent with previous results reported by one of us who tested 9 anti-D1, 1 anti-D2, and 1 anti-D4 anti-CD4 mAbs for binding to the mutants CD4 (18), we found no evidence of global distortion of the CD4 conformation and no obvious effect on the structure of D1. Moreover, these mutations do not affect the binding of gp120 to the CDR2-like loop in D1 (see Fig. 7). Only a local effect in the CDR3-like loop was found for double substitution mutants E87K,D88K and E91K,E92K, as detected by the loss of ST40 mAb binding to A2.01–2B and A2.01–3B cells.

We observed that cells expressing the CD4 mutants exhibit different ability to translocate NF-κB after HIV binding to CD4. Cells (A2.01–3B) expressing the E91K,E92K mutant CD4 molecule were refractory to HIV-induced activation and exhibited a significant reduction in virus production after exposure to low concentrations of HIV-1. According to the mAb binding results, these effects cannot be attributable to the density of CD4 molecules on the surface of the A2.01–3B cells nor to alterations of CD4 overall conformation although local conformational changes in the CDR3-like loop might play a role (see Fig. 2). They cannot be due either to an impaired accessibility of HIV-1 to CD4 as demonstrated by efficient binding of recombinant HIV-1-Lag gp120 to A2.01–3B cells (see Fig. 6) or to a defect in p56lck expression (see Fig. 3), or to a defect of NF-κB translocation, since NF-κB translocation was observed under PMA stimulation (see Fig. 5). The reduction in virus production thus apparently correlates with a defect in the capacity of the CD4 molecule to transduce an activation signal resulting in NF-κB translocation. The reduced reverse transcriptase activity in those cells was more pronounced when cells were exposed to low virus input (data not shown). Altogether these results indicate that negatively charged residues Glu-91 and Glu-92 in the CDR3-like loop play a role in activation signal...
controls consisted of RNA samples not submitted to reverse transcription. A control is shown in which an RNA-free sample was prepared for PCR. Other controls PCR were performed on pBRU plasmid DNA in HIV-1 exposed A2.01 (lane 1), A3.01 (lane 2), A2.01/CD4 (lane 3), A2.01-M1 (lane 4), A2.01-M2 (lane 5), A2.01-2B (lane 6), A2.01-3B (lane 7), and A2.01/CD4.403 (lane 8) cells was monitored at 24 h following virus exposure by PCR analysis using the M667/M668 oligonucleotide primer pair. The controls PCR were performed on pBRU plasmid (lane 9) and H2O (lane 10). The amplified products were electrophoresed, blotted, hybridized with a radiolabeled HIV-1 probe, and visualized by autoradiography. C, PCR analysis of HIV-1 spliced mRNAs in A2.01/CD4 and A2.01-3B infected cells. Total RNAs were extracted from cells 72 h after infection and retrotranscribed into DNA. PCR was then performed using the M671/VPR3 primer pair and various dilutions of retrotranscribed products (lanes 4–8 and lanes 11–15 corresponding to 1:1, 1:2, 1:5, 1:10, and 1:50 dilutions of sample, respectively). The amplified fragments were hybridized with a radiolabeled HIV-1 probe and visualized by autoradiography. A control is shown (lane 1) in which an RNA-free sample was prepared for PCR. Other controls consisted of RNA samples not submitted to reverse transcription (lanes 3 and 10) and of RNA extracted from uninfected cells (lanes 2 and 9). Products of PCR amplification of retrotranscribed thymidine kinase (TK) RNA, are shown as control.

In contrast to A2.01–3B cells, A2.01–2B cells expressing CD4 mutant E87K,D88K seemed to be constitutively activated and were shown to express high amounts of nuclear NF-κB in the absence of exogenous stimuli (Fig. 4). Although this result was unexpected, it was reproducible with two different clones of A2.01–2B cells. The fact that two clones demonstrated similar activation status suggests that the constitutive NF-κB translocation is probably not related to a putative integration of the CD4 construct in the vicinity of a cellular gene controlling cell activation. Chronic activation of these clones was further established by flow cytometry experiments indicating that they express elevated surface CD25 antigen. Interestingly, both mutants E91K,E92K and E87K,D88K exhibited a similar loss of ST40 mAb binding, indicating that these four negatively charged residues are involved in the ST40 epitope but play distinct functional roles in transduction of signals mediated by CD4.

