Activated lymphocytes synthesize and secrete substantial amounts of the β-chemokines macrophage inflammatory protein (MIP)-1α and MIP-1β/CCL4, both of which inhibit infection of cells with human immunodeficiency virus type 1 (HIV-1). The native form of MIP-1β secreted by activated human peripheral blood lymphocytes (MIP-1β(3–69)) lacks the two NH2-terminal amino acids of the full-length protein. This truncated form of MIP-1β has now been affinity-purified from the culture supernatant of such cells, and its structure has been confirmed by mass spectrometry. Functional studies of the purified protein revealed that MIP-1β(3–69) retains the abilities to induce down-modulation of surface expression of the chemokine receptor CCR5 and to inhibit the CCR5-mediated entry of HIV-1 in T cells. Characterization of the chemokine receptor specificity of MIP-1β(3–69) showed that the truncated protein not only shares the ability of intact MIP-1β to induce Ca2+ signaling through CCR5, but unlike the full-length protein, it also triggers a Ca2+ response via CCR1 and CCR2b. These results demonstrate that NH2-terminally truncated MIP-1β functions as a chemokine agonist with expanded receptor reactivity, which may represent an important mechanism for regulation of immune cell recruitment during inflammatory and antiviral responses.

Human macrophage inflammatory protein-1β (MIP-1β) is a proinflammatory chemokine that both promotes leukocyte accumulation in various inflammatory conditions and contributes to protective immunity against human immunodeficiency virus type 1 (HIV-1) (1–5). Studies of recombinant MIP-1β(1–69) have demonstrated that this full-length protein induces intracellular Ca2+ signaling, exerts its chemotactic activity, and inhibits HIV-1 infection through interaction with the chemokine receptor CCR5.

The specificity of chemokines for certain leukocyte subtypes depends on the expression by the latter of ligand-specific G protein-coupled receptors. Chemokines inhibit HIV-1 infection by blocking or down-regulating the receptors CCR5 or CXCR4, which also serve as coreceptors for the entry of HIV-1 (6–11). HIV-1-specific CD8+ T cell lines have been shown to secrete β-chemokines (1), and MIP-1α, MIP-1β, and RANTES/CCL5 (regulated on activation, normal T expressed and secreted) are the major anti-HIV-1 β-chemokines secreted by human peripheral T lymphocytes stimulated by the combination of interleukin (IL)-2 and either phytohemagglutinin (PHA) or IL-12 (4, 12). Recent studies have suggested that, among normal human peripheral blood lymphocytes (PBLs), perforin-low memory CD8+ T cells are the predominant producers of MIP-1β (13). We have shown previously that activated human PBLs secrete MIP-1β in a complex with MIP-1α (14). To date, however, the biological function of naturally produced MIP-1β has remained unclear.

We have now purified MIP-1β from the culture supernatant of stimulated human PBLs by affinity chromatography and used this material to study the biological activity of native MIP-1β. Our results demonstrate that the endogenous form of MIP-1β, which lacks the first two amino acid residues of the encoded protein, is active at CCR1 and CCR2 in addition to CCR5 and retains anti-HIV-1 activity.

EXPERIMENTAL PROCEDURES

Chemokines and Antibodies—Recombinant human MIP-1α, RANTES, and macrophage chemoattractant protein MCP-3/CCL7 were obtained from Peprotech (Rocky Hill, NJ), and recombinant human MIP-1β was from Sigma. Antibodies were from R&D Systems (Minneapolis, MN).

Cell Culture—Human PBLs and monocytes were isolated by elutria- tion from normal blood donors and cultured as described (15, 16). PBLs were maintained under 5% CO2 at 37 °C in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT), IL-2 (150 units/ml, R & D Systems), and PHA (2 μg/ml, Sigma). Monocytes were maintained for 2 days in macrophage serum-free medium (Invitrogen) supplemented with macrophage colony-stimulating factor (10 ng/ml, R&D Systems). HEK-293 and HOS cell transfecants were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum.

