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Inhibition of Tyrosine Kinase Activation Blocks the Down-Regulation of CXC Chemokine Receptor 4 by HIV-1 gp120 in CD4⁺ T Cells¹

Shao Bo Su,* Wanghua Gong,*[†] Michael Grimm,* Iku Utsunomiya,* Robert Sargeant,[†] Joost J. Oppenheim,* and Ji Ming Wang^{2*}

Because the binding of HIV-1 envelope to CD4 initiates a configurational change in glycoprotein 120 (gp120), enabling it to interact with fusion coreceptors, we investigated how this process interferes with the expression and function of CXC chemokine receptor 4 (CXCR4) in CD4⁺ T lymphocytes. A recombinant gp120 (MN), after preincubation with CD4⁺ T lymphocytes, significantly inhibited the binding and chemotaxis of the cells in response to the CXCR4 ligand stromal cell-derived factor-1 α (SDF-1 α), accompanied by a markedly reduced surface expression of CXCR4. gp120, but not SDF-1 α , induced rapid tyrosine phosphorylation of src-like kinase p56^{lck} in CD4⁺ T cells, whereas both gp120 and SDF-1 α caused phosphorylation of the CXCR4. The tyrosine kinase inhibitor herbimycin A abolished the phosphorylation of p56^{lck} and CXCR4 induced by gp120 in association with maintenance of normal expression of cell surface CXCR4 and a migratory response to SDF-1 α . Thus, a CD4-associated signaling molecule(s) including p56^{lck} is activated by gp120 and is required for the down-regulation of CXCR4. *The Journal of Immunology*, 1999, 162: 7128–7132.

Human immunodeficiency virus type 1 uses CD4 and seven transmembrane G protein receptors as fusion cofactors (1). T lymphocytotropic virus uses CXC chemokine receptor 4 (CXCR4)³, whereas monocytotropic virus uses CCR5 in addition to CD4 for cell entry (2, 3). Dual tropic viruses use both CXCR4 and CCR5 (4, 5) or a newly defined receptor, STRL33, for cell entry (6). The interaction of the envelope protein glycoprotein 120 (gp120) with CD4 and subsequently with CXCR4 or CCR5 is crucial to initiate the cell fusion (6). It has been proposed that after binding to CD4, gp120 develops a configurational change that enables it to bind chemokine receptor(s) (7, 8). This process, which culminates in the down-regulation of chemokine receptors, is not sensitive to pertussis toxin, suggesting that G protein of Gi type is not involved (9, 10). However, soluble gp120 from T lymphotropic HIV-1 has been reported to induce chemotaxis and calcium flux of both monocytes and T lymphocytes, which are biological activities involving cellular signaling events (11, 12). Recently, the precursor of gp120, gp160 of monocytotropic HIV-1, was also shown to activate T cells in this manner by using both CD4 and CCR5 (13). We can confirm that recom-

binant as well as purified natural gp120 from both monotropic and T cell-tropic HIV-1 strains induced a pertussis toxin-sensitive migration of human peripheral blood monocytes (14). We also found that after preincubation with human monocytes, gp120 of various HIV-1 strains markedly reduced the Ca²⁺ flux and chemotaxis response of the cells to a variety of chemokines, including the CXCR4 ligand stromal cell-derived factor-1 α (SDF-1 α) as well as the bacterial chemotactic peptide fMLP. The reduction of cell responses was associated with coreceptor down-regulation and internalization in a CD4-dependent manner (14). These observations prompted us to investigate whether the initial interaction of gp120 with CD4 on T lymphocytes activates the cells and whether this activation is required for the subsequent down-regulation of CXCR4. Our results show that down-regulation of CXCR4 on T cells by gp120 is associated with tyrosine phosphorylation of src-like kinase p56^{lck} and can be prevented by treating CD4 T cells with a tyrosine kinase inhibitor, herbimycin A (HA).

