



Leukocyte elastase negatively regulates Stromal cell-derived factor-1 (SDF-1)/CXCR4 binding and functions by amino-terminal processing of SDF-1 and CXCR4.

Agustín Valenzuela-Fernández, Thierry Planchenault, Françoise Baleux, Isabelle Staropoli, Karine Le-Barillec, Dominique Leduc, Thierry Delaunay, Françoise Lazarini, Jean-Louis Virelizier, Michel Chignard, et al.

► To cite this version:

Agustín Valenzuela-Fernández, Thierry Planchenault, Françoise Baleux, Isabelle Staropoli, Karine Le-Barillec, et al.. Leukocyte elastase negatively regulates Stromal cell-derived factor-1 (SDF-1)/CXCR4 binding and functions by amino-terminal processing of SDF-1 and CXCR4.. Journal of Biological Chemistry, 2002, 277 (18), pp.15677-89. 10.1074/jbc.M111388200 . pasteur-00166872

HAL Id: pasteur-00166872

<https://pasteur.hal.science/pasteur-00166872>

Submitted on 31 May 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Copyright

Leukocyte Elastase Negatively Regulates Stromal Cell-derived Factor-1 (SDF-1)/CXCR4 Binding and Functions by Amino-terminal Processing of SDF-1 and CXCR4*

Received for publication, November 29, 2001, and in revised form, February 19, 2002
Published, JBC Papers in Press, February 26, 2002, DOI 10.1074/jbc.M111388200

Agustín Valenzuela-Fernández,^{a,b,c} Thierry Planchenault,^{a,b} Françoise Baleux,^d
Isabelle Staropoli,^a Karine Le-Barillec,^{e,f} Dominique Leduc,^e Thierry Delaunay,^g
Françoise Lazarini,^h Jean-Louis Virelizier,^a Michel Chignard,^e Dominique Pidard,^{e,i}
and Fernando Arenzana-Seisdedos^{a,j}

From the ^aUnité d'Immunologie Virale, ^dUnité de Chimie Organique, ^eUnité de Défense Innée et Inflammation/INSERM U485 de l'Institut Pasteur, 28 Rue du Dr. Roux, 75724 Paris Cedex 15, ^fINRA, Station de Pathologie Végétale, BP81 33883 Villeneuve d'Ornon, and ^hUnité de Neurovirologie et Régénération du Système Nerveux de l'Institut Pasteur, 25 Rue du Dr. Roux, 75724 Paris Cedex 15, France

Activation of CXCR4 by the CXC chemokine stromal cell-derived factor-1 (SDF-1) requires interaction of the amino-terminal domains of both molecules. We report that proteinases released from either mononucleated blood cells or polymorphonuclear neutrophils degranulated by inflammatory stimuli generate an SDF-1 fragment that is deleted from amino-terminal residues Lys¹-Pro²-Val³, as characterized by mass spectrometry analysis. The proteolyzed chemokine fails to induce agonistic functions and is unable to prevent the fusogenic capacity of CXCR4-tropic human immunodeficiency viruses. Furthermore, we observed that exposure of CXCR4-expressing cells to leukocyte proteinases results in the proteolysis of the extracellular amino-terminal domain of the receptor, as assessed by flow cytometry analysis and electrophoretic separation of immunoprecipitated CXCR4. Blockade of SDF-1 and CXCR4 proteolysis by the specific leukocyte elastase inhibitor, *N*-methoxysuccinyl-alanine-alanine-proline-valine-chloromethyl ketone, identified elastase as the major enzyme among leukocyte-secreted proteinases that accounts for inactivation of both SDF-1 and CXCR4. Indeed, purified leukocyte elastase generated in either SDF-1 or CXCR4 a pattern of cleavage indistinguishable from that observed with leukocyte-secreted proteinases. Our findings suggest that elastase-mediated proteolysis of SDF-1/CXCR4 is part of a mechanism regulating their biological functions in both homeostatic and pathologic processes.

Chemokines also control other aspects of tissue homeostasis (1, 2). Most chemokines are members of one of the two subfamilies, CXC or CC, depending on whether or not the two conserved amino-terminal cysteines are spaced by an extra (X) amino acid. The biological activity of chemokines is regulated at both transcriptional and post-translational levels. Thus, stimuli from injured tissues, which disturb cell homeostasis, activate expression of inducible chemokines (3). At a post-translational level, chemokines are in some cases regulated by selective proteolysis after secretion (4–9). Regulation of leukocyte migration would ultimately be determined by the interplay of chemokines and proteinases (10, 11). Although natural proteolytic cleavage of chemokines occurs either at carboxyl or amino terminus, most of functionally relevant proteolysis takes place at the amino terminus, which encompasses both receptor-binding and signaling domains. Discrete amino-terminal proteolysis of chemokines changes their biological functions. In some cases limited proteolysis produces fragments with enhanced biological activity or active polypeptides from an inactive precursor (5, 6, 12). In other cases, cleavage of the amino terminus leads to the generation of potent antagonists as exemplified by monocyte chemoattractant protein (MCP)-2,¹ MCP-3 (7, 13), and regulated on activation normal T cell expressed and secreted (13).

Finally, deletion of amino-terminal residues can extend the specificity of the chemokine to other chemokine receptors, as reported for hemofiltrate CC chemokine-1 (14) and MCP-3 (9).

Chemokines regulate both basal and inflammation-induced trafficking of leukocytes. In non-immune cells such as neurons, smooth muscle, stromal, endothelial and epithelial cells, che-

* This work was supported in part by grants from ANRS (France) and Ensemble Contre le SIDA "SIDACTION" (France). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

^b Both authors contributed equally to this work.

^c Supported by a fellowship from the ANRS.

^f Present address: Unité de Cytokines et Développement Lymphoïde, de l'Institut Pasteur de Paris.

ⁱ Supported by CNRS.

^j To whom correspondence should be addressed: Dept. Médecine Moléculaire, Unité d'Immunologie Virale, Institut Pasteur, 28 Rue du Dr. Roux, 75724 Paris Cedex 15, France. Tel.: 33-1-45688263; Fax: 33-1-45688941; E-mail: farenzan@pasteur.fr.

¹ The abbreviations used are: MCP, monocyte chemoattractant protein; CXCR, CXC chemokine receptor; SDF-1, stromal cell-derived factor-1; LE, leukocyte elastase; CG, cathepsin G; MeOSuc-AAPV-CMK, *N*-methoxysuccinyl-alanine-alanine-proline-valine-chloromethyl ketone; PBMC, peripheral blood mononuclear cells; PMN, polymorphonuclear neutrophil leukocytes; RP-HPLC, reverse phase-high performance liquid chromatography; ESI-MS, electrospray ionization mass spectrometry; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; IL-8, interleukin-8; HIV-1, human immunodeficiency virus, type 1; SFV, Semliki Forest virus; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenoltetrazolium bromide; AEBBSF, 4-(2-aminoethyl)-benzenesulfonfyl fluoride hydrochloride; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PAR, proteinase-activated receptors; BSA, bovine serum albumin; PBS, phosphate-buffered saline; BHK, baby hamster kidney; mAb, monoclonal antibody; aa, amino acid; E64, *N*-[N-(L-3-*trans*-carboxirane-2-carbonyl)-L-leucyl]-agmatine; HBSS, Hanks' balance salt solution; HRP, horseradish peroxidase; FCS, fetal calf serum; PE, phycoerythrin; LTR, long terminal repeat; BM, bone marrow; SDF-1 α -bt, SDF-1 α -biotin; DPP IV, dipeptidyl peptidase IV; MMP, matrix metalloproteinases; G-CSF, granulocyte-colony-stimulating factor; HCP, hematopoietic cell precursors.

Given the redundancy and overlap of ligand/receptor specificities in the chemokine system, one might expect that the more striking *in vivo* functional consequences of selective proteolysis would occur among those chemokines that maintain non-promiscuous partnerships with their receptors. Such may be the case for the chemokine CXCL12 or stromal cell-derived factor-1 (SDF-1) which, through interaction with its unique receptor CXCR4, orchestrates cardiovascular (15–17), neuronal (17, 18), and hematopoietic embryonic development (15–18) as well as bone marrow (BM) stem cell engraftment (19–21), postnatal homeostasis of several hematopoietic lineages (21), and regulates T or B lymphocyte trafficking (21, 22). SDF-1 and CXCR4 are constitutively and broadly expressed in tissues (23, 24). In addition to their physiological properties, increasing evidence emphasizes the participation of SDF-1 α /CXCR4 in the pathogenesis of infectious and inflammatory processes. CXCR4 plays an important role in HIV infection, because CXCR4 (along with CCR5) is one of the two major HIV coreceptors (25), and its unique ligand SDF-1 α has the capacity to prevent cell entry of CXCR4-dependent viral isolates (X4 virus) (26, 27) by both occupying and promoting down-regulation of CXCR4 (28). Moreover, new evidence implicates the SDF-1 α /CXCR4 pair in the pathogenesis of allergic airway diseases (29), rheumatoid arthritis (30–32), and through induction of platelet aggregation the development of atherosclerosis and thrombo-occlusive diseases (33). Thus, although SDF-1 α and CXCR4 are involved in the regulation of homeostatic processes, it becomes evident that they are also constituents of adaptive responses by inflammatory cells. Proteolytic modification of either of the partners may be a mechanism that ultimately regulates the functional activity of the SDF-1 α /CXCR4 pair. Two proteolytic activities, dipeptidyl peptidase IV (DPP IV) (34) and cathepsin G (CG) (35), which are released by leukocytes, can cleave SDF-1 α *in vitro*. However, the large diversity of soluble or cell-bound proteinases expressed by leukocytes and the possibility that a given substrate may be the target of diverse enzymes suggest that other enzymes could be involved in the regulation of SDF-1 α /CXCR4 interactions. Therefore, it becomes important to define the relative contribution of putative proteolytic activities released by leukocytes that regulate SDF-1 α functions. Moreover, it is important to determine whether the capacity of CXCR4 to interact with its ligand is modified by proteolysis. In this study, we report that SDF-1 α and its receptor CXCR4 are modified by limited proteolysis in the presence of secreted leukocyte proteinases. We identify leukocyte elastase (LE) as the major enzyme accounting for this processing. Proteolysis occurs at the amino terminus of both molecules and results in abrogation of CXCR4/SDF-1 α interaction and function.

EXPERIMENTAL PROCEDURES

Reagents

Salt-free lyophilized human leukocyte elastase (LE) was purchased from Calbiochem and reconstituted at 35 μ M in 50 mM sodium acetate, 200 mM NaCl, pH 5.5. Hepes, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenoltetrazolium bromide (MTT), Indo-1 AM probe, Triton X-100, EDTA, EGTA, *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide (a neutrophil elastase substrate), and *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (a neutrophil cathepsin G (CG) substrate) were purchased from Sigma. The proteinase inhibitors 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF) and *N*-[*N*-(1-3-*trans*-carboxirane-2-carbonyl)-L-leucyl]-agmatine (E64) were purchased from Roche Molecular Biochemicals. Pepstatin, 1,10-phenanthroline, aprotinin, and eglin C were obtained from Sigma. Specific and irreversible LE inhibitor *N*-methoxy-succinyl-Ala-Ala-Pro-Val-chloromethyl ketone (MeOSuc-AAPV-CMK) was obtained from Calbiochem. MeOSuc-AAPV-CMK, E64, and 1,10-phenanthroline compounds were dissolved in dimethyl sulfoxide (Me₂SO) of which the final working concentration was less than 0.1% v/v. The specific, reversible, and competitive inhibitor of CD26, Lys-[Z(NO₂)]pyrrolidine (36), was a kind gift from Dr. A. Hovanessian

(Institut Pasteur, Paris, France). Cytochalasin B and *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) were purchased from Sigma. Bovine serum albumin (BSA) and dextran were purchased from Sigma. Hanks' balance salt solution (HBSS), without calcium and magnesium, was purchased from Invitrogen. Horseradish peroxidase (HRP)-conjugated streptavidin was purchased from Zymed Laboratories Inc. (San Francisco, CA).

Antibodies

K15C is an anti-SDF-1 mouse monoclonal antibody (mAb) (IgG2a κ) that recognizes an epitope containing the three first amino acids of the chemokine (37). Antibody 12G5 (IgG2a), used as a phycoerythrin conjugate (BD PharMingen), is a mouse mAb directed against the second extracellular loop of CXCR4, and mAb 6H8 (IgG1 κ) recognizes amino-terminal residues 22–25 of CXCR4 (38).

Chemokine Synthesis

Wild type SDF-1 α (aa 1–67) and SDF-1 α -biotin (SDF-1 α -bt, aa 1–68) were synthesized by the Merrifield solid phase method on a fully automated peptide synthesizer (Pioneer, Applied Biosystems, Inc., Foster, CA). SDF-1 α -bt carries a single biotin molecule conjugated to a carboxyl-terminal Lys residue (Lys⁶⁸). The procedures used for SDF-1 α and SDF-1 α -bt synthesis were described previously (37, 39). The concentration of each chemokine was determined by amino acid analysis in 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 Applied Biosystems, Inc. The capacity of SDF-1 α -bt and wild type SDF-1 α to bind to and activate CXCR4 was compared and found to be identical (39). The synthetic SDF-1 α lacking the Lys¹-Pro²-Val³ sequence (SDF-1 α -(4–67)) (40) was generously provided by Dr. I. Clark-Lewis (Biomedical Research Center, University of British Columbia, Vancouver, Canada).

