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Optimal Inhibition of X4 HIV Isolates by the CXC Chemokine Stromal Cell-derived Factor 1 α Requires Interaction with Cell Surface Heparan Sulfate Proteoglycans*

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Agustín Valenzuela-Fernández^{‡§¶}, Tania Palanche^{§¶*}, Ali Amara^{‡ §§}, Aude Magerus[‡], Ralf Altmeyer[‡], Thierry Delaunay^{§§}, Jean-Louis Virelizier[‡], Françoise Baleux^{¶¶}, Jean-Luc Galzi^{||}, and Fernando Arenzana-Seisdedos^{‡||}

From the [‡]Unité d'Immunologie Virale and ^{¶¶}Unité de Chimie Organique, Institut Pasteur, 28 Rue du Dr. Roux, 75724 Paris Cedex 15, France, the ^{§§}Département des Récepteurs et des Protéines Membranaires, Ecole Supérieure de Biotechnologie de Strasbourg, CNRS UPR 9050, Boulevard Sébastien Brant, 67400 Illkirch, France, and the ^{||}Institut National de la Recherche Agronomique, Station de Pathologie Végétale, BP81, 33883 Villeneuve d'Ornon, France

The chemokine stromal cell-derived factor 1 (SDF-1) is the natural ligand for CXC chemokine receptor 4 (CXCR4). SDF-1 inhibits infection of CD4⁺ cells by X4 (CXCR4-dependent) human immunodeficiency virus (HIV) strains. We previously showed that SDF-1 α interacts specifically with heparin or heparan sulfates (HSs). Herein, we delimited the boundaries of the HS-binding domain located in the first β -strand of SDF-1 α as the critical residues. We also provide evidence that binding to cell surface heparan sulfate proteoglycans (HSPGs) determines the capacity of SDF-1 α to prevent the fusogenic activity of HIV-1 X4 isolates in leukocytes. Indeed, SDF-1 α mutants lacking the capacity to interact with HSPGs showed a substantially reduced capacity to prevent cell-to-cell fusion mediated by X4 HIV envelope glycoproteins. Moreover, the enzymatic removal of cell surface HS diminishes the HIV-inhibitory capacity of the chemokine to the levels shown by the HS-binding-disabled mutant counterparts. The mechanisms underlying the optimal HIV-inhibitory activity of SDF-1 α when attached to HSPGs were investigated. Combining fluorescence resonance energy transfer and laser confocal microscopy, we demonstrate the concomitant binding of SDF-1 α to CXCR4 and HSPGs at the cell membrane. Using FRET between a Texas Red-labeled SDF-1 α and an enhanced green fluorescent protein-tagged CXCR4, we show that binding of SDF-1 α to cell surface HSPGs modifies neither the kinetics of occupancy nor activation in real time of CXCR4 by the chemokine. Moreover, attachment to HSPGs does not modify the potency of the chemokine to promote internalization of CXCR4. Attachment to cellular HSPGs may co-operate in the optimal anti-HIV activity of SDF-1 α by increasing the local concentration of the chemokine in the surrounding environment of CXCR4, thus facilitating

sustained occupancy and down-regulation of the HIV coreceptor.

The CXC chemokine stromal cell-derived factor 1 (SDF-1)¹ stimulates intracellular calcium flux and chemotaxis in monocytes, T lymphocytes, and neutrophils (1, 2), a characteristic shared with other chemokines. However, SDF-1, which is expressed in a broad range of tissues (3–5), exhibits biological properties that make it an original chemokine among members of CC and CXC families. Thus, SDF-1 plays an essential role during embryonic life, regulating organogenesis (neuronal patterning and cardiovascular development) and development of the hematopoietic system, namely B lymphopoiesis (6, 7). Three different SDF-1 products (α , β , and γ) generated by alternative splicing of a single gene have been identified (3, 8). SDF-1 is the only known ligand for CXCR4, a ubiquitously expressed member of the family of G-protein-coupled chemokine receptors (9). The interaction between SDF-1 and CXCR4 appears to be unique and nonpromiscuous, as suggested by the shared phenotype of defects shown by mice carrying either SDF-1- or CXCR4-knockout genes (7, 10, 11). Apart from these physiological functions, SDF-1 has the unique and selective capacity to inhibit entry of human immunodeficiency viruses (HIVs) that use CXCR4 as coreceptor into CD4 T lymphocytes, monocytes, and dendritic cells (1, 2, 12).

HIV enters cells through sequential interaction of the viral envelope (Env) glycoprotein with the cell surface CD4 molecule and either CXCR4 or CCR5, another G-protein-coupled chemokine receptor (13). HIV strains that infect macrophages and primary T cells use CCR5 (R5 viruses), whereas HIV strains that infect transformed CD4⁺ cell lines and primary T cells use CXCR4 (X4 virus). X4 HIV isolates, previously known as T lymphotropic, are characteristic of the late phases of infection and are frequently associated with a sharp decline on CD4 T cell counting and worsening of the clinical status (14, 15).

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|| To whom correspondence should be addressed. Tel.: 33-1-45688263; Fax: 33-1-45688941; E-mail: farenzana@pasteur.fr.

¹ The abbreviations used are: SDF, stromal cell-derived factor; GAG, glycosaminoglycan; HS, heparan sulfate; HSPG, HS proteoglycan; CXCR, CXC chemokine receptor; RANTES, regulated on activation normal T cell expressed and secreted; HIV-1, human immunodeficiency virus type 1; PBS, phosphate-buffered saline; PBMC, peripheral blood mononuclear cells; FRET, fluorescence resonance energy transfer; β -gal, β -galactosidase; CMTMR, 5- (and 6)-[[4-(chloromethyl)benzoyl]-amino] tetramethylrhodamine; CS, chondroitin sulfate; FCS, fetal calf serum; LTR, long terminal repeat; EGFP, enhanced green fluorescent protein; TR, Texas Red; HEK, human embryo kidney; cps, counts per second.

SDF-1 does not interfere with the primary binding of the surface subunit (gp120) of the X4 HIV-Env glycoprotein to CD4. Binding to CD4 promotes conformational changes of gp120 allowing subsequent interaction of the reorganized HIV-Env with CXCR4. Such interaction is thought to lead to exposure of the fusion domain of the HIV-Env transmembrane subunit (gp41), which eventually triggers fusion of CD4⁺/CXCR4⁺ cell targets with either cell-free HIV or HIV-infected cells (16–18). Occupancy of CXCR4 by SDF-1 prevents the interaction of X4 HIV Env with CXCR4, thus blocking the activation of the fusogenic capacity of gp41 and, ultimately, the entry of virions into the cell.

The biological functions of chemokines are thought to be influenced by their association with cellular or matrix extracellular glycosaminoglycans (GAGs). Usually attached to a core protein to form proteoglycans (19), the common GAGs (heparin, heparan sulfate (HS), dermatan sulfate, and chondroitin sulfate (CS)) are highly sulfated oligosaccharides. With the exception of CS, all of the above-mentioned GAGs are characterized by a high degree of structural heterogeneity (20, 21). Another common GAG is hyaluronic acid, but it is never attached to a core protein and is not a GAG sulfate (19).

We showed previously that SDF-1 α , the best characterized isoform of the chemokine, interacts selectively with HS, with relatively high affinity *in vitro* (22). HSs are also responsible for the binding of SDF-1 α to the membrane of CXCR4-negative epithelial or endothelial cells (22), which have been identified among the few cell types producing the chemokine in human tissues (4, 5). A cluster of basic residues (Lys²⁴, His²⁵, and Lys²⁷) in the first β -strand of SDF-1 α is necessary for the interaction of SDF-1 α with HS both *in vitro* and in intact cells. Indeed, a SDF-1 α derivative carrying three Ser substitutions in the Lys²⁴, His²⁵, Lys²⁷ cluster (SDF-1 3/6) is unable to bind HS. Inactivation of the putative HS-binding site does not affect the capacity of SDF-1 α to ligate CXCR4, suggesting that cellular HS proteoglycans (HSPGs) are dispensable for the HIV inhibitory effect of the chemokine. However, the contribution of cellular GAGs to the optimal, inhibitory activity of the CC chemokines RANTES or macrophage inflammatory protein 1 β against HIV R5 is supported by an increasing body of evidence. Surprisingly, in some cases, the optimal inhibition of HIV-1 infection shown by RANTES-HS complexes contrasts with the impairment of the agonistic capacity of the complex as compared with the chemokine alone (23). Consequently, it can be hypothesized that despite the apparent preserved functionality of HS-binding disabled SDF-1 α mutants, complexes of SDF-1 α with cellular HSPGs may also be determinant for maintaining the optimal capacity of the chemokine to prevent entry of X4 HIV isolates.

Analysis of the kinetics of SDF-1 α /CXCR4 interactions investigated by fluorescence resonance energy transfer (FRET) and real time measurement of CXCR4 activation indicates that complexing with HS does not modify the intrinsic capacities of SDF-1 α to interact with its cognate receptor. However, our findings indicate that selective interaction of SDF-1 α with cell membrane HS is required for optimal inhibition of CXCR4 envelope-mediated cell-HIV fusion. Neither SDF-1 α induced endocytosis nor recycling of CXCR4 is influenced by cell surface HSPGs. However, combining FRET and laser scanning confocal microscopy, we demonstrate the accumulation of large amounts of SDF-1 α at the cell surface, which is largely independent of CXCR4 and relies on the presence of HS. We propose that attachment of SDF-1 α to HSPGs increases the local concentration of the chemokine in the surrounding of CXCR4 thus facilitating sustained occupancy and down-regulation of the HIV coreceptor. SDF-1 α bound to cell membrane HS may

be a major constituent of a natural barrier limiting transmission and propagation of X4 HIV isolates *in vivo*.

