Chemokines are secreted proteins that function as chemoattractants for leukocytes. The chemokines macrophage inflammatory protein 1α and 1β (MIP-1α and MIP-1β) now have been shown to be secreted from activated human monocytes and peripheral blood lymphocytes (PBLs) as a heterodimer. Immunoprecipitation and immunoblot analysis revealed that antibodies to either MIP-1α or MIP-1β precipitated a protein complex containing both MIP-1α and MIP-1β under normal conditions from culture supernatants and lysates of these cells. Mass spectrometry of the complexes, precipitated from the culture supernatants of monocytes and PBLs, revealed the presence of NH2-terminal truncated MIP-1α (residues 5–70) together with either intact MIP-1β or NH2-terminal truncated MIP-1β (residues 3–69), respectively. The secreted MIP-1αβ heterodimers were dissociated into their component monomers under acidic conditions. Exposure of monocytes or PBLs to monensin induced the accumulation of heterodimers composed of NH2-terminal truncated MIP-1α and full-length MIP-1β in the Golgi complex. The mixing of recombinant chemokines in vitro demonstrated that heterodimerization of MIP-1α and MIP-1β is specific and that it occurs at physiological conditions, pH 7.4, and in the range of nanomolar concentrations. The data presented here provide the first biochemical evidence for the existence of chemokine heterodimers under natural conditions. Formation of heterodimers of MIP-1αβ may have an impact on intracellular signaling events that contribute to CCR5 and possibly to other chemokine receptor functions.

Chemokines constitute a family of small secreted proteins that were initially characterized on the basis of their chemo tactic effects on a variety of leukocytes (1, 2). They are produced locally in tissues and interact with selective G protein-coupled receptors expressed on the leukocyte surface. Chemokines also regulate leukocyte maturation, the trafficking and homing of lymphocytes, and the development of lymphoid tissues. Human immunodeficiency virus-type 1 (HIV-1) also targets chemokine receptors during entry into cells, and certain chemokines act as HIV-1 suppressive factors (3–5).

To date, at least 50 chemokines have been identified. Despite its large size, the chemokine family is remarkably homogeneous. Chemokines are divided into two subfamilies, α (CXC) and β (CC), on the basis of conserved cysteine residues. Four conserved cysteines form two essential disulfide bonds, Cys1-Cys3 and Cys2-Cys4, in all chemokines. The three-dimensional structures of three α chemokines (interleukin (IL)-8, growth-related oncogene-α, and platelet factor 4) and four β chemokines (macrophage inflammatory protein (MIP)-1α, MIP-1β, RANTES (regulated on activation normal T cell expressed), and macrophage chemoattractant protein-1 (MCP-1)) have been determined either by multidimensional nuclear magnetic resonance (NMR) or by x-ray crystallography (6–11). These studies have revealed that chemokines possess a short NH2-terminal domain preceding the first cysteine, a backbone that comprises three antiparallel β strands and a COOH-terminal α-helix. Whereas the backbone exhibits a well ordered structure, the structure of the NH2 terminus is disordered. The similarity in the three-dimensional structures of the chemokine monomers is consistent with the marked sequence homology of these proteins.

The quaternary structures of α and β chemokines, however, differ markedly from each other, and the dimer interfaces are formed by distinct sets of residues. Whereas the IL-8 dimer is globular, the homodimers formed by MIP-1α, MIP-1β, and RANTES are cylindrical (6, 7, 11, 12). Calculation of the solvation-free energies of dimerization and analysis of hydrophobic clusters of amino acids suggest that the formation and stabilization of the two different types of dimers result from the burial of hydrophobic residues and that the distinct quaternary structures of α and β chemokine dimers are preserved throughout the two subfamilies. The biological existence and significance of dimeric forms of chemokines, especially that of chemokine heterodimers, have remained unclear.

Human T lymphocytes and monocytes release anti-HIV-1 chemokines, predominantly the β chemokines MIP-1α, MIP-1β, and RANTES (3, 4, 13), in response to stimulation with mitogens, cytokines, or bacterial pathogens. We now show that human monocytes stimulated by lipopolysaccharide, human peripheral blood lymphocytes (PBLs) stimulated by cytokines (IL-2 and IL-12), or phytohemagglutinin produce a chemokine heterodimer containing MIP-1α and MIP-1β.

EXPERIMENTAL PROCEDURES
Reagents—Recombinant human MIP-1α (full-length), RANTES, MDC, MCP-1, and IP-10 were obtained from Peprotech (Rocky Hill, peripheral blood lymphocyte; MDC, macrophage-derived chemokine; IP-10, gamma interferon inducible protein-10; anti-α, antibodies to; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PHA, phytohemagglutinin A.