Materials and Methods

Reagents and cells

Recombinant gp120 of a laboratory-adapted HIV-1 strain, MN, which uses CD4 and CXCR4 as fusion coreceptors, was purchased from MicroGene-Sys (Meriden, CT). At the highest concentration tested, the gp120 preparations contained <0.2 ng/ml endotoxin. Recombinant SDF-1 α was purchased from PeptoTech (Rocky Hill, NJ). Radio-iodinated SDF-1 α was purchased from DuPont New England Nuclear (Boston, MA). Recombinant soluble human CD4 and an anti-CD4 mAb (clone E9) were purchased from Intracel (Cambridge, MA) and Biogenesis (Poole, U.K.), respectively. Anti-CXCR4 mAb (clone 12G5) was purchased from PharMingen (San Diego, CA). Monoclonal HRP-conjugated antiphosphotyrosine and polyclonal anti-human p56^{lck} Abs were obtained from Upstate Biotechnology (Lake Placid, NY). HA was purchased from Sigma (St. Louis, MO). Human peripheral CD4⁺ and CD8⁺ T cells were isolated from lymphocyte enriched buffy-coat (National Institutes of Health Clinical Center, Transfusion Medicine Department, Bethesda, MD) by CD4 or CD8 negative-selection columns (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The purity of the cell preparations was >95% for CD4⁺ T cells and >90% for CD8⁺ T cells. The CD4⁺ human CEM-SS T cell leukemia line, which is permissive to infection by the HIV-1 MN strain (15) (a kind gift of Dr. P. Nara, National Cancer Institute, Frederick,

*Laboratory of Molecular Immunoregulation, Division of Basic Sciences, [†]SAIC-Frederick National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, MD 21702; and [‡]Millennium Biotechnology, Ramona, CA 92065

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² Address correspondence and reprint requests to Dr. Ji Ming Wang, Laboratory of Molecular Immunoregulation, Division of Basic Sciences, National Cancer Institute, Frederick Cancer Research and Development Center, Building 560, Room 31-19, Frederick, MD 21702-1201. E-mail address: wangji@mail.ncicrf.gov

³ Abbreviations used in this paper: CXCR4, CXC chemokine receptor 4; gp, glycoprotein; SDF-1 α , stromal cell-derived factor-1 α ; HA, herbimycin A.

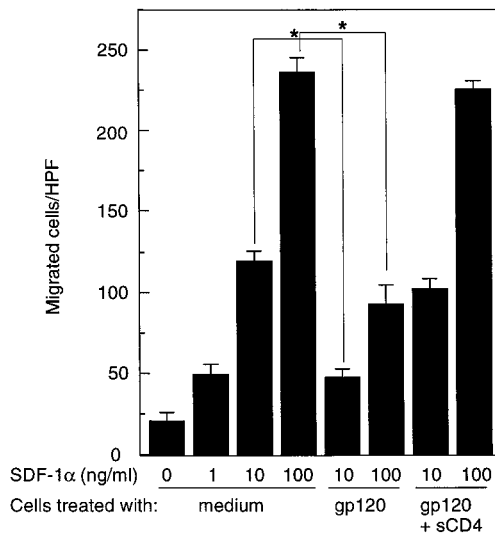


FIGURE 1. Effect of gp120 on SDF-1 α -induced CD4⁺ T cell migration. CEM-SS cells were preincubated with gp120 MN at 37°C for 1 h and subsequently tested for their chemotactic response to SDF-1 α as described in *Materials and Methods*. Results from one representative experiment of five performed are shown. An asterisk (*) indicates a statistically significant difference ($p < 0.05$). The same results were obtained with CD4⁺ blood T lymphocytes.

MD), was maintained in RPMI 1640 medium supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100 μ g/ml), and glutamine (20 mM).