Cells

The CXCR4-positive Jurkat lymphoblastoid T cell line was obtained from the American Type Cell Collection (ATCC) (Manassas, VA). The HeLa P4.2 cell clone is stably transfected with a human CD4 cDNA and an HIV-LTR-driven *Escherichia coli* β -galactosidase reporter gene (41). HeLa 243 cells co-expressing both Tat and Env HIV-1 proteins (derived from the X4 pLai proviral molecular clone) were obtained from Dr. M. Alison (Hôpital Cochin, Paris, France) (41). Cell cultures were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (FCS), Glutamax, antibiotics (100 units/ml penicillin and 100 μ g/ml streptomycin), and methotrexate (2 μ M). Baby hamster kidney cells (BHK-21) (ATCC CCL-10) were maintained in Glasgow's modified Eagle's medium (Invitrogen) containing 5% FCS, 20 mM Hepes, and 10% tryptose phosphate.

Isolation of Human PBMC or PMN and Preparation of Proteinase-enriched Supernatants

Human peripheral blood mononuclear cells (PBMC) were isolated from healthy donors using Ficoll-Paque (Amersham Biosciences AB) density gradient centrifugation. PBMCs (2×10^6 cells/ml) were cultured at 37 °C in AIM V culture medium (Invitrogen) without FCS and supplemented with Glutamax and antibiotics (100 units/ml penicillin and 100 μ g/ml streptomycin). Supernatants of PBMC cultures (PBMC-SN) were collected at various time points by centrifugation ($1,500 \times g$ for 5 min at 25 °C) and further cleared of cell debris by ultracentrifugation ($18,000 \times g$ for 20 min at 4 °C) before use in SDF-1 α degradation experiments. Polymorphonuclear neutrophil leukocytes (PMN) were isolated at room temperature within 3 h of venipuncture using differential sedimentations on dextran (Sigma) and Ficoll-Paque (Amersham Biosciences) as described previously (42). Isolated cells (>95% PMN) were resuspended (5×10^6 cells/ml) and equilibrated at 37 °C for 5 min in HBSS supplemented with 1.3 mM CaCl₂ and 1 mM MgCl₂. Proteinase-enriched supernatants (PMN-SN) were obtained from activated and degranulated PMN. For this purpose, PMN were incubated with 5 μ g/ml cytochalasin B for 5 min at 37 °C, followed by addition of 0.5 μ M fMLP for 5 min with gentle agitation. Cell suspensions were rapidly cooled to 4 °C and sedimented by centrifugation ($1,700 \times g$ for 10 min at 4 °C). Thereafter, PMN-SN were collected and ultracentrifuged ($18,000 \times g$, for 15 min at 4 °C) to remove cell debris and stored at –80 °C until use. Pretreatment with cytochalasin B is necessary to disrupt cytoplasmic microfilaments and to favor degranulation during PMN activation (43). Cytochalasin B and/or fMLP was omitted from control samples, whereas in some experiments, fMLP was replaced with

human IL-8 at 12 nM or SDF-1 α over a range 0.01–1 μ M. Enzymatic activities of human neutrophil LE and CG were measured in PBMC-SN or PMN-SN by hydrolysis of their specific synthetic substrates, *i.e.* *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide and *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide, respectively, as described previously (44). As standards, we used serial dilutions of each purified enzyme over a wide range of concentrations.

Exposure of SDF-1 α to Leukocyte Proteinases

SDF-1 α -bt (1 μ M) or SDF-1 α (1 μ M) were incubated for different times at 37 °C in cell-free PBMC-SN or PMN-SN or with purified LE (diluted in AIM V medium when needed). Proteinase inhibitors were added for 25 min at 37 °C prior to incubation with the chemokine. Proteolytic reactions were carried out in a volume of 50 μ L.

Western Blot Analysis of SDF-1 α Proteolysis

Degradation of SDF-1 α -bt or SDF-1 α exposed to leukocyte proteinases was stopped with Laemmli buffer. Proteins were separated by SDS-PAGE on 16% acrylamide Tricine gels (Invitrogen) at 4 °C for 4 h and at 25 mA per gel. Gels were transferred to polyvinylidene difluoride Immobilon-P[®] membranes (<10-kDa cut-off, Millipore Corp., Bedford, MA) by semi-dry transfer for 1 h at 120 mA, and membranes were blotted with anti-SDF-1 mAb K15C (2 μ g/ml) to detect the amino-terminal part of the chemokine. After stripping in 0.1 M glycine, pH 2.3, and subsequent incubation in a phosphate-buffered saline (PBS) solution containing 1 M NaCl, pH 7.4, membranes were blotted with HRP-conjugated streptavidin to identify the carboxyl terminus of the chemokine. Visualization and quantification of protein bands were performed using an electronically cooled LAS-1000 plus charge-coupled device (CCD) camera system, and Image Gauge 3.4 software (Fuji Photo Film Co., Tokyo, Japan).

ESI-MS Analysis of SDF-1 α Proteolysis

Immediately after exposure of SDF-1 α -bt or SDF-1 α to leukocyte proteinases, chemokine fragments were isolated by reverse phase-high performance liquid chromatography (RP-HPLC), and samples were further analyzed by electrospray ionization mass spectrometry (ESI-MS). Briefly, SDF-1 α -bt degradation mixtures (150 μ L) were filtered (0.45 μ m) and injected on a Nucleosil 5C18 300 Å semi-preparative column (250 \times 10 mm) (Macherey-Nagel, Düren, Germany). By using a 28–48% linear gradient of acetonitrile in 0.08% aqueous trifluoroacetic acid for 20 min at a 6 ml/min flow rate, intact SDF-1 α -bt or SDF-1 α and their respective fragments were eluted within the 9–11-min zone. Moreover, no peaks were detected in the 9–11-min elution zone upon injection of PMN-SN, PBMC-SN, or purified LE blanks. All samples collected were lyophilized and analyzed by ESI-MS on an API 365 triple-quadrupole mass spectrometer (PerkinElmer Life Sciences).

Inhibition of HIV-1 Env-mediated Cell-to-Cell Fusion by SDF-1 α

Co-culture of HeLa cells expressing HIV-1 Env and Tat proteins (HeLa 243 cell clone) with CD4+/CXCR4+ HeLa P4.2 cells leads to HIV-1 Env-mediated cell fusion and Tat-dependent activation of an HIV LTR-driven β -galactosidase reporter gene. The enzymatic activity of β -galactosidase reflects the magnitude of the HIV-1 Env-mediated cell fusion. To assess the capacity of proteolyzed SDF-1 α to prevent HIV-1 Env-mediated cell-to-cell fusion (45), co-culture of 243 and P4.2 HeLa cells was carried out in the presence of intact SDF-1 α or cleaved SDF-1 α in PBMC-SN. β -Galactosidase activity was measured in the cell lysates according to the manufacturer's instructions using the β -galactosidase Reporter Gene Assay (Roche Molecular Biochemicals).

Analysis of CXCR4 Endocytosis Induced by SDF-1 α —Jurkat CXCR4+ cells (1×10^6 cells/ml) were incubated with SDF-1 α for 30 min at 37 °C in PBS-0.1% BSA or medium. Cells were then incubated for 3 min at room temperature in an acidic buffer (50 mM glycine, pH 2.3) that stops receptor endocytosis and removes CXCR4-bound SDF-1 α molecules that would mask CXCR4 recognition by the mAb 12G5. Jurkat cells were washed twice with ice-cold PBS, 0.1% BSA, before incubation with PE-conjugated mAb 12G5 (1:100) for 1 h at 4 °C. Samples were analyzed on a FACSCalibur flow cytometer (BD Pharmingen). Basal cell fluorescence intensity was determined using cells stained with a PE-conjugated IgG2a isotype control alone.

Chemotaxis Assay

Migration of CEMx174 cells, a human lymphoblastoid CXCR4+/CD4+ T cell line, was assessed in 48-well chambers (Neuro Probe Inc., Cabin John, MD) as described previously (37). Briefly, SDF-1 α or SDF-1 α -(4–67) was added to the lower well at different concentrations, in a

total volume of 30 μ L in 25 mM Hepes-buffered Dulbecco's modified Eagle's medium at pH 7.4 (chemotaxis medium). The chemotaxis chamber was then assembled using polyvinylpyrrolidone-free polycarbonate membranes with 8 μ m pore size (Costar, Cambridge, MA), and 50 μ L of CEMx174 cells (1×10^6 cells/ml), in chemotaxis medium without chemokine, was added to the upper well. After incubation for 4 h at 37 °C in a 5% CO₂ humidified incubator, the chamber was disassembled, and cells that migrated through to the lower wells were transferred to a working 96-well plate. The migrated viable cells were then quantitated by a sensitive and quantitative colorimetric assay using MTT staining (46). Viable cells reduced MTT, and the degree of MTT reduction, which corresponds to the relative cell number, was measured automatically with an enzyme-linked immunosorbent assay reader reading the absorbance at 590 nm.

Measurement of Cytosolic Free Calcium

Intracellular calcium levels were measured in a Wallac VICTOR² multilabel counter (EG & G Wallac, Turku, Finland) using Indo-1 loaded CEMx174 cells. Briefly, cells (5×10^6 cells/ml) were loaded with 5 μ M Indo-1/AM in HBSS buffer (140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM MgSO₄, 1.2 mM CaCl₂, 10 mM Hepes, 5 mM glucose, 0.3 mM KH₂PO₄, and 2 mM Na₂HPO₄), pH 7.0, for 30 min at 37 °C. Thereafter, an equal volume of HBSS, pH 7.4, containing 10% heat-inactivated FCS, was added, and the cell suspension was incubated for 30 min. After washing with HBSS containing 5% heat-inactivated FCS, pH 7.2, cells were resuspended at 5×10^6 cells/ml and maintained at room temperature in the dark until use. For calcium measurements, aliquots of this cell suspension were preincubated for 5 min at 37 °C in a 96-well flat bottom plate, in a total volume of 200 μ L (1×10^6 cells/ml) in HBSS supplemented with 5% heat-inactivated FCS, pH 7.4. Different concentrations of SDF-1 α or SDF-1 α -(4–67) were added at the indicated times. Cell suspensions were excited at 355 nm, and calcium levels were determined by monitoring fluorescence emissions at 405 nm (Ca²⁺-bound dye) and 485 nm (Ca²⁺-free dye) every 2 s at 37 °C. Free calcium concentration values were calculated from the ratio of emission fluorescence (405/485 nm) using the equation described by Grynkiewicz *et al.* (47), with a K_d value of 250 nM for Indo-1. The R_{max} value was obtained by lysing the cells with 0.3% Triton X-100, followed by an addition of excess EGTA (300 mM in Tris-HCl, pH 7.4) for R_{min} . All experiments were performed in triplicate wells.

Analysis of CXCR4 Proteolysis

Immunoprecipitation and Electrophoretic Separation of CXCR4—BHK-21 cell monolayers (5×10^6 cells) were infected (multiplicity of infection 50) with a recombinant defective Semliki Forest virus (SFV) (48) encoding either full-length sequences of β -galactosidase or human CXCR4 fused to the TETSQVAPA sequence (C9 peptide) encoded in the bovine rhodopsin (49). Fourteen hours after infection, cells were pulse-labeled for 15 min with [³⁵S]methionine and [³⁵S]cysteine (200 μ Ci/ml) and cultured for an additional 2 h. Thereafter, cells were detached with PBS, 2 mM EDTA and exposed to PMN-SN for 2 h at 37 °C. CXCR4 proteolysis was stopped by adding 100 μ L of PBS containing 0.2 mg/ml eglin C. Cell pellets were lysed in solubilization buffer made of 100 mM (NH₄)₂SO₄, 20 mM Tris-HCl, pH 7.5, 10% glycerol, 1% (w/v) *n*-dodecyl- β -D-maltoside and a proteinase inhibitor mixture (Roche Molecular Biochemicals). Lysates were incubated at 4 °C for 30 min, and insoluble debris was removed by centrifugation (14,000 \times g for 30 min). CXCR4 was immunoprecipitated using either 12G5 (10 μ g/ml) or 1D4 (10 μ g/ml) mAbs coupled to protein-G-Sepharose beads. The 1D4 antibody (National Cell Culture Center, Minneapolis, MN) recognizes the C9 peptide (49). Proteins were separated by SDS-PAGE on 10% acrylamide gels at 4 °C, and radiolabeled bands were quantified in a Molecular Dynamics PhosphorImager (Amersham Biosciences).