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The abbreviations used are: HIV-1, human immunodeficiency virus-type 1; IL, interleukin; MIP, macrophage inflammatory protein; RANTES, regulated on activation normal T cell expressed; MCP, macrophage chemoattractant protein; LPS, lipopolysaccharide; PBL, peripheral blood lymphocyte; MDC, macrophage-derived chemokine; IP-10, gamma interferon inducible protein-10; anti-α, antibodies to; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PHA, phytohemagglutinin A.

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N.J.; recombinant human MIP-1β was from Sigma; and recombinant human (−4)MIP-1α and antigen affinity purified antibodies to chemokines were from R&D Systems (Minneapolis, MN).

**Cell Culture—**Human PBLs and monocytes were isolated by elutriation from normal blood donors and cultured as described previously (13, 14). PBLs were cultured under 5% CO₂ at 37°C in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 150 units/ml IL-2 (R&D Systems), and 10 ng/ml IL-12 (kindly provided by Genetics Institute, Boston, MA) or 2 μg/mo PHA (sigma). Monocytes were cultured in macrophage serum-free medium (Life Technologies, Inc.) supplemented with 1 μg/ml LPS (Sigma).

Cells were plated at a density of 1 × 10⁸ cells/ml in 6-well plates. They were then washed and lysed for 1 h in a solution containing 1% Triton X-100, 50 mM Tris-HCl, pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 10 mM E64, and 40 μg/ml leupeptin (Roche Molecular Biochemicals). After centrifugation of lysates at 6700 × g for 15 min, the resulting supernatants were subjected to immunoprecipitation either with anti-MIP-1α or anti-MIP-1β for mass spectrometric analysis.

**RESULTS**

**Identification of a Heterodimer of MIP-1α/β in Culture Supernatants of Human Monocytes and PBLs—**Supernatants were collected from cultures of either LPS-stimulated monocytes or PBLs stimulated with IL-2 and IL-12 after incubation for 1 or 6 days, respectively, times that correspond to peak chemokine secretion (data not shown). Enzyme-linked immunosorbent assays revealed that the culture supernatants contained large amounts of MIP-1α and MIP-1β but only a low concentration of RANTES (data not shown) (15). Immunoprecipitation of culture supernatants from both cell types with either anti-MIP-1α or anti-MIP-1β followed by immunoblot analysis with each of these antibodies revealed that each immunoprecipitate contained both MIP-1α and MIP-1β (Fig. 1A). The antigen affinity purified antibodies to each MIP-1 protein were specific and did not cross-react with the other isofrom on Western blot analysis (Fig. 1B) or with immunoprecipitation followed by mass spectrometric analysis (Fig. 2B).

To determine the molecular size of the chemokines precipitated by either anti-MIP-1α or anti-MIP-1β, we analyzed the precipitated proteins by mass spectrometry (MALDI-TOF). The two antibodies precipitated identical complexes from the culture supernatant of IL-2- and IL-12-stimulated PBLs contained (−4)MIP-1α and a protein with a molecular mass of 7658 Da, which corresponds to MIP-1β lacking the two NH₂-terminal residues ((−2)MIP-1β). Similar results were obtained with culture supernatants of PBLs stimulated with IL-2 and phytohemagglutinin (data not shown).

**Identification of a Native MIP-1α/β Heterodimer in the Golgi Complex—**We next investigated the kinetics and site of MIP-1α/β dimerization. Immunoprecipitation and mass spectrometric analysis revealed the presence of the MIP-1α/β heterodimer in the culture supernatants of monocytes or PBLs as early as 7–10 h after exposure to LPS or to IL-2 and IL-12, respectively (data not shown). To determine whether the heterodimerization of MIP-1α and
MIP-1β occurs intracellularly in the Golgi complex, we treated cells with monensin to prevent intracellular protein transport by inducing retention of proteins in the Golgi complex (15, 16). PBLs and monocytes were incubated with IL-2 and phytohemagglutinin A or with LPS, respectively, for 20 h, the last 10 h of which they were also exposed to monensin. Under these conditions, chemokines were produced primarily during the last 10 h of culture. The cells were then lysed and subjected to immunoprecipitation with anti-MIP-1α (upper panels) or anti-MIP-1β (lower panels). The molecular mass of the precipitated proteins was then determined by mass spectrometry. B, mass spectrometric analysis of immunoprecipitation of recombinant MIP-1α and recombinant MIP-1β with anti-MIP-1α or anti-MIP-1β antibody.