Affinity Purification of Native MIP-1β—The culture supernatant of PBLs stimulated with IL-2 and PHA for 6 days was subjected to pre-absorption with Sepharose 4B beads (Amersham Biosciences), adjusted to pH 4.0 with HCl, and then applied to an affinity column containing Sepharose 4B beads conjugated with a monoclonal antibody to MIP-1β. The column was washed with phosphate-buffered saline, after which proteins were eluted with 50 mM glycine (pH 2.0). The eluate was immediately adjusted to pH 7.0 with Tris. The purity and molecular size of the eluted MIP-1β were analyzed by mass spectrometry, and its concentration was determined with an enzyme-linked immunosorbent assay (R&D systems).

Mass Spectrometry—The molecular size of purified MIP-1β was determined by matrix-assisted laser desorption ionization and time-of-flight (MALDI-TOF) mass spectrometry (PerCective Biosystems, Boston, MA). α-Cyano-4-hydroxycinnamic acid (Sigma) and recombinant human MIP-1β were used as the matrix and internal standard, respectively. Mass data were analyzed with Data Explore software (PerCective Biosystems). Measurement of Cytosolic-free Ca2+ Concentration—Cells (1 × 10⁷/ml) in Ca2+ buffer (136 mM NaCl, 4.8 mM KCl, 1 mM CaCl₂, 5 mM...
glucose, 20 mM HEPES pH 7.4) were supplemented with 5 μM fura-2 acetoxy methyl ester (Molecular Probes, Eugene, OR) that had been premixed with 10% Pluronic F-127 and then were incubated for 45 min in the dark at 37 °C. The cells were washed and then resuspended at a density of 2 × 10⁶/ml in Ca²⁺ buffer supplemented with 0.1% bovine serum albumin. The cytosolic-free Ca²⁺ concentration was measured with a δ scan (Photon Technology Intl., Monmouth, NJ), with excitation at 340 and 380 nm and emission monitored at 510 nm. Data were recorded as the 340/380 nm fluorescence ratio.

Cell Surface Immunofluorescence Staining—After culture for 7 days in medium containing IL-2, PBLs were incubated for 1 h at 37 °C with full-length MIP-1β or MIP-1β(3–69). They were washed, blocked, and stained with a phycoerythrin-conjugated monoclonal antibody to CCR5 or an isotype control and were analyzed by flow cytometry as described (15).

PCR Amplification of Proviral DNA—PBLs cultured with IL-2 and PHA for 7 days were exposed to chemokines for 1 h at 37 °C. We have shown previously that resting human monocytes express the chemokine receptors CCR1, CCR2b, and CXCR4, whereas CCR5 is either unresponsive as of recombinant human MIP-1β(3–69) on Cell Surface Expression of CCR5—Chemokines RANTES, MIP-1α, and MIP-1β secreted by lipopolysaccharide-activated monocytes and by PBLs stimulated with IL-2 and IL-12 induce down-modulation of the cell surface expression of CCR5 and thereby inhibit HIV-1 infection (2, 4).

RESULTS

Purification and Characterization of Native Secreted MIP-1β—We have recently shown that human PBLs stimulated by the combination either of IL-2 and IL-12 or of IL-2 and PHA produce a truncated form (MIP-1β(3–69)) of MIP-1β (7658 Da) that lacks the two NH2-terminal amino acids of the full-length protein and is complexed with a truncated form (MIP-1α(5–70)) of MIP-1α (7459 Da) that lacks the four NH2-terminal residues (14). We also showed that this chemokine complex dissociates at low pH, suggesting that it is maintained by ionic interactions. To study the functions of the native form of MIP-1β, we first separated this chemokine from its complex with MIP-1α and then purified it by affinity chromatography. The culture supernatant of stimulated human PBLs was adjusted to pH 4.0 and then applied to an affinity column containing a monoclonal antibody to MIP-1β. Bound proteins were eluted from the column at pH 2.0 and analyzed by MALDI-TOF mass spectrometry. The column eluate consisted predominantly of the NH2-terminally truncated form (7658 Da) of MIP-1β (Fig. 1). Application of the PBL culture supernatant to the affinity column at pH 7.0 resulted in the copurification of the truncated forms of both MIP-1α and MIP-1β (Fig. 1), indicating that the chemokine complex remained intact.