Flow Cytometry Analysis—Jurkat or CXCR4-expressing BHK-21 cells were incubated in the presence of PMN-SN or purified LE in serum-free AIM V medium for 2 h at 37 °C. Inhibition of LE activity was achieved by incorporating into the samples either MeOSuc-AAPV-CMK (3 μ M) or eglin C (0.40 mg/ml) before incubation with cells. Proteinase-exposed cells were washed with ice-cold PBS, 0.1% BSA and labeled with either mAbs 6H8 or PE-conjugated 12G5. Labeling by 6H8 was revealed by a secondary goat PE-conjugated anti-mouse antibody (BD Pharmingen). Antibody-labeled cells were fixed in PBS, 1% formaldehyde and analyzed by flow cytometry.

Binding of SDF-1 α to CXCR4

Jurkat cells were exposed to purified LE (3 μ M) for 2 h at 37 °C, and proteolysis was stopped by adding MeOSuc-AAPV-CMK (3 μ M). CXCR4

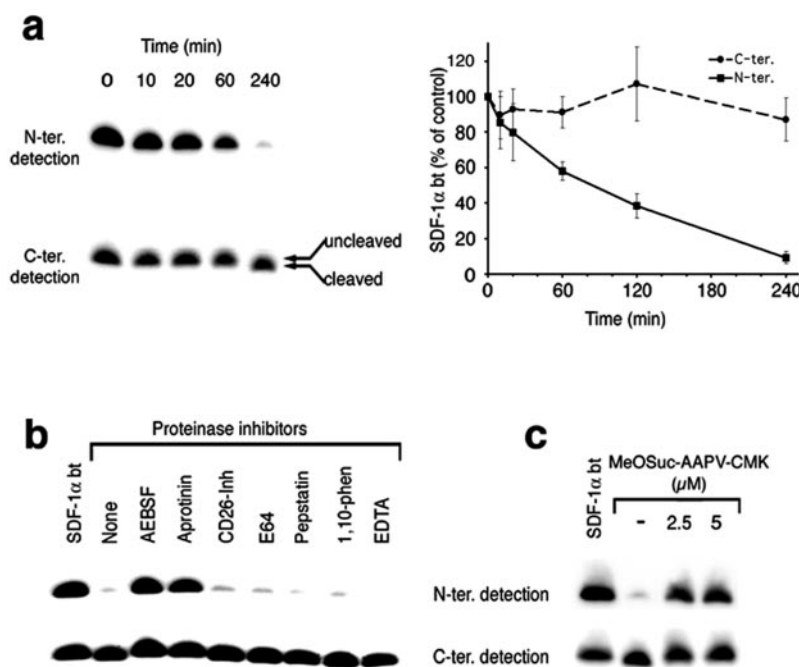


FIG. 1. Western blot analysis of SDF-1 α cleavage in PBMC-SN. *a*, synthetic SDF-1 α -bt (1 μ M) was incubated for up to 2 h in cell-free supernatants collected from 24-h PBMC cultures (2×10^6 cells/ml). Proteins were separated on Tricine/SDS-PAGE, transferred to polyvinylidene difluoride membranes, and blotted with anti-SDF-1 mAb K15C to detect the amino-terminal domain of the chemokine (*N-ter. detection*). After acid stripping, membranes were blotted with HRP-streptavidin to identify the carboxyl terminus of the chemokine (*C-ter. detection*). Quantification of chemiluminescence signals (*solid squares*, mAb K15C; *solid circles*, HRP-streptavidin) was performed using an electronically cooled CCD camera. Arrows indicate cleaved and uncleaved forms of the chemokine. *b*, SDF-1 α -bt was incubated for 12 h in PBMC-SN in the absence (*None*) or in the presence of the following proteinase inhibitors: 1 mM AEBSF; 10 μ M aprotinin; 30 μ M CD26 inhibitor (*CD26-Inh*); 2 μ g/ml E64; 1 μ M pepstatin; 5 mM 1,10-phenanthroline (*1,10-Phen.*); and 2.5 mM EDTA. *c*, SDF-1 α -bt was incubated in PBMC-SN for 4 h in the absence (—) or in the presence of the specific elastase inhibitor, MeOSuc-AAPV-CMK. *b* and *c*, amino-terminal and carboxyl-terminal domains of the chemokine were detected by Western blot analysis as described in *a*. The control (*SDF-1 α -bt lane*) was generated by incubating SDF-1 α -bt with AIM V medium instead of PBMC-SN. Illustrated is an experiment representative of six, in which LE and CG concentrations in PBMC-SN were 23 and 24 nM, respectively.

degradation was monitored by flow cytometry analysis with either 12G5 or 6H8 mAbs. Cells were incubated with 0.25 nM iodinated SDF-1 α (specific activity 2200 Ci/mmol; PerkinElmer Life Sciences) and competed with unlabeled SDF-1 α for 1 h at 4 $^{\circ}$ C in a final volume of 300 μ l. Incubations were terminated by centrifugation at 4 $^{\circ}$ C. Cell pellets were washed twice in ice-cold PBS. Nonspecific binding was determined in the presence of 1 μ M of unlabeled SDF-1 α . Cell pellet-associated radioactivity was counted using a microcomputer-controlled, 1272 ClinGamma counter (LKB-Wallac, Stockholm, Sweden). Data were analyzed using GraphPad Prism 2.0 software (GraphPad Software, Inc. San Diego, CA).

RESULTS

Proteolysis of SDF-1 α by Leukocyte Proteinases—Modification of SDF-1 α structure and function was investigated following *in vitro* exposure of the chemokine to human leukocytes. For this purpose, synthetic SDF-1 α carrying an additional Lys-conjugated biotin at the carboxyl terminus (SDF-1 α -bt, aa 1–68) was incubated with supernatants from blood mononucleated cells (PBMC-SN). SDF-1 α -bt was detected by Western blot analysis using either a monoclonal antibody (mAb) K15C recognizing an epitope encoded in the amino-terminal part of the chemokine (37) or by reacting HRP-coupled streptavidin with the carboxyl-terminal biotin. We observed that incubation of SDF-1 α -bt with PBMC-SN obtained from cells cultured for 24 h led to the rapid loss of the amino-terminal epitope recognized by the mAb K15C (Fig. 1*a*). Both the slight change on the electrophoretic mobility and the intactness of the carboxyl terminus region of SDF-1 α -bt, revealed by HRP-streptavidin labeling, proved the discrete modification undergone by the chemokine (Fig. 1*a*, arrows). These findings indicate the existence of a proteolytic mechanism that cleaves the amino-terminal domain of SDF-1 α .

To characterize the proteolytic activity accounting for the

discrete amino-terminal degradation of SDF-1 α -bt, chemokine was exposed to PBMC-SN in the presence of various proteinase inhibitors (Fig. 1*b*). Aspartic, cysteine, or metalloproteinase inhibitors (pepstatin, E64, and 1,10-phenanthroline or EDTA, respectively) failed to prevent degradation. A specific inhibitor of DPP IV, a proteolytic enzyme expressed by lymphocytes (CD26) shown previously to selectively cleave the amino-terminal part of SDF-1 α (34), also failed to inhibit degradation of the chemokine. In contrast, aprotinin and AEBSF efficiently prevented proteolysis of SDF-1 α -bt thus indicating that among leukocyte proteinases a serine proteinase accounts for degradation of SDF-1 α .

To identify the SDF-1 α residues cleaved by PBMC proteinases, we analyzed SDF-1 α -bt degradation products by electrospray ionization mass spectrometry (ESI-MS). For this purpose, SDF-1 α -bt (aa 1–68, Fig. 2*a*, *Control spectrum*) was incubated with PBMC-SN for 4 h at 37 $^{\circ}$ C and fractionated by RP-HPLC. Thereafter, eluted products were analyzed by ESI-MS. A polypeptide of molecular mass 7,973 Da was detected as the major molecular species generated upon cleavage and corresponded to an SDF-1 α -bt fragment lacking the amino-terminal Lys¹-Pro²-Val³ sequence (Fig. 2*a*, *PBMC-SN cleaved spectrum*). This finding confirms that the amino terminus of SDF-1 α is the target of the proteolytic attack by PBMC-secreted proteinases. To assess that the incorporation of a biotinylated Lys at the carboxyl-terminal end of the chemokine does not condition the pattern of proteolytic degradation undergone by SDF-1 α -bt, chemokine fragments generated by PBMC proteinases from either SDF-1 α -bt (8,298 Da) or a wild type, unmodified SDF-1 α (SDF-1 α ; aa 1–67, 7,831 Da) were analyzed by ESI-MS. The fragments were 7,973 Da and 7,507 Da, respectively, and corresponded in both cases to polypeptides lack-

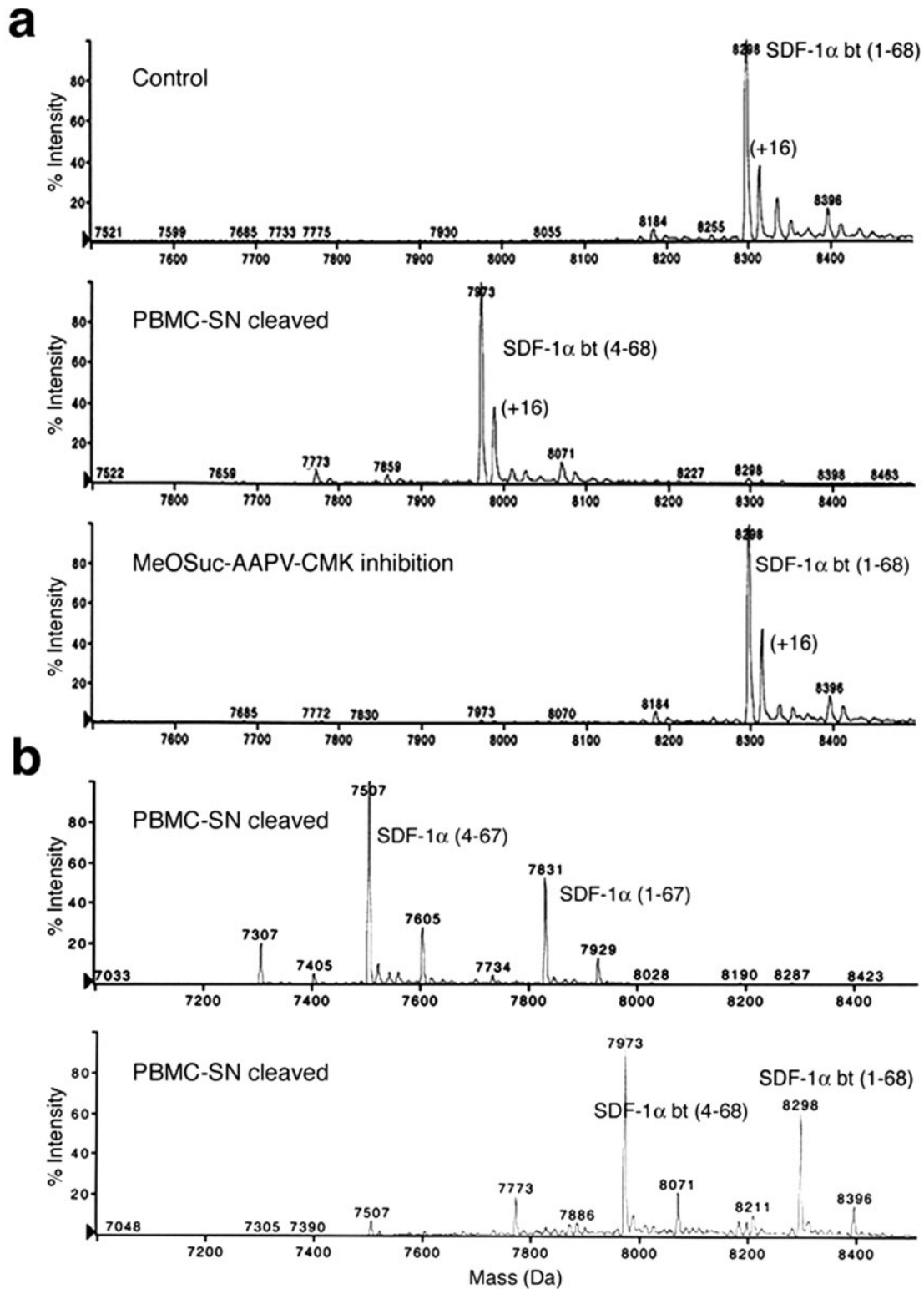


FIG. 2. **ESI-MS analysis of SDF-1 α -bt and SDF-1 α degradation in PBMC-SN.** *a*, synthetic SDF-1 α -bt (aa 1–68, 8,298 Da) (1 μ M) was incubated for 4 h at 37 $^{\circ}$ C in AIM V medium (*Control spectrum*), PBMC-SN (*PBMC-SN cleaved spectrum*), or PBMC-SN containing 0.5 μ M MeOSuc-AAPV-CMK inhibitor (*MeOSuc-AAPV-CMK inhibition spectrum*). Thereafter, chemokine derivatives were isolated by RP-HPLC and further analyzed by ESI-MS. *b*, SDF-1 α (aa 1–67, 7,831 Da) or SDF-1 α -bt (aa 1–68, 8,298 Da) (1 μ M) were incubated for 2 h at 37 $^{\circ}$ C in PBMC-SN and further analyzed by ESI-MS. *a* and *b*, SDF-1 α -bt-(1–68) of 8,298 Da and SDF-1 α -(1–67) of 7,831 Da represent the intact form of chemokines, whereas SDF-1 α -bt-(4–68) of 7,973 Da and SDF-1 α -(4–67) of 7,507 Da represent the corresponding cleaved forms of the molecules. The peaks noted (+16) correspond to the biotin-oxidized forms of SDF-1 α -bt that show an increase of 16 Da as compared with the nonoxidized counterparts. Illustrated is an experiment representative of six, in which LE and CG concentrations in PBMC-SN were 23 and 24 nM, respectively.

ing the Lys¹-Pro²-Val³ sequence (Fig. 2*b*, *PBMC-SN cleaved*, SDF-1 α -bt-(4–68) *lower* and SDF-1 α -(4–67) *upper* spectra). Thus, we conclude that both SDF-1 α -bt and the unmodified

SDF-1 α show identical susceptibility to the proteolytic attack by PBMC-secreted proteinases.