**Fig. 2.** Mass spectrometric analysis of MIP-1α/β complexes secreted by monocytes and PBLs. A, culture supernatants of either PBLs stimulated with IL-2 and IL-12 for 6 days (left panels) or monocytes stimulated with LPS for 1 day (right panels) were subjected to immunoprecipitation with anti-MIP-1α (upper panels) or anti-MIP-1β (lower panels). The molecular mass of the precipitated proteins was then determined by mass spectrometry. B, mass spectrometric analysis of immunoprecipitation of recombinant MIP-1α and recombinant MIP-1β with anti-MIP-1α or anti-MIP-1β antibody.

Fig. 3. Secretion of MIP-1α and MIP-1β from monocytes predominantly as a heterodimer. Culture supernatant of monocytes stimulated with LPS for 1 day was subjected to immunoprecipitation (IP) first with anti-MIP-1α and then either with anti-MIP-1α again or with anti-MIP-1β. The three precipitates were then subjected to immunoblot analysis with anti-MIP-1α or anti-MIP-1β as indicated. Recombinant human MIP-1α (7.4 kDa) or MIP-1β (7.8 kDa) were also probed with the corresponding antibodies.

**Fig. 3.** Secretion of MIP-1α and MIP-1β from monocytes predominantly as a heterodimer. Culture supernatant of monocytes stimulated with LPS for 1 day was subjected to immunoprecipitation (IP) first with anti-MIP-1α and then either with anti-MIP-1α again or with anti-MIP-1β. The three precipitates were then subjected to immunoblot analysis with anti-MIP-1α or anti-MIP-1β as indicated. Recombinant human MIP-1α (7.4 kDa) or MIP-1β (7.8 kDa) were also probed with the corresponding antibodies.
DISCUSSION

The possible existence and activity of native chemokine dimers have been controversial (21). The β chemokines MIP-1α, MIP-1β, and RANTES tend to self-associate and thereby form homodimers, tetramers, or larger aggregates in vitro. This process is dynamic and reversible, but it has been thought that chemokine concentrations in vivo may be too low for the formation of such multimers to occur. However, high local concentrations of chemokines may occur in vivo under certain conditions, such as during platelet degranulation, inflammatory disease, and local accumulation of chemokines on cell membranes mediated by receptors or by glycosaminoglycans.

We have now identified a naturally occurring MIP-1α/β heterodimer produced by activated monocytes and PBLs. Our data demonstrate that the MIP-1α/β heterodimer forms in the endoplasmic reticulum or Golgi complex, and that these two chemokines are secreted in the form of the heterodimer. Furthermore, the combination of MIP-1α with MIP-1β in vitro showed that these two chemokines indeed form heterodimers at physiological (nanomolar) concentrations.

Most studies on chemokine homodimerization have been performed in vitro, and the natural formation of either homodimers or heterodimers of chemokines has not been described previously. Native MIP-1 purified from LPS-stimulated mouse macrophage RAW 264.7 migrated on SDS-polyacrylamide gels as a doublet composed of peptides with similar physical characteristics. The NH₂-terminal sequences of the two peptides identified them as MIP-1α and MIP-1β (22). Although it was not shown that the co-purified mouse chemokines originally existed as a heterodimer, our data now suggest that these previous results might be explained by the formation of a native mouse MIP-1α/β heterodimer.

The three-dimensional structures of both α and β chemokines have been determined mostly with the molecules in the form of crystallized homodimers. Whereas the monomeric structures of both α and β chemokines are highly similar, the dimeric structures of members of these two subfamilies, as typified by IL-8 (12, 20, 23) and MIP-1β (9, 11, 20), respectively, differ markedly from each other with the dimer interfaces being formed by distinct sets of interacting residues. Whereas the IL-8 dimer is globular, the MIP-1β dimer is cylindrical. The three-dimensional structure of the α chemokine PF4 revealed it to be a tetramer composed of two dimers of the IL-8 type (18,
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MIP-1 wild-type protein, whereas a mutant lacking the NH2-terminal five residues forms a dimer similar to that formed by the other chemokines, including RANTES, MIP-1α, MCP-1, and MCP-2, exhibit a similar homodimeric structure (6, 8, 17, 25). MCP-3 remains monomeric at concentrations of up to 20 mg/ml (7), whereas I-309, another chemokine, was also shown to exist as a monomer at high concentrations during sedimentation (17).