Requirement for CD4 dimerization in transducing signals was suspected from the observation that cross-linking of CD4 can trigger autophosphorylation of p56\(^\text{cA}\) (27). We found here that mutants E91K,E92K and E87K,D88K were unable to bind the CD4 CDR3.AME-(82–89) analog that mimics the CD3-like loop and specifically binds to native CD4 (25). These results, together with molecular modeling studies, indicate that the CDR3.AME-(82–89) analog binds to the CD3-like loop (see Fig. 11) and that residues 87/88 and 91/92 can potentially be involved in this binding. Moreover our results strongly suggest that the CDR3-like loop constitutes a primary site for CD4 dimerization. Our model shows that residues 89–93 form the core of the dimeric interface and residues 87/88 at the periphery. The involvement of the CDR3-like region in CD4 dimerization has been previously proposed (24) based on the demonstration that CDR3-like loop-derived synthetic peptides can bind recombinant CD4 molecules. Further evidence in favor of this dimerization site has recently been obtained by some of us (25); it was found that CDR3.AME-(82–89) analog binding to CD4 inhibits CD4-HLA class II interaction and antigen-induced T cell activation, an effect consistent with prevention of CD4 homodimer formation and signal transduction.

In this regard, the defect of NF-κB translocation after HIV binding to CD4 and the reduction in virus production generated by the E91K,E92K mutant might result from prevention of CD4 dimerization. Indeed, calculation for the E91K,E92K mutant drastically changes the conformation of the CDR3-like loop. So it seems that these mutations not only disrupt the dimerization, it can also disrupt the CDR3-like loop. These data clearly indicate that both CD4 dimerization and CDR3-like loop structure are essential for efficient signaling and suggest that binding of multivalent molecules to the CDR2-like loop would induce CD4 dimerization at the CD3-like loop. In light of this model, molecules that react with the CDR3-like region (20, 41–43) might exert their anti-HIV properties by uncoupling CD4 from the signal transduction machinery, thereby preventing cell activation. It is very likely that the CDR3.AME-(82–89) analog would act like a "spacer" between the CD4 dimers accounting for its inhibitory effects on T cell activation in MHC class II restricted immune response (25); it was found that CDR3.AME-(82–89) analog binding to CD4 inhibits CD4-HLA class II interaction and antigen-induced T cell activation, an effect consistent with prevention of CD4 homodimer formation and signal transduction.

2 N. Signoret, unpublished observations.

2 and 9. Products of PCR amplification of retrotranscribed thymidine kinase (TK) RNA, are shown as control.
triaryl methane polymer AT-A that binds to part of the gp120 binding site induces conformational changes of the OKT4 epitope that is thought to arise from the tertiary structure of D1 through the juxtaposition of residues Gln-20, Lys-21, and Glu-91, via the S–S bond fixing the stem of the CDR3-like loop of D1 (45).

The opposite effects induced by the E87K,D88K mutation that seemed to generate a constitutive activation of A2.01–2B cells, which express high amounts of nuclear NF-kB in the absence of exogenous stimuli, are intriguing. Although residues Glu-87 and Asp-88 are at the periphery of the CD4 dimeric interface and exposed to solvents, their replacement by lysine does not show any significant changes in the CDR3-like loop. So it is possible that any instability caused by mutations can be compensated either by conformation changes or by solvents. Such subtle changes might account for the creation of new interaction sites facilitating mutant CD4 homodimer formation and consequently, constitutive cell activation, as detected in A2.01–2B cells. According to this hypothesis, the presence of CD4 dimers at the cell surface might prevent the CDR3.AME-(82–89) analog from binding to the E87K,D88K CD4 mutant. Alternatively, subtle changes in the charge balance might be responsible for its impaired binding. Further studies will be needed to determine whether E87K,D88K mutations favor NF-kB translocation or whether the two clones demonstrate high nuclear amounts of NF-kB for another reason which remains to be determined.

Although CD4 can likely form dimers (46), it is not known whether a dimeric CD4 state exists at the surface of helper T cell only under certain conditions or whether there is a monomer/dimer equilibrium. Native CD4 may be normally monomeric and oligomerize by interacting with HLA class II or HIV-1. Sweet and co-workers (47) have reported crystallographic studies of sCD4 suggesting that it oligomerizes by interaction between its D3–D4 regions. Most recently, Sakihama and co-workers (48) have reached the same conclusions, based on the observation that replacement of D3–D4 domains by D1–D2 of CD4 or the extracellular domain of CD2 prevented HLA class II binding to these chimeric membrane CD4 molecules. It is, however, not known how the D1–D2 domains are associated with D3–D4 on the cell surface. It is possible that D1 domain makes a primary contact followed by D3–D4 domain in a dimer formation. Moreover, it has been demonstrated that the membrane-proximal CD4 domain appears to be important for the overall conformation of CD4 (5), and replacement of this domain might have an indirect rather than a direct role in CD4 dimer formation. Our data and these results do not rule out that several domains of CD4, including the D1, D3, and D4, participate in CD4 oligomerization.

In conclusion, our results provide the first functional evidence indicating that negatively charged residues within the CDR3-like loop can play a role in CD4-mediated activation signals transduced in T cells after virus envelope binding to the CD2-like loop. Moreover, they strongly suggest that the CDR3-like loop represents a main site for CD4 dimerization.
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