The pattern of SDF-1 α cleavage by PBMC proteinases is

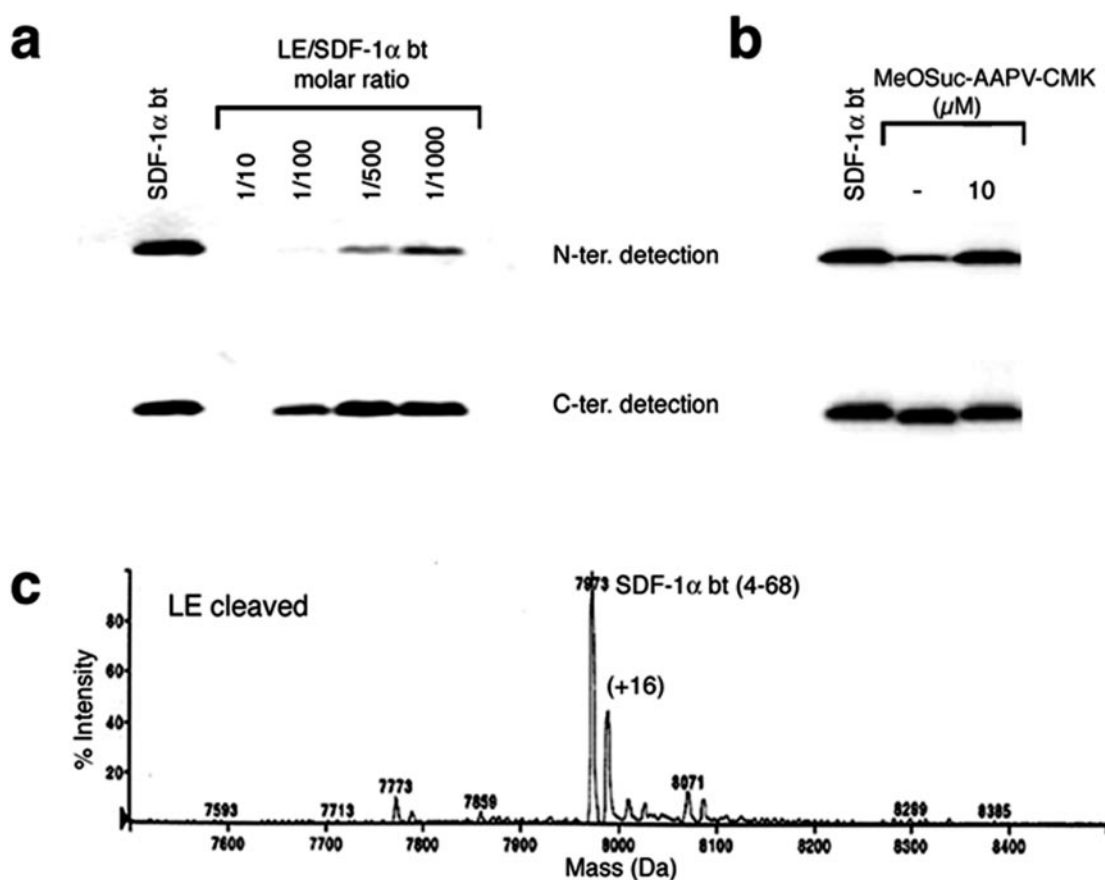


FIG. 3. Western blot and ESI-MS analysis of SDF-1 α -bt degradation by human purified LE. *a*, SDF-1 α -bt (1 μ M) was incubated for 6 h at 37 °C with purified human LE at different enzyme/chemokine molar ratios. *b*, SDF-1 α -bt (1 μ M) was incubated for 6 h at 37 °C with purified human LE at a 1:1000 (LE/SDF-1 α -bt) molar ratio in the absence (–) or in the presence of the specific LE inhibitor, MeOSuc-AAPV-CMK. *a* and *b*, the chemokine degradation was analyzed by Western blot as described in the legend to the Fig. 1, and the SDF-1 α -bt lanes represent controls of intact chemokine incubated in AIM V medium instead of LE. *c*, SDF-1 α -bt-(1–68) (1 μ M) was incubated for 6 h at 37 °C with purified human LE at a 1:100 (LE/SDF-1 α -bt) molar ratio, and ESI-MS analysis was performed as described in the legend to Fig. 2. SDF-1 α -bt-(4–68) of 7,973 Da represents the cleaved form of the chemokine obtained upon exposure to LE. Results from one representative experiment of three are shown.

compatible with the enzymatic activity characteristic of LE. LE is a serine proteinase that represents the major releasable enzyme in monocytes (50) and has been demonstrated to have proteolytic activity on various chemokines and cytokines (51). LE preferentially recognizes Pro-Val sequences and cleaves Val-X peptide bonds (52). Thus it is reasonable to postulate that LE may account for the removal of the Lys¹-Pro²-Val³ sequence of SDF-1 α when the chemokine is exposed to PBMC-SN, which has an average LE content of 18 nM \pm 2 nM (mean \pm S.E., n = 5). The cleavage of SDF-1 α -bt by purified human LE at low enzyme concentration (1 nM) supported this assumption (Fig. 3*a*), and ESI-MS analysis revealed a single degradation fragment of 7,973 Da (Fig. 3*c*), which corresponded to the SDF-1 α -bt-(4–68) polypeptide lacking the Lys¹-Pro²-Val³ sequence. Direct evidence identifying LE among other PBMC proteinases as the enzymatic activity hydrolyzing the SDF-1 α amino terminus was obtained by addition of the LE-specific inhibitor MeOSuc-AAPV-CMK (53) (Fig. 3*b*). Indeed, as shown by Western blot and ESI-MS analysis (Fig. 1*c* and Fig. 2*a*, MeOSuc-AAPV-CMK spectrum), degradation of SDF-1 α by PBMC-secreted proteinases was fully prevented in the presence of MeOSuc-AAPV-CMK. Using SDF-1 α we verified by ESI-MS analysis that biotinylation of the carboxyl terminus of SDF-1 α does not condition the susceptibility of the chemokine to proteolysis by purified LE (data not shown). Finally, when SDF-1 α -bt was directly exposed to PBMC suspensions (5×10^6 cells/ml), we observed that cleavage of the chemokine was identical to that described above for cell-free PBMC-SN or

purified LE, and MeOSuc-AAPV-CMK completely inhibited the degradation of the chemokine (data not shown). Together, these findings indicate that LE is the major proteolytic activity among PBMC-secreted proteinases that cleaves the amino-terminal end of SDF-1 α .

Although LE can be released by monocytes, PMN represents among leukocytes the major source of LE as well as of other serine proteinases such as cathepsin G (CG) and proteinase-3 (51). Stored in the azurophilic granules of PMN, the amount of LE is roughly 20-fold higher than in monocytes (50). PMN proteinases are released along with other microbicidal products upon inflammatory responses (54). This process may result in the proteolysis and regulation of the functional activities of SDF-1 α /CXCR4 as is the case for various cytokines and cytokine receptors (51). We thus examined the pattern of cleavage of SDF-1 α when exposed to PMN-secreted proteinases. PMNs were activated by the bacterial agonist fMLP, which promotes macrophages and PMN recruitment and activation at sites of inflammation (54). In keeping with previous reports (42, 44), measurement of enzymatic activities indicated that cell-free supernatants of fMLP-activated PMN (PMN-SN) contained both active LE and CG (see legend of Figs. 4–6). When SDF-1 α -bt was incubated in PMN-SN, Western blot analysis showed that cleavage of the chemokine at its amino terminus (Fig. 4*a*) occurred within minutes, with roughly 50% of the amino terminus of SDF-1 α -bt proteolyzed after 5 min of incubation, whereas the carboxyl terminus remained intact. As compared with PBMC-SN, PMN-SN degrades SDF-1 α -bt more efficiently,

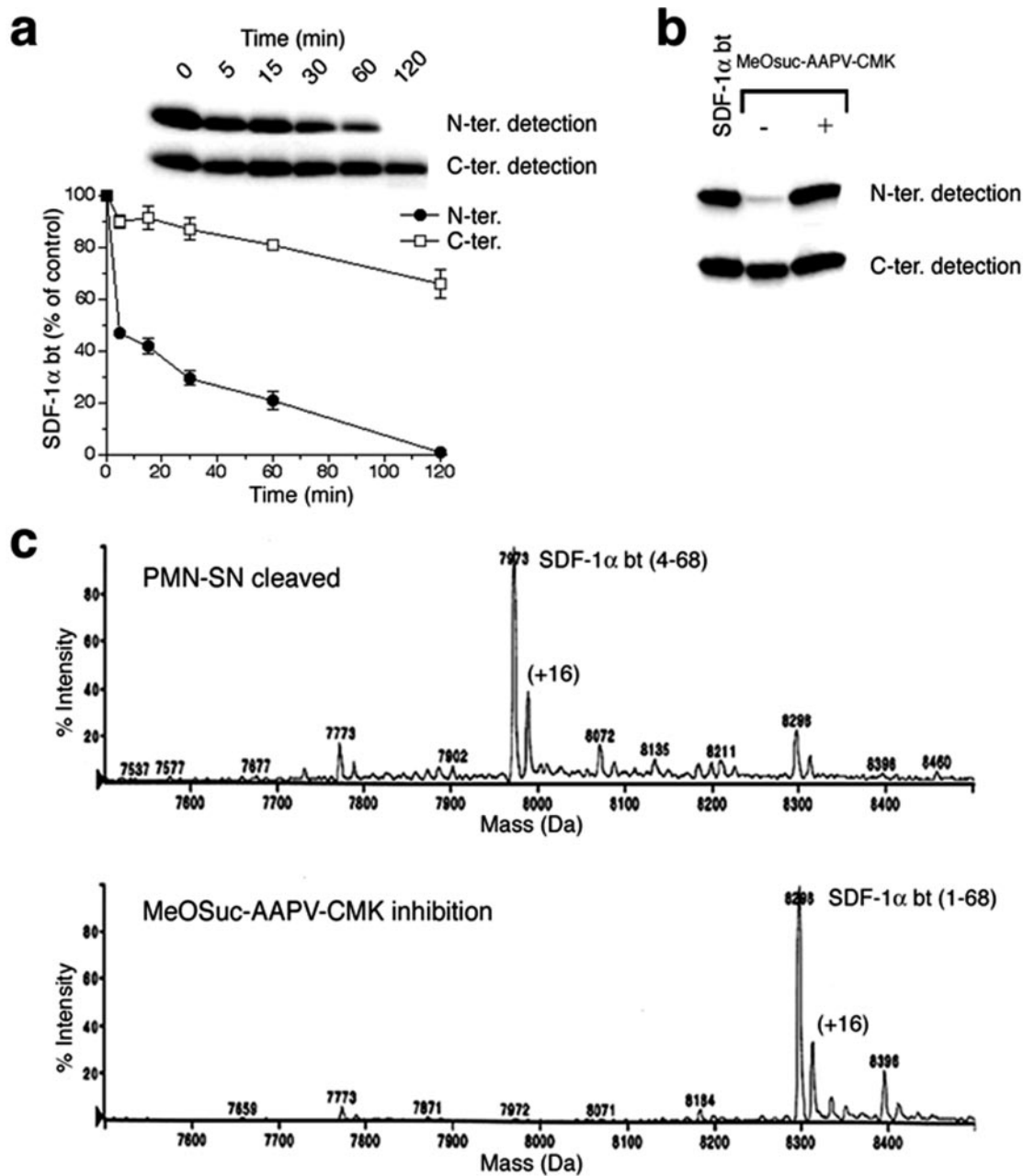


FIG. 4. Western blot and ESI-MS analysis of SDF-1 α -bt degradation by PMN-SN derived from fMLP-stimulated PMN. *a*, synthetic SDF-1 α -bt (1 μ M) was incubated for up to 2 h in cell-free supernatants from PMN (5×10^6 cells/ml) activated with 0.5 μ M fMLP in the presence of 5 μ g/ml cytochalasin B (PMN-SN). Chemokine degradation was analyzed by Western blot, and protein bands were quantitated as described in the legend to Fig. 1. Results are means \pm S.E. of three independent experiments. *b*, SDF-1 α -bt (1 μ M) was incubated for 2 h at 37 $^{\circ}$ C with PMN-SN in the absence (–) or in the presence (+) of 1 μ M MeOSuc-AAPV-CMK. The SDF-1 α -bt lane represents control of intact chemokine incubated in AIM V medium instead of PMN-SN. *c*, SDF-1 α -bt (1 μ M) was incubated for 4 h at 37 $^{\circ}$ C with PMN-SN either in the absence (PMN-cleaved spectrum) or in the presence of 1 μ M MeOSuc-AAPV-CMK (MeOSuc-AAPV-CMK inhibition spectrum). ESI-MS analysis was performed as described in the legend to Fig. 2. Illustrated is an experiment representative of three, in which LE and CG concentrations in PMN-SN were 390 and 230 nM, respectively.