MATERIALS AND METHODS

Reagents and Antibodies

Soluble HS was obtained from Sigma (catalogue number H5393). Heparitinase I (EC 4.2.2.8) and chondroitinase ABC (EC 4.2.2.4) were purchased from Seikagaku Corp. K15C is an anti-SDF-1 monoclonal antibody (IgG2a κ) against the amino-terminal region of SDF-1 (22). Q4120, an anti-CD4 monoclonal antibody (IgG1) that blocks the binding of gp120 to CD4, was obtained from the Medical Research Council AIDS Reagent Project. ¹²⁵I-SDF-1 α (specific activity, 2200 Ci/mmol) was purchased from PerkinElmer Life Sciences. Fluorescent calcein-AM, Indo-1/AM, and 5- (and 6)-[[4-chloromethyl]benzoyl]-amino tetramethylrhodamine (CMTMR) probes were obtained from Molecular Probes.

Cells

The HeLa P4.2 cell clone is stably transfected with a human CD4 cDNA and a HIV-LTR-driven β -gal reporter gene (24). HeLa 243 cells, provided by Dr. M. Alizon, co-express both Tat and Env HIV-1 proteins from an HIV-LTR-driven vector derived from the X4 pLai proviral molecular clone (25). Both cell cultures were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal calf serum (FCS), Glutamax, antibiotics (100 units/ml penicillin and 100 μ g/ml streptomycin, and 2 μ M of Methotrexate. Baby hamster kidney cells (BHK-21) (ATCC CCL-10) were maintained in Glasgow's modified Eagle's medium (Life Technologies, Inc.) containing 5% FCS, 20 mM Hepes, and 10% tryptose phosphate broth. To express HIV-Env Lai BHK-21, cell monolayers were infected with a defective Semliki Forest virus encoding a full-length sequence of the HIV-1 LAI *env* gene at multiplicity of infection of 10 for 1 h. Human peripheral blood mononuclear cells (PBMC) were isolated from healthy donors using Ficoll (Amersham Pharmacia Biotech) density gradient centrifugation. PBMC were cultured in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% heat-inactivated FCS. PBMC were activated during 3 days with 1 μ g/ml phytohemagglutinin (Murex Diagnostics S. A.). Adherent human embryo kidney (HEK) 293 cells were grown in minimum essential medium supplemented with 10% FCS.

Chemokine Synthesis

Wild type SDF-1 α , SDF-1 3/6 (K24S/H25S/K27S), SDF-1 2/6 (K24S/K27S), and SDF-1 α P2G were synthesized by the Merrifield solid phase method on a fully automated peptide synthesizer (Pioneer, Perspective Biosystems, and PerkinElmer Life Sciences). The procedure used for chemokine synthesis we described previously (22). The concentration of each chemokine was determined by amino acid analysis on a 6300 Beckman amino acid analyzer after hydrolysis for 20 h in 6 N HCl, 0.2% phenol in the presence of a known amount of norleucine as internal standard. All chemicals for the synthesis were purchased from Perspective Biosystems and PerkinElmer Life Sciences.

Binding of Chemokines to CXCR4

CEM cells (2.5×10^6 cells/ml) were incubated in PBS with 0.25 nM iodinated SDF-1 α (PerkinElmer Life Sciences, specific activity, 2200 Ci/mmol) and various concentrations of unlabeled SDF-1 α or SDF-1 2/6 for 1 h at 4 °C in a final volume of 300 μ l. Incubations were terminated by centrifugation at 4 °C. The cell pellets were washed twice in ice-cold PBS. Nonspecific binding was determined in the presence of 1 μ M unlabeled SDF-1 α . Cell pellet-associated radioactivity was counted using an LKB-Wallac microcomputer controlled 1272 ClineGamma counter. The binding data were analyzed using GraphPrad Prism 2.0 software.

Flow Cytometric Analysis of SDF-1 α , SDF-1 3/6, and SDF-1 2/6 Binding to Cells

Adherent cells were plated 2 days before binding experiments. Cells were detached with 2 mM EDTA in PBS and washed twice with ice-cold binding buffer (RPMI 1640, 20 mM Hepes, 1% bovine serum albumin). 4×10^5 cells were incubated in the presence of the indicated concentration of chemokines in a total volume of 200 μ l for 90 min at 4 °C with stirring. Unbound chemokine was removed by washing with binding buffer, and cell-bound SDF-1 α was detected by incubation with the anti-SDF-1 monoclonal antibody K15C (15 μ g/ml, diluted in PBS, 1% bovine serum albumin), which recognizes an epitope encompassing the amino-terminal end of the chemokine. After staining with phycoerythrin-conjugated anti-mouse immunoglobulins (Southern Biotech-

nology), cells were fixed in 1% formaldehyde buffer and analyzed by flow cytometry in a FACSCalibur (Becton Dickinson).

HIV-1 Env-mediated Cell-to-cell Fusion

β -Galactosidase Assay—HeLa 243 cells were co-incubated with HeLa P4.2 cells in a total volume of 250 μ l in 96-well plates at a 1:1 ratio during 18 h. After 18 h of co-culture, cells were washed twice with PBS at 37 °C and lysed with 50 μ l of lysis buffer (from Roche β -gal reporter gene assay kit) for 20 min, and the enzymatic activity was evaluated according to the instructions provided by the manufacturer (β -gal reporter gene assay (chemiluminescent), Roche Diagnostics).

Double Fluorescence Assay—This technique was modified from a procedure described previously by Puri *et al.* (26) and will be described in detail elsewhere. Briefly, BHK-21 expressing HIV-1 Lai Env and phytohemagglutinin-activated PBMC were loaded with aqueous calcein-AM and CMTMR, respectively. After for 15 h in culture, cell syncytia labeled with the two cytoplasmic dyes were analyzed by flow cytometry. When indicated, enzymatic cleavage of cell surface GAG was obtained by pretreating cells for 30 min before the onset of the culture with 5 milliunits/ml of either heparitinase I or chondroitinase ABC. GAG lytic enzymes were maintained in the cell cultures until the end of the experiment.

Intracellular Calcium Release Responses

HeLa P4.2 cells were incubated culture medium containing 5 μ M Indo-1/AM for 30 min at 37 °C followed by a 15-min incubation in probe-free medium. Measurements were made at 37 °C in Hepes buffer (5.6 mM glucose, 10 mM Hepes, 0.4 mM NaH_2PO_4 , 137.5 mM NaCl, 1.25 mM MgCl_2 , 1.25 mM CaCl_2 , 6 mM KCl, and 1% bovine serum albumin) supplemented with protease inhibitors (40 μ g/ml bestatin, 40 μ g/ml bacitracin, 50 μ g/ml chymostatin, 20 μ g/ml phosphoramidon, and 1 μ g/ml leupeptin). Fluorescence emission was detected at 405 and 475 nm (excitation, 338 nm). Real time intracellular calcium measurements from cell suspensions were made on a Fluorolog (SPEX) spectrofluorometer equipped with a 450-W xenon lamp, a double grating excitation monochromator, and two single grating emission monochromators. Slits were set to 4 or 6 mm, yielding bandwidths of 7.2 or 10.8 nm at excitation and 14.4 and 21.6 nm at emission, respectively. Data were acquired with two photon counting photomultipliers. Real time measurements were carried out with an Applied Photophysics SK1E rapid mixing apparatus modified for mixing with living cells (final concentration, 10^6 cells/ml). The observation chamber was an Hellma 176.002 (100 μ l) quartz circulation cuvette placed on the cuvette holder of the SPEX fluorolog spectrofluorometer. At a typical 12 bars air ram pressure, the flow rate was 4 ± 0.5 ml/s. Each measurement started after renewal of 7–8 times the volume of the observation cuvette. The dead volume (100 μ l) and dead time of the apparatus (25 ± 5 ms) were experimentally determined. Time-based recordings were typically sampled every 20–100 ms.

Actin Polymerization

Actin polymerization was tested as described by Bleul *et al.* (27). Activated PBMC were incubated in RPMI medium supplemented with 10% heat-inactivated FCS, Glutamax, and antibiotics (100 units/ml penicillin and 100 μ g/ml streptomycin) at 37 °C and induced with the chemokines as indicated. Forty-five s after induction, 100 μ l of a stop solution containing 4×10^{-7} M FITC-labeled phalloidin, 0.5 mg/ml 1- α -lysophosphatidylcholine (Sigma), and 15% formaldehyde in PBS were added to PBMC (in 400 μ l). Analysis of FITC-phalloidin-labeled cells was performed by flow cytometry.