Activity of MIP-1β(3–69) at CCR5—MIP-1β is a ligand for CCR5, its interaction with which induces a transient increase in the cytosolic-free Ca²⁺ concentration. We have shown previously that resting human monocytes express the chemokine receptors CCR1, CCR2b, and CXCR4, whereas CCR5 is either absent or expressed at a low level in these cells (17, 18). Stimulation with macrophage colony-stimulating factor induces the differentiation of monocytes into macrophages with the result that CCR5 is expressed at a high level and CCR2 at a low level. We therefore compared the effects of native MIP-1β(3–69) and the full-length recombinant protein on Ca²⁺ signaling in freshly isolated monocytes and in monocyte-derived macrophages.

The addition of 5 nM MIP-1β(3–69) or MIP-1β(3–69) to macrophages loaded with the fluorescent Ca²⁺ indicator fura-2 induced a rapid increase in the cytosolic-free Ca²⁺ concentration (Fig. 2A). Macrophages that were stimulated first with MIP-1β(3–69) did not show a second Ca²⁺ response when challenged with the full-length protein. In contrast, macrophages stimulated first with full-length MIP-1β retained the ability to respond to a subsequent challenge with MIP-1β(3–69), suggesting that MIP-1β(3–69) also interacts with a receptor other than CCR5.

MIP-1β(1–69) failed to induce Ca²⁺ mobilization in freshly isolated monocytes (Fig. 2B), confirming our previous observation (18) that monocytes purified by elutriation do not express CCR5. However, MIP-1β(3–69) both increased the cytosolic-free Ca²⁺ concentration in monocytes and fully desensitized the cells to the effect of RANTES (Fig. 2B), suggesting that the native form of MIP-1β signals through CCR1 or other receptors in addition to CCR5.

Expanded Receptor Reactivity of Native MIP-1β(3–69)—To identify the receptors targeted by MIP-1β(3–69), we examined its effects on Ca²⁺ signaling in cultured cells expressing recombinant human CCR5, CCR1, or CCR2b. Both MIP-1β(3–69) and the full-length protein exhibited similar abilities to induce a Ca²⁺ response in HEK-293 cells expressing recombinant CCR5 (Fig. 3A). In contrast, in HOS cells expressing recombinant CCR1, full-length MIP-1β failed to induce a Ca²⁺ response or to interfere with a subsequent Ca²⁺ response triggered by MIP-1β(3–69) (Fig. 3B). Finally, in HOS cells expressing recombinant CCR2b, MIP-1β(3–69) failed to induce a Ca²⁺ response or to interfere with a subsequent Ca²⁺ response triggered by MIP-1β(3–69) (Fig. 3C). Control cells transfected with empty vector alone did not respond to any of the ligands examined (data not shown). These data thus demonstrate that native secreted MIP-1β(3–69), unlike full-length MIP-1β, exhibits biological activity at CCR1 and CCR2b. Hence, MIP-1β(3–69) on Cell Surface Expression of CCR5—Chemokines RANTES, MIP-1α, and MIP-1β secreted by lipopolysaccharide-activated monocytes and by PBLs stimulated with IL-2 and IL-12 induce down-modulation of the cell surface expression of CCR5 and thereby inhibit HIV-1 infection (2, 4).
A major component of the mechanism through which chemokines protect cells from HIV infection is by inducing endocytosis of chemokine receptor. Both RANTES and aminooxypenta-ne(AOP)-RANTES induce rapid internalization of CCR5 (19–22). The effect of MIP-1β(3–69) on the cell surface expression of CCR5 was investigated by flow cytometry. PBLs were incubated for 7 days with IL-2 to induce expression of CCR5, exposed for 1 h at 37 °C to MIP-1β(1–69) or MIP-1β(3–69), and then stained with a phycoerythrin-conjugated monoclonal antibody (2D7) to CCR5. MIP-1β(3–69) and full-length MIP-1β each markedly reduced the extent of cell surface expression of CCR5 in a dose-dependent manner with approximately equal potencies (Fig. 4A). We have demonstrated previously that SDF-1α-mediated down-modulation of CXCR4 was not caused by the steric blocking of anti-CXCR4 monoclonal antibody 12G5 binding to CXCR4 by SDF-1α (16). To verify that receptor-bound MIP-1β did not interfere with the interaction of monoclonal antibody (2D7) with CCR5, we incubated cells with a high concentration (1 μg/ml) of MIP-1β(3–69) at 4 °C, which will allow MIP-1β(3–69) binding to CCR5 but prevent receptor endocytosis. Cells were then stained with 2D7 and analyzed by flow cytometry. Preincubation of MIP-1β(3–69) did not block the binding of 2D7 monoclonal antibody to CCR5 as shown in Fig. 4B.