with kinetics that correlate with the amount of secreted LE accumulated in the samples (compare Fig. 1*a* and 4*a*). ESI-MS analysis of isolated fragments identified a unique form showing a fragment of 7,973 Da that, as observed previously with PBMC-SN or purified LE, corresponds to a cleaved SDF-1 α polypeptide lacking the Lys¹-Pro²-Val³ sequence (Fig. 4*c*, PMN-SN cleaved spectrum). As shown by either Western blot or ESI-MS analysis, MeOSuc-AAPV-CMK fully prevented degradation of SDF-1 α -bt (Fig. 4, *b* and *c*, MeOSuc-AAPV-CMK inhibition spectrum), thus confirming the predominant role played by LE among PMN-secreted proteinases in the proteolysis of the chemokine. Similar findings were observed when

PMNs were induced by the inflammatory CXC chemokine IL-8, which orchestrates leukocyte trafficking during inflammatory responses and is known to induce PMN secretion of microbicidal products, including proteinases (55). As in the case of fMLP, the inhibitor MeOSuc-AAPV-CMK prevented proteolysis of SDF-1 α -bt exposed to PMN-SN from IL-8-induced PMN (data not shown). In keeping with previous reports showing that SDF-1 α , which is not an inflammatory chemokine and fails to induce degranulation from basophils (56), eosinophils (57), or mast cells (58), we observed that SDF-1 α did not induce protease release from PMN (data not shown).

The functional capacity of the LE-cleaved SDF-1 α was next

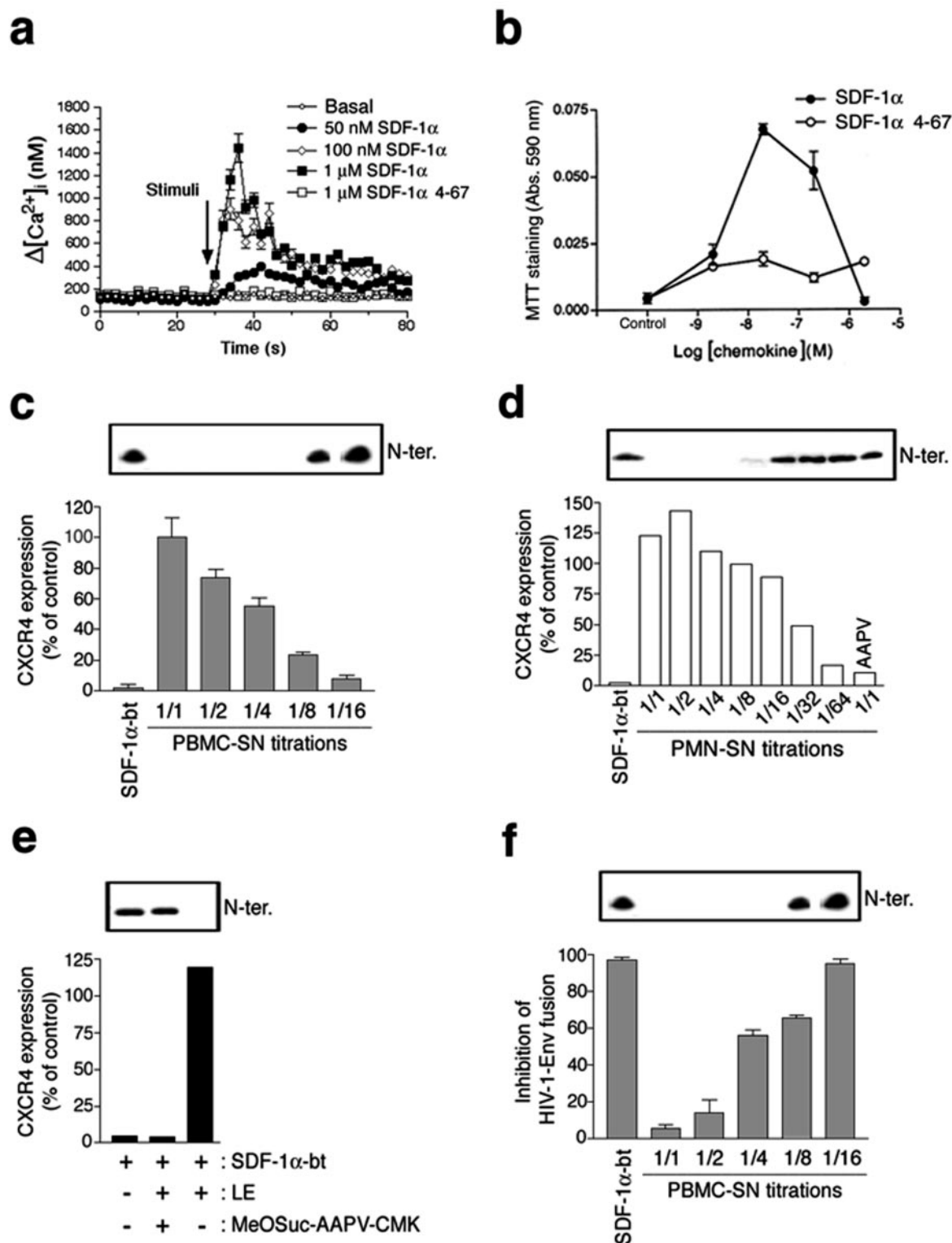


FIG. 5. Functional analysis of LE-cleaved SDF-1 α and synthetic SDF-1 α (4-67). *a*, intracellular calcium mobilization. A comparative analysis of SDF-1 α (4-67) versus SDF-1 α was performed using Indo-1-loaded CEMx174 cells at the indicated concentrations of chemokines. SDF-1 α (4-67) did not induce calcium mobilization at any concentration tested (ranging from 5 nM to 1 μ M), and the result obtained at 1 μ M is shown. Results are means \pm S.E. of three independent experiments. *b*, chemotaxis assay. Migration of CEMx174 lymphoblastoid cells was measured following stimulation with the indicated concentrations of SDF-1 α or SDF-1 α (4-67) for 3 h at 37 °C, and migrated viable cells were quantitated using a sensitive colorimetric MTT assay. Results are means \pm S.E. obtained from four independent experiments. *c*, *d*, and *e*, CXCR4 endocytosis. Jurkat cells were exposed to SDF-1 α -bt (1 μ M) previously treated for 2 h at 37 °C in serial dilutions of 24-h cell-free PBMC-SN (*c*) or serial dilutions of PMN-SN obtained from fMLP-activated PMN (*d*) or with 10 nM purified LE in the presence (+) or the absence (-) of MeOSuc-AAPV-CMK (0.05 μ M) (*e*). *d*, histogram labeled as AAPV refers to SDF-1 α -bt (1 μ M) treated by MeOSuc-AAPV-CMK (1 μ M) prior to incubation of the chemokine in PMN-SN (1/1 dilution). *c-e*, CXCR4 expression was analyzed by flow cytometry using a PE-conjugated 12G5 anti-CXCR4 mAb. A PE-conjugated IgG2a isotype was used as a control. Results are expressed as means \pm S.E. of three independent experiments and referred to CXCR4 expression in the absence of SDF-1 α -bt, taken as 100%. *f*, X4 HIV-1 Env-mediated cell fusion. Env- and Tat-expressing HeLa 243 cells were coincubated with CD4+/CXCR4+ HeLa P4.2 cells expressing a HIV LTR-driven β -galactosidase reporter gene. After fusion at a cell ratio of 1:1 for 16 h at 37 °C, β -galactosidase activity was measured in cell lysates. Results are means \pm S.E. of three independent

investigated. The non-promiscuous interaction between SDF-1 α and CXCR4 permits us to unambiguously assess the biological activity of the chemokine through the analysis of either the ligand-induced endocytosis or the HIV-coreceptor function of CXCR4 in the crude supernatants obtained from PBMC cultures or isolated PMN. These SDF-1 α -mediated functions require binding to CXCR4, are non-pertussis toxin-sensitive, and do not require G-protein-mediated signaling in lymphocytes (28).² To this purpose, 1 μ M SDF-1 α -bt was incubated with serial 2-fold dilutions of either PBMC-SN or PMN-SN, and samples were assessed for their capacity to promote CXCR4 endocytosis in Jurkat T cells. Expression of CXCR4 at the cell surface was investigated using a specific mAb (12G5) that recognizes a conformational epitope in the second extracellular loop of the receptor (59). Because binding of SDF-1 α to CXCR4 competes with the mAb 12G5 for CXCR4 occupation, residual chemokine bound to its receptor was removed from the cell surface by acidic washes before labeling with 12G5. Serial dilutions of PBMC-SN (Fig. 5c) or PMN-SN (Fig. 5d) resulted in a progressive increase in the capacity of the samples to promote CXCR4 endocytosis, which correlated with the amount of intact SDF-1 α -bt (see Western blots, *top insets* in Fig. 5, c and d). The capacity of SDF-1 α to prevent HIV-1 Env-dependent cell-to-cell fusion largely relies on the capacity of the chemokine to promote CXCR4 endocytosis (Fig. 5f). Following incubation of SDF-1 α -bt with PBMC-SN and in agreement with the impaired ability of the chemokine to induce CXCR4 endocytosis, the capacity of SDF-1 α to prevent HIV-1 Env-mediated cell fusion was severely reduced. It should be noted that when PBMC-SNs were used as a source of proteinases, a discrete receptor endocytosis and inhibition of HIV Env-dependent cell membrane fusion were observed in some samples in the absence of detectable intact SDF-1 α -bt by Western blot analysis, which is less sensitive than the functional assays (Fig. 5, c and f, see *dilution 1/4*). This phenomenon is likely accounted by remnant, intact chemokine not degraded by the relatively low accumulation of LE in PBMC-SN. This assumption was confirmed by the complete lack of CXCR4 endocytosis and HIV inhibitory capacity shown by the synthetic SDF-1 α -(4–67) (data not shown). Finally, exposure of 1 μ M SDF-1 α to 10 nM purified LE totally abrogated the capacity of the chemokine to induce CXCR4 endocytosis (Fig. 5e), and incubation of the undiluted PMN-SN with MeOSuc-AAPV-CMK (Fig. 5d, AAPV) preserved the CXCR4 endocytosis capacity of SDF-1 α -bt.

The capacity of the LE-cleaved SDF-1 α to trigger pertussis toxin-sensitive, G-protein-dependent intracellular signaling was assessed in lymphocytes by measuring cytosolic calcium mobilization and cell chemotaxis. Because of the potential presence of unidentified ligands different from SDF-1 α in the PBMC-SN or PMN-SN capable of inducing cell signaling independently of CXCR4 activation, these experiments necessitated the use of a synthetic SDF-1 α polypeptide lacking the Lys¹-Pro²-Val³ sequence cleaved by LE from SDF-1 α . Our results indicate that, in contrast to the unmodified SDF-1 α (aa 1–67), the truncated SDF-1 α -(4–67) (kindly provided by Dr. I. Clark-Lewis, University of British Columbia, Vancouver, Canada) failed to mobilize calcium from intracellular stores

(Fig. 5a) and to promote chemotaxis of CXCR4-expressing lymphoid cells (Fig. 5b).

We conclude that among proteinases expressed and secreted by human leukocytes, a serine proteinase indistinguishable from LE is responsible for the selective proteolysis of the amino terminus that inactivates SDF-1 α .

Degradation of CXCR4 by Leukocyte Proteinases—Similar to other G-protein-coupled receptors, the amino-terminal part of CXCR4 represents an exposed domain susceptible to the attack by proteinases. Proteinase-activated (60) and bradykinin B₂ (61) receptors are regulated through selective cleavage of their amino-terminal domains by serine proteinases. Because the extracellular CXCR4 amino terminus is a major determinant for high affinity binding of SDF-1 α , we investigated whether CXCR4 is prone to cleavage by leukocyte proteinases.