Kinetic Constants of SDF-1 α /CXCR4 Interactions

Detection and quantification of association and dissociation kinetics of SDF-1 α /CXCR4 interaction were measured by FRET, between enhanced green fluorescent protein (EGFP) and Texas Red (TR) chromophores on living cells. To this purpose, we engineered an EGFP-CXCR4 chimeric protein by fusing EGFP to the extracellular, amino-terminal end of CXCR4 as described (28) and synthesized a SDF-1 α derivative carrying a single TR molecule bound to Lys⁶⁸. A clone of HEK 293 cells stably expressing EGFP-CXCR4 was isolated. Upon excitation of EGFP-CXCR4 at 460–470 nm wavelength, association of the fluorescent ligand with the receptor was measured by monitoring the reduction of EGFP emission at 510 nm as described by Vollmer *et al.* (28). Similarly, dissociation was measured as the increase of EGFP emission at 510 nm, when bound SDF-1 α -TR was displaced by an excess of unlabeled SDF-1 α . Real time fluorescence data (in counts per second (cps)) were stored using the DM3000 software provided with the flu-

orolog 2 spectrofluorometer and analyzed with Kaleidagraph (Synergy Software) by various analytical expressions, such as exponentials, sum of exponentials, or sum of exponentials and linear relationships (29, 30). The uniqueness of the fit was checked by repeated calculations performed with distinct experimental data points (from several experiments) or with distinct values for initiation of the fitting procedure.

For a bimolecular reaction scheme,



describing ligand-receptor interactions, fluorescence traces were analyzed using the following relationship,

$$y = \lambda \exp(-k_{\text{app}} \times t) \quad (\text{Eq. 2})$$

where λ is the amplitude of the exponential fluorescence decline, t is the recording time, and k_{app} is the apparent rate constant of the reaction. The apparent rate constant k_{app} is equal to $k_{\text{app}} = k_1 \times L + k_{-1}$, where L is the concentration of ligand, k_1 is the forward rate constant, and k_{-1} is the backward rate constant of Equation 1.

Dynamic Confocal Microscopy

HEK 293 cells expressing EGFP-CXCR4 were grown on glass coverslips and incubated in Ringer solution (140 mM NaCl, 5 mM KCl, 2 mM MgCl_2 , 2 mM CaCl_2 , 10 mM Hepes, 11 mM glucose, pH 7.3) at 19 °C to avoid CXCR4 endocytosis. Images were taken on an inverted microscope (Nikon Eclipse TE300) equipped with a confocal imaging system (Bio-Rad MRC 1024 ES) using a Plan Apo $\times 40$ oil immersion objective (Nikon). Excitation light (488 nm) was obtained using 1 or 3% power from a 30-mW krypton-argon laser. Emitted fluorescence was recorded at 522 ± 5 and 605 ± 22 nm and was color-coded (256 gray levels). Images were acquired from a section located 3–6 μ m above the glass coverslip at 2–4 Hz and analyzed using Bio-Rad time course software. Ligands were applied in the bath using a perfusion pipette.

RESULTS AND DISCUSSION

We have previously identified a cluster of basic residues (Lys²⁴, His²⁵, and Lys²⁷) in the first β -strand of SDF-1 α necessary for interaction with HS, both *in vitro* and in intact cells (22). Indeed, a mutant SDF-1 α carrying the substitutions K24S/H25S/K27S (SDF-1 3/6) selectively failed to bind HS. To further delimit the boundaries of the HS-binding site, new SDF-1 α mutants were engineered by replacing each amino acid of the basic cluster with Ser. Binding of SDF-1 α derivatives to cell surface HS was assessed using an anti-SDF-1 α monoclonal antibody (K15C) that recognizes an epitope in the amino-terminal end of the chemokine. Each single-point mutant (K24S, H25S, or K27S) SDF-1 α induced CXCR4-dependent actin polymerization and bound to cell surface HS with efficiency and affinity comparable to wild type SDF-1 α (data not shown). In contrast, a mutant combining K24S/K27S substitutions (SDF-1 2/6) failed, like SDF-1 3/6, to bind to cell surface HS at concentrations up to 1 μ M (Fig. 1b). Gel chromatography and plasmon resonance spectroscopy confirmed that SDF-1 2/6 lost, to the same extent as SDF-1 3/6, the affinity for immobilized heparin. Like SDF-1 3/6, the SDF-1 2/6 mutant bound (Fig. 1a) and induced CXCR4 activation with affinity and efficiency comparable to the wild type counterpart (Fig. 1c). These results reinforce our conviction (22) that amino acid substitutions inactivating the putative HS-binding site of SDF-1 α preserve the overall three-dimensional conformation of the protein. Moreover, our findings confirm that the HS-binding site on SDF-1 α is indeed outside the amino-terminal domain involved in occupancy and signaling of CXCR4. However, they do not exclude the possibility that other residues, such as Arg⁴¹ and Lys⁴³, contribute to forming an accessible, positively charged surface for interaction with HS. In any case, Lys²⁴ and Lys²⁷ appear to be the critical residues, which either make physical contact with or critically shape the surface for attachment of HS.

The conserved ability of HS-binding-disabled derivatives of SDF-1 α to bind and activate CXCR4 prompted us to investigate

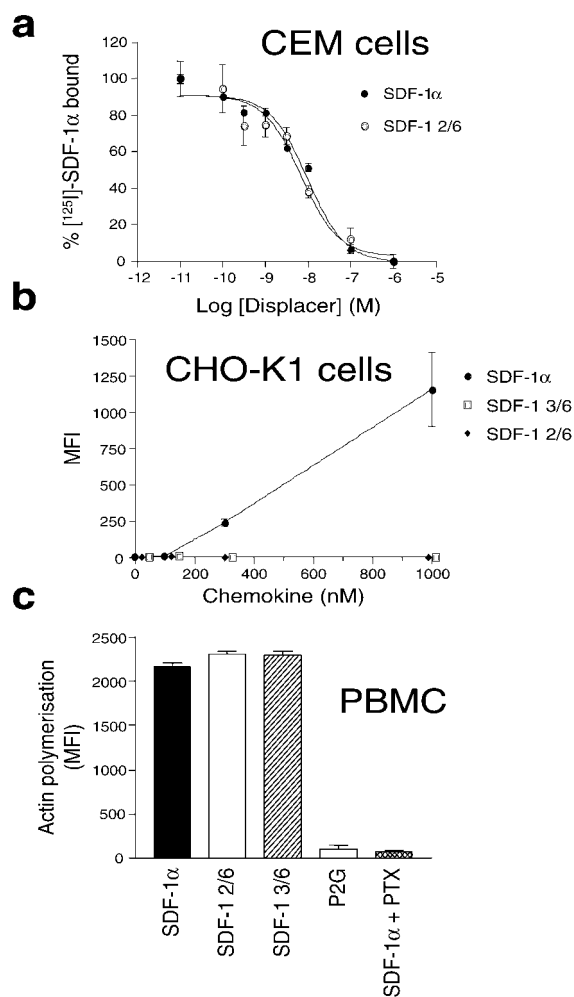


FIG. 1. Functional characteristics of wild type and HS-binding disabled SDF-1 α . *a*, binding of 125 I-labeled SDF-1 α (0.25 nM) to CXCR4+ CEM cells (2×10^6 cells/ml) was competed with either cold SDF-1 α or SDF-1 2/6. Results (mean \pm S.E.) are representative of six different experiments. Total specific binding was 183,265 cpm/mg of protein for SDF-1 α and 174,234 cpm/mg of protein for SDF-1 2/6. Scatchard analysis of the competition curves yielded linear plots with similar K_D values as follows: SDF-1 α , 1.38 ± 0.47 nM; SDF-1 2/6, 2.36 ± 0.102 nM. *b*, flow cytometric analysis of SDF-1 α or SDF-1 2/6 binding on CHO-K1 cells lacking CXCR4 expression. Cells were incubated with the either SDF-1 α or SDF-1 2/6 and with SDF-1 3/6 mutant as a negative control of chemokine binding to cellular HS. After removal of unbound chemokines, cells were labeled with the K15C monoclonal antibody and revealed by a secondary, anti-mouse FITC-labeled antibody. *c*, quantification of specific chemokine-induced actin polymerization in activated PBMC. Human PBMC were challenged with 3 nM of each chemokine (SDF-1 α , SDF-1 2/6, and SDF-1 3/6) for 45 s. Cell cultures were simultaneously fixed and labeled with FITC-labeled phalloidin, which selectively binds to polymerized forms of actin (F-actin). Mean fluorescence intensity (MFI) was calculated by deducing values from the control, unstimulated cell cultures. P2G (200 nM) is a SDF-1 α -derived (P2G), inactive mutant unable to elicit CXCR4-dependent cell activation (31). Pertussis toxin (PTX) (100 ng/ml), a selective inhibitor of G α_i proteins, was used to block induction by 100 nM SDF-1 α .

whether attachment of SDF-1 α to cell surface HSPGs contributes to the HIV inhibitory activity of the chemokine. For this purpose, an HIV-Env-mediated cell-cell fusion assay was performed by culturing HeLa CD4+ with HeLa cells co-expressing Tat and Env, both from the X4 HIV molecular clone pNL4-3. In fused cells, Tat activates transcription of an HIV-LTR-driven β -gal reporter gene integrated into the HeLa CD4+ cells, thus allowing quantification of the Env-mediated cell fusion. Assessment of X4 HIV-Env-mediated cell fusion was preferred to quantification of p24Gag HIV produced during viral replication