Binding assays

Binding assays were performed by preincubating T cells (2×10^6) with different concentrations of gp120 for 60 min at 37°C in 200 μ l/sample of binding medium (RPMI 1640, 1% BSA, 5 mM HEPES). Radiolabeled SDF-1 α (0.12 nM) was added to each sample. To parallel samples, different concentrations of gp120 or unlabeled SDF-1 α (as a control) were added simultaneously with radiolabeled SDF-1 α . After incubation at room temperature for 60 min, the cells were centrifuged through a 10% sucrose/PBS cushion; the cell-associated radioactivity was measured in a gamma counter. The nonspecifically bound radioactivity in the presence of unlabeled SDF-1 α (500-fold excess) was subtracted from the total bound radioactivity to yield specific binding. After subtraction of nonspecific binding, the inhibition of specific binding of ¹²⁵I-labeled SDF-1 α by gp120 MN was calculated as follows: % inhibition of specific binding = [(specific binding – binding of gp120 MN-treated cells)/specific binding] \times 100%.

Chemotaxis assays

Cell migration was assessed using 48-well microchemotaxis chambers and a fibronectin-coated polycarbonate membrane (pore size of 5 μ m) as described previously (16). The number of migrated T cells in three high power fields ($\times 400$ magnification) was counted by light microscopy after coding the samples. Results are expressed as the mean \pm SE value of the migrating cells in triplicate samples.

Immunofluorescence analysis

A total of 5×10^5 peripheral blood CD4⁺ T cells or CEM-SS cells treated with medium or gp120 were incubated with 5 μ g/ml anti-CXCR4 mAb (12G5) at 4°C for 30 min. Next, the cells were stained with FITC-conjugated anti-murine IgG. After washing, the cells were analyzed by a Coulter flow cytometer (courtesy of L. Finch, SAIC-Frederick, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD). For tyrosine kinase inhibitor treatment, the cells were preincubated with HA (2 μ M) for 18 h at 37°C, followed by staining with relevant Abs.

Tyrosine phosphorylation

CEM-SS or CD4⁺ T cells were stimulated with gp120 (50 nM) at 37°C for 5 min. After stimulation, cells were pelleted and lysed in a lysing buffer (50 mM HEPES (pH 7.2), 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 10% glycerol, and 1.5% Triton X-100) containing proteinase and phosphatase

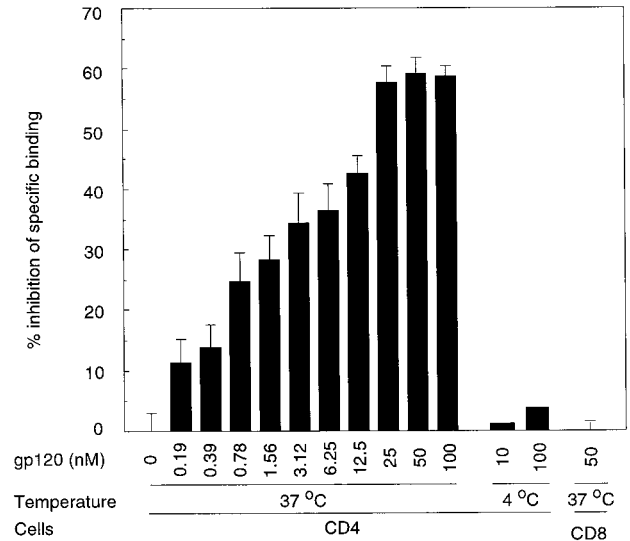


FIGURE 2. Effect of gp120 on T cell binding of ¹²⁵I-labeled SDF-1 α . CD4⁺ T lymphocytes were cultured with 2 μ M HA or medium alone at 37°C for 18 h and then incubated in triplicate with the indicated concentrations of gp120 MN for 60 min at 37°C. Binding assays with ¹²⁵I-labeled SDF-1 α were performed, and the percent inhibition of specific binding by gp120 MN was determined. One representative experiment of three performed is shown.