Anti-HIV-1 Activity of MIP-1β(3–69)—Finally, we examined the effect of MIP-1β(3–69) on the entry of M-tropic HIV-1 into T cells. PBLs were cultured in the presence of IL-2 and PHA for 7 days, incubated with full-length MIP-1β or MIP-1β(3–69) for 1 h, and then exposed to HIV-1Ba.L. Analysis of proviral DNA by semi-quantitative PCR analysis revealed that three independent preparations of MIP-1β(3–69) inhibited CCR5-mediated HIV-1 entry with potencies similar to that of intact MIP-1β (Fig. 5).

DISCUSSION

We have characterized the structure and function of the native form of MIP-1β that is secreted by activated human macrophages and monocytes in a complex with MIP-1β. It is a specific ligand for CCR5 and induces the migration of CCR5-positive macrophages and lymphocytes into tissues or lymph nodes. Our demonstration that MIP-1β(3–69) also interacts functionally with CCR1 and CCR2b suggests that secretion of this truncated protein with MIP-1α might also result in the attraction of cells that express CCR1 or CCR2, such as monocytes, immature dendritic cells, and lymphocytes, possibly through heterodimer-induced interactions between multiple receptors on the cell surface. The truncated form of MIP-1β may also act on regulatory T cells (CD4+ , CD25+), which are thought to respond primarily to MIP-1β (23). MIP-1β(3–69) appears to be produced only by activated T cells; it has not been detected in culture supernatants of monocytes or macrophages (14).

The NH2 termini of chemokines are important for receptor binding, activation, and specificity (17, 24–32). Relatively small changes in the NH2-terminal amino acid sequence thus markedly affect the biological activity of chemokines. Members of both the CC and CXC subfamilies of chemokines occur naturally as post-translationally modified proteins. We and others have shown that the dipeptidyl peptidase IV (DPPIV) (CD26) plays an important role in the processing of chemokines that contain a penultimate alanine or proline residue at the NH2-terminus (18).
termini and that such processing influences the functional properties of chemokines including RANTES, stromal cell-derived factor-1 (SDF-1)/CXCL12, eotaxin/CCL11, IP-10/CXCL10 (interferon-\(\gamma\)/H9253-inducible protein 10), granulocyte chemotactic protein/CXCL6, macrophage-derived chemokine (MDC)/CCL22, and MIP-1\(\beta\)/H9251/LD78/H9252 (17, 28, 29, 33–37). The removal of the NH\(_2\)-terminal dipeptide from RANTES, SDF-1, or MDC results in reduced receptor binding and signaling and consequent impairment of chemotactic responses. Such truncation of RANTES also results in a loss of the ability to signal through CCR1, in contrast to the situation with MIP-1\(\beta\), which gains activity at CCR1. RANTES(3–68), like MIP-1\(\beta\)(3–69), retains activity at CCR5 (17). Amino-terminal modification of other chemokines has been shown to result in gain of function. The NH\(_2\)-terminal truncation of IL-8, ENA-78 (epithelial cell-derived neutrophil-activating protein), and MIP-1\(\beta\)/LD78/CCL3 increases anti-HIV-1 activity (35, 36).