The effect of PMN-derived proteinases on the cell surface expression of CXCR4 was first assessed in BHK cells (Fig. 6a), transiently expressing functional human CXCR4 from an SFV-derived vector. Antibodies directed against specific regions of CXCR4 were used to look for molecular evidence of CXCR4 cleavage by leukocyte proteinases. The mAb 6H8 recognizes an epitope encompassing amino-terminal residues 22–25 (38). Binding of mAb 6H8 was reduced by 80% after exposure of BHK cells to PMN-SN containing proteinases and only to 50% when PMN-SN was diluted (Fig. 6a, 1/1 and 1/4 dilutions, respectively). In contrast, binding of mAb 12G5, which recognizes a conformational epitope located in the second extracellular loop, remained relatively unaffected. Like MeOSuc-AAPV-CMK, eglin C blocks LE but displays a broader inhibitory spectrum on other leukocyte serine proteinases (62). Preincubation of PMN-SN with MeOSuc-AAPV-CMK or eglin C efficiently prevented loss of mAb 6H8 binding to its amino-terminal epitope on CXCR4 (Fig. 6a), demonstrating involvement of a proteolytic mechanism in 6H8 epitope disappearance. In the presence of eglin C, maintenance of amino-terminal CXCR4 expression was slightly better than that obtained with MeOSuc-AAPV-CMK. Although the role of other serine proteinases cannot be formally excluded, it can be assumed that a proteolytic activity sensitive to MeOSuc-AAPV-CMK, *i.e.* LE, accounts for most of the loss of the amino-terminal domain of CXCR4. Experiments conducted in Jurkat T cells constitutively expressing CXCR4 led us to similar conclusions (Fig. 6b). Although the putative CXCR4 degradation occurs preferentially at the amino terminus, longer exposure (more than 2 h) of cells to undiluted PMN-SN resulted in progressive loss of the epitope defined by the mAb 12G5 (data not shown). The biological relevance of this phenomenon is uncertain and may reflect the attack of CXCR4 at secondary cleavage sites either by LE or other serine proteinases.

More direct evidence supporting the amino-terminal degradation of CXCR4 by leukocyte proteinases was obtained by electrophoretic separation of the metabolically radiolabeled receptor from BHK cells exposed to PMN-SN. Expression of ectopic genes from infectious SFV vectors allows robust and predominant expression of vector-encoded proteins, while causing almost complete shut-off of host cell protein synthesis (48). A protein migrating with a relative molecular mass of 40 kDa corresponded to the expected size of full-length CXCR4 (25) (Fig. 6c). The nature of the lower labeled band is unclear and

² A. Amara and F. Arenzana-Seisdedos, unpublished observations.

experiments. Values are represented as the percentage of fusion inhibition referred to the intact SDF-1 α -bt. c–e, control histograms labeled SDF-1 α -bt were obtained by incubating SDF-1 α -bt (1 μ M) in AIM V medium instead of cell supernatants. After degradation of SDF-1 α -bt in PBMC-SN (c and f), PMN-SN (d), or with purified LE (e), LE and CG activities were blocked by addition of 0.40 mg/ml of eglin C prior to incubation of the treated chemokine with CXCR4+ cells. SDF-1 α -bt was detected by Western blot using mAb K15C (c–f, *top inserts*) as described in the legend to Fig. 1. LE and CG concentrations in experiments shown in c, d, and f were 542 and 334 nM, respectively for PMN-SN, and 23 and 24 nM, respectively for PBMC-SN.

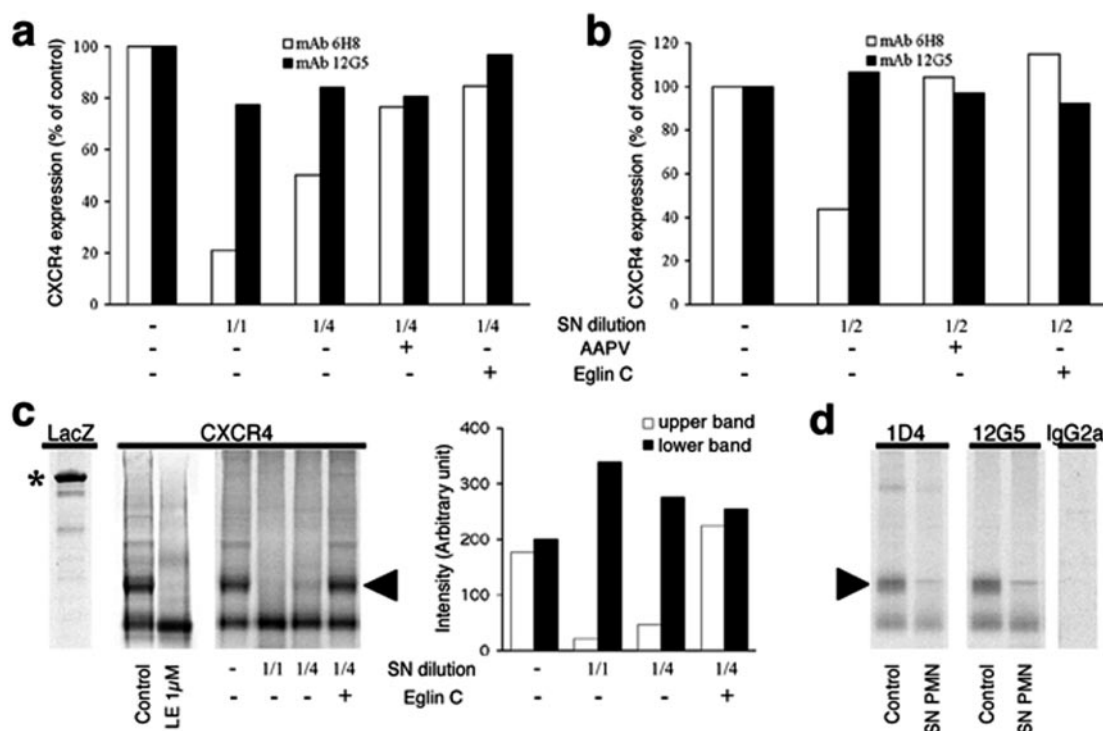


FIG. 6. Proteolysis of CXCR4 exposed to PMN-SN or purified LE. For flow cytometry analysis, CXCR4-expressing BHK (a) or Jurkat (b) cells (2×10^6 cells/ml) were exposed for 2 h at 37 °C to dilutions of PMN-SN obtained from fMLP-activated PMN. PMN-SN were treated (+) or not (-) either with 1 μ M MeOSuc-AAPV-CMK (AAPV) or 0.40 mg/ml eglin C (*Eglin C*) for 1 h at 37 °C prior to incubation with cells. The expression of CXCR4 was assessed with anti-CXCR4 mAbs 6H8 or PE-conjugated 12G5. In the case of 6H8, cells were stained with goat PE-conjugated anti-mouse immunoglobulins. Finally, cells were fixed and analyzed by flow cytometry. For SDS-PAGE immunoprecipitation, BHK cells expressing C9-tagged CXCR4 were 35 S-labeled before being exposed to PMN-SN (c and d) or to 1 μ M purified LE (c) for 2 h at 37 °C. PMN-SN were treated (+) or not (-) with 0.2 mg/ml eglin C (c, *Eglin C*) prior to incubation with cell cultures. CXCR4 from cell lysates were either directly separated by SDS-PAGE (c) or immunoprecipitated using either anti-CXCR4 mAb 12G5 or mAb 1D4 directed against the C9 tag (d). Irrelevant IgG2a were used as control (d). c, 35 S-labeled bands were quantitated in a PhosphorImager device, and the *LacZ* lane is a control obtained from cells infected with an SFV vector expressing *lacZ* instead of CXCR4. The asterisk indicates the band corresponding to the *lacZ* product. c and d, the full-length CXCR4 species are indicated by arrowheads and correspond to a mass of 40 kDa. Illustrated is an experiment representative of three, in which LE and CG concentrations in PMN-SN were 542 and 334 nM, respectively.

may correspond to a precursor of CXCR4 (Fig. 6c). Immunoprecipitation with either mAb 12G5 or mAb 1D4 directed against the C9 tag at the carboxyl terminus of the CXCR4 cytoplasmic tail confirmed that the two major radiolabeled bands corresponded to CXCR4 (Fig. 6d). Following exposure of cells to PMN proteinases, the amount of full-length CXCR4 was drastically reduced in the total cell lysates (Fig. 6c) and in 1D4 or 12G5 immunoprecipitates (Fig. 6d). Quantification of dried gels showed accumulation of a truncated protein generated upon cleavage of CXCR4 by PMN proteinases at the level of the lower CXCR4 form (Fig. 6c, *histograms*). Incubation in the presence of eglin C prevented degradation of full-length CXCR4 by PMN proteinases and restored the original ratio between the high 40-kDa full-length and the low M_r forms (Fig. 6c). Finally, direct incubation of CXCR4-expressing BHK cells with purified LE (1 μ M) resulted in a similar disappearance of full-length CXCR4 molecules and accumulation of the lower M_r form (Fig. 6c). Based on both antibody epitope mapping and biochemical profiles of CXCR4 degradation, it can be assumed that the CXCR4 amino terminus bears a primary cleavage site for proteolysis by the PMN serine proteinase elastase. Although our findings do not formally rule out the existence of discrete, alternative cleavage sites, they exclude the possibility of extensive CXCR4 degradation following proteolysis of other extracellular domains of the receptor.

The consequences of CXCR4 degradation on the binding of SDF-1 α were next investigated in Jurkat cells. Cells were exposed to purified LE (3 μ M), and cleavage of CXCR4 was monitored by antibody labeling followed by flow cytometry

analysis (Fig. 7a). As expected, LE-treated Jurkat cells lost the amino-terminal 6H8 epitope, whereas the epitope detected by mAb 12G5 was preserved (Fig. 7a). Cells were then exposed to LE for 2 h at 37 °C, washed, and incubated with MeOSuc-AAPV-CMK to inactivate any residual proteolytic activity before addition of iodinated SDF-1 α . Our findings show that LE-cleaved CXCR4 is profoundly impaired in its capacity to interact with SDF-1 α (Fig. 7b). Lack of specific binding by the digested receptor can be explained by cleavage of the SDF-1 α -binding motif at the CXCR4 amino terminus. This conclusion is reinforced by maintenance of SDF-1 α binding to CXCR4 (Fig. 7b) and receptor recognition by mAb 6H8 (Fig. 7a, *solid square*) when LE was previously inactivated by MeOSuc-AAPV-CMK. This result excludes the possibility that lack of high affinity interaction of SDF-1 α with cleaved CXCR4 was due to competition or steric hindrance mediated by interaction of LE with CXCR4.

DISCUSSION

Our findings show an unprecedented proteolytic inactivation of both a chemokine and its corresponding receptor. Our results demonstrate that limited degradation by leukocyte proteinases, restricted to the amino-terminal domains of both SDF-1 α and CXCR4, abrogates their interaction and function.

Among leukocyte proteinases, we identify LE as the predominant enzymatic activity responsible for limited proteolysis of the amino-terminal domains of both SDF-1 α and CXCR4. As concerns SDF-1 α , our conclusions are supported by manifold evidence. First, incubation with purified human LE led to rapid

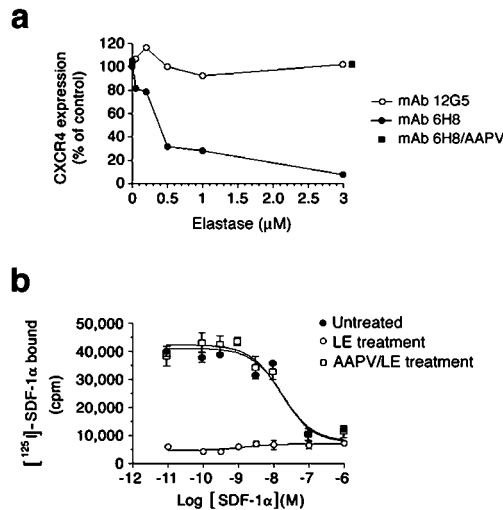


FIG. 7. Binding of ^{125}I -SDF-1 α to LE-treated CXCR4+ Jurkat cells. *a*, Jurkat cells (2×10^6 cells/ml) were pretreated with different concentrations of purified human LE for 2 h at 37 °C. Proteolytic reactions were then stopped with 3 μM MeOSuc-AAPV-CMK before addition of anti-CXCR4 mAbs 12G5 (open circles) or 6H8 (solid circles). The solid square corresponds to cells exposed to LE pretreated with 3 μM MeOSuc-AAPV and subsequently labeled with mAb 6H8. Flow cytometry was performed as described in the legend to Fig. 6. Results are representative of three independent experiments. *b*, Jurkat cells were either left untreated (solid circles), exposed to 3 μM purified LE for 2 h at 37 °C (open circles), or to LE previously inhibited with 3 μM MeOSuc-AAPV-CMK (open squares). Thereafter, cells were incubated with 0.25 nM ^{125}I -SDF-1 α and competed with unlabeled SDF-1 for 1 h at 4 °C. Incubations were terminated by centrifugation at 4 °C, and cell pellets were washed twice before counting the associated radioactivity. Nonspecific binding was determined in the presence of 1 μM of unlabeled SDF-1 α . Results are means \pm S.E. of six independent experiments. Non-linear analysis of the competition curves yielded IC_{50} binding values as follows: untreated cells (solid squares), 6.6 ± 0.23 nM; MeOSuc-AAPV-CMK inhibition (open squares), 5.7 ± 0.4 nM; LE-treated cells (open circles), unfitted curves.

degradation of the SDF-1 α amino terminus, detectable at LE concentrations in the low nanomolar range. Second, as observed for the purified enzyme, the effect of proteinases released by leukocytes is manifested rapidly and affects the amino terminus of SDF-1 α . The irreversible, highly specific leukocyte elastase inhibitor MeOSuc-AAPV-CMK (53), a chloromethyl ketone peptide devoid of known activity on other proteinases, abolished degradation of SDF-1 α exposed to leukocyte proteinases. Third, ESI-MS analysis of SDF-1 α exposed to either PBMC-SN or PMN-SN revealed a major degradation product lacking the three amino terminus residues Lys¹-Pro²-Val³, identical to that observed after incubation of the chemokine with purified LE. Fourth, only the uncleaved form of SDF-1 α was identified by ESI-MS analysis when the chemokine was exposed to leukocyte proteinases in the presence of the LE inhibitor, MeOSuc-AAPV-CMK.