inhibitors (1 mM PMSF, 10 μ g/ml of aprotinin and leupeptin, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM sodium vanadate, and 1 mM benzamide) and subsequently incubated on ice for 15 min. Lysates were clarified by centrifugation ($12,000 \times g$) and preabsorbed with protein A-Sepharose, followed by the addition of rabbit anti-human p56^{lck} Ab (Upstate Biotechnology) and protein A-Sepharose. The mixture was incubated at 4°C for 1 h while under constant rotation followed by extensive washing with lysing buffer. The immune complexes were eluted from protein A-Sepharose with SDS-PAGE sample buffer at 95°C for 10 min. The immunoprecipitates were subjected to 10% SDS-PAGE, and proteins were transferred to a nitrocellulose membrane. Next, proteins were stained with mouse antiphosphotyrosine (4G10) or anti-lck mAbs followed by the addition of an HRP-conjugated secondary Ab. Immunoreactive proteins were detected by an enhanced chemiluminescence kit (Amersham, Arlington Heights, IL). For HA treatment, the cells were preincubated with HA (2 μ M) for 18 h, washed, and stimulated with gp120.

Phosphorylation of CXCR4

The phosphorylation of CXCR4 was examined by culturing CEM-SS cells in 1% FCS-DMEM with or without HA (2 μ M) for 18 h at 37°C. The cells (5×10^6) were washed twice with phosphate-free DMEM and incubated with 150 μ Ci of [³²P]orthophosphate (Amersham) for 90 min. Next, the cells were stimulated with gp120 or SDF-1 α at 37°C for 15 min and placed on ice. All subsequent procedures, unless otherwise stated, were conducted at 4°C. The cells were washed with PBS and lysed with 1 ml of lysing buffer containing 157 mM NaCl, 50 mM Tris (pH 8.0), 1.0% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 5 mM EDTA, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM PMSF, and 10 μ g/ml of aprotinin and leupeptin. Lysates were clarified by centrifugation ($12,000 \times g$) and preabsorbed with protein A-Sepharose, followed by the addition of an immunoprecipitating rabbit polyclonal anti-CXCR4 Ab (YG9-1009, Millennium Biotechnology) and protein A-Sepharose. The mixture was incubated for 1 h under constant rotation, followed by extensive washing with lysing buffer. The immune complexes were eluted from protein A-Sepharose with SDS-PAGE sample buffer (Novex, San Diego, CA) at 95°C for 10 min. The immunoprecipitates were subjected to 10% SDS-PAGE and autoradiography.

Statistical analysis

All experiments were performed two to five times, and the representative results are shown. The statistical significance of the difference between the testing and control groups was calculated using the Student's *t* test.