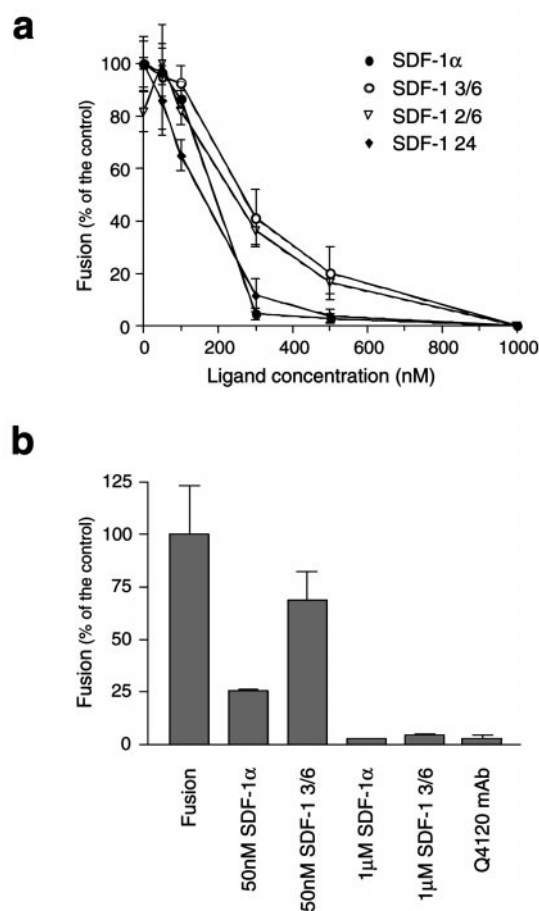


FIG. 2. Comparative analysis of the capacity of SDF-1 α and SDF-1 3/6 and 2/6 mutants to prevent the fusogenic activity of X4 HIV Env. *a*, cell-to-cell fusion experiments were performed by co-culturing HeLa CD4+ cells carrying an integrated HIV-LTR-driven β -gal reporter gene, with HeLa 243 cells co-expressing both the *tat* and *env* genes from the pLai X4 HIV-1 proviral molecular clone. After fusion, Tat protein induces in *trans* the transcription of an HIV-LTR-driven β -gal reporter gene. The enzymatic activity detected in the cell lysates reflects the magnitude of the Env-mediated cell fusion. *b*, cell-to-cell fusion experiments using CXCR4+ activated human PBMC against BHK-21 cells expressing the Env of the X4 HIV-1 Lai isolate. PBMC and BHK-21 cells were loaded with CMTMR and aqueous calcein-AM chromophores, respectively, and cell fusion was quantified by detection of cells simultaneously carrying both markers. All data are mean \pm S.E. of triplicates. Results from one experiment out of three are shown.

to evaluate the anti-HIV capacity of cell membrane-bound SDF-1 α ·HS complex. The fusion assay reflects directly and exclusively the interaction of the viral envelope with the complex of cellular receptors, permits rapid and accurate quantification of the phenomenon, and avoids problems related to the prolonged time of cell culture, such as the viability of the cells or the stability of the chemokine. Wild type SDF-1 α and SDF-1 24 (K24S) mutant, which have similar affinities for CXCR4 or HS, displayed equal capacity to inhibit Env-mediated cell fusion. Remarkably, the HS-binding SDF-1 α proteins proved to be more efficient than inactive SDF-1 2/6 or 3/6 mutants in preventing Env-CXCR4 interactions and subsequent induction of cell-cell fusion (Fig. 2*a*). Similarly, HS-binding SDF-1 α molecules showed greater efficacy in blocking HIV-Env-mediated cell fusion in an assay in which primary blood leukocytes were used as fusion partners with BHK-21 cells expressing an HIV (Lai isolate) X4 Env (Fig. 2*b*). The superior efficiency of HS-binding SDF-1 α molecules to prevent HIV Env-mediated cell fusion was manifest at nonsaturating concentrations (Fig. 2), and the difference between active and inactive HS-binding

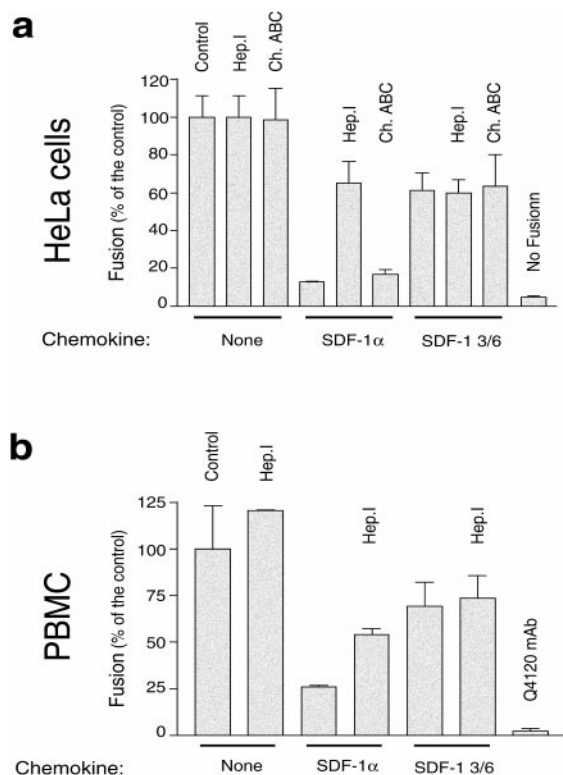


FIG. 3. Role of cell surface HS-proteoglycans in the SDF-1 α inhibition of the fusogenic capacity mediated by HIV X4 Env. Cell fusion assays were performed and assessed as described in Fig. 2, using as targets HeLa cells (a) or PBMC (b) fused to HeLa P4.2 or BHK-21 cells (both expressing X4 HIV Env), respectively. SDF-1 α and derivatives were used at 300 nM. Selective cleavage of either cell surface HS or CS was performed by enzymatic treatment with heparitinase I (*Hep.I*) (5 milliunits/ml) or chondroitinase ABC (*Ch. ABC*) (5 milliunits/ml). The antifusogenic effect of SDF-1 α was not impaired by exposure to concentrations of chondroitinase ABC of 100 milliunits/ml. The monoclonal antibody Q4120 (1 μ g/ml) prevents binding of HIV-1 to CD4 and therefore blocks HIV Env-mediated cell fusion.

SDF-1 α derivatives diminished as concentrations increased to reach CXCR4-saturating levels (Fig. 2a).

Further evidence supporting the contribution of HS-SDF-1 α interactions to the optimal HIV-inhibitory capacity of the chemokine was obtained from experiments in which HS was removed from plasma membrane proteoglycans by enzymatic cleavage. Indeed, exposure to heparitinase I substantially reduced the capacity of wild type SDF-1 α to inhibit HIV-Env-mediated cell fusion to the levels of the SDF-1 3/6 mutant (Fig. 3). In contrast, and as expected, the anti-HIV activity of SDF-1 3/6 was not affected by the enzymatic cleavage of cell surface HS. Chondroitinase ABC failed to promote any change in the activity of either wild type SDF-1 α or the 3/6 mutant, thus further proving the selective involvement of HS in the anchoring of the chemokine (Fig. 3a).

The enhanced capacity of wild type SDF-1 α , as compared with mutants unable to bind cell surface HS, to prevent HIV-Env-mediated cell fusion may be accounted for by activation of one or more mechanisms that we decided to explore systematically.

The first mechanism relies on the interaction of either CXCR4 or SDF-1 α with the amino-terminal and second extracellular loop domains of CXCR4 (31–35). Thus, inhibition of HIV infection critically depends on the capacity of SDF-1 α to occupy CXCR4 and impede the ligation of the HIV coreceptor by X4 envelopes by steric hindrance.

Previous studies from our laboratory showed that when an-

alyzed at equilibrium, the affinity constant (K_d) of SDF-1 α /CXCR4 binding is not modified by binding of the chemokine to HS (22). However, the formation of a complex with HS could modify the kinetics of SDF-1 α binding to CXCR4 and thereby the capacity to interfere with the HIV-Env. The inability to bind HS or the removal of HS from cell surfaces may result in either a slower rate of association to or a faster dissociation of SDF-1 α from CXCR4. This may in turn account for the reduced capacity to prevent HIV-Env-mediated cell fusion shown by low concentrations of the chemokine when either HS-disabled SDF-1 α mutants were used or binding to cell surface HS was precluded.

Kinetics of SDF-1 α /CXCR4 association were assessed by measuring in real time FRET between TR-labeled SDF-1 α and a EGFP-CXCR4 chimeric molecule expressed in living HEK 293 cells (Fig. 4). Both the fluorescent receptor and the fluorescent chemokine are functionally active as (i) the EGFP-CXCR4 receptor mediates intracellular responses and internalizes as a consequence of its interaction with agonists, (ii) the EGFP-CXCR4 receptor is a functional coreceptor for cell fusion-based assays, and (iii) SDF-1 α -TR is an agonist of both CXCR4 and EGFP-CXCR4 receptors that also leads to receptor internalization (data not shown).