A truncation mutant of MIP-1β lacking the NH2-terminal five residues forms a dimer similar to that formed by the wild-type protein, whereas a mutant lacking the NH2-terminal eight residues exists only as a folded monomer (11). An MCP-1 mutant lacking the NH2-terminal eight amino acids exists predominantly as a monomer (7). IL-8 and melanocyte growth stimulating activity/growth-related oncogene-α, and 100 μM for neutrophil-activating protein-2. MIP-1α also tends to aggregate, but this process is reversible, and MIP-1α exists as a monomer under physiological conditions (6, 7, 9, 17–20).

Comparison of our present data with those of previous studies of chemokine homodimers indicates that substantial differences exist between MIP-1α/β heterodimers and chemokine homodimers in terms of physical properties: (i) the formation of homodimers requires high concentrations of chemokines, whereas the MIP-1α/β heterodimer forms at physiological concentrations of monomers; (ii) the dissociation of homodimers occurs under physiological conditions, whereas dissociation of MIP-1α/β heterodimers is apparent only at low pH; (iii) homodimers have been detected only in solution under in vitro conditions, whereas MIP-1α/β heterodimers are secreted from primary monocytes and PBLS; and (iv) the formation of the MIP-1α/β heterodimer appears to be mediated by electrostatic interactions, whereas the formation of chemokine homodimers is thought to be mediated by hydrophobic interactions.

The existence of native chemokine homodimers remains to be demonstrated with the data having been obtained that are consistent or inconsistent with homodimers being the functionally active form of these proteins (12). Mutagenesis and cross-linking studies indicate that the active form of MCP-1 is a dimer (31). However, other studies have shown that IL-8 and MIP-1β derivatives that do not dimerize are fully active (32, 33).

With regard to the functional role of MIP-1α/β heterodimerization, it is possible that the formation of stable heterodimers protects these chemokines from enzymatic digestion and, thus, increases or stabilizes their activity. Preliminary data indicate that MIP-1α/β heterodimer-containing mixtures have potent activity in inducing down-regulation of the CCR5 receptor. The formation of the MIP-1α/β heterodimer under natural conditions may induce (possibly heterologous) receptor dimerization that may have an impact on intracellular signaling events, which contribute to CCR5 and possibly other chemokine receptor functions. Production of pure homogeneous preparations of heterodimers is required for further characterization of heterodimer activities and receptor binding properties.

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REFERENCES

1. Baggiolini, M., Dewald, B., and Moser, B. (1997) Annu. Rev. Immunol. 15, 675–705
2. Ward, S. G., and Westwick, J. (1998) Biochem. J. 333, 457–470
3. Cocchi, F., DeVico, A. L., Garzino-Demo, A., Arya, S. K., Gallo, R. C., and Lusso, P. (1995) Science 270, 1811–1815
4. Verani, A., Scarlatti, G., Comar, M., Tresoldi, E., Polo, S., Giacca, M., Lusso, P., Siceardi, A. G., and Vercelli, D. (1997) J. Exp. Med. 185, 805–816
5. Garzino-Demo, A., Moss, R. B., Margolick, J. B., Cleghorn, P., Sill, A., Blattner, W. A., Cocchi, F., Carlo, D. J., DeVico, A. L., and Gallo, R. C. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 11986–11991
6. Skelton, N. J., Aspiras, F., Ogez, J., and Schall, T. J. (1995) Biochemistry 34, 5329–5342
7. Kim, K. S., Rajaarathnam, K., Clark-Lewis, I., and Sykes, B. D. (1996) FEBS Lett. 395, 277–282
8. Chung, C. W., Cooke, R. M., Proudfoot, A. E., and Wells, T. N. (1995) Biochemistry 34, 9307–9314
9. Lodi, P. J., Garretti, D. S., Kuszewski, J., Tsang, M. L., Weatherbee, J. A., Leonard, W. J., Gronenborn, A. M., and Clore, G. M. (1984) Science 223, 1762–1767
10. Shao, W., Jerva, L. F., West, J., Lolis, E., and Schweitzer, B. I. (1998) Biochemistry 37, 8303–8313
11. Lawrance, J. S., Li, W. A., and Li, W. P. (1998) Biochemistry 37, 9346–9354
12. Covell, D. G., Smythers, G. W., Gronenborn, A. M., and Clore, G. M. (1994) Protein Sci. 3, 2064–2072
13. Wang, J., Guan, E., Rodriguez, G., and Norcross, M. A. (1999) J. Immunol. 163, 5763–5769
14. Shao, W., Jerva, L. F., West, J., Lolis, E., and Schweitzer, B. I. (1998) Biochemistry 37, 8303–8313
15. Lawrance, J. S., Li, W. A., and Li, W. P. (1998) Biochemistry 37, 9346–9354
16. Covell, D. G., Smythers, G. W., Gronenborn, A. M., and Clore, G. M. (1994) Protein Sci. 3, 2064–2072
17. Wang, J., Guan, E., Rodriguez, G., and Norcross, M. A. (1999) J. Immunol. 163, 5763–5769