A previous report (35) identified CG as the enzyme responsible for the cleavage of the amino-terminal domain of SDF-1 α when exposed to lymphocyte-associated proteinases. CG removes the five first residues of the chemokine by hydrolyzing the peptide bond between Leu⁵ and Ser⁶. However, in our hands, complete inhibition of SDF-1 α degradation by the specific LE inhibitor together with ESI-MS analysis of the fragment generated from SDF-1 α , *i.e.* SDF-1 α -(4–67), unambiguously identified LE among leukocyte proteinases as the enzyme accounting for cleavage and inactivation of SDF-1 α . The prominent role of CG reported by Delgado *et al.* (35) could be due to the fact that the search for SDF-1 α degradation was restricted to detergent-solubilized proteins extracted from fractionated lymphocyte membranes. The amino peptidase DPP IV/CD26

isolated from lymphoblastoid T cells (63) has been shown to remove Lys¹-Pro² residues from SDF-1 α . However this cleavage was not observed in our experiments even when the chemokine was exposed either to resting or phytohemagglutinin-blasted PBMC (data not shown), a treatment known to reinforce the expression of DPP IV/CD26 on PBMC (34). Low amounts of DPP IV/CD26 and/or the slow degradation kinetics of SDF-1 α shown by DPP IV/CD26 (63) may explain the undetectable DPP IV/CD26-mediated degradation of SDF-1 α when exposed to primary leukocytes.

Purified matrix metalloproteinases (MMP) MMP-2 and MMP-9 have been shown to cleave SDF-1 α between Ser⁴ and Leu⁵ residues (64).³ Following PMN degranulation induced by fMLP, we detected the presence of MMP-9 in the PMN-SN (data not shown). Incubation of SDF-1 α with either PBMC- or PMN-secreted proteinases did not generate the expected SDF-1 α -(5–67) fragment, and preincubation with 1,10-phenanthroline, a potent inhibitor of MMP, failed to prevent the amino-terminal degradation of SDF-1 α (Fig. 1b).³ The predominant accumulation of inactive, MMP-9 precursor forms in PMN-SN (data not shown) and/or the presence of natural MMP inhibitors (65) may explain the absence of SDF-1 α proteolysis by leukocyte-secreted MMP.

In this report we show that the discrete proteolysis of the SDF-1 α amino terminus by LE removes a domain encoding the critical Lys¹-Pro² residues involved in the activation of CXCR4 (40) and generates an inactive chemokine. Cleavage of the receptor activation motif of the chemokine is likely facilitated by the exposed backbone of the disordered amino-terminal region, which contains the binding site 2 (aa 1–8) for CXCR4. It has been reported that a synthetic SDF-1 α lacking the three amino-terminal residues (Lys¹-Pro²-Val³) removed by LE proteolysis presented a 100-fold decrease affinity for CXCR4 (40). Thus, it can be concluded that LE generates an SDF-1 α fragment lacking both the key motifs for both binding and activation of CXCR4. Besides the inability to trigger CXCR4 activation, LE-truncated SDF-1 α fails to prevent HIV-1 Env-mediated cell-to-cell fusion, a phenomenon that requires both occupancy and endocytosis of CXCR4. SDF-1 α shows a constitutive expression in many tissues including anatomical sites where HIV replication takes place (24, 66–68). It is reasonable to postulate that LE-mediated inactivation of SDF-1 α would result in the damage of the natural SDF-1 barrier that likely oppose spread of X4 HIV isolates.

Modification of G-protein coupled receptors by proteolysis has been noted previously. The proteinase-activated receptors (PAR) are the paradigm of this type of receptors (60). Typically, excision of part of their amino-terminal regions by thrombin (69) and other serine proteinases (70, 71) generates a tethered ligand with agonist capacity that ultimately activates the receptor. However, if activation is the physiological consequence of PAR proteolysis, proteinases can also inactivate the PAR upon cleavage and deactivate the tethered ligand (60). Interestingly, this is the case for LE that cleaves the thrombin receptor PAR-1 at peptide bonds other than the thrombin-activating site and removes the tethered ligand domain, thus preventing subsequent activation of the receptor (72). Similarly, proteinases accumulated in PMN-SN cleaves the CXCR4 amino terminus as indicated by the disappearance of both the epitope recognized by mAb 6H8 (aa 22–25) (38) and the characteristic 40-kDa form of the protein corresponding to the mature receptor. A similar pattern of CXCR4 excision was obtained with purified human LE. Moreover, the blockade of the

³ A. Valenzuela-Fernández, T. Planchenault, F. Baleux, and F. Arenzana-Seisdedos, unpublished results.

cleavage of CXCR4 by the specific inhibitor MeOSuc-AAPV-CMK confirmed the predominant role played by the PMN-secreted LE in the inactivation of the receptor.

LE cleaves peptide bonds preferentially after Val and Ala residues, although alternatively it can proteolyze Thr-X or Ile-X bonds (52, 72, 73). Several putative sites for LE-cleavage are encoded in the amino-terminal domain of the CXCR4 receptor among which hydrolysis at Ala³⁴ or Ile³⁹ is compatible with the phenotypic pattern of degradation that we observed. Cleavage of the amino-terminal domain of CXCR4 renders the receptor unable to bind SDF-1 α . In keeping with the two-site model of SDF-1 α /CXCR4 interaction (40), the absence of specific binding of SDF-1 α to the LE-cleaved receptor is likely determined by the lack of a functional CXCR4 amino terminus. According to this model, the binding of the SDF-1 α RFFESH loop (aa 12–17) with the amino-terminal domain of CXCR4 (binding site 1) permits initial docking of the chemokine. Subsequently, contact between the amino-terminal residues 1–8 of the docked chemokine and the groove at the top of the CXCR4 helices (binding site 2) determines CXCR4 activation (40). This model is supported by experimental evidence showing that point mutation of acidic residues or deletion of 20 amino-terminal residues of CXCR4 leads to a profound decrease of the receptor binding capacity for SDF-1 α (38, 74). Interestingly, although CXCR4 lacking a functional amino-terminal domain fails to bind SDF-1 α (38, 74), it retains substantial capacity to function as an HIV coreceptor (38, 59, 74). Thus, it is conceivable that proteolytic inactivation of SDF-1 α /CXCR4 interactions interferes with the natural barrier opposing infection by HIV X4 isolates while allowing virus propagation through a cleaved, yet functional HIV coreceptor. This could be favored by the higher susceptibility shown by SDF-1 α to LE-mediated proteolysis as compared with CXCR4, which requires higher concentrations of LE to undergo detectable proteolysis. Accumulation of LE at a concentration higher than that used in our experiments has been reported under physiopathologic conditions. Thus, in the confined injured tissue environment of inflammatory foci, the level of PMN may increase up to 100-fold (75) as compared with their circulating levels. Moreover, in the epithelial lining fluid of patients with cystic fibrosis, the concentration of catalytically active LE range from 1 to 80 μ M (76). It can be speculated that high levels of locally active LE could be also attained under supraphysiologic stimulation induced by injection of G-CSF that mobilizes and promotes accumulation of PMN in the bone marrow (77).

Beyond participation in inflammatory or infectious processes, disruption of the SDF-1 α /CXCR4 partnership by leukocyte proteinases could play a role in the regulation of the mobilization of hematopoietic cell precursors (HCP) from the BM. CXCR4 is expressed at the cell membrane of HCP including pluripotent CD34⁺ cells (78–81) and is required for confining HCP in hematopoietic organs for either myelopoiesis or B lymphopoiesis (21, 22). SDF-1 α is a potent attractant of immature and mature HCP (19, 82–85) and is expressed in BM by stromal and endothelial cells and osteoblasts (15, 86). Therefore, CXCR4/SDF-1 α cell signaling plays an essential role in BM-anchoring of HCP, chemotaxis of several hematopoietic cell lineages, and controls leukocyte recirculation. Recently, it has been shown that G-CSF administration induces BM accumulation of granulocytes (87, 88) and promotes LE- or CG-mediated proteolysis of BM stromal vascular cell adhesion molecule-1/CD106, which interacts with very late antigen 4 expressed at the surface of HCP (77). Concomitant degradation of SDF-1 α /CXCR4 by granulocyte-released LE may magnify perturbation of cell adhesion mechanisms and contribute to the release of HCP into the periphery. In this regard, the intriguing associ-

ation of LE gene (*ELA2*) inactivating mutations with severe sporadic (89) or familial congenital neutropenia (Kotlmann's syndrome (90)), as well as with cyclic neutropenia (89, 91), is compatible with the hypothesis that impairment of LE-mediated degradation of CXCR4 and/or SDF-1 α contributes to the pathogenic mechanisms of these syndromes. Based on these observations, we suggest that LE-mediated proteolysis of one or both partners would inactivate and terminate SDF-1 α /CXCR4-dependent cell signaling and may be a component of mobilization of PMN deployment from BM. Interestingly, genetically determined neutropenia are corrected by infusion of G-CSF (92). In this case, supraphysiologic exocytosis of other proteinases (*i.e.* CG or MMP) released as a consequence of IL-8 activation in response to G-CSF-administration (93) may account for the inactivation of SDF-1 α /CXCR4 interactions, thus permitting egress of cells from BM. At the light of the present findings, it may be expected that animal models based on the transgenic expression of proteinase genes or the administration of hematopoietic regulatory cytokines will shed light on both the complexity and biological relevance of the proteolytic regulation of the SDF-1 α /CXCR4 couple.

Acknowledgment—We thank Dr. Susan Michelson for helpful discussion and critical reading of the manuscript.