Upon ligation of EGFP-CXCR4 by SDF-1 α -TR, reduction of the basal level of CXCR4 fluorescence was directly proportional to the extent of occupancy of the receptor by the ligand (Fig. 4a). FRET experiments were carried out by exciting HEK 293 cells expressing EGFP-CXCR4 at the EGFP excitation wavelength (460–470 nm). Measurement of either the reduction of EGFP fluorescence when SDF-1 α -TR (emission wavelength, 522 nm) was added (Fig. 4a) or the increase of EGFP emission at 522 nm following displacement of bound SDF-1 α -TR by an excess of unlabeled SDF-1 α (Fig. 4b) allowed the estimation of association and dissociation rates. Addition of 50 nM SDF-1 α -TR to a suspension of 10^6 HEK 293 cells/ml expressing EGFP-CXCR4 resulted in a progressive decrease in EGFP fluorescence emission. Similarly, addition of the soluble SDF-1 α -TR-HS complexes led to a substantial reduction of EGFP emission with a rate comparable to that induced by the chemokine alone. For both untreated and heparitinase I-treated cells, binding traces were best fitted with a sum of two exponentials with a rapid phase representing 85% of relaxation amplitude at 12.5 nM and 55% at 50 nM ligand. The best fit was obtained with identical rates for heparitinase-treated and untreated cells ($k_{app} = 0.11$ s $^{-1}$ at 12.5 nM and 0.25 s $^{-1}$ at 50 nM SDF-1 α -TR), indicating that the presence or the absence of the HS moieties on proteoglycans was without effect on associations rates (Fig. 4a). Similarly, comparison of SDF-1 α -TR and SDF-1 α -TR-HS complex association rates revealed no variation in rate constants ($k_{app} = 0.25$ s $^{-1}$ at 50 nM SDF-1 α -TR) (Fig. 4c).

The specificity of EGFP-CXCR4 occupancy by labeled chemokines, either alone or complexed to HS, was probed by competition with a large molar excess of unlabeled SDF-1 α (500 nM) that fully restored EGFP emission to initial levels (Fig. 4b). Apparent dissociation values of SDF-1 α -TR either complexed or not to HS from EGFP-CXCR4 were also very similar ($k_{off} = 0.08 \pm 0.01$ s $^{-1}$). In both association and dissociation experiments, however, signal amplitudes exhibited sensitivity to proteoglycans, because after HS removal or addition of soluble HS, the extent of SDF-1 α -TR binding represented only 60–70% ($n = 4$) of control binding (Fig. 4, a and c). These data suggest that cell surface HS, under the present experimental conditions, contribute to enhance SDF-1 α -TR binding to CXCR4 without affecting the association and dissociation kinetics and thus the affinity of ligand-receptor interactions. These results support the idea that cell surface HS may concentrate SDF-

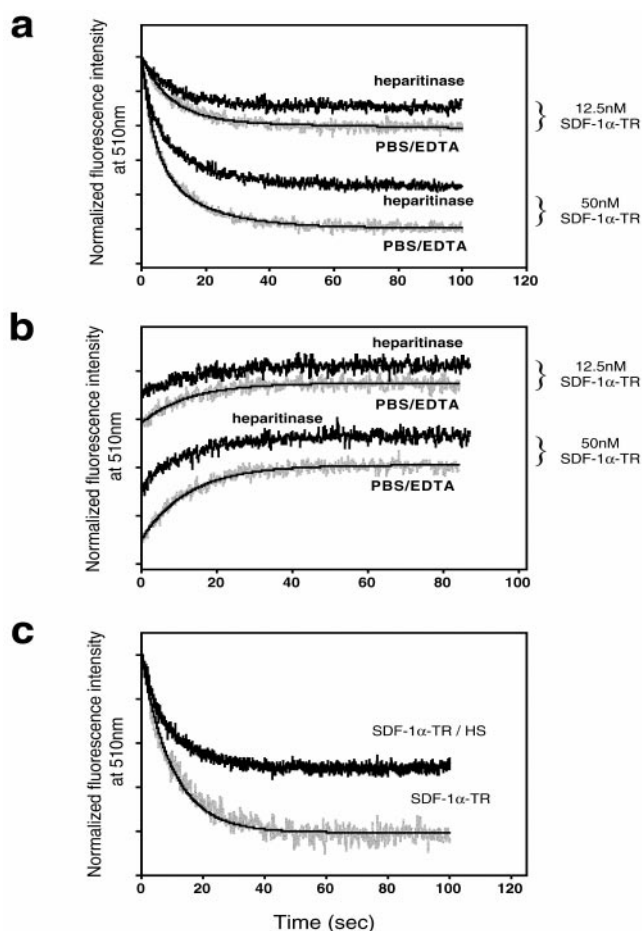


FIG. 4. Kinetics of CXCR4 occupancy by SDF-1 α . Role of cell surface HS. Association and dissociation kinetics were measured by FRET between EGFP-CXCR4 and SDF-1 α -TR. After detachment with PBS/EDTA (5 mM) buffer, cells stably expressing EGFP-CXCR4 (2×10^6 cells/ml) were treated or not with heparitinase I (5 milliunits/ml) and then rapidly mixed with SDF-1 α -TR at 21 °C. *a*, association time courses were measured in the presence of 12.5 or 50 nM of SDF-1 α -TR (final concentrations) as EGFP fluorescence extinction at 510 nm (excitation, 470 nm). Best fit of fluorescence traces were obtained with a single exponential fluorescence decay (Equation 1) or a single exponential decay plus a linear drift potentially reflecting a second, slowly developing, bimolecular reaction. In the presence of 12.5 nM SDF-1 α -TR, rate constants and amplitudes were as follows. PBS/EDTA: $k_{app} = 0.11 \text{ s}^{-1}$; amplitude, 2.8×10^5 cps; heparitinase: $k_{app} = 0.11 \text{ s}^{-1}$; amplitude, 3.8×10^5 cps. In the presence of 50 nM SDF-1 α -TR, rate constants and amplitudes were as follows. PBS/EDTA: $k_{app} = 0.25 \text{ s}^{-1}$; amplitude, 4.3×10^5 cps; heparitinase: $k_{app} = 0.25 \text{ s}^{-1}$; amplitude, 5.8×10^5 cps. *b*, after binding of SDF-1 α -TR (12.5 and 50 nM), dissociation was initiated by adding a molar excess of unlabeled SDF-1 α (500 nM) to cells treated or not with heparitinase I and monitored as the kinetic of restoration of EGFP fluorescence emission. *c*, binding of either SDF-1 α -TR-soluble HS complex or the chemokine alone to EGFP-CXCR4. The interaction of the ligands with the receptor was monitored by measuring their respective kinetics of extinction of EGFP fluorescence in HEK 293 EGFP-CXCR4+ cells.

1 α -TR in the proximity of the CXCR4 receptor, therefore increasing the occupancy of receptor sites.

Kinetics of SDF-1 α and SDF-1 α -HS interactions with CXCR4 were further investigated by real time analysis of intracellular calcium mobilization. HeLa cells constitutively expressing CXCR4 were loaded with Indo-1/AM and challenged by SDF-1 α , SDF-1 3/6, or SDF-1 α complexed with soluble HS (Fig. 5). Kinetics of intracellular calcium mobilization were determined by measuring Indo fluorescence every 100 ms. No differences were observed between SDF-1 α and 3/6 (Fig. 5*a*). Similar to HS cell-bound SDF-1 α , SDF-1 α complexed with HS mobilized intracellular calcium as efficiently as SDF-1 α alone (Fig. 5*b*).

These results are in keeping with findings obtained from the kinetic analyses of CXCR4 occupancy and lead to the conclusion that the intrinsic capacity of SDF-1 α to occupy and activate CXCR4 is not modified by attachment to soluble or cell-bound HS.

The second HIV-inhibitory mechanism mediated by SDF-1 α is functionally related to occupancy of CXCR4 by the ligand and relies on the down-regulation of CXCR4 from the cell surface (36, 37). The comparable potency shown by SDF-1 α and the HS-disabled mutants to induce an intracellular calcium flux suggests that association with HS should not modify the capacity of the chemokine to promote CXCR4 internalization. However, mobilization of intracellular calcium stores by SDF-1 α is inhibited by Pertussis toxin, a G α -protein specific inhibitor, whereas CXCR4 endocytosis is not affected (36, 38), thus indicating the existence of divergent signaling pathways, some of which may be preferentially induced by the ligand when bound to cell surface HS.

CXCR4 internalization following ligation with either SDF-1 α or SDF-1 α mutants unable to bind HS was investigated by exposing HeLa cells for 30 min to wild type, SDF-1 2/6, or SDF-1 3/6 (Fig. 5*c*). All the SDF-1 α molecules induced CXCR4 endocytosis to a similar extent, independently of their respective capacities to associate with cell surface HS.

Long term exposure to agonists is known to promote depletion of the cellular content of some G-protein-coupled receptors, likely mediated by alterations in the rates of degradation of internalized receptor and synthesis (reviewed in Ref. 39). It can be proposed that SDF-1 α -HS complexes may have a differential or more efficient capacity to promote sustained down-regulation of CXCR4 and optimal HIV inhibition. However, prolonged exposure for hours to either wild type or HS-binding disabled mutants did not reveal any significant difference on the amount of CXCR4 expressed in several cell lines (data not shown).