18. E. Guan, J. Wang, M. A. Norcross, unpublished observations.

Fig. 6. Specificity of MIP-1α/β heterodimerization in vitro. Recombinant (~4-MIP-1α (7450 Da), MIP-1β (7820 Da), and RANTES (7864 Da) dissolved in phosphate-buffered saline, pH 7.4, were mixed at final concentrations of 25 nM in the indicated combinations at room temperature and then subjected to immunoprecipitation with the indicated antibodies. The resulting precipitates were analyzed by mass spectrometry.

Most three-dimensional structural determinations have been performed at high concentrations (>1 mM) of chemokines with thermodynamics further strongly favoring dimerization. In contrast, physical data indicate that chemokines are fully dissociated into monomers at their normal physiological (nanomolar) concentrations. Dimer dissociation constants have been determined as 35 μM for RANTES, 33 μM for MCP-1, 58 μM for MCP-2, 40 nm for MIP-1β, 18 μM for IL-8, 73 μM for melanocyte growth stimulating activity/growth-related oncogene-α, and 100 μM for neutrophil-activating protein-2. MIP-1α also tends to aggregate, but this process is reversible, and MIP-1α exists as a monomer under physiological conditions (6, 7, 9, 17–20).
Identification of a Native MIP-1α/β Heterodimer

14. Wang, J., Rodriguez, G., Oravec, T., and Nercess, M. A. (1998) J. Virol. 72, 7642–7647
15. Tartakoff, A. M. (1983) Cell 32, 1026–1028
16. Korimilli, A., Gonzales, L. W., and Guttenberg, S. H. (2000) J. Biol. Chem. 275, 8672–8679
17. Paulini, J. F., Willard, D., Consler, T., Luther, M., and Krangel, M. S. (1994) J. Immunol. 153, 2704–2717
18. Mayo, K. H., and Chen, M. J. (1989) Biochemistry 28, 9469–9478
19. Chen, M. J., and Mayo, K. H. (1991) Biochemistry 30, 6402–6411
20. Clore, G. M., and Gronenborn, A. M. (1995) J. Leukocyte Biol. 57, 703–711
21. Clore, G. M., and Gronenborn, A. M. (1995) FASEB J. 9, 57–62
22. Sherry, B., Tekamp-olson, P., Gallegos, C., Bauer, D., Davatelis, G., Wolfe, S. D., Masiarz, F., Cuit, D., and Cerami, A. (1988) J. Exp. Med. 168, 2251–2259
23. Horcher, M., Rot, A., Aeschauer, H., and Besemer, J. (1998) Cytokine 10, 1–12
24. St. Charles, R., Wulz, D. A., and Edwards, B. F. (1989) J. Biol. Chem. 264, 2092–2099
25. Handel, T. M., and Domaille, P. J. (1996) Biochemistry 35, 6569–6584
26. Baldwin, E. T., Weber, I. T., St. Charles, R., Xuan, J. C., Appella, E., Yamada, M., Matsushima, K., Edwards, B. F., Clore, G. M., and Gronenborn, A. M. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 592–596
27. Fairbrother, W. J., Reilly, D., Colby, T., and Horuk, R. (1993) FEBS Lett. 330, 302–306
28. Kim, K. S., Clark-Lewis, I., and Sykes, B. D. (1994) J. Biol. Chem. 269, 32909–32915
29. Malkowski, M. G., Wu, J. Y., Lazar, J. B., Johnson, P. H., and Edwards, B. F. (1995) J. Biol. Chem. 270, 7077–7087
30. Zhang, X., Chen, L., Bancroft, D. P., Lai, C. K., and Maione, T. E. (1994) Biochemistry 33, 8361–8366
31. Zhang, Y., and Rollins, B. J. (1995) Mol. Cell. Biol. 15, 4851–4855
32. Rajarathnam, K., Sykes, B. D., Kay, C. M., Dewald, B., Geiser, T., Baggiolini, M., and Clark-Lewis, I. (1994) Science 264, 89–92
33. Laurence, J. S., Blanpain, C., Burgner, J. W., Parmentier, M., and Li Wang, P. J. (2000) Biochemistry 39, 3401–3409