REFERENCES

- Luster, A. D. (1998) *N. Engl. J. Med.* **338**, 436–445
- Locati, M., and Murphy, P. M. (1999) *Annu. Rev. Med.* **50**, 425–440
- Gerard, C., and Rollins, B. J. (2001) *Nat. Immunol.* **2**, 108–115
- Van Damme, J., Van Beeumen, J., Conings, R., Decock, B., and Billiau, A. (1989) *Eur. J. Biochem.* **181**, 337–344
- Yoshimura, T., Robinson, E. A., Appella, E., Matsushima, K., Showalter, S. D., Skeel, A., and Leonard, E. J. (1989) *Mol. Immunol.* **26**, 87–93
- Hebert, C. A., Lusinkas, F. W., Kiely, J. M., Luis, E. A., Darbonne, W. C., Bennett, G. L., Liu, C. C., Obin, M. S., Gimbrone, M. A., Jr., and Baker, J. B. (1990) *J. Immunol.* **145**, 3033–3040
- Proost, P., Struyf, S., Couvreur, M., Lenaerts, J. P., Conings, R., Menten, P., Verhaert, P., Wuyts, A., and Van Damme, J. (1998) *J. Immunol.* **160**, 4034–4041
- Wuyts, A., Govaerts, C., Struyf, S., Lenaerts, J. P., Put, W., Conings, R., Proost, P., and Van Damme, J. (1999) *Eur. J. Biochem.* **260**, 421–429
- McQuibban, G. A., Gong, J. H., Tam, E. M., McCulloch, C. A., Clark-Lewis, I., and Overall, C. M. (2000) *Science* **289**, 1202–1206
- Stetler-Stevenson, W. G., Liotta, L. A., and Kleiner, D. E., Jr. (1993) *FASEB J.* **7**, 1434–1441
- Oudenakker, G., Fibbe, W. E., and Van Damme, J. (1998) *Immunol. Today* **19**, 182–189
- Padrines, M., Wolf, M., Walz, A., and Baggiolini, M. (1994) *FEBS Lett.* **352**, 231–235
- Struyf, S., De Meester, I., Scharpe, S., Lenaerts, J. P., Menten, P., Wang, J. M., Proost, P., and Van Damme, J. (1998) *Eur. J. Immunol.* **28**, 1262–1271
- Detheux, M., Standker, L., Vakili, J., Munch, J., Forssmann, U., Adermann, K., Pohlmann, S., Vassart, G., Kirchhoff, F., Parmentier, M., and Forssmann, W. G. (2000) *J. Exp. Med.* **192**, 1501–1508
- Nagasawa, T., Hirota, S., Tachibana, K., Takakura, N., Nishikawa, S., Kitamura, Y., Yoshida, N., Kikutani, H., and Kishimoto, T. (1996) *Nature* **382**, 635–638
- Tachibana, K., Hirota, S., Iizasa, H., Yoshida, H., Kawabata, K., Kataoka, Y., Kitamura, Y., Matsushima, 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
- 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
- Aiuti, A., Webb, I. J., Bleul, C., Springer, T., and Gutierrez-Ramos, J. C. (1997) *J. Exp. Med.* **185**, 111–120
- Peled, A., Petit, I., Kollet, O., Magid, M., Ponomarev, T., Byk, T., Nagler, A., Ben-Hur, H., Many, A., Shultz, L., Lider, O., Alon, R., Zipori, D., and Lapidot, T. (1999) *Science* **283**, 845–848
- Ma, Q., Jones, D., and Springer, T. A. (1999) *Immunity* **10**, 463–471
- Kawabata, K., Ujikawa, M., Egawa, T., Kawamoto, H., Tachibana, K., Iizasa, H., Katsura, Y., Kishimoto, T., and Nagasawa, T. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 5663–5667
- Nagasawa, T., Kikutani, H., and Kishimoto, T. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 2305–2309
- Shirozu, M., Nakano, T., Inazawa, J., Tashiro, K., Tada, H., Shinohara, T., and Honjo, T. (1995) *Genomics* **28**, 495–500
- Feng, Y., Broder, C. C., Kennedy, P. E., and Berger, E. A. (1996) *Science* **272**, 872–877
- Bleul, C. C., Farzan, M., Choe, H., Parolin, C., Clark-Lewis, I., Sodroski, J., and Springer, T. A. (1996) *Nature* **382**, 829–833
- 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
28. 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
29. Gonzalo, J. A., Lloyd, C. M., Peled, A., Delaney, T., Coyle, A. J., and Gutierrez-Ramos, J. C. (2000) *J. Immunol.* **165**, 499–508
30. Buckley, C. D., Amft, N., Bradfield, P. F., Pilling, D., Ross, E., Arenzana-Seisdedos, F., Amara, A., Curnow, S. J., Lord, J. M., Scheel-Toellner, D., and Salmon, M. (2000) *J. Immunol.* **165**, 3423–3429
31. Nanki, T., Hayashida, K., El-Gabalawy, H. S., Suson, S., Shi, K., Girschick, H. J., Yavuz, S., and Lipsky, P. E. (2000) *J. Immunol.* **165**, 6590–6598
32. Burger, J. A., Zvaifler, N. J., Tsukada, N., Firestein, G. S., and Kipps, T. J. (2001) *J. Clin. Invest.* **107**, 305–315
33. Abi-Younes, S., Sauty, A., Mach, F., Sukhova, G. K., Libby, P., and Luster, A. D. (2000) *Circ. Res.* **86**, 131–138
34. De Meester, I., Korom, S., Van Damme, J., and Scharpe, S. (1999) *Immunol. Today* **20**, 367–375
35. Delgado, M. B., Clark-Lewis, I., Loetscher, P., Langen, H., Thelen, M., Baggiolini, M., and Wolf, M. (2001) *Eur. J. Immunol.* **31**, 699–707
36. Reinhold, D., Hemmer, B., Gran, B., Born, I., Faust, J., Neubert, K., McFarland, H. F., Martin, R., and Ansoerge, S. (1998) *J. Neuroimmunol.* **87**, 203–209
37. 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
38. Brelot, A., Heveker, N., Montes, M., and Alizon, M. (2000) *J. Biol. Chem.* **275**, 23736–23744
39. Sadir, R., Baleux, F., Grosdidier, A., Imbert, A., and Lortat-Jacob, H. (2001) *J. Biol. Chem.* **276**, 8288–8296
40. 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
41. Schwartz, O., Alizon, M., Heard, J. M., and Danos, O. (1994) *Virology* **198**, 360–365
42. Le-Barillec, K., Pidard, D., Balloy, V., and Chignard, M. (2000) *J. Leukocyte Biol.* **68**, 209–215
43. Wright, D. G. (1988) *Methods Enzymol.* **162**, 538–551
44. Renesto, P., and Chignard, M. (1993) *Blood* **82**, 139–144
45. Valenzuela-Fernandez, A., Palanche, T., Amara, A., Magerus, A., Altmeyer, R., Delaunay, T., Virelizier, J. L., Baleux, F., Galzi, J. L., and Arenzana-Seisdedos, F. (2001) *J. Biol. Chem.* **276**, 26550–26558
46. Shi, Y., Kornovski, B. S., Savani, R., and Turley, E. A. (1993) *J. Immunol. Methods* **164**, 149–154
47. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) *J. Biol. Chem.* **260**, 3440–3450
48. Liljestrom, P., and Garoff, H. (1991) *Bio/Technology* **9**, 1356–1361
49. Reeves, P. J., Thaurmond, R. L., and Khorana, H. G. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 11487–11492
50. Campbell, E. J., Silverman, E. K., and Campbell, M. A. (1989) *J. Immunol.* **143**, 2961–2968
51. Bank, U., and Ansoerge, S. (2001) *J. Leukocyte Biol.* **69**, 197–206
52. Travis, J. (1988) *Am. J. Med.* **84**, 37–42
53. Stein, R. L., and Trainor, D. A. (1986) *Biochemistry* **25**, 5414–5419
54. Lee, W. L., and Downey, G. P. (2001) *Am. J. Respir. Crit. Care Med.* **164**, 896–904
55. Taub, D. D., Anver, M., Oppenheim, J. J., Longo, D. L., and Murphy, W. J. (1996) *J. Clin. Invest.* **97**, 1931–1941
56. Iikura, M., Miyamasu, M., Yamaguchi, M., Kawasaki, H., Matsushima, K., Kitaura, M., Morita, Y., Yoshie, O., Yamamoto, K., and Hirai, K. (2001) *J. Leukocyte Biol.* **70**, 113–120
57. Nagase, H., Miyamasu, M., Yamaguchi, M., Fujisawa, T., Ohta, K., Yamamoto, K., Morita, Y., and Hirai, K. (2000) *J. Immunol.* **164**, 5935–5943
58. Juremalm, M., Hjertson, M., Olsson, N., Harvima, I., Nilsson, K., and Nilsson, G. (2000) *Eur. J. Immunol.* **30**, 3614–3622
59. Brelot, A., Heveker, N., Pleskoff, O., Sol, N., and Alizon, M. (1997) *J. Virol.* **71**, 4744–4751
60. Dery, O., Corvera, C. U., Steinhoff, M., and Bunnett, N. W. (1998) *Am. J. Physiol.* **274**, C1429–C1452
61. Hecquet, C., Tan, F., Marcic, B. M., and Erdos, E. G. (2000) *Mol. Pharmacol.* **58**, 828–836
62. Seemuller, U., Meier, M., Ohlsson, K., Muller, H. P., and Fritz, H. (1977) *Hoppe-Seyler's Z. Physiol. Chem.* **358**, 1105–1107
63. Shioda, T., Kato, H., Ohnishi, Y., Tashiro, K., Ikegawa, M., Nakayama, E. E., Hu, H., Kato, A., Sakai, Y., Liu, H., Honjo, T., Nomoto, A., Iwamoto, A., Morimoto, C., and Nagai, Y. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 6331–6336
64. McQuibban, G. A., Butler, G. S., Gong, J.-H., Bendall, L., Power, C., Clark-Lewis, I., and Overall, C. M. (2001) *J. Biol. Chem.* **276**, 43503–43508
65. Price, B., Dennison, C., Tschesche, H., and Elliott, E. (2000) *J. Biol. Chem.* **275**, 28308–28315
66. 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
67. Agace, W. W., Amara, A., Roberts, A. I., Pablos, J. L., Thelen, S., Ugucioni, 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
68. Coulomb-L'Hermine, A., Emilie, D., Durand-Gasselin, L., Galanaud, P., and Chaouat, G. (2000) *AIDS Res. Hum. Retroviruses* **16**, 1097–1098
69. Vu, T. K., Wheaton, V. I., Hung, D. T., Charo, I., and Coughlin, S. R. (1991) *Nature* **353**, 674–677
70. Suidan, H. S., Bouvier, J., Schaerer, E., Stone, S. R., Monard, D., and Tschopp, J. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 8112–8116
71. Vouret-Craviari, V., Grall, D., Chambard, J. C., Rasmussen, U. B., Pouyssegur, J., and Van Obberghen-Schilling, E. (1995) *J. Biol. Chem.* **270**, 8367–8372
72. Renesto, P., Si-Tahar, M., Moniatte, M., Balloy, V., Van Dorsselaer, A., Pidard, D., and Chignard, M. (1997) *Blood* **89**, 1944–1953
73. Samis, J. A., Kam, E., Nesheim, M. E., and Giles, A. R. (1998) *Blood* **92**, 1287–1296
74. 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
75. Colditz, I. G. (1987) *Leukocyte Emigration and Its Sequelae*, pp. 14–23, Karger Press, Basel, Switzerland
76. Vender, R. L. (1996) *J. Invest. Med.* **44**, 531–539
77. Levesque, J. P., Takamatsu, Y., Nilsson, S. K., Haylock, D. N., and Simmons, P. J. (2001) *Blood* **98**, 1289–1297
78. Bleul, C. C., Wu, L., Hoxie, J. A., Springer, T. A., and Mackay, C. R. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 1925–1930
79. D'Apuzzo, M., Rolink, A., Loetscher, M., Hoxie, J. A., Clark-Lewis, I., Melchers, F., Baggiolini, M., and Moser, B. (1997) *Eur. J. Immunol.* **27**, 1788–1793
80. Deichmann, M., Kronenwett, R., and Haas, R. (1997) *Blood* **89**, 3522–3528
81. Hargreaves, D. C., Hyman, P. L., Lu, T. T., Ngo, V. N., Bidgol, A., Suzuki, G., Zou, Y. R., Littman, D. R., and Cyster, J. G. (2001) *J. Exp. Med.* **194**, 45–56
82. Bleul, C. C., Fuhlbrigge, R. C., Casasnovas, J. M., Aiuti, A., and Springer, T. A. (1996) *J. Exp. Med.* **184**, 1101–1109
83. Imai, K., Kobayashi, M., Wang, J., Shinobu, N., Yoshida, H., Hamada, J., Shindo, M., Higashino, F., Tanaka, J., Asaka, M., and Hosokawa, M. (1999) *Br. J. Haematol.* **106**, 905–911
84. Jo, D. Y., Rafii, S., Hamada, T., and Moore, M. A. (2000) *J. Clin. Invest.* **105**, 101–111
85. Peled, A., Kollet, O., Ponomaryov, T., Petit, I., Franitza, S., Grabovsky, V., Slav, M. M., Nagler, A., Lider, O., Alon, R., Zipori, D., and Lapidot, T. (2000) *Blood* **95**, 3289–3296
86. Ponomaryov, T., Peled, A., Petit, I., Taichman, R. S., Habler, L., Sandbank, J., Arenzana-Seisdedos, F., Magerus, A., Caruz, A., Fujii, N., Nagler, A., Lahav, M., Szyper-Kravitz, M., Zipori, D., and Lapidot, T. (2000) *J. Clin. Invest.* **106**, 1331–1339
87. Hoglund, M., Hakansson, L., and Venge, P. (1997) *Eur. J. Haematol.* **58**, 195–202
88. Falanga, A., Marchetti, M., Evangelista, V., Manarini, S., Oldani, E., Giovanelli, S., Galbusera, M., Cerletti, C., and Barbui, T. (1999) *Blood* **93**, 2506–2514
89. Horwitz, M., Benson, K. F., Person, R. E., Aprikyan, A. G., and Dale, D. C. (1999) *Nat. Genet.* **23**, 433–436
90. Kostmann, R. (1956) *Acta Paediatr.* **45**, 1–78
91. Dale, D. C., Person, R. E., Bolyard, A. A., Aprikyan, A. G., Bos, C., Bonilla, M. A., Boxer, L. A., Kannourakis, G., Zeidler, C., Welte, K., Benson, K. F., and Horwitz, M. (2000) *Blood* **96**, 2317–2322
92. Welte, K., and Boxer, L. A. (1997) *Semin. Hematol.* **34**, 267–278
93. Watanabe, T., Kawano, Y., Kanamaru, S., Onishi, T., Kaneko, S., Wakata, Y., Nakagawa, R., Makimoto, A., Kuroda, Y., Takaue, Y., and Talmadge, J. E. (1999) *Blood* **93**, 1157–1163