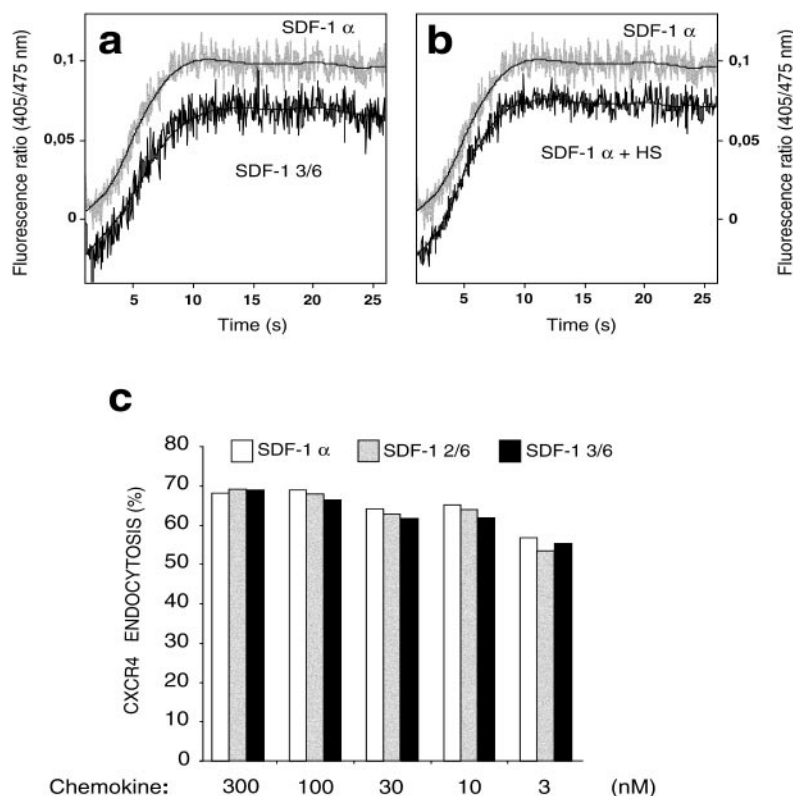
Taken together, those findings lead us to conclude that neither the rate of CXCR4 internalization nor its capacity to recycle to the cell surface is a target of the optimal anti-HIV fusogenic effect manifested by the complex formed by SDF-1 α bound to cellular HS.

A third mechanism can still be invoked to explain the reinforced HIV inhibitory effect of SDF-1 α when bound to cell surface HS. Indeed, the interaction of SDF-1 α with membrane-bound HSPGs may lead to the concentration of the chemokine in the surrounding environment of CXCR4 on HIV target cells. This, rather than modification of the intrinsic capacities of the chemokine to occupy and activate CXCR4, may underlie the optimal HIV-inhibitory activity of SDF-1 α -HS cell membrane-bound complex.

That assumption would imply the existence of functional SDF-1 α anchored at the cell membrane through specific interaction with cell surface HS. The failure of either HS-binding disabled SDF-1 α or the wild type counterpart upon selective cleavage of exposed HS to bind on cells lacking CXCR4 is in keeping with this hypothesis. However, the existence of functional SDF-1 α anchored at the cell membrane through specific interaction with cell surface HS still required formal demonstration. For this purpose, we developed an assay to visualize and quantify interactions of SDF-1 α with CXCR4 and/or cell surface GAGs, based on FRET between SDF-1 α -TR and EGFP-CXCR4 expressed in living cells.

The participation of cell surface GAGs in this phenomenon was investigated either by their enzymatic removal or the use of a molar excess of HS to form soluble complexes with SDF-1 α . HEK 293 cells expressing EGFP-CXCR4 were incubated with SDF-1 α -TR. Measurement of TR fluorescence allowed quanti-

FIG. 5. Role of cell surface HS in the activation of CXCR4 by SDF-1 α . Intracellular calcium mobilization induced by occupation of CXCR4 receptor by agonist. Comparative analysis of SDF-1 α versus SDF-1 3/6 (a) and SDF-1 α versus SDF-1 α -HS complex (b). HeLa P4.2 CXCR4+ cells were loaded with 5 μ M Indo-1/AM at 37 °C and rapidly mixed (2×10^6 cells/ml) with the corresponding ligands. Chemokine concentrations were 3 nM, and soluble HS concentration was 5 μ g/ml. Real time kinetic and amplitude of intracellular calcium mobilization were measured. Time to half peak for SDF-1 α ($t_{1/2} = 5.1 \pm 0.1$ s), SDF-1 3/6 ($t_{1/2} = 5.6 \pm 0.1$ s), and SDF-1 α -HS ($t_{1/2} = 4.9 \pm 0.3$ s) responses were calculated as a time to peak of the first derivative of the response. No differences were observed in response amplitude under the three experimental conditions. c, endocytosis of CXCR4 upon ligation of CXCR4 by either SDF-1 α , SDF-1 2/6, or SDF-1 3/6 for 30 min in HeLa cells as previously described (36).



fication of the amount of SDF-1 α -TR bound to EGFP-CXCR4 (recording at 522 nm, Fig. 6a, top row) and other cell surface structures (recording at 605 nm, Fig. 6a, bottom row). Thus, simultaneous recording of both fluorescence emissions allowed to discriminate in real time the fraction of SDF-1 α -TR selectively bound to CXCR4 (changes in EGFP fluorescence) from the total amount of ligand adsorbed to the cell surface (TR fluorescence). Under the standard experimental conditions we used, the relatively low intensity fluorescence of SDF-1 α -TR precluded discriminating between CXCR4 and HS-bound SDF-1 α -TR when TR fluorescence was selectively recorded.

SDF-1 α -TR (100 nM) was added to control cells when background EGFP fluorescence reached equilibrium (Fig. 6a, image 1). Twenty-five s after addition of SDF-1, EGFP-fluorescence intensity was reduced by 70% as a consequence of SDF-1 α -TR binding to EGFP-CXCR4 (Fig. 6a, image 2, with the corresponding time course shown in Fig. 6b, panel 1, green trace). Simultaneous measurement of SDF-1 α -TR fluorescence, resulting from both the occupancy of CXCR4 and attachment to HS, was recorded from the same cells at 605 nm. Before addition of SDF-1 α -TR, no fluorescence was detected (Fig. 6a, image 3). However, after addition of the ligand, a strong TR fluorescent labeling of the cell surface was observed (Fig. 6a, image 4, with a time course shown in Fig. 6b, panel 1, red trace), clearly indicating accumulation of SDF-1 α -TR at the cell surface. The agonistic capacity of SDF-1 α -TR induced upon binding to EGFP-CXCR4 was confirmed by measuring intracellular Ca^{2+} mobilization (data not shown).

Treatment with heparitinase I cleaves specifically HS moieties from cell surface proteoglycans. Cells exposed to heparitinase I lost virtually all the SDF-1 α -TR (200 nM) binding at the surface of HEK 293 cells expressing EGFP-CXCR4 (Fig. 6a, images 7 and 8). In contrast, removal of HS from the cell surface did not affect the specific interaction of SDF-1 α with CXCR4 (Fig. 6a, images 5 and 6), as revealed by the marked decrease of EGFP-fluorescence (recorded 8 s after addition of the ligand). The corresponding time-course recording of EGFP

(green trace) or TR fluorescence (red trace) is shown in Fig. 6b, panel 2.

Additional evidence supporting the formation of SDF-1 α -HS physical complexes at the surface of cells was obtained by the competitive effect of soluble HS in the binding of SDF-1 α -TR. SDF-1 α -TR (50 nM) was complexed with 5 μ g/ml of soluble HS. The complex bound to EGFP-CXCR4 with an efficiency comparable to that of SDF-1 α -TR alone, as revealed by a substantial reduction of EGFP fluorescence (Fig. 6a, images 9 and 10). Unlabeled SDF-1 α added in molar excess competed with soluble SDF-1 α -TR for binding to CXCR4 and restored the EGFP signal to initial level, thus proving that the interaction of soluble SDF-1 α -HS bound to the receptor is specific and reversible (Fig. 6a, image 11). Simultaneous assessment of TR fluorescence revealed that formation of soluble complexes with HS abolishes SDF-1 α -TR binding to the cell surface HS (Fig. 6a, images 12–14). Remarkably, the reduction of SDF-1 α -TR fluorescence adsorbed to the cell membrane in the presence of soluble HS was comparable to that caused by enzymatic cleavage of cell surface HS with heparitinase I (Fig. 6a, compare images 12 and 13 to images 7 and 8).

Those findings demonstrate the selective interaction of functional SDF-1 α with the HS moiety of HSPGs at the cell membrane. Remarkably, most of the SDF-1 α binding detected at the cell surface belonged to the fraction bound to HS. More importantly, SDF-1 α conserved the capacity to interact with its cognate receptor, CXCR4, while attached to HS. The evidence that we provide does not argue in favor of the modification of the functional capacities of SDF-1 α by interaction with HS. Instead, the optimal HIV-inhibitory activity shown by the chemokine when bound to cell surface HS likely reflects its immobilization and enhanced concentration in the surrounding CXCR4 environment.