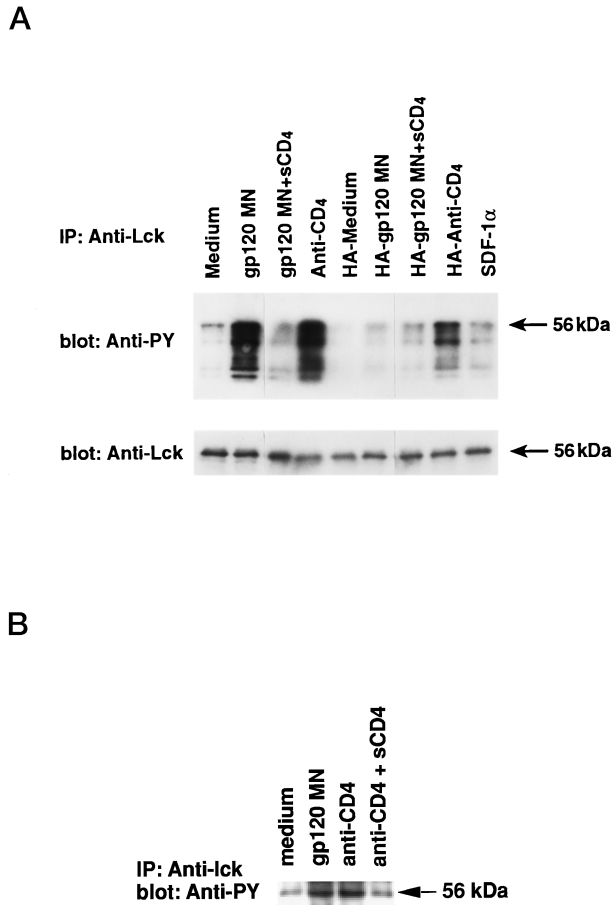


FIGURE 3. p56^{lck} phosphorylation induced by gp120. CD4⁺ CEM-SS cells were cultured with medium alone or HA (2 μ M) at 37°C for 18 h and subsequently stimulated with gp120 MN (50 nM), gp120 MN + soluble CD4 (100 nM), anti-CD4 (clone E9, 10 μ g/ml), or SDF-1 α (1 μ g/ml) at 37°C for 5 min. The cells were pelleted and lysed in lysing buffer. Tyrosine phosphorylation was examined by immunoprecipitation with anti-p56^{lck} followed by immunoblotting with antiphosphotyrosine Abs. *A*, Representative results obtained in three experiments. *B*, Anti-CD4 Ab was preincubated with soluble CD4 at 37°C for 60 min and subsequently tested for its effect on p56^{lck} tyrosine phosphorylation.

Results and Discussion

Because gp120 MN potently inhibited the cell response to chemoattractants including the ligand for CCR5 and CXCR4 in a CD4-dependent manner after preincubation with monocytes at 37°C for 1 h (14), we tested whether preincubation with gp120 could also affect the directional migration of T lymphocytes to SDF-1 α , the chemokine ligand for CXCR4. We initially used the CEM-SS cell line, which is derived from CD4⁺ T cells and has been widely used for the study of HIV-1 infection (17). Incubating CEM-SS cells with recombinant gp120 MN (50 nM) for 60 min at 37°C markedly reduced their chemotactic response to SDF-1 α (Fig. 1). The inhibition of CEM-SS cell migration by gp120 MN to SDF-1 α could be abrogated by preincubation of gp120 with soluble CD4 (Fig. 1), suggesting that soluble CD4 intercepts gp120 and prevents its binding to cell membrane-anchored CD4 and the subsequent interaction with CXCR4. gp120 of the monotropic strain CM did not interfere with CEM-SS cell migration in response to SDF-1 α (data not shown). Thus, the suppressive effect of gp120 on CXCR4 in CD4⁺ T cells appears to be restricted by the viral tropism.

We subsequently examined whether the inhibition by gp120 of CD4⁺ T cell migration in response to SDF-1 α was associated with

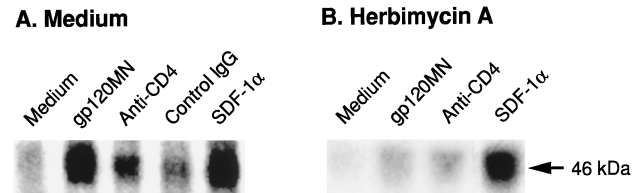


FIGURE 4. CXCR4 phosphorylation induced by gp120. CEM-SS cells were incubated at 37°C for 18 h in the absence (*A*) or presence (*B*) of 2 μ M HA. Next, the cells were washed and labeled with [³²P]orthophosphate followed by treatment with gp120, anti-CD4 Ab, or SDF-1 α for 15 min at 37°C. The cells were lysed, and immunoprecipitation was performed with anti-CXCR4 Ab followed by autoradiography. Two experiments yielded the same results.

the down-regulation of SDF-1 α binding sites on CD4⁺ T cells. Preincubating T cells with gp120 at 37°C for 60 min resulted in a significant reduction in the capacity of CEM-SS cells and CD4⁺ peripheral blood T cells to bind SDF-1 α (Fig. 2). In contrast, incubating CD8⁺ T cells with this gp120 did not affect their binding of SDF-1 α (Fig. 2), providing more evidence that the inhibition of SDF-1 α binding by gp120 MN is CD4-dependent.