The enzyme responsible for MIP-1\(\beta\) truncation has not been identified. Although the presence of a proline in the second NH\(_2\)-terminal position of MIP-1\(\beta\) suggests that DPPIV (CD26) is a candidate enzyme for this cleavage reaction, it has proved difficult to demonstrate processing of recombinant MIP-1\(\beta\) by DPPIV (24). Other proteases implicated in chemokine processing include cathepsin G and matrix metalloproteinase, both of which have been shown to cleave and inactivate SDF-1 (24, 29, 30).

Chemokine receptors and other molecules are differentially expressed by human T helper 1 (Th1) and Th2 cells. Th1 cell-associated molecules include CD26, interferon-\(\gamma\), lymphocyte activation gene-3 (LAG-3), and the chemokine receptors CCR5 and CXCR3, whereas Th2 cell-associated molecules include the chemokine receptors CCR3, CCR4, and CCR8 as well as CD62L and CD30. The expression of CD26 correlates with the production of interferon-\(\gamma\) in Th1 cell-associated granulomatous disorders and in inflamed synovia in individuals with rheumatoid arthritis (37–40). CD26 is also associated with the production of Th1 cytokines in CD4\(^+\) T cell clones (41). The differential expression of chemokine receptors by human Th1 and Th2 cells underlies the differential migration of these cells in response to various chemokines. Chemokines are thus important contributors to polarized Th1 cell- or Th2 cell-mediated immune responses. Truncation of the CCR5-specific chemokines RANTES and MIP-1\(\beta\), possibly mediated by CD26-expressed monoclonal antibody 2D7 to CCR5, and analyzed by flow cytometry. B, PBLs were incubated with 1 \(\mu\)g/ml MIP-1\(\beta\)(3–69) at 4 °C and stained with anti-CCR5 monoclonal antibody 2D7.

**Fig. 4.** Down-modulation of cell surface expression of CCR5 induced by MIP-1\(\beta\)(3–69). A, PBLs were stimulated with IL-2 for 7 days, incubated for 1 h at 37 °C with the indicated concentrations of full-length MIP-1\(\beta\) or MIP-1\(\beta\)(3–69), stained with phycoerythrin-la-

**Fig. 5.** Effect of MIP-1\(\beta\)(3–69) on CCR5-mediated HIV-1 entry into T cells. PBLs were stimulated with IL-2 and PHA for 7 days, incubated in the absence or presence of full-length MIP-1\(\beta\), MIP-1\(\beta\)(3–69) (preparations from three different individuals), or RANTES (positive control) at a concentration of 30 nM for 1 h, and exposed to the Ba-L strain of HIV-1 in the continued absence or presence of chemokine. Viral entry was then assessed by semi-quantitative PCR analysis; HIV-1 copy number standards are shown on the left. \(\Delta\), heat-inactivated Ba-L is used as a control to determine background levels of non-infectious uptake of HIV DNA in the viral stock.
pressed on activated lymphocytes, does not affect their activities at CCR5, whereas cleavage of IP-10 (17), a ligand for CXCR3, results in loss of its activity at this latter receptor. In contrast, chemokines (such as eotaxin and MDC) that are ligands for receptors on TH2 cells (CCR4 and CCR3) are inactivated by CD26 (24, 25). Under these conditions, the interaction of truncated MIP-1α and RANTES with CCR5 may constitute the predominant pathway for TH1 cell recruitment. Further studies are required to determine both the mechanism of MIP-1α truncation and the impact of the expanded chemokine receptor reactivity of MIP-1α (3–69) with regard to lymphocyte, monocyte, and dendritic cell migration and differentiation.

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Natural Truncation of the Chemokine MIP-1β/CCL4 Affects Receptor Specificity but Not Anti-HIV-1 Activity
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