The superior HIV inhibitory capacity shown by SDF-1 α -HS complexes is analogous to the improved efficiency shown by CCR5 ligands complexed to GAGs to prevent entry of R5 HIV isolates into target cells (23, 40). Indeed, it has been reported

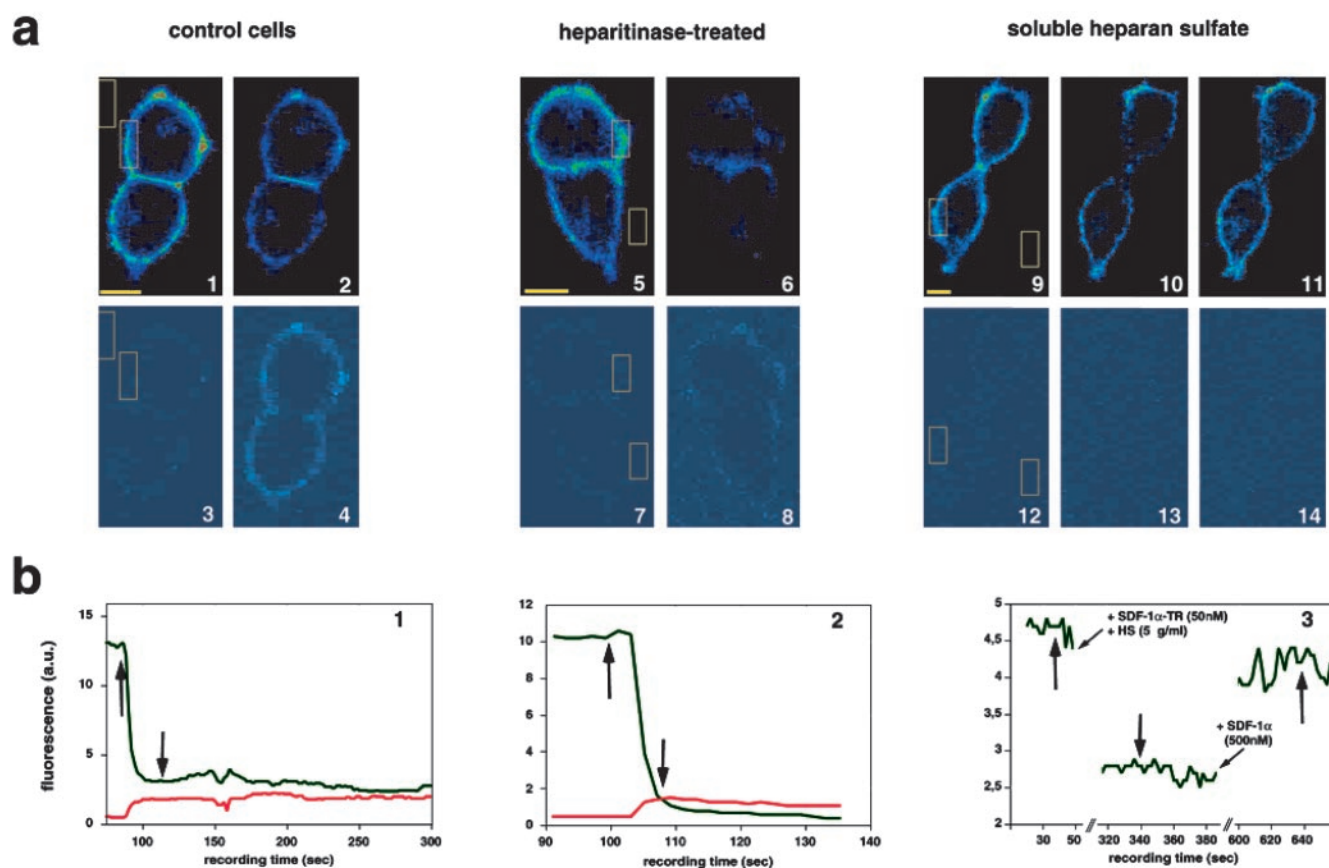


FIG. 6. Cell surface HSPG anchor SDF-1 α and enhance the local concentration of SDF-1 α in the surrounding environment of CXCR4 receptor. FRET between EGFP-CXCR4 and SDF-1 α -TR was dynamically studied by using confocal microscopy carried out at 19 °C on living cells (HEK 293 EGFP-CXCR4). Binding of SDF-1 α -TR to EGFP-CXCR4 was assessed by changes of EGFP fluorescence at 522 nm (*top row*). Simultaneously, assessment TR fluorescence permitted evaluation of both dependent and independent CXCR4 binding of the ligand (*bottom row*). Cleavage of cell surface HS by heparitinase I did not affect CXCR4-dependent binding of SDF-1 α -TR (*images 5 and 6*, before and after addition of SDF-1 α -TR, respectively) but prevented accumulation of the ligand at cell surfaces as reflected by the absence of detectable TR fluorescence (*images 7 and 8*, before and after addition of SDF-1 α -TR, respectively). SDF-1 α -TR complex with soluble HS acted as a functional ligand for CXCR4 (*images 9 and 10*, before and after addition of SDF-1 α -TR-HS complex, respectively). Addition of a molar excess of SDF-1 α (500 nM) reversed SDF-1 α -TR interaction with EGFP-CXCR4 (*image 11*). *b*, time course of SDF-1 α -TR interaction with EGFP-CXCR4 by FRET is shown as *green traces* for each experimental condition. Accumulation of SDF-1 α -TR at cell surfaces is shown as *red traces*. Quantification of fluorescence for *green* and *red traces* was carried out by measuring fluorescence over the whole images. More detailed quantification was performed by measuring fluorescence from the indicated constant size areas (delimited by *yellow rectangles* in *a*). As compared with background fluorescence (which was constant in *a*, *top row*), EGFP-CXCR4 fluorescence decreased by 8-fold in *a*, *image 2* (as compared with *image 1*), by 10-fold in *image 6* (as compared with *image 5*), and by 2-fold in *image 10* (as compared with *image 9*). Simultaneously, measurement of SDF-1 α -TR fluorescence in the same areas (*a*, *bottom row*) reveals that background fluorescence increased by 1.2-fold, whereas membrane fluorescence increased by 5-fold in *image 4* (as compared with *image 3*). Background fluorescence increased by 2.4-fold and membrane fluorescence by 2.8-fold in *image 8* (as compared with *image 7*), and both background and membrane fluorescence increased by 1.2-fold in *image 13* (as compared with *image 12*).

that removal of cell surface HS, but not CS, substantially diminishes the capacity of either RANTES or macrophage inflammatory protein 1 β to block R5 HIV infection in lymphoblastoid T CD4+ lymphocytes (41). In macrophages, most of CCR5-independent binding of RANTES to cell surface is accounted by CS (42), but its failure to bind cell surface HS has been proposed to explain the reduced capacity of RANTES to inhibit HIV entry in these cells (40, 41). However, the issue remains unclear because in other cases, cleavage of CS, but not HS, partially offsets the anti-HIV activity of the chemokine in macrophages (42). Precise identification of HS-binding domains in CCR5 will contribute to our understanding of CCR5/GAG interactions and, as in the case of SDF-1 α -HS complexes in this work, to elucidating the underlying mechanisms accounting for their optimal capacity to prevent HIV infection.

The accumulation of large amounts of SDF-1 α bound to cell surface HSPGs may be an essential component of a natural barrier against infection and propagation of X4 HIV isolates.

The anti-HIV activity of this complex at anatomic sites where viral replication occurs can be envisaged in two differ-

ent, not mutually exclusive, situations. First, SDF-1 α may passively accumulate on the plasma membranes of CD4+/CXCR4+ cells. Macrophages or T CD4+ lymphocytes, the major cell types that replicate HIV do not express the SDF-1 α gene. However, this gene is expressed in lymphoid organs, as demonstrated by detection of transcripts (43). The expression of HSPGs differs greatly between different T cells, and interestingly, HTLV-I infected cells express higher levels (44) and show HS-dependent binding of RANTES and macrophage inflammatory protein 1 β (41). This suggests that the expression of HSPG may be regulated depending on the activation and/or differentiation status of T lymphocytes. Attached to HSPGs, the accumulation of SDF-1 α in the surrounding environment of CXCR4 in HIV cell targets may contribute efficiently to the blockade of X4 HIV isolates. Second, the HIV inhibitory effect of SDF-1 α may be mediated by HSPGs-dependent binding of the chemokine at the cell membrane of non-HIV target cells. The epithelia from genital (endocervix and vagina) and digestive tract, including rectum (45), which express constitutively the most robust secretion of SDF-1 α detected in humans, may

be the paradigm of this situation. Epithelial cells from the digestive mucosa are well characterized by their high content of HSPGs that preferentially accumulate at the basolateral membrane (46–48). CD4⁺ T lymphocytes from mucosal tissues exposed to HIV transmission are involved during acute infection in the dissemination, replication, and propagation of chimeric simian immunodeficiency virus-HIV in macaques (49, 50), the animal model of HIV infection. Interestingly, mucosal T lymphocytes in intimate contact with SDF-1 α -producing cells exhibit a very low level of CXCR4, although they show abundant CCR5 expression (45, 51). The lack of CXCR4 expression in these cells seems to be largely determined by the cell environment, because shortly after their isolation from the tissue, they recover CXCR4 expression at the cell surface (45). It can be envisaged that HS-dependent anchoring of SDF-1 α to the cell surface promotes massive accumulation of the chemokine, thus enhancing its local concentration and facilitating persistent occupancy and sustained down-regulation of CXCR4 in the mucosal T lymphocytes. Similarly, this hypothesis would apply to dendritic and Langerhans cells. Indeed, these cells are scattered throughout epithelia in both digestive and genital mucosa, are in intimate contact with SDF-1 α -producing cells, and have been shown to be a primary target for simian immunodeficiency virus (52, 53). Eventually, the barrier created by the accumulation of SDF-1 α at these anatomic sites by both immobilization of the chemokine attached to the HS of the extracellular matrix and binding to cell surface HSPGs may restrain dissemination of X4 HIV isolates and contribute to the predominant propagation of R5 HIV isolates characteristic of the early phases of the infection.