In our experiments, preincubating CD4⁺ cells with gp120 MN at 4°C for \leq 4 h did not reduce the T cell binding of SDF-1 α (Fig. 2), suggesting that this gp120 did not directly compete with SDF-1 α for binding to the cells; rather, cell activation through CD4 might be required for gp120 to down-regulate the binding sites for SDF-1 α . Therefore, we investigated whether the initial interaction of gp120 with CD4 triggers cell signaling followed by CXCR4 down-regulation. The cytoplasmic domain of CD4⁺ is physically associated with p56^{lck} (18), a member of the src-tyrosine kinase family (19, 20), and HIV-1 gp120 has been reported to cross-link CD4, leading to tyrosine phosphorylation of p56^{lck} (21). We confirmed that the recombinant HIV-1 of the MN strain used in our study induced a rapid p56^{lck} tyrosine phosphorylation in CD4⁺ T cells, as shown in Fig. 3. An anti-CD4 mAb that was able to down-regulate CD4⁺ T cell binding of SDF-1 α (data not shown) similarly caused tyrosine phosphorylation of p56^{lck} in CD4⁺ T cells (Fig. 3A). Although this Ab has not been epitope-mapped, preincubation of the Ab with soluble CD4 abolished its effect on p56^{lck} tyrosine phosphorylation (Fig. 3B). Furthermore, the p56^{lck} phosphorylation induced by gp120 and anti-CD4 Ab was blocked by pretreating T cells with HA, a specific protein tyrosine kinase inhibitor. As expected, SDF-1 α did not induce tyrosine phosphorylation of p56^{lck} in CD4⁺ T cells (Fig. 3A). The tyrosine-phosphorylated species migrating faster than p56^{lck} are presumably phospho-proteins associated with activated p56^{lck} that could be recognized by antiphosphotyrosine Ab but not anti-p56^{lck} Ab. The precise nature of these protein species remains to be determined.

We further examined whether the tyrosine kinase activation caused by gp120 was required for CXCR4 down-regulation by analyzing phosphorylation and the surface expression of CXCR4 on CD4⁺ T cells after gp120 treatment. The CXCR4 phosphorylation that occurs on serine/threonine sites at the C terminus, which is typical for a seven-transmembrane G protein-coupled receptor (22), was measured by treating CD4⁺ CEM-SS cells with gp120 followed by immunoprecipitation and SDS-PAGE of the cell lysates. As shown in Fig. 4A, treating CEM-SS cells with both gp120 or SDF-1 α induced a specific phosphorylated protein species at a molecular mass of 46 kDa, immunoprecipitated by specific rabbit anti-CXCR4 Ab. This Ab could also precipitate a similar phosphoprotein of the same size in CXCR4-transfected CD4⁻/HEK293