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REFERENCES

- Oberlin, E., Amara, A., Bachelier, F., Bessia, C., Virelizier, J. L., Arenzana-Seisdedos, F., Schwartz, O., Heard, J. M., Clark-Lewis, I., Legler, D. F., Loetscher, M., Baggiolini, M., and Moser, B. (1996) *Nature* **382**, 833–835
- Bleul, C. C., Farzan, M., Choe, H., Parolin, C., Clark-Lewis, I., Sodroski, J., and Springer, T. A. (1996) *Nature* **382**, 829–833
- Shirozu, M., Nakano, T., Inazawa, J., Tashiro, K., Tada, H., Shinohara, T., and Honjo, T. (1995) *Genomics* **28**, 495–500
- Pablos, J. L., Amara, A., Boulloc, A., Santiago, B., Caruz, A., Galindo, M., Delaunay, T., Virelizier, J. L., and Arenzana-Seisdedos, F. (1999) *Am. J. Pathol.* **155**, 1577–1586
- Coulomb-L'Herminé, A., Amara, A., Durand-Gasselin, I., Foussat, A., Schiff, C., Ledee, N., Galanaud, P., Arenzana-Seisdedos, F., and Emilie, D. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 8585–8590
- Nagasawa, T., Hirota, S., Tachibana, K., Takakura, N., Nishikawa, S., Kitamura, Y., Yoshida, N., Kikutani, H., and Kishimoto, T. (1996) *Nature* **382**, 635–638
- Ma, Q., Jones, D., Borghesani, P. R., Segal, R. A., Nagasawa, T., Kishimoto, T., Bronson, R. T., and Springer, T. A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 9448–9453
- Gleichmann, M., Gillen, C., Czardybon, M., Bosse, F., Greiner-Petter, R., Auer, J., and Muller, H. W. (2000) *Eur. J. Neurosci.* **12**, 1857–1866
- Feng, Y., Broder, C. C., Kennedy, P. E., and Berger, E. A. (1996) *Science* **272**, 872–877
- Tachibana, K., Hirota, S., Iizasa, H., Yoshida, H., Kawabata, K., Kataoka, Y., Kitamura, Y., Matushima, K., Yoshida, N., Nishikawa, S., Kishimoto, T., and Nagasawa, T. (1998) *Nature* **393**, 591–594
- Zou, Y. R., Kottmann, A. H., Kuroda, M., Taniuchi, I., and Littman, D. R. (1998) *Nature* **393**, 595–599
- Granelli-Piperno, A., Moser, B., Pope, M., Chen, D., Wei, Y., Isdell, F., O'Doherty, U., Paxton, W., Koup, R., Mojssov, S., Bhardwaj, N., Clark-Lewis, I., Baggiolini, M., and Steinman, R. M. (1996) *J. Exp. Med.* **184**, 2433–2438
- Michael, N. L., and Moore, J. P. (2000) *Nat. Med.* **5**, 740–741
- Tersmette, M., Lange, J. M., de Goede, R. E., de Wolf, F., Eeftink-Schattenkerk, J. K., Schellekens, P. T., Coutinho, R. A., Huisman, J. G., Goudsmit, J., and Miedema, F. (1989) *Lancet* **1**, 983–985
- Scarlati, G., Tresoldi, E., Bjornal, A., Fredriksson, R., Colognesi, C., Deng, H. K., Malnati, M. S., Plebani, A., Siccardi, A. G., Littman, D. R., Fenyo, E. M., and Lusso, P. (1997) *Nat. Med.* **11**, 1259–1265
- Chan, D. C., Foss, D., Berger, J. M., and Kim, P. S. (1998) *Cell* **89**, 263–273
- Wyatt, R., and Sodroski, J. (1998) *Science* **280**, 1884–1888
- Berger, E. A., Murphy, P. M., and Farber, J. M. (1999) *Annu. Rev. Immunol.* **17**, 657–700
- Iozzo, R. V. (1998) *Annu. Rev. Biochem.* **67**, 609–652
- Kjellen, L., and Lindahl, U. (1991) *Annu. Rev. Biochem.* **60**, 443–475
- Hardingham, T. E., and Fosang, A. J. (1992) *FASEB J.* **6**, 861–870
- Amara, A., Lorthioir, O., Valenzuela, A., Magerus, A., Thelen, M., Montes, M., Virelizier, J. L., Delepiepierre, M., Baleux, F., Lortat-Jacob, H., and Arenzana-Seisdedos, F. (1999) *J. Biol. Chem.* **274**, 23916–23925
- Burns, J. M., Lewis, G. K., and DeVico, A. L. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 14499–14504
- Clavel, F., and Charneau, P. (1994) *J. Virol.* **68**, 1179–1185
- Schwartz, O., Alizon, M., Heard, J. M., and Danos, O. (1994) *Virology* **198**, 360–365
- Puri, A., Hug, P., Jernigan, K., Barchi, J., Kim, H. Y., Hamilton, J., Wiels, J., Murray, G. J., Brady, R. O., and Blumenthal, R. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 14435–14440
- Bleul, C. C., Fuhlbrigge, R. C., Casasnovas, J. M., Aiuti, A., and Springer, T. A. (1996) *J. Exp. Med.* **184**, 1101–1109
- Vollmer, J. Y., Alix, P., Chollet, A., Takeda, K., and Galzi, J. L. (1999) *J. Biol. Chem.* **274**, 37915–37922
- Heidmann, T., and Changeux, J. P. (1979) *Eur. J. Biochem.* **94**, 255–279
- Neubig, R. R., and Sklar, L. A. (1993) *Mol. Pharmacol.* **43**, 734–740
- Crump, M. P., Gong, J. H., Loetscher, P., Rajarathnam, K., Amara, A., Arenzana-Seisdedos, F., Virelizier, J. L., Baggiolini, M., Sykes, B. D., and Clark-Lewis, I. (1997) *EMBO J.* **16**, 6996–7007
- Heveker, N., Montes, M., Germeroth, L., Amara, A., Trautmann, A., Alizon, M., and Schneider-Mergener, J. (1998) *Curr. Biol.* **8**, 369–376
- Doranz, B. J., Orsini, M. J., Turner, J. D., Hoffman, T. L., Berson, J. F., Hoxie, J. A., Peiper, S. C., Brass, L. F., and Doms, R. W. (1999) *J. Virol.* **73**, 2752–2761
- Chabot, D. J., Zhang, P. F., Quinnan, G. V., and Broder, C. C. (1999) *J. Virol.* **73**, 6598–6609
- Brelot, A., Heveker, N., Montes, M., and Alizon, M. (2000) *J. Biol. Chem.* **275**, 23736–23744
- Amara, A., Gall, S. L., Schwartz, O., Salamero, J., Montes, M., Loetscher, P., Baggiolini, M., Virelizier, J. L., and Arenzana-Seisdedos, F. (1997) *J. Exp. Med.* **186**, 139–146
- Signoret, N., Oldridge, J., Pelchen-Matthews, A., Klasse, P. J., Tran, T., Brass, L. F., Rosenkilde, M. M., Schwartz, T. W., Holmes, W., Dallas, W., Luther, M. A., Wells, T. N., Hoxie, J. A., and Marsh, M. (1997) *J. Cell Biol.* **139**, 651–664
- Tanabe, S., Heesen, M., Yoshizawa, I., Berman, M. A., Luo, Y., Bleul, C. C., Springer, T. A., Okuda, K., Gerard, N., and Dorf, M. E. (1997) *J. Immunol.* **159**, 905–911
- Bohm, S. K., Grady, E. F., and Bunnett, N. W. (1997) *Biochem. J.* **322**, 1–18
- Wagner, L., Yang, O. O., Garcia-Zepeda, E. A., Ge, Y., Kalam, S. A., Walker, B. D., Pasternack, M. S., and Luster, A. D. (1998) *Nature* **391**, 908–911
- Oravecz, T., Pall, M., Wang, J., Roderiquez, G., Ditto, M., and Norcross, M. A. (1997) *J. Immunol.* **159**, 4587–4592
- Amzazi, S., Ylisastigui, L., Bakri, Y., Rabehi, L., Gattegno, L., Parmentier, M., Gluckman, J. C., and Benjouad, A. (1998) *Virology* **252**, 96–105
- Bleul, C. C., Schultze, J. L., and Springer, T. A. (1998) *J. Exp. Med.* **187**, 753–762
- Ohshiro, Y., Murakami, T., Matsuda, K., Nishioka, K., Yoshida, K., and Yamamoto, N. (1996) *Microbiol. Immunol.* **40**, 827–835
- Agace, W. W., Amara, A., Roberts, A. I., Pablos, J. L., Thelen, S., Uguccioni, M., Li, X. Y., Marsal, J., Arenzana-Seisdedos, F., Delaunay, T., Ebert, E. C., Moser, B., and Parker, C. M. (2000) *Curr. Biol.* **10**, 325–328
- Sanderson, R. D., and Bernfield, M. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 9562–9566
- Bernfield, M., and Sanderson, R. D. (1990) *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **327**, 171–186
- Ianelli, C. J., DeLellis, R., and Thorley-Lawson, D. A. (1998) *J. Biol. Chem.* **273**, 23367–23375
- Veazey, R. S., De Maria, M., Chalifoux, L. V., Shevtz, D. E., Pauley, D. R., Knight, H. L., Rosenzweig, M., Johnson, R. P., Desrosiers, R. C., and Lackner, A. A. (1998) *Science* **280**, 427–431
- Harousse, J. M., Gettie, A., Tan, R. C. H., Blanchard, J., and Cheng-Mayer, C. (1999) *Science* **284**, 816–819
- Agace, W. W., Roberts, A. I., Wu, L., Greineder, C., Ebert, E. C., and Parker, C. M. (2000) *Eur. J. Immunol.* **30**, 819–826
- Miller, C. J., and Hu, J. (1999) *J. Infect. Dis.* **179**, S413–S417
- Hu, J., Gardner, M. B., and Miller, C. J. (2000) *J. Virol.* **74**, 6087–6095