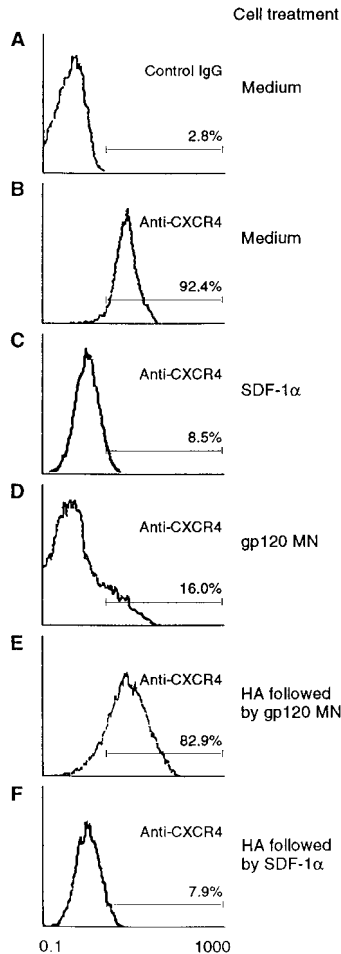


FIGURE 5. FACS analysis of CXCR4 expression on CD4⁺ lymphocytes pretreated with gp120 MN. Peripheral blood CD4⁺ lymphocytes were treated with HA (2 μM) or medium alone at 37°C for 18 h and subsequently incubated with medium alone (A and B), SDF-1α (1 μg/ml) (C), or gp120 MN (50 nM) (D and E) at 37°C for 60 min. After washing, cells were stained with control mouse IgG (A) or mAb to CXCR4 (12G5) (B–F), followed by FITC-conjugated goat anti-mouse IgG Ab. The percentage of cells positively stained with anti-CXCR4 Ab is shown in each panel. The data shown are from one experiment of three performed.

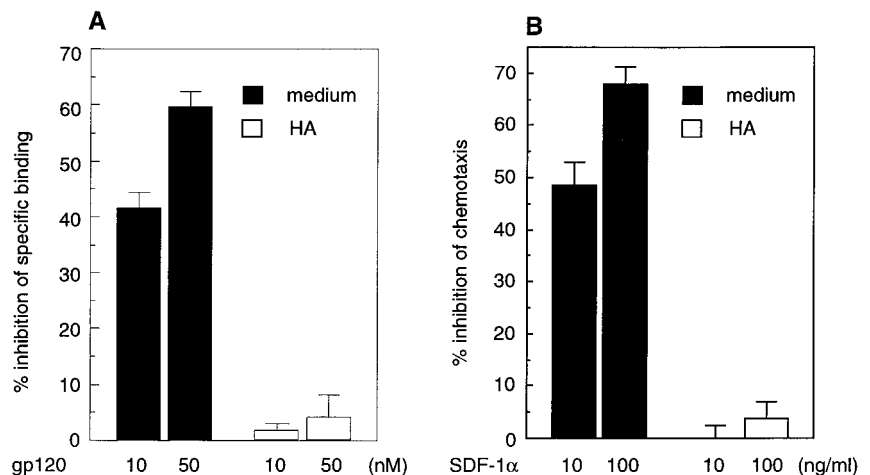
cells treated with SDF-1α but not with gp120, suggesting that this Ab specifically recognizes and precipitates CXCR4 (data not shown). In addition, gp120 but not SDF-1α failed to induce

CXCR4 phosphorylation in CEM-SS cells pretreated with HA (Fig. 4B). These results indicate that gp120-induced CXCR4 phosphorylation requires the involvement of tyrosine kinase activation. In contrast, the phosphorylation of CXCR4 caused by the native ligand SDF-1α did not require the activation of protein tyrosine kinase(s).

The phosphorylation of CXCR4 by gp120 was associated with a reduction of the cell surface CXCR4 as measured by FACS analysis with the anti-CXCR4 mAb 12G5, which has been widely used for the detection of CXCR4 expression in various reports (23). As shown in Fig. 5, 90% of the CD4⁺ T cells were CXCR4-positive (Fig. 5, A and B). The CXCR4 expression on the cell surface was significantly down-regulated by pretreatment of the cells with SDF-1α (Fig. 5C). In addition, pretreatment of the cells with gp120 MN at 37°C for 1 h markedly reduced the staining of CXCR4 on the cell membrane (Fig. 5D). T cells pretreated with HA were protected from the suppressive effect of gp120 MN and maintained the expression of CXCR4 on the cell surface at a level comparable with untreated cells (Fig. 5, B and E, 80% vs 90% positive cells). In contrast, the expression of CXCR4 on HA-treated CD4⁺ T cells was still markedly down-regulated by SDF-1α (Fig. 5F), suggesting that SDF-1α-mediated CXCR4 down-regulation does not involve tyrosine kinase activation as seen with gp120. In agreement with the protective effect of HA on gp120-induced p56^{lck} phosphorylation and CXCR4 down-regulation, CD4⁺ T cells treated with HA showed normal levels of SDF-1α binding sites and a functional chemotactic response compared with untreated cells (Fig. 6, A and B).

In this study, we showed for the first time that a gp120-mediated down-regulation of CXCR4 on CD4⁺ T cells requires the active participation of CD4. The activation of CD4-associated lck was reported to be responsible for the down-regulation of TCRs (24). Furthermore, cross-linking of CD4 by anti-CD4 Abs results in reduced expression of the α-chain of the IL-2R as well as in reduced transplantation rejection (25). We also observed that treatment of monocytes with gp120 similarly resulted in an inhibition of the expression and function of a variety of chemokine receptors as well as the receptor for the bacterial fMLP (14). The gp120-mediated down-regulation of chemokine receptors in monocytes was CD4-dependent and involved signaling events resembling the process of heterologous desensitization. In the case of monocytes, however, the suppressive effect of gp120 was reversible only by treatment of the cells with protein kinase C inhibitors such as staurosporine and calphostin C, but not by HA (14). It has been reported that HIV-1 gp120 and CD4/coreceptor-mediated cell

FIGURE 6. Effect of HA on binding and chemotaxis in response to SDF-1α of T cells treated with gp120. Human CD4⁺ T cells were preincubated with medium alone or HA for 18 h at 37°C. After washing, the cells were further treated with gp120 (strain MN) at 37°C for 1 h. Next, the cells were tested for their ability to bind ¹²⁵I-labeled SDF-1α (A) and to migrate in response to SDF-1α (B).



fusion and entry was resistant to pertussis toxin, suggesting that the virus competes for chemokine receptor occupancy without activating G protein signals (9, 10). Nevertheless, it has also been reported that HIV-1 envelope induced both tyrosine kinase- and G protein-mediated signaling in CD4/coreceptor-expressing cells (13, 26). Our present observation clearly indicates that gp120-induced CXCR4 down-regulation and phosphorylation in T cells is dependent upon CD4-mediated tyrosine kinase activation. It should be noted that although the gp120 MN could down-regulate the expression and function of CCR5 in studies with monocytes (14), this gp120 MN did not affect an already low level of expression of CCR5 on resting peripheral blood CD4⁺ T lymphocytes, suggesting that the effect of gp120 MN on CD4⁺ T cells is CXCR4 specific.

The pathophysiological relevance of this CD4-mediated down-regulation of CXCR4 needs further clarification. The demonstration in another study (14) that anti-CD4 Ab mimics this effect of gp120 suggests that these HIV-1 envelope proteins have subverted a CD4-dependent down-regulatory pathway. A negative regulatory role for CD4 is supported by reports showing the immunosuppressive effects of nondepleting anti-CD4 Abs (25). Furthermore, IL-16, a lymphokine that uses CD4 as a functional receptor for its chemotactic and calcium-mobilizing activity (27), is a potent inhibitor of mixed T lymphocyte reactions (27, 28). Thus, the interaction of CD4 with a number of other cell surface receptors has inhibitory consequences. This suppressive effect in our studies requires a period of preincubation and operationally resembles heterologous desensitization. Because CD4 is structurally unrelated to other receptors, perhaps this process should be termed "trans-deactivation" rather than desensitization. A similar trans-deactivation of CXCR4 was reported (29) after activation of TCR in a T cell line, which involved the activation of multiple intracellular molecules, including the tyrosine kinase lck. Thus, the scope of the cross-talk between CXCR4 and other cellular receptors may be broader than expected and may be important in the regulation of orchestrated immune responses.

Considerable quantities of soluble gp120 have been detected in HIV-1-infected patients (30) before the production of neutralizing Abs. By down-regulating chemokine coreceptors, these shed gp120 molecules could potentially interfere with subsequent HIV-1 entry, but may also disable host defenses by interfering with the mobilization of CD4⁺ T cells and monocytes in response to chemoattractants. These suppressive effects of gp120 may provide a molecular model for the design of antiinflammatory therapeutic agents.

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